IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

GENENTECH, INC. and CITY OF HOPE, *Plaintiffs*, v. AMGEN INC., *Defendant*.	)	C.A. No. 17-1407-CFC (CONSOLIDATED)
GENENTECH, INC., *Plaintiff and Counterclaim Defendant*, v. AMGEN INC., *Defendant and Counterclaim Plaintiff*.	)	C.A. No. 18-924-CFC

APPENDIX TO GENENTECH'S LETTER-BRIEF CONCERNING CONSTRUCTION OF "FOLLOWING FERMENTATION" AND SUPPORTING DECLARATION OF DR. HANSJÖRG HAUSER

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Kemp G., O'Neil P., *Large-Scale Production of Therapeutic Antibodies: Considerations for Optimizing Product Capture and Purification*, in: Subramanian G. (eds) Antibodies (2004) ("Kemp 2004")	Appx246
Dwivedi, *Validation of Cell Culture-Based Processes and Qualification of Associated Equipment and Facility*, in: Ozturk, Cell Culture Technology for Pharmaceutical and Cell-Based Therapies ("Dwivedi 2006")	Appx272
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Matijasevic et al., *Hypothermia causes a reversible, p53-mediated cell cyle arrest in cultured fibroblasts*, Oncol Res., 10(11-12): 605-610 (1998)	Appx354
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Hunt et al., *Low-Temperature Pausing of Cultivated Mammalian Cells*, Biotechnol Bioeng., 89(2):157-63, (2004)	Appx374
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(12) **United States Patent**
Kao et al.

(10) **Patent No.: US 8,574,869 B2**
(45) **Date of Patent: Nov. 5, 2013**

(54) **PREVENTION OF DISULFIDE BOND REDUCTION DURING RECOMBINANT PRODUCTION OF POLYPEPTIDES**

(75) Inventors: **Yung-Hsiang Kao**, San Mateo, CA (US); **Michael W. Laird**, San Ramon, CA (US); **Melody Trexler Schmidt**, San Carlos, CA (US); **Rita L. Wong**, Redwood City, CA (US); **Daniel P. Hewitt**, Sunnyvale, CA (US)

(73) Assignee: **Genentech, Inc.**, South San Francisco, CA (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **13/354,223**

(22) Filed: **Jan. 19, 2012**

(65) **Prior Publication Data**

US 2013/0017598 A1 Jan. 17, 2013

Related U.S. Application Data

(63) Continuation of application No. 12/217,745, filed on Jul. 8, 2008, now abandoned.

(60) Provisional application No. 60/948,677, filed on Jul. 9, 2007.

(51) **Int. Cl.**
C12P 1/00 (2006.01)
C12N 5/02 (2006.01)

(52) **U.S. Cl.**
USPC **435/41**; 435/325

(58) **Field of Classification Search**
None
See application file for complete search history.

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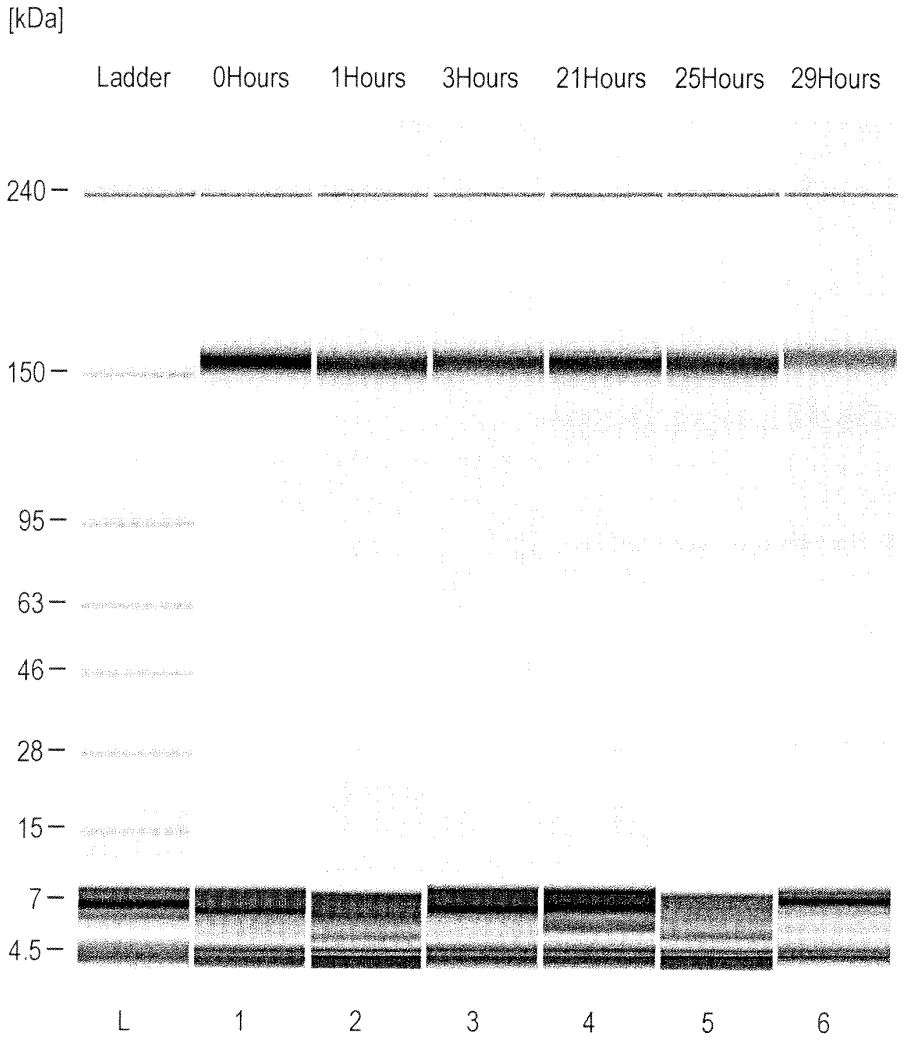
Primary Examiner — Suzanne M Noakes
Assistant Examiner — Jae W Lee
(74) *Attorney, Agent, or Firm* — Morrison & Foerster LLP

(57) **ABSTRACT**

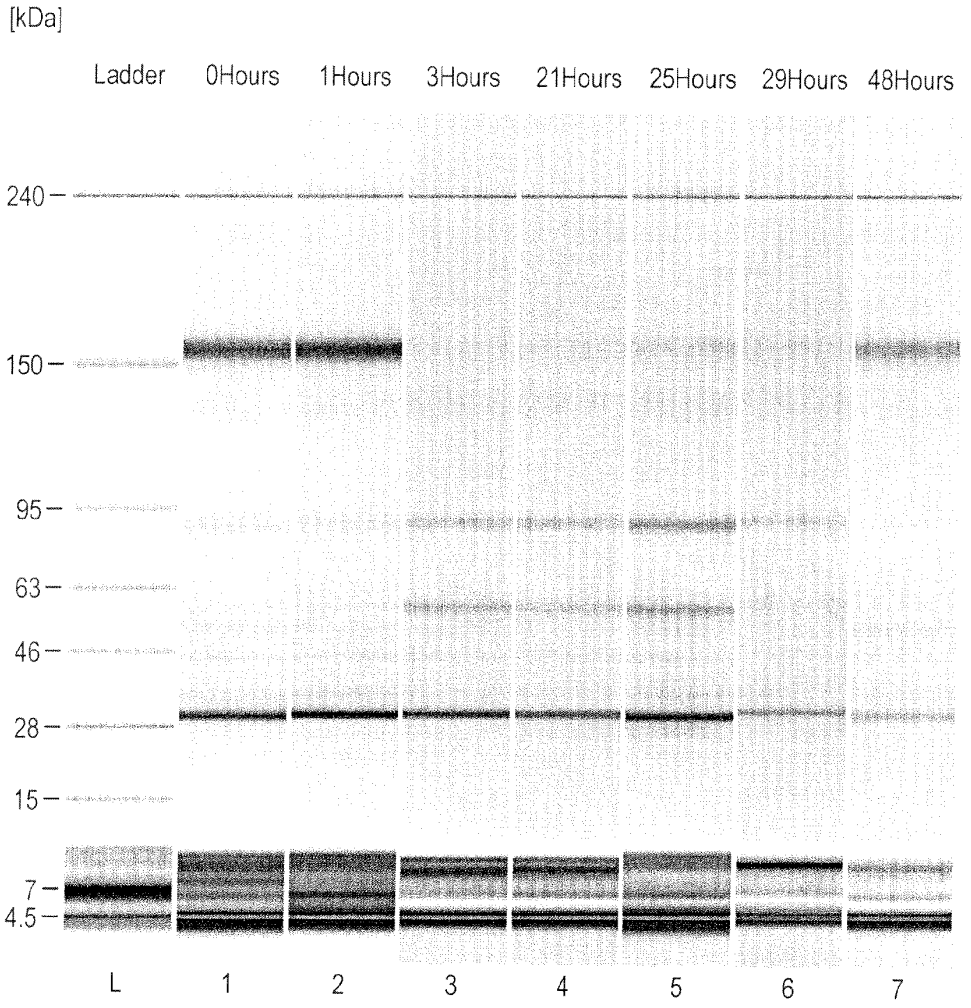
Provided herein are methods for preventing the reduction of disulfide bonds during the recombinant production of disulfide-containing polypeptides. In particular, the invention concerns the prevention of disulfide bond reduction during harvesting of disulfide-containing polypeptides, including antibodies, from recombinant host cell cultures.

10 Claims, 40 Drawing Sheets



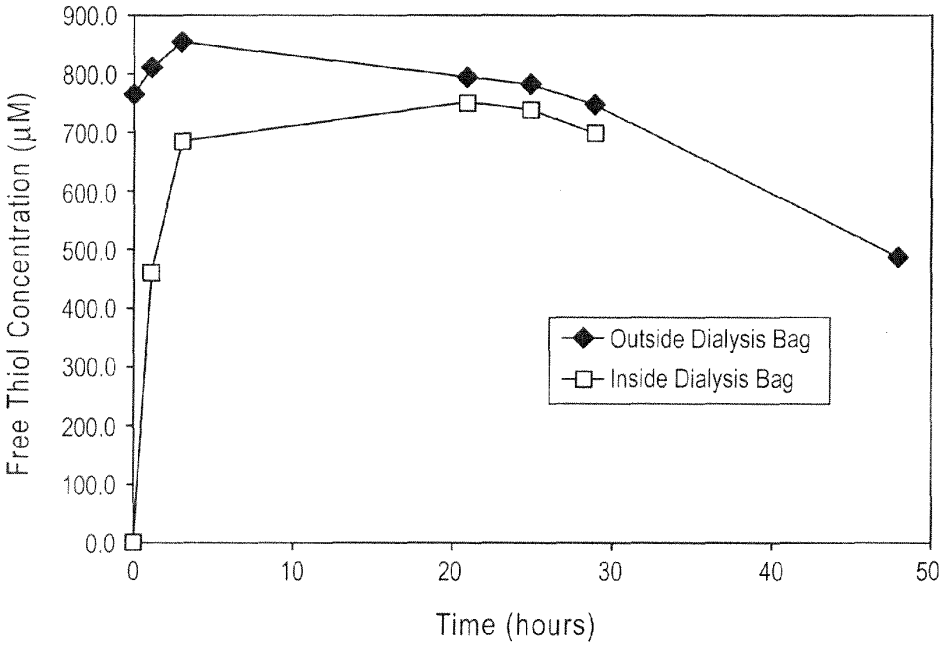
Dialysis Experiment

FIG. 1



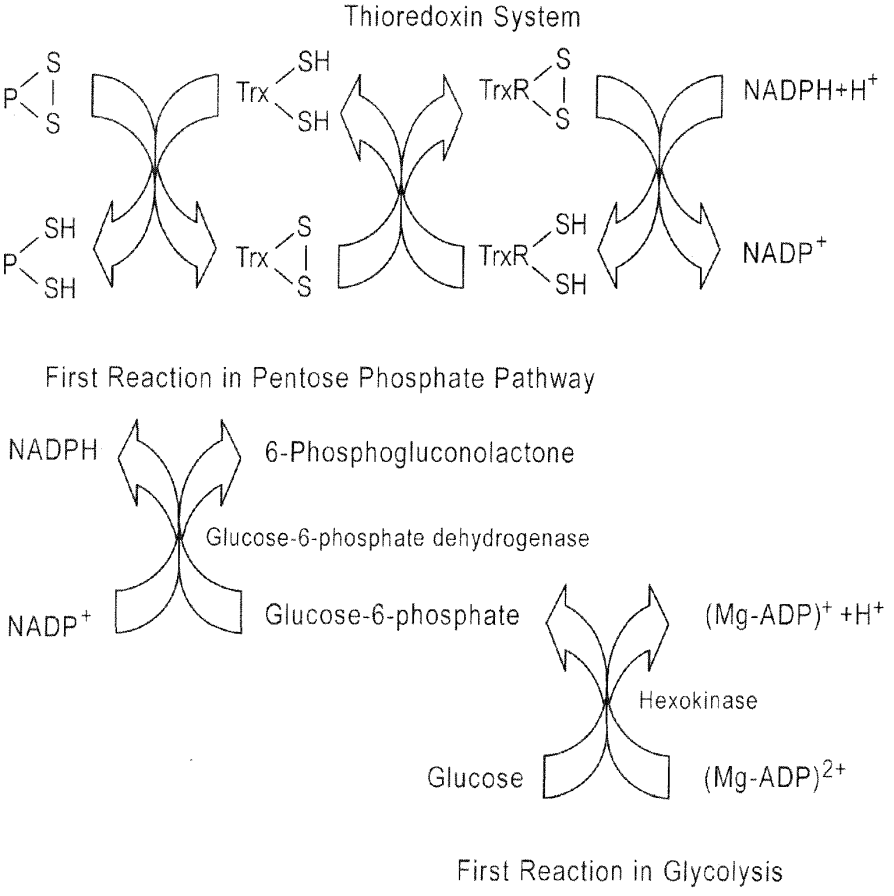
Dialysis Experiment

FIG. 2



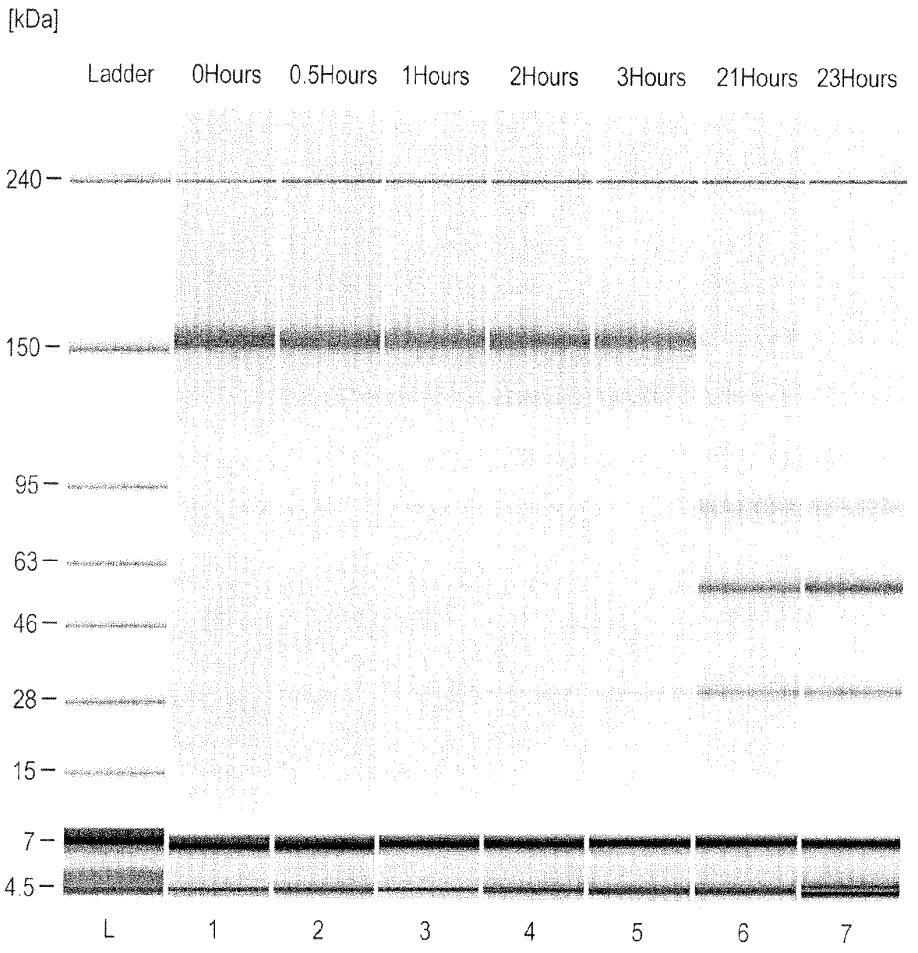
Free Thiol Levels from Dialysis Experiment

FIG. 3



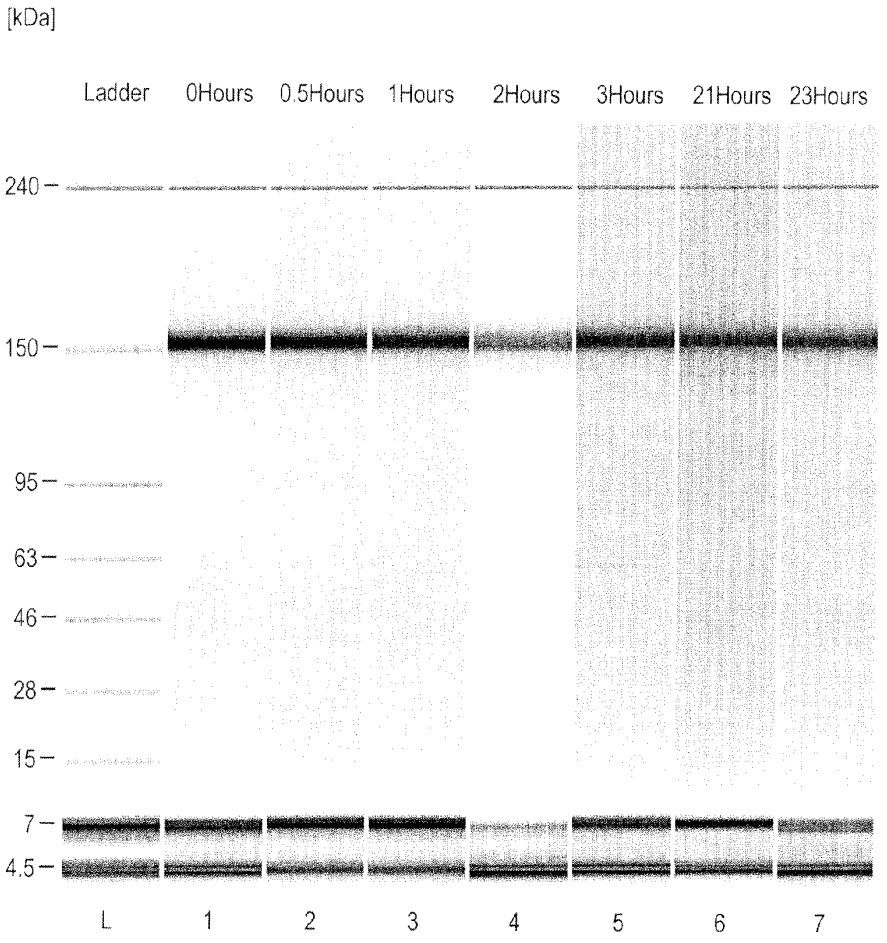
Thioredoxin System and Other Reactions Involved in Antibody Reduction

FIG. 4



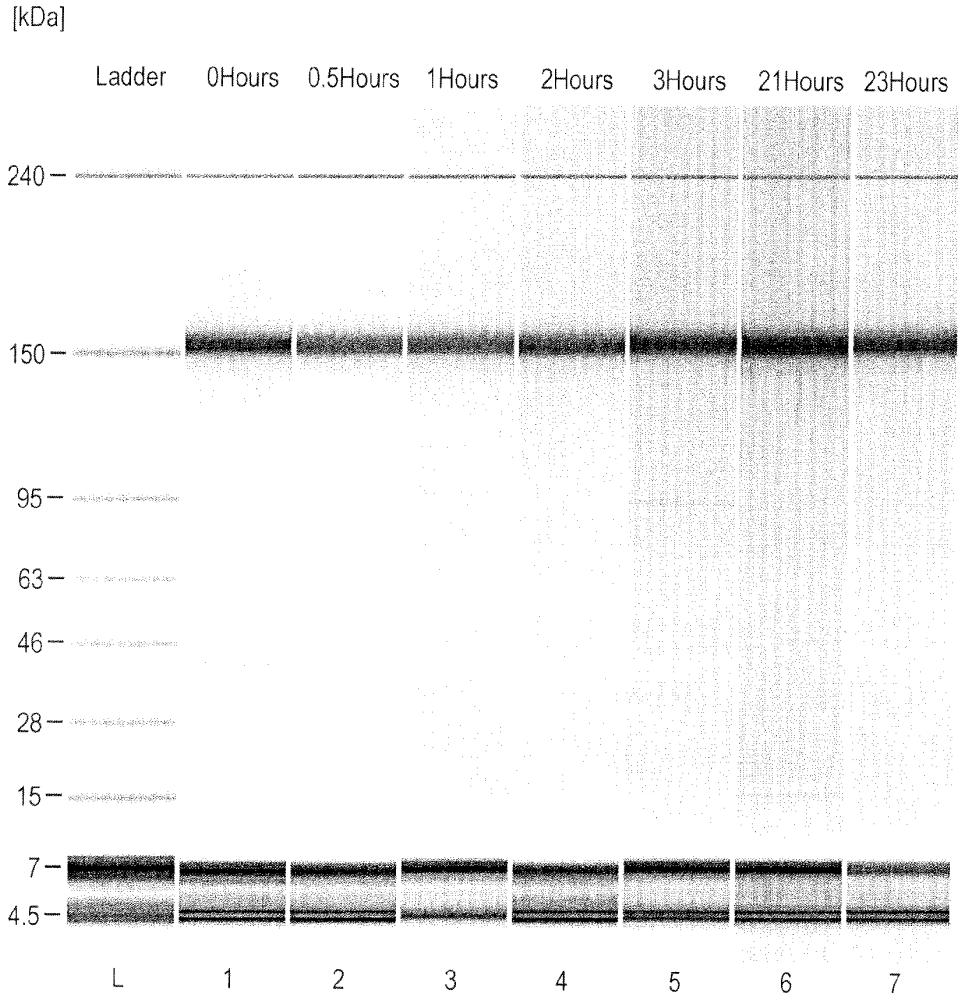
In *Vitro* Activity of Thioredoxin System

FIG. 5



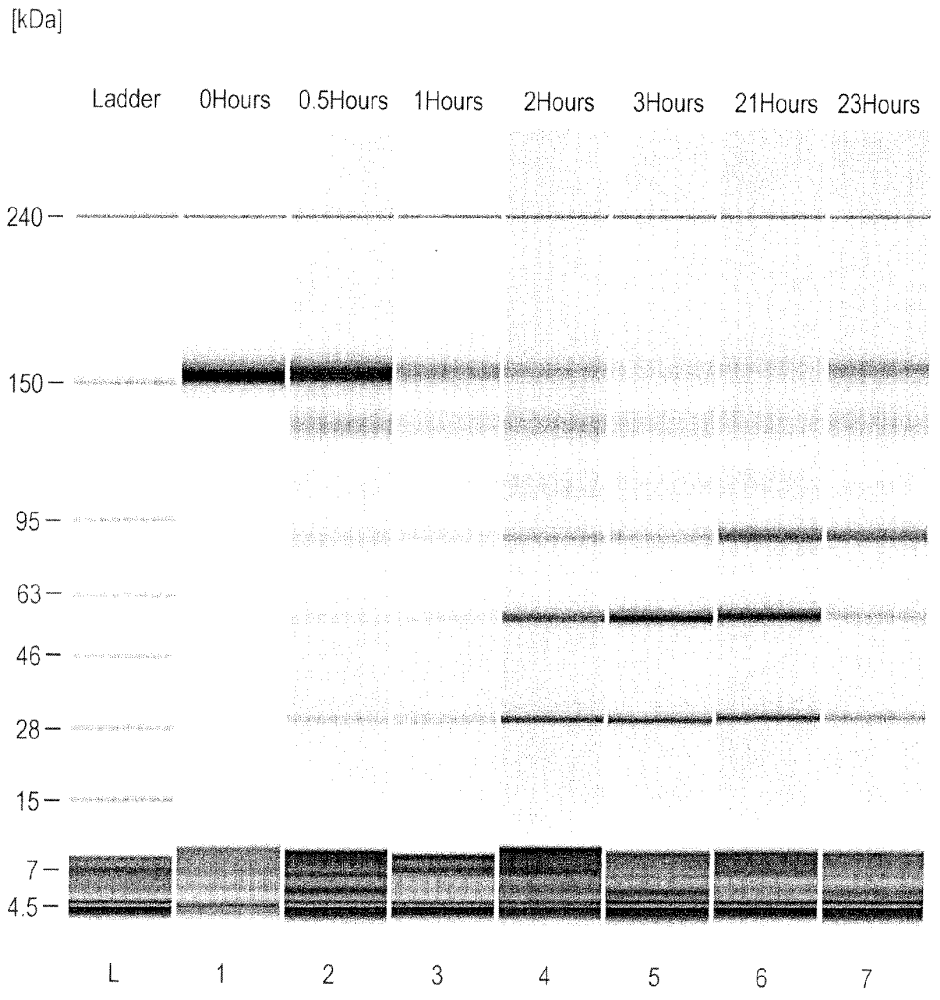
In *vitro* Activity of Thioredoxin System Inhibited by Aurothioglucose

FIG. 6



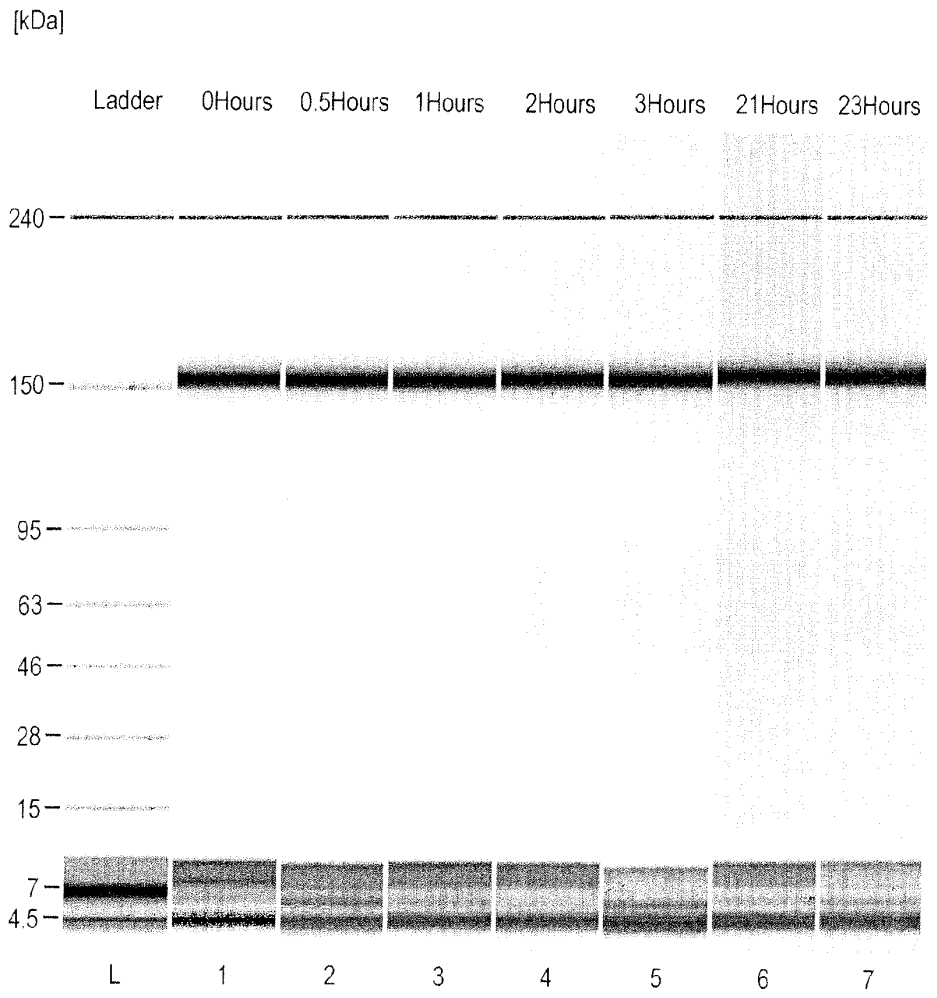
In *vitro* Activity of Thioredoxin System Inhibited by Aurothiomalate

FIG. 7



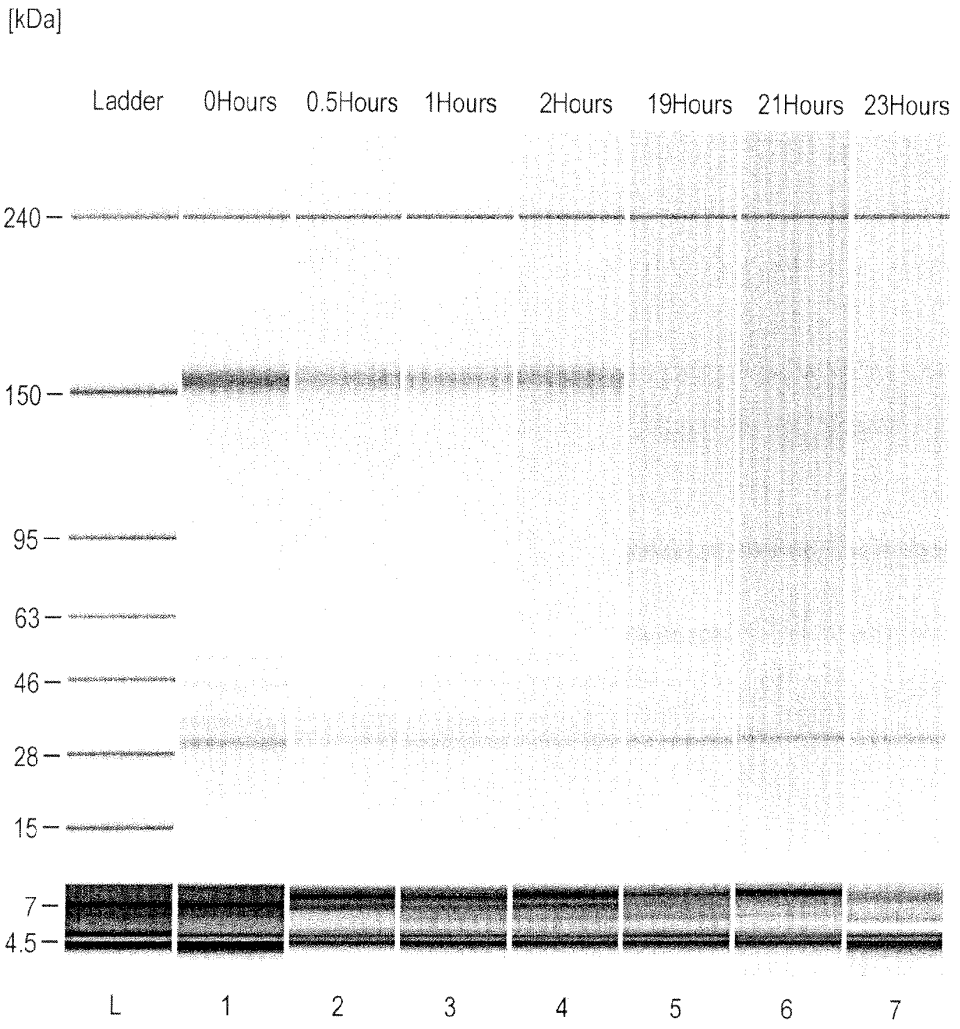
In *vitro* Activity of Thioredoxin System

FIG. 8



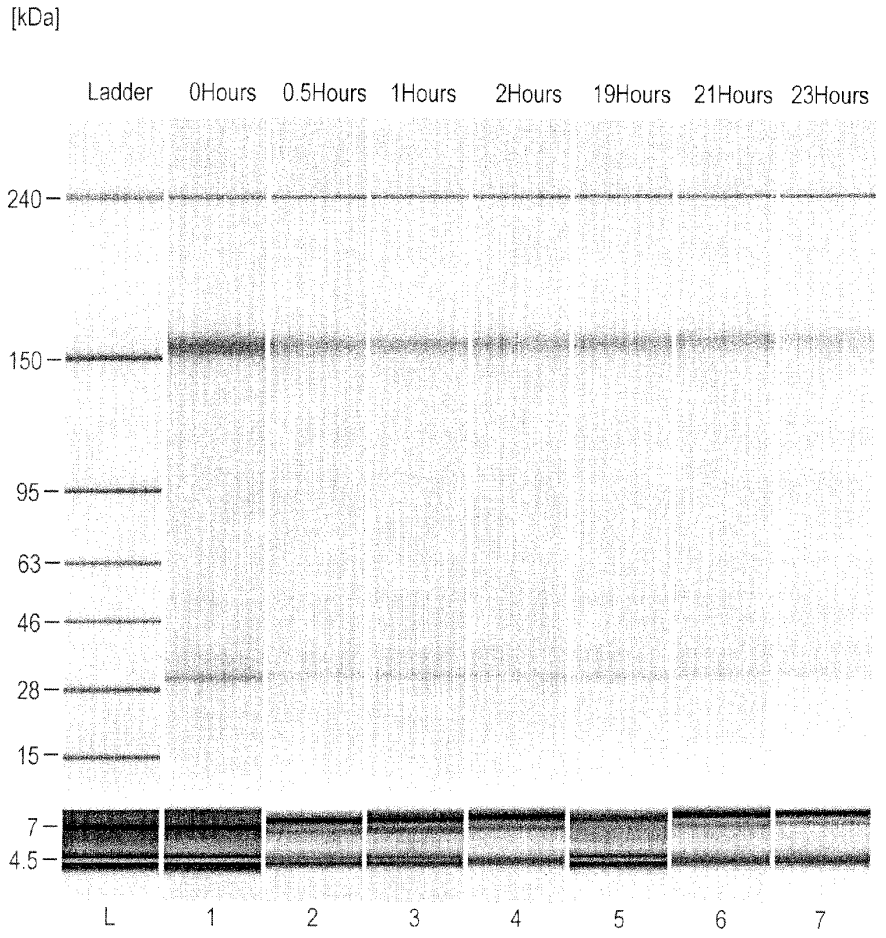
In *vitro* Activity of Thioredoxin System Inhibited by $CuSO_4$

FIG. 9



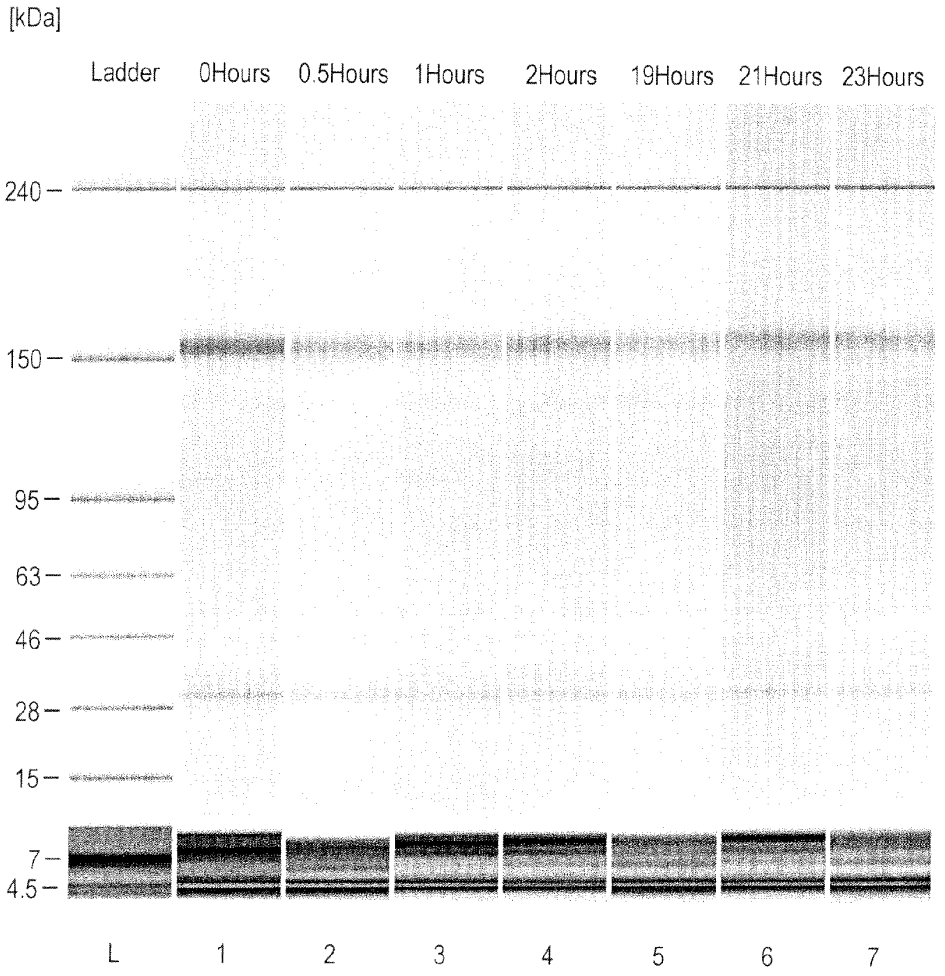
Ocrelizumab Reduction

FIG. 10



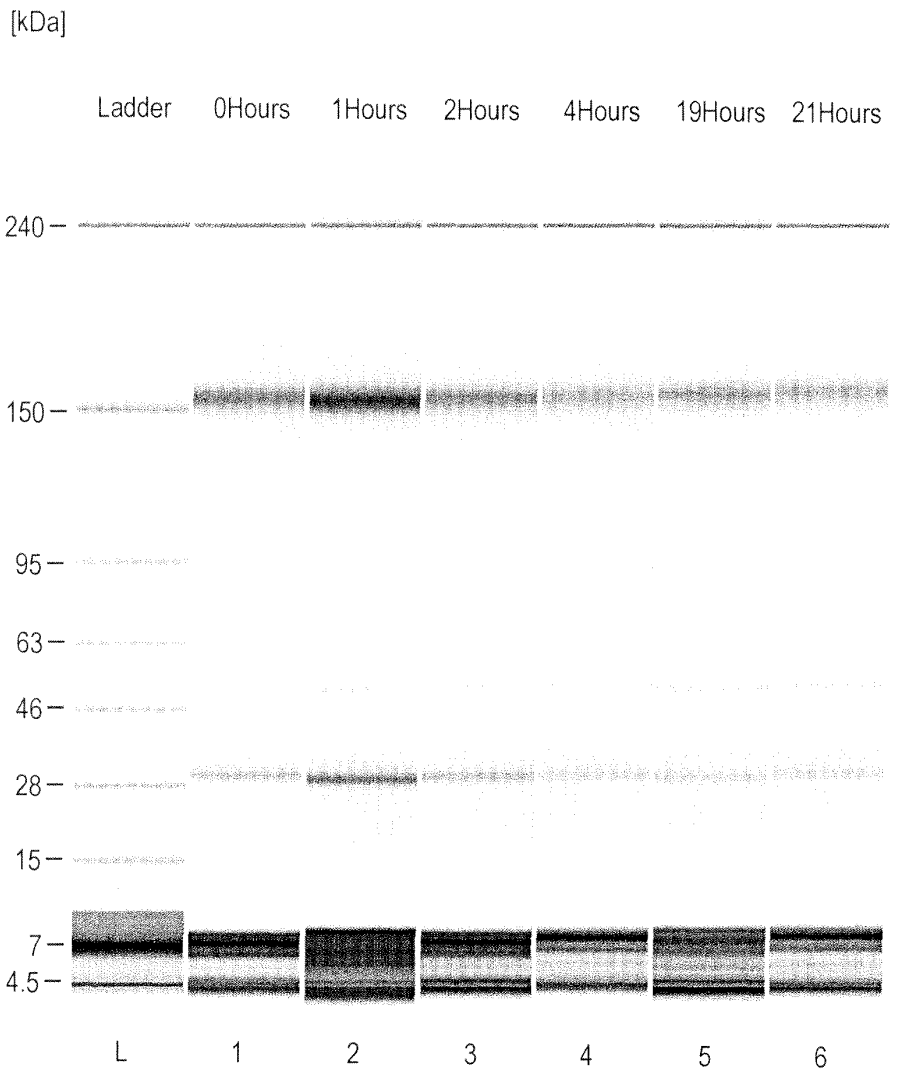
Inhibition of Ocrelizumab Reduction In HCCF by Aurothioglucose

FIG. 11



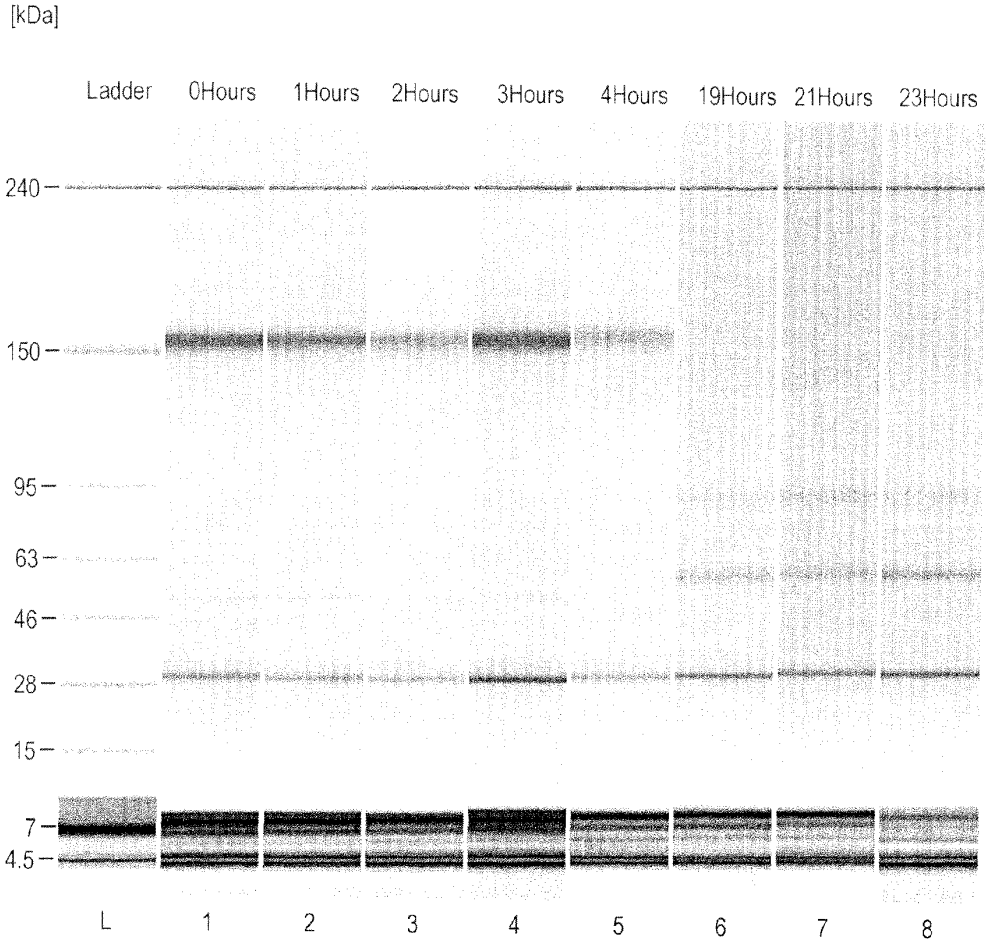
Inhibition of Ocrelizumab Reduction In HCCF by Aurothiomalate

FIG. 12



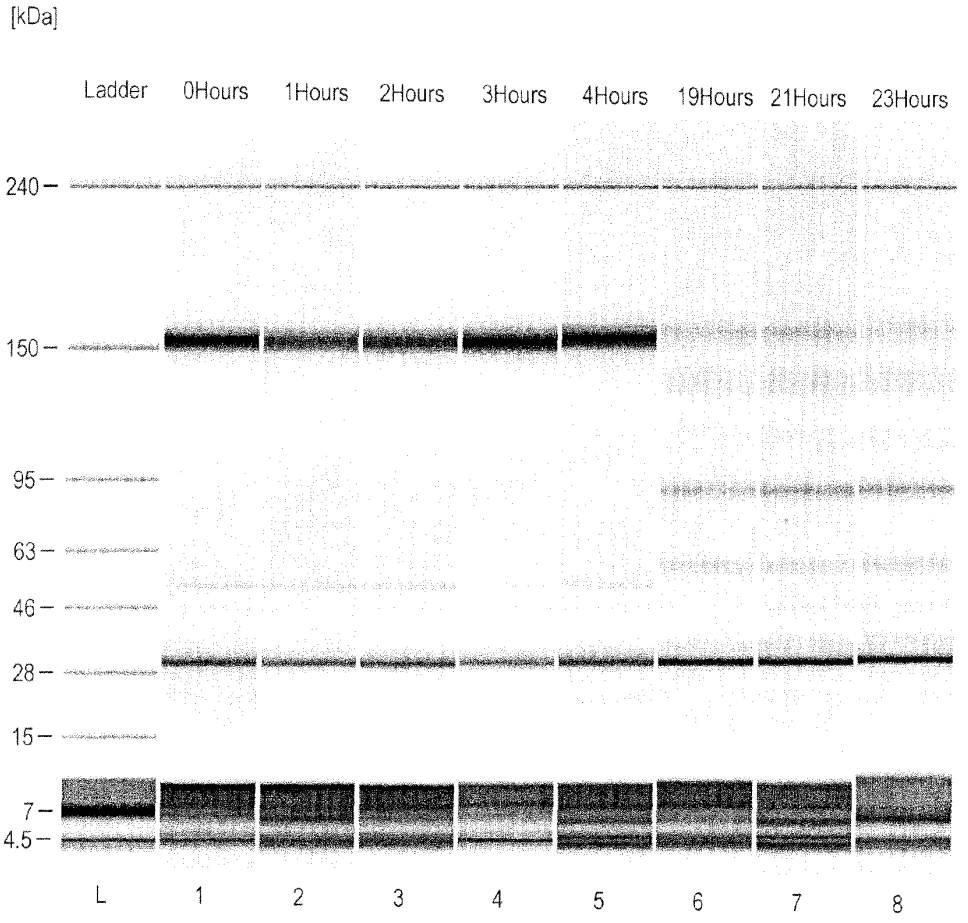
Losing Reduction Activity in HCCF

FIG. 13



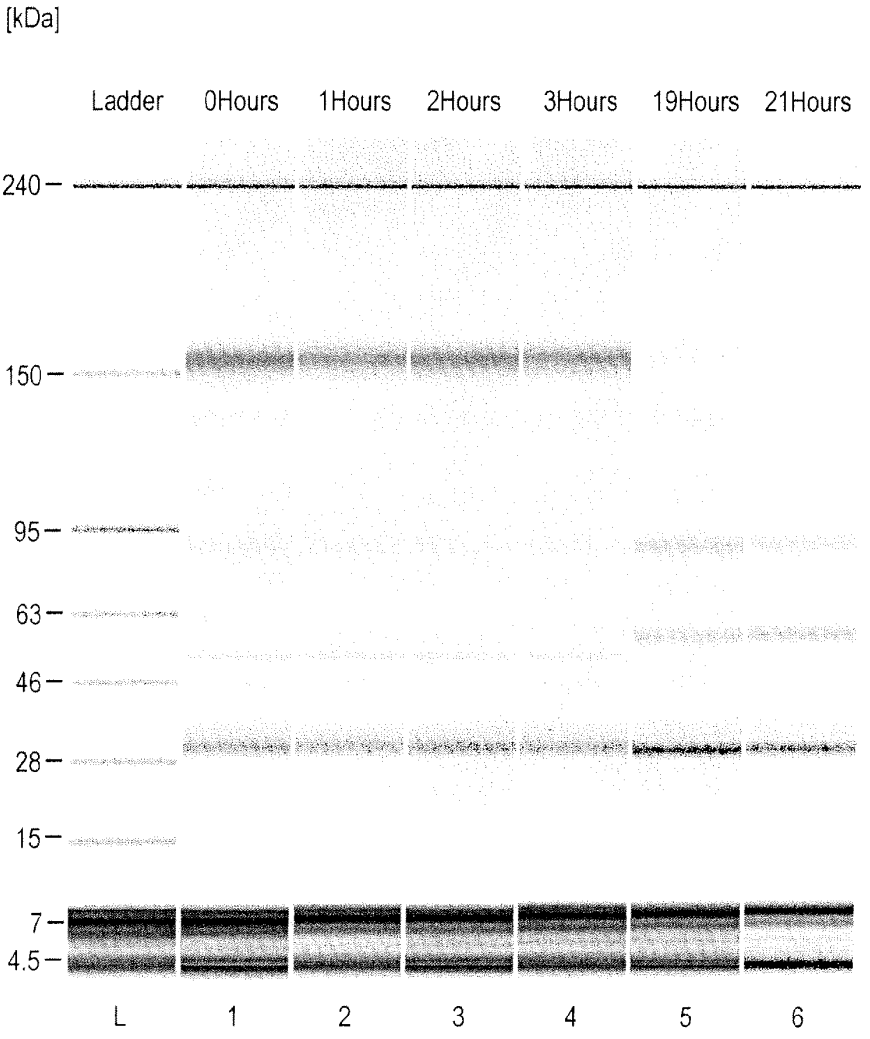
The Lost Reduction Activity in HCCF Restored by Addition of NADPH

FIG. 14



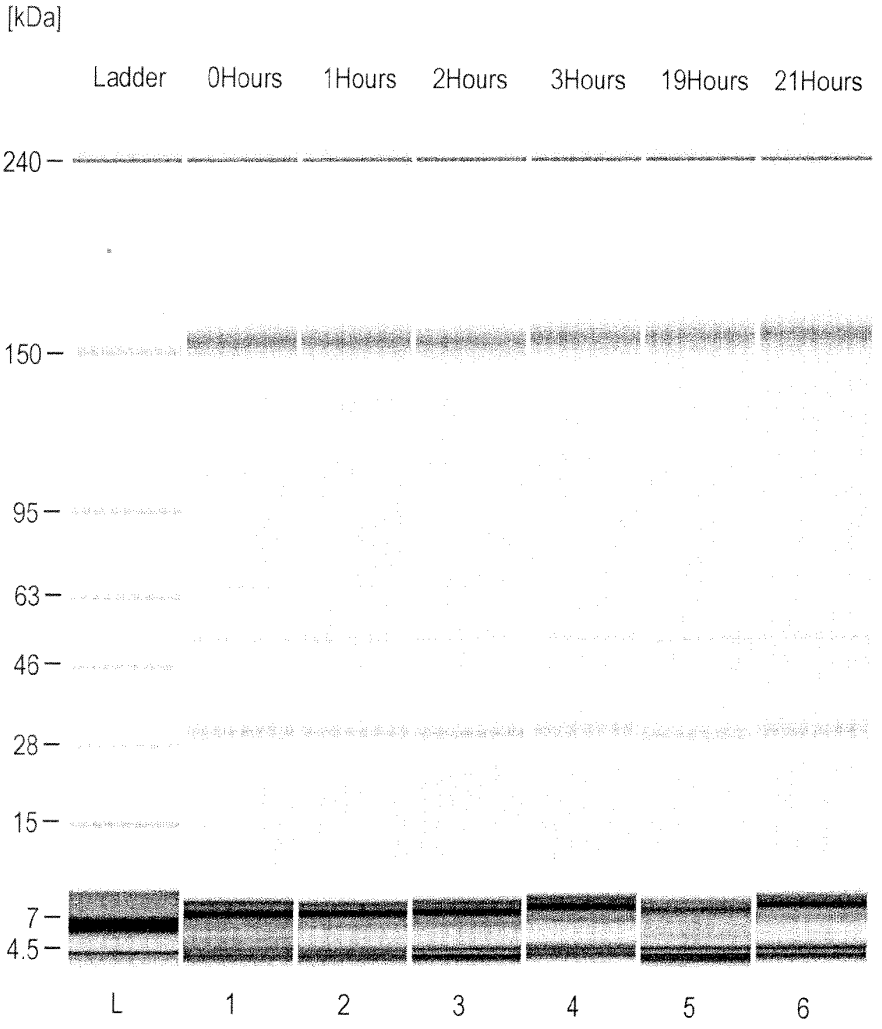
The Lost Reduction Activity in HCCF Restored by Addition of Glucose-6-Phosphate

FIG. 15



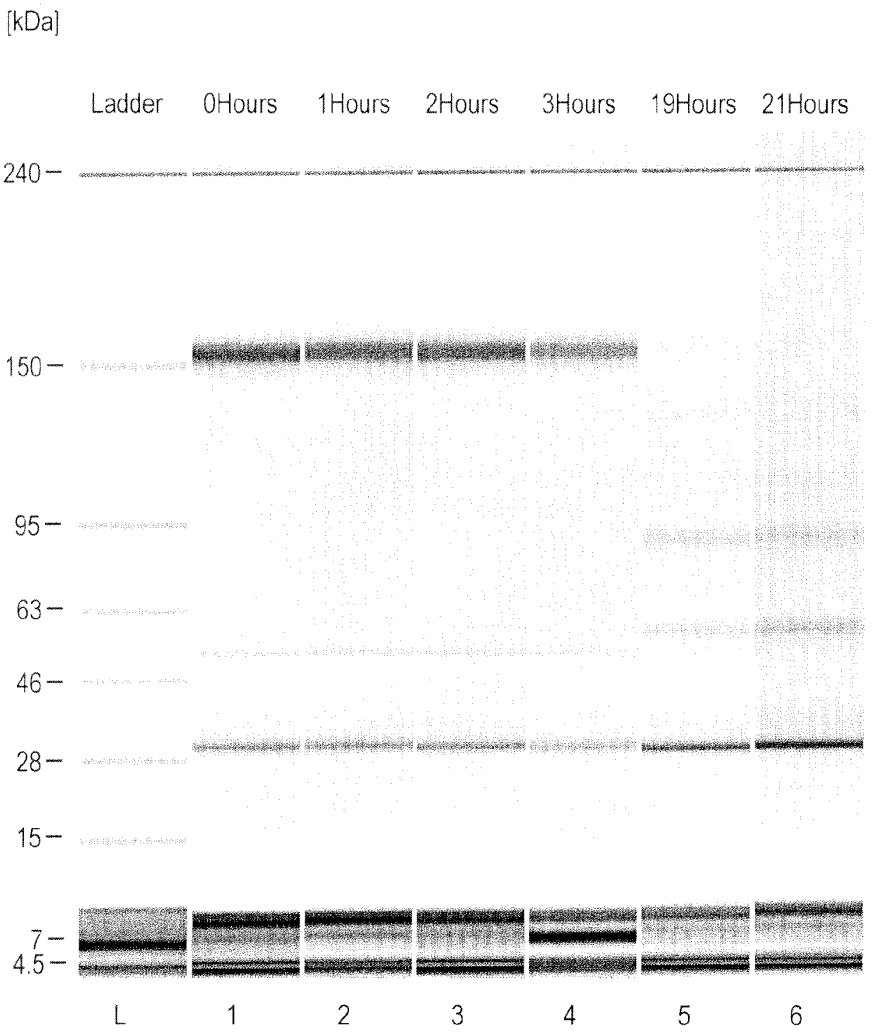
Ocrelizumab Reduction

FIG. 16



EDTA Inhibits Ocrelizumab Reduction

FIG. 17



The Lost Reduction Activity in Run 8 HCCF Restored by Addition of Glucose-6-Phosphate but No Inhibition of Reduction by EDTA

FIG. 18

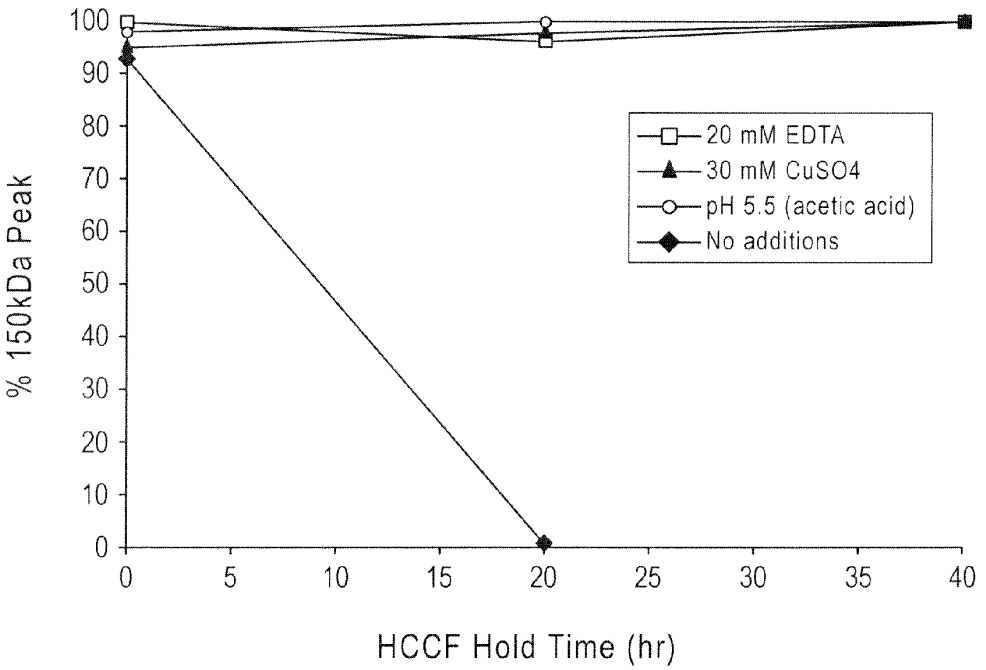

100
90
80
70
60
50
40
30
20
10
0
% 150kDa Peak
0
5
10
15
20
25
30
35
40
HCCF Hold Time (hr)
20 mM EDTA
30 mM CuSO4
pH 5.5 (acetic acid)
No additions

FIG. 19

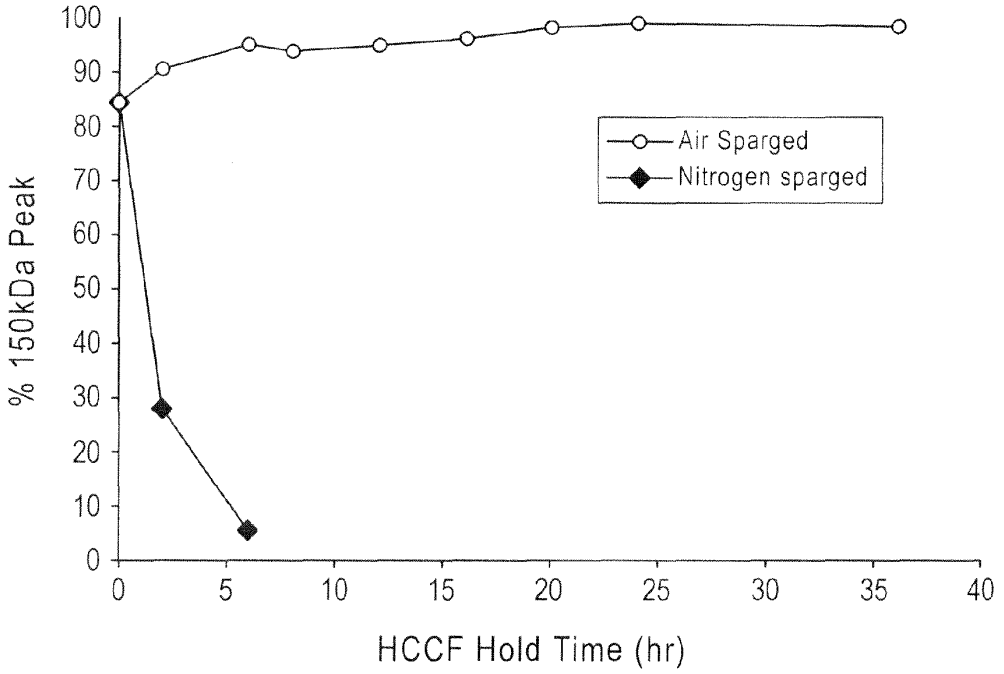

100
90
80
70
60
50
40
30
20
10
0
% 150kDa Peak
0
5
10
15
20
25
30
35
40
HCCF Hold Time (hr)
Air Sparged
Nitrogen sparged

FIG. 20

Light Chain

```
1               15                  30                  45
DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPK

46              60                  75                  90
LLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQ

91            105
HYTTPPTFGQGTKVEIK
```

FIG. 21

Heavy Chain

```
1               15                  30                  45
EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGL

46              60                  75                  90
EWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAED

91            105               120
TAVYYCSRWGGDGFYAMDYWGQGTLVTVSS
```

FIG. 22

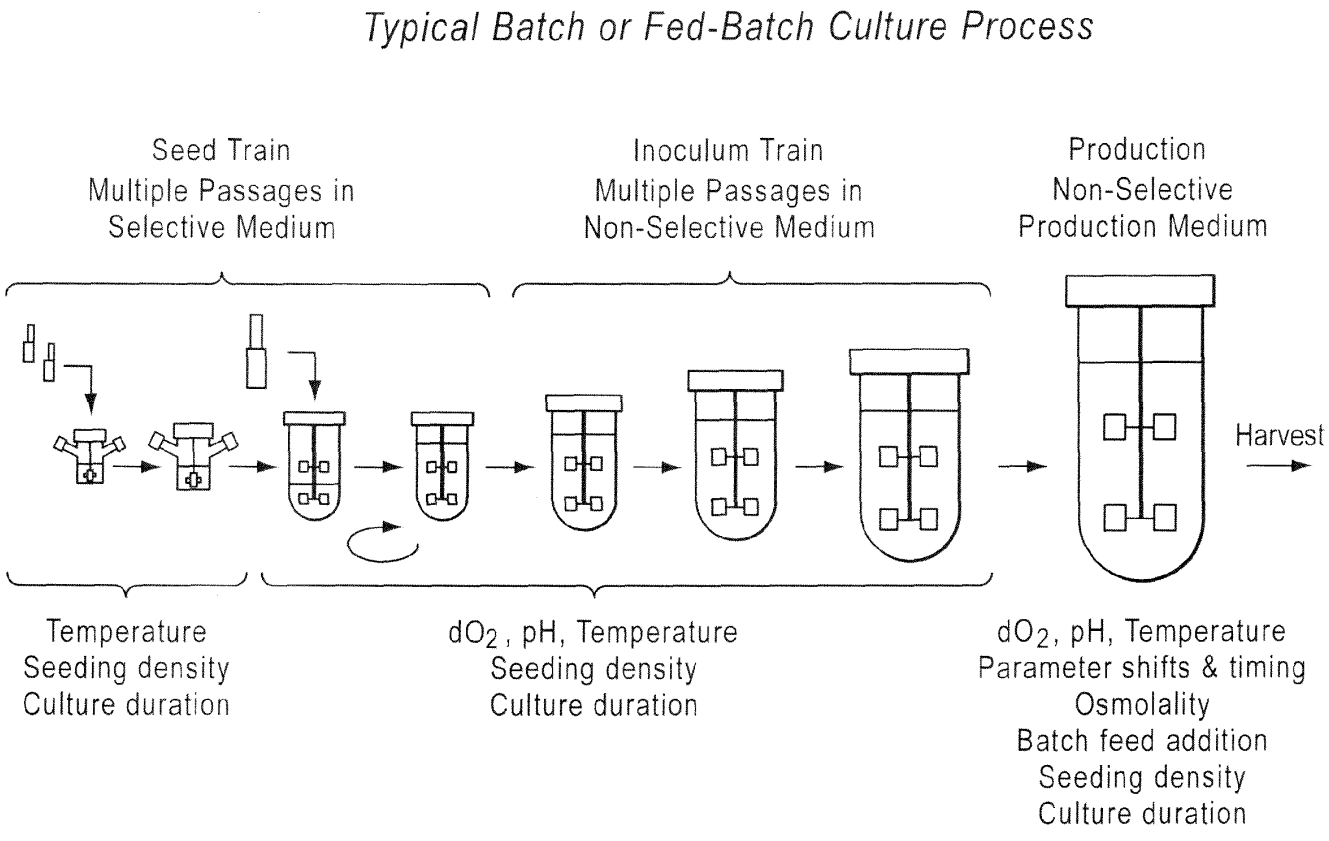
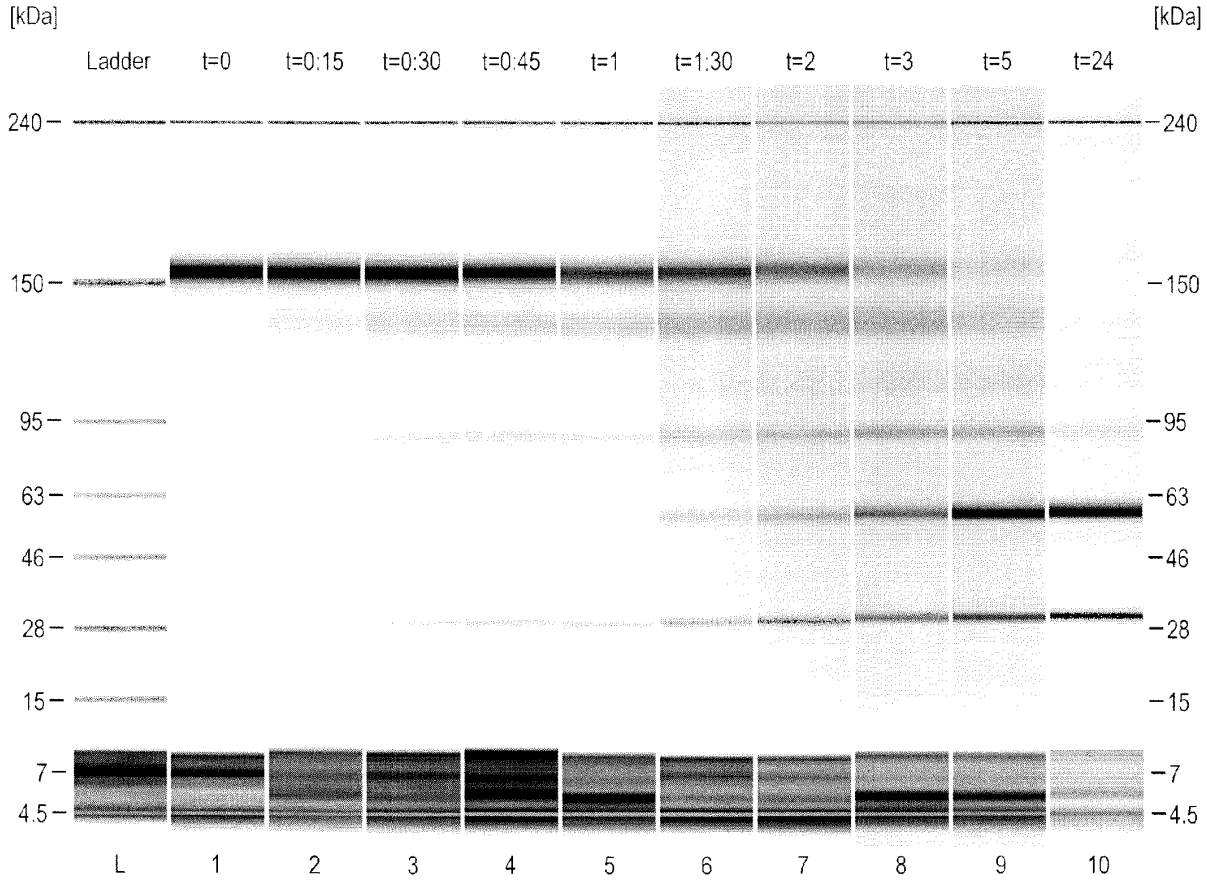

Typical Batch or Fed-Batch Culture Process
Seed Train
Multiple Passages in
Selective Medium
Inoculum Train
Multiple Passages in
Non-Selective Medium
Production
Non-Selective
Production Medium
Harvest
Temperature
Seeding density
Culture duration
dO2, pH, Temperature
Seeding density
Culture duration
dO2, pH, Temperature
Parameter shifts & timing
Osmolality
Batch feed addition
Seeding density
Culture duration

FIG. 23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t=1
t=1:30
t=2
t=3
t=5
t=24
240
150
95
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46
28
15
7
4.5
L
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FIG. 24

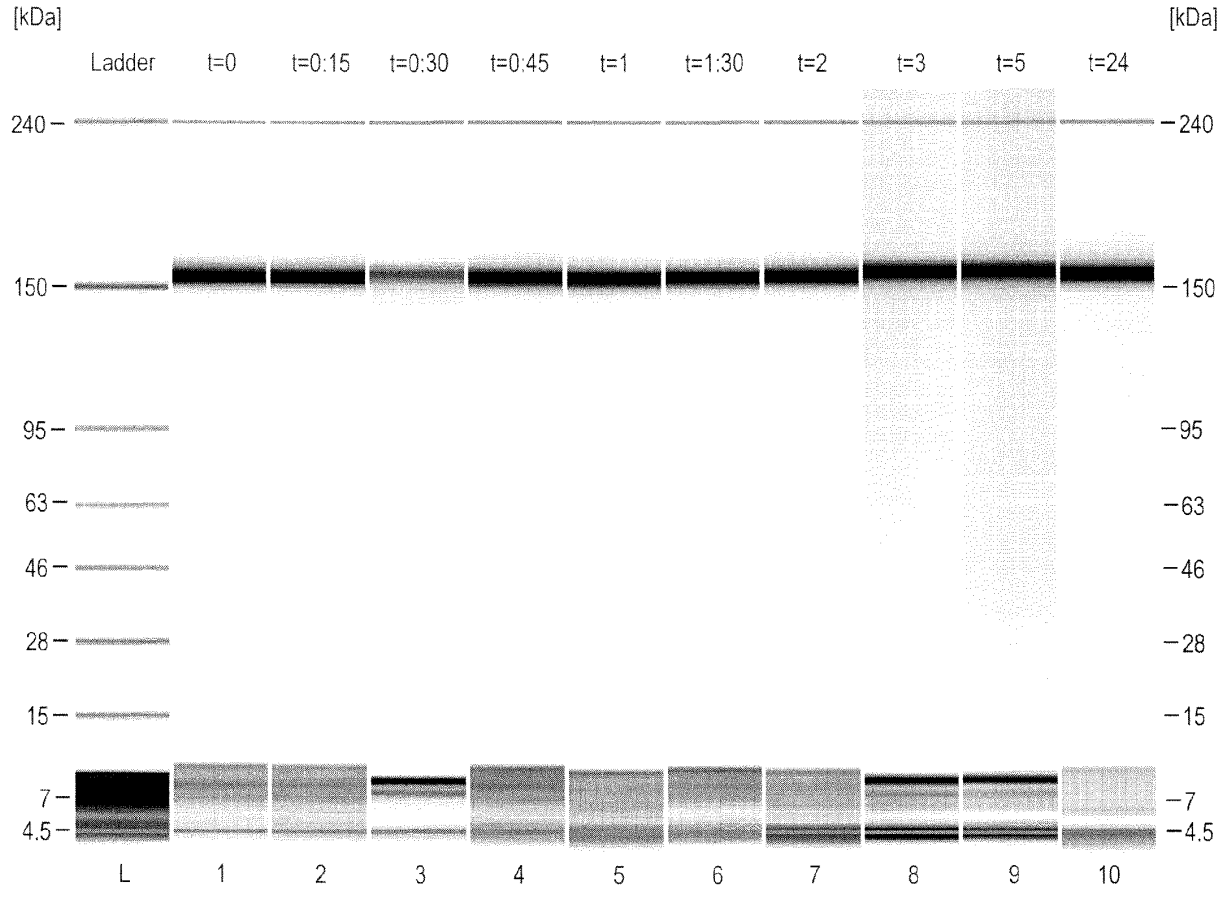
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FIG. 25

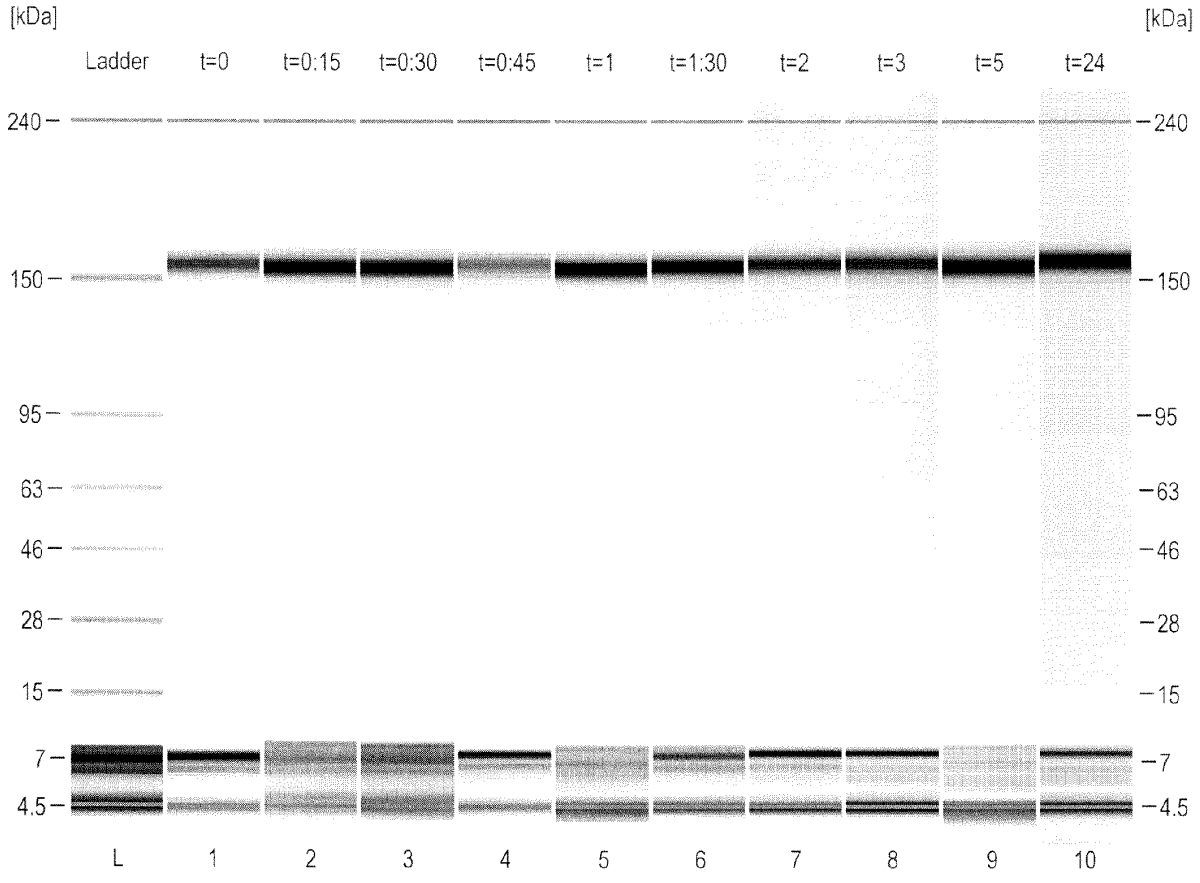
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FIG. 26

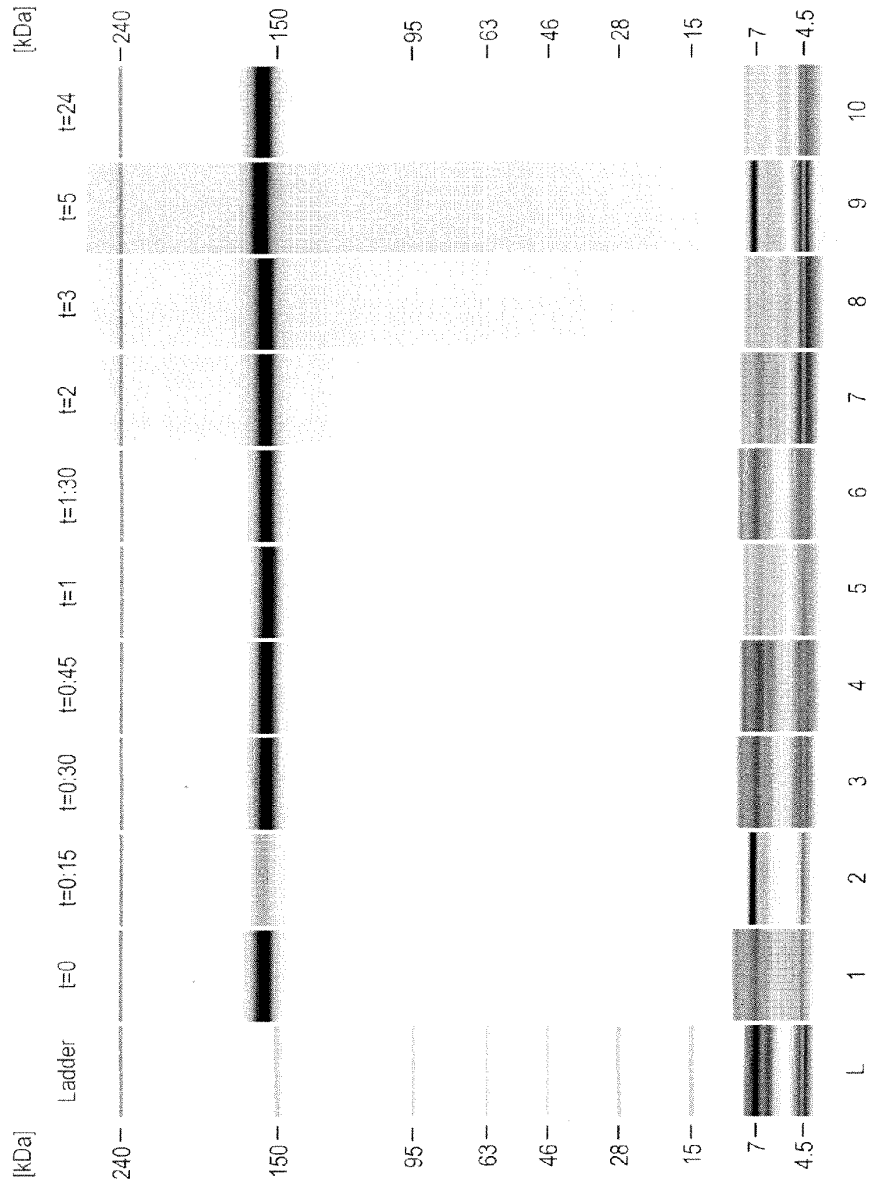
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FIG. 27

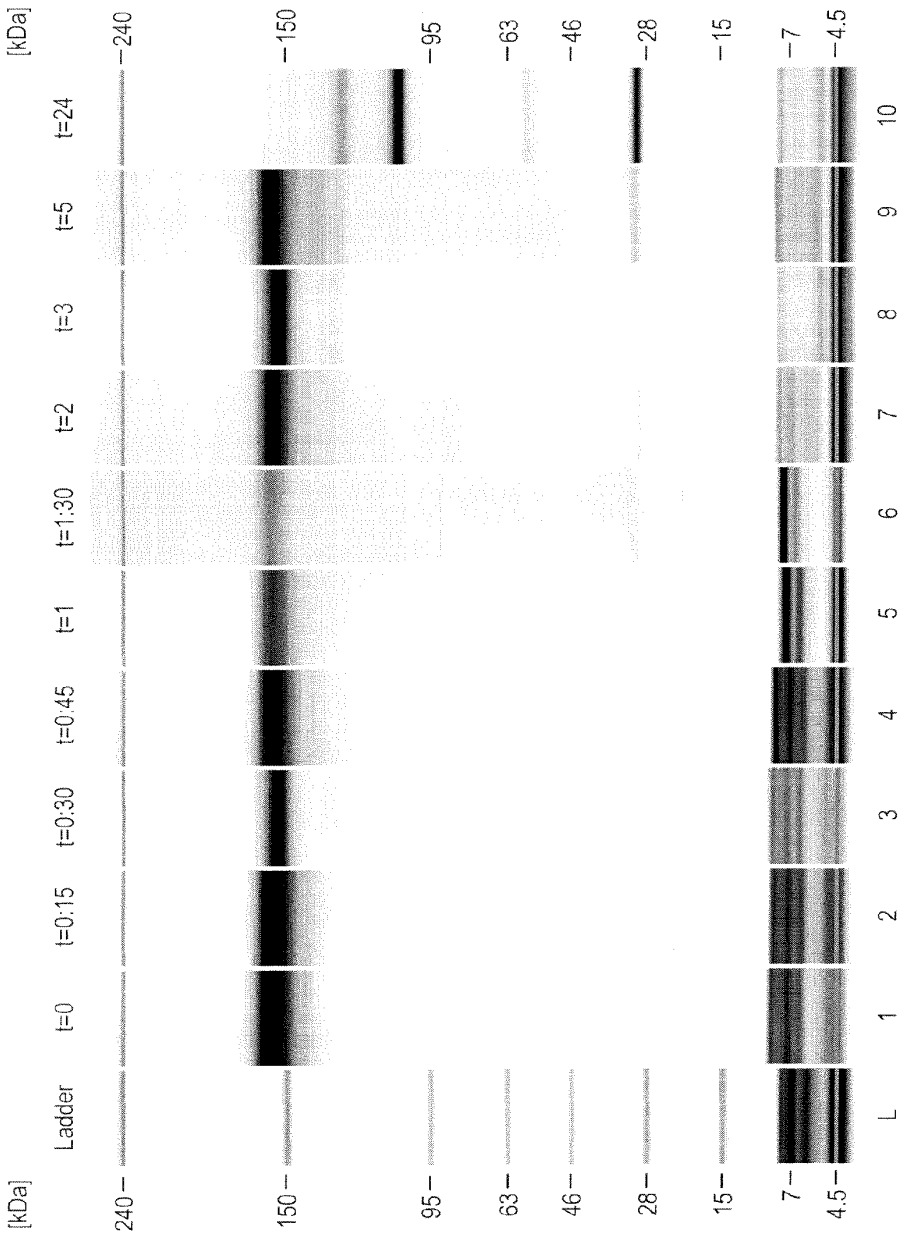
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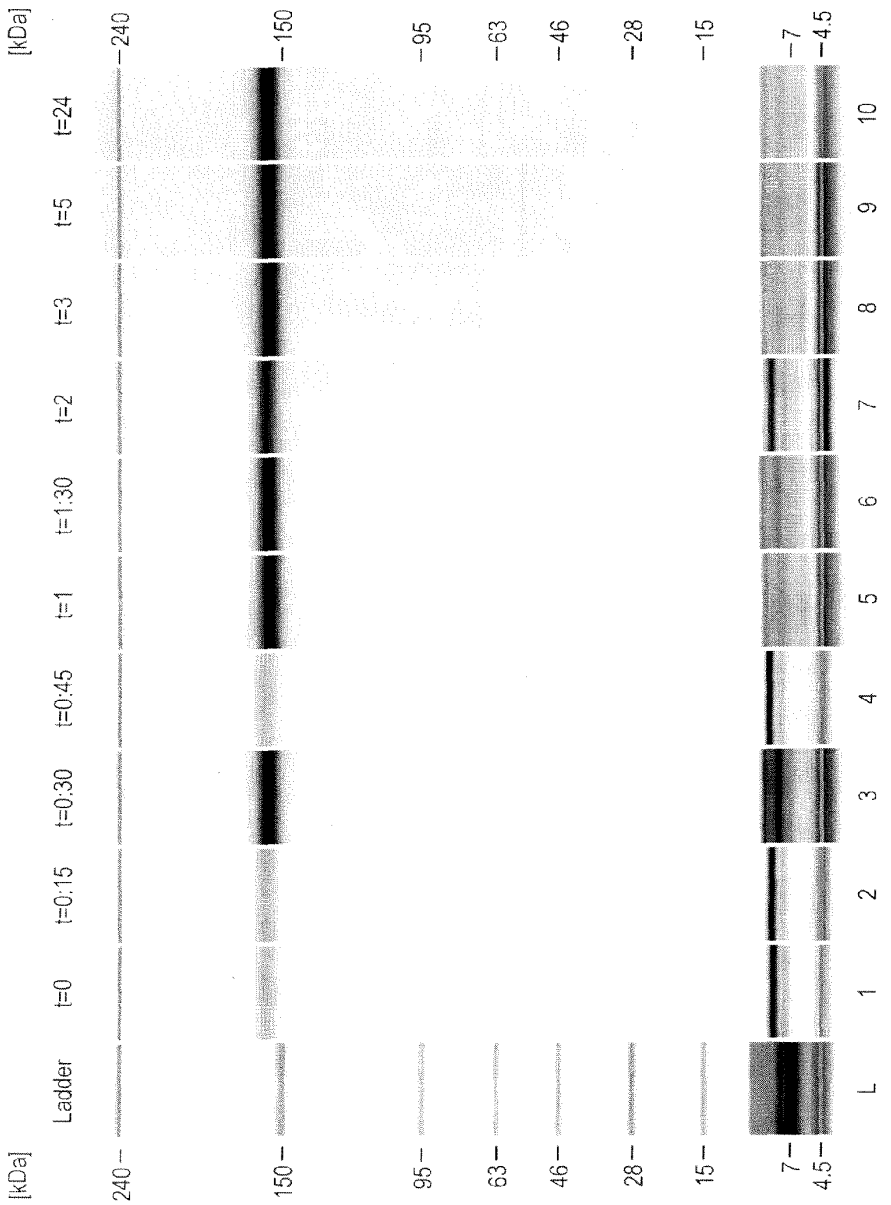
FIG. 28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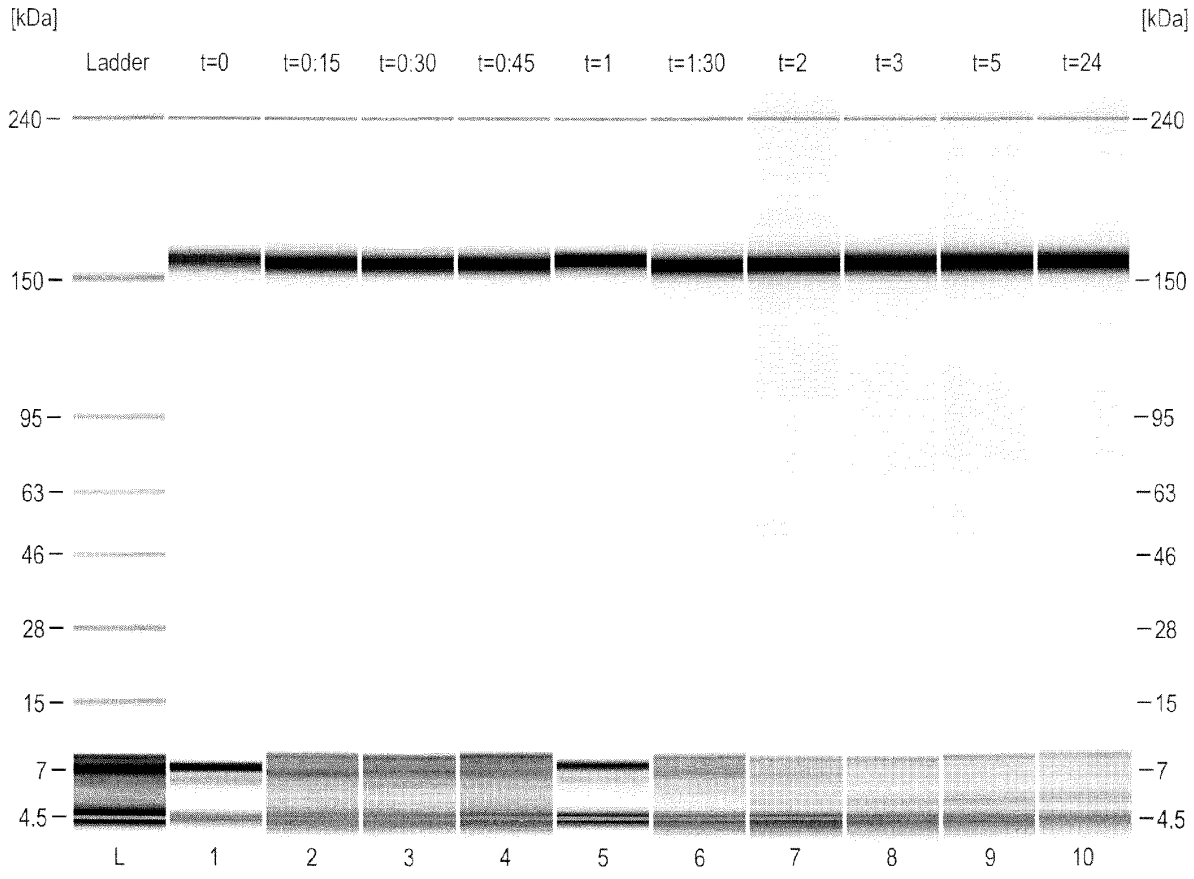
7
4.5

FIG. 29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t=0:45
t=1
t=1:30
t=2
t=3
t=5
t=24
240
150
95
63
46
28
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4.5
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FIG. 30

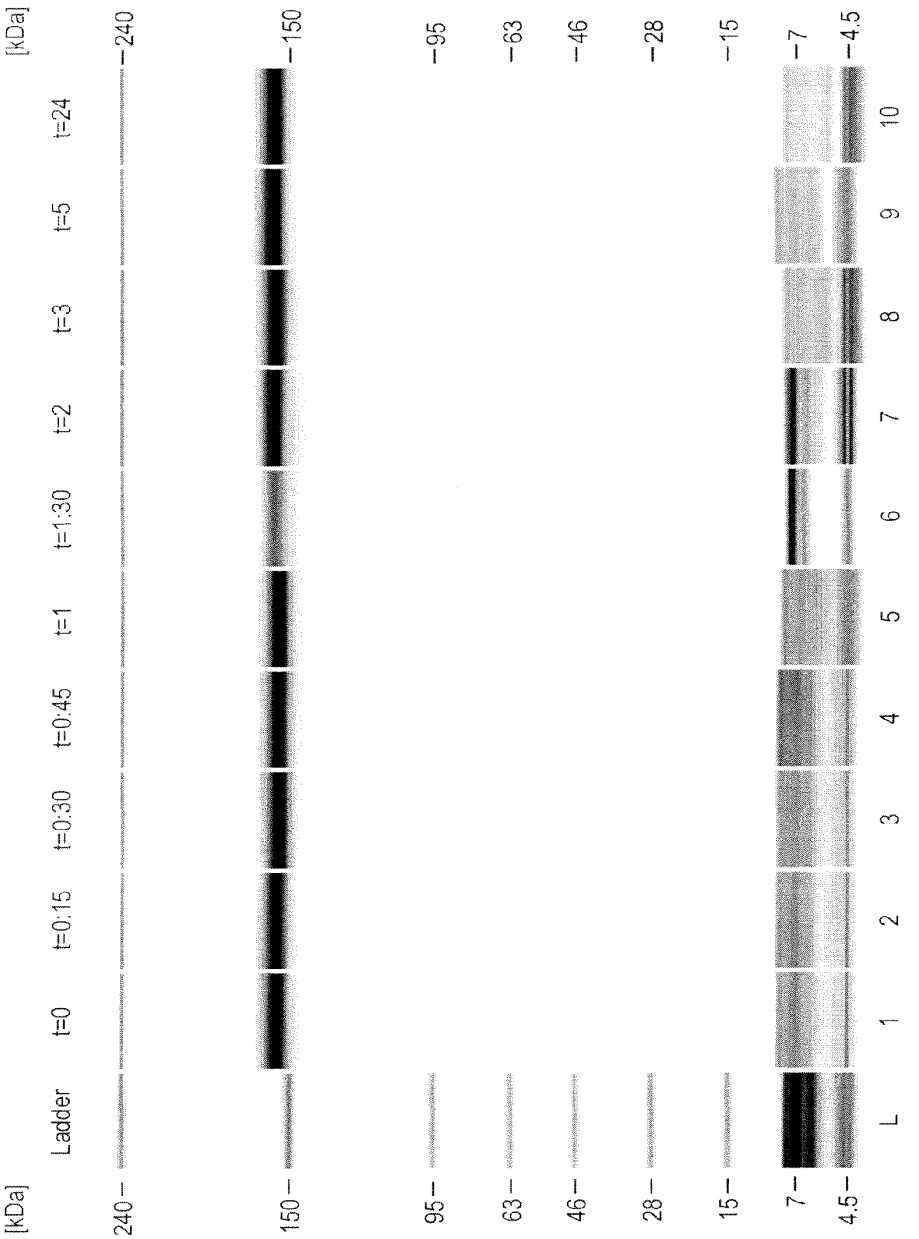
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FIG. 31

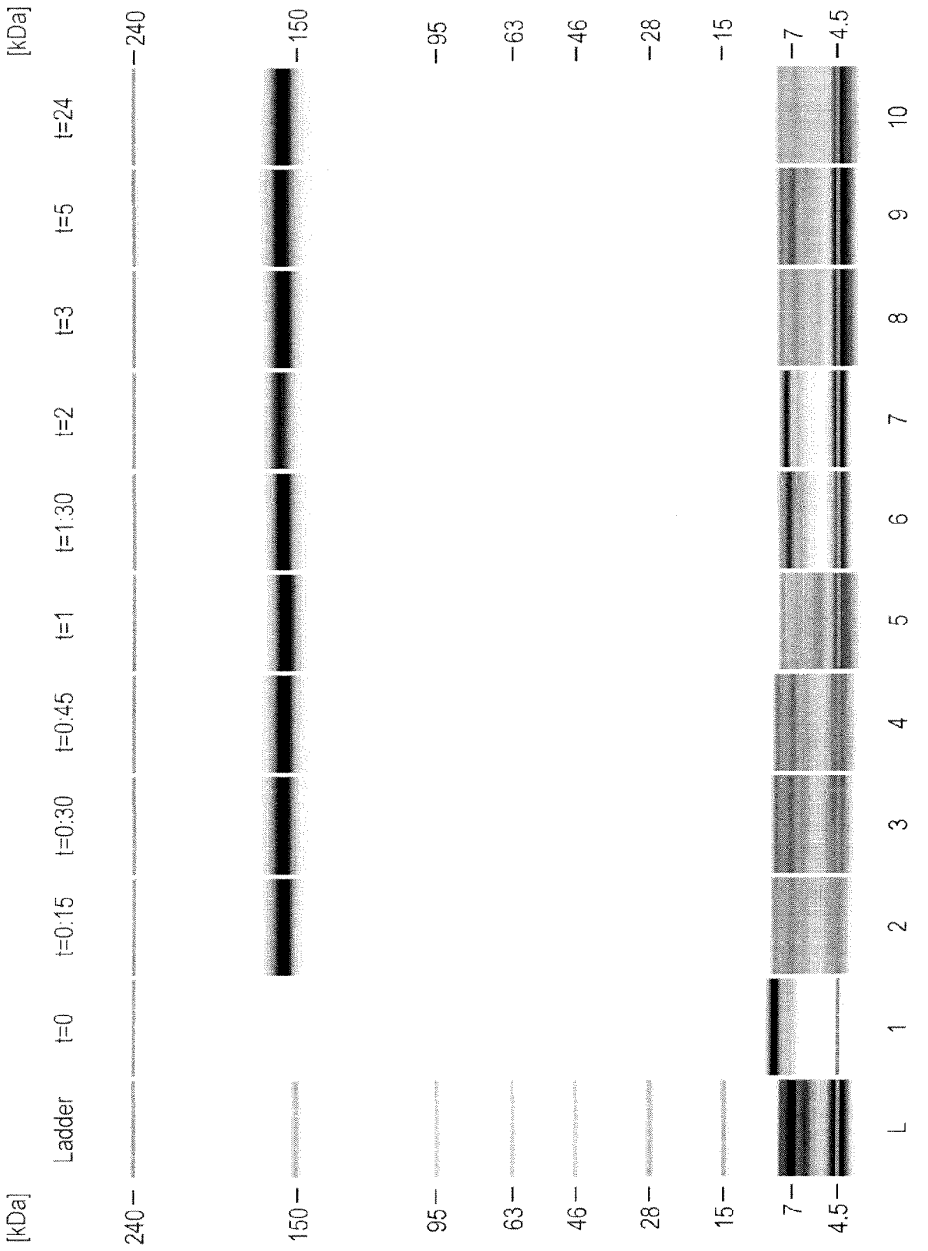
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FIG. 32

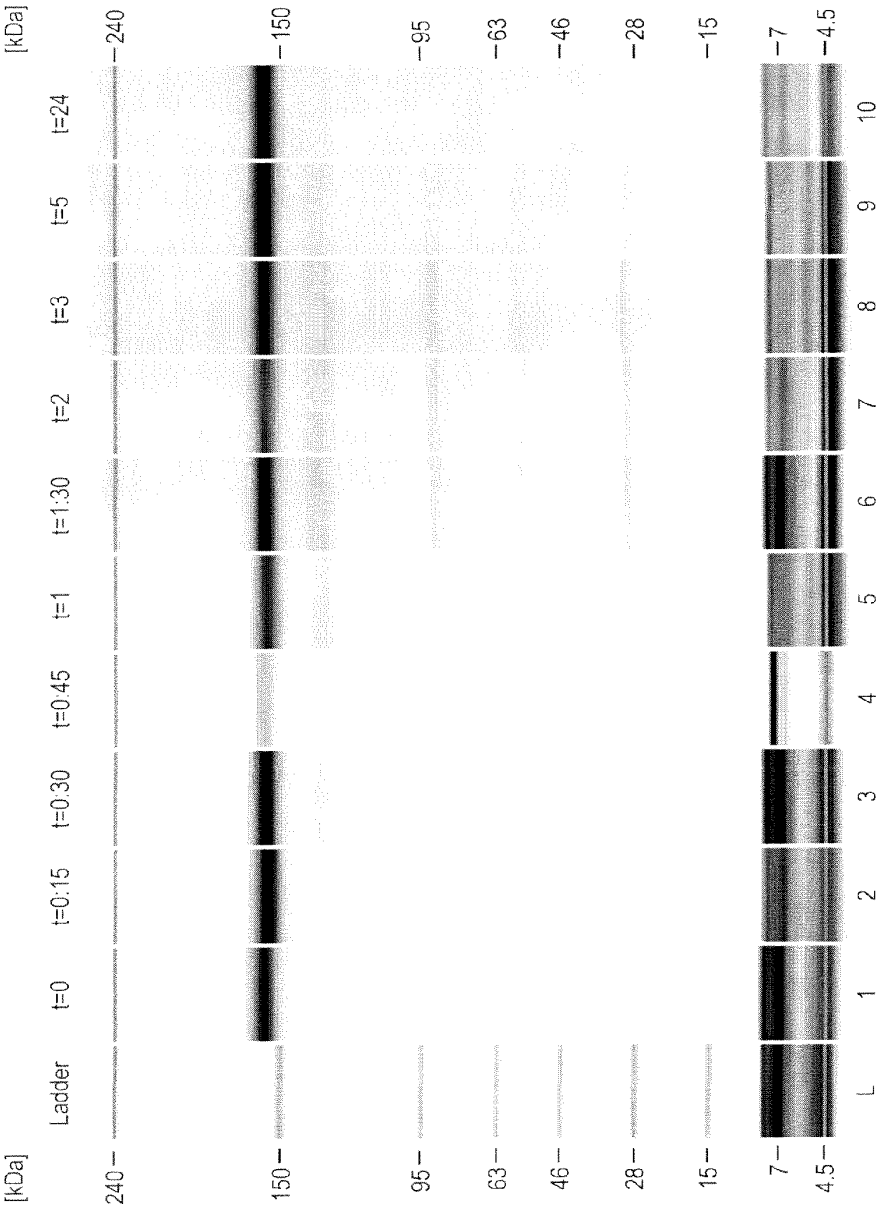
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FIG. 33

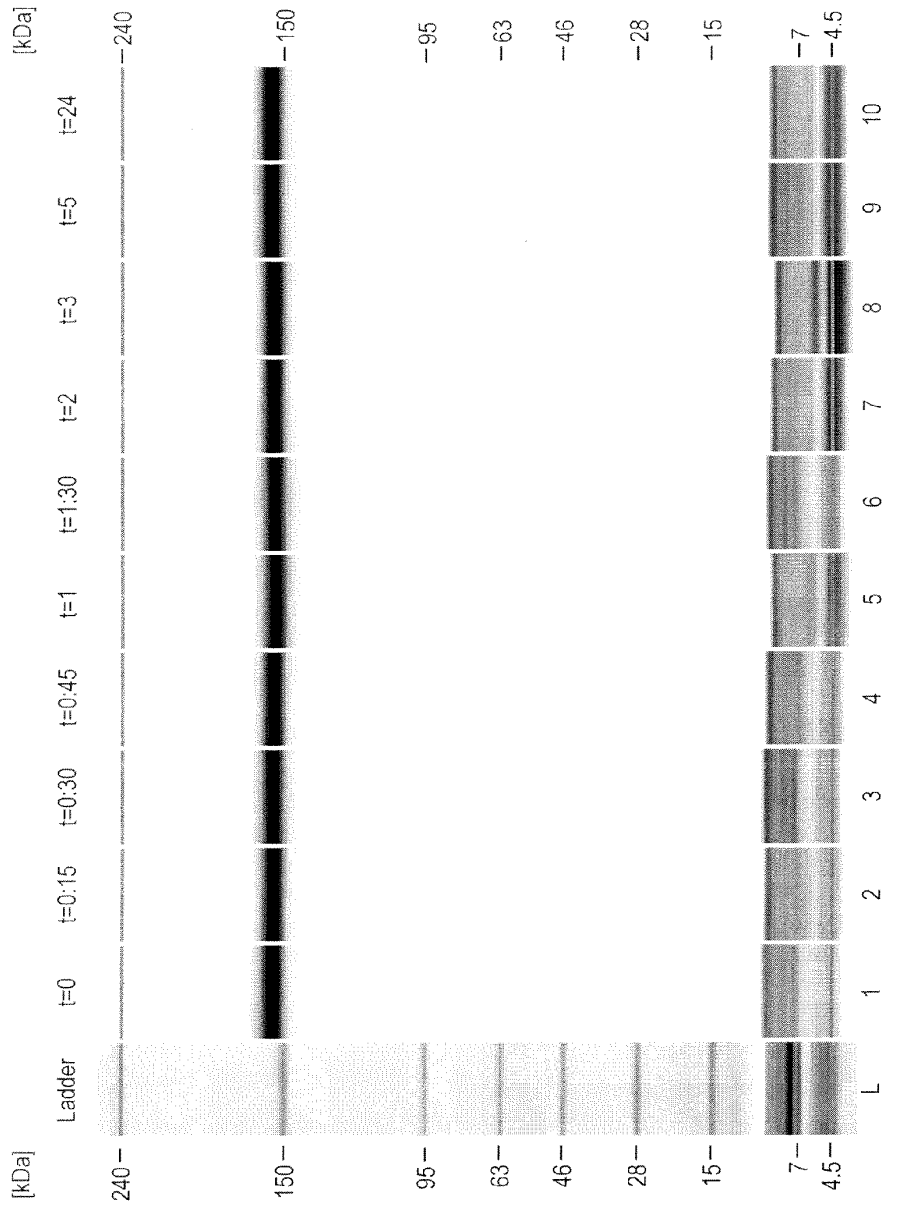
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FIG. 34

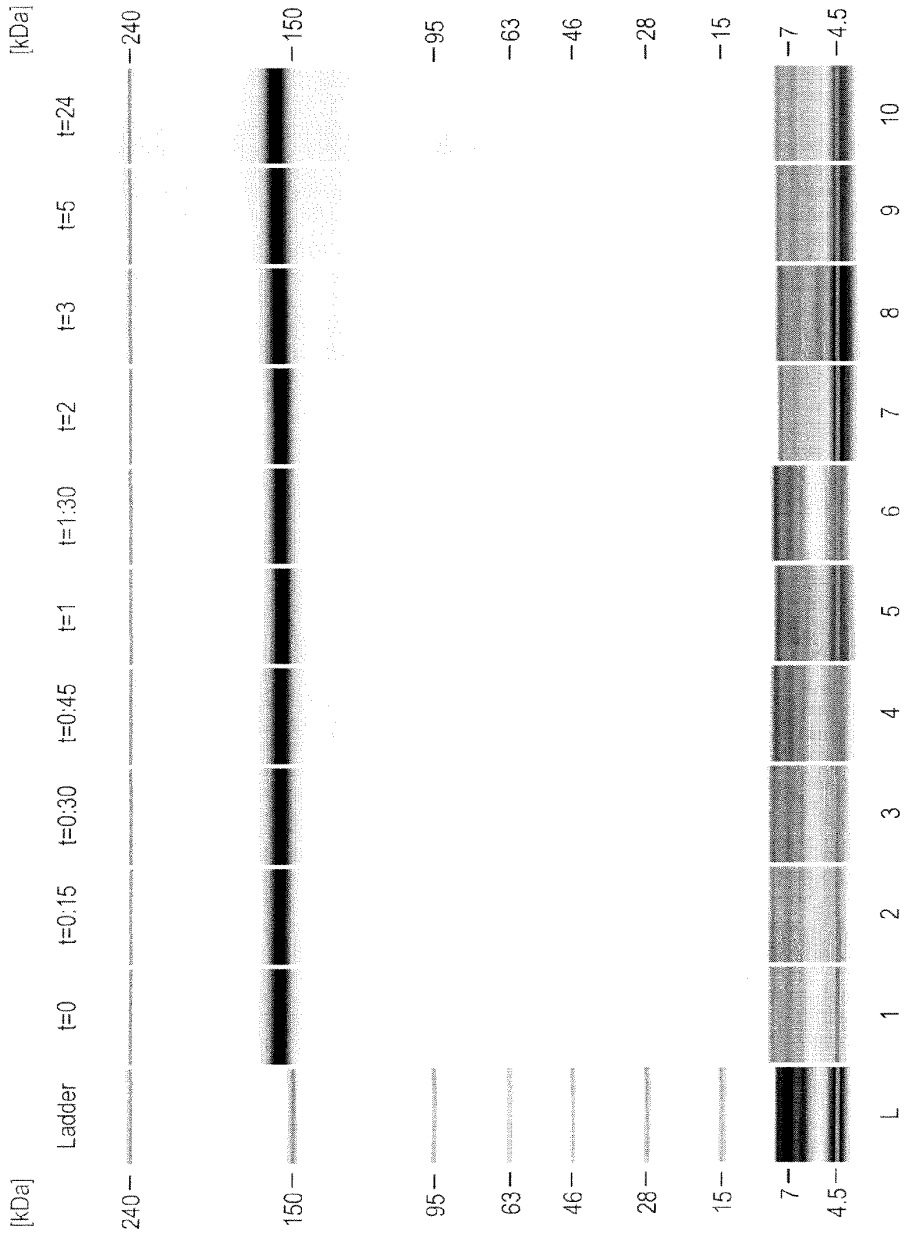
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FIG. 35

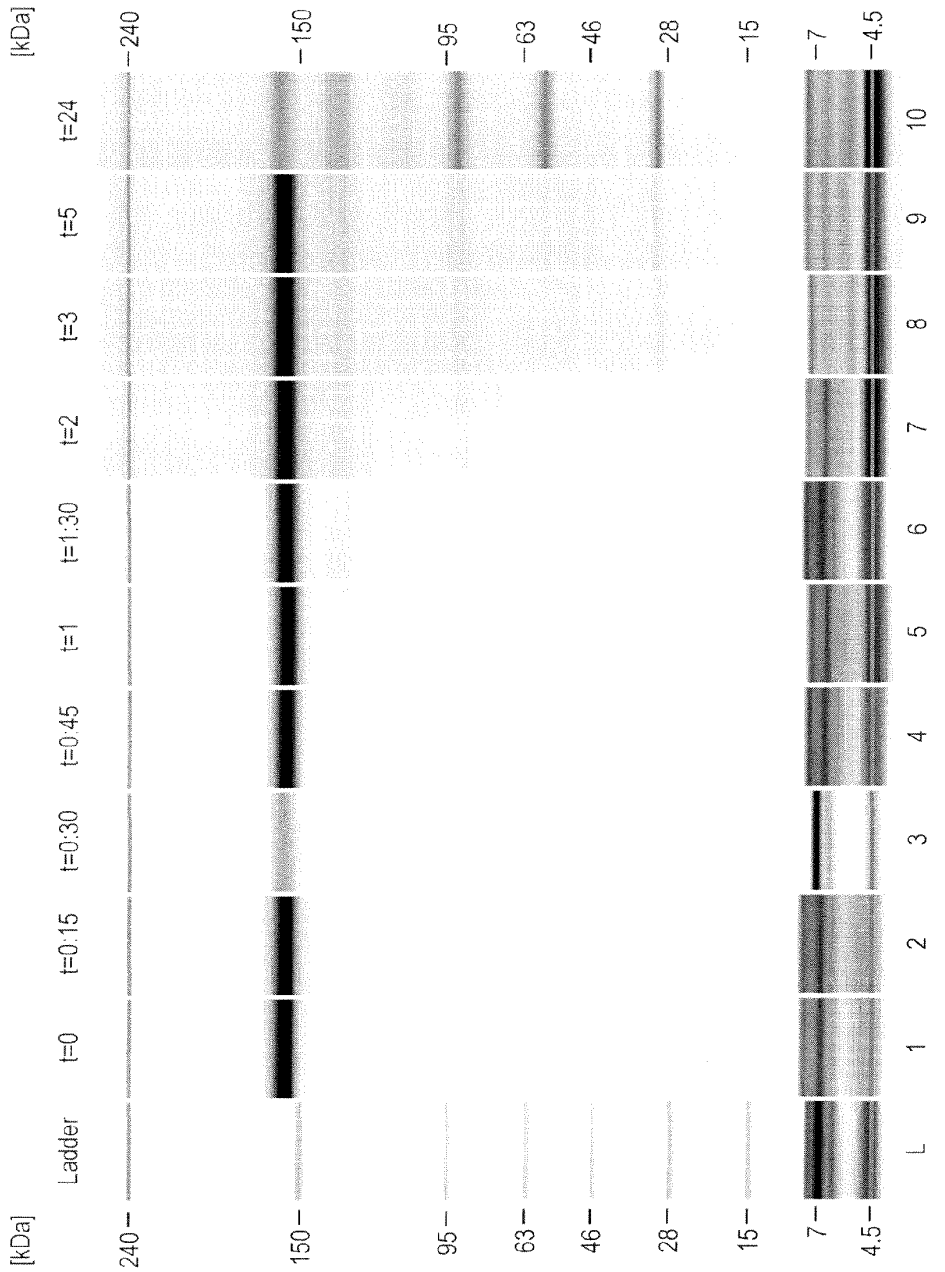
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FIG. 36

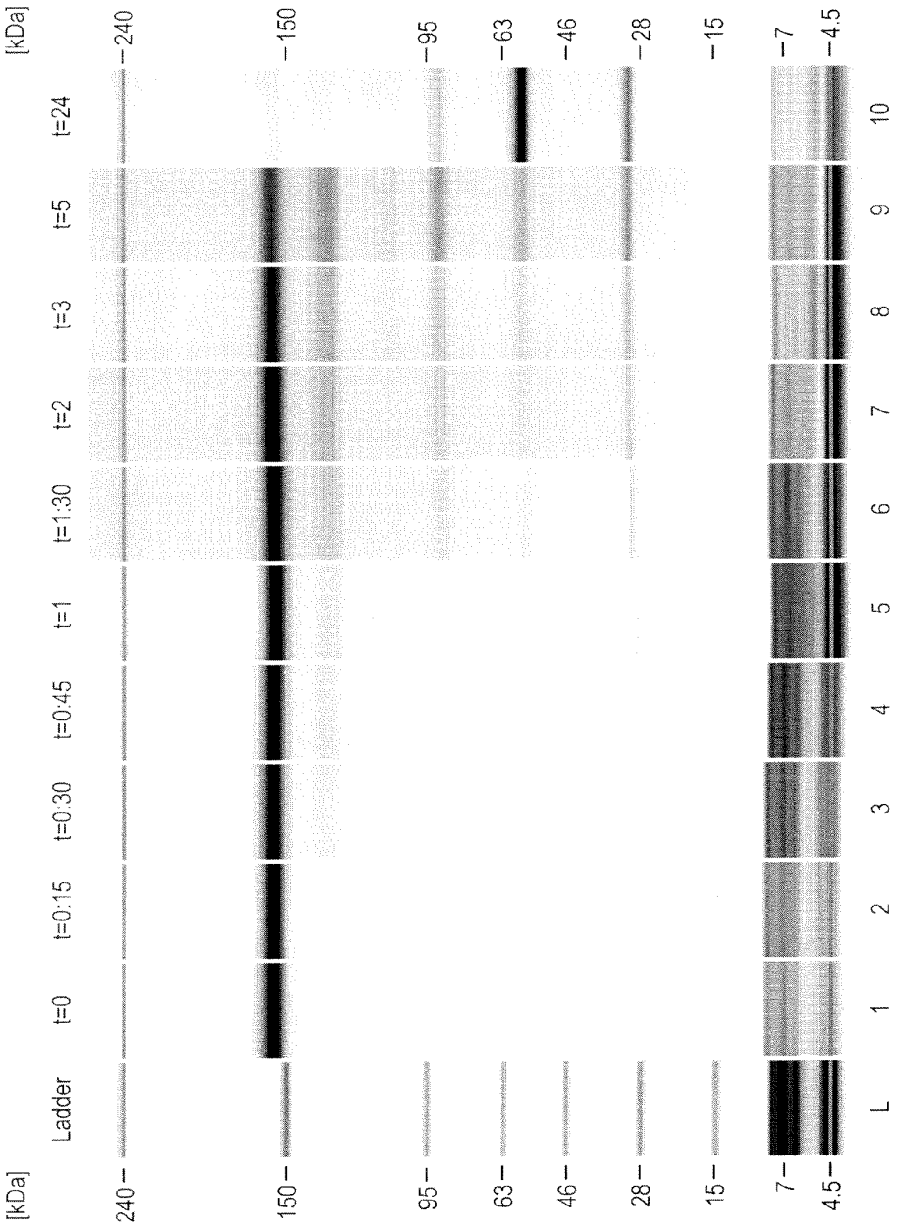
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FIG. 37

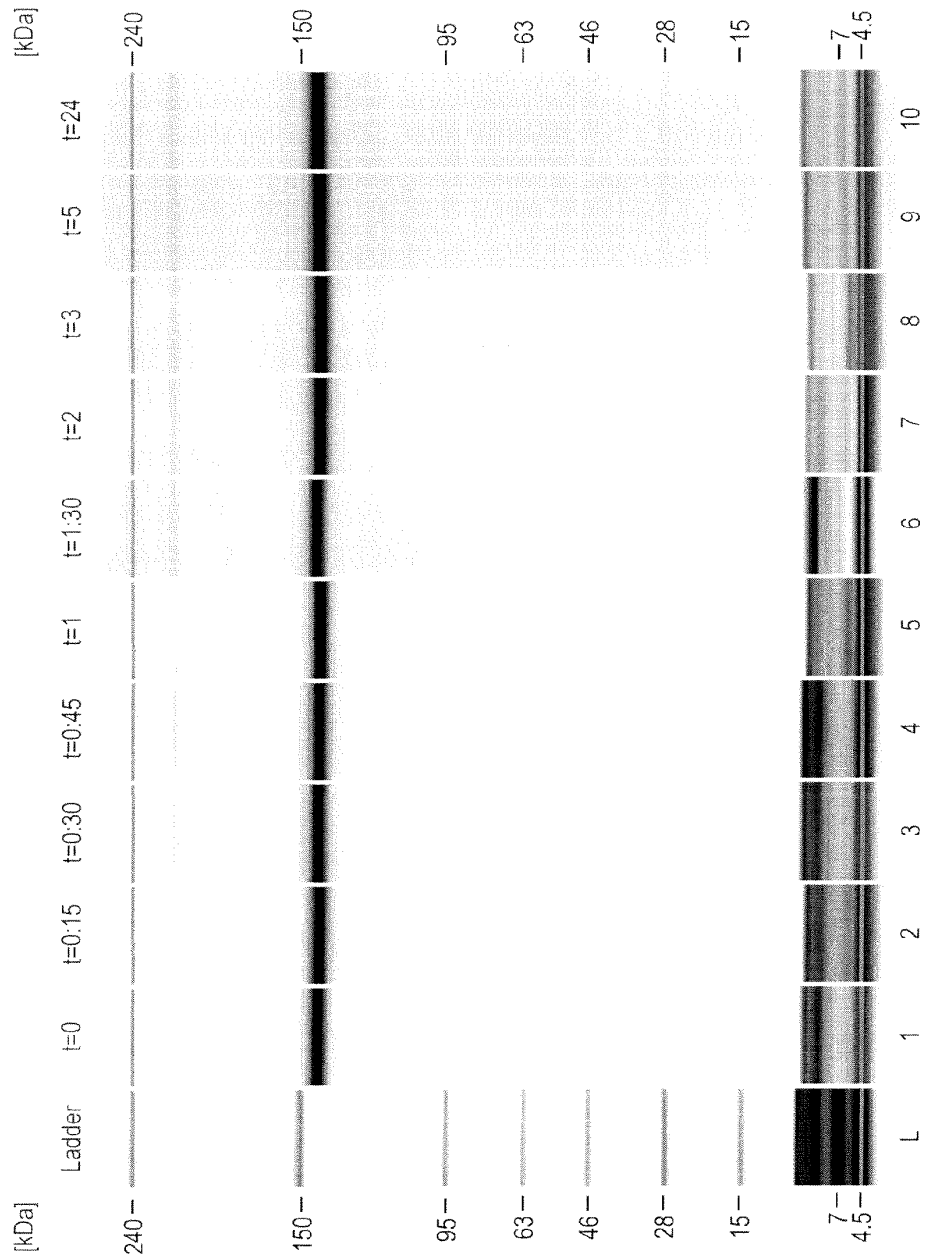
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FIG. 38

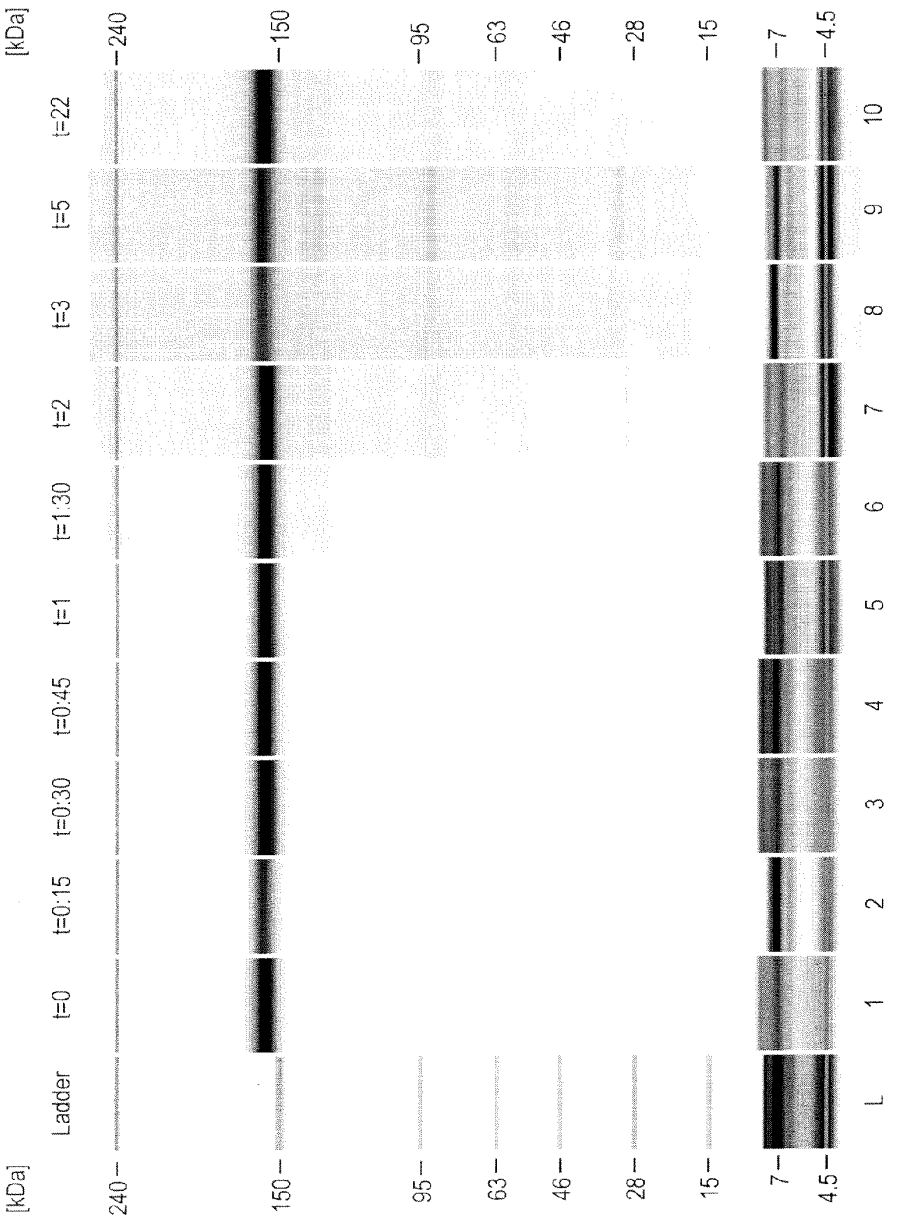
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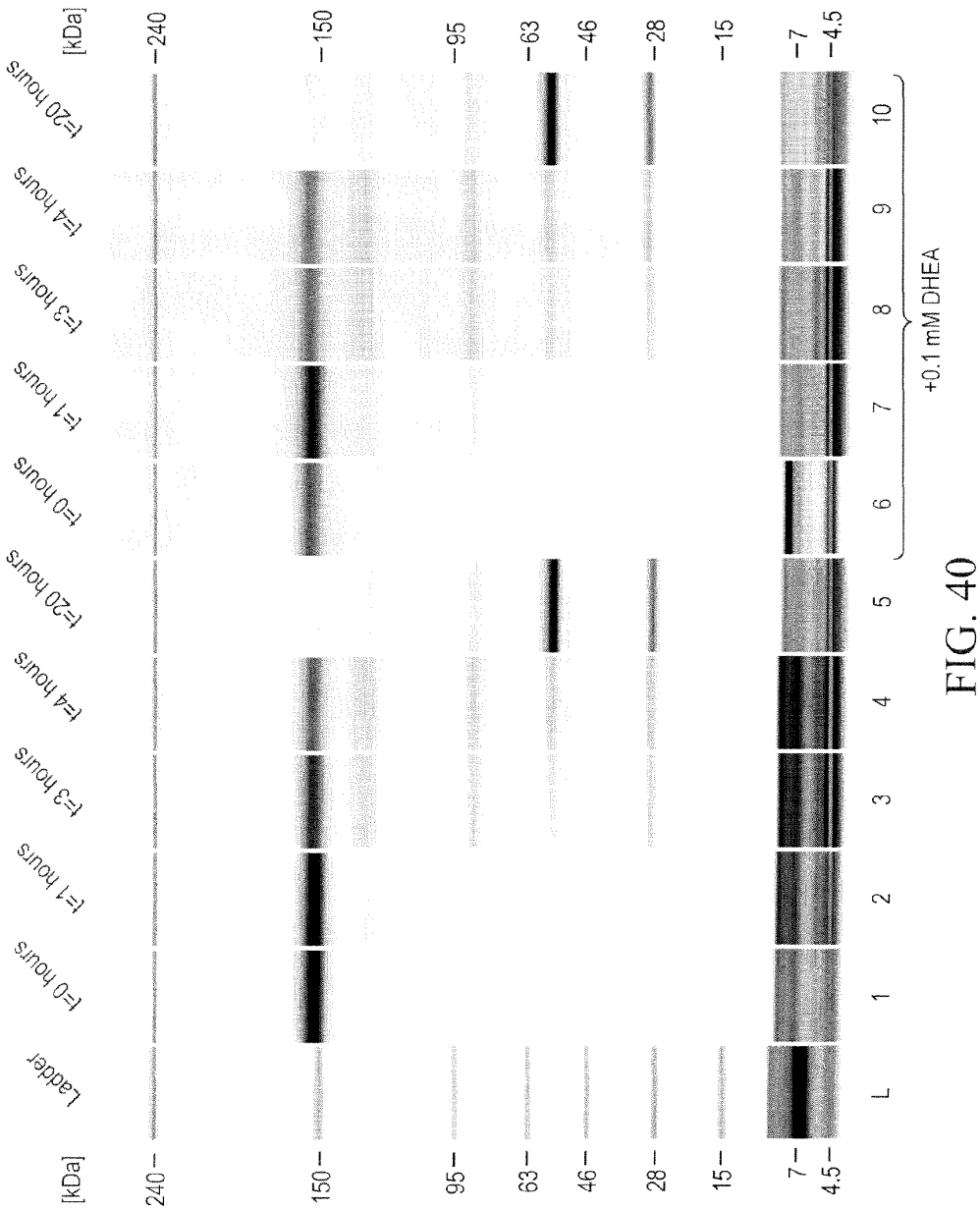
FIG. 39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+0.1 mM DHEA

FIG. 40

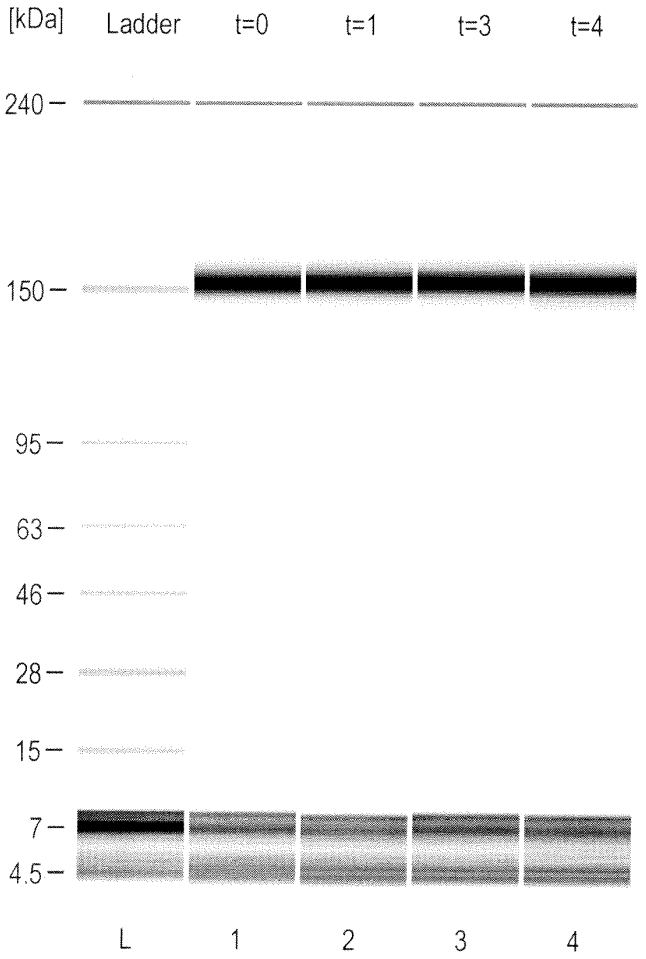

FIG. 41

PREVENTION OF DISULFIDE BOND REDUCTION DURING RECOMBINANT PRODUCTION OF POLYPEPTIDES

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. application Ser. No. 12/217,745, filed Jul. 8, 2008, which is a non-provisional application filed under 37 CFR 1.53(b)(1), claiming priority under 35 USC 119(e) to provisional Application No. 60/948,677 filed Jul. 9, 2007, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

The invention concerns methods and means for preventing the reduction of disulfide bonds during the recombinant production of disulfide-containing polypeptides. In particular, the invention concerns the prevention of disulfide bond reduction during harvesting of disulfide-containing polypeptides, including antibodies, from recombinant host cell cultures.

BACKGROUND OF THE INVENTION

In the biotechnology industry, pharmaceutical applications require a variety of proteins produced using recombinant DNA techniques. Generally, recombinant proteins are produced by cell culture, using either eukaryotic cells, such as mammalian cells, or prokaryotic cells, such as bacterial cells, engineered to produce the protein of interest by insertion of a recombinant plasmid containing the nucleic acid encoding the desired protein. For a protein to remain biologically active, the conformation of the protein, including its tertiary structure, must be maintained during its purification and isolation, and the protein's multiple functional groups must be protected from degradation.

Mammalian cells have become the dominant system for the production of mammalian proteins for clinical applications, primarily due to their ability to produce properly folded and assembled heterologous proteins, and their capacity for post-translational modifications. Chinese hamster ovary (CHO) cells, and cell lines obtained from various other mammalian sources, such as, for example, mouse myeloma (NS0), baby hamster kidney (BHK), human embryonic kidney (HEK-293) and human retinal cells, such as the PER.C6® cell line isolated from a human retinal cell, which provides human glycosylation characteristics, and is able to naturally produce antibodies that match human physiology, have been approved by regulatory agencies for the production of biopharmaceutical products.

Usually, to begin the production cycle, a small number of transformed recombinant host cells are allowed to grow in culture for several days (see, e.g., FIG. **23**). Once the cells have undergone several rounds of replication, they are transferred to a larger container where they are prepared to undergo fermentation. The media in which the cells are grown and the levels of oxygen, nitrogen and carbon dioxide that exist during the production cycle may have a significant impact on the production process. Growth parameters are determined specifically for each cell line and these parameters are measured frequently to assure optimal growth and production conditions.

When the cells grow to sufficient numbers, they are transferred to large-scale production tanks and grown for a longer period of time. At this point in the process, the recombinant protein can be harvested. Typically, the cells are engineered to secrete the polypeptide into the cell culture media, so the first step in the purification process is to separate the cells from the media. Typically, harvesting includes centrifugation and filtration to produce a Harvested Cell Culture Fluid (HCCF). The media is then subjected to several additional purification steps that remove any cellular debris, unwanted proteins, salts, minerals or other undesirable elements. At the end of the purification process, the recombinant protein is highly pure and is suitable for human therapeutic use.

Although this process has been the subject of much study and improvements over the past several decades, the production of recombinant proteins is still not without difficulties. Thus, for example, during the recombinant production of polypeptides comprising disulfide bonds, especially multi-chain polypeptides comprising inter-chain disulfide bonds such as antibodies, it is essential to protect and retain the disulfide bonds throughout the manufacturing, recovery and purification process, in order to produce properly folded polypeptides with the requisite biological activity.

SUMMARY OF THE INVENTION

The instant invention generally relates to a method for preventing reduction of a disulfide bond in a polypeptide expressed in a recombinant host cell, comprising supplementing the pre-harvest or harvested culture fluid of the recombinant host cell with an inhibitor of thioredoxin or a thioredoxin-like protein.

In one embodiment, the thioredoxin inhibitor is added to the pre-harvest culture fluid.

In another embodiment, the thioredoxin inhibitor is added to the harvested culture fluid.

In a further embodiment, the thioredoxin inhibitor is a direct inhibitor of thioredoxin.

In all embodiments, the thioredoxin inhibitor may, for example, be an alkyl-2-imidazolyl disulfide or a naphthoquinone spiroketal derivative.

In a further embodiment, the thioredoxin inhibitor is a specific inhibitor of thioredoxin reductase.

In a still further embodiment, the thioredoxin inhibitor is a gold complex, where the gold complex may, for example, be aurothioglucose (ATG) or aurothiomalate (ATM). While the effective inhibitory concentration may vary, it typically is between about 0.1 mM and 1 mM. Similarly, the minimum effective inhibitory concentration varies depending on the nature of the polypeptide and overall circumstances, and is typically reached when the ATG or ATG concentration is at least about four-times of thioreduxin concentration in the pre-harvest or harvested culture fluid.

In another embodiment of this aspect of the invention, the thioredoxin inhibitor is a metal ion, where the metal ion, without limitation, may be selected from the group consisting of Hg^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, and Mn^{2+}. When the metal ion is added in the form of cupric sulfate, the effective inhibitory concentration generally is between about 5 μM and about 100 μM, or between about 10 μM and about 80 μM, or between about 15 μM and about 50 μM. The minimum inhibitory concentration of cupric sulfate also varies, but typically is reached when cupric sulfate is added at a concentration at least about two-times of thioredoxin concentration in the pre-harves or harvested culture fluid.

In different embodiment, the thioredoxin inhibitor is an oxidizing agent, e.g., an inhibitor of G6PD, such as, for example, pyridoxal 5'-phosphate, 1 fluoro-2,4 dinitrobenzene, dehydroepiandrosterone (DHEA) or epiandrosterone (EA); cystine or cysteine. Typical effective inhibitor concen-

trations of DHEA are between about 0.05 mM and about 5 mM, or between about 0.1 mM and about 2.5 mM.

In a further embodiment, the thioredoxin inhibitor is an inhibitor of hexokinase activity, including, without limitation, chelators of metal ions, such as, for example, ethylenediamine tetraacetic acid (EDTA). EDTA is typically added and effective at a concentration between about 5 mM and about 60 mM, or about 10 mM and about 50 mM, or about 20 mM and about 40 mM.

In other preferred embodiments, the inhibitor of hexokinase activity is selected from the group consisting of sorbose-1-phosphate, polyphosphates, 6-deoxy-6-fluoroglucose, 2-C-hydroxy-methylglucose, xylose, and lyxose.

Other inhibitors include cystine, cysteine, and oxidized glutathione which are typically added at a concentration at least about 40-times of the concentration of the polypeptide in question in the pre-harvest or harvested culture fluid.

In a still further embodiment, the thioredoxin inhibitor is an siRNA, an antisense nucleotide, or an antibody specifically binding to a thioredoxin reductase.

In another embodiment, the thioredoxin inhibitor is a measure indirectly resulting in the inhibition of thioredoxin activity. This embodiment includes, for example, air sparging the harvested culture fluid of the recombinant host cell, and/or lowering the pH of the harvested culture fluid of the recombinant host cell.

In various embodiments, indirect means for inhibiting thioredoxin activity, such as air sparging and/or lowering of the pH, can be combined with the use of direct thioredoxin inhibitors, such as those listed above.

In all embodiments, the polypeptide may, for example, be an antibody, or a biologically functional fragment of an antibody. Representative antibody fragments include Fab, Fab', $F(ab')_2$, scFv, $(scFv)_2$, dAb, complementarity determining region (CDR) fragments, linear antibodies, single-chain antibody molecules, minibodies, diabodies, and multispecific antibodies formed from antibody fragments.

Therapeutic antibodies include, without limitation, anti-HER2 antibodies anti-CD20 antibodies; anti-IL-8 antibodies; anti-VEGF antibodies; anti-CD40 antibodies, anti-CD11a antibodies; anti-CD18 antibodies; anti-IgE antibodies; anti-Apo-2 receptor antibodies; anti-Tissue Factor (TF) antibodies; anti-human $\alpha_4\beta_7$ integrin antibodies; anti-EGFR antibodies; anti-CD3 antibodies; anti-CD25 antibodies; anti-CD4 antibodies; anti-CD52 antibodies; anti-Fc receptor antibodies; anti-carcinoembryonic antigen (CEA) antibodies; antibodies directed against breast epithelial cells; antibodies that bind to colon carcinoma cells; anti-CD38 antibodies; anti-CD33 antibodies; anti-CD22 antibodies; anti-EpCAM antibodies; anti-GpIIb/IIIa antibodies; anti-RSV antibodies; anti-CMV antibodies; anti-HIV antibodies; anti-hepatitis antibodies; anti-CA 125 antibodies; anti-$\alpha v\beta 3$ antibodies; anti-human renal cell carcinoma antibodies; anti-human 17-1A antibodies; anti-human colorectal tumor antibodies; anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma; and anti-human leukocyte antigen (HLA) antibodies, and anti-HLA DR antibodies.

In other embodiments, the therapeutic antibody is an antibody binding to a HER receptor, VEGF, IgE, CD20, CD11a, CD40, or DR5.

In a further embodiment, the HER receptor is HER1 and/or HER2, preferably HER2. The HER2 antibody may, for example, comprise a heavy and/or light chain variable domain sequence selected from the group consisting of SEQ ID NO: 16, 17, 18, and 19.

In another embodiment, the therapeutic antibody is an antibody that binds to CD20. The anti-CD20 antibody may, for example, comprise a heavy and/or light chain variable domain sequence selected from the group consisting of SEQ ID NOS: 1 through 15.

In yet another embodiment, the therapeutic antibody is an antibody that binds to VEGF. The anti-VEGF antibody may, for example, comprise a heavy and/or light chain variable domain sequence selected from the group consisting of SEQ ID NOS: 20 through 25.

In an additional embodiment, the therapeutic antibody is an antibody that binds CD11a. The anti-CD11a antibody may, for example, comprise a heavy and/or light chain variable domain sequence selected from the group consisting of SEQ ID NOS: 26 through 29.

In a further embodiment, the therapeutic antibody binds to a DR5 receptor. The anti-DR5 antibody may, for example, be selected from the group consisting of Apomabs 1.1, 2.1, 3.1, 4.1, 5.1, 5.2, 5.3, 6.1, 6.2, 6.3, 7.1, 7.2, 7.3, 8.1, 8.3, 9.1, 1.2, 2.2, 3.2, 4.2, 5.2, 6.2, 7.2, 8.2, 9.2, 1.3, 2.2, 3.3, 4.3, 5.3, 6.3, 7.3, 8.3, 9.3, and 25.3, and preferably is Apomab 8.3 or Apomab 7.3, and most preferably Apomab 7.3.

In other embodiments of the method of the present invention, the polypeptide expressed in the recombinant host cell is a therapeutic polypeptide. For example, the therapeutic polypeptide can be selected from the group consisting of a growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an

ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of said polypeptides.

In all embodiments, the recombinant host cell can be an eukaryotic host cell. such as a mammalian host cell, including, for example, Chinese Hamster Ovary (CHO) cells.

In all embodiments, the recombinant host cell can also be a prokaryotic host cell, such as a bacterial cell, including, without limitation, *E. coli* cells.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1**. Dialysis Experiment: Digital gel-like imaging obtained from Bioanalyzer analysis (each lane representing a time point) demonstrating that ocrelizumab (rhuMAb 2H7—Variant A) inside the dialysis bag remained intact during the incubation period.

FIG. **2**. Dialysis Experiment: Digital gel-like imaging obtained from Bioanalyzer analysis (each lane representing a time point) showing that ocrelizumab outside the dialysis bag was reduced during the incubation period. This is evidenced by the loss of intact antibody (~150 kDa) and the formation of antibody fragments depicted in the Figure. At the 48-hour time point (Lane 7), the reduced antibody appeared to be reoxidized, presumably as a result of loosing reduction activity in the Harvested Cell Culture Fluid (HCCF). The band appearing just above the 28 kDa marker arose from the light chain of antibody. There was a significant amount of free light already present in the HCCF before the incubation began. The presence of excess free light chain and dimers of light chain in the HCCF is typical for the cell line producing ocrelizumab.

FIG. **3**. Free Thiol Levels from Dialysis Experiment: Purified ocrelizumab in phosphate buffered saline (PBS) was inside the dialysis bag and HCCF containing ocrelizumab was outside the bag. Free thiols inside (boxes) and outside (diamonds) the dialysis bag reached comparable levels within a few hours, indicating a good exchange of small molecule components in the HCCF between inside and outside the dialysis bag.

FIG. **4**. Thioredoxin System and Other Reactions Involved in Antibody Reduction: The thioredoxin system, comprising thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH, functions as a hydrogen donor system for reduction of disulfide bonds in proteins. Trx is a small monomeric protein with a COX active site motif that catalyzes many redox reactions through thiol-disulfide exchange. The oxidized Trx can be reduced by NADPH via TrxR. The reduced Trx is then able to catalyze the reduction of disulfides in proteins. The NADPH required for thioredoxin system is provided via reactions in pentose phosphate pathway and glycolysis.

FIG. **5**. In Vitro Activity of Thioredoxin System: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) demonstrating that incubation of intact ocrelizumab (1 mg/mL) with 0.1 mM TrxR (rat liver), 5 mM Trx (human), and 1 mM NADPH in PBS resulted in the complete reduction of ocrelizumab; the ocrelizumab was completely reduced in less than 21 hours.

FIG. **6**. In Vitro Activity of Thioredoxin System Inhibited by Aurothioglucose: The addition of aurothioglucose (ATG) to the same reaction mixture as described in the caption for FIG. **5**, above, effectively inhibited the ocrelizumab reduction. This is seen by the digital gel-like image from Bioanalyzer analysis (each lane representing a time point).

FIG. **7**. In vitro Activity of Thioredoxin System Inhibited by Aurothiomalate: The addition of aurothiomalate (ATM) at a concentration of 1 mM to the same reaction mixture as described in the caption for FIG. **5**, above, effectively inhibited the ocrelizumab reduction. This is seen by the digital gel-like image from Bioanalyzer analysis (each lane representing a time point).

FIG. **8**. In Vitro Activity of Thioredoxin System: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that incubation of intact ocrelizumab (1 mg/mL) with 0.1 mM TrxR (rat liver), 5 mM Trx (human), and 1 mM NADPH in 10 mM histidine sulfate buffer resulted in the reduction of ocrelizumab in less than 1 hour.

FIG. **9**. In vitro Activity of Thioredoxin System Inhibited by $CuSO_4$: The addition of $CuSO_4$ at a concentration of 50 μM to the same reaction mixture as described in the caption for FIG. **8** effectively inhibited the ocrelizumab reduction as shown in the digital gel-like image from Bioanalyzer analysis (each lane representing a time point).

FIG. **10**. Ocrelizumab Reduction: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that ocrelizumab was reduced in an incubation experiment using HCCF from a homogenized CCF generated from a 3-L fermentor.

FIG. **11**. Inhibition of Ocrelizumab Reduction In HCCF by Aurothioglucose: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that the addition of 1 mM aurothioglucose to the same HCCF as used for the incubation experiment as shown in FIG. **10** inhibited the reduction of ocrelizumab.

FIG. **12**. Inhibition of Ocrelizumab Reduction In HCCF by Aurothiomalate: Digital gel-like image from Bioanalyzer (each lane representing a time point) analysis indicating that the addition of 1 mM aurothiomalate to the same HCCF as used for the incubation experiment shown in FIG. **10** inhibited the reduction of ocrelizumab.

FIG. **13**. Losing Reduction Activity in HCCF: The HCCF from one of the large scale manufacturing runs for ocrelizumab (the "beta" run) that was subject to several freeze/thaw cycles demonstrated no ocrelizumab reduction when used in an incubation experiment. This was shown by Bioanalyzer analysis (each lane representing a time point), and can be contrasted to the antibody reduction seen previously in the freshly thawed HCCF from the same fermentation batch.

FIG. **14**. The Lost Reduction Activity in HCCF Restored by Addition of NADPH: The reduction of ocrelizumab was observed again in the Bioanalyzer assay (each lane representing a time point) after the addition of NADPH at a concentration of 5 mM into the HCCF where the reduction activity has been eliminated under the conditions described above in FIG. **13**.

FIG. **15**. The Lost Reduction Activity in HCCF Restored by Addition of Glucose-6-Phosphate: The reduction of ocrelizumab was observed again in the Bioanalyzer assay (each lane representing a time point) after the addition of G6P at a concentration of 10 mM into the HCCF where the reduction activity has been eliminated due to the treatment described above in FIG. **13**.

FIG. **16**. Ocrelizumab Reduction: A digital gel-like image from Bioanalyzer analysis showing that ocrelizumab was reduced in an incubation experiment using a HCCF from a large scale manufacturing run (the "alpha" run).

FIG. **17**. EDTA Inhibits Ocrelizumab Reduction: Digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that the reduction of ocrelizumab was inhibited in an incubation experiment using a HCCF from the alpha run with EDTA added at a concentration of 20 mM to the HCCF whose reducing activity is demonstrated in FIG. **16**.

FIG. **18**. The Lost Reduction Activity in "Beta Run" HCCF Restored by Addition of Glucose-6-Phosphate but No Inhibition of Reduction by EDTA: The reduction of ocrelizumab was observed in the Bioanalyzer assay (each lane representing a time point) after the addition of G6P at a concentration of 5 mM and 20 mM EDTA into the HCCF whose reduction activity had been lost (see FIG. **13**). In contrast to the results shown in FIG. **17**, the presence of EDTA did not block the reduction of ocreliumab.

FIG. **19**. Inhibition of Ocrelizumab Reduction: by (i) addition of EDTA, (ii) addition of $CuSO_4$, or (iii) adjustment of pH to 5.5. All three different methods, (1) addition of EDTA, (2) addition of $CuSO_4$, and (3) adjustment of pH to 5.5, used independently, were effective in inhibiting ocrelizumab reduction. This was demonstrated by the depicted quantitative Bioanalyzer results that showed that nearly 100% intact (150 kDa) antibody remained in the protein A elution pools. In contrast, ocrelizumab was completely reduced in the control HCCF after 20 hours of HCCF hold time.

FIG. **20**. Inhibition of Ocrelizumab Reduction by Air Sparging: Sparging the HCCF with air was effective in inhibiting ocrelizumab disulfide bond reduction. This was demonstrated by the quantitative Bioanalyzer results showing that nearly 100% intact (150 kDa) antibody remained in the protein A elution pools. In contrast, ocrelizumab was almost completely reduced in the control HCCF after 5 hours of sparging with nitrogen.

FIG. **21** shows the V_L (SEQ ID NO. 24) amino acid sequence of an anti-Her2 antibody (Trastuzumab).

FIG. **22** shows the V_H (SEQ ID No. 25) amino acid sequence of an anti-Her2 antibody (Trastuzumab).

FIG. **23** is a schematic showing some steps of a typical large scale manufacturing process.

FIG. **24** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate.

FIG. **25** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 1 mM histidine sulfate+1 mM ATG.

FIG. **26** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.6 μM ATG (6:1 ATG:TrxR).

FIG. **27** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.4 μM ATG (4:1 ATG:TrxR).

FIG. **28** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.2 μM ATG (2:1 ATG:TrxR).

FIG. **29** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.1 mM autothiomalate (ATM).

FIG. **30** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+0.01 mM autothiomalate (ATM).

FIG. **31** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+20 μM $CuSO_4$ (4:1 Cu^{2+}:Trx).

FIG. **32** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+10 μM $CuSO_4$ (2:1 Cu^{2+}:Trx).

FIG. **33** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+5 μM $CuSO_4$ (1:1 Cu^{2+}:Trx).

FIG. **34** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+532 μM cystamine (20:1 cystamine:2H7 disulfide).

FIG. **35** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+266 μM cystamine (10:1 cystamine:2H7 disulfide).

FIG. **36** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+133 μM cystamine (5:1 cystamine:2H7 disulfide).

FIG. **37** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+26.6 μM cystamine (1:1 cystamine:2H7 disulfide).

FIG. **38** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate (pH=7.6)+2.6 mM cystine.

FIG. **39** is a digital gel-like image from Bioanalyzer analysis: 2H7 (Variant A)+1 mM NADPH+5 μM thioredoxin+0.1 μM thioredoxin reductase (recombinant) in 10 mM histidine sulfate+2.6 mM GSSG (oxidized glutathione).

FIG. **40** Reconstructed enzymatic reduction system. 1 mg/ml 2H7 (Variant A)+10 μg/mL hexokinase, 50 μg/mL glucose-6-phosphate dehydrogenase, 5 μM thioredoxin, 0.1 μM thioredoxin reductase, 2 mM glucose, 0.6 mM ATP, 2 mM Mg^{2+}, and 2 mM NADP in 50 mM histidine sulfate buffer at pH=7.38.

FIG. **41** The thioredoxin system requires NADPH. 1 mg/ml 2H7 (Variant A)+5 μM thioredoxin, 0.1 μM thioredoxin reductase, and 2 mM NADP in 50 mM histidine sulfate buffer at pH=7.38.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

I. Definitions

In the present invention, in the context of proteins, including antibodies, in general, or with regard to any specific protein or antibody, the term "reduction" is used to refer to the reduction of one or more disulfide bonds of the protein or antibody. Thus, for example, the terms "ocrelizumab reduction" is used interchangeably with the term "ocrelizumab disulfide bond reduction" and the term "antibody (Ab) reduction" is used interchangeably with the term "antibody (Ab) disulfide bond reuction."

The terms "reduction" or "disulfide bond reduction" are used in the broadest sense, and include complete and partial reduction and reduction of some or all of the disulfide bonds, interchain or intrachain, present in a protein such as an antibody.

By "protein" is meant a sequence of amino acids for which the chain length is sufficient to produce the higher levels of tertiary and/or quaternary structure. This is to distinguish from "peptides" or other small molecular weight drugs that do not have such structure. Typically, the protein herein will have a molecular weight of at least about 15-20 kD, preferably at least about 20 kD. Examples of proteins encompassed

within the definition herein include all mammalian proteins, in particular, therapeutic and diagnostic proteins, such as therapeutic and diagnostic antibodies, and, in general proteins that contain one or more disulfide bonds, including multi-chain polypeptides comprising one or more inter- and/or intrachain disulfide bonds.

The term "therapeutic protein" or "therapeutic polypeptide" refers to a protein that is used in the treatment of disease, regardless of its indication or mechanism of action. In order for therapeutic proteins to be useful in the clinic it must be manufactured in large quantities. "Manufacturing scale" production of therapeutic proteins, or other proteins, utilize cell cultures ranging from about 400 L to about 80,000 L, depending on the protein being produced and the need. Typically such manufacturing scale production utilizes cell culture sizes from about 400 L to about 25,000 L. Within this range, specific cell culture sizes such as 4,000 L, about 6,000 L, about 8,000, about 10,000, about 12,000 L, about 14,000 L, or about 16,000 L are utilized.

The term "therapeutic antibody" refers to an antibody that is used in the treatment of disease. A therapeutic antibody may have various mechanisms of action. A therapeutic antibody may bind and neutralize the normal function of a target associated with an antigen. For example, a monoclonal antibody that blocks the activity of the of protein needed for the survival of a cancer cell causes the cell's death. Another therapeutic monoclonal antibody may bind and activate the normal function of a target associated with an antigen. For example, a monoclonal antibody can bind to a protein on a cell and trigger an apoptosis signal. Yet another monoclonal antibody may bind to a target antigen expressed only on diseased tissue; conjugation of a toxic payload (effective agent), such as a chemotherapeutic or radioactive agent, to the monoclonal antibody can create an agent for specific delivery of the toxic payload to the diseased tissue, reducing harm to healthy tissue. A "biologically functional fragment" of a therapeutic antibody will exhibit at least one if not some or all of the biological functions attributed to the intact antibody, the function comprising at least specific binding to the target antigen.

The term "diagnostic protein" refers to a protein that is used in the diagnosis of a disease.

The term "diagnostic antibody" refers to an antibody that is used as a diagnostic reagent for a disease. The diagnostic antibody may bind to a target antigen that is specifically associated with, or shows increased expression in, a particular disease. The diagnostic antibody may be used, for example, to detect a target in a biological sample from a patient, or in diagnostic imaging of disease sites, such as tumors, in a patient. A "biologically functional fragment" of a diagnostic antibody will exhibit at least one if not some or all of the biological functions attributed to the intact antibody, the function comprising at least specific binding to the target antigen.

"Purified" means that a molecule is present in a sample at a concentration of at least 80-90% by weight of the sample in which it is contained.

The protein, including antibodies, which is purified is preferably essentially pure and desirably essentially homogeneous (i.e. free from contaminating proteins etc.).

An "essentially pure" protein means a protein composition comprising at least about 90% by weight of the protein, based on total weight of the composition, preferably at least about 95% by weight.

An "essentially homogeneous" protein means a protein composition comprising at least about 99% by weight of protein, based on total weight of the composition.

As noted above, in certain embodiments, the protein is an antibody. "Antibodies" (Abs) and "immunoglobulins" (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which generally lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

The term "antibody" is used in the broadest sense and specifically covers monoclonal antibodies (including full length antibodies which have an immunoglobulin Fc region), antibody compositions with polyepitopic specificity, bispecific antibodies, diabodies, and single-chain molecules such as scFv molecules, as well as antibody fragments (e.g., Fab, $F(ab')_2$, and Fv).

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible mutations, e.g., naturally occurring mutations, that may be present in minor amounts. Thus, the modifier "monoclonal" indicates the character of the antibody as not being a mixture of discrete antibodies. In certain embodiments, such a monoclonal antibody typically includes an antibody comprising a polypeptide sequence that binds a target, wherein the target-binding polypeptide sequence was obtained by a process that includes the selection of a single target binding polypeptide sequence from a plurality of polypeptide sequences. For example, the selection process can be the selection of a unique clone from a plurality of clones, such as a pool of hybridoma clones, phage clones, or recombinant DNA clones. It should be understood that a selected target binding sequence can be further altered, for example, to improve affinity for the target, to humanize the target binding sequence, to improve its production in cell culture, to reduce its immunogenicity in vivo, to create a multispecific antibody, etc., and that an antibody comprising the altered target binding sequence is also a monoclonal antibody of this invention. In contrast to polyclonal antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody of a monoclonal antibody preparation is directed against a single determinant on an antigen. In addition to their specificity, monoclonal antibody preparations are advantageous in that they are typically uncontaminated by other immunoglobulins.

The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by a variety of techniques, including, for example, the hybridoma method (e.g., Kohler et al., *Nature,* 256: 495 (1975); Harlow et al., *Antibodies: A Laboratory Manual*, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling et al., in: *Monoclonal Antibodies and T-Cell Hybridomas* 563-681 (Elsevier, N.Y., 1981)), recombinant DNA methods (see, e.g., U.S. Pat. No. 4,816,567), phage display technologies (see, e.g., Clackson et al., *Nature,* 352: 624-628 (1991); Marks et al., *J. Mol. Biol.* 222: 581-597 (1992); Sidhu et al., *J. Mol. Biol.* 338(2): 299-310 (2004); Lee et al., *J. Mol. Biol.* 340(5): 1073-1093 (2004); Fellouse, *Proc. Natl. Acad. Sci. USA* 101 (34): 12467-12472 (2004); and Lee et al., *J. Immunol. Methods* 284(1-2): 119-132 (2004), and technologies for producing human or human-like antibodies in animals that have parts or all of the human immunoglobulin loci or genes

encoding human immunoglobulin sequences (see, e.g., WO98/24893; WO96/34096; WO96/33735; WO91/10741; Jakobovits et al., *Proc. Natl. Acad. Sci. USA* 90: 2551 (1993); Jakobovits et al., *Nature* 362: 255-258 (1993); Bruggemann et al., *Year in Immunol.* 7:33 (1993); U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016; Marks et al., *BioTechnology* 10: 779-783 (1992); Lonberg et al., *Nature* 368: 856-859 (1994); Morrison, *Nature* 368: 812-813 (1994); Fishwild et al., *Nature Biotechnol.* 14: 845-851 (1996); Neuberger, *Nature Biotechnol.* 14: 826 (1996) and Lonberg and Huszar, *Intern. Rev. Immunol.* 13: 65-93 (1995).

The monoclonal antibodies herein specifically include "chimeric" antibodies in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567; and Morrison et al., *Proc. Natl. Acad. Sci. USA* 81:6851-6855 (1984)).

"Humanized" forms of non-human (e.g., murine) antibodies are chimeric antibodies that contain minimal sequence derived from non-human immunoglobulin. In one embodiment, a humanized antibody is a human immunoglobulin (recipient antibody) in which residues from a hypervariable region of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit, or nonhuman primate having the desired specificity, affinity, and/or capacity. In some instances, framework region (FR) residues of the human immunoglobulin are replaced by corresponding non-human residues. Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications may be made to further refine antibody performance. In general, a humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable loops correspond to those of a non-human immunoglobulin, and all or substantially all the FRs are those of a human immunoglobulin sequence. The humanized antibody optionally will also comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see Jones et al., *Nature* 321:522-525 (1986); Riechmann et al., *Nature* 332:323-329 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-596 (1992). See also the following review articles and references cited therein: Vaswani and Hamilton, *Ann. Allergy, Asthma & Immunol.* 1:105-115 (1998); Harris, *Biochem. Soc. Transactions* 23:1035-1038 (1995); Hurle and Gross, *Curr. Op. Biotech.* 5:428-433 (1994). The humanized antibody includes a Primatized™ antibody wherein the antigen-binding region of the antibody is derived from an antibody produced by immunizing macaque monkeys with the antigen of interest.

A "human antibody" is one which possesses an amino acid sequence which corresponds to that of an antibody produced by a human and/or has been made using any of the techniques for making human antibodies as disclosed herein. This definition of a human antibody specifically excludes a humanized antibody comprising non-human antigen-binding residues.

An "affinity matured" antibody is one with one or more alterations in one or more CDRs/HVRs thereof which result in an improvement in the affinity of the antibody for antigen, compared to a parent antibody which does not possess those alteration(s). Preferred affinity matured antibodies will have nanomolar or even picomolar affinities for the target antigen. Affinity matured antibodies are produced by procedures known in the art. Marks et al., Bio/Technology 10:779-783 (1992) describes affinity maturation by V_H and V_L domain shuffling. Random mutagenesis of CDR/HVR and/or framework residues is described by: Barbas et al., *Proc Nat. Acad. Sci. USA* 91:3809-3813 (1994); Schier et al., *Gene* 169:147-155 (1995); Yelton et al., *J. Immunol.* 155:1994-2004 (1995); Jackson et al., *J. Immunol.* 154(7):3310-9 (1995); and Hawkins et al., *J. Mol. Biol.* 226:889-896 (1992).

The "variable region" or "variable domain" of an antibody refers to the amino-terminal domains of the heavy or light chain of the antibody. The variable domain of the heavy chain may be referred to as "V_H." The variable domain of the light chain may be referred to as "V_L." These domains are generally the most variable parts of an antibody and contain the antigen-binding sites.

The term "variable" refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed throughout the variable domains of antibodies. It is concentrated in three segments called complementarity-determining regions (CDRs) or hypervariable regions (HVRs) both in the light-chain and the heavy-chain variable domains. The more highly conserved portions of variable domains are called the framework regions (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely adopting a beta-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the beta-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen-binding site of antibodies (see Kabat et al., *Sequences of Proteins of Immunological Interest*, Fifth Edition, National Institute of Health, Bethesda, Md. (1991)). The constant domains are not involved directly in the binding of an antibody to an antigen, but exhibit various effector functions, such as participation of the antibody in antibody-dependent cellular toxicity.

The "light chains" of antibodies (immunoglobulins) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino acid sequences of their constant domains.

Depending on the amino acid sequences of the constant domains of their heavy chains, antibodies (immunoglobulins) can be assigned to different classes. There are five major classes of immunoglobulins: IgA, IgD, IgE, IgG and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG_1, IgG_2, IgG_3, IgG_4, IgA_1, and IgA_2. The heavy chain constant domains that correspond to the different classes of immunoglobulins are called a, d, e, g, and m, respectively. The subunit structures and three-dimensional configurations of different classes of immunoglobulins are well known and described generally in, for example, Abbas et al., Cellular and *Mol. Immunology,* 4th ed. (2000). An antibody may be part of a larger fusion molecule, formed by covalent or non-covalent association of the antibody with one or more other proteins or peptides.

The terms "full length antibody," "intact antibody" and "whole antibody" are used herein interchangeably to refer to an antibody in its substantially intact form, not antibody fragments as defined below. The terms particularly refer to an antibody with heavy chains that contain the Fc region.

"Antibody fragments" comprise only a portion of an intact antibody, wherein the portion retains at least one, and as many

as most or all, of the functions normally associated with that portion when present in an intact antibody. In one embodiment, an antibody fragment comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, an antibody fragment, for example one that comprises the Fc region, retains at least one of the biological functions normally associated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, an antibody fragment is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For example, such an antibody fragment may comprise an antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen-combining sites and is still capable of cross-linking antigen.

The Fab fragment contains the heavy- and light-chain variable domains and also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxy terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains bear a free thiol group. F(ab')2 antibody fragments originally were produced as pairs of Fab' fragments which have hinge cysteines between them. Other chemical couplings of antibody fragments are also known.

"Fv" is the minimum antibody fragment which contains a complete antigen-binding site. In one embodiment, a two-chain Fv species consists of a dimer of one heavy- and one light-chain variable domain in tight, non-covalent association. In a single-chain Fv (scFv) species, one heavy- and one light-chain variable domain can be covalently linked by a flexible peptide linker such that the light and heavy chains can associate in a "dimeric" structure analogous to that in a two-chain Fv species. It is in this configuration that the three CDRs of each variable domain interact to define an antigen-binding site on the surface of the V_H-V_L dimer. Collectively, the six CDRs confer antigen-binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

"Single-chain Fv" or "scFv" antibody fragments comprise the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the scFv polypeptide further comprises a polypeptide linker between the V_H and V_L domains which enables the scFv to form the desired structure for antigen binding. For a review of scFv see Pluckthun, in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to small antibody fragments with two antigen-binding sites, which fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) in the same polypeptide chain (V_H-V_L). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies may be bivalent or bispecific. Diabodies are described more fully in, for example, EP 404,097; WO93/1161; Hudson et al., (2003) *Nat. Med.* 9:129-134; and Hollinger et al., *Proc. Natl. Acad. Sci. USA* 90: 6444-6448 (1993). Triabodies and tetrabodies are also described in Hudson et al., (2003) *Nat. Med.* 9:129-134.

The antibody may bind to any protein, including, without limitation, a member of the HER receptor family, such as HER1 (EGFR), HER2, HER3 and HER4; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD21, CD22, and CD34; cell adhesion molecules such as LFA-1, Mol, p150, 95, VLA-4, ICAM-1, VCAM and av/p3 integrin including either α or β or subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as vascular endothelial growth factor (VEGF); IgE; blood group antigens; flk2/flt3 receptor; obesity (OB) receptor; and protein C. Other exemplary proteins include growth hormone (GH), including human growth hormone (hGH) and bovine growth hormone (bGH); growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; α-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or tissue-type plasminogen activator (t-PA); bombazine; thrombin; tumor necrosis factor-α and -β; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-α); serum albumin such as human serum albumin (HSA); mullerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; DNase; inhibin; activin; receptors for hormones or growth factors; an integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-α and TGF-β, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I); insulin-like growth factor binding proteins (IGFBPs); erythropoietin (EPO); thrombopoietin (TPO); osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-α, -β, and -γ; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor (DAF); a viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; immunoadhesins; antibodies; and biologically active fragments or variants of any of the above-listed polypeptides. Many other antibodies and/or other proteins may be used in accordance with the instant invention, and the above lists are not meant to be limiting.

A "biologically functional fragment" of an antibody comprises only a portion of an intact antibody, wherein the portion retains at least one, and as many as most or all, of the functions normally associated with that portion when present in an intact antibody. In one embodiment, a biologically functional fragment of an antibody comprises an antigen binding site of the intact antibody and thus retains the ability to bind antigen. In another embodiment, a biologically functional fragment of an antibody, for example one that comprises the Fc region, retains at least one of the biological functions normally asso-

ciated with the Fc region when present in an intact antibody, such as FcRn binding, antibody half life modulation, ADCC function and complement binding. In one embodiment, a biologically functional fragment of an antibody is a monovalent antibody that has an in vivo half life substantially similar to an intact antibody. For example, such a biologically functional fragment of an antibody may comprise an antigen binding arm linked to an Fc sequence capable of conferring in vivo stability to the fragment.

The terms "thioredoxin inhibitor" and "Trx inhibitor" are used interchangeably, and include all agents and measures effective in inhibiting thioredoxin activity. Thus, thioredoxin (Trx) inhibitors include all agents and measures blocking any component of the Trx, G6PD and/or hexokinase enzyme systems. In this context, "inhibition" includes complete elimination (blocking) and reduction of thioredoxin activity, and, consequently, complete or partial elimination of disulfide bond reduction in a protein, such as an antibody.

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with research, diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In some embodiments, an antibody is purified (1) to greater than 95% by weight of antibody as determined by, for example, the Lowry method, and in some embodiments, to greater than 99% by weight; (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of, for example, a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using, for example, Coomassie blue or silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

The terms "Protein A" and "ProA" are used interchangeably herein and encompasses Protein A recovered from a native source thereof, Protein A produced synthetically (e.g. by peptide synthesis or by recombinant techniques), and variants thereof which retain the ability to bind proteins which have a C_H2/C_H3 region, such as an Fc region. Protein A can be purchased commercially from Repligen, GE Healthcare and Fermatech. Protein A is generally immobilized on a solid phase support material. The term "ProA" also refers to an affinity chromatography resin or column containing chromatographic solid support matrix to which is covalently attached Protein A.

The term "chromatography" refers to the process by which a solute of interest in a mixture is separated from other solutes in a mixture as a result of differences in rates at which the individual solutes of the mixture migrate through a stationary medium under the influence of a moving phase, or in bind and elute processes.

The term "affinity chromatography" and "protein affinity chromatography" are used interchangeably herein and refer to a protein separation technique in which a protein of interest or antibody of interest is reversibly and specifically bound to a biospecific ligand. Preferably, the biospecific ligand is covalently attached to a chromatographic solid phase material and is accessible to the protein of interest in solution as the solution contacts the chromatographic solid phase material. The protein of interest (e.g., antibody, enzyme, or receptor protein) retains its specific binding affinity for the biospecific ligand (antigen, substrate, cofactor, or hormone, for example) during the chromatographic steps, while other solutes and/or proteins in the mixture do not bind appreciably or specifically to the ligand. Binding of the protein of interest to the immobilized ligand allows contaminating proteins or protein impurities to be passed through the chromatographic medium while the protein of interest remains specifically bound to the immobilized ligand on the solid phase material. The specifically bound protein of interest is then removed in active form from the immobilized ligand with low pH, high pH, high salt, competing ligand, and the like, and passed through the chromatographic column with the elution buffer, free of the contaminating proteins or protein impurities that were earlier allowed to pass through the column. Any component can be used as a ligand for purifying its respective specific binding protein, e.g. antibody.

The terms "non-affinity chromatography" and "non-affinity purification" refer to a purification process in which affinity chromatography is not utilized. Non-affinity chromatography includes chromatographic techniques that rely on non-specific interactions between a molecule of interest (such as a protein, e.g. antibody) and a solid phase matrix.

A "cation exchange resin" refers to a solid phase which is negatively charged, and which thus has free cations for exchange with cations in an aqueous solution passed over or through the solid phase. A negatively charged ligand attached to the solid phase to form the cation exchange resin may, e.g., be a carboxylate or sulfonate. Commercially available cation exchange resins include carboxy-methyl-cellulose, sulphopropyl (SP) immobilized on agarose (e.g. SP-SEPHAROSE FAST FLOW™ or SP-SEPHAROSE HIGH PERFORMANCE™, from GE Healthcare) and sulphonyl immobilized on agarose (e.g. S-SEPHAROSE FAST FLOW™ from GE Healthcare). A "mixed mode ion exchange resin" refers to a solid phase which is covalently modified with cationic, anionic, and hydrophobic moieties. A commercially available mixed mode ion exchange resin is BAKERBOND ABX™ (J. T. Baker, Phillipsburg, N.J.) containing weak cation exchange groups, a low concentration of anion exchange groups, and hydrophobic ligands attached to a silica gel solid phase support matrix.

The term "anion exchange resin" is used herein to refer to a solid phase which is positively charged, e.g. having one or more positively charged ligands, such as quaternary amino groups, attached thereto. Commercially available anion exchange resins include DEAE cellulose, QAE SEPHADEX™ and FAST Q SEPHAROSE™ (GE Healthcare).

A "buffer" is a solution that resists changes in pH by the action of its acid-base conjugate components. Various buffers which can be employed depending, for example, on the desired pH of the buffer are described in *Buffers. A Guide for the Preparation and Use of Buffers in Biological Systems*, Gueffroy, D., ed. Calbiochem Corporation (1975). In one embodiment, the buffer has a pH in the range from about 2 to about 9, alternatively from about 3 to about 8, alternatively from about 4 to about 7 alternatively from about 5 to about 7. Non-limiting examples of buffers that will control the pH in this range include MES, MOPS, MOPSO, Tris, HEPES, phosphate, acetate, citrate, succinate, and ammonium buffers, as well as combinations of these.

The "loading buffer" is that which is used to load the composition comprising the polypeptide molecule of interest and one or more impurities onto the ion exchange resin. The loading buffer has a conductivity and/or pH such that the polypeptide molecule of interest (and generally one or more impurities) is/are bound to the ion exchange resin or such that the protein of interest flows through the column while the impurities bind to the resin.

The "intermediate buffer" is used to elute one or more impurities from the ion exchange resin, prior to eluting the polypeptide molecule of interest. The conductivity and/or pH of the intermediate buffer is/are such that one or more impurity is eluted from the ion exchange resin, but not significant amounts of the polypeptide of interest.

The term "wash buffer" when used herein refers to a buffer used to wash or re-equilibrate the ion exchange resin, prior to eluting the polypeptide molecule of interest. Conveniently, the wash buffer and loading buffer may be the same, but this is not required.

The "elution buffer" is used to elute the polypeptide of interest from the solid phase. The conductivity and/or pH of the elution buffer is/are such that the polypeptide of interest is eluted from the ion exchange resin.

A "regeneration buffer" may be used to regenerate the ion exchange resin such that it can be re-used. The regeneration buffer has a conductivity and/or pH as required to remove substantially all impurities and the polypeptide of interest from the ion exchange resin.

The term "substantially similar" or "substantially the same," as used herein, denotes a sufficiently high degree of similarity between two numeric values (for example, one associated with an antibody of the invention and the other associated with a reference/comparator antibody), such that one of skill in the art would consider the difference between the two values to be of little or no biological and/or statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values is, for example, less than about 50%, less than about 40%, less than about 30%, less than about 20%, and/or less than about 10% as a function of the reference/comparator value.

The phrase "substantially reduced," or "substantially different," as used herein with regard to amounts or numerical values (and not as reference to the chemical process of reduction), denotes a sufficiently high degree of difference between two numeric values (generally one associated with a molecule and the other associated with a reference/comparator molecule) such that one of skill in the art would consider the difference between the two values to be of statistical significance within the context of the biological characteristic measured by said values (e.g., Kd values). The difference between said two values is, for example, greater than about 10%, greater than about 20%, greater than about 30%, greater than about 40%, and/or greater than about 50% as a function of the value for the reference/comparator molecule.

The term "vector," as used herein, is intended to refer to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid," which refers to a circular double stranded DNA into which additional DNA segments may be ligated. Another type of vector is a phage vector. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "recombinant expression vectors," or simply, "expression vectors." In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" may be used interchangeably as the plasmid is the most commonly used form of vector.

"Percent (%) amino acid sequence identity" with respect to a reference polypeptide sequence is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in the reference polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for aligning sequences, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % amino acid sequence identity values are generated using the sequence comparison computer program ALIGN-2. The ALIGN-2 sequence comparison computer program was authored by Genentech, Inc., and the source code has been filed with user documentation in the U.S. Copyright Office, Washington D.C., 20559, where it is registered under U.S. Copyright Registration No. TXU510087. The ALIGN-2 program is publicly available from Genentech, Inc., South San Francisco, Calif., or may be compiled from the source code. The ALIGN-2 program should be compiled for use on a UNIX operating system, preferably digital UNIX V4.0D. All sequence comparison parameters are set by the ALIGN-2 program and do not vary.

In situations where ALIGN-2 is employed for amino acid sequence comparisons, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows:

100 times the fraction X/Y

where X is the number of amino acid residues scored as identical matches by the sequence alignment program ALIGN-2 in that program's alignment of A and B, and

where Y is the total number of amino acid residues in B.

It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. Unless specifically stated otherwise, all % amino acid sequence identity values used herein are obtained as described in the immediately preceding paragraph using the ALIGN-2 computer program.

"Percent (%) nucleic acid sequence identity" is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in a reference Factor D-encoding sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. Sequence identity is then calculated relative to the longer sequence, i.e. even if a shorter

sequence shows 100% sequence identity with a portion of a longer sequence, the overall sequence identity will be less than 100%.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. "Treatment" herein encompasses alleviation of the disease and of the signs and symptoms of the particular disease.

A "disorder" is any condition that would benefit from treatment with the protein. This includes chronic and acute disorders or diseases including those pathological conditions which predispose the mammal to the disorder in question. Non-limiting examples of disorders to be treated herein include carcinomas and allergies.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, non-human higher primates, other vertebrates, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

An "interfering RNA" or "small interfering RNA (siRNA)" is a double stranded RNA molecule less than about 30 nucleotides in length that reduces expression of a target gene. Interfering RNAs may be identified and synthesized using known methods (Shi Y., Trends in Genetics 19(1):9-12 (2003), WO/2003056012 and WO2003064621), and siRNA libraries are commercially available, for example from Dharmacon, Lafayette, Colo. Frequently, siRNAs can be successfully designed to target the 5' end of a gene.

II. Compositions and Methods of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology and the like, which are within the skill of the art. Such techniques are explained fully in the literature. See e.g., *Molecular Cloning: A Laboratory Manual*, (J. Sambrook et al., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989); *Current Protocols in Molecular Biology* (F. Ausubel et al., eds., 1987 updated); *Essential Molecular Biology* (T. Brown ed., IRL Press 1991); *Gene Expression Technology* (Goeddel ed., Academic Press 1991); *Methods for Cloning and Analysis of Eukaryotic Genes* (A. Bothwell et al., eds., Bartlett Publ. 1990); *Gene Transfer and Expression* (M. Kriegler, Stockton Press 1990); *Recombinant DNA Methodology II* (R. Wu et al., eds., Academic Press 1995); PCR: *A Practical Approach* (M. McPherson et al., IRL Press at Oxford University Press 1991); *Oligonucleotide Synthesis* (M. Gait ed., 1984); *Cell Culture for Biochemists* (R. Adams ed., Elsevier Science Publishers 1990); *Gene Transfer Vectors for Mammalian Cells* (J. Miller & M. Calos eds., 1987); *Mammalian Cell Biotechnology* (M. Butler ed., 1991); *Animal Cell Culture* (J. Pollard et al., eds., Humana Press 1990); *Culture of Animal Cells,* 2nd Ed. (R. Freshney et al., eds., Alan R. Liss 1987); *Flow Cytometry and Sorting* (M. Melamed et al., eds., Wiley-Liss 1990); the series *Methods in Enzymology* (Academic Press, Inc.); Wirth M. and Hauser H. (1993); *Immunochemistry in Practice,* 3rd edition, A. Johnstone & R. Thorpe, Blackwell Science, Cambridge, Mass., 1996; *Techniques in Immunocytochemistry*, (G. Bullock & P. Petrusz eds., Academic Press 1982, 1983, 1985, 1989); Handbook of Experimental Immunology, (D. Weir & C. Blackwell, eds.); *Current Protocols in Immunology* (J. Coligan et al., eds. 1991); *Immunoassay* (E. P. Diamandis & T. K. Christopoulos, eds., Academic Press, Inc., 1996); Goding (1986) *Monoclonal Antibodies: Principles and Practice* (2d ed) Academic Press, New York; Ed Harlow and David Lane, *Antibodies A laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1988; *Antibody Engineering,* 2nd edition (C. Borrebaeck, ed., Oxford University Press, 1995); and the series *Annual Review of Immunology*; the series *Advances in Immunology.*

1. Prevention of Disulfide Bond Reduction

The present invention concerns methods for the prevention of the reduction of disulfide bonds of proteins during recombinant production. In particular, the invention concerns methods for preventing the reduction of disulfide bonds of recombinant proteins during processing following fermentation. The methods of the invention are particularly valuable for large scale production of disulfide bond containing proteins, such as at a manufacturing scale. In one embodiment, the methods of the invention are useful for large scale protein production at a scale of greater than 5,000 L.

It has been experimentally found that disulfide bond reduction occurs during processing of the Harvested Cell Culture Fluid (HCCF) produced during manufacturing of recombinant proteins that contain disulfide bonds. Typically, this reduction is observed after cell lysis, especially mechanical cell lysis during harvest operations, when it reaches a certain threshold, such as, for example, from about 30% to about 70%, or from about 40% to about 60%, or from about 50% to about 60% total cell lysis. This threshold will vary, depending on the nature of the protein (e.g. antibody) produced, the recombinant host, the production system, production parameters used, and the like, and can be readily determined experimentally.

Theoretically, such reduction might result from a variety of factors and conditions during the manufacturing process, and might be caused by a variety of reducing agents. The present invention is based, at least in part, on the recognition that the root cause of this reduction is an active thioredoxin (Trx) or thioredoxin-like system in the HCCF.

The Trx enzyme system, composed of Trx, thioredoxin reductase (TrxR) and NADPH, is a hydrogen donor system for reduction of disulfide bonds in proteins. Trx is a small monomeric protein with a COX active site motif that catalyzes many redox reactions through thiol-disulfide exchange. The oxidized Trx can be reduced by NADPH via TrxR. The reduced Trx is then able to catalyze the reduction of disulfides in proteins. The NADPH required for thioredoxin system is provided via reactions in pentose phosphate pathway and glycolysis. The results presented herein demonstrate that NADPH, which is required for activity of the Trx system is provided by glucose-6-phosphate dehyrogenase (G6PD) activity, which generates NADPH from glucose and ATP by hexokinase (see FIG. **4**). These cellular enzymes (Trx system, G6PD, and hexokinase) along with their substrates are released into the CCF upon cell lysis, allowing reduction to occur. Accordingly, disulfide reduction can be prevented by inhibitors of the Trx enzyme system or upstream enzyme systems providing components for an active Trx system, such as G6PD and hexokinase activity.

For further details of these enzyme systems, or regarding other details of protein production, see, for example: Babson, A. L. and Babson, S. R. (1973) Kinetic Colorimetric Measurement of Serum Lactate Dehydrogenase Activity. *Clin. Chem.* 19: 766-769; Michael W. Laird et al., "Optimization of BLyS Production and Purification from *Eschericia coli*," *Protein Expression and Purification* 39:237-246 (2005); John C. Joly et al., "Overexpression of *Eschericia coli* Oxidoreductases Increases Recombinant Insulin-like Growth Factor-I Accumulation," Proc. Natl. Acad. Sci. USA 95:2773-2777 (March 1998); Dana C. Andersen et al., "Production Technologies for Monoclonal Antibodies and Their Fragments,"

Current Opinion in Biotechnology 15:456-462 (2004); Yariv Mazor et al., "Isolation of Engineered, Full-length Antibodies from Libraries Expressed in *Escherichia coli*," *Nature Biotech.* 25, 563-565 (1 Jun. 2007); Laura C. Simmons et al., "Expression of Full-length Immunoglobulins in *Escherichia coli*: Rapid and Efficient Production of Aglycosylated Antibodies," *Journal of Immunological Methods* 263:133-147 (2002); Paul H. Bessette et al., "Efficient Folding of Proteins with Multiple Disulfide Bonds in the *Escherichia coli* cytoplasm," Proc. Natl. Acad. Sci. 96(24):13703-08 (1999); Chaderjian, W. B., Chin, E. T., Harris, R. J., and Etcheverry, T. M., (2005) "Effect of copper sulfate on performance of a serum-free CHO cell culture process and the level of free thiol in the recombinant antibody expressed," *Biotechnol. Prog.* 21: 550-553; Gordon G., Mackow M. C., and Levy H. R., (1995) "On the mechanism of interaction of steroids with human glucose 6-phosphate dehydrogenase," Arch. Biochem. Biophys. 318: 25-29; Gromer S., Urig S., and Becker K., (2004) "TheTrx System—From Science to Clinic," Medicinal Research Reviews, 24: 40-89; Hammes G. G. and Kochavi D., (1962a) "Studies of the Enzyme Hexokinase. I. Steady State Kinetics at pH 8," J. Am. Chem. Soc. 84:2069-2073; Hammes G. G. and Kochavi D., (1962b) "Studies of the Enzyme Hexokinase. III. The Role of the Metal Ion," J. Am. Chem. Soc. 84:2076-2079; Johansson C., Lillig C. H., and Holmgren A., (2004) "Human Mitochondrial Glutaredoxin Reduces S-Glutathionylated Proteins with High Affinity Accepting Electrons from Either Glutathione or Thioredoxin Reductase," J. Biol. Chem. 279:7537-7543; Legrand, C., Bour, J. M., Jacob, C., Capiaumont J., Martial, A., Marc, A., Wudtke, M., Kretzmer, G., Demangel, C., Duval, D., and Hache J., (1992) "Lactate Dehydrogenase (LDH) Activity of the Number of Dead Cells in the Medium of Cultured Eukaryotic Cells as Marker," J. Biotechnol., 25: 231-243; McDonald, M. R., (1955) "Yeast Hexokinase: ATP+ Hexose→Hexose-6-phosphate+ADP," Methods in Enzymology, 1: 269-276, Academic Press, NY; Sols, A., DelaFuente, G., Villar-Palasi, C., and Asensio, C., (1958) "Substrate Specificity and Some Other Properties of Bakers' Yeast Hexokinase," Biochim Biophys Acta 30: 92-101; Kirkpatrick D. L., Kuperus M., Dowdeswell M., Potier N., Donald L. J., Kunkel M., Berggren M., Angulo M., and Powis G., (1998) "Mechanisms of inhibition of the Trx growth factor system by antitumor 2-imidazolyl disulfides," Biochem. Pharmacol. 55: 987-994; Kirkpatrick D. L., Watson S., Kunkel M., Fletcher S., Ulhaq S., and Powis G., (1999) "Parallel syntheses of disulfide inhibitors of the Trx redox system as potential antitumor agents," Anticancer Drug Des. 14: 421-432; Milhausen, M., and Levy, H. R., (1975) "Evidence for an Essential Lysine in G6PD from *Leuconostoc mesenteroides*," Eur. J. Biochem. 50: 453-461; Pleasants, J. C., Guo, W., and Rabenstein, D. L., (1989) "A comparative study of the kinetics of selenol/diselenide and thiol/disulfide exchange reactions," J. Am. Chem. Soc. 111: 6553-6558; Whitesides, G. M., Lilburn, J. E., and Szajewski, R. P., (1977) "Rates of thioldisulfide interchange reactions between mono- and dithiols and Ellman's reagent," J. Org. Chem. 42: 332-338; and Wipf P., Hopkins T. D., Jung J. K., Rodriguez S., Birmingham A., Southwick E. C., Lazo J. S., and Powis G, (2001) "New inhibitors of the Trx-TrxR system based on a naphthoquinone spiroketal natural product lead," *Bioorg. Med. Chem. Lett.* 11: 2637-2641.

According to one aspect of the present invention, disulfide bond reduction can be prevented by blocking any component of the Trx, G6PD and hexokinase enzyme systems. Inhibitors of these enzyme systems are collectively referred to herein as "thioredoxin inhibitors," or "Trx inhibitors." The Trx inhibitors are typically added to the cell culture fluid (CCF), which contains the recombinant host cells and the culture media, and/or to the harvested cell culture fluid (HCCF), which is obtained after harvesting by centrifugation, filtration, or similar separation methods. The HCCF lacks intact host cells but typically contains host cell proteins and other contaminants, including DNA, which are removed in subsequent purification steps. Thus, the Trx inhibitors may be added before harvest and/or during harvest, preferably before harvest.

Alternatively or in addition other, non-specific methods can also be used to prevent the reduction of disulfide bond reduction following fermentation during the recombinant production of recombinant proteins, such as air sparging or pH adjustment. Certain reduction inhibition methods contemplated herein are listed in the following Table 1.

TABLE 1

Reduction Inhibition Methods

Method[1]	Purpose
Addition of EDTA, EGTA, or citrate	To inhibit hexokinase
Addition of sorbose-1-phosphate, polyphosphates, 6-deoxy-6-fluoroglucose, 2-C-hydroxy-methylglucose, xylose, or lyxose	To inhibit hexokinase
Addition of epiandrosterone or dehydroepiandrosterone (DHEA)	To inhibit G6PD
Addition of pyridoxal 5'-phosphate or 1-fluoro-2,4-dinitrobenzene	To inhibit G6PD
Addition of metal ions such as Cu^{2+}, Zn^{2+} Hg^{2+}, Co^{2+}, or Mn^{2+}	To inhibit Trx system
Addition of alkyl-2-imidazolyl disulfides and related compounds (e.g., 1 methylpropyl-2-imidazolyl disulfide[2]) or naphthoquinone spiroketal derivatives (e.g. palmarumycin CP_1[2])	To inhibit Trx
Addition of aurothioglucose (ATG) or aurothiomalate (ATM)	To inhibit TrxR
Air sparging	To deplete G6P and NADPH; oxidizing agent
Cystine	Oxidizing agent
Oxidized glutathione	Oxidizing agents
pH Adjustment to below 6.0	To reduce thiol-disulfide exchange rate and Trx system activity

[1]Applied to CCF prior to harvest or in HCCF immediately after harvest.
[2]Currently not available commercially.

"Trx inhibitors" for use in the methods of the present invention include, without limitation, (1) direct inhibitors of Trx, such as alkyl-2-imidazolyl disulfides and related compounds (e.g., 1 methylpropyl-2-imidazolyl disulfide) (Kirkpatrick et al., 1998 and 1999, supra) and naphthoquinone spiroketal derivatives (e.g., palmarumycin CP_1) (Wipf et al., 2001, supra); (2) specific inhibitors of TrxR, including gold complexes, such as aurothioglucose (ATG) and aurothiomalate (ATM) (see, e.g., the review by Gromer et al., 2004), which are examples of irreversible inhibitors of TrxR; (3) metal ions, such as Hg^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, and Mn^{2+}, which can form readily complexes with thiols and selenols, and thus can be used in embodiments of the instant invention as inhibitors of TrxR or Trx; (4) inhibitors of G6PD, such as, for example, pyridoxal 5'-phosphate and 1 fluoro-2,4 dinitrobenzene (Milhausen and Levy 1975, supra), certain steroids, such as dehydroepiandrosterone (DHEA) and epiandrosterone (EA) (Gordon et al., 1995, supra); and (4) inhibitors of hexokinase activity (and thereby production of G6P for the G6PD), including chelators of metal ions, e.g. Mg^{2+}, such as EDTA, and compounds that react with SH groups, sorbose-1-phosphate, polyphosphates, 6-deoxy-6-fluoroglucose,

2-C-hydroxy-methylglucose, xylose and lyxose (Sols et al., 1958, supra; McDonald, 1955, supra); further hexokinase inhibitors are disclosed in U.S. Pat. No. 5,854,067 entitled "Hexokinase Inhibitors." It will be understood that these inhibitors are listed for illustration only. Other Trx inhibitors exists and can be used, alone or in various combinations, in the methods of the present invention.

"Trx inhibitors" for use in the methods of the present invention also include reagents whereby the reduction of recombinantly produced antibodies or proteins may be reduced or prevented by decreasing the levels of enzymes of the Trx system, the pentose phosphate pathway or hexokinase at various points during the production campaign. In some embodiments, this reduction of enzyme levels may be accomplished by the use of targeted siRNAs, antisense nucleotides, or antibodies. To design targeted siRNAs or antisense nucleotides to the genes as found in CHO cells, these gene sequences are available from public databases to select sequences for targeting enzymes in different organisms. See Example 9 below for examples of the genes of the *E. coli* and mouse Trx system.

In addition to using inhibitors discussed above, it is also possible in certain embodiments of the instant invention to prevent the reduction of a recombinant protein to be purified by sparging the HCCF with air to maintain an oxidizing redox potential in the HCCF. This is a non-directed measure that can deplete glucose, G6P and NADPH by continuously oxidizing the reduced forms of Trx and TrxR. Air sparging of the HCCF tank can be performed, for example, with an air flow of about 100 liters to about 200 liters, such as, for example, 150 liters per minutes. Air sparging can be performed to reach an endpoint percentage of saturation; for example, air sparging can be continued until the HCCF is about 100% saturated with air, or it can be continued until the HCCR is about 30% saturated with air, or until it is between about 100% saturated to about 30% saturated with air. The minimum amount of dissolved oxygen (dO_2) required for the desired inhibitory effect also depends on the antibody or other recombinant protein produced. Thus, for example, about 10% dO_2 (or about 10 sccm for continuous stream) will have the desired effect during the production of antibody 2H7 (Variant A), while Apomab might require a higher (about 30%) dO_2.

In further embodiments of the instant invention, another non-directed method usable to block the reduction of the recombinant protein is lowering the pH of the HCCF. This embodiment takes advantage of particularly slow thiol-disulfide exchange at lower pH values (Whitesides et al., 1977, supra; Pleasants et al., 1989, supra). Therefore, the activity of the Trx system is significantly lower at pH values below 6, and thus the reduction of the recombinant protein, such as ocrelizumab, can be inhibited.

The non-directed approaches can also be combined with each other and/or with the use of one or more Trx inhibitors.

Disulfide bond reduction can be inhibited (i.e., partially or fully blocked) by using one or more Trx inhibitors and/or applying non-directed approaches following completion of the cell culture process, preferably to CCF prior to harvest or in the HCCF immediately after harvest. The optimal time and mode of application and effective amounts depend on the nature of the protein to be purified, the recombinant host cells, and the specific production method used. Determination of the optimal parameters is well within the skill of those of ordinary skill in the art.

For example, in a mammalian cell culture process, such as the CHO antibody production process described in the Examples herein, if cupric sulfate ($CuSO_4$ in the form of pentahydrate or the anhydrous form) is used as a Trx inhibitor, it can be added to supplement the CCF or HCCF in the concentration range of from about 5 μM to about 100 μM, such as from about 10 μM to about 80 μM, preferably from about 15 μM to about 50 μM. Since some cell cultures already contain copper (e.g. about 0.04 μM $CuSO_4$ for the CHO cell cultures used in the Examples herein), this amount is in addition to the copper, if any, already present in the cell culture. Any copper (II) salt can be used instead of $CuSO_4$ as long as solubility is not an issue. For example, copper acetate and copper chloride, which are both soluble in water, can be used instead of $CuSO_4$. The minimum effective concentration may also depend on the antibody produced and the stage where the inhibitor is used. Thus, for example, when cupric sulfate is added pre-lysis, for antibody 2H7 (Variant A) the minimum effective concentration is about 30 μM, for Apomab is about 75 μM, and for antibody Variant C (see Table 2) is about 50 μM. When cupric sulfate is added in CC medium, for antibody 2H7 (Variant A) the minimum effective concentration is about 15 μM, for Apomab is about 25 μM, and for antibody Variant C is about 20 μM. One typical minimal $CuSO_4$ inhibitor concentration of 2×Trx concentration (or Trx equivalence).

EDTA can be used in a wide concentration range, depending on the extent of cell lysis, the recombinant host cell used, and other parameters of the production process. For example, when using CHO or other mammalian host cells, EDTA can be typically added in a concentration of between about 5 mM to about 60 mM, such as from about 10 mM to about 50 mM, or from about 20 mM to about 40 mM, depending on the extent of cell lysis. For lower degree of cell lysis, lower concentrations of EDTA will suffice, while for a cell lysis of about 75%-100%, the required EDTA concentration is higher, such as, for example, from about 20 mM to about 40 mM. The minimum effective concentration may also depend on the antibody produced. Thus, for example, for antibody 2H7 (Variant A) the minimum effective EDTA concentration is about 10 mM.

DHEA as a Trx inhibitor is typically effective at a lower concentration, such as for example, in the concentration range from about 0.05 mM to about 5 mM, preferably from about 0.1 mM to about 2.5 mM.

Other Trx inhibitors, such as aurothioglucose (ATG) and aurothiomalate (ATM) inhibit reduction of disulfide bonds in the μM concentration range. Thus, for example, ATG or ATM may be added in a concentration between about 0.1 mM to about 1 mM. While the minimum inhibitory concentration varies depending on the actual conditions, for ATG and ATM typically it is around 4×TrxR concentration.

It is noted that all inhibitors can be used in an excess amount, therefore, it is not always necessary to know the amount of Trx or TrxR in the system.

In a preferred embodiment, the mammalian host cell used in the manufacturing process is a chinese hamster ovary (CHO) cell (Urlaub et al., *Proc. Natl. Acad. Sci. USA* 77:4216 (1980)). Other mammalian host cells include, without limitation, monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line (293 or 293 cells subcloned for growth in suspension culture), Graham et al., *J. Gen Virol.* 36:59 (1977)); baby hamster kidney cells (BHK, ATCC CCL 10); mouse sertoli cells (TM4, Mather, *Biol. Reprod.* 23:243-251 (1980)); monkey kidney cells (CV1 ATCC CCL 70); African green monkey kidney cells (VERO-76, ATCC CRL-1587); human cervical carcinoma cells (HELA, ATCC CCL 2); canine kidney cells (MDCK, ATCC CCL 34); buffalo rat liver cells (BRL 3A, ATCC CRL 1442); human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); mouse mammary

tumor (MMT 060562, ATCC CCL51); TR1 cells (Mather et al., *Annals N.Y. Acad. Sci.* 383:44-68 (1982)); MRC 5 cells; FS4 cells; a human hepatoma line (Hep G2); and myeloma or lymphoma cells (e.g. Y0, J558L, P3 and NS0 cells) (see U.S. Pat. No. 5,807,715).

A preferred host cell for the production of the polypeptides herein is the CHO cell line DP12 (CHO K1 dhfr$^-$). This is one of the best known CHO cell lines, widely used in laboratory practice (see, for example, EP 0,307,247, published Mar. 15, 1989). In addition, other CHO-K1 (dhfr$^-$) cell lines are known and can be used in the methods of the present invention.

The mammalian host cells used to produce peptides, polypeptides and proteins can be cultured in a variety of media. Commercially available media such as Ham's F10 (Sigma), Minimal Essential Medium ((MEM), Sigma), RPMI-1640 (Sigma), and Dulbecco's Modified Eagle's Medium ((DMEM, Sigma) are suitable for culturing the host cells. In addition, any of the media described in Ham and Wallace (1979), Meth. in Enz. 58:44, Barnes and Sato (1980), Anal. Biochem. 102:255, U.S. Pat. Nos. 4,767,704; 4,657,866; 4,927,762; or 4,560,655; WO 90/03430; WO 87/00195; U.S. Pat. No. Re. 30,985; or U.S. Pat. No. 5,122,469, the disclosures of all of which are incorporated herein by reference, may be used as culture media for the host cells. Any of these media may be supplemented as necessary with hormones and/or other growth factors (such as insulin, transferrin, or epidermal growth factor), salts (such as sodium chloride, calcium, magnesium, and phosphate), buffers (such as HEPES), nucleosides (such as adenosine and thymidine), antibiotics (such as Gentamycin™ drug), trace elements (defined as inorganic compounds usually present at final concentrations in the micromolar range), and glucose or an equivalent energy source. Any other necessary supplements may also be included at appropriate concentrations that would be known to those skilled in the art. The culture conditions, such as temperature, pH, and the like, are those previously used with the host cell selected for expression, and will be apparent to the ordinarily skilled artisan.

A protocol for the production, recovery and purification of recombinant antibodies in mammalian, such as CHO, cells may include the following steps:

Cells may be cultured in a stirred tank bioreactor system and a fed batch culture, procedure is employed. In a preferred fed batch culture the mammalian host cells and culture medium are supplied to a culturing vessel initially and additional culture nutrients are fed, continuously or in discrete increments, to the culture during culturing, with or without periodic cell and/or product harvest before termination of culture. The fed batch culture can include, for example, a semi-continuous fed batch culture, wherein periodically whole culture (including cells and medium) is removed and replaced by fresh medium. Fed batch culture is distinguished from simple batch culture in which all components for cell culturing (including the cells and all culture nutrients) are supplied to the culturing vessel at the start of the culturing process. Fed batch culture can be further distinguished from perfusion culturing insofar as the supernate is not removed from the culturing vessel during the process (in perfusion culturing, the cells are restrained in the culture by, e.g., filtration, encapsulation, anchoring to microcarriers etc. and the culture medium is continuously or intermittently introduced and removed from the culturing vessel).

Further, the cells of the culture may be propagated according to any scheme or routine that may be suitable for the particular host cell and the particular production plan contemplated. Therefore, a single step or multiple step culture procedure may be employed. In a single step culture the host cells are inoculated into a culture environment and the processes are employed during a single production phase of the cell culture. Alternatively, a multi-stage culture can be used. In the multi-stage culture cells may be cultivated in a number of steps or phases. For instance, cells may be grown in a first step or growth phase culture wherein cells, possibly removed from storage, are inoculated into a medium suitable for promoting growth and high viability. The cells may be maintained in the growth phase for a suitable period of time by the addition of fresh medium to the host cell culture.

In certain embodiments, fed batch or continuous cell culture conditions may be devised to enhance growth of the mammalian cells in the growth phase of the cell culture. In the growth phase cells are grown under conditions and for a period of time that is maximized for growth. Culture conditions, such as temperature, pH, dissolved oxygen (dO_2) and the like, are those used with the particular host and will be apparent to the ordinarily skilled artisan. Generally, the pH is adjusted to a level between about 6.5 and 7.5 using either an acid (e.g., CO_2) or a base (e.g., $Na2CO_3$ or NaOH). A suitable temperature range for culturing mammalian cells such as CHO cells is between about 30° C. to 38° C., and a suitable dO_2 is between 5-90% of air saturation.

At a particular stage the cells may be used to inoculate a production phase or step of the cell culture. Alternatively, as described above the production phase or step may be continuous with the inoculation or growth phase or step.

The cell culture environment during the production phase of the cell culture is typically controlled. Thus, if a glycoprotein is produced, factors affecting cell specific productivity of the mammalian host cell may be manipulated such that the desired sialic acid content is achieved in the resulting glycoprotein. In a preferred aspect, the production phase of the cell culture process is preceded by a transition phase of the cell culture in which parameters for the production phase of the cell culture are engaged. Further details of this process are found in U.S. Pat. No. 5,721,121, and Chaderjian et al., *Biotechnol. Prog.* 21(2):550-3 (2005), the entire disclosures of which are expressly incorporated by reference herein.

Following fermentation proteins are purified. Procedures for purification of proteins from cell debris initially depend on the site of expression of the protein. Some proteins can be caused to be secreted directly from the cell into the surrounding growth media; others are made intracellularly. For the latter proteins, the first step of a purification process involves lysis of the cell, which can be done by a variety of methods, including mechanical shear, osmotic shock, or enzymatic treatments. Such disruption releases the entire contents of the cell into the homogenate, and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration. The same problem arises, although on a smaller scale, with directly secreted proteins due to the natural death of cells and release of intracellular host cell proteins and components in the course of the protein production run.

Once a clarified solution containing the protein of interest has been obtained, its separation from the other proteins produced by the cell is usually attempted using a combination of different chromatography techniques. These techniques separate mixtures of proteins on the basis of their charge, degree of hydrophobicity, or size. Several different chromatography resins are available for each of these techniques, allowing accurate tailoring of the purification scheme to the particular protein involved. The essence of each of these separation methods is that proteins can be caused either to move at different rates down a long column, achieving a

physical separation that increases as they pass further down the column, or to adhere selectively to the separation medium, being then differentially eluted by different solvents. In some cases, the desired protein is separated from impurities when the impurities specifically adhere to the column, and the protein of interest does not, that is, the protein of interest is present in the "flow-through." Thus, purification of recombinant proteins from the cell culture of mammalian host cells may include one or more affinity (e.g. protein A) and/or ion exchange chomarographic steps.

Ion exchange chromatography is a chromatographic technique that is commonly used for the purification of proteins. In ion exchange chromatography, charged patches on the surface of the solute are attracted by opposite charges attached to a chromatography matrix, provided the ionic strength of the surrounding buffer is low. Elution is generally achieved by increasing the ionic strength (i.e. conductivity) of the buffer to compete with the solute for the charged sites of the ion exchange matrix. Changing the pH and thereby altering the charge of the solute is another way to achieve elution of the solute. The change in conductivity or pH may be gradual (gradient elution) or stepwise (step elution). In the past, these changes have been progressive; i.e., the pH or conductivity is increased or decreased in a single direction.

For further details of the industrial purification of therapeutic antibodies see, for example, Fahrner et al., *Biotechnol. Genet. Eng. Rev.* 18:301-27 (2001), the entire disclosure of which is expressly incorporated by reference herein.

In addition to mammalian host cells, other eukaryotic organisms can be used as host cells for expression of the recombinant protein. For expression in yeast host cells, such as common baker's yeast or *Saccharomyces cerevisiae*, suitable vectors include episomally-replicating vectors based on the 2-micron plasmid, integration vectors, and yeast artificial chromosome (YAC) vectors. Other yeast suitable for recombinant production of heterologous proteins include *Schizosaccharomyces pombe* (Beach and Nurse, Nature, 290: 140 (1981); EP 139,383 published 2 May 1985); *Kluyveromyces* hosts (U.S. Pat. No. 4,943,529; Fleer et al., Bio/Technology, 2: 968 975 (1991)) such as, e.g., *K. lactis* (MW98-8C, CBS683, CBS4574; Louvencourt et al., J. Bacteriol., 737 (1983)), *K. fragilis* (ATCC 12,424), *K. bulgaricus* (ATCC 16,045), *K. wickeramii* (ATCC 24,178), *K. waltii* (ATCC 56,500), *K. drosophilarum* (ATCC 36,906; Van den Berg et al., Bio/Technology, 8: 135 (1990)), *K. thermotolerans*, and *K. marxianus; yarrowia* (EP 402,226); *Pichia pastoris* (EP 183,070; Sreekrishna et al., J. Basic Microbiol., 28: 265 278 (1988)); *Candida; Trichoderma reesia* (EP 244,234); *Neurospora crassa* (Case et al., Proc. Natl. Acad. Sci. USA, 76: 5259 5263 (1979)); *Schwanniomyces* such as *Schwanniomyces occidentalis* (EP 394,538 published 31 Oct. 1990); and filamentous fungi such as, e.g., *Neurospora, Penicillium, Tolypocladium* (WO 91/00357 published 10 Jan. 1991), and *Aspergillus* hosts such as *A. nidulans* (Ballance et al., Biochem. Biophys. Res. Commun., 112: 284 289 (1983); Tilburn et al., Gene, 26: 205 221 (1983); Yelton et al., Proc. Natl. Acad. Sci. USA, 81: 1470 1474 (1984)) and *A. niger* (Kelly and Hynes, EMBO J., 4: 475 479 (1985)). Methylotropic yeasts are suitable herein and include, but are not limited to, yeast capable of growth on methanol selected from the genera consisting of *Hansenula, Candida, Kloeckera, Pichia, Saccharomyces, Torulopsis*, and *Rhodotorula*. A list of specific species that are exemplary of this class of yeasts may be found in C. Anthony, The Biochemistry of Methylotrophs, 269 (1982). Expression systems for the listed and other yeasts are well known in the art and/or are commercially available.

For expression in insect host cells, such as Sf9 cells, suitable vectors include baculoviral vectors. For expression in plant host cells, particularly dicotyledonous plant hosts, such as tobacco, suitable expression vectors include vectors derived from the Ti plasmid of *Agrobacterium tumefaciens.*

The methods of the present invention also extend to cultures of prokaryotic host cells. Prokaryotic host cells suitable for expressing antibodies and other proteins to be protected by means of the instant invention include Archaebacteria and Eubacteria, such as Gram-negative or Gram-positive organisms. Examples of useful bacteria include *Escherichia* (e.g., *E. coli*), *Bacilli* (e.g., *B. subtilis*), *Enterobacteria, Pseudomonas* species (e.g., *P. aeruginosa*), *Salmonella typhimurium, Serratia marcescans, Klebsiella, Proteus, Shigella, Rhizobia, Vitreoscilla*, or *Paracoccus*. In one embodiment, gram-negative cells are used. Examples of *E. coli* strains include strain W3110 (Bachmann, Cellular and Molecular Biology, vol. 2 (Washington, D.C.: American Society for Microbiology, 1987), pp. 1190-1219; ATCC Deposit No. 27,325) and derivatives thereof, including strain 33D3 having genotype W3110 ΔfhuA (ΔtonA) ptr3 lac Iq lacL8 ΔompTΔ(nmpc-fepE) degP41 kanR (U.S. Pat. No. 5,639,635). Other strains and derivatives thereof, such as *E. coli* 294 (ATCC 31,446), *E. coli* B, *E. coli* 1776 (ATCC 31,537) and *E. coli* RV308 (ATCC 31,608) are also suitable. These examples are illustrative rather than limiting. Methods for constructing derivatives of any of the above-mentioned bacteria having defined genotypes are known in the art and described in, for example, Bass et al., Proteins, 8:309-314 (1990). It is generally necessary to select the appropriate bacteria taking into consideration replicability of the replicon in the cells of a bacterium. For example, *E. coli, Serratia*, or *Salmonella* species can be suitably used as the host when well known plasmids such as pBR322, pBR325, pACYC177, or pKN410 are used to supply the replicon. Typically the host cell should secrete minimal amounts of proteolytic enzymes, and additional protease inhibitors may desirably be incorporated in the cell culture.

Methods for the production, recovery and purification of recombinant proteins from non-mammalian host cell cultures are also well known in the art. If the polypeptide is produced in a non-mammalian cell, e.g., a microorganism such as fungi or *E. coli*, the polypeptide will be recovered inside the cell or in the periplasmic space (Kipriyanov and Little, *Molecular Biotechnology,* 12: 173 201 (1999); Skerra and Pluckthun, *Science,* 240: 1038 1040 (1988)). Hence, it is necessary to release the protein from the cells to the extracellular medium by extraction such as cell lysis. Such disruption releases the entire contents of the cell into the homogenate, and in addition produces subcellular fragments that are difficult to remove due to their small size. These are generally removed by differential centrifugation or by filtration.

Cell lysis is typically accomplished using mechanical disruption techniques such as homogenization or head milling. While the protein of interest is generally effectively liberated, such techniques have several disadvantages (Engler, *Protein Purification Process Engineering*, Harrison eds., 37 55 (1994)). Temperature increases, which often occur during processing, may result in inactivation of the protein. Moreover, the resulting suspension contains a broad spectrum of contaminating proteins, nucleic acids, and polysaccharides. Nucleic acids and polysaccharides increase solution viscosity, potentially complicating subsequent processing by centrifugation, cross-flow filtration, or chromatography. Complex associations of these contaminants with the protein of interest can complicate the purification process and result in unacceptably low yields. Improved methods for purification of heterologous polypeptides from microbial fermentation

broth or homogenate are described, for example, in U.S. Pat. No. 7,169,908, the entire disclosure of which is expressly incorporated herein by reference.

It is emphasized that the fermentation, recovery and purification methods described herein are only for illustration purposes. The methods of the present invention can be combined with any manufacturing process developed for the production, recovery and purification of recombinant proteins.

2. Antibodies

In a preferred embodiment, the methods of the present invention are used to prevent the reduction of inter- and/or intrachain disulfide bonds of antibodies, including therapeutic and diagnostic antibodies. Antibodies within the scope of the present invention include, but are not limited to: anti-HER2 antibodies including Trastuzumab (HERCEPTIN®) (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285-4289 (1992), U.S. Pat. No. 5,725,856); anti-CD20 antibodies such as chimeric anti-CD20 "C2B8" as in U.S. Pat. No. 5,736,137 (RITUXAN®), a chimeric or humanized variant of the 2H7 antibody as in U.S. Pat. No. 5,721,108B1, or Tositumomab (BEXXAR®); anti-IL-8 (St John et al., *Chest,* 103:932 (1993), and International Publication No. WO 95/23865); anti-VEGF antibodies including humanized and/or affinity matured anti-VEGF antibodies such as the humanized anti-VEGF antibody huA4.6.1 AVASTIN® (Kim et al., *Growth Factors,* 7:53-64 (1992), International Publication No. WO 96/30046, and WO 98/45331, published Oct. 15, 1998); anti-PSCA antibodies (WO01/40309); anti-CD40 antibodies, including S2C6 and humanized variants thereof (WO00/75348); anti-CD11a (U.S. Pat. No. 5,622,700, WO 98/23761, Steppe et al., *Transplant Intl.* 4:3-7 (1991), and Hourmant et al., *Transplantation* 58:377-380 (1994)); anti-IgE (Presta et al., *J. Immunol.* 151:2623-2632 (1993), and International Publication No. WO 95/19181); anti-CD18 (U.S. Pat. No. 5,622,700, issued Apr. 22, 1997, or as in WO 97/26912, published Jul. 31, 1997); anti-IgE (including E25, E26 and E27; U.S. Pat. No. 5,714,338, issued Feb. 3, 1998 or U.S. Pat. No. 5,091,313, issued Feb. 25, 1992, WO 93/04173 published Mar. 4, 1993, or International Application No. PCT/US98/13410 filed Jun. 30, 1998, U.S. Pat. No. 5,714,338); anti-Apo-2 receptor antibody (WO 98/51793 published Nov. 19, 1998); anti-TNF-α antibodies including cA2 (REMICADE®), CDP571 and MAK-195 (See, U.S. Pat. No. 5,672,347 issued Sep. 30, 1997, Lorenz et al., *J. Immunol.* 156(4):1646-1653 (1996), and Dhainaut et al., *Crit. Care Med.* 23(9):1461-1469 (1995)); anti-Tissue Factor (TF) (European Patent No. 0 420 937 B1 granted Nov. 9, 1994); anti-human $\alpha_4\beta_7$ integrin (WO 98/06248 published Feb. 19, 1998); anti-EGFR (chimerized or humanized 225 antibody as in WO 96/40210 published Dec. 19, 1996); anti-CD3 antibodies such as OKT3 (U.S. Pat. No. 4,515,893 issued May 7, 1985); anti-CD25 or anti-tac antibodies such as CHI-621 (SIMULECT®) and (ZENAPAX®) (See U.S. Pat. No. 5,693,762 issued Dec. 2, 1997); anti-CD4 antibodies such as the cM-7412 antibody (Choy et al., *Arthritis Rheum* 39(1):52-56 (1996)); anti-CD52 antibodies such as CAMPATH-1H (Riechmann et al., *Nature* 332:323-337 (1988)); anti-Fc receptor antibodies such as the M22 antibody directed against FcγRI as in Graziano et al., *J. Immunol.* 155(10):4996-5002 (1995); anti-carcinoembryonic antigen (CEA) antibodies such as hMN-14 (Sharkey et al., *Cancer Res.* 55(23 Suppl): 5935s-5945s (1995); antibodies directed against breast epithelial cells including huBrE-3, hu-Mc 3 and CHL6 (Ceriani et al., *Cancer Res.* 55(23): 5852s-5856s (1995); and Richman et al., *Cancer Res.* 55(23 Supp): 5916s-5920s (1995)); antibodies that bind to colon carcinoma cells such as C242 (Litton et al., *Eur J. Immunol.* 26(1):1-9 (1996)); anti-CD38 antibodies, e.g. AT 13/5 (Ellis et al., *J. Immunol.* 155(2):925-937 (1995)); anti-CD33 antibodies such as Hu M195 (Jurcic et al., *Cancer Res* 55(23 Suppl): 5908s-5910s (1995) and CMA-676 or CDP771; anti-CD22 antibodies such as LL2 or LymphoCide (Juweid et al., *Cancer Res* 55(23 Suppl): 5899s-5907s (1995)); anti-Ep-CAM antibodies such as 17-1A (PANOREX®); anti-GpIIb/IIIa antibodies such as abciximab or c7E3 Fab (REOPRO®); anti-RSV antibodies such as MEDI-493 (SYNAGIS®); anti-CMV antibodies such as PROTOVIR®; anti-HIV antibodies such as PRO542; anti-hepatitis antibodies such as the anti-Hep B antibody OSTAVIR®; anti-CA 125 antibody OvaRex; anti-idiotypic GD3 epitope antibody BEC2; anti-αvβ3 antibody VITAXIN®; anti-human renal cell carcinoma antibody such as ch-G250; ING-1; anti-human 17-1A antibody (3622W94); anti-human colorectal tumor antibody (A33); anti-human melanoma antibody R24 directed against GD3 ganglioside; anti-human squamous-cell carcinoma (SF-25); and anti-human leukocyte antigen (HLA) antibodies such as Smart ID10 and the anti-HLA DR antibody Oncolym (Lym-1). The preferred target antigens for the antibody herein are: HER2 receptor, VEGF, IgE, CD20, CD11a, and CD40.

Many of these antibodies are widely used in clinical practice to treat various diseases, including cancer.

In certain specific embodiments, the methods of the present invention are used for the production of the following antibodies and recombinant proteins.

Anti-CD20 Antibodies

Rituximab (RITUXAN®) is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen. Rituximab is the antibody called "C2B8" in U.S. Pat. No. 5,736,137 issued Apr. 7, 1998 (Anderson et al.). Rituximab is indicated for the treatment of patients with relapsed or refractory low-grade or follicular, CD20-positive, B cell non-Hodgkin's lymphoma. In vitro mechanism of action studies have demonstrated that rituximab binds human complement and lyses lymphoid B cell lines through complement-dependent cytotoxicity (CDC) (Reff et al., *Blood* 83(2):435-445 (1994)). Additionally, it has significant activity in assays for antibody-dependent cellular cytotoxicity (ADCC). More recently, rituximab has been shown to have anti-proliferative effects in tritiated thymidine incorporation assays and to induce apoptosis directly, while other anti-CD19 and CD20 antibodies do not (Maloney et al., *Blood* 88(10):637a (1996)). Synergy between rituximab and chemotherapies and toxins has also been observed experimentally. In particular, rituximab. sensitizes drug-resistant human B cell lymphoma cell lines to the cytotoxic effects of doxorubicin, CDDP, VP-1 6, diphtheria toxin and ricin (Demidem et al., *Cancer Chemotherapy & Radiopharmaceuticals* 12(3):177-186 (1997)). In vivo preclinical studies have shown that rituximab depletes B cells from the peripheral blood, lymph nodes, and bone marrow of cynomolgus monkeys, presumably through complement and cell-mediated processes (Reff et al., *Blood* 83(2):435-445 (1994)).

Patents and patent publications concerning CD20 antibodies include U.S. Pat. Nos. 5,776,456, 5,736,137, 6,399,061, and 5,843,439, as well as U.S. patent application Nos. US 2002/0197255A1, US 2003/0021781A1, US 2003/0082172 A1, US 2003/0095963 A1, US 2003/0147885 A1 (Anderson et al.); U.S. Pat. No. 6,455,043B1 and WO00/09160 (Grillo-Lopez, A.); WO00/27428 (Grillo-Lopez and White); WO00/27433 (Grillo-Lopez and Leonard); WO00/44788 (Braslawsky et al.); WO01/10462 (Rastetter, W.); WO01/10461 (Rastetter and White); WO01/10460 (White and Grillo-Lopez); U.S. application No. US2002/0006404 and WO02/04021 (Hanna and Hariharan); U.S. application No. US2002/0012665 A1 and WO01/74388 (Hanna, N.); U.S. application

No. US 2002/0058029 A1 (Hanna, N.); U.S. application No. US 2003/0103971 A1 (Hariharan and Hanna); U.S. application No. US2002/0009444A1, and WO01/80884 (Grillo-Lopez, A.); WO01/97858 (White, C.); U.S. application No. US2002/0128488A1 and WO02/34790 (Reff, M.); W) 02/060955 (Braslawsky et al.); WO2/096948 (Braslawsky et al.); WO02/079255 (Reff and Davies); U.S. Pat. No. 6,171,586B1, and WO98/56418 (Lam et al.); WO98/58964 (Raju, S.); WO99/22764 (Raju, S.); WO99/51642, U.S. Pat. No. 6,194,551B1, U.S. Pat. No. 6,242,195B1, U.S. Pat. No. 6,528,624B1 and U.S. Pat. No. 6,538,124 (Idusogie et al.); WO00/42072 (Presta, L.); WO00/67796 (Curd et al.); WO01/03734 (Grillo-Lopez et al.); U.S. application No. US 2002/0004587A1 and WO01/77342 (Miller and Presta); U.S. application No. US2002/0197256 (Grewal, I.); U.S. application No. US 2003/0157108 A1 (Presta, L.); U.S. Pat. Nos. 6,090,365B1, 6,287,537B1, 6,015,542, 5,843,398, and 5,595,721, (Kaminski et al.); U.S. Pat. Nos. 5,500,362, 5,677,180, 5,721,108, and 6,120,767 (Robinson et al.); U.S. Pat. No. 6,410,391B1 (Raubitschek et al.); U.S. Pat. No. 6,224,866B1 and WO00/20864 (Barbera-Guillem, E.); WO01/13945 (Barbera-Guillem, E.); WO00/67795 (Goldenberg); U.S. application No. US 2003/01339301 A1 and WO00/74718 (Goldenberg and Hansen); WO00/76542 (Golay et al.); WO01/72333 (Wolin and Rosenblatt); U.S. Pat. No. 6,368,596B1 (Ghetie et al.); U.S. application No. US2002/0041847 A1, (Goldenberg, D.); U.S. application No. US2003/0026801A1 (Weiner and Hartmann); WO02/102312 (Engleman, E.); U.S. patent application No. 2003/0068664 (Albitar et al.); WO03/002607 (Leung, S.); WO 03/049694 and US 2003/0185796 A1 (Wolin et al.); WO03/061694 (Sing and Siegall); US 2003/0219818 A1 (Bohen et al.); US 2003/0219433 A1 and WO 03/068821 (Hansen et al.) each of which is expressly incorporated herein by reference. See, also, U.S. Pat. No. 5,849,898 and EP application no. 330,191 (Seed et al.); U.S. Pat. No. 4,861,579 and EP332,865A2 (Meyer and Weiss); U.S. Pat. No. 4,861,579 (Meyer et al.) and WO95/03770 (Bhat et al.).

Publications concerning therapy with Rituximab include: Perotta and Abuel "Response of chronic relapsing ITP of 10 years duration to Rituximab" Abstract #3360 *Blood* 10(1) (part 1-2): p. 88B (1998); Stashi et al., "Rituximab chimeric anti-CD20 monoclonal antibody treatment for adults with chronic idiopathic thrombocytopenic purpura" *Blood* 98(4): 952-957 (2001); Matthews, R. "Medical Heretics" New Scientist (7 Apr. 2001); Leandro et al., "Clinical outcome in 22 patients with rheumatoid arthritis treated with B lymphocyte depletion" *Ann Rheum Dis* 61:833-888 (2002); Leandro et al., "Lymphocyte depletion in rheumatoid arthritis: early evidence for safety, efficacy and dose response. *Arthritis & Rheumatism* 44(9): 5370 (2001); Leandro et al., "An open study of B lymphocyte depletion in systemic lupus erythematosus", *Arthritis & Rheumatism* 46(1):2673-2677 (2002); Edwards and Cambridge "Sustained improvement in rheumatoid arthritis following a protocol designed to deplete B lymphocytes" *Rheumatology* 40:205-211 (2001); Edwards et al., "B-lymphocyte depletion therapy in rheumatoid arthritis and other autoimmune disorders" *Biochem. Soc. Trans.* 30(4):824-828 (2002); Edwards et al., "Efficacy and safety of Rituximab, a B-cell targeted chimeric monoclonal antibody: A randomized, placebo controlled trial in patients with rheumatoid arthritis. *Arthritis & Rheumatism* 46(9): S197 (2002); Levine and Pestronk "IgM antibody-related polyneuropathies: B-cell depletion chemotherapy using Rituximab" *Neurology* 52: 1701-1704 (1999); DeVita et al., "Efficacy of selective B cell blockade in the treatment of rheumatoid arthritis" *Arthritis & Rheumatism* 46:2029-2033 (2002); Hidashida et al., "Treatment of DMARD-Refractory rheumatoid arthritis with rituximab." Presented at the Annual Scientific Meeting of the American College of Rheumatology; October 24-29; New Orleans, La. 2002; Tuscan, J. "Successful treatment of Infliximab-refractory rheumatoid arthritis with rituximab" Presented at the Annual Scientific Meeting of the American College of Rheumatology; October 24-29; New Orleans, La. 2002. Sarwal et al., *N. Eng. J. Med.* 349(2):125-138 (Jul. 10, 2003) reports molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling.

In various embodiments, the invention provides pharmaceutical compositions comprising humanized 2H7 anti-CD20 antibodies. In specific embodiments, the humanized 2H7 antibody is an antibody listed in Table 2.

TABLE 2

Humanized anti-CD20 Antibody and Variants Thereof

2H7 variant	V_L SEQ ID NO.	V_H SEQ ID NO.	Full L chain SEQ ID NO.	Full H chain SEQ ID NO.
A	1	2	6	7
B	1	2	6	8
C	3	4	9	10
D	3	4	9	11
F	3	4	9	12
G	3	4	9	13
H	3	5	9	14
I	1	2	6	15

Each of the antibody variants A, B and I of Table 2 comprises the light chain variable sequence (V_L):

(SEQ ID NO: 1)
DIQMTQSPSSLSASVGDRVTITCRASSSVSYMHWYQQKPGKAPKPLIYA

PSNLASGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQWSFNPPTFG

QGTKVEIKR;
and

the heavy chain variable sequence (V_H):

(SEQ ID NO: 2)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA

IYPGNGDTSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV

YYSNSYWYFDVWGQGTLVTVSS.

Each of the antibody variants C, D, F and G of Table 2 comprises the light chain variable sequence (V_L):

(SEQ ID NO: 3)
DIQMTQSPSSLSASVGDRVTITCRASSSVSYLHWYQQKPGKAPKPLIYAP

SNLASGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQWAFNPPTFGQG

TKVEIKR,
and

the heavy chain variable sequence (V_H):

(SEQ ID NO: 4)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSASYWYFDVWGQGTLVTVSS.

The antibody variant H of Table 2 comprises the light chain variable sequence (V_L) of SEQ ID NO:3 (above) and the heavy chain variable sequence (V_H):

(SEQ ID NO: 5)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSYRYWYFDVWGQGTLVTVSS

Each of the antibody variants A, B and I of Table 2 comprises the full length light chain sequence:

(SEQ ID NO: 6)
DIQMTQSPSSLSASVGDRVTITCRASSSVSYMHWYQQKPGKAPKPLIYAP
SNLASGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQWSFNPPTFGQG
TKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVD
NALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGL
SSPVTKSFNRGEC.

Variant A of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 7)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGDTSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSNSYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK.

Variant B of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 8)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGDTSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSNSYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIAATISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK.

Variant I of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 15)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGDTSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSNSYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVLHQDWLNGKEYKCKVSNAALPAPIAATISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK.

Each of the antibody variants C. D, F, G and H of Table 2 comprises the full length light chain sequence:

(SEQ ID NO: 9)
DIQMTQSPSSLSASVGDRVTITCRASSSVSYLHWYQQKPGKAPKPLIYAP
SNLASGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQWAFNPPTFGQG
TKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVD
NALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQGL
SSPVTKSFNRGEC.

Variant C of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 10)
EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSASYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIAATISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK.

Variant D of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 11)

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSASYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVLHQDWLNGKEYKCAVSNKALPAPIEATISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK.

Variant F of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 12)

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSASYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVLHQDWLNGKEYKCKVSNAALPAPIAATISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK.

Variant G of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 13)

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSASYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVLHQDWLNGKEYKCKVSNAALPAPIAATISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHWHYTQKSLSLSP
GK.

Variant H of Table 2 comprises the full length heavy chain sequence:

(SEQ ID NO: 14)

EVQLVESGGGLVQPGGSLRLSCAASGYTFTSYNMHWVRQAPGKGLEWVGA
IYPGNGATSYNQKFKGRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARVV
YYSYRYWYFDVWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCL
VKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGT
QTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPP
KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ
YNATYRVVSVLTVLHQDWLNGKEYKCKVSNAALPAPIAATISKAKGQPRE
PQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTP
PVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSP
GK.

In certain embodiments, the humanized 2H7 antibody of the invention further comprises amino acid alterations in the IgG Fc and exhibits increased binding affinity for human FcRn over an antibody having wild-type IgG Fc, by at least 60 fold, at least 70 fold, at least 80 fold, more preferably at least 100 fold, preferably at least 125 fold, even more preferably at least 150 fold to about 170 fold.

The N-glycosylation site in IgG is at Asn297 in the C_H2 domain. Humanized 2H7 antibody compositions of the present invention include compositions of any of the preceding humanized 2H7 antibodies having a Fc region, wherein about 80-100% (and preferably about 90-99%) of the antibody in the composition comprises a mature core carbohydrate structure which lacks fucose, attached to the Fc region of the glycoprotein. Such compositions were demonstrated herein to exhibit a surprising improvement in binding to FcγRIIIA(F158), which is not as effective as FcγRIIIA (V158) in interacting with human IgG. FcγRIIIA (F158) is more common than FcγRIIIA (V158) in normal, healthy African Americans and Caucasians. See Lehrnbecher et al., *Blood* 94:4220 (1999). Historically, antibodies produced in Chinese Hamster Ovary Cells (CHO), one of the most commonly used industrial hosts, contain about 2 to 6% in the population that are nonfucosylated. YB2/0 and Lec13, however, can produce antibodies with 78 to 98% nonfucosylated species. Shinkawa et al., *J Bio. Chem.* 278 (5), 3466-347 (2003), reported that antibodies produced in YB2/0 and Lec13 cells, which have less FUT8 activity, show significantly increased ADCC activity in vitro. The production of antibodies with reduced fucose content are also described in e.g., Li et al., (GlycoFi) "Optimization of humanized IgGs in glycoengineered *Pichia pastoris*" in Nature Biology online publication 22 Jan. 2006; Niwa R. et al., *Cancer Res.* 64(6):2127-2133 (2004); US 2003/0157108 (Presta); U.S. Pat. No. 6,602,684 and US 2003/0175884 (Glycart Biotechnology); US 2004/0093621, US 2004/0110704, US 2004/0132140 (all of Kyowa Hakko Kogyo).

A bispecific humanized 2H7 antibody encompasses an antibody wherein one arm of the antibody has at least the antigen binding region of the H and/or L chain of a humanized 2H7 in antibody of the invention, and the other arm has V region binding specificity for a second antigen. In specific embodiments, the second antigen is selected from the group consisting of CD3, CD64, CD32A, CD16, NKG2D or other NK activating ligands.

Anti-HER2 Antibodies

A recombinant humanized version of the murine HER2 antibody 4D5 (huMAb4D5-8, rhuMAb HER2, trastuzumab or HERCEPTIN®; U.S. Pat. No. 5,821,337) is clinically active in patients with HER2-overexpressing metastatic breast cancers that have received extensive prior anti-cancer therapy (Baselga et al., *J. Clin. Oncol.* 14:737-744 (1996)). Trastuzumab received marketing approval from the Food and Drug Administration (FDA) Sep. 25, 1998 for the treatment of patients with metastatic breast cancer whose tumors overexpress the HER2 protein. In November 2006, the FDA approved Herceptin as part of a treatment regimen containing doxorubicin, cyclophosphamide and paclitaxel, for the adjuvant treatment of patients with HER2-positive, node-positive breast cancer.

In one embodiment, the anti-HER2 antibody comprises the following V_L and V_H domain sequences:

humanized 2C4 version 574 antibody V_L (SEQ ID NO: 16)

DIQMTQSPSSLSASVGDRVTITC**KASQDVSIGVA**WYQQKPGKAPKLLIYS

ASYRYTGVPSRFSGSGSGTDFTLTISSLQPEDFATYYC**QQYYIYPYT**FGQ

GTKVEIK.

and humanized 2C4 version 574 antibody V_H (SEQ ID NO: 17)

EVQLVESGGGLVQPGGSLRLSCAAS**GFTFTDYTMD**WVRQAPGKGLEWVA**D**

VNPNSGGSIYNQRFKGRFTLSVDRSKNTLYLQMNSLRAEDTAVYYCARN**L**

GPSFYFDYWGQGTLVTVSS.

In another embodiment, the anti-HER2 antibody comprises the V_L (SEQ ID NO:18) and V_H (SEQ ID NO:19) domain sequences of trastuzumab as shown in FIG. **21** and FIG. **22**, respectively.

Other HER2 antibodies with various properties have been described in Tagliabue et al., *Int. J. Cancer* 47:933-937 (1991); McKenzie et al., *Oncogene* 4:543-548 (1989); Maier et al., *Cancer Res.* 51:5361-5369 (1991); Bacus et al., *Molecular Carcinogenesis* 3:350-362 (1990); Stancovski et al., *PNAS* (*USA*) 88:8691-8695 (1991); Bacus et al., *Cancer Research* 52:2580-2589 (1992); Xu et al., *Int. J. Cancer* 53:401-408 (1993); WO94/00136; Kasprzyk et al., *Cancer Research* 52:2771-2776 (1992); Hancock et al., *Cancer Res.* 51:4575-4580 (1991); Shawver et al., *Cancer Res.* 54:1367-1373 (1994); Arteaga et al., *Cancer Res.* 54:3758-3765 (1994); Harwerth et al., *J. Biol. Chem.* 267:15160-15167 (1992); U.S. Pat. No. 5,783,186; and Klapper et al., *Oncogene* 14:2099-2109 (1997).

Anti-VEGF Antibodies

The anti-VEGF antibodies may, for example, comprise the following sequences:

In one embodiment, the anti-VEGF antibody comprises the following V_L sequence (SEQ ID NO:20):

DIQMTQTTSS LSASLGDRVI ISCSASQDIS NYLNWYQQKP

DGTVKVLIYF TSSLHSGVPS RFSGSGSGTD YSLTISNLEP

EDIATYYCQQ YSTVPWTFGG GTKLEIKR;
and

the following V_H sequence (SEQ ID NO:21):

EIQLVQSGPE LKQPGETVRI SCKASGYTFT NYGMNWVKQA

PGKGLKWMGW INTYTGEPTY AADFKRRFTF SLETSASTAY

LQISNLKNDD TATYFCAKYP HYYGSSHWYF DVWGAGTTVT VSS.

In another embodiment, the anti-VEGF antibody comprises the following V_L sequence (SEQ ID NO:22):

DIQMTQSPSS LSASVGDRVT ITCSASQDIS NYLNWYQQKP

GKAPKVLIYF TSSLHSGVPS RFSGSGSGTD FTLTISSLQP

EDFATYYCQQ YSTVPWTFGQ GTKVEIKR;
and

the following V_H sequence (SEQ ID NO:23):

EVQLVESGGG LVQPGGSLRL SCAASGYTFT NYGMNWVRQA

PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY

LQMNSLRAED TAVYYCAKYP HYYGSSHWYF DVWGQGTLVT VSS.

In a third embodiment, the anti-VEGF antibody comprises the following V_L sequence (SEQ ID NO:24):

DIQLTQSPSS LSASVGDRVT ITCSASQDIS NYLNWYQQKP

GKAPKVLIYF TSSLHSGVPS RFSGSGSGTD FTLTISSLQP

EDFATYYCQQ YSTVPWTFGQ GTKVEIKR;
and

the following V_H sequence (SEQ ID NO:25):

EVQLVESGGG LVQPGGSLRL SCAASGYDFT HYGMNWVRQA

PGKGLEWVGW INTYTGEPTY AADFKRRFTF SLDTSKSTAY

LQMNSLRAED TAVYYCAKYP YYYGTSHWYF DVWGQGTLVT VSS.

Anti-CD11a Antibodies

The humanized anti-CD11a antibody efalizumab or Raptiva® (U.S. Pat. No. 6,037,454) received marketing approval from the Food and Drug Administration on Oct. 27, 2003 for the treatment for the treatment of psoriasis. One embodiment provides for an anti-human CD11a antibody comprising the V_L and V_H sequences of HuMHM24 below:

V_L (SEQ ID NO: 26):
DIQMTQSPSSLSASVGDRVTITCRASKTISKYLAWYQQKPGKAPKLLIYS

GSTLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQHNEYPLTFGQ

GTKVEIKR;
and

V_H (SEQ ID NO: 27):
EVQLVESGGGLVQPGGSLRLSCAASGYSFTGHWMNWVRQAPGKGLEWVGM

IHPSDSETRYNQKFKDRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARGI

YFYGTTYFDYWGQGTLVTVSS.

The anti-human CD11a antibody may comprise the V_H of SEQ ID NO:27 and the full length L chain of HuMHM24 having the sequence of:

(SEQ ID NO: 28)
DIQMTQSPSSLSASVGDRVTITCRASKTISKYLAWYQQKPGKAPKLLIYS

GSTLQSGVPSRFSGSGSGTDFTLTISSLQPEDFATYYCQQHNEYPLTFGQ

GTKVEIKRTVAAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKV

DNALQSGNSQESVTEQDSKDSTYSLSSTLTLSKADYEKHKVYACEVTHQG

LSSPVTKSFNRGEC,
or

the L chain above with the H chain having the sequence of:

(SEQ ID NO: 29)
EVQLVESGGGLVQPGGSLRLSCAASGYSFTGHWMNWVRQAPGKGLEWVGM

IHPSDSETRYNQKFKDRFTISVDKSKNTLYLQMNSLRAEDTAVYYCARGI

YFYGTTYFDYWGQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV

KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQ

TYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPELLGGPSVFLFPPK

PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY

NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREP

QVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP

VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

K.

Antibodies to the DR5 receptor (anti-DR5) antibodies can also be produced in accordance with the present invention. Such anti-DR5 antibodies specifically include all antibody variants disclosed in PCT Publication No. WO 2006/083971, such as the anti-DR5 antibodies designated Apomabs 1.1, 2.1, 3.1, 4.1, 5.1, 5.2, 5.3, 6.1, 6.2, 6.3, 7.1, 7.2, 7.3, 8.1, 8.3, 9.1, 1.2, 2.2, 3.2, 4.2, 5.2, 6.2, 7.2, 8.2, 9.2, 1.3, 2.2, 3.3, 4.3, 5.3, 6.3, 7.3, 8.3, 9.3, and 25.3, especially Apomab 8.3 and Apomab 7.3, preferably Apomab 7.3. The entire content of WO 2006/083971 is hereby expressly incorporated by reference.

3. Other Disulfide-Containing Proteins

In addition to antibodies, the methods of the present invention find utility in the manufacturing of other polypeptides including disulfide bonds. Representative examples of such polypeptides include, without limitation, the following therapeutic proteins: tissue plasminogen activators (t-PAs), such as human tissue plasminogen activator (htPA, alteplase, ACTIVASE®), a thrombolytic agent for the treatment of myocardial infarction; a TNKase™, a ht-PA variant with extended half-life and fibrin specificity for single-bolus administration; recombinant human growth hormone (rhGH, somatropin, NUTROPIN®, PROTROPIN®) for the treatment of growth hormone deficiency in children and adults; and recombinant human deoxyribonuclease I (DNase I) for the treatment of cystic fibrosis (CF).

Examples of disulfide-containing biologically important proteins include growth hormone, including human growth hormone and bovine growth hormone; growth hormone releasing factor; parathyroid hormone; thyroid stimulating hormone; lipoproteins; alpha-1-antitrypsin; insulin A-chain; insulin B-chain; proinsulin; follicle stimulating hormone; calcitonin; luteinizing hormone; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrands factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or human urine or tissue-type plasminogen activator (t-PA); bombesin; thrombin; hemopoietic growth factor; tumor necrosis factor-alpha and -beta; enkephalinase; RANTES (regulated on activation normally T-cell expressed and secreted); human macrophage inflammatory protein (MIP-1-alpha); a serum albumin such as human serum albumin; Muellerian-inhibiting substance; relaxin A-chain; relaxin B-chain; prorelaxin; mouse gonadotropin-associated peptide; a microbial protein, such as beta-lactamase; DNase; IgE; a cytotoxic T-lymphocyte associated antigen (CTLA), such as CTLA-4; inhibin; activin; vascular endothelial growth factor (VEGF); receptors for hormones or growth factors; Protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF-β; platelet-derived growth factor (PDGF); fibroblast growth factor such as aFGF and bFGF; epidermal growth factor (EGF); transforming growth factor (TGF) such as TGF-alpha and TGF-beta, including TGF-β1, TGF-β2, TGF-β3, TGF-β4, or TGF-β5; insulin-like growth factor-I and -II (IGF-I and IGF-II); des(1-3)-IGF-I (brain IGF-I), insulin-like growth factor binding proteins; CD proteins such as CD3, CD4, CD8, CD19, CD20, CD34, and CD40; erythropoietin; osteoinductive factors; immunotoxins; a bone morphogenetic protein (BMP); an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; integrins such as CD11a, CD11b, CD11c, CD18, an ICAM, VLA-4 and VCAM; a tumor associated antigen such as HER2, HER3 or HER4 receptor; and fragments of any of the above-listed polypeptides.

4. General Methods for the Recombinant Production of Antibodies

The antibodies and other recombinant proteins herein can be produced by well known techniques of recombinant DNA technology. Thus, aside from the antibodies specifically identified above, the skilled practitioner could generate antibodies directed against an antigen of interest, e.g., using the techniques described below.

Antigen Selection and Preparation

The antibody herein is directed against an antigen of interest. Preferably, the antigen is a biologically important polypeptide and administration of the antibody to a mammal suffering from a disease or disorder can result in a therapeutic benefit in that mammal. However, antibodies directed against nonpolypeptide antigens (such as tumor-associated glycolipid antigens; see U.S. Pat. No. 5,091,178) are also contemplated. Where the antigen is a polypeptide, it may be a transmembrane molecule (e.g. receptor) or ligand such as a growth factor. Exemplary antigens include those proteins described in section (3) below. Exemplary molecular targets for antibodies encompassed by the present invention include CD proteins such as CD3, CD4, CD8, CD19, CD20, CD22, CD34, CD40; members of the ErbB receptor family such as the EGF receptor, HER2, HER3 or HER4 receptor; cell adhesion molecules such as LFA-1, Mac1, p150, 95, VLA-4, ICAM-1, VCAM and αv/133 integrin including either α or β subunits thereof (e.g. anti-CD11a, anti-CD18 or anti-CD11b antibodies); growth factors such as VEGF; IgE; blood group

antigens; flk2/flt3 receptor; obesity (OB) receptor; mpl receptor; CTLA-4; protein C, or any of the other antigens mentioned herein. Antigens to which the antibodies listed above bind are specifically included within the scope herein.

Soluble antigens or fragments thereof, optionally conjugated to other molecules, can be used as immunogens for generating antibodies. For transmembrane molecules, such as receptors, fragments of these (e.g. the extracellular domain of a receptor) can be used as the immunogen. Alternatively, cells expressing the transmembrane molecule can be used as the immunogen. Such cells can be derived from a natural source (e.g. cancer cell lines) or may be cells which have been transformed by recombinant techniques to express the transmembrane molecule.

Other antigens and forms thereof useful for preparing antibodies will be apparent to those in the art.

Polyclonal Antibodies

Polyclonal antibodies are preferably raised in animals by multiple subcutaneous (sc) or intraperitoneal (ip) injections of the relevant antigen and an adjuvant. It may be useful to conjugate the antigen to a protein that is immunogenic in the species to be immunized, e.g., keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, or soybean trypsin inhibitor using a bifunctional or derivatizing agent, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, $SOCl_2$, or $R^1N{=}C{=}NR$, where R and R^1 are different alkyl groups.

Animals are immunized against the antigen, immunogenic conjugates, or derivatives by combining, e.g., 100 μg or 5 μg of the protein or conjugate (for rabbits or mice, respectively) with 3 volumes of Freund's complete adjuvant and injecting the solution intradermally at multiple sites. One month later the animals are boosted with ⅕ to 1/10 the original amount of antigen or conjugate in Freund's complete adjuvant by subcutaneous injection at multiple sites. Seven to 14 days later the animals are bled and the serum is assayed for antibody titer. Animals are boosted until the titer plateaus. Preferably, the animal is boosted with the conjugate of the same antigen, but conjugated to a different protein and/or through a different cross-linking reagent. Conjugates also can be made in recombinant cell culture as protein fusions. Also, aggregating agents such as alum are suitably used to enhance the immune response.

Monoclonal Antibodies

Monoclonal antibodies may be made using the hybridoma method first described by Kohler et al., *Nature,* 256:495 (1975), or may be made by recombinant DNA methods (U.S. Pat. No. 4,816,567).

In the hybridoma method, a mouse or other appropriate host animal, such as a hamster or macaque monkey, is immunized as hereinabove described to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the protein used for immunization. Alternatively, lymphocytes may be immunized in vitro. Lymphocytes then are fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)).

The hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. For example, if the parental myeloma cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine (HAT medium), which substances prevent the growth of HGPRT-deficient cells.

Preferred myeloma cells are those that fuse efficiently, support stable high-level production of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. Among these, preferred myeloma cell lines are murine myeloma lines, such as those derived from MOPC-21 and MPC-11 mouse tumors available from the Salk Institute Cell Distribution Center, San Diego, Calif. USA, and SP-2 or X63-Ag8-653 cells available from the American Type Culture Collection, Rockville, Md. USA. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies (Kozbor, *J. Immunol.,* 133:3001 (1984); Brodeur et al., *Monoclonal Antibody Production Techniques and Applications*, pp. 51-63 (Marcel Dekker, Inc., New York, 1987)).

Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. Preferably, the binding specificity of monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an in vitro binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA).

After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown in vivo as ascites tumors in an animal.

The monoclonal antibodies secreted by the subclones are suitably separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, Protein A-Sepharose, hydroxyapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography. Preferably the Protein A chromatography procedure described herein is used.

DNA encoding the monoclonal antibodies is readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of the monoclonal antibodies). The hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as *E. coli* cells, simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells.

The DNA also may be modified, for example, by substituting the coding sequence for human heavy- and light-chain constant domains in place of the homologous murine sequences (U.S. Pat. No. 4,816,567; Morrison, et al., *Proc. Natl. Acad. Sci. USA,* 81:6851 (1984)), or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide.

Typically such non-immunoglobulin polypeptides are substituted for the constant domains of an antibody, or they are substituted for the variable domains of one antigen-combining site of an antibody to create a chimeric bivalent antibody comprising one antigen-combining site having specificity for an antigen and another antigen-combining site having specificity for a different antigen.

In a further embodiment, monoclonal antibodies can be isolated from antibody phage libraries generated using the techniques described in McCafferty et al., *Nature,* 348:552-554 (1990). Clackson et al., *Nature,* 352:624-628 (1991) and Marks et al., *J. Mol. Biol.,* 222:581-597 (1991) describe the isolation of murine and human antibodies, respectively, using phage libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks et al., *Bio/Technology,* 10:779-783 (1992)), as well as combinatorial infection and in vivo recombination as a strategy for constructing very large phage libraries (Waterhouse et al., *Nuc. Acids. Res.,* 21:2265-2266 (1993)). Thus, these techniques are viable alternatives to traditional hybridoma techniques for isolation of monoclonal antibodies.

Humanized and Human Antibodies

A humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers (Jones et al., *Nature,* 321:522-525 (1986); Riechmann et al., *Nature,* 332:323-327 (1988); Verhoeyen et al., *Science,* 239:1534-1536 (1988)), by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

The choice of human variable domains, both light and heavy, to be used in making the humanized antibodies is very important to reduce antigenicity. According to the so-called "best-fit" method, the sequence of the variable domain of a rodent antibody is screened against the entire library of known human variable-domain sequences. The human sequence which is closest to that of the rodent is then accepted as the human FR for the humanized antibody (Sims et al., *J. Immunol.,* 151:2296 (1993)). Another method uses a particular framework derived from the consensus sequence of all human antibodies of a particular subgroup of light or heavy chains. The same framework may be used for several different humanized antibodies (Carter et al., *Proc. Natl. Acad. Sci. USA,* 89:4285 (1992); Presta et al., *J. Immunol.,* 151:2623 (1993)).

It is further important that antibodies be humanized with retention of high affinity for the antigen and other favorable biological properties. To achieve this goal, according to a preferred method, humanized antibodies are prepared by a process of analysis of the parental sequences and various conceptual humanized products using three-dimensional models of the parental and humanized sequences. Three-dimensional immunoglobulin models are commonly available and are familiar to those skilled in the art. Computer programs are available which illustrate and display probable three-dimensional conformational structures of selected candidate immunoglobulin sequences. Inspection of these displays permits analysis of the likely role of the residues in the functioning of the candidate immunoglobulin sequence, i.e., the analysis of residues that influence the ability of the candidate immunoglobulin to bind its antigen. In this way, FR residues can be selected and combined from the recipient and import sequences so that the desired antibody characteristic, such as increased affinity for the target antigen(s), is achieved. In general, the CDR residues are directly and most substantially involved in influencing antigen binding.

Alternatively, it is now possible to produce transgenic animals (e.g., mice) that are capable, upon immunization, of producing a full repertoire of human antibodies in the absence of endogenous immunoglobulin production. For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region (J_H) gene in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production.

Transfer of the human germ-line immunoglobulin gene array in such germ-line mutant mice will result in the production of human antibodies upon antigen challenge. See, e.g., Jakobovits et al., *Proc. Natl. Acad. Sci. USA,* 90:2551 (1993); Jakobovits et al., *Nature,* 362:255-258 (1993); Bruggermann et al., *Year in Immuno.,* 7:33 (1993); and Duchosal et al., *Nature* 355:258 (1992). Human antibodies can also be derived from phage-display libraries (Hoogenboom et al., *J. Mol. Biol.,* 227:381 (1991); Marks et al., *J. Mol. Biol.,* 222:581-597 (1991); Vaughan et al., *Nature Biotech* 14:309 (1996)).

Antibody Fragments

Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived via proteolytic digestion of intact antibodies (see, e.g., Morimoto et al., *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan et al., *Science,* 229:81 (1985)). However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form $F(ab')_2$ fragments (Carter et al., Bio/Technology 10:163-167 (1992)). According to another approach, $F(ab')_2$ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner. In other embodiments, the antibody of choice is a single chain Fv fragment (scFv) (see WO 93/16185).

Multispecific Antibodies

Multispecific antibodies have binding specificities for at least two different antigens. While such molecules normally will only bind two antigens (i.e. bispecific antibodies, BsAbs), antibodies with additional specificities such as trispecific antibodies are encompassed by this expression when used herein.

Methods for making bispecific antibodies are known in the art. Traditional production of full length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Millstein et al., *Nature,* 305:537-539 (1983)). Because of the random assortment of immunoglobulin heavy and light chains, these hybridomas (quadromas) produce a potential mixture of 10 different antibody molecules, of which only one has the correct bispecific structure. Purification of the correct molecule, which is usually done by affinity chromatography steps, is rather cumbersome, and the product yields are low. Similar procedures are disclosed in WO 93/08829, and in Traunecker et al., *EMBO J.,* 10:3655-3659 (1991).

According to another approach described in WO96/27011, the interface between a pair of antibody molecules can be engineered to maximize the percentage of heterodimers which are recovered from recombinant cell culture. The preferred interface comprises at least a part of the C_H3 domain of an antibody constant domain. In this method, one or more small amino acid side chains from the interface of the first

antibody molecule are replaced with larger side chains (e.g. tyrosine or tryptophan). Compensatory "cavities" of identical or similar size to the large side chain(s) are created on the interface of the second antibody molecule by replacing large amino acid side chains with smaller ones (e.g. alanine or threonine). This provides a mechanism for increasing the yield of the heterodimer over other unwanted end-products such as homodimers.

Bispecific antibodies include cross-linked or "heteroconjugate" antibodies. For example, one of the antibodies in the heteroconjugate can be coupled to avidin, the other to biotin. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells (U.S. Pat. No. 4,676,980), and for treatment of HIV infection (WO 91/00360, WO 92/200373, and EP 03089). Heteroconjugate antibodies may be made using any convenient cross-linking methods. Suitable cross-linking agents are well known in the art, and are disclosed in U.S. Pat. No. 4,676,980, along with a number of cross-linking techniques.

Techniques for generating bispecific antibodies from antibody fragments have also been described in the literature. For example, bispecific antibodies can be prepared using chemical linkage. Brennan et al., *Science,* 229: 81 (1985) describe a procedure wherein intact antibodies are proteolytically cleaved to generate $F(ab')_2$ fragments. These fragments are reduced in the presence of the dithiol complexing agent sodium arsenite to stabilize vicinal dithiols and prevent intermolecular disulfide formation. The Fab' fragments generated are then converted to thionitrobenzoate (TNB) derivatives. One of the Fab'-TNB derivatives is then reconverted to the Fab'-thiol by reduction with mercaptoethylamine and is mixed with an equimolar amount of the other Fab'-TNB derivative to form the bispecific antibody. The bispecific antibodies produced can be used as agents for the selective immobilization of enzymes.

Recent progress has facilitated the direct recovery of Fab'-SH fragments from *E. coli*, which can be chemically coupled to form bispecific antibodies. Shalaby et al., *J. Exp. Med.,* 175: 217-225 (1992) describe the production of a fully humanized bispecific antibody $F(ab')_2$ molecule. Each Fab' fragment was separately secreted from *E. coli* and subjected to directed chemical coupling in vitro to form the bispecific antibody. The bispecific antibody thus formed was able to bind to cells overexpressing the ErbB2 receptor and normal human T cells, as well as trigger the lytic activity of human cytotoxic lymphocytes against human breast tumor targets.

Various techniques for making and isolating bispecific antibody fragments directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers. Kostelny et al., *J. Immunol.,* 148(5):1547-1553 (1992). The leucine zipper peptides from the Fos and Jun proteins were linked to the Fab' portions of two different antibodies by gene fusion. The antibody homodimers were reduced at the hinge region to form monomers and then re-oxidized to form the antibody heterodimers. This method can also be utilized for the production of antibody homodimers. The "diabody" technology described by Hollinger et al., *Proc. Natl. Acad. Sci. USA,* 90:6444-6448 (1993) has provided an alternative mechanism for making bispecific antibody fragments. The fragments comprise a heavy-chain variable domain (V_H) connected to a light-chain variable domain (V_L) by a linker which is too short to allow pairing between the two domains on the same chain. Accordingly, the V_H and V_L domains of one fragment are forced to pair with the complementary V_L and V_H domains of another fragment, thereby forming two antigen-binding sites. Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported. See Gruber et al., *J. Immunol.,* 152:5368 (1994). Alternatively, the antibodies can be "linear antibodies" as described in Zapata et al., *Protein Eng.* 8(10):1057-1062 (1995). Briefly, these antibodies comprise a pair of tandem Fd segments (V_H-C_H1-V_H-C_H1) which form a pair of antigen binding regions. Linear antibodies can be bispecific or monospecific.

Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared. Tutt et al., *J. Immunol.* 147: 60 (1991).

Immunoadhesins

The simplest and most straightforward immunoadhesin design combines the binding domain(s) of the adhesin (e.g. the extracellular domain (ECD) of a receptor) with the hinge and Fc regions of an immunoglobulin heavy chain. Ordinarily, when preparing the immunoadhesins of the present invention, nucleic acid encoding the binding domain of the adhesin will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence, however N-terminal fusions are also possible.

Typically, in such fusions the encoded chimeric polypeptide will retain at least functionally active hinge, C_H2 and C_H3 domains of the constant region of an immunoglobulin heavy chain. Fusions are also made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the C_H1 of the heavy chain or the corresponding region of the light chain. The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion, or binding characteristics of the immunoadhesin.

In a preferred embodiment, the adhesin sequence is fused to the N-terminus of the Fc domain of immunoglobulin G_1 (IgG_1). It is possible to fuse the entire heavy chain constant region to the adhesin sequence. However, more preferably, a sequence beginning in the hinge region just upstream of the papain cleavage site which defines IgG Fc chemically (i.e. residue 216, taking the first residue of heavy chain constant region to be 114), or analogous sites of other immunoglobulins is used in the fusion. In a particularly preferred embodiment, the adhesin amino acid sequence is fused to (a) the hinge region and C_H2 and C_H3 or (b) the C_H1, hinge, C_H2 and C_H3 domains, of an IgG heavy chain.

For bispecific immunoadhesins, the immunoadhesins are assembled as multimers, and particularly as heterodimers or heterotetramers. Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of four basic units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each of the four units may be the same or different.

Various exemplary assembled immunoadhesins within the scope herein are schematically diagrammed below:

AC_L-AC_L;

AC_H-(AC_H, AC_L-AC_H, AC_L-V_HC_H, or V_LC_L-AC_H);

AC_L-AC_H-(AC_L-AC_H, AC_L-V_HC_H, V_LC_L-AC_H, or V_LC_L-V_HC_H)

AC_L-V_HC_H-(AC_H, or AC_L-V_HC_H, or V_LC_L-AC_H);

V_LC_L-AC_H-(AC_L-V_HC_H, or V_LC_L-AC_H); and

$(A\text{-}Y)_n$-$(V_LC_L\text{-}V_HC_H)_2$,

wherein each A represents identical or different adhesin amino acid sequences;

V_L is an immunoglobulin light chain variable domain;

V_H is an immunoglobulin heavy chain variable domain;

C_L is an immunoglobulin light chain constant domain;

C_H is an immunoglobulin heavy chain constant domain;

n is an integer greater than 1;

Y designates the residue of a covalent cross-linking agent.

In the interests of brevity, the foregoing structures only show key features; they do not indicate joining (J) or other domains of the immunoglobulins, nor are disulfide bonds shown. However, where such domains are required for binding activity, they shall be constructed to be present in the ordinary locations which they occupy in the immunoglobulin molecules.

Alternatively, the adhesin sequences can be inserted between immunoglobulin heavy chain and light chain sequences, such that an immunoglobulin comprising a chimeric heavy chain is obtained. In this embodiment, the adhesin sequences are fused to the 3' end of an immunoglobulin heavy chain in each arm of an immunoglobulin, either between the hinge and the C_H2 domain, or between the C_H2 and C_H3 domains. Similar constructs have been reported by Hoogenboom, et al., *Mol. Immunol.* 28:1027-1037 (1991).

Although the presence of an immunoglobulin light chain is not required in the immunoadhesins of the present invention, an immunoglobulin light chain might be present either covalently associated to an adhesin-immunoglobulin heavy chain fusion polypeptide, or directly fused to the adhesin. In the former case, DNA encoding an immunoglobulin light chain is typically coexpressed with the DNA encoding the adhesin-immunoglobulin heavy chain fusion protein. Upon secretion, the hybrid heavy chain and the light chain will be covalently associated to provide an immunoglobulin-like structure comprising two disulfide-linked immunoglobulin heavy chain-light chain pairs. Methods suitable for the preparation of such structures are, for example, disclosed in U.S. Pat. No. 4,816,567, issued 28 Mar. 1989.

Immunoadhesins are most conveniently constructed by fusing the cDNA sequence encoding the adhesin portion in-frame to an immunoglobulin cDNA sequence. However, fusion to genomic immunoglobulin fragments can also be used (see, e.g. Aruffo et al., *Cell* 61:1303-1313 (1990); and Stamenkovic et al., Cell 66:1133-1144 (1991)). The latter type of fusion requires the presence of Ig regulatory sequences for expression. cDNAs encoding IgG heavy-chain constant regions can be isolated based on published sequences from cDNA libraries derived from spleen or peripheral blood lymphocytes, by hybridization or by polymerase chain reaction (PCR) techniques. The cDNAs encoding the "adhesin" and the immunoglobulin parts of the immunoadhesin are inserted in tandem into a plasmid vector that directs efficient expression in the chosen host cells.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way.

All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated. The source of those cells identified in the following examples, and throughout the specification, by ATCC accession numbers is the American Type Culture Collection, Manassas, Va.

Example 1

Description of Materials and Methods

The following materials and methods were used in Examples 2-8 below.

Materials

Materials and devices used in the experiments described in the experimental examples include: stainless steel vials (mini-tanks, Flow Components, Dublin, Calif.; short (50 cc) and tall (55 cc)); dialysis tubing (Spectra/Por, 6-8000 MWCO, cat. #132645), 0.22 μm filter (Millipore Millipak Gamma Gold cat. # MPGL04GH2); phosphate buffered saline (PBS, EMD, cat. #6506); ethylenediaminetetraacetic acid (EDTA, Sigma, cat. # E4884); α-nicotinamide adenine dinucleotide phosphate (NADPH, Calbiochem, cat. #481973); dehydroepiandrosterone (DHEA, TCI, cat. # D0044); cupric sulfate (Sigma, cat. # C8027), glucose-6-phosphate (G6P, Calbiochem, cat. #346764); aurothioglucose (ATG, USP, cat. #1045508); aurothiomalate (ATM, Alfa Aesar, cat. #39740); reduced glutathione (GSH, J. T. Baker, cat. # M770-01); monobromobimane (mBB, Fluka, cat. #69898); histidine (J. T. Baker, cat. #2080-05); sodium sulfate (J. T. Baker, cat. #3897-05); Trx (Sigma, cat. # T8690); TrxR (Sigma, cat. # T9698). All chemicals and reagents were used as received with no further purification. Stock solutions of EDTA (250 mM, pH 7.5), $CuSO_4$ (10 mM), ATG (30 mM), ATM (30 mM), NADPH (75 mM), G6P (300 mM) were prepared for use in the mini-tank time course studies.

Generation of Cell Culture Fluid (CCF)

In order to generate ocrelizumab CCF for the various reduction studies, a representative small-scale fermentation process was utilized similar to the methods described previously (Chaderjian et al., 2005). Briefly, 3 liter glass stirred-tank Applikon® bioreactors fitted with pitched blade impellers were used for the inoculum-train and production cultures with the ocrelizumab media components. The bioreactors were outfitted with calibrated dissolved oxygen (DO), pH and temperature probes. DO, pH, temperature, and agitation rate were controlled using digital control units to the defined parameters of the ocrelizumab manufacturing process. The working volume for both the inoculum-train and production cultures was 1.5 L. Daily samples were analyzed on a NOVA Bioprofile blood gas analyzer to ensure the accuracy of the on-line value for pH and dissolved oxygen as well as to monitor the glucose, lactate, ammonium, glutamine, glutamate, and sodium concentrations in the cultures. Daily samples were also taken to monitor cell growth, viability, and titer. Cell growth was measured both by viable cell counts using a ViCell as well as on a packed cell volume (PCV) basis. Culture viability was determined by trypan blue exclusion on a ViCell instrument. Supernatant samples were assayed by an HPLC-based method to measure ocrelizumab titer values.

Harvested Cell Culture Fluid (HCCF) Preparation

Complete lysis of CCF was achieved by high pressure homogenization using a Microfluidics HC-8000 homogenizer. The pressure regulator of the instrument was set to 4,000-8,000 psi, and the CCF was pulled in through the homogenizer to obtain complete cell lysis (membrane breakage) after a single pass. The CCF homogenate was collected once water was purged through the system. The homogenate was transferred to centrifuge bottles and centrifuged in a Sorval RC-3B rotor centrifuge at 4,500 rpm for 30 minutes at 20° C. The centrate was decanted and then depth filtered followed by 0.22 μm sterile filtration using a peristaltic pump with silicon tubing to generate the final HCCF from the homogenized CCF (100% cell lysis). Alternatively, the CCF

was centrifuged straight from the fermentor without any homogenization and then the centrate was filtered with a sterile 0.22 μm filter to generate the HCCF.

Mini-Tank Handling

A laminar flow hood was used in handling all mini-tanks and all materials used in the HCCF incubation experiments were either autoclaved or rinsed using 70% isopropanol to minimize bacterial contamination.

Lactate Dehydrogenase Assay

For lactate dehydrogenase assay, see Babson & Babson (1973) and Legrand et al., (1992), which are hereby incorporated by reference.

Dialysis Experiment

A dialysis experiment was carried out in order to determine whether the components causing reduction of ocrelizumab were small molecules or macromolecules (i.e. enzymes). A sample of 3 mL of purified and formulated ocrelizumab (30.2 mg/mL) was dialyzed against 1 L of phosphate buffered saline (PBS, 10 mM pH 7.2) for 24 hours and the PBS was changed after 8 hours. The concentration of the ocrelizumab sample was then adjusted to 1 mg/mL using the absorbance at 280 nm. Aliquots were stored at −70° C. prior to use. Dialysis tubing was hydrated overnight in a 0.05% azide solution and rinsed with sterile water prior to use. The HCCF obtained from homogenization of CCF from a 3-L fermentor was thawed and filtered through a 0.22 μm Millipak filter using a peristaltic pump. Six short mini-tanks were filled with 30 mL of HCCF each. To each mini-tank, 500 μL of ocrelizumab sample in sealed dialysis tubing was added. The mini-tanks were sealed and loaded into a bench top mixer (Barnstead Lab-Line MAX Q 4000) operating at 35 rpm and ambient temperature. For each time-point, one mini-tank was removed from the mixer, and aliquots of the HCCF (in the mini-tank) and ocrelizumab sample (in the dialysis bag) were taken and stored at −70° C. until analyzed with the free thiol assay and the Bioanalyzer assay (described below).

Test Inhibitors for Reduction in a Small-Scale In Vitro System

A tall mini-tank was filled with 27 mL of HCCF. Depending on the experiment design, various reagents (NADPH, G6P, inhibitors of G6PD or TrxR) were added to the desired concentration, and the final volume in the mini-tank was brought to 30 mL with PBS (10 mM pH 7.2). The mini-tanks were sealed and loaded into a bench top mixer running at 35 rpm and ambient temperature. At each-time point for sampling, the exteriors of the mini-tanks were sterilized with 70% IPA and opened in a laminar flow hood for the removal of an aliquot. The mini-tanks were then re-sealed and loaded back into the bench top mixer. All aliquots were stored at −70° C. until analyzed with the free thiol assay and Bioanalyzer assay (described below).

In Vitro Trx/TrxRreductase Studies

A commercial TrxR (rat liver) solution (4 μM) was diluted with water to yield a 2.86 μM solution. Lyophilized Trx (human) was reconstituted with PBS (10 mM, pH 7.2) yielding a 500 μM solution. A solution of 20 mM NADPH and 10 mM ATG and ATM solutions were prepared in water.

In a black polypropylene 1.5 mL micro centrifuge tube, 437 μL PBS, 25 μL NADPH, 16 μL formulated ocrelizumab solution (30.2 mg/mL) and 5 μL Trx were gently mixed. The reaction was initiated by the addition of 17.5 μL TrxR. The reaction was incubated at room temperature for 24 hours. Aliquots of 20 μL were taken at each sampling time-point and stored at −70° C. until analyzed by the Bioanalyzer assay (see below). Controls were performed to determine if the enzymatic pathway was active when an enzyme was omitted by substituting an equal volume of PBS for either Trx and/or TrxR in the reaction mixture.

Inhibition of the Trx system was demonstrated using the same reaction conditions described above with the addition of 5 μL ATG or ATM. To demonstrate the inhibition of Trx system by Cu^{2+}, 2.5 μL of $CuSO_4$ (10 mM) was added to reaction mixture using the same enzymes but a different buffer (10 mM histidine, 10 mM Na_2SO_4, 137 mM NaCl, 2.5 mM KCl, pH 7.0) to prevent formation of insoluble $Cu_3(PO_4)_2$.

Free Thiol Assay

A standard curve using GSH was generated in PBS (10 mM, pH 6.0±0.05). From a 110 mM GSH solution, standards were prepared at concentrations of 0, 5.5, 11, 22, 44, 55, 110 and 550 μM through serial dilution. From an acetonitrile stock solution of mBB (10 mM stored at −20° C.), a 100 μM solution of mBB was prepared in PBS (10 mM, pH 10.0±0.05) and stored away from light.

In a black, flat bottomed 96 well plate, 100 μL of mBB was dispensed into each well. For the standard curve, 10 μL of standard GSH solution was added yielding a working pH of 8.0±0.2. For samples, 10 μL of sample was added to the wells. All wells were prepared in triplicate. The plate was incubated at room temperature for 1 hour in the dark then read using a fluorescence plate reader (Molecular Devices SpectraMax® Gemini XS) with an excitation wavelength of 390 nm and an emission wavelength of 490 nm. A linear standard curve was generated using the average result of the three standard wells plotted versus GSH concentration. Free thiol levels in samples were calculated from the linear equation of the standard curve using the average value of the three sample wells.

Bioanalyzer Assay

Capillary electrophoresis measurements were acquired using the Agilent 2100 Bioanalyzer. Sample preparation was carried out as described in the Agilent Protein 230 Assay Protocol (manual part number G2938-90052) with minor changes. HCCF samples were diluted, 1:4 and Protein A samples were diluted to 1.0 g/L with water prior to preparation. For HCCF samples at the denaturing step, 24 μL of a 50 mM iodoacetamide (IAM), 0.5% SDS solution was added in addition to the 2 μL of denaturing solution provided. For Protein A samples, 0.5% SDS with no JAM and 2 μL of denaturing solution were used. Digital gel-like images were generated using Agilent 2100 Expert software.

Stock Solutions for HCCF Hold Time Studies

Three separate stock solutions were used in the lab scale HCCF hold time studies: (1) 250 mM stock solution of EDTA (pH 7.4) prepared using EDTA, disodium dihydrate (Mallinckrodt, cat. #7727-06 or Sigma, cat. # E-5134) and EDTA, tetrasodium dihydrate (Sigma, cat. #E-6511), (2) 50 mM stock solution of cupric sulfate pentahydrate ($CuSO_4$, Sigma, cat. # C-8027), and (3) 1 M acetic acid solution (Mallinckrodt, cat. # V193).

Inhibitor Additions and Cell Culture Fluid (CCF) Blending

A stock solution of either 250 mM EDTA or 50 mM $CuSO_4$ was added to the CCF prior to homogenization to evaluate a range of final concentrations to prevent antibody disulfide reduction. Once the final HCCF was generated from the homogenized CCF, these solutions were then mixed with the HCCF generated from the non-homogenized CCF (also containing EDTA or $CuSO_4$) in order to dilute and decrease the total level of cell lysis to below the 100% maximum. Alternatively, a stock solution of 1 M acetic acid was added to a final blended HCCF solution (homogenized CCF and non-homogenized CCF) to decrease the pH of the solution to prevent antibody disulfide reduction.

Approximately 30-50 mL of each HCCF solution (containing EDTA, $CuSO_4$, acetic acid, or no addition for the control) was held in a 50 mL 316L stainless steel vial. The vial was sealed with a clamp, and the solution was not aerated or agitated. The vial was stored at room temperature (18-22° C.). At pre-determined time points, the solution was removed and purified over a lab scale protein A affinity resin.

Similar results can be obtained with other oxidizing agents, such as, for example, cystine and oxidized glutathione.

Air Sparging

To evaluate air sparging of the HCCF generated from homogenized CCF to prevent antibody disulfide reduction, 3-L glass or 15-L stainless steel vessels were utilized. Approximately 1-5 L of HCCF was 0.22 μm sterile filtered into each sterilized vessel. Experimental conditions were maintained at 18-22° C. and 50 (15-L fermentor) or 275 rpm (3-L fermentor) agitation either with or without pH control by the addition of carbon dioxide. Solutions were either sparged with air to increase the dissolved oxygen level to air saturation or with nitrogen (control) to remove any dissolved oxygen in solution. Gas flow to each vessel was variable dependent upon whether a constant aeration rate was used or a minimum level of dissolved oxygen was maintained. At pre-determined time points, 25-50 mL samples were removed from both vessels and purified over a lab scale protein A affinity resin prior to analysis.

Protein A Processing

Antibody in harvested cell culture fluid samples can be captured and purified using a specific affinity chromatography resin. Protein A resin (Millipore, Prosep-vA High Capacity) was selected as the affinity resin for antibody purification. The resin was packed in a 0.66 cm inner diameter glass column (Omnifit®) with a 14 cm bed height resulting in a 4.8 mL final column volume. Chromatography was performed using an AKTA Explorer 100 chromatography system (GE Healthcare).

The resin was exposed to buffers and HCCF at a linear flow rate between 350-560 cm/hr. The resin was equilibrated with 25 mM Tris, 25 mM NaCl, 5 mM EDTA, pH 7.1. For each purification, the resin was loaded between 5-15 mg antibody per mL of resin. The antibody concentration in the HCCF was determined using an immobilized protein A HPLC column (Applied Biosystems, POROS A). After loading, the resin was washed with 25 mM Tris, 25 mM NaCl, 5 mM EDTA, 0.5 M TMAC, pH 7.1, and then the antibody was eluted using 0.1M acetic acid, pH 2.9. Elution pooling was based on UV absorbance at 280 nm measured inline after the column. The purified elution pools were pH-adjusted using 1 M Sodium HEPES to pH 5.0-5.5. After regeneration of the resin with 0.1M phosphoric acid, the same or similar packed resins were used for subsequent purification of other HCCF solutions.

The antibody concentration in the purified protein A pool was measured using UV spectrometry at 280 nm. The purified protein A elution pools were analyzed by the Bioanalyzer assay to quantitate the percentage of intact antibody at 150 kDa molecular weight.

Example 2

Dialysis Experiment

A dialysis experiment was designed and carried out to determine if the reduction of ocrelizumab was caused by small reducing molecules or macromolecules (e.g., enzymes). In this dialysis experiment, purified intact ocrelizumab was placed in a dialysis bag with a molecular weight cut off (MWCO) of 7000 and incubated the dialysis bag in HCCF containing ocrelizumab in a stainless steel mini-tank. As shown in FIGS. **1** and **2**, the ocrelizumab inside the bag was not reduced after the incubation period (FIG. **1**), whereas the ocrelizumab outside the bag in the HCCF was significantly reduced soon after the incubation started. This was evidenced by the loss of intact ocrelizumab (~150 kDa) and the formation of ocrelizumab fragments (various combinations of heavy and light chains) (FIG. **2**). The mass spectrometry analysis of the ocrelizumab in the protein A elution pools from the reduced manufacturing runs indicated that those observed fragments were formed by reduction of only the inter-chain disulfide bonds.

The free thiol measurement showed that no free thiols were present inside the dialysis bag at the beginning of the incubation; however the levels of free thiols inside and outside the dialysis bag become comparable in less than five hours after the incubation started, indicating that the small molecule components in the HCCF are fully equilibrated inside and outside the dialysis bag (FIG. **3**). Since the reduction was observed only outside but not inside the dialysis bag with a MWCO of 7000 Da, the molecular weight of the reducing molecule(s) must be greater than 7000 Da. Thus, an enzymatic reaction is responsible for the reduction of ocrelizumab.

Example 3

Reduction of Ocrelizumab (rhuMAb 2H7, Variant A) by Trx/TrxR In Vitro

The Trx system was tested for its ability to reduce ocrelizumab in vitro by incubating intact ocrelizumab with Trx, TrxR, and NADPH. The Bioanalyzer results indicate that ocrelizumab was reduced in vitro by the Trx system (FIG. **5**). The rate of reduction in this in vitro system appears to be slower than that in the HCCF (for example when compared to the reduction shown in FIG. **2**). This is likely due to lower concentrations of the enzymes (Trx and Trx-R) and/or the buffer system used in the in vitro reaction because reaction rate of Trx system is dependent on both the enzyme concentrations and buffer systems.

Example 4

Inhibitors of the Trx System

(i) Inhibition of Reduction of Recombinant Antibody by Cupric Sulfate

Cupric sulfate is known for its ability to provide oxidizing redox potential and has been used in the cell culture processes to minimize free thiol (i.e., minimize unpaired cysteine) levels in recombinant antibody molecules (Chaderjian et al., 2005, supra). Cupric sulfate was tested for efficacy in inhibiting the Trx system in vitro and the subsequent reduction of ocrelizumab. In this in vitro reduction experiment, the buffer system was changed from PBS to histidine sulfate to avoid the formation of insoluble $Cu_3(PO_4)_2$. FIG. **8** shows that ocrelizumab was readily reduced by the Trx system in the histidine sulfate buffer (even faster than in PBS buffer). The addition of $CuSO_4$ to this reaction clearly inhibits the ocrelizumab reduction (FIG. **9**).

(ii) Inhibition of Reduction of Recombinant Antibody in HCCF by ATG and ATM

Two commercially available specific inhibitors of TrxR, aurothioglucose (ATG) and aurothiomalate (ATM), were tested for their ability to inhibit the Trx system in vitro and the reduction of ocrelizumab. Both ATG and ATM can effectively

inhibit the reduction of ocrelizumab in the assay described above (see FIGS. **6** and **7**). The addition of aurothioglucose or aurothiomalate, at a concentration of 1 mM to the same reaction mixture as described in the caption for FIG. **5** effectively inhibited the ocrelizumab reduction as shown in the digital gel-like image from Bioanalyzer analysis.

If the Trx system was active in the HCCF and reduced ocrelizumab as observed in the manufacturing runs resulting in reduced antibody molecules or in the lab scale experiments, both gold compounds (ATG and ATM) should be able to inhibit the reduction of ocrelizumab in HCCF. FIG. **10** shows that ocrelizumab was readily reduced in an HCCF from homogenized CCT generated from a 3-L fermentor after a period of incubation. However, the ocrelizumab reduction event was completely inhibited when either 1 mM ATG or ATM was added to the HCCF (FIGS. **11** and **12**). These results demonstrated that the Trx system is active in the HCCF and is directly responsible for the reduction of ocrelizumab.

Example 5

The Source of NADPH for Trx System Activity and the Roles of G6P and Glucose in Reduction Mechanism

The reduction of disulfides by the Trx system requires the reducing equivalents from NADPH (FIG. **4**). The main cellular metabolic pathway that provides NADPH for all reductive biosynthesis reactions is the pentose phosphate pathway. For the antibody reduction event to occur, the enzymes in this pathway must be still active in the HCCF in order to keep the Trx system active. At a minimum, the first step in the pentose phosphate pathway (catalyzed by G6PD) must be active to reduce $NADP^+$ to NADPH while converting G6P to 6-phosphogluconolactone. In addition, G6P is most likely produced from glucose and adenosine 5'-triphosphate (ATP) by the hexokinase activity in HCCF. The overall mechanism of ocrelizumab reduction is summarized in FIG. **4**.

The reducing activity in the HCCF appeared to be transitory in some cases and may be inhibited over time under certain storage conditions or after multiple freeze/thaw cycles. HCCF that has fully lost reducing activity provided an opportunity to explore the role of NADPH and G6P in the reduction of ocrelizumab by Trx system.

An HCCF from a large scale manufacturing run (the "beta" run) was subjected to several freeze/thaw cycles and used in an experiment designed to measure reduction; no ocrelizumab reduction was observed (FIG. **13**) despite its ability to bring about antibody reduction seen previously in freshly-thawed HCCF from this same fermentation. NADPH was added to this non-reducing HCCF at a concentration of 5 mM and the reduction event returned (FIG. **14**). Therefore, the Trx system is still intact and active in the HCCF where reduction no longer occurs, and capable of reducing protein and/or antibody if supplied with cofactors. Additionally, the reducing activity was lost over time as the NADPH source was depleted (presumably due to the oxidation of NADPH by all of the reductive reactions that compete for NADPH), and not because the Trx system was degraded or inactivated.

This was verified by another experiment. 10 mM G6P was added to a HCCF that had been repeatedly freeze-thawed from the beta run. This G6P addition reactivated the Trx system which subsequently reduced ocrelizumab in the HCCF incubation experiment (FIG. **15**). This demonstrated that the reduction of ocrelizumab in the HCCF was caused by the activities of both the Trx system and G6PD. Furthermore, G6PD is still active in a repeatedly freeze/thawed HCCF of the beta run; the loss of reduction activity in this a repeatedly freeze/thawed HCCF beta run appears to be due to the depletion of G6P, which thus eliminated the conversion of $NADP^+$ to NADPH.

In our studies, we have observed that EDTA can effectively inhibit the ocrelizumab reduction in the HCCF incubation experiment. As shown in FIG. **16**, the ocrelizumab was reduced after incubating the HCCF from a 12,000 L scale ocrelizumab manufacturing run (not repeatedly freeze/thawed and no loss of reducing activity) at ambient temperature for more than 19 hours. However, the reduction was completely inhibited when 20 mM EDTA was added to the 12 kL HCCF and held in a separate stainless steel minitank (FIG. **17**). In the first step of glycolysis, the hexokinase catalyzes the transfer of phosphate group from Mg2+-ATP to glucose, a reaction that requires the complexation of Mg2+ with ATP (Hammes & Kochavi, 1962a & 1962b, supra). Since EDTA is a metal ion chelator, especially for Mg2+, it can be an effective inhibitor of hexokinase. The observation that an excess amount of EDTA can effectively block the reduction indicates the involvement of hexokinase (i.e. providing G6P) in the mechanism of ocrelizumab reduction. Without being bound by this, or any other theory, EDTA blocks the reduction of ocrelizumab by eliminating the hexokinase activity and thereby reducing the G6P level available for G6PD, and subsequently the NADPH level available for the Trx system.

Although EDTA is every effective in blocking the reduction of ocrelizumab in fresh HCCF, it was unable to prevent the reduction of ocerlizumab in the beta run HCCF in which the Trx system activity was lost then reactivated by the addition of G6P. For example, the reduction of ocrelizumab was observed in an HCCF incubation experiment in which 5 mM G6P and 20 mM EDTA (final concentrations) were added to the beta run HCCF that had fully lost reducing activity (FIG. **18**). However, no reduction was seen in the control incubation experiment in which no G6P and EDTA were added. Without being bound by this or any other theory, the EDTA used in this manner may therefore inhibit neither the Trx system nor the G6PD, and may function as an inhibitor for hexokinase, which produces the G6P for the G6PD. Without G6P, the Trx system would not be supplied with the necessary NADPH for activity.

Example 6

Inhibition of Reduction of Recombinant Antibody by DHEA

Dehydroepiandrosterone (DHEA), as well as other similar G6PD inhibitors, effectively blocks G6PD activity (Gordon et al., 1995, supra). G6PD inhibitors also prevent the reduction of an antibody in HCCF, for example, ocrelizumab, by blocking the generation of NADPH. The ability of DHEA to inhibit the reduction of orcelizumab is demonstrated in an HCCF incubation experiment. Adding DHEA to a HCCF prevents antibody reduction.

DHEA is typically used in the concentration range from about 0.05 mM to about 5 mM. DHEA is also typically used in the concentration range from about 0.1 mM to about 2.5 mM.

Example 7

Inhibition of Reduction of Recombinant Antibody by (i) EDTA, (ii) Cupric Sulfate, and (iii) Acetic Acid Additions

Four different HCCFs were stored and held in the stainless steel vials. The solutions were similar in the amount of cell

lysis, which were generated by diluting HCCF from homogenized CCF with HCCF from non-homogenized CCF. For example, 150 mL of the first lysed solution was mixed with 50 mL of the second solution, respectively. The four HCCF mixtures evaluated in this study contained either: (1) 20 mM EDTA, (2) 30 μM $CuSO_4$, (3) 15 mM acetic acid (pH 5.5), and (4) no chemical inhibitor was added for the control solution. The ocrelizumab antibody from all four mixtures was purified immediately (t=0 hr) using protein A chromatography and then again after 20 hr and 40 hr of storage in the stainless steel vials. Purified protein A elution pools were analyzed by the Bioanalyzer assay to quantitate the percentage of intact antibody (150 kDa). The results showed that greater than 90% intact antibody was present in all four mixtures at the initial time point (FIG. **19**). However, at the 20 hr time point, intact antibody was not detected in the control mixture (without any addition) indicating reduction of the antibody disulfide bonds. In the three other mixtures, over 90% intact antibody was still detected at both 20 hr and 40 hr time points, demonstrating the prevention of disulfide bond reduction by all three inhibitors tested.

Example 8

Inhibition of Reduction of Recombinant Antibody by Air Sparging the HCCF

One HCCF mixture generated from homogenized CCF was stored and held in two separate 10 L stainless steel fermentors. One vessel was sparged with air while the other vessel was sparged with nitrogen gas. The ocrelizumab antibody was purified immediately (t=0 hr) from the initial mixture using protein A chromatography. At selected time points, 50 mL samples were removed from each vessel and the antibody was purified using protein A chromatography. Purified protein A elution pools were then analyzed by the Bioanalyzer assay to quantitate the percentage of intact antibody at 150 kDa. The results showed that approximately 85% intact antibody was present in the initial solution (FIG. **20**), indicating some early reduction of the antibody disulfide bonds prior to exposure to oxygen (i.e. sparged air in the fermentor). Once the mixture was sparged with air for two hours, greater than 90% intact antibody was measured for the remainder of the 36 hr study. In contrast, when the mixture was sparged with nitrogen gas, the antibody reduction event continued as measured at 2 hr (28% 150 kDa peak) and 6 hr (5% 150 kDa peak). These results demonstrated the prevention of disulfide bond reduction in the antibody when the HCCF mixture generated from homogenized CCF was exposed to oxygen.

Example 9

Design of Targeted siRNA or Antisense Nucleotide Trx Inhibitors

The design of targeted siRNAs or antisense nucleotides to the genes as found in CHO cells may be done by using publicly available sequences such as those for *E. coli* thioredoxin TrxA (SEQ ID NO:30), *E. coli* thioredoxin reductase TrxB (SEQ ID NO:31); mouse thioredoxin 1 (SEQ ID NO:32), mouse thioredoxin 2 (SEQ ID NO:33), mouse thioredoxin reductase 1 (SEQ ID NO:34), and mouse thioredoxin reductase 2 (SEQ ID NO:35). One of ordinary skill in the art can use these sequences to select sequences to design Trx inhibitors for targeting enzymes in different organisms and/or cells, such as CHO cells.

The sequence of *E. coli* Thioredoxin TrxA is:

(SEQ ID NO: 30)

```
ATG TTA CAC CAA CAA CGA AAC CAA CAC GCC AGG CTT ATT CCT GTG GAG

TTA TAT ATG AGC GAT AAA ATT ATT CAC CTG ACT GAC GAC AGT TTT GAC ACG

GAT GTA CTC AAA GCG GAC GGG GCG ATC CTC GTC GAT TTC TGG GCA GAG

TGG TGC GGT CCG TGC AAA ATG ATC GCC CCG ATT CTG GAT GAA ATC GCT

GAC GAA TAT CAG GGC AAA CTG ACC GTT GCA AAA CTG AAC ATC GAT CAA

AAC CCT GGC ACT GCG CCG AAA TAT GGC ATC CGT GGT ATC CCG ACT CTG CTG

CTG TTC AAA AAC GGT GAA GTG GCG GCA ACC AAA GTG GGT GCA CTG TCT

AAA GGT CAG TTG AAA GAG TTC CTC GAC GCT AAC CTG GCG TAA.
```

The sequence of *E. coli* Thioredoxin TrxB is:

(SEQ ID NO: 31)

```
ATG GGC ACG ACC AAA CAC AGT AAA CTG CTT ATC CTG GGT TCA GGC CCG

GCG GGA TAC ACC GCT GCT GTC TAC GCG GCG CGC GCC AAC CTG CAA CCT

GTG CTG ATT ACC GGC ATG GAA AAA GGC GGC CAA CTG ACC ACC ACC ACG

GAA GTG GAA AAC TGG CCT GGC GAT CCA AAC GAT CTG ACC GGT CCG TTA

TTA ATG GAG CGC ATG CAC GAA CAT GCC ACC AAG TTT GAA ACT GAG ATC

ATT TTT GAT CAT ATC AAC AAG GTG GAT CTG CAA AAC CGT CCG TTC CGT CTG

AAT GGC GAT AAC GGC GAA TAC ACT TGC GAC GCG CTG ATT ATT GCC ACC

GGA GCT TCT GCA CGC TAT CTC GGC CTG CCC TCT GAA GAA GCC TTT AAA GGC
```

-continued

CGT GGG GTT TCT GCT TGT GCA ACC TGC GAC GGT TTC TTC TAT CGC AAC CAG

AAA GTT GCG GTC ATC GGC GGC GGC AAT ACC GCG GTT GAA GAG GCG TTG

TAT CTG TCT AAC ATC GCT TCG GAA GTG CAT CTG ATT CAC CGC CGT GAC GGT

TTC CGC GCG GAA AAA ATC CTC ATT AAG CGC CTG ATG GAT AAA GTG GAG

AAC GGC AAC ATC ATT CTG CAC ACC AAC CGT ACG CTG GAA GAA GTG ACC

GGC GAT CAA ATG GGT GTC ACT GGC GTT CGT CTG CGC GAT ACG CAA AAC

AGC GAT AAC ATC GAG TCA CTC GAC GTT GCC GGT CTG TTT GTT GCT ATC GGT

CAC AGC CCG AAT ACT GCG ATT TTC GAA GGG CAG CTG GAA CTG GAA AAC

GGC TAC ATC AAA GTA CAG TCG GGT ATT CAT GGT AAT GCC ACC CAG ACC

AGC ATT CCT GGC GTC TTT GCC GCA GGC GAC GTG ATG GAT CAC ATT TAT CGC

CAG GCC ATT ACT TCG GCC GGT ACA GGC TGC ATG GCA GCA CTT GAT GCG

GAA CGC TAC CTC GAT GGT TTA GCT GAC GCA AAA TAA.

The sequence of mouse thioredoxin 1 is:

(SEQ ID NO: 32)

ATGGTGAAGCTGATCGAGAGCAAGGAAGCTTTTCAGGAGGCCCTGGCCGC

CGCGGGAGACAAGCTTGTCGTGGTGGACTTCTCTGCTACGTGGTGTGGAC

CTTGCAAAATGATCAAGCCCTTCTTCCATTCCCTCTGTGACAAGTATTCC

AATGTGGTGTTCCTTGAAGTGGATGTGGATGACTGCCAGGATGTTGCTGC

AGACTGTGAAGTCAAATGCATGCCGACCTTCCAGTTTTATAAAAAGGGTC

AAAAGGTGGGGGAGTTCTCCGGTGCTAACAAGGAAAAGCTTGAAGCCTCT

ATTACTGAATATGCCTAA.

The sequence of mouse thioreodoxin 2 is:

(SEQ ID NO: 33)

ATGGCTCAGCGGCTCCTCCTGGGGAGGTTCCTGACCTCAGTCATCTCCAG

GAAGCCTCCTCAGGGTGTGTGGGCTTCCCTCACCTCTAAGACCCTGCAGA

CCCCTCAGTACAATGCTGGTGGTCTAACAGTAATGCCCAGCCCAGCCCGG

ACAGTACACACCACCAGAGTCTGTTTGACGACCTTTAACGTCCAGGATGG

ACCTGACTTTCAAGACAGAGTTGTCAACAGTGAGACACCAGTTGTTGTGG

ACTTTCATGCACAGTGGTGTGGCCCCTGCAAGATCCTAGGACCGCGGCTA

GAGAAGATGGTCGCCAAGCAGCACGGGAAGGTGGTCATGGCCAAAGTGGA

CATTGACGATCACACAGACCTTGCCATTGAATATGAGGTGTCAGCTGTGC

CTACCGTGCTAGCCATCAAGAACGGGGACGTGGTGGACAAGTTTGTGGGG

ATCAAGGACGAGGACCAGCTAGAAGCCTTCCTGAAGAAGCTGATTGGCTG

A.

The sequence of mouse thioredoxin reductase 1 is:

(SEQ ID NO: 34)

ATGAATGGCTCCAAAGATCCCCCTGGGTCCTATGACTTCGACCTGATCAT

CATTGGAGGAGGCTCAGGAGGACTGGCAGCAGCTAAGGAGGCAGCCAAAT

TTGACAAGAAAGTGCTGGTCTTGGATTTTGTCACACCGACTCCTCTTGGG

-continued

ACCAGATGGGGTCTCGGAGGAACGTGTGTGAATGTGGGTTGCATACCTAA

GAAGCTGATGCACCAGGCAGCTTTGCTCGGACAAGCTCTGAAAGACTCGC

GCAACTATGGCTGGAAAGTCGAAGACACAGTGAAGCATGACTGGGAGAAA

ATGACGGAATCTGTGCAGAGTCACATCGGCTCGCTGAACTGGGGCTACCG

CGTAGCTCTCCGGGAGAAAAAGGTCGTCTATGAGAATGCTTACGGGAGGT

TCATTGGTCCTCACAGGATTGTGGCGACAAATAACAAAGGTAAAGAAAAA

ATCTATTCAGCAGAGCGGTTCCTCATCGCCACAGGTGAGAGGCCCCGCTA

CCTGGGCATCCCTGGAGACAAAGAGTACTGCATCAGCAGTGATGATCTTT

TCTCCTTGCCTTACTGCCCGGGGAAGACCCTAGTAGTTGGTGCATCCTAT

GTCGCCTTGGAATGTGCAGGATTTCTGGCTGGTATCGGCTTAGACGTCAC

TGTAATGGTGCGGTCCATTCTCCTTAGAGGATTTGACCAAGACATGGCCA

ACAAAATCGGTGAACACATGGAAGAACATGGTATCAAGTTTATAAGGCAG

TTCGTCCCAACGAAAATTGAACAGATCGAAGCAGGAACACCAGGCCGACT

CAGGGTGACTGCTCAATCCACAAACAGCGAGGAGACCATAGAGGGCGAAT

TTAACACAGTGTTGCTGGCGGTAGGAAGAGATTCTTGTACGAGAACTATT

GGCTTAGAGACCGTGGGCGTGAAGATAAACGAAAAAACCGGAAAGATACC

CGTCACGGATGAAGAGCAGACCAATGTGCCTTACATCTACGCCATCGGTG

ACATCCTGGAGGGGAAGCTAGAGCTGACTCCCGTAGCCATCCAGGCGGGG

AGATTGCTGGCTCAGAGGCTGTATGGAGGCTCCAATGTCAAATGTGACTA

TGACAATGTCCCAACGACTGTATTTACTCCTTTGGAATATGGCTGTTGTG

GCCTCTCTGAAGAAAAAGCCGTAGAGAAATTTGGGGAAGAAAATATTGAA

GTTTACCATAGTTTCTTTTGGCCATTGGAATGGACAGTCCCATCCCGGGA

TAACAACAAATGTTATGCAAAAATAATCTGCAACCTTAAAGACGATGAAC

GTGTCGTGGGCTTCCACGTGCTGGGTCCAAACGCTGGAGAGGTGACGCAG

GGCTTTGCGGCTGCGCTCAAGTGTGGGCTGACTAAGCAGCAGCTGGACAG

CACCATCGGCATCCACCCGGTCTGTGCAGAGATATTCACAACGTTGTCAG

TGACGAAGCGCTCTGGGGGAGACATCCTCCAGTCTGGCTGCTGA

The sequence of mouse thioredoxin reductase 2 is:

(SEQ ID NO: 35)

ATGGCGGCGATGGTGGCGGCGATGGTGGCGGCGCTGCGTGGACCCAGCAG

GCGCTTCCGGCCGCGGACACGGGCTCTGACACGCGGGACAAGGGGCGCGG

CGAGTGCAGCGGGAGGGCAGCAGAGCTTTGATCTCTTGGTGATCGGTGGG

GGATCCGGTGGCCTAGCTTGTGCCAAGGAAGCTGCTCAGCTGGGAAAGAA

GGTGGCTGTGGCTGACTATGTGGAACCTTCTCCCCGAGGCACCAAGTGGG

GCCTTGGTGGCACCTGTGTCAACGTGGGTTGCATACCCAAGAAGCTGATG

CATCAGGCTGCACTGCTGGGGGGCATGATCAGAGATGCTCACCACTATGG

CTGGGAGGTGGCCCAGCCTGTCCAACACAACTGGAAGACAATGGCAGAAG

CCGTGCAAAACCATGTGAAATCCTTGAACTGGGGTCATCGCGTCCAACTG

CAGGACAGGAAAGTCAAGTACTTTAACATCAAAGCCAGCTTTGTGGATGA

GCACACAGTTCGCGGTGTGGACAAAGGCGGGAAGGCGACTCTGCTTTCAG

CTGAGCACATTGTCATTGCTACAGGAGGACGGCCAAGGTACCCCACACAA

GTCAAAGGAGCCCTGGAATATGGAATCACAAGTGACGACATCTTCTGGCT

GAAGGAGTCCCCTGGGAAAACGTTGGTGGTTGGAGCCAGCTATGTGGCCC

TAGAGTGTGCTGGCTTCCTCACTGGAATTGGACTGGATACCACTGTCATG

ATGCGCAGCATCCCTCTCCGAGGCTTTGACCAGCAAATGTCATCTTTGGT

CACAGAGCACATGGAGTCTCATGGCACCCAGTTCCTGAAAGGCTGTGTCC

CCTCCCACATCAAAAAACTCCCAACTAACCAGCTGCAGGTCACTTGGGAG

GATCATGCTTCTGGCAAGGAAGACACAGGCACCTTTGACACTGTCCTGTG

GGCCATAGGGCGAGTTCCAGAAACCAGGACTTTGAATCTGGAGAAGGCTG

GCATCAGTACCAACCCTAAGAATCAGAAGATTATTGTGGATGCCCAGGAG

GCTACCTCTGTTCCCCACATCTATGCCATTGGAGATGTTGCTGAGGGGCG

GCCTGAGCTGACGCCCACAGCTATCAAGGCAGGAAAGCTTCTGGCTCAGC

GGCTCTTTGGGAAATCCTCAACCTTAATGGATTACAGCAATGTTCCCACA

ACTGTCTTTACACCACTGGAGTATGGCTGTGTGGGGCTGTCTGAGGAGGA

GGCTGTGGCTCTCCATGGCCAGGAGCATGTAGAGGTTTACCATGCATATT

ATAAGCCCCTAGAGTTCACGGTGGCGGATAGGGATGCATCACAGTGCTAC

ATAAAGATGGTATGCATGAGGGAGCCCCCACAACTGGTGCTGGGCCTGCA

CTTCCTTGGCCCCAACGCTGGAGAAGTCACCCAAGGATTTGCTCTTGGGA

TCAAGTGTGGGGCTTCATATGCACAGGTGATGCAGACAGTAGGGATCCAT

CCCACCTGCTCTGAGGAGGTGGTCAAGCTGCACATCTCCAAGCGCTCCGG

CCTGGAGCCTACTGTGACTGGTTGCTGA.

Example 10

In Vitro Trx/Trx Reductase Studies

Materials and Methods

A commercial TrxR (rat liver) solution (4 μM) was diluted with water to yield a 2.86 μM solution. Lyophilized Trx (human) was reconstituted with PBS (10 mM, pH 7.2) yielding a 500 μM solution. A solution of 20 mM NADPH and 10 mM ATG and ATM solutions were prepared in water.

In a black polypropylene 1.5 mL micro centrifuge tube, 437 μL reaction buffer (10 mM histidine, 10 mM Na2SO4, 137 mM NaCl, 2.5 mM KCl, pH 7.0), 25 μL NADPH, 16 μL formulated ocrelizumab solution (30.2 mg/mL) and 5 μL Trx were gently mixed. The reaction was initiated by the addition of 17.5 μL TrxR. The reaction was incubated at room temperature for 24 hours. Aliquots of 20 μL were taken at each sampling time-point and stored at −70° C. until analyzed by the Bioanalyzer assay.

Inhibition of the Trx system was demonstrated using the same reaction conditions described above with the addition of various inhibitors.

1. In Vitro Activity of Thioredoxin System

FIG. **24** shows a digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that incubation of intact ocrelizumab ("2H7," a humanized anti-CD20 antibody, referred to as "Variant A" above) (1 mg/mL) with 0.1 μM TrxR (rat liver), 5 μM Trx (human) and 1 mM NADPH in 10 mM histidine sulfate buffer results in the reduction of ocrelizumab in less than one hour.

2. In Vitro Activity of Thioredoxin System Inhibited by Aurothioglucose

Aurothioglucose (ATG) was added to the ocrelizumab mixture described above, at the following concentrations: 1 mM; 0.6 μM (6:1 ATG:TrxR); 0.4 μM (4:1 ATG:TrxR); and 0.2 μM (2:1 ATG:TrxR).

As attested by the digital gel-like images from Bioanalyzer analysis shown in FIGS. **25-27**, aurothioglucose added at concentrations 1 mM, 0.6 μM, and 0.4 μM effectively inhibits the reduction of ocrelizumab by the thioredoxin system. However, as shown in FIG. **28**, under these experimental conditions aurothioglucose added at a concentration of 0.2 μM cannot inhibit ocrelizumab reduction after 24 hours.

3. In Vitro Activity of Thioredoxin System Inhibited by Aurothiomalate

Aurothiomalate (ATM) was added to the ocrelizumab mixture described above, at concentrations of 0.1 mM and 0.01 mM. As attested by the digital gel-like images from Bioanalyzer analysis shown in FIGS. **29** and **30**, ATM effectively inhibits the reduction of ocrelizumab by the thioredoxin system at both concentrations tested.

4. In Vitro Activity of Thioredoxin System Inhibited by $Cuso_4$

$CuSO_4$ was added to the ocrelizumab mixture described above, at concentrations of 20 μM (4:1 Cu^{2+}:Trx); 10 μm (2:1 Cu^{2+}:Trx); and 5 μM (1:1 Cu^{2+}:Trx). As shown in FIGS. **31-33**, $CuSO_4$ effectively inhibits thioredoxin-induced reduction of ocrelizumab at concentrations of 20 μM and 10 μM (FIGS. **31** and **32**), but the 5 μM concentration is insufficient to result in a complete inhibition of reduction (FIG. **33**).

5. In Vitro Activity of Thioredoxin System Inhibited by Cystamine

Cystamine was added to the ocrelizumab mixture describe above at the following concentrations: 532 μM (20:1 cystamine:2H7 (Variant A) disulfide); 266 μM (10:1 cystamine: 2H7 (Variant A) disulfide); 133 μM (5:1 cystamine:2H7 disulfide); and 26.6 μM (1:1 cystamine:2H7 (Variant A) disulfide). As shown in FIGS. **34-37**, cystamine effectively inhibits thioredoxin-induced reduction of ocrelizumab at concentrations of 532 μM (20:1 cystamine:2H7 (Variant A) disulfide) and 266 μM (10:1 cystamine:2H7 (Variant A)) (FIGS. **34** and **35**) but the 133 μM (5:1 cystamine:2H7 (Variant A) disulfide) and 26.6 μM (1:1 cystamine:2H7 (Variant A) disulfide) concentrations are insufficient to inhibit the reduction of ocrelizumab after 24 hours (FIGS. **36** and **37**).

6. In Vitro Activity of Thioredoxin System Inhibited by Cystine

Cystine was added to the ocrelizumab mixture described above at a concentration of 2.6 mM. As shown in FIG. **38**, at this concentration cystine effectively inhibits reduction of ocrelizumab by the thioredoxin system. It is noted that the minimum effective concentration of cystine (just as the effective minimum concentration of other inhibitors) depends on the actual circumstances, and might be different for different proteins, such as antibodies, and might vary depending on the timing of addition. Thus, for example, if cystine is added pre-lysis, the minimum effective concentration for antibody 2H7 (Variant A) is about 1.3 mM, for Apomab about 1 mM and for antibody Variant C about 4.5 mM. When cystine is added in the cell culture medium, the minimum effective concentration typically is somewhat higher, and is about 5.2 mM for 2H7 (Variant A), 6 mM for Apomab and 9 mM for antibody Variant C. Usually, for cystine, cystamine and oxidized glutathione (see below) the minimum effective inhibitory concentration is about 40× of the antibody concentration (in μM).

7. In Vitro Activity of Thioredoxin System Inhibited by Oxidized Glutathione (GSSG)

GSSG was added to the ocrelizumab mixture described above at a concentration of 2.6 mM. As shown in FIG. **39**, at this concentration GSSG effectively inhibits reduction of ocrelizumab by the thioredoxin system. It is noted, however, that the minimum effective concentration of oxidize glutathione (just as that of the other inhibitors) depends on the actual circumstances, such as, for example, on the nature of the protein (e.g. antibody) produced and the timing of addition. For example, for antibody 2H7 (Variant A) the minimum effective concentration is about 1.3 mM for addition prior to lysis.

8. In Vitro Activity of Enzymatic Reduction System

FIG. **40** shows a digital gel-like image from Bioanalyzer analysis (each lane representing a time point) showing that incubation of intact ocrelizumab ("2H7," a humanized anti-CD20 antibody, Variant A) (1 mg/mL) with 10 μg/mL hexokinase, 50 μg/mL glucose-6-phosphate dehydrogenase, 5 μM thioredoxin, 0.1 μM thoredoxin reductase, 2 mM glucose, 0.6 mM ATP, 2 mM Mg^{2+}, and 2 mM NADP in 50 mM histidine sulfate buffered at pH 7.38 results in the reduction of ocrelizumab in about one hour. Addition of 0.1 mM HDEA, a known glucose-6-phosphate dehydrogenase inhibitor does not inhibit the reduction.

9. In Vitro Activity of Enzymatic Reduction System Requires NADPH

As shown in the digital gel-like image from Bioanalyzer analysis of FIG. **41**, incubation of intact ocrelizumab (1 mg/mL) with 5 μM thioredoxin, 0.1 μM thioredoxin reductase, and 2 mM NADP in 50 mM histidine sulfate buffer at pH 7.38 does not result in the reduction of the ocrelizumab antibody. Reduction of ocrelizumab could not occur without hexokinase and glucose-6-phosphate dehydrogenase and their substrates to generate NADPH.

The invention illustratively described herein can suitably be practiced in the absence of any element or elements, limitation or limitations that is not specifically disclosed herein. Thus, for example, the terms "comprising," "including," "containing," etc. shall be read expansively and without limitation. Additionally, the terms and expressions employed herein have been used as terms of description and not of limitation, and there is no intention in the use of such terms and expressions of excluding any equivalent of the invention shown or portion thereof, but it is recognized that various modifications are possible within the scope of the invention claimed. Thus, it should be understood that although the present invention has been specifically disclosed by preferred embodiments and optional features, modifications and variations of the inventions embodied herein disclosed can be readily made by those skilled in the art, and that such modifications and variations are considered to be within the scope of the inventions disclosed herein. The inventions have been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure also form the part of these inventions. This includes within the generic description of each of the inventions a proviso or negative limitation that will allow removing any subject matter from the genus, regardless or whether or not the material to be removed was specifically recited. In addition, where features or aspects of an invention are described in terms of the Markush group, those schooled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. Further, when a reference to an aspect of the invention lists a range of individual members, as for example, 'SEQ ID NO:1 to SEQ ID NO:100, inclusive,' it is intended to be equivalent to listing every member of the list individually, and additionally it should be understood that every individual member may be excluded or included in the claim individually.

The steps depicted and/or used in methods herein may be performed in a different order than as depicted and/or stated. The steps are merely exemplary of the order these steps may occur. The steps may occur in any order that is desired such that it still performs the goals of the claimed invention.

From the description of the invention herein, it is manifest that various equivalents can be used to implement the concepts of the present invention without departing from its scope. Moreover, while the invention has been described with specific reference to certain embodiments, a person of ordinary skill in the art would recognize that changes can be made in form and detail without departing from the spirit and the scope of the invention. The described embodiments are considered in all respects as illustrative and not restrictive. It should also be understood that the invention is not limited to the particular embodiments described herein, but is capable of many equivalents, rearrangements, modifications, and substitutions without departing from the scope of the invention. Thus, additional embodiments are within the scope of the invention and within the following claims.

All U.S. patents and applications; foreign patents and applications; scientific articles; books; and publications mentioned herein are hereby incorporated by reference in their entirety as if each individual patent or publication was specifically and individually indicated to be incorporated by reference, including any drawings, figures and tables, as though set forth in full.

SEQUENCE LISTING

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<210> SEQ ID NO 1
<211> LENGTH: 107
<212> TYPE: PRT

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<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: light chain variable

<400> SEQUENCE: 1

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Met
20 25 30

His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro Leu Ile Tyr
35 40 45

Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
50 55 60

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
65 70 75 80

Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Phe Asn Pro Pro Thr
85 90 95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
100 105

<210> SEQ ID NO 2
<211> LENGTH: 122
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: heavy chain variable

<400> SEQUENCE: 2

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
20 25 30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
50 55 60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Val Val Tyr Tyr Ser Asn Ser Tyr Trp Tyr Phe Asp Val Trp
100 105 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser
115 120

<210> SEQ ID NO 3
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: light chain variable

<400> SEQUENCE: 3

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Leu
20 25 30

His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro Leu Ile Tyr
35 40 45

-continued

```
Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
    50                  55                  60

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
65                  70                  75                  80

Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp Ala Phe Asn Pro Pro Thr
                85                  90                  95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
            100                 105


<210> SEQ ID NO 4
<211> LENGTH: 122
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: heavy chain variable

<400> SEQUENCE: 4

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Ala Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120


<210> SEQ ID NO 5
<211> LENGTH: 122
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: heavy chain variable

<400> SEQUENCE: 5

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Tyr Arg Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120
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<210> SEQ ID NO 6
<211> LENGTH: 213
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length light chain

<400> SEQUENCE: 6

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Met
20 25 30

His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro Leu Ile Tyr
35 40 45

Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
50 55 60

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
65 70 75 80

Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp Ser Phe Asn Pro Pro Thr
85 90 95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro
100 105 110

Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr
115 120 125

Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys
130 135 140

Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
145 150 155 160

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
165 170 175

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
180 185 190

Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe
195 200 205

Asn Arg Gly Glu Cys
210

<210> SEQ ID NO 7
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain sequence

<400> SEQUENCE: 7

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
20 25 30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
50 55 60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

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Ala Arg Val Val Tyr Tyr Ser Asn Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
                325                 330                 335

Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450


<210> SEQ ID NO 8
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain

<400> SEQUENCE: 8

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
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1               5                  10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Asn Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
                325                 330                 335

Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430
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Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450


<210> SEQ ID NO 9
<211> LENGTH: 213
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length light chain

<400> SEQUENCE: 9

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Ser Ser Val Ser Tyr Leu
            20                  25                  30

His Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Pro Leu Ile Tyr
        35                  40                  45

Ala Pro Ser Asn Leu Ala Ser Gly Val Pro Ser Arg Phe Ser Gly Ser
    50                  55                  60

Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro Glu
65                  70                  75                  80

Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Trp Ala Phe Asn Pro Pro Thr
                85                  90                  95

Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala Pro
            100                 105                 110

Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly Thr
        115                 120                 125

Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala Lys
    130                 135                 140

Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln Glu
145                 150                 155                 160

Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser Ser
                165                 170                 175

Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
            180                 185                 190

Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser Phe
        195                 200                 205

Asn Arg Gly Glu Cys
    210


<210> SEQ ID NO 10
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain

<400> SEQUENCE: 10

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60
```

-continued

```
Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Ala Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro
                325                 330                 335

Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450


<210> SEQ ID NO 11
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
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<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain sequence

<400> SEQUENCE: 11

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Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Ala Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Ala Val Ser Asn Lys Ala Leu Pro Ala Pro
                325                 330                 335

Ile Glu Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400
```

-continued

```
Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450


<210> SEQ ID NO 12
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain sequence

<400> SEQUENCE: 12

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Ala Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
```

```
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Ala Ala Leu Pro Ala Pro
                325                 330                 335

Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450


<210> SEQ ID NO 13
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain sequence

<400> SEQUENCE: 13

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Ala Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220
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Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Ala Ala Leu Pro Ala Pro
                325                 330                 335

Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Trp His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450


<210> SEQ ID NO 14
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain

<400> SEQUENCE: 14

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Ala Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Tyr Arg Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125
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Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Ala Ala Leu Pro Ala Pro
                325                 330                 335

Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450


<210> SEQ ID NO 15
<211> LENGTH: 452
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: full length heavy chain

<400> SEQUENCE: 15

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
            20                  25                  30

Asn Met His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
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        35                  40                  45

Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Val Val Tyr Tyr Ser Asn Ser Tyr Trp Tyr Phe Asp Val Trp
            100                 105                 110

Gly Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro
        115                 120                 125

Ser Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr
    130                 135                 140

Ala Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr
145                 150                 155                 160

Val Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro
                165                 170                 175

Ala Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr
            180                 185                 190

Val Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn
        195                 200                 205

His Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser
    210                 215                 220

Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu
225                 230                 235                 240

Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu
                245                 250                 255

Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser
            260                 265                 270

His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu
        275                 280                 285

Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ala Thr
    290                 295                 300

Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn
305                 310                 315                 320

Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Ala Ala Leu Pro Ala Pro
                325                 330                 335

Ile Ala Ala Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln
            340                 345                 350

Val Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val
        355                 360                 365

Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val
    370                 375                 380

Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro
385                 390                 395                 400

Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr
                405                 410                 415

Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val
            420                 425                 430

Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu
        435                 440                 445

Ser Pro Gly Lys
    450
```

<210> SEQ ID NO 16
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 16

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Lys Ala Ser Gln Asp Val Ser Ile Gly
20 25 30

Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
35 40 45

Tyr Ser Ala Ser Tyr Arg Tyr Thr Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Tyr Ile Tyr Pro Tyr
85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
100 105

<210> SEQ ID NO 17
<211> LENGTH: 119
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 17

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Thr Asp Tyr
20 25 30

Thr Met Asp Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
35 40 45

Ala Asp Val Asn Pro Asn Ser Gly Gly Ser Ile Tyr Asn Gln Arg Phe
50 55 60

Lys Gly Arg Phe Thr Leu Ser Val Asp Arg Ser Lys Asn Thr Leu Tyr
65 70 75 80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
85 90 95

Ala Arg Asn Leu Gly Pro Ser Phe Tyr Phe Asp Tyr Trp Gly Gln Gly
100 105 110

Thr Leu Val Thr Val Ser Ser
115

<210> SEQ ID NO 18
<211> LENGTH: 107
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: light chain

<400> SEQUENCE: 18

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Val Asn Thr Ala
20 25 30

```
Val Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Ser Ala Ser Phe Leu Tyr Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Arg Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Tyr Thr Thr Pro Pro
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
            100                 105


<210> SEQ ID NO 19
<211> LENGTH: 120
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: heavy chain

<400> SEQUENCE: 19

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Asn Ile Lys Asp Thr
            20                  25                  30

Tyr Ile His Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Ala Arg Ile Tyr Pro Thr Asn Gly Tyr Thr Arg Tyr Ala Asp Ser Val
    50                  55                  60

Lys Gly Arg Phe Thr Ile Ser Ala Asp Thr Ser Lys Asn Thr Ala Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ser Arg Trp Gly Gly Asp Gly Phe Tyr Ala Met Asp Tyr Trp Gly Gln
            100                 105                 110

Gly Thr Leu Val Thr Val Ser Ser
        115                 120


<210> SEQ ID NO 20
<211> LENGTH: 108
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 20

Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu Gly
1               5                   10                  15

Asp Arg Val Ile Ile Ser Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr
            20                  25                  30

Leu Asn Trp Tyr Gln Gln Lys Pro Asp Gly Thr Val Lys Val Leu Ile
        35                  40                  45

Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile Ser Asn Leu Glu Pro
65                  70                  75                  80

Glu Asp Ile Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Thr Val Pro Trp
                85                  90                  95

Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg
            100                 105
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<210> SEQ ID NO 21
<211> LENGTH: 123
<212> TYPE: PRT
<213> ORGANISM: artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 21

Glu Ile Gln Leu Val Gln Ser Gly Pro Glu Leu Lys Gln Pro Gly Glu
1 5 10 15

Thr Val Arg Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asn Tyr
20 25 30

Gly Met Asn Trp Val Lys Gln Ala Pro Gly Lys Gly Leu Lys Trp Met
35 40 45

Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe
50 55 60

Lys Arg Arg Phe Thr Phe Ser Leu Glu Thr Ser Ala Ser Thr Ala Tyr
65 70 75 80

Leu Gln Ile Ser Asn Leu Lys Asn Asp Asp Thr Ala Thr Tyr Phe Cys
85 90 95

Ala Lys Tyr Pro His Tyr Tyr Gly Ser Ser His Trp Tyr Phe Asp Val
100 105 110

Trp Gly Ala Gly Thr Thr Val Thr Val Ser Ser
115 120

<210> SEQ ID NO 22
<211> LENGTH: 108
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 22

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1 5 10 15

Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr
20 25 30

Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Val Leu Ile
35 40 45

Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65 70 75 80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Thr Val Pro Trp
85 90 95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
100 105

<210> SEQ ID NO 23
<211> LENGTH: 123
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 23

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1 5 10 15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Thr Phe Thr Asn Tyr

```
            20                  25                  30

Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe
    50                  55                  60

Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser Lys Ser Thr Ala Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Lys Tyr Pro His Tyr Tyr Gly Ser Ser His Trp Tyr Phe Asp Val
            100                 105                 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120


<210> SEQ ID NO 24
<211> LENGTH: 108
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 24

Asp Ile Gln Leu Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Ser Ala Ser Gln Asp Ile Ser Asn Tyr
            20                  25                  30

Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Val Leu Ile
        35                  40                  45

Tyr Phe Thr Ser Ser Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln Tyr Ser Thr Val Pro Trp
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
            100                 105


<210> SEQ ID NO 25
<211> LENGTH: 123
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 25

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Asp Phe Thr His Tyr
            20                  25                  30

Gly Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Trp Ile Asn Thr Tyr Thr Gly Glu Pro Thr Tyr Ala Ala Asp Phe
    50                  55                  60

Lys Arg Arg Phe Thr Phe Ser Leu Asp Thr Ser Lys Ser Thr Ala Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Lys Tyr Pro Tyr Tyr Tyr Gly Thr Ser His Trp Tyr Phe Asp Val
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            100                 105                 110

Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120


<210> SEQ ID NO 26
<211> LENGTH: 108
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 26

Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                   10                  15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Lys Thr Ile Ser Lys Tyr
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Ser Gly Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Asn Glu Tyr Pro Leu
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
            100                 105


<210> SEQ ID NO 27
<211> LENGTH: 121
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 27

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                   10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr Gly His
            20                  25                  30

Trp Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Met Ile His Pro Ser Asp Ser Glu Thr Arg Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Asp Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Gly Ile Tyr Phe Tyr Gly Thr Thr Tyr Phe Asp Tyr Trp Gly
            100                 105                 110

Gln Gly Thr Leu Val Thr Val Ser Ser
        115                 120


<210> SEQ ID NO 28
<211> LENGTH: 214
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 28
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Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly
1               5                  10                  15

Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Lys Thr Ile Ser Lys Tyr
            20                  25                  30

Leu Ala Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
        35                  40                  45

Tyr Ser Gly Ser Thr Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly
    50                  55                  60

Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Pro
65                  70                  75                  80

Glu Asp Phe Ala Thr Tyr Tyr Cys Gln Gln His Asn Glu Tyr Pro Leu
                85                  90                  95

Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala
            100                 105                 110

Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
        115                 120                 125

Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala
    130                 135                 140

Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln
145                 150                 155                 160

Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser
                165                 170                 175

Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr
            180                 185                 190

Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
        195                 200                 205

Phe Asn Arg Gly Glu Cys
    210


<210> SEQ ID NO 29
<211> LENGTH: 451
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: antibody

<400> SEQUENCE: 29

Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln Pro Gly Gly
1               5                  10                  15

Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Tyr Ser Phe Thr Gly His
            20                  25                  30

Trp Met Asn Trp Val Arg Gln Ala Pro Gly Lys Gly Leu Glu Trp Val
        35                  40                  45

Gly Met Ile His Pro Ser Asp Ser Glu Thr Arg Tyr Asn Gln Lys Phe
    50                  55                  60

Lys Asp Arg Phe Thr Ile Ser Val Asp Lys Ser Lys Asn Thr Leu Tyr
65                  70                  75                  80

Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys
                85                  90                  95

Ala Arg Gly Ile Tyr Phe Tyr Gly Thr Thr Tyr Phe Asp Tyr Trp Gly
            100                 105                 110

Gln Gly Thr Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser
        115                 120                 125

Val Phe Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala
    130                 135                 140

Ala Leu Gly Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
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145                 150                 155                 160

Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala
                165                 170                 175

Val Leu Gln Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val
            180                 185                 190

Pro Ser Ser Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His
        195                 200                 205

Lys Pro Ser Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys
    210                 215                 220

Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly
225                 230                 235                 240

Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met
                245                 250                 255

Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp Val Ser His
            260                 265                 270

Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val
        275                 280                 285

His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr
    290                 295                 300

Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly
305                 310                 315                 320

Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile
                325                 330                 335

Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val
            340                 345                 350

Tyr Thr Leu Pro Pro Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser
        355                 360                 365

Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu
    370                 375                 380

Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro
385                 390                 395                 400

Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val
                405                 410                 415

Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met
            420                 425                 430

His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser
        435                 440                 445

Pro Gly Lys
    450
```

<210> SEQ ID NO 30
<211> LENGTH: 384
<212> TYPE: DNA
<213> ORGANISM: E. coli Thioredoxin TrxA

<400> SEQUENCE: 30

```
atgttacacc aacaacgaaa ccaacacgcc aggcttattc ctgtggagtt atatatgagc     60

gataaaatta ttcacctgac tgacgacagt tttgacacgg atgtactcaa agcggacggg    120

gcgatcctcg tcgatttctg ggcagagtgg tgcggtccgt gcaaaatgat cgccccgatt    180

ctggatgaaa tcgctgacga atatcagggc aaactgaccg ttgcaaaact gaacatcgat    240

caaaaccctg gcactgcgcc gaaatatggc atccgtggta tcccgactct gctgctgttc    300

aaaaacggtg aagtggcggc aaccaaagtg ggtgcactgt ctaaaggtca gttgaaagag    360

ttcctcgacg ctaacctggc gtaa                                           384
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<210> SEQ ID NO 31
<211> LENGTH: 966
<212> TYPE: DNA
<213> ORGANISM: E. coli thioredoxin reductase TrxB

<400> SEQUENCE: 31

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ggtctaacag taatgcccag cccagcccgg acagtacaca ccaccagagt ctgtttgacg 180

acctttaacg tccaggatgg acctgacttt caagacagag ttgtcaacag tgagacacca 240

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cacacagacc ttgccattga atatgaggtg tcagctgtgc ctaccgtgct agccatcaag 420

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gaagaacatg gtatcaagtt tataaggcag ttcgtcccaa cgaaaattga acagatcgaa 780

gcaggaacac caggccgact cagggtgact gctcaatcca caaacagcga ggagaccata 840

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ggcttagaga ccgtgggcgt gaagataaac gaaaaaaccg gaaagatacc cgtcacggat 960

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cccacctgct ctgaggaggt ggtcaagctg cacatctcca agcgctccgg cctggagcct   1560

actgtgactg gttgctga                                                 1578
```

What is claimed is:

1. A method for the prevention of the reduction of a disulfide bond in an antibody expressed in a recombinant host cell, comprising, following fermentation, sparging the pre-harvest or harvested culture fluid of said recombinant host cell with air, wherein the amount of dissolved oxygen (dO_2) in the pre-harvest or harvested culture fluid is at least 10%.

2. The method of claim **1** wherein said air sparging is continued until the pre-harvest or harvested culture fluid is at least 30% saturated with air.

3. The method of claim **1** wherein said air sparging is continued until the pre-harvest or harvested culture fluid is between about 100% saturated to about 30% saturated with air.

4. The method of claim **1** wherein the amount of dissolved oxygen (dO_2) in the pre-harvest or harvested culture fluid is at least 30%.

5. The method of claim **1** wherein the antibody is a therapeutic antibody.

6. The method of claim **1** wherein the antibody is a biologically functional fragment of an antibody.

7. The method of claim **1** wherein the host cell is eukaryotic host cell.

8. The method of claim **7** wherein the eukaryotic host cell is a mammalian host cell.

9. The method of claim **1** wherein the host cell is prokaryotic host cell.

10. The method of claim **9** wherein the prokaryotic host cell is a bacterial cell.

* * * * *

MOLECULAR BIOLOGY OF

THE CELL

ALBERTS JOHNSON LEWIS RAFF ROBERTS WALTER

FOURTH EDITION

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Front cover Human Genome: Reprinted by permission from *Nature*, International Human Genome Sequencing Consortium, 409:860–921, 2001 © Macmillan Magazines Ltd. Adapted from an image by Francis Collins, NHGRI; Jim Kent, UCSC; Ewan Birney, EBI; and Darryl Leja, NHGRI; showing a portion of Chromosome 1 from the initial sequencing of the human genome.

Back cover In 1967, the British artist Peter Blake created a design classic. Nearly 35 years later Nigel Orme (illustrator), Richard Denyer (photographer), and the authors have together produced an affectionate tribute to Mr Blake's image. With its gallery of icons and influences, its assembly created almost as much complexity, intrigue and mystery as the original. *Drosophila, Arabidopsis,* Dolly and the assembled company tempt you to dip inside where, as in the original, "a splendid time is guaranteed for all."
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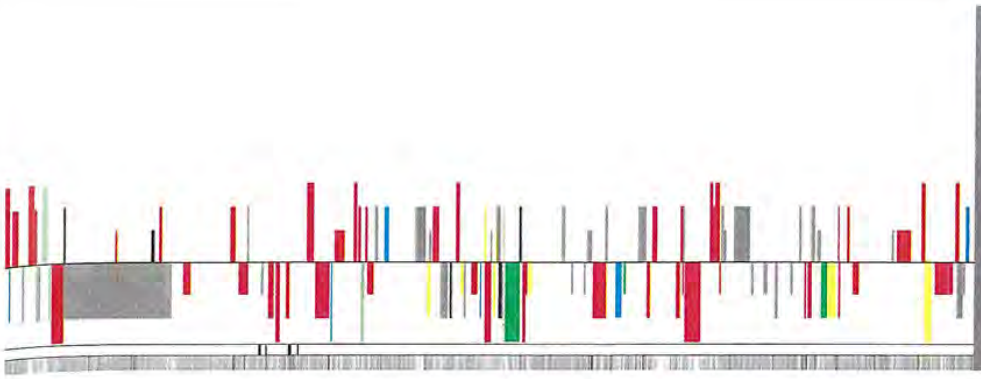


3

PROTEINS

When we look at a cell through a microscope or analyze its electrical or biochemical activity, we are, in essence, observing proteins. Proteins constitute most of a cell's dry mass. They are not only the building blocks from which cells are built; they also execute nearly all cell functions. Thus, enzymes provide the intricate molecular surfaces in a cell that promote its many chemical reactions. Proteins embedded in the plasma membrane form channels and pumps that control the passage of small molecules into and out of the cell. Other proteins carry messages from one cell to another, or act as signal integrators that relay sets of signals inward from the plasma membrane to the cell nucleus. Yet others serve as tiny molecular machines with moving parts: *kinesin*, for example, propels organelles through the cytoplasm; *topoisomerase* can untangle knotted DNA molecules. Other specialized proteins act as antibodies, toxins, hormones, antifreeze molecules, elastic fibers, ropes, or sources of luminescence. Before we can hope to understand how genes work, how muscles contract, how nerves conduct electricity, how embryos develop, or how our bodies function, we must attain a deep understanding of proteins.

THE SHAPE AND STRUCTURE OF PROTEINS

From a chemical point of view, proteins are by far the most structurally complex and functionally sophisticated molecules known. This is perhaps not surprising, once one realizes that the structure and chemistry of each protein has been developed and fine-tuned over billions of years of evolutionary history. We start this chapter by considering how the location of each amino acid in the long string of amino acids that forms a protein determines its three-dimensional shape. We will then use this understanding of protein structure at the atomic level to describe how the precise shape of each protein molecule determines its function in a cell.

The Shape of a Protein Is Specified by Its Amino Acid Sequence

Recall from Chapter 2 that there are 20 types of amino acids in proteins, each with different chemical properties. A **protein** molecule is made from a long chain of these amino acids, each linked to its neighbor through a covalent peptide bond (Figure 3–1). Proteins are therefore also known as *polypeptides*. Each type of protein has a unique sequence of amino acids, exactly the same from one molecule to the next. Many thousands of different proteins are known, each with its own particular amino acid sequence.

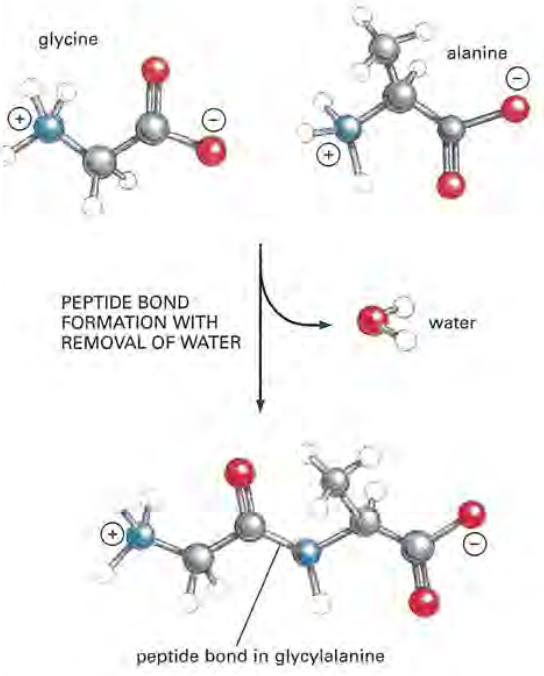


Figure 3–1 A peptide bond. This covalent bond forms when the carbon atom from the carboxyl group of one amino acid shares electrons with the nitrogen atom *(blue)* from the amino group of a second amino acid. As indicated, a molecule of water is lost in this condensation reaction.

The repeating sequence of atoms along the core of the polypeptide chain is referred to as the **polypeptide backbone**. Attached to this repetitive chain are those portions of the amino acids that are not involved in making a peptide bond and which give each amino acid its unique properties: the 20 different amino acid **side chains** (Figure 3–2). Some of these side chains are nonpolar and hydrophobic ("water-fearing"), others are negatively or positively charged, some are reactive, and so on. Their atomic structures are presented in Panel 3–1, and a brief list with abbreviations is provided in Figure 3–3.

As discussed in Chapter 2, atoms behave almost as if they were hard spheres with a definite radius (their *van der Waals radius*). The requirement that no two atoms overlap limits greatly the possible bond angles in a polypeptide chain (Figure 3–4). This constraint and other steric interactions severely restrict the variety of three-dimensional arrangements of atoms (or *conformations)* that are possible. Nevertheless, a long flexible chain, such as a protein, can still fold in an enormous number of ways.

The folding of a protein chain is, however, further constrained by many different sets of weak *noncovalent bonds* that form between one part of the chain and another. These involve atoms in the polypeptide backbone, as well as atoms in the amino acid side chains. The weak bonds are of three types: *hydrogen bonds*, *ionic bonds*, and *van der Waals attractions*, as explained in Chapter 2 (see p. 57). Individual noncovalent bonds are 30–300 times weaker than the typical covalent bonds that create biological molecules. But many weak bonds can act in parallel to hold two regions of a polypeptide chain tightly together. The stability of each folded shape is therefore determined by the combined strength of large numbers of such noncovalent bonds (Figure 3–5).

A fourth weak force also has a central role in determining the shape of a protein. As described in Chapter 2, hydrophobic molecules, including the nonpolar side chains of particular amino acids, tend to be forced together in an aqueous environment in order to minimize their disruptive effect on the hydrogen-bonded network of water molecules (see p. 58 and Panel 2–2, pp. 112–113). Therefore, an important factor governing the folding of any protein is the distribution of its polar and nonpolar amino acids. The nonpolar (hydrophobic) side chains in a protein—belonging to such amino acids as phenylalanine, leucine, valine, and tryptophan—tend to cluster in the interior of the molecule (just as hydrophobic oil droplets coalesce in water to form one large droplet). This enables them to

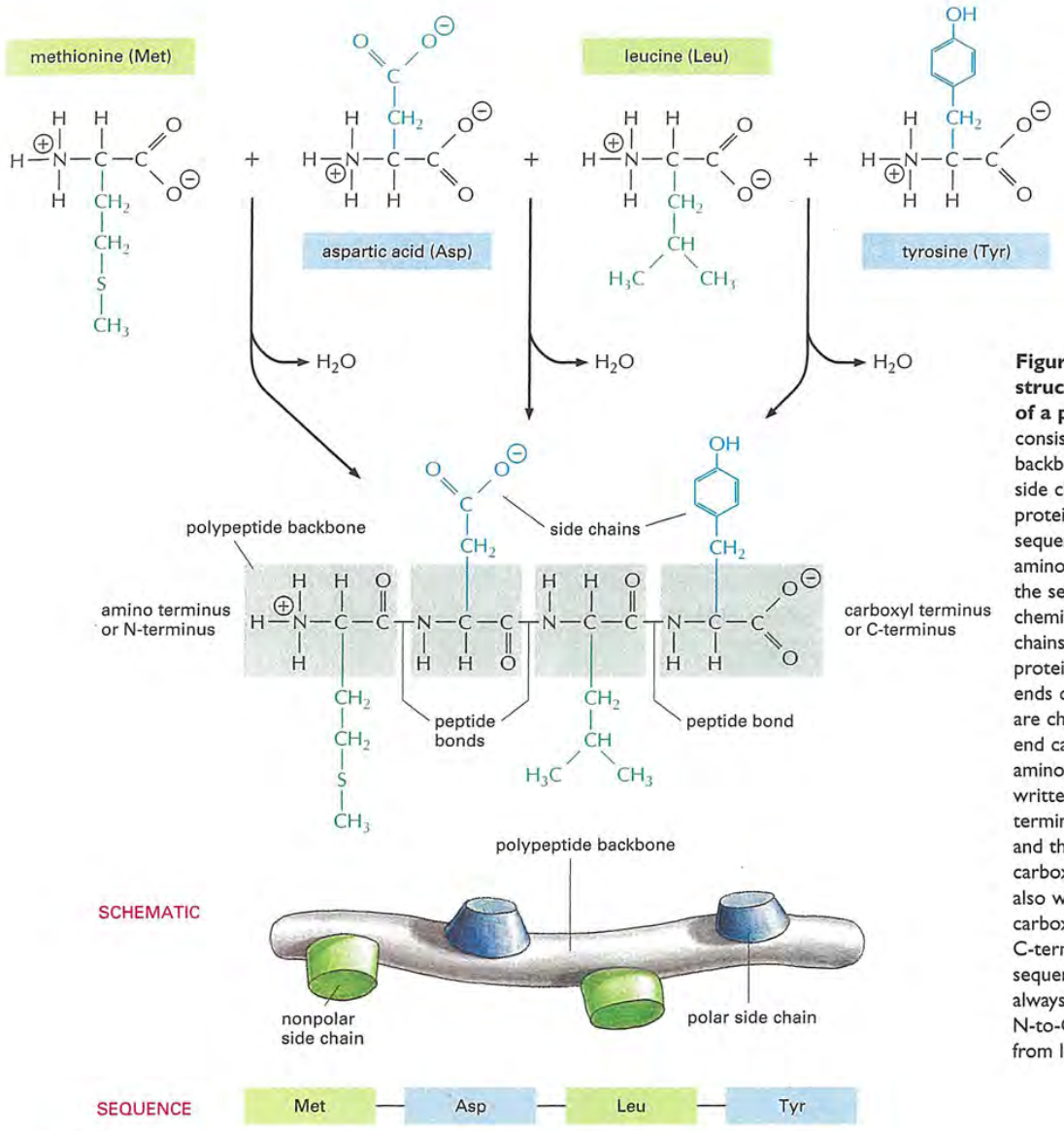


Figure 3–2 The structural components of a protein. A protein consists of a polypeptide backbone with attached side chains. Each type of protein differs in its sequence and number of amino acids; therefore, it is the sequence of the chemically different side chains that makes each protein distinct. The two ends of a polypeptide chain are chemically different: the end carrying the free amino group (NH_3^+, also written NH_2) is the amino terminus, or N-terminus, and that carrying the free carboxyl group (COO^-, also written COOH) is the carboxyl terminus or C-terminus. The amino acid sequence of a protein is always presented in the N-to-C direction, reading from left to right.

AMINO ACID			SIDE CHAIN
Aspartic acid	Asp	D	negative
Glutamic acid	Glu	E	negative
Arginine	Arg	R	positive
Lysine	Lys	K	positive
Histidine	His	H	positive
Asparagine	Asn	N	uncharged polar
Glutamine	Gln	Q	uncharged polar
Serine	Ser	S	uncharged polar
Threonine	Thr	T	uncharged polar
Tyrosine	Tyr	Y	uncharged polar

POLAR AMINO ACIDS

AMINO ACID			SIDE CHAIN
Alanine	Ala	A	nonpolar
Glycine	Gly	G	nonpolar
Valine	Val	V	nonpolar
Leucine	Leu	L	nonpolar
Isoleucine	Ile	I	nonpolar
Proline	Pro	P	nonpolar
Phenylalanine	Phe	F	nonpolar
Methionine	Met	M	nonpolar
Tryptophan	Trp	W	nonpolar
Cysteine	Cys	C	nonpolar

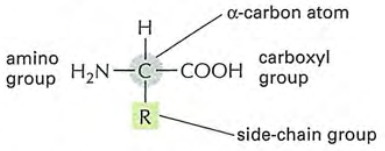
NONPOLAR AMINO ACIDS

Figure 3–3 The 20 amino acids found in proteins. Both three-letter and one-letter abbreviations are listed. As shown, there are equal numbers of polar and nonpolar side chains. For their atomic structures, see Panel 3–1 (pp. 132–133).

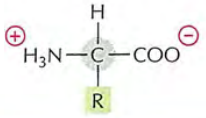
PANEL 3–1 The 20 Amino Acids Found in Proteins

THE AMINO ACID

The general formula of an amino acid is

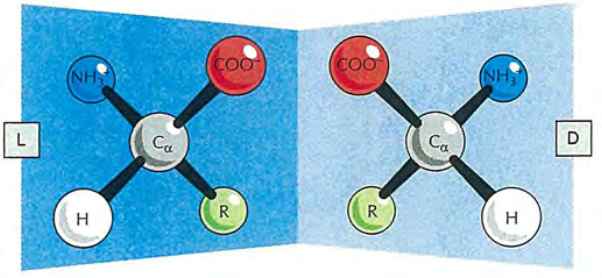


R is commonly one of 20 different side chains. At pH 7 both the amino and carboxyl groups are ionized.



OPTICAL ISOMERS

The α-carbon atom is asymmetric, which allows for two mirror image (or stereo-) isomers, L and D.



Proteins consist exclusively of L-amino acids.

FAMILIES OF AMINO ACIDS

The common amino acids are grouped according to whether their side chains are

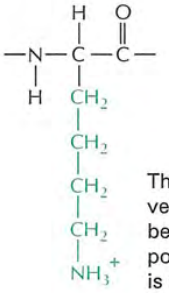
- acidic
- basic
- uncharged polar
- nonpolar

These 20 amino acids are given both three-letter and one-letter abbreviations.

Thus: alanine = Ala = A

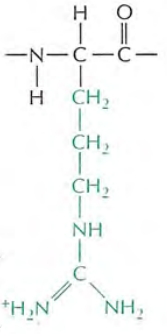
BASIC SIDE CHAINS

lysine (Lys, or K)

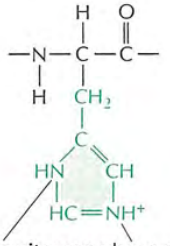


This group is very basic because its positive charge is stabilized by resonance.

arginine (Arg, or R)



histidine (His, or H)

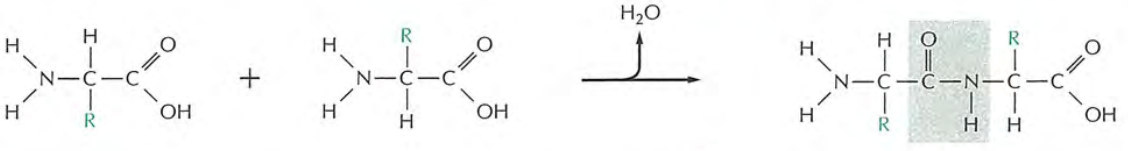


These nitrogens have a relatively weak affinity for an H^+ and are only partly positive at neutral pH.

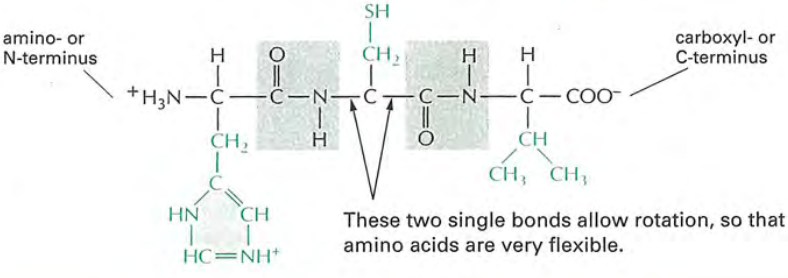
PEPTIDE BONDS

Amino acids are commonly joined together by an amide linkage, called a peptide bond.

Peptide bond: The four atoms in each *gray box* form a rigid planar unit. There is no rotation around the C–N bond.



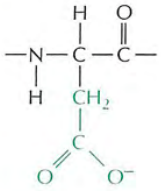
Proteins are long polymers of amino acids linked by peptide bonds, and they are always written with the N-terminus toward the left. The sequence of this tripeptide is histidine-cysteine-valine.



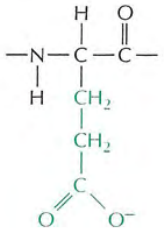
These two single bonds allow rotation, so that long chains of amino acids are very flexible.

ACIDIC SIDE CHAINS

aspartic acid (Asp, or D)

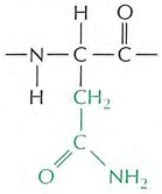


glutamic acid (Glu, or E)

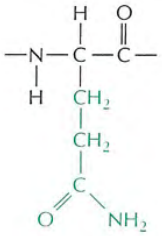


UNCHARGED POLAR SIDE CHAINS

asparagine (Asn, or N)

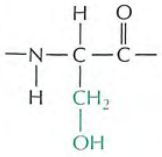


glutamine (Gln, or Q)

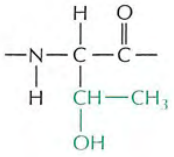


Although the amide N is not charged at neutral pH, it is polar.

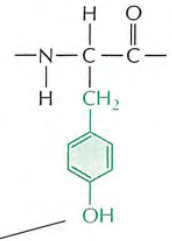
serine (Ser, or S)



threonine (Thr, or T)



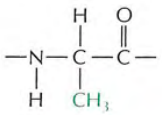
tyrosine (Tyr, or Y)



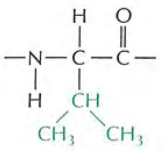
The –OH group is polar.

NONPOLAR SIDE CHAINS

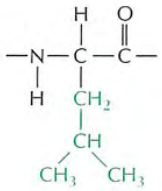
alanine (Ala, or A)



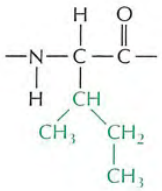
valine (Val, or V)



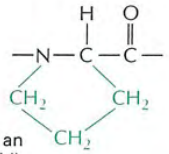
leucine (Leu, or L)



isoleucine (Ile, or I)

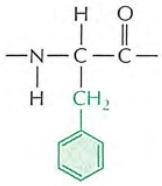


proline (Pro, or P)

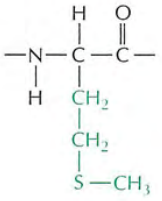


(actually an imino acid)

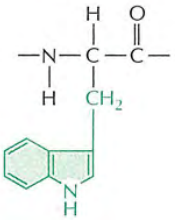
phenylalanine (Phe, or F)



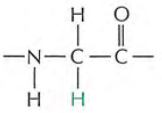
methionine (Met, or M)



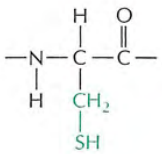
tryptophan (Trp, or W)



glycine (Gly, or G)



cysteine (Cys, or C)



Disulfide bonds can form between two cysteine side chains in proteins.

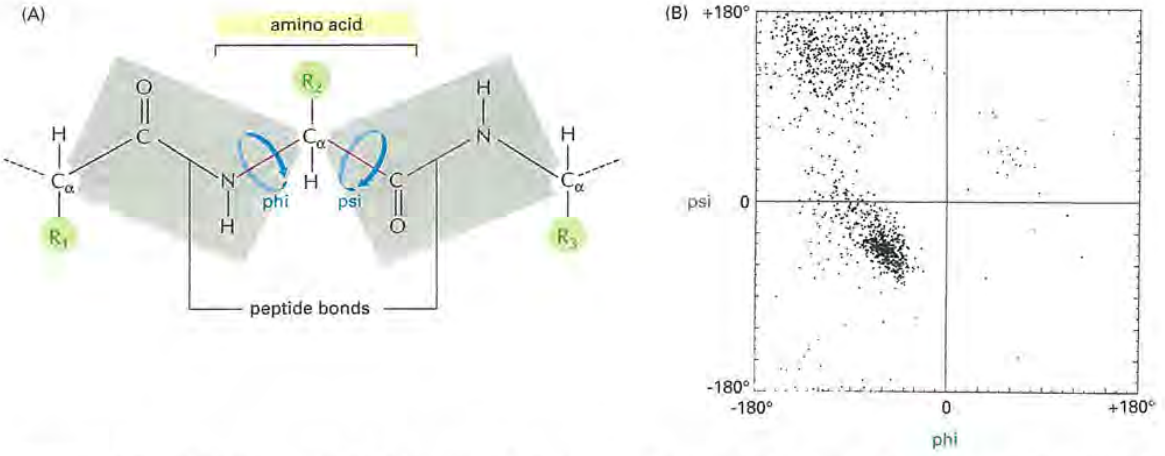


Figure 3–4 Steric limitations on the bond angles in a polypeptide chain. (A) Each amino acid contributes three bonds *(red)* to the backbone of the chain. The peptide bond is planar *(gray shading)* and does not permit rotation. By contrast, rotation can occur about the C_α–C bond, whose angle of rotation is called psi (ψ), and about the N–C_α bond, whose angle of rotation is called phi (ϕ). By convention, an R group is often used to denote an amino acid side chain *(green circles)*. (B) The conformation of the main-chain atoms in a protein is determined by one pair of ϕ and ψ angles for each amino acid; because of steric collisions between atoms within each amino acid, most pairs of ϕ and ψ angles do not occur. In this so-called Ramachandran plot, each dot represents an observed pair of angles in a protein. (B, from J. Richardson, *Adv. Prot. Chem.* 34:174–175, 1981. © Academic Press.)

avoid contact with the water that surrounds them inside a cell. In contrast, polar side chains—such as those belonging to arginine, glutamine, and histidine—tend to arrange themselves near the outside of the molecule, where they can form hydrogen bonds with water and with other polar molecules (Figure 3–6). When polar amino acids are buried within the protein, they are usually hydrogen-bonded to other polar amino acids or to the polypeptide backbone (Figure 3–7).

Proteins Fold into a Conformation of Lowest Energy

As a result of all of these interactions, each type of protein has a particular three-dimensional structure, which is determined by the order of the amino acids in its chain. The final folded structure, or **conformation**, adopted by any polypeptide chain is generally the one in which the free energy is minimized. Protein folding has been studied in a test tube by using highly purified proteins. A protein can be unfolded, or *denatured*, by treatment with certain solvents, which disrupt the noncovalent interactions holding the folded chain together. This treatment converts the protein into a flexible polypeptide chain that has lost its

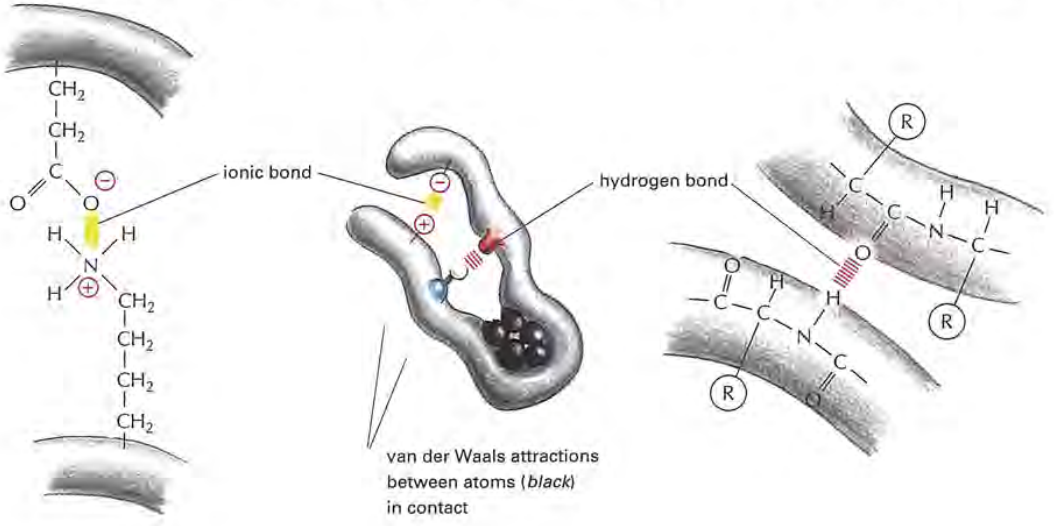


Figure 3–5 Three types of noncovalent bonds that help proteins fold. Although a single one of these bonds is quite weak, many of them often form together to create a strong bonding arrangement, as in the example shown. As in the previous figure, R is used as a general designation for an amino acid side chain.

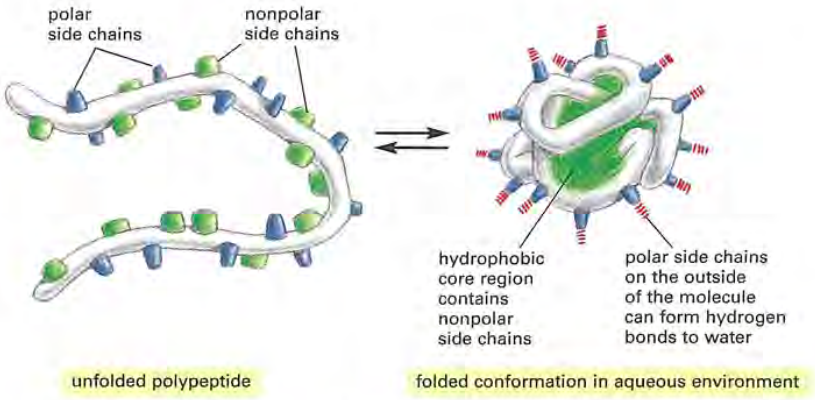


Figure 3–6 How a protein folds into a compact conformation. The polar amino acid side chains tend to gather on the outside of the protein, where they can interact with water; the nonpolar amino acid side chains are buried on the inside to form a tightly packed hydrophobic core of atoms that are hidden from water. In this schematic drawing, the protein contains only about 30 amino acids.

natural shape. When the denaturing solvent is removed, the protein often refolds spontaneously, or *renatures*, into its original conformation (Figure 3–8), indicating that all the information needed for specifying the three-dimensional shape of a protein is contained in its amino acid sequence.

Each protein normally folds up into a single stable conformation. However, the conformation often changes slightly when the protein interacts with other molecules in the cell. This change in shape is often crucial to the function of the protein, as we see later.

Although a protein chain can fold into its correct conformation without outside help, protein folding in a living cell is often assisted by special proteins called *molecular chaperones*. These proteins bind to partly folded polypeptide chains and help them progress along the most energetically favorable folding pathway. Chaperones are vital in the crowded conditions of the cytoplasm, since they prevent the temporarily exposed hydrophobic regions in newly synthesized protein chains from associating with each other to form protein aggregates (see p. 357). However, the final three-dimensional shape of the protein is still specified by its amino acid sequence: chaperones simply make the folding process more reliable.

Proteins come in a wide variety of shapes, and they are generally between 50 and 2000 amino acids long. Large proteins generally consist of several distinct *protein domains*—structural units that fold more or less independently of each other, as we discuss below. The detailed structure of any protein is complicated; for simplicity a protein's structure can be depicted in several different ways, each emphasizing different features of the protein.

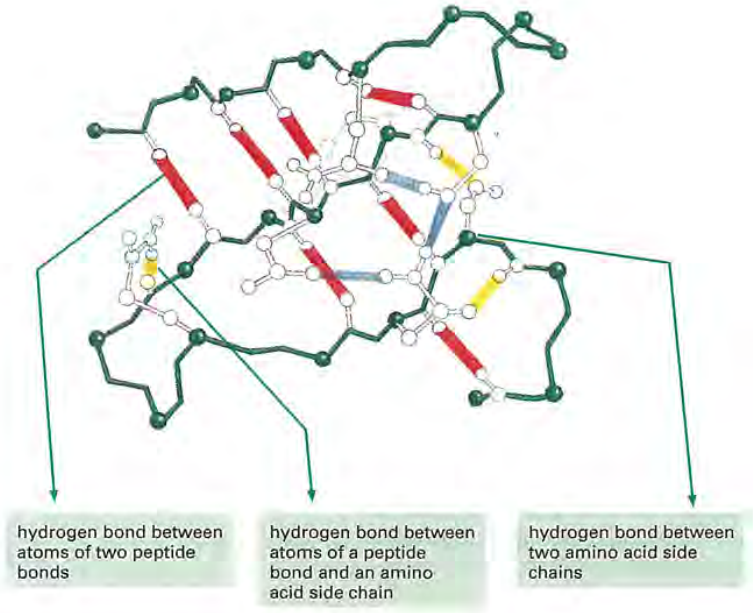


Figure 3–7 Hydrogen bonds in a protein molecule. Large numbers of hydrogen bonds form between adjacent regions of the folded polypeptide chain and help stabilize its three-dimensional shape. The protein depicted is a portion of the enzyme lysozyme, and the hydrogen bonds between the three possible pairs of partners have been differently colored, as indicated. (After C.K. Matthews and K.E. van Holde, Biochemistry. Redwood City, CA: Benjamin/Cummings, 1996.)

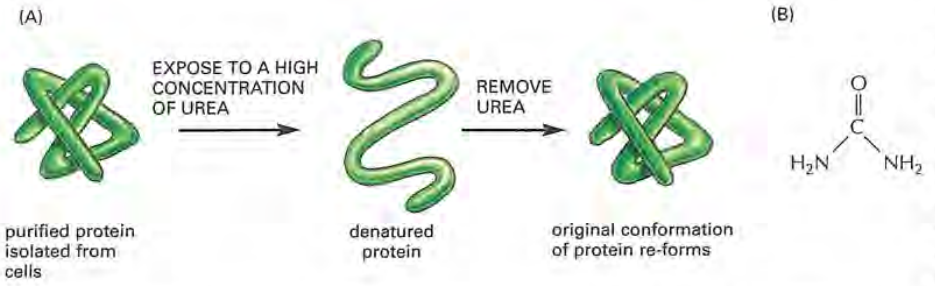


Figure 3–8 The refolding of a denatured protein. (A) This experiment demonstrates that the conformation of a protein is determined solely by its amino acid sequence. (B) The structure of urea. Urea is very soluble in water and unfolds proteins at high concentrations, where there is about one urea molecule for every six water molecules.

Panel 3–2 (pp. 138–139) presents four different depictions of a protein domain called SH2, which has important functions in eucaryotic cells. Constructed from a string of 100 amino acids, the structure is displayed as (A) a polypeptide backbone model, (B) a ribbon model, (C) a wire model that includes the amino acid side chains, and (D) a space-filling model. Each of the three horizontal rows shows the protein in a different orientation, and the image is colored in a way that allows the polypeptide chain to be followed from its N-terminus *(purple)* to its C-terminus *(red)*.

Panel 3–2 shows that a protein's conformation is amazingly complex, even for a structure as small as the SH2 domain. But the description of protein structures can be simplified by the recognition that they are built up from several common structural motifs, as we discuss next.

The α Helix and the β Sheet Are Common Folding Patterns

When the three-dimensional structures of many different protein molecules are compared, it becomes clear that, although the overall conformation of each protein is unique, two regular folding patterns are often found in parts of them. Both patterns were discovered about 50 years ago from studies of hair and silk. The first folding pattern to be discovered, called the **α helix**, was found in the protein *α-keratin*, which is abundant in skin and its derivatives—such as hair, nails, and horns. Within a year of the discovery of the α helix, a second folded structure, called a **β sheet**, was found in the protein *fibroin*, the major constituent of silk. These two patterns are particularly common because they result from hydrogen-bonding between the N–H and C=O groups in the polypeptide backbone, without involving the side chains of the amino acids. Thus, they can be formed by many different amino acid sequences. In each case, the protein chain adopts a regular, repeating conformation. These two conformations, as well as the abbreviations that are used to denote them in ribbon models of proteins, are shown in Figure 3–9.

The core of many proteins contains extensive regions of β sheet. As shown in Figure 3–10, these β sheets can form either from neighboring polypeptide chains that run in the same orientation (parallel chains) or from a polypeptide chain that folds back and forth upon itself, with each section of the chain running in the direction opposite to that of its immediate neighbors (antiparallel chains). Both types of β sheet produce a very rigid structure, held together by hydrogen bonds that connect the peptide bonds in neighboring chains (see Figure 3–9D).

An α helix is generated when a single polypeptide chain twists around on itself to form a rigid cylinder. A hydrogen bond is made between every fourth peptide bond, linking the C=O of one peptide bond to the N–H of another (see Figure 3–9A). This gives rise to a regular helix with a complete turn every 3.6 amino acids. Note that the protein domain illustrated in Panel 3–2 contains two α helices, as well as β sheet structures.

Short regions of α helix are especially abundant in proteins located in cell membranes, such as transport proteins and receptors. As we discuss in Chapter 10, those portions of a transmembrane protein that cross the lipid bilayer usually cross as an α helix composed largely of amino acids with nonpolar side chains. The polypeptide backbone, which is hydrophilic, is hydrogen-bonded to itself in the α helix and shielded from the hydrophobic lipid environment of the membrane by its protruding nonpolar side chains (see also Figure 3–77).

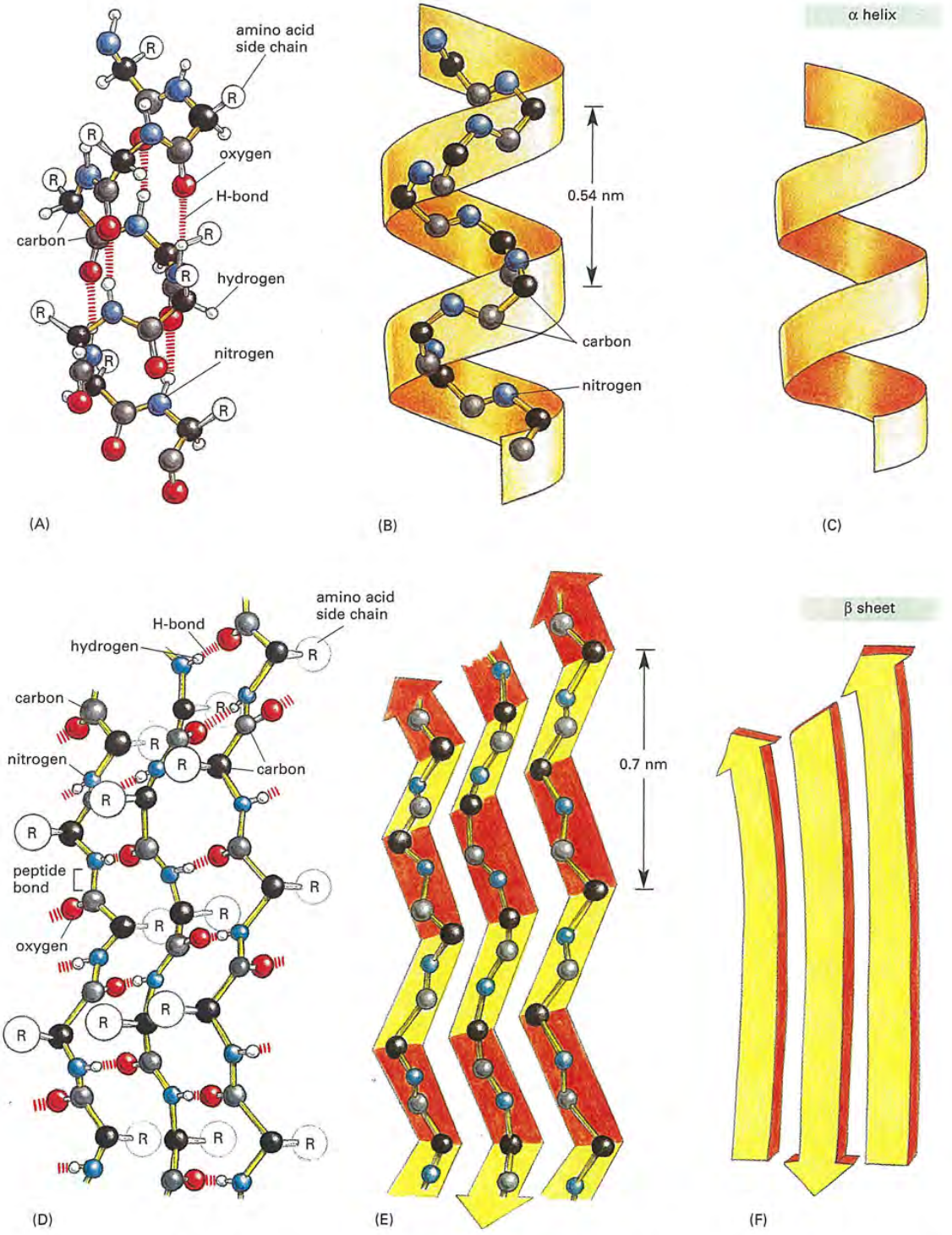
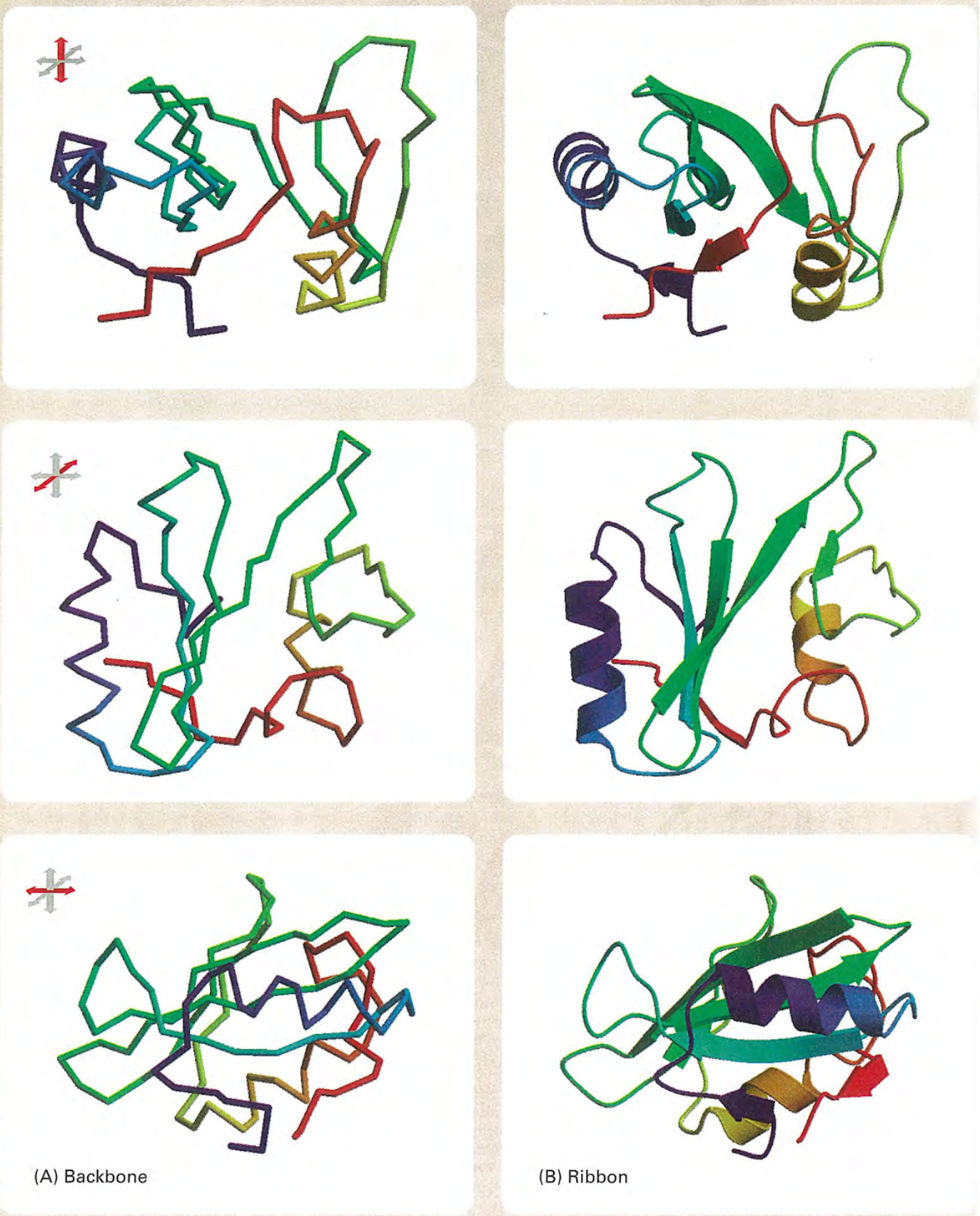


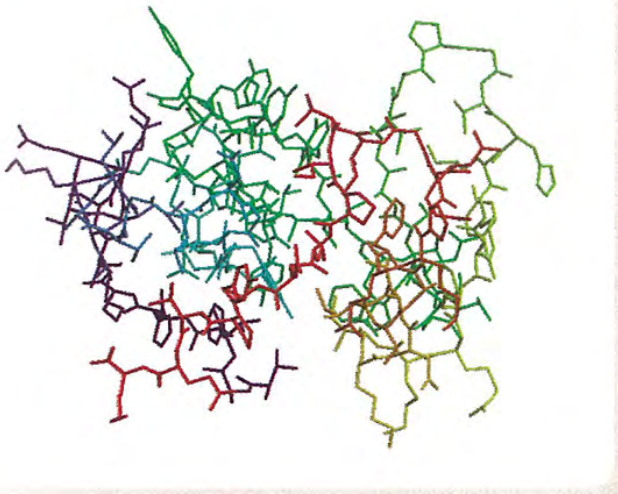
Figure 3–9 The regular conformation of the polypeptide backbone observed in the α helix and the β sheet. (A, B, and C) The α helix. The N–H of every peptide bond is hydrogen-bonded to the C=O of a neighboring peptide bond located four peptide bonds away in the same chain. (D, E, and F) The β sheet. In this example, adjacent peptide chains run in opposite (antiparallel) directions. The individual polypeptide chains (strands) in a β sheet are held together by hydrogen-bonding between peptide bonds in different strands, and the amino acid side chains in each strand alternately project above and below the plane of the sheet. (A) and (D) show all the atoms in the polypeptide backbone, but the amino acid side chains are truncated and denoted by R. In contrast, (B) and (E) show the backbone atoms only, while (C) and (F) display the shorthand symbols that are used to represent the α helix and the β sheet in ribbon drawings of proteins (see Panel 3–2B).

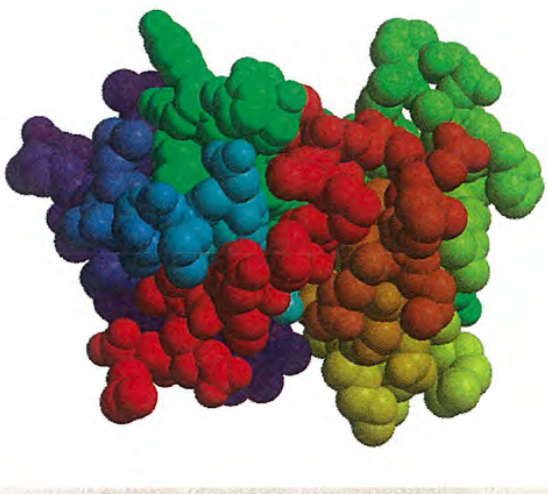
PANEL 3–2 Four Different Ways of Depicting a Small Protein Domain: the SH2 Domain. (Courtesy of David Lawson.)

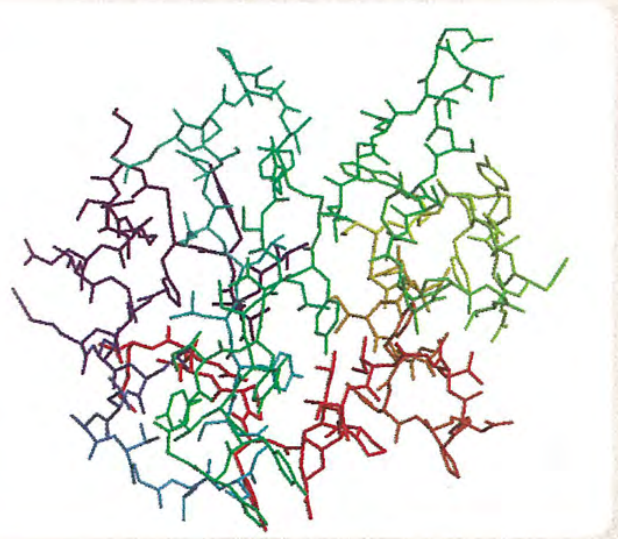


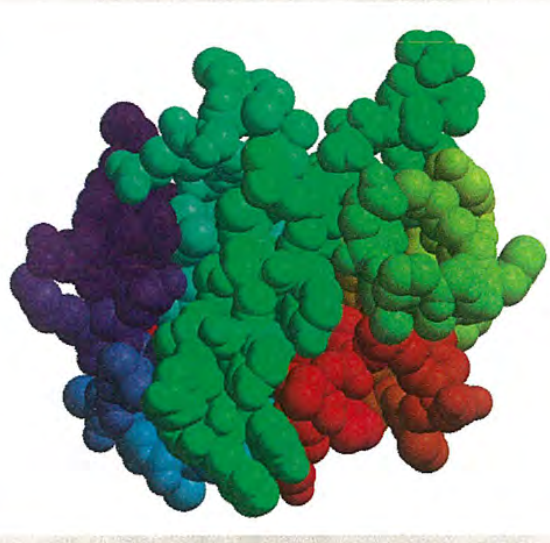
(A) Backbone

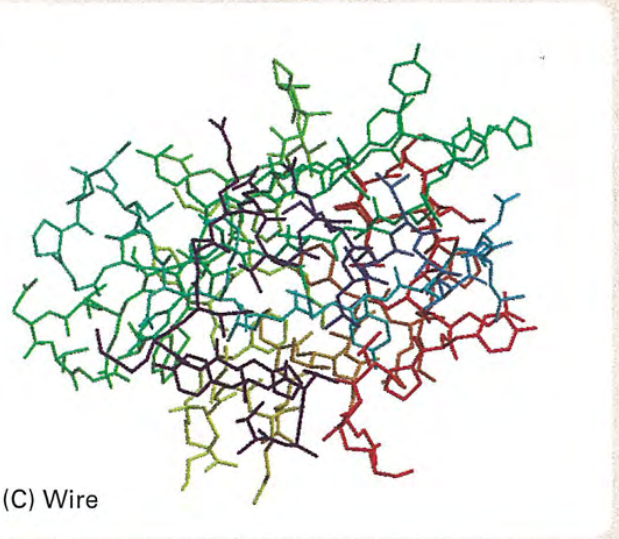
(B) Ribbon



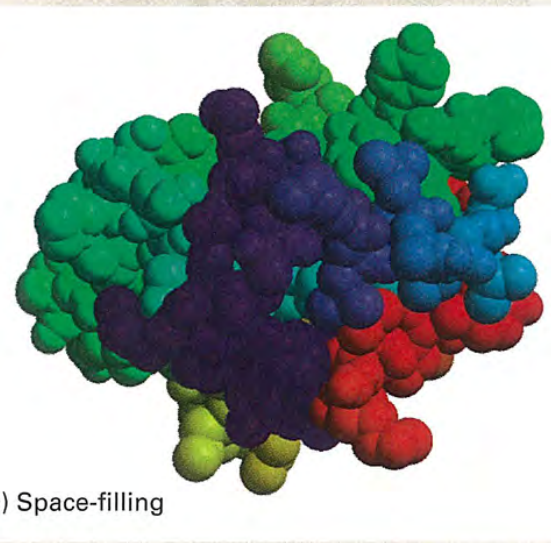








(C) Wire



(D) Space-filling

In other proteins, α helices wrap around each other to form a particularly stable structure, known as a **coiled-coil**. This structure can form when the two (or in some cases three) α helices have most of their nonpolar (hydrophobic) side chains on one side, so that they can twist around each other with these side chains facing inward (Figure 3–11). Long rodlike coiled-coils provide the structural framework for many elongated proteins. Examples are α-keratin, which forms the intracellular fibers that reinforce the outer layer of the skin and its appendages, and the myosin molecules responsible for muscle contraction.

The Protein Domain Is a Fundamental Unit of Organization

Even a small protein molecule is built from thousands of atoms linked together by precisely oriented covalent and noncovalent bonds, and it is extremely difficult to visualize such a complicated structure without a three-dimensional display. For this reason, various graphic and computer-based aids are used. A CD-ROM produced to accompany this book contains computer-generated images of selected proteins, designed to be displayed and rotated on the screen in a variety of formats.

Biologists distinguish four levels of organization in the structure of a protein. The amino acid sequence is known as the **primary structure** of the protein. Stretches of polypeptide chain that form α helices and β sheets constitute the protein's **secondary structure**. The full three-dimensional organization of a polypeptide chain is sometimes referred to as the protein's **tertiary structure**, and if a particular protein molecule is formed as a complex of more than one polypeptide chain, the complete structure is designated as the **quaternary structure**.

Studies of the conformation, function, and evolution of proteins have also revealed the central importance of a unit of organization distinct from the four just described. This is the **protein domain**, a substructure produced by any part of a polypeptide chain that can fold independently into a compact, stable structure. A domain usually contains between 40 and 350 amino acids, and it is the

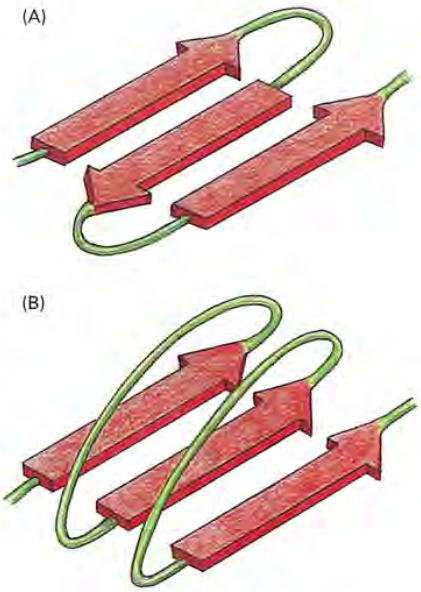


Figure 3–10 Two types of β sheet structures. (A) An antiparallel β sheet (see Figure 3–9D). (B) A parallel β sheet. Both of these structures are common in proteins.

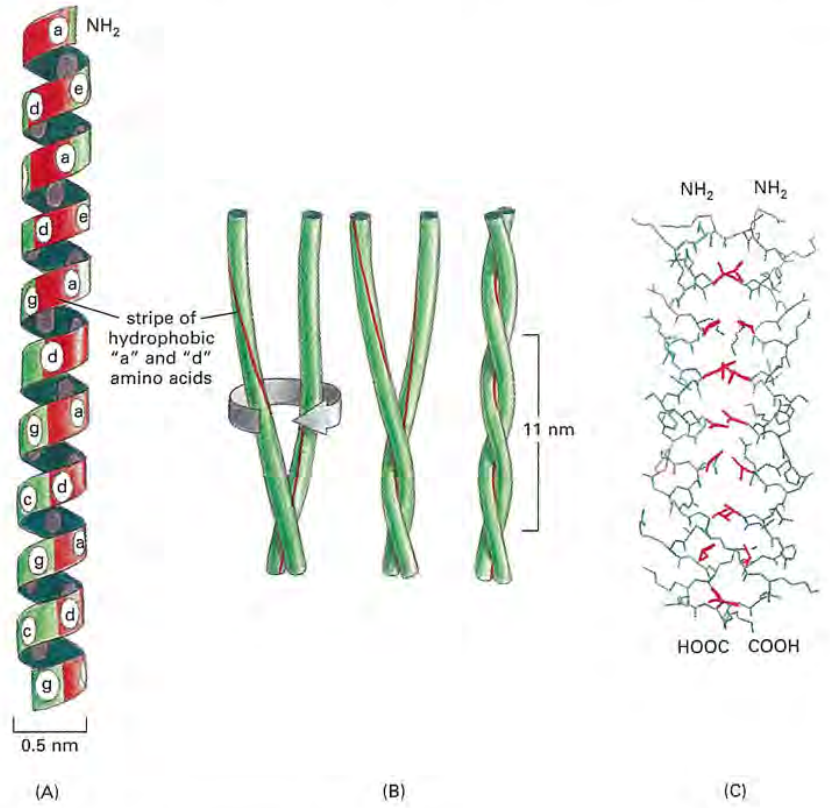


Figure 3–11 The structure of a coiled-coil. (A) A single α helix, with successive amino acid side chains labeled in a sevenfold sequence, "abcdefg" (from bottom to top). Amino acids "a" and "d" in such a sequence lie close together on the cylinder surface, forming a "stripe" (*red*) that winds slowly around the α helix. Proteins that form coiled-coils typically have nonpolar amino acids at positions "a" and "d." Consequently, as shown in (B), the two α helices can wrap around each other with the nonpolar side chains of one α helix interacting with the nonpolar side chains of the other, while the more hydrophilic amino acid side chains are left exposed to the aqueous environment. (C) The atomic structure of a coiled-coil determined by x-ray crystallography. The *red* side chains are nonpolar.

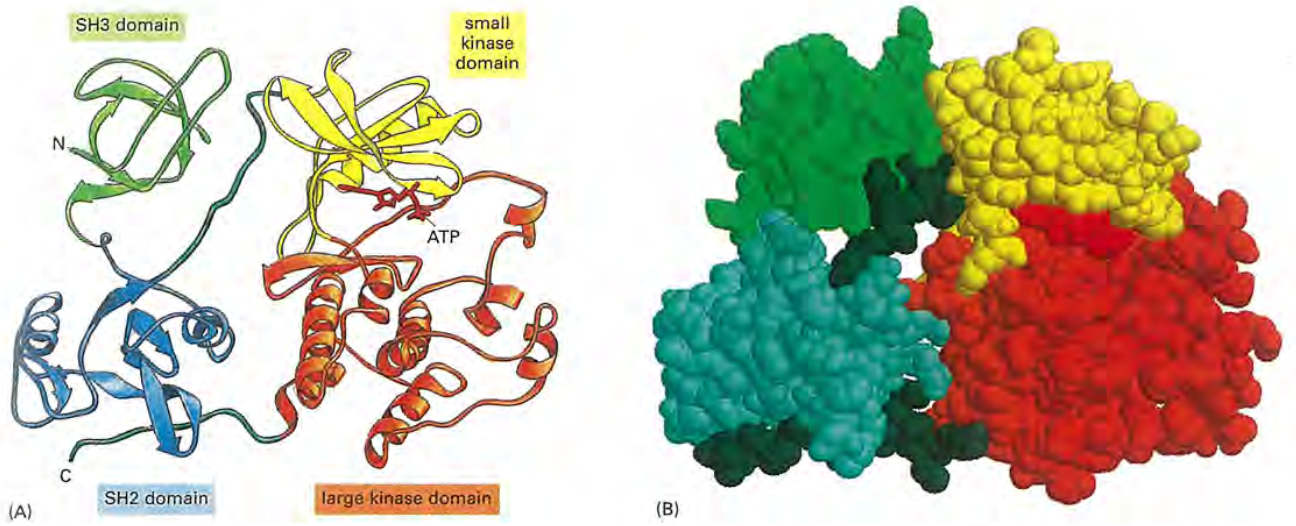


Figure 3–12 A protein formed from four domains. In the Src protein shown, two of the domains form a protein kinase enzyme, while the SH2 and SH3 domains perform regulatory functions. (A) A ribbon model, with ATP substrate in *red*. (B) A spacing-filling model, with ATP substrate in *red*. Note that the site that binds ATP is positioned at the interface of the two domains that form the kinase. The detailed structure of the SH2 domain is illustrated in Panel 3–2 (pp. 138–139).

modular unit from which many larger proteins are constructed. The different domains of a protein are often associated with different functions. Figure 3–12 shows an example—the Src protein kinase, which functions in signaling pathways inside vertebrate cells (Src is pronounced "sarc"). This protein has four domains: the SH2 and SH3 domains have regulatory roles, while the two remaining domains are responsible for the kinase catalytic activity. Later in the chapter, we shall return to this protein, in order to explain how proteins can form molecular switches that transmit information throughout cells.

The smallest protein molecules contain only a single domain, whereas larger proteins can contain as many as several dozen domains, usually connected to each other by short, relatively unstructured lengths of polypeptide chain. Figure 3–13 presents ribbon models of three differently organized protein domains. As these examples illustrate, the central core of a domain can be constructed from α helices, from β sheets, or from various combinations of these two fundamental folding elements. Each different combination is known as a *protein fold*. So far, about 1000 different protein folds have been identified among the ten thousand proteins whose detailed conformations are known.

Few of the Many Possible Polypeptide Chains Will Be Useful

Since each of the 20 amino acids is chemically distinct and each can, in principle, occur at any position in a protein chain, there are $20 \times 20 \times 20 \times 20 = 160{,}000$ different possible polypeptide chains four amino acids long, or 20^n different possible polypeptide chains n amino acids long. For a typical protein length of about 300 amino acids, more than 10^{390} (20^{300}) different polypeptide chains could theoretically be made. This is such an enormous number that to produce just one molecule of each kind would require many more atoms than exist in the universe.

Only a very small fraction of this vast set of conceivable polypeptide chains would adopt a single, stable three-dimensional conformation—by some estimates, less than one in a billion. The vast majority of possible protein molecules could adopt many conformations of roughly equal stability, each conformation having different chemical properties. And yet virtually all proteins present in cells adopt unique and stable conformations. How is this possible? The answer lies in natural selection. A protein with an unpredictably variable structure and biochemical activity is unlikely to help the survival of a cell that contains it. Such proteins would therefore have been eliminated by natural selection through the enormously long trial-and-error process that underlies biological evolution.

Because of natural selection, not only is the amino acid sequence of a present-day protein such that a single conformation is extremely stable, but this conformation has its chemical properties finely tuned to enable the protein to

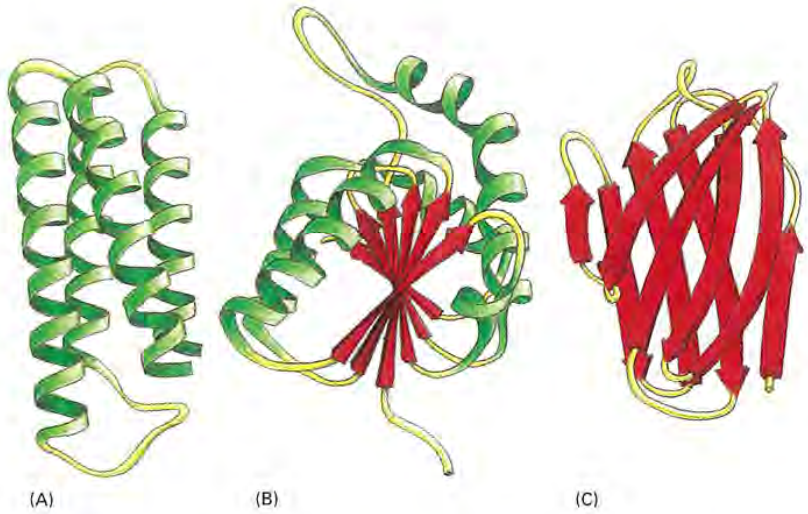


Figure 3–13 Ribbon models of three different protein domains. (A) Cytochrome b_{562}, a single-domain protein involved in electron transport in mitochondria. This protein is composed almost entirely of α helices. (B) The NAD-binding domain of the enzyme lactic dehydrogenase, which is composed of a mixture of α helices and β sheets. (C) The variable domain of an immunoglobulin (antibody) light chain, composed of a sandwich of two β sheets. In these examples, the α helices are shown in *green*, while strands organized as β sheets are denoted by *red arrows*.

Note that the polypeptide chain generally traverses back and forth across the entire domain, making sharp turns only at the protein surface. It is the protruding loop regions *(yellow)* that often form the binding sites for other molecules. (Adapted from drawings courtesy of Jane Richardson.)

perform a particular catalytic or structural function in the cell. Proteins are so precisely built that the change of even a few atoms in one amino acid can sometimes disrupt the structure of the whole molecule so severely that all function is lost.

Proteins Can Be Classified into Many Families

Once a protein had evolved that folded up into a stable conformation with useful properties, its structure could be modified during evolution to enable it to perform new functions. This process has been greatly accelerated by genetic mechanisms that occasionally produce duplicate copies of genes, allowing one gene copy to evolve independently to perform a new function (discussed in Chapter 7). This type of event has occurred quite often in the past; as a result, many present-day proteins can be grouped into protein families, each family member having an amino acid sequence and a three-dimensional conformation that resemble those of the other family members.

Consider, for example, the *serine proteases*, a large family of protein-cleaving (proteolytic) enzymes that includes the digestive enzymes chymotrypsin, trypsin, and elastase, and several proteases involved in blood clotting. When the protease portions of any two of these enzymes are compared, parts of their amino acid sequences are found to match. The similarity of their three-dimensional conformations is even more striking: most of the detailed twists and turns in their polypeptide chains, which are several hundred amino acids long, are virtually identical (Figure 3–14). The many different serine proteases nevertheless have distinct enzymatic activities, each cleaving different proteins or the peptide bonds between different types of amino acids. Each therefore performs a distinct function in an organism.

The story we have told for the serine proteases could be repeated for hundreds of other protein families. In many cases the amino acid sequences have diverged much further than for the serine proteases, so that one cannot be sure of a family relationship between two proteins without determining their three-dimensional structures. The yeast α2 protein and the *Drosophila* engrailed protein, for example, are both gene regulatory proteins in the homeodomain family. Because they are identical in only 17 of their 60 amino acid residues, their relationship became certain only when their three-dimensional structures were compared (Figure 3–15).

The various members of a large protein family often have distinct functions. Some of the amino acid changes that make family members different were no doubt selected in the course of evolution because they resulted in useful changes in biological activity, giving the individual family members the different functional properties they have today. But many other amino acid changes are effectively "neutral," having neither a beneficial nor a damaging effect on the

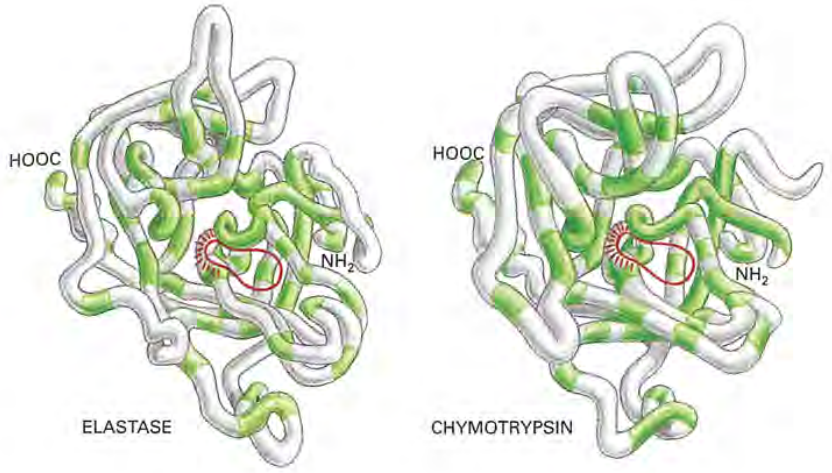


Figure 3–14 The conformations of two serine proteases compared. The backbone conformations of elastase and chymotrypsin. Although only those amino acids in the polypeptide chain shaded in *green* are the same in the two proteins, the two conformations are very similar nearly everywhere. The active site of each enzyme is circled in *red;* this is where the peptide bonds of the proteins that serve as substrates are bound and cleaved by hydrolysis. The serine proteases derive their name from the amino acid serine, whose side chain is part of the active site of each enzyme and directly participates in the cleavage reaction.

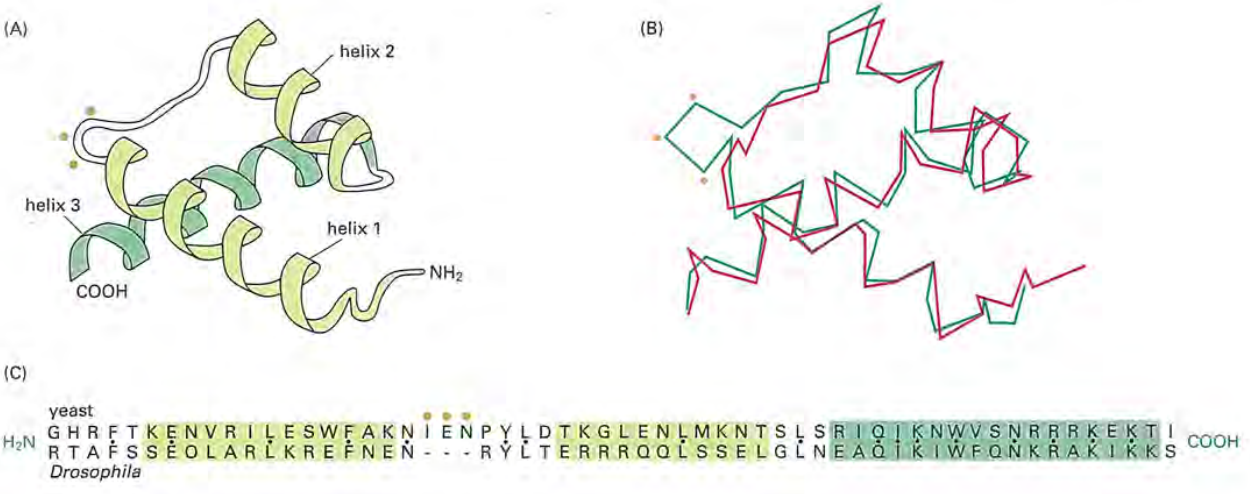
basic structure and function of the protein. In addition, since mutation is a random process, there must also have been many deleterious changes that altered the three-dimensional structure of these proteins sufficiently to harm them. Such faulty proteins would have been lost whenever the individual organisms making them were at enough of a disadvantage to be eliminated by natural selection.

Protein families are readily recognized when the genome of any organism is sequenced; for example, the determination of the DNA sequence for the entire genome of the nematode *Caenorhabditis elegans* has revealed that this tiny worm contains more than 18,000 genes. Through sequence comparisons, the products of a large fraction of these genes can be seen to contain domains from one or another protein family; for example, there appear to be 388 genes containing protein kinase domains, 66 genes containing DNA and RNA helicase domains, 43 genes containing SH2 domains, 70 genes containing immunoglobulin domains, and 88 genes containing DNA-binding homeodomains in this genome of 97 million base pairs (Figure 3–16).

Proteins Can Adopt a Limited Number of Different Protein Folds

It is astounding to consider the rapidity of the increase in our knowledge about cells. In 1950, we did not know the order of the amino acids in a single protein, and many even doubted that the amino acids in proteins are arranged in an exact sequence. In 1960, the first three-dimensional structure of a protein was determined by x-ray crystallography. Now that we have access to hundreds of

Figure 3–15 A comparison of a class of DNA-binding domains, called homeodomains, in a pair of proteins from two organisms separated by more than a billion years of evolution. (A) A ribbon model of the structure common to both proteins. (B) A trace of the α-carbon positions. The three-dimensional structures shown were determined by x-ray crystallography for the yeast α2 protein *(green)* and the *Drosophila* engrailed protein *(red)*. (C) A comparison of amino acid sequences for the region of the proteins shown in (A) and (B). *Black dots* mark sites with identical amino acids. *Orange dots* indicate the position of a three amino acid insert in the α2 protein. (Adapted from C. Wolberger et al., *Cell* 67:517–528, 1991.)



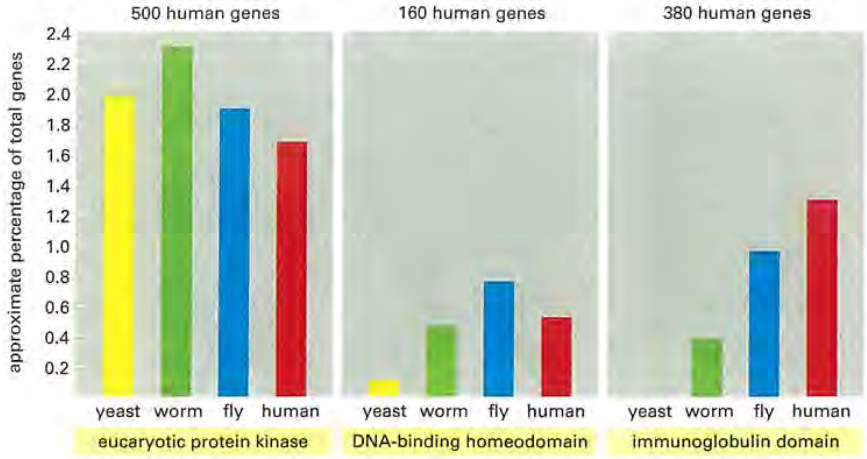


Figure 3–16 Percentage of total genes containing one or more copies of the indicated protein domain, as derived from complete genome sequences. Note that one of the three domains selected, the immunoglobulin domain, has been a relatively late addition, and its relative abundance has increased in the vertebrate lineage. The estimates of human gene numbers are approximate.

thousands of protein sequences from sequencing the genes that encode them, what technical developments can we look forward to next?

It is no longer a big step to progress from a gene sequence to the production of large amounts of the pure protein encoded by that gene. Thanks to DNA cloning and genetic engineering techniques (discussed in Chapter 8), this step is often routine. But there is still nothing routine about determining the complete three-dimensional structure of a protein. The standard technique based on x-ray diffraction requires that the protein be subjected to conditions that cause the molecules to aggregate into a large, perfectly ordered crystalline array—that is, a protein crystal. Each protein behaves quite differently in this respect, and protein crystals can be generated only through exhaustive trial-and-error methods that often take many years to succeed—if they succeed at all.

Membrane proteins and large protein complexes with many moving parts have generally been the most difficult to crystallize, which is why only a few such protein structures are displayed in this book. Increasingly, therefore, large proteins have been analyzed through determination of the structures of their individual domains: either by crystallizing isolated domains and then bombarding the crystals with x-rays, or by studying the conformations of isolated domains in concentrated aqueous solutions with powerful nuclear magnetic resonance (NMR) techniques (discussed in Chapter 8). From a combination of x-ray and NMR studies, we now know the three-dimensional shapes, or conformations, of thousands of different proteins.

By carefully comparing the conformations of known proteins, structural biologists (that is, experts on the structure of biological molecules) have concluded that there are a limited number of ways in which protein domains fold up—maybe as few as 2000. As we saw, the structures for about 1000 of these protein folds have thus far been determined; we may, therefore, already know half of the total number of possible structures for a protein domain. A complete catalog of all of the protein folds that exist in living organisms would therefore seem to be within our reach.

Sequence Homology Searches Can Identify Close Relatives

The present database of known protein sequences contains more than 500,000 entries, and it is growing very rapidly as more and more genomes are sequenced—revealing huge numbers of new genes that encode proteins. Powerful computer search programs are available that allow one to compare each newly discovered protein with this entire database, looking for possible relatives. Homologous proteins are defined as those whose genes have evolved from a common ancestral gene, and these are identified by the discovery of statistically significant similarities in amino acid sequences.

With such a large number of proteins in the database, the search programs find many nonsignificant matches, resulting in a background noise level that makes it very difficult to pick out all but the closest relatives. Generally speaking, a 30% identity in the sequence of two proteins is needed to be certain that a match has been found. However, many short signature sequences ("fingerprints") indicative of particular protein functions are known, and these are widely used to find more distant homologies (Figure 3–17).

These protein comparisons are important because related structures often imply related functions. Many years of experimentation can be saved by discovering that a new protein has an amino acid sequence homology with a protein of known function. Such sequence homologies, for example, first indicated that certain genes that cause mammalian cells to become cancerous are protein kinases. In the same way, many of the proteins that control pattern formation during the embryonic development of the fruit fly *Drosophila* were quickly recognized to be gene regulatory proteins.

Computational Methods Allow Amino Acid Sequences to Be Threaded into Known Protein Folds

We know that there are an enormous number of ways to make proteins with the same three-dimensional structure, and that—over evolutionary time—random mutations can cause amino acid sequences to change without a major change in the conformation of a protein. For this reason, one current goal of structural biologists is to determine all the different protein folds that proteins have in nature, and to devise computer-based methods to test the amino acid sequence of a domain to identify which one of these previously determined conformations the domain is likely to adopt.

A computational technique called threading can be used to fit an amino acid sequence to a particular protein fold. For each possible fold known, the computer searches for the best fit of the particular amino acid sequence to that structure. Are the hydrophobic residues on the inside? Are the sequences with a strong propensity to form an α helix in an α helix? And so on. The best fit gets a numerical score reflecting the estimated stability of the structure.

In many cases, one particular three-dimensional structure will stand out as a good fit for the amino acid sequence, suggesting an approximate conformation for the protein domain. In other cases, none of the known folds will seem possible. By applying x-ray and NMR studies to the latter class of proteins, structural biologists hope to able to expand the number of known folds rapidly, aiming for a database that contains the complete library of protein folds that exist in nature. With such a library, plus expected improvements in the computational methods used for threading, it may eventually become possible to obtain an approximate three-dimensional structure for a protein as soon as its amino acid sequence is known.

Some Protein Domains, Called Modules, Form Parts of Many Different Proteins

As previously stated, most proteins are composed of a series of protein domains, in which different regions of the polypeptide chain have folded independently to form compact structures. Such multidomain proteins are believed to have originated when the DNA sequences that encode each domain accidentally became joined, creating a new gene. Novel binding surfaces have often been created at the juxtaposition of domains, and many of the functional sites where

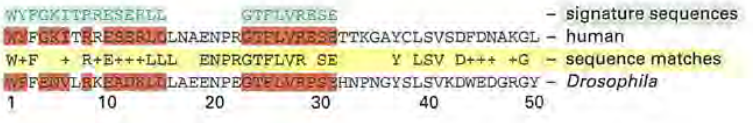


Figure 3–17 The use of short signature sequences to find homologous protein domains. The two short sequences of 15 and 9 amino acids shown *(green)* can be used to search large databases for a protein domain that is found in many proteins, the SH2 domain. Here, the first 50 amino acids of the SH2 domain of 100 amino acids is compared for the human and *Drosophila* Src protein (see Figure 3–12). In the computer-generated sequence comparison *(yellow row)*, exact matches between the human and *Drosophila* proteins are noted by the one-letter abbreviation for the amino acid; the positions with a similar but nonidentical amino acid are denoted by +, and nonmatches are blank. In this diagram, wherever one or both proteins contain an exact match to a position in the *green* sequences, both aligned sequences are colored *red*.

proteins bind to small molecules are found to be located there (for an example see Figure 3–12). Many large proteins show clear signs of having evolved by the joining of preexisting domains in new combinations, an evolutionary process called *domain shuffling* (Figure 3–18).

A subset of protein domains have been especially mobile during evolution; these so-called **protein modules** are generally somewhat smaller (40–200 amino acids) than an average domain, and they seem to have particularly versatile structures. The structure of one such module, the SH2 domain, was illustrated in Panel 3–2 (pp. 138–139). The structures of some additional protein modules are illustrated in Figure 3–19.

Each of the modules shown has a stable core structure formed from strands of β sheet, from which less-ordered loops of polypeptide chain protrude *(green)*. The loops are ideally situated to form binding sites for other molecules, as most flagrantly demonstrated for the immunoglobulin fold, which forms the basis for antibody molecules (see Figure 3–42). The evolutionary success of such β-sheet-based modules is likely to have been due to their providing a convenient framework for the generation of new binding sites for ligands through small changes to these protruding loops.

A second feature of protein modules that explains their utility is the ease with which they can be integrated into other proteins. Five of the six modules illustrated in Figure 3–19 have their N- and C-terminal ends at opposite poles of the module. This "in-line" arrangement means that when the DNA encoding such a module undergoes tandem duplication, which is not unusual in the evolution of genomes (discussed in Chapter 7), the duplicated modules can be readily linked in series to form extended structures—either with themselves or with other in-line modules (Figure 3–20). Stiff extended structures composed of a series of modules are especially common in extracellular matrix molecules and in the extracellular portions of cell-surface receptor proteins. Other modules, including the SH2 domain and the kringle module illustrated in Figure 3–19, are

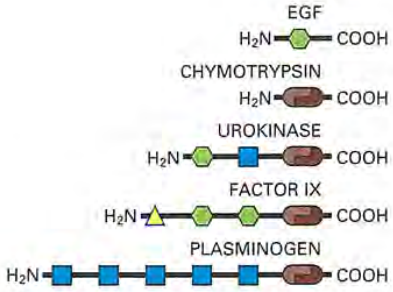


Figure 3–18 Domain shuffling. An extensive shuffling of blocks of protein sequence (protein domains) has occurred during protein evolution. Those portions of a protein denoted by the same shape and color in this diagram are evolutionarily related. Serine proteases like chymotrypsin are formed from two domains *(brown)*. In the three other proteases shown, which are highly regulated and more specialized, these two protease domains are connected to one or more domains homologous to domains found in epidermal growth factor (EGF; *green*), to a calcium-binding protein *(yellow)*, or to a "kringle" domain *(blue)* that contains three internal disulfide bridges. Chymotrypsin is illustrated in Figure 3–14.

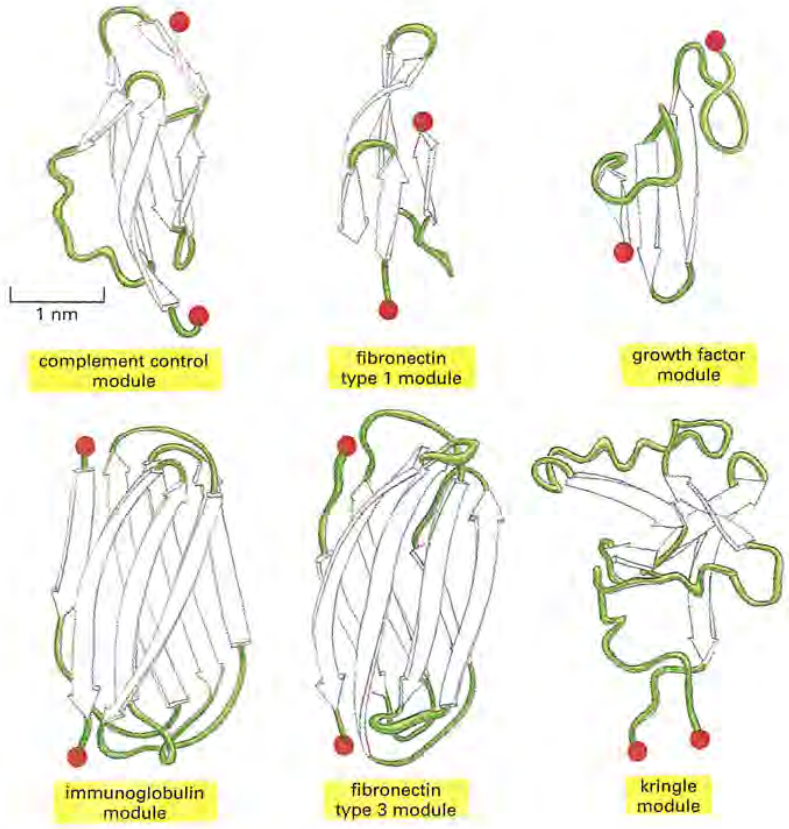


Figure 3–19 The three-dimensional structures of some protein modules. In these ribbon diagrams, β-sheet strands are shown as *arrows*, and the N- and C-termini are indicated by *red spheres*. (Adapted from M. Baron, D.G. Norman, and I.D. Campbell, *Trends Biochem. Sci.* 16:13–17, 1991, and D.J. Leahy et al., *Science* 258:987–991, 1992.)

of a "plug-in" type. After genomic rearrangements, such modules are usually accommodated as an insertion into a loop region of a second protein.

The Human Genome Encodes a Complex Set of Proteins, Revealing Much That Remains Unknown

The result of sequencing the human genome has been surprising, because it reveals that our chromosomes contain only 30,000 to 35,000 genes. With regard to gene number, we would appear to be no more than 1.4-fold more complex than the tiny mustard weed, *Arabidopsis,* and less than 2-fold more complex than a nematode worm. The genome sequences also reveal that vertebrates have inherited nearly all of their protein domains from invertebrates—with only 7 percent of identified human domains being vertebrate-specific.

Each of our proteins is on average more complicated, however. A process of domain shuffling during vertebrate evolution has given rise to many novel combinations of protein domains, with the result that there are nearly twice as many combinations of domains found in human proteins as in a worm or a fly. Thus, for example, the trypsinlike serine protease domain is linked to at least 18 other types of protein domains in human proteins, whereas it is found covalently joined to only 5 different domains in the worm. This extra variety in our proteins greatly increases the range of protein–protein interactions possible (see Figure 3–78), but how it contributes to making us human is not known.

The complexity of living organisms is staggering, and it is quite sobering to note that we currently lack even the tiniest hint of what the function might be for more than 10,000 of the proteins that have thus far been identified in the human genome. There are certainly enormous challenges ahead for the next generation of cell biologists, with no shortage of fascinating mysteries to solve.

Larger Protein Molecules Often Contain More Than One Polypeptide Chain

The same weak noncovalent bonds that enable a protein chain to fold into a specific conformation also allow proteins to bind to each other to produce larger structures in the cell. Any region of a protein's surface that can interact with another molecule through sets of noncovalent bonds is called a **binding site**. A protein can contain binding sites for a variety of molecules, both large and small. If a binding site recognizes the surface of a second protein, the tight binding of two folded polypeptide chains at this site creates a larger protein molecule with a precisely defined geometry. Each polypeptide chain in such a protein is called a **protein subunit**.

In the simplest case, two identical folded polypeptide chains bind to each other in a "head-to-head" arrangement, forming a symmetric complex of two protein subunits (a *dimer*) held together by interactions between two identical binding sites. The *Cro repressor protein*—a gene regulatory protein that binds to DNA to turn genes off in a bacterial cell—provides an example (Figure 3–21). Many other types of symmetric protein complexes, formed from multiple copies of a single polypeptide chain, are commonly found in cells. The enzyme

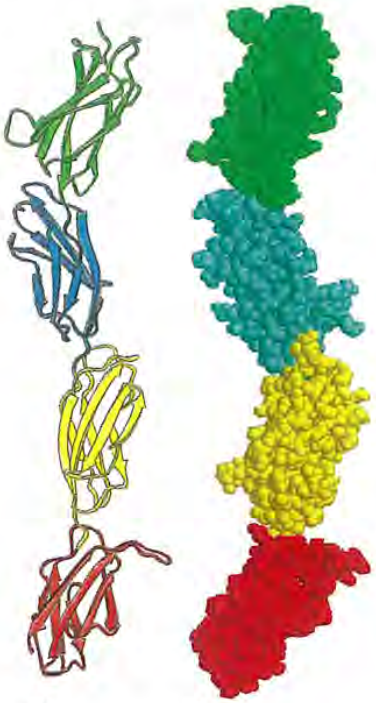


Figure 3–20 An extended structure formed from a series of in-line protein modules. Four fibronectin type 3 modules (see Figure 3–19) from the extracellular matrix molecule fibronectin are illustrated in (A) ribbon and (B) space-filling models. (Adapted from D.J. Leahy, I. Aukhil, and H.P. Erickson, *Cell* 84:155–164, 1996.)

Figure 3–21 Two identical protein subunits binding together to form a symmetric protein dimer. The Cro repressor protein from bacteriophage lambda binds to DNA to turn off viral genes. Its two identical subunits bind head-to-head, held together by a combination of hydrophobic forces *(blue)* and a set of hydrogen bonds *(yellow* region). (Adapted from D.H. Ohlendorf, D.E. Tronrud, and B.W. Matthews, *J. Mol. Biol.* 280:129–136, 1998.)

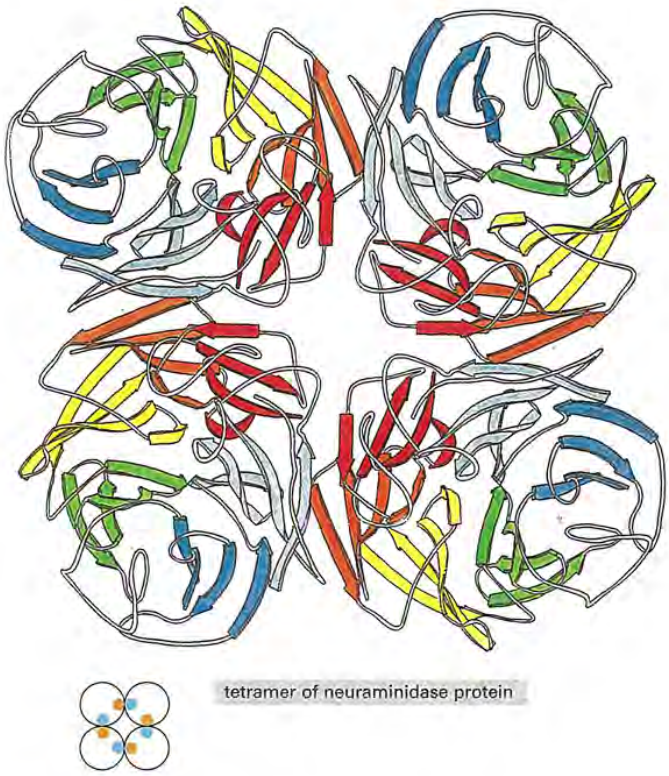


Figure 3–22 A protein molecule containing multiple copies of a single protein subunit. The enzyme neuraminidase exists as a ring of four identical polypeptide chains. The small diagram shows how the repeated use of the same binding interaction forms the structure.

neuraminidase, for example, consists of four identical protein subunits, each bound to the next in a "head-to-tail" arrangement that forms a closed ring (Figure 3–22).

Many of the proteins in cells contain two or more types of polypeptide chains. *Hemoglobin*, the protein that carries oxygen in red blood cells, is a particularly well-studied example (Figure 3–23). It contains two identical α-globin subunits and two identical β-globin subunits, symmetrically arranged. Such multisubunit proteins are very common in cells, and they can be very large. Figure 3–24 provides a sampling of proteins whose exact structures are known, allowing the sizes and shapes of a few larger proteins to be compared with the relatively small proteins that we have thus far presented as models.

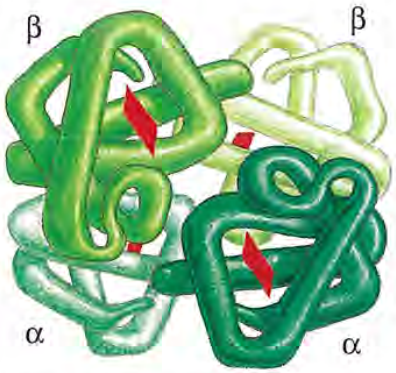


Figure 3–23 A protein formed as a symmetric assembly of two different subunits. Hemoglobin is an abundant protein in red blood cells that contains two copies of α globin and two copies of β globin. Each of these four polypeptide chains contains a heme molecule *(red)*, which is the site where oxygen (O_2) is bound. Thus, each molecule of hemoglobin in the blood carries four molecules of oxygen.

Some Proteins Form Long Helical Filaments

Some protein molecules can assemble to form filaments that may span the entire length of a cell. Most simply, a long chain of identical protein molecules can be constructed if each molecule has a binding site complementary to another region of the surface of the same molecule (Figure 3–25). An actin filament, for example, is a long helical structure produced from many molecules of the protein *actin* (Figure 3–26). Actin is very abundant in eucaryotic cells, where it constitutes one of the major filament systems of the cytoskeleton (discussed in Chapter 16).

Why is a helix such a common structure in biology? As we have seen, biological structures are often formed by linking subunits that are very similar to each other—such as amino acids or protein molecules—into long, repetitive chains. If all the subunits are identical, the neighboring subunits in the chain can often fit together in only one way, adjusting their relative positions to minimize the free energy of the contact between them. As a result, each subunit is positioned in exactly the same way in relation to the next, so that subunit 3 fits onto subunit 2 in the same way that subunit 2 fits onto subunit 1, and so on. Because it is very rare for subunits to join up in a straight line, this arrangement generally results in a helix—a regular structure that resembles a spiral staircase,

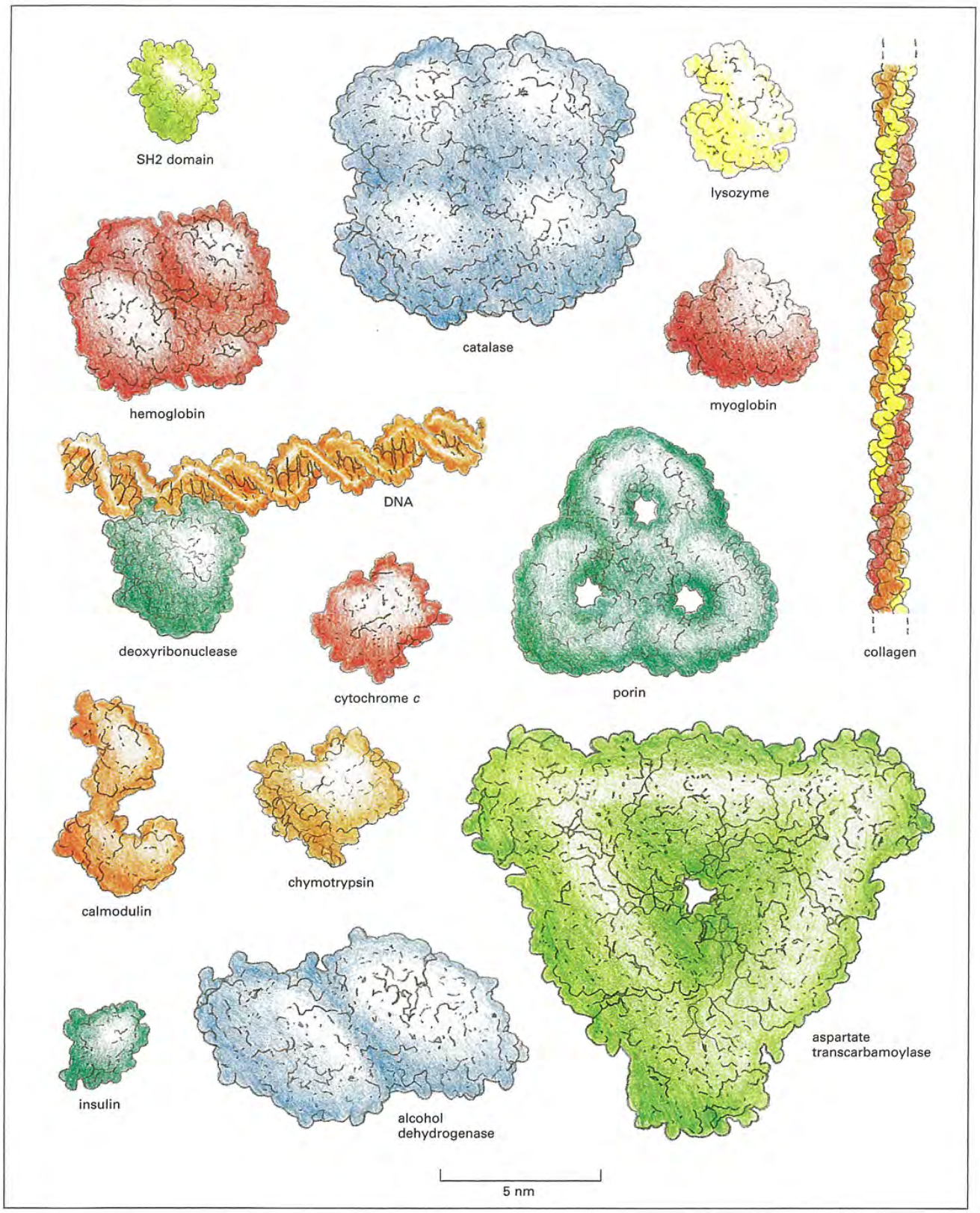


Figure 3–24 A collection of protein molecules, shown at the same scale. For comparison, a DNA molecule bound to a protein is also illustrated. These space-filling models represent a range of sizes and shapes. Hemoglobin, catalase, porin, alcohol dehydrogenase, and aspartate transcarbamoylase are formed from multiple copies of subunits. The SH2 domain *(top left)* is presented in detail in Panel 3–2 (pp. 138–139). (After David S. Goodsell, Our Molecular Nature. New York: Springer-Verlag, 1996.)

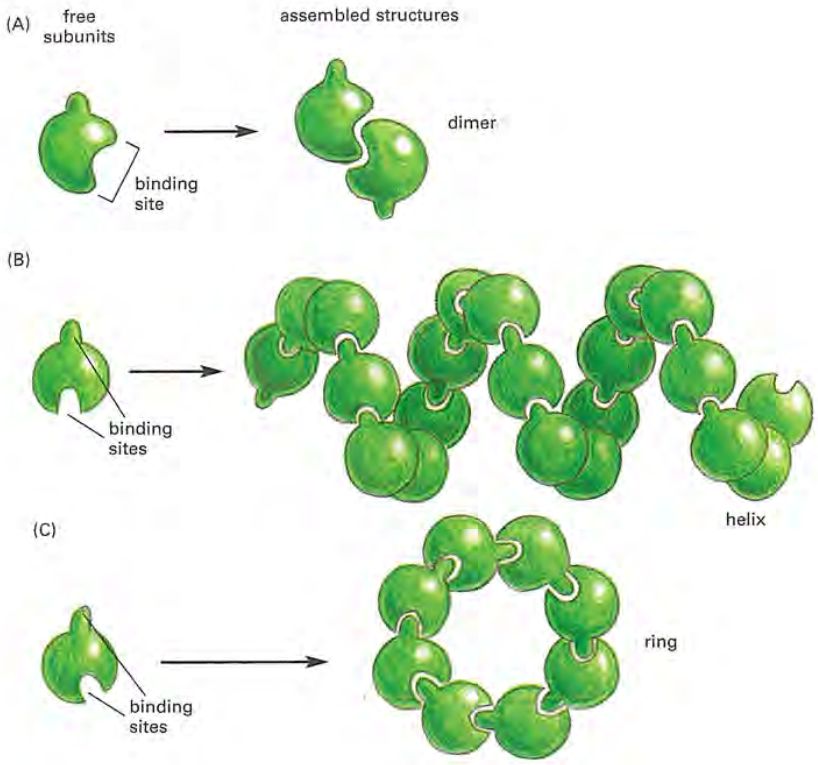


Figure 3–25 Protein assemblies. (A) A protein with just one binding site can form a dimer with another identical protein. (B) Identical proteins with two different binding sites often form a long helical filament. (C) If the two binding sites are disposed appropriately in relation to each other, the protein subunits may form a closed ring instead of a helix. (For an example of A, see Figure 3–21; for an example of C, see Figure 3–22.)

as illustrated in Figure 3–27. Depending on the twist of the staircase, a helix is said to be either right-handed or left-handed (Figure 3–27E). Handedness is not affected by turning the helix upside down, but it is reversed if the helix is reflected in the mirror.

Helices occur commonly in biological structures, whether the subunits are small molecules linked together by covalent bonds (for example, the amino acids in an α helix) or large protein molecules that are linked by noncovalent forces (for example, the actin molecules in actin filaments). This is not surprising. A helix is an unexceptional structure, and it is generated simply by placing many similar subunits next to each other, each in the same strictly repeated relationship to the one before.

A Protein Molecule Can Have an Elongated, Fibrous Shape

Most of the proteins we have discussed so far are *globular proteins,* in which the polypeptide chain folds up into a compact shape like a ball with an irregular surface. Enzymes tend to be globular proteins: even though many are large and complicated, with multiple subunits, most have an overall rounded shape (see Figure 3–24). In contrast, other proteins have roles in the cell requiring each individual protein molecule to span a large distance. These proteins generally have a relatively simple, elongated three-dimensional structure and are commonly referred to as *fibrous proteins.*

One large family of intracellular fibrous proteins consists of α-keratin, introduced earlier, and its relatives. Keratin filaments are extremely stable and are the main component in long-lived structures such as hair, horn, and nails. An α-keratin molecule is a dimer of two identical subunits, with the long α helices of each subunit forming a coiled-coil (see Figure 3–11). The coiled-coil regions are capped at each end by globular domains containing binding sites. This enables this class of protein to assemble into ropelike *intermediate filaments*—an important component of the cytoskeleton that creates the cell's internal structural scaffold (see Figure 16–16).

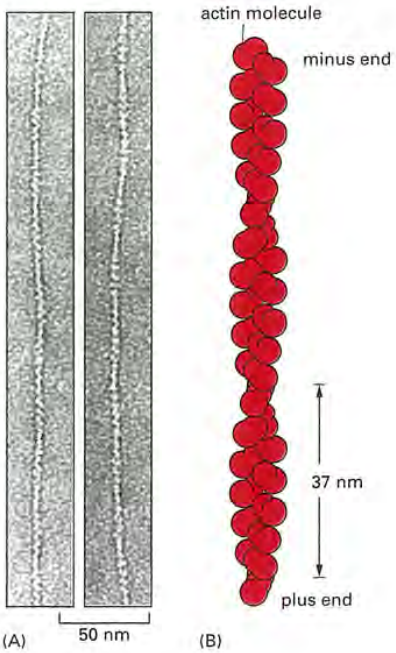


Figure 3–26 Actin filaments. (A) Transmission electron micrographs of negatively stained actin filaments. (B) The helical arrangement of actin molecules in an actin filament. (A, courtesy of Roger Craig.)

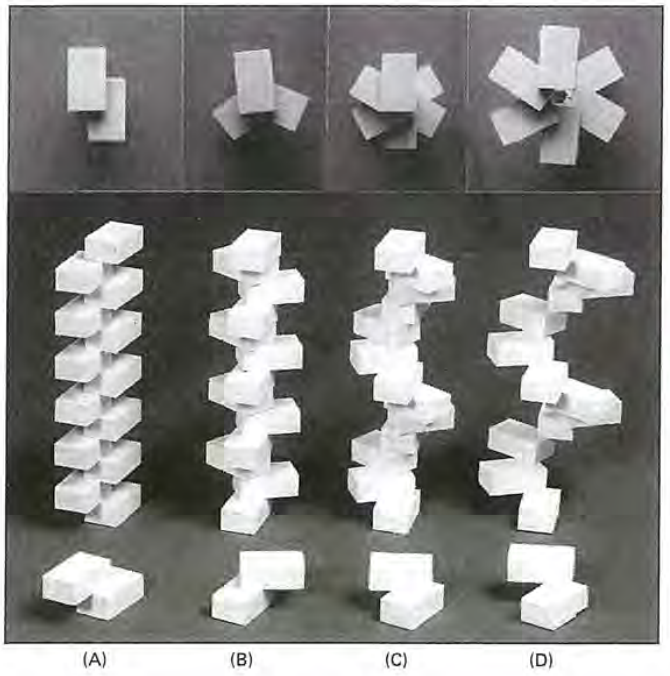


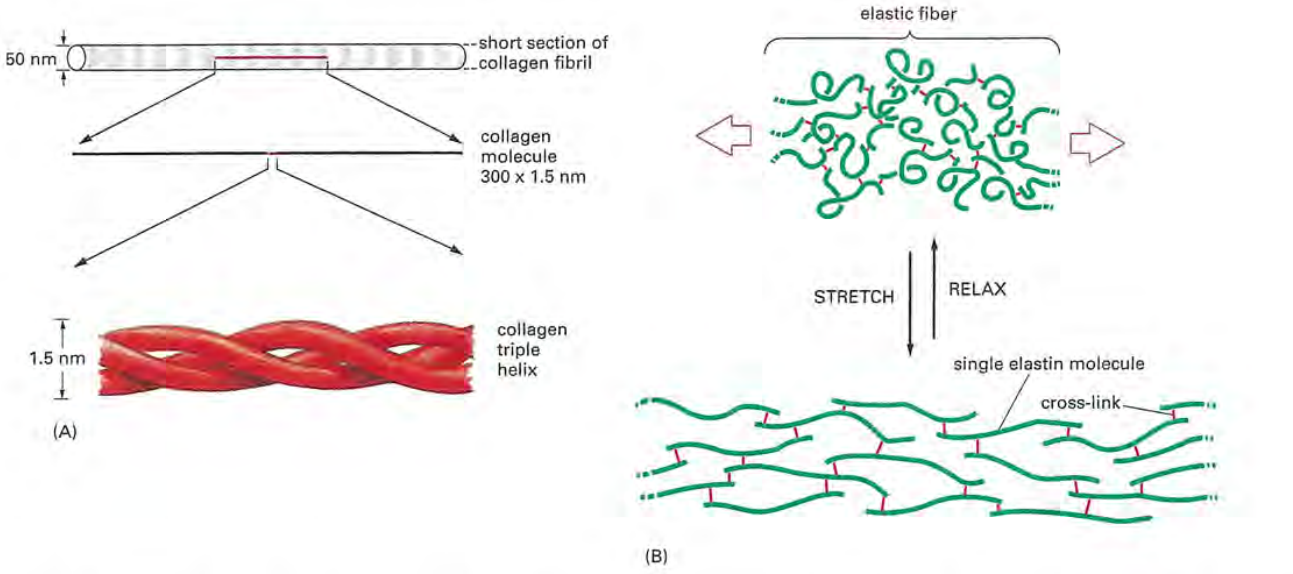
Figure 3–27 Some properties of a helix. (A–D) A helix forms when a series of subunits bind to each other in a regular way. At the bottom, the interaction between two subunits is shown; behind them are the helices that result. These helices have two (A), three (B), and six (C and D) subunits per helical turn. At the top, the arrangement of subunits has been photographed from directly above the helix. Note that the helix in (D) has a wider path than that in (C), but the same number of subunits per turn. (E) A helix can be either right-handed or left-handed. As a reference, it is useful to remember that standard metal screws, which insert when turned clockwise, are right-handed. Note that a helix retains the same handedness when it is turned upside down.

Fibrous proteins are especially abundant outside the cell, where they are a main component of the gel-like *extracellular matrix* that helps to bind collections of cells together to form tissues. Extracellular matrix proteins are secreted by the cells into their surroundings, where they often assemble into sheets or long fibrils. *Collagen* is the most abundant of these proteins in animal tissues. A collagen molecule consists of three long polypeptide chains, each containing the nonpolar amino acid glycine at every third position. This regular structure allows the chains to wind around one another to generate a long regular triple helix (Figure 3–28A). Many collagen molecules then bind to one another side-by-side and end-to-end to create long overlapping arrays—thereby generating the extremely tough collagen fibrils that give connective tissues their tensile strength, as described in Chapter 19.

In complete contrast to collagen is another protein in the extracellular matrix, *elastin*. Elastin molecules are formed from relatively loose and unstructured polypeptide chains that are covalently cross-linked into a rubberlike elastic meshwork: unlike most proteins, they do not have a uniquely defined stable structure, but can be reversibly pulled from one conformation to another, as illustrated in Figure 3–28B. The resulting elastic fibers enable skin and other tissues, such as arteries and lungs, to stretch and recoil without tearing.

Extracellular Proteins Are Often Stabilized by Covalent Cross-Linkages

Many protein molecules are either attached to the outside of a cell's plasma membrane or secreted as part of the extracellular matrix. All such proteins are directly exposed to extracellular conditions. To help maintain their structures, the polypeptide chains in such proteins are often stabilized by covalent cross-linkages. These linkages can either tie two amino acids in the same protein together, or connect different polypeptide chains in a multisubunit protein. The most common cross-linkages in proteins are covalent sulfur–sulfur bonds. These *disulfide bonds* (also called *S–S bonds*) form as proteins are being prepared for export from cells. As described in Chapter 12, their formation is catalyzed in the endoplasmic reticulum by an enzyme that links together two pairs of –SH groups of cysteine side chains that are adjacent in the folded protein (Figure 3–29). Disulfide bonds do not change the conformation of a protein but



instead act as atomic staples to reinforce its most favored conformation. For example, lysozyme—an enzyme in tears that dissolves bacterial cell walls—retains its antibacterial activity for a long time because it is stabilized by such cross-linkages.

Disulfide bonds generally fail to form in the cell cytosol, where a high concentration of reducing agents converts S–S bonds back to cysteine –SH groups. Apparently, proteins do not require this type of reinforcement in the relatively mild environment inside the cell.

Figure 3–28 Collagen and elastin. (A) Collagen is a triple helix formed by three extended protein chains that wrap around one another *(bottom)*. Many rodlike collagen molecules are cross-linked together in the extracellular space to form unextendable collagen fibrils *(top)* that have the tensile strength of steel. The striping on the collagen fibril is caused by the regular repeating arrangement of the collagen molecules within the fibril. (B) Elastin polypeptide chains are cross-linked together to form rubberlike, elastic fibers. Each elastin molecule uncoils into a more extended conformation when the fiber is stretched and recoils spontaneously as soon as the stretching force is relaxed.

Protein Molecules Often Serve as Subunits for the Assembly of Large Structures

The same principles that enable a protein molecule to associate with itself to form rings or filaments operate to generate much larger structures in the cell—supramolecular structures such as enzyme complexes, ribosomes, protein filaments, viruses, and membranes. These large objects are not made as single, giant, covalently linked molecules. Instead they are formed by the noncovalent assembly of many separately manufactured molecules, which serve as the subunits of the final structure.

The use of smaller subunits to build larger structures has several advantages:

1. A large structure built from one or a few repeating smaller subunits requires only a small amount of genetic information.

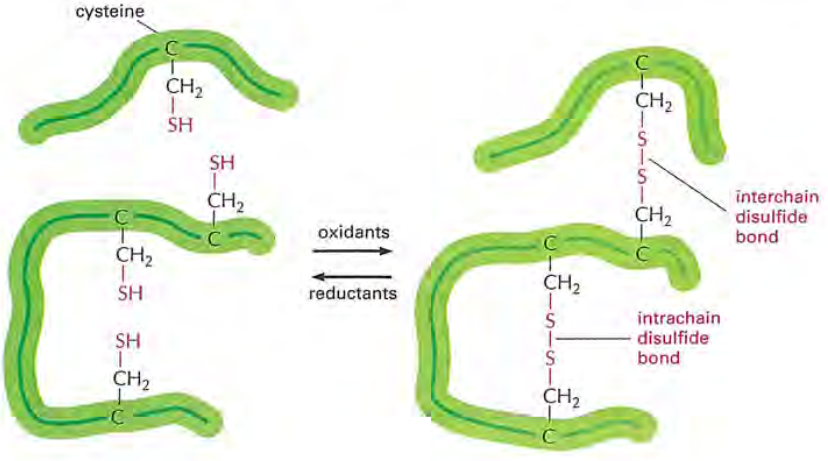


Figure 3–29 Disulfide bonds. This diagram illustrates how covalent disulfide bonds form between adjacent cysteine side chains. As indicated, these cross-linkages can join either two parts of the same polypeptide chain or two different polypeptide chains. Since the energy required to break one covalent bond is much larger than the energy required to break even a whole set of noncovalent bonds (see Table 2–2, p. 57), a disulfide bond can have a major stabilizing effect on a protein.

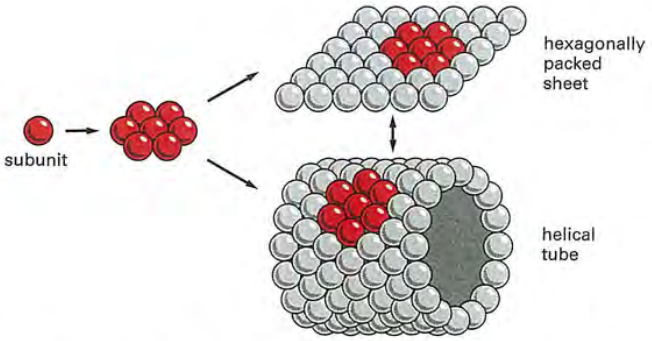


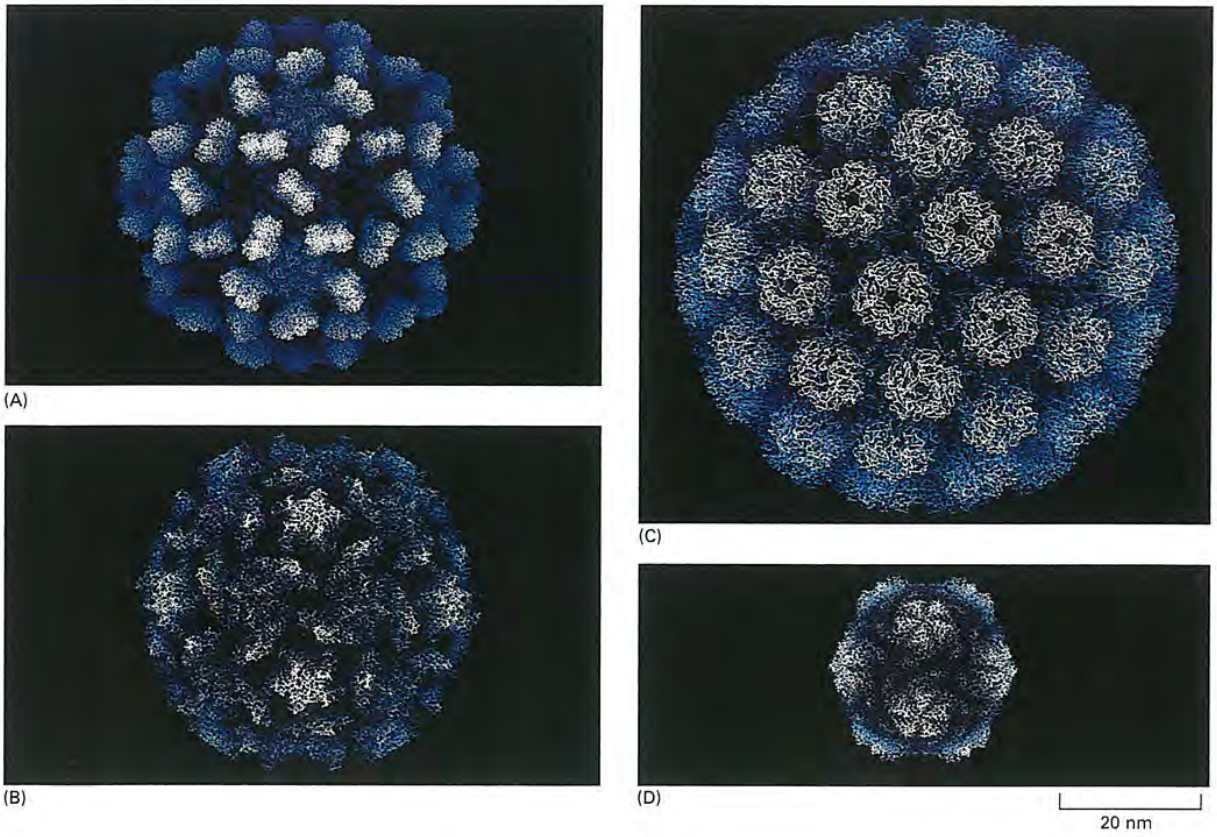
Figure 3–30 An example of single protein subunit assembly requiring multiple protein–protein contacts. Hexagonally packed globular protein subunits can form either a flat sheet or a tube.

2. Both assembly and disassembly can be readily controlled, reversible processes, since the subunits associate through multiple bonds of relatively low energy.
3. Errors in the synthesis of the structure can be more easily avoided, since correction mechanisms can operate during the course of assembly to exclude malformed subunits.

Some protein subunits assemble into flat sheets in which the subunits are arranged in hexagonal patterns. Specialized membrane proteins are sometimes arranged this way in lipid bilayers. With a slight change in the geometry of the individual subunits, a hexagonal sheet can be converted into a tube (Figure 3–30) or, with more changes, into a hollow sphere. Protein tubes and spheres that bind specific RNA and DNA molecules form the coats of viruses.

The formation of closed structures, such as rings, tubes, or spheres, provides additional stability because it increases the number of bonds between the protein subunits. Moreover, because such a structure is created by mutually dependent, cooperative interactions between subunits, it can be driven to assemble or disassemble by a relatively small change that affects each subunit individually. These principles are dramatically illustrated in the protein coat or *capsid* of many simple viruses, which takes the form of a hollow sphere (Figure 3–31).

Figure 3–31 The capsids of some viruses, all shown at the same scale. (A) Tomato bushy stunt virus; (B) poliovirus; (C) simian virus 40 (SV40); (D) satellite tobacco necrosis virus. The structures of all of these capsids have been determined by x-ray crystallography and are known in atomic detail. (Courtesy of Robert Grant, Stephan Crainic, and James M. Hogle.)



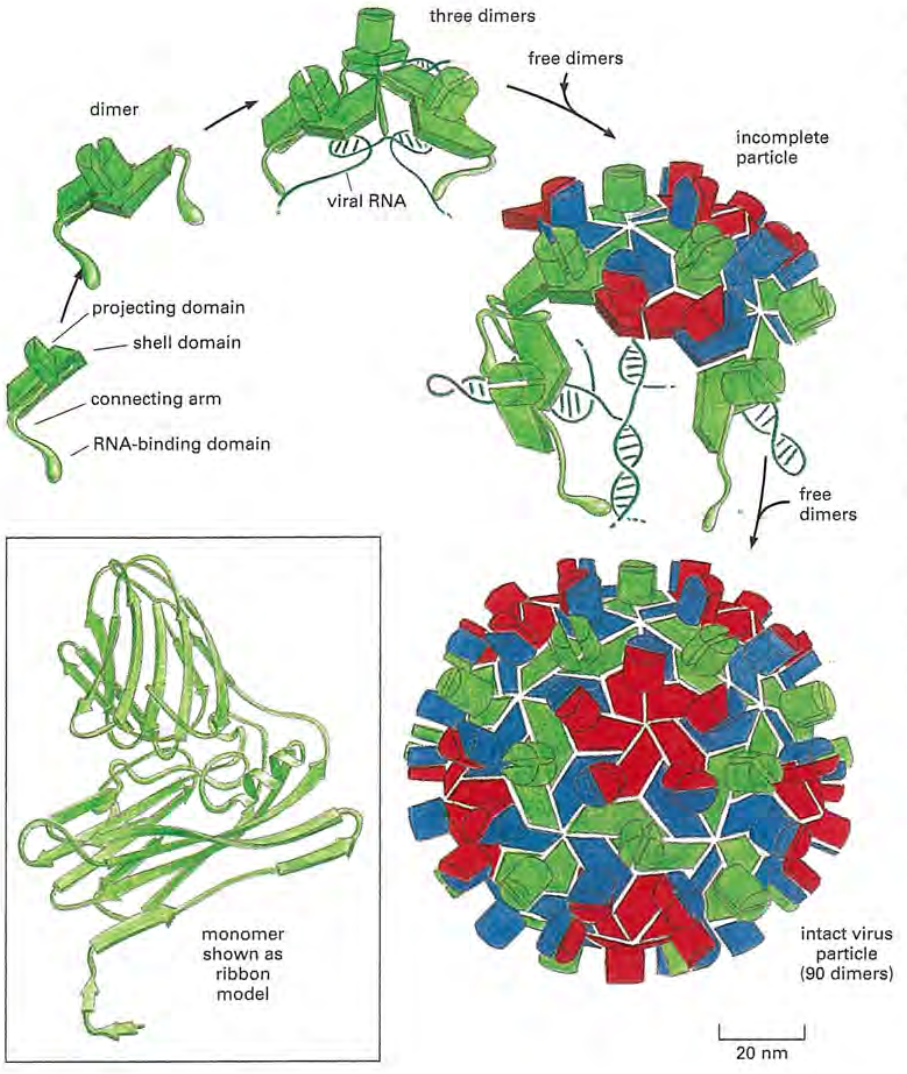


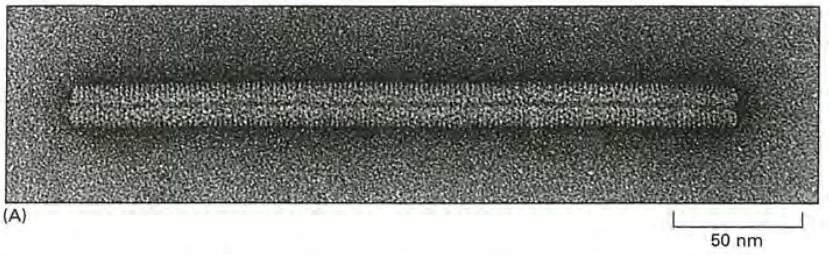
Figure 3–32 The structure of a spherical virus. In many viruses, identical protein subunits pack together to create a spherical shell (a capsid) that encloses the viral genome, composed of either RNA or DNA (see also Figure 3–31). For geometric reasons, no more than 60 identical subunits can pack together in a precisely symmetric way. If slight irregularities are allowed, however, more subunits can be used to produce a larger capsid. The tomato bushy stunt virus (TBSV) shown here, for example, is a spherical virus about 33 nm in diameter formed from 180 identical copies of a 386 amino acid capsid protein plus an RNA genome of 4500 nucleotides. To construct such a large capsid, the protein must be able to fit into three somewhat different environments, each of which is differently colored in the virus particle shown here. The postulated pathway of assembly is shown; the precise three-dimensional structure has been determined by x-ray diffraction. (Courtesy of Steve Harrison.)

Capsids are often made of hundreds of identical protein subunits that enclose and protect the viral nucleic acid (Figure 3–32). The protein in such a capsid must have a particularly adaptable structure: it must not only make several different kinds of contacts to create the sphere, it must also change this arrangement to let the nucleic acid out to initiate viral replication once the virus has entered a cell.

Many Structures in Cells Are Capable of Self-Assembly

The information for forming many of the complex assemblies of macromolecules in cells must be contained in the subunits themselves, because purified subunits can spontaneously assemble into the final structure under the appropriate conditions. The first large macromolecular aggregate shown to be capable of self-assembly from its component parts was *tobacco mosaic virus (TMV)*. This virus is a long rod in which a cylinder of protein is arranged around a helical RNA core (Figure 3–33). If the dissociated RNA and protein subunits are mixed together in solution, they recombine to form fully active viral particles. The assembly process is unexpectedly complex and includes the formation of double rings of protein, which serve as intermediates that add to the growing viral coat.

Another complex macromolecular aggregate that can reassemble from its component parts is the bacterial ribosome. This structure is composed of about



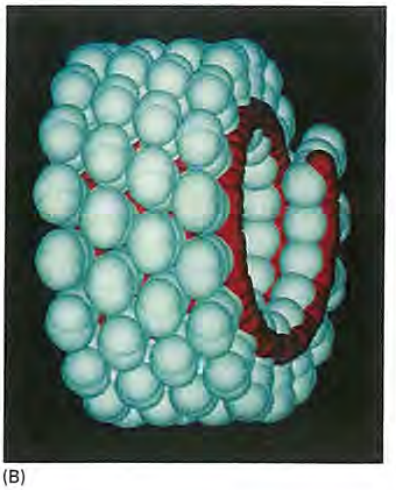


Figure 3–33 The structure of tobacco mosaic virus (TMV). (A) An electron micrograph of the viral particle, which consists of a single long RNA molecule enclosed in a cylindrical protein coat composed of identical protein subunits. (B) A model showing part of the structure of TMV. A single-stranded RNA molecule of 6000 nucleotides is packaged in a helical coat constructed from 2130 copies of a coat protein 158 amino acids long. Fully infective viral particles can self-assemble in a test tube from purified RNA and protein molecules. (A, courtesy of Robley Williams; B, courtesy of Richard J. Feldmann.)

55 different protein molecules and 3 different rRNA molecules. If the individual components are incubated under appropriate conditions in a test tube, they spontaneously re-form the original structure. Most importantly, such reconstituted ribosomes are able to perform protein synthesis. As might be expected, the reassembly of ribosomes follows a specific pathway: after certain proteins have bound to the RNA, this complex is then recognized by other proteins, and so on, until the structure is complete.

It is still not clear how some of the more elaborate self-assembly processes are regulated. Many structures in the cell, for example, seem to have a precisely defined length that is many times greater than that of their component macromolecules. How such length determination is achieved is in many cases a mystery. Three possible mechanisms are illustrated in Figure 3–34. In the simplest case, a long core protein or other macromolecule provides a scaffold that determines the extent of the final assembly. This is the mechanism that determines the length of the TMV particle, where the RNA chain provides the core. Similarly, a core protein is thought to determine the length of the thin filaments in muscle, as well as the length of the long tails of some bacterial viruses (Figure 3–35).

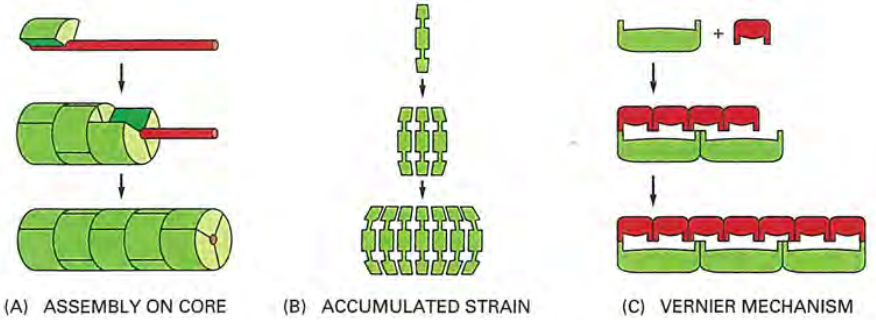


Figure 3–34 Three mechanisms of length determination for large protein assemblies. (A) Coassembly along an elongated core protein or other macromolecule that acts as a measuring device. (B) Termination of assembly because of strain that accumulates in the polymeric structure as additional subunits are added, so that beyond a certain length the energy required to fit another subunit onto the chain becomes excessively large. (C) A vernier type of assembly, in which two sets of rodlike molecules differing in length form a staggered complex that grows until their ends exactly match. The name derives from a measuring device based on the same principle, used in mechanical instruments.

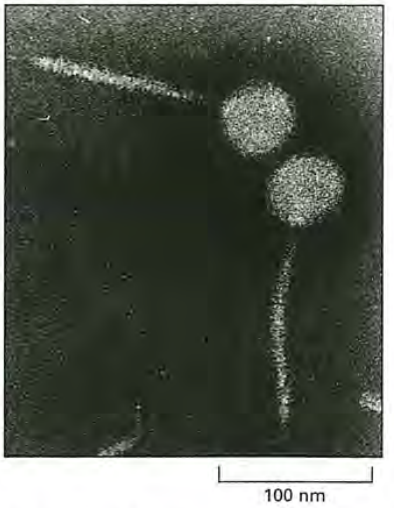


Figure 3–35 An electron micrograph of bacteriophage lambda. The tip of the virus tail attaches to a specific protein on the surface of a bacterial cell, after which the tightly packaged DNA in the head is injected through the tail into the cell. The tail has a precise length, determined by the mechanism shown in Figure 3–34A.

The Formation of Complex Biological Structures Is Often Aided by Assembly Factors

Not all cellular structures held together by noncovalent bonds are capable of self-assembly. A mitochondrion, a cilium, or a myofibril of a muscle cell, for example, cannot form spontaneously from a solution of its component macromolecules. In these cases, part of the assembly information is provided by special enzymes and other cellular proteins that perform the function of templates, guiding construction but taking no part in the final assembled structure.

Even relatively simple structures may lack some of the ingredients necessary for their own assembly. In the formation of certain bacterial viruses, for example, the head, which is composed of many copies of a single protein subunit, is assembled on a temporary scaffold composed of a second protein. Because the second protein is absent from the final viral particle, the head structure cannot spontaneously reassemble once it has been taken apart. Other examples are known in which proteolytic cleavage is an essential and irreversible step in the normal assembly process. This is even the case for some small protein assemblies, including the structural protein collagen and the hormone insulin (Figure 3–36). From these relatively simple examples, it seems very likely that the assembly of a structure as complex as a mitochondrion or a cilium will involve temporal and spatial ordering imparted by numerous other cell components.

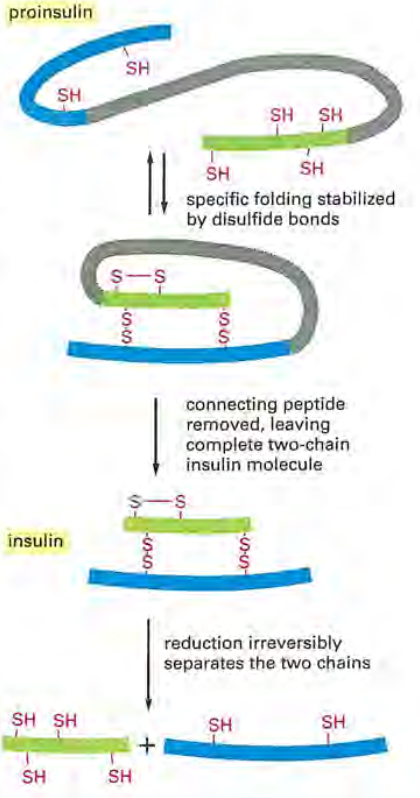


Figure 3–36 Proteolytic cleavage in insulin assembly. The polypeptide hormone insulin cannot spontaneously re-form efficiently if its disulfide bonds are disrupted. It is synthesized as a larger protein *(proinsulin)* that is cleaved by a proteolytic enzyme after the protein chain has folded into a specific shape. Excision of part of the proinsulin polypeptide chain removes some of the information needed for the protein to fold spontaneously into its normal conformation once it has been denatured and its two polypeptide chains separated.

Summary

The three-dimensional conformation of a protein molecule is determined by its amino acid sequence. The folded structure is stabilized by noncovalent interactions between different parts of the polypeptide chain. The amino acids with hydrophobic side chains tend to cluster in the interior of the molecule, and local hydrogen-bond interactions between neighboring peptide bonds give rise to α helices and β sheets.

Globular regions, known as domains, are the modular units from which many proteins are constructed; such domains generally contain 40–350 amino acids. Small proteins typically consist of only a single domain, while large proteins are formed from several domains linked together by short lengths of polypeptide chain. As proteins have evolved, domains have been modified and combined with other domains to construct new proteins. Domains that participate in the formation of large numbers of proteins are known as protein modules. Thus far, about 1000 different ways of folding up a domain have been observed, among more than about 10,000 known protein structures.

Proteins are brought together into larger structures by the same noncovalent forces that determine protein folding. Proteins with binding sites for their own surface can assemble into dimers, closed rings, spherical shells, or helical polymers. Although mixtures of proteins and nucleic acids can assemble spontaneously into complex structures in a test tube, many biological assembly processes involve irreversible steps. Consequently, not all structures in the cell are capable of spontaneous reassembly after they have been dissociated into their component parts.

PROTEIN FUNCTION

We have seen that each type of protein consists of a precise sequence of amino acids that allows it to fold up into a particular three-dimensional shape, or conformation. But proteins are not rigid lumps of material. They can have precisely engineered moving parts whose mechanical actions are coupled to chemical events. It is this coupling of chemistry and movement that gives proteins the extraordinary capabilities that underlie the dynamic processes in living cells.

In this section, we explain how proteins bind to other selected molecules and how their activity depends on such binding. We show that the ability to bind to other molecules enables proteins to act as catalysts, signal receptors, switches, motors, or tiny pumps. The examples we discuss in this chapter by no means exhaust the vast functional repertoire of proteins. However, the specialized

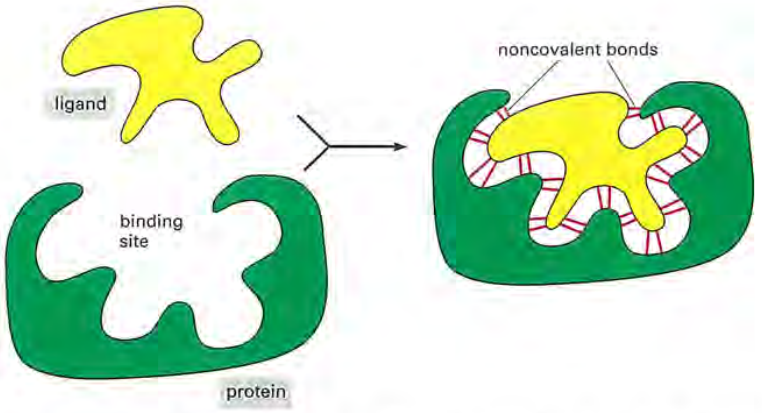


Figure 3–37 The selective binding of a protein to another molecule. Many weak bonds are needed to enable a protein to bind tightly to a second molecule, which is called a *ligand* for the protein. A ligand must therefore fit precisely into a protein's binding site, like a hand into a glove, so that a large number of noncovalent bonds can be formed between the protein and the ligand.

functions of many of the proteins you will encounter elsewhere in this book are based on similar principles.

All Proteins Bind to Other Molecules

The biological properties of a protein molecule depend on its physical interaction with other molecules. Thus, antibodies attach to viruses or bacteria to mark them for destruction, the enzyme hexokinase binds glucose and ATP so as to catalyze a reaction between them, actin molecules bind to each other to assemble into actin filaments, and so on. Indeed, all proteins stick, or *bind*, to other molecules. In some cases, this binding is very tight; in others, it is weak and short-lived. But the binding always shows great *specificity*, in the sense that each protein molecule can usually bind just one or a few molecules out of the many thousands of different types it encounters. The substance that is bound by the protein—no matter whether it is an ion, a small molecule, or a macromolecule— is referred to as a **ligand** for that protein (from the Latin word *ligare*, meaning "to bind").

The ability of a protein to bind selectively and with high affinity to a ligand depends on the formation of a set of weak, noncovalent bonds—hydrogen bonds, ionic bonds, and van der Waals attractions—plus favorable hydrophobic interactions (see Panel 2–3, pp. 114–115). Because each individual bond is weak, an effective binding interaction requires that many weak bonds be formed simultaneously. This is possible only if the surface contours of the ligand molecule fit very closely to the protein, matching it like a hand in a glove (Figure 3–37).

The region of a protein that associates with a ligand, known as the ligand's *binding site,* usually consists of a cavity in the protein surface formed by a particular arrangement of amino acids. These amino acids can belong to different portions of the polypeptide chain that are brought together when the protein folds (Figure 3–38). Separate regions of the protein surface generally provide binding sites for different ligands, allowing the protein's activity to be regulated, as we shall see later. And other parts of the protein can serve as a handle to place the protein in a particular location in the cell—an example is the SH2 domain discussed previously, which is often used to move a protein containing it to sites in the plasma membrane in response to particular signals.

Although the atoms buried in the interior of the protein have no direct contact with the ligand, they provide an essential scaffold that gives the surface its contours and chemical properties. Even small changes to the amino acids in the interior of a protein molecule can change its three-dimensional shape enough to destroy a binding site on the surface.

The Details of a Protein's Conformation Determine Its Chemistry

Proteins have impressive chemical capabilities because the neighboring chemical groups on their surface often interact in ways that enhance the chemical reactivity of amino acid side chains. These interactions fall into two main categories.

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BMC Proceedings

MEETING ABSTRACT Open Access

Disulphide bond reduction of a therapeutic monoclonal antibody during cell culture manufacturing operations

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Background

Disulphide bonding is critical to maintaining immunoglobulin (IgG) tertiary and quaternary structure for therapeutic monoclonal antibodies (MAb). Both inter- and intra-chain disulphide bonds are formed intracellularly in the expression host prior to secretion and purification during MAb production processes. Disulphide bond shuffling has previously been reported for IgG_2[1,2] and disulphide-mediated arm-exchange for IgG_4[3,4], reflecting innate behaviour of these IgG classes. However, atypical and significant reduction of disulphide bonds has been recently observed in IgG_1[5,6] that present significant issues for manufacturing of therapeutic MAbs.

During manufacturing of preliminary lots of a recently transferred MAb manufacturing process (IgG_1), gross disulphide bond reduction following affinity capture chromatography of clarified production bioreactor material was observed. Investigations leading to the identification of the nature of this reduction process, and process steps to mitigate against its future occurrence, are described here. The MAb was co-developed with MacroGenics, Rockville, MD.

Methods

Production Bioreactor material for downstream processing was supplied from a 16 day, fed-batch, GS-CHO culture [7]. The Production Bioreactor was a single-use (Wave System) with a 100L (full scale) or 10L (lab model) working volume. Clarified harvest intermediate (CHI) hold studies were performed in either 560L Lev-Mix units (full scale) or 5L Braun benchtop bioreactors (lab model). Purification to produce Affinity Capture chromatography eluted mainstream was performed using a 20cm x 20cm MAbselect Protein A resin (GE Healthcare) column, and an AKTA Process skid (GE Healthcare).

LC-MS analysis was performed on a Polymer Laboratories PLRP-S HPLC column and analyzed using an Agilent 1100 HPLC system coupled to an Applied Biosystems QSTAR XL mass spectrometer, following sample preparation. CE-SDS analysis was performed using a Beckman Coulter PA800 capillary electrophoresis instrument fitted with bare-fused silica capillary and UV detection at 220 nm, following sample preparation. Microchip CE-SDS analysis was performed using a Lab-on-chip microanalyser (Agilent). Free thiols were quantified using Ellman's reagent. Metabolic analysis was conducted by Metabolon (Durham, NC).

Results

Identification of disulphide reduction of IgG during Primary Recovery

The IgG manufacturing process as transferred from the co-developing partner included a cell culture settling step following the Production Bioreactor and prior to Primary Recovery.

Disulphide bond reduction was first detected during initial development runs by routine Non-reduced (NR) CE-SDS in-process analysis after Affinity capture chromatography (data not shown). NR-CE-SDS analysis identified elevated levels of free light chain and half antibody molecules, when compared to Reference Standard.

Additional analysis, employing microchip-based NR-CE-SDS methods indicated that the antibody reduction occurred during the primary recovery cell settling step

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(results not shown). This was confirmed by LC-MC analysis (results not shown). Assessment of disulphide bonding pattern and intactness by LC-MS peptide mapping identified both inter- and intra-chain disulphide scrambling (results not shown).

Delineation of events leading to IgG reduction

Initial investigations to understand process behaviour during primary recovery identified that reducing species, including free thiols (which increase over the course of the Production Bioreactor, up to 1mM), were present at the end of the Production Bioreactor (Figure 1a). Dissolved oxygen was also shown to deplete during the cell settling phase following harvest (data not shown). From this, an initial working hypothesis was formed that reducing species, including free thiols, became reactive at low dissolved oxygen concentrations and led to IgG_1 disulphide bond reduction.

A revised process control strategy was implemented (see below) to prevent oxygen depletion and maintain dissolved oxygen levels above a minimum level. This involved including an aerated and agitated hold for Clarified Harvest Intermediate (CHI) in the process.

Further studies identified that O_2 is critical to maintaining a stable environment for oxidised (i.e., normally disulphide bonded) IgG_1 in CHI. When O_2 was present, IgG_1 remained intact under all conditions evaluated. Only when O_2 was deliberately absent, or stripped away, would the harvest material or CHI demonstrate potential for reduction (Figure 1b).

Metabolic behaviour of reducing intermediates

The working hypothesis was that by maintaining sufficient levels of dissolved oxygen in the CFM, the thiol species could be reacted out (oxidised) and a stable environment for oxidised IgG_1 created (Figure 1a). However, the relationship between IgG reduction and thiol redox state is not first order (Figure 1c), and the rate of thiol oxidation was found to be dependent on the source of Production Bioreactor material (i.e., varied with different harvest lots). This indicated the involvement of an additional component, potentially catalytic, which has not yet been identified in our studies. Thioredoxins have been identified as such a catalytic component by others [5,6], and these need to be recycled after one redox cycle via Thioredoxin Reductase / NADP(H).

Metabolic analysis of cell and media material from Production Bioreactors indicated high levels of oxidised homocysteine and cysteine (both reactive redox molecules), which correlated with decreasing levels of folate

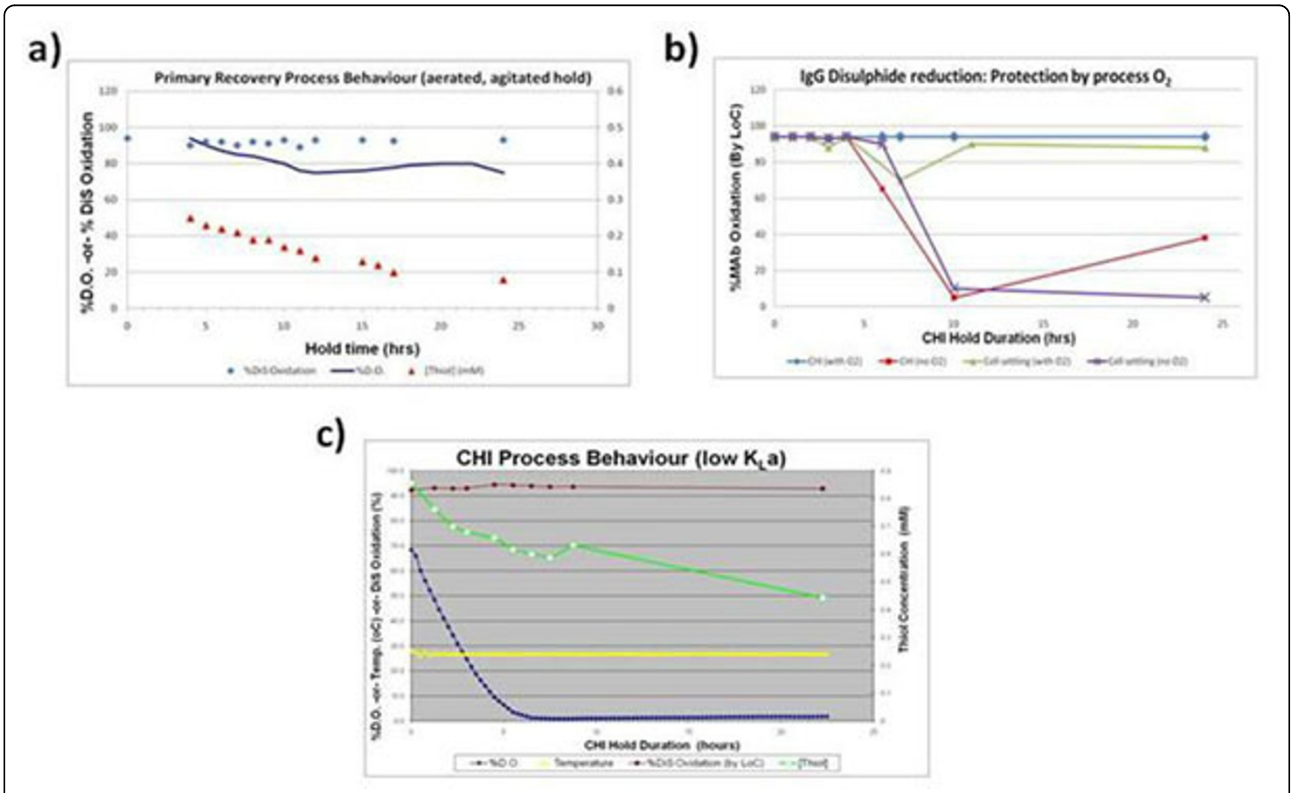

Figure 1 IgG disulphide bond reduction under various conditions for cell-settled and immediately clarified harvest material. CHI, Clarified harvest intermediate; DiS, Disulphide; LoC, Lab-on-a-Chip (Agilent).

(B6) and cobalamine (B12), both of which are involved in recycling homocysteine. Overall, this analysis identified numerous options for media optimisation to mitigate against IgG reduction. However, given the success of process controls (described below), and the late stage of process development (pre-validation) these media optimisation options were not pursued.

Process controls to mitigate disulphide reduction of IgG

A process control strategy was implemented including:

- Establishing a minimal dissolved oxygen level in the Production Bioreactor prior to harvesting
- Immediately clarifying the Production Bioreactor material (i.e., eliminating the cell settling step)
- Holding the CHI in a hold vessel (LevMix container, agitated hold) that had been partially pre-filled with process air.

Conclusions

- Gross disulphide bond reduction was observed during late stage development of an IgG_1 monoclonal antibody being commercialised for a therapeutic indication;
- Disulphide bond reduction had a second, or higher, order link to low dissolved oxygen levels in process intermediates, and the involvement of a catalytic factor was also indicated;
- Implementation of an appropriate control strategy (and associated process analytics) informed by process development has ensured no recurrence of this issue (for n=15 full scale lots).

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ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Identification and Prevention of Antibody Disulfide Bond Reduction During Cell Culture Manufacturing

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ABSTRACT: In the biopharmaceutical industry, therapeutic monoclonal antibodies are primarily produced in mammalian cell culture systems. During the scale-up of a monoclonal antibody production process, we observed excessive mechanical cell shear as well as significant reduction of the antibody's interchain disulfide bonds during harvest operations. This antibody reduction event was catastrophic as the product failed to meet the drug substance specifications and the bulk product was lost. Subsequent laboratory studies have demonstrated that cells subjected to mechanical shear release cellular enzymes that contribute to this antibody reduction phenomenon (manuscript submitted; Kao et al., 2009). Several methods to prevent this antibody reduction event were developed using a lab-scale model to reproduce the lysis and reduction events. These methods included modifications to the cell culture media with chemicals (e.g., cupric sulfate ($CuSO_4$)), pre- and post-harvest chemical additions to the cell culture fluid (CCF) (e.g., $CuSO_4$, EDTA, L-cystine), as well as lowering the pH and air sparging of the harvested CCF (HCCF). These methods were evaluated for their effectiveness in preventing disulfide bond reduction and their impact to product quality. Effective prevention methods, which yielded acceptable product quality were evaluated for their potential to be implemented at manufacturing-scale. The work described here identifies numerous effective reduction prevention measures from lab-scale studies; several of these methods were then successfully translated into manufacturing processes.

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KEYWORDS: antibody; disulfide; reduction; centrifugation; shear; lysis

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Introduction

Recombinant monoclonal antibodies (rMAb) have become prevalent as a clinical therapy over the last 20 years for oncology and immunological diseases (Reichert, 2001, 2002; Reichert and Pavolu, 2004; Reichert et al., 2005). Currently for commercialized products in the biopharmaceutical industry, rMAbs are typically produced in mammalian cell culture in large stainless steel fermentors up to 25-kL in scale (Benton et al., 2002; Kelley, 2007). After initiating a production bioreactor, various process additions and parameter manipulations are performed to maximize growth and antibody production and yield suitable product quality (Andersen and Krummen, 2002; Andersen and Reilly, 2004; Birch et al., 2005; Birch and Racher, 2006; Wurm, 2004). The rMAbs are expressed and secreted extracellularly into the cell culture fluid (CCF). At the end of the production phase, the feedstock is usually harvested by disc stacked centrifugation followed by depth filtration or by tangential flow microfiltration (Kempken et al., 1995; Roush and Lu, 2008). The resulting harvested cell culture fluid (HCCF) is then purified using protein A affinity chromatography, a series of alternative chromatography steps, and finally formulated by ultrafiltration/diafiltration or size exclusion chromatography (Fahrner et al., 2001; Kelley, 2007).

During process development of the centrifugation step, clarification efficiency during harvest operations is typically determined by measurement of centrate turbidity, particle size distribution, and/or filterability (Kempken et al., 1995; Roush and Lu, 2008). In addition, mechanical cell lysis is also an important consideration during large-scale harvest operations. Excessive mechanical shear during harvest may impact product quality (e.g., aggregates) (Hutchinson et al., 2006) and/or the release of undesirable intracellular components into the HCCF that could degrade the product

of interest or be difficult to clear downstream. Sources of cell shear during harvest could be from the centrifuge equipment alone including feed zones or from any flow restrictions between the fermentor and centrifuge such as an inlet feed pump and inlet flow control valve.

During scale-up of a rMAb process, we observed significant reduction of the antibody's interchain disulfide bonds during harvest operations as well as significant mechanical cell lysis. Subsequent laboratory studies demonstrated that cells subjected to mechanical shear release cellular components that contribute to this antibody reduction event. When a sufficient amount of these active enzymes were present, the extent of product disulfide bond reduction was then dependent on HCCF incubation time (duration), temperature, and dissolved oxygen level. The intrachain disulfide bonds were not reduced as determined by both mass spectrometry and reversed-phase HPLC analysis. Previous work by Zhang and Czupryn (2002) identified the presence of free sulfhydryls and non-covalently associated antibody fragments after purification from CHO-derived HCCF. However, the authors proposed that the origin of these species was related to incomplete disulfide bond formation in the endoplasmic reticulum as a consequence of inefficient assembly of heavy and light chains and not the result of degradation via cellular components.

The disulfide reduction event described here in this work was somewhat difficult to monitor in the HCCF, but was readily analyzed in the purified protein A pool when protein A affinity chromatography was used as the capture step. The enzymatic reduction activity was established through observations of an increase in the level of free thiols during HCCF incubation as well as the required involvement of macromolecules in a dialysis study (data not shown). The currently understood cellular mechanism for rMAb reduction is discussed in a subsequent manuscript (manuscript submitted; Kao et al., 2009).

Here in this work, we describe that mechanical cell shear of highly viable cells is required to induce the antibody reduction phenomenon observed during large-scale manufacturing. Multiple approaches were tested in a lab-scale reduction susceptibility model to determine effective conditions for preventing disulfide reduction during large-scale manufacturing. The methods capable of preventing disulfide reduction were evaluated for their process feasibility as well as their impact to other product quality attributes. Our strategy is to inactivate the reducing enzymes in the HCCF to prevent antibody reduction. It is not desirable to oxidize the reduced interchain disulfides after allowing antibody reduction to occur because the impact of uncontrolled re-oxidation of the antibody poses a potential risk to product quality. For example, reduced antibody might react with other thiol containing molecules in the HCCF or form aggregates through cross linking between reduced cysteines in two different antibodies. This report discusses the antibody reduction events, prevention, and implementation in large-scale manufacturing.

Materials and Methods

Generation of CCF and Production of rMAb

Mammalian cell culture fluids derived from Chinese hamster ovary (CHO) cells were generated using a representative small-scale fermentation process similar to the methods described previously (Chaderjian et al., 2005). Cell culture process indicators (e.g., pH, temperature, dissolved oxygen (DO), agitation rate) were monitored on-line while other culture indicators such as glucose, lactate, ammonium, glutamine, glutamate, and sodium were measured daily. Samples were also taken to monitor cell growth, viability, and rMAb concentration every 24 h.

Lab-Scale HCCF Preparation

At the end of the production culture, complete mechanical lysis of CCF was achieved by high pressure homogenization using a Microfluidics HC-8000 homogenizer. The pressure regulator of the instrument was set to 4,000–8,000 psi, and complete cell lysis (membrane breakage) was achieved after a single pass, as determined by a lactate dehydrogenase (LDH) assay. The homogenate was then blended with the original CCF (non-mechanically lysed) in order to obtain final pools with desired target amounts of total lysis levels. The blends of homogenate and CCF were centrifuged in a Sorval RC-3B rotor centrifuge at 4,500 rpm for 30 min at 20°C. The centrate was decanted, depth filtered and then sterile filtered (0.22 μm) to generate HCCF. For a non-homogenized control, a CCF sample was centrifuged at lab-scale and the centrate was sterile filtered (0.22 μm).

Chemical Inhibitor Additions

The following separate stock solutions were used in the lab-scale HCCF hold time studies: (1) 250 mM EDTA, pH 7.4 prepared using EDTA, disodium dihydrate (Sigma-Aldrich, St. Louis, MO) and EDTA, tetrasodium dihydrate (Sigma); (2) 50 or 80 mM cupric sulfate pentahydrate ($CuSO_4$) (Sigma); (3) 1.0 M acetic acid solution (Mallindkrodt, Hazelwood, MO); and (4) 200 mM L-cystine (Fluka, Sigma-Aldrich, St Louis, MO). The EDTA, $CuSO_4$, acetic acid, or L-cystine stock solutions were added to either the CCF prior to homogenization or directly to the HCCF (post-harvest) to evaluate a range of final concentrations to prevent antibody disulfide reduction. Additions made prior to homogenization were to mimic a pre-harvest addition in a manufacturing setting.

HCCF Pool Incubation in Mini-Tanks

The HCCF pools were generated from either manufacturing-scale runs via large-scale centrifugation and depth filtration or from lab-scale studies as discussed in the previous section. The HCCF was held in 50 mL 316L stainless steel mini-tank containers (Flow Components,

Dublin, CA). The stainless steel mini-tanks were filled with approximately 30–50 mL HCCF, sealed with a clamp to avoid air exposure, and stored at the desired incubation temperature (usually ambient, 18–22°C). The solutions held in the mini-tanks were not aerated or agitated. For experiments with only HCCF sample analysis, samples were taken at pre-determined time-points and immediately frozen at <−70°C until analysis. For experiments with protein A purification, at pre-determined time points (usually after 1- and 2-day holds), the HCCF solution was removed from the mini-tank and immediately purified over a lab-scale protein A affinity column. The generated protein A pools were immediately frozen at <−70°C until analysis.

HCCF Air Sparging

For air sparging studies, 15-L stainless steel vessels (Sartorius Stedim Biotech, Aubagne, France) were used. Approximately 4 L of HCCF was 0.22 μm sterile filtered into each sterilized vessel. HCCF temperature was controlled to 20°C, and the HCCF was agitated during incubation at a rate of 50 rpm (15-L fermentor). Dissolved oxygen, oxidative-reduction potential (ORP), and pH were monitored on-line throughout each study using three separate probes manufactured by Broadley-James, Irvine, CA. The pH of the HCCF was not controlled in these studies. The HCCF was constantly sparged with either air to increase the dissolved oxygen level or with nitrogen (control) to remove any dissolved oxygen in solution. Gas flow to each vessel ranged between 0.01 and 0.02 vvm (~50 mL/min) in order to be representative of a potential future flow rate for a manufacturing process. At pre-determined time points, 50 mL samples were removed from both vessels and purified over a lab-scale protein A affinity column prior to analysis.

Protein A Processing

Antibody purification from the HCCF samples was achieved by Protein A affinity chromatography (Millipore, Billerica, MA, Prosep-vA High Capacity or GE Healthcare, Uppsala, Sweden, MabSelect SuRe). The resin was packed in a 0.66 cm inner diameter glass column (Omnifit, Diba Industries, Danbury, CT) with a 14–20 cm bed height resulting in a 4.8–6.8 mL final column volume. Chromatography was performed using an AKTA Explorer 100 chromatography system (GE Healthcare) at ambient temperature. The protein A purification process varied, depending on the rMAb of interest and the resin. An acidic buffer (~pH 3) was used for elution of product, and each pool was pH adjusted to pH 5–7 using a stock solution of Sodium HEPES or Tris base. Protein A elution pool samples aliquots were stored at <−70°C until analysis by the Bioanalyzer assay to quantitate the percentage of non-reduced antibody at 150 kDa.

Cell Lysis Measurements

Percent cell lysis was determined by measuring the level of LDH, an enzyme that catalyzes the oxidation of lactate to pyruvate (Babson and Babson, 1973; Legrand et al., 1992). All sample aliquots were stored in 0.1 g/L saponin at <−70°C until analysis. The lysis percentage in the CCF supernatant, centrate, and HCCF samples was calculated by dividing the LDH level in the selected sample by the LDH level in the whole cell sample.

Monoclonal Antibody Protein Concentration Assays

For HCCF samples, rMAb concentration was assayed by an HPLC-based protein A method to measure rMAb titer values. The antibody concentration in the purified protein A pool was measured using UV spectrometry at 280 nm and the known extinction coefficient for that particular rMAb.

Disulfide Bond Reduction Assay

For non-reduced SDS-PAGE analyses (Laemmli, 1970), samples were mixed with 4× NuPAGE LDS sample buffer (Life Technologies, Invitrogen, Carlsbad, CA), heated 5–10 min at 70°C, and then loaded onto a precast NuPAGE 4–12% Bis-Tris gel (Invitrogen) with MOPS running buffer. The molecular weight marker was Mark12 Unstained Standard (Invitrogen, LC5677). Gels were stained with either Coomassie Blue (12 μg protein per well) or SYPRO® Ruby stain (2–3 μg product per well) with a 5–7 s exposure. Microchip capillary electrophoresis (CE) (Agilent 2100 Bioanalyzer) was used to quantitate the level of non-reduced antibody. Sample preparation was carried out as described in the Agilent Protein 230 Assay Protocol (G2938-90052) with minor changes defined here. Samples were diluted to 1 g/L with water prior to preparation, and then 0.5% SDS without iodoacetamide (IAM) and 2 μL of denaturing solution were used in the denaturing step. Samples for CE analysis were heated for 5 min at 70°C.

Results

Detection of Antibody Disulfide Reduction

During a manufacturing campaign for a clinical IgG1 monoclonal antibody product, two out of five runs exhibited interchain disulfide reduction in the protein A pool (Fig. 1). In the runs where the antibody was reduced (Runs C and E), a decrease in the amount of the intact IgG band (150 kDa) and an increase in the amount of heavy chain (50 kDa) and light chain (25 kDa) fragments was observed. There was also a slight increase in the heavy-heavy-light band (125 kDa) compared to Runs A, B, and D. Given that only the Fc portion of a monoclonal antibody should bind to the protein A ligand, it was unexpected to observe the presence of light chain in the protein A pool. This occurrence is most likely due to a non-covalent

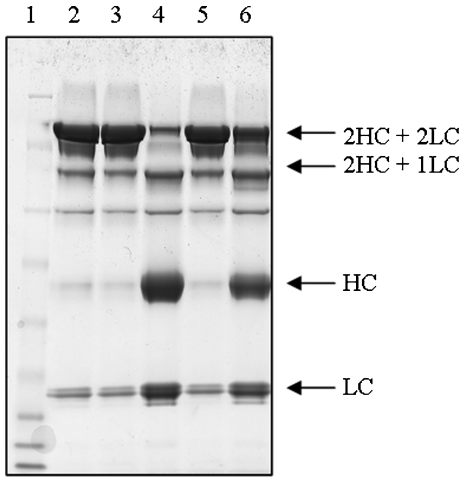

Figure 1. Non-reduced SDS-PAGE (Coomassie Blue stain) analysis of five protein A pools from large-scale manufacturing. **Lane 1**, Molecular weight marker (Invitrogen, LC5925): 191, 97, 64, 51, 39, 28, 19, 14 kDa; **Lane 2**, Run A; **Lane 3**, Run B; **Lane 4**, Run C; **Lane 5**, Run D; **Lane 6**, Run E.

interaction of the heavy and light chain of the antibody even with reduced interchain disulfide bonds. Future work will investigate the role of the protein A step in disulfide reduction including addition of an alykating agent such as iodoacetamide (IAM) to the HCCF post-incubation and prior to protein A purification to eliminate the purification process from influencing the amount of reduced antibody.

Isolation of Reducing Component(s) Activity

HCCF generated from a manufacturing run was purified over a lab-scale protein A column and in addition to the collection of the elution pool, the load flow-through was also collected in order to isolate the reducing enzymes during the protein A process. The lab-scale protein A pool was adjusted from pH 5.3 to pH 7.0 to maintain a similar pH to the HCCF pH where antibody reduction was typically observed. Pure antibody was spiked into the protein A load flow-through at a concentration of ~1 g/L to mimic the concentration in the original HCCF. All three solutions (original HCCF; proA pool; antibody spiked protein A load flow-through) were held separately in stainless steel mini-tanks at 25°C for 22 h. Two mini-tanks were used per solution to start the "$t = 0$" time point at different times of the day in order to obtain hold time data at more frequent intervals. Figure 2 shows non-reduced SDS-PAGE for all three holds where the loss of intact IgG (150 kDa) and additional fragments were observed in both the HCCF and the antibody spiked protein A load flow-through samples after ~14 h of incubation. Excess light chain (25 kDa) was also observed and is the result of excessive expression during the cell culture process. Antibody reduction was not observed in the protein A pool incubation, thus confirming that the reducing enzymes released by the lysed cells are removed during the load phase of the protein A operation and therefore removed from the manufacturing process. In addition, spiking of various purified antibodies into the active protein A load flow-through solution also resulted in disulfide reduction confirming that the event was not molecule structure specific to this particular IgG1 product (data not shown). When the protein A load flow-through, collected using manufacturing-scale HCCF, was incubated at 20°C in 50 mL Falcon tubes for 4 days, the activity of reducing enzymes was lost. However, after incubation of protein A load flow-through at <-70°C and 5°C for 4 days, the reducing activity was preserved as evident in disulfide reduction of antibody after the spike (data not shown).

Lab-Scale Antibody Reduction Model

In order to develop reduction prevention strategies that could be implemented into the manufacturing process, it was imperative to first develop a lab-scale model to accurately reproduce the event. Although the HCCF generated from the manufacturing process can exhibit disulfide reduction during lab-scale incubation, the reducing components may lose activity during long-term HCCF storage at 5°C, after HCCF freeze/thaw, and/or after exposure to oxygen from air surface transfer. In addition, reductant-active HCCF from the manufacturing process may have been generated just on the threshold of lysis required for disulfide reduction, thereby limiting the release of intracellular reducing components. This resulted in experimental inconsistencies when reproducing the reduction event in a lab setting. These complications make it difficult to troubleshoot and inhibit the disulfide reduction event that occurred during large-scale harvest operations. Since conducting studies at large-scale is not practical, a lab-scale reduction susceptibility model was developed to reproduce the large-scale reduction event and eliminate most, if not all, of the variables previously observed.

Homogenization was used to fully lyse the mammalian cells (100% lysis) once the cell culture process was complete. The homogenate was then diluted with CCF supernatant (non-homogenized) to achieve the desired levels of total cell lysis. Figure 3a shows the reduction susceptibility curves using CCF from different cell culture runs for two different products at various amounts of total lysis. The HCCF generated from the blending procedures was incubated at 20°C for 1 day and then purified using protein A chromatography. For Product A, the final cell culture viability measured by Trypan Blue cell counts ranged between 70%, 80%, and 90% and the initial cell lysis measured by the LDH assay was 25%, 20%, and 10%, respectively. For Product B, both cell culture materials tested were ~90% viable by cell count and ~10% initial cell lysis by LDH. Therefore, cell death measurements using both assays matched as expected. From this data, Product A exhibited disulfide bond reduction at 60–70% total lysis for the three different cultures, while Product B reduced at ~40% total lysis for the

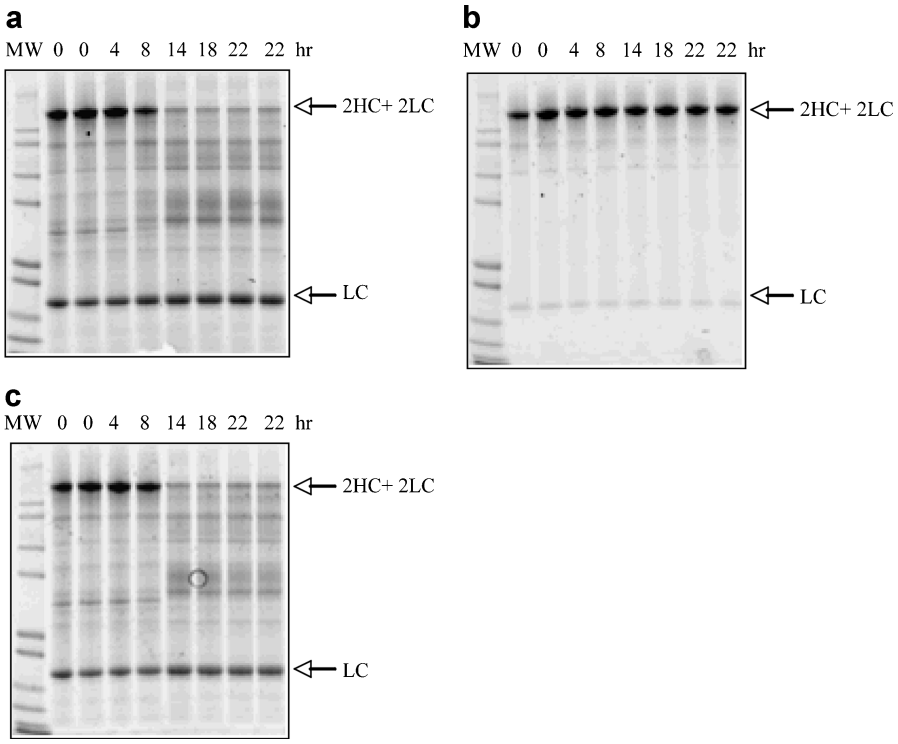

Figure 2. Non-reduced SDS-PAGE analysis (SYPRO® Ruby stain) during mini-tank incubation at 25°C. Molecular weight marker (Invitrogen, LC5677; 200, 116.3, 97.4, 66.3, 55.4, 36.5, 31.0, 21.5, 14.4, 6.0 kDa) is shown and then time of pool incubation (h) is listed above each gel. Duplicate times represent samples taken from a duplicate mini-tank. **a**: Manufacturing-scale generated HCCF. **b**: Lab-scale generated protein A pool. **c**: Lab-scale generated protein A load flow-through spiked with pure antibody.

two different cultures shown. Note that different rMAb processes as well as different cell culture conditions and/or cell culture performance within each rMAb process can lead to differences in susceptibility to disulfide bond reduction. It has also been observed that healthier, actively growing cells contain more reducing components than cultures containing mostly non-viable cells and cultures exhibiting sharp declines in cell viability. With so many factors affecting reduction susceptibility, comparing products in terms of percent lysis is currently the most practical approach although future analysis could include normalization to other factors such as cell density or total cellular protein. For Product A, disulfide reduction was observed during manufacturing runs at $\geq$50% total lysis which is slightly lower than that observed in the lab-scale model. This could be due to differences in the HCCF dissolved oxygen level in the manufacturing-scale HCCF hold tank and the lab-scale mini-tank.

Another way to evaluate these results is to plot the additional lysis instead of total lysis since this represents the mechanical lysis contribution from the harvest operations in a manufacturing run (Fig. 3b). This data plot is then heavily dependent on the initial level of cell lysis for each study. Product A reduced at ~50% additional lysis for the three different cultures shown, while Product B reduced at ~30% additional lysis for the two different cultures shown. For Product A, disulfide reduction was observed during manufacturing runs at ~30% additional lysis, again suggesting differences in the HCCF dissolved oxygen level between manufacturing-scale and lab-scale. One disadvantage of the lab-scale blending model to determine reduction susceptibility is that homogenization requires taking the cells to extreme lysis and then diluting back to a particular percentage compared to the manufacturing process where mechanical cell lysis from the harvest operation is added to the starting level of lysis at the end of the cell culture process.

Chemical Inhibition of Disulfide Reduction

Chemical additions were evaluated as a method to prevent disulfide reduction during HCCF incubation. Since rMAb reduction is caused by reducing enzymes, inhibitors for any of the enzymes involved in the pathway may prevent reduction. Chemical inhibition methods that were evaluated included (1) an increase in the $CuSO_4$ concentration in the cell culture basal media as previously evaluated in a separate study (Chaderjian et al., 2005), (2) the addition of EDTA to pre-harvest CCF or HCCF, (3) the addition of $CuSO_4$ to pre-harvest CCF or HCCF, (4) lowering the pH of the pre-harvest CCF or HCCF to pH 5.0–5.5, and (5) the addition of L-cystine to the pre-harvest CCF. As a known

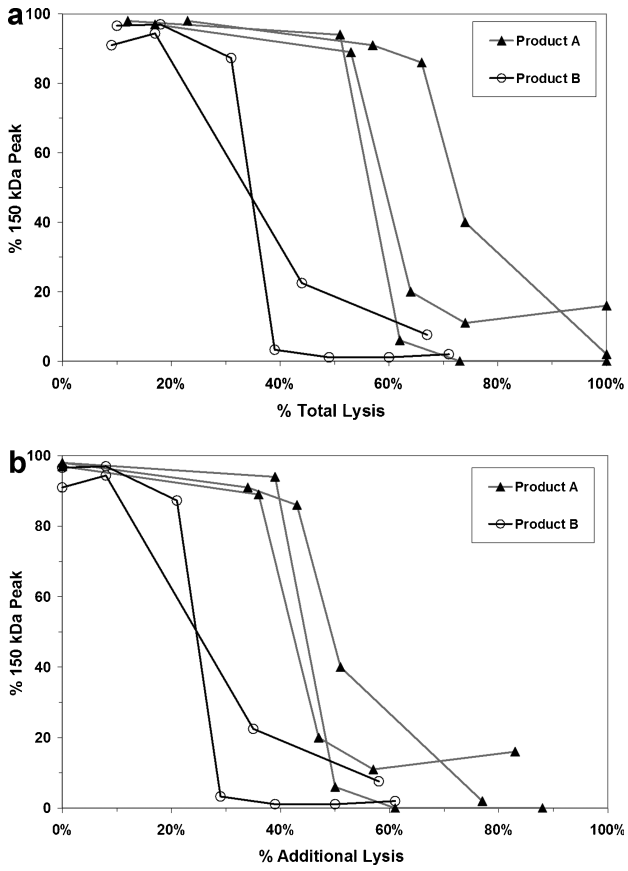

Figure 3. Reduction susceptibility curves for two different rMAb products. Protein A pools were analyzed after 1 day of HCCF incubation at 20°C. **a**: Graphed with total lysis on the *x*-axis. **b**: Graphed with additional mechanical lysis post-fermentation on the *x*-axis. Total lysis is calculated by combining the end of fermentation natural lysis value (e.g., an 80% viable culture exhibits 20% natural lysis) plus the added lysis value obtained from the mechanical shear. The percent intact antibody is calculated via the Bioanalyzer analysis.

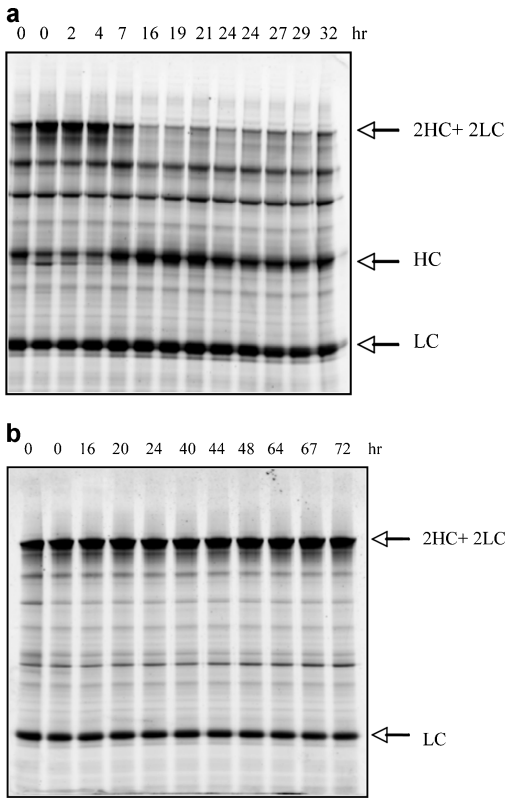

Figure 4. Non-reduced SDS-PAGE analysis (SYPRO® Ruby stain) during HCCF incubation in mini-tanks at 20°C. Time of incubation (h) is listed above each gel. **a**: No EDTA addition. **b**: 12 mM EDTA addition to HCCF prior to incubation.

metal chelator and protease inhibitor, EDTA was added to sequester the metal ions that that may be required for activities of enzymes involved in rMAb reduction. The addition of copper can prevent rMAb reduction by maintaining the reducing components in their oxidized form and/or acting as a direct enzyme inhibitor. L-Cystine may function as a competitive inhibitor against the reducing components. Finally, lowering the CCF/HCCF pH to below pH 6.0 stabilizes the protonated form of free thiols and thus decreases the reduction activity.

HCCF generated from a manufacturing run was incubated in mini-tanks for 32 h in the absence of EDTA or for 72 h in the presence of 12 mM EDTA. In the absence of EDTA, disulfide reduction was observed after only 7 h of incubation at 20°C via a noticeable decrease in the 150 kDa band (intact IgG) and an increase in the 50 kDa band (heavy chain) as shown in Figure 4. By 16 h, there was complete reduction of antibody. However, in the presence of EDTA, reduction was inhibited for the entire 72 h incubation. In a separate study, $CuSO_4$ was added to manufacturing-generated HCCF and incubated in mini-tanks at 20°C for 48 h. At selected time points, the HCCF was purified over protein A to analyze the product in the absence of reducing components. Figure 5 shows the inhibition from the addition of 50 μM $CuSO_4$ compared to the control case where no $CuSO_4$ was added. While analysis of crude HCCF samples was faster and more time points were able to be obtained for analyzing reaction kinetics, it was determined that protein A purification of the incubated HCCF samples was optimal for robust reduction analysis by avoiding the presence of reducing components that could interfere with the analytical measurement.

Once a lab-scale reduction susceptibility model was implemented and proven to be robust and reproducible, chemical inhibitors were then tested in this model. Lab-scale generated HCCF via cell lysis not only can enable testing inhibitor robustness at higher levels of cell lysis, but the large-scale manufacturing generated HCCF tested usually has lower reducing activity due to freeze/thaw. In order to test chemical inhibitors in the lab-scale model, additions can occur to the cell culture basal media, pre-homogenization, or to the final blended HCCF itself. As shown in Figure 6a, four different inhibition methods: (1) addition

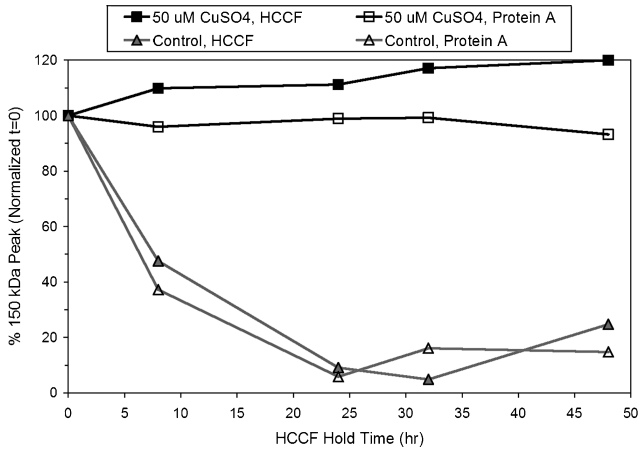

Figure 5. $CuSO_4$ was added to manufacturing-generated HCCF and incubated in mini-tanks at 20°C for 48 h. HCCF samples were taken and frozen as well as purified over protein A chromatography at the shown time points. No $CuSO_4$ was added to the control conditions. Percent 150 kDa peak was quantitated for both the HCCF and protein A pools, and all results were normalized to the $t=0$ h time point for consistency.

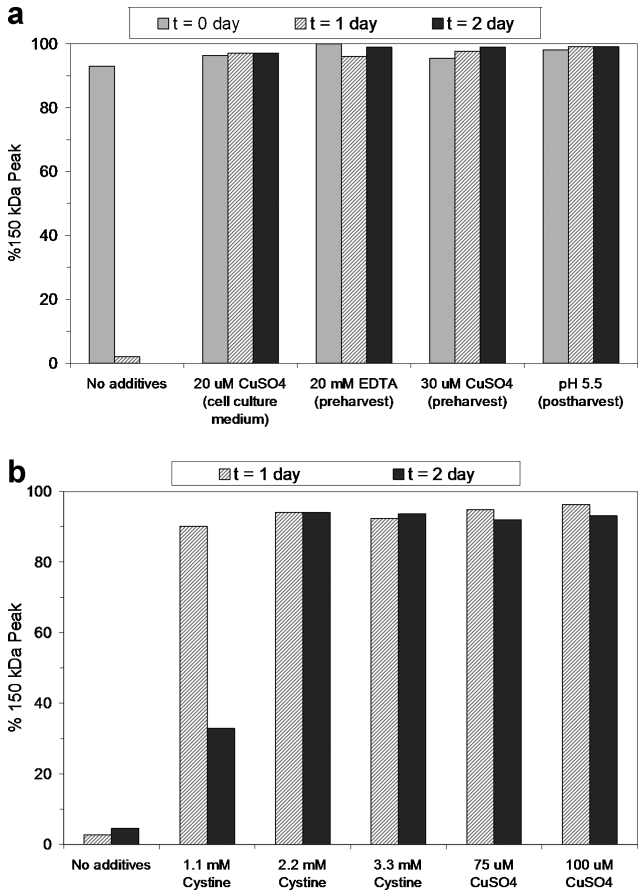

Figure 6. Protein A pool results are shown where different reduction inhibition methods were tested for two different products. Stock solutions of $CuSO_4$, EDTA, or L-cystine were added to the initial cell culture medium or pre-homogenization. To decrease the HCCF pH, a stock solution of glacial acetic acid was added to the HCCF to decrease the pH from pH ~7.0 to pH 5.5. The final HCCF pools were incubated at 20°C and purified using protein A chromatography after 1- and 2-day holds. **a**: Results from one product tested at ~75% total lysis. The 2-day time point from the HCCF without additions was not analyzed. **b**: Results from a second product tested at ~60% total lysis. The 0-day time point was not analyzed for any of the conditions tested.

of EDTA pre-harvest, (2) addition of $CuSO_4$ to the cell culture batch medium, (3) addition of $CuSO_4$ pre-harvest, and (4) adjustment of the HCCF from pH ~7.0 to 5.5, used independently for one feedstock, were effective in preventing disulfide reduction at ~75% total lysis during 2 days of HCCF hold at 20°C. Without an inhibitor addition, the product was completely reduced after just 1 day of HCCF incubation at 20°C. A separate study demonstrated that concentrations of 20 mM EDTA and 50 μM $CuSO_4$ added pre-homogenization were not robust at completely inhibiting reduction when tested at the extreme condition of 100% total lysis (data not shown).

For another rMAb product, L-cystine was also evaluated as a competitive inhibitor for the reducing enzymes as shown in Figure 6b. L-Cystine concentrations ≥2.2 mM or $CuSO_4$ concentrations ≥75 μM were effective at preventing reduction at 60% total lysis, whereas 1.1 mM L-cystine was not effective. Maximum expected levels of total lysis expected in the manufacturing process with the products shown is 50–60% (equivalent to ~30% additional lysis for 70–80% viable cell cultures) under standard harvest conditions, or 30–40% total lysis (equivalent to ~10% additional lysis for 70–80% viable cell cultures) under improved harvest conditions that minimize cell shear. Since each product's final cell viability at the end of the cell culture production phase will vary, it is also important to track and evaluate additional cell lysis during harvest operations.

To evaluate chemical additive robustness to inhibit reduction across multiple feed streams, three different feedstocks consisting of three separate starting lab-scale cell culture runs with the same product and cell culture process parameters were processed through the lab-scale lysis model. Levels of total lysis targeted in these three studies were approximately 65% and/or 75% total lysis. Stock solutions of either EDTA or $CuSO_4$ were added either pre-homogenization or to the HCCF directly to achieve final concentrations of 20 mM EDTA or 30–35 μM $CuSO_4$ in the HCCF. As shown in Figure 7, both 20 mM EDTA and 30–35 μM $CuSO_4$ were robust at inhibiting reduction during lab-scale incubations at 20°C. Feedstock variability in terms of reduction susceptibility was observed in which Feedstock B did not completely reduce to <5% 150 kDa peak at either 65% or 75% total cell lysis compared to Feedstocks A and C.

Lowering the pre-harvest CCF or the HCCF pH from pH ~7.0 to 5–6 causes a large amount of precipitation of host cell proteins and DNA (Lydersen et al., 1994; Roush and Lu, 2008). Additional studies were conducted to evaluate if the loss of reducing activity was due to removal of reducing components via this precipitation event instead of protonated thiols at the lower pH. After pH adjustment and

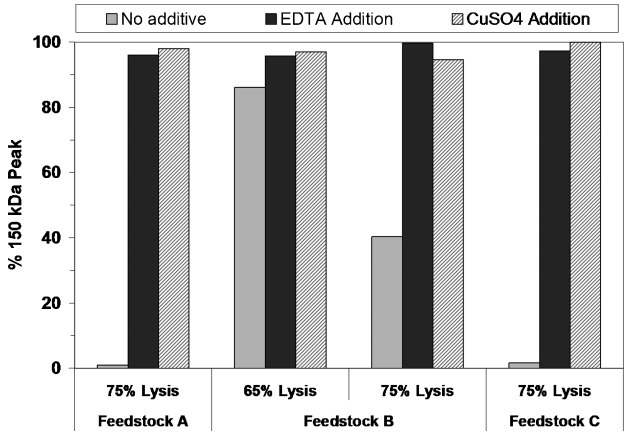

Figure 7. Protein A pool results are shown where three different feedstocks (**A**–**C**), consisting of three separate starting lab-scale cell culture runs of the same product were processed through the lab-scale lysis model. Approximately 65% and/or 75% total lysis was targeted in these three studies. Stock solutions of either EDTA or $CuSO_4$ were added either pre-homogenization or to the HCCF directly to achieve final concentrations of 20 mM EDTA or 30–35 μM $CuSO_4$ in the HCCF. The final HCCF pools were incubated at 20°C for 1 day prior to protein A purification.

filtration, the HCCF pool was pH adjusted back to pH 7.0 using 1 M Sodium HEPES and then incubated in mini-tanks for 36 h at 20°C. Figure 8 shows the SDS-PAGE results from this study where the rMAb still reduced. The pH 5.0 HCCF incubation did not exhibit reduced rMAb as expected (data not shown).

Inhibition of Reduction of Recombinant Antibody by HCCF Air Sparging

Air sparging was also evaluated as a method to prevent disulfide reduction and is effective by maintaining the dissolved oxygen concentration above levels permissive of disulfide reduction. HCCF generated from 100% lysed CCF was held in two separate stainless steel 15-L fermentors and either air or nitrogen sparged for 36 h at 20°C. The results showed that approximately 85% intact antibody was present in the initial solution (Fig. 9a) indicating some early reduction of the antibody's disulfide bonds prior to exposure to oxygen which is most likely due to the extreme lysis method of cell homogenization employed as well as experimental timing logistics to account for homogenization, filtration, and transfer to the 15-L aerated vessel. Once the mixture was sparged with air for 2 h, greater than 90% intact antibody was measured for the remainder of the 36 h study. In contrast, when the mixture was sparged with nitrogen gas, antibody reduction continued as measured at 2 h (28% 150 kDa peak) and 6 h (5% 150 kDa peak). Figure 9b shows the corresponding dissolved oxygen and oxidative-reduction potential readings in both vessels in this study. There was an increase in dO_2 level in the air sparged vessel while the ORP level remained fairly constant at approximately −50 mV. Due to the associated variability in

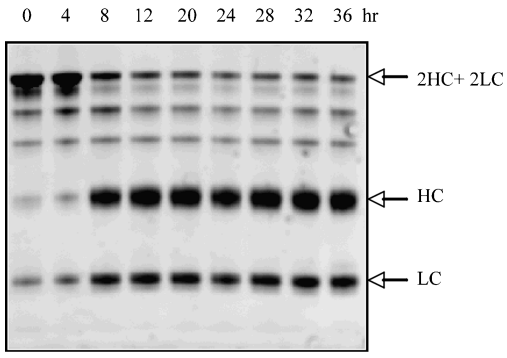

Figure 8. Non-reduced SDS-PAGE analysis (SYPRO® Ruby stain) during HCCF incubation at pH 7.0 in mini-tanks at 20°C. The time of incubation (h) is listed above each gel. The manufacturing HCCF was adjusted to pH 5.0 and then back to pH 7.0 prior to incubation at 20°C.

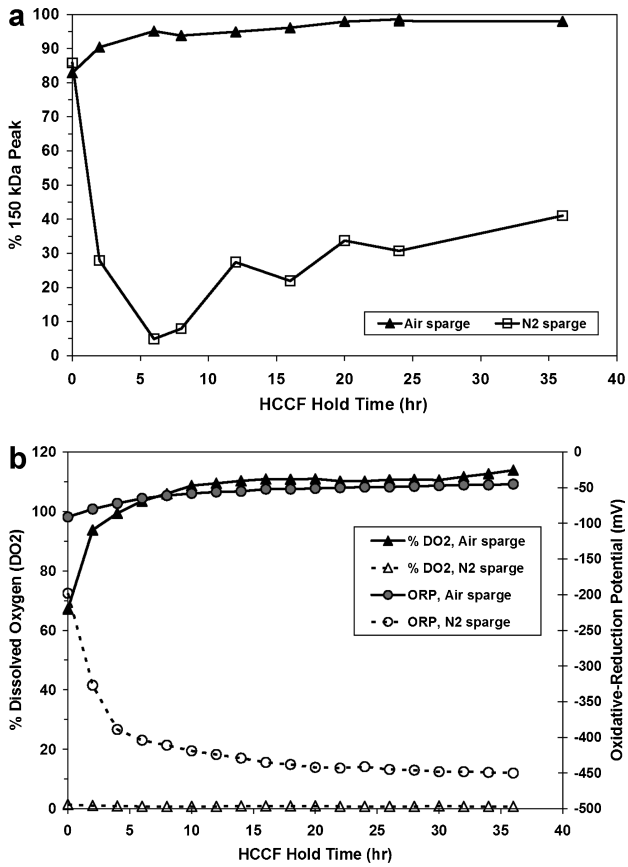

Figure 9. HCCF generated from lab lysis and held under either air or nitrogen sparged conditions at 20°C. **a**: Intact IgG profiles from protein A pool analysis. **b**: Percent dissolved oxygen (% dO_2) and oxidative-reduction potential (ORP) profiles.

calibrating the dO_2 probe in air saturated water, the dO_2 level in the saturated HCCF may read >100%, as in this study. In the nitrogen sparged vessel, the dO_2 level decreased immediately to 0%, and ORP decreased from −200 mV to −450 mV. However, after 8 h of constant nitrogen sparging, antibody re-oxidation was observed gradually increasing from 5% to 40% over the next 30 h. These results were unexpected since the HCCF contained 0% dO_2 and negative ORP, but could be due to loss of reductant activity over time.

The HCCF pH in both the air and nitrogen sparged vessels increased up to 0.7 pH units (∼pH 7.1 to ∼pH 7.8) by the end of the 36 h study with a shift of 0.4–0.5 pH units after 20 h of HCCF hold. The increase in pH was expected due to the stripping of carbon dioxide by the continuous stream of gas inlet flow. Product quality analyses demonstrated an increase in aggregate by size exclusion chromatography (SEC) of +0.2%/day and an increase in acidic variants by cation exchange chromatography (CEC) of +2%/day of HCCF hold (data not shown). Antibody amino acid oxidation was not observed by peptide map analysis, and the changes in the SEC and CEC profiles were not considered to be significant.

Discussion

Various levels of chemical inhibitors, low HCCF pH, and air (oxygen) were all found to effectively inhibit antibody disulfide reduction. The concentration of inhibitor required to fully prevent reduction is dependent on the absolute level of the reducing components, which are then subject to cell culture performance (e.g., culture viability, cell density, antibody expression level, etc.) and the amount of mechanical cell lysis. For example, different cell culture processes yielded CCF that was more susceptible to disulfide reduction than others. In addition, differences in reduction susceptibility could be observed even from fermentation to fermentation for the same product depending on culture performance. Therefore, it may be necessary to repeat reduction prevention testing several times using multiple feedstocks in a multivariate design of experiments to ensure the optimal inhibitor concentration is selected for robust inhibition at expected future levels of mechanical cell lysis.

The effectiveness of each inhibitor discussed was evaluated on the low end of range to find the minimum level for reduction inhibition as well as the high end to ensure no negative impact to other product quality attributes, or vice versa for the low HCCF pH option. Downstream purification performance was also evaluated, and with the exception of the decreased HCCF pH, there was no impact to impurity removal with the pre-harvest or post-harvest additions of EDTA, $CuSO_4$, or L-cystine, or with HCCF air sparging (data not shown). For the lower HCCF pH of 5.0, lower host cell protein and DNA was observed in the HCCF and the protein A pool, but comparable impurities levels were still observed in the final chromatography pool compared to the control (data not shown). For all the prevention methods tested, cation exchange chromatography (CEC), size exclusion chromatography (SEC), and endoproteinase Lys-C peptide map analyses (by RP-HPLC) were employed to evaluate the impact of the reduction prevention methods on product quality. These assays were also included in the product release testing. CEC assay was used to monitor charge variants (e.g., C-terminal lysine on heavy chain). SEC was used to monitor antibody aggregation and fragmentation. Endoproteinase Lys-C peptide map was used to detect changes in other modifications (e.g., oxidation, deamidation, etc.). Product quality was assessed not only on lab-scale lysed material, but also on non-lysed material to also ensure no impact even in an environment of low reduction potential. As previously discussed for the air sparging method and for pre-harvest and post-harvest additions of EDTA, $CuSO_4$, or L-cystine, there was no impact to size or charge heterogeneity nor was there evidence of amino acid oxidation. Additions of excess $CuSO_4$ or L-cystine to the cell culture batch media (data not shown) resulted in slight increases in aggregate and acidic variants however again there was no evidence of amino acid oxidation. Additional work is required to further evaluate the impact to product quality when lowering the HCCF pH. Antibody degradation was observed in some of our studies and may be more frequent when the acid is added to the CCF versus the cell-free HCCF.

Besides product quality and patient safety, there also needs to be considerations on target chemical concentration from a manufacturing viewpoint including technician safety, stock solution solubility and volume of addition to the manufacturing tank, cost of goods, raw material supply, environmental disposal limits, downstream clearance, and whether the chemicals are GRAS (generally regarded as safe) or not. For the lower pH option, CCF/HCCF filterability due to impurity precipitation also needs to be considered. Time of addition or implementation is also very important, with pre-harvest additions regarded as more robust compared to post-harvest additions in case of any extreme lysis conditions or manufacturing delays that might occur post-harvest.

The decision on target concentration of chemical inhibitor or dissolved oxygen level will ultimately be based on expected levels of total cell lysis or additional mechanical cell lysis during large-scale harvest operations. From a processing standpoint, it is not necessary to plan for 100% worst case lysis which may result in adding unnecessary high levels of chemicals. Instead, a safety factor should be used while considering the particular product's susceptibility to cell lysis and susceptibility to reduction. For air sparging, additional studies with a variety of cell lines have shown different dissolved oxygen demands in HCCF generated from mechanically lysed cells (data not shown). The most robust method for future implementation would be to use one-sided dissolved oxygen control in order to avoid over-sparging the HCCF pool unnecessarily.

Finally, beyond chemical additions or air sparging, there are various other disulfide reduction mitigation methods that can be implemented in manufacturing-scale processes. These include a hermetic style centrifuge, which minimizes potential sources of cell shear such as a feed pump and inlet flow control valve, increased agitation and air overlay of the HCCF to increase the dissolved oxygen level, chilling the CCF prior to harvest to 10–25°C to decrease the HCCF temperature more rapidly and thus slowdown enzymatic activity, and/or decreased processing time between harvest and the first capture step. From this work, we have successfully implemented pre-harvest additions of EDTA, $CuSO_4$, and L-cystine during clinical production campaigns as reduction mitigation methods. In addition, we have installed hermetic centrifuges, which have dramatically decreased cell lysis of many different production cell lines.

While it is not ideal to homogenize the CCF to reach extreme lysis levels followed by diluting back to a target level of total lysis, this method has been found to be the most reproducible for disulfide reduction compared to alternative lysis methods such as lysis through a partially closed valve or vessel pressurization. This is potentially due to the undesired, preliminary exposure of the solution to air, thereby oxidizing the reducing components during material preparation. Future work is also needed to develop a smaller-scale model to enable high throughput screening of additional inhibitors of disulfide reduction as well as to evaluate modified cell culture media and new clinical products.

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ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Mechanism of Antibody Reduction in Cell Culture Production Processes

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ABSTRACT: We recently observed a significant disulfide reduction problem during the scale-up of a manufacturing process for a therapeutic antibody using a CHO expression system. Under certain conditions, extensive reduction of inter-chain disulfide bonds of an antibody produced by CHO cell culture may occur during the harvest operations and/or the protein A chromatography step, resulting in the observation of antibody fragments (light chain, heavy chain, and various combination of both) in the protein A pools. Although all conditions leading to disulfide reduction have not been completely identified, an excessive amount of mechanical cell lysis generated at the harvest step appears to be an important requirement for antibody reduction (Trexler-Schmidt et al., 2010). We have been able to determine the mechanism by which the antibody is reduced despite the fact that not all requirements for antibody reduction were identified. Here we present data strongly suggesting that the antibody reduction was caused by a thioredoxin system or other reducing enzymes with thioredoxin-like activity. The intracellular reducing enzymes and their substrates/cofactors apparently were released into the harvest cell culture fluid (HCCF) when cells were exposed to mechanical cell shear during harvest operations. Surprisingly, the reducing activity in the HCCF can last for a long period of time, causing the reduction of inter-chain disulfide bonds in an antibody. Our findings provide a basis for designing methods to prevent the antibody reduction during the manufacturing process.

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KEYWORDS: antibody; disulfide; thioredoxin; reduction; mechanism

Introduction

A large number of recombinant monoclonal antibodies (mAb) have been approved by regulatory agencies for treating human diseases during the last 15 years (Reichert, 2001, 2002; Reichert and Pavolu, 2004; Reichert et al., 2005). Many more are currently in various stages of development and expected to become available in the near future. A very important structural feature of an antibody is the disulfide bonds that link its light and heavy chains (inter-chain disulfides) together to form a quaternary complex (Davies et al., 1975). In a mammalian cell culture system for large-scale therapeutic antibody production (e.g., CHO), all of the disulfide bonds (both inter- and intra-chain) of the antibody are correctly paired before the product is secreted into the cell culture fluid (CCF). Recently, we encountered a significant antibody disulfide reduction problem during the manufacturing process of a therapeutic antibody using a CHO expression system (Trexler-Schmidt et al., 2010). Under specific conditions, extensive reduction of the antibody's inter-chain disulfides bonds was observed after the harvest operations (centrifugation and filtration) and/or the first purification step (i.e., protein A chromatography). On one such instance, it was estimated that as little as 10% of the antibody remained intact after the protein A step. This antibody reduction is not a unique issue to a specific antibody or a particular cell line as this phenomenon has been observed for multiple antibodies both at manufacturing and laboratory scales.

The exact conditions resulting in antibody reduction remain elusive as the reduction phenomenon is not always reproducible at the manufacturing scale. However, our investigations have shown that an excessive amount of mechanical cell lysis generated at the harvest step was an important factor for the antibody reduction and the reducing activity was observed in the protein A load flow-through (Trexler-Schmidt et al., 2010). In this report, we present evidence indicating that the reduction was caused by an active thioredoxin (Trx) system or other reducing enzymes with thioredoxin-like activity in the harvested cell culture fluid (HCCF; post-centrifugation and filtration). The Trx system, consisting of thioredoxin (Trx),

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thioredoxin reductase (TrxR), and NADPH, is a ubiquitous antioxidative enzymatic system that plays an important role in maintaining the cellular redox balance and keeping intracellular protein disulfides generally reduced (Gromer et al., 2004). This system is also known to be involved in various cellular processes including gene expression, signal transduction, proliferation, and apoptosis (Arnér and Holmgren, 2000; Matsuo et al., 2002). Considering the importance of the Trx system and the cell lysis caused by the harvest operations, the presence of the Trx system in the HCCF is not surprising. However, it was surprising that the Trx system, fueled by NADPH, remained active for an extended period of time after the cell culture harvest. Our data indicated that the NADPH required for Trx and TrxR activities was provided by glucose-6-phosphate dehydrogenase (G6PD) activity, which generated NADPH from glucose-6-phosphate (G6P), and $NADP^+$. Furthermore, G6P is likely produced from glucose and ATP by hexokinase (phosphorylation of glucose; the first step of glycolysis). Together, these cellular enzymes (Trx system, G6PD, and hexokinase) along with their substrates are released into the HCCF upon cell lysis allowing the reduction event to occur.

The observed antibody reduction is an outcome of a highly coupled reaction network in HCCF. Its kinetics is a very complex problem and dependent on many factors, such as viability of the production cell culture, the additional cell lysis from harvest operation, and the dissolved oxygen (DO) level in HCCF. While the antibody reduction kinetics is an important subject, the focus of this study is to determine the reduction mechanism. The kinetics of antibody reduction will be addressed in future studies.

Moreover, we also describe strategies that can be applied to prevent undesired disulfide reduction in the manufacturing process for recombinant antibodies. Based on the identified reduction mechanism, any inhibitors, methods, or processes that can eliminate the activities of Trx system, G6PD, or hexokinase can be included as a mode to prevent disulfide reduction of recombinant proteins.

Materials and Methods

Materials

Materials and devices used in the experiments described in this manuscript include: 50 and 55 cm^3 stainless steel vials (mini-tanks; Flow Components, Dublin, CA); dialysis tubing (6,000–8,000 MWCO; Spectrum Laboratories, Rancho Dominguez, CA); 0.22 μm filter (Millipak Gamma Gold; Millipore, Billerica, MA); phosphate-buffered saline (PBS; EDM Chemicals, Gibbstown, NJ); ethylenediaminetetraacetic acid (EDTA; Sigma–Aldrich, St. Louis, MO); α-nicotinamide adenine dinucleotide phosphate (NADPH; EDM Chemicals); dehydroepiandrosterone (DHEA; TCI, Portland, OR); cupric sulfate (Sigma–Aldrich), G6P (EDM Chemicals); aurothioglucose (ATG; USP, Rockville, MD); aurothiomalate (ATM; Alfa Aesar, Ward Hill, MA); reduced glutathione (GSH; Mallinckrodt Baker, Phillipsburg, NJ); monobromobimane (mBB; Sigma–Aldrich); histidine (Mallinckrodt Baker); sodium sulfate (Mallinckrodt Baker); thioredoxin (Trx; Sigma–Aldrich); thioredoxin reductase (TrxR; Sigma–Aldrich). All chemicals and reagents were used as received with no further purification.

Generation of Cell Culture Fluid (CCF)

Mammalian CCF containing an antibody produced by Chinese hamster ovary (CHO) cells were generated using a representative small-scale fermentation process according to the methods described by Chaderjian et al. (2005). Cell culture process indicators (e.g., pH, temperature, DO, agitation rate) were monitored on-line while other culture indicators such as glucose, lactate, ammonium, glutamine, glutamate, and sodium were measured daily. Samples were also taken to monitor cell growth, viability, and rMAb concentration every 24 h.

Harvested Cell Culture Fluid (HCCF) Preparation

At the end of the production culture, complete mechanical lysis of CCF was achieved by high-pressure homogenization using a Microfluidics (Newton, MA) HC-8000 homogenizer. The pressure regulator of the instrument was set to 4,000–8,000 psi, and complete cell lysis (membrane breakage) was achieved after a single pass, as determined by a lactate dehydrogenase (LDH) assay. The homogenate was centrifuged in a Sorval (Thermo Scientific, Asheville, NC) RC-3B rotor centrifuge at 4,500 rpm for 30 min at 20°C. The centrate was decanted, depth filtered, and then sterile filtered (0.22 μm) to generate HCCF. For a non-homogenized control, a CCF sample was centrifuged at lab-scale and the centrate was sterile filtered (0.22 μm).

Dialysis Experiment

A dialysis experiment was carried out in order to determine whether the components causing reduction of the antibody were small molecules or macromolecules (i.e., enzymes). A sample of 3 mL of purified and formulated antibody (30.2 mg/mL) was dialyzed against 1 L of PBS (10 mM, pH 7.2) at 25°C for 24 h and the PBS was changed after 8 h. Dialysis tubing was hydrated overnight in a 0.05% azide solution and rinsed with sterile water prior to use. After dialysis, the concentration of the antibody sample was determined by the absorbance at 280 nm and adjusted to 1 mg/mL. Aliquots were stored at −70°C prior to use. The HCCF, obtained from homogenization of CCF from a 3-L fermentor, was thawed and filtered through a 0.22 μm Millipak filter using a peristaltic pump. Six 50 cm^3 mini-tanks were filled with 30 mL of HCCF each. In each mini-tank, a purified antibody sample (500 μL) placed in a

dialysis bag was submerged in the HCCF. The mini-tanks were sealed and loaded into a bench top mixer (Lab-Line MAX Q 4000; Thermo Scientific) operating at 35 rpm and ambient temperature. For each time point, one mini-tank was removed from the mixer, and aliquots of the HCCF (in the mini-tank) and the antibody sample (in the dialysis bag) were taken and stored at −70°C until analyzed with the free thiol assay and the microchip based capillary electrophoresis assay (described below).

Liquid Chromatography–Mass Spectrometry (LC–MS) Analysis of Reduced Antibody

The protein A elution pool from a manufacturing run that showed reduced antibody product was analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) using a Poroshell column (300SB-C8 1.0 mm × 75 mm, 5 μm, Agilent Technologies, Santa Clara, CA). The sample was eluted with a formic acid/trifluoroacetic acid/acetonitrile gradient for direct on-line electrospray ionization-mass spectrometry (ESI-MS) using a QStar Pulsar i mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA). Spectra were derived from multiple charged ions and deconvoluted using the Analyst QS 1.0/BioAnalyst 2.0 software package (Applied Biosystems/MDS Sciex). The sample was also analyzed after treated with tris(2-carboxyethyl)phosphine (TCEP) to fully reduce all disulfide bonds in the antibody.

HCCF Incubation Experiments to Probe the Role of NADPH and Glucose-6-Phosphate and to Test Inhibitors for Reduction

A 55 cm^3 mini-tank was filled with 27 mL of HCCF. Depending on the experiment design, various reagents such as NADPH, G6P, inhibitors of G6PD, or inhibitors of TrxR were added to the desired concentration, and the final volume in the mini-tank was brought to 30 mL with PBS (10 mM, pH 7.2). The mini-tanks were sealed and loaded into a bench top mixer running at 35 rpm and ambient temperature. At each time point for sampling, the exteriors of the mini-tanks were sterilized with 70% isopropyl alcohol and opened in a laminar flow hood for the removal of an aliquot. The mini-tanks were then re-sealed and loaded back into the bench top mixer. All aliquots were stored at −70°C until analyzed with the free thiol assay and microchip-based capillary electrophoresis assay.

In Vitro Trx/TrxR Studies

In a polypropylene 1.5 mL microcentrifuge tube, 437 μL PBS, 25 μL NADPH (20 mM), 16 μL formulated the antibody solution (30.2 mg/mL), and 5 μL human Trx solution (500 μM in 10 mM PBS, pH 7.2) were gently mixed. The reaction was initiated by the addition of 17.5 μL rat liver TrxR solution (4 μM). The reaction was incubated at room temperature for 24 h in the absence of light. Aliquots of 20 μL were taken at each sampling time point and stored at −70°C until analyzed by the microchip-based capillary electrophoresis assay. Control reactions were performed to determine if the enzymatic pathway was active when an enzyme was omitted by substituting an equal volume of PBS for either Trx and/or TrxR in the reaction mixture. Inhibition of the Trx/TrxR activity was demonstrated using the same reaction conditions described above with the addition of 5 μL ATG (10 mM) or ATM (10 mM). To demonstrate the inhibition of Trx system by Cu^{2+}, 2.5 μL of $CuSO_4$ (10 mM) was added to reaction mixture using the same enzymes but a different buffer (10 mM histidine, 10 mM Na_2SO_4, 137 mM NaCl, 2.5 mM KCl, pH 7.0) to prevent formation of insoluble $Cu_3(PO_4)_2$.

Measuring Free Thiol Contents in HCCF Samples

A series of reduced GSH standard samples were prepared in PBS (10 mM, pH 6.0) in order to generate a standard curve for quantifying free thiols. From a 110 mM GSH solution, standards were prepared at concentrations of 0, 5.5, 11, 22, 44, 55, 110, and 550 μM through serial dilution. From an acetonitrile stock solution of mBB (10 mM stored at −20°C), a 100 μM solution of mBB was prepared in PBS (10 mM, pH 10.0) and stored protected from light.

In a flat-bottomed 96-well plate, 100 μL of mBB was dispensed into each well. A 10 μL aliquot of GSH standard solution or test sample was then added to the wells. All wells were prepared in triplicate. The plate was incubated at room temperature for 1 h and then analyzed using a fluorescence plate reader (SpectraMax Gemini XS; Molecular Devices, Sunnyvale, CA) with an excitation wavelength of 390 nm and an emission wavelength of 490 nm. A linear standard curve was generated using the average result of the three standard wells plotted versus GSH concentration. Free thiol levels in samples were calculated from the linear equation of the standard curve using the average value of the three sample wells.

Microchip-Based Capillary Electrophoresis Assay

Reduction of antibody was monitored by capillary electrophoresis using the Agilent 2100 Bioanalyzer (Agilent Technologies). Sample preparation was carried out as described in the Agilent manual (Protein 230 Assay Protocol) with minor changes. For HCCF samples a 1:4 dilution was performed on the samples prior to preparation. At the denaturing step, in addition to mixing the test sample with 2 μL denaturing solution as described in the protocol, a 24-μL solution containing 50 mM iodoacetamide (IAM) and 0.5% SDS solution was added. Digital gel-like images were generated using Agilent 2100 Expert software.

Results and Discussion

Dialysis Experiment

The reducing activity in HCCF was found to remain in the protein A load flow-through as evidenced by the reduction of various purified intact antibodies spiked into the protein A load flow-through, indicating that reducing components could be readily removed from the antibody manufacturing process (Trexler-Schmidt et al., 2010). However, it was not immediately clear whether the reducing components were small molecules or macromolecules (e.g., enzymes). To elucidate this question, a dialysis experiment was designed to determine the nature of reducing molecules involved in antibody reduction. In this dialysis experiment, an intact antibody was placed in a dialysis bag with a MWCO of 7,000 Da and incubated in HCCF contained in a stainless steel mini-tank. As shown in Figure 1, the antibody inside the bag was not reduced after the incubation period (Fig. 1a), whereas the antibody outside the bag in the HCCF was significantly reduced soon after the incubation started as indicated by the loss of intact antibody (~150 kDa) and the formation of the antibody fragments (various combinations of heavy and light chains) (Fig. 1b).

The free thiol measurement (by using mBB to derivatize and quantify the sulfhydryl groups) showed that no free thiols were present inside the dialysis bag at the beginning of the incubation (Fig. 2). The levels of free thiols inside the dialysis bag did not reach the exact same level as observed outside the bag, most likely because some free thiols were present on macromolecules and unable to enter the dialysis bag. Nevertheless, the levels of free thiols inside and outside the dialysis bag became comparable in <5 h after the incubation was initiated, suggesting that the free thiol containing small molecules in the HCCF entered the dialysis bag without restraints and small molecule components from the HCCF were able to reach equilibrium inside and outside the dialysis bag. Thus, the antibody reduction occurred outside the dialysis bag was not caused by the free thiol containing small molecules. Since the reduction was observed only outside the dialysis bag with a MWCO of 7,000 Da, the molecular weight of the reducing molecule(s) must be >7,000 Da. The result from this dialysis experiment suggested that an enzymatic reaction was responsible for the observed antibody reduction.

Liquid Chromatography–Mass Spectrometry (LC–MS) Analysis of Reduced Antibody

An LC–MS analysis was performed on the protein A elution pool from a manufacturing run that showed reduced antibody product. Under non-reducing assay conditions, the major species detected were antibody light chains and heavy chains with very little intact antibody observed, indicating the presence of reduced antibody in the protein A

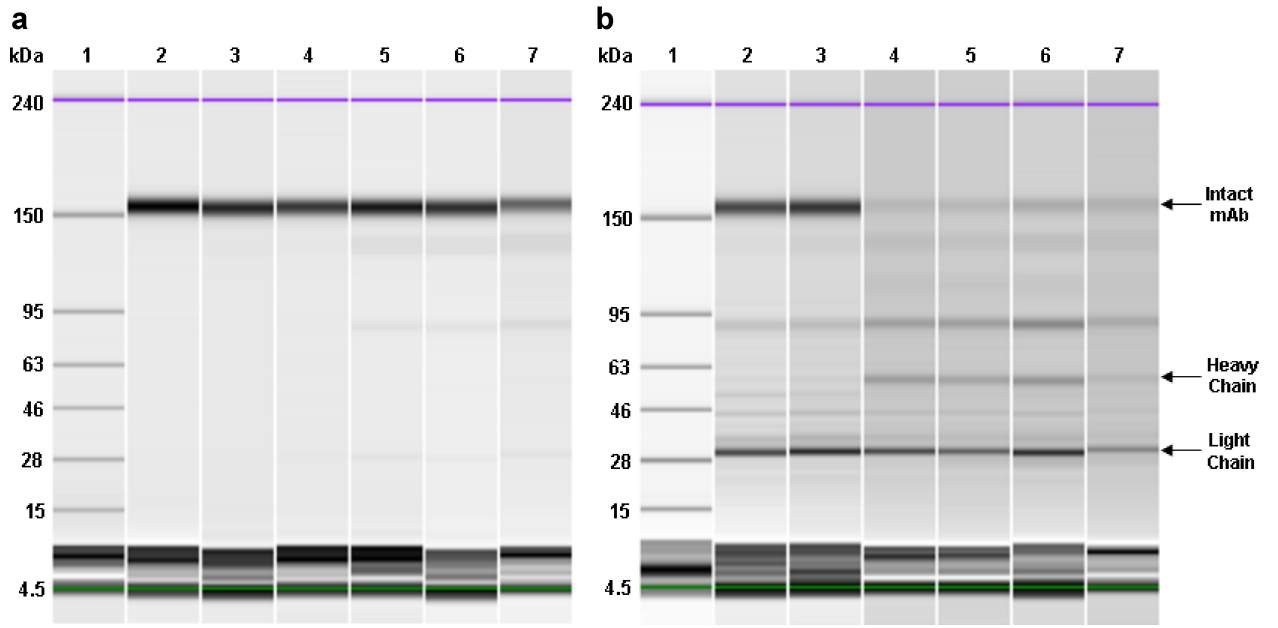

Figure 1. Dialysis experiment: digital gel-like images obtained from the microchip-based capillary electrophoresis analysis (each lane representing a time point: 0, 1, 3, 21, 25, and 29 h from lanes 2–7). **a**: The antibody inside the dialysis bag remained intact during the incubation period. **b**: The antibody outside the dialysis bag was reduced during the incubation period as evidenced by the loss of intact antibody (~150 kDa) and the formation of antibody fragments. The band appearing just above the 28 kDa marker arises from the light chain of antibody. There was a significant amount of free light chain already present in the HCCF before the incubation began. The presence of excess free light chain and dimers of light chain in the HCCF is typical for the cell line producing antibody. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

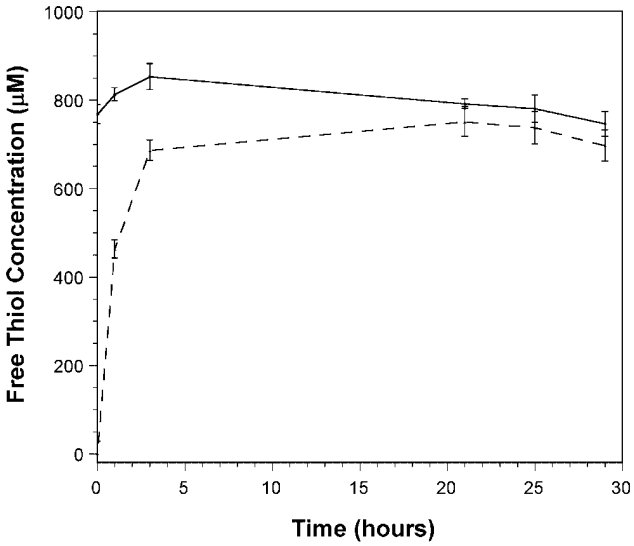

Figure 2. Free thiol levels from dialysis experiment: free thiols inside (dashed line) and outside (solid line) the dialysis bag reach comparable levels within a few hours, indicating a good exchange of small molecule components in the HCCF between inside and outside the dialysis bag. The error bars are based on the standard deviation of three independent free thiol measurements of the each sample.

pool. The observed mass for the light chains in the protein A pool sample was 23,234 Da. Since the heavy chain in an IgG molecule contains an N-linked glycsoylation site, several masses arising from the heterogeneity of glycan structures were observed for the heavy chains in the protein A pool sample. The predominant heavy chain species had a mass of 50,996 Da. For light chain, the observed mass was lower than the expected mass of the fully reduced light chain (23,238 Da) by 4 Da. For heavy chain, the main observed mass was lower than the expected mass of the fully reduced heavy chain containing an asialo, agalacto biantennary oligosaccharide structure with a core fucose (G0 glycoform; 51,004 Da) by 8 Da. In an IgG molecule, there are two and four intra-chain disulfide bonds in the light chain and heavy chain, respectively. The mass differences observed in the LC–MS analysis suggested that the light chain and heavy chain in the protein A pool sample must contain two and four disulfide bonds, respectively, because the formation of each disulfide would reduce the mass number by 2 Da (loss of two protons). These mass spectrometry data clearly indicated that only the inter-chain disulfide bonds were reduced, resulting in the presence of light chain and heavy chain in the protein A pool. The intra-chain disulfide bonds in the light chain and heavy chain, however, were still intact. Subsequent peptide map analysis with LC–MS detection also confirmed that reduction occurred only at the inter-chain disulfide bonds. The fact that only the inter-chain disulfide bonds were reduced is consistent with the results from dialysis experiment showing that antibody reduction in the HCCF was caused by reducing macromolecules since the intra-chain disulfides are generally located in the interior of an antibody and inaccessible to the reducing enzymes.

Reduction of Antibody by Trx/TrxR In Vitro

The Trx system (i.e., Trx and TrxR) is one of the two intracellular enzymatic systems (the other is the glutaredoxin [Grx] system) that regulates the cellular redox status and maintains a reducing environment in the cytosol (Gromer et al., 2004). The Trx system can reduce disulfide bonds within a protein at the expense of NADPH (Fig. 3). Since the Grx system activity is only limited to the reduction of *S*-glutathionylated substrates (i.e., GSH-mixed disulfides) (Johansson et al., 2004), the Trx system is most likely the primary enzymatic system that reduces the inter-chain disulfides in the antibody. An in vitro experiment was conducted to test if the Trx system can reduce the antibody by incubating the intact antibody with Trx, TrxR, and NADPH. As expected, the microchip-based capillary electrophoresis results indicate that the antibody can be reduced in vitro by the Trx system (Fig. 4). Similar to the reduction observed in the HCCF, the LC–MS analysis also showed that only inter-chain disulfide bonds were reduced in vitro by the Trx system (data not shown).

There are many known Trx and TrxR inhibitors (Gromer et al., 2004). For example, gold complexes are among the

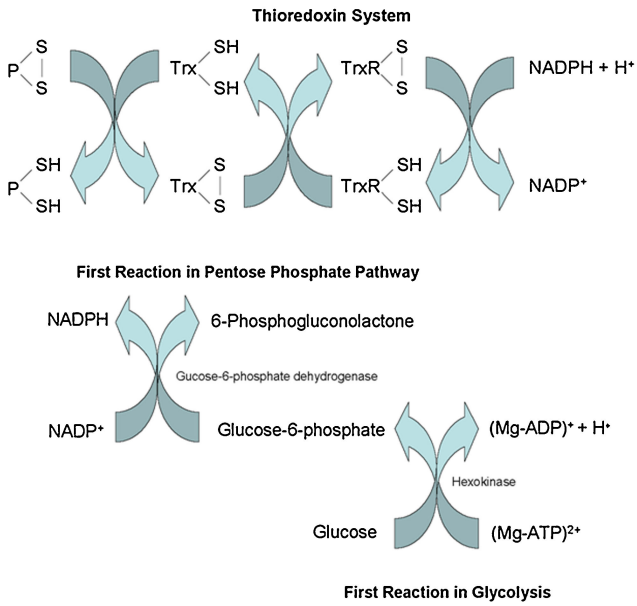

Figure 3. Thioredoxin system and other reactions involved in antibody reduction: the thioredoxin system, composed of thioredoxin (Trx), thioredoxin reductase (TrxR), and NADPH, is a hydrogen donor system for reduction of disulfide bonds in proteins. Trx is a small monomeric protein with a CXXC active site motif that catalyzes many redox reactions through thiol-disulfide exchange. The oxidized Trx can be reduced by NADPH via TrxR. The reduced Trx is then able to catalyze the reduction of disulfides in proteins. The NADPH required for thioredoxin system is provided via reactions in pentose phosphate pathway and glycolysis. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

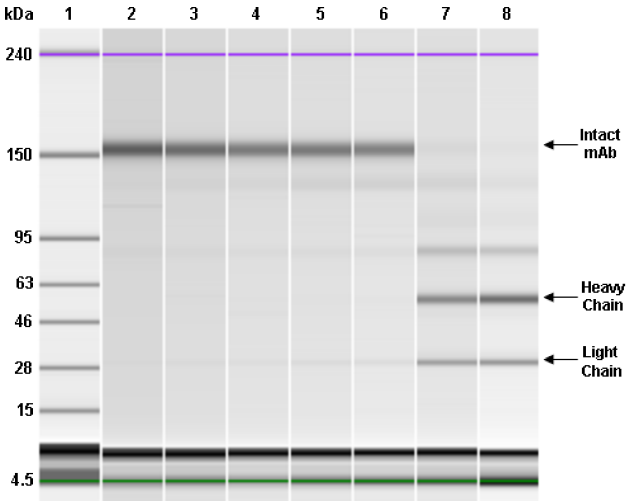

Figure 4. In vitro activity of thioredoxin system: digital gel-like image from the microchip-based capillary electrophoresis analysis (each lane representing a time point: 0, 0.5, 1, 2, 3, 21, and 23 h from lanes 2–8). The incubation of intact antibody (1 mg/mL) with 0.1 μM TrxR (rat liver), 5 μM Trx (human), and 1 mM NADPH in PBS resulted in antibody reduction (completely reduced in <21 h). [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

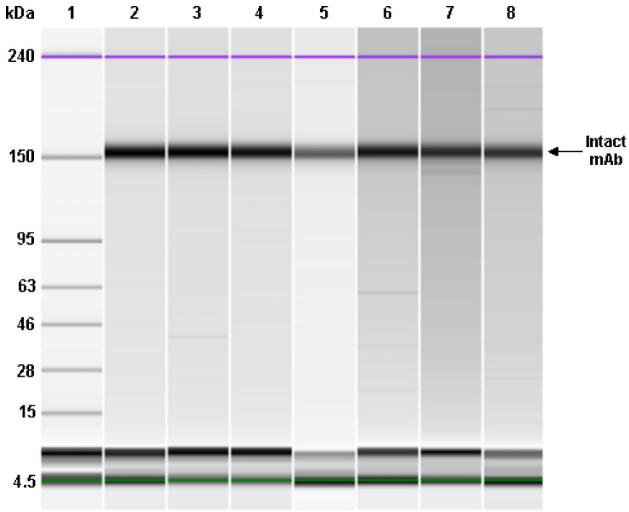

Figure 5. In vitro activity of thioredoxin system inhibited by ATG: the addition of ATG at a concentration of 1 mM to the reaction mixture as described in the caption for Figure 4 effectively inhibited the antibody reduction as shown in the digital gel-like image from the microchip-based capillary electrophoresis analysis (each lane representing a time point: 0, 0.5, 1, 2, 3, 21, and 23 h from lanes 2–8). Similarly, the addition of aurothiomalate (ATM) to the reaction mixture has the same inhibitory effect on antibody reduction (data not shown). [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

most effective and selective inhibitors of mammalian TrxRs known to date. Two commercially available specific inhibitors of TrxR, ATG, and ATM, were tested for their ability to inhibit the Trx system in vitro and the antibody reduction. As expected, both ATG and ATM can effectively inhibit the antibody reduction in the assay described above (Fig. 5).

Cupric sulfate is known for its ability to provide oxidizing redox potential and has been used in the cell culture processes to minimize free thiol (i.e., minimize unpaired cysteine) levels in recombinant antibody molecules (Chaderjian et al., 2005). We have also tested whether cupric sulfate can inhibit the Trx system in vitro and the subsequent reduction of the antibody. In this in vitro reduction experiment, the buffer system was changed from PBS to histidine sulfate in order to avoid the formation of insoluble $Cu_3(PO_4)_2$. Figure 6 showed that the antibody was readily reduced by the Trx system in the histidine sulfate buffer (even faster than in PBS buffer), but the addition of $CuSO_4$ to this reaction clearly inhibited the antibody reduction.

Inhibition of Antibody in HCCF by ATG and ATM

The two gold compounds shown to be capable of inhibiting reduction of the antibody by the Trx system in vitro are specific inhibitors for TrxR (Fig. 5). If the Trx system was active in the HCCF and caused the antibody reduction in the failed antibody manufacturing runs and in the lab scale reduction experiments, both gold compounds (ATG and ATM) should be able to inhibit the reduction of antibody in HCCF as well. Figure 7 showed that the antibody was readily reduced in a homogenized HCCF generated from a 3-L fermentor after a period of incubation. However, the antibody reduction event was completely inhibited when either 1 mM ATG or ATM was added to the HCCF. These results demonstrated that the Trx system was active in the HCCF and directly responsible for the antibody reduction.

The Source of NADPH for Trx System Activity and the Roles of G6P and Glucose in the Reduction Mechanism

The reduction of disulfides by the Trx system requires the reducing equivalents from NADPH (Fig. 3). The main cellular metabolic pathway that provides NADPH for all reductive biosynthesis reactions is the pentose phosphate pathway. For the antibody reduction event to occur, the enzymes in this pathway must be still active in the HCCF in order to keep the Trx system active. At a minimum, the first step in the pentose phosphate pathway (catalyzed by G6PD) must be active to reduce $NADP^+$ to NADPH while converting G6P to 6-phosphogluconolactone. In addition, G6P is most likely produced from glucose and adenosine 5′-triphosphate (ATP) by the hexokinase activity in HCCF. The overall mechanism of the antibody reduction in HCCF is summarized in Figure 4.

The reducing activity in the HCCF appeared to be transitory in some cases and may be lost over time under certain storage conditions or after multiple freeze/thaw cycles. The HCCF that had lost reducing activity actually

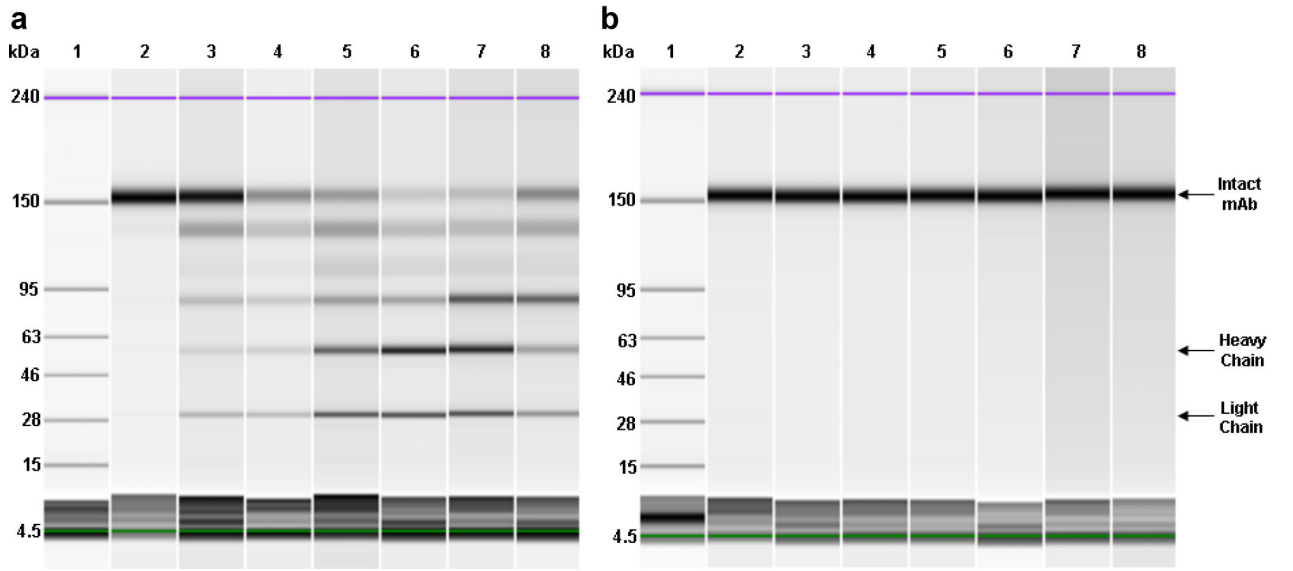

Figure 6. In vitro activity of thioredoxin system inhibited by $CuSO_4$: digital gel-like images from the microchip-based capillary electrophoresis analysis (each lane representing a time point: 0, 0.5, 1, 2, 3, 21, and 23 h from lanes 2–8). **a**: The incubation of intact antibody (1 mg/mL) with 0.1 μM TrxR (rat liver), 5 μM Trx (human), and 1 mM NADPH in 10 mM histidine sulfate buffer resulted in antibody reduction in <1 h. **b**: The addition of $CuSO_4$ at a concentration of 50 μM to the reaction mixture effectively inhibited the antibody reduction. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

provided an opportunity to explore the role of NADPH and G6P in the antibody reduction by Trx system. An HCCF which already experienced several freeze/thaw cycles was found to have lost its reducing activity (Fig. 8a) despite that the antibody reduction was seen previously in the freshly thawed HCCF from the same fermentation (data not shown). To determine if the Trx system was still active in this non-reducing HCCF, NADPH was added to the HCCF at a concentration of 5 mM. The antibody reduction event was observed again after the addition of NADPH (Fig. 8b).

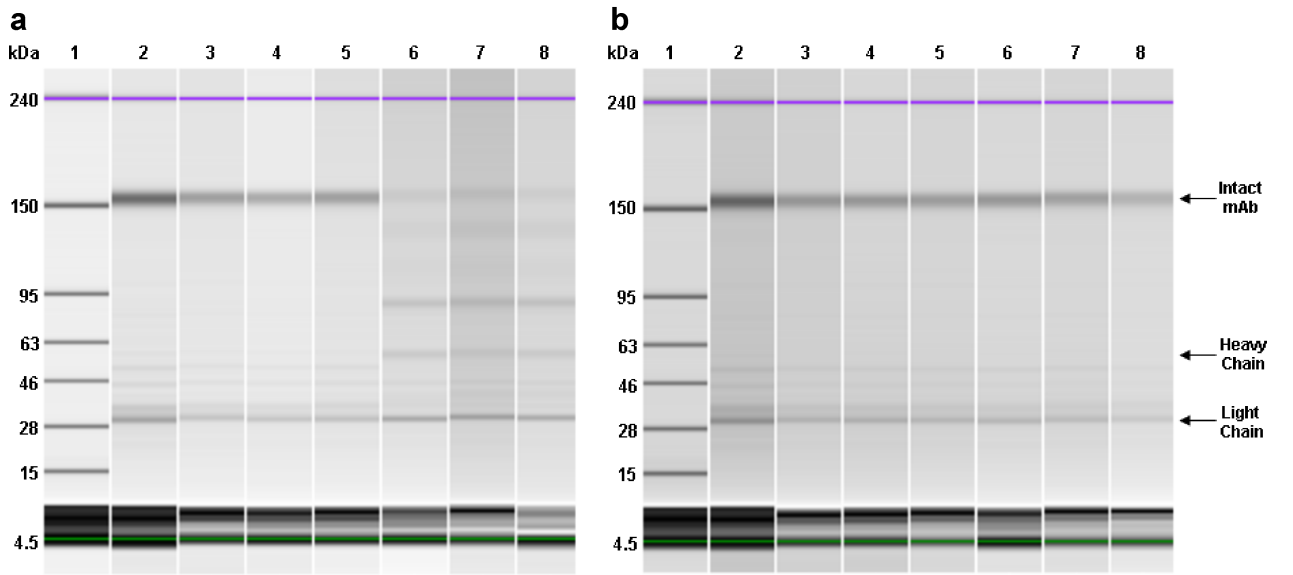

Figure 7. Inhibition of the antibody reduction in HCCF by aurothioglucose: digital gel-like images from the microchip-based capillary electrophoresis analysis (each lane representing a time point: 0, 0.5, 1, 2, 19, 21, and 23 h from lanes 2–8). **a**: The antibody was reduced in an incubation experiment using an HCCF generated from homogenized CCF from a 3-L fermentor. **b**: Addition of 1 mM aurothioglucose to the HCCF effectively inhibited antibody reduction. The addition of aurothiomalate (ATM) to the HCCF has the same inhibitory effect on antibody reduction (data not shown). [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

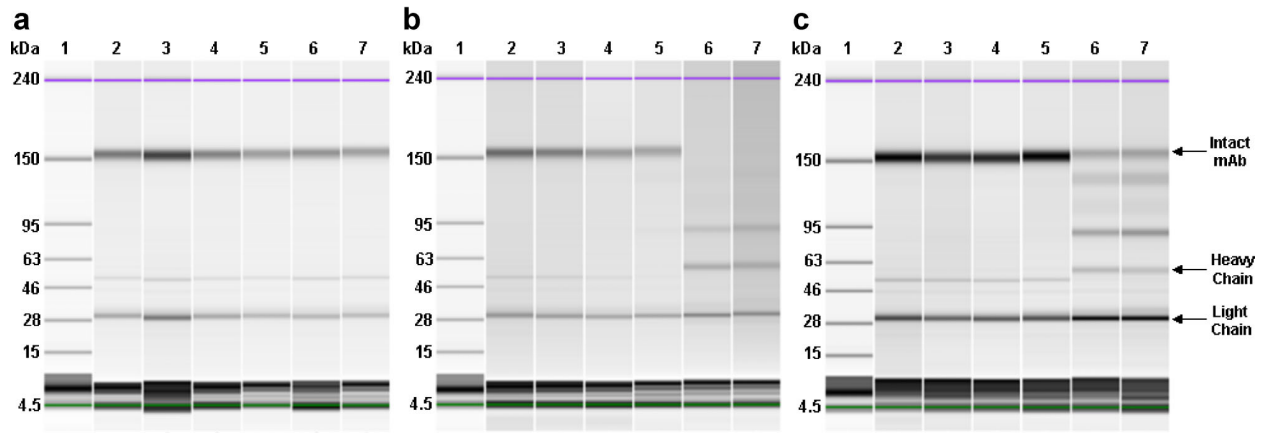

Figure 8. Losing and restoring reduction activity in HCCF: digital gel-like images obtained from the microchip-based capillary electrophoresis analysis (each lane representing a time point: 0, 1, 2, 4, 19, and 21 h from lanes 2–7). **a**: The HCCF from one of the manufacturing runs that already experienced several freeze/thaw cycles was used in an incubation experiment. Surprisingly, no antibody reduction was observed in the microchip-based capillary electrophoresis analysis despite the antibody reduction seen previously in the freshly thawed HCCF from this same fermentation (data not shown). **b**: The reduction of antibody was observed again after the addition of NADPH at a concentration of 5 mM into the HCCF whose reduction activity had been previously lost. **c**: The reduction of antibody was also observed again in the microchip-based capillary electrophoresis assay after the addition of G6P at a concentration of 10 mM into the HCCF. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

These results suggested that the Trx system was still intact in the HCCF that had lost it reducing activity. In addition, the reducing activity was lost in this HCCF over time because the NADPH source was depleted, presumably as a result of the oxidation of NADPH by all of the reductive reactions that competed for NADPH. Another experiment to support this hypothesis involved the addition of G6P (10 mM) to the same non-reducing HCCF. The results showed that the G6P addition was also able to reactivate the Trx system and subsequently reduce the antibody in the HCCF incubation experiment (Fig. 8c). This is a very important result as it indicated that the antibody reduction in HCCF was caused by the activities of both the Trx system and G6PD. Furthermore, G6PD was still active in the non-reducing HCCF and the loss of reduction activity in this HCCF appeared to be due to the depletion of G6P, which disabled the conversion of $NADP^+$ to NADPH.

It has been observed that EDTA can effectively inhibit the antibody reduction in the HCCF incubation experiment. As shown in Figure 9a, the antibody was reduced after incubating an HCCF at ambient temperature for more than 19 h. However, the reduction was completely inhibited when EDTA was added at a concentration of 20 mM to the HCCF (Fig. 9b). These results provided some insight into the role of glucose in antibody reduction. In the first step of glycolysis, the hexokinase catalyzes the transfer of a phosphate group from Mg^{2+}-ATP to glucose, a reaction that requires the complexation of Mg^{2+} with ATP (Hammes and Kochavi, 1962a,b). Since EDTA is a metal ion chelator, especially for Mg^{2+}, it can be an effective inhibitor of hexokinase. The observation that an excess amount of EDTA can effectively block the reduction indicates the involvement of hexokinase (i.e., providing G6P) in the mechanism of the antibody reduction. EDTA blocks antibody reduction apparently by eliminating the hexokinase activity and subsequently blocking the production of G6P and NADPH that are required for the activation of the Trx system.

Although EDTA was every effective in blocking antibody reduction in the fresh HCCF with reducing activity, it was unable to prevent the reduction of antibody in the HCCF in which the reducing activity of Trx system was lost then reactivated by the addition of G6P. For example, antibody reduction was observed in an HCCF incubation experiment in which 5 mM G6P and 20 mM EDTA (final concentrations) were added to the non-reducing HCCF (Fig. 10). The reduction was not observed in the control incubation experiment in which G6P and EDTA were not added. This observation suggested that EDTA inhibited a reaction that is upstream from the production of G6P in the reduction pathway (Fig. 3) and supported the hypothesis discussed above that EDTA is an inhibitor for hexokinase.

Strategies for Inhibiting Antibody Reduction in Manufacturing Processes

The mechanism for antibody disulfide bonds reduction occurred during the failed manufacturing runs has been elucidated as described above and shown in Figure 4. Data presented here strongly suggest that the activities of the Trx system, G6PD, and hexokinase are the key factors involved in antibody reduction. Based on these findings, any methods that can effectively block one of the three enzyme systems (Trx system, G6PD, or hexokinase) may potentially be used during manufacturing processes to prevent antibody reduction. Additionally, modulating the redox potential of the HCCF can provide an additional means of eliminating antibody reduction. Moreover, these methods may be

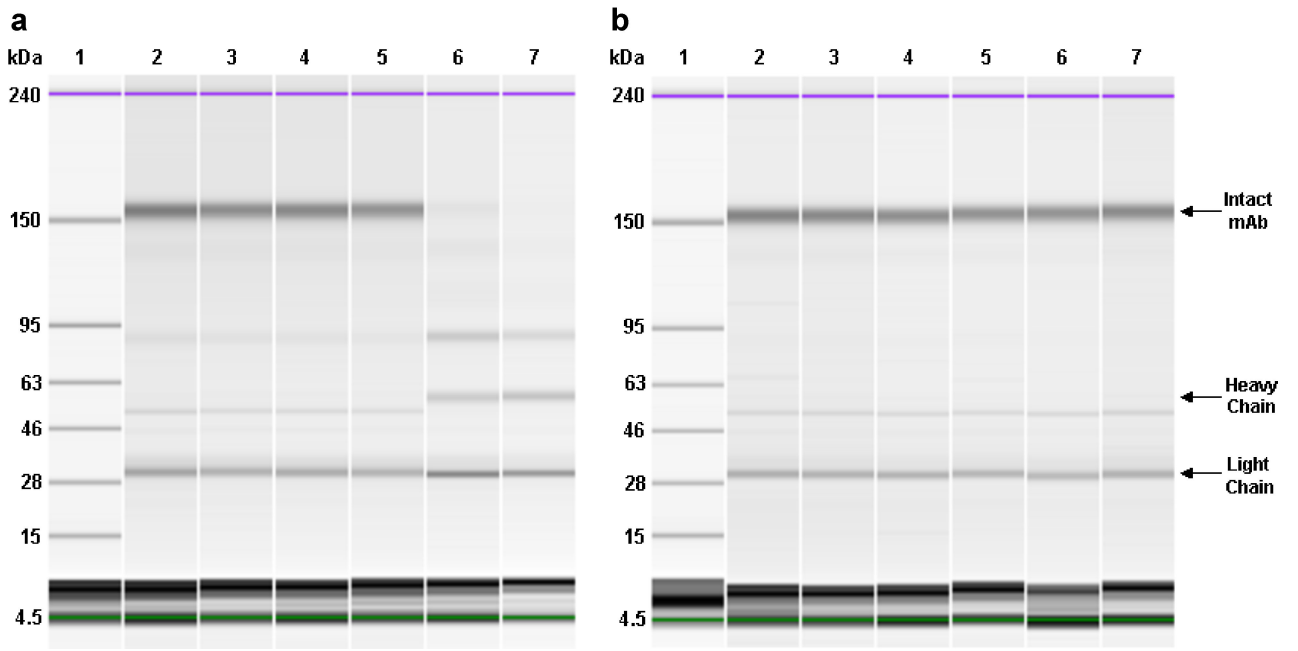

Figure 9. EDTA inhibits antibody reduction: digital gel-like images obtained from the microchip-based capillary electrophoresis analysis (each lane representing a time point: 0, 1, 2, 3, 19, and 21 h from lanes 2–7). **a**: The antibody was reduced in HCCF after incubation. **b**: Addition of EDTA at a concentration of 20 mM effectively inhibited the reduction of antibody. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

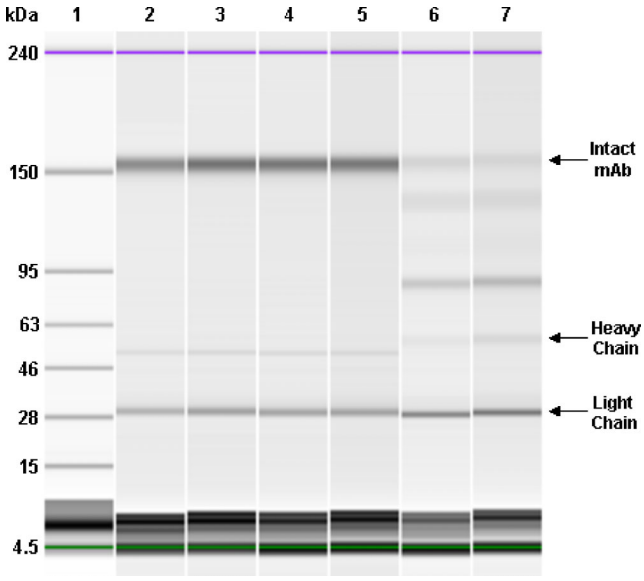

Figure 10. EDTA is unable to inhibit the reducing activity restored by the addition of glucose-6-phosphate: digital gel-like images obtained from the microchip based capillary electrophoresis analysis (each lane representing a time point: 0, 1, 2, 3, 19, and 21 h from lanes 2–7). The reduction of antibody was observed after the addition of 5 mM G6P and 20 mM EDTA into the HCCF whose reduction activity had been previously lost (see Fig. 8a). In contrast to the results shown in Figure 9, the presence of EDTA did not block the reduction of antibody in the presence of G6P. [Color figure can be seen in the online version of this article, available at wileyonlinelibrary.com.]

applied to the manufacturing processes for other recombinant proteins that have a similar disulfide bond reduction issue in the production cell line.

A list of some potential reduction inhibition methods that can be used in the production of recombinant proteins is provided in Table I. In general, there are three ways to inhibit antibody reduction by blocking enzymatic pathways: (1) use inhibitors for Trx system, (2) use inhibitors for G6PD, and (3) use inhibitors for hexokinase. In addition, some non-specific measures that can slow or stop antibody reduction are included in Table I as well. There are only a few known Trx inhibitors including alkyl-2-imidazolyl disulfides and related compounds (Kirkpatrick et al., 1998, 1999) and naphthoquinone spiroketal derivatives (Wipf et al., 2001). Numerous inhibitors are available for TrxR (Gromer et al., 2004). The two gold compounds, ATG and ATM, used in this study are examples of irreversible inhibitors of TrxR. Metal ions, such as Hg^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, and Mn^{2+}, can form readily complexes with thiols and selenols. Thus, these metal ions can be inhibitors for TrxR or Trx. Furthermore, there are also various inhibitors for G6PD. For example, pyridoxal 5′-phosphate and 1-fluoro-2,4-dinitrobenzene are two known inhibitors (Milhausen and Levy, 1975). In addition, certain steroids, such as DHEA and epiandrosterone (EA), are potent inhibitors of G6PD (Gordon et al., 1995). We have preliminary data suggesting that DHEA can inhibit the G6PD in HCCF. Thus, it is possible that these G6PD

Table I. Potential reduction inhibition methods.

Method[a]	Purpose
Addition of EDTA, EGTA, or citrate	To inhibit hexokinase (this study)
Addition of sorbose-1-phosphate, polyphosphates, 6-deoxy-6-fluoroglucose, 2-C-hydroxy-methylglucose, xylose, or lyxose	To inhibit hexokinase (McDonald, 1955; Sols et al., 1958)
Addition of dehydroepiandrosterone or epiandrosterone	To inhibit G6PD (Gordon et al., 1995)
Addition of pyridoxal 5′-phosphate or 1-fluoro-2,4-dinitrobenzene	To inhibit G6PD (Milhausen and Levy, 1975)
Addition of metal ions such as Cu^{2+}, Zn^{2+} Hg^{2+}, Co^{2+}, or Mn^{2+}	To inhibit Trx system (Gromer et al., 2004)
Addition of alkyl-2-imidazolyl disulfides and related compounds (e.g., 1—methylpropyl-2-imidazolyl disulfide[b]) or naphthoquinone spiroketal derivatives (e.g., palmarumycin CP_1[,b])	To inhibit Trx (Kirkpatrick et al., 1998, 1999; Wipf et al., 2001)
Addition of aurothioglucose or aurothiomalate	To inhibit TrxR (Gromer et al., 2004)
Air sparging	To deplete G6P and NADPH (Trexler-Schmidt et al., 2010)
pH adjustment to below 6.0	To reduce thiol-disulfide exchange rate and Trx system activity (Trexler-Schmidt et al., 2010)

[a]Applied to CCF prior to harvest or in HCCF immediately after harvest.
[b]Currently not available commercially.

inhibitors can block antibody reduction as well. As discussed above, EDTA is capable of inhibiting hexokinase activity. Other metal chelators, such as EGTA and citrate, are likely to inhibit both the hexokinase activity and the reduction of antibody as well. Additionally, hexokinase can be inhibited by compounds that react with thiol groups. Other hexokinase inhibitors include sorbose-1-phosphate, polyphosphates, 6-deoxy-6-fluoroglucose, 2-C-hydroxy-methylglucose, xylose, and lyxose (McDonald, 1955; Sols et al., 1958). Using any of these inhibitors against the Trx/TrxR system, G6PD, and/or hexokinase is a potential method to block disulfide bond reduction in a recombinant protein manufacturing process.

Several different inhibitors for preventing antibody reduction based on the strategy discussed above have been evaluated for implementation into manufacturing (Trexler-Schmidt et al., 2010). In addition to reduction prevention effectiveness, inhibition methods need to be assessed based on impact to product quality, downstream purification performance, downstream clearance, and manufacturability (e.g., environmental disposal limits, technician safety, cost of goods, raw material supply). For initial evaluations, inhibitors commonly used in biological production processes were tested. These methods included modifications to the cell culture media with chemicals (e.g., cupric sulfate, cysteine) and pre- and post-harvest additions to the CCF (e.g., cupric sulfate, EDTA, L-cystine). It is also possible to prevent antibody reduction by sparging the HCCF with air to maintain an oxidizing redox potential in the HCCF (Mun et al., 2010, manuscript in preparation). This is a non-specific method that can deplete glucose, G6P, and NADPH by continuously oxidizing the reduced forms of Trx and TrxR. Another non-specific method to block antibody reduction is to lower the pH of HCCF. Thiol-disulfide exchange is a reaction that is particularly slow at lower pH values (Pleasants et al., 1989; Whitesides et al., 1977). Therefore, the activity of the Trx system is significantly lower at pH values below 6 whereby the antibody reduction can be inhibited. Finally, the commercial effectiveness and robustness of each reduction inhibition method will need to be evaluated on a case-by-case basis including the assessment of site-specific manufacturing feasibility.

Conclusions

The disulfide bond reduction presents a significant challenge for antibody manufacturing. Here, we have identified the mechanism by which the antibody is reduced. The data presented in this report strongly suggest that the root cause of the antibody reduction is an active Trx system in the HCCF. Our data also indicate that NADPH, required for activity of the Trx system, is provided by the functions of G6PD and hexokinase. Based on the identified reduction mechanism, any inhibitors, methods, or processes that can block one of the three enzyme systems involved (Trx system, G6PD, and hexokinase) can be used to prevent disulfide bond reduction in recombinant protein production.

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ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Air Sparging for Prevention of Antibody Disulfide Bond Reduction in Harvested CHO Cell Culture Fluid

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ABSTRACT: During the scale-up of several Chinese Hamster Ovary (CHO) cell monoclonal antibody production processes, significant reduction of the antibody interchain disulfide bonds was observed. The reduction was correlated with excessive mechanical cell shear during the harvest operations. These antibody reduction events resulted in failed product specifications and the subsequent loss of the drug substance batches. Several methods were recently developed to prevent antibody reduction, including modifying the cell culture media, using pre- and post-harvest chemical additions to the cell culture fluid (CCF), lowering the pH, and air sparging of the harvested CCF (HCCF). The work described in this paper further explores the option of HCCF air sparging for preventing antibody reduction. Here, a small-scale model was developed using a 3-L bioreactor to mimic the conditions of a manufacturing-scale harvest vessel and was subsequently employed to evaluate several air sparging strategies. In addition, these studies enabled further understanding of the relationships between cell lysis levels, oxygen consumption, and antibody reduction. Finally, the effectiveness of air sparging for several CHO cell lines and the potential impact on product quality were assessed to demonstrate that air sparging is an effective method in preventing antibody reduction.

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KEYWORDS: antibody; disulfide; reduction; lysis; sparging; dissolved oxygen; air

Introduction

Recombinant monoclonal antibodies (rMAb) are commonly produced in CHO cells in the biotherapeutics industry. In large-scale manufacturing of rMAb, cells are generally cultured in stainless steel bioreactors (Andersen and Krummen, 2002; Wurm, 2004) and the product is separated from the cells via centrifugation (Kempken et al., 1995; Roush and Lu, 2008). The product then undergoes further downstream purification (Fahrner et al., 2001; Kelley, 2007) prior to the bulk formulation steps. During the centrifugation process, cells are subjected to mechanical shear (Hutchinson et al., 2006) which can lead to the disruption of cell membrane integrity. Reduction of antibody interchain disulfide bonds has been observed in the scale-up of CHO rMAb production processes at Genentech as a result of excessive cell shear during the centrifugation process (Trexler-Schmidt et al., 2010). The reduction events were caused by intracellular components, identified as thioredoxin (Trx/TrxR) or thioredoxin-like enzymes, their associated enzyme pathway intermediates (e.g., glucose-6-phosphate dehydrogenase, hexokinase), and an energy source (e.g., NADPH) (Kao et al., 2010; Koterba et al., 2011), which were released upon cell lysis. Studies have shown that disulfide bond reduction is correlated to levels of mechanical cell lysis of viable, actively growing cells (Hutterer et al., 2013; Trexler-Schmidt et al., 2010) which results in the release of active thioredoxin system components. In contrast, reduction enzymes released upon cell death may not be active at the time of harvest and the energy source may be depleted. Multiple strategies have been developed to prevent this antibody reduction event including the addition of chemical inhibitors to the CCF and HCCF, as well as maintaining a minimum dissolved oxygen (dO_2) level in the HCCF via air sparging to promote an oxidizing environment for the antibody (Trexler-Schmidt et al., 2010).

Mammalian cells rely on several systems to maintain the oxidation reduction potential of each intracellular compartment at the appropriate state. Some of the sulfhydryl-containing oxidoreductase systems commonly studied include thioredoxin/thioredoxin reductase (Trx/TrxR) and glutathione/glutathione disulfide (GSH/GSSG). These systems regulate cellular events such as cell signaling (Filomeni et al., 2002), formation of disulfide bonds (Cumming et al., 2004; Jessop and Bulleid, 2004) and gene transcription (Sen and Packer, 1996), and also serve to protect the cells from reactive oxygen species (ROS) (Linke and Jakob, 2003; Nordberg and Arner, 2001; Shen et al., 2005). Oxygen plays a role as

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the terminal electron acceptor in these oxidoreductase systems (Shimizu and Hendershot, 2009) and oxygen supplementation has previously been applied in protein refolding to promote the correct formation of disulfide bonds (Fischer et al., 1993; Menzella et al., 2002). With this understanding in mind and coupled with the knowledge gained from the studies described by Trexler-Schmidt et al. (2010), the dO_2 trends of HCCF derived from manufacturing-scale harvests were evaluated. Surprisingly, the dO_2 level dropped to 0% within a few hours. This appears to be linked to mechanical cell lysis and plays an important role in the subsequent disulfide bond reduction event. Therefore, implementation of air sparging in the HCCF vessel to promote an oxidizing environment should aid in preventing reduction of disulfide bonds.

Dissolved oxygen control strategies are widely applied in cell culture bioreactors. A common sparging strategy employs a dO_2 probe which provides feedback to a PID control loop to scale the air and/or oxygen sparge output to a rate appropriate to maintain the desired dO_2 set-point. Many factors are considered in bioreactor design in order to provide adequate gas transfer to maintain high cell densities while minimizing potential harmful shear effects from agitation or bubble rupture that could damage the cells or protein (Al-Rubeai et al., 1995; Chisti, 2001; Merchuk, 1991; Trinh et al., 1994).

This paper explores the method of air sparging as an antibody reduction mitigation strategy through the development and utilization of a small-scale model to evaluate process requirements and characteristics. First, a small-scale model was established using a 3-L bioreactor to mimic the conditions of an HCCF tank. This small-scale model was then used to evaluate several air sparging strategies for effectiveness at preventing disulfide bond reduction and potential impact on product quality. Several CHO cell lines were tested with this method to ensure robustness for manufacturing and to determine if this could be universally applied to prevent antibody reduction. These studies also permitted further understanding of the relationships between cell lysis levels, oxygen consumption, and antibody reduction.

Materials and Methods

Generation of CCF and Production of rMAb in Bioreactors

CHO cells were cultured in 3-L glass stirred tank bioreactors (Applikon) in conditions similar to those previously described by Chaderjian et al. (2005). Culture conditions (e.g., temperature, pH, dO_2, and agitation) were controlled and monitored on-line. Off-line measurements of pH, dissolved gases (pO_2, pCO_2), sodium, and metabolite concentrations (glucose, lactate, ammonia) were obtained with a NOVA Bioprofile Analyzer. Daily samples were taken to monitor cell growth, viability, and titer.

Small-Scale Lysis Model to Generate HCCF With Reduction Activity

CCF at the end of the production culture was homogenized using a Microfluidics HC-8000 homogenizer, centrifuged, and filtered as described previously by Trexler-Schmidt et al. (2010). The non-mechanically lysed material (also referred to as "non-lysed") has cell lysis levels present from the end of the production culture. "Non-lysed" cell lysis levels were consistently between 10–30% for the feedstocks used for these studies. The lysis levels reported in the data figures throughout this work reflect total lysis levels ("non-lysed" + mechanical lysis).

HCCF Pool Incubation in Small-Scale Vessels for Sparging Studies

HCCF pools were incubated at a 2-L working volume in sterile 3-L glass stirred tank bioreactors (Applikon) with downflow pitched blade impellers for up to two days. Conditions were monitored with dO_2 (Mettler Toledo), pH (Broadley James), and temperature probes. Digital control units (B. Braun) were used to control agitation rate and maintain dO_2 at or above the specified set-points by delivering air through an open pipe sparger as required. Air sparge rates ranged from 0–50 standard cubic centimeters per minute (sccm), which corresponds to 0–0.025 vvm. No N_2 sparge was used to decrease the dO_2 level if it exceeded set-point. The pH levels were maintained at specified set-points if called for by the experiment design by addition of CO_2 or 1 M Na_2CO_3. HCCF was held at ambient temperature without temperature control (18–22°C). The agitation rate was controlled at 50–100 rpm. Unless otherwise noted, a 50 sccm N_2 overlay was supplied for all cases with dO_2 control, while no N_2 overlay was employed for non-sparged holds. After 1 and 2 day holds, HCCF was purified over a lab-scale Protein A affinity column and the pools were frozen at < -70°C until analysis.

HCCF Pool Incubation in Mini-Tanks and Protein A Processing

HCCF was held in 50 mL 316-L stainless steel mini-tank containers (Flow Components, Dublin, CA) as described previously by Trexler-Schmidt et al. (2010). After 1 and 2 day holds, the HCCF was purified over a lab-scale Protein A affinity column as described by Trexler-Schmidt et al. (2010).

Disulfide Bond Reduction Assay

Microchip capillary electrophoresis (CE) (Agilent 2100 Bioanalyzer) was used to quantitate the level of non-reduced antibody as described previously by Trexler-Schmidt et al. (2010). The non-reduced, intact antibody migrates at 150 kDa.

Mass Transfer Measurements

Volumetric gas transfer coefficients (k_La) were determined for the sparge vessel conditions using a dynamic method. Sparge vessels were filled with 2-L of cell-free culture media and set to the desired agitation and sparge rates at ambient temperature (18–22°C). N_2 or air was added to the bioreactor through the sparger and/or headspace to drive the dO_2 to ~50 or ~100%, respectively. dO_2 readings were measured with a probe and recorded using FermWorks software (Jova Solutions). Based on the equation $\ln(C^* - C_L) = -k_La \times t + \text{constant}$, data were plotted in a semi-log fashion with $(C^* - C_L)$ versus time. Linear regressions were

performed with the slope representing the k_La value for each condition.

Cell Lysis Measurements (LDH Assay)

Percent cell lysis was determined by measuring the level of lactate dehydrogenase (LDH) as described previously by Trexler-Schmidt et al. (2010).

Monoclonal Antibody Protein Concentration Assays

For HCCF samples, an HPLC-based Protein A method was used to determine rMAb concentration in order to calculate purification load density and yield. The rMAb concentration in the purified Protein A pool was measured using UV spectrometry at 280 nm and the extinction coefficient for that particular rMAb.

IEC-HPLC Assay

Ion-exchange chromatography (IEC) was used to assess charge heterogeneity. Samples were treated with carboxypeptidase B to remove C-terminal lysine before analysis by cation-exchange chromatography using a Dionex ProPac WCX-10 column (4×250 mm). The mobile phase used in the separation consisted of a potassium phosphate/potassium chloride pH 6.9 buffer.

Perturbation Oxygen Uptake Rate (OUR) Measurements

HCCF pools were incubated in 3-L glass stirred tank bioreactors with dO_2 controlled at 30%. At specified intervals throughout the hold period (e.g., every 2–4 h), dO_2 control and air sparging were turned off and the rate of change in dO_2 was calculated over a 5% drop. Agitation remained at set-point throughout the hold period and during the perturbation.

Results

Establishment of a Small-Scale Sparge Model

An initial experiment was performed to investigate hold conditions for the small-scale sparge model. Lysed HCCF (85% lysis) was held at a 2-L working volume in 3-L bioreactors at two conditions: 50 rpm agitation with 50 sccm N_2 overlay and 100 rpm agitation with no N_2 overlay. The rMAb reduction levels in these two hold conditions were measured using the Agilent Bioanalyzer assay and compared to the control mini-tank hold. As shown in Figure 1, rMAb reduction was observed in the mini-tank control after a two-day hold time. In contrast, no rMAb reduction occurred with the 100 rpm agitation and no N_2 overlay conditions and reduction was accelerated with the 50 rpm agitation and N_2 overlay conditions, with reduction occurring after a one-day hold time. In addition, a significant difference in dO_2 levels was observed between the two sparge vessel hold conditions due to surface transfer from the headspace. The presence of a N_2 overlay led to removal of O_2 from the liquid and helped drive the dO_2 levels to 0%. While 0% dO_2 was maintained for the entire hold period, re-formation of the antibody disulfide bonds was observed once the reduction activity (i.e., intracellular components) was depleted after the two-day hold period. Re-formation of the disulfide bonds could be the result of the antibody attaining a more stable conformation of the lowest energy for its given environment. However, other as yet to be defined mechanisms may also be at work here. In the case without a N_2 overlay, dO_2 levels increased over time due to surface transfer from air in the headspace, which was also facilitated by the higher agitation rate. Based on these results, 50 rpm was selected as the agitation rate for future studies and a N_2 overlay was employed for most sparge experiments, unless otherwise specified, since it represented a worst case environment for antibody reduction. As an exception, no N_2 overlay was used for non-sparged hold cases where dO_2 trends were being observed. Surface transfer is expected to be less significant in a manufacturing-scale tank due to a smaller surface area-to-volume ratio.

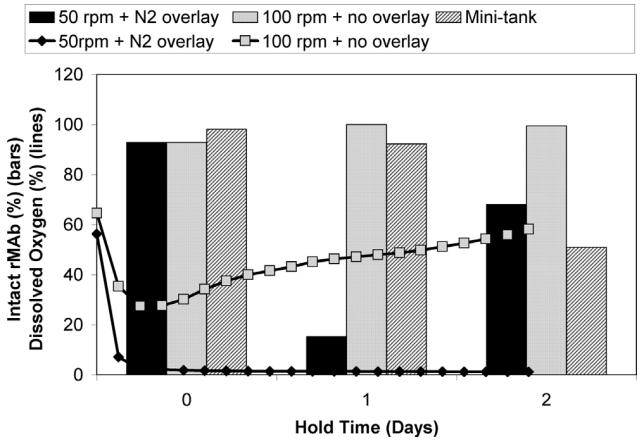

Figure 1. Lysed HCCF (85% total lysis; 65% mechanical lysis) was held in small-scale sparge vessels at room temperature (18–22°C) at two different hold conditions. Levels of rMAb reduction and dO_2 were compared between conditions as well as to the control mini-tank hold.

Gas transfer in bioreactors occurs through two interfaces, one between the liquid and sparged bubbles and the other between the liquid surface and tank headspace. To characterize the small-scale sparge model, k_La values were measured at varying agitation and sparge rates through both the sparger and headspace. As shown in Table I, the sparger k_La was 2.5-fold higher at a sparge rate of 50 sccm versus 10 sccm. In addition, the lower agitation rate decreased the headspace k_La slightly but did not significantly impact gas transfer through the sparger. These results further confirm the selection of a 50 rpm agitation rate in order to minimize the surface transfer yet provide adequate mixing. No difference was observed in the headspace k_La with a 50 sccm or 250 sccm overlay, so 50 sccm was chosen for future studies. Overall, the headspace k_La values were relatively low compared to the sparger k_La values within the range of sparge rates discussed in this work. This suggests that sparge rates were the main driver in the observed results and that headspace transfer played a relatively minor role.

In order to optimize the sparge vessel conditions to adequately maintain a dO_2 set-point of 30%, a study was performed to compare a 10 or 50 sccm maximum air sparge rate. Lysed HCCF (85% lysis) was held in sparge vessels with an agitation rate of 50 rpm and a

Table I. Sparger and headspace k_La values in the small-scale sparge model at varying agitation and sparge rates.

Agitation (rpm)	Sparge rate (sccm)	Sparger k_La (hr^{-1})	Headspace k_La (hr^{-1})
50	10	0.28	—
	50	0.75	0.12
	250	—	0.13
100	10	—	—
	50	0.68	0.15
	250	—	0.16

50 sccm N_2 overlay and dO_2 trends were monitored for each condition. As shown in Figure 2, a maximum sparge rate of 10 sccm provided insufficient gas transfer to maintain the dO_2 set-point early in the hold, while a maximum sparge rate of 50 sccm provided immediate and reliable control at the dO_2 set-point. Therefore, a maximum sparge rate of 50 sccm, which corresponds to a k_La of 0.75 hr^{-1}, was chosen for future studies.

Profile of Lysed HCCF in the Sparge Model

To characterize the behavior of lysed HCCF, material at several lysis levels was held in small-scale sparge vessels with 50 rpm agitation, no air sparge, and no N_2 overlay. Correlations were observed between cell lysis levels, the amount of oxygen consumed, and rMAb reduction for several CHO production cell lines. Figure 3A shows that an increase in cell lysis levels resulted in an increase in oxygen consumption. For the non-lysed (10% lysis) and 20% lysis cases, oxygen consumption was minimal and the levels of dO_2 remained relatively high. Conversely, at higher total cell lysis levels of 45 and 65%, the oxygen demand was high and resulted in the depletion of dO_2. These trends suggest that HCCF dO_2 levels could be used as an indicator for lysis levels and the potential for rMAb reduction at manufacturing-scale. However, absolute values from the small-scale model cannot be applied to manufacturing-scale due to differences in behavior and operations between scales (e.g., surface transfer, overlay pressure, hydrostatic pressure). The maximum oxygen consumption rate observed in the small-scale model was approximately 60%/h for the same cell line tested in Figure 3A, using a perturbation oxygen uptake rate (OUR) algorithm at 100% total lysis (data not shown). Concurrent implementation of other reduction mitigation strategies, such as lower temperature or chemical inhibitors, has also been shown to impact dO_2 levels (data not shown).

In addition to dO_2 levels, cell lysis levels were also found to impact the level of rMAb reduction, which is consistent with previous findings (Trexler-Schmidt et al., 2010). Different threshold lysis levels are required for rMAb reduction to occur in different cell lines (data not shown). In Figure 3B, the results after a one-day hold show that intact rMAb levels were lower at the higher lysis levels. Higher levels of intact rMAb were observed after the two-day hold presumably due to a decrease in sample reduction activity following the depletion of enzyme pathway reducing agents after an extended hold time, which allowed the rMAb to re-oxidize to its lowest energy state. Results from previous experiments also support that the maximum reduction activity is observed within the first 24 h of incubation with active reducing components (data not shown).

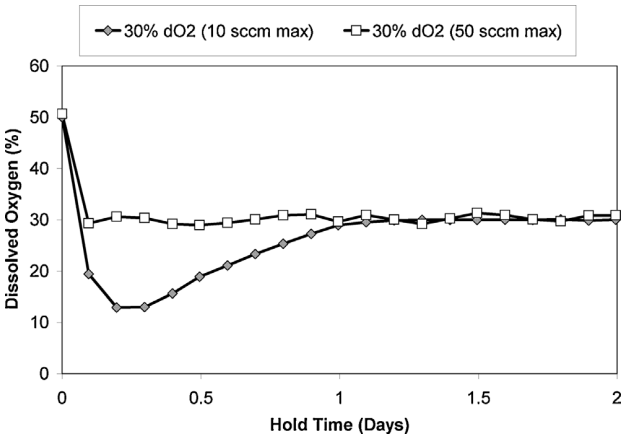

Figure 2. dO_2 trends for HCCF (85% total lysis; 65% mechanical lysis) controlled at 30% dO_2 set-point with two maximum sparge rates in the small-scale sparge vessel.

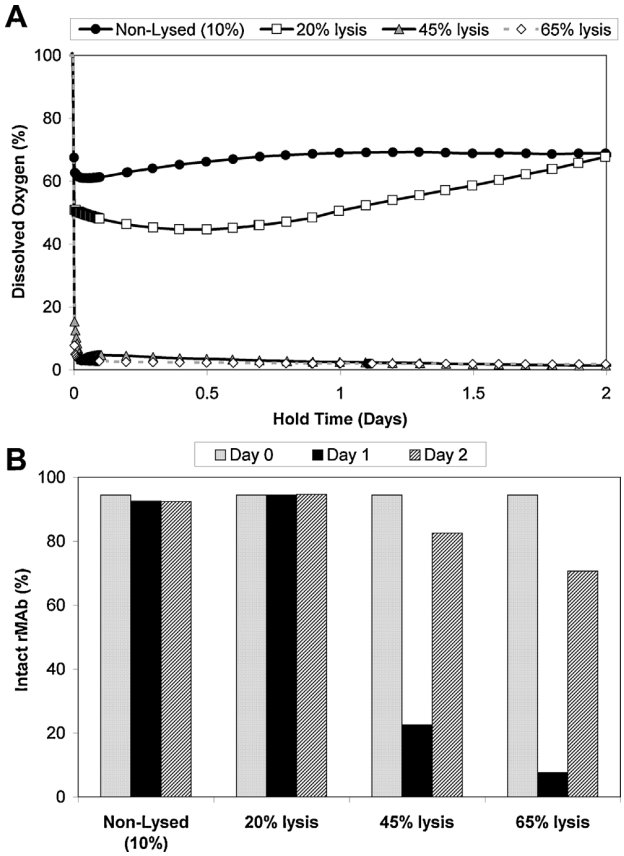

Figure 3. Results showing the correlation between cell lysis levels and (**A**) Dissolved oxygen levels; (**B**) rMAb reduction. Lysis values listed reflect total lysis levels ("non-lysed" (10%) + mechanical lysis). In this study, the CCF was harvested at 90% viability, which was confirmed by the LDH assay and represented 10% lysis. Data were collected over a two-day hold in the sparge vessels.

While high lysis conditions result in both oxygen depletion (Fig. 3A) and rMAb reduction (Fig. 3B), oxygen depletion alone is not sufficient to trigger reduction. Material with low reduction activity was sparged with N_2 to drive down the oxygen levels to 0% and no rMAb reduction was observed (data not shown). Therefore, a combination of sufficient reduction activity and low dO_2 levels are required for the reduction event to occur.

Impact of Air Sparging Strategies on rMAb Reduction Inhibition

Two potential air sparging strategies were explored: constant sparge rates with variable dO_2 levels and constant dO_2 levels with variable sparge rates. In order to determine the minimum level of air sparging required to inhibit rMAb reduction, a range of air flow rates of 5–50 sccm and a range of dO_2 levels of 10–50% were tested with lysed HCCF (65–85% lysis) that was shown to be susceptible to disulfide bond reduction in the non-sparged control vessel. The dO_2 control was one-sided with air supplied up to a maximum sparge rate of 50 sccm to maintain the set-point. All cases had a N_2 overlay except for the 50 sccm constant sparge case. Figure 4A shows the levels of intact rMAb over a two-day hold period for the various sparge strategies tested. Slight reduction was observed for the 5 sccm constant sparge case after a two-day hold time, however, the degree of reduction was decreased compared to the non-sparged control. All other sparge rates and dO_2 set-points were found to be sufficient to prevent disulfide bond reduction.

As shown in Figure 4B, the dO_2 levels for the non-sparged control case and the 5 sccm sparge case were depleted within one day. This indicates that 5 sccm air sparge was unable to provide sufficient oxygen supply to maintain the dO_2 level above 0% in competition with the rate of oxygen consumption by the lysed HCCF. All other sparge cases maintained dO_2 levels >0% since the sparge rates met or exceeded the oxygen consumption rates. Climbing dO_2 levels were observed for the 50 sccm sparge case as the oxygen delivery rate exceeded the consumption rate.

As shown in Figure 4C, the HCCF pH increased by up to 1.3 units over two days as a result of CO_2 stripping. The rate of pH increase was accelerated at the higher air sparge rates or higher dO_2 set-points due to increased CO_2 mass transfer. Lower rates of CO_2 stripping are expected in a manufacturing-scale HCCF tank because the lower liquid surface-to-volume ratio reduces CO_2 mass transfer (Matsunaga et al., 2009).

When choosing an appropriate HCCF air sparging strategy, it is desirable to minimize the amount of air sparge delivered to decrease CO_2 stripping and the subsequent increase in pH and potential impact on product quality. The use of a minimum dO_2 set-point provides feedback control and scales the air output appropriately to meet demand. This ensures that adequate air is supplied to maintain dO_2 levels >0% and prevent antibody reduction, while also preventing the addition of excess air sparge. Therefore, a sparge strategy of dO_2 control was selected as the preferred antibody reduction mitigation strategy for further evaluation. A 30% minimum dO_2 set-point was chosen because it is commonly used in mammalian cell culture production bioreactors and has been shown to be robust and achievable, leaving a sufficient safety factor on the lower end, while not over-sparging.

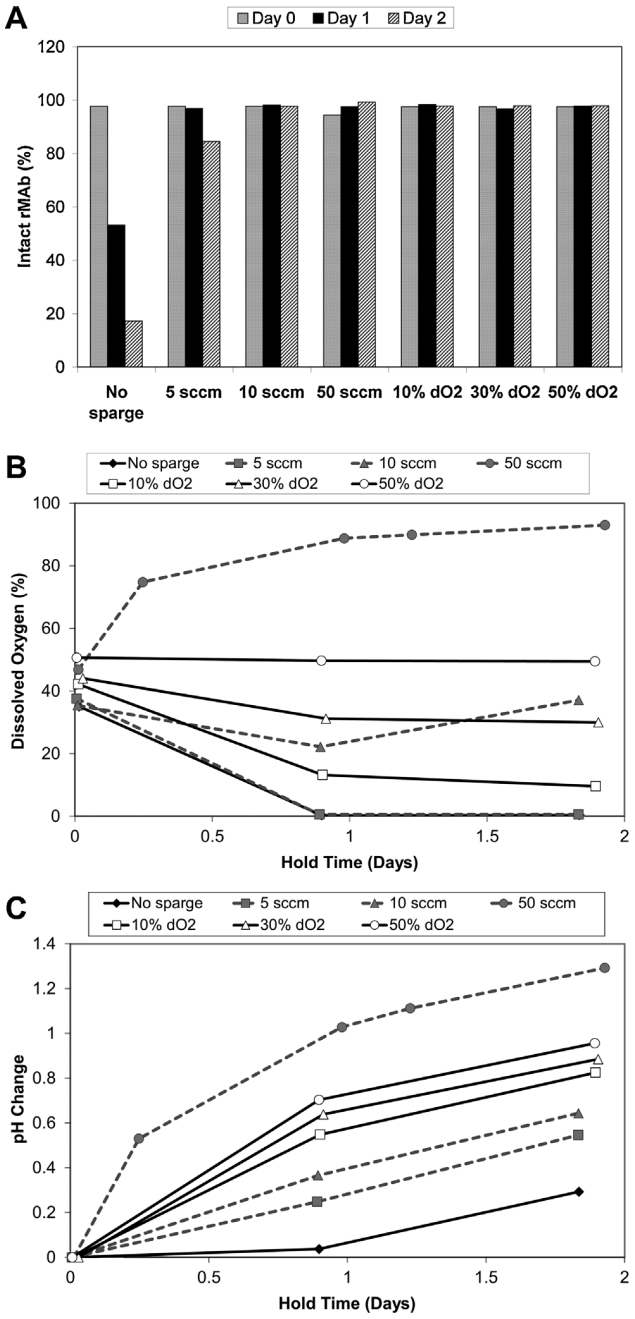

Figure 4. HCCF with 65–85% total lysis (40–55% mechanical lysis) was tested with multiple air sparge strategies. A N_2 overlay was used in all cases except for the 50 sccm constant sparge case. Results show trends of (**A**) rMAb reduction, (**B**) Dissolved oxygen, (**C**) pH change.

Air Sparge Requirement Per Lysed Cell

Figure 5A,B shows an assessment of the amount of air that is required to maintain a 30% dO_2 set-point for a single cell line at three lysis levels. As seen in Figure 3A, the amount of oxygen consumed is dependent upon the level of cell lysis. In this experiment, lysed HCCF was held in sparge vessels with or without a N_2 overlay to assess extreme conditions with respect to additional

oxygen transfer from the liquid surface. These conditions bracket the expected surface oxygen transfer rate of a manufacturing-scale HCCF tank. As predicted, larger volumes of air were required to maintain the 30% dO_2 set-point for cases with higher levels of cell lysis (Fig. 5A). In the presence of a N_2 overlay, additional air sparge was required to maintain the dO_2 set-point. Most of the air sparge was required during the first 24 h of the hold time, corresponding to the period of maximum reduction activity.

The air sparge requirement through a 24-hour hold is plotted in Figure 5B versus the number of mechanically lysed viable cells. For this cell line, the sparge requirement per lysed cell in the small-scale model is 3.3×10^{-9} L air/lysed cell or 6.9×10^{-10} L O_2/lysed cell. This value could be used to determine oxygen demand for a particular lysis level or gauge relative reduction activity between cell lines or culture conditions. Additionally, since a linear correlation is observed between required air sparge and the number of lysed cells, these data provide confirmation that the oxygen-consuming compounds in the HCCF are being released from the cells upon lysis. An alternative approach to defining a dissolved oxygen control strategy at manufacturing-scale would be to measure the oxygen uptake rate of the cell lysate in order to estimate the required mass transfer coefficient in a HCCF vessel.

Air Sparging Effectiveness for Multiple Cell Lines

Since air sparging was found to be a robust and effective method in preventing rMAb reduction for one product, three additional products produced in CHO cell lines were tested in the small-scale model and the results are summarized in Figure 6. For all four products, air sparging to maintain a minimum 30% dO_2 level was able to fully prevent reduction and maintain >90% intact rMAb. These results show that air sparging can be used as a mitigation strategy to prevent rMAb reduction for multiple cell lines with varying levels of reduction activity.

Air Sparging Impact on Product Quality

In the manufacture of rMAbs, appropriate process controls are important to ensure consistent product quality. Protein product quality can be sensitive to variations in cell culture process parameters (e.g., temperature, pH, or dO_2) and changes could result in a shift in product attributes such as glycan distribution, aggregate formation, or charge variant profile (Andersen and Goochee, 1994; Cromwell et al., 2006; Yoon et al., 2004). Additionally, product quality can be impacted by cell-free hold conditions during recovery operations or in the final formulation buffer (Cromwell et al., 2006; Peters and Trout, 2006; Wang, 1999; Wakankar and Borchardt, 2006). Implementation of air sparging during the HCCF hold step has the potential to impact product quality. Therefore, as with any

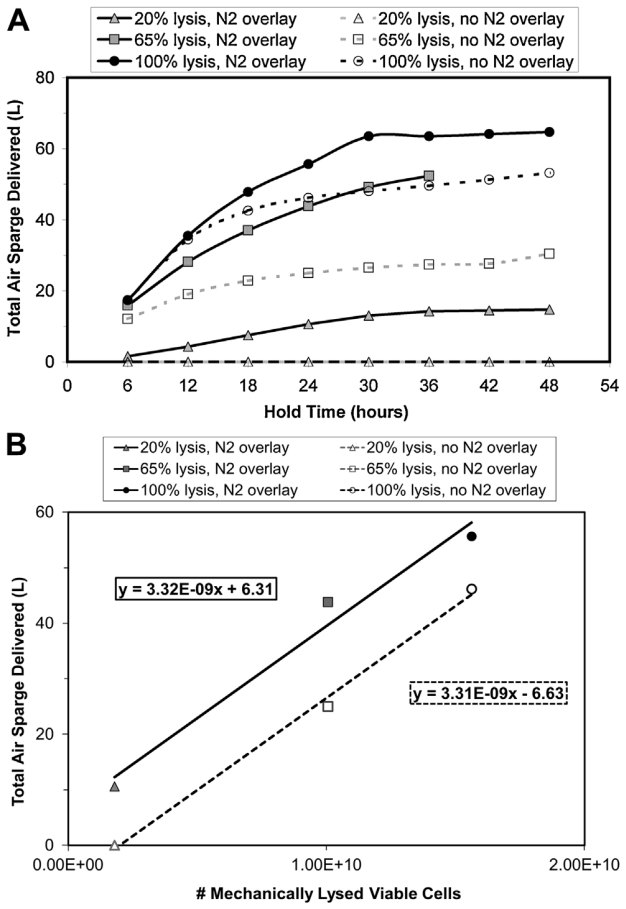

Figure 5. Totalized volume of air sparge required to maintain a 30% dO_2 set-point for varying lysis levels in the sparge model with and without a N_2 overlay. Lysis values listed reflect total lysis levels ("non-lysed" (10%) + mechanical lysis). (**A**) Plotted with time on the x-axis; (**B**) Totalized air sparge over 24 h on the y-axis and number of mechanically lysed cells on the x-axis. The slope of each line represents the volume of air sparge required over 24 h per mechanically lysed cell to maintain a 30% dO_2 set-point.

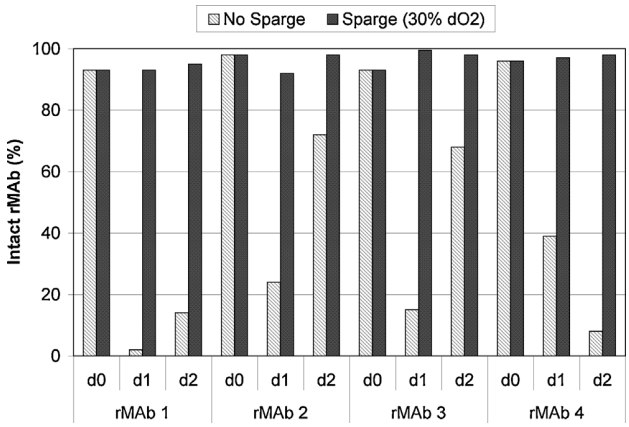

Figure 6. Results showing the effectiveness of air sparging on preventing reduction with four different rMAb products. The dashed bars indicate the no sparge condition and the solid bars indicate the sparge condition (30% dO_2 set-point with up to a maximum 50 sccm sparge rate). Products were tested at a total lysis level conducive to reduction (65–85% total lysis; 55–65% mechanical lysis). The HCCF was sampled from the sparge vessels and purified over Protein A after 0, 1, and 2 days.

other change to a process, the appropriate product quality analyses should be performed to ensure minimal impact.

Sparged material purified by Protein A was evaluated using SEC-HPLC, IEC-HPLC, CE-glycan, and peptide map to evaluate any changes to aggregate levels, charge variant distribution, glycan distribution, and oxidation, respectively. Based on these analyses, air sparging was found to have no significant impact with respect to aggregate, glycan, or oxidation profiles (data not shown). However, a decrease in the levels of main peak by IEC-HPLC was observed with a concurrent increase in the levels of acidic variants. Experiments were performed to identify the cause of these acidic variants by specifically evaluating the effects of sparging and cell lysis. Figure 7 displays the effect of 0–50 sccm air sparging on main peak levels for HCCF material with a low lysis percentage of 20% total lysis (10% mechanical lysis), which is expected to be representative of the lysis levels of manufacturing-generated HCCF. Lower main peak was observed with increasing sparge rate. Overall, the non-sparged control material had a drop of 1% main peak per day while the sparged cases showed an additional 0.5–1.5% decrease per day. Previous studies observed a rate of ~2% decrease per day which was not considered significant (Trexler-Schmidt et al., 2010).

Next, the effect of cell lysis on the charged variant profile was examined. In this experiment, HCCF at different lysis levels, including a non-lysed control, was sparged at a constant rate to ensure that the product in each case had equivalent air exposure. A high sparge rate of 50 sccm was selected to assess worst case. The results shown in Figure 8 demonstrate that cell lysis contributes to the decrease in main peak with as much as a 2% additional decrease per day over the non-lysed control for the 100% lysis case. Taken together, the results from Figures 7 and 8 indicate that the highest levels of acidic variant increase (main peak decrease) would be expected for material with both high lysis and high sparge rates.

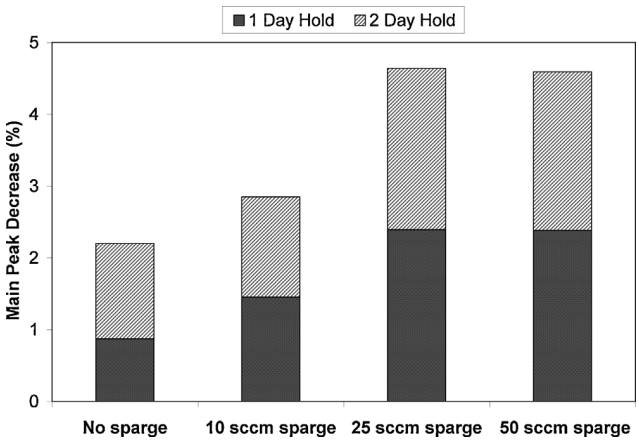

Figure 7. Non-lysed + 10% mechanical lysis (20% total lysis) material was tested at different levels of constant air sparge (no N_2 overlay) to assess the impact of air sparging on IEC-HPLC main peak. The HCCF was sampled from the sparge vessels and purified using Protein A after 1 and 2 days and submitted for IEC-HPLC analysis. The data were compared to non-sparged hold conditions.

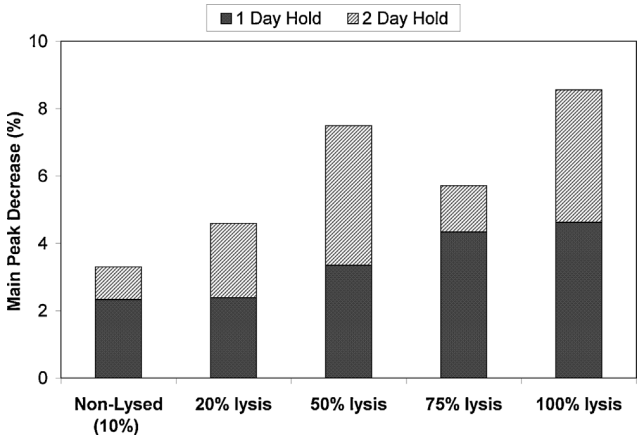

Figure 8. Material at different lysis percentages was sparged at a constant sparge rate of 50 sccm (no N_2 overlay) to assess the impact of cell lysis on IEC-HPLC main peak. Lysis values listed reflect total lysis levels ("non-lysed" (10%) + mechanical lysis). The HCCF was sampled from the sparge vessels and purified over Protein A after 1 and 2 days and submitted for IEC-HPLC analysis. The data were compared to a non-lysed control.

Additional studies to help understand the source of the acidic variant formation are ongoing. One factor that has been explored is the possibility that the pH increase during air sparging, due to CO_2 stripping from the HCCF in the small-scale sparge vessel, leads to accelerated deamidation and therefore an increase in acidic variants (Patel and Borchardt, 1990; Peters and Trout, 2006).

Discussion

Air sparging has been shown to be a robust and universal mitigation strategy to prevent rMAb reduction. While the complete mechanism of rMAb reduction prevention has not yet been fully elucidated, the consumption of oxygen by cellular metabolic pathways results in the exhaustion of nutrient and energy supplies. For example, oxygen may convert NADPH, the proposed primary fuel for the reduction pathway, into NADP+ thereby preventing it from being used as fuel for the reduction pathway. As the energy that fuels the reduction pathway is depleted, the oxygen consumption rate is decreased and less HCCF air sparging is required.

When selecting an appropriate HCCF air sparging strategy for manufacturing implementation there are several important factors to consider. Both a constant air sparge rate or maintaining a minimum dO_2 level are effective at preventing reduction; however each strategy has its own advantages and disadvantages. Implementation of a constant air sparge rate would minimize facility modifications since a dO_2 control loop would not be required. Furthermore, this strategy would provide a predictable pH increase which could potentially be beneficial from a process consistency standpoint. However, there is a potential for providing inadequate oxygen and leaving the rMAb at risk for reduction or conversely over-sparging the HCCF and potentially impacting product quality. Overall, this strategy lacks robustness since the air sparge rate would not be responsive to variation in oxygen

requirements based on substrate conditions (i.e., lysis percentage, cell density, culture viability, etc.). In contrast, a minimum dO_2 set-point with a one-sided dO_2 control loop provides feedback control and scales the air output appropriately to meet the demand. This ensures adequate air is supplied to maintain dO_2 levels $>0\%$ and prevent antibody reduction while also avoiding the unnecessary addition of excess air sparge. This preferred method is sensitive and responsive to the dynamic nature of oxygen consumption and compensates for differences in reduction activity of the material due to variation from cell lysis levels or the cell culture process.

An appropriate minimum dO_2 target should be selected such that it inhibits antibody reduction and provides an adequate safety factor at the lower end of the range while not being set too high as to over-sparge the HCCF. Since oxygen consumption is observed immediately upon cell lysis, it is important to have the air sparge strategy engaged immediately upon entry of the material into the HCCF tank. In addition, k_La measurements of the manufacturing-scale HCCF tank should be performed to ensure the oxygen demand of the HCCF material from varying cell lines and lysis levels can be met through appropriate agitation and sparge rates.

While shifts in the charge variant profile have been observed in these studies, changes at manufacturing-scale are expected to be significantly smaller. The experimental factors tested with the small-scale model are not fully representative of manufacturing-scale HCCF tank environments and are likely worst case for acidic variant formation. First, the lysis levels and duration of sparge at scale are expected to be lower. Cell lysis levels could be minimized with the use of a hermetic-style centrifuge (Liu et al., 2010), and the HCCF would not be sparged for longer than one day assuming typical manufacturing processing times. Second, implementation of a minimum dO_2 level instead of a constant air sparge would minimize the amount of air exposure to the product. Third, a smaller pH perturbation from air sparging is expected in a large-scale tank due to the decreased CO_2 stripping relative to O_2 transfer observed at larger scales. Further studies are required to better understand how a combination of factors may influence acidic variant formation in the small-scale sparge model (e.g., increase in pH, intracellular factors released during cell lysis, rate of air sparge, and dO_2 levels). It would be ideal to perform studies at manufacturing-scale with representative conditions to assess the degree of product quality impact that might be expected with air sparging. The significance of any product quality shift as a result of air sparging should be considered in the context of the given product's clinical experience, potency impact, and potential downstream purification clearance of these species.

Finally, we have observed a correlation between oxygen consumption and rMAb reduction, suggesting that perhaps the rate of HCCF oxygen consumption could be used as a surrogate for reduction activity. Future work will focus on identifying the oxygen-consuming components in the HCCF to help elucidate the complete reduction pathway. In addition, oxygen consumption rates will be measured for multiple cell lines and conditions to gain a greater understanding of this phenomenon and enable manufacturing-scale implementation of HCCF air sparging.

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Monoclonal antibody disulfide reduction during manufacturing

Untangling process effects from product effects

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Keywords: antibody disulfide reduction, free cysteine, harvest, capillary electrophoresis, CE-SDS

Abbreviations: CCF, cell culture fluid; CHO, Chinese hamster ovary; DO, dissolved oxygen; DTT, dithiothreitol; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent); EOP, end of production; HCCF, harvested cell culture fluid; IAM, iodoacetamide; IgG, immunoglobulin G; mAb(s), monoclonal antibody(ies); MEA, micro-extractor automated instrument; NADPH, nicotinamide adenine dinucleotide phosphate; NEM, N-ethylmaleimide; NR CE-SDS, non-reduced capillary electrophoresis with sodium dodecyl sulfate; PAT, process analytical technology; PPP, pentose phosphate pathway; SDS, sodium dodecyl sulfate; t0, initial time point

Manufacturing-induced disulfide reduction has recently been reported for monoclonal human immunoglobulin gamma (IgG) antibodies, a widely used modality in the biopharmaceutical industry. This effect has been tied to components of the intracellular thioredoxin reduction system that are released upon cell breakage. Here, we describe the effect of process parameters and intrinsic molecule properties on the extent of reduction. Material taken from cell cultures at the end of production displayed large variations in the extent of antibody reduction between different products, including no reduction, when subjected to the same reduction-promoting harvest conditions. Additionally, in a reconstituted model in which process variables could be isolated from product properties, we found that antibody reduction was dependent on the cell line (clone) and cell culture process. A bench-scale model using a thioredoxin/thioredoxin reductase regeneration system revealed that reduction susceptibility depended on not only antibody class but also light chain type; the model further demonstrates that the trend in reducibility was identical to DTT reduction sensitivity following the order IgG1λ > IgG1κ > IgG2λ > IgG2κ. Thus, both product attributes and process parameters contribute to the extent of antibody reduction during production.

Introduction

The target specificity, favorable pharmacokinetics and pharmacodynamics, and stability of monoclonal human immunoglobulin gamma (IgG) antibodies have resulted in their widespread use in the biopharmaceutical industry.[1,2] Commercial therapeutic antibody production is a complex but fairly well established process, typically involving expression in Chinese hamster ovary cells (CHO), harvesting of the secreted protein, and a series of chromatography steps to remove impurities. Reduction of antibody interchain disulfide bonds during manufacturing operations has recently been the subject of much interest.[3-5] This phenomenon is observed when extending the time that the antibody remains in the cell culture fluid (CCF) or harvested cell culture fluid (HCCF) in the "harvest" step of production. This harvest step includes separation of cells from the media prior to the first column purification.

Process-induced antibody disulfide bond reduction has been observed inconsistently at large scale processes and is not typically observed with standard bench-scale (up to 10 L) models.[5] This reduction has been attributed to certain enzymes that are released from the intracellular compartments of lysed cells. Components in the thioredoxin reduction pathway, including thioredoxin reductase and NADPH, have been proposed as the principal underlying contributor for this antibody disulfide bond reduction.[3,4] Reduction has been shown to be virtually eliminated by maintaining dissolved oxygen (DO) levels during harvest operations.[5] In addition, the cysteine/cystine redox couple, which is present in the growth media, may affect disulfide bond formation, reduction, and rearrangement.[6] Likewise, many other media components, such as certain metal ions and their complexes, are likely to affect the reduction potential during the harvest procedure.[5,6]

In these studies, cell lysis and an anaerobic environment both promoted antibody reduction during harvest;[5,6] therefore,

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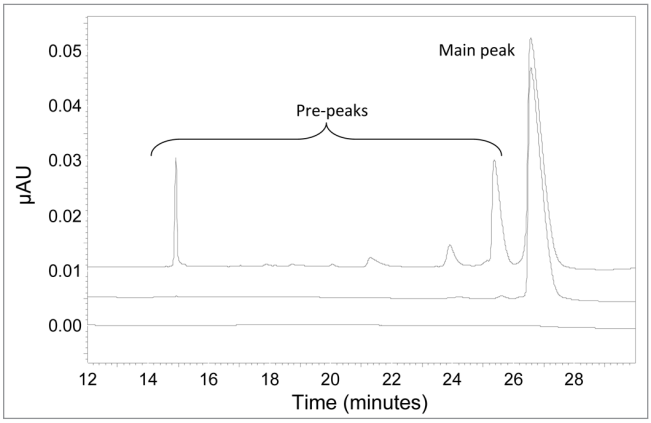

Figure 1. NR CE-SDS Electropherograms. Partially Reduced mAb (top), Purified mAb (middle), and Blank (bottom).

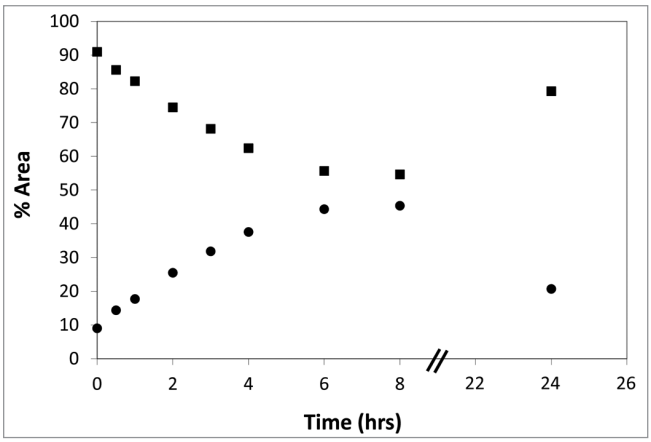

Figure 2. Reduction of mAb A in Small Scale Reduction Model as a Function of Time. Pre-peaks (circles) and Main peak (squares).

it is clear that adequate process understanding and control is necessary to minimize or eliminate disulfide bond reduction induced by manufacturing procedures. In addition to variation due to manufacturing processes, differences between products were observed.[5] Because cell cultures, cell lines, and the products themselves can vary in cell cultures expressing two different antibody products, the underlying causes for these reduction differences could not be determined. The study presented here explores the relationship between reduction and process variables, separating the influence of process and products to demonstrate that CHO cell line or cell culture process can dramatically influence reduction during harvest operations and that the antibody class and light chain type also influences the extent of that reduction.

Results

Small scale model. Harvest-related disulfide reduction has been reported as highly dependent on process scale and has been reported in some scaled-up, but not bench-scale, processes.[5] This effect of scale may be attributed to the maintenance of oxygen in small-scale harvests, which may preserve disulfide bonds. Typically, bench-scale experiments are open to the air, which allows more efficient oxygen transfer than typical manufacturing-scale (15,000 to 20,000 L) cell culture production. Bench-scale experiments also use different centrifuge equipment, introducing the possibility of different degrees of cell shearing during removal of debris. To facilitate harvest reduction experiments, a small-scale model, similar to previously described models,[5] was developed. A "worst case" reduction model of cell culture extract was generated by mechanically shearing 2 L of whole cell culture fluid (CCF) used for production of an IgG1κ mAb (mAb A), transferring the sheared CCF into a 3 L bioreactor, and sparging the resultant slurry with nitrogen to simulate the anaerobic environment of the commercial scales. Samples were taken at 0, 0.5, 1, 2, 4, 8, and 24 h and immediately frozen at –70°C. Non-reduced capillary electrophoresis with sodium dodecyl sulfate (NR CE-SDS) was performed on all samples to measure the degree of interchain disulfide bond breakage. Representative electropherograms of a partially reduced antibody, a properly disulfide-linked antibody, and a blank are shown in **Figure 1**. This figure shows that the peaks in the pre-peak region of the electropherogram increase in intensity relative to the main, properly disulfide-linked, peak. These pre-peaks have been shown to be light chain (L), heavy chain (H), and combinations of the two chains (HL, HH, HHL).[7] Because size exclusion chromatography indicates that reduction does not result in disassembly of the antibody chains, the NR CE-SDS pre-peaks represent properly assembled antibodies with one or more broken interchain disulfide bonds. The relative area associated with the pre-peaks and main peak were used to monitor interchain disulfide reduction in a series of harvest experiments. Results showing antibody reduction in mAb A for up to 24 h after cell shearing in the small-scale model are shown in **Figure 2**. An increase in the percentage of pre-peaks over time is observed, from 9% at the initial time point to ~45% at 8 h. This increase in the percentage of pre-peaks replicates previously published results[5] and demonstrates that the small-scale model is capable of inducing and monitoring disulfide bond reduction. It is worth noting that the pre-peak level decreases after 8 h, and it is only ~12% by 24 h, indicating that disulfide bonds can reform. This observation is consistent with previously published results.[5]

Product, cell line and process. Partial disulfide bond reduction behavior was probed with multiple Amgen therapeutic antibodies and cell lines. Three products, an IgG2λ (mAb B), an IgG2κ (mAb C), and mAb A, the IgG1κ discussed above, were tested by shearing end of production cells and subjecting the lysed CCF to nitrogen sparging in the small-scale model at 25°C. **Figure 3** displays the relative amount of intact antibody for each of these products as a function of time. Although the IgG1κ results demonstrate that an interchain disulfide can be reduced in this antibody type using this small-scale model, no changes were seen in mAb C (IgG2κ) or mAb B (IgG2λ). This lack of reduction under these reduction promoting conditions has not been previously reported, and indicates tight controls of air sparging

or cell shearing are not necessary for all antibody production processes. This comparison of end of production CCF shows stark differences in behavior, but does not distinguish between the effects of product, cell line, or cell culture process. While the mAb C (IgG2κ) titer and cell density are fairly low, and that might account for the difference, both the mAb A (IgG1κ) and the mAb B (IgG2λ) have relatively high titers and cell densities, as shown in **Table 1**. IgG2s, such as the mAb B, are known to be less susceptible to reduction by thioredoxin;[8] however, there could also be differences between the cell lines or processes that could contribute to these observations.

Direct comparison between cell lines is complicated due to the differences that may arise through the transfection process. Both copy number and insertion site can vary from clone to clone, and both of these parameters may also affect cell growth, viability, productivity, and metabolism.[12,13] Therefore, to partially disentangle the effect of product, cell line and process on the level of reduction, end of production cells from these three products were lysed, and the original product was removed via Protein A affinity to create soluble cellular component material. The reduction activity was shown to be maintained through this type of processing by Trexler-Schmidt et al.[5] The results in **Table 2** illustrate the difference in NR CE-SDS % Main peak between the t0 and 8 h samples for several combinations of cells and purified products. To determine whether this material remained active, purified mAb A was spiked back into its own soluble cellular components and held under nitrogen overlay for 8 h. When mAb A sheared cell broth was reconstituted in this manner, the difference in NR CE-SDS % Main peak was ~45%, identical to the small-scale model results, which indicates that the reducing activity was preserved through this processing step. This is consistent with the experiment performed by Trexler-Schmidt et al.[5] When purified mAb D (IgG2κ) was spiked into mAb A soluble cellular components, little reduction (0.9% reduction in % main peak) was observed, showing that mAb D reduction is more resistant to these conditions. Thus, the product will influence the degree of reduction observed in the harvest process. To test the effects of cell line and cell culture process independent of product, purified mAb A was spiked into mAb B and mAb C soluble cellular components. Although mAb A is susceptible to reduction in its own soluble cellular components, little reduction was observed when it was incubated in either mAb C or mAb B components (1.5% and 2.7% reduction in % main peak, respectively). All of the materials were carefully sparged with nitrogen during processing, and the soluble cellular components were prepared and used within 30 min of cell lysis. In addition, repeat analysis of the (mAb B) IgG2λ spike into the mAb A (IgG1κ) yielded identical results, as did intermediate time points for the other conditions. The lack of reduction for these conditions must be due to differences in the reducing power of cellular component samples because the product is identical. Disufide reducing ability of the cellular component sample could arise from differences in the cell line, such as differences in expression of thioredoxin or thioredoxin reductase, or differences in availability of NADPH due to regulation of the pentose phosphate pathway (PPP). Differences in reducing power of the soluble cellular component sample could also arise from

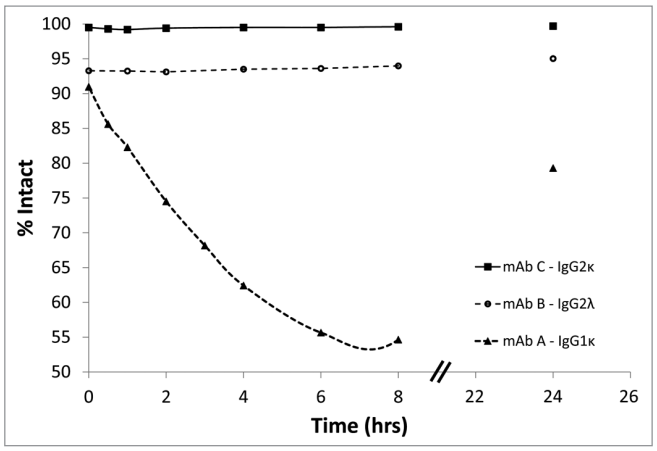

Figure 3. Reduction Behavior of Different Products and Cell Lines over time. mAb A (triangles), mAb B (circles), and mAb C (squares).

the cell culture process differences, either indirectly, by influencing expression of thioredoxin, expression of thioredoxin reductase, and utilization of the PPP, or directly, by differing levels of redox active media components such as cystine/cysteine and copper. In the case of mAb C, the measured thioredoxin reductase activity is lower than that for mAb A (**Table 1**); however, this could be due to the lower cell density. In contrast, the cell density is similar for mAb B, and therefore the cell line and process can be determined to have a significant effect on reducing power. The presence of substantial thioredoxin reductase activity in these cell lysates is not unexpected because some apoptosis, which will release intracellular contents, inevitably occurs during cell culture. This means, however, that the difference in reducing power cannot be attributed to thioredoxin system activity alone because the thioredoxin reductase activity was higher in the mAb B lysate than the mAb A lysate. Therefore, either the other redox active components of the system have a major affect or substantial differences in the availability of NADPH exist. Taken together, the results suggest that the cell line (clone) or cell culture process play a key role in harvest-related reduction.

Product properties. As described above, the reconstituted extract model, demonstrates that striking differences exist in susceptibility to reduction among antibody products. Previously published studies have shown that antibody sub-classes differ in sensitivity to disulfide bond reduction.[8] Differences in reduction susceptibility due to light chain type have not previously been observed for thioredoxin catalyzed reduction, but have been shown using chemical reductants.[9-11] A chemical model system was developed to investigate antibody type (IgG1 and IgG2) and light chain type sensitivity to thioredoxin catalyzed reduction. As illustrated in **Figure 4**, reduction sensitivity is dependent on both antibody class and light chain type. Reduction sensitivity, in decreasing order, is IgG1λ, IgG1κ, IgG2λ, IgG2κ. This trend held true for all of the additional molecules we have tested, and for different stoichiometric ratios of the reagents and antibody (data not shown). Sensitivity to antibody subclass has also been reported for other reductants, such as DTT.[9,10] The reduction sensitivity trend

Table 1. End of production cell densities, viability and titer for mAbs A, B and C

Product	Viable Cell Density (cells/mL)	Viability (%)	Titer (g/mL)	Thioredoxin Reductase Activity (μmol/min/mL)
IgG2κ (mAb C)	$7.0x10^6$	75.4	1.3	Below detection limit
IgG2λ (mAb B)	$17.2x10^6$	62.4	4.7	0.16
IgG1κ (mAb A)	$27.0x10^6$	78.9	4.4	0.04

Table 2. Influence of product and cell line/process on reduction

Soluble Cellular Component	Purified mAb	Difference in NR CE-SDS % Main peak
IgG1κ (mAb A)	IgG1κ (mAb A)	46.3%
IgG1κ (mAb A)	IgG2κ (mAb D)	0.9%
IgG2κ (mAb C)	IgG1κ (mAb A)	1.5%
IgG2λ (mAb B)	IgG1κ (mAb A)	2.7%

Difference in NR CE-SDS % Main peak, 8 h, relative to initial.

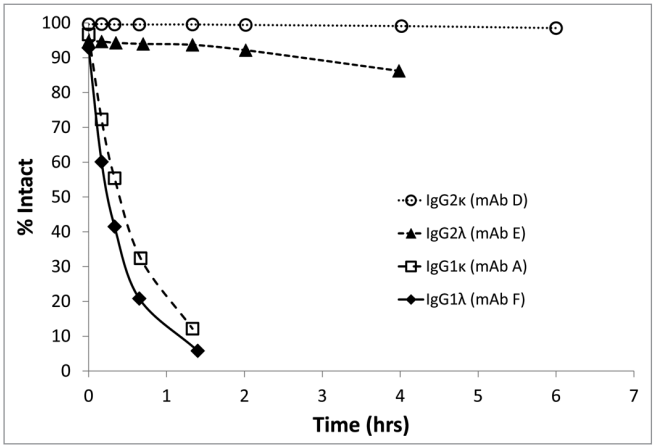

Figure 4. Influence of Product on Reduction using Thioredoxin System. Intact antibody, as measured by % Main peak in the NR CE-SDS analysis as a function of time.

was similar between the thioredoxin system and with DTT (**Fig. 4 and 5**). This comparison indicates that differences in reduction during harvest are the result of overall reducing potential of the system and the antibody type, and not any specific interactions between thioredoxin and certain antibody types.

Equipped with the knowledge of the reducibility trend IgG1λ > IgG1κ > IgG2λ > IgG2κ, a general understanding of the product contribution to the risk of process-induced reduction can be made prior to expression of products. This general understanding can be further refined by putting a small amount of purified product into either the chemically-defined thioredoxin reducing system, or by making kinetic measurements of the reducibility of the product by DTT.

Discussion

Process-induced partial antibody disulfide reduction is an active topic of discussion in the literature and with regulatory authorities. This phenomenon is caused by shearing of cells, resulting in the release of intracellular components, and requires an anaerobic environment. Different cell lines and processes have been demonstrated to have strikingly different reduction responses, e.g., mAb B soluble cellular components having less than 1/10th the reducing power of those of mAb A. The difference in reducing power cannot be attributed to differences in thioredoxin and thioredoxin reductase levels, as measurement of thioredoxin reductase shows that it is higher in some of the cell lysates that show no reduction. Therefore, these differences must stem from other redox active components in the media, or more likely, from differences in NADPH availability and regulation of the PPP.

The susceptibility of products to reduction by thioredoxin has been demonstrated to be dependent on antibody class and light chain type, IgG1λ > IgG1κ > IgG2λ > IgG2κ, with potentially some sequence dependency within each range. The susceptibility of the antibody classes to thioredoxin catalyzed reduction follows the same trend as antibody disulfide reduction by DTT. Therefore, a general understanding of product reducibility is available prior to expression of the product, and a more refined understanding of its susceptibility to reduction is possible with only micrograms of material in a chemically-defined system.

With the understanding of the reducing power of the cell line and process, screening of cell lines and cell culture conditions is possible. Combining process knowledge with the antibody class, a good understanding of the overall reduction behavior can be obtained early in process development.

Materials and Methods

Materials. Cell culture fluid and purified antibodies were produced at Amgen using standard manufacturing procedures. Reagents were obtained from Sigma-Aldrich unless otherwise specified.

Cell shearing. Complete cell lysis of end of production (EOP) cell culture fluid, which contains both cells and the media containing product, was achieved by high-pressure homogenization using a Microfluidics M-110Y high shear fluid processor. Homogenization was performed with a single pass at 8,000–10,000 psi. Complete lysis was verified using the Roche Innovatis Cedex AS20 cell counter.

Small-scale reduction model. A 3 L glass stirred-tank bioreactor (Applikon Corporation) controlled by a customized DeltaV distributed control system (DCS) was used to evaluate harvest conditions. Processed cells were transferred to this bioreactor. Agitation was set at 250 rpm. Temperature was controlled to 8–10°C by passing chilled water through a thermal well in the bioreactor. Room temperature conditions were

unregulated, at ~22°C. Dissolved oxygen (DO) was measured using a Mettler Toledo DO probe connected to a Rosemont Transmitter. DO was lowered by sparging nitrogen gas through a drilled tube sparger with a flow sufficient to achieve a zero response for dissolved oxygen. To achieve oxygen at the 100% level, air was passed through the drilled tube sparger at 100 to 200 mL/min. DO, temperature, airflow and agitation data were collected by the DeltaV DCS and archived into a PI data historian (OSIsoft).

Reconstituted extract model. Cell culture fluid (CCF) depleted in antibody product was generated using a batch binding process to remove existing monoclonal antibodies (mAbs). CCF was transferred to a 250 mL polycarbonate bottle (Nalgene) and homogenized using a Tissue Tearor™ (Biospec Products) for one minute of homogenization to ensure complete cell breakage. During homogenization, a nitrogen (N_2) gas overlay was applied. MabSelect SuRe™ Protein A affinity resin (GE Healthcare) was washed twice with an equilibration buffer of 100 mM NaCl, 25 mM Tris, pH 7.4, dried by vacuum over a nylon membrane, and applied in excess directly to the bottle. The mixture was placed on a rocker for 10 min to facilitate binding. The solution was centrifuged for 5 min x 1000 rpm in 50 mL conical tubes to pellet the resin. The supernatant was extracted from each tube and sparged with N_2 to form the soluble cellular components, and used within 30 min of production.

Sample antibody drug substance was added to a separate 15 mL polypropylene centrifuge tube and brought to a total volume of 7 mL with the soluble cellular component material to give a final antibody concentration of 3 mg/mL. An N_2 overlay was applied to each tube. The tubes were covered with laboratory paraffin film and placed in a digitally controlled water bath set at 10°C. One mL aliquots were pulled at 0, 4 and 8 h and immediately frozen at –80°C prior to analysis by NR CE-SDS.

Reduction by thioredoxin system. The roles of thioredoxin and thioredoxin reductase (TR) have previously been described as nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular protein disulfide reductases.[14,15] An in vitro lab-scale model using this complex was optimized using recombinant human thioredoxin (Sigma), NAPDH (Calbiochem), in excess and thioredoxin reductase from rat liver (Sigma). A polypropylene 2 mL cryogenic vial (Corning) was sparged for 1 min with N_2 prior to being sealed in a borosilicate septa vial (I-Chem). In a separate 1.5 mL microcentrifuge tube, 825 µL phosphate buffered saline, 14 µL NADPH (10 mM), 10 µL human thioredoxin solution (0.5 mg/mL) and antibody drug substance were combined to give a final volume of 1 mL and antibody concentration of 4 mg/mL. The reaction was initiated with the addition of 18 µL TR solution (7 µM) and transferred immediately into the sealed vial using a syringe. The septa vials were placed within a temperature-controlled water bath at 10°C with an applied overlay of N_2 to exclude oxygen. Aliquots of 100 µL were taken at each time point, quenched immediately with 8.7 µL N-ethylmaleimide (NEM) at 250 mM and frozen at –80°C prior to analysis by NR CE-SDS.

Reduction by DTT. Partially reduced mAbs, with majority of the interchain disulfide bonds broken, were generated by incubating mAbs at the concentration of 2 mg/mL in 50 mM TRIS-HCl, pH 7.5 (Teknova) with 2 mM DTT (Geno Technology) at ambient temperature. Aliquots were taken at multiple time points and the reduction was quenched by immediately adding NEM (MP biomedical) to a final concentration of 25 mM. A non-reducing Caliper CE-SDS assay was performed to measure the level of reduction.

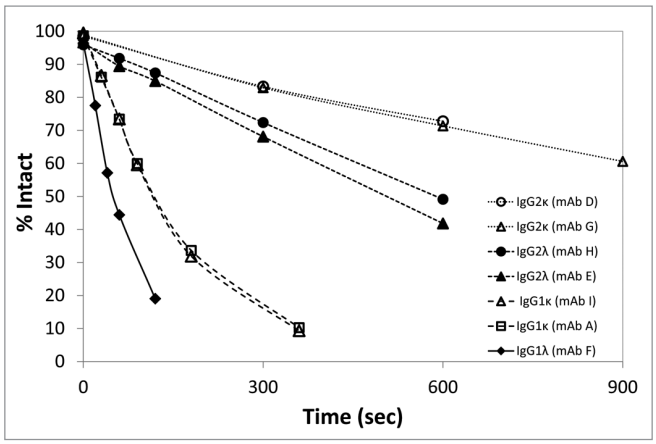

Figure 5. Influence of Product on Reduction using DTT. Intact antibody, as measured by % Main peak in the NR CE-SDS analysis as a function of time.

Thioredoxin reductase activity. Thioredoxin reductase activity of lysates was assessed using a colorimetric 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) kit (Cayman Chemical). Briefly, lysed CCF was added to a pH 7 sample buffer containing 50 mM potassium phosphate, 50 mM potassium chloride, 1 mM EDTA, and 0.2 mg/mL bovine serum albumin, at a final dilution factor of 1:10. For each sample a matrix control was made by adding 20 µM sodium aurothiomalate (final concentration). Excess NADPH and 0.5 mM DTNB (final concentration) were added to each sample, matrix control, blank, and positive control (rat liver thioredoxin reductase). Light absorbance was monitored at 405 nm for 5 min, and the activity of thioredoxin reductase in µmol/min/mL was calculated by taking the difference in slopes between the sample and the matrix control, dividing by the extinction coefficient and path length, and multiplying by the dilution factor.

Non-reduced CE-SDS. Harvested cell culture fluid samples were prepared using an automated robotic platform, as previously described.[16] Briefly, samples were centrifuged at 13,000 rpm for 1 min and loaded onto a Micro-Extractor Automated Instrument (MEA, PhyNexus). PhyTip® 200 µL Columns with 20 µL protein A affinity resin protein A tips were used to remove host cell proteins. Non-reducing sample buffer with a final concentration of 7 mM NEM, 57 mM sodium phosphate, 1.9% SDS, pH 6.5 was added to the purified samples. Incubation was set for 5 min at 60°C and samples were injected onto a 30 cm bare fused silica capillary with a 20 cm effective length and 50 µm inner diameter using electrokinetic injection. Separation was performed using CE-SDS gel (Beckman Coulter) and 15 kV effective voltage, and detection was by UV light absorbance at 220 nm.

Non-reduced Caliper CE-SDS. A LabChip 90 (Caliper Life Sciences) was used to separate SDS bound proteins through a sieving polymer based on the hydrodynamic size of the SDS-protein complex.[17] HT Protein Express Sample Buffer (Caliper Life Sciences) was combined with iodoacetamide (IAM) to a final IAM concentration of approximately 5 mM. A total of 5 μL antibody sample at approximately 1 mg/mL was mixed with 100 μL of the IAM containing sample buffer. The samples were incubated at 75°C for 10 min. The denatured proteins were analyzed by LabChip 90 with the "HT Protein Express 200" program.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Effects of Antibody Disulfide Bond Reduction on Purification Process Performance and Final Drug Substance Stability

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ABSTRACT: Antibody disulfide bond reduction during monoclonal antibody (mAb) production is a phenomenon that has been attributed to the reducing enzymes from CHO cells acting on the mAb during the harvest process. However, the impact of antibody reduction on the downstream purification process has not been studied. During the production of an IgG_2 mAb, antibody reduction was observed in the harvested cell culture fluid (HCCF), resulting in high fragment levels. In addition, aggregate levels increased during the low pH treatment step in the purification process. A correlation between the level of free thiol in the HCCF (as a result of antibody reduction) and aggregation during the low pH step was established, wherein higher levels of free thiol in the starting sample resulted in increased levels of aggregates during low pH treatment. The elevated levels of free thiol were not reduced over the course of purification, resulting in carry-over of high free thiol content into the formulated drug substance. When the drug substance with high free thiols was monitored for product degradation at room temperature and 2–8°C, faster rates of aggregation were observed compared to the drug substance generated from HCCF that was purified immediately after harvest. Further, when antibody reduction mitigations (e.g., chilling, aeration, and addition of cystine) were applied, HCCF could be held for an extended period of time while providing the same product quality/stability as material that had been purified immediately after harvest.

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Introduction

Monoclonal antibodies (mAbs) are an important class of biomolecules that are used in the treatment of various diseases such as cancer, multiple sclerosis, rheumatoid arthritis, lupus, and respiratory diseases (Choy et al., 1998; Cobleigh et al., 1999; Haynes et al., 2009; Helliwell and Coles, 2009; Robak and Robak, 2009). During mAb process development, aggregates and fragments have to be removed to adequate levels due to their associated risks with increased immunogenicity and potential effects on drug efficacy (Fan et al., 2012; Rosenberg, 2006). Further, the presence of these product variants can also affect the stability of the product during storage leading to reduced shelf life.

Many commercial manufacturing processes for mAbs involve the use of Chinese Hamster Ovary (CHO) cells for product expression and depth filtration or centrifugation for harvest, followed by purification, and formulation to produce the drug substance. There were several recently reported instances in literature whereby reduction of mAb disulfide (S-S) bonds is observed during different parts of the process. Hutchinson and co-workers observed significant mAb fragmentation of an IgG4 molecule with increasing

centrifugation shear conditions. They hypothesized that the mechanical forces in the centrifuge were responsible for reducing the molecule into half-antibodies (Hutchinson et al., 2006). Trexler-Schmidt and co-workers further demonstrated that antibody disulfide reduction can be attributed to the cell lysis and the release of intra-cellular reducing enzymes (primarily thio-redoxin reductase/thioredoxin) as a result of harsh centrifugation conditions (Trexler-Schmidt et al., 2010). Hutterer and co-workers attributed the extent of antibody reduction to be dependent on the cell line and cell culture process (Hutterer et al., 2013). Various methods of minimizing antibody reduction were reported. Maintaining a highly oxidative environment through air sparging was proposed as a solution to shift the equilibrium of the reversible redox reaction toward oxidation (Mun et al., 2015). Addition of chemical inhibitors (e.g., cystine, copper sulfate, EDTA) to act as a competitive inhibitor, directly inhibit responsible enzymes, or remove the metal ions required in the enzymatic pathway was suggested as means of minimizing enzymatic activity (Trexler-Schmidt et al., 2010). However, while the manufacturing process conditions that cause the antibody reduction (Hutterer et al., 2013), the impact on the molecular structure, as well the susceptibility of various mAb isoforms to reduction (Magnusson et al., 1997; Wang et al., 2015) were well-characterized, the impact of antibody reduction on purification process performance and long term drug substance stability has not been reported.

During process development of an IgG_2 antibody, antibody reduction was observed in the harvested cell culture fluid (HCCF) and was accompanied by an increase in the aggregate levels after the low-pH viral inactivation step. The long term drug substance stability was also affected as a higher rate of aggregation was observed. This study investigated the link between antibody reduction in the starting HCCF material and aggregation in the downstream process as well as in the drug substance storage stage. Various reduction strategies to prevent the antibody reduction were tested and their impact on process performance and drug substance stability were examined.

Materials and Methods

Cell Culture, Purification, and Formulation Procedures

Cell culture fluid was generated using CHO cells in a 50 L scale fed-batch process in a stainless steel bioreactor (Applkon; Delft, The Netherlands) using proprietary media and nutrient feeds with an initial working volume of 42 L. Cell separation was performed by an LAPX 404 continuous centrifuge (Alfa Laval; Lund, Sweden). The centrate was then filtered using X0HC POD depth filters followed by SHC sterile membrane filters (Millipore; Billerica, MA). Storage vessels for HCCF include 1 and 2 L PETG bottles (VWR, Bridgeport, NJ, 89096–292 and 89095–290) for small volume aliquots and disposable sterile bags (Sartorius Stedim, Bohemia, NY, FXB110922) for large volume aliquots. For storage conditions where headspace is required in the sterile bags, air was introduced through a 0.2 μm Acro 50 sterile filter (Pall, 4250) until the bag was fully inflated. HCCF was allowed to warm up to room temperature before initiation of purification if it had been stored chilled. Purification was performed at bench scale using an ÄKTA Explorer 100 (GE Healthcare, Piscataway, NJ, 18111241) systems and Vantage L (EMD Millipore, Billerica, MA, 96220250) chromatography columns. Protein A capture was performed using MabSelectSuRe resin (GE Healthcare Piscataway, NJ, 17-5438-05) followed by low pH inactivation with 300 mM Glycine, pH 2.35. Intermediate polishing was carried out using Super Q 650-M resin (Tosoh Bioscience, King of Prussia, PA, 17229) and final polishing utilized POROS 50HS resin (Thermo Fisher Scientific, Waltham, MA, 1335908). Hold studies were performed using 50 mL Flexboy storage bags from Sartorius Stedim. After purification, the samples were concentrated through centrifugation at 4000*g* to approximately 120 mg/mL using an Amicon Ultra-15 centrifugal filter unit with an Ultracel-30 membrane, (Millipore, UFC903096). Samples were then dialyzed overnight into 10 mM histidine buffer at pH 6. The final formulation to 60 mg/mL protein in 10% (w/v) trehalose dihydrate, 0.02% (w/v) polysorbate 80, 10 mM histidine at pH 6 was achieved by mixing in concentrated buffer.

Antibody Aggregation Analysis

The percentage of antibody aggregates was determined using a standard size exclusion chromatography (HP-SEC) method. An Agilent HPLC system (Agilent 1200 series) was used with a 7.8 mm × 300 mm TSKgel G3000SW XL column (Tosoh Bioscience, 08541) at 1 mL/min flow rate using a mobile phase buffer of 0.1 M sodium phosphate, 0.1 M sodium sulfate, pH 6.8. The absorbance at 280 nm was used to quantify the results.

Reduced Antibody Species Analysis

Samples were diluted to 2.0 mg/mL in 1X PBS and mixed in non-reducing sample buffer containing N-Ethylmaleimide (NEM). All samples were heated on a heating block at 100°C for 2 min and the protein ladder was heated on a heating block at 100°C for 5 min. Following denaturation, samples, and the ladder were diluted with ultra-pure water and loaded on a 96-well plate. The plate and a chip that contained the gel dye, the destain solution, and the protein express lower maker were placed into a LabChip GX system (Perkin Elmer, Waltham, MA, 124582) for analysis. The GX LabChip was placed in a LabChip GXII analyzer (Perkin Elmer, 124582/b) and read using LabChip GXII software. Protein and fragments were detected by laser-induced fluorescence and translated into gel-like images (bands) and electropherograms (peaks).

Free Thiol Quantitation in Harvested Cell Culture Fluid (HCCF)

The amount of free thiol at each site of IgG from HCCF was determined by Lys-C peptide mapping method under non-reducing condition. The free cysteine was capped with NEM, and the free thiol per each cysteine-containing peptide was calculated as the percentage of NEM-capped peptide. The HCCF was first buffer-exchanged to phosphate buffer using a 30 kDa

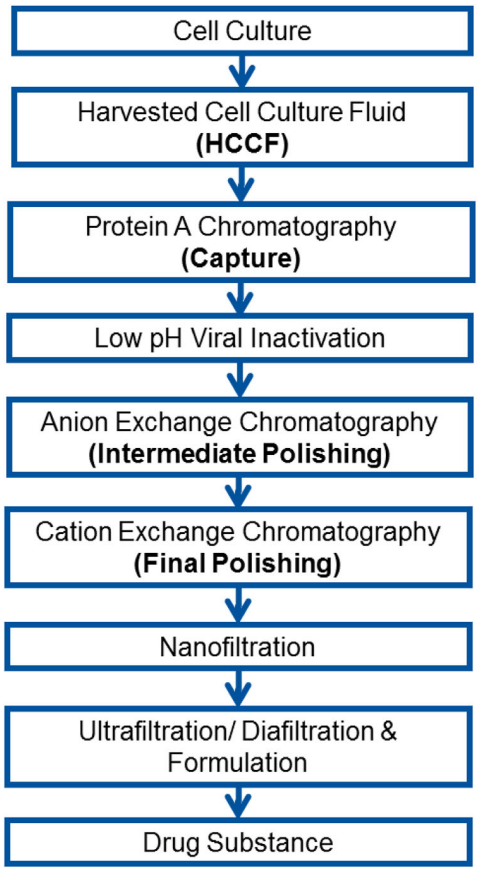

Figure 1. Schematic of platform mAb purification process.

MW cut-off centrifugal device. Prior to digestion with a serine protease, sample was mixed with NEM and guanidine to cap the free cysteine and denature the protein. Following protease digestion, half of each reaction mixture was reduced by the addition of 1,4-dithiothreitol (DTT). The reduced and non-reduced digests were both separated by a 1.7 μm, 2.1 × 150 mm Acquity UPLC HSS C18 column (Waters, 176001126) and analyzed by a tunable UV (TUV) detector and an Orbitrap mass spectrometer. The mobile phase A was 0.02% trifluoroacetic acid (TFA) in water and the mobile phase B was 0.02% TFA in acetonitrile. The peptides were eluted at a flow rate of 0.2 mL/min with a gradient of mobile phase B from 0% to 95% over 90 min.

Colorimetric Free Thiol Quantitation in Purification Process Intermediates

The free thiol assay evaluates the integrity of the disulfide connections in a protein by measuring the levels of free thiol groups on unpaired cysteine residues. Samples are incubated under native and denatured conditions with 5, 5′-dithiobis-(2-nitrobenzoic acid (DTNB) that binds to free thiol and releases a colored thiolate ion. The colored thiolate ion is detected with a UV-visible spectrophotometer. The concentration of free thiol is interpolated from a standard curve and the free thiol-to-antibody molar ratio is reported.

Results and Discussion

Observation of Reduced Antibody Species Formation

A platform mAb purification process (Fig. 1) was used to purify the IgG_2 monoclonal antibody that had been stored chilled in a storage bag with no headspace for 30 days before purification was initiated. As shown in Table I, Purification Run 1, high fragment levels (89%) were observed in the HCCF and gradually decreased with each step of the purification process. Aggregates levels increased by 1.0% after low pH viral inactivation and were removed during the subsequent polishing steps. As shown in Figure 2a and b, NR-GX images of the capture product and final chromatography polishing intermediate detected the presence of fragment bands with molecular weights corresponding to combinations of heavy chain (H) and light chain (L) fragments of the intact antibody that can arise as a result of reduction (L, H, L-L, H-L, H-H, H-H-L), showing that the inter-chain disulfide bonds were being reduced. As seen in Figure 2c, similar bands of the reduced species were also detected when the harvested cell culture fluid (HCCF) was analyzed by NR-GX, indicating that the mAb reduction phenomenon was occurring either in the bioreactor or at the harvest step. The cell culture material was clarified using centrifugation harvest and it has previously been reported by Trexler-Schmidt and co-workers that harsh centrifugation conditions can impact product quality through increased cell rupture leading to the release of intra-cellular host cell proteins such as thioredoxin and thioredoxin reductase. This can subsequently lead to cleavage of inter-chain disulfide bonds (Trexler-Schmidt et al., 2010). In addition to thioredoxin and thioredoxin reductase, other reducing enzymes present in the lysed HCCF including gluthathione reductase and protein disulfide isomerase can also cleave disulfide bonds (Guzman, 1997; Ikebuchi et al., 1992).

Based on the hypothesis that the loss in product quality was driven primarily by the release of intra-cellular reducing enzymes (either from dead cells in the bioreactor or through harsh centrifuge conditions) disrupting disulfide bonds in the mAb molecule, controls were put in place to slow down the enzymatic reaction by the temperature of the HCCF to 2–8°C. In this study, (Table I, Purification Run 2), storage of the material in a vessel with air-containing headspace was also employed in an attempt to provide a more oxidative environment. It was reported that providing an oxidative environment can allow for re-oxidation of reduced disulfide bonds (Mullan et al., 2011; Mun et al., 2015; Wang et al., 2015). As a rule of thumb, the volume of the storage vessel employed was twice the volume of HCCF (e.g., 2 L PETG bottle were used to store 1 L of HCCF, storage bags were filled to half the recommended maximum volume and inflated with filtered air). In addition, the HCCF was purified within 3 h after harvest to minimize the exposure of the mAb to reducing enzymes. As re-oxidation of reduced species can occur across the duration of the purification process, multiple 1 mL aliquots of purification process intermediates were made as soon as they

Table I. Comparison of aggregate and fragment levels in the purification process pre- and post-process optimization.

Purification Run no.	Purification conditions	Purity analysis	Purification process intermediate					
			HCCF	Capture	Post-low pH viral inactivation	Intermediate polishing	Final polishing	Drug substance
Purification Run 1	Purification process with HCCF subjected to: (i) 2–8°C storage in 50 L bag without headspace (ii) purification after 30 day hold	HP-SEC	NA	96.2 (%mon) 2.2 (%agg) 1.6 (%frag)	95.7 (%mon) 3.2 (%agg) 1.1 (%frag)	96.0 (%mon) 2.8 (%agg) 1.2 (%frag)	98.2 (%mon) 0.5 (%agg) 1.3 (%frag)	NA
		NR-GX	6.4 (%mon) 93.6 (%frag)	13.1 (%mon) 86.9 (%frag)	63.5 (%mon) 36.5 (%frag)	NA	87.6 (%mon) 13.4 (%frag)	NA
Purification Run 2	Purification process with HCCF subjected to: (i) 2–8°C storage in 50 L bag with headspace (ii) immediate purification	HP-SEC	NA	97.7 (%mon) 2.3 (%agg) 0.0 (%frag)	97.2 (%mon) 2.8 (%agg) 0.0 (%frag)	97.2 (%mon) 2.8 (%agg) 0.0 (%frag)	99.6 (%mon) 0.4 (%agg) 0.0 (%frag)	99.5 (%mon) 0.5 (%agg) 0.0 (%frag)
		NR-GX	87.8 (%mon) 12.2 (%frag)	99.0 (%mon) 1.0 (%frag)	99.3 (%mon) 0.7 (%frag)	99.4 (%mon) 0.6 (%frag)	99.1 (%mon) 0.9 (%frag)	NA
Purification Run 3	Purification process with HCCF subjected to: (i) 0.4 mM cys/g mAb spike (ii) 2–8°C storage in 50 L bag with headspace (iii) purification after 4 day hold	HP-SEC	NA	97.9 (%mon) 2.0 (%agg) 0.1 (%frag)	95.9 (%mon) 4.0 (%agg) 0.1 (%frag)	96.3 (%mon) 3.6 (%agg) 0.1 (%frag)	99.0 (%mon) 1.0 (%agg) 0.0 (%frag)	98.4 (%mon) 1.5 (%agg) 0.1 (%frag)
		NR-GX	83.5 (%mon) 16.5 (%frag)	93.1 (%mon) 6.9 (%frag)	99.8 (%mon) 0.2 (%frag)	100.0 (%mon) 0.0 (%frag)	100.0 (%mon) 0.0 (%frag)	99.8 (%mon) 0.2 (%frag)
Purification Run 4	Purification process with HCCF subjected to: (i) 0.8 mM cys/g mAb spike (ii) 2–8°C storage in 2 L bottle with headspace (iii) purification after 2 week hold	HP-SEC	NA	98.2 (%mon) 1.8 (%agg) 0.0 (%frag)	97.8 (%mon) 2.2 (%agg) 0.0 (%frag)	NA	99.2 (%mon) 0.8 (%agg) 0.0 (%frag)	99.0 (%mon) 1.0 (%agg) 0.0 (%frag)
		NR-GX	87.6 (%mon) 13.4 (%frag)	99.3 (%mon) 0.7 (%frag)	99.3 (%mon) 0.7 (%frag)	99.3 (%mon) 0.7 (%frag)	99.3 (%mon) 0.7 (%frag)	NA
		Free thiol	2.1% (inter-chain) 5.4% (intra-chain LC) 30.8% (intra-chain HC)	0.1 (mol/mol)	0.14 (mol/mol)	NA	0.05 (mol/mol)	NA

Aggregate levels were determined by HP-SEC. Fragment levels were determined by HP-SEC and non-reduced GX (NR-GX). Free thiol levels in the HCCF and subsequent process intermediates were determined by LC-MS and a colorimetric assay, respectively (NA, not analyzed).

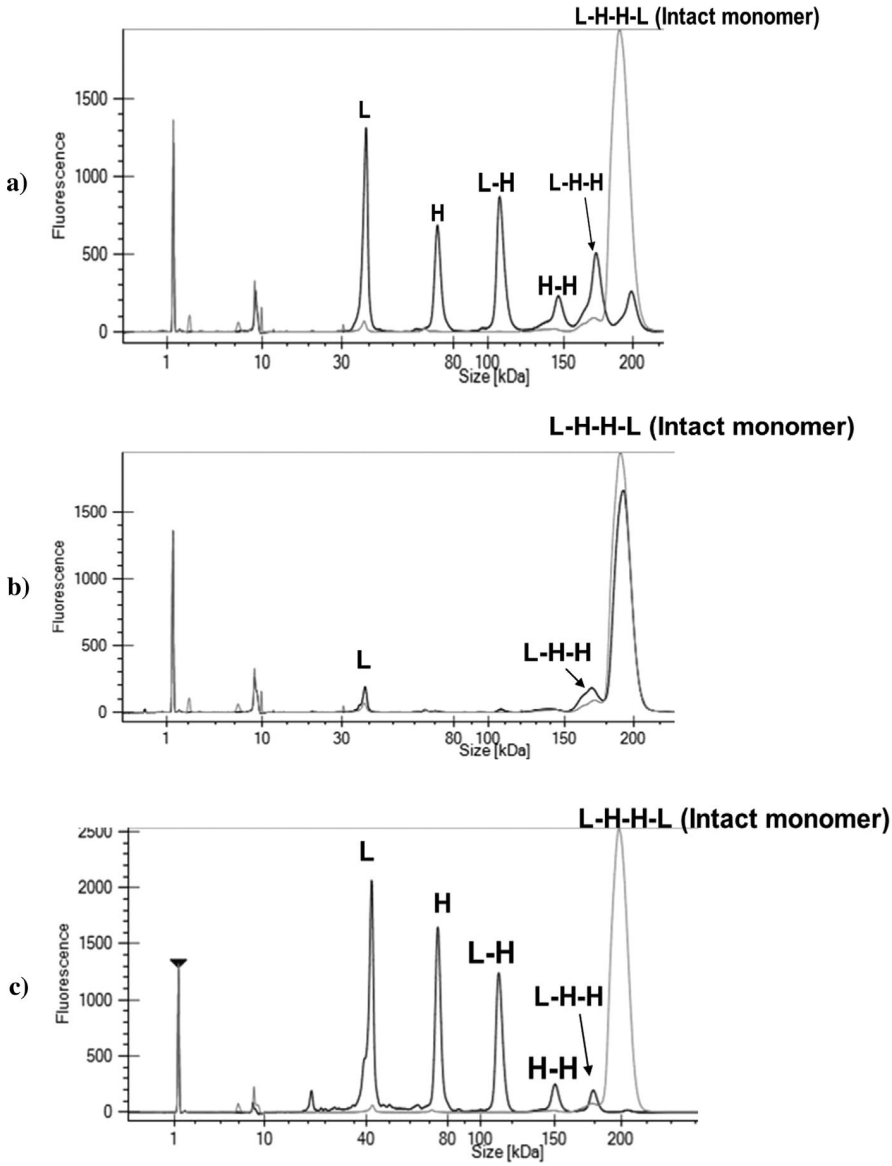

Figure 2. Non-reduced GX (NR-GX) electropherograms of purification Run 1 (Table I) process intermediates **(a)** capture product, **(b)** final polishing product, **(c)** harvested cell culture fluid (HCCF). Black trace: sample; gray trace: reference standard; L: light chain fragment; H: heavy chain fragment.

were available, flash-frozen (by immersion into a solution of ethanol containing dry ice) and stored at −80°C. When thawed, the aliquots were analyzed immediately in order to get a representative readout of the product quality at each step of the purification process. The approach of sample handling was applied to Purification Run 2 and all subsequent purification Runs.

As seen in Figure 3 and Table I, NR-GX analysis of the intermediates showed no reduced species (fragments) present across the purification process. In contrast to Purification Run 1 which started with 86.9% fragment in the HCCF and ended with 13.4% at the final polishing step, Purification Run 2 had fragment levels of 12.2% and 1.0% in the HCCF and final polishing step, respectively. Aggregate levels increased by 0.5% (from 2.3% pre-low pH treatment to 2.8% post-low pH treatment), which was lower than the 1% increase observed in Purification Run 1. Hence, it is likely that prevention of reduction in the HCCF also helped in minimizing aggregate formation during low pH treatment.

While it is possible to control for reduction through the combined approach in Table I, Purification Run 2 (chilled HCCF, air-containing headspace, immediate processing), its implementation imposes severe limitations on manufacturing flexibility. Chilling of the HCCF requires increases processing time. Head space aeration potentially becomes less effective as product surface area to volume ratios decrease with increasing processing scale. Larger product hold tanks may be required and lower capacity limits may have to be set due to the need for adequate headspace. While alternatives like

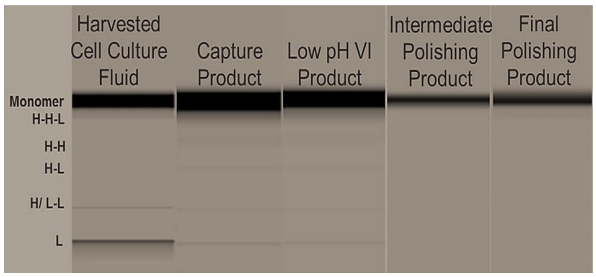

Figure 3. NR-GX images of purification process intermediates from purification Run 2 (Table I). HCCF subjected to chilling, headspace aeration, and immediate purification after harvest (L: light chain fragment; H: heavy chain fragment—disulfide link between fragments).

air sparging in the hold vessel may be a solution, these may not be available at every facility. Immediate purification of the HCCF becomes challenging when column size limitations in the manufacturing plant may require multiple cycles of the capture step to be performed.

Impact of Cystine Addition on Reduction Control

In order to allow for extended HCCF holds to assure manufacturing flexibility without impacting product quality, L-cystine spikes into the HCCF were evaluated. L-cystine was reported to be a potential competitive inhibitor of the reducing enzymes or to act as a surrogate substrate for the enzyme in place of the mAb product (Trexler-Schmidt et al., 2010).

As part of an initial assessment of cystine levels on reduction control, the level of L-cystine in the harvested HCCF was adjusted to 0, 0.4, and 0.8 mM L-cystine per gram of mAb (mM Cys/g mAb) through the addition of L-cystine into the HCCF immediately after harvest. To provide a worst case scenario for antibody reduction (low oxidative environment), the HCCF aliquots were sealed in flexboy storage bags without headspace and held for 2 weeks at 2–8°C before being analyzed. Figure 4 shows reduced species were not detected at the start of the hold

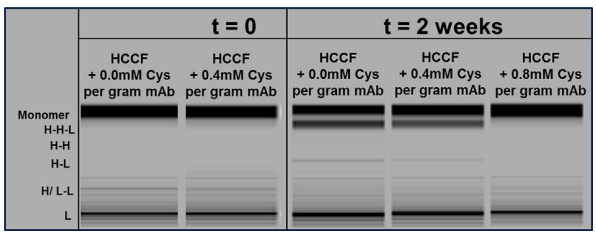

Figure 4. NR-GX images of harvested cell culture fluid (HCCF) at $t=0$ and 2 weeks, containing 0, 0.4, and 0.8 mM cystine per gram mAb. During 2 week hold, HCCF was held at 2–8°C in air-tight bags. (Note: a separate aliquot of the same HCCF at $t=0$ was spiked to 0.4 mM cystine per gram mAb and purified after a 4 day hold at 2–8°C in a vessel with headspace and is listed as purification Run 3 in Table I).

study, but by the end of the hold study, bold bands representing H-H-L fragments and faint bands of H-L fragments were identified in the samples containing 0 (no cystine) and 0.4 mM Cys/g mAb while the sample containing 0.8 mM Cys/g mAb still showed no signs of reduction. Decrease in band intensity with increasing cystine levels also demonstrates that 0.4 mM Cys/g mAb slowed down the rate of reduction during the 2 week hold, but 0.8 mM Cys/g mAb was required for complete prevention of reduction. Disulfide mapping mass spectrometry (MS) provided further evidence that supports the hypothesis of 0.4 mM Cys/g mAb being inadequate for complete reduction mitigation (Fig. 5). Higher levels of inter-chain free thiol were observed in the 0 and 0.4 mM Cys/g mAb samples by the end of the hold but not in the 0.8 mM Cys/g mAb sample nor in the control starting material (HCCF + 0 mM cys, $t=0$). The largest increase in free thiol content occurred at the hinge cysteines, followed by the inter-chain cysteines (LC Cys 216, HC Cys134). The intra-chain cysteines on the light chain of the mAb showed smaller increases in free thiol than the inter-chain cysteines. Little or no increase in free thiol was detected on the intra-chain cysteines of the mAb heavy chain.

To assess the impact of cystine addition on purification process performance, HCCF was spiked to 0.4 mM cystine/g mAb (same material as used in the cystine hold study above) and purified after a 4 day hold at 2–8°C in a vessel with headspace (Table I, Purification Run 3). At the start of purification, higher levels of fragment were observed in the HCCF (16.5% frag) as compared to Purification Run 2 (HCCF: 12.2% frag). After low pH treatment, a 2% increase in aggregate was observed. In comparing Purification Runs 1, 2, and 3 in Table I, a trend is observed wherein higher levels of fragment in the HCCF results in higher levels of aggregate after low pH treatment. Given that antibody disulfide bond reduction was observed for Runs 1 and 3 but not Run 2, it can be hypothesized that reduction in the HCCF is related to the higher levels of aggregate formation during low pH treatment.

The purification performance of Run 3 coupled with the observation of reduction for the same HCCF during the cystine hold study indicates that chilling, provision of headspace in the hold vessel and spiking of cys to 0.4 mM/g mAb was insufficient in the prevention of antibody reduction during the HCCF hold. To better understand the contributions of headspace provision to preventing antibody disulfide reduction, a 0.75 mL aliquot of the HCCF from Run 3 was stored at 2–8°C in a 1.5 mL Eppendorf tube for 2 weeks before being analyzed by NR-GX. Fragment bands were detected in the sample at the end of the 2 week hold but not at the beginning indicated that the provision of headspace was playing a less significant role to the prevention of antibody disulfide bond reduction as compared to spiking adequate levels of cystine. Although it was not evaluated in this study, it is possible that agitating the HCCF (for large volumes) through storage of HCCF in vessels with mixing impellers could make the provision of an oxidative environment a more effective strategy in preventing antibody disulfide bond reduction due to improved oxygen transfer into the HCCF as compared to the small scale systems where constant agitation is difficult to achieve.

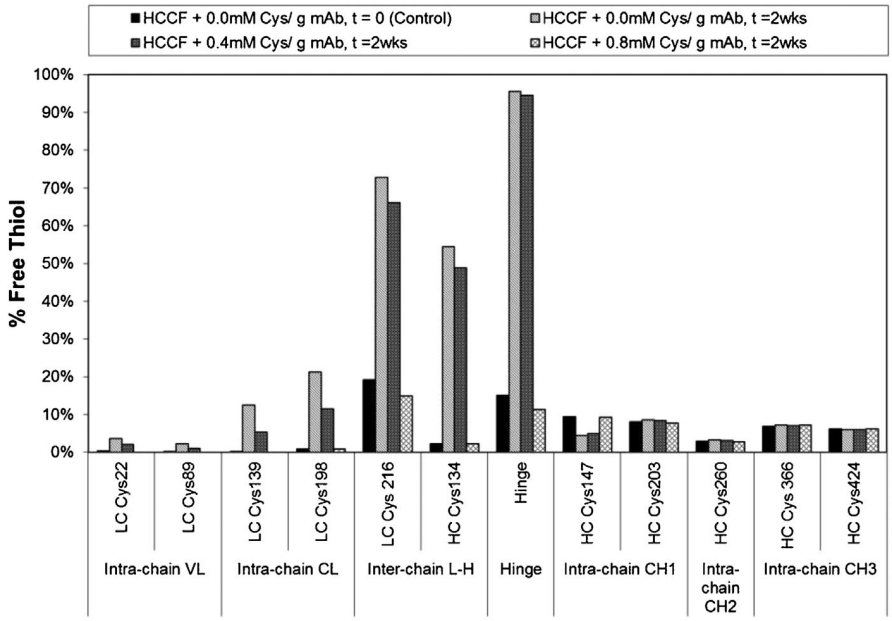

Figure 5. Mass spectrometry quantification of change in free thiol levels for HCCF held for 2 weeks in the presence of 0, 0.4, and 0.8 mM cystine per gram of mAb.

Impact of Reduction During HCCF Hold on Aggregation During Low pH Treatment

When the capture product from Purification Run 2 was held in low pH buffers ranging from pH 3.2 to 3.6, an increase in free thiol content was observed with decreasing pH (Fig. 6a). As reducing enzymes such as thioredoxin reductase, glutathione reductase, and protein disulfide isomerase generally exhibit decreased enzymatic activity with decreasing pH (Guzman, 1997; Ikebuchi et al., 1992; Xia et al., 2003), the increase in free thiol is unlikely to be due to enzymatic action but rather due to exposure of the antibody to low pH.

An increase in aggregate content was also seen with decreasing pH (Fig. 6b), indicating that there could be a correlation between free thiol content and aggregate content. Franey et al. (2010) have reported similar findings that an increase in free thiol level leads to a corresponding increase in aggregate formation for monoclonal antibodies, with the impact on IgG_2 molecules being more severe compared to other IgG formats due to higher number of inter-chain disulfide bonds. It has been established earlier that reduction during the HCCF hold leads to the generation of reduced species along with increased free thiol. The reduced species with heavy chain subunits (H-L, H-H, H-H-L) are likely recovered in the protein A product along with intact monomer and undergo low pH treatment. With a higher starting level of free thiol in the protein A product, coupled with further free thiol formation during low pH treatment, increased aggregate formation occurs. Although the exact mechanism was not investigated in this study, Buchanan and co-workers have demonstrated through site-directed mutagenesis that having unpaired cysteines on the surface of a mAb can lead to significantly increased rates of aggregation (Buchanan et al., 2013). Hence, a larger increase in aggregate content was observed during low pH treatment for Purification Runs 1 and 3 (which showed reduction in the HCCF), but not Purification Run 2 (no reduction in HCCF due to immediate purification after harvest).

Effect of Increased Cystine Levels and HCCF Hold Conditions on Product Quality

As shown above, spiking of HCCF to 0.4 mM cys/g mAb in combination with low temperature hold and provision of headspace was only able to slow down the rate of reduction and not prevent reduction completely. This resulted in high free thiol levels (as determined by disulfide mapping MS) which caused increased aggregation during low pH treatment. In contrast, 0.8 mM cys/g mAb seemed to provide better reduction mitigation and minimize aggregate formation. This hypothesis was evaluated through the study outlined in Figure 7. To one sample, no L-cystine (0 mM cystine) was spiked into the mixture; to the other sample, the mixture was spiked to 0.8 mM cystine/g mAb. Half of each sample mixture was purified and formulated to 50 mg/mL immediately, while the other half was held in vessels with headspace for 2 weeks at 2–8°C before purification and formulation. After formulation, all four lots were monitored for aggregation stability for up to 17 months at 2–8°C and 1 month at 25 and 40°C.

Table I, Purification Run 4 shows the purification performance for the HCCF lot that was spiked to 0.8 mM cystine/g mAb and stored chilled in a vessel with headspace for 2 weeks before being purified. Fragment levels in the HCCF (13.4% frag) were lower as compared to Run 3 (16.5% frag) even though the material was held for a longer duration under similar conditions. After low pH treatment, aggregate levels only increased by 0.4% (comparable to Run 2), further supporting the theory that prevention of reduction

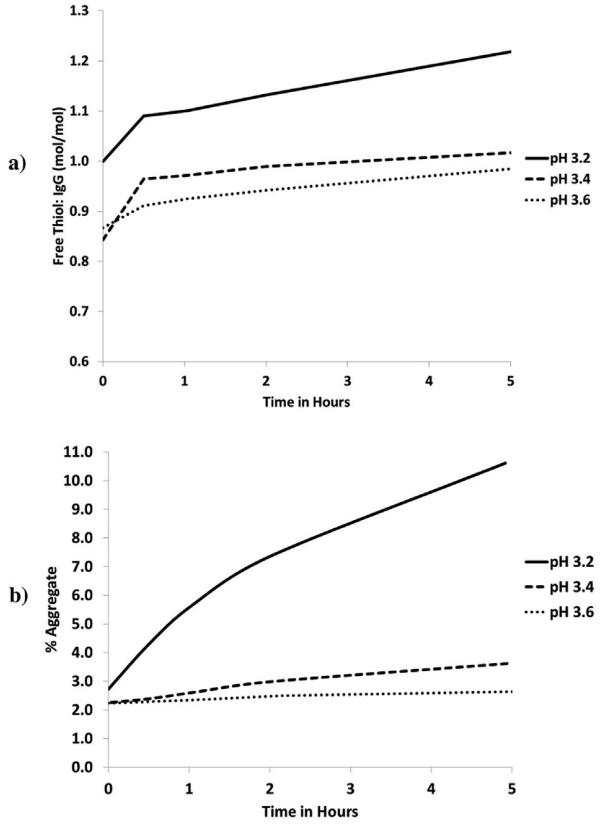

Figure 6. Change in **(a)** free content and **(b)** aggregate content with time when protein a product from purification Run 2 is incubated at pH 3.2, 3.4, and 3.6.

in the HCCF is important in minimizing aggregate formation during low pH treatment.

The free thiol content of the process intermediates generated from the conditions outlined in Figure 7 were measured by mass spectrometry (for HCCF) and a colorimetric assay (for subsequent process intermediates). As seen in Figure 8a, an increase in inter-chain free thiol content was observed for the HCCF held for 2 weeks

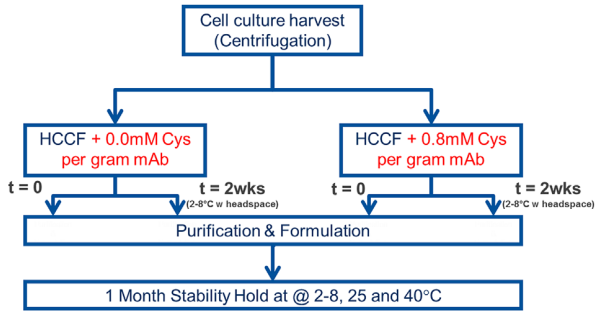

Figure 7. Evaluation schematic to assess the impact of reduction mitigation on purification process performance and formulated product stability.

at 2–8°C in the absence of cystine. In contrast, HCCF that was spiked to 0.8 mM cys/g mAb and held under similar conditions showed no change in free thiol levels. Similar trends in free thiol levels were also observed in the subsequent process intermediates (Fig. 8b). Capture product generated from HCCF held for 2 weeks in the absence of cystine showed the highest free thiol level. Free thiol content increased for all aliquots after low pH treatment but the capture product with the highest starting free thiol level also showed the largest increase during low pH treatment. Though it was not investigated in this study, existing levels of free thiol in a sample can potentially determine the rate of increase during low pH treatment.

Free thiol levels decreased for all aliquots between the low pH treatment and final polishing chromatography process steps. There are two possible explanations for this observed decrease: (i) fragments and aggregates containing free thiols were removed by the polishing chromatography steps; and (ii) exposure to the atmosphere during purification allowed for disulfide bond formation (i.e., re-oxidation) between free thiols leading to the reformation of larger fragments, monomer, and aggregates. The intermediate polishing unit operation consisted of an anion exchange (AEX) chromatography step that is operated in flow-through mode which does not retain any fragment, monomer, or aggregate. The final polishing unit operation consists of a cation exchange (CEX) chromatography step (operated in bind and elute mode) that was designed for aggregate removal. Shown in Supplementary Figure S1a and b are CEX chromatograms from Purification Run 1 (showed the highest degree of antibody disulfide bond reduction and high fragment levels) and Run 2 (showed no signs of antibody disulfide bond reduction and low fragment levels), respectively. In Purification Run 1, a step yield 48% was obtained when the column was loaded to a capacity of 21g/L. In contrast, purification Run 2 gave a step yield of 63% for the same column loaded to a capacity of 16g/L. Protein breakthrough did not occur in the loading and re-equilibration steps, indicating that all loaded protein was collected back in the elution and strip pool. Compared to Run 2 which showed the typical CEX chromatographic profile for the process, the elution peak (usually composed of monomer) in Run 1 had a lower peak height with significant tailing, while the strip peak (usually composed of monomer and aggregate) was larger than expected. Despite the lack of peaks in the load and re-equilibration steps, fragment levels by NR-GX fell from 36.5% at the end of low pH inactivation to 13.4% at the end of CEX. These observations supports the hypothesis that re-oxidation of the free thiol leading to the formation of monomer or aggregate is likely playing a larger role in causing the observed decrease in free thiol and fragment level for this mAb molecule. During the reoxidation of fragment, different monomer, and aggregate isoforms can arise depending on the site of re-oxidation on the antibody fragment. These structural differences could result in different retention behavior on the CEX column and potentially explain for the tailing effect observed during the elution step of Run 1.

With higher free thiol levels, there is the increased likelihood that all re-oxidized species can still possess free thiol residues. The purification process may be optimized for the removal of fragment

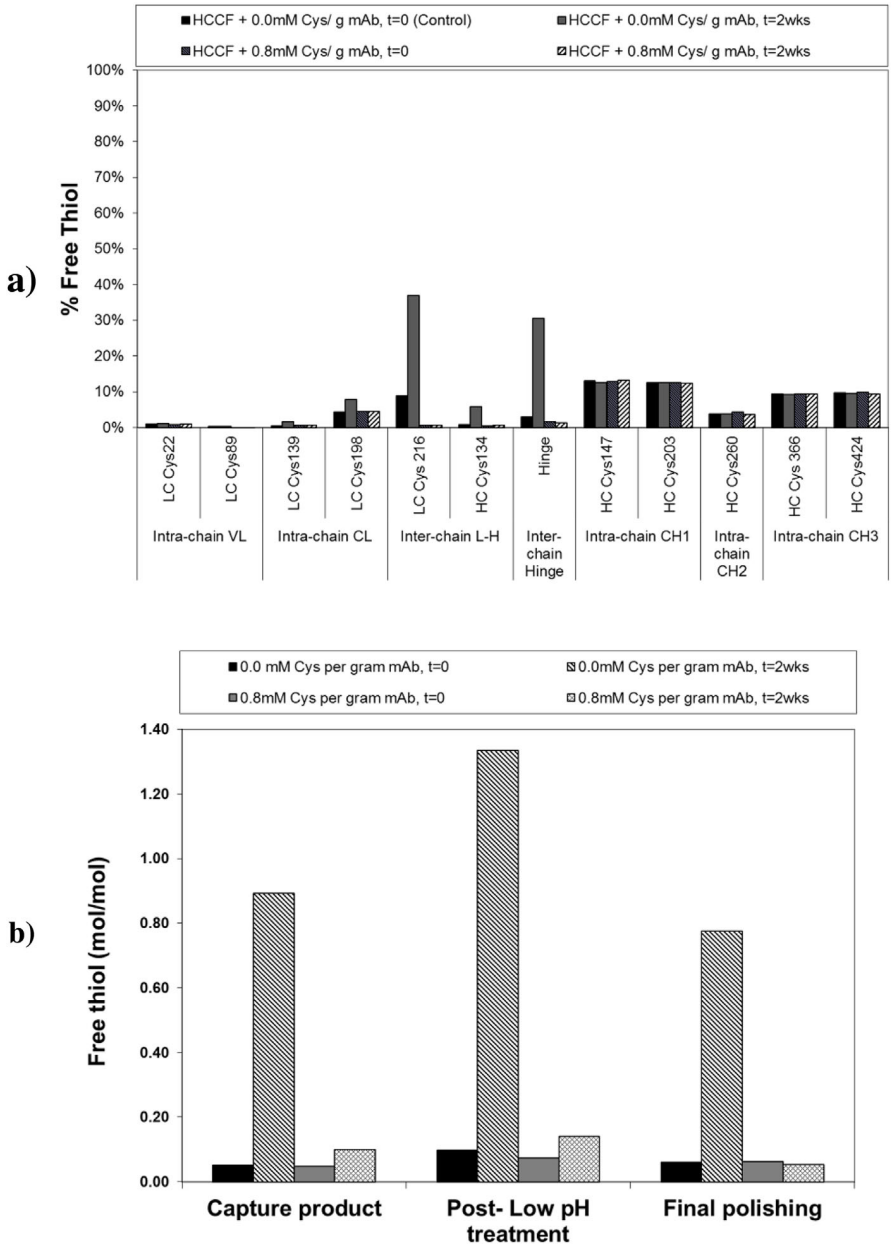

Figure 8. **(a)** Mass spectrometry quantification of change in free thiol levels for HCCF at $t = 0$ and after a 2 week hold at 2–8°C in the presence of 0 and 0.8 mM cystine per gram mAb. **(b)** Free thiol levels determined by colorimetric assay for purification process intermediates generated from HCCF purified immediately and held for 2 weeks in the presence of no cystine (0 mM) and 0.8 mM cystine per gram mAb.

and aggregate, but may not be able to resolve monomers with different free thiol levels that may subsequently form more aggregate. The formation of the impurities during processing also increases the impurity burden (both fragment and aggregate) on the polishing steps, making it more challenging to achieve robust process control. Further, antibody disulfide bond reduction lead to significantly fragment formation and lower overall process yield as observed in Purification Run 1. Hence, it is important to implement adequate reduction mitigation early on in the process.

The formulated drug substance from each of the four runs purified as described above was held at 25 and 40°C for 1 month and 5°C for 17 months. Aggregate levels were measured using HP-SEC. As seen in Figure 9a–c, at all temperatures, the material generated from the HCCF that was held for 2 weeks in the absence of cystine had higher starting and final aggregate levels despite going through the same purification and formulation process. In contrast, the material generated from the HCCF that was spiked to 0.8 mM cys/g mAb and held for 2 weeks showed similar starting and final

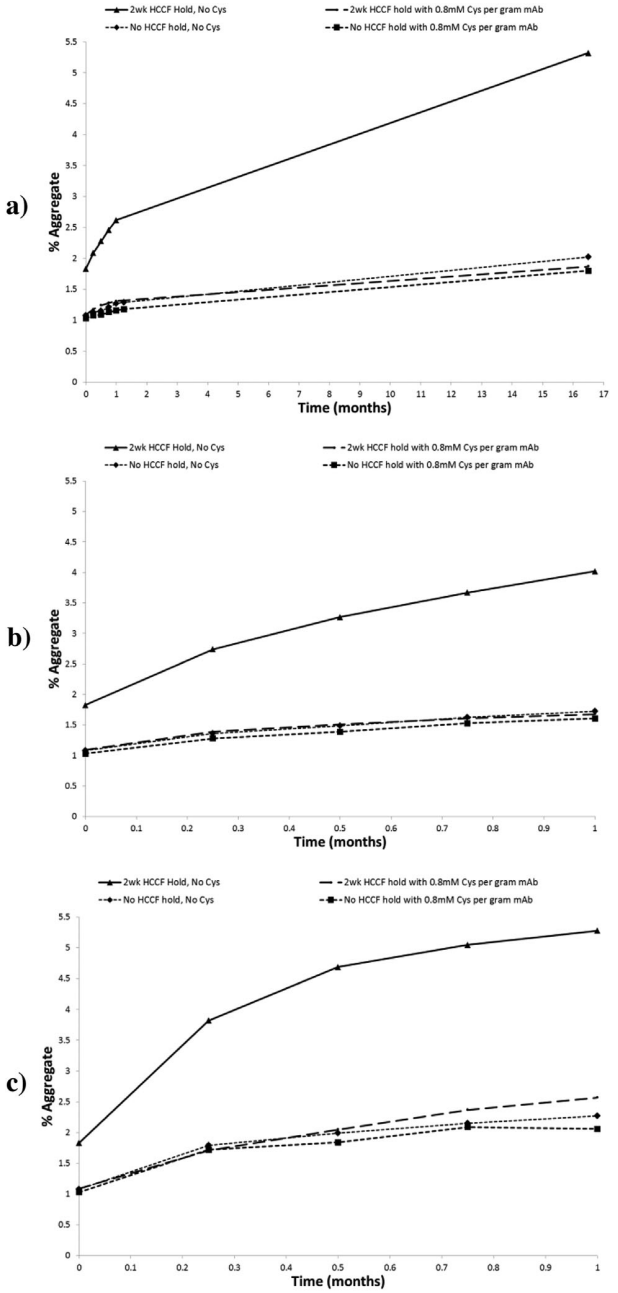

Figure 9. Change in percent aggregate levels over time for drug substance generated from HCCF held in the absence of cystine (0 mM) or in the presence of 0.8 mM cystine per gram mAb at **(a)** 2–8°C for 17 months **(b)** 25°C for 1 month **(c)** 40°C for 1 month.

purity levels as the drug substance generated from the HCCF that had been purified immediately. In addition, the rate of aggregate formation was also significantly higher for the sample without cystine and with 2 weeks hold when compared to other samples. Remarkably, the observed trend in the aggregation rates of the drug substances is consistent with the free thiol levels of their corresponding final polishing intermediates. Hence, antibody reduction during the hold of HCCF not only has consequences on the performance of the purification process, it also has a carry-over effect on the stability of the final drug substance, potentially leading to shorter product shelf life.

Conclusions

During purification of an IgG2 antibody, high level of fragment in the process intermediates was detected by HP-SEC. Further investigations subsequently traced the cause to be antibody reduction in the HCCF. In addition, when the HCCF was reduced, larger increase in aggregate content was observed during low pH treatment. Four different strategies were employed to mitigate antibody reduction by minimizing reducing enzyme activity: (i) shorter hold duration (immediate purification after harvest); (ii) oxidative environment (exposure to air); (iii) low temperature; and (iv) inhibitor addition (cystine addition).

When purification was initiated immediately after harvest, antibody reduction was not observed in the HCCF and aggregate formation during low pH treatment was minimized. This can be attributed to the shorter exposure time of the antibody to reducing enzymes in the HCCF. However, immediate purification may not always be a feasible option, particularly as process scale increases.

Increasing cystine concentrations resulted in lower free thiol levels when the HCCF was held. When HCCF is spiked to 0.8 mM cystine/g mAb, free thiol levels remained constant across a 2 week hold. When a combination of low temperature, exposure to air and 0.4 mM cystine/g mAb spike was applied to a HCCF before purification, antibody reduction progressed at a slower rate, which still resulted in increased aggregate formation during low pH treatment. However, when the cystine concentration was increased to 0.8 mM cystine/g mAb, reduction was not observed. Less aggregate was formed during the low pH step, suggesting a potential link between antibody reduction in the HCCF and aggregate formation during low pH treatment. A pH hold study subsequently demonstrated increasing free thiol levels and aggregate with decreasing pH. This indicates that some reduced species with free thiol which get co-purified with the intact monomer during protein A chromatography can exacerbate aggregate formation during low pH treatment.

Subsequent decrease in free thiol levels from the low pH treatment step to final polishing chromatography can be attributed to clearance of fragments and aggregates with high free thiol content, or the formation of disulfide bonds between free thiols due to aeration due to exposure to the atmosphere, or potentially both mechanisms in combination. Nonetheless, high free thiol levels increases the likelihood of forming subclasses of monomer that still possess free thiol moieties which may be difficult to remove during polishing, and also adversely affect drug substance stability and lot-to-lot consistency.

The carry-over of product species with high free thiol content from the final polishing product into the drug substance had a subsequent effect on the final product stability with faster rates of aggregation occurring at all temperatures. However, when free thiol levels in the purified material was kept low, similar rates of aggregation were observed regardless of whether the original HCCF was purified immediately or held for an extended period before purification.

Therefore, higher free thiol content after harvest not only increases the burden on the purification process due to higher levels of aggregate, but also limits the stability and shelf-life of the molecule. As the cause of free thiol increase was ultimately identified to be a result of mAb reduction, proper mitigations should always be considered in the process to prevent antibody reduction during development and manufacturing. By making the product quality less dependent on lot-to-lot differences in HCCF hold conditions, greater control of product consistency and quality can be achieved.

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Industrial Purification of Pharmaceutical Antibodies: Development, Operation, and Validation of Chromatography Processes

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Industrial Purification of Pharmaceutical Antibodies: Development, Operation, and Validation of Chromatography Processes

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Introduction

Recombinant monoclonal antibodies are becoming a great success for the biotechnology industry. They are currently being studied in many clinical trials for treating a variety of diseases, and recently several have been approved for treating cancer (Carter *et al.*, 1992; Anderson *et al.*, 1996; Baselga *et al.*, 1996; Bodey *et al.*, 1996; Longo, 1996). Although there are several types of antibodies produced in different types of cell lines, the most clinically significant antibodies are full-length humanized IgG_1 produced in CHO cells. This review describes the methods used to purify these antibodies at industrial scale, focusing on chromatography processes, and with particular reference to recent work at Genentech.

Routine laboratory purification of antibodies has been well described (for example see Scott *et al.*, 1987), but the considerations for large-scale production of pharmaceutical-grade antibodies are much different than those for laboratory scale. There are extreme purity requirements for pharmaceutical antibodies, and routine large-scale production requires high yield and process reliability. To gain regulatory approval, the process must be completely validated to run consistently within specified limits, so the process should be designed to facilitate validation.

Large-scale production of antibodies as pharmaceutical products is a complex

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Abbreviations: CV, column volume; HCCF, harvested cell culture fluid; CHOP, Chinese hamster ovary proteins; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; BSA, bovine serum albumin; CE, capillary electrophoresis; HPLC, high-performance liquid chromatography; ppm, parts per million (ng/mg); LOQ, limit of quantitation; SEC, size exclusion chromatography; pI, isoelectric point; GMP, good manufacturing practice; g/l, when describing column loads this is grams of antibody per litre of column volume.

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endeavour, including a manufacturing process with multiple steps and significant analytical support. Antibody manufacturing includes cell banking and cell culture, recovery, filling (possibly including lyophilization), finishing, and packaging. Product recovery includes harvest, which is removal of cells and cell debris by tangential flow filtration or centrifugation (van Reis *et al.*, 1991), chromatography for antibody purification, and formulation by tangential flow filtration. Here we focus on process chromatography, which must reliably produce highly purified antibody.

To satisfy the stringent purity requirements for pharmaceutical antibodies, an extensive analytical control system is integrated with the manufacturing process at all steps, particularly on release of the final product. The analytical control system includes assays for product-related variants (including charge and glycosylation variants), often using ion exchange HPLC or CE (Hunt *et al.*, 1996; Hunt and Nashabeh, 1999), but these variants are typically controlled during cell culture and are not removed during chromatography. To ensure that no variants are formed during purification, antibody stability is controlled during chromatography by limiting extremes of pH, temperature, and other process variables to reduce the amount of oxidation, deamidation, aggregation, and other variant-formation routes.

Many pharmaceutical proteins require a significant clearance of product-related variants. An example of this is insulin-like growth factor, where several product-related variants (such as a single amino acid oxidation and clipped forms) are removed to <1% during purification (Fahrner *et al.*, 1998, 1999b). The acceptable level of product-related variants is an issue which dates to the first proteins produced by recombinant DNA technology. The resolution and sensitivity of current analytical technology permits the definition of very minor differences among the product protein population. The fact that variants can be discovered does not automatically indicate that they need to be removed or even controlled. For example, the DNA sequence for IgG_1 antibodies codes for a lysine at the C-terminus of each heavy chain. During cell culture, one or both of these lysines are usually removed, leading to three charged populations (zero, one, or two lysines). This variability has no impact on the ability of the antibody to bind its target antigen or effect any biological activity. Therefore, the product definition would allow for all three species. The same approach can be extended to other product variants. It is necessary to characterize the molecular source of the variation and demonstrate that the variation has no effect on potency or safety.

From a recovery standpoint, one of the most significant advantages to using antibodies produced in CHO cells is that the level of product-related variants can be effectively controlled during cell culture so that little or no variants must be removed during recovery. This level of control during cell culture allows the use of a streamlined, three-step recovery process. Instead of focusing on the removal of product-related variants, the process is concerned with the clearance of pharmaceutical impurities such as virus, DNA, host cell proteins, endotoxin, and small molecules. This recovery process consists of protein A affinity chromatography, cation exchange chromatography, and anion exchange chromatography.

Antibody recovery

Since no single chromatography step can achieve the necessary antibody purity, the

three process steps must be integrated to meet the requirements of purity, yield, and throughput. In addition, the process must be robust, reliable, and amenable to validation.

The primary consideration is purity. While yield and throughput may be necessary for an economically viable product, without meeting the purity requirements for biological pharmaceuticals there will be no product at all. Throughput and yield are becoming more important as many clinical indications for antibodies require very high doses. At our manufacturing plant, we typically use processes that purify a 5–10 kg antibody batch in less than three days with greater than 65% overall process yield.

PURITY CONSIDERATIONS

Although pharmaceutical antibodies do not require the removal of product-related variants that complicate the purification of some proteins, other purity requirements are extreme. There are six main purity considerations for the recovery of pharmaceutical antibodies.

1. Host cell proteins

Host cell proteins are present in high amounts (sometimes >1,000,000 ng/mg) in the harvested cell culture fluid. They are typically removed during purification <5 ppm, a total reduction of at least 10^5. In our studies, the level of host cell proteins was measured quantitatively by ELISA (Chen, 1996) and qualitatively by SDS-PAGE.

For the ELISA, affinity purified goat anti-CHOP antibodies were immobilized on microtitre plate wells. Dilutions of the pool samples were incubated in the wells, followed by an incubation with peroxidase-conjugated goat anti-CHOP. The horseradish peroxidase enzymatic activity was quantified with *o*-phenylenediamine. Samples were serially diluted 2-fold in assay diluent so that the absorbance reading fell within the range of the standard curve (1.5 ng/ml to 400 ng/ml).

To analyse the antibody by SDS-PAGE, the pool samples were run under reducing and non-reducing conditions on one-dimensional Novex 8–16% Tris–glycine gels. Samples were loaded at 2.5 µg/lane for non-reducing conditions and 5.0 µg/lane for reducing conditions. The gels were silver-stained using the Novex silver express kit. The samples were compared to a reference standard for identification of product related bands.

2. DNA

The World Health Organization set a requirement for DNA in biopharmaceutical formulations of <10 ng/dose. DNA is present at high levels in the harvested cell culture fluid (>1,000,000 pg/mg) and must be removed to <10 ng/dose levels. During validation studies DNA may be spiked into the load to demonstrate clearance.

In our studies, the level of DNA was measured using the Molecular Devices Threshold DNA assay kit. The typical range of detection of the Threshold Total DNA assay was between 6.3 and 400 pg/ml. Samples were assayed at a minimum of 3 dilutions with and without a 100 pg spike of DNA. This procedure was used to evaluate DNA recovery because some buffers, impurities and proteins are known to

inhibit the detection of DNA and inhibit spike recovery. The mean value for all sample dilutions falling within the range of the standard curve and meeting spike recovery acceptance criteria was used.

3. *Aggregate*

The main product-related variant that must be reduced is aggregated forms of the antibody (mostly dimer) because of the possible immunogenicity of the aggregate. The aggregate content in the HCCF is about 5–15% for many antibodies, and it is typically reduced to below 0.5% in the final bulk. The primary step used to remove aggregate is cation exchange chromatography.

In our studies, aggregate was measured by size-exclusion chromatography. A Bio Sil SEC-250 7.5 × 300 mm column from BioRad was run at 1 ml/min using a mobile phase containing 50 mM NaH_2PO_4/50 mM Na_2HPO_4/0.15 M NaCl, pH 6.8. The column was equilibrated with the mobile phase buffer and 20 µl volumes of blank, standard, control and study samples were sequentially injected and run on the SEC for analysis.

4. *Small molecules*

The harvested cell culture fluid contains many small molecules, originating from the media components and created during cell culture by the CHO cells. Rather than determining the level of all small molecules, a few representative marker molecules are measured. Here we present the results from measurements of insulin and Pluronic F-68.

The level of Pluronic F-68 was measured using a 500 MHz NMR. NMR detects hydrogen-containing molecules based on magnetic moments. Pluronic has a characteristic peak in the spectrum with a chemical shift of 1.1 ppm, which was used for quantification. Peak areas in samples were compared with the standards. The Pluronic F-68 standard curve was run in process buffers, and covered the range of 25 µg/ml to 1024 µg/ml. As controls, the conditioned protein A pool was analysed unspiked and spiked with 25 µg/ml Pluronic F-68.

The level of insulin in the pool samples was determined by a competition ELISA. The monoclonal antibody to insulin was immobilized on microtitre plate wells. Diluted samples and biotinylated insulin were placed in the antibody immobilized wells. The insulin and biotinylated-insulin compete for binding to the antibody. The amount of bound biotinylated-insulin was detected with alkaline phosphatase–streptavidin and *p*-nitrophenyl phosphate substrate. All samples were assayed in wells coated with non-immune mouse antibody in place of the specific monoclonal antibody. This control showed that binding to the plate is mediated by the specific monoclonal antibody and not by a non-specific interaction.

5. *Leached protein A*

During protein A affinity chromatography, some protein A leaches from the column and ends up in the antibody pool. Because protein A can be immunogenic and cause other physiological reactions (Gagnon, 1996), leached protein A must be cleared during downstream chromatography.

The level of protein A in our samples was determined by a sandwich ELISA (Lucas *et al.*, 1988). Chicken anti-protein A antibody was immobilized on microtitre wells; Protein A binds to the coat antibody. The amount of bound protein A was detected with chicken anti-protein A labelled with biotin, followed by streptavidin–HRP and then the substrate *o*-phenylenediamine dihydrochloride and hydrogen peroxide. The reaction was stopped by adding sulphuric acid. The product was quantified by reading an absorbance at 490 nm. All samples were initially diluted to 0.2 mg/ml antibody in assay diluent. Samples were then serially diluted 2-fold with sample/standard diluent which contained 0.2 µg/ml antibody. Samples were assayed as a dilution series to ensure that antibody excess was reached. Values were calculated as the average of all results within the reporting range (0.78–25 ng/ml).

6. Virus

Harvested cell culture fluid may have 10^4 or more retrovirus-like particles per ml, and biological pharmaceuticals are allowed to have 1 theoretical virus particle per 10^6 doses, so the recovery process must provide significant virus clearance. The validation and test procedures for viral clearance are complicated and are beyond the scope of this chapter. However, the process is capable of clearing virus to acceptable levels. In general, the protein A affinity chromatography step provides 10^7 ('7 logs') of virus clearance (10^4 by removal and 10^3 by low-pH inactivation in the elution pool), and the anion exchange chromatography step provides 10^4 (4 logs) of viral clearance by removal. If this level of viral clearance is not sufficient, additional process steps such as viral filtration may be required.

Purity calculations

For all quantitative assays, the level of impurity in the sample is calculated by multiplying the measured value by the sample dilution. Since samples may be diluted to differing extents to avoid matrix interference, the absolute sensitivity (LOQ) of the assay will be influenced by the required sample dilution. Because values are often reported in ppm or ng of impurity per mg of product (not ng/ml), the reported sensitivity will also depend on the product concentration in the sample.

THREE-STEP RECOVERY PROCESS

The purity, yield, and throughput requirements can be achieved using three chromatography steps: protein A affinity chromatography, followed by cation exchange chromatography, followed by anion exchange chromatography. Protein A and cation exchange chromatography are run in bind-and-elute modes, while the anion exchange chromatography is run in flow-through mode (for antibodies with pI greater than about 8). Running in these modes in this order produces a high-yield process capable of meeting the purity requirements (*Table 12.1*).

We present methods that may be applied to many antibodies, but it is important to note that some antibodies may have specific considerations, such as susceptibility to aggregation, oxidation, deamidation, or other stability problems. In these cases, adjustments to the process may have to be made. For example, an antibody that is

Table 12.1. Typical yield and purity values for the three-step antibody recovery process

	Yield (%)	Host cell proteins (ng/mg)	DNA (pg/mg)	Endotoxin (EU/mg)	Protein A (ng/mg)	Aggregate (%)
HCCF	–	250,000–1,000,000	100,000–1,500,000	5–100	–	5–15
Protein A	>95	200–3000	100–1000	<0.005	3–35	5–15
Cation	75–90	25–150	<10	<0.005	<2	<0.5
Anion	>95	<5	<10	<0.005	<2	<0.5

highly prone to deamidation may require a limit on its exposure to high pH (>8) during recovery. An important part of process development is determining the stability of the antibody, since product stability will strongly influence the specific parameters used during recovery.

The first step in the process is protein A affinity chromatography (*Figure 12.1*). The majority of the purification occurs during protein A affinity chromatography (*Table 12.1*), which clears host cell proteins, DNA, and endotoxin. In addition, it removes insulin and Pluronic F-68 to less than detectable levels. However, it does not clear aggregate, and it adds protein A into the pool.

Protein A is a bacterial cell wall protein that binds specifically to antibodies, and it binds particularly well to human IgG_1. When immobilized onto chromatography

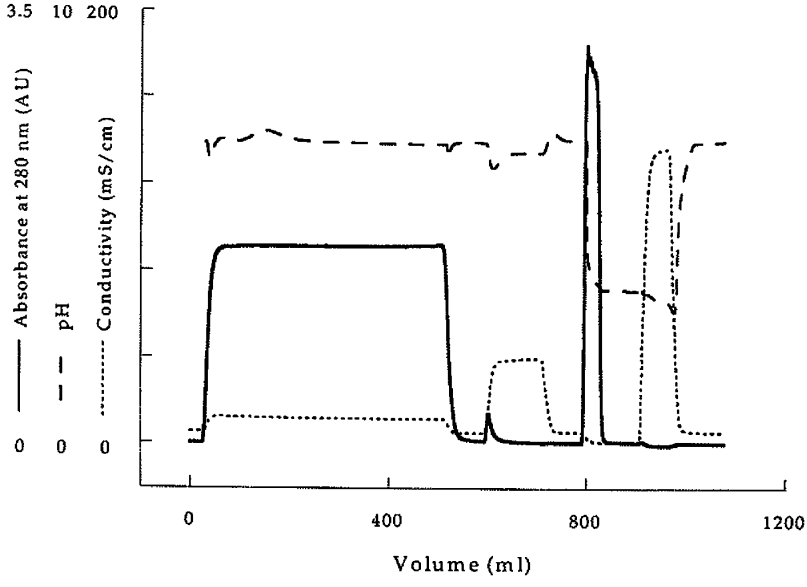

Figure 12.1. Chromatogram from a typical protein A affinity chromatography run. A 1.0 cm inner diameter × 20 cm length column was packed with Prosep A chromatography media. Four buffers were used. Buffer A was 25 mM Tris, 25 mM NaCl, 5 mM EDTA, pH 7.1; buffer B was 25 mM Tris, 25 mM NaCl, 5 mM EDTA, 0.5 M tetramethylammonium chloride pH 7.0; buffer C was 0.1 M acetic acid, pH 3.5; and buffer D was 2 M guanidine HCl, 10 mM Tris, pH 7.5. The column was equilibrated with 5 column volumes of buffer A, loaded to 20 g/l, washed with 3 column volumes of buffer A, washed with 3 column volumes buffer B, washed with 3 column volumes of buffer A, eluted with 5 column volumes of buffer C, and regenerated with 3 column volumes of buffer D. The column was run at 550 cm/h.

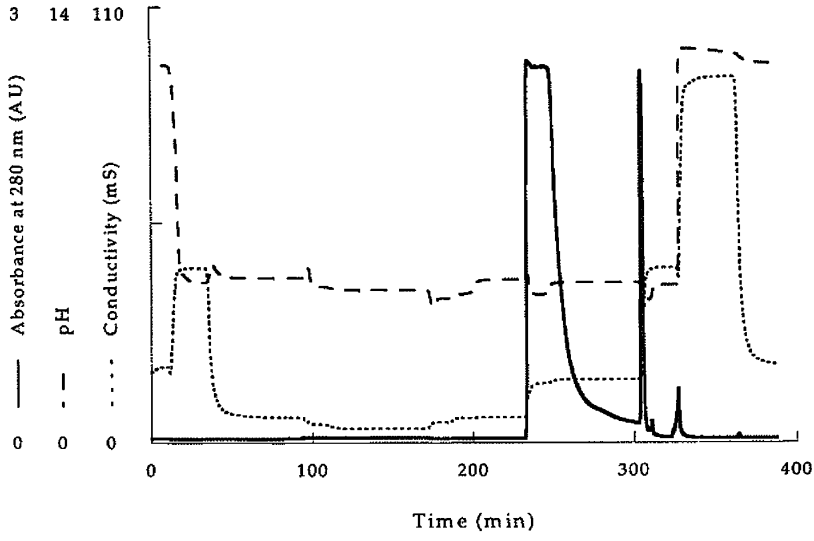

Figure 12.2. Chromatogram from a typical cation exchange chromatography run. The column was 0.66 cm inner diameter × 20 cm length, packed with Poros 50 HS. The column was washed with 2 CV of 0.016 M MES/0.004 M NaMES/0.500 M NaCl, pH 5.5, then equilibrated with 5 CV of 0.016 M MES/0.004 M NaMES/0.060 M NaCl, pH 5.5, loaded to 40 g/l, washed with 5 CV of 0.016 M MES/0.004 M NaMES/0.060 M NaCl, pH 5.5, eluted with 5 CV of 0.016 M MES/0.004 M NaMES/0.160 M NaCl, pH 5.5, regenerated with 2 CV of 0.016 M MES/0.004 M NaMES/0.500 M NaCl, pH 5.5, sanitized with 2 CV of 0.5 N NaOH, and stored in 3 CV of 0.1 N NaOH.

media, protein A provides a technique for purifying recombinant antibodies because it can selectively bind antibodies in complex solutions, allowing impurities to flow through (Ey *et al.*, 1978; Surolia *et al.*, 1982; Lindmark *et al.*, 1983; Reis *et al.*, 1984). Protein A affinity chromatography is by far the most effective type of chromatography for removal of host cell proteins and small molecules, and this is the main reason that it is used for antibody purification.

In the past, the harvested cell culture fluid was often concentrated before the first chromatography step to decrease the loading time. With the development of high-titre cell culture (typically >0.5 g/l) and protein A affinity chromatography media capable of high capacity at high flow rate (typically 20 g/l at 40 CV/h), the need to concentrate the harvested cell culture fluid has been eliminated. In our three-step process, the harvested cell culture fluid is loaded directly onto the protein A column. Because protein A affinity chromatography media is expensive, a smaller column is cycled several times to purify a single batch. This is possible because of the high flow rates that can be achieved for protein A columns.

Cation exchange chromatography (*Figure 12.2*) is the second step. It uses a negatively charged group (typically sulphopropyl) immobilized to the chromatography media. Cation exchange chromatography clears host cell proteins, aggregate, and leached protein A (*Table 12.1*). The antibody binds to the negatively charged sites on the column, and it is eluted with a step gradient to high salt. Host cell proteins, aggregate, and leached protein A elute in the regeneration phase, after the antibody

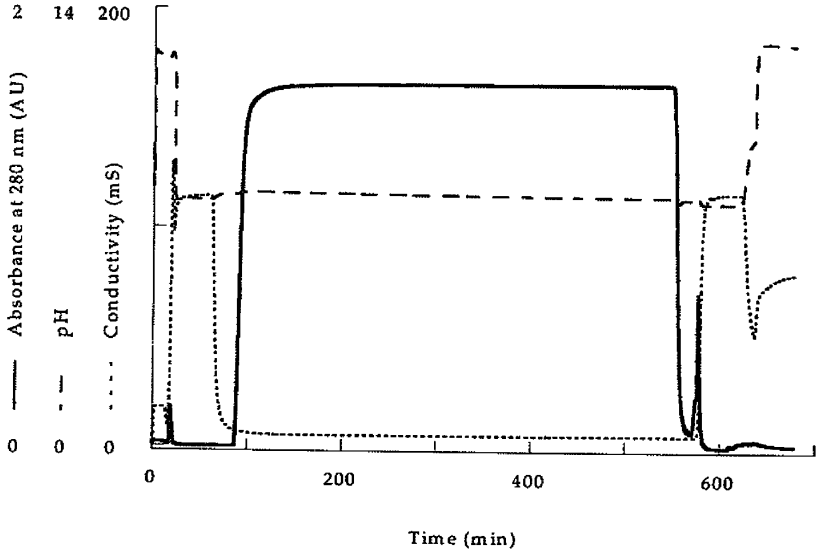

Figure 12.3. Chromatogram from a typical anion exchange chromatography run. The column was 0.66 cm inner diameter × 20 cm length, packed with Q Sepharose Fast Flow. The column was washed with 3 CV of 0.180 M Tris HCl/0.07 M Tris Base/2.0 M NaCl, pH 8.0, equilibrated with 4 CV of 0.018 M Tris HCl/0.007 M Tris Base/0.05 M NaCl, pH 8.0, loaded to 100 g/l, washed with 7 CV of 0.180 M Tris HCl/0.07 M Tris Base/2.0 M NaCl, pH 8.0, regenerated with 3 CV of 0.25 M Tris/2.0 M NaCl, pH 8.0, sanitized with 2 CV of 0.5 NaOH, and stored in 3 CV of 0.1 NaOH, 3 CV.

has eluted. Cation exchange columns can be loaded to >40 g/l, which allows the batch of antibody to be purified in a single cycle on a reasonably sized column.

Anion exchange chromatography (*Figure 12.3*) is the last chromatography step. It uses a positively charged group (typically quaternary amine) immobilized on the chromatography media. Anion exchange chromatography can be run in flow-through mode, which means that the antibody product flows through the column while the impurities bind. It removes DNA and residual host cell proteins. These impurities are removed from the column with a regeneration step, typically 0.5–1 M NaOH.

These three steps together comprise a process that, while meeting stringent purity and throughput restrictions, still produces a high yield of antibody (*Table 12.1*). The protein A affinity step has >95% yield, the cation exchange step has >75% yield, and the anion step has >95% yield, for an overall >65% process yield, which is exceptional for an industrial process with these extreme purity requirements. By choosing and sizing columns correctly and running them under conditions for high capacity, the throughput requirements can be met.

PROCESS VALIDATION

Validation is a regulatory requirement to demonstrate that a process, when operated within set parameters, can consistently produce a specified product. The complete validation plan is extensive and includes validation of process equipment, software, utilities, equipment cleaning, and analytical methods.

According to the U.S. FDA, 'Process validation is establishing documented evidence which provides a high degree of assurance that a specific process will consistently produce a product meeting its pre-determined specifications and quality attributes' (FDA's Guidelines on General Principles of Process Validation, May 1, 1987). For chromatography processes, this means in part that processes must be validated at extremes of operating parameters such as load, conductivity, pH, and column lifetime.

An important part of the validation effort is developing and writing validation protocols. A validation protocol is 'a written plan stating how validation will be conducted, including test parameters, product characteristics, production equipment, and decision points on what constitutes acceptable test results' (ibid.). The chromatography validation studies should be carefully designed in advance, and data generated during process development is often used to determine validation ranges and critical process variables.

Some validation studies must be performed at manufacturing scale. This includes the validation of process purity, where the levels of impurities are measured at each process step over several (usually three) runs. Validating the removal of impurities can eliminate the necessity to measure these impurities in each batch prior to release. The specific impurities to be measured are determined in advance, and a table similar to *Table 12.1* is constructed showing the measured levels across the process. Consistent results can then be demonstrated for consecutive runs.

Several studies that are not practical to do at manufacturing scale may be performed at laboratory scale (Sofer, 1996). These include viral clearance, hold times for product pools and buffers used in production, and column parameter and re-use. In the following sections, we present data from studies that validated the column operating ranges (parameter validation) and the column lifetime (re-use validation). This data also serves to illustrate the constraints under which the processes must operate, which may in turn affect the development effort.

Parameter validation determines the effects of the variation of process conditions on the product and the process, because processes must be robust within the licensed operating parameters (Kelley *et al.*, 1997). Typical variables that are studied during characterization are load, buffer conductivity, and buffer pH. The effect on the product and process is measured by yield and purity. Column lifetime should be prospectively determined, and re-use validation determines a limit on the number of times a chromatography column may be re-used or cycled (Seely *et al.*, 1994).

Both parameter and re-use validation were performed at laboratory scale. When using laboratory scale studies as part of the overall chromatography validation plan, every parameter except column diameter must be the same as manufacturing scale. To ensure comparability to the manufacturing process, all process parameters, including buffers, volumes (measured in CV), and column heights were the same as the manufacturing process. Only the column diameter was changed. The buffers were prepared according to the manufacturing batch records using GMP raw materials.

Protein A affinity chromatography

DEVELOPMENT AND OPERATION

The basic protocol of a protein A affinity column is straightforward: bind at neutral pH and elute at acid pH. This simple bind/elute chemistry does not leave much room

for purification optimization, but since protein A affinity chromatography provides extreme purification in a single step, even an unoptimized process can produce a highly purified antibody. The optimization effort typically focuses not on purity but on throughput.

Protein A affinity media is expensive compared to ion exchange media – more than 30 times the cost. While the ion exchange process columns are sized so that a batch of antibody can be purified on a single cycle on the column, protein A affinity columns are sized to run several cycles to purify a single batch in order to minimize the cost of the column (as well as minimizing the cost of replacing the column if it is damaged). This cycling requires throughput optimization in order to purify the antibody in a reasonable amount of time. One important factor in optimizing throughput is the column capacity.

Capacity is affected by many variables, including the type of protein A affinity chromatography media, ligand density, the antibody concentration in the load, the column temperature and column length, the buffer, conductivity, and pH of the load, and the flow rate (Katoh *et al.*, 1978; Tu *et al.*, 1988; Fuglistaller, 1989; Kamiya *et al.*, 1990; Kang and Ryu, 1991; Schuler and Reinacher, 1991; Van Sommeren *et al.*, 1992). Of these variables, the simplest to control for production and the ones that will have the most significant impact on capacity are the column length, the flow rate, and the chromatography media. Bed height and flow rate both affect the breakthrough capacity; together bed height and flow rate determine the residence time (Fahrner *et al.*, 1999a).

Several types of chromatography media are available for process applications. They include Sepharose Fast Flow (crosslinked agarose), Poros 50 (polystyrene-divinylbenzene), and Prosep (controlled-pore glass). In a study comparing these sorbents (Fahrner *et al.*, 1999c), we found that the sorbent type and flow rate had a strong effect on breakthrough capacity (*Figure 12.4*). Flow rate had the strongest effect on Sepharose; while both Poros and Prosep were less strongly affected by flow rate, Poros had a higher capacity at all flow rates. The type of media had a strong effect on breakthrough capacity, but it did not strongly affect the purity of the antibody (*Table 12.2*). For example, the amount of host cell proteins in the purified antibody pools ranged from 2.5 mg/g to 4.9 mg/g. The amount of host cell proteins in the load was approximately 950 mg/g (950,000 ppm), so these numbers represent a range from 380-fold clearance to 190-fold clearance. The Poros sorbent may have the least non-

Table 12.2. Comparison of protein A affinity chromatography sorbents

	Poros 50	Prosep	Sepharose
Pressure drop (psi h $cm^{-2} \times 10^{-3}$)	3.2	0.3	1.1
Purified antibody			
Yield (%)	104 ± 1	103 ± 2	100 ± 2
DNA (ng/mg)	41 ± 3	40 ± 4	29 ± 2
Host cell proteins (mg/g)	2.5 ± 0.2	3.7 ± 0.2	4.9 ± 1.2
Protein A (ng/mg)	4.6 ± 0.5	3.1 ± 0.5	5.7 ± 1.7

(Data from Fahrner *et al.*, 1999c.) Values for yield (percent loaded antibody in the purified pool), host cell proteins (mg host cell proteins per g antibody), DNA (ng DNA per mg antibody), and protein A (ng protein A per mg antibody) were for runs using a 10 cm column length and 500 cm/h flow rate (50 CV/h), loaded to their capacity determined at 1% breakthrough. Values are the average of three runs, plus or minus one standard deviation. Load material was clarified Chinese hamster ovary cell culture fluid.

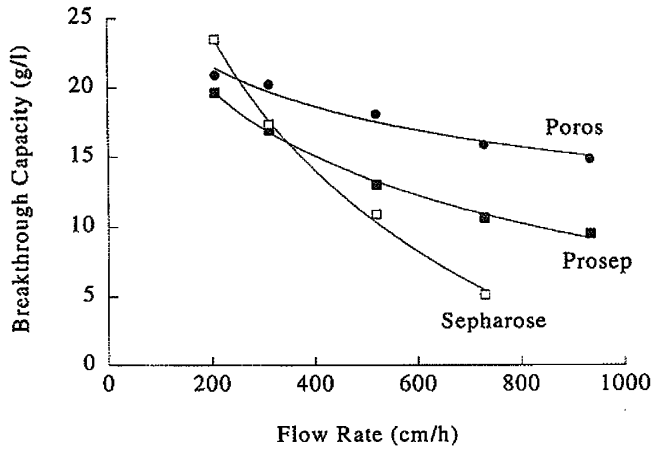

Figure 12.4. Effect of sorbent and flow rate on the breakthrough capacity for protein A affinity chromatography. Data from Fahrner *et al.* (1999c).

specific binding since Poros 50 has the lowest value for host cell proteins. However, even the lowest clearance (for Sepharose) represents a 99.48% removal of host cell proteins, leaving a small amount of host cell proteins that can be cleared downstream.

In our work, we use Prosep A (controlled-pore glass) because it provides good purity, low protein A leakage, and good throughput. It is also easy to pack due to a fast settling rate and high permeability. We have found that it withstands re-use reasonably well, and for several antibodies we have validated its lifetime to 300–400 cycles.

Although the development of protein A affinity chromatography does not focus on purity, purity is still a concern. One way to decrease the amount of host cell proteins in the elution pool when using Prosep media is by using an intermediate wash with tetramethylammonium chloride (TMAC). Since the base material for Prosep is controlled-pore glass that is made from silica, any exposed silica surface may bind proteins. TMAC is known to elute proteins from silica (Chandha and Sulkowski, 1981; Reifsnyder *et al.*, 1996), and in the chromatogram in *Figure 12.1*, a peak is apparent when TMAC is introduced into the column. This peak is not nearly as large when washing with other salts such as sodium chloride or sodium sulphate, which supports the theory that TMAC is eluting host cell proteins that are bound to the exposed silica.

Development of the elution stage is concerned with the elution buffer (Narhi *et al.*, 1997). For elution at pH <3, either citrate or acetate may be used. Some antibodies may partially or completely precipitate in one of these buffers, but generally either citrate or acetate will produce high recovery yield. The elution buffer should be used in an amount that will produce a pool of pH <3.8, so that no adjustment is required for viral inactivation, which requires pH <3.8 for 15–30 min.

The protein A column may typically be run in 4–10 cycles to purify a single batch. Since each cycle is only about 1 hour long, this cycling allows rapid throughput while reducing the cost of the column. The load material must be assayed for antibody concentration prior to loading, usually by an analytical protein A affinity assay. After

Table 12.3. Results from the protein A affinity chromatography re-use study

Cycle	Yield (%)	CHOP (ng/mg)	DNA (ng/mg)	Insulin (ng/mg)	Protein A (ng/mg)	Pluronic F-68 (ng/mg)
5	101	516	1.3	<1.5	6.0	<1246
150	103	525	1.5	<2.0	17.9	<1743
300	96	1455	0.5	<1.7	18.9	<1475
340	90	1604	0.1	<1.8	34.3	<1989

determining the antibody concentration, the load is split up into the minimum amount of cycles that can purify the batch. This may underload the column for each cycle. Because the measurement of the load volume may be inaccurate, it is important to have an air sensor on the load line to end loading of the last cycle if the load runs out early. During elution, the pool begins when the absorbance reaches a predetermined value. The pooling may end when either absorbance or volume reaches a predetermined value. Typically, the pH of the elution pool is adjusted to >5 before holding for an extended amount of time because the antibody may be unstable in the elution pool due to the low pH.

RE-USE VALIDATION

Because protein A affinity chromatography media is expensive, column re-use is a significant concern. For one of our antibodies, we validated the life of the column to 340 cycles. A laboratory scale study was performed to demonstrate that the quality of the product purified by the protein A affinity chromatography step was not affected by multiple re-use of the Prosep A resin. Representative cycles (5, 150, 300, and 340) over the course of the study were selected and the pools were analysed for yield and purity (host cell proteins, DNA, protein A, insulin, and Pluronic F-68). These pools were also analysed by SDS-PAGE under reducing and non-reducing conditions. Chromatograms for these selected cycles were compared to a reference chromatogram.

The yield and purity (host cell proteins, DNA, insulin, protein A and Pluronic F-68) for the four representative cycles are shown in *Table 12.3*. The yields ranged from 90% to 103%. Although there is an apparent decrease in yield with increasing cycle number, a yield of 90% is acceptable. The levels of DNA ranged from 0.1 to 1.5 ng/mg. The levels of host cell proteins ranged from 516 to 1604 ng/mg. An approximate three-fold variation in host cell protein levels in the protein A pool does not significantly affect the final product purity, given that the process has been validated to consistently clear host cell proteins by greater than one thousand-fold in subsequent steps. Insulin levels were consistently below the limit of quantitation (LOQ <2 ng/mg). Protein A levels ranged from 6.0 to 34.3 ng/mg. These levels were less than the manufacturing action limit of 50 ng/mg. The levels of Pluronic F-68 were consistently below the LOQ (<2000 ng/mg). Although the change in host cell proteins and leached protein A suggested ageing of the column, the extent of this was not sufficient to warrant reducing the column lifetime to less than 340 cycles.

Pool samples run on SDS-PAGE, under reducing and non-reducing conditions, were consistent over the course of the 340 cycles (*Figures 12.5A* and *12.5B*). A small band appears in cycle 300 (lane 10, non-reduced) but does not appear in cycle 340

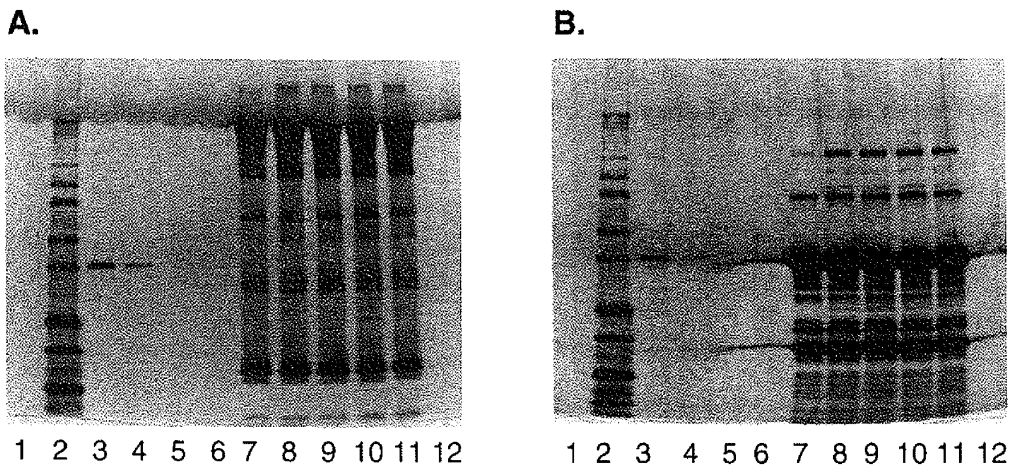

Figure 12.5. (A) Non-reduced and (B) Reduced SDS-PAGE of elution pools from the protein A affinity chromatography re-use validation study. For both gels, lane 1: buffer blank, 2: Molecular weight standards (200, 116, 97, 66, 55, 36, 31, 21, 14 kD), 3: 20 ng BSA, 4: 2 ng BSA, 5: buffer blank, 6: buffer blank, 7: antibody standard, 8: pool from cycle 5, 9: cycle 150, 10: cycle 300, 11: cycle 340.

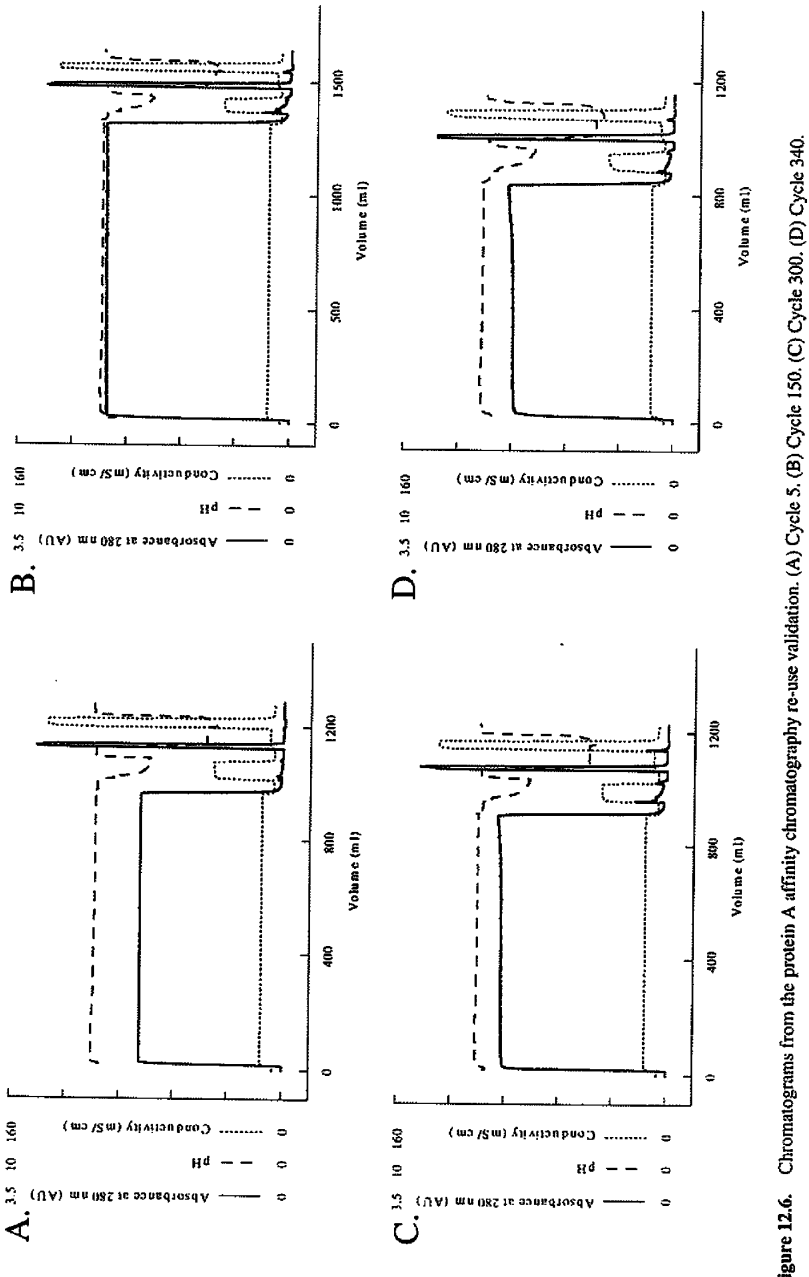

Figure 12.6. Chromatograms from the protein A affinity chromatography re-use validation. (A) Cycle 5. (B) Cycle 150. (C) Cycle 300. (D) Cycle 340.

(lane 11, non-reduced), indicating that this is not a trend. The chromatograms from the representative cycles were compared with the chromatogram from cycle 5, the reference chromatogram (*Figure 12.6*). The peak shape and the absorbance, pH, and conductivity profiles of the reference chromatogram were consistent throughout the study.

Cation exchange chromatography

DEVELOPMENT AND OPERATION

Cation exchange chromatography is used as the intermediate purification step for recombinant antibodies. Although the protein A affinity step greatly reduces the amount of host cell proteins, DNA, and endotoxin, these impurities must be further removed. In addition, the protein A affinity step does not reduce the level of aggregate, and it introduces protein A molecules into the purified antibody. Cation exchange chromatography reduces the level of host cell proteins, DNA, endotoxin, aggregate, and leached protein A. It will also reduce the level of any misformed antibody (for example, antibody with two heavy chains and one light chain). Some host cell proteins flow through the column during load, some elute with the antibody, but the majority elute during the regeneration phase. During chromatography, the leached protein A and aggregate also elute during the regeneration phase.

Development of the cation exchange step focuses on several aspects: the chromatography media, the wash and elution conditions, and the load onto the column. For large-scale use, several vendors can supply chromatography media, including Pharmacia of Uppsala, Sweden (which makes Sepharose) and PerSeptive Biosystems of Framingham, MA, U.S.A. (which makes Poros). We have found that the best media depends on the antibody. For the antibody shown in *Figure 12.2*, Poros 50 HS separated aggregate better than SP Sepharose Fast Flow, providing higher yield at equivalent purity.

There are two critical variables to investigate when developing the wash and elution conditions: the buffer pH and the amount of salt in each buffer. The pH of the separation will be determined in part by the stability of the antibody, and before finalizing the separation pH the stability of the antibody at that pH should be evaluated. We have found that a pH of 5.5 is often optimal for bind-and-elute cation exchange chromatography of antibodies. This pH is high enough that antibody stability is not a problem, and it is low enough to provide sufficient capacity. The typical column capacity at pH 5.5 and a load conductivity <8 mS/cm is about 40 g/l. The conductivity of the protein A affinity chromatography pool is low (<5 mS/cm), so capacity is not greatly affected by pH until the pH is >7 (about 2 pH units below the antibody pI), when capacity may decrease.

The elution conditions are optimized in series of experiments where the column is loaded to capacity and eluted using varying concentrations of sodium chloride or another salt. The collected pool is analysed for yield, purity (aggregate, host cell proteins, DNA, and protein A), and peak width. Generally, at lower salt concentrations the antibody may not completely elute and yield will be low, and at higher salt concentrations the yield will be high but aggregate and host cell proteins will begin eluting with the antibody. This balance between purity and yield will then be the focus

of the development effort. The width of the eluted peak is important for process applications, since a large peak will need a large tank for collection, and the peak volume will affect the throughput of the next step since a larger peak will take a longer time to load. At higher salt concentrations the width will be narrow and at lower salt concentrations the peak will be broader.

If pH is also a factor during development of the elution conditions, the varying salt conditions are applied at varying pH values. This will produce a complex interaction between pH, salt concentration, yield, and purity.

Impurities are removed from the column by increasing the conductivity with a regeneration buffer wash. For development purposes, this wash may be 1 M NaCl or some other appropriate salt so that the regeneration fraction may be assayed for host cell proteins, DNA, and antibody to ensure mass balance. Later, in production, this high-salt wash is generally replaced with sanitization buffer such as 0.5 or 1 M NaOH. In addition to eluting proteins due to the high ionic strength, NaOH also degrades proteins and other molecules and strips them from the column.

Operation at large scale is straightforward except for two factors: column size and peak collection. Since the run time for a typical cation exchange column is about 8 hours, column cycling is not desirable. Therefore the column is usually sized to purify a batch of antibody in a single cycle. The maximum column diameter may be determined by plant size or other considerations, so often the bed height (or column length) will need to be adjusted to have a column size sufficient to purify the batch of antibody. The bed height may affect the separation, so this parameter should be defined during development or the effect of the bed height on the separation should be evaluated during development. The bed height is typically 20–30 cm.

Peak collection begins when the absorbance reaches a pre-set value. It ends when either a pre-set absorbance or volume is reached. When developing elution conditions, the balance between purity, yield and peak width may result in elution conditions where the aggregate is not baseline resolved from the antibody. In this case, rather than eluting in the regeneration, some aggregate may elute in the tail of the main antibody peak. When this occurs, special attention needs to be paid to the pooling conditions so that an antibody peak low in aggregate can be collected. Often, by ending the pool at a relatively high absorbance, a low-aggregate peak can be collected without greatly affecting yield.

PARAMETER VALIDATION

Because the salt concentration and pH of the elution buffer has a strong effect on the antibody separation, the effect of these variables was carefully validated. A laboratory scale cation exchange column was packed (6.84 ml volume, 0.66 cm diameter and 20 cm bed height). The column was packed at the maximum flow rate of the study (200 cm/h). The packed column was sanitized with 0.5 N NaOH (2 CV) and stored with 0.1 N NaOH (3 CV). The control chromatography run was performed with a load of 40 g/l and a flow rate of 100 cm/h. Values measured during the parameter validation are compiled in *Table 12.4*.

Variation in antibody loaded onto the cation exchange resin (10 to 50 g antibody per litre of resin) had no significant effects on the chromatography, recovery, or the quality of the product in the pool. Several load-dependent effects were evident in the

Table 12.4. Results from the cation exchange parameter chromatography validation study

Load (g/l)	Flow rate (cm/h)	Buffer pH	Buffer addition	Yield (%)	Pool concentration (g/l)	Aggregate (%)	DNA (log clearance)
10	–	–	–	82	1.63	0.00	>1.85
20	–	–	–	88	3.48	0.00	>2.18
30	–	–	–	91	5.44	0.57	>2.37
40*	100	5.5	–	86	6.74	0.51	>2.47
50	–	–	–	89	8.58	0.66	2.27
–	200	–	–	87	7.00	0.71	2.3
–	50	–	–	86	6.92	0.59	>2.48
–	–	5.6	–	91	7.26	1.55	>2.5
–	–	5.4	–	82	6.43	0.00	2.04
–	–	–	– 5 mM NaCl	89	6.84	0.49	>2.47
–	–	–	+ 5 mM NaCl	90	6.87	0.95	>2.48

* indicates the control run
– indicates that the baseline condition was used

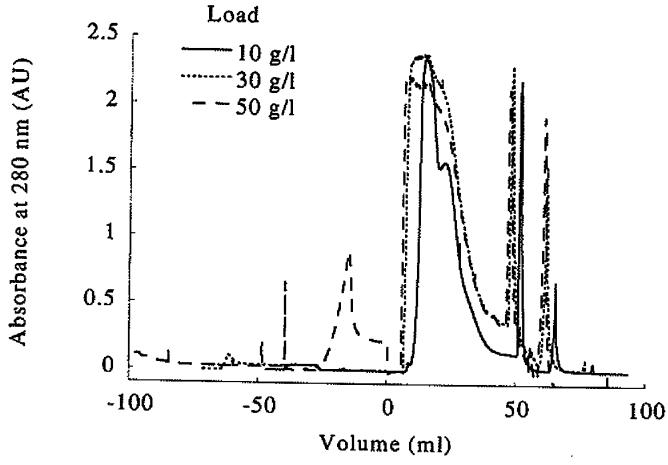

Figure 12.7. Overlaid chromatograms from the cation exchange load validation study. The x-axis is volume from the start of elution. Except for the amount of antibody loaded, conditions are the same as in *Figure 12.2.*

chromatograms (*Figure 12.7*). An increase in absorbance during loading showed some breakthrough at 50 g/l loads, but the amount of protein lost due to breakthrough was minimal (1.8% of the product loaded on the column) and had no significant effect on the recovery of product in the elution pool. Although the elution peak showed some broadening with increasing loads (initially on the tailing edge, then on the leading edge), the broadening of the elution peak does not effect the performance of the chromatography step since the defined pooling criteria (five column volumes) accommodates the largest load. As the overall mass of the load increased, the components which elute in the regeneration and sanitization peaks increased as well.

The antibody yield in the elution pool was independent of the mass of antibody loaded on the column and was approximately 85% of the protein loaded. This consistent recovery of product in the pre-set pool volume results in a concentration of antibody in the pool proportional to the antibody loaded.

The antibody quality in the pool was not affected by the amount of antibody loaded, as determined by size exclusion chromatography and DNA analysis. In addition, the relative SDS-PAGE purity of these pool samples was equivalent to the antibody produced at manufacturing scale, and a significant reduction of the highest molecular weight band seen in the non-reduced load sample was observed in each pool sample. The later finding is consistent with the SEC data of the load and pool samples. Monomeric antibody was measured at 95.4% in the load and 99.3% in the pool samples. This improvement in product quality is a result of aggregate protein being retained on the cation exchange resin during elution and is unaffected by load amount. Furthermore, the measured reduction of DNA from the load to the pool samples was a minimum of 2 logs regardless of the antibody applied to the cation exchange resin.

At the flow rate range of 50 to 200 cm/h, there was no difference in the cation exchange chromatography of antibody. There was no peak broadening either on the leading or tailing edge of the elution profile due to change in flow rate. The yield,

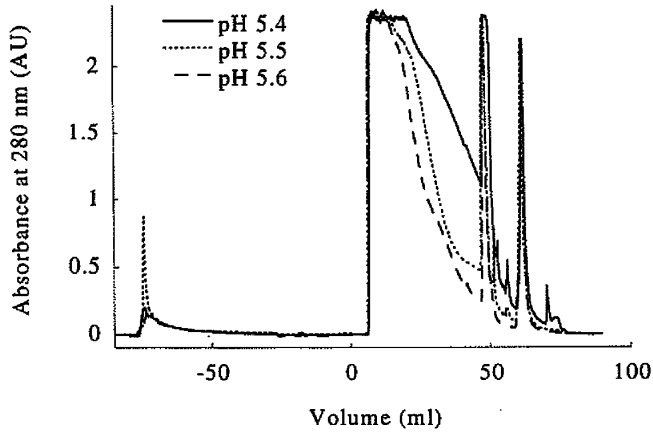

Figure 12.8. Overlaid chromatograms from the cation exchange pH validation study. The x-axis is volume from the start of elution. Except for the pH of the buffers, conditions are the same as in *Figure 12.2*.

SDS-PAGE, SEC, and DNA were consistent for all flow rates. The yield of antibody in the pools ranged from 87% to 89%. The reduced and non-reduced SDS-PAGE gel for pool samples demonstrated equivalence to the cation exchange pools produced at manufacturing scale. Finally, the DNA reduction from the load to the pool of the cation exchange chromatography was a minimum of 2.3 logs regardless of the flow rate applied to the system.

At the pH range of 5.4 to 5.6, the cation exchange chromatography and the antibody quality was not significantly affected. While buffer pH change within this range does have an effect on the elution profile (*Figure 12.8*), the recovery of product and the antibody quality in the eluate pool was acceptable. Although increasing buffer pH leads to an elution profile which approaches baseline at a faster rate, the product recovery yield of antibody in the cation exchange pool is at least 82.6%. In addition, the amount of monomeric antibody as determined by SEC is at least 98.5%. Finally, the DNA is reduced by a minimum log factor of 2.04 regardless of the buffer pH used to perform cation exchange chromatography of antibody.

With conductivity changes of ± 5 mM NaCl in each buffer used for cation exchange chromatography, the chromatography and the antibody quality was not significantly affected. With these changes in buffer conductivity, a minimal shift in the elution profile was noted. However, the resulting pool is acceptable as measured by product recovery yield, SDS-PAGE, SEC, and DNA analysis. The yield was consistent among runs, ranging from 87.1% to 89.9%. The SDS-PAGE and the SEC indicate product equivalent to the reference product. The amount of monomer is at least 99.0% in the pools produced by change of conductivity. Finally, the amount of DNA in these pools is more than 2.5 log less than the amount of DNA present in conditioned protein A pool and independent of the buffer conductivity.

The findings of this study demonstrated that this chromatography can withstand the extremes of each parameter tested to produce pools of acceptable antibody yield and purity.

Table 12.5. Results from the cation exchange chromatography re-use study

Cycle	Yield (%)	CHOP (ppm)	DNA (ppm)	Insulin (ppm)	Protein A (ppm)	Aggregate (%)
1	88.7	75	<0.006	<4.61	<3.9	0.67
11	90.4	90	0.060	<3.9	0.68	
21	90.0	85	0.012	<4.56	<3.9	0.44
31	90.6	63	0.058	<3.9	0.44	
41	95.9	35	0.015	<4.40	<3.9	0.33
50	88.3	34	0.011	<4.74	<3.9	0.30
Average	90.6	64	0.48			
Standard deviation	2.4	22	0.15			

RE-USE VALIDATION

To demonstrate that the quality of the product purified by the cation exchange chromatography step is not affected by multiple re-use of the column, a laboratory scale study was performed. A 1.6 cm inner diameter × 20 cm length column (column volume = 38 ml) was packed with cation exchange media. 50 cycles were performed on the laboratory scale column using the same operational parameters and setpoints/ranges as used in manufacturing. Because the column used at manufacturing scale is often repacked between manufacturing campaigns, the laboratory scale column was periodically repacked. The column was placed in storage buffer after each cycle. During re-use, to simulate varying process conditions encountered in production, the load of the column was changed each cycle, varying between 10–50 g/l.

Six representative pools over the course of the study were analysed for total protein and purity (SDS-PAGE, aggregate, protein A, DNA, and host cell proteins), and four representative pools were analysed for insulin (*Table 12.5*).

Yield varied from 88.7% to 95.9%, with no significant change from cycle 1 (88.7%) to cycle 50 (88.3%). DNA varied from <0.006 ppm to 0.06 ppm, with no significant change from cycle 1 (<0.006 ppm) to cycle 50 (0.011 ppm). Host cell proteins varied from 34 ppm to 90 ppm, with no significant change from cycle 1 (75 ppm) to cycle 50 (34 ppm). The level of protein A in the pools was less than detectable (<3.9 ppm). The levels of insulin were less than detectable. Aggregate varied from 0.30% to 0.67%, with no significant change from cycle 1 (0.67%) to cycle 50 (0.30%). None of the observed fluctuations are significant enough to impact the product quality or the downstream process.

The SDS-PAGE gels, both reduced and non-reduced, are shown in *Figures 12.9A* and *12.9B*. No significant new bands or change in the profile of protein distribution appeared over the course of 50 cycles, and bands on the pool samples were of comparable density to the reference material. In addition, the chromatograms were consistent with respect to absorbance, pH, and conductivity.

Yield, host cell proteins, aggregate, and DNA did not change significantly through the cycles. Insulin and protein A were reduced to non-detectable levels in all cycles assayed. The results of this study demonstrated that the cation exchange column performs consistently throughout 50 cycles. Product quality, as measured by yield, host cell proteins, DNA, insulin, and protein A, remained consistent throughout 50 cycles. Column performance, as measured by comparing chromatograms and by column integrity, remained consistent throughout 50 cycles.

Figure 12.9. (A) Non-reduced and (B) Reduced SDS-PAGE of elution pools from the cation exchange chromatography re-use validation study. (A). Lane 1: Molecular weight standards (200, 116, 97, 66, 55, 36, 31, 21, 14 kD), 2: 2 ng BSA, 3: 50 ng BSA, 4: antibody standard, 5: buffer blank, 6: pool from cycle 1, 7: cycle 11, 8: cycle 21, 9: cycle 31, 10: cycle 41, 11: cycle 50. (B). Lane 1: Molecular weight standards, 2: 2 ng BSA, 3: 50 ng BSA, 4: buffer blank, 5: buffer blank, 6: antibody standard, 7: buffer blank, 8: pool from cycle 1, 9: cycle 11, 10: cycle 21, 11: cycle 31, 12: cycle 41, 13: cycle 50.

Anion exchange chromatography

DEVELOPMENT AND OPERATION

The isoelectric point of many antibodies is high (often >8 and sometimes >9), so anion exchange chromatography run in flow-through mode offers a high-yield method for final purification of antibodies with a high pI. For antibodies with a pI lower than about 8, the anion exchange chromatography step may be run in bind-and-elute mode, which may offer advantages over the flow-through mode, such as additional clearance of leached protein A.

For anion exchange chromatography run in flow-through mode, the pH of the load material is raised to about 0.5–1 pH unit below the pI of the antibody, the conductivity of the load material is adjusted to <7.5 mS/cm, and the antibody is then pumped through the column. Because the pH of the load is less than the pI of the antibody, the antibody will flow through. Since the pI of most host cell proteins is less than the pH of the load, most host cell proteins will bind to the column. Under these conditions, DNA and endotoxin will also bind strongly to the column. The purified antibody is collected after it flows through the column, and the impurities are removed from the column during the regeneration phase.

Development of the flow-through step is straightforward. The antibody is run through the column at various levels of pH and conductivity. In general, at any value of pH, decreasing the conductivity will increase the clearance of host cell proteins. Since the conductivity of the cation exchange pool may be relatively high (>12 mS/cm), the conductivity of the load onto the anion exchange column is controlled by dilution of the cation exchange pool with water. After dilution, the pH is adjusted to the appropriate value.

Since the throughput of the anion exchange column will be determined by the volume of the load, it is best to have the dilution as small as possible. Running the column pH just below antibody binding will allow the least dilution (highest conductivity), so the column is typically run at a pH that is 0.5–1 pH units below the antibody pI. When the pH is only about 0.2 units below the pI of the antibody, decreasing the conductivity below a critical value will allow the antibody to bind, resulting in yield loss. For our antibodies, we typically need a 2× dilution to reduce the conductivity to <7.5 mS/cm and the step is run at pH 8.

Like the cation exchange column, contaminants are removed from the column by increasing the conductivity with a regeneration buffer wash. For development purposes, this wash may be 1 M NaCl (or some other appropriate salt) so that the regeneration fraction may be assayed for host cell proteins, DNA, and antibody to ensure mass balance. Later, in production, this high-salt wash is generally replaced with sanitization buffer such as 1 M NaOH. In addition to eluting proteins due to the high ionic strength, it also degrades proteins and other molecules and strips them from the column.

In production, the column is sized for throughput. Because only a small amount of host cell proteins, DNA, and endotoxin bind to the column, the column dimensions could in principle be very small. However, with most commonly used anion exchange resins, the flow rate is limited. For example, when packed into a process-scale column the flow rate limit on Q Sepharose Fast Flow is about 200 cm/h. With this low flow

rate, using a column sized for binding capacity of the impurities would dramatically increase the run time. For this reason, anion exchange columns are typically about the same size as the cation exchange (about 100–200 l), and are loaded with about 100 g/l antibody.

PARAMETER VALIDATION

This study examined the effects in purification performance induced by changes in the process parameters used in the antibody anion exchange chromatography step. We examined the effect of total amount of antibody to be loaded, flow rate, buffer pH, and buffer conductivity.

A laboratory scale anion exchange column (0.66 cm diameter, 19 cm bed height and a column volume of 6.5 ml) was prepared. The column was sanitized with 0.5 N NaOH (2 CV) and stored in 0.1 N NaOH (3 CV).

Separate samples were conditioned (as performed in manufacturing) and loaded to study each parameter change. The conductivity of the load was adjusted during the conditioning by increasing or decreasing the dilution of the load with purified water. The conductivity of the load was matched to the conductivity of the elution/wash buffer and the conductivity of the elution/wash buffer was altered by changing the salt concentration in the buffer. The pH of the load and buffers was adjusted by titration with 1.5 M Tris.

A control chromatography was performed with an antibody load of 100 g/l and at a flow rate of 76 cm/h as defined for manufacturing scale. Each of the remaining chromatography experiments was performed with one variable changed from the control conditions. The results from this study are compiled in *Table 12.6*.

Analysis of the recovery yields for the different antibody loads showed no significant differences. The recovery yields for the anion exchange step were comparable and range between 95 and 100%. SDS-PAGE analysis of the anion exchange pools from the different loads showed no additional bands when the load of antibody increased from 50 g/l to 125 g/l. These results show that the capacity of the anion exchange column to remove contaminants is unchanged even at the highest load of 125 g/l. The shape of the chromatograms during the different load experiments show no unexpected discrepancies, and the level of host cell proteins was <4 ppm.

Table 12.6. Results from the anion exchange chromatography parameter validation study

Load (g/l)	Flow rate (cm/h)	Buffer pH	Buffer adjustment	Yield (%)	Host cell proteins (ng/mg)	DNA (pg/mg)
125	–	–	–	96.6	<4	<9
100*	76	8.0	(50 mM)	97.6	<4	<9
75	–	–	–	101.1	<4	<9
50	–	–	–	101.4	<4	<9
–	–	8.5	–	101.5	<4	<9
–	–	7.5	–	101.2	<4	<9
–	–	–	+ 50 mM	98.9	<4	<9
–	–	–	– 25 mM	99.2	<4	<9
–	140	–	–	100.0	<4	<9
–	40	–	–	99.1	<4	<9

* indicates the control run

The performance of the anion exchange step was evaluated at two flow rates (40 cm/h and 140 cm/h). The product recovery yields at the low and high flow rate showed no significant changes when compared to the standard anion exchange chromatography step yield. SDS-PAGE analysis of the anion exchange pools from the different flow rate experiments showed no additional bands when compared to the standard pool, and the level of host cell proteins was consistently <4 ppm. These results showed that the capacity of the anion exchange column to remove protein contaminants is not affected over the flow rate range tested. The shape of the chromatograms from the different flow rate experiments is equivalent with only variations in load volume. No discrepancies were found in the chromatograms.

The performance of the anion exchange step was evaluated at two conductivity values. The load conductivity and the equilibration/elution buffer conductivity were changed to 4.4 mS and 13.32 mS in separate experiments. The product recovery yields at the low and high conductivities showed no significant changes when compared to the control anion exchange chromatography step yield. SDS-PAGE analysis of the anion exchange pools from the different conductivity experiments showed no additional bands when compared to the standard anion exchange pool, and host cell proteins were <4 ppm. These results demonstrated that the capacity of the anion exchange column to remove protein contaminants is not affected over the conductivity range tested. The performance of the anion exchange step was evaluated at two pH ranges. The product recovery yields at the low and high pH showed no significant changes when compared to the control anion exchange chromatography step yield. SDS-PAGE analysis of the anion exchange pools from the different pH experiments showed no additional bands when the pH was changed from 8.5 to 7.5, and the level of host cell proteins was consistently <4 ppm. The results demonstrated that the anion exchange step performs adequately over the range of all the parameters tested.

RE-USE VALIDATION

A 1.6 cm inner diameter × 19 cm length column (column volume = 38 ml) was packed with Q Sepharose chromatography media. After the column was cycled 50 times, the column was unpacked and an aliquot of the used resin was packed into a 0.66 cm inner diameter × 19 cm length column (column volume = 6.5 ml), and a DNA spike challenge was performed on the column.

Several measured values for the re-use study are shown in *Table 12.7*. Yield varied from 101.0% to 109.7%, with no significant change from cycle 1 (107.6%) to cycle

Table 12.7. Results from the anion exchange chromatography re-use study

Cycle	Yield (%)	CHOP (ng/mg)	DNA (ng/mg)
1	107.6	<7	<3
11	106.4	<7	<3
21	108.5	<7	<3
31	109.7	<3	<3
41	101.0	<3	<3
50	106.6	<3	<3
Average	106.6		
Standard deviation	2.8		

50 (106.6%). The level of host cell proteins was <7 ppm for all cycles. DNA in the Q-Sepharose pool was less than detectable for all pools.

In the SDS-PAGE gels, both reduced and non-reduced, no significant new bands appeared over the course of 50 cycles, and bands on the pool samples were of comparable intensity to the reference material. The chromatograms were consistent with respect to absorbance, pH, and conductivity.

The results of this study demonstrate that the anion exchange column performs consistently throughout 50 cycles. Product quality, as measured by yield and host cell proteins, remained consistent throughout 50 cycles.

Conclusions

With the increasing use of monoclonal antibodies as pharmaceuticals, there is a need for robust, reliable, cost-effective processes. As a central part of an overall manufacturing system, the three-step recovery process we described can meet the requirements for purity, throughput, and yield. Integrating the protein A affinity, cation exchange, and anion exchange chromatography steps can provide sufficient clearance of host cell proteins, DNA, endotoxin, virus, small molecules, and aggregate. With correctly sized columns and process equipment, each batch of antibody can be recovered in less than three days. By developing and running each step correctly, an overall process yield of 70% can be achieved. The process is robust, reliable, and can be validated to operate within a range of operating parameters.

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Antibody production☆

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Abstract

The clinical and commercial success of monoclonal antibodies has led to the need for very large-scale production in mammalian cell culture. This has resulted in rapid expansion of global manufacturing capacity [1], an increase in size of reactors (up to 20,000 L) and a greatly increased effort to improve process efficiency with concomitant manufacturing cost reduction. This has been particularly successful in the upstream part of the process where productivity of cell cultures has improved 100 fold in the last 15 years. This success has resulted from improvements in expression technology and from process optimisation, especially the development of fed-batch cultures. In addition to improving process/cost efficiencies, a second key area has been reducing the time taken to develop processes and produce the first material required for clinical testing and proof-of-principle. Cell line creation is often the slowest step in this stage of process development. This article will review the technologies currently used to make monoclonal antibodies with particular emphasis on mammalian cell culture. Likely future trends are also discussed.

Keywords: Fed-batch culture; CHO; NS0; Gene expression systems; Downstream processing; Fermentation; Cell line selection

Contents

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1. Introduction

Currently, there are 18 monoclonal antibodies approved for therapeutic use [2]. The majority (15) of these antibodies are produced by recombinant DNA technology although three are murine antibodies made in hybridomas. The recombinant antibodies are produced in mammalian cell expression systems using Chinese hamster ovary (CHO) or murine lymphoid cell lines (e.g., NS0, Sp2/0-Ag14). Most products in clinical trial are whole antibodies made in mammalian cell systems but some are antibody fragments, which can be made in microorganisms such as *E. coli*. For example, CIMZIA™ [2] is a pegylated Fab′ fragment made in a microbial system and is currently in phase III trials. Reichert et al. [2] report that, of the 15 antibodies they identified in phase III trials, six were single chain or Fab fragments. In this article, we review the technologies used to manufacture antibodies focusing particularly on the current status of mammalian cell culture and approaches taken in process development. There are two crucial issues, which have to be faced in process development. The first is to minimise the time taken to provide material for clinical studies and the second is to develop a process which can deliver sufficient drug substance to meet market demands at an acceptable price per dose.

The industry continues to look at new technologies and process development strategies that will reduce timelines. The resulting processes must be easily scaleable, robust and meet quality and safety criteria. One approach to shortening the timelines is the use of platform technologies for cell culture processes, for example using standard media, feeds and growth conditions. Cell line construction and selection is often a critical path activity and needs to be completed rapidly without compromising quality criteria. Ideally, one would like to rapidly create a highly productive cell line that could be used for long-term manufacture obviating the need to create an improved second generation cell line at a later stage of development.

Productivity of mammalian cell processes has improved dramatically in recent years [3] and modern cell culture processes can achieve antibody concentrations exceeding 5 g/L [4,5]. This has resulted from improvements in expression technology and from process optimisation, particularly of the upstream, cell culture stage. Most current processes are based on fed-batch culture and the development of feeds in particular has made a significant contribution to increased antibody yields. Highly productive cell lines result from using a host cell line that has the desired characteristics, an appropriate expression system, and a good transfection and selection protocol. A number of expression systems with the potential to produce cell lines with high specific production rates (Q_p) are available. The challenge is to create cell lines that not only have high Q_p but also

have the growth characteristics that will lead to high productivity in the manufacturing process. This can be achieved by selecting cell lines with the combination of a high Q_p, an ability to achieve high space–time yields of viable biomass and consequent high volumetric production rate in a screen designed to mimic the final production process. The resulting cell lines are also selected for stability, growth in suspension culture using a chemically defined, animal component-free (CDACF) medium and the ability to make the desired post-translational modifications.

Purification strategies are based on chromatographic procedures usually including a protein A affinity step. With increasing upstream concentrations, significant attention is now being paid to improving downstream efficiency.

2. Expression systems

The ability of a cell line to achieve high volumetric productivities results from a combination of characteristics. Efficient transcription of the antibody genes is achieved by using an appropriately designed expression vector. Secondly, one requires a cell line capable of efficiently translating antibody mRNAs, assembling and modifying the antibody at high rates with minimal accumulation of incorrectly processed polypeptides, and having sufficient secretory capacity for secreting the resulting assembled antibody. It is probable that with the current generation of cell lines, productivity is not limited by transcription. It has been reported that, for a panel of antibody-producing GS-NS0 cell lines with Q_p values varying between 0.05 and 0.95 pg/(100 pg cell protein h), there was no correlation between Q_p and mRNA levels [6]. These data suggest that events downstream of transcription are the limiting factors. Thirdly, the cell line must be capable of achieving high viable cell concentration within an acceptable time. An additional criterion is that the cell line must produce antibody with the desired product quality characteristics, such as glycosylation.

2.1. Expression vectors

The expression vector systems most frequently used for the production of therapeutic monoclonal antibodies are the Glutamine Synthetase (GS) Gene Expression System (Lonza Biologics; [5]) and those based on dihydrofolate reductase (DHFR) genes. A selection of expression vectors developed for the expression of immunoglobulin genes is shown in Table 1. To achieve high levels of gene expression, GS and DHFR vectors usually have strong promoters to drive expression of the antibody genes. The promoters are typically of viral origin (e.g., human cytomegalovirus) or they are derived from genes that are highly expressed in a mammalian cell [10,11]. Historically, the vectors have also included favourable RNA processing signals such as polyA tail, 5′ and 3′ untranslated region, presence of an intron to encourage export from the nucleus and a splice site to remove this intron. Coding sequences may also be optimised to remove, for example, cryptic splice sites or cryptic polyA tails, or sequences that lead to unfavourable folding of the mRNA. To increase mRNA processing and improve secretion, codon usage can be optimised for the target cell type, GC content increased and signal sequences used to target the heavy and light chain polypeptides to the correct part of the secretory pathway.

2.1.1. DHFR expression systems

DHFR expression systems use the folate analogue methotrexate (MTX) to inhibit the function of DHFR, an essential metabolic enzyme. Transfection with an expression vector containing a DHFR gene prevents MTX poisoning of transfected cells. The antibiotic resistance gene frequently used in DHFR expression vectors acts as the selectable marker: the primary function of the DHFR gene is then to facilitate vector amplification. The DHFR gene is usually under the control of a weak promoter, such as one from SV40. The use of a weak promoter to regulate DHFR

Table 1
Expression vector systems for use with expression of immunoglobulin genes

Expression vectors and selectable markers	References
GS vectors	www.lonza.com, [5]
DHFR intron vector/hygromycin	[7]
Neomycin	[8]
DHFR bicistronic vector	[9]
CHEF/DHFR	[10,11]
DHFR/neomycin	[12]

Where resistance to an antibiotic is used to select transfectants, the target antibiotic is shown.

gene expression should reduce promoter interference (due to read-through from the upstream promoter inhibiting expression from the downstream promoter) thus increasing expression of the immunoglobulin genes.

2.1.2. Glutamine Synthetase (GS) Expression System

GS synthesises glutamine from glutamate and ammonium. Since glutamine is an essential amino acid, transfection of cells that lack endogenous GS with the GS vector confers the ability to grow in glutamine-free media. GS expression vectors contain the GS gene downstream of a SV40 promoter, which offers similar benefits to those seen when a weak promoter is used to drive DHFR gene expression.

2.2. Increasing specific production rate by improving transcription

Transcription is probably not limiting antibody secretion in the current generation of cell lines. This is because they were constructed using expression vector systems developed to give high mRNA levels. Several options exist to increase transcription. In early expression systems, this was generally by gene amplification. Gene amplification is usually achieved by constructing the expression vector so that the genes of interest are linked to an amplifiable gene (e.g., thymidine kinase, adenosine deaminase, GS or DHFR). Tansfected cells are then exposed to increasing levels of a specific enzyme inhibitor at concentrations substantially higher than those used for selection of transfectants. If the drug inhibits an enzyme (e.g., GS or DHFR) essential for the survival of the cell, only cells that overproduce this enzyme will survive. The overproduction of the enzyme commonly results from increased levels of its particular mRNA, resulting from either an increase in gene copy number (i.e., amplification), or from more efficient transcription [13]. Often more DNA (up to 1000 kb) than just the target gene is amplified. Therefore, when the transfected genes are amplified, other tightly linked sequences, including the immunoglobulin genes, on the vector are co-amplified. The high copy numbers of the expression vector seen upon amplification, especially with the DHFR expression system, may increase Q_p but it can also have a detrimental effect on other cellular properties. Amplification of the transgenes will frequently result in poor growth performance of the resulting cell population and may alter cellular metabolism. Amplification and the resulting variation in copy number can also alter the inherent stability of expression and often requires the continued presence of the selective agent. If the selective agent is required in the production bioreactor, it will be necessary to demonstrate that the purification process removes this compound from the bulk drug substance.

The GS system [5] and some variants of the DHFR one [12] do not rely upon amplification to achieve high productivities. Instead, these systems rely upon insertion of the antibody construct into a transcriptionally active region to achieve high productivities, selecting against insertion into the heterochromatin.

One approach is to use site specific recombination of the gene(s) of interest into a known transcriptionally active locus. Expression vectors can be constructed that contain a specific targeting sequence that will direct the vector to integrate by homologous recombination into a particular active site. Such a sequence has been identified in the immunoglobulin locus of the murine myeloma cell line NS0 [14]. Vectors containing this sequence are targeted to the immunoglobulin locus in more than 75% of high producing NS0 cell lines.

A corollary of this approach is to take the sequences flanking the transcriptionally active locus and incorporate them into the expression vector. Thus, the vector should create a favourable environment for expression independent of its integration site into the genome. Vectors incorporating ubiquitous chromatin opening elements [15], matrix attachment regions and anti-repressor sequences [16,17] or the flanking sequences of the Chinese hamster elongation factor-1α gene [10,11] have been shown to increase and maintain transgene expression and are being actively evaluated with immunoglobulin genes.

An alternative approach is to transfect the cells with a conventional expression vector (i.e., randomly integrate the expression vector into the genome) but then bias the selection method so that only transfectants where the vector integrated into a transcriptionally active site are selected. This can be done by using a selection system that only allows transfectants producing sufficient levels of the selectable marker

gene product to proliferate. Expression systems using a selectable marker gene with either the weak SV40 promoter [5] or an impaired Kozak sequence upstream of the marker gene [12] are included in this class of selection system. Linkage of the antibody construct to the selectable marker gene results in the over-production of antibody as both genes are integrated into a transcriptionally active locus. The choice of selection conditions is extremely important for the success of this approach [5].

3. Cell lines

The key issues affecting the choice of a cell line for use in a manufacturing process are: the capability to produce high antibody concentrations in the chosen production system, the ability to consistently produce a product of uniform characteristics, and the speed with which a high yielding cell line can be obtained. The availability of a suitable expression system and the importance of post-translational modifications of the recombinant antibodies may also affect this choice.

There are 18 therapeutic antibodies currently licensed for use of which 10 are manufactured in Chinese hamster ovary (CHO) cell lines and 8 are made in murine lymphoid cells (including NS0 and Sp2/0-Ag 14). These parental cell lines are also the ones most commonly used for antibodies currently in clinical trials. In addition, murine hybridomas and other cell lines such as the human cell line PER.C6 are used.

3.1. CHO

CHO cells are widely used to produce recombinant antibodies using both the DHFR and GS expression systems. The most commonly used CHO strains with DHFR expression vectors are DUKX-B11 and DG44, which both lack dhfr. The GS system uses the CHO-K1 strain, or a derivative of the CHO-K1, CHOK1SV. Although both CHO-K1 and CHOK1SV [18] express functional GS enzyme, inclusion of the GS inhibitor methionine sulphoximine (MSX) in the medium allows use of the GS expression vectors. Endogenous GS in CHO cells is inhibited by 3 μM MSX, which is a cytotoxic concentration. By selecting GS-CHO transfectants in the presence of 50 μM MSX, only those cell lines that have stably incorporated the expression vector into a transcriptionally active locus will form transfectant colonies. These cells produce enough GS enzyme to titrate out the MSX whilst leaving sufficient functional enzyme to meet the cellular demand for glutamine. Recombinant CHO cell lines show efficient post-translational processing of complex proteins, while the glycosylation patterns of native and CHO-derived recombinant proteins are similar.

The preferred culture format for large-scale (substantially greater than 10 L) is single cell suspension, ideally using chemically defined, animal component-free (CDACF) media. Wild type CHO strains have adherent cell morphology and require serum supplementation for growth. Adaptation of recombinant CHO cell lines from adherent to suspension culture formats and adaptation to CDACF media can take up to 9 months, which is not compatible with short development timelines. The industry trend has been to pre-adapt the parental CHO cell line to suspension culture in CDACF media, reducing timelines by about 6 months [18,19].

3.2. Murine lymphoid cell lines

The host cell lines NS0 and Sp2/0-Ag14 are widely used for antibody production. Both cell lines were derived from a plasmacytoma cell line originating from a BALB/c mouse. The starting cell line underwent numerous rounds of cloning, and in the case of Sp2/0-Ag14, fusion with spleen cells from another BALB/c mouse, to generate these two parental cell lines [20,21]. Both the cell lines lack the ability to synthesise and secrete immunoglobulin proteins. The parental cell type of the two cell lines is a differentiated B cell, which is inherently capable of high levels of immunoglobulin production. These two characteristics favour their use for manufacturing antibodies. The genotype of NS0 cells makes them particular suited for use with the GS expression system. Unlike other cell types, NS0 cells are obligate glutamine auxotrophs: glutamine independence can be conferred upon NS0 cells following transfection with a functional GS gene. In the case of Sp2/0-Ag14, mutants that no longer require glutamine occur spontaneously with relatively high frequency. This is not observed with NS0 cells [22].

3.3. Hybridomas

In addition to murine antibodies, it is now possible to make human antibodies using murine hybridoma technology. However, unlike the original hybridomas [23], the spleen cells are taken from a transgenic mouse which has the murine immunoglobulin locus replaced by the human genes.

3.4. Other cell lines

There are reports of other types of cell line being used to produce monoclonal antibodies including the hamster line BHK21 [24] and the human PER.C6 cell line [8]. The PER.C6 cell line is derived from human embryonic retinal cells by transfection with the adenovirus E1 region followed by selection for transfectants with an immortal phenotype. Analysis of the glycosylation profiles of IgG_1 antibody revealed no high mannose or hybrid structures; all were biantennary with core fucose. Galactosylation was similar to human serum IgG_1.

4. Screening of cell lines

The function of the expression vectors described in the previous sections is to generate cell lines with high Q_p values. Transfectants with high Q_p are rare events and this is the reason to use expression technologies that provide stringent selection and/or an increased frequency of high producers. However, a transfectant with a high Q_p does not necessarily result in a cell line that performs well in the production process. Hence, a sufficient number of cell lines need to be generated to allow for the attrition in numbers when screening for other desired characteristics.

The issue is, therefore, how can the hit rate for finding highly productive cell lines be increased? The simplest approach is to screen more transfectants, but how many? Simulations run by one of us (AJR, unpublished) suggest that several thousand should be screened, even after being enriched with a stringent selection system, to be confident of getting multiple transfectants with the desired productivity characteristics [5].

Conventional methods for the screening of cell lines are labour intensive, which limits the number of cell lines that can be screened. Increasingly robotics is being used to automate the liquid handling and cell transfer stages. This does not address the need to screen large numbers of transfectants to identify sufficient high producers to screen against the additional growth criteria that contribute to high productivity in a manufacturing process. The number can be reduced by using a fluorescence-activated cell sorting (FACS) technique to identify cells secreting high levels of antibody and sort them away from the lower producers. One such method, based on the capture of the secreted antibody by a capture matrix and its detection by a labelled probe, has been described by Holmes and Al-Rubeai [25]. The cells can be sorted into large populations ("bulk sorting"), from which cell lines can be isolated by conventional cloning methods, or by single cell sorting using FACS. Strictly, these approaches enrich for cells with a high Q_p since the secreted monoclonal antibody is captured close to the cell surface and secretion occurs over a short time period. Q_p is not the only phenotypic characteristic contributing to productivity and cell lines need to be screened for other characteristics. Typically, several criteria are used to select the production cell line including a high Q_p, growth characteristics such as the magnitude of the time integral of the viable cell concentration and maximum cell concentration, antibody concentration at harvest, cell line stability and product quality. An important feature of any screening scheme is that it incorporates a technique that is a predictive model of the manufacturing process. This screening step is often carried out in shake flask cultures, which may include the feeding techniques used in the final process.

5. Transient and other expression systems for production of development material

In order to produce quantities of material for process development studies, rapid expression technologies are frequently used that allow the generation of milligrams to grams of material in advance of a stable manufacturing cell line becoming available. These methods include the use of uncloned transfectant pools and the application of transient expres-

sion technology. Large-scale (up to 100 L) transient expression systems are being developed to meet this demand [26].

6. Cell engineering to increase productivity or modify product characteristics

High monoclonal antibody concentrations are the result of high Q_p values and space–time yield of viable cells. As discussed above, Q_p is probably limited by events downstream of transcription, only some of which can be addressed by vector engineering. An alternative approach is to modify the translational or secretory pathways where antibody production is considered limited at folding and assembly reactions. Dinnis and James [27] recently reviewed ways of increasing Q_p through cell line engineering. These authors proposed that, since foldases and chaperones exist as large multi-protein complexes, global expansion of all components of the secretory pathway is required for generic improvement of antibody secretion rather than over-expression of selected proteins. This would be similar to the events occurring during the differentiation of B cells into plasma cells, where the unfolded protein response (UPR), an important intracellular signalling pathway, is induced. Protein expression in differentiating B cells is coordinated by components of the UPR to achieve maximum antibody production. It has been proposed that there may be benefit in over-expression of proteins known or suspected of having a key role in modulating signalling pathways, e.g., BLIMP-1, or initiation of ER expansion (XBP-1, ATF6) [27]. An alternative approach to over-expression of specific genes is to use randomised zinc finger protein-transcription factor (ZFP-TF) libraries [28]. Theoretically, the ZFP-TF libraries can modulate the expression of any gene, so that a specific phenotype can potentially be created without a detailed knowledge of the molecular basis of the phenotype.

High space–time yields of viable biomass are achieved by using a cell line capable of growing to a very high viable cell concentration and then maintaining it for extended periods. The maintenance of high viability for such cultures requires minimisation of the death rate. The major cause of cell death in animal cell cultures is by apoptosis pathways. Since apoptosis can be induced by various chronic insults and is mediated by a number of pathways, numerous strategies have been developed to limit cell death [5,29]. Nutrient limitation can induce apoptosis, so one strategy for limiting apoptosis is to prevent nutrient limitation. Although operating the culture in fed-batch mode can delay the onset and reduce the extent of apoptosis, the cells will still eventually die by apoptosis. Alternatively, resistance to apoptosis can be engineered into the cell lines. As activation of the apoptotic pathways is lethal to the cell, the pathways must be tightly regulated. The best understood regulatory mechanism involves the Bcl-2 family of proteins. The anti-apoptotic properties of Bcl-2 family members have been used to protect industrially important cell lines from insults typically experienced during cell culture operations. However, the results are contradictory with respect to productivity and there are few reports describing the behaviour of cell lines engineered to have increased apoptosis resistance in modern antibody manufacturing processes.

In addition to changing characteristics related to productivity, there are also examples where it has been advantageous to alter the cell's ability to carry out particular post-translational steps such as glycosylation. This has been driven by increasing awareness of the role of glycosylation in effector functions such as antibody-dependent cellular cytotoxicity (ADCC). ADCC is believed to play a role in the function of some therapeutic monoclonal antibodies, with various studies showing that oligosaccharide engineering may optimise ADCC. The degree of galactosylation and fucosylation and the proportion of bisecting GlcNAc residues have all been implicated in modulating effector functions. Oligosaccharide engineering has thus become an important research area for increasing antibody potency. Yamane-Ohnuki et al. [30] created a FUT8 double knockout of the CHO DG44 host that lacks α-1, 6-fucosyltransferase activity and cannot synthesise fucosylated antibodies. The ADCC of the resulting antibody was increased 100-fold compared to the fucosylated form. In a different approach, over-expression of *N*-acetylglucosaminyltransferase III in CHO increased the proportion of bisecting GlcNAc residues, increasing the ADCC substantially compared to the parental molecule [31,32].

Increasingly, our ability to isolate useful variant cells or to engineer them will come from a better understanding of the biology which defines the

desired phenotype. The "omics" tools which have become available in recent years will play a major part in providing this knowledge base and we are already seeing examples of the power of these methods. Recently, Smales et al. [6] compared the proteomes of GS-NS0 cell lines with varying monoclonal antibody production rates and were able to demonstrate changes in abundance of several proteins associated with changes in productivity.

7. Reactor systems used for large-scale antibody production

A consequence of the rapidly growing demand for monoclonal antibodies has been a dramatic increase in capacity in the industry [1] and an increase in the scale of reactors used for production. Two types of culture system are used for large-scale manufacture, fed-batch and continuous perfusion culture [33]. The principles of these types of reactor are shown in Figs. 1 and 2. Fed-batch processes are by far the most common and are now operated at scales up to 20000 L working volume. Several authors [33,34] have reviewed the types of cell culture reactor systems and processes in industrial use.

In fed-batch culture, small volumes (in our case less than 10% of the reactor volume) of key nutrients are fed to the culture during the fermentation process to maintain nutrient sufficiency, and the culture is harvested at the end of the batch cycle. Optimisation of feeding strategies has been a major factor contributing to improvements in growth and productivity in recent years.

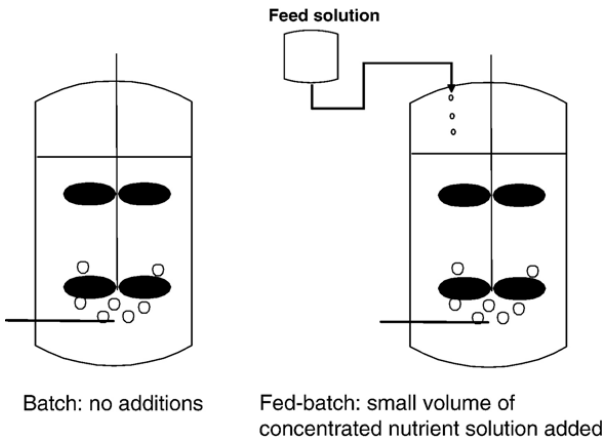

Fig. 1. Schematic representation of batch and fed-batch culture systems. The fed-batch system is supplied with a concentrated nutrient solution: no spent culture medium is removed. In a batch system, no additions of nutrient solutions are made.

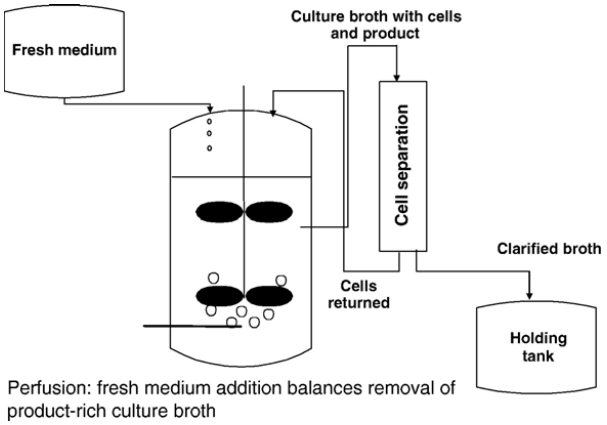

Fig. 2. Schematic representation of a perfusion culture system. Fresh nutrient solution is supplied to the vessel at the same rate that it is withdrawn. Before the spent culture is removed, however, the biomass is removed and returned to the culture.

In perfusion culture systems fresh medium is added continuously to the reactor and spent medium, containing product, is continuously removed. In these systems, cells are retained in the reactor and a variety of retention devices have been described which may be internal or external to the reactor [35]. An example of a 500 L industrial perfusion system for monoclonal antibody production is described by Deo et al. [36]. They were able to operate this type of system for 15 to 35 days for the production of multikilogram quantities of antibody. The authors claim that the system has a throughput of antibody, which is approximately 10 times higher than can be achieved in a batch or fed-batch system. A disadvantage of the perfusion system is the additional time and complexity involved in developing the process.

At small scale, increasing use is being made of disposable bag systems including reactors and holding tanks, particularly to meet early development needs. One such reactor system which uses wave induced agitation and is commercially available is described by Singh [37]. The use of disposables reduces the need for cleaning and sterilisation with significant economic benefits [38]. Such systems are also finding application in, for example, the inoculum stages of large-scale culture.

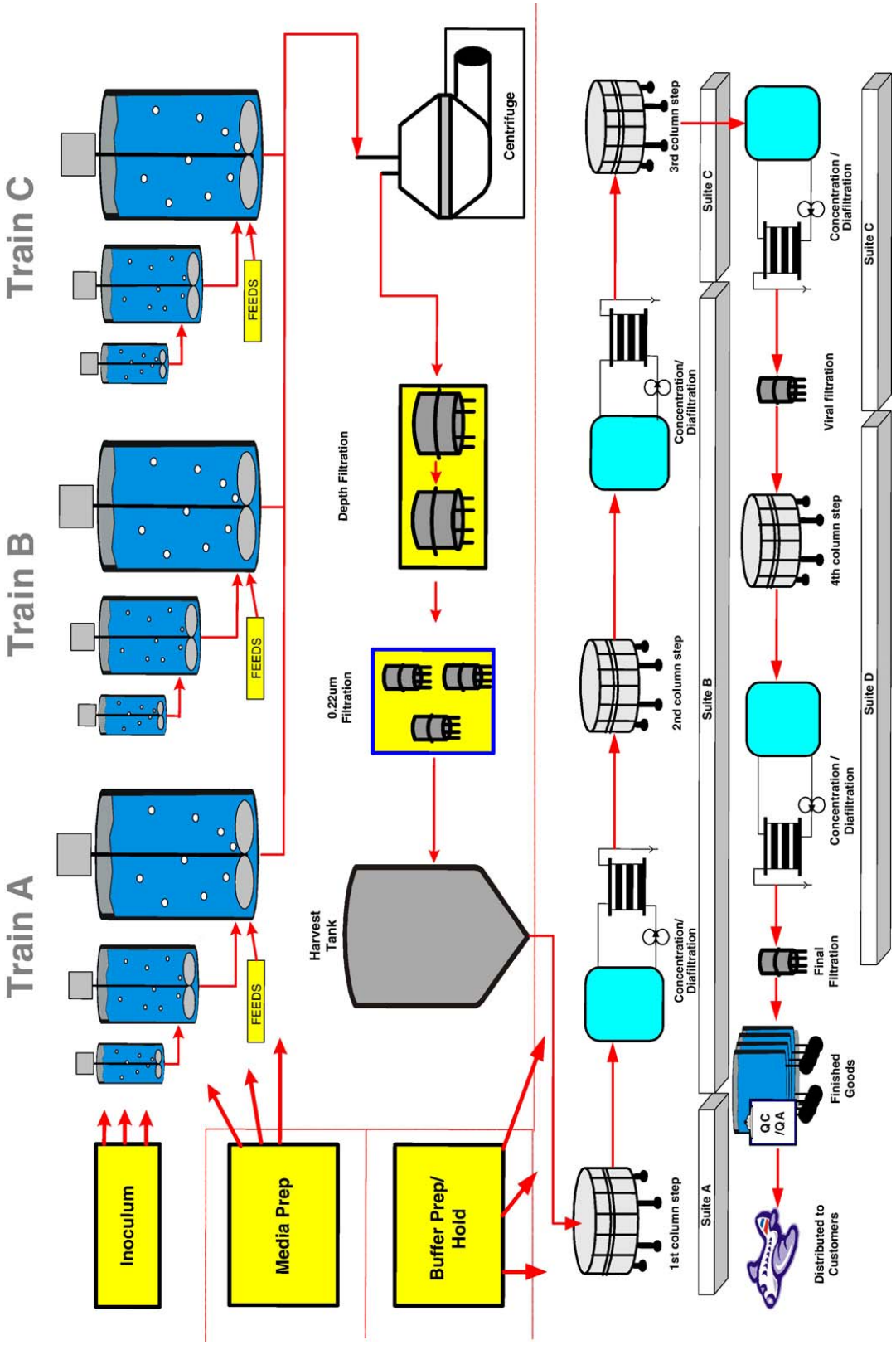

Fig. 3. Process flow for a large-scale facility for manufacturing proteins (reproduced courtesy of Lonza Biologics Inc.).

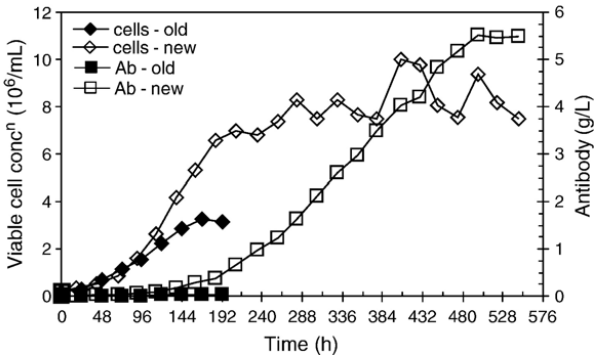

Fig. 4. Growth and productivity of two GS-CHO cell lines making the same chimeric antibody: comparison of a process from 1990 ('old') and from 2005 ('new').

7.1. Steps in a fed-batch process

Cell culture is typically done in stirred stainless steel tanks. Airlift reactors up to 5000 L scale have also been used but are much less common [39]. Detailed aspects of the issues taken into account in reactor design are summarised by Varley and Birch [40]. It is normal to control pH, dissolved oxygen concentration and temperature in reactors and careful consideration has to be given to the design of vessels to ensure adequate mixing and mass transfer of oxygen and CO_2. By way of example, addition of pH control agents to the surface of the culture can lead to high local departures from the setpoint pH value [41]. This can be avoided by adding the agent in a well mixed zone of the reactor. It is important to have laboratory and pilot reactors that can be used to predict large-scale reactor performance. Dissolved oxygen concentration is regulated by the controlled sparging of air into the fermenter and pH is controlled by the addition of CO_2 gas or an alkaline solution (e.g., NaOH) as relevant.

Fig. 3 shows the steps in a production process based on Lonza's facility in New Hampshire, USA which has three 20,000 L reactors. Cells from a frozen working cell bank are expanded through small reactors into inoculum vessels in the production train. The 20,000 L vessels are operated in fed-batch mode and, at the end of the batch cycle, the contents of the reactor are clarified through a centrifuge and through filters prior to purification in a series of chromatography steps.

7.2. Culture media

There has been rapid progress in recent years in the development of serum-free media, driven principally by concerns about the possible introduction of adventitious agents in animal derived ingredients. Initially, serum was replaced by serum proteins such as albumin, transferrin and insulin, and latterly it has been possible, in many cases, to develop chemically defined, protein-free media which contain no animal derived materials. Frequently, the serum protein was acting as a carrier of an essential nutrient such as a lipid, which could be used to substitute for the protein. Defined media are inherently less expensive and make downstream processing more straightforward as there are fewer contaminants to monitor and remove. Process optimisation is also easier in a defined environment. Protein-free culture media for the commonly used industrial cell lines such as CHO and NS0 are commercially available and there are descriptions of such media in the literature (e.g., [33,42,43]). For suspension culture, it is common to add a synthetic polymer to the culture medium to act as a protectant against the damage to cells that would otherwise be caused by sparging gases into the reactor [44]. Antifoam is used to prevent foaming caused by sparging.

Table 2
The evolution of a fed-batch process for antibody producing GS-CHO cell lines showing the improvement in productivity

Process	Antibody (mg/L)	Antibody (fold increase)	Volumetric productivity (mg/L/day)
Original cell line (CHO-K1), original process	139	–	13
Iteration 1	334	2	33
Iteration 2	585	4	42
New cell line (CHOK1SV), iteration 2	1917	14	128
Iteration 3	2829	20	189
Iteration 4	3560	26	237
Iteration 5	4301	31	215
New clone, iteration 5	5520	40	240

7.3. Optimisation of culture conditions

Improvements in recombinant protein production in cell culture have been dramatic. In a review of progress in this area, Wurm [3] noted that concentrations have increased 100-fold since 1980. Some of this progress has resulted from improvements in expression technology and strain selection as noted earlier, but an equally significant contribution has come from optimisation of the fermentation processes. In Fig. 4, we show current growth and productivity profiles for a GS-CHO cell line making a chimeric antibody compared with data for a process we were using in 1990. Productivity is over 100-fold higher in the current process. It can be seen that there has been a dramatic increase in cell concentration in the reactor and the cells can be maintained in a viable state for a much longer period. This is crucial because productivity is a function of the specific production rate of the cells and the integral of the viable cell count with respect to time (IVC). Cell concentrations exceeding 10^7/mL are now commonly seen in reactors.

Much of this improvement has come from the optimisation of media and feeds based on a better

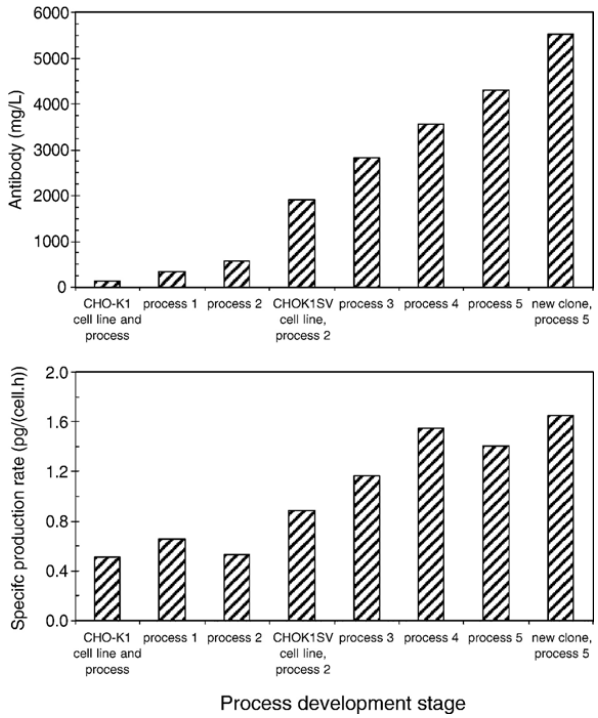

Fig. 5. Improvement in Q_p and antibody concentration during the development of a fed-batch process for GS-CHO cell lines.

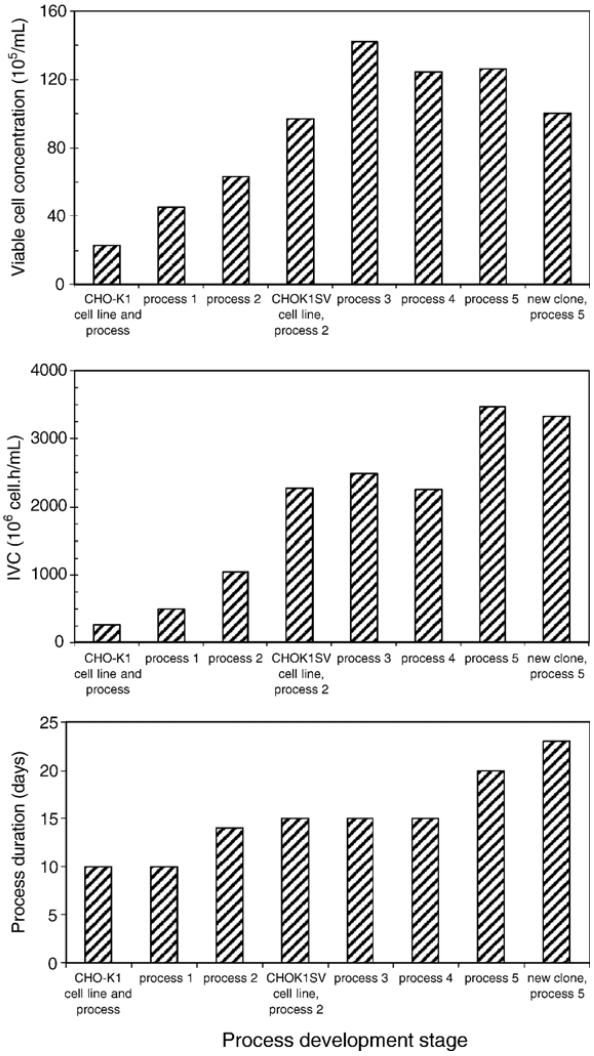

Fig. 6. Improvement in growth kinetics for an antibody-producing GS-CHO process using a fed-batch culture system.

quantitative understanding of cells' nutritional requirements. This understanding is often based on iterative analysis of nutrient depletion followed by supplementation of the relevant nutrient in the medium or feed. Strategies for medium and feed development have been reviewed [5,45]. The overall aim of the fed-batch approach is to increase maximum cell concentrations in the reactor and to prolong the production phase. Dempsey et al. [46] and Sauer et al. [47] give examples of NS0 and Sp2/0 processes in which they improved productivity by approximately an order of magnitude.

Another factor to be taken into consideration in designing media and feeds is the potentially toxic effect

of accumulated catabolites such as ammonium and lactate. Various strategies have been adopted to minimise accumulation of these growth inhibitors. It is possible to use stoichiometric feeding of key energy substrates (glutamine and glucose) to reduce accumulation of catabolites [47,48,49]. In the case of ammonium, the problem can be substantially reduced by eliminating the main source (glutamine) from the culture medium. This is one of the advantages of using cell lines, which have glutamine synthetase and do not require glutamine. It has also been found that CO_2 concentrations can reach inhibitory levels in cell culture (e.g., [50]). It is important to ensure that gas exchange systems in reactors can remove CO_2 adequately. It has also been found that osmolality can reach inhibitory levels for example because of addition of feeds and pH control agents. The situation is made more complex by the fact that many parameters interact. For example elevated osmolality can lead to an increase in rates of production of lactate and ammonium [50].

Table 2 shows the effect of iterative process improvement for a GS-CHO process making a chimeric IgG_4 antibody. A 40-fold improvement in productivity was achieved in part as a result of optimising the culture process and in part due to the introduction of a more productive cell line based on a suspension variant of CHO.

Figs. 5 and 6 show the effect of optimisation on various aspects of growth and productivity. It can be seen that improvements were made to antibody concentration, Q_p, maximum viable cell concentration and IVC. The improvements were achieved without a dramatic increase in process duration. The overall objective is to increase throughput in the plant and hence attention is focused on those parameters that increase yield without increasing culture duration, e.g., Q_p and maximum cell population density.

7.4. Impact of process improvements on product

Any process improvement programme must take account of possible product changes, such as aggregation, altered glycosylation or degradation resulting from modifications to the process. There are several examples in the literature of such changes. Patel et al. [51] for example showed that the glycosylation pattern of an IgG made from a murine hybridoma varied depending on the culture system which was used. Muthing et al. [52] discuss the effect of pH on glycosylation of a murine antibody. Galactosylation of antibody glycans varied with pH.

8. Alternative production systems

All licensed antibodies and the majority of those in clinical development are currently made in mammalian cell culture. There is however continuing interest in alternative processes that might provide less complex and less expensive alternatives in the longer term. Good progress has been made in the production of both whole antibodies and antibody fragments in bacteria and in fungi. Humphreys [53] and Bowering [54] describe methods for producing antibody fragments in E. coli including procedures for subsequent pegylation of the fragments to decrease rate of clearance in vivo. Antibody fragments are more readily produced in microbes than are whole antibodies. As stated above, Reichert et al. [2] note that of the 15 antibodies they identified in Phase III trial, three were single chain fragments and three were Fabs. The production of whole antibodies, albeit non-glycosylated, in *E. coli* has been described by Simmons et al. [55]. Antibody concentrations exceeding 100 mg/L were reported in a process in which the antibody was secreted into the periplasm of the bacterium. This approach may be useful for indications where glycosylation is not needed for biological activity. Production of antibodies has also been studied in yeasts and in filamentous fungi such as *Aspergillus* [56]. There is already experience in using these organisms for protein production, including very large-scale manufacture of industrial enzymes in the case of *Aspergillus*. Although fungi can glycosylate proteins, the resultant glycans are not the structures one would normally find on human proteins. However, good progress is being made in the resolution of this problem and Choi et al. [57] have described the use of a genetic engineering approach to humanize the glycosylation of the yeast *Pichia pastoris*. Apart from microbial systems considerable effort has gone into the evaluation of transgenic production systems particularly those based on plants [58] and transgenic milk from animals [59]. Most recently, eggs have been discussed as a possible production route. Given the rapid

progress made in cell culture it remains to be seen what impact the transgenic route will make, particularly given the fact that downstream costs make up a significant proportion of total manufacturing costs and are likely to be similar regardless of the production method. Chadd and Chamow [60] discuss the economics of different production routes.

9. Downstream processing of antibodies

Large-scale purification of monoclonal antibodies is based on chromatography. Protein A affinity purification is used in the majority of cases in combination with at least one ion exchange step. It is common for there to be at least one and sometimes more polishing steps using ion exchange, hydrophobic interaction and/or size exclusion chromatography. The steps are designed to remove contaminant proteins from cells or media to ppm levels and DNA to ppb levels. Depending on the process there may be additional specific contaminants (e.g., leached protein A) to be removed. In addition to contaminants, it may also be necessary to remove undesirable derivatives of the product itself such as degradation products and aggregates. For mammalian cell processes, one also has to take account of potential virus risks and establish the ability of the purification steps to remove a range of virus types. In addition, at least two virus removal/inactivation steps are included, typically based on filtration, low pH treatment and, sometimes, use of solvent/detergent. Typical yields from a purification process for antibodies are in the range 60–80% depending on the number of steps. For a general discussion of purification process steps and their relevance to removal of different contaminants, see Birch et al. [39] and Berthold and Walter [61].

With increasing upstream concentrations increasing attention is being paid to downstream recovery because this becomes a significant proportion of total cost and it can also limit overall plant throughput. If we consider just the issue of buffers for chromatography, the volumes used can be an order of magnitude higher than those upstream. Hence, we are seeing improvements to reduce volumetric handling as well as, for example, the introduction of improved chromatography matrices, which allow increased throughput and the use of rapid membrane separation steps.

10. Conclusions

The demand for monoclonal antibodies seems set to increase for the foreseeable future. Pavlou and Belsey [62] project that the market will grow by 20.9% per year to reach $16.7 bn in 2008. This is likely to be matched by increased global capacity for mammalian cell culture on the one hand and by technological progress on the other. It is likely that mammalian cell culture will be dominant for the immediate future and that improvements will continue to be made in process efficiency. This will result from improvements to the inherent productivity of cell lines which in the future will be driven to a greater extent by a better understanding of the fundamental biology of the cell, informed by the systems biology that is evolving from the various ‘omics’ approaches. It is generally believed that such improvements combined with further progress in media and feed development will lead to antibody concentrations of at least 10 g/L. As concentrations increase, downstream processing will become a much more significant component of cost and this will be a driver to develop more efficient processes, potentially using radically different approaches to those used now. It also seems likely that other expression technologies, especially those based on bacteria and/or fungi will become more dominant not just for fragments but potentially for whole antibodies as well. A final factor that will impact on process volumes and cost of goods is product potency. Some of the recent progress in improving effector functions by glycosylation engineering demonstrates the potential in this area.

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a state of ferment : AGITATE, EXCITE, FOMENT ⟨quick-spreading rumors ~*ed* the city and violence soon broke out⟩

²fer·ment \'fər,ment, 'fə,m-, 'fə,m- *sometimes* ,=ˈ= *or* _fə(r)'m- *or chiefly Brit* '=_mənt\ *n* -S [ME, fr. L *fermentum* leaven, yeast — more at BARM] **1 a :** an agent capable of bringing about fermentation and other metabolic processes: (1) **:** a living organism (as a yeast or bacterium) that acts by virtue of its enzymes — called also *organized ferment;* used chiefly commercially; compare STARTER 3d (2) **:** ENZYME — called also *unorganized ferment* **b :** a person or thing that stimulates agitation or the active working out of change in an individual or society ⟨the possessive instinct, the most violent of ~s —Havelock Ellis⟩ ⟨the active ~ at work in China . . . was that of nationalism —*Times Lit. Supp.*⟩ **2 a :** FERMENTATION 1 **b :** a state of unrest **:** AGITATION, EXCITEMENT, TUMULT ⟨that ~ in the air which accompanies an election —John Buchan⟩ ⟨she was thrown into a ~ by his unexpected arrival⟩; *also* **:** a process of active often disorderly development in an individual life or in a society **:** the painful or disturbing transition from old to new ⟨a continent in ~, awakening to a new era after centuries of stagnation —Tad Szulc⟩ ⟨the great period of creative ~ in literature —William Barrett⟩

fer·ment·abil·i·ty \(,)fər,mentə'bilədē, -latē, -i\ *n* -ES **:** the quality or state of being fermentable

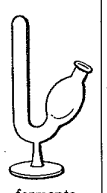
fer·ment·able \(,)fər'mentəbəl\ *adj* **:** capable of undergoing esp. alcoholic fermentation

fer·men·tal \(')fər'ment²l\ *adj* [²*ferment* + *-al*] **:** FERMENTATIVE

fermentate *vt* -ED/-ING/-S [L *fermentatus*, past part.] *obs* **:** to cause to ferment

fer·men·ta·tion \,fərmən·'tāshən, ,fōm-, ,fə̄m-, ,fə̄m-, ,men-\ *n* -S [ME *fermentacioun*, fr. LL *fermentation-, fermentatio*, fr. L *fermentatus* (past part. of *fermentare* to cause to rise or ferment) + *-ion-, -io* -ion — more at FERMENT] **1 a :** a chemical change accompanied by effervescence and suggestive of changes produced in organic materials by yeasts **b :** any of various enzymatic transformations of organic substrates (as the formation of alcohol from sugars or of vinegar from cider or the souring of milk); *esp* **:** a transformation of a carbohydrate material that yields such products as alcohols, acids, and carbon dioxide and that typically involves decomposition without the participation of oxygen — see ALCOHOLIC FERMENTATION; compare GLYCOLYSIS **c** (1) **:** any of various controlled aerobic or anaerobic processes used for the manufacture of certain products (as alcohols, acids, vitamins of the B complex, or antibiotics) by the action usu. of yeasts, molds, or bacteria (2) **:** any of various industrial processes for improving esp. flavor, aroma, or quality (as of tea, tobacco, or cheese) by means of fermentation **2 :** FERMENT 2b

fermentation tube *n* **:** a modified culture tube with an upright closed arm for collecting gas formed in broth cultures by microorganisms



fermentation tube

fer·ment·ative \fə(r)'mentəd·iv\ *also* **fer·men·tive** \-entiv\ *adj* **1 :** causing or having power to cause fermentation ⟨the ~ substance in yeast⟩ **2 :** of or produced by fermentation ⟨the ~ process⟩ ⟨~ gases⟩ **3 :** FERMENTABLE

fer·ment·er *pronunc at* ¹FERMENT+ə(r)\ *n* -S **1 :** one that ferments: as **a :** a worker who attends a fermentation process (as of moistened tobacco or of mash for beer) **b :** an organism that causes fermentation **2** *or* **fer·men·tor** \"\ **a :** a vessel in which mash is fermented during the brewing process **:** a fermenting tank **b :** a laboratory apparatus for carrying out fermentation

fer·men·tes·ci·ble \,fərmən·'tesəbəl\ *or* **fer·men·tis·ci·ble** \-tis-\ *adj* [*fermentescible* fr. L *fermentescere* to swell, rise, ferment (fr. L *fermentum* leaven, yeast + *-escere*, incho. verb ending) + *-ible; fermentiscible* irreg. fr. L *fermentescere* + E *-ible* — more at BARM] **:** FERMENTABLE

fer·ment·tol·o·gist \,fər=·'täləjəst\ *n* -S **:** a specialist in fermentology; *specif* **:** a chemist who experiments with ingredients and production processes of alcoholic beverages in order to control and improve taste, color, odor, and other characteristics — called also *oenologist*

fer·men·tol·o·gy \-əjē\ *n* -ES [²*ferment* + *-alogy*] **:** a science that deals with ferments and fermentation — compare ENZYMOLOGY

ferments *pres 3d sing of* FERMENT, *pl of* FERMENT

fer·me·ture \'fərmə,chu̇(ə)r\ *n* -S [F, lit., act of closing, apparatus for closing, fr. MF, fr. ML *firmatura* lock, clasp, fr. L *firmatus* (past part. of *firmare* to make fast) + *-ura* -ure — more at FIRM (v.)] **:** the mechanism closing the breech of a breech-loading firearm

fer·mi·di·rac distribution \,fer(,)mēdə,rak—\ *n, usu cap F&D* [after Enrico *Fermi* †1954 Ital. physicist and Paul A. M. *Dirac b*1902 Eng. physicist] **:** an assumed statistical distribution of speeds among the electrons responsible for thermal conduction in metals

fermi-dirac statistics *also* **fermi statistics** *n, usu cap F&D* **:** quantum-mechanical statistics according to which subatomic particles of a given class (as electrons, protons, and neutrons) have a quantum-mechanical symmetry that makes it impossible for more than one particle to occupy any particular quantum-mechanical state — compare BOSE-EINSTEIN STATISTICS

fer·miere \fermyeer\ *adj* [F *(à la) fermière* in the manner of the farmer's wife] *of a food* **:** prepared in plain country style

fer·mi·on \'fermē,än, 'fər-\ *n* -S [Enrico *Fermi* + E *-on*] **:** a particle (as an electron, proton, or neutron) having a half-odd-integer number of quantum units of spin and conforming to the Fermi-Dirac statistics

fer·mi·um \-ēəm\ *n* -S [NL, fr. Enrico *Fermi* + NL *-ium*] **:** a radioactive metallic element artificially produced (as by bombardment of plutonium with neutrons) — symbol *Fm;* see ELEMENT table

fer·mor·ite \'fərmə,rīt\ *n* -S [Lewis L. *Fermor* †1954 Eng. geologist + E *-ite*] **:** a mineral $(Ca,Sr)_5[(As,P)O_4]_3$ that consists of an arsenate, phosphate, and fluoride of calcium and strontium and that is related to apatite and found in white crystalline masses

fern \'fərn, 'fən, 'fəin\ *n* -S *often attrib* [ME *fern, ferne*, fr. OE *fearn;* akin to OHG *farn* fern, MIr *raith* fern, Skt *parṇa* wing, feather, leaf, and perh. to OE *faran* to travel — more at FARE] **1 a :** any of numerous nonflowering vascular plants constituting a class (Filicineae) of the division Tracheophyta; *esp* **:** a plant of the order Filicales resembling seed plants in being differentiated into root, stem, and leaflike fronds and in having vascular tissue but differing in reproducing by spores that are borne usu. in sori on fertile fronds or fertile portions of vegetative fronds and that upon germination commonly produce a flat typical thallus which produces antheridia and archegonia upon its surface, the egg of the archegonium giving rise to the sporophyte which is the conspicuous generation in the life cycle — see FROND illustration **b :** a frond of a fern **c :** a growth or quantity of ferns ⟨admiring the ~ of the park⟩ ⟨decorated with white roses banked with ~⟩ **2 :** any of various plants with fernlike foliage — usu. used in combination ⟨asparagus ~⟩ ⟨sweet ~⟩

fern ally *n* **1 a :** a pteridophyte other than a member of the order Filicales **b :** any of various pteridophytes (as horsetails or club mosses) that are not leafy in habit as distinguished from the leafy true ferns **2 :** WATER FERN 1

fer·nam·bu·co wood \,fərnəm,b(y)ü(,)kō-, ,fernəm,bü(,)kō-\ *n, usu cap F* [fr. *Fernambuco* (now *Pernambuco*), state of Brazil, fr. Pg] **:** PERNAMBUCO WOOD

fer·nan·de·ño \,fərnən'dān(,)yō, ,fer-\ *n, pl* **fernandeño** *or* **fernandeños** *usu cap* [Sp *fernandeño*, fr. San *Fernando*, Franciscan mission in Los Angeles county, Calif. + Sp *-eño* (suffix added to place names to form names of inhabitants)] **1 :** a Shoshonean people of the valley of the Los Angeles river, California **2 :** a member of the Fernandeño people

fer·nan·di·nite \,fərnən'dē,nīt\ *n* -S [Eulagio E. *Fernandini*, 20th cent. Peruvian mine owner + E *-ite*] **:** a mineral consisting of a massive dull green hydrous calcium vanadyl vanadate

fern asparagus *n* **:** ASPARAGUS FERN

fern ball *n* **:** a ball composed of the compacted rhizomes of several small drooping ferns that is usu. imported in a dry dormant condition from Japan for use in house decoration — see BALL FERN

fernbird \'=,=\ *n* [*fern* + *bird*] **:** a small passerine bird (*Bowdleria punctata*) of New Zealand that frequents marshy ground and is becoming rare

fernbrake \'=,=\ *n* [*fern* + *brake* (thicket)] **:** a dense growth of ferns

fern-bush \'=,=\ *n* **:** a low densely branched very leafy white-flowered shrub (*Chamaebatiaria milbefolium*) of the family Rosaceae that is widely distributed in dry uplands of the western U.S. — called also *desert sweet*

fern clubmoss *n* **:** an epiphytic Australasian fern ally (*Tmesipteris tannensis*) with large lanceolate green leaves that grows on trunks of tree ferns

fern cycad *n* **:** CYCAD FERN

ferned \'fərnd, 'fənd, 'fəind\ *adj* **:** abounding in or covered with ferns

fer·nent \fə(r)'nent\ *or* **fer·ninst** \-'nin(t)st, -'ninzt\ *var of* FORNENT

fern·ery \'fərn(ə)rē, 'fən-, 'fəin-, -ri\ *n* -ES **1 a :** a place where ferns are growing **b :** a planter for ferns **2 :** a collection of growing ferns

fernery

fern·flö·te \'fern,flœ̄tə\ *n* -S [G, fr. *fern* far (fr. OHG *ferrana* from far, fr. *ferro* far) + *flöte* flute — more at FAR, BLOCKFLÖTE] **:** a very soft organ pipe of flute tone and 8-foot or 4-foot pitch

fern fruit *n* **:** SORUS a

ferngale \'=,=\ *n* [*fern* + *gale* sweet gale, fr. ME *gale, gayl*, fr. OE *gagel;* akin to MLG & MD *gagel* sweet gale] **:** SWEET FERN 1a

fern green *n* **:** a moderate yellow green that is greener and paler than average moss green, duller than average pea green, and duller and very slightly greener than apple green (sense 1)

fernleaf \'=,=\ *n, pl* **fernleaf 1 :** a delicate red alga (*Callithamnion gracillimum*) with finely divided thallus **2 :** a disease of tomatoes caused by the cucumber mosaic virus and characterized by mottling and fernlike narrowing of the leaves

fernlike \'=,=\ *adj* **:** resembling a fern esp. in leaf shape

fern moss *n* **:** any of various fernlike mosses esp. of the genus *Thuidium*

fern owl *n* **:** a nightjar (*Caprimulgus europaeus*)

fern palm *n* **:** any of several cycads with palmlike foliage

fern poisoning *or* **fern staggers** *n* **:** BRACKEN POISONING

ferns *pl of* FERN

fern scale *n* **:** a tropical armored scale (*Pinnaspis aspidistrae*) common on potted ferns and in greenhouses

fern seed *n* **:** the dustlike asexual spores of ferns that were formerly taken for seeds and reputed to render one invisible

fern·tick·le \'fern,tikəl\ *n* -S [ME *ferntikel, ferntikill*, fr. *fern* + *-tikel, -tikill*, prob. fr. L *-ticula* (as in *lenticula* lentil, group of freckles) — more at LENTIL] *n, chiefly Scot* **:** FRECKLE — **fern·tick·led** \-kəld\ *adj*

fern tree *n* **:** TREE FERN

fern weevil *n* **:** a weevil (*Syagrius fulvitarsis*) of Australia and the Pacific islands that feeds on ferns

fernwort \'=,=\ *n* **:** a plant belonging to the Pteridophyta **:** FERN ALLY **:** FERN

ferny \'fərnē, 'fənē, 'fəinē, -ni\ *adj, usu* -ER/-EST [ME, fr. *fern* + *-y*] **1 :** of or abounding in ferns **2 :** FERNLIKE ⟨the ~ shadows of locust leaves —W.V.T.Clark⟩

fer·o·cac·tus \,fer·ə'kaktəs, ,fir-\ *n, cap* [NL, fr. *fero-* (fr. L *ferus* wild, fierce) + *cactus*] **:** a genus of nearly globular deeply ribbed cacti of Mexico and the adjacent U.S. having numerous spines, large funnel-shaped flowers, and dry fruits

fe·ro·ce \fā'rō(,)chā\ *adj* [It, fr. L *feroc-, ferox*] **:** FIERCE, FEROCIOUS — used as a direction in music

fe·ro·cious \fə'rōshəs\ *adj* [L *feroc-, ferox* fierce (fr. *ferus* wild, fierce + *-oc-, -ox* looking, appearing — akin to L *oculus* eye) + E *-ious* — more at FIERCE, EYE] **1 a :** characterized by wild or extreme rapacity, cruelty, acrimony, or destructiveness **:** violently aggressive **:** BLOODTHIRSTY ⟨a ~ tiger⟩ ⟨the raiders' ~ butchery of women and children⟩ **:** BITTER ⟨the ~ word battles he has had with other editors⟩ **:** DEVASTATING ⟨the ~ torrents of the flood⟩ **b :** suggesting a ferocious character or mood **:** FORMIDABLE ⟨a ~ beard⟩ ⟨a ~ smile⟩ **2 :** very great **:** EXTREME, EXCESSIVE ⟨he was a ~ bore⟩ **:** FURIOUS ⟨sought to forget his troubles through ~ activity⟩ ⟨a ~ wind swept the sea⟩ **syn** see FIERCE

fe·ro·cious·ly *adv* **:** in a ferocious manner ⟨pounces ~ on a trivial error of fact —C.W.Shumaker⟩ ⟨a ~ logical system⟩

fe·ro·cious·ness *n* -ES **:** FEROCITY

fe·roc·i·ty \fə'räsəd·ē, -sətē, -i\ *n* -ES [F & L; F *férocité*, fr. L *ferocitas*, fr. *feroc-, ferox* fierce + *-itas* -ity] **1 :** the quality or state of being ferocious **:** savage wildness **:** FURY ⟨turned on them with a ~ which made a savage of him on the spot —Virginia Woolf⟩ **:** extreme or furious intensity **:** ARDOR ⟨a wild ~ of joy overcame him —Liam O'Flaherty⟩ **2 :** an instance of ferocity ⟨has his great sentimentalities to compensate for his chronic *ferocities* —Edmund Wilson⟩

-fer·ous _f(ə)rəs\ *adj comb form* [ME, fr. L *-fer* & MF *-fere* (fr. L *-fer*) + E *-ous* — more at -FER] **:** bearing **:** producing **:** yielding ⟨auri*ferous*⟩ ⟨ovuli*ferous*⟩ — almost always preceded by *i* — **-fer·ous·ly** *adv comb form* — **-fer·ous·ness** *n comb form* -ES

fer·rai·o·lo·ne \fə,rīə'lōnē\ *n* -S [It, aug. of *ferraiolo* large mantle, cloak, prob. fr. Ar *feryūl* wool cape, fr. L *palliolum* small Greek mantle, dim. of *pallium* Greek mantle — more at PALL] **:** a large full length cloak having a large flat collar, varying in color according to the wearer's rank, and forming the necessary complement of full ecclesiastical dress among Roman Catholic clergy on nonliturgical occasions (as an academic ceremony or papal audience)

fer·ra·ra \fə'rärə\ *adj, usu cap* [fr. *Ferrara*, Italy] **:** of or from the city of Ferrara, Italy **:** of the kind or style prevalent in Ferrara

¹fer·ra·rese \fə'rä,rēz, -ēs, ,ferə'r- *adj, usu cap* [It, adj. & n., fr. *Ferrara* Italy + It *-ese*] **1 :** of, relating to, or characteristic of Ferrara, a city in Italy **2 :** of, relating to, or characteristic of the people of Ferrara

²ferrarese \"\ *n, pl* **ferrarese** *cap* **:** a native or resident of Ferrara, Italy

ferrash *var of* FERASH

fer·rate \'fe,rāt\ *n* -S [ISV *ferr-* (fr. L *ferrum* iron) + *-ate*] **:** any of various classes of compounds containing iron and oxygen in the anion or regarded as so constituted: as **a :** a strongly oxidizing dark red salt analogous to the chromates and sulfates and formed in various ways (as by heating iron filings with a nitrate) ⟨potassium ~ K_2FeO_4⟩ — called also *ferrate(VI)* **b :** FERRITE

fer·rei·ro \fə'rā(,)rō\ *n* -S [Pg, lit., blacksmith, fr. L *ferrarius*, fr. *ferrum* iron + *-arius* -ary — more at FARRIER] **:** a Brazilian tree frog (*Hyla faber*) that produces notes resembling measured beating on a copper plate

fer·rel \'ferəl\ *archaic var of* FERRULE

fer·rel's law \'ferəlz-\ *n, usu cap F* [after William *Ferrel* †1891 Am. meteorologist, its formulator] **:** a statement in meteorology: a wind in any direction tends to deflect to the right in the northern hemisphere and to the left in the southern with a force that is directly proportional to the mass of wind in question, its velocity, the sine of the latitude, and the angular velocity of the earth's rotation

fer·re·ous \'ferēəs\ *adj* [L *ferreus*, fr. *ferrum* iron] **:** of, like, or containing iron

ferrer *n* -S [ME *ferrour* blacksmith who shoes horses, veterinarian — more at FARRIER] **1** *obs* **:** IRONSMITH **2** *obs* **:** FARRIER

¹fer·ret \'ferət, *usu* -ədˌ+V\ *n* -S [ME *feret, ferret, furet*, fr. MF *furet, fuiret*, fr. (assumed) VL *furittus*, lit., small thief, dim. of L *fur* thief — more at FURTIVE] **1 a :** a semidomesticated variety of the European polecat sometimes treated as a separate species (*Mustela furo*) that is usu. albino with red eyes and is much used for hunting rodents and sometimes rabbits in Europe and occas. in the U.S. **b :** BLACK-FOOTED FERRET **2 :** a person who searches actively and persistently (as for incriminating information) ⟨German ~s who constantly spied on the Allied prisoners of war⟩ **3 :** an airplane equipped to detect a radar installation and analyze its signals

²ferret \"\ *vb* -ED/-ING/-S [ME *fereten, fureten*, fr. *feret, furet*, n.] *vt* **1 :** to hunt with a ferret: **a :** to hunt over ⟨they have *ferreted* the duke's fields⟩ **b :** to hunt for **:** TAKE *esp* **:** to drive esp. from covert ⟨they *ferreted* a number of rabbits⟩ **2 :** to worry or harry as with a ferret ⟨the king kept *ferreting* the rebellious baron⟩ ~ *vi* **1 :** to hunt game or drive out vermin with a ferret ⟨some U.S. states have laws against *ferreting*⟩ **2 :** to search carefully or diligently and sometimes presumptuously **:** search about **:** PRY ⟨old-fashioned . . . to go *ferreting* into people's pasts —Virginia Woolf⟩

³ferret \"\ *also* **fer·ret·ing** \-əd·iŋ\ *n* -S [*ferret* fr. earlier *ferret silk*, prob. modif. of It *fioretti* floss silk, fr. pl. of *fioretto* small flower, dim. of *fiore* flower, fr. L *flor-, flos; ferreting* fr. *ferret* + *-ing* — more at BLOW (blossom)] **1 :** a narrow silk tape or ribbon for trimming or decorative lacing **2 :** a strong tape of cotton or wool for binding or shoelaces

ferret-badger \'=,=\ *n* **:** any of several heavy-bodied mammals (as *Helictis moschata*) of southeastern Asia that resemble the weasel — called also *pahmi*

fer·ret·er \'ferəd·ə(r)\ *n* -S [ME *fereter, fureter*, fr. *feret, furet* ferret + *-er*] **:** one that ferrets ⟨paid a ~ to drive out the rats⟩

ferret out *vt* **:** to find or uncover with keen, diligent, crafty, or shrewd search ⟨*ferret out* the enemies of the country⟩ ⟨*ferret* the facts *out* after hours of painstaking examination of records⟩ **syn** see SEEK

ferret-polecat \'=ˌ=ˈ=,=\ *n* **:** an unusually vicious ferret valuable as a rodent destroyer, closely resembling the wild European polecat, and said to result from interbreeding the domestic ferret with the wild polecat

fer·rety \'ferəd·ē\ *adj* [¹*ferret* + *-y*] **:** suggestive of a ferret ⟨into his ~ eyes there came a gentler look —Norman Douglas⟩

ferri- *comb form* [L *ferri-*, fr. *ferrum* iron] **1 :** iron ⟨*ferri*ferous⟩ **2** [*ferric*] **:** containing ferric iron ⟨ferrihemoglobin⟩

fer·ri·age *or* **fer·ry·age** \'ferēij, -ri·ij\ *n* -S [ME *feriage*, fr. *ferien* to ferry + *-age* — more at FERRY] **1 :** the act or business of transporting by ferry ⟨cross the larger streams by ~⟩ **2 :** the fare to be paid for a ferry passage ⟨no money to pay the ~⟩

fer·ri·an \'ferēən\ *adj* [*ferri-* + *-an*] **:** containing ferric iron

fer·ric \-rik\ *adj* [L *ferrum* iron + E *-ic* — more at FARRIER] **:** of, relating to, or containing iron — used esp. of compounds in which this element has a higher valence, usu. three, than in the ferrous compounds or of iron with such a valence

ferric acetate *n* **:** either of two acetates of iron used chiefly in the textile industry as mordants and formerly in medicine as tonics: **a :** the normal acetate $Fe(C_2H_3O_2)_3$ known best in solution **b :** a basic acetate $Fe(OH)(C_2H_3O_2)_2$ obtained as brownish red scales or powder

ferric ammonium citrate *n* **:** a complex salt containing varying amounts of iron, one type being obtained as red crystals or a brownish yellow powder and another type as green crystals or powder and both being used in medicine for treating iron-deficiency anemia and in photography for making blueprints

ferric chloride *n* **:** a deliquescent salt $FeCl_3$ that is obtained in anhydrous form (as by heating iron in chlorine) as dark crystals appearing red by transmitted light and green by reflected light, that forms several crystalline hydrates (as the yellow hexahydrate $FeCl_3.6H_2O$), and that is used chiefly as an oxidizing agent, as a catalyst, as an etching agent in photoengraving, as a coagulant in treating industrial wastes, and in medicine in a water solution or tincture usu. as an astringent or styptic; iron trichloride

ferric hydroxide *n* **:** any of several hydrates $Fe_2O_3.nH_2O$ of ferric oxide that are capable of acting both as bases and weak acids **:** hydrated ferric oxide: as **a :** reddish brown gelatinous precipitate obtained by adding an alkali to a ferric salt solution and often regarded as the trihydrate $Fe(OH)_3$ **b :** a red to reddish brown crystalline oxide and hydroxide FeO(OH) occurring in nature as lepidocrocite

ferric oxide *n* **:** the red or black crystalline sesquioxide of iron Fe_2O_3 that is found in nature both as hematite and as hydrated forms (as rust and limonite) and is also obtained synthetically (as by calcining ferrous sulfate or hydrated ferric oxide) and that is used chiefly as a pigment and polishing material and in the removal of hydrogen sulfide from gases — called also *iron(III) oxide;* compare IRON OXIDE a, IRON RED, ROUGE 2

ferric sulfate *n* **:** a salt $Fe_2(SO_4)_3$ that is found in nature as the hydrated minerals coquimbite and quenstedtite and is also obtained synthetically (as by oxidation of ferrous sulfate) in the white anhydrous form and that is used chiefly in making iron alums, in pickling metals, as a mordant in dyeing, and as a coagulant in treating industrial wastes

fer·ri·cyanic acid \'fe,rī, ,ferē+. . .-\ *n* [*ferri-* + *cyanic*] **:** a brown crystalline unstable acid $H_3Fe(CN)_6$ obtained by treating ferricyanides with strong acids

fer·ri·cyanide \"+\ *n* -S [ISV *ferri-* + *cyanide*] **:** a salt of ferricyanic acid obtained usu. by oxidation of a ferrocyanide — see IRON BLUE ⟨cupric ~ $Cu_3[Fe(CN)_6]_2$⟩

fer·ri·did·dle \'ferē,did²l\ *n* -S [origin unknown] *dial* **:** CHIPMUNK

ferried *past of* FERRY

¹fer·ri·er \'ferēə(r), -eriə-\ *n* -S [ME *ferier*, fr. *ferien* to ferry + *-er* — more at FERRY] **:** FERRYMAN

²ferrier \"\ *var of* FARRIER

ferries *pres 3d sing of* FERRY, *pl of* FERRY

fer·rif·er·ous \fə'rif(ə)rəs, (')fe,r-\ *adj* [*ferri-* + *-ferous*] **:** containing iron **:** iron-bearing ⟨highly ~ carbonates⟩

fer·ri·hemoglobin \'fe,rī, ,ferē+\ *n* [*ferri-* + *hemoglobin*] **:** METHEMOGLOBIN

fer·ri·magnetic \"+\ *adj* [ISV *ferri-* + *magnetic*] **:** of or relating to a class of substances (as ferrite) characterized by magnetization in which the polarization in one group of magnetic ions is antiparallel to the polarization in another group — **fer·ri·magnetism** \"+\ *n*

fer·ri·molybdite \"+\ *n* [ISV *ferri-* + *molybdite*] **:** a mineral $Fe_2(MoO_4)_3.8H_2O(?)$ consisting of hydrated iron molybdate

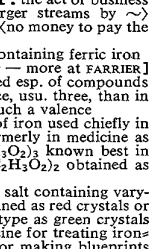
fer·ri·natrite \,fe,rī'nā·,trīt, ,ferē'-\ *n* -S [alter. (influenced by *ferri*) of *ferronatrite*, fr. *ferro-* + *natron* + *-ite*] **:** a mineral $Na_3Fe(SO_4)_3.3H_2O$ consisting of a greenish or white sodium ferric iron double sulfate usu. occurring in spherical forms

fer·ri·porphyrin \,fe,rī, ,ferē+\ *n* [*ferri-* + *porphyrin*] **:** a red-brown to black ferric derivative of a porphyrin that differs from a ferroporphyrin by the additional combination of a univalent anion (as chloride) with the iron atom

fer·ri·protoporphyrin \"+\ *n* [*ferri-* + *protoporphyrin*] **:** a ferriporphyrin in which the porphyrin is protoporphyrin — see HEMATIN, HEMIN

fer·ri·sicklerite \"+\ *n* [ISV *ferri-* + *sicklerite*] **:** a mineral $(Li,Fe,Mn)(PO_4)$ consisting of phosphate of lithium, ferric iron, and manganese with more iron than manganese and isomorphous with sicklerite

fer·ris wheel \'ferəs-\ *n, usu cap F* [after George Washington Gale *Ferris* †1896 Am. engineer who designed such a wheel for the World's Columbian Exposition in Chicago in 1893] **:** an amusement device consisting of a large power-driven wheel made in two parallel sections having seats suspended between the sections, the seats maintaining a horizontal position while the wheel rotates in a vertical plane



Ferris wheel

fer·rite \'fe,rīt\ *n* -S [L *ferrum* iron + E *-ite*] **1 :** any of several compounds formed usu. by treating hydrated ferric oxide with an alkali or by heating ferric oxide with a metallic oxide and regarded in some cases as salts of a ferric hydroxide acting in its capacity of an acid and in other cases as spinels ⟨sodium ~ $NaFeO_2$⟩ ⟨zinc ~ $ZnFe_2O_4$ has a spinel structure⟩ — called also *ferrate, ferrate(III)* **2 :** a solid solution in which alpha iron is the solvent

fer·rit·ic \fə'rid·ik, (')fe,r-\ *adj* **:** composed chiefly of ferrite ⟨~ stainless steel⟩

fer·ri·tin \'ferət²n\ *n* -S [ISV *ferr-* (fr. L *ferrum* iron) + *-ite* + *-in*] **:** an amber-colored crystalline protein that contains more than 20 percent of iron in the form of a ferric hydroxide-phosphate complex with apoferritin, that is abundant esp. in the liver and spleen, and that constitutes a body mechanism for the storage of reserves of iron

fer·ri·tize \-rə,tīz\ *vt* -ED/-ING/-S **:** to convert (as steel) into ferrite

Webster's Third New International Dictionary

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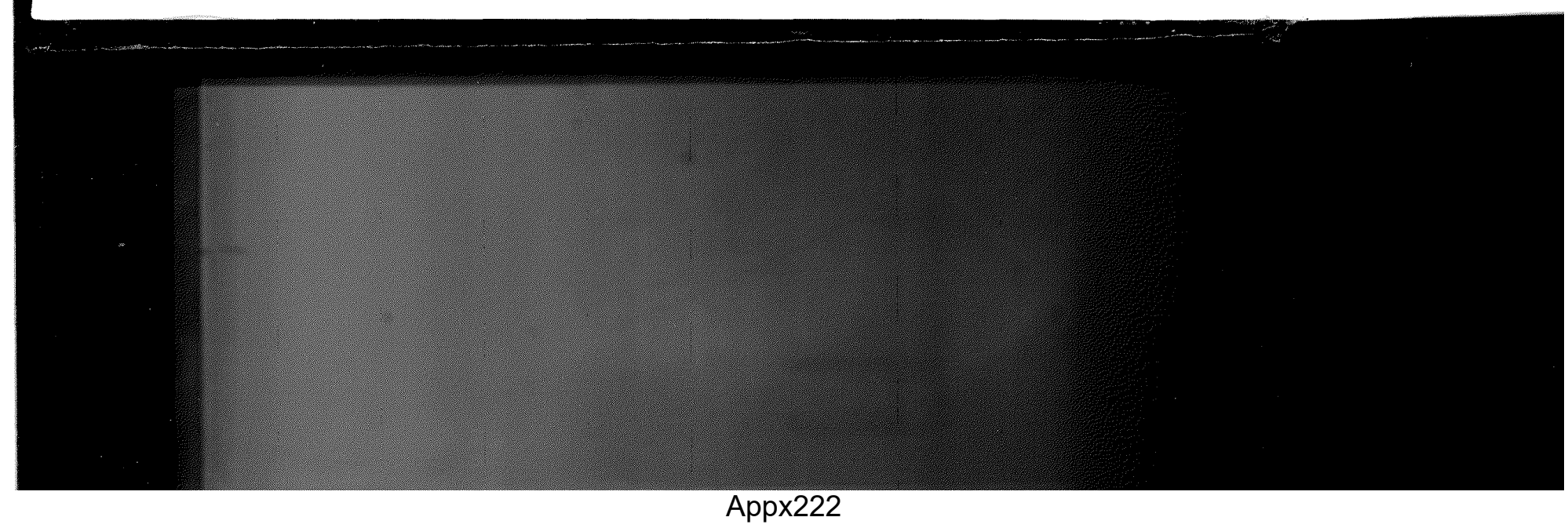

Biotechnology Inspection Guide (11/91)

BIOTECHNOLOGY INSPECTION GUIDE REFERENCE MATERIALS AND TRAINING AIDS

November 1991

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CONTENTS

BIOTECHNOLOGY INSPECTION GUIDE FOR INVESTIGATORS

INTRODUCTION

Biotechnology, defined as "the application of biological systems and organisms to technical and industrial processes", is not new. The use of yeast to ferment grain into alcohol has been ongoing for centuries. Likewise, farmers and breeders use a form of "genetic engineering" to produce improved crops and stock by selecting for desirable characteristics in plants and animals. Only recently have "new" biotechnology techniques enabled scientists to modify an organism's genetic material at the cellular or molecular level. These methods are more precise, but the results are similar to those produced with classical genetic techniques involving whole organisms. Biotechnology - derived products (BDP) used in this Guide refers to those products derived from the new biotechnology techniques.

The development of BDP and the inspection of the manufacture and control of these products offer many challenges. Because of the diversified manufacturing and control processes that are continuously being developed, considerable effort is required to achieve a level of technical competence to inspect these operations. Although the level of technology is increasing, it must be recognized that the same basic regulations and requirements are applicable to the manufacture and control of biotechnically- derived substances and devices as for "conventionally" manufactured products.

The same criteria have been used for many years in the inspection of manufacturers of antibiotics, enzymes and other high molecular weight substances including insulin, heparin, and albumin. This Guide will address some of the basic problems identified during inspections of manufacturers of BDP. Production systems may include animals, cell clones (e.g. hybridomas), mammalian and insect cell cultures, yeast, and bacteria or combinations of these systems.

A. **Objective**

b. Confirm that the sterilization cycle has been properly validated to ensure that the media will be sterile.

c. Verify that all raw materials have been tested by quality control. Determine the origin of all bovine material.

d. Document instances where the media failed to meet all specifications.

e. Verify that expired raw materials have not been used in manufacture.

f. Check that media and other additives have been properly stored.

C. **Culture Growth**

1. Inoculation and Aseptic Transfer

   Bioreactor inoculation, transfer, and harvesting operations must be done using validated aseptic techniques. Additions or withdrawals from industrial bioreactors are generally done through steam sterilized lines and steam-lock assemblies. Steam may be left on in situations for which the heating of the line or bioreactor vessel wall would not be harmful to the culture.

2. Monitoring of Growth Parameters and Control

   It is important for a bioreactor system to be closely monitored and tightly controlled to achieve the proper and efficient expression of the desired product. The parameters for the fermentation process must be specified and monitored. These may include: growth rate, pH, waste byproduct level, viscosity, addition of chemicals, density, mixing, aeration, foaming, etc. Other factors which may affect the finished product include shear forces, process-generated heat, and effectiveness of seals and gaskets.

   Many growth parameters can influence protein production. Some of these factors may affect deamidation, isopeptide formation, or host cell proteolytic processing. Although nutrient-deficient media are used as a selection mechanism in certain cases, media deficient in certain amino acids may cause substitutions. For example, when E. coli is starved of methionine and/or leucine while growing, the organism will synthesize norleucine and incorporate it in a position normally occupied by methionine, yielding an analogue of the wild-type protein. The presence of these closely related products will be difficult to separate

fragment.

DNA LIBRARY - Set of cloned DNA fragments which together represent the entire genome or the transcription of a particular tissue.

DNA POLYMERASE - An enzyme which catalyses the synthesis of double-stranded DNA from single- stranded DNA.

DNA SYNTHESIS - The formation of DNA by the sequential addition of nucleotide bases.

DNase - An enzyme which produces single- stranded nicks in DNA. DNase is used in nick translation.

ELUTION - The removal of adsorbed material from an adsorbent such as the removal of a product from an enzyme bound on a column.

ENDONULEASES - Enzymes which cleave bonds within nucleic acid molecules.

ENDOTOXIN - A heat- stable lipopolysaccharide associated with the outer membrane of certain gram- negative bacteria. It is not secreted and is released only when the cells are disrupted. When injected into humans, endotoxins produce a febrile response, leading to severe clinical problems, including death. An endotoxin unit (EU) is defined in comparison to the current USP Reference Standard Lot EC- 5. One vial of lot EC- 5 contains 10,000 EU. The official test for endotoxin is found in the USP.

ENZYMES - Proteins that act as a catalyst in biochemical reactions.

EXONUCLEASES - Enzymes that catalyze the removal of nucleotides from the ends of a DNA molecule.

FERMENTATION - An anaerobic bioprocess. Fermentation is used in various industrial processes for the manufacture of products such as alcohols, acids, and cheese by the action of yeasts, molds, and bacteria. The fermentation process is used also in the production of monoclonal antibodies.

FUSION OF PROTOPLASTS - Fusion of two cells whose walls have been eliminated, making it possible to redistribute the genetic heritage of micro-organisms.

GENE - The basic unit of heredity, which plays a part in the expression of a specific characteristic. The expression of a gene is the mechanism by which the genetic information that it contains is transcribed and translated to obtain a

PRODUCTION OF BIOLOGICALS FROM ANIMAL CELLS IN CULTURE

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MAMMALIAN CELL FERMENTATION:
Are specialized bioreactors a necessity?

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INTRODUCTION.
The expanding research in the field of mammalian cell fermentation has carried along the entry of a lot of different fermentation equipment on the market (1). Despite all the great efforts and hard work done so far, none of these systems has proven to be superior. The question therefore emerge, if such specialized bioreactors are necessary at all, for a satisfactory research on mammalian cell fermentations. We doubt it, at least as concerns laboratory-scale fermentation. Obviously there are some major problems with the cells in the area of shear-sensitivity and others (2,3), but nevertheless we believe, that taking the cells through a gentle stepwise adaption, one can grow mammalian cells in such simple equipments as shake-flasks, big spinnerflasks, spin-bar devices and modified bacterial fermentors, with allmost the same production yield, as one can get from most of the specialized mammalian cell reactors, available on the market today.
In this paper we report on some preliminary fermentations made with non-adapted cells in several different "home-made" reactors. The results are compared with fermentations done under same conditions in a Biostat MC bioreactor.
Our results show, that mammalian cells not necessesarily are so fragile and shear-sensitive as believed, and that one might change attitude toward the basic considerations for shear-forces etc. in mammalian cell fermentations.
Also it is interesting to see, that maximum specific production rate (i.e. max. production/cell in growth phase/hr) is higher in the "low-technology" systems, than in the Biostat MC.

MATERIALS & METHODS:
Cell lines: All preliminary studies was carried out with a x63-Ag8.653-derived mouse/mouse hybridoma cell line, NUC 1-4, a low-producer of monoclonal antibodies against the enzyme nuclease. The cell line was established on The Technical University of Denmark (DTH) in 1987. Further investigations used other cell lines, e.g. NUC 1-2, STI 4C6 (mouse/mouse hybridomas (DTH)) and BHK-21 cells (GBF, W.-Germany)).
Media: Standard DMEM (Biochrom) with 10% fetal calf serum was used in all experiments. There was no antibiotics added to the media.
Fermentation parameters: Batch fermentations carried out under following standard conditions: Temperature: 37°C; pH: 7,2 - 7,4; DO: 40% with regard to atm. air; CO_2: 5% and agitation was varied in the range: 60 - 180 rpm.
Equipment: As reference system we used a Biostat MC (B.Braun,

Melsungen, W.-Germany), and results from other systems (**fig. 1**, made at DTH) has been compared to the values optained from the Biostat MC. The working volume was in all cases 300-600 ml (i.e. small scale systems).

Equipment	Vessel	Temp.contr.	Stirring	speed (rpm)	Aeration
Biostat MC	round bottom	water jacket	marine impeller	80	silicone tubing
Blue-cap bottle	flat bottom	incubator	spin-bar	180	small bubbles
Plastic jar	flat bottom	incubator	spin-bar device	80	surface
Ordinary shakeflask	flat bottom	incubator	orbital shaker	100	surface
Bacterial fermentor	flat bottom	incubator	turbine impeller	80	silicone tubing

Fig. 1: *Different kind of equipment used*

RESULTS:

For the clearness of this report, only results from fermentations with the cell line NUC 1-4 is shown. It is clear from **fig. 2** that while the cells in the "low-technology" systems need quite a lag fase, before they begin to grow, the Biostat MC very fast brings the cells into growth phase. Also we see, that the amount of (viable) cells obtained in the specialized reactor are several fold higher than even the best home-made system. The high cell number brings along a relative good amount of product (**fig. 3**), but both the cell number and the yield (IgG) decreases rapidly after about 144 hours of fermentation. In contrast to this, we find, that despite the extended lag phase in the simple systems, they do support cell growth and a fairly good antibody production. Also it looks as if the product-degradation rate is slower in most of the simple systems, than in the Biostat MC.

Looking behind the figures in the graphs in figs. 1 & 2, and calculating the maximum specific production rate (i.e. max. production/cell in growth fase/hr), the situation is as pictured in **table 1.** From this table it is obvious that cells grown in socalled "low-technology"-systems -(with one exeption)- have a much higher maximum specific production rate, than found in the specialized Biostat MC. Even the blue-cap bottle, which has a quite simple stirring device that runs with a relatively high speed shows better figures than the Biostat MC.

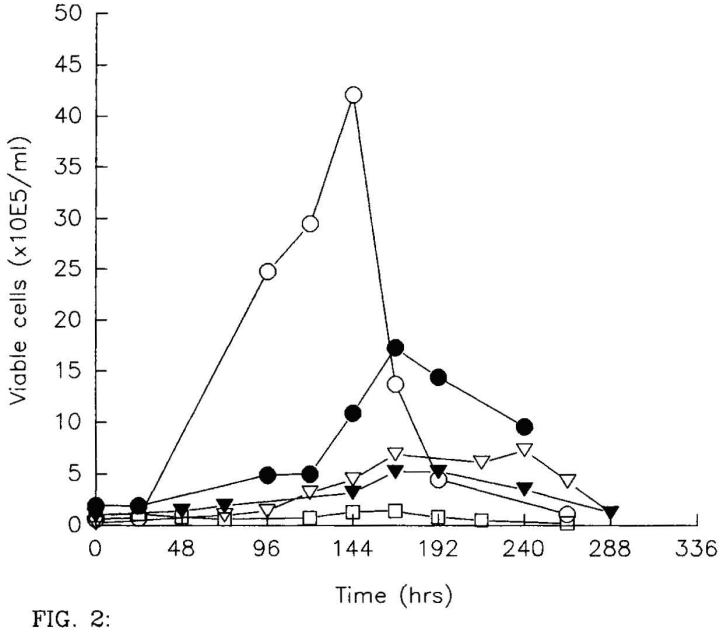

FIG. 2:

○ Biostat MC
● Bluecap-bottle with bubble-aeration & spinbar
▽ Plastic-jar with surface-aeration & spinbar-device
▼ Traditional shakeflask
□ Bacterial fermentor with bubble-free aeration

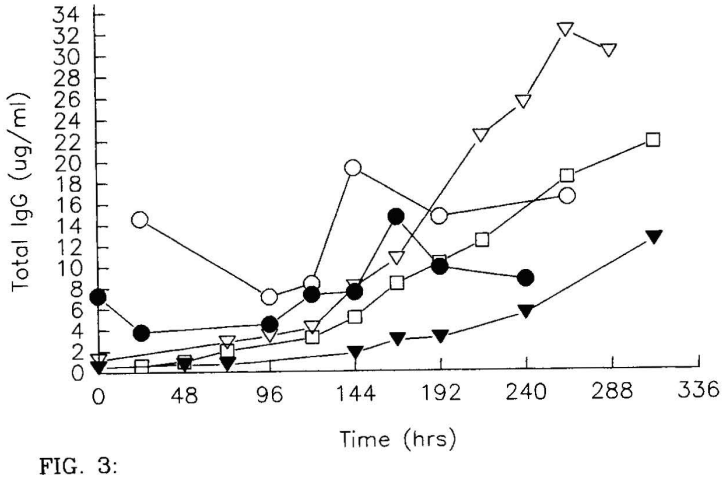

FIG. 3:

DISCUSSION:

Our results support the general assumption that specialized bioreactors for mammalian cell fermentations improve cell growth and production yields (4). In our case it is obvious, that the Biostat MC support cell growth and antibody-production perceptibly, compared to the "low-technology"-systems tested. A guess is that this is due to a better pH- and DO- control in the Biostat MC, since high-speed stirring (>200 rpm) in the Biostat MC (data not shown) do not influence considerably on these figures. However, in case of maximum specific production rate, the situation is totally different: Here we find, that allmost all the simple systems show significant better figures, than the Biostat MC. Thus even if the cells grow rather slow with a long lag period (adaption?), they are completely capable of producing good amounts of antibodies. Since we must assume to find inferior growth conditions in the "home-made" equipment, compared to the specialized systems, this means, that the cells presumably are more resistant to conditions such as high shear, pH- and/or DO-fluctuations etc., than formerly assumed. This again means that a slow and gentle adaption of the cells might improve cell growth and product formation even in rather simple fermentation equipment. Therefore we believe, that at least in laboratory-scale, one can reach very far by concentrating on cell adaption instead of making huge investments in specialized fermentation systems. On the other hand: Because of the superior monitoring- and control abilities build into most specialized reactors today, we certainly need such systems, when it comes to continuous fermentation, pilot- and production-scale fermentations and similar arrangements. However, also in this area we believe, that cell adaption might solve some up-scaling problems (e.g. shear, inhomogenious medias etc.) in large-scale mammalian cell fermentation today.

Table 1:

Equipment	Max. Spec. Prod. Rate (grams/cell/hr)
Biostat MC	1.60 x10E-13
Blue-cap bottle	2.75 x10E-13
Plastic jar	5.08 x10E-13
Ordinary shakeflask	1.56 x10E-13
Bacterial fermentor (modified)	10.90 x10E-13

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ANIMAL CELL TECHNOLOGY
Products of Today, Prospects for Tomorrow

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PRODUCTION OF RECOMBINANT FACTOR VIII FROM PERFUSION CULTURES: I. LARGE-SCALE FERMENTATION

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Keywords: Factor VIII, fermentation, continuous perfusion, high cell density, production, validation

Introduction

Coagulation Factor VIII, which is deficient in hemophilia A patients, represents the largest and most complex glycoprotein successfully cloned and expressed in mammalian cells (1-4). One of the expression systems is a Baby Hamster Kidney cell line transfected with the human gene encoding for factor VIII (4). The recombinant factor VIII (rFVIII) from these cells underwent a successful clinical development (5) and is produced at commercial scale using a continuous, high cell density perfusion process with a maximum run length of 6 months (6). The product was licensed in the USA and is marketed under the tradename Kogenate®. It represents the first FDA-approved biopharmaceutical produced from recombinant mammalian cells using continuous perfusion cultures. In this paper, we describe the cell cultivation and perfusion operation in deep tank fermenters, including process control and validation issues for the production of Kogenate® in detail. An accompanying paper focuses on purification issues (7).

Results and Discussion

1. Cell Cultivation and Fermentation Operation

Each production campaign starts from an ampoule of the frozen cell bank stored in liquid nitrogen. Cell cultivation from initial cell bank expansion to final scale fermentation is carried out with specifically designed culture medium. It contains the 3 protein additives insulin, transferrin, and human serum albumin. This rFVIII medium supports cell growth, gives high rFVIII yields, and stabilizes the factor VIII molecule. It is bovine serum-free and does not need von Willebrand factor for stabilization of the molecule. Scaleup of the BHK-derived production cells starts with an initial cultivation at low cell density in tissue culture flasks and roller bottles. Many roller bottles are then combined to inoculate the first 15-L fermenter. Further scaleup is performed through the fermenter cascade 50 L, 100 L, 200 L, and finally 500 L. All fermenters are operated under perfusion conditions at high cell density. High cell density is achieved by separating the cells from the harvest fluid in external cell retention systems and recycling them to the fermenter. The perfusion rate is adjusted according to the actual cell density in the

fermenter to provide steady-state nutrient exchange. The fermentation conditions are shown in Table 1. The maximum allowed run length is 185 days of fermentation operation. Since even the small scale fermenters are run at high cell density, it is possible to inoculate the larger fermenters with very high initial cell concentrations of 1-5x10^6 cells/mL, avoiding any lag phase after inoculation.

Table 1: Fermentation for rFVIII

• Deep tank fermenter run under controlled conditions (pH, oxygen, temperature, agitation)
• Continuous perfusion for physiological steady state conditions
• Maximal run length of 185 days
• High cell density by cell retention
• Maximum fermenter scale 500 L

The controlled and monitored culture parameters are shown in Table 2. For commercial production it is very important to tightly control the perfusion culture, to reproducibly get product of the same quality and consistency. Important tools are the cellular parameters specific perfusion, specific glucose consumption, and specific rFVIII production, which indicate how the cells perform during the cultivation period. The specific perfusion, which represents the addition of fresh medium per cell and time, is normally kept constant at approximately 0.3 nL/cell/day for rFVIII production. Under these conditions, the cells develop a constant metabolic activity indicated by the specific glucose consumption rate and a constant specific rFVIII production rate. The culture is performing well when these two parameters remain constant. If they change, something has changed in the culture that might influence quality of the product. In most cases, these changes can be attributed to bad medium or modified fermenter operation

Table 2: rFVIII Fermentation Controlled and Monitored Culture Parameter

Measured parameters	*Calculated parameters*
•Oxygen concentration	• Specific rFVIII production (per cell/day)
•pH	• Specific glucose consumption
•Temperature	• Specific perfusion rate
•Agitation speed	• Volumetric rFVIII production (per L fermenter volume per day)
•Glucose concentration	
•rFVIII concentration	
•Perfusion rate	
•Cell density	
•Sterility	
•Absence of mycoplasmas and viruses	

2. Comparison of continuous high cell density perfusion cultures with batch

There are two major advantages of the continuous perfusion cultures compared with batch using rFVIII production from BHK cells, which requires in excess of one million liter of harvest to be processed for the full market demand. The first advantage is a high degree of culture control. In a perfusion culture, conditions for optimal medium components can be adjusted and kept constant. This results in real steady-state physiological conditions during fermentation. In batch or repeated batch cultures, on the other hand, there are gradients in medium nutrients during each cultivation cycle, ranging from optimal conditions at the beginning to poor conditions at the end of cultivation. The second advantage of the perfusion culture at high cell density is the large reduction of fermentation volume. Because the specific rFVIII production is not affected by the actual cell density, a perfusion with a 30-fold increased cell density produces 30-fold more rFVIII than culture without cell retention. This results in very small "compact" fermenters of 100 L to 500 L scale, which are much easier to operate, clean, sterilize, and maintain than are 5000 L to 15000 L batch fermenters.

The main disadvantage of the perfusion culture compared with batch is the long and extensive process validation. Table 3 shows the different requirements for perfusion fermentation validation ranging from stability, product quality, to cell characterization issues.

Table 3: Validation of Continuous Perfusion Fermentation

- **Stability of fermentation process**
  - viability
  - product release
  - cell-related performance
- **Comparability of product quality**
  - fermènter scale
  - fermentation time
- **Cell characterization at final and extended run length**
  - genetic stablitiy
  - cell safety

Validating a 185-day fermentation process, i.e., showing reproducibility and consistency of product and process, is a long process. Genetically stable expression systems, as well as stability of the cells during fermentation are prerequisites. In case of rFVIII production, for example, viability remains above 90% for the entire fermentation campaign. It is very important and time-consuming to prove that quality and consistency of the product are constant at different fermentation time and fermenter scale. This was validated for rFVIII production by running several full-length campaigns and producing representative final container lots from the early, middle, and late phase of fermentation.

Conclusion

The continuous perfusion process at high cell density is a very efficient and economical large-scale process for producing rFVIII from transfected BHK cells. Compared to batch cultures, it has several advantages, which compensate for the longer and more extensive process validation requirements. The recent approval of rFVIII (Kogenate®) in the USA and the former approval of the monoclonal antibody Centoxin® in Europe indicate that the continuous perfusion culture is establishing itself as an alternative method for the production of biopharmaceuticals from mammalian cell lines with growing acceptance by the international regulatory agencies.

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Real-Time Monitoring of Protein Secretion in Mammalian Cell Fermentation: Measurement of Monoclonal Antibodies Using a Computer-Controlled HPLC System (BioCad/RPM)

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On-line, "real-time" monitoring of product concentration is important for mammalian cell culture fermentation. The continuous measurement of monoclonal antibodies allows for instantaneous determination of cell productivity and effective manipulation of the fermentor operating conditions for optimal production. This article will present the evaluation and application of a BioCad/RPM system (PerSeptive Biosystems) for rapid analysis of IgG concentration for hybridoma cell cultivation. Several commercial crossflow filtration devices are tested for low protein retention and fouling properties. A protein G column is used successfully for analyzing about 400 samples of IgG_1 without significant loss in separation efficiency. The ImmunoDetection system is integrated into a computer-controlled 15-L fermentor. This fermentor could be operated in batch and perfusion modes with cell densities up to 20 million cells/mL. A continuous cell-free sample stream obtained by a hollow fiber filter system is introduced to the BioCad/RPM for analysis. The speed of this system allows for real-time monitoring even at high densities with fast dynamics. A murine hybridoma cell (A10G10) is cultivated in batch and continuous reactors and antibody concentration is measured continuously with complete sterility. The results are compared to off-line measurements with good agreement. © 1995 John Wiley & Sons, Inc.

Key words: one-line monitoring • fermentation • cell culture • monoclonal antibodies • real-time immunoassays • BioCad/RPM

INTRODUCTION

Successful operation and control of mammalian cell fermentation requires an extensive collection of data on bioreactor conditions and metabolite and product concentrations.[9,19] The rapid assessment of the cellular environment is especially important for high-cell-density continuous perfusion bioreactors, because the culture environment can quickly change due to the higher cellular activities.[10] In batch cultures, rapid process monitoring is needed to determine where productivity or product concentration reaches a maximum. In fed-batch cultures, feeding strategies can be better implemented if continuous information is available for cell activities.[19] The success of any feedback control in both batch and perfusion systems is also determined on the rate of data collection from the fermentor. Typically, only pH, dissolved oxygen, and temperature are continuously monitored and controlled on-line in mammalian cell bioreactors. Metabolite and product concentrations, on the other hand, require off-line sampling. This is routinely performed on a daily basis and fermentor conditions are manually adjusted accordingly. In most cases, the assays, especially for product, take at least 1 day and it is impractical to use the information for manipulation of the fermentor-operating conditions for optimal product yield.

On-line measurements ideally require in situ sterilizable probes. Recent developments in optical probes has allowed for successful monitoring of cell densities.[11,18,19] Cellular respiration rates can also be measured on-line for mammalian cells.[12,19] For metabolite and product concentrations, on the other hand, more research is needed. Because mammalian cell fermentation involves a complex mixture of components, selectivity and sensitivity are present obstacles for probe development.[1] If samples can be drawn from the reactor aseptically, then they can be analyzed in "real-time" using rapid analyzers. Several investigators used crossflow filtration devices for removal of cell-free samples from mammalian cell bioreactors and analyzed them for several metabolites including glucose, lactate, and glutamine.[3,6,13]

Product concentration is an important bioreactor parameter for the manufacturing of mammalian cell derived biologicals. In batch or fed-batch operation, on-line product concentration would aid manufacturing with the decision of when to end the fermentation for collecting the harvest. In batch or continuous operation, real-time monitoring of product concentration allows the manipulation of bioreactor conditions in a rapid fashion using a feedback control. Several investigators reported success for the feasibility of automated flow injection analyzer systems for the detection of monoclonal antibodies. These studies involved turbidity assays, affinity chromatography,[2,4,7,8,14,17] capacitance mea-

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surements,[5] and sensors based on grating couplers.[14] More work is needed for improving these techniques for long-term cell culture fermentation.[16] Some of the previous studies used "model" systems instead of fermentor conditions where other proteins (present in the feeding media or produced by the cells) complicate the analysis. In some cases, the length of the evaluation was too short to validate the system for real fermentor operations.[14] While these developments are in progress, we have investigated the use of commercially available high-speed affinity chromatography for the monitoring of monoclonal antibodies. In this article, we demonstrate the real-time measurement of monoclonal antibodies during a murine hybridoma (A10G10) fermentation using high performance liquid chromatography (HPLC) (PerSeptive Biosystem BioCad/RPM, Cambridge, MA) integrated into a 15-L bioreactor. A cell-free sample stream was generated using a hollow fiber crossflow filter and the product was measured with HPLC during both batch and perfusion fermentations.

MATERIALS AND METHODS

Cell Line

The murine hybridoma cell line, A10G10, producing IgG monoclonal antibody, was used in this study. Cells were recovered from the master working cell bank (MWCB# 18096-43) and expanded in tissue culture flasks for approximately 14 days before inoculation.

Medium

Monoclonal antibody (MoAb) production medium, a DMEM/F12-based medium supplemented with 3.0 mg/mL glucose, 5 mM glutamine, 0.2% human serum albumin, 10μg/mL transferrin, μg/mL insulin, 1μg/mL cholesterol, and 5μg/mL oleic acid, was used for the fermentation.

Fermentation

A 15-L MBR (Switzerland) reactor was used for fermentation. In this reactor mixing was provided by marine impellers and oxygenation was accomplished through silicone tubing. The reactor was integrated with a cell retention device for perfusion operation. The reactor was also operated in batch mode without the cell retention device. The fermentors were operated at 37°C (pH 7.0) 50% air saturation, and agitation at 80 rpm. Dissolved oxygen was maintained by changing the nitrogen/air/carbon dioxide/oxygen gas mixture in a silicone tubing aeration basket. pH was maintained by the addition of carbon dioxide or 0.3 *M* sodium hydroxide. For the perfusion culture, the harvest flow rate was controlled by the computer utilizing on-line optical density for control. A constant volume in the reactor was achieved by controlling the weight of the reactor using a feed pump. Cell densities and glucose and lactate concentrations in this fermentor were also measured continuously by optical cell density probes[14] (Aquasant, Switzerland), and by a commercial YSI analyzer (Yellow Springs, OH),[10] respectively. The data acquisition and implementation of the control software were achieved using object-oriented control software (LabView, National Instruments, Austin, TX) and a Macintosh (Apple Computers, Cupertino, CA) computer.

Off-Line Sampling

During the course of the study, samples were taken at least once daily for off-line analysis. A YSI glucose/lactate analyzer (Yellow Springs, OH) was used for determining the concentration of these metabolites. Cell density and viability were determined by the trypan blue exclusion method. A Nova Biomedical (Newton, MA) blood/gas analyzer was used for checking fermentor pH and dissolved oxygen values. Off-line analysis of antibody was performed using ELISA and nephalometric methods.

On-Line Measurement of Monoclonal Antibody Concentration

BioCad Perfusion Chromatography

The PerSeptive Biosystems (Cambridge, MA) BioCad/RPM chromatography workstation, a fully integrated, computer-controlled automated HPLC system, was used for product quantitation. This system can analyze very small samples (5μm) in a very short time (2.5 min) using a small column filled by porous beads. The system used a PerSeptive Biosystems 2.1×3.0 mm POROS Protein G affinity column for product capture. Samples were quantitated every 50 or 33 min using a 200-μL injection volume. Phosphate-buffered saline (PBS) (pH 7.3) was used for the wash/equilibration buffer and 12 m*M* HCl was used for the elution buffer. Twenty-four percent EtOH, 4% acetic acid was used for column cleaning after each cycle. Column calibrated curve generated using serially diluted IgG_1 standards.

On-Line Aseptic Sampling

Culture fluid was circulated through a hollow fiber tangential filter system (Xampler, A/G Technologies, Newton, MA) and a cell-free sample was pumped at a constant flow rate of 10 to 20 mL/h to the BioCad/RPM system. Fermentor cell suspension was recirculated through the filter at 60 mL/min. Other filtration devices evaluated included a Microgon 0.2-μm "MiniKros" hollow fiber and Gelman 0.2-μm Tuffryn (polysulfone) and Sartorius 0.45-μm cellulose acetate membranes in a Applicon A-Sep housing. A diagram of the integrated fermentor, sampling device, and BioCad workstation is depicted in Figure 1.

Figure 2 depicts the single column set-up utilized in this

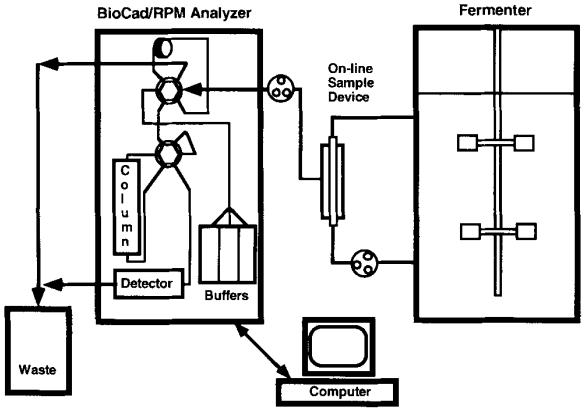

Figure 1. Experimental setup for real-time measurements of product concentrations using the BioCad/RPM immunoaffinity system.

work, with buffer flows represented for each part of the cycle. The column is loaded and the sample was analyzed in the first 5.5 min. A cleaning of the tubing (sample loop in injection valve) is followed by a cleaning of the column by cleaning solution (24% EtOH, 4% acetic acid). The column was equilibrated between the samples for the next sample.

System Sanitization/Sterility Control

All system tubing was either autoclaved or sanitized with hypoclorite prior to the start of the fermentations. Sterility in the system was maintained by sanitizing the lines after each cycle utilizing the BioCad valves and software. The system tubing was flushed at high flow rate (20 mL/min) with 24% EtOH, 4% acetic acid after each cycle. Small inner-diameter (i.d.) tubing (0.2 in.) was used to insure a high linear flow rate at 3 mL/min, helping to prevent contaminating microorganisms from moving "up" the tubing to the reactor. Furthermore, the peristaltic pump and microfiltration membrane represent two additional barriers to microorganisms. By these precautions, no contamination was observed for over 60 days.

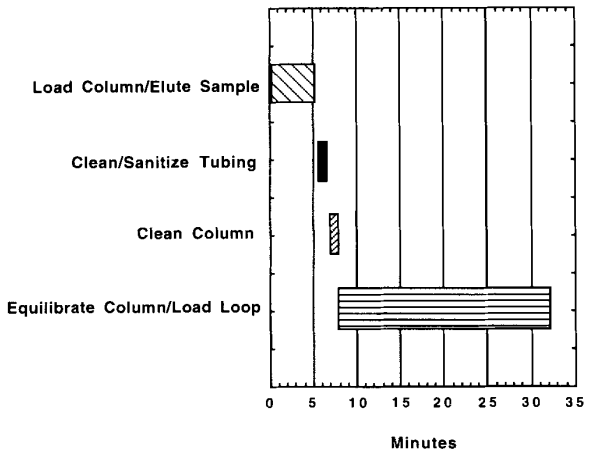

Figure 2. Diagrammatic representation of a 33-min cycle for product concentration.

RESULTS AND DISCUSSION

Affinity Chromatography

Figure 3 depicts a representative chromatography for protein G chromatography using the BioCad/RPM. Cell culture supernatant was loaded on the column and washed with PBS. During this period, the IgG stayed the column binding to protein G, while other proteins were eluted (first peak). The IgG is then eluted by 12 m*M* HCl (second peak). These buffers gave good separation of flow-through and product with very little baseline shift. It should be noted that Tris-containing buffers commonly used for this type of separation resulted in some shift in baseline at this wavelength (220 nm) for cell culture samples.

Column Evaluation

Column lifetime and accuracy were evaluated by running consecutive cycles using prefiltered (0.2-μm) cell culture harvest. Four-hundred cycles were performed with the column being cleaned halfway through backflushing with 24%

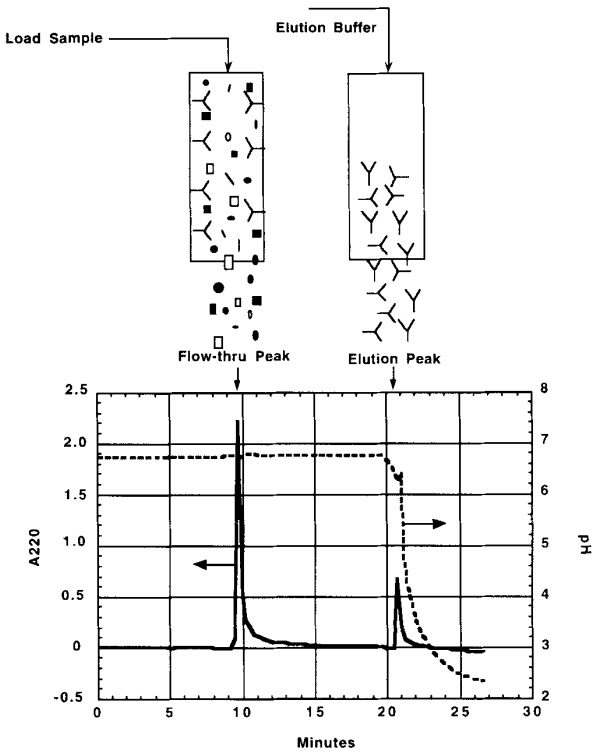

Figure 3. Typical chromatograph from on-line BioCad/RPM protein G chromatography.

EtOH, 4% acetic acid. Product concentration remained consistent over the course of the test (Fig. 4) with a coefficient of variation of 5.6%. Backpressure increased, starting at approximately 2.4 MPa, and increasing to about 13.6 MPa at the end of the test. This increase in backpressure was partially circumvented in the actual fermentor monitoring by cleaning the column after each cycle.

Filter Evaluation

Filtration devices for on-line sample clarification were also evaluated (Fig. 5). A cell-free harvest in a 5-L bottle was recirculated with a flow rate of 60 mL/min for 5 days through the filters and sampled daily with antibody concentration determined by nephalometry. Figure 5 shows the results of this study. The 0.45-μ "Xampler" (A/G Technology, Newton, MA) hollow fiber filter outperformed the other filters (<90% of reservoir concentration) and was chosen as the sample clarification device. Other filters fouled in time, probably due to accumulation of protein and lipid layers on the pores, and retained the antibody. Note that none of the filters presented in Figure 5 had difficulty analyzing metabolites such as glucose and lactate (data not shown).

In addition to media components (lipids and proteins), cells also contributed to the fouling of the filters. High circulation rates (60 mL/min) helped prolong filter life; however, in a long run (30 days) at high cell densities (>10 million cells/mL) we had to replace the filter. Evaluating the filter by daily injections of sampled fermentation broth helped to determine when to change the filter. If a difference was observed between the on-line and the manually injected off-line sample, then the filter was changed.

Column Calibration Drift

Like other probes used for fermentation monitoring (pH and so forth) a calibration drift was observed for the column calibration (Fig. 6). After 80 cycles, the calibration curve shifted by 15%. This drift was corrected by calibrating the column periodically.

Current work with the system has indicated that a column plumbing scheme, which allows for column loading in one direction and cleaning in the reverse direction, increases column life and reduces the increase in column backpressure previously seen over time. The column is backflushed with cleaning buffer after each cycle for prolonging the column life and minimizing the calibration drifts.

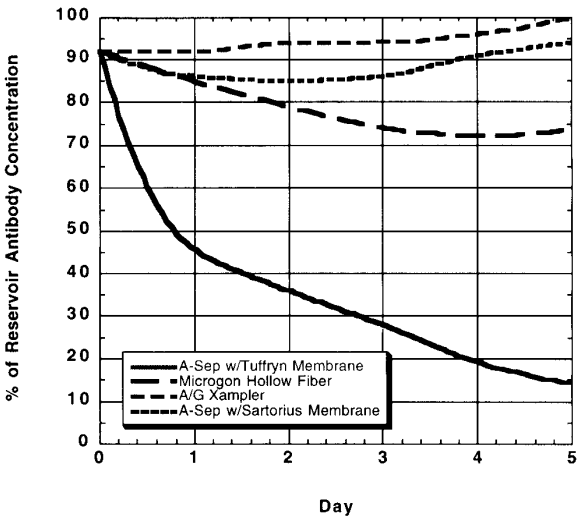

Figure 5. Crossflow filter evaluation for on-line sample clarification.

On-Line Antibody Concentration During Batch Culture

The system was evaluated during batch fermentation of a murine hybridoma cell (A10G10) line. Batch culture, with

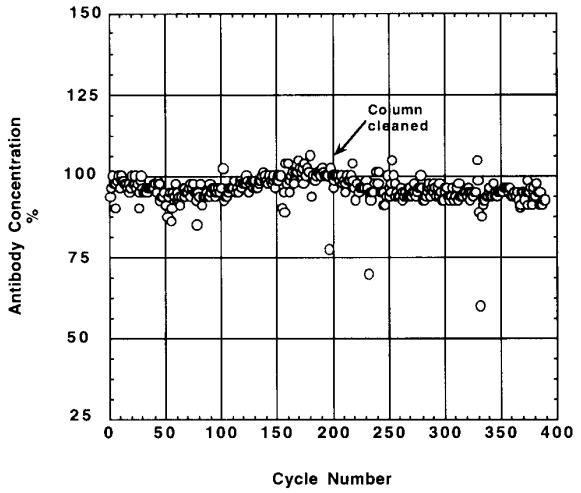

Figure 4. A 400-cycle evaluation of the Perseptive Biosystems POROS 2.1 × 30 mm Protein G column.

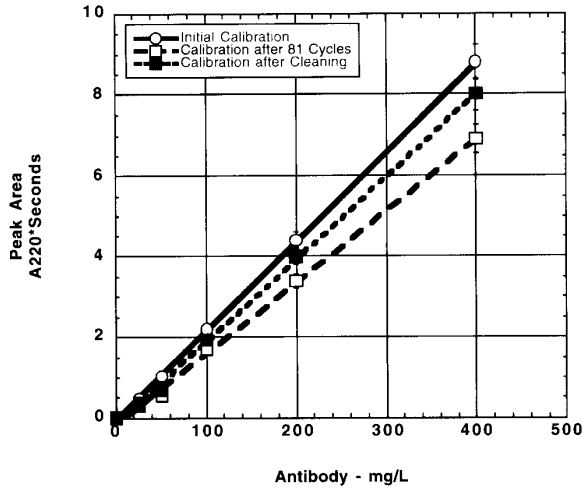

Figure 6. Calibration curves for a POROS 2.1 × 30 mm protein G column after several cycles.

antibody concentration values changing throughout the course of the fermentation, provides a useful tool for evaluating the sensitivity and responsiveness of the system. Samples were analyzed every 50 min for a total of 121 cycles and compared with off-line (nephalometry and ELISA) results from daily sampling. Figure 7 shows the antibody concentration results of this fermentation. The values of IgG were normalized using average titers for typical batch cultures. Antibody concentration increased continually during the culture. The batch culture was run for 5 days until the antibody concentration showed no further increase. Cell growth stopped on day 3 for this culture (data not shown). However, antibody levels continued to rise, even in the decline phase, demonstrating that for this cell line the production was not growth associated. Figure 7 also presents off-line measurements that were analyzed at the end of the fermentation. A good agreement between the real-time BioCad/RPM results and off-line data over the range of values was observed.

On-Line Antibody Concentration During Continuous Perfusion Culture

Figure 8 shows antibody concentration results from continuous perfusion fermentation B039. The fermentation was run at steady state for 16 days with cell densities around 13 million cells/mL. In this fermentor, cell density and glucose and lactate were also measured continuously using an optical cell density probe,[11,18] and a YSI glucose/lactate analyzer,[13] respectively. An optical density of OD = 65 correspond to a cell density of 13 million cells/mL. The antibody concentrations remained at around 92% of the batch culture titers under the steady-state conditions. Glucose and lactate levels were also at steady state around 1.6 g/L and 0.85 g/L, respectively. The cell density in the fermentor was decreased to 2.5 million cells/mL (OD = 17) by purging cells from the fermentor over a 2-day period. During the run, the perfusion was achieved by setting the harvest rate proportional to cell density. The volume of the reactor was maintained constant by the feed pump. The removal of cell suspension from the fermentor caused extra addition of fresh medium to the fermentor. This caused an increase in glucose and a decrease in lactate levels in the fermentor. Antibody concentration also decreased as a result of this event following closely the decrease in cell and lactate concentrations. After day 18, purging of the cells was stopped. The perfusion control algorithm decreased the harvest rate and cells were allowed to accumulate in the fermentor. In 3 days all parameters reached their steady-state levels. The new steady-state levels were slightly different than the previous one, which was mainly due to a slight drift in optical density probe calibration not taken care of at the time of the experiments. The antibody concentration responded to operating conditions, dropping as cells were purged and recovering as the cell density recovered. As in the case of batch culture, a good agreement was observed with real-time and off-line measurements for monoclonal antibody concentration. However, as the assays for off-line measurement could only be performed at least 2 to 3 days later in our facility, the whole dynamics of the antibody production could have been missed without the real-time measurements to determine proper actions for fermentor operation.

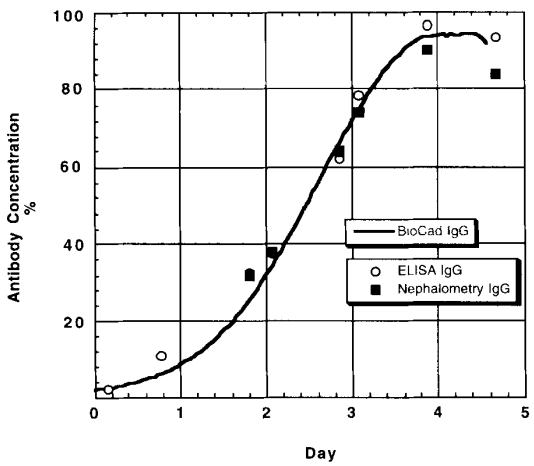

Figure 7. Batch fermentation for hybridoma cell A10G10 (MPD-B038). On-line BioCad/RPM antibody concentration results compared with daily off-line ELISA and nephalometry results.

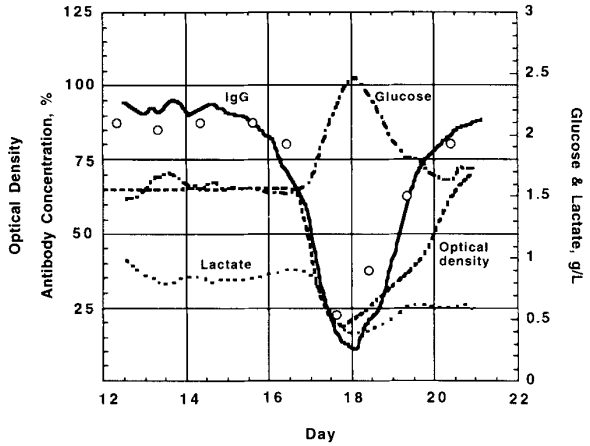

Figure 8. Perfusion culture of A10G10 hybridoma cell line. Comparison of on-line antibody concentrations to off-line analysis (circles).

CONCLUSIONS

In this article we have demonstrated the real-time measurement of murine IgG with an on-line sampling device and HPLC (PerSeptive Biosystems BioCad/RPM chromatographic workstation) integrated into a 15-L bioreactor. A PerSeptive Biosystems POROS Protein-G column was successfully used for product capture and was tested off-line by reproducibly processing over 400 samples. The use of perfusion chromatography such as that used in this system

allowed rapid analysis of monoclonal antibodies from bath batch and perfusion cultures. Compared with other systems discussed in the literature, the HPLC system offers some advantages. The technique is already established and the system is commercially available and easy to operate. However, we found certain considerations that were critical for accurate quantitation of fermentor antibody concentrations. We described our experience in this system, in terms of sterility, sample filteration, extending column life, and maintaining the accuracy of the measurements. The most important was the choice of the filtration device.

For our application it was found that the "Xampler" (A/G Technologies) performed best. Care also needed to be taken in evaluating the on-line filter for product retention and changing to a new one when appropriate. Calibration drift is also a consideration and needed to be evaluated and adjusted on a regular basis. A plumbing scheme that allowed for sample loading in one direction and cleaning in the opposite direction prolonged column life and reduced the increase in backpressure over time. Batch and perfusion cultures of murine hybridoma cells (A10G10) demonstrated the utility of high-speed chromatography for real-time monitoring of monoclonal antibodies. On-line BioCad/RPM antibody concentration values coincided with off-line ELISA and nephalometer results, allowing for real-time determination of antibody concentration and production kinetics. Although the application described here involves the determination of monoclonal antibodies, the system presented can be used for any immunoanalytical detection of mammalian cell derived products. For instance, using the current system, antibodies for a particular product can be immobilized to the column and the product can be detected in real-time.

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Chapter 3

LARGE-SCALE PRODUCTION OF THERAPEUTIC ANTIBODIES: CONSIDERATIONS FOR OPTIMIZING PRODUCT CAPTURE AND PURIFICATION

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1. INTRODUCTION

The first therapeutic proteins produced by fermentation of genetically engineered bacteria emerged two decades ago. With the notable exception of insulin, most of the initial wave of biotechnology products launched in the 1980s and 1990s were peptide hormones and enzymes licensed for indications with relatively small patient populations and for short–term rather than chronic use. Consequently, the market requirement for these products was frequently in the low kilogram range.

Products based on monoclonal antibody technology have now reached the market and are creating new pressures for production technology. Two factors combine to increase product requirements significantly – larger patient populations and long term use in chronic indications. There are now several monoclonal therapeutics both on the market and in clinical trials for which the requirements are at or approaching hundreds of kilograms. This has had ramifications across the industry and has impacted all aspects of drug development. Most notably, this demand has spurred drug developers to dramatically expand fermentation capacity, locate capacity at contract manufacturers or explore alternatives such as microbial expression or transgenic technology. Fermentation groups are reporting expression levels into the gram range by different techniques, and drug developers are

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exploring ways to boost the potency of antibodies with directed evolution or by coupling with cytotoxins or radiochemicals, indirectly reducing requirements for bulk product. Despite these measures, pressure will continue to be placed on the downstream process to cope with ever-larger amounts of antibody.

The requirement for an ever-increasing mass of antibody is only part of the story. The key driver before product approval is the 'time to market'. It is critical to get product in time and in sufficient amounts and with high enough purity for clinical trials to take place. Once product approval has been gained, there is usually a fundamental shift in emphasis towards a reduced cost of goods and increased process efficiency, both in terms of yield and reliability. This has occurred in several companies with large scale antibody products on the market and will undoubtedly occur for many more companies when they too reach this stage of growth. One of the key lessons to be learned from this is that the 'product' from a pilot scale group should not only be clinical trial material but also a process that is robust, scalable and cost effective. In order to achieve this, process optimization should be considered from the very earliest stages in the pilot plant.

Well designed processes will not only yield sufficiently pure protein but will also maximize throughput and minimize process costs. Efficient processes can decrease the requirement for fermentation capacity significantly, with a direct impact on capital resources for the size of the recovery operations and facilities, and further savings in cost of goods once a plant comes online. There is of course a compromise in terms of time to market, but investment in intensive process development can lead to eventual savings in capital costs and cost of goods sold.

Recent developments in process design and implementation have focused on strategies to improve overall economics. These include: *improvements in throughput*, which decrease scale of individual process steps, shorten turnaround times and improve utilization of equipment, and *process compression* to combine one or more steps into a smaller unit area and decrease raw material consumption, hardware requirements and overall footprint. These approaches have been used both in isolation and in combination.

In this chapter we discuss strategies for increasing process throughout and examine how these improvements can be applied to large-scale antibody purification, focussing particular attention on optimizing product capture and trace contaminant removal. We then review a series of new or emerging methodologies being applied to enhance process efficiency and improve economics.

2. PURIFICATION PROCESSES

2.1 Production/Purification Goals and Challenges

Modern therapeutic monoclonal antibodies and their derivatives are produced as recombinant proteins most often via mammalian cell fermentation. These production systems are capable of relatively high levels of product expression and with a degree of glycosylation sufficient to enhance biological activity at the therapeutic application. Relative to more physically stable microbial cultures, mammalian cell fermentation results in somewhat more challenging feed streams due in large part to host cell-derived contaminants liberated by cell attrition. In addition to host cells and cell debris, contaminants include host cell proteins, media additives (serum supplements or protein/other additives such as growth promoters or stabilizers), adventitious agents such as viruses and bacterial pathogens or breakdown products of same (e.g., endotoxins), and any potential leachates from contact surfaces of equipment. Impurities may include incompletely expressed product, remnants of proteolysis of target or other proteins, and aggregates of the target product. Increasingly, there is a trend towards higher expression levels resulting in a lower relative content of impurities. However the higher stress placed on the cells can result in a change in the profile of the contaminants which may in turn increase stress on the downstream purification process.

Table 3.1. Common process compounds and methods of removal or purification

Component	Culture harvest level	Final product level	Conventional method
Therapeutic Antibody	0.1-1.5 g/l	1-10 g/l	UF/Cromatography
Isoforms	Various	Monomer	Chromatography
Serum and host proteins	0.1-3.0 g/l	< 0.1-10 mg/l	Chromatography
Cell debris and colloids	10^6/ml	None	MF
Bacterial pathogens	Various	$<10^{-6}$/dose	MF
Virus pathogens	Various	$<10^{-6}$/dose (12 LRV)	virus filtration
DNA	1 mg/l	10 ng/dose	Chromatography
Endotoxins	Various	<0.25 EU/ml	Chromatography
Lipids, surfactants	0-1 g/l	<0.1-10 mg/l	Chromatography
Buffer	Growth media	Stability media	UF
Extractables/leachables	Various	<0.1-10 mg/l	UF/ Chromatography
Purification reagents	Various	<0.1-10mg/l	UF

Conventionally, a combination of several filtration and chromatographic methods are employed and work in aggregate and, ideally, in concert, to improve the purity and maintain the quality of the target biotherapeutic. Table 3.1 lists the downstream processing method that is primarily responsible for achieving reduction of each contaminant class.

2.2 Generic processing

The current practice of large-scale monoclonal purification, with protein A affinity capture as the cornerstone, represents perhaps the closest the industry comes to a generic downstream process. In addition to reducing development costs, it simplifies manufacturing implementation and enhances reliability at all phases of manufacturing. Applying a generic approach for similar molecules also simplifies some of the regulatory considerations. For example, while the effectiveness of a given processing step must be proven for each therapeutic and each facility, development of the protocols and systems employed to generate validation data need only occur once.

For the purposes of this discussion, processes are divided into the following general phases: (1) *initial capture*, in which the harvested culture is clarified and the target product is rapidly isolated and concentrated on an adsorbing medium, and (2) *contaminant removal*, in which contaminants remaining at sub parts per hundred are removed by a series of purification and finishing or polishing steps.

A summary of methods developed to address the downstream processing needs of monoclonal antibody production is provided in Table 3.2. Most of these are applied today in conventional large scale processing, but others that are not yet fully employed are listed for completeness. A more thorough description of the most widely used of these methods and the relative effectiveness and practicality of each is discussed below.

Table 3.2. Commonly used downstream processing methods

Processing method	Attributes	Benefits	Limitations
Clarification:			
Sedimentation based clarification	Continuous centrifugation	Capable of handling very large harvest volumes	Open process-contamination and safety issues
Normal flow Filtration	Microporous		Volume and throughput limited
	Charged filter media		
	Cellulose pads		
Tangential flow filtration	Contained systems	Capable of handling large harvest volumes	

Processing method	Attributes	Benefits	Limitations
Capture:			
Chromatography	Protein A Affinity	High throughput, high purity	High initial cost
	Other affinity ligands	High throughput	Purity, regulatory acceptance
	Cation exchange	Low cost media	Low throughput, feedstock preconditioning
Simultaneous clarification and capture	Expanded bed adsorption (EBA)	Reduces unit operations	Sensitive to feed variations and fouling. Challenge for sanitization
Purification:			
Chromatography	Ion exchange, HIC,hydroxyapatite, IMAC	Variety of selectivities, high capacity, robust	Often flow rate limited
Adsorptive membrane	Charged membranes	High throughput, contained, suited to trace contaminant removal	Low capacities

2.3 Initial Capture (clarification and initial recovery)

The initial steps after fermentation are designed to remove solids and particulates, to reduce volumes and to bring product to a stable holding point as quickly as possible. In practice, this means harvesting a 10,000-liter fermenter and processing product through at least the first recovery step to prevent degradative exposure to proteolytic enzymes and/or product breakdown due to instability.

Centrifugation can be used to clarify cell culture feedstocks. Stacked disk centrifuges are capable of handling large volumes of liquid and are well suited to removal of cells; however, there may be disruption of the cells during the process, increasing the burden placed on the subsequent downstream step. It can be a challenge to maintain sterility within a stacked disk centrifuge and pre- and post-use preparation of the equipment is laborious. More recently developed formats for large scale spinning bowl centrifuges are now available which are designed for use in a clean environment and offer significant advantages over disk stack centrifuges for clarification. Advances include automated semi-continuous operation and programmable cleaning cycles.

A number of filtration approaches are possible to achieve clarification of mammalian cell harvest. At industrial scale, a succession of methods are employed, grouped within 3 stages: primary, secondary and sterilizing. Primary clarification removes whole cells and large particles by centri-

fugation or microfiltration in either the tangential or normal flow mode. Secondary clarification clears colloidal particulate and any other material that can shorten the life of finer filters downstream. The sterilizing filter, frequently 0.22 um pore size, eliminates bacteria and other remaining bioburden prior to the first chromatography unit operation.

2.4 Clarification Process Compression

Traditionally, secondary filtration has been accomplished with a series of depth filters of diminishing pore size, typically in two or three stages (e.g., 1 um followed by 0.2 um, etc.). Considerable development/optimization can be required, as efficient pre-filtration must be achieved to ensure adequate capacity at each subsequent filtration step. This has been especially problematic for mammalian cell cultures both because they are more costly to produce than other systems and they tend to carry higher colloid loads.

The excessive consumption of facility floor space and the inevitable loss of yield at each step have motivated several suppliers to design advanced composite, multi-layered alternatives for increased filter capacity. These composite membranes, with biphasic characteristics, incorporate an initial charged depth filter to remove coarse particulates, followed by a finer filtration surface within the same device. Further development has resulted in devices which also include a charge on one of the coarse filter layers to act as a further pre-conditioning step prior to downstream purification (Figure 3.1). The incorporation of the coarse elements protect the finer filter and prolong usage while the integration into a single device reduces the amount of handling required at this process stage. The result is compression of the process train and highly effective protection of the sterilizing filter.

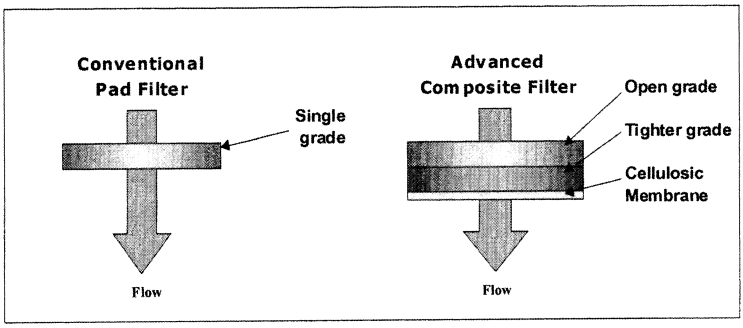


Figure 3.1 Composite membrane construction of Millistack HC+ (Millipore).

3. CAPTURE CHROMATOGRAPHY

Affinity chromatography using immobilized Protein A provides the foundation for all current large scale antibody processes due to its exquisite selectivity, an ability to load/bind product with minimal pre-treatment of the feed, and relatively simple and generic protocols. Such highly selective initial capture steps can be scaled for the target compound and need not have significant excess capacity to handle additional material in the sample. Some of the largest columns installed today range up to several hundred liters in scale, but the alternatives using ion exchange would be an order of magnitude larger. Protein A capture achieves removal of bulk contaminants (host cell protein, DNA, virus, and elements of the cell culture medium) resulting in a purity of >95% and product concentration in a single step. An additional benefit of the use of protein A affinity capture is the integrated hold in low pH elution buffer, providing partial compliance with product safety requirements of two orthogonal methods/steps to accomplish virus clearance (as discussed in section 3.4.4).

3.1 Protein A Affinity chromatography process considerations

Increasing productivity by good process design has become a significant area of research (Kamiya *et al.* 1990, Yamamoto and Sano 1992, Kemp *et al.* 2001). The rate limiting parameter of initial capture chromatography is traditionally the flow rate capability of the capture media at a point in the process in which process volumes are greatest, and the potential for product loss via proteolytic degradation is highest. Process economics for the capture step at commercial scale are dictated by the rate at which the target product is isolated (*productivity*), the media and buffer volumes consumed, and the footprint or plant area occupied by the operation.

Over recent years, increases in antibody expression levels have been shifting the bottleneck in affinity protein A capture from volume limitation to time limitation. As seen in Table 3.3, a 10-fold increase in antibody expression level from 100mg/litre to 1000mg/litre will be accompanied by a 10-fold decrease in the volume of loading material. In such cases, very high flow rate media offer less of an advantage over moderate flow rate media. Thus it can be seen that a reduction from a 20 hour load to a 7 hour load is highly desirable, but a reduction from 2 hours to 40 minutes, although useful, is less dramatic. Hence, for high concentrations of IgG, the emphasis is placed on high capacity media over high flow ratecapabilities. However, for expression levels of IgG below 1g/litre, which is still commonly the case, especially in the early development phases, high flow rate operation will still confer a significant throughput advantage. New

versions of the two most widely used industrial scale protein A affinity media have recently been introduced (MabSelect, Amersham Biosciences, PROSEP Ultra, Millipore) which offer both high capacity and increased flow capability compared to previous media.

Table 3.3. Effect of expression levels on process parameters and throughput

Expression level:	100mg/litre	100mg/litre	1000mg/litre	1000mg/litre
Media:	PROSEP A HC	FF Sepharose A	PROSEP A HC	FF Sepharose A
Operating flow velocity	600 cm/hr	200 cm/hr	600 cm/hr	200 cm/hr
Column size	250mm diameter x 200mm bed	250mm diameter x 200mm bed	250mm diameter x 200mm bed	250mm diameter x 200mm bed
Dynamic binding capacity	20 mg/ml	20 mg/ml	20 mg/ml	20 mg/ml
Load volume	2000 litres	2000 litres	200 litres	200 litres
Load time	6.8 hours	20.4 hours	0.68 hours	2 hours
Wash/elute/regen time	0.68 hours	2 hours	0.68 hours	2 hours
Cycle time (hrs)	7.5	22.4	1.4	4
IgG Throughput	26.8 g /hr	8.9 g/hr	147 g/hr	50 g/hr

3.2 Optimizing Protein A media for maximal productivity

3.2.1 Throughput and productivity

The key parameters to consider when optimizing a protein A capture step are throughput, defined as: the mass of antibody produced per unit time, and productivity, defined as: the throughput per litre of media per unit cost. Throughput is relatively easy to determine. First, the optimum combination of dynamic binding capacity and flow rate should be established. In general, as the flow rate is increased, the dynamic binding capacity will decrease; clearly these two parameters are in conflict. A common mistake in optimizing the protein A capture process is to try and achieve the highest dynamic binding capacity possible. This can only happen at low flow rates, i.e. long residence times for the antibody within the packed bed. However, long residence times will result in long cycle times which are inefficient in terms of throughput (mass of antibody *per unit time*). From a plot of residence time versus dynamic binding capacity (Figure 3.2) for variants of

PROSEP A affinity media (Millipore), it can be seen that, for residence times above 3 minutes, the relative increase in binding capacity is disproportionate to the time this will add to the process cycles. Similarly, decreasing the residence time too far will require a larger number of very short cycles. The practical considerations of equipment capabilities and QC burden generally make the use of very large numbers of short cycles less economically efficient, even though they may be very efficient in terms of throughput.

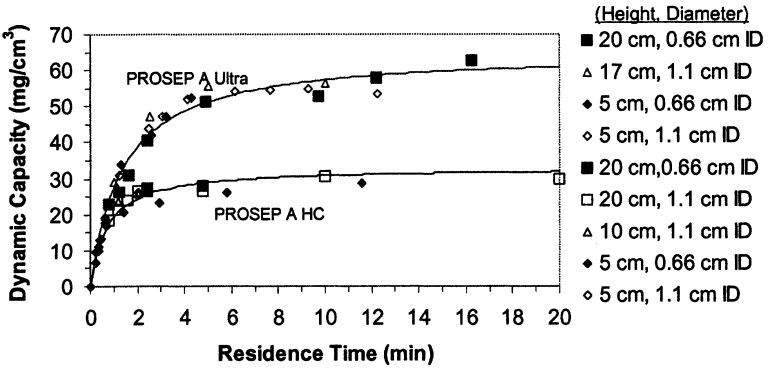


Figure 3.2. Effect of residence time on dynamic binding capacity for hIgG.

The relationship between flow rate and capacity can be modeled. Several authors have reported using eqn 1 or close derivations to characterize the performance of media. The values of Qmax and tau will be specific for each media. It can also be seen that flow rate is expressed indirectly, in terms of residence time (eqn 2). This allows the flow rate to be normalized to any column bed height. In practice, very short beds (<5cm) tend to show distribution anomalies, whereas very long beds (>60cm) will require unreasonably high flow rates to achieve even modest residence times; thus operation tends to remain within these nominal boundaries

$$Q_d = \frac{Q_{\max} \times t_r}{\tau + t_r} \qquad \text{eqn 1}$$

Qd is the dynamic capacity (mg/ml or g/l)
Qmax is the theoretical maximum dynamic capacity
t_r is the residence time (in minutes or seconds)
τ (tau) is the residence time required for Qd = 1/2 x Qmax

$$t_r = \frac{60 \times CSA \times h}{U} \qquad \text{eqn 2}$$

t_r = Residence time in seconds
CSA = cross section area of the column in cm^2
h = bed height in cm
U = Flow rate in ml per minute

High throughput and favorable process economics in process-scale antibody purification trains are achieved through the use of chromatography media possessing high capacity, high permeability, and good chemical stability (i.e. re-usable for multiple cycles). A fully rigid matrix clearly offers high flow rate capability but, in order for this to translate into higher productivity, the adsorptive media must also allow rapid mass transfer of target solutes to and from available binding sites to enable high dynamic capacity. When extremely high capture rates are enabled by these media properties, then very large fermenter harvest volumes (>10,000 l) can be accommodated economically by rapid cycling of the protein A capture step using a proportionately reduced capture column size (Table 3.4). The use of smaller columns offers capital cost savings via both by reducing equipment costs and by minimizing the space consumed in the purification suite. Since the column will experience more frequent use, the immediately obvious savings in media costs must be substantiated by proving long media lifetime (expressed as usable cycle #).

Table 3.4. Productivity and scheduling

Parameter	Case 1	Case 2
Sample volume (litres)	10,000	10,000
Sample concentration (g/l)	1	1
Loading flow velocity (cm/hr)	500	750
Elution regeneration flow (cm/hr)	500	1000
Column diameter (cm)	80	60
Bed height (cm)	20	25
Column volume (litres)	100	71
Number of cycles	5	6
Time (hours)	7.8	7.7

Production rates in the protein A capture step have been the focus of several recent studies (Fahrner *et al.* 1999b, H. Iyer 2002, Garcia *et al.* 2003). Iyer *et al.* demonstrated that a capture affinity medium enabling 2.5-fold higher processing rate offset that medium's lower adsorption capacity and provided higher productivity and reduction in overall processing costs

for the purification of a humanized monoclonal antibody. The above authors all point to the ultimate importance of considering productivity (mass of IgG x h^{-1} x L bed $volume^{-1}$), rather than dynamic binding capacity (mass IgG/ L bed volume) alone, when establishing overall processing costs. In studies comparing productivity within the permeability-dependent flow constraints of various protein A media, Fahrner, *et al.* (1999a). showed that the highest productivity resulted from use of rigid media. Production rates (P_r) for different human IgG feed concentrations and residence times were determined for PG 700 and PG 1000 using equation 3 adopted from Fahrner *et al.* (1999b):

$$P_r = \frac{1}{1000\left(\frac{1}{C_o U_L} + \frac{N}{Q_d U_E}\right)} \qquad \text{eqn 3}$$

where production rate is a function of five parameters: dynamic capacity (Q_d), protein concentration in the feed (C_o); number of column volumes for product recovery and column regeneration for repeat cycling, including wash, elute, clean and equilibration steps (N), load velocity (U_L), and regeneration velocity (U_E). Twenty (N=20) column volumes were assumed for recovery and regeneration.

Having determined the relationship between residence time and dynamic binding capacity, and knowing the permeability (pressure drop) of a media it is possible to use this equation to model the productivity over a range of flow rates and bed heights. Limits can be set to reflect maximum pressure capabilities of the system -- either the media or the hardware, whichever is the lower -- and also by selecting a maximum cycle time beyond which the process would be deemed uneconomical. Such a plot provides an indication of the envelope of operation for a given media. It can be seen from Figure 3.3 that a rigid permeable media with efficient mass transfer properties will afford the process developer a much greater scope of operation, allowing taller packed beds and faster flow rates to be fully exploited.

As noted above, the trend towards higher expression levels in mammalian fermentation systems is changing the optimal performance window for capture media. To address this, manufacturers have developed higher capacity media which are still capable of operating at high flow rates. One such media is PROSEP A Ultra. This is based on a controlled pore glass as is the case for standard PROSEP A HC but, in order to achieve a higher capacity, a controlled pore glass bead with a smaller pore diameter was selected. The pore size was reduced from 1000A down to 700A (Figure 3.4). This resulted in an increase in surface area from $23m^2/ml$ to $35m^2/ml$. The glass beads were activated and protein A immobilized using the same

chemistry as PROSEP A HC. Dynamic binding capacity tests were performed to confirm that the additional capacity was available for binding.

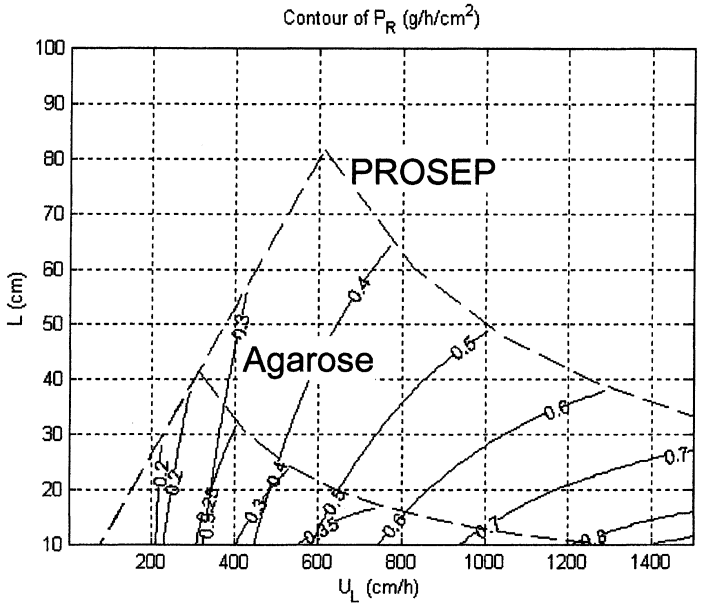
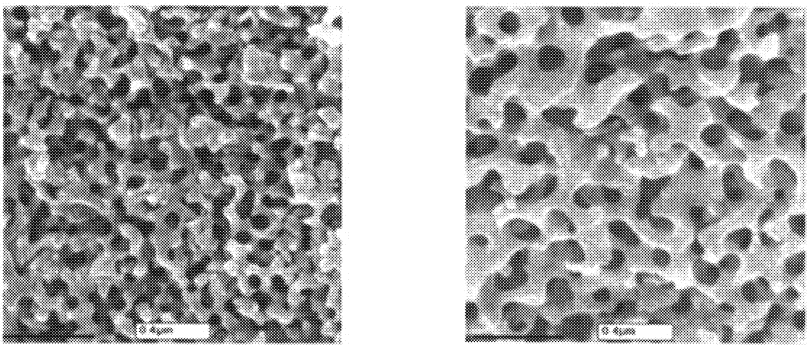


Figure 3.3. Productivity profiles



PROSEP A Ultra
(700 Å pore diameter)

PROSEP A HC
(1000 Å pore diameter)

Figure 3.4.Scanning electron micrographs of 700A and 1000A pore size glass beads.

The productivity models discussed above were then used to determine the effect, if any, of this increase in available binding capacity.

Plotting production rates obtained for a range of feed concentrations and load residence times showed that PROSEP A Ultra and PROSEP A HC achieved similar production rates at hIgG feed concentrations ≤ 1.0 mg/cm^3. However, as the hIgG feed concentration increased, the production rate for PROSEP A Ultra became greater than for PROSEP A HC. The reason for this is that, at lower expression levels, the greater volume required to saturate the PROSEP A Ultra media cancels out the benefits of higher capacity by requiring a significantly longer time to load. However, as the concentration of antibody increased, the relative impact of load time was reduced and the productivity increased. This is in agreement with the trends shown in Table 3.3.

Additional studies conducted by the authors have shown a similar trend for the purification of an Fc fusion protein. It is possible, however, that for antibody constructs that are larger than IgG (e.g., conjugates or high MW fusion proteins), the PG 1000 medium would exhibit higher productivity than the PG 700, due to greater accessibility to internal pore surface area and binding sites.

3.3 Scaling-up by changing bed height

A consensus practice has formed which teaches scale up of the protein A capture step based on residence time, rather than by holding velocity and bed height constant (Malmquist at al. 2000, Kemp *et al.* 2001). This is in contrast to the conventional scale-up procedure in which the bed height is held constant and scale up is by increasing the column diameter alone (Figure 3.5). In this latter case, the volumetric flow rate is adjusted according the to the ratio of cross sectional areas of the columns. When scaling up by increasing bed height, the important parameter to maintain constant is the residence time within the column for a notional IgG molecule. It can be seen that scale up by diameter is, in effect, a special subset of scale up by maintaining residence time. Conceptually linked to this practice is an understanding that flexibility in column geometry delivers measurable economic benefit by the use of smaller diameter, taller beds. Savings are derived from lower equipment costs and facility space savings. Taller columns of smaller diameter, which nevertheless are of the same bed volume, can cost half as much as wider, shorter alternatives, and can occupy one half the space required for packing and operating.

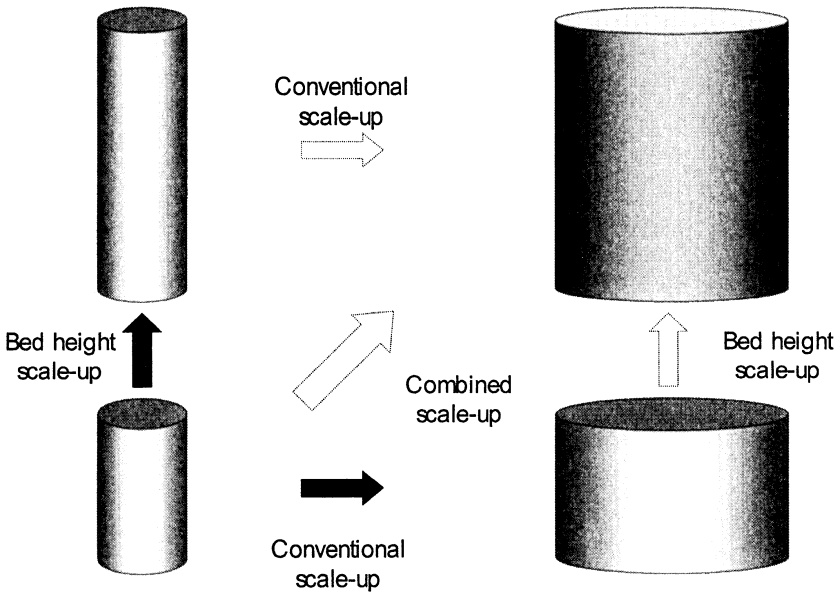


Figure 3.5 Scale-up options for protein A affinity chromatography

When scaling up by increasing bed height, care should be taken not to exceed the pressure limits of the equipment or media. In the case of compressible media such as agarose, the limiting factor will usually be the mechanical strength of the media itself. With rigid media the maximum operating pressure will usually be dictated by the pressure limitation of the column.

3.4 Other considerations for the Protein A capture step

3.4.1 Elution conditions

Antibody fragmentation and aggregation can be significant contributors to product yield loss. The harshness of acidic elution conditions is one of the most common causes for aggregation. Therefore, thorough methods development should include establishing the minimum pH required to recover product and to optimize its isolation from aggregates, contaminating antibodies, or antibody fragments.

3.4.2 Media lifetime

When considering the cost impact of the protein A capture step, a key factor is the lifetime of the media. This should always be considered in conjunction with the initial acquisition of the media when making comparisons against other methods. As the number of use cycles increases, the initial media purchase cost becomes much less significant in proportion to other process costs such as labour, process buffers and plant overheads. Often these costs are not obvious during development stages.

Protein A is a robust protein capable of withstanding exposures to pH as low as 1.5. However, it is less stable in high pH environments. Although immobilization increases the alkaline stability of protein A, the use of high concentrations of NaOH sufficient to sanitize the media are still not feasible. This means that, unlike ion-exchange media and hydrophobic interaction media, cleaning and sanitizing methods using 0.5-1.0M NaOH are not appropriate. A common cleaning procedure is to use a very low pH wash of pH1.5 acid, usually phosphoric or hydrochloric, after each elution. This is complemented by a more rigorous cleaning step, using 4-6M guanidine hydrochloride, at the end of each batch or prior to storage in 20% ethanol or 0.1% benzyl alcohol. Production processes using such cleaning regimes have been validated to 400 purification cycles and are routinely used in excess of 200 cycles. However, the efficacy of cleaning will always be dependent upon the feedstock and it is essential to perform lifetime studies on each product and for the specific media to be used. Agarose media have lower chemical stability at low pH (<2.0) compared to polymeric resins or controlled pore glass. While very dilute solutions of NaOH (0.01M) have been advocated recently as cleaning agents (Amersham Biosciences) for agarose-based protein A media, these should be accompanied by at least 2.0M NaCl to afford some protection to the protein A. Even under these conditions, some inactivation of the protein A is to be expected. Moreover, hydroxide at this relatively low concentration is not effective as a sanitant.

3.4.3 Intermediate washing

Once sample has been loaded onto the protein A column, an intermediate wash step is included to remove any feedstock in the interstitial spaces between the chromatography beads or remaining within the pores of the beads. This is also an opportunity to reduce any non-specific binding which occurs. The main contaminating group in any feedstock is usually host cell proteins. These represent a diverse range of proteins with an equally diverse range of physicochemical properties. Non-specific binding can occur due to interaction between host cell proteins and the protein A ligand, the activation chemistry, or the base media itself. Non-specific binding can also occur

between the chromatography media and non-protein constituents such as DNA, endotoxin or culture media additives. All modern media are treated during manufacture to minimize non-specific binding. However, the incorporation of specific wash steps between loading and elution can reduce the load of non-specifically bound contaminants even further. Typical washes may include NaCl (1.0M) or organic modifiers such as tetramethyl ammonium chloride (Sulkowski 1987) and may have to be fine tuned for the specific impurity binding.

3.4.4 Virus removal

Protein A chromatography provides a very effective step for virus removal (Brorson *et al.* 2003). Two mechanisms are used which will provide clearance of virus particles. The first is the physical separation of viruses from the antibody by partition on the column. The second is through chemical inactivation during the low pH elution. By using PCR to monitor the reduction in virus nucleic acid in conjunction with enumeration of viable virus particles, it is possible to quantify and validate each of these components separately and present evidence for two orthogonal virus removal steps. When used in combination with a physical method of removal, such as a virus filtration membrane, this provides sufficient validatable virus removal to satisfy the regulatory authorities.

3.4.5 Ligand leaching

It is a fact of chemistry that all protein A affinity media will leach to some extent (Fuglistaller 1989, Fahrner 1999b). The amount of leaching can be affected by several factors: the physical and chemical strength of the backbone support, the method of immobilization and the presence of proteases. The assay of leached protein A is made more difficult by the fact that it is in the presence of a large excess of IgG which can also bind to the protein A and interfere with detection. In general, the assay system of choice is an enzyme-linked immunosorbent assay (ELISA). Several ELISA kits are now available which are specifically designed to quantify residual levels of protein A in the presence of human IgG. For each ELISA, the nature of the protein A itself – e.g., natural, full recombinant, truncated recombinant - will affect the assay methodology. As a general rule, the control protein A used for the assays of leached protein A should always be from the same source as the protein A used to manufacture the affinity media.

4. POST AFFINITY PURIFICATION STEPS

4.1 Contaminant removal

While dramatically enriched for the product antibody, the eluted pool from the protein A capture step requires additional processing to remove remaining trace contaminants -- including and residual host cell protein, DNA, virus and endotoxin -- before it can be administered parenterally. As discussed above, there will also probably be trace levels of leached protein A ligand present with antibody at a relative concentration of 10-100 PPM antibody. A number of methods may be used to remove these trace impurities. The most common are discussed below. One method not covered here, but often mentioned in older literature, is size exclusion chromatography. While this method can give excellent result in the lab and at pilot scale, it is usually deemed too slow and cumbersome for use at very large scale use.

4.2 Ion exchange

Ion exchange chromatography, immediately following protein affinity capture, is generally regarded as the most effective orthogonal means of achieving significant reduction in remaining trace contaminants. It is typically preferred at commercial scale because it is largely non-denaturing and, with sufficient methods development, offers a robust, flexible, and reasonably selective purification tool. As the majority of monoclonal antibodies are basic proteins (pI >7) and predominating contaminants are acidic, cation exchange media represents a better choice for the second purification column. While anion exchange media may be operated under conditions that bind MAb, capacity co-consumption by the mostly acidic contaminants would necessitate larger columns than desired. Instead, anion exchange functionality is better applied downstream for IgG flow-through steps for removal of trace remaining contaminants, typically clearing 2-5 logs endotoxin and 3-5 logs DNA.

Table 3.5. Typical contaminant clearance values

Contaminant	Affinity load	Intermediate purification load	Polishing load
Host cell protein (ng/ml)	10^5	10^3	10
Endotoxin (EU/ml)	10^6	10	<1
DNA (pg/ml)	10^6	10^3	10^2

Chromatographic selectivity, defined here as the degree to which soluble protein A and host cell protein are resolved from the antibody, is a major focus of methods optimization. When optimizing the cation exchange step, the factors to take into account include the loading conditions, the elution method and the rate of operation.

Ideally, the loading conditions should allow the IgG eluted from the protein A column to be loaded directly, with no pre-conditioning. In practice, this is not always possible. The low pH elution buffer may be too low for long term stability of the IgG activity. Although a hold period at low pH for virus inactivation is common after elution from the protein A column, the buffer pH is then usually adjusted up to nearer neutral pH. The conductivity of the feed for the cation exchange column is also critical to the performance of the media and so limits should be tested during process development. Usually the feed should have conductivity in the range of 4-10mS. Below this value, precipitation of the immunoglobulins can occur; above this conductivity, the binding capacity of the media may be adversely affected.

4.3 Other Chromatographic Purification Methods

Although ion exchange is the most common chromatography method used following protein A affinity, two other alternative methods have been widely exploited: hydrophobic interaction chromatography (HIC) and hydroxyapatite chromatography. These are especially useful when the isoelectric point of the monoclonal antibody is lower than pH 6. When this is the case, the monoclonal antibody and many of the host cell proteins and leached protein A will tend to co-elute from ion exchange media. Hydroxyapatite itself is a crystalline form of calcium phosphate. In this form it is friable and not suited to large scale chromatography. However, it is also available commercially in a ceramic bead form which confers sufficient mechanical robustness to make large scale use possible. The separation is based on a combination of cation, anion and calcium-induced interactions. The novel selectivity of hydroxyapatite makes it difficult to predict optimal operating conditions without recourse to direct experimentation (Stapleton *et al.* 1996, Aoyama and Chiba 1993).

Hydrophobic interaction chromatography is somewhat under-utilized at an industrial scale, yet it is capable of very effective purification of monoclonal antibodies. Perhaps the main disadvantage of HIC is the requirement to add lyotropic salts to promote binding. This can present significant problems at large scale due to the quantity, expense and handling difficulties involved with high purity ammonium or sodium sulphate solutions. In the lab, HIC has traditionally been used as the step following initial precipitation since this reduces the amount of feedstock conditioning

required. The use of hydrophobic interaction for the purification of monoclonal antibodies has been well reviewed by Gagnon (1996).

5. POLISHING

Following one or two intermediate purification steps, a common orthogonal method applied for final trace contaminant removal is anion exchange chromatography operated in the flow through mode. As DNA, endotoxin, many viruses, and a large percentage of host cell proteins are negatively charged at neutral pH, they are bound while the typically basic (i.e. positively charged at neutral pH) antibody species is not. Adsorptive membranes provide an alternative to conventional strong anion exchange beaded media but with three primary advantages: they are less kinetically limited (i.e., permit rapid mass transfer), less hydraulically limited, and imminently more convenient to install. When utilizing conventional packed-

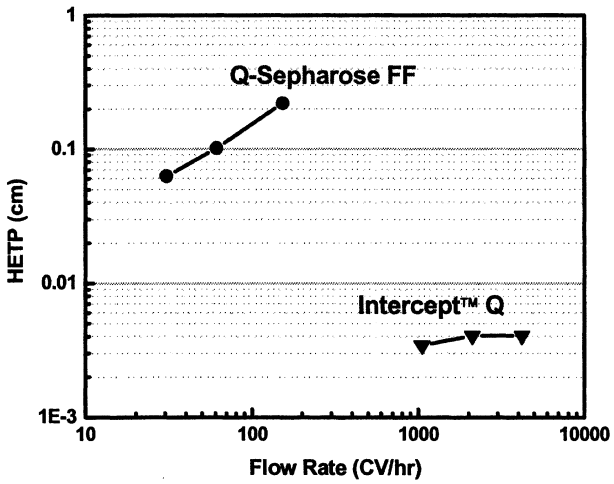


Figure 3.6. Comparison of anion exchange membrane and chromatography media performance.

bed chromatography, columns of very large diameter are required to permit high volumetric flow rates in order to prevent a process bottleneck at the polishing step. As proper flow distribution in production columns requires minimum bed depths of approximately 10 cm, bed volume becomes significant. The result is that such columns are dramatically under-utilized.

In comparison, the capacity of an adsorptive membrane presented in a multi-layered cartridge format is more completely utilized. The physical nature of such devices also lends itself to rapid processing (Figure 3.6). While device integrity testing is advisable, one avoids the manually intensive tasks of packing and testing for flow dispersion properties (e.g., measurement of HETP, asymmetry).

6. OTHER PURIFICATION METHODS

6.1 Crystallization

Crystallization and liquid-solid separation is considered a possible means of addressing future production requirements for monoclonal antibodies (Harris 1995, Visuri 2002). Some precedent exists for the partial purification of IgG by this method as an element of the Cohn plasma fractionation scheme and in the preparation of IgG and antibody fragments for x-ray diffraction structural studies. As the flexibility of the whole IgG molecule imparted by the hinged regions of the heavy chains is thought to challenge the reproducible recovery of stable and active antibody by this method, it may be more practical for isolating subunits such as Fab fragments than for whole IgG. Practical, large-scale purification of therapeutic monoclonal antibodies by crystallization has not yet been reported. Among the issues warranting more study is the process-friendliness of the required salts or organic modifiers, their contributions to cost at large scale, and overall process economies in the face of unknown recoveries/yields, the possible need for costly temperature controls and other equipment requirements.

6.2 Enhanced Ultrafiltration (EUF)

Novel approaches have been applied to improve process economy of tangential flow ultrafiltration steps. In all ultrafiltration there is a trade-off between product yield and throughput that affects the economics of the unit operation. While a lower molecular weight cut-off membrane is more retentive for the target product, and thereby permits higher yield, the permeability of such membranes is typically low. The price for that higher yield is paid in the form of increased membrane area requirements and longer processing times. Similarly, use of a higher molecular weight membrane offers higher flux but at the cost of product yield.

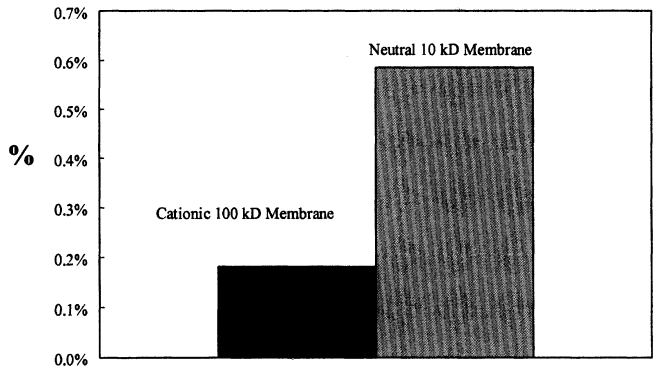


Figure 3.7 – Difference in percent sieving of IgG for neutral and charged 100KkDa membrane

The relationship between yield and permeability can be favorably impacted with the use of charged membranes (Van Reis 1999, Christy *et al.* 2002). The presence of a diffuse counter-ion cloud layered around the charged protein makes it effectively larger in size than if uncharged. If it is then presented to a membrane that has the same charge, the membrane's permeability for that species is reduced both because of its larger effective size and because of electrostatic repulsion from the membrane and its pores. As a result, a membrane having a MW cut-off much closer to that of the retained species can be used, thereby permitting higher flux rates without sacrificing retention and yield. This effect has been demonstrated for IgG using a cationic membrane of a nominal MW cut-off of 100kD (Figure 3.7). Typically, it would be advisable to employ a 30 kD MWCO membrane to achieve the best retention and yield of IgG. However, as a result of the phenomena described above, retention of IgG with the charged 100kD membrane was greater than for an uncharged 30 kD version.

The effects are most evident when the antibody is in a buffer having low ionic strength to minimize the potential that other ions present dampen repulsion effects by means of ion pairing (e.g., below 50mM buffer and other salts). Also, enhancement is greatest at low concentration of charged protein impurities, to avoid loss of membrane permeability by the fouling effects of oppositely charged impurities. Therefore, enhanced ultrafiltration (UEF) will be most practically applied following one or more post-clarification purification steps. It is ideally suited for post-polishing diafiltration operations just preceding product formulation.

6.3 Expanded Bed Adsorption (EBA)

An approach which continues to show promise is the use of stabilized expanded beds. The system is based on adsorption media that are considerably more dense than conventional media. It is contained in suspension in a chromatography column with certain design modifications to vary bed height/expansion volume and to allow one to monitor the height of the expanded bed. The unprocessed fermentation broth is applied directly through this device and the sample of interest is retained as other materials pass through to waste. The potential benefits lie in removing one or more filtration or centrifugation steps, improving yields and lowering costs.

In principle, expanded beds allow the operator to ignore bioburden. However, in practice it is not quite so simple and the overall bioburden can have a significant impact in the life length of the gel, so conditions must be optimized carefully to minimize binding of contaminants (Thommes *et al.* 1996, Feuser *et al.* 1999, Fernandez-Lahore *et al.* 2000). Another factor that has limited the application of EBA is that the density of agarose based materials (in composite with quartz particles) available for expanded bed use is not significantly greater than the buffers used, so the allowable flow rate range is typically limited to 200-300 cm/h. Such flow velocities are at least half of those that can be achieved using a conventionally packed bed of controlled pore glass based media. The use of significantly more dense base media such as zirconia or stainless steel particles may permit flow rates an order of magnitude higher. Equipment design also has had a significant impact on the successful implementation of the technique, and although there are quite useful designs at pilot scale, the cost of similar designs at full scale can be 2-3 times that of a conventional column. Lastly, the material eluting form an expanded bed column still must undergo sterile filtration.

Notwithstanding these challenges, there are several cases where expanded bed processes have been scaled up successfully to production scale with products purified to homogeneity. In such examples, it is appropriate to examine process economy and other benefits. In the case of monoclonal antibody purification, workers at Genentech have compared EBA to conventional processes (Fahrner *et al.* 1999c). They showed that contaminant profiles varied and that, although yields were better after the first step, contaminants were also present at greater levels. This could result in the need for an additional purification step later in the train in certain situations. A separate study concerning media lifetime showed that EBA media lifetimes were considerably lower than would be expected for a conventional process. As these authors point out, the development of robust, selective ligands, which can withstand extensive cleaning, would greatly assist the widespread utilization of expanded bed technology.

6.4 Future directions

Despite the strong position of protein A affinity chromatography within the monoclonal antibody industry there is still intensive research to find lower cost, more robust alternatives. Some of these stem from work previously carried out on thiophilic media (See Boschetti 2001 for a review of thiophilic chromatography). The basis of thiophilic media is that a small discrete chemical ligand can have an unexpectedly specific interaction with immunoglobulins. Further work in this field has identified some alternative small chemical ligands, which also have high specificity towards immunoglobulins. The general mode of action for the ligands had been described as hydrophobic charge induction (Schwartz *et al.* 2001, Boschetti 2002). So far, these ligand have proved to be interesting but their general applicability is reduced by the degrees of non-specific binding they can show, especially towards hydrophobic contaminants -- such as cellular lipid and antifoams -- and their poor performance at the flow rates required for high throughput processes.

The search for alternative ligands to Protein A has also utilised methods more commonly applied to drug discovery, such as phage display screening (Erlich 2001) or protein engineering (Gülich 2000), in addition to the increased research into alternative bacterial proteins such as Protein L (Graille 2001). However, the weight of regulatory compliance, accepted practice and accumulated knowledge still makes protein A the ligand of choice.

As expression levels increase and growth media become better defined and free of animal products, alternative paradigms may be required for the purification for monoclonal antibodies. The emphasis will move away from capture in the first instance, towards clarification and concentration. Subsequent purification steps will be more analogous to polishing whereby trace impurities are removed from the product stream, often by negative, or flow-through, chromatography. In such a case, the absolute capacity will not need to be as high as for capture steps and the use of charged membranes may become favoured. As noted in the introduction, there is a significant pressure on process developers to meet the requirement for ever-increasing amounts of antibodies. There is also strong cost pressure on monoclonal therapeutics. It can be expected that these pressures will drive monoclonal production towards still higher throughputs more commonly achieved in the conventional pharmaceutical and fine chemical industry. Early research is already under way underway on forms of simulated moving bed (SMB) chromatography which may yield pseudo-continuous processes for antibody purification. SMB processes have already gained acceptance in the chemical industry and may prove to be the future direction of the biotechnology industry too.

7. CONCLUSION

The fundamental goal of process validation is to ensure that the detailed quality specifications of the product are consistently met, even in the face of variability in the process inputs. Inputs include raw materials, such as the many elements of the fermentation system (cell line, media components), and resulting harvest, as well as any in-process materials contacting the product. The latter includes all chromatographic media, filter media, and equipment.

It is worth noting that a process can be robust and validated without necessarily being cost effective. In general, purification processes will be fixed before producing Phase III clinical trial material. This process will then remain fixed until after product approval is gained. Even after product approval, there is inevitably great reluctance to implement process changes. Although the approach of the FDA is becoming more amenable to scale-up and post-approval changes (SUPAC) of complex biologicals, such changes still tend to be fraught with difficulty and uncertainty and usually prove to be both time consuming and expensive. In short, it is important to appreciate that the successful validation of a cost-*in*efficient process will greatly impact future returns on investment and may prove difficult to change. Hence the requirement for process efficiencies in terms of throughput and cost of goods produced to be factored in from the earliest stages of process development.

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