commercializing new delivery modalities. It is therefore reasonable to expect that incremental improvements in existing technologies will continue to dominate the near-term future of depot delivery. These improvements may include new manufacturing process techniques, new approaches to sterilization, novel packaging technologies, and novel combinations of existing technologies. Recent examples of these include the emerging use of supercritical fluid technologies to make polymeric microspheres (169), evaluation of electron-beam and ethylene oxide as methods of sterilization (170), increasing use of delivery devices, such as the Lupron Depot-PED® dual-chamber syringe, to enhance convenience during administration, and the integration of acid-neutralizing excipients in PLGA formulations to counteract acidification by hydrolysis products (44). Further value may be extracted from these technologies if leads are optimized during discovery specifically for sustained release, emphasizing potency and stability as key criteria.

Introduction of New Excipients

The acceptability of materials for parenteral use, from both the safety and regulatory points of view, continues to be a major constraint in the development of new depot delivery technologies. The hurdles to introduction of new excipients are significant, and few companies are willing to invest the significant time and money required to bring new or novel-use excipients through development to the market. PLGA enjoys the status of being a proven and well-accepted excipient, and continues to be the most common polymer used in parenteral sustained-release systems, further entrenching it in this application. Although PLGA is attractive in many respects, new polymeric materials are needed to provide a wider range of properties and potential release profiles, and to enhance the range of actives compatible with sustained-release approaches. In the short term, the most promising new candidates for approval are likely to be copolymers of currently-approved materials, such as copolymers of PLA and PEG, which can be expected to degrade to known materials. Longer-term, one approach to speed the introduction of new excipients could be the formation of jointly-funded industrial consortia, to advance the preclinical evaluation of novel materials.

Enhanced Control over Drug Release

Despite their many advances over the years, marketed depot delivery systems continue to offer a relatively limited ability to control release rate, relying on the intrinsic properties of the formulation (e.g., matrix degradation, API dissolution or partition, osmotic pressure, etc.) to govern drug release. The ability to rationally change drug release during dosing would represent a major step forward, and continues to comprise an active area of scientific inquiry. The ultimate goal is responsive systems, or smart delivery systems, which incorporate the ability to sense their surroundings and alter their function in response to specific signals generated in the body (171). Such systems will be particularly valuable in the treatment of diabetes and other metabolic disorders, and may also be useful in chronotherapy (172,173).

Several approaches have been evaluated in the pursuit of this goal, including environmentally responsive polymers and microprocessor-based devices. Novel polymers have been synthesized, which are capable of changing their properties in response to changes in their environment, including pH, temperature, ionic strength, solvent composition or electromagnetic radiation (174 178). These include the pH-sensitive methacrylates, which change in their degree of swelling as pH changes, and temperature-sensitive systems such as poly (*N*-isopropylacrylamide) (174). Microelectromechanical solutions include an electro-thermally activated implantable silicon chip, under development by MicroCHIPS (179). The device is segmented into multiple wells, which can be sealed prior to implantation and then opened on demand. Depot delivery systems of the future will likely include integrated sensing of biomarkers, metabolites, or actives, feedback-control over drug release, and real-time output of information relating to the underlying pathology and treatment (168).

New Applications

A number of new applications for depot delivery are emerging, including targeted delivery, gene delivery, and tissue engineering. Fabrication of nanoparticles from PLGA offers a new platform for targeted delivery, amenable to IV administration (180). These systems are being

developed and studied for the targeted delivery of a range of therapeutics, from small molecules to nucleic acids. Nucleic acid delivery via sustained-release systems is an increasingly active field of research given the recent advent of RNAi technology and continued interest in local gene delivery (181,182). Tissue engineering and regenerative medicine strategies often require controlled delivery of bioactive molecules, with particular sensitivity to spatial and temporal control of release (183), to a particular cell type or in a particular region of the body (184). There are many potent growth factors including nerve growth factor, bone morphogenic protein and vascular endothelial growth factor, which are under investigation (185). Approaches for regenerating nerve tissues, repairing bone defects from fractures, infections and cancers, and the ability to accelerate blood vessel formation are all areas of active research. The field of parenteral sustained release promises to be an exciting and active area of research for many years to come, offering the potential to significantly increase the value of both existing and new therapeutics and address important unmet medical needs.

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8 Biophysical and biochemical characterization of peptide and protein drug product

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INTRODUCTION

Classes of Biotherapeutics

The biotherapeutics class of drugs that are commercially available encompass a range of compounds including recombinant or purified proteins, monoclonal antibodies (also proteins), peptides, conjugated or fused peptides, antibody conjugates, protein vaccines, oligonucleotides, protein-lipid complexes, enzymes, antibody fragments (Fabs), glycosylated proteins, and carbohydrates (Fig. 1). Additional molecule types are in preclinical and clinical development.

The biotherapeutics class contains a wide variety of recombinant proteins derived from microbial, mammalian, and yeast sources (Table 1). There are few products that are extracted from natural sources. The biotherapeutics class of drugs uses a variety of technologies for extending half-life such as conjugating to polyethylene glycol (PEG), fusion with antibody or Fab, and employing the antibody itself. This is especially true for peptides and other small entities that would be cleared via the kidneys without a half-life enhancing strategy such as conjugation or fusion. Table 1 illustrates the wide variety of biotherapeutics entities on the market.

Regulatory Guidance on Structural Characterization

Regulatory approval of a biotherapeutic entity requires meeting the guidelines for chemistry, manufacturing, and controls (CMC) put forth by the relevant regulatory agency. A complete CMC package includes a description of the characterization of the biotherapeutic entity, which includes the Elucidation of Structure and Impurities sections, which, for biological entities can be quite complex. It is expected that the applicant have a detailed understanding of the structure, heterogeneity, and stability of the biotherapeutic entity using a variety of analytical methods. Regulatory guidance on the characterization of biotherapeutic molecules can be found in several sources. The U.S. Food and Drug Administration (FDA), the European Medicines Agency (EMEA), and other regulatory agencies around the world often provide guidance documents on specific topics relating to the review and approval of drugs, and these can be excellent sources of information for applicants (www.fda.gov, www.emea.europa.eu). The International Committee on Harmonization (ICH) (www.ich.org) provides guidance documentation agreed on by the regulatory agencies of the United States, Europe, and Japan. The ICH guideline Q5 deals specifically with biotechnology products, and some information concerning characterization is available in this section, particularly Q5E on comparability. Q6B deals with specifications of biotechnology products, and provides further relevant information for biotherapeutic entities.

Proof of Structure

As part of the Elucidation of Structure section of a CMC package, a detailed analysis of the structure of the biotherapeutic is required. This evaluation is in addition to the normal batch release assays used for the product which ensure the safety and efficacy of each batch. The characterization assays included in this section are used for confirmation of the predicted primary structure, higher order structures, post-translational modifications, and degradation products that may form or increase on stability. The presence and levels of variant forms needs to be measured, and their impact on the safety and efficacy of the product needs to be assessed. The attributes investigated may be assessed using multiple analytical methods for each, as discussed in some detail below.

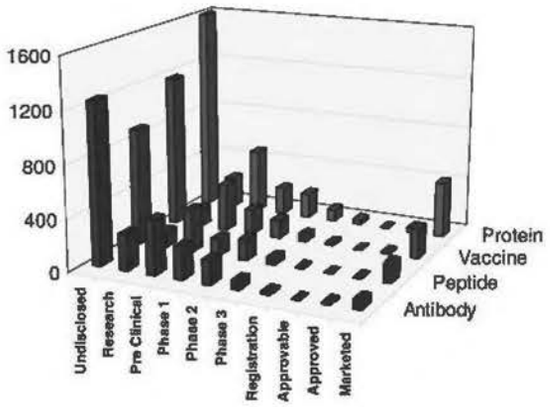

Figure 1 Portfolio of selected biotherapeutic class of drugs and drug candidates in various stages of development (data from PharmaCircle, March 2009). Numbers do not represent unique molecule types in any of the classes.

The confirmation of primary structure may include assays that demonstrate the product has the expected amino acid sequence, such as amino acid sequencing, mass spectrometry (MS), and electrophoresis. These methods ensure that there are no translation variants such as amino acid substitutions, terminal extensions, or unprocessed introns present in the product. Higher order structure may be assessed by biophysical and spectroscopic methods such as circular dichroism (CD) and fluorescence spectroscopy. This may include a determination of the disulfide bond connectivity, which can be critical for a protein to maintain its active conformation. Many post-translational modifications of proteins are possible, such as glycosylation. Other modifications may include related species formed as a consequence of degradation, such as oxidation and deamidation. For conjugated products, variants due to the conjugation process and degradation products of these need to be assessed and understood. In total, biotherapeutics may include a heterogeneous mixture due to all of the variant forms possible, and the applicant needs to demonstrate an understanding of the species present.

Potency Determination

For biologics, in most cases, a relevant potency assay for the biological entity is required for its approval. The assay needs to demonstrate "the specific ability or capacity of a product to achieve a defined biological effect." (ICH, Q6B, specifications: test procedures and acceptance criteria for biotechnological/biological products). One or more bioassays are typically included as part of batch release, and range from binding assays, cell-based assays, or in vivo animal assays. As part of characterization, it is expected that variant forms of the biological entity be assessed for potency. This involves isolation of the variant form and testing in the relevant bioassay(s) for the product. For species that form or increase on stability because of degradation, stress conditions can be used to generate sufficient material to perform potency assays.

Formulation Characterization

Most therapeutic biologics currently are administered via parenteral (intravenous or subcutaneous) route. The goal of biologics drug product formulation development is to minimize various degradation pathways to achieve a minimum shelf-life of 18 to 24 months at the intended storage condition. An emerging strategy in the biotherapeutics industry is to minimize investment in the early stages of preclinical and clinical development, and therefore, drug product formulation for early clinical trials may not be characterized in detail. Additionally, long-term stability data may be rarely available in early stage. However it is necessary to make an assessment of potential chemical and physical labilities that may impact long-term stability. A part of this assessment can be achieved by Preformulation work which is a combination of experimental and bioinformatics studies conducted in early stage prior to nominating a drug product formulation. "Formulation characterization" refers to

Table 1 Examples of Biotherapeutics Class of Molecules Types Sources Technologies and Molecules

Name of drug	Name of active substance	Class of molecule	Technology	Source	ndication	Company
Genotropin	Somatropin	Protein	Single polypeptide	*Escherichia coli* (rDNA)	Growth hormone deficiency Turner syndrome and others	Pfizer
Somavert	Pegvisomant	Conjugated protein	Single polypeptide PEGylated at multiple sites	*E. coli* (rDNA)	Acromegaly	Pfizer
PEG- ntron Redipen	Peginterferon α-2b	Conjugated protein	Covalent conjugate of PEG to protein	*E. coli* (rDNA)	nfections hepatitis C	Schering-Plough
Nplate	Romiplostim	Fusion protein	Fc-peptide fusion protein (peptibody)	*E. coli* (rDNA)	Thrombocytopenic purpura	Amgen
Survanta	Beractant	Lipid-protein mixture	Natural bovine lung extract containing lipids and surfactant-associated proteins and added lipids	Bovine lung extract	Respiratory distress syndrome	Mitsubishi Tanabe Ross (Abbott)
DigiFab	Digoxin immune Fab	Antibody fragment (Fab)	digoxin-specific Fab	Ovine serum	Digoxin toxicity or overdose	BTG nternational Ltd Nycomed
Lucentis	Ranibizumab	Antibody fragment (Fab)	Humanized gG1 κ	*E. coli* (rDNA)	Age-related macular degeneration	Genentech and partners
Cimzia	Certolizumab pegol (CDP-870)	Antibody fragment (Fab) conjugate	Humanized antibody fragment Pegylated	*E. coli* (rDNA)	Crohn's disease	UCB and partners
Enbrel	Etanercept	Fusion protein	Dimeric fusion protein (extracellular portion of human tumor necrosis factor receptor linked to gG1 Fc	Mammalian cell (CHO) (rDNA)	Rheumatoid arthritis plaque psoriasis and others	Amgen Wyeth Takeda
Herceptin	Trastuzumab	Full length antibody	Humanized gG1 κ	Mammalian cell (CHO) (rDNA)	Cancer (breast stomach pancreatic)	Genentech and partners

Vectibix	Panitumumab	Full length antibody	Humanized gG1 κ	Mammalian cell (CHO) (rDNA)	Cancer (colorectal)	Amgen
Gardasil	Human papillomavirus quadrivalent vaccine	Protein vaccine (VLP)	Self-assembled VLP of capsid protein of HPV types 6 11 16 and 18—adsorbed into aluminium-containing adjuvant	*Saccharomyces cerevisiae* (yeast) (rDNA)	Prevention of several diseases caused by HPV	Merck
Prevnar	Pneumococcal 7-valent conjugate vaccine	Vaccine (glycoconjugate)	Saccharides of capsular antigens of *Streptococcus pneumoniae* serotypes 4 6B 9V 14 18C 19F and 23F each conjugated to diphtheria CRM197 protein	Serotype from soy peptone broth CRM197 from *Corynebacterium diphtheriae*	mmunisation against several diseases caused by *S. pneumoniae*	Wyeth (Pfizer)
Fragmin	Dalteparin sodium injection	Carbohydrate	Controlled depolymerization of sodium heparin	Porcine intestinal mucosa	Deep vein thrombosis and others	Pfizer
Pulmozyme	Dornase α	Enzyme (glycoprotein)	Recombinant human deoxyribonuclease	Mammalian cell (CHO) (rDNA)	Cystic fibrosis	Genentech
Fabrazyme	Agalsidase β	Enzyme (glycoprotein)	Recombinant human α-galactosidase A	Mammalian cell (CHO) (rDNA)	Fabry disease	Genzyme

Abbreviations gG immunoglobulin Fab antibody fragments PEG polyethylene glycol VLP virus-like particles mAb monoclonal antibody

characterization of drug product formulation using biochemical and biophysical methods for adequate understanding of structural and functional correlations to stability in a stage appropriate manner. It should be noted that depending on the type of biologics candidate and its stability profile, it may be necessary to conduct additional formulation characterization studies especially when stability is poor and/or stability-bioactivity correlation is complex. In later stages of clinical development as well as for biologics license applications (BLA) it is expected that extensive formulation characterization studies are conducted.

Determination of Hot Spots

An important and first step in formulation characterization is to determine the potential liabilities in the amino acid sequence and other parts (for contents other than amino acid) of the biotherapeutic candidate. These liabilities are often referred to as "hot spots." There are some amino acids or groups of amino acids that exhibit common occurrences of chemical or physical degradation events such as oxidation and deamidation. For example, the amino acid methionine (Met) undergoes oxidation, especially in the presence of oxygen and when it is on the protein surface exposed to bulk solvent. Similarly, a surface-exposed pair of asparagine-glycine (Asn-Gly) when present in a loosely formed structural domain in the protein may be prone to deamidation under certain formulation conditions (1).

Linear sequence vs. folded structure. Determination of hot spots may not be trivial for all protein types. Prediction of lability of an amino acid based on primary structure [i.e., amino acid linkage (Table 2)] does not work well for folded proteins because surface exposure and flexibility in the three-dimensional structure are among the important criteria dictating propensity of degradation. For certain classes of biotherapeutics where adequate correlation between structural and chemical degradation is available, it might be possible to more accurately predict hot spots. For example, immunoglobulins (IgGs) of a given subtype may contain common hot spots in the conserved part of the sequence (Table 2). Similarly, degradation behavior of a nonconserved amino acid in a conserved structural motif in IgGs may be partially predicted on the basis of structural flexibility of the motif (unordered vs. helical or β sheet). While these approaches are quite useful in enlisting the common hot spots for chemical degradation, they may not predict physical degradation (aggregation) hot spots or unique chemical degradation events [e.g., tyrosine (Tyr)/tryptophan (Trp) oxidation].

The determination of hot spots needs information on folded structure but many biotherapeutic candidates will not have its crystal structure or other solution-based (e.g., NMR) structure available. In the absence of structure, homology modeling may be beneficial to derive qualitative structure using bioinformatics tools. In a recent study, Wang et al. (14) employed a novel use of bioinformatics tools to delineate common sequence segments across several antibodies and hypothesized that such segments may contribute to aggregation propensity on the basis of certain physicochemical properties of the contributing amino acids in these segments (rich in aliphatic/aromatic residues). Using full antibody atomistic molecular dynamics simulations, Chennamsetty et al. (15) identified the antibody regions prone to aggregation by using a technology called spatial aggregation propensity. Development of such bioinformatics tools is a good first step in understanding aggregation propensity, however it remains to be experimentally tested how accurately and widely such tools can be used for reliable prediction appropriate for drug development.

Physical and Chemical Degradations

Following determination of hot spots as described above, the next step in formulation characterization is to experimentally determine the major degradation pathway(s) and to understand the mechanism of degradation. Unlike small molecule drugs, protein-based biotherapeutics candidates have added complexity of several degrees of structure such as secondary, tertiary and quaternary structures that are critical to its stability and intended function. The degradations observed and/or predicted can be categorized into two types chemical and physical degradations. Majority of the degradations cited in Table 2 are of

Table 2 Protein and Peptide Degradation Hot Spots

Labile groups	Type of degradation	Occurrence in IgG and other proteins
Asn Gly	Deamidation, Isomerization	$NN^{386}G$ in CH3 (IgG2a) (2) $QN^{156}G$ in CL (IgG2a) (2) $LN^{316}G$ in CH2 (IgG1) (3) $SN^{385}G$ in CH3 (IgG1) (3)
Asn Ser, Asn Asn, Asn Thr, Asn Lys, Asn His, Asn Asp	Deamidation, Isomerization	$RN^{423}S$ in CH3 (IgG2a) (2) $PEN^{390}NY$ in CH3 (3) $VN^{30}T$ in CDR1 of LC (4) $SN^{329}K$ in CH2 (5)
Asp Pro Asp Gln	Clipping (peptide bond)	D^{274} P^{275} (IgG1) (5) D K in hinge (IgG1) (5) H T in hinge (IgG1) (5)
Asp Lys His Thr		
Asp	Isomerization	$D^{102}G$ in CDR3 of HC (IgG1) (4)
Met	Oxidation	M^{34} in CDR1 of HC (IgG1) (6) M^{101} in CDR3 of HC (IgG1) (6)
Cys	Oxidation (to form disulfide)	C^{105} in CDR3 of HC (IgG2a) (2)
Trp	Oxidation	W^{54}, W^{55} in CDR2 of HC (IgG1) (6) W^{105} in CDR3 of HC (IgG1) (6)
Tyr	Oxidation	Oxidation of lens protein forms dihydroxyphenylalanine, o and m Tyr, and di Tyr (7)
Pro	Proline isomerization	Trans P^{32} isomer formation in β2 microglobulin (8)
Lys	Glycation	K^{49} in LC (IgG1) (9)
Fe His/Asp/Tyr	Metal bond breakage	Iron loss by acidic pH, chelator in transferrin (10)
His Fe (heme)	Metal bond breakage	Low pH Fe His breakage in hemoglobin (11)
Met Fe (heme)	Metal bond breakage	Labile Fe S (Met) bond in cytochrome c breaks under various conditions (12)
Amine and other reactive amino acids	Reaction with buffer/excipients	May form adducts such as carboxylate adduct with citrate/succinate (13)
Various hydrophobic segments	Aggregation	Potential hot spots for aggregation in IgG predicted using bioinformatics tools (14,15)

Abbreviations: IgG, immunoglobulin; LC, light chain of IgG; HC, heavy chain of IgG; Tyr, tyrosine; Met, methionine.

chemical nature, whereas physical degradation includes aggregation, particulate formation, and related structural degradation events associated with adsorption, misfolding, denaturation (by heat, chemicals, chaotropes, etc.), partial misfolding, nucleating species, and sometimes chemical degradation. Physical degradation is complex and may involve a wide variety of causative factors that may involve protein-protein interaction, native state conformational distortion, air-water interfacial tension, and conformational changes induced by solvents, additives, and processing. Therefore, a multitude of biophysical tools (in addition to biochemical characterization) is often necessary to achieve a comprehensive formulation characterization.

ASSESSMENT OF PRIMARY STRUCTURE

Simply put, the primary structure of a protein consists of its amino acid sequence. For recombinant proteins, the amino acid sequence can be predicted from the cDNA used in its production. This basic attribute of a protein determines the entirety of its biophysical and biochemical properties. The amino acid sequence of a protein determines its ability to fold properly, and thus determines its ability to maintain its function. Therefore, a small change in the primary structure, depending on its location, may have a range of effects on a protein's activity, from no effect to a very large impact. The amino acid sequence can also impact the chemical and physical stability of a protein, even when there is no measurable impact on activity. Thus, confirming the amino acid sequence of a protein is fundamental to understanding its overall structure and properties.

During production of recombinant proteins, several modifications to the primary structure are possible. These include errors in transcription or translation, generating such variant forms as amino acid substitutions, N- and C-terminal extensions, splice variants, and internal sequence extensions. Other changes to the primary structure may occur as a consequence of biochemical instability, such as deamidation or oxidation. All of these variant forms can have large impacts on the properties of the protein, and need to be detected and controlled during production and storage.

Amino Acid Composition Analysis

One of the most basic assessments of primary structure is the confirmation of the expected amino acid composition of the polypeptide. Recombinantly produced proteins have amino acid sequences predicted from the DNA sequence used in their production. The amino acid composition, therefore, is a predictable attribute, and can be confirmed using amino acid composition analysis. The technique can be broken down into three steps: complete hydrolysis of the polypeptide into its constituent amino acids, chemical labeling of the free amino acids with a chromophore or fluorophore, and separation of the amino acids by liquid chromatography (LC), with quantification of the individual amino acids by UV absorbance or fluorescence detection (16,17). Typically, overnight digestion with 6N HCl or other acids at high temperature or vapor phase hydrolysis with trifluoroacetic acid is used for complete hydrolysis. Derivatization can be achieved either prior to separation (precolumn) or after separation but prior to detection (post-column). Typical chemical labels include fluorescamine, o-phthalaldehyde (OPA), ninhydrin, and phenyl isothiocyanate (PITC). Separation can be accomplished for all twenty naturally occurring amino acids using reversed-phase or ion exchange chromatography (IEC), the former typically used with precolumn derivatization methods and the latter used in combination with post-column derivatization approaches.

The harsh conditions used for complete hydrolysis of the polypeptide can lead to destruction of particularly sensitive residues. Trp and cysteine residues are typically destroyed during acid hydrolysis, and cannot be confidently quantified using this approach. Also, amino acids with side chain amide groups, glutamine and asparagine, are modified to form their analogous amino acids with side chain acid groups. The levels of these amino acids are added to the levels for the glutamic acid and aspartic acid residues, and can be quantified as combinations of glutamine plus glutamic acid and as asparagine plus aspartic acid (Glx and Asx).

The relative amounts of the amino acids present in the protein are determined by comparison with quantitative standards. This is one of the most accurate methods for determining the protein quantity. The amino acid composition of a sample can be compared with the theoretical composition on a residue-by-residue basis. Each of the amino acid residues may have a different precision depending on the relative stability of the residue during hydrolysis and the chromatographic properties of the residue on a given system.

In combination with accurate absorbance measurements, amino acid composition analysis is commonly used for accurate determination of protein molar absorptivity, or extinction coefficient (18,19). Once an accurate extinction coefficient is determined for a given protein, the concentration of the protein in formulated solutions can be determined consistently using UV absorbance spectroscopy according to the Beer-Lambert law

$$A = \varepsilon l C \tag{1}$$

in which A is the measured absorbance at a given wavelength, ε is the molar absorption coefficient in $M^{-1}cm^{-1}$ at that wavelength, l is the pathlength used in measuring the absorbance in cm, and C is the protein concentration in M. So, after determining the molar absorptivity at, for example, 280 nm, the protein concentration can be reliably determined by measuring the absorbance at 280 nm.

N-Terminal Sequencing by Edman Degradation

Confirming the termini of polypeptides is fundamental to their characterization, and N-terminal sequencing using Edman degradation is a robust technology for achieving

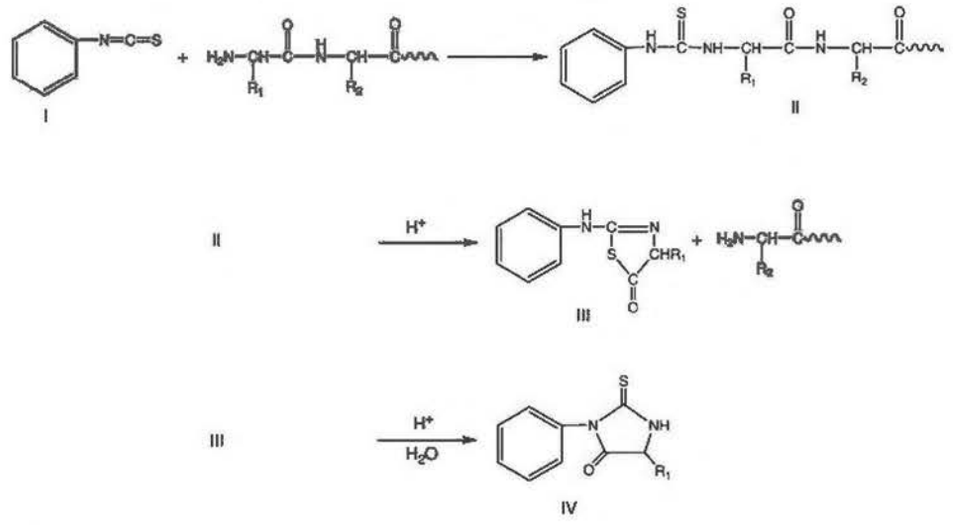

Figure 2 Edman degradation chemistry.

confirmation of the N-terminal residues (20). This technology uses amine-specific chemistry to remove the N-terminal residue, followed by chromatographic separation of the residue. By comparison of the retention time of the released residue with amino acid standards, the identity of the residue can be determined. After release of the N-terminal residue, a new N-terminal amine is generated, and the chemistry can be repeated in multiple cycles to deduce the N-terminal sequence of the protein. The reaction sequence is illustrated in the scheme below (Fig. 2). There are three steps to the Edman degradation reaction: (*i*) coupling of the Edman reagent, PITC, (*ii*) cleavage of the phenylthiocarbamyl polypeptide (*iii*) under acidic conditions to form an anilinothiazolinone (ATZ) derivative of the N-terminal amino acid, and a new N-terminus on the n-1 polypeptide, and (*iv*) conversion of the ATZ amino acid under acidic conditions to form a stable thiohydantoin (PTH) derivative of the N-terminal amino acid. The chemistry can be repeated after extraction of the PTH amino acid to determine the next amino acid in the polypeptide, and so on. Sequencing instruments are available such that the entire process is automated.

The number of cycles that can be repeated for a protein is highly dependent on the sequence of the protein, the amount of protein in the sample, and the conditions of the reaction. Typically, up to twenty cycles is easily attainable for a recombinant protein.

In many instances, the free amine on the N-terminus of the protein may be blocked, typically by acetylation or cyclization, thus preventing the Edman degradation reaction from occurring (21). N-terminal acetylation is a common post-translational modification which can prevent Edman sequencing. There are strategies for unblocking or removing acetylated N-terminal residues using enzymatic or chemical methods, but these methods are not generally considered to be very efficient. If the N-terminal residue is glutamine, these residues undergo spontaneous cyclization, blocking the free amine to form pyroglutamic acid (22). Less common is cyclization of glutamic acid to form pyroglutamic acid (23). Pyroglutamic acid can be efficiently removed using a pyroglutaminase enzyme, generating a free N-terminus on the n +1 residue that can then be sequenced using Edman degradation (24).

Proteolytic Mapping

Proteolytic mapping of proteins is the most comprehensive method for the determination of primary structure. This method employs the use of residue-specific enzymes to cleave the protein into smaller peptides, which can then be separated using high-performance liquid chromatography (HPLC). The resulting chromatogram, or proteolytic map, can be extremely

reproducible and specific for the protein, and can be used as an identity method when compared with a reference standard of the protein. It is often used as a batch release test for this purpose (25). Proteolytic maps can be extremely efficient at detecting changes in the protein primary structure, since a single change of an amino acid at the peptide level can often generate a detectable shift in the retention time of the peptide. In combination with MS, it is often used as a characterization tool for detecting and quantifying impurities and degradants (26).

The proteolytic enzyme appropriate for use for a given protein depends on the amino acid sequence. An analysis can be performed utilizing theoretical digestion on the basis of the specificity of the enzyme to determine the most appropriate enzyme for a given protein. The goal is to generate a sufficient number of peptides that can be well separated chromatographically, typically using reversed-phase chromatography, to generate a highly specific proteolytic map.

The specificity of proteolytic enzymes suitable for mapping include trypsin (C-terminal to Arg and Lys), endoproteinase Lys-C (C-terminal to Lys), V8 protease (C-terminal to Glu and Asp), and endoproteinase Asp-N (N-terminal to Asp). There are many other less common or less specific proteases that can be used when appropriate. Trypsin is a very common enzyme used for proteolytic mapping because of its high fidelity for its substrate sites and its generation of highly specific proteolytic maps for many proteins. It has the added advantage of generating peptides with C-terminal Arg or Lys residues, which can be detected with high sensitivity when analyzed using MS because of the high ionization efficiency of basic peptides in the positive ion mode.

Mass Spectrometry

MS is a powerful method for confirmation of the primary structure of proteins and peptides (27). The use of MS for characterization of therapeutic proteins is typically performed as a part of structural elucidation for regulatory submissions, and not as a routine batch release test. Current MS instrumentation is capable of measuring the molecular mass of proteins to within 100 ppm for intact proteins, depending on the instrumentation used and the molecular mass being measured. This is sufficient mass accuracy to confirm the predicted molecular mass on the basis of the amino acid sequence and expected post-translational modifications. For example, a protein with a predicted molecular mass of 20 kDa can be measured to within 2 Da at 100 ppm mass accuracy. With this mass accuracy, many modifications of the primary structure can be detected and examined further if present. The exception to this is alterations in the sequence of amino acids, or modifications or substitutions that lead to mass changes of 2 Da or less, such as deamidation of asparagine residues (a 1 Da mass change). These types of changes require proteolytic mapping in combination with MS or other orthogonal methods for their detection.

There are many types of MS methods that can be used for analysis of biotherapeutic proteins and peptides. MS is categorized by the type of ionization method and the type of mass analyzer used. For proteins and peptides, either electrospray ionization (ESI) or matrix-assisted laser desorption/ionization (MALDI) are used almost exclusively as ionization methods. ESI is more commonly used, as it is directly compatible with LC/MS as long as volatile mobile phase components are used for the separation (28,29). For analysis of intact proteins, either ionization method can be used. There are many types of mass analyzers used for therapeutic proteins. MALDI is typically coupled with time-of-flight (TOF) mass analyzers, and is characterized by very good sensitivity and a fairly high tolerance of salts and other buffer excipients (30). Compared with ESI coupled with TOF analyzers, however, the resolution of MALDI-TOF is significantly lower, meaning that the mass accuracy is not as good and the ability to detect variant forms is diminished. ESI is very intolerant of salts and buffer components; adducts of alkali metal ions are common for impure samples, which can lower the effective sensitivity and lead to an inability to accurately determine the molecular mass. Therefore, significant sample preparation to desalt the sample is required. However, when coupled with LC/MS, the separation effectively ensures that a pure sample is introduced into the ionization source so that high-quality spectra can be acquired. An efficient approach to analyzing intact proteins is to use LC/MS in which a de-salting column is used prior to introduction of the sample into the

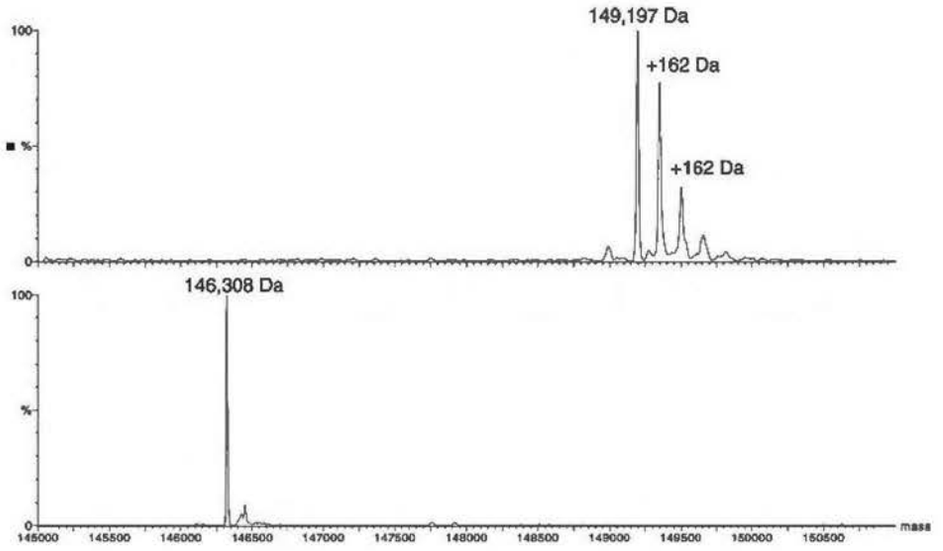

Figure 3 Mass spectra of an IgG (*top*) and the same IgG treated with PNGaseF to remove the N linked glycans (*bottom*).

ionization source. A common approach for characterizing proteins and peptides using MS is to first analyze the molecule intact, and then perform analyses on samples which have been purposefully degraded in some way to generate smaller species which can be thoroughly characterized. For example, a multichain glycoprotein can be analyzed intact, reduced, deglycosylated, reduced and deglycosylated, etc., with proteolytic mapping as the final "degradation" approach. An example of this is shown below in Figure 3, which shows a therapeutic monoclonal antibody (mAb) before and after deglycosylation analyzed using LC/MS. In the top spectrum, the glycan heterogeneity is evident from the multiple signals observed in the spectrum, most of which differ by the mass of a single hexose moiety, 162 Da. The mass difference between the untreated and deglycosylated samples yields the molecular mass of the N-linked glycans removed from the mAb, in this case 2889 Da. This difference corresponds to the molecular weight of two G0 glycans, each with a monosaccharide composition of four *N*-acetylglucosamine residues, three mannose residues, and one fucose residue. This is a common type of glycan for IgG molecules. On the basis of this analysis, the molecular mass of the expected primary structure can be confirmed, and information concerning some of the modifications, in this case glycosylation, can be determined as well.

When used in combination with proteolytic mapping, MS is invaluable for detecting unpredicted modifications to the primary structure and post-translational modifications (26). By digesting the protein into smaller pieces, more detailed information can be obtained for any modifications to the primary structure. For a tryptic peptide of molecular mass 1000 Da, for example, a mass change of 1 Da from the predicted molecular mass can be easily determined. An example is shown in Figure 4, which shows an expanded version of the 214 nm UV absorbance chromatograms of a tryptic digest of an IgG (top) compared with the same molecule subjected to a pH of 8 for three days to induce deamidation (bottom). The control sample shows a peak, labeled Peak A, which has a molecular weight consistent with an expected tryptic peptide with a sequence of GFYPSDIAVEWESNGQPENNYK. Two new peaks show up in the stressed sample, labeled B and C. Figure 5 shows the mass spectra of these peaks. Peaks B and C show a 1 Da difference relative to Peak A. This is consistent with deamidation of an asparagine residue. The predicted peptide contains three asparagine residues, making this a reasonable interpretation of the data. Tandem MS, in which an ion

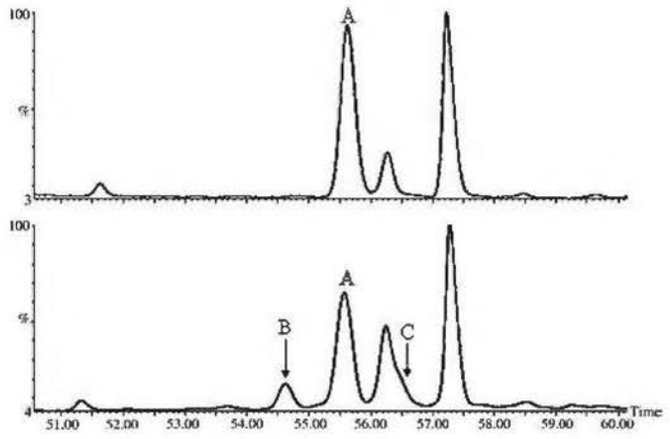

Figure 4 Expanded views of the tryptic maps (214 nm) of an IgG sample (*top*) and the sample treated with pH 8 for three days (*bottom*).

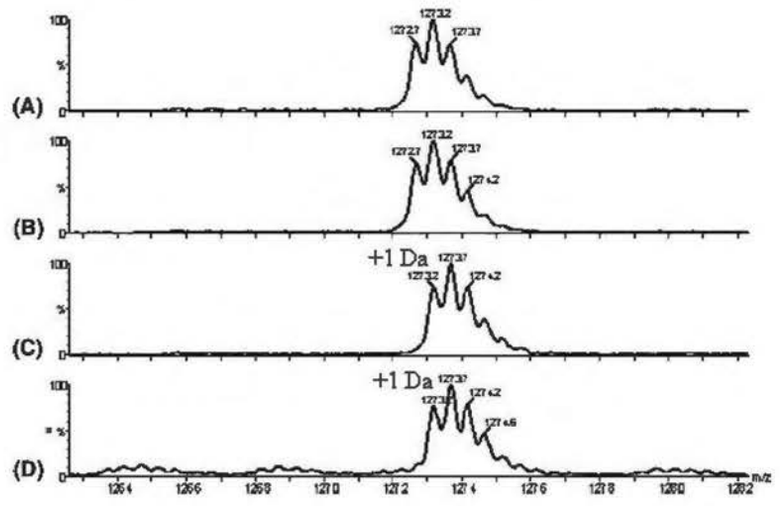

Figure 5 Mass spectra of the peaks shown in Figure 3. (**A**) IgG peak A. (**B**) Stressed IgG peak A. (**C**) Stressed IgG peak B. (**D**) Stressed IgG peak C.

formed in the ionization source is subjected to fragmentation and the resulting fragment ions are measured, is a powerful tool for determining the sites of modifications. In the example above, the precursor ion for the putatively deamidated peptide can be subjected to tandem MS to determine which asparagine in the peptide is the site of deamidation for each of the peaks.

Tandem mass spectrometry (MS/MS) can be accomplished using multiple modes of fragmentation. Most commonly used is collisionally activated dissociation (CAD), in which the precursor ion is accelerated in a collision cell in the mass spectrometer which is filled with a collision gas, such as argon, to impart internal energy into the ion, leading to fragmentation. For peptides, fragmentation tends to occur along the peptide backbone at the amide bonds. This leads to fragment ion spectra which differ in mass by the residue mass of the amino acids present in the peptide. In this way, the sequence of the peptide and the site of any modifications to the peptide can be determined. Fragmentation of the peptide can also be generated using other means, including electron transfer dissociation (ETD) in ion trap

instrument, or electron capture dissociation (ECD) or multiphoton dissociation (MPD) in ion cyclotron resonance (ICR) instruments.

ASSESSMENT OF SECONDARY STRUCTURE

Circular Dichroism

CD spectroscopy measures differences in the absorption of left-handed and right-handed circularly polarized light which arises from an optically active (chiral) molecule. The amide bonds in a protein absorb in the far ultraviolet (far UV), approximately 180 to 250 nm, where the peptide contributions dominate. The intrinsic CD of a protein in the far-UV region is influenced by the asymmetric environment as a consequence of the three-dimensional structure adopted by the molecule, and therefore is sensitive to the secondary structure (e.g., α-helical, β-sheet, β-turn) of the protein. This gives rise to characteristic CD profiles for each secondary structure type (31,32). Therefore, any changes in protein secondary structure due to unfolding or structural conversion can be conveniently monitored by CD spectroscopy.

In the wavelength range at greater than ~240 nm, typically ~240 to 300 nm (near UV), the amino acids Cys (at >240 nm and >320 nm), Phe (peaks at ~262 nm and ~268 nm), Tyr (maxima at ~275 282 nm) and Trp (~280 293 nm and ~265 nm) exhibit CD bands that are characteristic of the tertiary structure of the biomolecule. These bands can be used to monitor local conformational changes as well as large scale structural changes in the protein (31,32).

The biomolecules that contain non amino acid groups in their active site (ligand- or substrate-binding site) such as porphyrin, heme, metal centers (Fe, Mo, Cu, etc.) coordinated to amino acids, Fe-S cluster, and many other groups may display characteristic CD bands depending on the local structure of the chromophore and its chiral properties. Such CD bands can be extremely useful probes for studying structure-function relationship in these proteins, especially the redox-induced events.

CD of protein-based biologics such as human growth hormone or monoclonal antibodies is measured in aqueous buffered solutions. A protein solution of approximately 0.1 to 1.0 mg/mL can be used, depending on protein molecular weight, its CD strength, and pathlength of measurement cell to measure far-UV CD spectra. Because the near-UV CD signal of proteins is far less intense than in the far UV, typically a ~10× higher concentration is needed for the near-UV range. Alternatively, with higher protein concentration, various pathlengths of sample cell (e.g., cuvette) can be used to accommodate CD measurements in wider wavelength ranges. One can push the measurable limit of high protein concentrations using conventional CD instrumentation by reducing the cell pathlength to much less than 1 mm. However, concerns of surface denaturation of protein due to interfacial tension, artifacts of solution drying, and inaccuracy of pathlength need to be considered to ensure the quality of CD data. To consistently acquire good quality CD spectra down to ~190 nm, the spectrometer along with the UV lamp and mirrors must be carefully maintained and purged with high quality nitrogen flow.

Far-UV and near-UV CD data are often used for assessment of secondary and tertiary structure, respectively, of a biologics candidate for analytical reference material characterization as well as drug product formulation characterization. It is also used to establish comparability of drug substance between campaigns and/or batches. It should be noted that the near-UV CD spectral signature by itself generally does not point to any particular tertiary structural type of a protein, but instead can be used to compare changes between batches of recombinantly produced protein.

The CD spectrum of proteins in the far-UV range has distinct signatures for α-helical and β-sheet structures. For example, a majority α-helical content (e.g., human growth hormone) displays strong negative bands at ~208 nm and ~222 nm, and a positive band at ~192 nm, while a majority β-structure content (e.g., mAb) shows a negative peak at ~216 nm and a positive peak at ~200 nm depending on the mix of β-sheet (parallel or antiparallel β-sheet) and β-turn components, and any α-helical contributions. Because α-helical structure contributes much stronger to the CD spectrum in the far UV, the presence of even a small percentage of α-helical structure content can significantly change the CD peak positions of a majority β-sheet protein. Unordered (random coil, e.g., unfolded protein) structures can exhibit a strong negative band at ~195 to 200 nm (32). The CD spectrum in the far-UV range can be used to

make an empirical estimate of secondary structure using several algorithms including least squares fitting, singular value decomposition, and self-consistent method (SELCON) (31,33). SELCON is quite popular for secondary structural estimates, and it deconvolutes decent structural information for both α-helical and β-sheet/turn structural components. However, depending on the quality of CD spectra, the estimation of structure can vary significantly, and therefore such estimates should not be used for comparability purposes. A better protocol for drug substance comparability is to compare and overlap normalized CD spectra of protein samples of which accurate protein concentration data (of the identical samples that are used in CD measurements) are available. Normalized CD values can also be expressed in molar residue ellipticity (i.e., also normalized for number of amino acid residues) that is useful to compare CD value/spectra between different proteins belonging to the homologous structural class.

Although CD spectra in far and near UV are very useful in assessing as well as comparing the secondary and tertiary structures of a protein, it is very challenging to reproducibly detect small structural changes. Because of uncertainties introduced by the measurement protocol as well as interference from the drug product formulation matrix, it is difficult to determine an accurate limit of quantitation of the method.

Fourier Transform Infrared

Fourier transform infrared (FTIR) spectroscopy is another tool for probing secondary structure of protein- and peptide-based biologics candidates (34,35). The vibrational motions in a molecule when coupled with a change in dipole moment can be observed, in principle, in an FTIR spectrum. However, several factors including overlap with rotational motions result in significant band broadening under normal conditions relevant to biologics formulations. Additionally, the changes in dipole moment need to be sufficient for actually observing a vibrational frequency. For peptides and proteins, typically the amide region is tracked for secondary structure determinations (34,35). The amide region has multiple frequencies but practically three of them (Amide I, Amide II, and Amide III) are most useful. Modern FTIR spectrometers are capable of producing high quality spectra in the mid-IR range of approximately 1000 to 1800 cm^{-1} that is useful for protein secondary structures. The low frequency range (<1000 cm^{-1}), if desired for detecting out-of-plane bending modes in polypeptides, can be studied using accessories with appropriate IR grade materials (crystal). For example, an attenuated total reflectance (ATR) accessory with diamond crystal and compatible optics can go down to approximately 200 cm^{-1}. In addition to protein-related vibrational bands, one can choose to probe signature bands from excipients (e.g., sucrose) and other additives present in biologics formulations. Finally, the CO stretching vibration of carboxylic acid-containing side chains and other vibrational modes from polar and aromatic side chains of amino acids also can be seen in FTIR spectra of proteins, but these are typically much weaker than amide I and II bands (35).

Protein FTIR spectra show a strong amide I band in the 1600 to 1700 cm^{-1} range arising from primarily C = O stretching of the polypeptide backbone. Amide II (~1480 1580 cm^{-1}) and III (~1230 1300 cm^{-1}) bands are comprised of CN stretching and NH bending modes. The amide bands are sensitive to type of secondary structure (e.g., α-helical, β-sheet, β-turn) and therefore the band pattern (intensity and frequency) in the amide region of an FTIR spectrum can be used to distinguish protein structural types. The origin of this sensitivity (frequency and intensity pattern) is attributed to hydrogen bond strength of amide CO and NH groups, and associated dipole orientations (collectively) present in a particular secondary structure type.

Unfortunately, interference from water (water bending frequency at ~1645 cm^{-1} overlaps with amide I) is a major issue for most biologics formulations, especially for aqueous solutions. Water being the major component (~55 M) in aqueous formulations gives rise to a strong band that requires careful subtraction by a reference spectrum. Obviously, the water issue is minimized when the biologics formulation is freeze-dried to make lyophilized powder with low water content. Water interference as well as other measurement errors can lead to erroneous assignment of secondary structure types. Several practical measures have been proposed to avoid some of the artifacts (36). These include ensuring (a) appropriate amide I/II ratio (1.2 1.7), (b) presence of amide III bands, (c) presence of C-H stretching modes,

Table 3 Fourier Transform Infrared Frequencies of Amide I Band in Polypeptides

Structure type	Amide I frequency, cm ^{1}
α Helix	~1654 (range 1640 1660)
β Sheet[a]	~1633 (range 1620 1641)
	~1684 (range 1670 1695)[a]
β Turn[b]	~1672 (range 1650 1690)
3_{10} Helix	~1660 1670
Unordered structure (random coil)	~1654 (range 1640 1660)
Denatured aggregate[c]	~1615
	~1695

[a]β sheet amide I is often characterized by a shoulder at ~1670 to 1695 cm ^{1} in addition to the major band at ~1620 to 1640 cm ^{1}.
[b]Assignment of amide I for β turn is highly variable and should therefore be used with caution.
[c]Aggregates formed by native state or nearly native state of proteins may not exhibit amide I frequencies similar to denatured aggregates.
Source: From Refs. 35 38.

(d) appropriate subtraction of vapor bands, (e) no artifact from protein adsorption on sample cell or ATR crystal, (f) appropriate baseline of spectrum, and (g) mismatch of pathlength between sample and reference spectra. An ATR accessory is particularly useful for versatile applications including lyophilized powder, suspensions, liquid, etc., that provide adequate surface contact on the crystal. Diamond crystal is scratch resistant and may help avoid excessive protein adsorption, therefore eliminating some of the artifacts noted above.

Determination of secondary structure is often achieved by examining the amide I frequency or group of frequencies (Table 3). This is possible when a protein or peptide has predominant helical or β structure. However, if structure content is mixed, it is difficult to readily assign a structural type. Additionally, as seen in Table 3, the range of amide I frequencies of multiple structure types overlap significantly (e.g., frequency overlap of α-helix and unordered structure). For an unknown structure, one can get a qualitative estimate by using various algorithms including curve fitting, and pattern recognition such as factor analysis. The derived numbers for content of structural component types are only qualitative, and they can be quite sensitive to the quality of an FTIR spectrum. Therefore structure content determinations may not be suitable for QC (quality control) environment.

Collecting FTIR spectra of low-concentration protein formulations (1 mg/mL or less) can be challenging for the detection of amide bands. However, many of the commercial and clinical biologics formulations employ relatively high active concentrations; therefore FTIR can be suitably used. In fact, for very high concentration protein formulations, FTIR is one of the very few techniques that do not require sample dilution. To prepare samples for FTIR measurements, KBr pelleting has been extensively used. This could be a problem for some sensitive proteins. The modern applications (using ATR and other state-of-the-art accessories), however, do not require sample manipulation, and therefore enables higher throughput as well as application to a wide variety of biologics samples.

ASSESSMENT OF TERTIARY STRUCTURE

Disulfide Bond Determination

The tertiary structure of a protein is often highly dependent on the formation of disulfide bonds. Disulfide bonds confer physical stability to the protein as well as ensuring that it maintains its active form. For recombinantly produced proteins, the confirmation of disulfide bonds is a fundamental part of the elucidation of structure, and any variants present because of incorrectly paired disulfides needs to be assessed. The number and arrangement of cysteine residues in a protein can lead to significant complexity for the determination of the disulfide connectivity.

A typical approach for the determination of disulfides in a protein involves proteolytic mapping under nonreducing conditions, followed by detection of the resulting disulfide-bound

peptides formed, often using mass spectrometric detection (39). For small proteins with few cysteines, this may be straightforward. For larger proteins with many cysteine residues, the complexity may require additional analyses to map all of the disulfides. A parallel analysis, in which all of the disulfides are reduced, with a comparison of which peaks have changed upon reduction, can aid in the detection of which peptides are involved in disulfide bonding. IgG molecules, which are a major class of biotherapeutics in the form of monoclonal antibodies (mAbs), have several disulfides predicted in the constant and variable regions of the molecule. These disulfides serve to connect the heavy and light chains together and to form the intrachain loops necessary for the IgG to maintain its functions. Most of the commercial therapeutic mAbs are IgG1 molecules, which is the major subclass of the IgG class of molecules. The disulfide bonding of IgG1 molecules has been well established. IgG2 molecules have been under development as biotherapeutic entities for some indications because of their low level of secondary activity, such as antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). One IgG2 molecule, panitumumab, which is an antiepidermal growth factor receptor (EGFR) mAb, has been approved for use for the treatment of metastatic colorectal carcinoma (40). It was recently discovered that IgG2 molecules have an intrinsic heterogeneity in their disulfide connectivity, which leads to a mixture of at least three forms of disulfide isomers (41). These disulfide mediate isomers differ in the interchain disulfide bonds. As therapeutic entities, the levels of each form and their relative activities and properties are attributes that need to be determined.

Protein Intrinsic Fluorescence

Fluorescence spectroscopy is a powerful and widely used tool to monitor higher order structures in proteins (42). Most proteins have intrinsic fluorescence that originates primarily from Trp residues. Tyr and Phe residues also contribute to total protein fluorescence, although quantum yield of Tyr is much less than for Trp, and Phe is the weakest among the three. Fluorescence may also originate from other cofactors present in a protein such as flavin, porphyrin, etc. For most therapeutic proteins, Trp is widely used as a fluorescence probe because of its frequent presence in proteins as well as ease of use and wide applicability in formulation screening and characterization studies. The advanced uses of fluorescence include fluorescence lifetime measurement (time-correlated single photon counting method, phase modulation method in frequency domain), fluorescence resonance energy transfer (FRET), fluorescence correlation spectroscopy (FCS), single-molecule fluorescence, rotational correlation time by time-resolved anisotropy, decay-associated spectrum (DAS), and others. Discussion of these advanced uses is generally out-of-scope for this section.

When a fluorophore (e.g., Trp) is excited using a light source matching its absorption band (excitation wavelength) electrons are promoted from ground electronic state (S_0) to excited states (S_1, S_2, etc.). The cascade of events following excitation is often described by the Jablonski diagram (42). Fluorescence emission occurs from the lowest vibrational level of the excited state (S_1), and exhibits a red shift because of loss of energy in the process.

Trp(s) in proteins exhibit a relatively broad absorption band at approximately 280 nm. When excited at 280 nm, Trp emission occurs over a range of wavelengths up to approximately 450 nm and appears as a very broad band. Most folded proteins show Trp fluorescence emission maxima in the 320 to 350 nm wavelength range. Exceptions include azurin in which the Trp located in a highly hydrophobic environment exhibits an emission maximum at 308 nm the most blue-shifted spectrum known of Trp in a protein. Typically, protein unfolding causes exposure of buried Trp to bulk solvents, and hence a red shift of the emission maxima to approximately 350 nm is observed.

A typical steady-state (i.e., not a fluorescence lifetime study) fluorescence measurement is quite straightforward. However, several precautions should be taken to avoid artifacts. An appropriate concentration of the protein or peptide in solution should be chosen to ensure that absorbance at 280 nm or the chosen excitation wavelength is not far greater than approximately 0.1 OD. High absorbance at or following the excitation wavelength causes nonlinearity and an artificial reduction of emission intensity called "inner filter effect" (loss of emitted photons due to absorption). Inner filter effect is caused by high absorbance of any

component in solution including protein, excipients and other additives if it overlaps with the emission wavelengths, and may lead to incorrect conclusions from fluorescence data, reported in literature [caused by sodium dithionite absorbance, (43,44)]. It is possible to collect partial emission spectra of higher concentration protein solutions (up to ~0.5 OD at 280 nm) at >310 nm emission range and using a higher wavelength excitation (e.g., at 295 nm). However data interpretation must be conducted with great care keeping in mind that emission intensity may not be proportional to lower protein concentrations.

Appropriate baseline correction should be performed by subtraction of a reference spectrum of matching solvent. This is particularly important for low-concentration protein solutions and when quantum yield of Trp is very low (such as quenched by heme/metal, or Trp is oxidized). In these cases, the relative intensity of the water Raman band may appear as a prominent shoulder or peak in the emission spectrum. Location of the Raman band (~3450 cm^{-1}) in a fluorescence spectrum depends on the excitation wavelength (for 295 nm excitation, it appears at ~329 nm).

Choice of excitation wavelength depends on what fluorophore is used as a probe. If a protein contains both Trp and Tyr residues, one can use either 280 nm or 295 nm to collect fluorescence contribution, respectively, from Trp plus Tyr or Trp only. If there are multiple Trps present in a protein, they all contribute to the emission spectrum. Therefore, if a change in fluorescence intensity and/or emission maximum is observed in a multi-Trp protein (such as a mAb), it is not easy to interpret the data because of the large number of possibilities as causative factors including local conformational change, global structural change, solvent effect (if relevant), quenching due to charge, quenching by oxygen/additives/side chain/bound groups/disulfide bond, change of quenching efficiency of quenchers present in native state, and many others.

Measurement of fluorescence lifetime is generally recognized as providing a more quantitative estimate of some of the fluorescence events. For example, if a fluorescence dye partitions itself between hydrophobic and solvent-exposed environments, simplistically it may yield two distinct lifetimes and one can determine the percentage population of each of the components. Measurement of Trp lifetime may not always help because each single Trp displays two prominent lifetime components arising from two rotamers (42,45,46). Therefore multi-Trp proteins are comprised of (theoretically) several lifetime components; however, there are practical difficulties of how many discrete lifetimes can be retrieved from fluorescence decay data. Analysis involving more than four lifetime components is unreliable, but one can employ lifetime distribution analysis aided by sophisticated mathematical algorithms such as Maximum Entropy Method (47). Trp lifetime data can sometimes help in understanding the impact of solvent relaxation and dynamic quenching.

The sensitivity of Trp fluorescence emission maximum in proteins is generally interpreted as excited Trp (indole ring) interacting with its microenvironment (45,46). For example, in azurin (also noted above), Trp side chain is surrounded by a nonpolar environment, whereas if the excited state interacts with a polar solvent or charged/polar side chains, it emits in the red. Emission maxima as well as quantum yield are also influenced by intramolecular quenching (for example, Fe-porphyrin in cytochrome c, Cu in hemocyanin).

For practical applications of Trp fluorescence in formulation characterization as well as for comparability purposes, steady-state fluorescence studies are quite sufficient to probe conformational changes or unfolding of a therapeutic biological candidate because of high sensitivity of fluorescence signal to local environment of Trp and high signal-to-noise ratio of fluorescence signal. The major goal in the application of Trp fluorescence spectroscopy in a comparability study is to interpret the fluorescence properties such as emission maxima and fluorescence intensity in terms of changes in protein structure. In other words, it is expected that comparison of fluorescence spectra will detect any significant changes in folding and structure of a biologics candidate arising from changes in manufacturing and process. Fluorescence quenching studies using acrylamide and sodium iodide provide valuable information on surface exposure of Trp. A conformational transition may change the exposure of Trp to solute quenchers (acrylamide, iodide, or CsCl), hence can be monitored by measuring Trp quenching (45). Steady-state fluorescence anisotropy is another fluorescence protocol that can be used to study rigidity (or lack of) of a protein segment and relative size of a protein.

Anisotropy value can change upon unfolding of a protein or complexation of a protein (e.g., aggregation, antigen binding).

Tyr fluorescence is less commonly studied because of its weaker fluorescence relative to Trp. Tyr absorption band appears at ~277 nm (tyrosinate at ~294 nm) and the corresponding fluorescence emission maximum is at ~303 nm (~340 nm for tyrosinate emission). Although the microenvironment of Tyr may have a strong effect on its emission intensity, the emission maximum of Tyr is relatively insensitive to local environment (48), in sharp contrast to the behavior of Trp.

Use of external fluorescence probes is very popular in all areas of biology, biological chemistry, and protein chemistry. There are literally thousands of fluorescent dyes for various purposes. For formulation characterization, a few of them are worth noting in this section. ANS (8-anilino-1-naphthalenesulfonic acid) and bis-ANS are used traditionally to probe hydrophobicity and change in surface exposure of hydrophobic groups in a protein. Thioflavin T and Congo red are generally used to look for the presence of amyloid-like structure (aggregate). Nile red is also known to be sensitive for aggregate detection.

POST-TRANSLATIONAL MODIFICATIONS

Most proteins are modified in some way after translation of the polypeptide chain. These modifications may impart specific function to the protein and can be integral to the protein activity or stability. For biotherapeutic proteins, common post-translational modifications include disulfide bond formation, N-terminal acetylation, or glycosylation. Degradation of amino acid residues can be considered as post-translational modifications, but are typically discussed separately as part of stability. However, the tools used for analysis of many types of post-translational modifications are the same. The types and propensity of these modifications are dependent on both the protein and the expression system used for its production. Some of the most common modifications and degradation products observed for biotherapeutic proteins are discussed below.

Glycosylation Analysis

Glycosylation of proteins is a common post-translational modification which can affect the physical properties and activity of the biotherapeutic protein. Glycosylation has been shown to affect the activity, *in vivo* clearance, immunogenicity, and stability of biotherapeutic proteins (49,50). For these reasons, the levels and types of glycosylation need to be determined and controlled for biotherapeutic proteins.

Glycoproteins can be either N-linked or O-linked, depending on the type of covalent modification of the glycan to the protein. The type of glycosylation is dependent on both the protein sequence and the expression system used to produce it. Glycosylation may commonly occur for proteins expressed in mammalian or yeast expression systems, but is not observed for proteins expressed in bacterial systems. N-linked glycosylation occurs only at asparagine residues in the consensus sequence of Asn-Xxx-Ser or Asn-Xxx-Thr, where Xxx is any amino acid except proline. While the presence of this sequon does not guarantee glycosylation, it makes N-linked glycosylation a predictable attribute. The amino acid sequence can be easily scanned for this sequon to determine if N-linked glycosylation is a possibility for a given biotherapeutic protein. Analysis of N-linked glycosylation, therefore, begins with an assessment of the site occupancy levels of any possible N-linked glycosylation sites in molecule, referred to as the macroheterogeneity. This can be accomplished using analytical methods which can distinguish size variants, such as electrophoretic or chromatographic separations, or MS. For glycoproteins with multiple glycosylation sites, macroheterogeneity can lead to complex mixtures. For example, the therapeutic glycoprotein interferon γ (IFN-γ) has two sequons for N-linked glycosylation. Therefore, there are four theoretical forms on the basis of occupancy alone: unoccupied, two different singly occupied forms, and one fully occupied form.

The identities of the glycans at a specific site can be extremely varied as well, contributing to additional complexity termed microheterogeneity. Microheterogeneity can be assessed by isolating the glycans associated with a given site and determining the glycan identity. There are a wide variety of analytical methods and approaches for assessing the levels

and identities of glycans present in a biotherapeutic protein. The methods used depend highly on the specific molecule being analyzed, the type of instrumentation and skill available in the laboratory performing the analyses, and the level of detail required for regulatory approval. For routine batch release of a glycoprotein, profiling for consistency may be appropriate, while more detailed structural characterization may be required to satisfy Elucidation of Structure expectations.

For N-linked glycans, there are enzymes such as PNGaseF, which are efficient at removing glycans, which can then be identified using orthogonal methods. Typically, chemical labeling of released glycans is necessary, since they lack a chromophore and thus a sensitive detection method. A common method for quantifying released N-linked glycans, termed glycan size profiling, employs enzymatic release of the glycans, removal of the protein by precipitation or filtration, labeling of the glycans with a fluorophore, and separation of the labeled glycans using normal-phase HPLC (NPLC) with fluorescence detection (51). This method is highly quantitative, since each glycan has one fluorescent label. For this reason, it can be used for routine batch release to ensure consistency in the types and levels of glycans.

Charge profiling is a common method for the determination of the relative amount of charged, or sialic acid containing, glycans. In this method, the glycans are prepared identically to size profiling: enzymatic release of the N-linked glycans followed by fluorescent labeling. The glycans are then separated by anion exchange chromatography, which separates neutral from singly charged from doubly charged glycans. This yields the relative levels of sialic acids in the glycan population.

There are several types of sialic acids possible, and these types depend on the production cell line. For example, murine cell lines such as NS0 produce mainly *N*-glycolylneuraminic acid, while CHO cell lines produce mainly *N*-acetylneuraminic acid. These sialic acid types can be distinguished using sialic acid typing, in which the sialic acid residues are removed from the glycans by acid hydrolysis, labeled with a fluorescent tag, and separated by reversed-phase HPLC. The identities of the sialic acids are determined by comparison of the retention times to a sialic acids reference panel of standards.

Glycan structure determination includes the assessment of monosaccharide composition, the sequence of the monosaccharides, the branching heterogeneity, and the linkage heterogeneity. While known structures can be confirmed using authentic standards, unknowns require a combination of methods, including MS and linkage-specific enzymes. The identities of the glycans can be determined using MS or by analysis of authentic standards. MALDI-TOF or electrospray MS of the released, labeled glycans yields accurate masses, which can be compared with the theoretical masses for confirmation of identity. For detailed structural information, tandem MS may be performed, which can be used for the determination of the sequence, linkage, and branching (52). In combination with chemical labeling methods such as permethylation or peracetylation, linkage information can be determined as well. The types of MS instruments utilized for tandem MS experiments include quadrupole time-of-flight (Q-TOF) hybrid instruments, which have an advantage of yielding accurate mass of product ions, or ion trap instruments, which are capable of multiple stages of fragmentation (MS^n) for potentially increased structural information. The degree of characterization performed is dependent on the nature and requirements of the molecule being developed.

Charge Heterogeneity

Biotherapeutic proteins may have intrinsic heterogeneity based on charge variants. These variants may be due to a variety of sources, including but not limited to, glycosylation with acidic or basic glycans, variably processed or modified N- or C-termini, degradation due to deamidation or cyclic imide formation, other modifications to basic or acidic residues, or peptide bond hydrolysis.

Deamidation may be a major degradation pathway for peptides and proteins containing asparagine residues. The mechanism for asparagine deamidation, shown in Figure 6, involves loss of NH_3 via a cyclic intermediate. The cyclic imide intermediate can be hydrolyzed to yield two potential products: an aspartic acid or an isoaspartic acid, which is a β amino acid. Both of these products are acidic variants of the original polypeptide and can be separated using charge-based separation methods, and both generate a change in mass of 1 Da relative to the

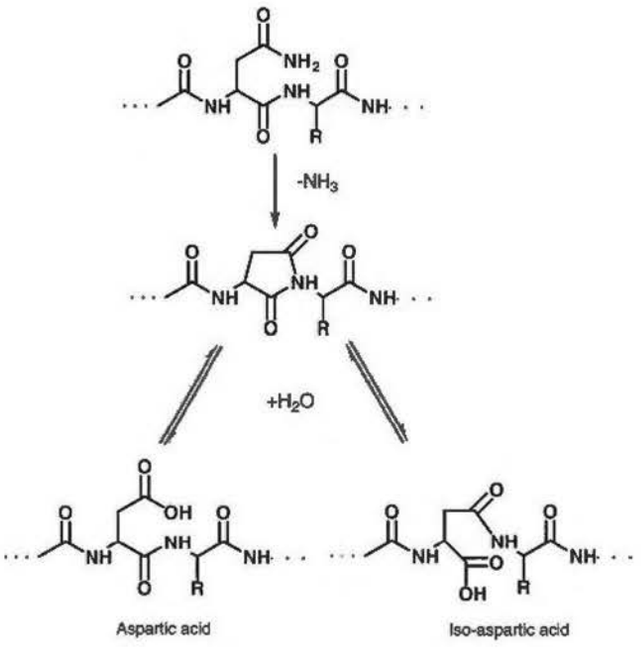

Figure 6 Mechanism for deamidation of an asparagine residue to form aspartic acid and isoaspartic acid via a cyclic imide intermediate.

original polypeptide. The degree of degradation is dependent on many factors, including neighboring residues, surface accessibility and conformation, and the pH of the formulation. The susceptibility and rate of deamidation of a given asparagine residue has been shown to be greatly influenced by the N+1 residue (1). Glycine in the N+1 position has been shown to give the highest rate of deamidation, followed by His, Ser, and Ala. A similar mechanism may occur for aspartic acid residues, in which cyclization followed by hydrolysis to yield either the starting material or its isomer, the isoaspartic acid residue may form. In this case, there is no difference in charge or mass relative to the original molecule. Exceptions to this are examples in which the cyclic imide intermediate is stable. For these species, the cyclic imide has a net basic shift in charge relative to the aspartic acid starting molecule, and is 18 Da lower in molecular mass.

Pyroglutamic acid formation is a common modification for proteins, and occurs spontaneously when the N-terminal residue is a glutamine, or less commonly, a glutamic acid. The formation of pyroglutamic acid from an N-terminal glutamine residue generates a net acidic shift and a loss of 17 Da. This is due to cyclization with the N-terminus with the loss of NH_3 from the side chain, which blocks the N-terminal amine. For monoclonal antibodies, N-terminal glutamine and glutamic acid residues are common for both heavy and light chains, and pyroglutamic acid formation is a very common post-translational modification for IgG molecules (22).

For monoclonal antibodies, variable levels of C-terminal lysine on the heavy chains lead to charge heterogeneity as well. The conserved heavy chain sequence of IgG molecules predicts a C-terminal lysine residue. This residue has been observed to be removed as a post-translational modification, and is thought to be due to proteolysis in the cell leading to a heterogeneous population (53). Typically, a mixture of species exists in which zero, one, or two heavy chains have the lysine removed.

Other modifications leading to charge heterogeneity include glycation of lysine (acidic shift), carbamylation of lysine (acidic shift), C-terminal amidation (basic shift), and N-terminal acetylation (acidic shift). These potential modifications of biotherapeutic proteins need to be detected, quantified, and controlled using analytical methods appropriate for their detection and quantification.

There are a variety of methods that are useful for detecting, characterizing, and quantifying charge variants in proteins. These include isoelectric focusing (IEF), capillary IEF, and ion exchange chromatography (IEC). The advantages of these methods are that they can separate and quantify overall charge heterogeneity. However, they give little or no information concerning the types or sites of charge heterogeneity present in the molecule. For monitoring stability, inherent charge variability may interfere with the ability to monitor degradation using these methods. An example would be the assessment of deamidation in a glycoprotein in the presence of significant heterogeneity in sialic acid levels. So, while these methods may be appropriate for routine batch release and monitoring of consistency, more detailed characterization is required to gain information on the presence of specific modifications leading to charge heterogeneity. Site-specific information can be assessed using approaches involving proteolysis and LC/MS. This approach can be used to characterize and quantify, for example, deamidation at a specific site in the presence of inherent heterogeneity elsewhere in the molecule.

IEF is a gel-based method which separates analytes in an immobilized pH gradient (54). Proteins will migrate in an electric field to their isoelectric point (pI), which is the pH at which the overall charge is net neutral. Charge variants can be well separated using this technique, with resolution as high as 0.01 pH units. The resolution can be determined by the gradient used in the separation. For high resolution separations a very narrow pH gradient may be used with long focusing times. IEF offers a reproducible method for establishing consistency of batches with regard to charge variants, as well as a powerful method for monitoring stability of protein therapeutics. This method is tried and true, but is not highly quantitative because of the need for general protein staining and densitometry, both of which may be highly variable.

Capillary IEF offers the possibility of high resolution with a more reproducible quantitation (55). In capillary IEF, the species are focused in a capillary to their pI, and then migrate to the detector using either electroosmotic flow or differential pressure. Detection can be performed by UV absorbance, offering reproducible and automated quantitation. For more sensitive detection, laser-induced fluorescence (LIF) detection can be used. Another mode of capillary IEF, termed imaged capillary electrophoresis (iCE), offers detection in the capillary without a mobilization step (56). This leads to increased resolution because no band broadening occurs as a consequence of the mobilization step. Also, detection is based on imaging of the entire capillary, so quantitation is generally more reproducible. This method offers high quality, quantitative data for charge variants. The acidic and basic species can be reproducibly quantified using this method.

IEC is a powerful method for separating charge species in an HPLC format (57). IEC separates charged species on the basis of electrostatic interactions of the analyte with a column resin. Anion exchange resins are positively charged, and bind negatively charged analytes, while cation exchange resins are negatively charged, and bind positively charged analytes. In an anion exchange separation, more acidic, or negatively charged analytes, will be retained more strongly and will elute later than less acidic species. Ion exchange resins can be considered strong or weak, depending on the type of resin used. A typical strong anion exchange resin contains a quaternary amine, which has a fixed positive charge or other strong basic species, and tightly binds negatively charges species. Weak anion exchange resins have basic species such as diethylamine functional groups, which bind negatively charged species, but not as tightly as strong anion exchange resins. Conversely, cation exchange resins are either strong acids, such as sulfate groups, or weak acids, such as carboxymethyl groups. Elution of analytes from ion exchange resins can be obtained using a salt gradient to compete with the charge on the resin, or by changing the charge on the analyte by changing the pH of the mobile phase over the course of the separation. As a consequence of IEC being an HPLC method, it has advantages over IEF methods in terms of throughput, ease of use, and quantitative precision. HPLC methods are extremely valuable in that individual fractions of separated species can be collected and characterized further. In an IEC separation, for example, an acidic variant can be separated and quantified, collected, assessed for potency relative to the parent molecule, and further characterized using orthogonal methods to determine the specific site(s) of modification in the molecule. This type of further characterization would be difficult or impossible using capillary electrophoretic methods.

Size Heterogeneity

Size heterogeneity of recombinant proteins may refer to truncated variants because of peptide bond hydrolysis or to the formation of aggregates.

Truncated Species

For truncated variants due to peptide bond cleavage, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a common method used for analysis. SDS-PAGE is based on the migration of a protein in an electric field. The proteins are first treated with SDS, which coats the proteins with a polyanion. In this way, the proteins are coated to similar size to charge ratios, and migration through a polyacrylamide gel is dependent on the size of the protein, with smaller proteins migrating faster through the gel, and larger proteins migrating a shorter distance. By comparison with standards, the molecular weight of the analytes can be estimated on the basis of the migration distance through the gel. Just about any protein analysis laboratory is set up to perform routine SDS-PAGE analysis as a first step in characterization. It gives a visual assessment of the quality of the material in terms of the purity and size heterogeneity. It can be extremely flexible in the type of detection used. General protein stains such as Coomassie blue are reliable and give a visual readout for the detection. For more sensitive detection, silver stain or some of the fluorescent stains such as Sypro Ruby offer the highest sensitivity. Finally, gels offer the possibility of immunoblotting or immunostaining of analytes once they are separated, which provides some additional functional information of the species being separated. Like other gel methods, SDS-PAGE is labor intensive and suffers from a difficulty in reliable quantitation. For routine characterization, SDS-PAGE is a reliable method for assessing the size heterogeneity of a formulated protein product, and is a powerful method for comparison of batches or stability in different formulations.

Capillary gel electrophoresis (CGE), also referred to SDS-CE, has the ability to resolve proteins from 10 to 200 kDa. It offers similar resolution to gel-based separations, but is more easily quantifiable because of the detection methods used (UV absorbance). Like SDS-PAGE, the proteins are coated with SDS and are separated on the basis of migration through an electric field, although in this case through a capillary. Since this method utilizes UV absorbance detection, the sensitivity may be limited for low levels of size variants that in gels could be detected with sensitive staining techniques such as silver stain. LIF detection can help overcome this limitation when applied to CGE. In combination with fluorescence labeling, LIF can lead to sensitive detection of separated species, including low level impurities and truncated variant species (58).

Aggregates and Particulates

Aggregation is a process in which one or more drug molecules combine physically and/or chemically to form nonnative oligomers which may remain soluble or become insoluble depending on their size and other physical properties. Protein aggregates and particulates form in a wide range of sizes (nanometer to centimeter, thereby spanning nearly million-folds in dimension) and shapes making it extremely challenging to comprehensively characterize particulates in a biologics formulation (59,60). Dimers and other smaller size aggregates are soluble in nature and typically range in size from few nanometers to tens of nanometers. The aggregate species that are in the size range of hundreds of nanometers may still remain soluble in the sense that they may not exhibit any change in appearance of the formulation. Some of the multimers can grow huge in size and may eventually appear as visible particulates. Although all multimeric species (referring to degradation products only and not a purposefully created multimeric therapeutic candidate) are generally termed as aggregates, particulates refer to the large size aggregates in the size range of tens of microns or larger species that are visibly detected. Subvisible particulates range in size from few microns to many tens of microns. An approximate size boundary for a particulate to be visibly detected is 100 μm (59).

Characterization of aggregates typically includes detection of soluble aggregates on the basis of size, and determination of physicochemical properties and nature of aggregated

species such as covalent, noncovalent, reversible, irreversible, etc. Covalent aggregates are generally irreversible. Example of a covalent aggregate is disulfide scrambled species, which often are dissociable by a reducing agent. Noncovalent aggregates can be held together either by strong association (i.e., not dissociated by simple dilution or mild treatments) or weak association (i.e., may be reverted to monomer by dilution). Aggregates in both of these structural categories can cover a wide range of sizes. Therefore, it may be more convenient to classify the aggregates in terms of their size in reference to the capability of various biophysical and particle analysis technologies. Insoluble aggregate is also referred to as particulate (subvisible and visible as noted above), and precipitate (large size species that easily sediments). Insoluble aggregates require somewhat specialized protocols including enumeration (using light obscuration, light scattering, and light microscope) and characterization (imaging-based techniques, and spectroscopic methods such as FTIR or Raman), depending on the types of aggregates observed. Finally, finding the root cause of aggregation may involve all of the above and additional custom-designed protocols.

Formation of aggregates may occur under conditions such as storage, shipping, handling, manufacturing, processing, and freezing-thawing. One of the most challenging areas in aggregation, lately, is studying aggregate formation induced by freezing and thawing of biologics. It should be noted that the freeze-thaw induced aggregation phenomenon should not be confused with cold denaturation. Cold denaturation classically refers to denaturation induced by thermal factors per se without a change in the state of the bulk, and is linked to thermodynamically favored hydration of the hydrophobic core at low temperature (61). Freeze-thaw induced aggregation has been linked to secondary factors such as ice surface denaturation, freeze-induced change in solute concentration and pH, etc. (62), but theoretically may also include effects of cold denaturation. The study of freeze-thaw-induced protein denaturation and aggregation requires specialized equipments and protocols that can probe events in the frozen state.

Several aggregate separation methods are available depending on the type of information sought. Separation methods may either detect the presence of various species in a drug formulation [such as dynamic light scattering (DLS) and analytical ultracentrifugation (AUC)], or fractionate various species [such as size exclusion chromatography (SEC) and asymmetric flow field flow fractionation (aFFF)]. Fractionated species, if desired, may be collected for further analysis.

SEC is considered a "work horse" technique, especially for biologics, and major advantages include high throughput, automation, amenability to several detection systems, reproducibility, reliability, and operational compatibility in both the development and QC environments. Major applications include separation of drug monomer from higher molecular weight species that might accumulate during storage stability and processing. Disadvantages of SEC include concern for potential alteration/dissociation of aggregated species as a result of column/mobile phase interactions. Also, for a given biologics, the dynamic range for separation of various aggregated species is rather limited leaving large aggregates unfractionated or lost.

All four methods noted above (SEC, aFFF, AUC, DLS) are used in formulation characterization to monitor aggregate formation and to delineate the aggregation mechanism. Only SEC is used in a QC environment such as in GMP stability studies. Use of the other three techniques (AUC, aFFF, and DLS) in a QC environment is quite challenging because of the difficulty in adequately validating the methods and/or their low throughput. More detail of these techniques is covered in the last section of this chapter.

Although a relatively smaller number of techniques are available to study large particulates including protein precipitates, additional characterization can be accomplished by solubilizing the particulates using dispersing/denaturing solvents. Multiple biochemical assays can be utilized with solubilized particulates including SDS-PAGE or CGE. This characterization approach can be employed to estimate aggregate size after solubilization and determine if there are covalent linkages between protein molecules. However, the influence of hydrodynamic size may result in an inaccurate estimate of molecular mass for certain molecules, such as conjugated or pegylated proteins.

FORMULATION CHARACTERIZATION METHODS

Selected formulation characterization methods are described below. Use of these methods depends on the type of formulation (e.g., liquid, lyophilized powder, etc.), stage of clinical development, and type of information sought (e.g., to solve a process-related issue, to characterize a degradant, or delineate a stability issue).

Analytical Ultracentrifugation

AUC is an orthogonal method for size-based separation of high and low molecular weight species that employs centrifugal principles to determine size and shape (60). Two principal types of experimentations are conducted in AUC sedimentation velocity and sedimentation equilibrium. Sedimentation rate (velocity) of the protein species (monomer, dimer, etc.) present in solution is measured as sedimentation coefficient which is governed by several factors including molecular mass, conformation and solvent properties.

AUC measurement does not involve any matrix (column, membrane) interactions, does not dilute the measured sample, and covers a wide size range, especially when multiple measurements are conducted using various centrifugal speeds. AUC typically uses absorbance as a probe. Additionally, interference (for higher concentration) and fluorescence (low-concentration) probes are also available. Therefore, AUC offers an independent confirmation of the presence of any aggregate species in a biologics formulation measured by SEC.

Equilibrium studies employ low centrifugal force to achieve a diffusion-controlled equilibrium, and are typically used to determine molecular mass as well as equilibrium binding constants (e.g., monomer-dimer reversible transition). One of the important applications of equilibrium studies in biotherapeutics is to detect any self-association (reversibly aggregated species).

AUC suffers from low throughput of measurement, lack of robustness, and artifacts from solvent and high concentration formulations. The majorities of the commercial as well as clinical biologics formulations cannot be studied "as is" with the absorbance probe, and require dilution. Although the concentration limit can be pushed higher by the use of an interference probe, several sources of "nonideality" (high concentration, sensitivity to excipients, protein shape factor) can cripple data interpretation. Because the sedimentation profiles by themselves do not provide an estimate of the protein species present, one needs good data analysis software to derive relative quantity of individual species. This is in contrast to SEC quantification, which relies on relative UV absorbance. Several instrument configuration parameters (rotor, cell, loading, probe alignment, wavelength, etc.) add sensitivity to analyzed data, and therefore it can be quite challenging to achieve consistent quantitative results.

Asymmetric Flow Field Flow Fractionation

aFFF uses cross flow onto a membrane in a channel with parallel flow where the smaller particles are transported more rapidly along the channel than the larger particles, hence achieving separation. Prior to migration of particles is initiated, the injected sample is focused onto a narrow area. aFFF is considered an orthogonal method of aggregate separation, using no column (a difference from SEC), and to achieve a wider dynamic range. However, potential interactions with membrane and concern of aggregate formation during its focusing step make aFFF rather unreliable (63). Like SEC, aFFF can also use one or more probes such as light scattering, UV, and refractive index to detect and characterize the fractionated species by hydrodynamic size, molecular weight, and conformational difference.

Light Scattering (Static and Dynamic)

Static Light Scattering

Static Light Scattering measures time-averaged value of scattered light intensity from a sample, typically over many seconds. SLS is used typically in conjunction with separation techniques such as SEC or aFFF. The intensity of the scattered light depends on protein concentration as well as scattering angle, and it is related to radius of gyration, hence molecular mass. SLS provides quite reliable determination of molecular mass of protein monomers and aggregates. For large size particles (such as protein aggregates larger than ~60 nm, depending on wavelength of incident laser) angular dependence is significant, and measurement at several

angles can produce useful data on size. For most protein monomers ($< \sim 10$ nm) such angular dependence is diminished, and measurement at a 90° angle can be used to determine mass.

Dynamic Light Scattering

DLS relies on measuring fluctuations (microsecond and longer time scale) of the scattered light caused by Brownian motion of molecules in solution, and therefore relates to diffusion coefficient (63). With spherical approximation, hydrodynamic radius (Rh) can be extracted from diffusion coefficient values. DLS provides a relatively easy and fast measurement of size (Rh), and covers a large dynamic range (~ 1 nm to ~ 1 μm) in one single measurement. Additionally, the measurement can be done with liquid/suspension of formulated API or drug product without any alteration/dilution (unless strength is greater than approximately 0.3 mM). However, it can resolve species of various sizes only if their hydrodynamic sizes differ by more than 2-fold to 5-fold. This is a serious disadvantage because, for example, monomer and dimer cannot be separated by DLS, instead an average value of size will be measured.

DLS is also referred to as photon correlation spectroscopy (PCS) or, quasielastic light scattering (QELS). Some of the DLS equipments are also configured to measure zeta potential.

Imaging (Static and Dynamic)

Microscopy is an established technique for studying protein particulates. Typically, it requires the particulates to be filtered and examined in static mode. Microscope images can be used for enumeration (pharmacopeia method) as well as for directly visualizing size and shape. Advances in imaging technology enable analysis in dynamic mode where the particles remain suspended in fluid either in stationary or flow modes (59). Digital images of particulates are collected and analyzed to provide a digital archive of particle parameters such as Feret diameter, aspect ratio, circularity, and intensity. Also, if particulate formation in a protein formulation is relatively slow, the dynamic nature of size distribution can be tracked over time. Such data are valuable to characterize particulate formation during biologics formulation development as well as to find potential prevention strategies. Disadvantages include the inherent complexity in determining a true size distribution from imaging data for biologics particulates because of their often extreme irregularity in size and shape. Finally, the size distribution and particulate count from dynamic imaging cannot be directly compared with such information obtained from light obscuration or laser-diffraction analyses (63).

Raman Spectroscopy

Raman spectroscopy, discovered by C.V. Raman (64), is a powerful tool to record the vibrational frequency pattern of a molecule that can be used as a fingerprint for identification. Raman spectroscopy uses laser excitation in modern applications, and frequency shifts caused by the probed molecule relative to the excitation frequency are recorded to generate a Raman spectrum. It is a powerful tool for small molecule pharmaceutical applications that include API identification, determination of tablet depth, and study of polymorphs. Unlike FTIR, Raman spectra have minimal interference from water and therefore the technique is quite suitable for studying aqueous biologics formulations. But protein fluorescence is a significant problem, and Raman bands might completely disappear with elevated background from highly fluorescing proteins, especially in the near-UV region. For proteins, amide bands can be conveniently detected in the ~ 1200 to 1700 cm^{-1} spectral range (analogous to FTIR spectrum) for secondary structure determination. A more advanced application of Raman spectroscopy is Resonance Raman that uses a laser frequency excitation overlapping with a particular protein absorption band (chromophore). This enables detailed structural analysis (including local tertiary structure) of the desired chromophore (e.g., Tyr).

Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) is capable of measuring thermally induced transitions, and particularly the structural transitions of biological macromolecules, such as between the folded and the unfolded structure of a protein. DSC measures the excess heat capacity of a protein solution (Cp) as a function of temperature and the structural transition is recognized as

a sharp endothermic peak centered at the melting temperature (T_m). Generally, DSC is useful to study the energetics of protein thermal unfolding. The T_m of liquid protein formulations is often used as a probe for protein physical stability, that is, higher T_m value may indicate greater physical stability. However, it is recognized that comparisons of physical stability of different classes of proteins by T_m may not hold true. T_m values are also known to be sensitive to the solution matrix such as excipients, pH, buffer and surfactants. However, no clear correlation exists. While some of the stabilizing components may increase the T_m value, some of the stabilizers (e.g., surfactants) may actually result in lower T_m values. Because T_m cannot reliably predict physical stability, establishment of critical parameters in formulation screening and characterization should not be based solely on DSC data. Finally, for monoclonal antibodies, quite often multiple T_m values are observed which are typically assigned arbitrarily to structural domains on the basis of available T_m data of isolated domains in similar protein class. This makes structural interpretation of changes in T_m values as a function of pH or other additives very challenging.

Isothermal Titration Calorimetry

ITC measures heat change from binding interactions, such as antibody-antigen binding or receptor-ligand binding. It is quite versatile and can be applied to a wide variety of molecules in solution without any pretreatment (such as fixation of matrix). ITC can also detect weak interactions with dissociation constants in the sub-millimolar range. Appropriate control experiments must be conducted as several sources of heat change (e.g., heat of dilution) can introduce artifacts.

Near-Infrared Spectroscopy

A near-infrared (NIR) spectrum (12,000 14,000 cm^{-1}) represents combination and overtone bands that are harmonics of absorption frequencies in the mid-infrared region. Because each material has a unique NIR spectrum, NIR spectroscopy can be used as a positive identification of material. NIR is a versatile technique with reduced or eliminated sample preparation, decreased cost and analysis time, and the ability to record spectra through glass and packaging materials.

NIR measures vibrational spectra of a wide variety of materials including solids, liquids, powders, pastes and tablets. NIR has a variety of applications in the area of microbial and cell culture system monitoring and control. An important pharmaceutical application in injectables development is moisture analysis of freeze-dried samples without opening the vials. Determination of water content employs the strong water absorption bands in the NIR region, most prominently the first overtone of OH stretching at around 6800 to 7100 cm^{-1} and the combination band of OH stretching and bending at around 5100 to 5300 cm^{-1}. Karl Fischer method is the most commonly used method for measuring moisture content but it is a destructive method, may need method development, and requires careful handling of sample to not allow additional moisture when a vial is opened. On the other hand, NIR offers increased efficiency in measurement time (higher throughput than Karl Fischer) and reduced cost (especially for expensive biologics products) because the vials can be reused to conduct other assays following NIR measurement. It should be noted however that a standard curve must be generated and requires method development to establish a robust NIR protocol for moisture analysis. If formulation composition is changed, NIR spectrum may also change and additional method development may be necessary.

Powder X-Ray Diffraction

The primary use of powder X-ray diffraction (XRD) in characterizing biologics formulation is to probe the presence of amorphous and any crystalline states in the freeze-dried form. It also can detect the presence of polymorphs of certain excipients such as mannitol. Additionally, the X-ray diffraction can be used to study the phase behavior of the frozen state of protein solutions as well as placebos, using low-temperature accessories (65). Low-temperature XRD is a powerful tool to identify the phases that crystallize during cooling and annealing of frozen solutions.

Freeze-dried powder is often characterized for its crystalinity or amorphousness by the presence or absence of sharp diffraction peaks in the XRD profile. If formulations contain

mannitol, it is important to establish if any of the mannitol polymorphs are present. Mannitol is known to crystallize in multiple forms such as α, β, and δ forms. It also forms a metastable hydrate form that might negatively impact the stability of the freeze-dried formulation.

SUMMARY

Biotherapeutic entities include a variety of macromolecular compounds, each with distinct biochemical and biophysical properties. Extensive structural characterization must be performed for these molecules to be approved as drugs by worldwide regulatory agencies. Characterization should typically include assays to demonstrate that the molecule has the expected primary, secondary, tertiary structure, as well as the expected bioactivity. Any post-translational modifications or variant forms need to be adequately described in terms of the levels and any therapeutic effects such as potency or in vivo half-life. Similarly, the sponsor must demonstrate that the molecule has adequate chemical and physical stability for the proposed shelf-life in its formulation, and that the degradation pathways are well-understood. The analytical toolbox required for these investigations can be extensive, and a suite of assays specific for the biotherapeutic entity can be tailored to provide the required information.

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9 Formulation of protein- and peptide-based parenteral products

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INTRODUCTION

Since the early 1970s, scientific advances in molecular biology and genetic engineering have led to enormous success in protein- and peptide-based therapeutics for the treatment of many human diseases. They cover almost all therapeutic categories, including cardiovascular hemostasis, antineoplastic, diabetes and endocrinology, anti-infective, neuropharmacological, enzyme replacement, wound healing, respiratory, and bone cartilage. Protein-based therapeutics are emerging as a major class of new molecular entities in the pharmaceutical industry. Over 200 biotechnology and pharmaceutical companies are developing protein-based therapeutics. More than 150 biologics are currently marketed, and over 400 are in advanced stages of testing and clinical trials (1).

Unlike small molecules, which are typically synthesized through chemical processes, proteins are produced in living systems. The main technology used to produce proteins utilizes recombinant DNA techniques to produce protein molecules in a host cell. Several types of host cells have been employed, including *Escherichia coli*, yeast, mammalian cells [e.g., Chinese hamster ovary (CHO) cells and human fibroblasts], and plant-derived cells. Several other technologies are also used to produce therapeutic proteins. Small proteins and peptides, such as calcitonin, may be produced by chemical synthesis. Most human serum albumin is sourced from human blood, urokinase from urine, and streptokinase from fungi. Recombinant human antithrombin (ATryn®), a new product approved by the FDA in 2009, is produced by transgenic animals.

CHARACTERISTICS OF PROTEINS AND PEPTIDES

Compared with small-molecule drugs, protein-based pharmaceuticals are not only larger in molecular weight, but they also contain more complex compositions and higher order structures. Intrinsically, most proteins have poor stability and a very short half-life in vivo. Because of their poor oral bioavailability, most proteins require parenteral administration routes. In some cases, they require specific delivery systems targeting the specific site of action to achieve sufficient efficacy. Therefore, formulating these proteins as therapeutic agents with proper efficacy and safety profiles has been a challenging task. For successful product development, one needs to have a thorough understanding of the protein's physicochemical and biological characteristics, including stability, immunogenicity, and pharmacokinetic properties. The characterization of proteins is therefore an important step in formulation development.

Molecular Composition, Structure, and Heterogeneity

A protein, or polypeptide, is formed through the linkage of peptide bonds of amino acids. Generally, protein structures are described at four levels: primary, secondary, tertiary, and quaternary. Details about these can be found in the preceding chapter of this volume.

Because of their complex manufacturing process, from cell culture to downstream purification, protein products generally contain multiple species in terms of molecular weight or size, which could be due to various modifications to the polypeptide side chains or glycans, reversible or irreversible formation of oligomers by either noncovalent or covalent linkages, and formation of large soluble and/or insoluble aggregates. It is important to characterize and quantify all species, as they may directly affect product efficacy, safety, and immunogenicity.

Depending on its size and the nature of its associations, several analytical techniques can be used to characterize a protein's size. Routinely, electrophoretic and chromatographic (with multiangle light-scattering detector) techniques have been used to estimate protein size up to

oligomers. By combining a denaturing electrophoretic technique (sodium dodecyl sulfate polyacrylamide gel electrophoresis [SDS-PAGE]) with size-exclusion high-performance liquid chromatography (HPLC) or a native electrophoretic technique (Native PAGE), the size of proteins and the nature of their associations (covalent vs. noncovalent) in native and denatured states can also be estimated. To more accurately determine the size of proteins, mass spectrometry, such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) or liquid chromatography mass spectrometry (LC-MS), is often used. However, because of the matrix effect and the high energy applied, the molecular weight or size determined by this technique may not be the true size in solution.

To measure the size of a protein in solution up to 100 nm, several biophysical techniques may be feasible, including analytical ultracentrifugation (AUC), field flow fractionation (FFF), and dynamic light scattering. It should be noted that the size distribution of proteins in solution, especially for reversible association, may be highly dependent on the solution properties, including pH, salt concentration, and protein concentration. Therefore, the mobile phase used in these analyses is preferably the same as the formulation vehicle, and the impact of the dilution factor during analysis should be assessed.

Insoluble aggregates or particles larger than 100 μm can be observed by visual inspection with the unaided eye. Their size can be estimated by microscopy. Subvisible insoluble aggregates between 10 and 100 μm in size can be quantified and sized either by a light obscuration test or by a microscopic particle count test per USP method <788>. It is still technically challenging to accurately quantify and size particles between 0.1 and 10 μm. A technique using Micro-Flow™ imaging (MFI) has been used for particles as small as 0.75 μm (2).

Isoelectric Point

Proteins that contain both positively and negatively charged amino acids are amphoteric molecules. One property that characterizes a protein's charge profile is its isoelectric point, or pI. The pI of a protein is the pH at which it carries no net electrical charge. At a pH below its pI, a protein is positively charged; above its pI, it is negatively charged.

The pI may be approximately calculated from the amino acid composition data, that is, $pI = (pK_1 + pK_2 + pK_3 \ldots + pK_n)/n$ for n ionizable groups. However, because the dielectric constant in the immediate vicinity of an ionizable group depends on protein structure, and because hydrogen bonding may alter dissociation constants (K_a), the true pI can differ significantly from the calculated one. Several websites provide theoretical estimations of pI for proteins (e.g., http://www.scripps.edu/~cdputnam/protcalc.html, http://www.expasy.ch/tools/pi tool.html, and http://www.nihilnovus.com/Palabra.html).

Some proteins have multiple species with different charge profiles, and each species has its own pI, so these proteins appear to have more than one pI. Some glycoproteins in particular exhibit complicated pI patterns because of the heterogeneity in their glycan composition. Also, some proteins comprise multiple deamidation species, which also results in complicated charge profiles that could be characterized by several techniques, including isoelectric focusing (IEF), ion exchange chromatography (IEC), and capillary electrophoresis (CE).

Proteins show a broad range of pIs, mostly in the range of 2 to 12. The pI of a protein may play an important role in solubility and stability. In general, protein solubility is at its minimum when the pH is near its pI. Also, because zero net charge at pI should presumably allow maximum interaction between salt bridges and exert the least interaction between protein molecules, it could be expected to be the most stable condition for conformation. However, studies have shown that the optimal pH for conformational stability can be quite different from the pI and in many cases is found at a pH corresponding to a large net charge of the protein (3).

Solubility

The varieties of functional groups (charged, hydrophobic, etc.) on the side chain of amino acids and glycans (for glycoproteins) make protein solubility dependent on the pH, salt concentration, and polarity of the solvent. The overall size of the protein does not necessarily influence solubility. For example, antibodies, which have molecular weights of approximately 150 kDa, can often achieve aqueous solubility greater than 100 mg/mL.

The aqueous solubility of a peptide or protein is not easy to determine because peptides and proteins at high concentrations may form gels, or may develop aggregates upon concentrating, thus making solubility assessment difficult. In addition, solubility varies significantly depending on the conformation. The solubility determined by most methods is apparent solubility, because the true solubility of a protein as a hydrocolloid is difficult to define. A common approach is to concentrate a protein solution using a semipermeable membrane with centrifugation until the highest protein concentration is reached. Another approach is to lyophilize a protein or peptide and then add water to the point where undissolved material is barely present. When a limited amount of protein is available, one approach is to determine solubility in polyethylene glycol (PEG) solution (typically 1 9%) and then extrapolate the solubility to 0% PEG to determine aqueous solubility (4).

The factors that determine a protein's solubility include its intrinsic properties and the composition of the solvent. The intrinsic properties are the composition of amino acids, the folded structure, and for glycoproteins, the composition and structure of glycans. Generally, a protein made of a large proportion of hydrophobic amino acids such as Phe, Tyr, and Trp will have low water solubility, and adding glycans increases water solubility. The solvent properties, including pH, salt concentration, and specific ligands, can also significantly affect the solubility. Protein solubility as a function of pH is typically in the shape of a U or V, where the minimum is at the pI. However, there are exceptions. The solubility of a protein at low ionic strength generally increases with the salt concentration, which is called the salting-in effect. As the salt concentration increases, the additional counter-ions shield the ionic charge and thereby increase the protein solubility. As salt concentration continues to increase, protein solubility decreases (the salting-out effect). At high salt concentration, the salts begin to compete with the ionic moieties of the protein for the solvation of the polar solvent, which results in decreasing solubility. A specific ligand or stabilizer that binds to the protein may also influence solubility. For example, increased solubility of fibroblast growth factor was observed in the presence of heparin or heparin-like substances (5). Also, alteplase solubility was increased by the addition of arginine (6). However, one needs to assess whether the ligand or excipient is acceptable for the intended clinical use before adding it into the final formulation.

Thermal Transition Midpoint

Because native proteins exhibit folded structure in solution, they can undergo transition from native form to unfolded or denatured form with increasing temperature. The thermal transition midpoint (T_m), defined as the temperature at which equal amounts of native and denatured forms exist in equilibrium, is an important characteristic of proteins, measuring their thermal stability. Generally, a higher T_m value indicates better thermal stability.

The most commonly used technique to determine T_m is differential scanning calorimetry (DSC), as this method not only provides an accurate measurement of T_m but also can assess reversibility of transition and estimate apparent enthalpy. Temperature-controlled spectrometry, including circular dichroism (CD), fluorescence, and ultraviolet (UV) absorbance spectroscopy, is also sometimes used to differentiate the transitions by tertiary structure from those by secondary structure.

Measurement of T_m has been widely used in preformulation and formulation development. The profile of T_m as a function of pH provides important information in selecting the optimal pH for formulation. This method has also been used in screening different stabilizers, as an excipient that elevates T_m is expected to be a potential stabilizer (7). However, it should be noted that in choosing the formulation, one also needs to consider other information, as T_m alone is only indicative of thermal stability.

Proteins in solid state also exhibit thermal transitions upon heating. These are typically determined by DSC. However, it is difficult to measure the true thermal transitions of solid protein, because in most cases other components present in the solid dosage form also contribute to the overall thermal transition. Recently, glass transition temperatures (T_g) for proteins have been estimated by extrapolating excipient concentration to zero using T_g values measured at a very fast scanning rate in binary mixtures of protein and another glass form excipient, such as sucrose, over a range of excipient concentrations (8).

Instability: Key Degradation Pathways

The structural complexity of proteins makes them susceptible to processing and handling conditions that can result in structural and functional modifications. A protein can undergo a variety of covalent and noncovalent reactions or modifications, which may be generally classified into two main categories: (*i*) physical or non covalent bond degradation pathways and (*ii*) chemical or covalent bond degradation pathways. Common physical degradation pathways include denaturation or unfolding, adsorption, and aggregation due to noncovalent forces. Chemical degradation pathways include covalent-bonded aggregation, disulfide exchange, deamidation, isomerization, racemization, fragmentation, oxidation, β-elimination, Maillard reaction, diketopiperazine formation, and so on. Oftentimes, physical degradation facilitates chemical degradation, and vice versa. The fundamentals of these degradation pathways have been extensively described in several review articles and book chapters (9 14). A brief description of each degradation pathway, the factors responsible for degradation in some proteins, and remedies are presented below.

Denaturation

Denaturation is the process of altering protein structure (i.e., secondary, tertiary, or quaternary structures) from its native folded state. Denaturation may result in an unfolded state, which could further facilitate other physical and chemical degradations. Because a specific structure is required for proteins to exert physiological and pharmacological activities, denaturation causes loss of efficacy and incurs the risk of safety such as immunogenicity.

Many times, the denaturation process can be described as $N \leftrightarrow I \leftrightarrow D$. The folded native structure (N) unwinds and passes through a partially unfolded or intermediate state (I) to a denatured state (D). This process may be reversible or irreversible, depending on conditions. For reversible denaturation, the unfolded protein will regain its native state once the denaturing condition is removed.

Many factors can cause denaturation, including heat, freezing, extreme pHs, organic solvents, high salt concentration, lyophilization, surface adsorption, and mechanical stress. These denaturing conditions disrupt a protein's higher order structure, which is held together by intramolecular forces including hydrogen bonding, salt bridges or electrostatic forces, hydrophobic interactions, and van der Waals forces.

Hydrogen bonds are critical in determining overall protein conformation, since they are the major forces that stabilize the secondary α-helices and β-sheets, as well as the overall folded structure. Water, the nearly ubiquitous medium for proteins, contributes to this hydrogen bonding. Cosolvents such as ethanol and acetone and chaotropic agents such as urea and guanidine hydrochloride disrupt the hydrogen bonds and thus readily denature proteins.

The ionic side chains of aspartic acid, glutamic acid, lysine, arginine, and histidine, normally found on the surface of the protein, contribute to the stability of the native conformation by forming salt bridges. The pH of the solvent will determine the charge of the side chains on these amino acids and the extent of ionic bonding. Therefore, an extreme pH shift can disrupt these salt bridges and lead to denaturation. Furthermore, organic solvents will reduce dielectric constant and increase ionic forces or salt bridges, so inappropriate exposure to organic solvents can also result in denaturation.

Because hydrophobic side chains (i.e., phenyl, indole, and hydrocarbon chains) are usually tucked inside the protein's globular structure, significant stabilizing effects result from their hydrophobic interactions. These interactions, too, are sensitive to the effects of solvents. Disruption of hydrophobic interactions is also considered the mechanism of denaturation by surfactant, extreme temperature, and mechanical stress, all of which commonly occur during manufacturing processes.

Adsorption

Proteins are amphiphilic polyelectrolytes, so they tend to adsorb at liquid-solid, liquid-gas, and liquid-liquid interfaces. When adsorption of proteins occurs, the molecules exchange their interactions with the solvent and other solutes for interactions with the surface. Two mechanisms are primarily responsible for protein adsorption. One mechanism is charge-charge

or electrostatic interaction. For example, salmon calcitonin, as a positively charged protein, strongly binds to the negative potential of a glass surface through electrostatic interaction (15). The other mechanism is hydrophobic interaction. One example is bovine serum albumin, which near its isoelectric point has shown the highest affinity to the hydrophobic surface of polystyrene through hydrophobic interactions (16). Other interactions, including charge-dipole, dipole-dipole, and van der Waals forces, may also contribute to the adsorption.

These interactions may lead to altered structures, including secondary, tertiary, and quaternary structures, which could further facilitate other physical and chemical degradation, including aggregation and covalently bonded modification. Therefore, depending on the nature of the protein and of the contact surface, interfacial adsorption can significantly impact a protein drug's potency, stability, and safety, particularly in a low-concentration dosage form.

The key strategy to minimize or inhibit protein adsorption is either to adjust formulation parameters or to modify or avoid certain contact surfaces. The formulation parameters that potentially control adsorption include protein concentration, pH, ionic strength, and addition of specific excipients such as surfactants or albumin. For example, modification of the contact surface of siliconized vials has minimized interferon adsorption on the glass surface (17). When these approaches do not prevent significant adsorption, alternative contact surfaces should be considered during process or storage. In some cases, when the level of adsorption can be predicted, overage is required in the vials.

Aggregation by Noncovalent Linkage

Non covalently linked aggregation often results from some degree of denaturation of proteins, since unfolding leads to the exposure of hydrophobic moieties previously buried in the protein interior, which is followed by the association of unfolded molecules via noncovalent interactions to form aggregates. Non covalently linked aggregation can be a reversible or an irreversible process, depending on conditions.

Reversible aggregation is highly dependent on protein concentration, pH, salt concentration, and other formulation components. Generally, proteins tend to form high molecular weight species (HMWS) at high protein concentration. Upon dilution, these HMWS or oligomers may dissociate into monomers or dimers. This self-association phenomenon may be characterized by AUC (18) or by static light scattering (19).

Irreversible aggregates can be soluble or insoluble, depending on the size and nature of the molecules. Generally, these aggregates can be induced by single or multiple stress conditions, including heat, extreme pH, mechanical pumping, high pressure, shaking or agitation, freezing, and freeze-drying. For example, acidic pH and a temperature of 37°C have resulted in irreversible aggregation of albumin (20).

To minimize aggregation, besides tight control of the process parameters, adjusting formulation parameters, such as adding sucrose, should be assessed. Sucrose and other polyols maintain protein molecules in a native compact form, so as to be resistant to external stress.

Aggregation by Covalent Linkage

The most commonly observed protein aggregation by covalent linkage occurs through intermolecular disulfide linkage, also called disulfide bond formation and scrambling. This intermolecular aggregation may occur to any protein containing cysteine or cystine.

Generally, proteins with a free thiol group tend to form aggregates more easily through disulfide bonds, especially when the free thiol group is solvent-exposed on the surface of the protein. Free thiol groups buried within the tertiary structure are less reactive. The formation of disulfide bonds in protein aggregates with free thiol groups can take place either through the two free thiol groups available on the surface of each of two protein molecules, or through thiol-disulfide exchange, whereby a reactive thiol group in one molecule attacks an existing disulfide bond in another molecule to form a new disulfide bond between the two molecules.

Proteins without free thiol groups may still form aggregates by disulfide bond scrambling through intermolecular disulfide exchange, especially in alkaline conditions. A cystine or disulfide bond in one molecule can be reduced into two free cysteines, which can react with cysteine or cystine in another molecule to form a new disulfide bond.

Aggregates formed through disulfide bonds may, through multiple disulfide scrambling reactions, result in high-molecular-weight aggregates, which could eventually precipitate from solution. In addition, the formation of a new disulfide bond may change the native conformation to a denatured form, which could further aggregate through noncovalent hydrophobic interactions due to exposed hydrophobic residues.

In neutral or alkaline pH conditions, disulfide-bonded aggregation generally becomes more severe as the thiol group becomes more reactive. However, extremely acidic pH may also cause disulfide-bonded aggregation (21).

To prevent or minimize disulfide-bonded aggregates, the main formulation parameter is pH. A slightly lower pH (e.g., pH 5) may significantly reduce aggregation. The addition of reducing agents such as cysteine, or of stabilizers that alter conformation such that free cysteine or reactive cystine becomes more buried into tertiary structure, may also minimize aggregation. For proteins with severe disulfide aggregation, lyophilized formulation should be considered, as reactivity in the solid state is reduced significantly.

Nonreducible aggregates through nondisulfide linkages have also been reported. The reactions involving these covalent linkages include (*i*) oxidation-induced reactions through Trp or Tyr linkage (22); (*ii*) reaction through transamidation, whereby an amino group of amino acids (e.g., lysine residue or N-terminal of a protein) in one molecule forms an isopeptide bond with the carbonyl group of either Asn or Gln in another molecule [examples are insulin (23) and lyophilized ribonuclease A (24); and (*iii*) reaction through a reactive dehydroalanine generated from β-elimination at alkaline pH, which forms nonreducible cross linkages with other amino acids such as Tyr, Lys, His, Arg, and Cys.

Intramolecular Disulfide Exchange

Disulfide exchange can also take place within a protein molecule when a cystine (disulfide) bond is reduced into two cysteines; one of the cysteine residues then reacts with another cysteine to form a new disulfide bond. Improper linkages of disulfide bonds were responsible for a reduction in biological activity of interleukin-2 (IL-2) (25). There are three cysteines in IL-2 at positions 58, 105, and 125. The native protein forms a disulfide linkage between the two cysteines at 58 and 105. The cleavage of this disulfide in IL-2 and the subsequent formation of two less active isomers with disulfide bonds at incorrect positions (Cys^{58}-Cys^{125} and Cys^{105}-Cys^{125}) are promoted by high pH and copper ions (25). Intramolecular disulfide exchange has also been reported for monoclonal antibodies.

To minimize this type of degradation, it is important to select a low formulation pH and minimize any impurities, such as peroxides or heavy metals, known to promote redox reactions.

Deamidation/Isomerization

Deamidation refers to the removal of ammonia from the amide ($RCONH_2$) moiety of an Asn or Gln side chain, resulting in a carboxylic acid. Deamidation is a major cause of instability of proteins and peptides in aqueous solution. In lyophilized solid state, the deamidation rate is slower than in solution.

Deamidation occurs through different pathways at different pH levels. In an acidic pH of 2 to 5, deamidation occurs by direct hydrolysis, which causes Asn or Gln residue to change into Asp or Glu residue, respectively. The type of neighboring amino acids does not affect the deamidation rate. Hydrolytic mechanisms in neutral or alkaline pHs are more complex, however. Under these conditions, the side chain carbonyl group on the Asn or Gln residue reacts with the nitrogen atom on the peptide backbone to form a cyclic imide (succinimide) intermediate (Asu). Depending on which bond in the cyclic imide breaks, the reaction product can be (*i*) the des-amido peptide (Asp), (*ii*) the isopeptide (IsoAsp), or (*iii*) D-isomers. The formation of isopeptides is called isomerization, or sometimes referred to as transpeptidation, because an extra methylene group is inserted to the peptide backbone. When deamidation occurs, the IsoAsp to Asp ratio is typically 3. Detailed descriptions of deamidation and isomerization can be found in a review by Wakankar and Borchardt (26).

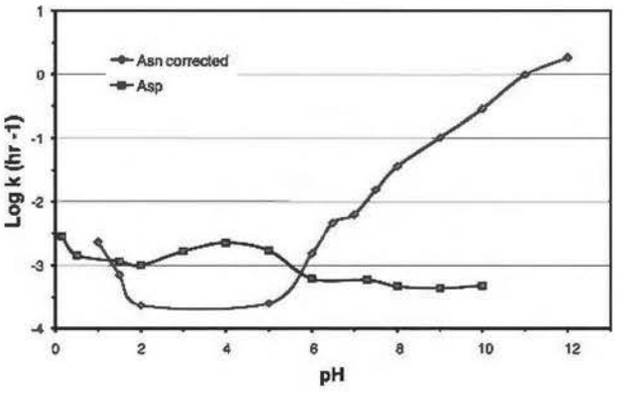

Figure 1 Comparison of Asn deamidation (*diamond*) and Asp dehydration (*square*) rates in a model peptide (Val Tyr Pro Asx Gly Ala) at 37°C. Dehydration is the rate limiting step for isomerization. *Source*: From Refs. 104 and 105.

At neutral to alkaline pHs, the rate of deamidation is significantly affected by the size of the amino acid on the C-terminal side of the Asn or Gln residue. In general, Asn is more labile than Gln and is most labile when adjacent to glycine, which is least obstructive to the formation of a cyclic imide. Since Asn-Gly is most susceptible to deamidation, protein engineers make significant efforts to avoid constructing a protein drug candidate with such a hot spot.

The deamidation rate profile as a function of pH is V-shaped, usually with a minimum rate at a pH of about 4 to 5 (Fig. 1). In a number of synthetic peptides, the half-life of deamidation reactions of Asn residues at 37°C in pH 7.2 phosphate buffer ranges from two days to nine years (27). Not all Asn residues are equally labile; those buried within the interior portion of a protein are inaccessible to water and thus less reactive. Secondary and tertiary structures play an important role in determining the site and the rate of deamidation. In insulin, for example, there are three asparagines. At acidic pH, the prevailing deamidated species was monodesamido-(A21)-insulin. At neutral pH, deamidation occurred to Asn at the B3 position (28). For growth hormone containing nine asparagines, deamidation occurred primarily at the Asn-149 position (29). These proteins and others pramlintide (30), epidermal growth factor (31), IgG (32), IgG1 (33) represent a small fraction of proteins that have shown deamidation.

Isomerization at Asp goes through the same cyclic imide intermediate, Asu. Because of the effect of pH on the leaving group (-OH), the rate-pH profile is significantly different from that of deamidation of Asn. Examples of protein drugs that undergo IsoAsp formation include insulin aspart (34), hirudin (35), and porcine somatropin (36). On the basis of the study of two monoclonal antibodies (37), the degradants from isomerization were detected by hydrophobic interaction chromatography.

The best way to avoid deamidation and isomerization is to mutate Asn-Gly or Asp-Gly sequence in the solvent-exposed region of the protein, if the mutation at these sites does not affect the biological activity. Otherwise, an appropriate pH (in the range of 5 6) should be selected to minimize the degradation rate. As the formation of cyclic imide intermediates does not depend on water, these reactions may occur even in anhydrous conditions such as 100% dimethyl sulfoxide (DMSO) (38)

Racemization

The racemization reaction is catalyzed by both acid and base. Racemization of peptides and proteins results in the formation of diastereomers. Racemization under basic conditions is hypothesized to proceed by abstraction of the α-proton from an amino acid in a peptide to yield a negatively charged planar carbanion. A proton can then be returned to this optically inactive intermediate, thus producing a mixture of D- and L-enantiomers for the individual amino acid. Since a peptide is composed of multiple chiral centers, the product formed is a diastereomer. Racemization is biologically significant because a peptide composed of D-amino acids is generally metabolized much more slowly than a naturally occurring peptide made

only of L-amino acids. For this reason, many new synthetic peptides, both agonists and antagonists, incorporate D-amino acids. A pH dependency for racemization was demonstrated in an aqueous degradation study of a decapeptide, RS-26306 (39), which found that at neutral and alkaline pHs, racemization contributed to more degradation than did deamidation.

Fragmentation

The peptide bond (RNH CO R) can undergo hydrolysis, resulting in peptide fragments. Generally, most peptide bonds are relatively stable. For example, oxytocin injection was reported to be stable at room temperature for five years (40). Protirelin, a tripeptide (PyrGlu-His-Pro), is stable for 20 hours at 80°C at both pH 3.3 and pH 6 (41).

The formulation factor that most influences the hydrolytic rate is solution pH. The rate of hydrolysis is in direct proportion to the activity of hydronium or hydroxide ions, when in acidic or alkaline pHs, respectively. Generally, the reaction becomes much faster in either extremely acidic or extremely alkaline conditions. Fragmentation of therapeutic peptides, including nafarelin (42), secretin (43), captopril, (44), and urokinase (45), has been reported in various pH conditions.

The type of neighboring amino acids also affects the susceptibility of the linkage to fragmentation. For example, the bond between aspartic acid and proline is sensitive to acid hydrolysis. A cleavage at Asp-Pro was found in basic fibroblast growth factor (bFGF) (46) and macrophage colony-stimulating factor (47). The resultant products are peptides with aspartic acid at the C-terminus. The C-terminal peptide bond adjacent to serine is also a reactive one, because of the neighboring-group effect of the alcohol on serine (48).

Another example of fragmentation is the cleavage at hinge regions of antibodies resulting in a Fab fragment. The hinge region in IgG1 heavy chain comprises about eight amino acids, including two cysteines responsible for the two disulfide linkages between the two heavy chains. Cleavage takes place at multiple sites, with the majority between Asp and Lys and between His and Thr (49). The extreme flexibility at the hinge region and the solvent exposure are the driving forces for such cleavage. The rate of hydrolysis at hinge region is minimal at pH 6 (50).

Besides chemically induced fragmentation, protein hydrolysis can also be mediated by some residual proteases remaining from production. The cleavage site in this case is dependent on the type of proteases present. As the proteolytic activity of proteases is typically pH dependent, the degradation rate is also dependent on solution pH. For example, a cathepsin D protease derived from CHO was identified as being responsible for degradation of an Fc-fusion recombinant protein. This protease belongs to an aspartic protease family and is preferentially active at acidic pH (51).

Oxidation

Several amino acid residues including Cys, Met, Trp, His, and Tyr are potential sites of oxidation. These residues can be oxidized by atmospheric oxygen or by peroxide impurities from a number of raw materials including formulation excipients such as polysorbates. Oxidation can also be induced by exposure to light or catalyzed by transition metal ions such as Cu^{2+} and Fe^{3+}. The most commonly observed oxidations in therapeutic peptides and proteins are described below.

Oxidation of cysteine. Under neutral or basic conditions, the free thiol (-SH) group of a cysteine is the most reactive moiety of all amino acid components. The disulfide (-S S-) bond formed from the oxidation of two thiol groups results in significant changes in conformation both intramolecularly and intermolecularly.

Oxidation of the thiol group is promoted at both neutral and basic pH. The rate-pH profile for captopril, a quasi-dipeptide, shows an increase in oxidation rate starting at pH 5 (44). This reaction can also be catalyzed by heavy metals. For example, this reaction was effectively retarded by the addition of a metal chelating agent such as EDTA in FGF formulation (5).

Oxidation of methionine. The thiolether ($-CH_2-S-CH_3$) moiety on methionine is susceptible to oxidation to form sulfoxide ($-CH_2-SO-CH3$) derivatives. The susceptibility of methionine to

oxidation is highly dependent on its site in a protein. For example, of the three methionines in human growth hormone, Met-125 is most reactive, Met-14 is less, and Met-170 is not reactive at all (52). The reactive methionine is likely to be the one exposed on a protein surface, and the unreactive one buried within the core. Air in the headspace of formulated and freeze-dried growth hormone can cause 40% of the growth hormone molecules to be oxidized during a six-month storage period (53). Examples of other pharmaceutical proteins exhibiting Met oxidation are listed below.

- Interleukin-2 (54)
- Murine antibody (Orthoclone OKT3) (55)
- Herceptin® (trastuzumab) (56)
- Recombinant interferon γ (Actimmune) and recombinant tissue plasminogen activator (alteplase, Activase®) (57)
- Recombinant human granulocyte colony-stimulating factor (58,59)
- Parathyroid hormone (60)

As methionine can be easily oxidized by atmospheric oxygen, replacing oxygen with nitrogen or argon during manufacturing or in the headspace of the final product container is a common practice to minimize oxidation. In addition, adding free methionine as an antioxidant is also an effective approach to reducing oxidation (61).

Oxidation of tryptophan. Oxidation of tryptophan can generate multiple oxidized species. Stability studies of Trp amino acid alone in aqueous solution (62) and Trp residues in small peptides and lysozymes (63) and in bovine α-crystallin (64) clearly identified the main degradants as 5-hydroxy-Trp, oxy-indole alanine, kynurenine, and *N*-formylkynurenine. There are very few articles reporting the oxidation of Trp in therapeutic proteins. Davies et al. reported oxidized bovine serum albumin with oxygen radicals generated from cobalt radiation (65); Trp oxidation in monoclonal antibodies was recently reported by Yang et al. (66) and Wei et al. (67), and ozone and UV irradiation were used as stress conditions in these studies. That Trp oxidation has not been studied in depth is perhaps due to the fact that no model oxidizing condition has been adopted, and a system that promotes Trp oxidation is not easy to reproduce. In the case of Met oxidation, tert-butyl hydroperoxide (tBHP) and H_2O_2 are commonly used reagents to generate oxidized Met species. Most recently, a free radical generator, AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride) was found to effectively oxidize tryptophan in peptides or large proteins (68). By using AAPH as an oxidation stressing agent, one can predict the vulnerability of a protein or the specific Trp residue in a protein.

As Trp oxidation appears to be mediated through oxygen radicals because of light exposure or peroxide residues from excipients, it is important for mitigation of Trp oxidation to limit direct light exposure and to use high-quality excipients.

β Elimination

The disulfide bond between two peptide chains can be cleaved disproportionally, catalyzed by hydroxide ions to produce dehydroalanine and thiocysteine through the mechanism of β-elimination. The thiolate ion (HS-) is very reactive and will continue to react with other disulfide bonds, causing chain reactions. This is one of the operative mechanisms for the covalent aggregates in solid phase for bovine serum albumin (69) and ANP, a 25 amino acid peptide (70). β-elimination has also been attributed to the fragmentation of hinge regions in antibodies (71).

Maillard Reaction

The first substantial investigation of the reaction of reducing sugars with amino acids was carried out about 75 years ago by Maillard. The chemical reactions involved are, first, the reversible formation of a Schiff base between the aldehydic function group of reducing sugars (e.g., glucose) and the amino group of lysine residues in proteins, followed by a relatively

slow, but essentially irreversible, Amadori rearrangement, with the formation of ketoamines, which forms the hemiketal structure. As the Schiff base may also be involved in cross-linking, this reaction commonly leads to nonenzymatic browning, also called a browning reaction. This reaction occurs most readily in neutral to weakly alkaline conditions.

In protein formulation, reducing sugars such as glucose, maltose, fructose, and lactose should be avoided to prevent potential Maillard reactions. In addition, one needs to be concerned with acidic pH, which may cause hydrolysis of sucrose into glucose and fructose, leading to glycation of the protein. One example is freeze-dried bFGF. When the cake collapsed at elevated temperature, the acidity from its buffer, citric acid, caused hydrolysis of sucrose, which resulted in glycation of the protein with glucose (46).

Diketopiperazine Formation

Rearrangement of the N-terminal dipeptide results in the splitting off of a cyclic diketopiperazine at high pH. Proline and glycine in the N-terminal promote the reaction. Aspartame degrades through a similar mechanism (72), with the cyclization of aspartame minimal at a pH of 6 to 7, moderate at 7 to 8.5, and rapid above 8.5. Diketopiperazine formation has also been reported in the case of vascular endothelial growth factor (73).

PHARMACEUTICAL DEVELOPMENT PRINCIPLES

Because of their poor permeability to epithelium cell membranes and instability in gastrointestinal tract, proteins and peptides have very poor oral bioavailability and are therefore primarily formulated as injectable or parenteral products for intravenous (IV) infusion or subcutaneous (SC), intramuscular (IM), or intrathecal (IT) administration. Some proteins are also delivered through noninvasive administration routes, such as nasal and pulmonary formulations, to improve patient compliance. For local therapeutic effect, proteins such as growth factors are formulated for topical application and bone matrices.

Most protein products are in aqueous solution or in solid, freeze-dried form. Some are also formulated as suspensions in a crystalline form or in other lipid- or polymer-based delivery systems such as microspheres, liposomes, and nanoparticles. Rarely, some proteins, such as bovine growth hormone, are formulated in oleaginous vehicle.

The formulation development process for products that involve lipids or polymers as carriers is more complicated than the process for simple conventional dosage forms of liquid and lyophilized formulations. The principles described below focus mainly on the development process for the conventional dosage forms, some of which have also been discussed in several book chapters and review articles (74 79).

General Formulation Development Process in Industry

Generally, the manufacturing process for most protein products consists of two steps. One is the manufacture of drug substance, *aka* the active pharmaceutical ingredient (API), which is typically stored in bulk containers, such as plastic or stainless steel containers, in frozen condition for long-term storage purposes. The other step is the manufacture of the final drug product from drug substance. This is the final presentation to patients and healthcare professionals, typically stored in glass vials, prefilled syringes, or injection devices, at refrigerated condition for convenient use and distribution.

Formulations for drug substance and drug product may not be the same. Typically, the drug substance formulation is developed earlier, as it is the first step of the manufacturing process. However, drug substance should ideally be in a formulation vehicle that can be readily further formulated into drug product. Therefore, it is important to develop the drug product formulation as early as possible, so that the manufacturing process from drug substance to drug product will be harmonized and straightforward.

Prior to formulation development, it is important to conduct preformulation development activities, which serve to identify the key degradation pathways of the molecule and to develop stability-indicating assays to support formulation development studies. In general, preformulation activities should evaluate the biophysical and biochemical properties of the molecule under pharmaceutically relevant stress conditions, which include chemical-related

factors (such as extremes of pH, salts, buffers, oxygen, and peroxides) and process-related factors (such as high temperature, freezing, thawing, light, agitation, pressure, and shear stress). In these studies, biophysical characterization assays such as UV-visible spectroscopy, DSC, CD, Fourier transform infrared spectroscopy (FT-IR), AUC, and fluorescence spectroscopy are often used to determine which conditions result in the highest thermal stability and the least conformational changes. Biochemical methods such as size-exclusion HPLC, reversed-phase HPLC, hydrophobic interaction chromatography, capillary electrophoresis, peptide map, gel electrophoresis, ion-exchange HPLC, and potency assays are used to identify the key chemical degradation pathways, primarily through covalent bond changes, under these stress conditions.

Results from preformulation studies should identify the potential sources of the instability of the molecules and the key stability-indicating assays to monitor the degradation products, as these are needed for formulation screening and optimization studies. Once the leading formulation(s) are identified from the screening studies, appropriate container closure for final product presentation should be evaluated. In addition, the manufacturing process should be developed.

The choice of formulation, dosage form, and final product presentation is dependent on several factors. These choices not only need to offer the best stability and safety profiles, but they must also be easy to scale up for manufacturing and convenient to use during administration. The decisions are also dependent on the development stage of the product. At preclinical and early clinical stages, the goal of the project is to evaluate the proof of concept in product efficacy and safety as early as possible, so the shelf-life of early clinical products may only need to be 12 months or even less, and the storage or shipping conditions may not require the refrigerated or ambient temperature that is typically used for commercial products. Therefore, frozen or lyophilized formulations are typically chosen for preclinical and early clinical studies. At late clinical and commercial stages, there are more stringent requirements. At these stages, not only stability but also cost and marketing competitiveness are very important in making the final choice of the formulation. A very critical factor in a field of many competing, similar biotech products is patient compliance and acceptance, so prefilled syringes and auto-injection devices are in many cases essential for the success of the product's introduction to the market. As a result, evaluation of these dosage forms and devices must be initiated during phase III or pivotal clinical trials.

Overall, formulation development is a critical and evolving process step in product development. It is important to initiate the development effort as early as possible. It is also important to have input from multiple functional areas before finalizing the formulation choice.

Evaluation of Critical Formulation Parameters

To prepare a stable formulation suitable for patient use, the critical formulation parameters should be evaluated, including protein concentration, pH, buffers, stabilizers, tonicity modifiers, bulk agents (for lyophilized products only), and preservatives (for multidose products only). A discussion of evaluating each formulation parameter follows.

Protein Concentration

Protein concentration not only serves as a critical parameter in finalizing product presentation, but is also a key parameter in product stability. The following considerations are important in the selection of a viable protein concentration in a formulation:

- It should be below the protein's solubility in the selected formulation vehicle. Since protein solubility varies in different formulation vehicles and at different temperatures, the selected protein concentration should be below the "true" or thermodynamic solubility in the vehicle at the intended long-term storage temperature. However, measurement of true protein solubility is challenging, so this is an empirical exercise where real-time data needs to be evaluated on potential precipitation of the protein at the selected concentration and stored for the desired duration. This

assessment should be made for both drug substance and drug product before a protein concentration is finalized.

- It should maintain the protein's stability during long-term storage. Generally, proteins at high concentrations may lead to aggregation and precipitation. On the other hand, low protein concentrations may lead to significant loss of protein content due to adsorption onto various product-contacting surfaces (e.g., container closures during storage, filter membranes and silicone tubing during processing). Also, a higher relative ratio of any reactive impurities (such as traces of heavy metals, peroxides from surfactants, and sterilants used in the aseptic filling process) to the protein could cause degradation.
- It should be operable under manufacturing conditions. High protein concentrations achieved at lab-scale may not be operable at manufacturing scale. For example, a formulation with a high protein concentration may have high viscosity, requiring an undesirably long processing time for filtration or the ultrafiltration and diafiltration (UFDF) step. On the other hand, at low concentrations there may be significant protein loss from the filtration membrane due to the larger surface area at manufacturing scale. Therefore, it is critical to assess the scalability of the manufacturing process before choosing a protein concentration.
- It should minimize product waste during the manufacturing process, testing, and clinical use. Material losses during manufacturing (e.g., line loss), sample testing, and dose preparation for clinical use (e.g., residual in containers) are all volume based, so a high protein concentration will result in a large amount of protein waste. Therefore, the lowest protein concentration that delivers the required dose and maximizes production yield should be identified.
- It should meet the requirements for product presentation. Product presentation is selected on the basis of factors such as dose (size and frequency), administration route, convenience of dose preparation, patient weight distribution (for weight-based dosing schemes), number of manufacturing lots desired or manageable per year, and commercial considerations of cost and product differentiation. From these analyses, the amount of protein per vial is specified, and subsequently, the optimal volume and corresponding protein concentration is determined on the basis of factors such as solubility, stability, minimal protein waste, and manufacturability.
- It should take into account manufacturing process cycle time and cost. For lyophilized products, the major contributor to manufacturing cost is the lyophilization time. Reducing the fill volume by increasing protein concentration can significantly reduce the lyophilization time.

In summary, protein concentration, as a critical formulation parameter, should be chosen on the basis of multiple factors, including solubility, stability, manufacturability, cost, product presentation, and commercial considerations.

pH

As proteins containing both positively and negatively charged amino acids are amphoteric molecules, solution pH has a direct impact on the overall stability of proteins. When solution pH is far from the pI of the protein, electrostatic repulsions between like charges in the protein increase, causing a tendency to unfold. In addition to electrostatic interactions, pH also affects other interactions within proteins. Therefore, changing pH directly affects the conformational stability of proteins and their solubility in aqueous solution. In addition, since certain chemical reactions are highly pH dependent, solution pH also affects the chemical stability of proteins. The following areas should be assessed when selecting an optimal pH for a protein formulation:

- Profiles of conformational stability, chemical stability, and solubility as functions of pH in solution may not overlap each other for a given protein. The pH at maximum solubility may not be the same as the pH at maximum conformational stability or

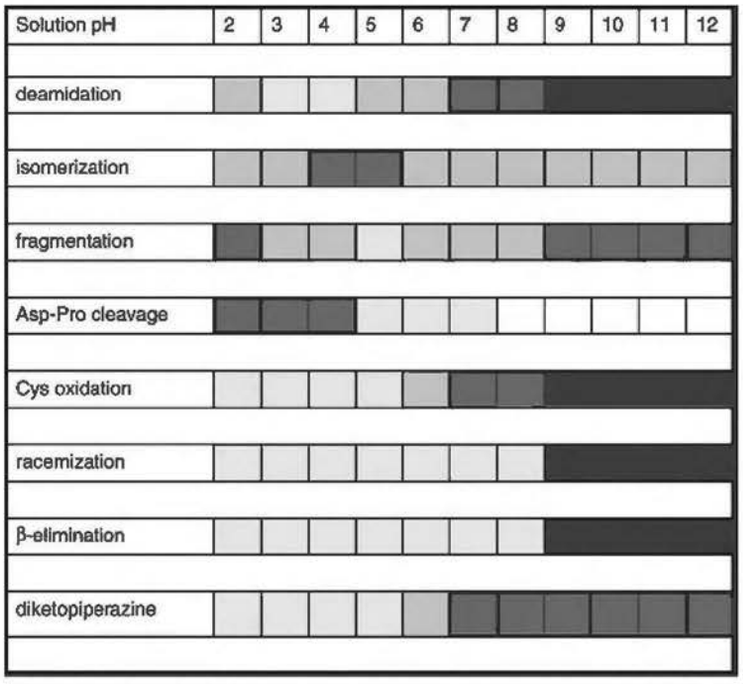

Figure 2 Reaction rates as a function of pH. (Darker color indicates a faster reaction rate at designated pH range.)

chemical stability. It is important to define an optimal pH that is a good compromise among all these characteristics.

- As chemical stability involves several different degradation pathways and each degradation pathway may have a different stability profile as a function of pH in solution (Fig. 2), it is important to balance all the degradation profiles before finalizing the optimal pH for the formulation. The degradation pathways that lead to significant loss in potency or biological activity or that cause immunoreactivity should be kept to a minimum when selecting the pH.
- The optimal pH selected in solution may not be optimal for proteins in solid dosage forms, so it is important to reassess the effect of pH when the protein dosage form changes from liquid to lyophilized form. For example, opposite trends in pH-dependent stability were observed for lyophilized and liquid formulations (80).
- The selected pH should not have any impact on the stability of other excipients in the formulation. Certain excipients may not be stable in a certain pH range under long-term storage conditions. For example, acidic pH has caused the hydrolysis of sucrose, a commonly used stabilizer in protein formulations (81,82).
- The selected pH should be compatible with product-contacting surfaces during manufacturing and storage. It has been reported that acidic pH caused corrosion of stainless steel in the presence of chloride ions, which generated iron ions that catalyzed methionine oxidation in a monoclonal antibody (83). Also, high or low pH may cause more leachables from stoppers, which serve as primary container closure.
- The selected pH needs to be safe to use for its intended administration route. A certain pH range may be suitable for IV injection but cause side effects when used for SC, IM, or IT injection.

For most protein formulations, the selected pH is in the range of 6 to 7, as this range is close to the physiological pH and also provides the optimal stability for most proteins. For

peptides, formulation pH is mostly in the acidic range of 4 to 5, as this pH range provides better aqueous solubility and less adsorption.

Buffers

Buffers are often used to control the formulation pH, keeping it within a narrow range to prevent small changes that can affect the stability and solubility of proteins. The selection of a proper buffer type and concentration for proteins should be based on the following considerations:

- The buffer species and concentration should not cause protein instability. For example, sodium phosphate buffer may result in a significant pH drop upon freezing, which has been found to cause instability of some proteins, particularly at high buffer concentration or low protein concentration conditions (84).
- The buffer species should have a pK_a near the target pH, preferably within one pH unit. Table 1 lists the pH control ranges for some commonly used buffer species.
- When buffer concentration has no effect on the protein stability, it should be kept to a minimum, but high enough to provide sufficient buffering capacity to control the formulation pH. High buffer concentration may cause some pain or discomfort upon SC or IM injection. In addition, for lyophilized product, a high concentration of buffer species such as sodium phosphate or citrate may lead to a lower glass transition temperature, which would require a longer lyophilization cycle.
- Buffers, like salts of ionic compounds, contribute to the overall ionic strength of the formulation solution. Therefore, buffer concentration also influences other properties that are dependent on ionic strength, such as protein solubility and stability.
- Certain buffer species, besides controlling solution pH, may also serve as a stabilizing agent in some protein formulations. For example, citrate may serve as a chelating agent to remove any heavy metals that potentially catalyze oxidation. Histidine also has an antioxidant effect (85).
- The buffer species and concentration should be safe to use for its intended administration route. Some buffer species and concentrations may be suitable for IV injection but may not be compatible when used for SC, IM, or IT injections. For example, citrate was found to cause more pain than histidine as a buffer when administered by SC injection (86).

It should be noted that as zwitterions, proteins have their own buffering capacity, especially at high concentration, so a buffer may not be required in formulations if the pH can be maintained by the protein itself. It has been reported that monoclonal antibodies at 60 mg/mL have a strong self-buffering capacity and that the long-term stability of self-buffered formulations is comparable to that of conventionally buffered formulations (87).

Ionic Strength/Salt Concentration

As proteins carry both negatively and positively charged groups, ionic strength in formulation solution may directly affect the solubility and stability. Ionic strength in parenteral formulation

Table 1 Buffers for Protein Formulations

Buffer	Acid	Base	pH range control	Examples
Phosphate	Monosodium phosphate	Disodium phosphate	5.8 7.8	Elaprase®, Remicade®
Acetate	Acetic acid	Sodium acetate	3.8 5.8	Avonex®, Neupogen®
Citrate	Citric acid	Sodium citrate	3.0 7.4	Amevive®, Rituxan®
Succinate	Succinic acid	Sodium succinate	3.3 6.6	Actimmune®
TRIS	TRIS HCl	TRIS	7.1 9.1	Wellferon®, Enbrel®
Histidine	Histidine HCl	Histidine	5.1 7.0	Xolair®, Raptiva®
Carbonate	Sodium bicarbonate	Sodium carbonate	5.4 7.4, 9.3 11.3	Fuzeon™

is mainly adjusted using NaCl. NaCl affects electrostatics in a protein either by nonspecific (Debye-Huckel) electrostatic shielding or by specific ion binding to the protein. At low concentration, salts affect electrostatic shielding and weaken ionic repulsion/attraction as counter-ions, so this shielding effect may be stabilizing when there are major repulsive interactions leading to protein unfolding, or could be destabilizing when there are major stabilizing salt bridges or ion pairs in the proteins. At high concentrations, electrostatic shielding is saturated. The dominant effect of salt, as of other additives, is on the solvent properties of the solution. The stabilizing salts seem to increase surface tension at the water-protein interface and strengthen hydrophobic interaction by keeping hydrophobic groups away from water molecules, inducing preferential hydration of proteins (13). Therefore, ionic strength or salt concentration affects both the solubility and the stability of proteins. The following areas should be assessed when selecting a proper ionic strength or salt concentration:

- Similar to the effect of pH, the profiles of solubility and stability as functions of ionic strength may not overlap each other for a specific protein, so the optimum ionic strength at maximum solubility may not be the same as at maximum stability. It is important to define an optimal ionic strength that is a good compromise between these two characteristics.
- Ionic strength or NaCl concentration has been reported to have an impact on the viscosity of formulations, especially at high protein concentrations (88). It may therefore serve an important factor in adjusting the viscosity of the product. Viscosity is an important parameter for syringeability of high protein concentration formulations used in SC and IM administration.
- Ionic strength or NaCl concentration should be compatible with other excipients in the formulation and with product-contacting surfaces. It has been reported that high salt concentration combined with acidic pH may cause rusting of stainless steel, resulting in an elevated level of iron ions responsible for oxidation of the protein (83).
- Ionic strength or NaCl is certainly a key contributor to the overall tonicity of the formulation, and it is important to keep the concentration or tonicity suitable for the intended administration routes.

Stabilizers

When adjusting the parameters discussed above protein concentration, pH, buffer, and ionic strength still does not result in sufficient protein stability, the addition of stabilizers should be considered. Several types of stabilizers, listed in Table 2, are commonly used in protein formulations to stabilize proteins against various stresses. It is important to consider the following aspects when choosing a stabilizer:

- The choice of stabilizer type and concentration should be rational. An experimental laboratory-scale model should be developed to screen the stabilizer type and concentration for specific degradation against specific stress. For example, to identify a stabilizing excipient against shaking stress, surfactants and concentration ranges should first be tested through an established shaking model. To find a stabilizer against freezing/thawing stress, an appropriate freeze/thaw stress model should be used to screen various cryoprotectants and their concentration ranges.
- The number of stabilizers in a single formulation should be minimal and based on needs. An ideal stabilizer inhibits or minimizes multiple degradation pathways. For example, conformational stabilizers, such as sucrose, which enhance conformational stability, minimizes not only the aggregation but also other chemical degradations such as oxidation and fragmentation that occur when the reactive sites are exposed in absence of sucrose.
- The type and concentration of stabilizers should be compatible with other excipients in the formulation. For example, Ca^{2+} may be a good stabilizer, but if the buffer is phosphate and the pH is above neutral, precipitation of calcium phosphate may occur.
- Any stabilizers that may cause interference with protein assays should be avoided. For examples, polymers and albumins used as stabilizers in formulations may interfere

Table 2 Commonly Used Stabilizers in Protein Formulations

Type	Hypothesized stabilizing mechanism(s) (reference number)	Examples
Sugars	Stabilize proteins by preferential hydration in solution; serve as cryoprotectant and/or lyoprotectant; certain sugars such as glucose may chelate heavy metals, thus serving as antioxidants (89).	Sucrose: Follistim®, Panglubulin®, Ovidrel®, Xigris™ Trehalose: Advate, Herceptin® Lactose: Factrel®, Glucagon® Glucose: Gammagard® S/D Maltose: Bexxar®, Gamimune®
Polyols	Stabilize proteins by preferential hydration in solution; may serve as cryoprotectant and/or lyoprotectant; certain polyols such as mannitol may also serve as antioxidants by chelating metal ions (89).	Mannitol: DigiFab™, Fabrazyme®, Cerezyme® Sorbitol: Digibind®, Neulasta™, Neupogen® Glycerol: Humalog®, Humulin®R
Surfactants	Reduce agitation induced aggregation by reducing surface tension; facilitate refolding by specific or nonspecific binding to protein; minimize adsorption and prevent other degradation by preferentially binding to interfaces (air liquid, ice or solid surfaces).	Polysorbate 20: Neulasta™, Replagal®, Raptiva® Polysorbate 80: PEG Intron, Remicade®, WinRho SDF® Poloxamer 188: Elitek™
Amino acids	Suppress protein aggregation and protein protein or protein surface interactions; arginine increases the surface tension of water, thus favorably interacting with most amino acid side chains and peptide bonds (90).	Glycine: Gamunex®, Synagis®, Neumega® Arginine: Activase®, TNKase® Cysteine: Acthrel®, SecreFlo® Histidine: BeneFIX®
Metal chelators	Chelate heavy metals to prevent metal ion catalyzed oxidation of cysteine and methionine residues in proteins.	EDTA: Kineret™, Ontak®
Divalent metal cations	Stabilize protein conformation by specific binding to certain sites of protein.	Ca^{++}: Pulmozyme®, ReFracto®, Kogenate® Zn^{++}: Nutropin Depot™, Aralast™
Polymers or proteins	Stabilize proteins by mechanisms similar to those of surfactants; serve as cryoprotectant or lyoprotectant.	Albumin: Intron®A, Rebif®, Zevalin™, Procrit® PEG: Autoplex®T, Hemofil®M, Monarc M™, Prolastin® Carboxylmethylcellulose: Plenaxis™ Heparin: Thrombate III®, Autoplex®T Dextran: Mylotarg™

with protein assays, particularly in UV, HPLC methods and gel electrophoresis, creating complications for release and stability testing.

- Any stabilizers that may introduce potential contaminants, especially animal or human derived, should be avoided. Albumin, for example, is an excellent stabilizer for many therapeutic proteins and was widely used in early products; however, because of concerns about blood source contamination, it has seldom been used as a stabilizer in recent products. However, availability of pharmaceutical grade recombinant human serum albumin may change this dynamics.
- Stabilizers of high quality from a reputable vendor should be used for lab screening studies. Like any excipients, stabilizers may contain different levels and types of impurities when they are made from different sources or processes. These impurities

could cause inconclusive results from screening studies, as they may result in instability even while the stabilizer itself has a stabilizing effect. A case in point is the peroxide level in polysorbates: varying peroxide levels in polysorbates used in formulation studies often confound the study results.
- The type and concentration of stabilizers chosen should be safe to use for intended administration routes. It is important to assess the safety and toxicity of any new excipient prior to clinical studies.

As shown, choosing the proper type and concentration of stabilizer is a challenging process. It is important to consider all aspects, including solubility, stability, compliance, safety, and operational challenges (such as posing analytical difficulties).

Tonicity Modifiers

For parenteral administration, the final product is generally formulated to be isotonic or iso-osmotic, which is equivalent to 0.9% or 150 mM NaCl with an osmolality of 289 mOsm/kg. The following aspects should be considered when finalizing the type and concentration of tonicity modifiers in the final product:

- Commonly used tonicity modifiers in protein formulations include NaCl, mannitol, and sorbitol. An excipient already selected for the formulation, such as salt or stabilizer, is the preferred choice when increasing concentration does not have an effect on the overall properties of the formulation.
- The requirement of isotonicity depends on the administration route and dose preparation. If the product is diluted into IV fluid such as normal saline solution prior to administration, the formulation may not be required to be isotonic. However, if the product is directly injected without any dilution, isotonicity is preferred, particularly for SC, IM, and IT injections.
- For lyophilized product, the formulation prior to lyophilization may not be required to be isotonic even when the reconstituted solution is required to be isotonic. Isotonicity in final reconstituted solution can be achieved by choosing the proper type and volume of diluent to reconstitute the lyophilized product.

It should be noted that some recent studies have shown that infusion of solutions with iso-osmolality but hypotonicity may cause some adverse effects (91). This suggests that although the terms "isotonic" and "isoosmotic" have been used interchangeably, they may have different effects on safety, particularly for products that will be infused in large quantity.

Bulking Agents (for Lyophilized Product Only)

For lyophilized product, bulking agents are required to provide enough solids to maintain good cake structure during lyophilization and long-term storage. To choose the proper type and amount of bulking agent, the following aspects should be considered:

- The type and amount of bulking agent added to the formulation should be compatible with the protein and other excipients. It should not cause significant protein instability during lyophilization or storage. An excipient already selected for the formulation, such as a stabilizer (sucrose or trehalose), should be preferred as the bulking agent when increasing the concentration does not have an effect on the overall properties of the formulation (e.g., high sucrose concentration may require an extremely long lyophilization cycle or result in partially collapsed cakes).
- Mannitol and glycine are commonly used bulking agents as they provide better cake appearance and do not require a longer lyophilization cycle. However, because of their crystalline nature, they may cause phase separation during storage, leading to stability issues. In addition, a high content of mannitol may lead to vial breakage during freezing (92) due to volume expansion during crystallization. This vial breakage phenomenon has been also observed with NaCl crystallization during lyophilization (93).

Table 3 Preservatives for Protein Formulation

Type	Example(s)	Comments
Phenol	Antivenin Aplisol® Nutropin AQ®	Air and light sensitive; may act as a reducing agent.
Benzyl alcohol	Epogen® Nutropin® Pegasys® Factrel®	Usually in diluent for reconstitution of lyophilized product.
m Cresol	Humatrope NovoLog®	Used in both liquid multiuse products and diluents for lyophilized products.
Thimerosal	Antivenin	Not commonly used for recent products because of mercury related toxicity.
Chlorobutanol	Desmopressin	Widely used preservative in pharmaceuticals, including injectables. Typically used at 0.5%.

- Polymeric bulking agents [e.g., hydroxyethyl starch (HES) or dextran] and proteins (e.g., albumin) may significantly reduce the length of lyophilization cycle by raising T_g', however, some of them may interfere with certain protein assays. Therefore, potential complications in analytical testing should be considered when choosing this type of bulking agents.
- For high-protein-concentration formulations, bulking agents may not be required, as a protein itself serves as a bulking agent to provide good cake structure.

Preservatives (for Multidose Products)

Most injectable protein products are intended for single-dose injection, which does not require inclusion of antimicrobial preservatives in the formulation. However, some products are intended for multidose administration, which requires preservatives in the formulation to prevent any microbial growth from the time the product is opened for use to the time the last dose is administered.

Table 3 lists the preservatives that have been used for protein formulations. To choose the appropriate type and concentration of preservatives in a formulation, the following aspects should be considered:

- The type and concentration of preservatives selected for a formulation should make the final product meet the antimicrobial effectiveness testing required by USP and BP/EP at the time of product release and at the end of shelf-life and last dosing. One needs to be aware that requirements in BP/EP are more stringent than those in USP (94).
- Adding preservatives generally results in protein instability. This is not surprising, as the bactericidal or bacteriostatic effect is derived from the preservative's interaction with proteins or DNA in microorganisms. It is important to screen for a compatible type of preservative for specific formulations or proteins.
- Minimizing the contact time between preservative and protein is a general approach to reducing the preservative's stability impact. In this approach, preservatives are typically added to the diluent (for lyophilized product) or to the product upon preparation for the first dose.

General Strategies for Formulation Screening and Optimization

As discussed above, protein formulation has multiple parameters, including the protein itself, buffers, pH, stabilizers, and other excipients, and each parameter has its own functions. Some of these parameters may interact with each other; for example, pH and stabilizers both affect

Table 4 Formulation Parameters, Evaluation Models, and Critical Attributes for Design of Experiments

Formulation parameters	Evaluation models	Critical attributes
Protein concentration	Thermal stress	Appearance
pH	Freeze/thaw cycle	Content
Buffer type/concentration	Peroxides exposure	Aggregation
Stabilizer type/concentration	Light exposure	Fragmentation
Tonicity modifier type/concentration	Shaking/agitation	Oxidation
Preservative type/concentration		Deamidation
		Potency

the stability of the molecule. In addition, the product has to be exposed to multiple process-related stresses during manufacturing, storage, and handling, such as extreme temperatures, freeze/thaw cycles, agitation, and pressure. Therefore, it is challenging to develop a stable formulation containing many parameters against various process-related stresses.

Design of experiments (DOE) has proven an effective tool in dealing with such a complicated development process involving multiple variables. DOE, as part of the concept of quality by design (QbD) recently introduced by regulatory agencies for pharmaceutical development, is a tool to establish the design space through statistical analysis. The design space forms a link between development and manufacturing design (ICH Q8, Pharmaceutical Development). For formulation development, the design space refers to the defined range of formulation parameters and quality attributes that have been demonstrated to provide assurance of quality.

Formulation development using a DOE approach typically has two stages: formulation screening and optimization/robustness studies. The goal of the screening study is to identify the key formulation parameters, while the optimization/robustness study defines the optimal or robust range of the selected key parameters.

To design a proper space for statistical DOE studies, it is important to collect all the information from preformulation development activities and any prior knowledge on the protein. This information helps in identifying the critical formulation parameters and the key degradation pathways that potentially affect the critical quality attributes (CQAs) of the product for the design space, and in selecting a proper evaluation model that can be used for screening or optimization of formulations. In addition, the target product profile (TPP) should also be established prior to DOE studies.

Table 4 lists the parameters and attributes for DOE studies. Once the study is completed and data are collected, statistical analysis should be performed to establish the design space. This established design space can not only justify the choice of formulation ranges and help identify the robust region of the formulation, but can also enable study of the interactions between each formulation parameter. Several case studies using the DOE concept have been described in a book chapter written by Ng and Rajagopalan (95).

Choice of Container and Closure System

Because proteins may interact with the contact surfaces, the compatibility of immediate packaging material with protein product needs to be evaluated during selection of the container closure system.

As described in section "General Formulation Development Process in Industry," most protein products are formulated as drug substance and drug product, which are typically stored at frozen and refrigerated conditions, respectively. In choosing an appropriate container closure system for drug substance, the following aspects should be considered:

- Commonly used container closure systems for drug substance include plastic bottles or bags and stainless steel vessels. Various types of plastics have been used in packaging protein drug substance, including Teflon, polyolefin, glycol-modified polyethylene terephthalate (PETG), polypropylene (PP), polycarbonate (PC), polyvinylchloride (PVC), polyvinyl alcohol (PVA), and polyethylene (PE). It is important to

evaluate whether the product remains stable under intended storage conditions. For example, hydrophobic proteins tend to adsorb more on hydrophobic polymers such as Teflon. In addition, the material's gas permeability and leachables should also be evaluated. Plastics with high gas permeability may affect product stability during long-term storage if the product is oxygen sensitive. Plastics such as PVC that contain a substantial amount of plasticizer may generate more leachables than other plastics. When stored in a stainless steel vessel, acidic pH and chloride ions may cause corrosion. With an increased amount of dissolved metal ions, protein oxidation is a concern (83).

- The material should retain its function at the intended storage condition. Since most protein drug substances are stored frozen, it is important to assess the brittleness point of the plastics at the intended storage temperature. If the brittleness point is above the intended storage temperature, container closure integrity may be compromised. In addition, breakage of container closure may occur upon impact, such as an accidental drop. Therefore, PC and Teflon are preferred because of their lower brittleness points.
- The size of the containers should be selected on the basis of product stability, potential expansion of product, and cost effectiveness. For example, a sufficient amount of headspace should be allowed to accommodate volume expansion upon freezing for frozen drug substance. If the product is stored as a liquid, then minimal headspace should be considered to minimize the potential instability caused by agitation upon handling and shipping. With large containers, it may be difficult to control the freezing process. Stainless steel cryo-vessels with temperature-controlling systems have many advantages over plastic containers, such as controlled freezing and thawing rates and nonbreakable characteristics. However, they are expensive and need to undergo cleaning validation for multiuse purposes. In addition, they may need frequent passivation to retain resistance to potential corrosion.

To choose an appropriate container closure system for drug product, the following aspects should be considered:

- Unlike drug substance, most drug products are stored in glass vials with rubber stopper systems. Some products are also packaged in prefilled syringes, cartridges, and dual chamber Lyo-Ject® syringes, which all consist of glass barrels or tubing and rubber stoppers. Plastic vials or tubing have also recently been introduced. It is important to evaluate the product's compatibility with various types of glass, plastics, and rubber stoppers, as protein adsorption and other degradations may occur.
- Container closure integrity should be retained to ensure the product's sterility throughout its shelf-life.
- Most glass vials are washed and then depyrogenated prior to use. It has been reported that siliconized vials may minimize adsorption of the product (17). However, the possibility of silicone oil causing protein aggregation also needs to be examined. For prefilled syringes, the glass barrels should be siliconized for proper syringeability. It is important to recognize, however, this step may affect not only syringeability but also product stability. For lyophilized product, the stoppers may need to be dried in an oven or autoclave following steam sterilization, because retained moisture may be released to the lyophilized cake during long-term storage and cause instability issues, which are critical for moisture-sensitive products.
- The type of container closure system should be decided on the basis of the product's stability, the development stage, the intended use (indication and administration route), and marketing competitiveness. For an early clinical development stage, a vial and stopper system is often chosen, as it involves less technical complexity and thus requires less development time. For late development or commercial stages, the performance of a container closure system such as improving ease of administration, minimizing drug wastage, and conforming with patient needs (e.g., self-administration) becomes more important. Prefilled syringes, self-injection devices using cartridges, and dual chamber lyo-ject syringes may help the drug product to gain a greater market share because of their convenience of use, which leads to better patient compliance.

- The size of container closure should be decided on the basis of several factors, including dosing regimen, product stability, cost effectiveness, and compliance for patient use. For example, vial size affects the headspace, which is a critical parameter for most liquid products as it may impact product stability upon agitation during shipping and handling. For prefilled syringes, the size of the syringe also affects the headspace and the movement of the stoppers upon exposure to reduced pressure during airplane shipping. Cartridges tend to have less headspace.

For both drug substance and drug product, the suppliers of the container closure system should be reputable and well established. Suppliers that have established Drug Master Files (DMFs) for the packaging components should be preferred. In addition, for commercial products, a second source for the container closure system may need to be established in case issues arise with the primary source.

Manufacturing Process Development

The next step after selection of an appropriate formulation and container closure system is to develop a manufacturing process that maintains protein stability under process conditions such as mixing, filtration, filling, and lyophilization. Instability of proteins under these conditions is often observed, and therefore it is important to evaluate and define the optimal process conditions prior to cGMP manufacturing. From these studies, the critical process parameters and acceptable operation ranges should be defined using a DOE approach similar to that used in formulation development. The CQAs impacted by these process conditions should be evaluated. The following aspects should be considered when developing a robust and suitable manufacturing process:

- All product-contacting surfaces during manufacturing should be compatible with the formulation. Generally, the product-contacting surfaces during drug product manufacturing include silicone tubing, a Teflon-coated stir bar, a stainless steel tank or impeller mixer, rubber gaskets, plastic connectors, plastic housing and filter membranes, and other glass or plastic containers. It is important to evaluate the compatibility of the protein with these contact surfaces prior to the start of manufacturing using these materials.
- The container closure system should be compatible with the fill line at the manufacturing site. Typically, machinability needs to be conducted prior to cGMP manufacturing to ensure that the filling and stoppering operations run smoothly, with low rejection rates, and that the final container closure system meets the container closure integrity test criteria (integrity is typically tested by dye leak or vacuum decay method).
- The mixing condition should not result in product degradation. If mixing by a magnetic stir bar causes protein precipitation, alternative mixing methods such as an impeller should be considered.
- If the product is sensitive to dissolved oxygen in solution, several manufacturing process steps should be designed appropriately. Degassing the solutions and overlaying inert gas (nitrogen or argon) in the headspace of vials may be required to minimize oxidation due to dissolved oxygen in the product.
- The filter size for sterile filtration should be large enough not to give high back pressure during filtration. For aqueous protein formulations, a polyvinylidene fluoride (PVDF) membrane is the most commonly used. A filter sizing study should be performed to define the proper size of filter for cGMP manufacturing.
- The filling conditions should be compatible with the product. For solution filling, several filling machines are commonly used, including peristaltic pump, stainless steel piston syringe, ceramic piston syringe, rolling diaphragm, and rotary time pressure filling systems. While a syringe-filling system typically provides better accuracy, it applies high shear stress between the piston and barrel during movement, which could lead to precipitation of proteins. In addition, the filling speed needs to be controlled to avoid foaming or splashing during filling.

- The lyophilization parameters developed at laboratory scale should be robust enough to produce consistent product quality. Sometimes when the lyophilization cycle developed for the laboratory-scale lyophilizer is directly transferred to the production-scale lyophilizer, product quality may not be the same. This could be caused by the poor robustness of lyophilization cycle, as different lyophilizer designs may lead to different levels of heat and mass transfer, which could cause changes in product quality, such as cake collapse, if the selected lyophilization parameters are on the edge of the process design.
- Hold conditions (time and temperature) for all process intermediates should be established to meet the needs of routine production operations and to support potential excursions. An operation deviation may result in longer hold time for the process intermediate than during routine manufacturing; examples could be deviations prior to the formulation step or filling into final product, or after lyophilization. Supporting data for defining acceptable hold conditions need to address product quality from both chemical stability and microbiological perspective. Assessment of the microbiological acceptability of a process intermediate hold time is tied to manufacturing process operations and to the microbial growth potential of the intermediate composition. Support for extended or cumulative hold times generally comes from development-scale studies, while support for microbiological properties comes from manufacturing-scale studies.

Stability Studies

After the manufacture of drug substance and drug product, the following stability studies are generally conducted to support the shelf-life during long-term storage and the product quality during distribution and use at clinics:

- *Long term stability studies to support shelf life.* Several ICH guidelines outline the content and testing requirements for stability studies supporting shelf-life at long-term storage conditions (ICH Q1A, Q1C, Q1D, Q1E, and Q5C). Depending on the intended long-term storage condition, accelerated and stress conditions are often required for the clinical stability program and lots made during process validation. For postapproval commercial stability programs, one lot of drug substance and one lot of drug product are generally required to be placed on stability annually, and only at the long-term storage condition.
- *Temperature cycling studies to support excursions during distribution.* From completion of manufacturing to the time when the product is dosed into patients, the drug product experiences exposure to various temperatures, different from the intended long-term storage temperature range, which is typically the refrigerated temperature. It is important to conduct studies to evaluate the effect of these temperature variations on product stability. The design of these studies has been recommended in the Parenteral Drug Association (PDA) Technical Report No. 39 (96).
- *Shipping studies to support the exposure to vibration and reduced pressure during air and ground transportation.* It is important to recognize that shaking studies conducted at laboratory scale may not be representative of the actual shipping conditions to which the product is exposed, since the shaking studies may not have vibration amplitudes and frequencies similar to those generated during shipping. In addition, reduced pressure occurs during air shipment. This is a concern particularly for prefilled syringes, as stopper movement has been observed during multiple cycles of reduced pressure, which may affect the sterility of the product (97). Therefore, it is important to demonstrate that the product remains intact or within the designed space using actual or simulated shipping conditions, including representative secondary packaging and product orientation.
- *Confirmatory photostability studies to support exposure to light.* Sensitivity to light is highly dependent on the composition, structure, and formulation of the product. Most proteins and peptides are sensitive to intense light, particularly UV light, which

typically results in oxidation. However, they are relatively stable under normal indoor light. In addition, protein products are typically stored in refrigerated condition, in which there is no direct exposure to light. Commercial products normally have secondary packages, which prevent the product's exposure to light. To demonstrate that the product remains stable, it is important to conduct a confirmatory photostability study using representative commercial packaging per the ICH guideline Q1B.

- *In use and compatibility studies with the administration system to support product stability and to assess compatibility with product contacting surfaces and stresses during administration*. For IV infusion, some products may need to be diluted in an IV bag and then infused into patients through an IV infusion apparatus by a pump or other mechanism. It is critical to assess product stability after dilution prior to dosing, as well as the compatibility of the product in contact with the surfaces of the administration apparatus and infusion system, such as pumps. For lyophilized products, the stability of reconstituted solution should be evaluated to ensure the product remains stable during the ambient exposure period after reconstitution.

FORMULATIONS OF MARKETED PROTEIN PRODUCTS

In the United States, by law all marketed injectable products must disclose quantitative formulation, so the details on each marketed product are public. Sources on prescription information include the Physicians' Desk Reference (PDR) and numerous websites, including the FDA's http://www.accessdata.fda.gov/scripts/cder/drugsatfda and websites for specific products (which commonly take the form www.*tradename*.com e.g., www.simponi.com). The formulations of discontinued products can be found in older editions of the PDR.

The excipients used in parenteral products were first reviewed and collated by Wang and Kowal in 1980 (98). Subsequently, there were reviews by Nema et al. (99) and Powell et al. (100). Specifically for biotech products, they were first reviewed by Wang and Hansen (101), and recently by Gokarn et al. (102).

The types of excipients used in protein formulations have evolved over time. For example, in the early years (1980s 1990s), human serum albumin (HSA) was commonly used as a stabilizer in many protein formulations, particularly low-concentration, high-potency products such as interferons, Factor VIII, and other growth factors. However, because of concerns about potentially contaminated blood that might compromise the quality of albumin, most products have been reformulated into HSA-free formulations. For example, Eprex® (epoetin α), originally containing albumin, was reformulated to use polysorbate 80 as an HSA-free product in prefilled syringes. Avonex® (interferon β), originally having albumin in lyophilized form, was reformulated to a polysorbate-20-containing liquid formulation in prefilled syringes.

Another example of evolving parenteral formulation excipients is the type of polysorbate. Because of concerns about potential auto-oxidation of the unsaturated double bond in polysorbate 80 (103), used predominantly in the early days of protein formulations, the trend seems to be moving toward the use of polysorbate 20. For example, Neupogen® is formulated with polysorbate 80, but the surfactant in its newer version, PEGylated protein (Neulasta®) was changed to polysorbate 20. Polysorbate 80 included in the Activase® formulation was also changed to polysorbate 20 in its newer variant TNKase®.

To show how formulations have evolved over time, examples of recombinant human growth hormones and monoclonal antibodies (including Fab, Fab-PEG, and Fc-fusion proteins) are listed in Tables 5 and 6, respectively. For human growth hormone, changes of formulation have been minimal since its first approval in 1985 to one recently approved in 2008. For lyophilized monoclonal antibodies, the buffer species used has changed from phosphate in early approved products to histidine in recently approved products. Sucrose is the most commonly used sugar in lyophilized formulations, and only a few products use trehalose or maltose. In addition, amino acids including arginine and glycine are used in both liquid and lyophilized formulations. For liquid monoclonal antibodies, sodium phosphate appears to be the most commonly used buffer, likely because of its good buffering capacity

Table 5 Formulations for Human Growth Hormone (in order of approval year)

Trade Name	Approval year	Dosing route	Presentation	Dosage form	Formulation
Protropin®	1985	SC	Vial/stopper	Lyophilized	5 mg vial[a] 40 mg mannitol 0 1 mg monobasic sodium phosphate 1 6 mg dibasic sodium phosphate pH 4 5–7 0
Humatrope®	1987	SC/ M	Vial/stopper	Lyophilized	5 mg/5 mL vial 25 mg mannitol 5 mg glycine 1 13 mg dibasic sodium phosphate pH 7 5
			Pen cartridge	Lyophilized	6 mg/2 mL cartridge[a] same formulation as in vial/stopper
Norditropin®	1987	SC	Pen cartridge	Liquid	5 mg/1 5 mL cartridge[a] 1 mg histidine 4 5 mg Poloxamer 188 4 5 mg phenol 60 mg mannitol
Nutropin®	1993	SC	Vial/stopper	Lyophilized	5 mg/1 mL vial[a] 45 mg mannitol 0 4 mg sodium phosphate monobasic 1 3 mg sodium phosphate dibasic 1 7 mg glycine pH 7 4
			Vial/stopper	Liquid	10 mg/2 mL vial 17 4 mg sodium chloride 5 mg phenol 4 mg polysorbate 20 10 mM sodium citrate pH 6 0
			Pen cartridge	Liquid	5 mg/1 mL cartridge[a] same formulation as in vial/stopper
Tev-T opin®	1995	SC	Vial/stopper	Lyophilized	5 mg/5 mL vial 30 mg mannitol pH 7–9
Genotropin®	1995	SC	2-chamber cartridge	Lyophilized	5 mg/1 mL cartridge[a] 5 8 mg somatropin 2 2 mg glycine 1 8 mg mannitol 0 32 mg sodium dihydrogen phosphate anhydrous 0 31 mg disodium phosphate anhydrous pH 6 7
Saizen/Serostim®	1996	SC	Vial/stopper	Lyophilized	4 mg vial[a] 27 3 mg sucrose 0 9 mg phosphoric acid pH 6 5–8 5
Zorbtive®	2003	SC	Vial/stopper	Lyophilized	4 mg vial[a] 27 3 mg sucrose 0 9 mg phosphoric acid pH 7 4–8 5
Omnitrope®	2006	SC	Vial/stopper	Lyophilized	1 5 mg/1 13 mL vial[a] 0 88 mg disodium hydrogen phosphate heptahydrate 0 21 mg sodium dihydrogen phosphate dihydrate 27 6 mg glycine
			Pen cartridge	Liquid	5 mg/1 5 mL cartridge[a] 1 3 mg disodium hydrogen phosphate heptahydrate 1 6 mg sodium dihydrogen phosphate dihydrate 3 0 mg poloxamer 188 52 5 mg mannitol 13 5 mg benzyl alcohol
Valtropin®	2007	SC	Vial/stopper	Lyophilized	5 mg vial/1 mL 10 mg glycine 45 mg mannitol 0 22 mg monobasic sodium phosphate 2 98 mg dibasic sodium phosphate pH 6 0
Accretropin®	2008	SC	Vial/stopper	Liquid	5 mg vial 0 75% NaCl 0 34% phenol 0 2% Pluronic F-68 10 mM sodium phosphate pH 6 0

[a] ndicates a product that has multiple dose strengths but only the lowest strength is listed

Table 6 Formulations for Antibodies Fc Fusion and Fab Conjugates (in order of approval year)

Trade name	Nonproprietary name	Approval year	Dosing route	Presentation	Dosage form	Formulation
Orthoclone OKT-3®	Muromonab-CD3	1986	V	Ampule	Liquid	5 mg/5 mL ampule 2 25 mg monobasic sodium phosphate 9 0 mg dibasic sodium phosphate 43 mg sodium chloride 1 0 mg polysorbate 80 pH 6 5–7 5
Reopro®	Abciximab (Chimeric Fab)	1994	V	Vial/stopper	Liquid	10 mg/5 mL vial 0 01 M sodium phosphate 0 15 M sodium chloride 0 001% polysorbate 80 pH 7 2
Rituxan®	Rituximab	1997	V	Vial/stopper	Liquid	100 mg/10 mL vial[a] 9 mg/mL sodium chloride 7 35 mg/mL sodium citrate dihydrate 0 7 mg/mL polysorbate 80 pH 6 5
Enbrel®	Etanercept	1998	SC	Vial/stopper	Lyophilized	25 mg/0 5 mL vial 40 mg mannitol 10 mg sucrose 1 2 mg tromethamine pH 7 1–7 7
				PFS	Liquid	25 mg/0 5 mL PFS[a] 1% sucrose 100 mM sodium chloride 25 mM L-arginine hydrochloride 25 mM sodium phosphate pH 6 1–6 5
				Autoinjector	Liquid	Same as Liquid in PFS
Remicade®	nfliximab	1998	V	Vial/stopper	Lyophilized	100 mg/10 mL vial 500 mg sucrose 0 5 mg polysorbate 80 2 2 mg monobasic sodium phosphate monohydrate 6 1 mg dibasic sodium phosphate dihydrate pH 7 2
Simulect®	Basiliximab	1998	V	Vial/stopper	Lyophilized	10 mg/2 5 mL vial[a] 3 61 mg monobasic potassium phosphate 0 5 mg disodium hydrogen phosphate (anhydrous) 0 80 mg sodium chloride 10 mg sucrose 40 mg mannitol 20 mg glycine
Synagis®	Palivizumab	1998	M	Vial/stopper	Liquid	50 mg/0 5 mL vial[a] 1 9 mg histidine 0 06 mg glycine 0 2 mg chloride pH 6 0
Herceptin®	Trastuzumab	1998	V	Vial/stopper	Lyophilized	440 mg/20 mL vial 400 mg α α-trehalose dihydrate 9 9 mg L-histidine HCl 6 4 mg L-histidine 1 8 mg polysorbate 20 pH 6 0
Zenapax®	Daclizumab	1999	V	Vial/stopper	Liquid	25 mg/5 mL vial 3 6 mg sodium phosphate monobasic monohydrate 11 mg sodium phosphate dibasic heptahydrate 4 6 mg sodium chloride 0 2 mg polysorbate 80 pH 6 9
Mylotarg®	Gemtuzumab ozogamicin (calicheamicin)	2000	V	Vial/stopper	Lyophilized	5 mg/5 mL vial dextran 40 sucrose sodium chloride monobasic and dibasic sodium phosphate[b]
Campath®	Alemtuzumab	2001	V	Vial/stopper	Liquid	30 mg/1 mL vial 8 0 mg sodium chloride 1 44 mg dibasic sodium phosphate 0 2 mg potassium chloride 0 2 mg monobasic sodium phosphate 0 1 mg polysorbate 80 0 0187 mg disodium edetate dihydrate pH 6 8–7 4

Humira® (United Stataes) Trudexa (European Union)	Adalimumab	2002	SC	PFS	Liquid	40 mg/0 8 mL in PFS 4 93 mg sodium chloride 0 69 mg monobasic sodium phosphate dihydrate 1 22 mg dibasic sodium phosphate dihydrate 0 24 mg sodium citrate 1 04 mg citric acid monohydrate 9 6 mg mannitol 0 8 mg polysorbate 80 pH 5 2
Zevalin®	britumomab tiuxetan (^{90}Y)	2002	V	Vial/stopper	Liquid	3 2 mg/2 mL vial 0 9% NaCl 50 mM sodium acetate
Amevive®	Alefacept	2003	V/ M	Vial/stopper	Lyophilized	7 5 mg/0 5 mL vial[a] 12 5 mg sucrose 5 mg glycine 3 6 mg sodium citrate dihydrate 0 06 mg citric acid monohydrate pH 6 9
Bexxar®	Tositumomab (gG and gG-^{131})	2003	V	Vial/stopper	Liquid	35 mg/2 5 mL vial[a] 10% maltose 145 mM sodium chloride 10 mM phosphate pH 7 2
Xolair®	Omalizumab	2003	SC	Via/stopper	Lyophilized	202 5 mg/1 4 mL vial 145 5 mg sucrose 2 8 mg L-histidine hydrochloride monohydrate 1 8 mg L-histidine 0 5 mg polysorbate 20
Raptiva®	Efalizumab	2003	SC	Vial/stopper	Lyophilized	150 mg/1 3 mL vial 123 2 mg sucrose 6 8 mg L-histidine hydrochloride monohydrate 4 3 mg L-histidine 3 mg polysorbate 20 pH 6 2
Avastin®	Bevacizumab	2004	V	Vial/stopper	Liquid	100 mg/4 mL vial[a] 240 mg α α-trehalose dihydrate 23 3 mg sodium phosphate (monobasic monohydrate) 4 8 mg sodium phosphate (dibasic anhydrous) 1 6 mg polysorbate 20 pH 6 2
Erbitux®	Cetuximab	2004	V	Vial/stopper	Liquid	100 mg/50 mL vial[a] 8 48 mg/mL sodium chloride 1 88 mg/mL sodium phosphate dibasic heptahydrate 0 41 mg/mL sodium phosphate monobasic monohydrate pH 7 0–7 4
Tysabri®	Natalizumab	2004	V	Vial/stopper	Liquid	300 mg/15 mL vial 123 mg sodium chloride 17 0 mg sodium phosphate monobasic monohydrate 7 24 mg sodium phosphate dibasic heptahydrate 3 0 mg polysorbate 80 pH 6 1
Vectibix™	Panitumumab	2005	V	Vial/stopper	Liquid	100 mg/50 mL vial[a] 29 mg sodium chloride 34 mg sodium acetate pH 5 6–6 0
Orencia®	Abatacept	2005	V	Vial/stopper	Lyophilized	250 mg/10 mL vial 500 mg maltose 17 2 mg monobasic sodium phosphate 14 6 mg sodium chloride pH 7 2–7 8
Lucentis®	Ranibizumab	2006	ntravitreal	Vial/stopper	Liquid	0 5 mg/0 05 mL vial 10 mM histidine HCl 10% α α-trehalose dihydrate 0 01% polysorbate 20 pH 5 5

(*continued*)

Table 6 Formulations for Antibodies Fc Fusion and Fab Conjugates (in order of approval year) (*continued*)

Trade name	Nonproprietary name	Approval year	Dosing route	Presentation	Dosage form	Formulation
Soliris®	Eculizumab	2007	V	Vial/stopper	Liquid	300 mg/30 mL vial 13 8 mg sodium phosphate monobasic 53 4 mg sodium phosphate dibasic 263 1 mg sodium chloride 6 6 mg polysorbate 80 pH 7 0
Cimzia®	Certolizumab pegol	2008	SC	Vial/stopper	Lyophilized	200 mg/1 mL vial 100 mg sucrose 0 9 mg lactic acid 0 1 mg polysorbate pH 5 2
Arcalyst®	Rilonacept	2008	SC	Vial/stopper	Lyophilized	160 mg/2 3 mL vial histidine arginine polyethylene glycol 3350 sucrose glycine pH 6 2–6 8[b]
Nplate®	Romiplostim	2008	SC	Vial/stopper	Lyophilized	250 μg/0 72 mL vial[a] 30 mg mannitol 15 mg sucrose 1 2 mg L-histidine 0 03 mg polysorbate 20 sufficient HCL to bring pH to 5 0
Simponi®	Golimumab	2009	SC	PFS	Liquid	50 mg/0 5 mL in PFS 50 mg golimumab antibody 0 44 mg L-histidine and L-histidine monohydrochloride monohydrate 20 5 mg sorbitol 0 08 mg polysorbate 80 pH 5 5
Ilaris®	Canakinumab	2009	SC	Vial/stopper	Lyophilized	180 mg/1 mL vial sucrose L-histidine L-histidine HCL monohydrate polysorbate 80[b]

[a] ndicates a product that has multiple dose strengths but only the lowest strength is listed
[b]Quantitative formulation is not disclosed not consistent with 21 CFR 201 100 (b) (5) iii
Abbreviations SC subcutaneous M intramuscular V intravenous

around pH 6 to 7, the pH at which most monoclonal antibodies are formulated. Polysorbate 80 or 20 is also present in many monoclonal antibody formulations.

CONCLUSION

In this book chapter we have attempted to provide an overview of formulation development for peptide- and protein-based therapeutics. For successful formulation development, it is important first of all to understand and characterize the unique characteristics of the protein or peptide, including molecular composition, structures, size, charge profile (pI), solubility, thermal transition midpoint, and key degradation pathways. Preformulation activities are also critical in identifying the key instability issues and potential pharmaceutically relevant sources responsible for specific degradation pathways. This chapter provides general principles and examples of major pharmaceutical development activities, including evaluation of critical formulation parameters, selection of container closure, development of the manufacturing process, and stability studies to support shelf-life and clinical use conditions. The results from these development activities are generally required in completing the pharmaceutical development sections of regulatory filings. Finally, trends in the evolution of formulation development since the early 1980s are described on the basis of several examples, including human growth hormone and monoclonal antibodies.

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10 | Development of ophthalmic formulations

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INTRODUCTION

Ophthalmic formulations are those that are intended for treating conditions of the eye; they may be intended to alleviate the signs or symptoms associated with a certain disease state, to provide relief from minor discomfort and irritation, or for treatment of the cause of a disease of the eye itself. In general, the best way to treat ophthalmic diseases is with a local treatment such as a topically administered eye drop. However, the biological design of the eye is optimized to keep the surface of the eye clear of all foreign substances and to provide a substantial barrier to transport of materials into or out of the eye. As a result, ophthalmic drug delivery presents a significant technical challenge. The ophthalmic formulator, therefore, must begin with a good understanding of the physiology of the eye and understand what ophthalmic drug delivery possibilities are available. The formulator must also understand the nature of the drug substance that needs to be delivered to the eye and its limitations. The ultimate job of the ophthalmic formulator is to discover the best way to bring the drug and the eye together in a fashion that will provide the optimal benefit to the patient.

This chapter will focus on the anatomy and physiology of the eye and the challenges in drug delivery to this organ. The goal will be to familiarize the reader with the biopharmaceutical aspects of drug delivery to the eye, the various strategies for targeting different tissues within the eye, and to provide a guide to a rational approach to formulation development for ophthalmic drug delivery. We will also provide a brief overview of ophthalmic formulation preservation, manufacturing and packaging, and regulatory pathways for bringing a formulation to market. Finally, the chapter will discuss some recent advances in drug delivery and the future of ophthalmic drug delivery.

STRUCTURE AND FLUID COMPOSITION OF THE EYE

The eye globe is continually cleansed and hydrated by the secretions of the nasolacrimal system. The eye globe can be divided into three concentric tunics: the fibrous tunic comprising the cornea and sclera, the vascular tunic (consisting of the iris, ciliary body, and choroid), and the retinal tunic. Other internal components of the eye include the lens, aqueous humor, and vitreous humor. Figure 1 illustrates the relative locations of these tissues in the eye. When a drug is delivered to the outer surface of the eye, it may need to diffuse through many of these tissues before it can reach the target tissue. The following is a very basic review of the main components of the eye relevant to the drug delivery:

Nasolacrimal System

The nasolacrimal system consists of three parts: the secretory system (lacrimal glands, meibomian glands, and goblet cells), the distributive system (eyelid movements and blinking), and the excretory system (lacrimal puncta; superior, inferior and common canaliculi; lacrimal sac; and nasolacrimal duct). The nasolacrimal system plays a major role in protecting and hydrating the eye surface. It also has significant impact on the amount of drug absorbed to the eye from topical administration.

Tear Fluid Secretion and Volume

Tears are continuously secreted by the lacrimal glands and the goblet cells. The normal (basal) secretion rate is about 1.2 mcL/min (1), however, under reflex tearing the secretion rate may increase to as high as 300 to 400 mcL/min (2). The normal volume of tear fluid on the eye is about 6 to 7 mcL. When additional fluid is added to the eye surface, the eye can hold about 25 mcL of fluid but will appear "watery" because of the added liquid. With greater additions of fluid, the excess tear fluid will immediately overflow at the lacrimal lake (1) or be splashed into the eyelashes by reflex blinking (3).

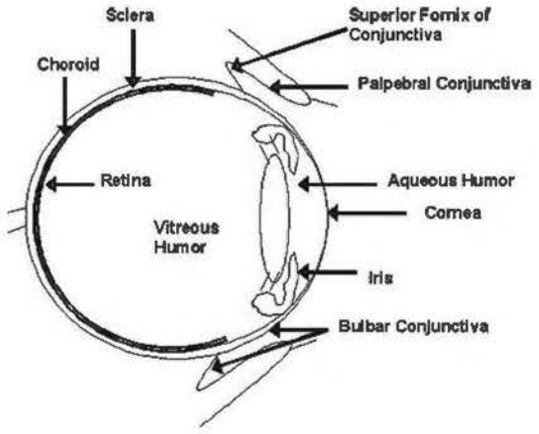

Figure 1 Schematic cross section of human eye.

Tear Fluid Lipid Content

The lipid layer of the tear film is secreted by the meibomian glands. The lipid layer of the tear film serves many functions including reducing evaporation from the ocular surface, lubricating the ocular surface, controlling the surface energy of the tear film, and providing a barrier function at the lid margin to inhibit the flow of skin lipids into the eye and tear fluid out of the eye (4,5). The meibomian secretions are primarily wax esters and sterol esters (about 59%), phospholipids (about 15%), and the remainder is diglycerides, triglycerides, free fatty acids, free sterols, and hydrocarbons (4,5). The polar lipids (phospholipids) are primarily phosphatidylcholine (40%) and phosphatidylethanolamine (18%) (6). The meibomian secretions are produced at a rate of about 400 mcg/hr and are excreted onto the lid margin and the anterior surface of the tear film by the normal blinking action. The thickness of the oil film on the tear fluid has been measured by various interference techniques giving values of 32 to 80 nm in thickness (5). From this thickness, the steady state amount of oil present on the surface of the tear fluid is calculated to be about 9 mcg per eye (5).

Tear Fluid Proteins and Enzymes

Tear fluid contains proteins in high concentration (about 8 μg/μL). Major components include lysozyme (an antibacterial enzyme), lactoferrin (which sequesters iron), secretory immunoglobin A (an antibody important for mucosal immunity), serum albumin, lipocalin and lipophilin (7). In addition, over 400 other proteins have been identified that serve various roles in the tear fluid (7).

Tear Fluid Mucus Layer

The mucus layer is secreted onto the eye surface by the goblet cells. Mucus consists of glycoproteins, proteins, lipids, electrolytes, enzymes, mucopolysaccharides, and water. The primary component of mucus is mucin, a high-molecular-weight glycoprotein that is negatively charged at physiological pH. The mucus layer forms a gel layer with viscoelastic properties which protects and lubricates the eye. The mucus gel traps bacteria, cell debris, and foreign bodies. The mucus layer may hinder drug delivery by forming a diffusional barrier to macromolecules, but it may also bind other substances (i.e., cationic or mucoadhesive) and prolong residence on the surface of the eye.

Tear Fluid pH and Buffer Capacity

The pH of normal tear fluid is 7.4 ± 0.2 (8). The primary buffering components present in the tear fluid are bicarbonate and proteins (8,9). The buffering capacity of the tear fluid is not symmetric around the mean pH. Rather, the tear fluid has more than twice the buffer capacity to resist drops in pH than it has to resist increases in pH (9). As a result of this asymmetric

buffering capacity, unbuffered solutions in the pH 4.0 to 8.5 range will cause minimal shift of the pH on the surface of the eye and will be easily neutralized by the tear fluid (9). Solutions with higher buffer capacities, particularly if greater than that of the tear fluid, may be uncomfortable to the eye if they result in a significant shift of the tear fluid pH.

Tear Fluid Osmolality

The tear fluid osmolality normally ranges from about 300 to 320 mOsm (3,8) and most of the osmolality in the tear fluid can be attributed to the salt content of the lacrimal fluid which is primarily sodium chloride, sodium bicarbonate, potassium chloride, calcium chloride, and magnesium chloride (3). Normally, there is about five times more sodium than potassium in the tear fluid and the levels of calcium and magnesium are less than 1/200th of the sodium levels (3). Higher than normal osmolality in the tear fluid is often seen in patients with dry eye syndrome. Abnormally high evaporation of tear fluid increases the salt levels and results in higher osmolality. As a result, many products for treatment of dry eye are often formulated with lower than normal osmolality.

Tear Fluid Viscosity and Surface Tension

The viscosity of the tear fluid would be expected to be primarily controlled by the higher molecular weight proteins dissolved in the lacrimal fluid. The viscosity of human tears has seldom been determined because of the difficulty of collecting enough sample for a determination. Schuller, et al., (10) found the viscosity of human tears ranges from 1.3 to 5.9 cps with a mean value of 2.9 cps. The viscosity of ophthalmic solutions may be increased in an effort to improve retention on the ocular surface. Hung, et al., (11) estimated that a painful sensation would be elicited if the tear fluid viscosity is increased above 300 cps at the shear conditions of the closing eyelid (shear rate of 20 000/sec). The surface tension of the tear fluid depends on the presence of soluble mucins, lipocalins, and lipids. The mean surface tension value is about 44 mN/min (12).

Fibrous Tunic

Cornea

The cornea is a transparent structure responsible for the refraction of light entering the eye. It forms the anterior one-sixth of the eyeball. The cornea is thinnest at its center (0.5 0.6 mm) and thicker in the periphery (1.2 mm) (13). The cornea is an avascular tissue that is supplied with oxygen and nutrients via the lacrimal fluid, aqueous humor, and the blood vessels at the cornea/sclera junction. The cornea is composed of five layers.

1. The epithelium a stratified squamous epithelium made of 5 layers of cells (10 layers at the corneoscleral junction, i.e., the limbus) that has total thickness of around 50 to 100 μm. At the limbus, the corneal epithelium is continuous with the bulbar conjunctiva. The epithelial cells are connected through tight junctions which limit drug permeability significantly.
2. Bowman's membrane lies between the basement membrane of the epithelium and the stroma, and is composed of acellular interwoven collagen fibers.
3. The stroma accounts for 90% of the cornea thickness and is mainly composed of water and collagenous lamellae that gives the strength and structure for this layer and yet allows the penetration of light. Generally, it does not significantly limit drug permeability.
4. The Descemet's membrane composed of collagen fibers, it lies between the stroma and the endothelium.
5. The endothelium composed of a single layer of flattened cells that are connected via tight junctions. It controls the hydration of the cornea by limiting access of water from the aqueous humor and by active transport mechanisms.

The cornea provides a limited surface area of about 1 cm^2 for drug diffusion, and is a significant barrier to both hydrophilic and lipophilic compounds. Lipophilic molecules will

diffuse more easily through the epithelium and the endothelium, but hydrophilic molecules will diffuse more easily through the highly aqueous stroma.

Conjunctiva

The conjunctiva is a thin mucus secreting membrane that lines the posterior layer of the eyelids (palpebral conjunctiva), the anterior sclera (bulbar conjunctiva), and the superior and inferior conjunctival fornices (joining areas between the palpebral and bulbar conjunctiva). The conjunctiva is composed of two layers: an outer epithelium layer (which is continuous with the corneal epithelium) and an underlying stroma layer.

The conjunctival epithelium is made of 5 to 15 layers of stratified epithelial cells that are connected at the apical side with tight junctions and it plays a major role in limiting drug penetration (14). Nevertheless, the human conjunctiva is 2 to 30 times more permeable to drugs than the cornea (15).

The stroma layer of the conjunctiva contains the nerves, lymphatics, and blood vessels and it attaches loosely to the sclera. The conjunctiva contributes to the tear film formation by secreting electrolytes, fluid, and mucin (14).

Sclera

The sclera covers five-sixths of the eyeball surface and has a mean surface area of 16.3 cm^2 (16). It connects to the cornea anteriorly at the limbus. The sclera is mainly composed of collagen fibers with varying sizes and orientation that are embedded in a glycosaminoglycan matrix. Scleral thickness varies by location; the mean thickness is 0.53 mm near the limbus, is 0.39 mm near the equator, and is about 0.9 to 1.0 mm near the optic nerve (17). The sclera is composed of three main components.

1. Episclera the outermost layer made of loosely arranged collagen fibers that is connected to the eyeball sheath (Tenon's capsule).
2. Stroma composed of larger collagen fibers and elastic tissue.
3. Lamina fusca the innermost layer of sclera that forms the uveal tract with the choroids. It is composed of loosely coherent collagen bundles and melanocytes.

Aqueous Humor

Aqueous humor is a clear fluid that is secreted by the ciliary body via the filtration of blood passing the through the ciliary body capillaries. It has several functions including maintaining the shape of the eye by controlling its pressure, providing nutrition to the cornea and lens, and providing transport of waste materials away from surrounding tissues. The aqueous humor is composed mainly of water, high concentrations of ascorbic acid, glucose, amino acids, and limited levels of proteins. Aqueous humor flows from the ciliary body in the posterior chamber (behind the iris) into the anterior chamber (between the iris and the cornea). Aqueous humor flows out of the eye through the trabecular meshwork (a network of collagen fibers and endothelial-like trabecular cells) into Schlemm's canal, and through the uveoscleral route (18). The entire volume of the aqueous humor is about 0.2 mL and is replaced every one to two hours (13). Maintaining the intraocular pressure (IOP) of 10 to 20 mmHg is a balancing act of production and drainage.

Vitreous Humor

Vitreous humor is a gel-like material that occupies the space between the lens and the retina. The vitreous humor is composed mainly of water (98 99.7%), collagen fibrils and hyaluronic acid (19). It supports the posterior surface of the lens and helps keep the neural part of the retina in place. The normal aging process can lead to liquefaction of the vitreous (>50% by age 80 90) and posterior vitreous detachment. The close proximity of the vitreous to the retina and choroid makes this cavity a direct place for drug delivery to the posterior tissues.

Vascular Tunic

Blood Retina Barrier

The blood-retina barrier is composed of two parts which regulate the transport to the retina: the outer retina barrier formed by retinal pigment epithelium (RPE) and the inner retina barrier formed by the endothelial cells of the retinal vessels.

Two vascular beds supply the retina. Retinal vessels supply the inner two thirds, while the outer retina is avascular and receives oxygen and nutrients from the choriocapillaris. The choriocapillaris is fenestrated to enhance nutrients transport to the underlying retina. Plasma leaks from the choriocapillaris and diffuses through the Bruch's membrane and through the RPE to the outer retina. RPE tight junctions constitute the outer blood-retinal barrier.

Retinal vessels are supplied by the central retinal vessel. Retinal capillaries are composed of a single layer of endothelial cells surrounded by a basement membrane and pericytes. The endothelial cells are attached to each other by tight junctions forming the inner blood-retina barrier. These narrow tight junctions, similar to those present in the brain vessels, impair the paracellular transport of hydrophilic compounds and necessitate their passage through the intracellular routes (20).

Choroid

The choroid is a highly vascularized tissue between the retina and the sclera. It consists of: the vessel layer, the choriocapillaris, and Bruch's membrane (which is in direct contact with the RPE). Between the sclera and the choroid there is the suprachoroidal, or perichoroidal, space. This is a very thin space consisting of various connective tissue lamellae and is characterized as sponge tissue. Substantial amounts of the aqueous humor that leaves the eye via the uveoscleral route ends up in the suprachoroidal space and is finally drained out from the eye through porosites in the sclera.

The choriocapillaris is found in the inner portion just below the RPE and it provides nutrition to the RPE and the outer one third of the retina. Between the RPE and the choriocapillaris is the Bruch's membrane. Bruch's membrane is composed of five layers: the basement membrane of the RPE, an inner collagenous layer, the elastic layer, the outer collagenous zone, and the basement membrane of the capillary endothelial cells.

Retinal Tunic

The retina is composed of neural retina and RPE. The inner surface of the neural retinal is facing the vitreous humor while the outer border is next to the RPE. The neural retinal is composed of nine layers containing the nerve fibers and the photoreceptors responsible for light detection. The RPE is composed of a single layer of cells connected by tight junctions.

Biopharmaceutics and Routes of Administration

Drug penetration into the eye is a challenging task and can follow different pathways to reach the ocular target tissues depending on the route of administration and the drug's physicochemical properties. The target tissue within the eye is different for each drug and indication. In general, the focus of drug delivery to the eye can be divided into delivery to the anterior segment of the eye and to the posterior segment of the eye.

Delivery to the Anterior Segment of the Eye (Topical)

The anterior segment includes the conjunctiva, the cornea, the anterior sclera, the iris, the ciliary body, and the aqueous humor. Topical administration of drugs is considered the most common and acceptable route of administration for these target tissues. Drugs applied topically as an eye drop of a solution or suspension or as an ointment are easy to administer and noninvasive. However, drug penetration via this route is inefficient bioavailability is generally less than 5% of the administered dose. Accordingly, the majority of the dose will end up in the systemic circulation and may have systemic effects (21,22). In certain conditions, particularly when sustained prolonged drug release is preferred, a subconjunctival injection or implant may be used to target these tissues. Drug penetration to the anterior tissues from topical administration faces significant barriers that limit its ocular bioavailability (Fig. 2).

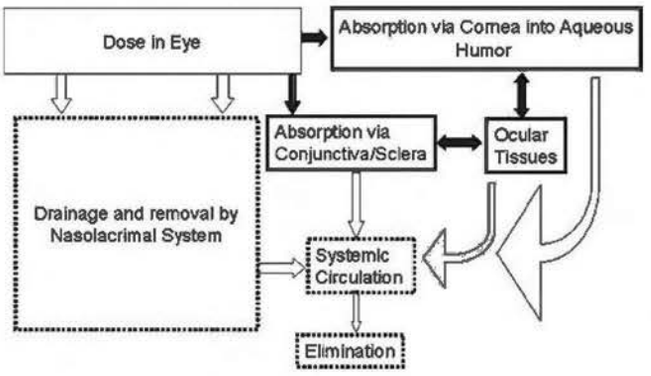

Figure 2 Schematic representation of compartmental drug penetration and elimination from topical administration.

Nasolacrimal Drainage

One of the most important attributes of a good ophthalmic topical formulation is that it needs to remain on the surface of the eye long enough to deliver a therapeutic amount of the medication. This necessarily means that the formulation needs to mix with or replace a portion of the natural tear fluid and should be as comfortable on the surface of the eye as the natural tear fluid. If the formulation evokes discomfort on the eye in any way (pH, osmolality, viscosity), it will lead to reflex tearing and blinking as the body attempts to flush the offending agent from the surface of the eye. Therefore the first step to understanding how to formulate an ophthalmic formulation is to understand the characteristics of the tear film (discussed above) that should be appropriately mimicked by the formulation. In addition, the formulation should be designed so that it does not adversely interact with the components in the natural tear fluid.

Most of the topical dose is lost through the nasolacrimal drainage before it can reach the eye. The limited volume that the eye surface can accommodate (30 μL), the high tear turnover rate (0.5 2.2 μL/min), and blinking rate are all natural ways of the eye to protect itself and limit penetration through its surface. The introduction of an eye drop (average volume of 39 μL), and possibly its composition will induce more tear secretion and increased blinking that will enhance the drainage out of the eye surface and reduce amount of drug available for absorption (23).

Corneal Absorption

The cornea offers the major site of drug diffusing into the anterior chamber of the eye, especially for small molecules. Drug penetration through the cornea can be by passive diffusion or by active transport mechanisms. The two main factors influencing the passive diffusion are lipophilicity and molecular size. Small lipophilic compounds generally penetrate through the epithelium via the intracellular route, while small hydrophilic compounds are limited to the paracellular route (partitioning of small lipophilic compounds into the cornea causes it to act as a depot). Large hydrophilic compounds (5000 Da) are generally excluded by the epithelium tight junctions (24). The fraction of a lipophilic compound penetrating through the cornea is 20 times more than a hydrophilic molecule of similar molecular size (25). A logD value of 2 to 3 for β-blockers was reported to provide optimal corneal permeation (26). Molecular size is also an important factor for small hydrophilic and lipophilic compounds. Increasing the molecular size from 0.35 nm to 0.95 nm reduces the permeability through the cornea, and conjunctiva significantly (25).

Active transport in the cornea can carry drug molecules from the eye surface into the aqueous humor and vice versa. However, saturation of the active transporter is possible and may limit the significance of this route during the limited residence time of the formulation on the surface of the eye. A prodrug approach targeting certain transporters in the cornea to enhance the permeation of acyclovir has been recently reported (27,28). Mannermaa, et al., (29) has provided a detailed review on the emerging role of transporters in ocular delivery.

Conjunctival and Scleral Absorption

Permeation through the conjunctival epithelium is limited by the tight junctions. However, the pore size of 5.5 nm in the conjunctiva allows larger molecular weights up to 38 600 Da to passively diffuse (30). The high surface area of diffusion of the conjunctiva compared with the cornea (17:1) contributes the importance of this route especially for hydrophilic compounds and large molecules (31). Compounds penetrating through the conjunctiva can continue the penetration into the eye through the sclera. Scleral permeation does not depend on the compound lipophilicity, but depends on the molecular radius (32). The presence of blood vessels in the conjunctiva can act as a sink condition that limits drug penetration to the sclera, carrying drug instead to the systemic circulation. As with the cornea, active transporters in the conjunctiva have been reported and reviewed (33).

Elimination from the Anterior Segment of the Eye

Drug molecules reach the aqueous humor though the corneal route or the iris/ciliary body through the conjunctiva/sclera route can be cleared through the aqueous humor drainage and through the blood vessels penetrating the eye to the systemic circulation.

Delivery to the Posterior Segment of the Eye

Posterior drug delivery may target the retina, choroids, and vitreous humor. Targeting the posterior tissues of the eye has gained significant interest in recent years with the advent of new agents for treatment of age-related macular degeneration and diabetic retinopathy.

Several routes can be used to direct drug molecules to the posterior tissue (Fig. 3). The following is a summary of these administration and possible penetration routes:

Topical

Several compounds have been reported to reach the posterior segment of the eye from topical administration (34,35). As with the delivery to the anterior segment of the eye, there are two main pathways for drugs to reach the posterior segment of the eye: the corneal route, and the conjunctival/sclera route. Once the drug molecules reach the anterior segment tissue it can penetrate to the rest of the ocular tissues via several routes as explained in Figure 4. Penetration through the lens into the vitreous is limited and generally observed with lipophilic compounds (36). Alternatively drug molecules can diffuse against the aqueous humor outflow to into the vitreous, or through the uveoscleral route. Drug penetration through the conjunctiva/sclera route is believed to be most significant in reaching the retina and choroids. Drugs reaching the sclera can diffuse laterally around the orbit and into the choroids and retina. Systemic recirculation plays a role in reintroducing the drug molecules lost to the systemic circulation back to the ocular tissue as observed with the effect of topical β-blockers on the contralateral eye (37).

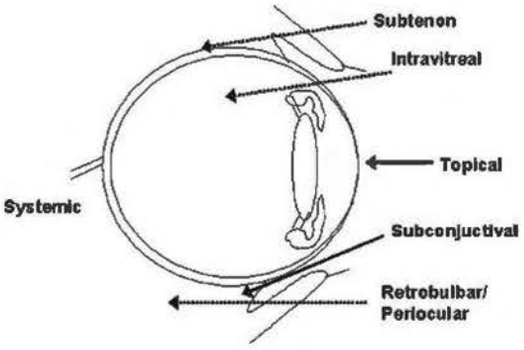

Figure 3 Different routes of ocular administration.

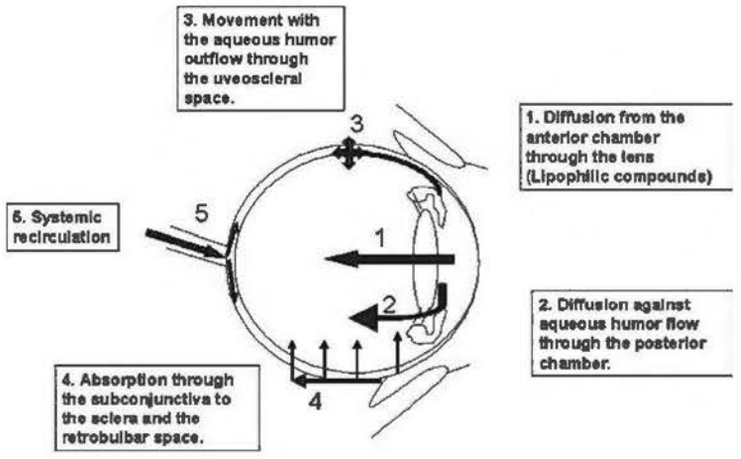

Figure 4 Schematic representation of drug penetration pathways to the posterior segment of the eye.

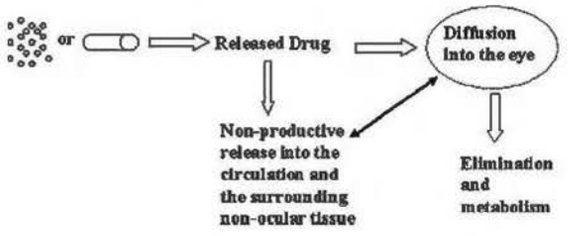

Figure 5 Schematic representation of drug release and diffusion into the eye from controlled delivery system (microsphere or implant) after periocular administration.

Subconjunctival/Transscleral Delivery

This is an important and promising route of delivering compounds to the back of the eye. It includes subconjunctival, peribulbar, retrobulbar, and subtenon injections. In all these injections the major permeability and loss to the systemic circulation limitations through the conjunctiva is avoided. Additionally, the drug has more time to diffuse through the sclera to the choroids and retina than that with topical administration. Scleral permeability, as discussed before, is not affected by lipophilicity of the compound but with the molecular radius. Large molecules up to 70 kDa are still able to penetrate the sclera (38). The large surface area of the sclera offers great potential for both small and large molecules to diffuse into the choroids, retina, and vitreous. In the periocular delivery, drug release from various delivery system and elimination can be depicted as in Figure 5. Once drug molecules diffuse through the sclera, it has to diffuse through the suprachoroidal space to the choriocapillaris, and then through Bruch's membrane to the RPE (outer retina-blood-barrier). The major limitation of drug to diffuse to the retina is the RPE. The majority of drug dissolved or released will be lost to nonocular tissue and eventually to the systemic circulation. Minimal loss to the choroidal circulation is expected (39). The ability of the nanoparticles to penetrate through the sclera-choroid-retina has been recently reported to have nonsignificant transport across these tissues with the majority of the nanoparticles being lost to periocular circulation and lymphatics (40). Differences between the various injections (subconjunctival, peribulbar, retrobulbar, and subtenon) exist with regard to penetration into posterior tissues (41). More drug is available in the vitreous and subretinal fluid when given as a subconjunctival injection compared with peribulbar injection (42,43). This can be due to the close proximity to the eye in case of subconjunctival injection. Subtenon injection also utilized for the delivery of active compounds behind the macula for effective delivery to the choroids and retina. The advantage of this

injection is the potential ability of Tenon's capsule to capture the delivery system (suspension, microspheres, or nanoparticles) in place where drug release/dissolution will continue for extended period of time. The transscleral route is most promising and less invasive route when compared with intravitreal delivery, especially with the advancement in the controlled release delivery systems.

Intravitreal Injection/Implant

Delivery through the intravitreal route is the most direct way to the retina. When delivered via this route, drug molecules only need to the diffuse through the vitreous to reach the retina, and through the RPE to reach the choroids. The low systemic exposure with intravitreal injection is a major advantage for this route. However, repeated injections of medication are often required which may lead to increased risk of endophthalmitis, damage to the lens, and retinal detachment. Intravitreal injections are typically administered in the inferotemporal quadrant, approximately 4 mm from the limbus (44). Controlled release formulations and implants can be used to decrease the frequency of administration required. Drug elimination and distribution from intravitreal delivery is controlled by the position of an intravitreal injection, and the lipophilicity and molecular size of the drug (45). Disposition from the vitreous humor can be through retinal absorption (retina/choroid/sclera) or via the posterior chamber (annular gap between lens and the ciliary body) then through the aqueous humor. Compounds with high lipophilicity are believed to be cleared via the retina pathway, while small hydrophilic and macromolecules are cleared anteriorly through the aqueous humor flow. Clearance and localization of polymeric nanoparticles after intravitreal injection was reported to depend on the size of the particles (46,47).

Ophthalmic Indications and Diseases

Table 1 lists several examples of marketed ophthalmic formulations used to target disease conditions in both the anterior and posterior tissues. While it is beyond the scope of this chapter to provide a comprehensive listing of ophthalmic diseases and indications, we will briefly discuss the most common indications.

Anti-infective Agents

There are many drugs available to treat bacterial, viral, and fungal infections of the eye. The antibiotic drugs that are available are generally broad spectrum. Ophthalmic formulations in this category are in the form of ointments and suspensions in addition to conventional solution eye drops. Many of the products are combinations of drugs and the relative efficacy of the formulations is judged on the frequency of instillation and duration of treatment.

Broadly the following are the major types of ocular infections that are treated by antibiotics or a combination of antibiotics and anti-inflammatory agents (48):

> Conjunctivitis (viral, bacterial, neonatal); episcleritis; keratitis (viral, bacterial, keratitis due to light exposure); uveitis (anterior, intermediate, posterior, and retinitis); hordeolum and chalazion; dacryocystitis; and periorbital and orbital cellulitis.

During the day, patients are usually treated using eye drops (sometimes up to several times a day) and at night they may be additionally directed to use an ophthalmic ointment (e.g., in the case of severe infections and blepharitis).

Anti-inflammatory Agents

Inflammation is the manifestation of vascular and cellular response of the host tissue to injury. Injury to the tissue may be inflicted by physical or chemical agents, invasion of pathogens, ischemia, and excessive (hypersensitivity) or inappropriate (autoimmunity) operation of immune mechanisms. In ocular tissues, inflammatory reactions are mediated by arachidonic acid cascade products formed via the cyclooxygenase pathway. There are two types of anti-inflammatory agents: corticosteroids and nonsteroidal anti-inflammatory drugs (NSAIDs). Both corticosteroids and NSAIDs may be administered orally as well as topically, but topical

Table 1 Examples of Marketed Ophthalmic Formulations for Treatment of Anterior and Posterior ndications

ndication	Active ingredient	Class/mechanism	Formulation type	Product example
Anterior drug delivery examples				
Acute infection—blepharitis	Bacitracin	Miscellaneous antibiotic	Ointment	Ciloxan (Alcon)
Acute infection—conjunctivitis	Tobramycin	Aminoglycoside	Suspension	Tobrex (Alcon)
Acute infection—conjunctivitis	Azithromycin	Macrolide	Mucoadhesive solution	AzaSite (nspire Pharma)
Acute infection—keratitis	Ofloxacin	Quinolone	Solution	Ocuflox (Allergan)
Acute pain/inflammation	Prednisolone acetate	Corticosteroid	Suspension	Pred-Forte (Allergan)
Acute pain/inflammation	Diclofenac sodium	NSA D	Solution	Voltaren (Novartis)
Acute pain/inflammation	Flurbiprofen sodium	NSA D	Solution	Ocufen (Allergan)
Acute pain/inflammation	Loteprednol etabonate	Soft steroid	Suspension	Lotemax (Bausch & Lomb)
Allergy OTC	Ketotifen fumarate	Antihistamine	Solution	Alaway (Bausch and Lomb)
Allergy OTC	Naphazoline HCl pheniramine maleate	Decongestant/vasoconstrictor	Solution	Opcon-A (Bausch & Lomb)
Allergy Rx	Azelastine HCl	Antihistamine	Solution	Optivar (MedPointe)
Allergy Rx	Olopatadine HCl	Antihistamine	Solution	Pataday (Alcon)
Allergy Rx	Loteprednol etabonate	Soft steroid	Suspension	Alrex (Bausch & Lomb)
Dry eye OTC	PEG400 propylene glycol	Aqueous tear-fluid replacement/ stabilizer	n situ gelling solution	Systane (Alcon)
Dry eye OTC	Glycerin propylene glycol	Aqueous tear-fluid replacement/ stabilizer	Mucoadhesive solution	Soothe (Bausch & Lomb)
Dry eye OTC	Light mineral oil mineral oil	Ocular lipid replacement	Emulsion	Soothe XP (Bausch & Lomb)
Dry eye Rx	Cyclosporine	mmunomodulator/anti-inflammatory	Emulsion	Restasis (Allergan)
Glaucoma	Brimonidine tartrate	α2 adrenergic agonist	Mucoadhesive solution	Alphagan P (Allergan)
Glaucoma	Betaxolol HCl	β-blocker β1	Mucoadhesive complexed solution	Betoptic S (Alcon)
Glaucoma	Timolol maleate	β-blocker β1 and β2	n situ gelling solution	Timoptic XE (Merck)
Glaucoma	Dorzolamide HCl	Carbonic anhydrase inhibitor	Solution	Trusopt (Bausch & Lomb)
Glaucoma	Latanoprost	Prostaglandin	Solution	Xalatan (Pfizer)
Posterior drug delivery examples				
"Wet" AMD	Ranibizumab injection	Monoclonal antibody fragment/VEGF inhibitor	Solution intravitreal injection	Lucentis (Genetech)
Wet AMD	Pegaptanib sodium	Oligonucleaotide/VEGF inhibitor	Solution intravitreal injection	Macugen (Pfizer)
Chronic uveitis	Fluocinolone acetonide	Corticosteroid	mplant	Retisert (Bausch & Lomb)
Cytomegalovirus retinitis	Ganciclovir	Antiviral	mplant	Vitrasert (Bausch & Lomb)

Abbreviations NSA D nonsteroidal anti-inflammatory drug VEGF vascular endothelial growth factor

administration is the preferred route for management of ocular inflammation as it provides high ocular drug concentrations and reduces the systemic side effects.

Corticosteroids work by blocking the enzyme phospholipase A2 to inhibit arachidonic acid production, thereby preventing the synthesis and release of prostoglandins, thromboxanes, and eicosanoids. Some concerning side effects of corticosteroid treatment are an increase in IOP, suppression of the immune system response to pathogens, slowed wound healing, and formation of cataracts. Steroids have been used extensively before and post surgery as a result of their broad effects and are generally more potent than NSAIDs for treatment of severe inflammation. Recently, soft steroids have been introduced in an effort to maintain the potent efficacy of the cortiosteroids while reducing the undesirable side effects. One of these soft steroids is loteprednol etabonate, which has less effect on IOP because of its rapid metabolic deactivation. Most of the steroids have low aqueous solubility and, hence, most are formulated as suspensions or emulsions.

NSAIDs exert their anti-inflammatory action by inhibiting the cyclooxygenase enzymes (viz., COX-1, COX-2, and COX-3). NSAIDs are commonly used to treat postoperative inflammation, in the prevention and treatment of cystoid macular edema and for relief in allergic conjunctivitis. Treatment with NSAIDs is preferred because of the lower occurrence of side effects associated with steroidal drugs; however, most of the NSAIDs for ophthalmic use are weakly acidic compounds (49) which have a tendency to lower the pH of the formulations making the formulations somewhat irritating. In addition, many of them have poor water solubility; thus, they are often used in the form of their more soluble salt forms (e.g., sodium, potassium, tromethamine, or lysine salts) or are formulated with solubilizers like surfactants or cyclodextrins.

Antiallergy Agents

Ocular allergic disorders include seasonal allergic conjunctivitis (SAC), perennial allergic conjunctivitis (PAC), vernal keratoconjunctivitis (VKC), giant papillary conjunctivitis (GPC) and atopic keratoconjunctivitis (AKC). The treatment of acute and more chronic forms of allergic conjunctivitis has been mainly focused on symptomatic relief of symptoms such as, redness, itching, and burning. They are primarily antihistamines, that is, H1 blockers. In some cases the allergic condition may require the use of topical corticosteroids as well. Some of the antiallergy eye drops are available OTC, but many are still only available as prescription medications.

Dry Eye Treatments

Dry-eye syndrome results from problems originating in the nasolacrimal system resulting in inadequate quantity and quality of tears or ocular surface abnormalities. Signs and symptoms of dry eye include itchiness, redness, foreign body sensation, and grittiness. Most treatments alleviate the signs and symptoms of dry eye rather than treating the cause. Most OTC dry-eye treatments are designed to replace and stabilize the aqueous portion of the tear film. A couple of OTC treatments are also available to replace the lipid portion of the tear film which may be inadequate to prevent evaporation of the aqueous portion of the tear film. A pharmaceutical approach to treating dry eye may involve the use hormones (or analogs) to increase the lipid production of the meibomian glands or to treat inflammation of the lacrimal glands to increase the secretion of the lacrimal glands (50). Regardless of the type of treatment, most dry-eye products are often dosed several times a day and therefore need to be mild and contain little or no preservatives or preservatives that are nonirritating.

Antiglaucoma Agents

Glaucoma is a sight-threatening optic neuropathy. The disease is characterized by increased IOP, excavation of the optic nerve head, reduction in the number of retinal ganglion cells, and a resultant progressive loss of visual field. Elevated IOP is a major risk factor and available antiglaucoma drugs treat this facet of the disease. The most common form of the disease is open-angle glaucoma in which IOP rises as a result of decreased outflow of aqueous humor through the trabecular meshwork and Schlemm's canal. Antiglaucoma drugs may act by decreasing aqueous humor production or increasing aqueous humor outflow (via the

trabecular meshwork or the uveoscleral pathway) (51). Drugs that affect aqueous humor production include β2-adrenergic receptor agonists, β1-adrenergic receptor agonists, α2 adrenergic receptor agonists and carbonic anhydrase inhibitors. The newest category of drugs used in the treatment of glaucoma is the prostaglandin analogs which affect aqueous humor outflow (52,53). Most of these products need to be dosed once or twice daily. The prostaglandin analogs however, have certain side effects associated with them namely, iris hyperpigmentation and change in the length, color and thickness of eyelashes, hyperemia and pruritis.

Posterior Indications

"Wet" age-related macular degeneration is a condition where blood vessels behind the retina start to grow and leak blood and fluid. This causes damage to the macula (the center of the retina) and results in central vision loss that can occur quickly. The medications available to treat wet AMD work by inhibiting the action of vascular endothelial growth factors (VEGF). Delivering these actives to the retina involves an intravitreal injection every 6 to 12 weeks.

Intravitreal implants are available for the treatment of posterior diseases as well and offer the ability to reduce the dosing frequency for these posterior treatments to once per year. However, there are two significant issues with intravitreal implants. Firstly, current intravitreal implants require a surgical procedure that is more invasive than an intravitreal injection. Secondly, a formulation that delivers drug over the course of a year requires a much longer (and, hence, more costly) clinical trial. Therefore, the use of intravitreal implants is limited.

Formulations for Ophthalmic Delivery

The following section discusses the various components and factors to be considered in the development of the different types of formulations for ophthalmic medications.

Excipients for Use in Ophthalmic Formulations

A suitable ophthalmic formulation must include excipients to control the osmolality, pH, and stability of the formulation. Control of the formulation stability includes chemical, physical, and antimicrobial stability. In addition, some excipients may be added to a formulation to enhance the drug delivery of the formulation by modifying the solubility of the active ingredient or increasing the retention of the active ingredient on the surface of the eye. When a formulator begins to design a new formulation for an active ingredient, they must be aware of the additional development effort or risk that might be associated with the use of novel formulation ingredients. In some cases, the risk of using novel ingredients will be warranted in an effort to gain patent protection or overcome difficult drug delivery issues. In other cases, the risk may not be warranted as it could lead to longer and more costly development programs. In either case, the formulator should always begin their formulation development efforts by selecting ingredients from those that have previously been used in ophthalmic formulations. In the U.S. market, the Food and Drug Administration (FDA) inactive ingredients database provides a convenient listing of these materials. A listing of these ophthalmic excipients and their potential use is provided in Table 2. No such database is currently available from the other primary regulatory agencies.

Solutions

An ophthalmic solution formulation is always the first choice if a drug substance has suitable aqueous solubility and stability in the range from pH 5 to 8. A good example of the simplest approach to a topical solution formulation is the marketed latanoprost formulation. The formulation is a neutral pH, phosphate-buffered saline preserved with 200 ppm benzalkonium chloride (BAK). This formulation example demonstrates that even the simplest formulation should provide control of pH and osmolality, and provide antimicrobial stability.

The osmolality and pH of the formulation should always be matched as closely as possible to that of the tear fluid; however, significant ranges for both of these variables have been found to be acceptable in practice. The eye is better able to tolerate pH excursions on the acidic side rather than on the basic side, hence, the range of acceptable pH values is skewed more to the acidic side of the mean tear fluid pH. There are many topical formulations in the pH 5.5 to 7.5 range, and a few that go as low as 4.0 and as high as 8.0. This asymmetry of the

Table 2 Excipients Listed in Food and Drug Administration Inactive Ingredients Database

Category of excipient	Ingredient name	Use level in database	Compendial listing(s)
Wetting and solubilizing Agents/emulsifying agents	Benzalkonium chloride	2%	NF, PhEur, JP
	Benzethonium chloride		USP, PhEur, JP
	Benzododecinium bromide	0.01%	
	Carbomer 1342	0.05%	NF, PhEur, JPE
	Cetyl alcohol	0.5%	NF, PhEur, JP
	Cholesterol		NF, PhEur, JP
	Cocamidopropyl betaine	0.002%	
	Glyceryl monostearate	0.5%	NF, PhEur, JP
	Lanolin alcohols	10%	NF, JPE
	Lauralkonium chloride	0.005%	
	N lauroylsarcosine	0.03%	
	Nonoxynol 9	0.12%	USP, JPE
	Octoxynol 40	0.01%	JPE
	Poloxamer 188	0.1%	NF, PhEur, JPE
	Poloxamer 407	0.2%	NF, PhEur, JPE
	Polyoxyl 35 castor oil	5%	NF, PhEur, JPE
	Polyoxyl 40 hydrogenated castor oil	0.5%	NF, PhEur, JPE
	Polyoxyl 40 stearate	7%	NF, PhEur, JP
	Polysorbate 20	0.05%	NF, PhEur, JPE
	Polysorbate 60	15%	NF, PhEur, JPE
	Polysorbate 80	4%	NF, PhEur, JP
	Sorbitan monolaurate		NF, PhEur, JPE
	Tyloxapol	0.3%	USP
Suspending and/or viscosity increasing agents	Carbomer 1342	0.05%	NF
	Carbomer 934P	0.45%	NF, JPE
	Carbomer 940	4%	NF, JPE
	Carbomer 974P	0.5%	NF, PhEur, JPE
	Carboxymethylcellulose sodium	0.5%	NF, PhEur, JP
	Gellan gum	0.6%	NF
	Hydroxyethyl cellulose	0.5%	NF, PhEur, JPE
	Hypromellose 2906	0.5%	USP, PhEur, JP
	Hypromellose 2910	0.5%	USP, PhEur, JP
	Methylcellulose	0.5%	USP, PhEur, JP
	Polycarbophil	0.9%	USP
	Polyethylene glycol 8000	2%	NF, PhEur
	Polyvinyl alcohol	1.4%	USP, PhEur, JPE
	Povidone K30	2%	USP, PhEur, JP
	Povidone K90	1.2%	USP, PhEur, JP
	Xanthan gum	0.6%	NF, PhEur, JPE
Acidfying agents/alkalizing agents (pH adjustment)	Acetic acid	0.2%	NF, PhEur, JP
	Ammonium hydroxide		
	Citric acid	0.2%	USP, PhEur, JP
	Diethanolamine		NF, JPE
	Hydrochloric acid	1.06%	NF, PhEur, JP
	Nitric acid		NF, PhEur
	Phosphoric acid		NF, PhEur, JPE
	Sulfuric acid	0.02%	NF, JPE
		1.1%	NF, PhEur, JP
		1%	NF, PhEur, JP
		0.1%	NF, PhEur, JP
	Sodium borate		
	Sodium bisulfate		
	Sodium carbonate		
	Sodium hydroxide		
Buffering agents (pH control)	Acetic acid	0.2%	NF, PhEur, JP
	Boric acid	37.2%	NF, PhEur, JP
	Citric acid	0.2%	USP, PhEur, JP

(*Continued*)

Table 2 Excipients Listed in Food and Drug Administration Inactive Ingredients Database (*Continued*)

Category of excipient	Ingredient name	Use level in database	Compendial listing(s)
	Phosphoric acid		NF, PhEur, JPE
	Potassium acetate	4%	USP, PhEur, JPE
	Potassium phosphate, monobasic	0.44%	NF
	Potassium sorbate	0.47%	NF, PhEur, JPE
	Sodium acetate	1.27%	USP, PhEur, JP
	Sodium borate	1.1%	NF, PhEur, JP
	Sodium carbonate	1%	NF, PhEur, JP
	Sodium citrate	2.2%	USP, PhEur, JP
	Sodium phosphate, dibasic	1.4%	USP, PhEur, JP
	Sodium phosphate, monobasic	0.78%	USP, PhEur, JPE
	Sorbic acid	0.2%	NF, PhEur, JPE
	Tromethamine	0.93%	USP
Humectants/tonicity agents/salts (ionic strength and osmolality control)	Calcium chloride	0.04%	USP, PhEur, JP
	Glycerin	2.6%	USP, PhEur, JP
	Magnesium chloride	0.03%	USP, PhEur
	Mannitol	23%	USP, PhEur, JP
	Polyethylene glycol 300		NF, PhEur, JPE
	Polyethylene glycol 400	4.99%	NF, PhEur, JP
	Potassium chloride	22.2%	USP, PhEur, JP
	Propylene glycol	10%	USP, PhEur, JP
	Sodium chloride	55%	USP, PhEur, JP
	Sodium nitrate	1.18%	
	Sodium sulfate	1.2%	USP, PhEur, JPE
	Sorbitol	40%	NF, PhEur, JP
Ointment base	Lanolin	3%	USP, JP
	Light mineral oil		NF, PhEur, JP
	Mineral oil	59.5%	USP, PhEur, JP
	Petrolatum	85%	USP, PhEur, JP
	Petrolatum, white	89%	USP, PhEur, JP
Antioxidants/chelating agents/sequestering agents (chemical stability control)	Citric acid	0.2%	USP, PhEur, JP
	Creatinine	0.5%	NF, JPE
	Divinylbenzene styrene copolymer	0.75%	USP, JP
	Edetate sodium	10%	USP, PhEur, JP
	Sodium bisulfite	0.1%	JP
	Sodium citrate	2.2%	USP, PhEur, JP
	Sodium metabisulfite	0.25%	NF, PhEur
	Sodium sulfite	0.2%	NF, PhEur, JPE
	Sodium thiosulfate	5%	USP, PhEur, JP
	Tocophersolan (Vit E TPGS)	0.5%	NF
Antimicrobial Preservatives	*Quaternary ammonium compounds*		
	Benzalkonium chloride	8.8%	NF, PhEur, JP
			USP, PhEur, JP
		0.01%	
	Benzethonium chloride	0.0005%	
	Benzododecinium bromide		
	Polyquaternium 1	0.2%	NF, PhEur, JP
		37.2%	NF, PhEur, JP
		4%	USP, PhEur, JPE
		0.47%	NF, PhEur, JPE
		1.27%	USP, PhEur, JP
		1.1%	NF, PhEur, JP
		0.2%	NF, PhEur, JPE
		0.65%	NF, PhEur, JP
		0.5%	USP, JPE
		0.0008%	NF, PhEur
		0.002%	NF, PhEur
		1%	USP, PhEur
		0.05%	NF, JP
		0.01%	NF, JP

(*Continued*)

Table 2 Excipients Listed in Food and Drug Administration Inactive Ingredients Database (*Continued*)

Category of excipient	Ingredient name	Use level in database	Compendial listing(s)
		0.005%	
		0.0025%	USP, PhEur, JP
	Acid/base compounds		
	Acetic acid		
	Boric acid		
	Potassium acetate		
	Potassium sorbate		
	Sodium acetate		
	Sodium borate		
	Sorbic acid		
	Alcohols		
	Chlorobutanol		
	Phenylethyl alcohol		
	Organic mercurial compounds		
	Phenylmercuric acetate		
	Phenylmercuric nitrate		
	Thimerosal		
	Parabens		
	Methylparaben		
	Propylparaben		
	Oxidizing agents		
	Sodium chlorite		
	Metal salts		
	Zinc chloride		

Note: Compendial listings for ingredients are also noted.

acceptable pH range is partially due to the buffering capacity of the tear fluid, but is also a result of the fact that excursions to high pH can result in saponification of lipids in the tissues and immediately compromise the barrier properties of the tissues (2). to minimize the discomfort caused by a formulation, at the extreme low or high end of this pH range, it is advisable to minimize the buffer capacity of a formulation. For example, a 50 mM pH 5 acetate buffer would be more easily neutralized by the tear fluid than a 50 mM pH 5 citrate, but a 5 mM pH 5 acetate buffer would be even better if it could provide sufficient pH stability for the formulation. For osmolality, reflex tearing is generally not seen in the range of 200 to 400 mOsm/Kg (2,3), but some studies suggest that hypotonic formulations may be better tolerated than hypertonic formulations and have been shown to enhance drug delivery in some instances (3). The osmolality of the formulation may be controlled equally well using electrolytes or nonelectrolytes and a comfortable formulation may be designed using either. In general, it may be preferable to rely on nonelectrolytes for osmolality control and target osmolalities slightly less than that of the tear fluid to avoid aggravating the hyperosmotic conditions that afflict the population of people with evaporative dry eye.

Additional formulation components that may be beneficial for solution formulations include surfactants and viscosity increasing agents. Even if not required for its solubilization, the addition of a small amount of surfactant may help with the wetting and spreading characteristics of the formulation on the surface of the eye. The addition of a viscoelastic polymer to the formulation can also be beneficial in prolonging the retention of the formulation on the surface of the eye. Increasing the viscosity of a solution formulation to 12 to 15 cps was shown to provide optimal benefit higher viscosities show diminishing improvements in slowing the drainage rate (54).

Ointments

After solution formulations, the next most complicated formulation to design and manufacture is an ointment. Ointment formulations are generally suspensions of drugs in a base of mineral

oil and petrolatum. These formulations are generally suspensions because mineral oil and petrolatum are not good solvents for most drug compounds. The ointment base of petrolatum softens at body temperature and melts between 38°C to 60°C. The melting and softening behavior of the petrolatum base may be modified by the addition of mineral oil or light mineral oil. Ophthalmic ointments typically contain from 40% to 90% petrolatum with 60% to 10% mineral oil. The simplest ointment formulation will have only the active ingredient suspended in this mineral oil/petrolatum base. The active ingredient will need to be suitably controlled with respect to its particle size; hence, the drug substance is typically micronized before addition to the ointment base. The mineral oil/petrolatum base is not supportive of microbial growth (i.e., due to the low water activity), and does not affect the pH or osmolality of the eye, hence, additional excipients are not required.

This simple petrolatum and mineral oil base is the best option for drug substances that are hydrolytically unstable, but the drug delivery from such an ointment may suffer because of the fact that the ointment base is not readily miscible with the tear fluid. To improve the drug delivery characteristics of an ointment, a formulation may include a water-in-oil surfactant such as lanolin. This creates what is termed an absorption ointment base and is intended to improve the drug absorption from the ointment as well as improve the ability to incorporate hydrophilic drugs. The absorption ointment base is more likely to require the addition of a preservative such as chlorobutanol or parabens.

Suspensions

Suspensions are a necessary formulation option for cases where the aqueous solubility of the drug substance is extremely low or when the stability of the drug substance is significantly enhanced by keeping it as a suspended particle rather than dissolved (e.g., to reduce hydrolysis). Suspension formulations have the additional concerns of particle size distribution, sedimentation and resuspendability, and content uniformity of the delivered formulation.

Particle Size Distribution

The particle size distribution in an ophthalmic suspension must be controlled to assure the comfort of the formulation on the surface of the eye as well as assure that the drug delivery characteristics are consistent. Particle size of the active agent also plays a key role in physical stability of the drug product. The rate of sedimentation, agglomeration and resuspendability are affected by particle size. Table 3 lists the current compendial requirements for ophthalmic suspensions.

The most common method for controlling the particle size is mechanical comminution of previously formed larger, crystalline particles (e.g., by grinding with a mortar-pestle, air-jet micronization, or wet-milling with ceramic beads). Another method is the production of small particles using a controlled association process (e.g., spray drying, precipitation from supercritical fluid, or controlled crystallization). The process used to obtain the desired particle size distribution may have significant effects on the properties of the drug product. For example, comminution methods may generate heat that can create amorphous regions or polymorphic changes in the active pharmaceutical ingredient (API) particles which can, in turn, affect dissolution and drug delivery characteristics. In addition, if a change is made from one comminution method to another during the course of development, the API behavior may change significantly. For example, jet-air micronization can result in triboelectrification

Table 3 Summary of Compendial Requirements for Suspension Particles Size Distributions

USP	"It is imperative that such suspensions contain the drug in a micronized form to prevent irritation and/or scratching of the cornea. Ophthalmic suspensions should never be dispensed if there is evidence of caking or aggregation."
EP	NMT 20 particles > 25 μm/10 mcg solid NMT 2 particles > 50 μm/10 mcg solid No particles > 90 μm/10 mcg solid
JP	No particles > 75 μm

(i.e., charging) of the API particles. This charging of the particles may impact the aggregation and processing requirements of the formulation. Because is not practical to fully optimize the method for controlling the particle size independent of the formulation, a formulator must keep in mind how the process may change during the planned development and scale-up activities and be prepared for those necessary changes. The ultimate goal is to develop the formulation and particle size control method that will be used for manufacturing the marketed drug product and, therefore, the earlier this compatibility can be tested and verified, the better.

When formulation research is started, the formulation scientist typically has very little API available for evaluation of particle size methods. Some simple, small-scale experiments may help indicate what particle size control methods are viable options. For example, grinding a small amount of drug substance with a mortar-pestle to evaluate how easily a material can be ground (brittleness) and evaluation of the crystallinity of the drug substance before and after grinding may indicate if comminution methods are viable. Likewise, small-scale experiments with dissolving and precipitating the drug may indicate if a controlled precipitation process will produce a suitable crystalline particle.

Physical Stability (Sedimentation and Resuspendability)

It is important to understand that suspensions are kinetically stable but thermodynamically unstable systems. When left undisturbed for a long period of time the suspension particles will aggregate, sediment, and eventually cake. When a suspension is very well dispersed (i.e., deflocculated), the particles will settle as small individual particles. This settling will be very slow and will result in a low-volume, high-density sediment that may be difficult or impossible to redisperse. When the particles are held together in a loose open structure, the system is said to be in the state of flocculation. The flocculated particles will settle rapidly and form a large-volume, low-density sediment that is readily dispersible. Relative properties of flocculated and deflocculated particles in suspension are provided in Table 4.

The flocculation state of a suspension product is primarily controlled by the nature of the surface of the suspended particles. The surface charge (i.e., zeta potential) of the particle may be adjusted to move between a flocculated and deflocculated state. Also, adsorption of surface active polymers or surfactants can stabilize suspensions by preventing the removal of water from between the particles. A textbook example (55) illustrates how to modify the zeta potential of a suspension to switch between a deflocculated and a flocculated state. First, the adsorption of a cationic surfactant (e.g., BAK) to the surface of a suspended particle provides charge-charge repulsion resulting in a deflocculated suspension. Then, an oppositely charged flocculating agent (e.g., phosphate) is added at increasing levels to shield these surface charges and reduce the zeta potential close to zero, at which point flocculation is observed. A list of the formulation factors that can be adjusted to affect the physical stability of a suspension formulation includes the following:

- Flocculation/deflocculation: (*i*) add charged surface active polymer or surfactant, (*ii*) add an oppositely charged flocculation agent, (*iii*) add a nonionic surface active

Table 4 Relative Property of Flocculated and Deflocculated Particles in Suspension

Deflocculated	Flocculated
Little to no aggregation. Particles are present as primary particles.	Particles form loose aggregates (flocculants).
Sedimentation is slow.	Sedimentation is fast.
Sedimentation volume is small as particles may pack more efficiently.	Sedimentation volume is typically large.
Sediment may become a hard cake that is difficult or impossible to redisperse. Resuspendability is typically poor.	A dense cake does not form. The sediment is easy to redisperse, so as to reform the original suspension. Resuspendability is typically excellent.

polymer or surfactant, (*iv*) adjust the ionic strength of vehicle, and (*v*) if drug has a pKa, adjust pH to modify the surface charge.

- Sedimentation rate: (*i*) increase the viscosity of the vehicle, (*ii*) decrease the particle size of the drug, and (*iii*) develop a structured vehicle, which does not settle.

Content Uniformity in Delivery from the Selected Container/Closure

Another difference between a suspension formulation and a solution formulation is that when a suspension drop is delivered from the controlled-tip dropper bottle, it is not guaranteed to be uniform. Several factors, which may affect the uniformity of the drop delivered to the patient's eye, include compatibility between the formulation and the package, resuspendability of the formulation in the selected package, and the patient's ability to properly resuspend the formulation within the selected package. Typically, patients are not willing to vigorously shake a bottle of suspension for more than a few seconds. In addition, the resuspendability of a suspension formulation may be significantly affected by the material of the container (e.g., polyethylene vs. glass). It is advisable that careful, early evaluation of the resuspendability of suspension formulations be performed under simulated use conditions in the selected container/closure system to identify and fix physical stability issues as early as possible. An evaluation like this should indicate that a drop delivered from the selected package will have the appropriate potency (e.g., 90 110% of label) when delivered according to the label instructions.

Emulsions

Although emulsion formulations are not very novel and have been used extensively in topical (dermatological) and oral delivery routes, there are currently only two marketed formulations for ophthalmic use [Restasis® (cyclosporine emulsion in castor oil) and Durezol® (difluprednate emulsion in castor oil)]. The potential advantages of emulsions for ophthalmic drug delivery include being able to provide a greater driving force for drug delivery of low solubility compounds and being able to eliminate many of the quality control issues associated with suspended drug particles. The disadvantages of the emulsion formulations are that they have proven to be difficult formulations to preserve and difficult to manufacture under sterile conditions. These disadvantages are being overcome and there will undoubtedly be many new ophthalmic emulsions brought to the market over the next several years.

The emulsion formulation has an aqueous continuous phase that must comply with the same requirements as the solution formulations discussed above. In addition to the aqueous continuous phase, the emulsion formulation contains an oil (lipid) phase, which is dispersed in the continuous phase with suitable emulsifiers. The oil phase for an emulsion should be selected to provide adequate solubilization of the drug substance. The oil-in-water emulsifiers may include surfactants (e.g., polysorbate 80 or polyoxyl 35 castor oil), Carbomer 1342, or both.

The difficulty in preserving an emulsion formulation is evident from the fact that most of the antimicrobial preservatives readily available for ophthalmic use are incompatible with some aspect of the emulsion formulation. The emulsion formulation generally contains either high levels of surfactants or Carbomer 1342. High levels of surfactant can deactivate parabens, BAK, alcohols, and organic acids. Carbomer 1342 is an anionic polymer that may interact strongly with the quaternary amines. In addition, any surface active or lipophilic preservatives may partition into the oil phase and become unavailable for preservation of the aqueous phase. In the Restasis formulation, the preservative problem was solved by designing a preservative-free, single-dose formulation. For the Durezol formulation, the use of a combination of three water-soluble antimicrobial acids (sorbic, acetic, boric) provided sufficient preservative efficacy.

Enhanced Drug Delivery Systems

After topical administration, typically less than 5% of the applied drug penetrates the cornea and reaches intraocular tissues. The primary problem for topical delivery of ophthalmic drugs is the rapid and extensive precorneal loss caused by drainage and high tear fluid turnover. A major portion of the formulation efforts have been aimed at maximizing ocular drug absorption through prolongation of the drug residence time in the cornea and the conjunctival sac. Improved ocular residence of liquid formulations has been accomplished through the use

of viscosity-increasing and mucoadhesive agents, *in situ* gelation of the formulation, and use of charge-charge interactions between cationic components in the formulation and the anionic surface of the eye. Even greater residence improvements can be made by using polymeric inserts for drug delivery. Enhanced drug delivery from these formulations may allow the treatment of posterior indications with topical administration. In addition, various polymers may be used to produce prolonged-delivery systems which allow less frequent injections for posterior treatment.

High Viscosity Liquid Formulations

A high-viscosity formulation can improve the retention of a drug substance on the surface of the eye, however, if the viscosity is too high under the shear conditions of the closing eyelid (about 20,000/sec) it may cause discomfort and reflex tearing (56). Many commonly used viscosity-increasing agents result in Newtonian viscoelastic behavior so that the viscosity increases similarly at both low and high shear. Polymers that thicken this way include hydroxypropylmethyl cellulose (HPMC), hydroxyethylcellulose (HEC), polyvinylpyrrolidone (PVP), and polyvinyl alcohol. Patton, et al., (54) found that increasing the viscosity to about 12 to 15 cps using either HPMC or PVA resulted in significant improvement in ocular retention whereas further increases in viscosity resulted in only small improvements. Other polymers may be used to produce non-Newtonian viscoelastic fluids that are either shear thinning or thixotropic. Polymers resulting in shear-thinning behavior include Carbomers and sodium carboxymethylcellulose. Polymers that shear thin more dramatically and can be considered thixotropic include polycarbophil and xanthan gum. In the AzaSite® (polycarbophil suspension of azithromycin) formulation, the polycarbophil creates a low-shear viscosity of over 2000 cps, but the formulation is still well tolerated in the eye because the viscosity of the formulation during the eye blink is much less (i.e., less than 300 cps).

Mucoadhesive Liquid Formulations

Mucoadhesion refers to the tendency of a polymer to specifically bind with the mucins of mucus membranes and lead to enhanced retention or viscosity as a result of the polymer-polymer interactions. The mucoadhesive performance of the ophthalmically-used polymers can be qualitatively ranked as follows (12): carbomers, polycarbophil > hyaluronan > carboxymethylcellulose sodium > sodium alginate > poloxamers, HPMC, methylcellulose, PVA, PVP.

Examples of formulations taking advantage of the ability of mucoadhesion to enhance the retention of a formulation include the Pilopine HS® (Carbomer 940 gel of pilocarpine HCl), and Alphagan® P (NaCMC solution of brimonidine tartrate).

In Situ Gelling Liquid Formulations

In situ gelation can be induced on the surface of the eye because of the change in pH, temperature, or ionic strength that occurs after the formulation is administered and mixes with the tear fluid. The change in pH can be used to induce *in situ* gelation between borates and polyol-containing polymers. The OTC dry-eye treatment Systane® takes advantage of the gelation between borates and HP-Guar as the pH is increased after administration. *In situ* thermal gelation with poloxamers has also been investigated, but is not currently applied in any marketed products. The gelation induced by interaction with the salt content of the tear fluid is used by the Timoptic-XE® (timolol maleate solution in gellan gum) product.

Cationic Liquid Formulations

Because the surface of the eye is generally anionic, the application of cationic drugs or drug delivery systems should interact electrostatically with the mucins on surface of the eye and lead to enhanced retention. Some formulations demonstrating this approach include cationic nanoparticles, cationic emulsions, and formulations using of cationic suspending or mucoadhesive agents. Nanoparticles may enhance delivery of poorly water-soluble drugs, but without improved retention on the eye nanoparticles are unlikely to result in delivery

superior to a solution. Preparation of cationic nanoparticles can be accomplished using either cationic Eudragit® polymers, chitosan polymers, or by incorporating cationic surfactants into solid-lipid nanoparticles (57). Chitosan polymers and cationic cellulosic polymers (e.g., polyquaternium-10) have also been used as cationic suspending agents and have been shown to provide good mucoadhesion properties (12). Cationic emulsions have been prepared by incorporating cationic surfactants at the solid-liquid interface of the emulsion to enhance drug delivery (58).

Prolonged Delivery Polymeric Systems

Topical eye drop administration is mainly suitable for treatment of ocular conditions in the anterior segment of the eye. Targeting the posterior segment of the eye presents a far greater challenge and represents an area of unmet medical needs. Many of the newer drugs aimed at treating conditions such as diabetic retinopathy and age-related macular degeneration are administered via repeated intravitreal injections. Alternative approaches that would improve patient acceptance such as biodegradable inserts or micro- and nanoparticulate delivery systems present a growing field in the area of ophthalmic drug delivery.

Controlled release of drugs can be obtained by encapsulating the drug in micro-(1 10,000 μm) or nano(1 1000 nm)particles. These are usually given as intravitreal injections. They can provide sustained delivery over few weeks up to several months (59). However, the intravitreal injections of these particulates can cause vitreal clouding. Microparticles tend to sink to the lower part of the vitreal cavity, while nanoparticles are more susceptible to cause clouding in the vitreous (59).

Biodegradable and biocompatible polymers such as polylactide and PLGA [poly-(lactic-co-glycolic acid)] (both approved by the FDA) are typically used. In these materials, the drug is released by bulk erosion of the matrix following cleavage of the polymeric chains via autocatalytic acid/base and/or enzymatic hydrolysis; the products lactic and glycolic acids, are metabolized to carbon dioxide and water. Low molecular weight polymers tend to degrade rapidly; copolymers such as PLGA degrade faster than the corresponding homopolymers. Some microsphere formulations have shown promise in preclinical studies but have yet to undergo clinical trials. A microsphere formulation of PKC412 (protein kinase C inhibitor + receptors for VEGF) was administered via pericoular injection to treat choroid neovascularization. The studies showed a significant suppression of neovascularization using this delivery system.

Poly(anhydrides) and poly-(ortho-ester)s are also promising polymers for drug delivery; their release properties are regulated mainly by surface erosion rather than diffusion (60). Poly (orthoester)s have shown excellent ocular biocompatibility and have been used to demonstrate the sustained release of 5-fluorouracil (61).

Nanoparticulates are of importance since colloidal delivery systems are particularly suitable for poorly water-soluble drugs. However, the major impedence to the use of nano-particles has been the availability of a universally acceptable method of making the nanoparticles especially on large scale and the stabilization and sterilization of the formulations. Some nanosytems based on surface-charge segregated particles containing chitosan or polyethylene glycol have been found to be stable and also in overcoming preclinical barriers.

Intraocular implants are usually placed intravitreally, at the pars plana of the eye and therefore, requires minor surgery. However, the use of implants have the benefit of by-passing the blood-ocular barriers to deliver constant therapeutic levels of drug at the site of action, avoidance of repeated administration and use of smaller doses of drugs (62). Implants may be nonbiodegradable or biodegradable depending on the material from which they are fabricated. Biodegradable implants of a poly (DL-lactic-co-glycolic acid) implant containing a novel aldose reductase inhibitor, fabricated with 50% drug loading have been shown to give sustained drug release in vitro and in vivo in rats (63). Nonbiodegradable implants provide more accurate/reproducible dosing lasting over longer periods of time than biodegradable inserts (62). The nonbiodegradable implants however, require surgical removal after completion of therapy. Vitrasert® and Retisert® (Bausch and Lomb) are two clinically used nonbiodegradable implants for the treatment of CMV retinitis (AIDS-related) and chronic uveitis, respectively.

Other implant systems in different phases of clinical trials include Medidur® (Alimera Sciences) for treatment of diabetic macular edema; Surodex® and Posurdex® (Allergan, U.S.A.) containing dexamethasone.

Transporter Mediated Drug Delivery

Transporter-mediated drug delivery involves targeting of drug molecules to the membrane transporters to enable efficient passage across the cell membranes. Various transporters may be utilized to facilitate the passage of drugs across cell membranes (64); these include nutrient transporters for peptides, aminoacids, monocarboxylic acids, folates and organic anion and cation transporters, etc. Various peptide and amino acid transporters have been utilized for retinal drug delivery. Majumdar et. al. have studied the role of various dipeptide prodrugs of gancyclovir to improve its ocular bioavailability after topical administration and found good corneal permeability with a Val-Val dipeptide gancyclovir prodrug (65). The approach of using various transporter mechaninsms in the eye for improved intraocular delivery following topical administration, is interesting and provides newer opportunities for ophthalmic drug delivery.

Intraocular Irrigation Solutions

An ophthalmic irrigation solution is used for the application on the external surface of the eyes topically and in ocular surgeries to rinse, as well as to keep the operated ocular tissues moist. Replacement of the aqueous or vitreous humors with the irrigation solution occurs as the consequence of ocular surgeries including corneal transplant (penetrating keratoplasty), cataract extraction, intraocular lens implantation and vitrectomy. In these instances, the irrigation solution remains in the eyes after surgery until the components are either deprived by the surrounding tissues or the solution is eventually equilibrated with body fluids, with subsequent clearance through the circulation. Thus, it is essential that the irrigation solution used should be physiologically compatible, including tonicity and pH, and desirably should also contain components enabling the cells to sustain their viability and capability to perform physiological functions.

Irrigation solutions used during and after surgery are of particular importance to the cornea and the lens. Both organs are avascular. The cornea obtains its nourishment mainly from the fluid in the anterior chamber, and to a lesser extent, from the tear. The lens obtains its nourishment from fluids, both in the anterior chamber and in the vitreous. The retina, ciliary body and iris are vascularized tissues; they obtain their nourishment through the circulating plasma of the blood vessel network. Therefore, the components of the irrigation solution may not exert an effect on these tissues as significant as that on the cornea and the lens. A proper electrolyte balance as well as addition of certain nutrients such as glucose, amino acids, etc., may add to the beneficial nature of an irrigation solution. Often irrigation solutions are used to simply bathe and soothe the eye and help wash away impurities and contaminants from the environment. There are two intraocular irrigation solutions presently being used in ophthalmic surgeries. These two irrigation solutions are BSS and BSS Plus (both by Alcon Labs Inc.). BSS is a balanced salt solution that incorporates a sodium citrate of a balanced salt solution with a bicarbonate buffering system, with Dextrose added as an additional osmotic agent and energy source. An additional component, oxidized glutathione is reduced by the ocular cells and serves as an antioxidant. In addition, some intraocular irrigation solutions may contain viscoelastic components or viscosity enhancers such as sodium hyaluronate, chondroitin sulfate, hydroxypropylmethyl cellulose, and polyacrylamide. However, the use of these agents may lead to an elevation of IOP (66).

PRESERVATION OF OPHTHALMIC FORMULATIONS

Ophthalmic formulations must not only be sterile products but need to be adequately preserved from microbial contamination once the package is opened. Most ophthalmic products are multidose products packaged in semi-permeable containers. The repeated opening and closing of the containers as well as frequent contact with the ocular surface (e.g., for dropper tips) exposes the contents of the package to a variety of microorganisms from

the external environment. Many of the microorganisms can cause severe reactions (inflammation, itching, pain, loss of visual acuity, etc.) including, in the most severe cases, blindness. The choice of the preservative is dictated by the nature of the formulation itself, whether it is a suspension, solution or gel system. Often the choice of buffer/vehicle composition will also affect preservative efficacy. It is well known that the borate buffer system itself has good antimicrobial properties (67) and can help boost the antimicrobial efficacy of some preservatives. Additionally it is known that high salt concentrations can decrease preservative efficacy. The specific composition of the formulation not only affects the efficacy and stability of the preservative system but, may also alter the tolerability of the preservative system. For example, incorporation of viscosity-increasing agents can increase the irritation potential of a preservative because of increased residence on the eye this has been demonstrated in BAK-containing systems with hydroxyethylcellulose (68). In addition the incorporation of surfactants and polymers that bind the preservative(s) will result in decreased antimicrobial efficacy. The use of preservatives in chronic-use products such as antiglaucoma and dry-eye medications is of concern because of the cumulative toxicity of certain agents on the corneal epithelium (69). Thus, such medications should ideally be preservative-free or contain preservatives that have little to no chance of accumulating in ocular tissues.

Antimicrobial Effectiveness Testing

Antimicrobial effectiveness testing (AET) is used to ensure that a product is adequately protected from microbial contamination during patient use. The AET method is described in the major compendia the USP in chapter <51> (70), the PhEur in chapter 5.1.3, and the JP in chapter <19>. The bacterial challenge organisms used in the AET are *Escherichia coli* (ATCC8739), *Pseudomonas aeruginosa* (ATCC9027) and *Staphylococcus aureus* (ATCC6538), and the yeast/mold challenge organisms are *Candida albicans* (ATCC10231) and *Aspergillus brasiliensis* (ATCC16404). Criteria for the effectiveness of a preservative system are expressed as the percentage of reduction in viable cells in a specific amount of time. At this time, there is not one harmonized criteria that is accepted globally for product preservation. Table 5 summarizes the criteria that are most widely used for antimicrobial preservative testing from the USP, PhEur, and JP. The PhEur criteria are the most stringent among the three and guide the development of globally-acceptable pharmaceutical formulations.

Preservatives Used in Ophthalmic Formulations

There are a wide variety of agents that alone or in combination with each other can act to effectively reduce the chances of contamination of a formulation by microbial growth. The section below addresses some of the more widely accepted ophthalmic preservatives that are used today. Many previously used preservatives such as the organic mercurial compounds (e.g., thimerosal) have seen a decline in use because of evidence of hypersensitivity and ocular toxicity upon long-term use (71,72).

Table 5 Criteria of Acceptance for Antimicrobial Effectiveness Testing (USP Category "1" Products, PhEur Parenteral and Ophthalmic Formulations, and JP Category IA Products)

		Log_{10} reduction				
	Innoculum (CFU/mL)	6 hr	24 hr	7 days	14 days	28 days
USP: bacteria	10^5 10^6			1.0	3.0	No increase[a]
JP: bacteria	10^5 10^6				3	No increase
PhEur A: bacteria	10^5 10^6	2	3			No recovery
PhEur B: bacteria	10^5 10^6		1	3		No increase
USP: yeast/mold	10^5 10^6			No increase	No increase	No increase
JP: yeast/mold	10^5 10^6				No increase	No increase
PhEur A: yeast/mold	10^5 10^6			2		No increase
PhEur B: yeast/mold	10^5 10^6				1	No increase

[a]No increase implies no decrease in the log reduction values for microbial growth from previous time point.

Quaternary Ammonium Compounds

Quaternary ammonium compounds are small, positively-charged molecules. It is believed that they act by perturbing the cell membrane of gram-positive and gram-negative bacteria specifically via intercalating into the lipid bilayers and displacing ions, such as calcium and magnesium, that play a crucial role in stabilization of the bacterial cytoplasmic membrane (73). These agents can interact with the teichoic acid and polysaccharide elements in gram positive bacteria and the lipopolysaccharide element in Gram-negative bacteria. It is believed that chelating agents such as ethylenediamine tetraacetic acid or EDTA and ethylene glycol tetraacetic acid or EGTA (used in concentrations from 0.01 0.1% w/w), further potentiate the antimicrobial effect of these agents. The most commonly used agent in this category is benzalkonium chloride (BAC or BAK) followed by cetyltrimethyl ammonium bromide (cetrimide). These agents are usually used in concentrations from 20 to 200 ppm, have good ocular tolerability profiles, good stability, and a long history of use. They are incompatible with high concentrations of anionic components or surfactants in a formulation. Although widely used, it is generally agreed that there may be concern regarding the cumulative toxicity of these agents when present in chronic use products such as dry-eye medications and antiglaucoma medications. Therefore, there is a growing preference for other, more gentle antimicrobials.

Polyquaternary Ammonium Compounds

Polyquaternium is the International Nomenclature for Cosmetic Ingredients designation for several polycationic polymers that are used in the personal care and pharmaceutical industry. Polyquaternium is a generic term used to emphasize the presence of multiple quaternary ammonium centers in the polymer. INCI has approved at least 37 different polymers under the polyquaternium designation. Because of their large size, they are generally thought to be less permeable across the corneal epithelium and, hence, pose less risk of accumulation in ocular tissues leading to chronic toxicity issues. Their mode of action is similar to the monoquaternary compounds in that they also destabilize the outer membrane of bacteria and cause leakage of intracellular components leading to cell death (73).

Two commonly used polyquaterniums are polyquaternium-1 (PQ-1) and polyquaternium-42 (PQ-42) and the molecular weight of these compounds can go up to several thousand Daltons. Chemically, PQ-1 is ethanol, 2,2′,2″-nitrilotris-, polymer with 1,4-dichloro-2-butene and *N,N,N′,N′*-tetramethyl-2-butene-1,4-diamine; typically, it has an average molecular weight of around 6 kDa. It can be used in concentrations of 1 to 10 ppm and its efficacy against yeast and fungi is improved at higher pHs. Chemically, PQ-42 is [polyoxyethylene(dimethylimino) ethylene-(dimethylimino)ethylene dichloride]. It has been used in ophthalmic formulations such as Freshkote, Dwelle, and Dakrina eye drops and Nutra-tear. It is also used in a lens care solution for rigid gas-permeable (RGP) lenses (Total Care CLS by AMO) at a concentration of 6 ppm by weight. PQ-1 is a more potent antimicrobial agent than PQ-42.

Biguanides and Polymeric Biguanides

Biguanides refer to the class of compounds that are derivatives of imidodicarbonimidic diamide. The most commonly known biguanide is chlorhexidine [1,6-bis(4′-chloro-phenyl-biguanide)hexane; usually used a its digluconate salt] which has a broad spectrum of activity. However, its action is pH dependent and greatly reduced by the presence of organic matter. It can only be used in very low concentrations in ophthalmic formulations because of its irritation potential. Chlorhexidine is believed to exert its action by membrane destabilization leading to the leakage of intracellular components; at high concentrations it can cause protein and nucleic acid precipitation (74). It is generally used at concentrations of 5 to 10 ppm by weight. Because of its weak activity against yeast, fungi, and *Serratia marcescens*, it is usually used in combination with other agents such as EDTA, BAK, etc.

Polymeric biguanides are also available, the most widely used one being polyaminopropyl biguanide or PAPB (also known as polyhexamethylene biguanide or PHMB, or polyhexanide) and is commercially available under the trade names of Cosmocil and Vantocil. PAPB has a broad spectrum of activity and can be used in concentrations as low as 0.5 ppm up

to 5 ppm. Lower concentrations may be used in combination with other antimicrobial agents. PAPB activity is reduced by anionic polymeric agents such as hyaluronic acid, carboxymethylcellulose, alginates, etc., and cellulosic polymers.

Alcohols

Phenylethyl alcohol and chlorobutanol are antimicrobial alcohols. Phenylethyl alcohol (up to 0.5%) is usually used in combination with another preservative but is limited in its application because of its volatility and tendency to permeate through plastic packaging. Chlorobutanol is a commonly used ophthalmic preservative and is generally considered to be quite safe (75). It is mostly used in ophthalmic ointments because it has good solubility in petrolatum. It can be used at concentrations up to 0.5%, but it is unstable at pH > 6, high temperature, susceptible to absorption into packaging components and may be lost through the headspace of semi-permeable packaging because of its volatility.

Parabens

Parabens are esters of p-hydroxybenzoic acid. They have been widely used in pharmaceuticals and as ophthalmic preservatives. They have a well established safety and tolerability profile. The useful concentration is typically limited by the water solubility, and therefore a combination of parabens can be used together to enhance their activity (e.g., 0.05% methylparaben + 0.01% propylparaben). Parabens are effective in the pH range of 4 to 8, but they are more susceptible to hydrolysis at high pH. Parabens may permeate into packaging components, and may be inactivated by high concentrations of surfactants or polymers.

Acids

Antimicrobial acids have a useful pH range around the pKa of the acid and the optimal antimicrobial activity will typically occur very close to this pKa. The most commonly used acid for preservation of ophthalmic formulations is sorbic acid (or potassium sorbate) which has a pKa of 4.76. Sorbic acid is primarily antifungal, but does have antibacterial activity. Sorbic acid is useful in the range of pH 4.5 to 6 and is usually combined with EDTA or other preservatives for broad-spectrum preservation. Sorbic acid is sensitive to oxidation, which results in discoloration of the product, and is more rapidly degraded at temperatures above 38°C. Boric acid is another useful acid for preservation in ophthalmic formulations; however, its activity is classified as bacteriostatic rather than biocidal.

Oxidizing Agents

Oxidizing agents are generally deemed much safer and well tolerated than most other preservatives because the preservatives "disappear" over time and pose little or no chance of accumulation in ocular tissues over repeated use. The two most widely used preservative systems in this category are stabilized hydrogen peroxide systems and hypochlorites. Hydrogen peroxide provides its antimicrobial action via generation of the hydroxyl radical which can readily attack bacterial cell membrane lipids and intracellular DNA (76). Hydrogen peroxide is effective against a wide variety of microorganisms and relatively unaffected by pH. Aside from hydrogen peroxide itself, other peroxide-generating compounds that are useful include sodium perborate, percarbonates and carbamate peroxide. The use of hypochlorites in ophthalmic formulations was introduced in 1996. The stabilized oxychloro complex (SOC) (i.e., Purite) is a hypochlorite preservative consisting of 99.5% chlorite; 0.5% chlorate and a trace amount of chlorine dioxide. The formation of chlorine dioxide in the microbial acidic environments leads to disruption of protein synthesis. However, the components of the preservative system dissipate readily in the eye into components already found in human tears (Na^+, Cl^-, O_2, and H_2O).

CONTACT LENS CARE SOLUTIONS AND REWETTING DROPS

Contact lenses may be rigid gas-permeable lenses (RGP) or soft contact lenses. to properly use contact lenses, they must be kept clean and free from microbial contamination when stored. Contact lens solutions are mainly multipurpose solutions (MPS) that achieve

cleaning, disinfection, and lubrication (for insertion comfort) all in one step. The development of new contact lens multipurpose solution compatible with an increasing array of soft contact lens materials on the market is very challenging. In addition to being able to effectively clean and disinfect the contact lenses, solutions are required to provide patient comfort when the cleaned lens is inserted back into the eye. The products must also maintain their ability to effectively clean and disinfect when stored in unopened containers over a period of 18 months to two years. An even greater challenge is designing a product that is robust enough to counter noncompliance of patients in their contact lens cleaning regimens (77) where compliance requires discarding opened solutions after three months; changing the contact lens cleaning case and never resoaking lens in previously used solution. The market needs are constantly evolving such that there is a continuous need for newer and better products. Biocidal efficacy is tested against five organisms (three bacteria: *Pseudomonas aeruginosa*, *S. aureus*, and *S. marcescens*; one yeast: *C. albicans*; and one fungus: *Fusarium solani*) in the presence of organic soil (required for United States and not for Europe) with a defined (e.g., four hours) exposure time. At least a 3 log reduction in CFUs per mililiter for the bacteria and 1 log reduction in CFUs per mililiter for the yeast and fungi are required (initially and throughout shelf-life) to be considered as passing the biocidal efficacy testing (78).

The key components of MPS are: surfactants or cleaning agents (such as the block copolymers Tetronic 1107 or 1304), lubricating agents (e.g., hyaluronic acid, hydroxypropyl guar, cellulosic polymers), disinfectants (viz., PQ-1, PAPB, SOC, sodium perborate, PQ-42), chelators (e.g., EDTA or hydroxyalkylphosphoates) and other agents that help in moisture retention (e.g., dextran, glycerin). In addition, buffers, electrolytes and stabilizers (e.g., antioxidants) are also included. The ionic strength of the formulations is of particular importance in the formulations because of the significant effect on lens shrinking and swelling. Preservatives can also be taken up by the lenses resulting in changes in lens dimensions.

Contact lens rewetting drops contain a suitable wetting agent (surfactant), an ocular demulcent, a preservative system in a suitable vehicle containing buffers, electrolytes and stabilizers. Rewetting drops help relieve symptoms of ocular discomfort (dryness, foreign body sensation, itching, blurry vision, etc.) in contact lens wearers during use.

MANUFACTURING AND PACKAGING OF OPHTHALMIC FORMULATIONS

Sterile Manufacturing

In 1953, the FDA announced that all ophthalmic products must be manufactured sterile (79). The sterility requirements for ophthalmic formulations first appeared in USP XVIII, third supplement, 1972. In general, ophthalmic formulations are described in the USP as "sterile dosage forms essentially free from foreign particles suitably compounded and packaged for instillation in the eye" (80). The formulations should be terminally sterilized by autoclaving whenever possible. As an alternative to steam sterilization, formulations may be sterilized by sterile filtration through 0.22-μm filters. If neither steam sterilization nor filtration is an option, then aseptic processing of presterilized components is required (81). The requirements and guidance for the compounding of sterile preparations is outlined in detail in USP <797>. In addition to the quality of raw materials and packaging components, and the condition of manufacturing components, a major factor in ensuring the quality of the final product is the environment in which it is manufactured and filled. For ophthalmic formulations, manufacturing must be carried out in an ISO class 5 (previously class 100) environment. There are also high standards that are described with regard to personnel garbing and gloving; personnel training and testing in aseptic manipulations, environmental quality specifications and monitoring and disinfection of gloves and surfaces. Formulation compounding may involve several steps rather than the simplified idea of putting all ingredients into a sterilized mixing vessel and mixing. In many cases the manufacturing may consist of a multistep process where the thermostable portion of the formulation is autoclaved and then the heat sensitive components are added aseptically (through a sterile filter) to the autoclaved portion (after cooling down to acceptable temperatures). When

developing a formulation it is very important to focus on the following general points to establish a manufacturing process that will be scaleable, reproducible, and cost and time efficient.

1. The order of addition of the components in the formulation.
2. The time (and temperature) required for mixing and type of mixing that may be desirable.
3. Possible interaction of formulation components with the manufacturing components, including tubing, filters, filter housing, cleaning agents that may be used to clean manufacturing components, sources of trace metal contamination, etc.
4. Filter choice is of particular importance when dealing with potent drugs and preservatives since these are prone to significant loss because of binding by the filter. In such cases several developmental batches may be necessary to determine the flush and discard volume (prior to beginning of the filling operation) to saturate the filters and minimize losses to the filter. Often a certain overage is included in the formulation to account for losses during manufacturing.

Other things to focus on are formulation specific, that is, different manufacturing requirements for suspensions/emulsions, regular solutions versus viscous formulations (gels), ointments, etc. Below are two examples of formulations requiring special compounding procedures for manufacturing and filling.

Manufacturing Example 1: an Aqueous Solution Sterilized by Filtration
The active ingredient, a lipid-soluble drug substance, was weighed into a glass vial with a calculated overage to compensate for loss to filters and the process surfaces during manufacturing. A cationicpreservative, which also serves as a solubilizer for the drug, was added as a concentrate solution to a glass beaker. The drug was transferred from the glass vial into the glass beaker with rinsing and this drug/preservartive concentrate was mixed thoroughly for a sufficient length of time. In a large stainless steel manufacturing vessel the other formulation ingredients were dissolved with constant stirring in ~80% of the water for injection (WFI) for the batch. After all ingredients were dissolved, the concentrated premix solution was quantitatively transferred to the manufacturing vessel and the solution in the vessel was stirred continuously. In-process pH adjustment was performed and the formulation brought to its final weight with WFI. The filling operation was performed in an ISO class 5 environment. The filling line had a 5-μm pore size clarifying filter followed by two, serial, 0.22-μm sterilizing filters. Several liters of formulation were purged through the filling lines and filter assembly to saturate the filters with drug and preservative before the formulation was filled into presterilized plastic bottles. Presterilized tips were inserted and presterilized caps were applied in the ISO class 5 environment. Additional labeling and packaging was performed in an ISO class 7 manufacturing environment.

Manufacturing Example 2: Sterile Addition of a Drug Suspension to an Autoclaved Gel
A drug having very low solubility was suspended in a Carbomer gel to enhance drug delivery. The Carbomer was first dispersed, in an ISO class 7 manufacturing environment, at a high concentration in a clean compounding vessel using high speed homogenizers. The Carbomer phase was then transferred using a diaphragm pump to the manufacturing vessel in an ISO class 5 manufacturing environment. The Carbomer phase was autoclaved in the manufacturing vessel with continuous mixing and then cooled to ~40°C. The micronized drug substance, which was sterilized by γ irradiation, was aseptically added, with continuous mixing, to the Carbomer phase in the ISO class 5 environment. Approximately 50% of the water in the formulation was used to dissolve the chlorobutanol preservative and this solution was sterile filtered through a 0.22-μm filter into the manufacturing vessel. The pH was adjusted to 4.5 using autoclaved sodium hydroxide to produce a thick gel. The final formulation was aseptically filled into presterilized tubes and closed. The final labeling and packaging operations were completed in an ISO class 7 manufacturing environment (82).

Table 6 Cap Color Coding for Ophthalmic Products

Therapeutic class	Color
β blockers	Yellow, blue, or both
Mydriatics and cycloplegics	Red
Miotics	Green
Nonsteroidal anti inflammatory drugs	Grey
Anti infectives	Brown, tan
Carbonic anhydrase inhibitors	Orange
Prostoglandin analogs	Teal

Packaging

Packaging of ophthalmic formulations is very important since the shelf-life of a product is inherently tied in with packaging choice in many cases. The vast majority of ophthalmic formulations (except the injectable and specialized delivery systems) are packaged in polyolefin containers predominantly high-density polyethylene (HDPE), LDPE (low-density polyethylene), polypropylene (PP) and may also include materials such as polyethylene terephthalate (PET). Topical eye drops are typically packaged in 5 to 15 mL LDPE or HDPE bottles with tips that can be of linear low-density polyethylene (LLDPE) or HDPE or PP and caps that are usually HDPE or PP. LDPE is generally preferred for eye drop bottles because of their pliability which affects the ease with which a drop can be dispensed. The quality of the product may be affected by additives in the polymer which may interact with formulation components (e.g., binding of preservatives and actives, formation of insoluble complexes resulting in haze over time, etc.) or they may appear as contaminants in the form of extractables and leachables. Extractables and leachables may also be contributed by labels and secondary packaging components such as cartons and package inserts. The FDA is highly sensitive to the presence of extractables and leachables in ophthalmic products. To ensure the best quality of the product, bottles have some form of tamper evident seal. All primary packaging components must be sterile. Sterilization of plastics may be by ethylene oxide vapors (typically for LDPE and PP) or by γ irradiation (HDPE, LLDPE) and the sterilization method for packaging components must be validated. In blow-fill-seal (BFS), or form-fill-seal, operations product is filled into the bottle as it is being formed (in a sterile environment); because of the high temperature of the polymer as it is molded, it is assumed to be sterile and no further sterilization of the end product is generally required. The dropper tips may be molded as part of the operation or separate preformed, presterilized tips may be inserted followed by capping. There are specific color-coding requirements for different ophthalmic drugs as outlined in Table 6.

Preservative-free Multidose Devices

Although the great majority of eye drops are available as preserved multidose formulations in traditional LDPE dropper bottles, there are some patient populations that are sensitive to the presence of preservatives in formulations. That is the reason many formulations are also packaged as "unit-dose" or single-use vials. These are usually small volumes (0.5 mL or less) in LDPE form-fill-seal containers with twist off caps. Once opened these containers can not be stored beyond a single day of use and need to be discarded because of risk of contamination. The unit-dose presentations are more expensive to manufacture and as such are more costly for patients. As a result the market has seen the advent of preservative-free multidose devices (PFMD). The ABAK® system is a patented preservative free multidose eye drop dispenser (Fig. 6). It contains a 0.2 nylon fiber micro membrane that filters the solution. The pressure exerted causes the solution to pass through the antibacterial filter in the ABAK® system, forming a drop that falls from the tip of the dispenser. When pressure is released, the solution is reabsorbed and filtered from bacteria and air, ensuring the protection of the solution throughout its use. The ABAK® system filter provides a double protection: without using preservatives, it protects the solution inside the bottle from microbial contamination. The system has been used for the delivery of preservative-free timolol formulations to the eye and

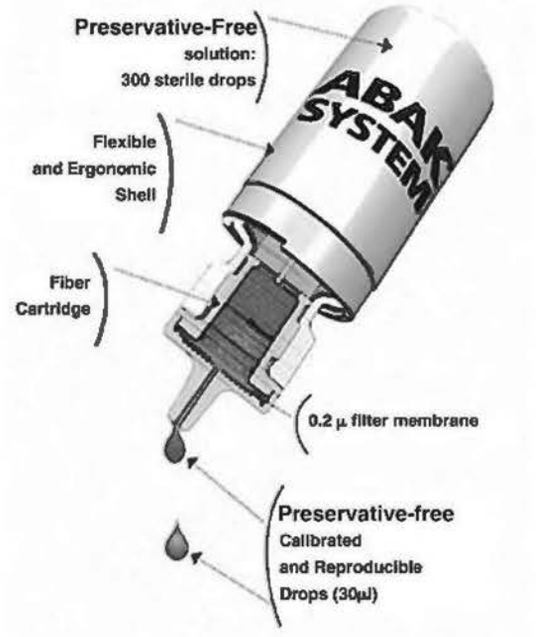

Figure 6 A preservative free delivery device: ABAK®. *Source*: From Ref. 85.

is available in certain markets under the trade name of Timabak (Thea, France; Nitten Pharmaceutical Ltd., Japan). Administration of timolol via Timabak® showed marked decrease in cytotoxicity in human corneal epithelial cells when compared with preserved formulations containing 0.2% benzalkonium chloride (83). Alternatively, devices which employ a valve-mechanism that prevents the suction of air back into the container, which could contain bacteria, can also be employed for multiuse applications of preservative-free formulations. PFMDs have recently been used for several dry-eye therapies including Artelac-advanced-MDO and Hycosan® (both marketed by Bausch and Lomb) and Hylo-Comod® (Ursapharm). With growing regulatory and safety concerns regarding the use of preservatives in ophthalmic formulations, especially those intended for chronic use, PFMDs are likely to gain more popularity.

Regulatory Pathways for Ophthalmic Formulations

Ophthalmic New Drug Applications

The cost, monetarily and in time, for developing a new ophthalmic formulation will be determined primarily by the number and complexity of in vivo studies required. These regulatory requirements, as well as the potential market exclusivity of the new formulation, may influence whether a decision is made to develop a generic formulation, a new formulation, or to not develop a formulation at all. The benefit of developing a generic formulation is that a product may be developed without any in vivo studies, or, possibly, with one small in vivo study. Typical development time for a generic formulation is two to four years. Once approved, the generic formulation may then be prescribed for any indications for which the reference-listed drug (RLD) is approved. This pathway has very little risk with regards to safety/efficacy of the active, little risk with regards to clinical efficacy, and moderate risk with regards to regulatory approval. However, this pathway results in no market exclusivity except for the potential 180-day market exclusivity granted to the "first-to-file" generic developer when the patent has expired. Alternatively, if faced with the prospect of performing in vivo studies to demonstrate clinical efficacy of a new formulation in a particular indication, it may make more sense to produce a new, nonequivalent formulation of a

Table 7 Summary of Regulatory Pathways, Risks, and Costs for New Formulation Development

	Generics	Supergenerics	Proprietary
Filing	ANDA 505(j)	NDA 505(b)(2)	NDA 505(b)(1)
Exclusivity	None or 180 days	3 yr	Composition or application patent
Cost	Low	Medium	High
Time	2 4 yr	3 5 yr	10 12 yr
Development	0.5 1.5 yr	0.5 1.5 yr	1 3 yr
Animal safety		0 0.5 yr	3 yr
Human safety		0 1 yr	1 yr
Human efficacy	0 1.5 yr	0.5 1.5 yr	3 yr
Regulatory review	1 2.5 yr	1 2.5 yr	2.5 yr
Risks			
Safety/efficacy risk	Low	Low	High
Clinical risk	Low	Medium	High
Regulatory risk	Low	Medium	High
Revenue/margins	Low	Medium	High
Commercial advantage	Price	Benefit to patient/price	Benefit to patient
Required stability data	3 mo real time on 1 batch, accelerated 3 mo	12 mo real time on 3 batches, accelerated 6 mo	12 mo real time on 3 batches, accelerated 6 mo

previously approved drug. Typical development time for this pathway is three to four years, but involves more costly in vivo studies. This regulatory pathway also has little risk with regards to the safety/efficacy of the active, but does have moderate risk with regards to clinical efficacy and regulatory approval. The potential benefit of this development pathway is three-year market exclusivity for the new formulation or new indication. In addition, there is always the potential that the new formulation may be patentable and provide additional exclusivity for the formulation composition that is developed. The longest and most costly development pathway is for a new pharmaceutical ingredient. This pathway has the same clinical and regulatory risks as developing a new formulation for an approved drug, but this is compounded with significant risk with regards to the safety/efficacy of the active ingredient. The typical development time for this pathway is 10 to 12 years. Table 7 summarizes the differences between these regulatory/development pathways.

Ophthalmic Medical Devices [510(k)]

Section 510(k) of the Food, Drug and Cosmetic Act requires device manufacturers who must register to notify FDA, at least 90 days in advance, of their intent to market a medical device. A medical device, according to the U.S. FDA, is an instrument, apparatus, implement, machine, contrivance, implant, in vitro reagent or other similar or related article, including component part or accessory. Thus, certain nonmedicated OTC products (e.g., contact lens rewetting drops), irrigation solutions and contact lens solutions would generally be filed in this category. There are three classes of medical device class I (general controls are sufficient to show safety and efficacy); class II (general and special controls are required; special controls may include labeling requirements, requirements for postmarketing surveillance, etc.); and class III (general controls and premarket approval (PMA) are required to demonstrate safety and effectiveness). Most of the ophthalmic products that enter the market through the 510(k) filing fall into class II and III medical devices. The process involves detailed scientific review by FDA for PMA including clinical studies protocol that has been agreed by FDA. There is no regulatory time-limit for PMA review but, the FDA does target completion of approval within 180 working days of receipt, if it can be approved as received, or 320 days if additional information is required. The filing of a 510(k) requires identification of a "predicate" device (with very similar composition, packaging, and use) and a detailed comparison with the predicate device.

Stability Storage and Testing of Ophthalmic Formulations

The stability testing requirements for various types of ophthalmic products (eye drops, eye ointments, ophthalmic inserts, injections, irrigating solutions, lens care products, etc.) are not always straightforward. The International Conference on Harmonization (ICH) guidelines do not address all of the stability requirements for the diverse array of products. For the large number of ophthalmic formulations that are packaged in semi-permeable containers, "stress conditions" are present at high temperatures and low humidity. Thus, accelerated testing of these products is carried out under these conditions as per ICH guidelines. The specific conditions include long-term stability testing at 25°C/40%RH; intermediate accelerated (if 40°C fails) testing at 30°C/40% RH (FDA guidelines) or 30°C/60%RH (ICH guidelines) and accelerated testing at 40°C/15%RH (84). For specialized formulations and packaging systems, a well-planned, customized stability protocol will have to be written that properly addresses important product characteristics during use and storage. To increase the chances of product approval it is important to develop a well-defined stability protocol that aims to address all international, regional and local requirements that is approved by the regulatory authorities prior to start of stability studies.

FUTURE DIRECTIONS

Many new ophthalmic drug delivery technologies which offer potential advantages are currently available, and more will be discovered in the near future. However, the development of a new ophthalmic drug or new ophthalmic drug delivery technology is an expensive and time-consuming project. Therefore, the decision of whether or not to develop a new formulation will continue to be based on the added value that a new product will offer to the patient. A new technology may offer more comfortable, less invasive treatment of a disease, less frequent dosing of a product, or safer, more effective treatment of a particular indication.

The advances in drug delivery technology that promise to reduce the dosing frequency of a drug substance (e.g., moving from four times per day to twice per day), or offer more comfortable formulations will likely be developed for the treatment of chronic indications, such as glaucoma and dry eye. The benefits of lower dosing frequency and offering more comfortable treatment are not outweighed by the cost increase for treatment of acute indications, but over years of treatment, a patient will be willing to pay more for an improved formulation technology. It is also likely that combination products, which offer the convenience of delivering a single drop rather than multiple drops, will continue to be developed for chronic indications, but will not likely be developed for acute indications.

Although noninvasive methods such as use of an oral tablet or a topical formulation for posterior treatment (rather than an intravitreal injection) will always be of significant interest for any ophthalmic indication, they will most likely be of greatest use in the treatment of acute indications. Invasive technologies involving implants and intraocular injections will be of importance in treating chronic indications and where patient compliance is likely to be low because of a frequent dosing schedule (e.g., in elderly glaucoma patients).

Whenever feasible, new drug substances will continue to be brought to market, at least initially, in simple, low-risk formulations like solutions and suspensions.

In the future, the ophthalmic formulator will need to continue to have a firm understanding of the structure of the eye, the nature of the drug substance that needs to be delivered to treat the eye, and the options for how to bring the two together in the best way possible. The final choice of drug substance, formulation type, delivery method and manufacturing and packaging for the final product will need to take into account the overall market potential of the product as well as the cost and benefit to the patients.

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11 | Glass containers for parenteral products

Robert Swift

INTRODUCTION

Glass containers have a long history as packaging materials for foods, beverages and medicinal products. Among other properties, glass compositions suitable for use as pharmaceutical containers offer sufficient inertness to minimize product interactions, impermeability to prevent ingress of contaminants, dimensional stability at temperatures needed for sterilization or depyrogenation as well as for lyophilization or frozen storage and transparency to allow product inspection. Where needed, coloration for light protection is possible. From the business side, a wide range of glass container styles and sizes is readily available in large quantities at reasonable cost. Increasingly, many manufacturers also offer preinspection, sterilization, barrier coatings or other specialized services needed for specific applications.

Despite general familiarity with glass in everyday life, detailed knowledge about the chemistry and manufacture of glass containers and, specifically, glass containers used for parenteral medications is limited. To provide basic information about glass, this chapter explores the characteristics of the glassy state, the broad range of industrial glass compositions and applications, the function of the various types of constituents that are included in commercial glasses and the manufacturing process steps that are common to the production of virtually all glass articles. This is followed by a more specific discussion of types of glass compositions used for pharmaceutical applications, how they are categorized and tested in the major pharmacopoeia, the various design families of containers used for parenteral products and the manufacturing processes by which they are produced. Some key aspects of quality control also are mentioned. The chapter concludes with a series of topics that are relevant to pharmaceutical formulation development, pharmaceutical filling, inspection and packaging operations and the quality of parenteral drug products that are filled into glass containers: the chemical, thermal and physical properties of containers and an overview of some quality blemishes and defects that can arise at various points throughout the supply chain.

When the first edition of this work was published in 1984, molded bottles for both small volume parenterals (SVPs) and large volume parenterals (LVPs) were in widespread use. By the time the second edition was published, in 1992, a significant proportion of LVPs had shifted to flexible containers. The LVP container information in the earlier editions is largely unchanged and still may be relevant in some markets. However, recent market trends for SVPs have increased interest in single dose vials, prefilled pens and prefilled syringes. This edition addresses these containers more fully.

THE GLASSY STATE

Glassy materials have been described or defined several ways by numerous authors and organizations. For example, Boyd (1) quotes Morey, ASTM C162 and Shelby, while Pfaender (2) provides three popular answers to the question "What is glass?" Some common themes can be summarized as follows:

- A supercooled liquid that has solidified or frozen without crystallization
- A solid material with amorphous, liquid-like structure
- A liquid with such high viscosity at room temperature that it behaves as a solid
- A material which lacks long-range molecular order but exhibits the stress-strain characteristics of a brittle, elastic solid.

While a wide range of materials, including organic molecules can be induced to form glasses, commercial container glasses are inorganic silicates produced by melting. With this

restriction, one can say that glass is an inorganic material or mixture of materials that has been heated to a molten liquid state then cooled without crystallization to a solid state.

The backbone of any glass formulation is a network former. There are several metallic oxides that readily cool without crystallization to form glasses. Special purpose glasses are produced using oxides of boron (B_2O_3), phosphorus (P_2O_5) or germanium (GeO_2) as the network former (3). However, the primary network former in glass formulations for commercial applications including parenteral containers is silica (silicon dioxide, SiO_2).

The basic network building block for silicate glasses is a tetrahedral form of silica, (SiO_4) (Fig. 1). Ideally, each silicon atom has shared bonds with four oxygen atoms and each oxygen atom has shared bonds with two silicon atoms. This configuration leads to a cross-linked, 3-D network (Fig. 2) of shared covalent bonds. The spatial interaction of these bonds causes viscosity to increase rapidly with decreasing temperature and inhibits the molecular reordering needed for the material to make the transition from a randomly ordered structure of the liquid state to the regular, long-range order of a crystalline solid. As a result, the network cools to rigidity in the glassy state. When processed under the appropriate conditions silica will crystallize as quartz (Fig. 3).

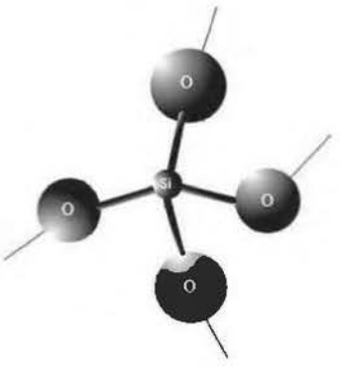

Figure 1 The SiO_4 tetrahedron is the basic network building block for silicate glasses. Each oxygen atom binds to the central silicon atom and either bridges to an adjacent silicon atom or exists as a nonbridged oxygen anion.

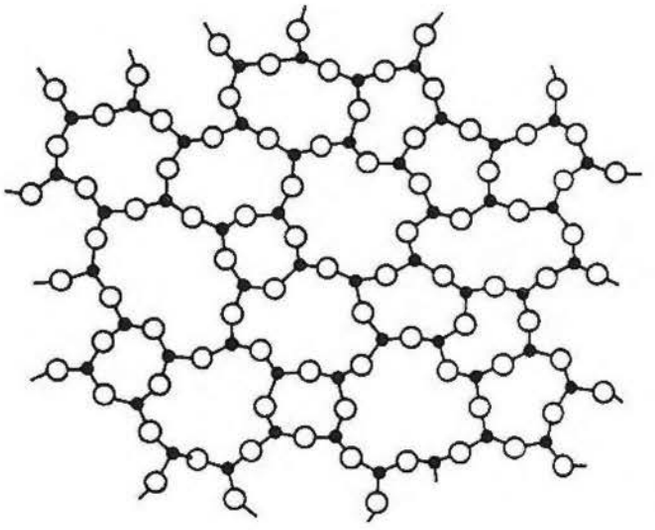

Figure 2 Two dimensional schematic representation of glassy silicon dioxide in a random 3 D network of tetrahedral silica. *Source*: From Ref. 5.

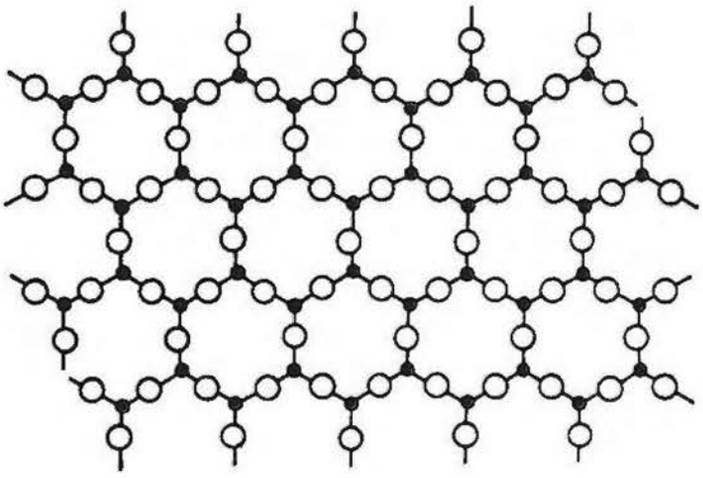

Figure 3 Two dimen sional schematic represen tation of 3 D crystalline quartz with long range structure composed of tet rahedral silica. *Source*: From Ref. 5.

SILICATE GLASS FORMULATION FAMILIES

Both vitreous (glassy) silica and crystalline silica (quartz) are found in nature. However, commercial glass melting techniques require the viscosity of the melt to be in the range of 1 dPa-sec. For pure silica, this viscosity corresponds to about 2300°C, which is not practical for industrial production. Similarly, upon cooling, the viscosity of molten silica increases too quickly to be formed into containers using conventional production processes. As a result, practical glass formulations for containers are mixtures of silica and other minerals that lower the melting point and modify the properties of the glass to improve workability.

In fact, the network modifiers have the greatest influence on the physical and chemical properties of the glass and resulting finished glass articles. For this reason, glass formulations can be divided into broad families on the basis of the primary network modifiers used. The following sections describe the glass families used for containers and the role of the various network modifiers.

Soda-Lime-Silicate Glasses

The oldest and most widely melted glasses are known as soda-lime-silicate glasses. In the raw material mixture, or batch, these oxides typically are supplied as soda ash (sodium carbonate) and limestone (calcium carbonate) hence, the common description "soda-lime" glass (4). In a glass formulation, soda and lime refer to sodium oxide and calcium oxide, which are the primary network modifiers and comprise roughly 25% of the composition by weight. Glasses in this family may include some magnesium oxide by the addition of dolomite (calcium magnesium carbonate). Potassium oxide, supplied as potash (potassium carbonate) may also be used. Within the silica matrix, the monovalent cations, sometimes called alkaline oxides, (Na^+ and K^+) satisfy the charges of nonbridged oxygen atoms (Modifier cation M_1 in Fig. 4). This reduces the extent of cross-linking in the silica backbone, which lowers the melting point. However, the sodium or potassium cations are relatively mobile and can be leached from the surface which limits chemical durability of the glass. The bivalent cations, also known as alkaline earth oxides, (Ca^{2+} and Mg^{2+}) interact with the silica matrix in a similar way occupying locations adjacent to two nonbridged oxygen atoms (Modifier cation M_2 in Fig. 4) and are more resistant to leaching. Usually, 2% to 3% aluminum oxide (Al_2O_3) is added to facilitate melting and to improve chemical durability. The aluminum cations (Al^{3+}) are able to form covalent bonds within the silica matrix (Modifier cation M_3 in Fig. 4) and, thus, are much more resistant to leaching. When light protection is needed, ferric oxide (Fe_2O_3) is added to produce amber glasses, which absorb ultraviolet wavelengths more effectively than colorless

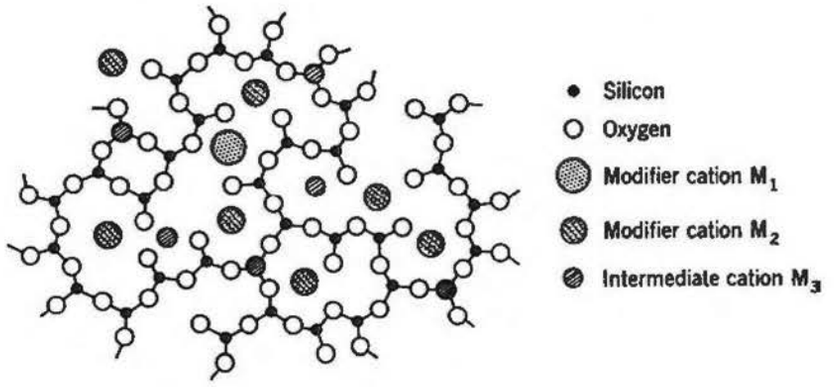

Figure 4 Two dimensional schematic representation of the 3 D structure of a multicomponent glass. Monovalent and divalent cations exist in interstitial space and balance the negatively charged nonbridged oxygen atoms. Trivalent cations integrate into the silica network. At surfaces, nonbridging oxygen atoms are dominant and yield a net negative charge. *Source*: From Ref. 5.

glasses. The nominal compositions and properties of several soda-lime container glass formulations are shown in Table 1.

Borosilicate Glasses

In the late 19th century, Otto Schott, a German chemist and glass researcher, conducted systematic research to investigate the effects of various minerals and oxides on the optical, chemical and thermal properties of silicate glasses. He discovered that replacing some of the sodium and calcium with boron oxide (B_2O_3) resulted in glasses with exceptional chemical durability and heat resistance including resistance to abrupt temperature changes, or thermal shock (7). Over time, a wide range of borosilicate glasses and other special glasses (8) have been developed for various applications including pharmaceutical containers as well as the familiar Duran®, Kimax®, and Pyrex® brands of laboratory glassware. Thermal shock and the related property of the thermal expansion coefficient will be developed more fully in the section Mechanical and Thermal Properties later in this chapter.

Borosilicate glasses require higher melting and forming temperatures than soda-lime glasses. However, with roughly ten-fold improvement in durability, as measured by extractable alkali, and high tolerance for thermal processes such as depyrogenation, lyophilization and terminal sterilization, borosilicate glasses now account for nearly all containers used for small volume parenterals. Ferric oxide (Fe_2O_3) and titanium oxide (Ti_2O_3) or manganese oxide (MnO) can be added to produce amber borosilicate glasses for protection from ultraviolet light. The nominal composition and properties of representative borosilicate container glasses also are shown in Table 1.

Compendial Classifications and Test Methods

Pharmacopoeias around the world acknowledge these two families of glass compositions as suitable materials for drug product containers. In fact, the compendia designate glass "types" based on these composition categories and reference the composition family in the general description of each type. For example, in the USP (9), type I containers have the description "Highly resistant, borosilicate glass" while type III containers are described as "soda-lime glass." Test methods used to differentiate between borosilicate and soda-lime containers and classify them according to type rely on the substantially lower quantity of alkaline ions that can be extracted from borosilicate glass or containers.

For example, the USP "powdered glass" test assesses the intrinsic chemical resistance of the glass formulation by crushing containers to obtain powder of a defined grain size and performing an extraction from the powdered glass into water by autoclaving. The alkali

Table 1 Nominal Compositions and Properties of Representative Soda-Lime and Borosilicate Glasses Used for Blow-Molded and Tubular Containers for Parenterals

Chemical composition (weight %)	Borosilicate							Soda-lime	
	Tubing					Molded		Molded	
	Clear			Amber		Clear	Amber	Clear	Amber
Silicon (SiO_2)	81	75	74 7	69	70	69	66	73	72
Boron (B_2O_3)	13	10 5	11 1	10	7	12	11		0 5
Sodium (Na_2O)	4	7	7 3	6	7	10	8	14	14 2
Aluminum (Al_2O_3)	2	5	6 1	6	6	6	6	2	2
Calcium (CaO)		1 5	0 4	0 5	< 1	1	1	10 5	10
Potassium (K_2O)			0 8	2	1				
Magnesium (MgO)				0 5					
Barium (BaO)				2		2	1		
Titanium (TiO_2)				3	5				
Manganese (MnO)							5		
ron (Fe_2O_3)					1	<0 1	1	<0 05	0 3
Zinc (ZnO)				1					
Chloride (Cl^-) and fluoride (F^-)			0 4						
Sulfate (SO_3)				0 2				0 2	
Physical properties									
Thermal expansion 0–300°C ($\times 10^{-7}$)	33	49	51	54	55	60	62	88	91
Softening point (°C) ($10^{7.6}$ dPA-s)	825	785	783	765	770	739	745	729	713
Annealing point (°C) (10^{13} dPa-s)	560	565	566	558	560	567	530	548	536
Strain point (°C) ($10^{14.5}$ dPa-s)	525	Not published	518	520	Not published	528	515	510	496
Density (g/cm^3)	2 23	2 34	2 32	2 39	2 42	2 41	2 48	2 48	2 5

Source From Ref 6

content of the resulting extract solution is determined by titration with acid. Although the test details differ, other compendia include similar methods. These methods can differentiate between type I borosilicate glass and type III soda-lime glass because of the significantly higher quantity of alkali that can be extracted from soda-lime glass. This is expected given the much higher levels of sodium, calcium and other alkaline and alkaline earth oxides present in soda-lime glass. The USP glass powder test and similar methods assume that the fresh surface exposed by crushing the container is representative of the inner surface of the container which will contact the drug product. This assumption is not always justified, as will be discussed in the section "Glass Chemistry" later in this chapter.

There are chemical treatments that can be applied to the inner surface of freshly formed containers to react with the alkaline ions at or near the surface. Sulfur dioxide (SO_2) or sulfur trioxide (SO_3) gas, or, more conveniently, ammonium sulfate [$(NH_4)_2SO_4$] pellets or solution may be injected into the containers before annealing. At elevated temperature and in the presence of water vapor, these substances produce sulfuric acid which reacts with alkaline ions on the glass surface to form various salt residues that are readily removed by rinsing prior to use. The removal of alkaline ions from the inner surface in this way significantly reduces the level of alkali available for leaching into the drug after filling. When type III containers made from soda-lime glass are treated in this way, the surface resistance is improved to such an extent that the pharmacopoeias recognize them separately as type II glass or containers. For example, USP <660> (9) designates Type II glass and provides the general description "Treated soda-lime glass." Similar classifications and descriptions are found in the European Pharmacopoeia (10).

Since only the surface resistance is improved by the treatment process, glass powder test methods cannot differentiate between treated or nontreated containers or assess the effectiveness of the treatment process. For treated containers, alternative test methods such as the USP Water Attack at 121° Test, the USP Surface Test, the Ph. Eur. Test for Surface Hydrolytic Resistance, or similar method must be used. In these tests, the extraction into water is performed using intact, filled containers rather than glass powder. As with glass powder methods, the results usually are determined by titration of the extract with acid. Some methods allow the use of spectroscopy to quantify directly the concentration of extracted alkaline ions.

If the composition family of the container glass is known (e.g., soda-lime glass), one may perform any of the surface test methods, apply the corresponding limit values and confirm the use and effectiveness of a chemical treatment process. When neither the glass formulation family nor use of chemical treatment is known, it may be necessary to perform both a surface test and a glass powder test to classify the containers correctly. However, many pharmaceutical companies confirm the container type on the basis of the supplier's test results and certificate of conformance.

As will be explained in section "Surface Chemistry," later in this chapter, there is another reason that the chemical resistance of the inner surface may be different from the intrinsic resistance of the glass formulation. The container forming process can cause degradation of the physical and chemical properties of the inner surface even when borosilicate glass is used. The Ph. Eur. test for surface hydrolytic resistance, the USP Surface Test or other similar methods may be used to evaluate residual surface alkalinity of containers made from borosilicate glass to confirm that the inner surface retains the level of chemical resistance expected in type I containers. The compendia are silent on test methods for and classification of containers made from borosilicate glass that are subsequently chemically treated to reduce alkaline surface residues deposited during forming. Users of "treated" borosilicate containers are advised to consult with their supplier to understand how the forming and "dealkalization" processes are controlled to ensure consistent results.

There is a tendency to assume that the terms soda-lime and borosilicate, especially as used in the pharmacopeias, refer to specific glass formulations. In fact, within the broad categories of soda-lime and borosilicate glasses, a wide range of glass formulations have been developed for specific applications. This point is especially relevant to borosilicates where two major subfamilies are important for parenteral containers. Within the industry, these subfamilies are often identified as "33 expansion" and "51 expansion." These terms are derived from the thermal expansion coefficient of some typical formulations in each group.

The American Society for Testing and Materials has published ASTM E-438-92 (11) defining nominal composition ranges and physical properties for glassware used in laboratory apparatus. This standard differentiates between the two borosilicate subfamilies by the designations "Type I, Class A" and "Type I, Class B." Although these designations are not used in the pharmacopeias, they are understood by glass manufacturers. Thus, for example, a specification defining the material requirement as ASTM E-438 Type I, Class A ensures that a 33-expansion borosilicate glass will be used.

GLASS PRODUCTION

Regardless of the glass composition, production of all glass containers begins with the transformation of inorganic raw materials into molten glass in large furnaces lined with refractory brick. A simplified cross section is shown in Figure 5.

The conversion of granular high purity silica sand, alumina, various carbonates and, for borosilicates, sodium borate into molten glass suitable for forming involves a series of complex physical and chemical reactions well beyond the scope of this chapter. However, the main process steps can be summarized as follows. The raw materials, or batch, are weighed, blended and conveyed continuously to the melting furnace. Typically, the batch includes a controlled percentage of internally recycled crushed glass known in the industry as cullet, which facilitates melting of the other batch materials. As the materials are heated and the melting process begins, carbon dioxide is liberated by decomposition of the carbonates and dissolved water is released. A substantial portion of the overall melting process is the refining process during which the CO_2, H_2O, and other gases coalesce, rise through the molten glass and escape into the furnace atmosphere. Bubbles that do not escape can be carried through to the forming process as seeds or blisters in molded bottles or as air lines in tubular containers. During refining, convection currents within the glass serve to homogenize the melt. Finally, the refined, homogenized molten glass must be slowly and uniformly cooled to reach the viscosity needed for the forming process which follows.

Glass composition is controlled primarily through careful monitoring of the raw material composition and corresponding minor adjustments to the batch proportions. Complete chemical analysis of glass composition is difficult and time consuming. Therefore, day-to-day monitoring of the melting process is accomplished by measuring physical properties such as density and thermal expansion coefficient that are extremely sensitive to changes in composition. Homogeneity and relative absence of bubbles are monitored by quality control inspections of the molded containers or tubing.

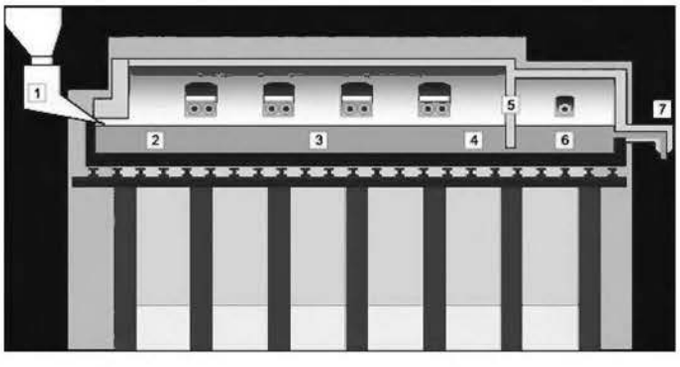

Figure 5 Longitudinal cross section of a large industrial glass melting furnace. Raw materials are added continuously at the batch feeder (1). Melting (2), refining (3), and homogenization (4) occur gradually as the melt progresses through the main furnace chamber. The molten glass flows under the bridge wall (5) into the conditioning section (6) before passing into the forehearth (7) for transfer to the blow molding or tube draw process. *Source*: From Ref. 2.

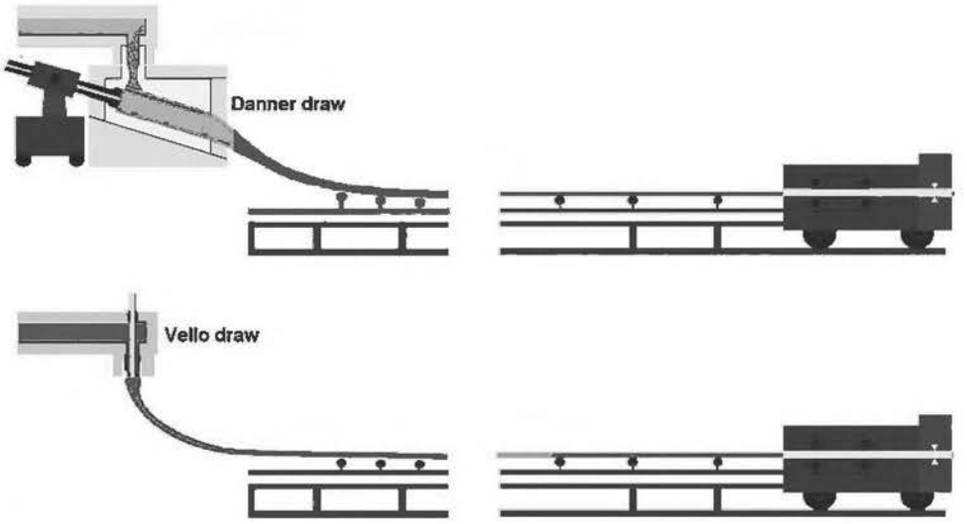

Figure 6 Glass tubing production for small volume parenteral containers uses either the Danner process of the Vello process. Both processes receive a continuous vertical stream of molten glass at the appropriate viscosity and transform it into discrete lengths of glass tubing with precisely controlled outer diameter and wall thickness. *Source*: From Ref. 4.

For both types of forming processes, the continuous output of the melting process is molten glass that has been cooled to reach a suitable viscosity. For blow-molded containers, a reciprocating plunger in the forehearth pumps the glass so that it can be cut into discrete charges, or "gobs," of molten glass that are guided through chutes to the forming machine. For all other styles of parenteral containers, the molten glass flows continuously from the furnace and is drawn into tubular form. The diameter and wall thickness of the tube are as needed for the body of the container into which the tube will later be formed. Glass tubing for parenteral containers is produced using either the Danner process or the Vello process (Fig. 6).

In the Vello process, a mandrel with the approximate shape of a bell is positioned in the stream of molten glass flowing from the orifice ring located in a bowl-shaped "drain" in the bottom of the forehearth. The molten glass flows out of the furnace and over the bell. Compressed air is blown through the center of the bell to form and maintain the stream as a tube.

The Danner process is similar except that the molten glass streams from the furnace onto a ceramic mandrel as a ribbon. The mandrel rotates slowly and is inclined slightly downward. As the glass flows down the length of the mandrel, it cools to the appropriate viscosity. Compressed air is blown through the center of the mandrel to form the tube.

With either process, the tractor belts of the drawing machine, located up to 120 m (~400 ft) downstream, provide a pulling motion which redirects the glass stream into a horizontal orientation. As the continuously moving glass tube cools and solidifies, it is supported on carbon rollers or air beds. The diameter and wall thickness are controlled by a delicate balance of the glass flow rate out of the furnace, the pressure of the blowing air and the speed of the drawing machine. The glass flow rate cannot be controlled directly but is the result of precise control of forehearth temperature, glass level within the furnace and, for Danner, the mandrel temperature or, for Vello, the relative dimensions and positions of the bell and ring. For either process, just after the drawing machine, the continuous tube is cracked off into discrete lengths, the ends are flame-smoothed or trimmed and fire-polished to prevent chipping and cracking and the tubes are packaged for shipment to the container producer.

In state of the art production facilities for pharmaceutical grade glass tubing, tubing diameter and melting defects such as knots, stones, and air lines are continuously inspected on

the tubing alley between the furnace and the drawing machine using laser or camera-based instruments. Additional checks of all other tubing dimensions and attributes are performed through automated or visual inspection of finished tubes. In general, acceptable quality level (AQL) sampling plans are used.

The next sections will discuss the various styles of container designs, some advantages and disadvantage of each and provide some details about the container forming processes.

CONTAINER DESIGNS AND MANUFACTURING PROCESSES

There are four main categories of container designs used for parenteral medications. Each is available in a range of sizes and shapes from multiple manufacturers.

Ampoules

An ampoule is a complete one-piece container system made entirely of glass and produced from tubing. The most common capacity range for ampoules is 0.5 to 2 mL. Capacities up to 20 mL or larger are possible for special applications. Some sizes and configurations have become de facto standards in certain markets. In addition, the International Organization for Standardization (ISO) has defined materials, dimensions, capacities, performance and packaging requirements for glass ampoules for injectable products in ISO 9187-1 (12).

The main steps of the forming process are illustrated in Figure 7. The wall of the tip or stem is thin and tightly controlled in the forming process. Similarly, the wall of the constriction is also tightly controlled. The constriction generally is preweakened by scoring or by the application of a color-break band to facilitate breaking the ampoule in the constriction at the time of use. Color-break bands are ceramic enamels with a slightly different thermal expansion coefficient. The mismatch prestresses the constriction to reduce the force needed to open the ampoule. An improved ampoule opening system called "One Point Cut" (OPC) has been developed. In this system, a small score of precise width and depth is cut at a single point of the constriction. OPC is claimed to provide more consistent opening force and fewer glass particles. ISO 9187-2 describes the requirements for ampoules using this design.

Quality control for ampoule manufacturing may include online 100% gauging of critical dimensions. State of the art producers use feedback control of the flames to maintain tight

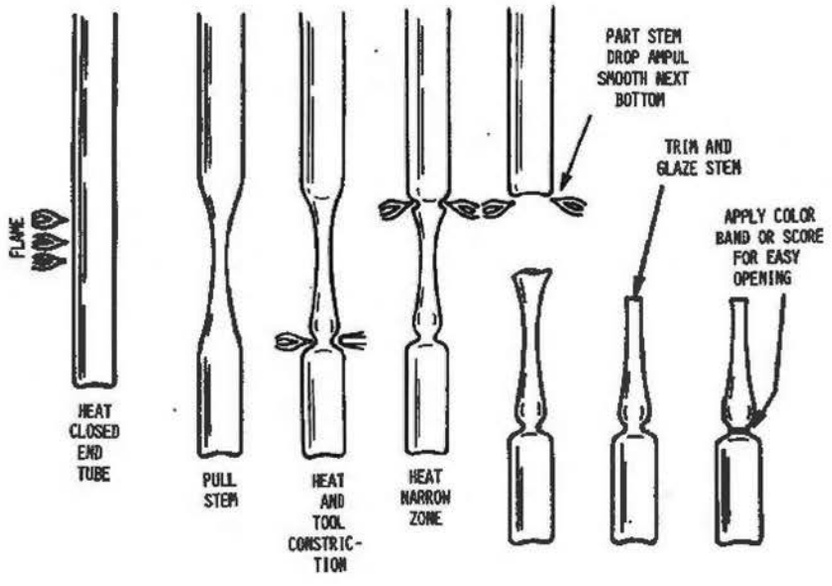

Figure 7 Typical process steps to form ampoules from glass tubing. *Source*: From Ref. 6.

control over the diameter and wall thickness of the stem. This minimizes variability in the sealing process during pharmaceutical processing. Some manufacturers offer supplemental 100% camera-based inspection to eliminate minor cosmetic blemishes at the point of manufacture to reduce container-related rejection of ampoules after filling and sealing. Additional quality control checks for dimensional and cosmetic attributes and breaking strength are performed periodically on finished ampoules. In general, AQL sampling plans are used.

After filling by the pharmaceutical producer, the stem of the ampoule is melted and usually pulled to seal the container. The combination of geometry and preweakening at the constriction allows the user to snap off the tip at the time of use and withdraw the contents of the ampoule into a disposable syringe so that the dose can be administered.

The main advantage of the ampoule container system is the simplicity of a single product contact material, highly inert borosilicate glass, throughout the shelf life of the drug product. However, breaking glass to gain access to the contents is not considered to be user-friendly. One also must consider the safety aspects of the sharp edges created when opening an ampoule and the possible need to use a filter when transferring the dose to a disposable syringe for administration. Therefore, while ampoules still are widely used for generic drugs and in developing countries, it is rare for new products to be developed in ampoule format.

Bottles and Vials

The most recognizable container system for parenteral products is a glass bottle or vial that has been closed with an elastomeric stopper and aluminum crimp seal. The glass container may be produced from glass tubing. Tubing vial capacities generally are limited to 30 mL. ISO has defined the materials, shape, dimensions, capacities and performance requirements for injection vials made from glass tubing up to 30 mL in ISO 8362-1 (13). A wide range of other heights, diameters and wall thickness also are produced. In addition, with specially designed forming machines and tubing up to 50 mm (~2 in) in diameter, it is possible to produce tubing vials up to 100 mL capacity or larger.

The production steps to form a vial from a glass tube are shown in Figure 8. The dimensions of the container body are unchanged by the forming process and retain the diameter and wall thickness of the original tube. As such, the wall thickness and diameter are uniform and well-controlled. This may allow higher filling and packaging line speeds and facilitate the use of high speed, camera-based inspection of filled containers. Forming the shoulder and bottom of the vial can cause occasional slight dimensional variation which may affect processing efficiency. In addition, the lighter weight of tubing vials may cause handling problems on lines orginally designed for heavier molded bottles.

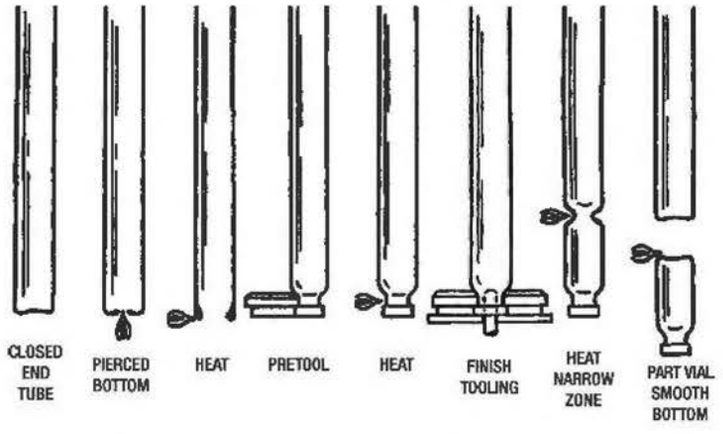

Figure 8 Typical process steps to form vials from glass tubing. *Source*: From Ref. 6.

Leading producers generally use camera-based systems directly after forming to perform 100% inspection of dimensions affecting the interface with stopper and seal. As with ampoules, some manufacturers offer supplemental 100% camera-based inspection to eliminate minor cosmetic blemishes at the point of manufacture to reduce container-related rejection of vials after filling and sealing. Additional quality control checks for dimensional and cosmetic attributes are performed periodically on finished vials using AQL-based sampling plans.

Containers for use with elastomer stoppers and aluminum seals also may be blow-molded bottles. Molded bottles for parenterals are available with capacities from 2 mL to 1 L or more. ISO has defined materials, shape, dimensions, capacities and performance requirements for injection vials made of molded glass in ISO 8362-4 (14). As with tubing vials, a wide range of other sizes and shapes are available. Typical process steps are shown in Figure 9. Compared with forming lines for tubular vials, molded bottle production lines have higher tooling costs longer changeover times and also must be located adjacent to the melting furnace. As such, production campaigns for molded bottles may be longer but less frequent. Production planning and inventory levels can be adjusted accordingly. Quality control steps for molded bottles are similar to those for tubular vials.

The nature of the blow-molding process is such that the wall thickness of a molded bottle will be heavier and more variable than the wall thickness for a tubing vial of similar capacity. Optical distortion caused by wall thickness variation can complicate inspection of the contents, especially when using automated, camera-based inspection systems. To accommodate the longer overall working time needed, borosilicate glass formulations suitable for blow-molding tend to have slightly higher sodium and boron content when compared with similar tubing glass formulations. On the other hand, heavier wall molded bottles may be more resistant to breakage caused by accidental abuse or mishandling.

For bottles and vials intended to be used with elastomeric stoppers and aluminum seals, the bottle or vial is only part of the overall container-closure system. Three-dimensional parameters of the mouth or finish are of particular functional importance at the interface with the stopper and seal. The neck inner diameter must ensure an appropriate interference fit with the plug of the stopper. Similarly, the outer diameter and thickness of the lip or finish must be suited to the diameter and skirt length of the aluminum seal. Other details of angles and radii also are important in matching the three components and the sealing equipment to create a robust container-closure system. While all of these parameters matter, by convention, the size designation is based on the nominal outer diameter of the finish. For small volume parenterals, typical container systems use finishes with either 13 mm or 20 mm nominal flange diameter.

ISO 8362-1 and ISO 8362-4 standards for injection vials provide dimensional details for the finish area as well as design parameters for complete containers, that is, diameter, total height, wall thickness, capacity, etc. ISO 8362 parts 2, 3, 5, and 6 are companion standards for elastomeric closures, aluminum caps and aluminum-plastic combination caps. This family of standards is intended to facilitate suitability of components from different suppliers in different but related industries. The roots of these ISO standards can be traced to German DIN standards. As such, the nominal dimensions and tolerances were developed in millimeters.

Historically, in the United States, container finish dimensions and matching closures have been based on the "2710 Biological Finish" standard developed in the 1940s by the Glass Container Manufacturer's Institute (GCMI), now known as the Glass Packaging Institute (GPI). The dimensions and tolerances of the GPI 2710 standard (15) are in inches but the size designations also are based on the nominal outer diameter of the finish in millimeters. As a consequence, both the ISO family of standards and the GPI 2710 standard include finish designs having finish outer diameters of about 13 and 20 mm. The important dimensions are similar but not identical. When selecting components, one must be aware, for example, that a "20-mm" stopper and seal from a U.S. producer may not be optimized for use with a 20-mm vial from a European producer. Discrepancies of this nature may also exist in published or de facto standards that may be widely used in other markets. Care must be taken to ensure the selected components are suitable for use as an integrated container-closure system.

As pharmaceutical filling line speeds have increased, container and closure manufacturers have worked with their customers to optimize processing efficiency. When stoppers are inserted into filled vials, the stopper plug often seals the neck of the vial before the stopper is

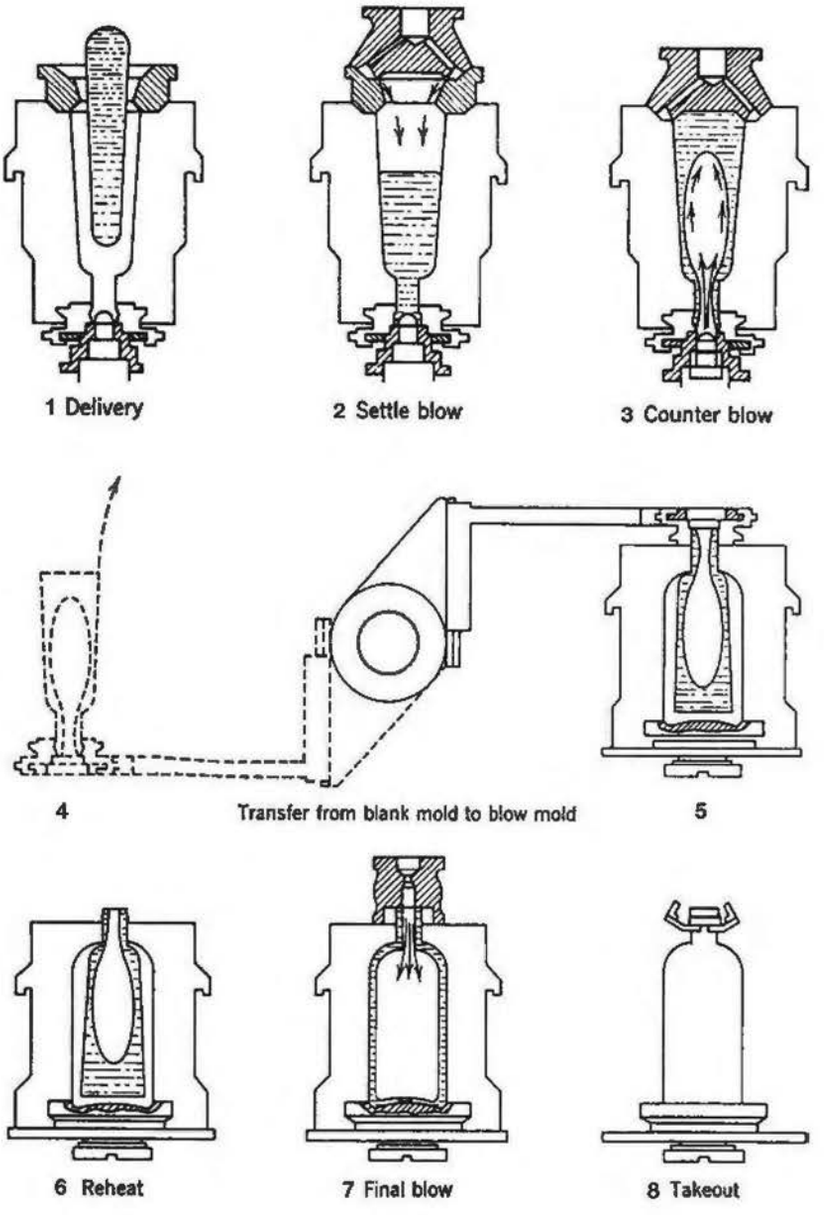

Figure 9 Typical process steps in the production of molded bottles by the blow blow process. A charge or gob of molten glass is delivered by a chute from the furnace to the preform mold (**1**). Compressed air blows the glass into the mold to form the container mouth and neck (**2**). Compressed air then counterblows to shape the preform (**3**). The preform mold retracts (**4**), allowing preform to be transferred to the final mold which closes around it (**5**). The outer surface of preform that has been cooled by the preform mold reheats from residual heat in the molten core (**6**). Compressed air blows the glass out to the shape of the final mold (**7**). After some cooling time, the finished bottle is removed from the mold (**8**) and conveyed to the annealing (stress relief) tunnel. *Source*: From Ref. 5.