Engineered antibodies now represent over 30% of biopharmaceuticals in clinical trials, as highlighted by recent approvals from the US Food and Drug Administration. Recombinant antibodies have been reduced in size, rebuilt into multivalent molecules and fused with , for example radionuclides, toxins, enzymes, liposomes and viruses. The emergence of recombinant technologies has revolutionized the selection, humanization and production of antibodies, superseding hybridoma technology and allowing the design of antibody-based reagents of any specificity and for very diverse purposes.

Engineered antibodies

PETER J. HUDSON & CHRISTELLE SOURIAU

The discovery of hybridoma technology by Kohler and Milstein[1] in 1975 heralded a new era in antibody research and clinical development. Mouse hybridomas were the first reliable source of monoclonal antibodies and were developed for a number of *in vivo* therapeutic applications (Table 1; see also FDA product approval information at http://www.fda.gov/cber/efoi/approve.htm). Throughout the 1990s, innovative recombinant DNA technology, including chimerization and humanization, enhanced the clinical efficiency of mouse antibodies and led to the recent wave of approvals by the FDA for therapeutic immunoglobulin (Ig) and Fab molecules (monovalent antibody fragment produced by proteolysis) (Table 1 and Fig. 1; refs. 2,3). These developments have continued, and in 2002 the FDA approved the first radiolabeled antibody for cancer immunotherapy (Zevalin)[4].

The list of approved antibody therapeutics against cancer and against viral and inflammatory diseases is growing rapidly (Table 1), with more than 30 antibodies in late-phase clinical trials[2,3]. Most recently, innovative structural designs have improved *in vivo* pharmacokinetics, expanded immune repertoires and permitted screening against refractory targets and complex proteome arrays, while new molecular evolution strategies have enhanced affinity, stability and expression levels. This review describes these emerging technologies and discusses the creation of a vast range of engineered, antibody-based reagents that specifically target biomarkers of human health and disease[1–3]. We review antibodies designed as intact molecules and recombinant fragments and then focus on the latest technologies for attaching additional therapeutic payloads, such as radionuclides, drugs, enzymes and vaccine-inducing epitopes.

Intact antibodies, humanization and de-immunization

Intact antibodies provide high-specificity, high-affinity targeting reagents and are usually multivalent (Fig. 1). Their simultaneous binding to two adjacent antigens increases functional affinity and confers high retention times, for example, on cell surfaces. In addition, intact antibodies comprise Fc domains, which can be important for cancer immunotherapy through their abilities both to recruit cytotoxic effector functions[2,5] and to extend the serum half-life, mediated by the neonatal Fc receptor[6]. Unmodified mouse monoclonal antibodies formed the first wave of FDA-approved immunotherapeutic reagents (Table 1), although their *in vivo* applications were limited because repeated administrations provoked an anti-mouse immune response[2]. Simple strategies have been developed to avoid, mask or redirect this human immune surveillance; these strategies include fusion of mouse variable regions to human constant regions as 'chimeric' antibodies, 'de-immunization' mouse surface residues onto human acceptor antibody frameworks (Table 1; see also Supplementary Note online)[2]. Modern alternative strategies now allow selection of fully human antibodies directly from natural or synthetic repertoires, including live transgenic mice producing purely human antibodies[7]. Human antibody-display libraries were used to transform a mouse antibody *in vitro* into a fully human derivative (D2E7), which is likely to be the first FDA-approved fully human anti-inflammatory antibody. Many other fully human antibodies, including Efalizumab for the treatment of psoriasis, are in clinical evaluation (see http://www.fda.gov/cber/efoi/approve.htm).

Design of 'antibody fragments' for unique clinical applications

For cytokine inactivation, receptor blockade or viral neutralization, the Fc-induced effector functions are often unwanted and can be simply removed by proteolysis of intact antibodies to yield monovalent Fab fragments (ReoPro, Remicade; Table 1). Proteolysis, however, does not easily yield molecules smaller than a Fab fragment, and microbial expression of single-chain Fv (scFv) is currently the favored method of production (Fig. 1). In scFvs, the variable (V_H and V_L) domains are stably tethered together with a flexible polypeptide linker (Fig. 1)[2,3]. In comparison with whole antibodies, small antibody fragments such as Fab or scFv exhibit better pharmacokinetics for tissue penetration and also provide full binding specificity because the antigen-binding surface is unaltered. However, Fab and scFv are monovalent and often exhibit fast off-rates and poor retention time on the target[8,9]. Therefore, Fab and scFv fragments have been engineered into dimeric, trimeric or tetrameric conjugates to increase functional affinity through the use of either chemical or genetic cross-links (Fig. 1)[2,3,10,11]. Various methods have been devised to genetically encode multimeric scFvs, of which the most successful design was the simple reduction of scFv linker length to direct the formation of bivalent dimers (diabodies, 60 kDa), triabodies (90 kDa) or tetrabodies (120 kDa) (Fig. 1)[11]. Indeed, the first clinical trials of scFv fragments are likely to be as multivalent reagents, because they exhibit high functional affinity and have been very successful in preclinical studies[8,12–14].

Pharmacokinetics of intact antibodies versus fragments

The efficiency of antibodies *in vivo*, for example in cancer therapy, lies in their capacity to discriminate among tumor-associated antigens at low levels. Immunotherapy has been more successful against circulating cancer cells than solid tumors because of better cell accessibility. This is illustrated by the FDA approval of intact antibodies: Rituxan for the treatment of non-Hodgkin lymphoma and Campath and Mylotarg for the treat-

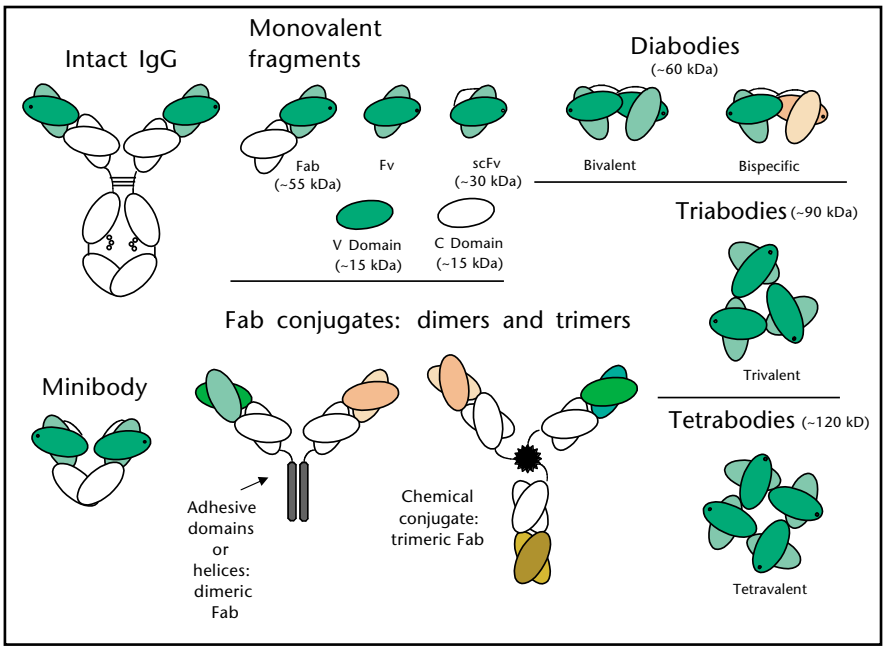

Fig. 1 Schematic representation of an intact Ig together with Fab and Fv fragments and single V (colored ovals; dots represent antigen-binding sites) and C domains (uncolored). Engineered recombinant antibodies are shown as scFv monomers, dimers (diabodies), trimers (triabodies) and tetramers (tetrabodies), with linkers represented by a black line. Minibodies are shown as two scFv modules joined by two C domains. Also shown are Fab dimers (conjugates by adhesive polypeptide or protein domains) and Fab trimers (chemically conjugated). Colors denote different specificities for the bispecific scFv dimers (diabodies) and Fab dimers and trimers.

have been approved for the treatment of solid tumors: Herceptin for the treatment of breast carcinoma and PanoRex for colon cancer (in Germany). Although the mechanisms of action are still under investigation, Herceptin appears to utilize Fc receptors and angiogenesis[16], whereas Rituxan activates apoptosis through receptor dimerization[17].

Radiolabeled antibodies are important clinical reagents for both tumor imaging and therapy and also provide an effective evaluation of pharmacokinetics[18]. The choice of radionuclide dictates the application. For example, Zevalin is approved for lymphoma therapy as a rapidly cleared, intact mouse antibody to match the clearance rates of yttrium-90 (ref. 4). Therapeutic administration requires a balance between long dissociation rates at the target site and slow blood clearance, which can lead to accumulation in the liver and high radiation exposure of other tissues. Biodistribution studies in solid tumors have also revealed that whole IgG molecules are too large (150 kDa) for rapid tumor penetration. The best tumor-targeting reagents comprise an intermediate-sized multivalent molecule, providing rapid tissue penetration, high target retention and rapid blood clearance[8,12–14]. For example, diabodies (60 kDa) are efficacious with short-lived radioisotopes for clinical imaging as a result of the fast clearance rates[12–14]. Larger molecules, such as minibodies (90 kDa), are used with long-lived radioisotopes and are suitable for tumor therapy because they achieve a higher total tumor 'load'[14]. Fab dimers (110 kDa) have also been effective in preclinical studies[19].

The short half-life of antibody fragments can also be extended by 'pegylation', that is, a fusion to polyethylene glycol (PEG)[20]. Renal and hepatic localization of intact radiolabeled antibody fragments constitutes a major problem. An important study demonstrated that a previously undescribed radioiodination reagent could liberate radionuclide from the antibody fragment before incorporation into renal cells[21]. The radionuclide is excreted rapidly, thus decreasing the total renal radiation dose[21]. The development of new metabolizable chelates will further improve the pharmacokinetics of recombinant antibodies for cancer targeting. Modifications to surface charge designed to alter the isoelectric point (pI), such as glycolation, can also reduce the tissue (kidney) uptake[22]. The improved functional affinity, tumor penetration and biodistribution of these engineered antibody fragments will stimulate the development of a new generation of reagents for imaging

Engineering multiple specificity in antibody fragments

Bispecific antibodies contain two different binding specificities fused together and, in the most simple example, bind to two adjacent epitopes on a single target antigen, thereby increasing the avidity. Alternatively, bispecific antibodies can cross-link two different antigens and are powerful therapeutic reagents, particularly for recruitment of cytotoxic T cells for cancer treatment[23,24]. Bispecific antibodies can be produced by fusion of two hybridoma cell lines into a single 'quadroma' cell line; however, this technique is complex and time-consuming, and it produces unwanted pairing of the heavy and light chains. Far more effective methods to couple two different Fab modules incorporate either chemical or genetic conjugation or fusion to adhesive heterodimeric domains, including designed CH3 domains[23,24]. Bispecific diabodies provide an innovative alternative therapeutic[25] (Fig. 1).

Bifunctional antibodies. The original 'magic bullet' concept is still alive: antibodies have been fused to a vast range of molecules that provide important ancillary functions after target binding. These include radionuclides (discussed earlier) and also cytotoxic drugs, toxins, peptides, proteins, enzymes and viruses, the latter for targeted gene therapy[26–30]. For cancer therapy, bifunctional antibodies are engineered to effectively target tumor-associated antigens at low levels and then deliver a cytotoxic payload to tumor cells. The latest antibody-toxin conjugates are stable *in vivo* and minimally immunogenic[28,29]. Antibodies have also been fused to lipids and PEGs[20], both to enhance *in vivo* delivery and pharmacokinetics and to direct drug-loaded liposomes[31,32]. As immunoliposomes, anti-transferrin receptor antibodies have been used to deliver drugs to the brain, passing through the blood-brain barrier[32]. Antibody-enzyme fusions have also been developed for prodrug activation, primarily for cancer therapy[33].

Antibody libraries: construction, display and selection. Library display has superseded hybridoma technology for the selection of human antibodies through the creation of large nat-

libraries, specific high-affinity antibodies can be selected by linking phenotype (binding affinity) to genotype, thereby allowing simultaneous recovery of the gene encoding the selected antibody. Antibodies are usually displayed as monovalent Fab or scFv fragments and then, as required, reassembled into intact Ig or multivalent variants after selection[34,37]. If the repertoire is sufficiently large, a high-affinity Fab or scFv can be selected directly or, more frequently, the recovered gene can be subjected to cycles of mutation and further selection to enhance affinity (Fig. 2). Furthermore, new methods of selection and screening have been designed to specifically isolate antibodies with desired characteristics, such as enhanced stability, high expression or capacity to activate receptors[38,39].

Bacteriophage display. Fd phage and Fd phagemid technologies are currently the most widely used *in vitro* methods for the display of large repertoires and for the selection of high-affinity recombinant antibodies against a range of clinically important target molecules[35,37,40,41]. Innovative selection methods have proved powerful for isolating antibodies against previously refractory antigens, such as new tumor-associated antigens, cell surface receptors and HLA-A1-presented peptides[35,42]. Important improvements in selection technology have included array screening for high-avidity antibodies[43] and recovery of internalized phage from live cells to select against internalizing (human) receptors[38]. Phage technology has been applied to complete proteome analysis using membrane-based screening[41]. The latest methods for generating large phage libraries and avoiding the limitations imposed by bacterial cell transformation are discussed in the Supplementary Note online.

Libraries of mRNA-protein complexes. Ribosome display relies on stabilized complexes of antibody, ribosome and mRNA to replace bacteriophage as the display platform[39,44,45]. Ribosome complexes are constructed totally *in vitro*, thereby eliminating the need for cell transformation and allowing the production of large libraries, $\leq 10^{14}$ members. The system is limited only by the requirement of a ribonuclease-free environment for selection and buffer compositions suitable for antibody folding. Indeed, picomolar affinity antibodies have been selected and rapid affinity maturation cycles carried out using this innovative *in vitro* method[39,45]. Covalent display using puromycin-stabilized mRNA-protein complexes is an alternative strategy to ribosome display[46,47].

Cell surface libraries. Before the advent of bacteriophage systems, antibodies had been displayed on or in bacterial cells, although replica plating had limited screening to libraries of $<10^8$. The recent development of high-speed flow cytometers has re-activated the efforts in cell surface display, and several high-affinity antibodies have been isolated by this method[48,49].

Transgenic mice. Transgenic mice have been produced that lack the native mouse immune repertoire and instead harbor most of the human antibody repertoire in the germline. Injection of antigens into these mice leads to the development of human antibodies that have undergone mouse somatic hypermutation and selection to relatively high affinity[7]. Antibodies can be recovered by classic hybridoma technology or, for more efficient affinity enhancement, by *in vitro* display and selection technologies (Fig. 2).

Production, stability and expression levels

Production of antibodies for preclinical and clinical trials has been evaluated in numerous expression systems, including bacteria, yeast, plant, insect and mammalian cells. Bacteria are favored for expression of small, non-glycosylated Fab and scFv fragments, usually with terminal polypeptides such as c-Myc, His or FLAG, for affinity purification[11]. Mammalian or plant cells are favored for intact antibodies and, occasionally, also for expression of scFvs, diabodies and minibodies[14,50] There is still hope that eukaryotic cell cultures, such as those of the yeast *Pichia pastoris*, will allow efficient production of fully processed scFvs, albeit with high-mannose oligosaccharides[51]. Additional expression methods are discussed in the Supplementary Note online.

Affinity maturation

Both transgenic mice and display libraries typically produce human antibodies with binding affinities (K_D) ranging from 10^{-7} to 10^{-9} M. Obtaining higher-affinity antibodies is important for efficient binding to the antigenic target for *in vitro* diagnosis, viral neutralization, cell targeting and *in vivo*

Table 1 FDA-approved therapeutic antibodies

Product name[a]	Specificity	Product type	Indication	Year
Orthoclone OKT3	CD3	Mouse	Transplant rejection	1986
ReoPro	GpIIb/gpIIa	Chimeric Fab	Cardiovascular disease	1994
Rituxan	CD20	Chimeric	Non-Hodgkin lymphoma	1997
Zenapax	CD25	Humanized	Transplant rejection	1997
Remicade	TNF-α	Chimeric	Crohn disease, rheumatoid arthritis	1998, 1999
Simulect	CD25	Chimeric	Transplant rejection	1998
Synagis	RSV	Humanized	Respiratory syncytial virus	1998
Herceptin	Her-2	Humanized	Metastatic breast cancer	1998
Mylotarg	CD33	Humanized	Acute myeloid leukemia	2000
CroFab	Snake venom	Ovine Fab	Rattlesnake antidote	2000
DigiFab	Digoxin	Ovine Fab	Digoxin overdose	2001
Campath	CD52	Humanized	Chronic lymphocytic leukemia	2001
Zevalin	CD20	Mouse	Non-Hodgkin lymphoma	2002

[a]Product names are registered trademarks. Recent developments in FDA approvals can be obtained from http://www.fda.gov/cber/efoi/approve.htm. Updates on products relevant to lymphoma immunotherapy can be obtained from http://www.lymphomainfo.net/therapy/immunotherapy/. The latest product developments, antibody formulation and prescribing details can usually be obtained from http://www.productname.com or the manufacturer's Internet site.

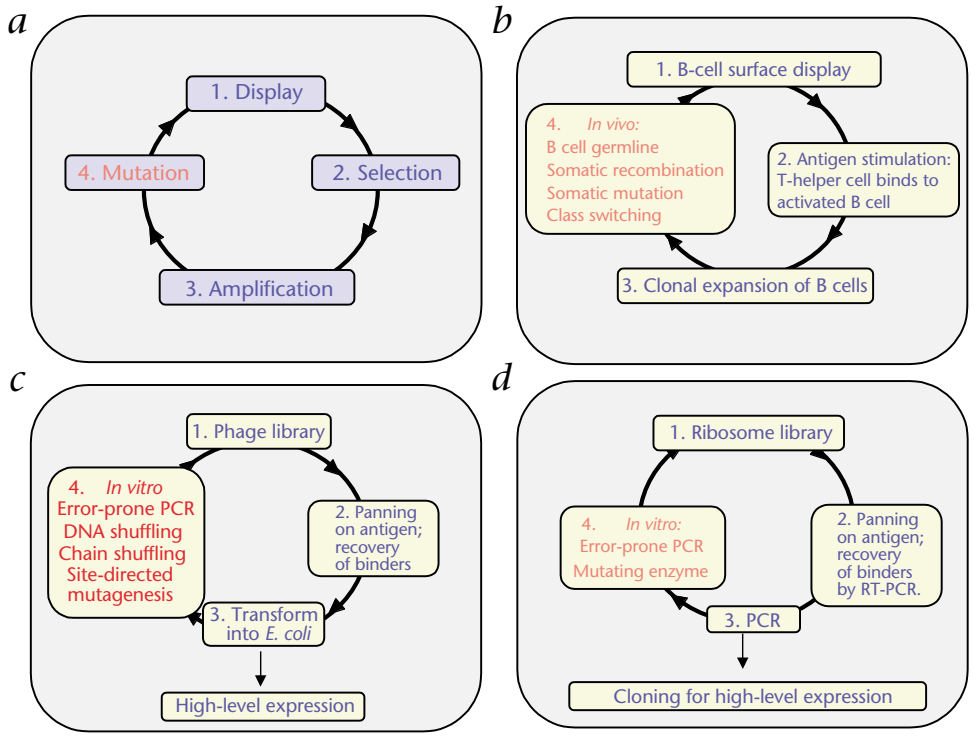

Fig. 2 Affinity maturation cycles. ***a***, General strategy of protein display, selection (gene recovery), amplification and gene mutation. For the specific affinity maturation cycles (*b–d*), alternative technologies are listed for each component of the cycle. ***b***, Mammalian *in vivo* process of antibody maturation[52]. ***c***, Bacteriophage display of engineered antibody repertoires (library) using an *in vitro* cycle of mutation and selection. ***d***, Ribosome display of engineered antibody repertoires (library) with an *in vitro* cycle of mutation and selection.

imaging. To improve antibody affinity, various *in vitro* strategies have recently been optimized to mimic the mammalian *in vivo* process of somatic hypermutation and selection[52] (Fig. 2). These include site-specific mutagenesis based on structural information, combinatorial mutagenesis of complementarity-determining regions (CDRs), random mutagenesis of the entire gene or chain shuffling[39,48,53,54].

After a decade of developing library display strategies, it is now obvious that the most successful methods rely on several cycles of mutation, display, selection (recovery) and gene amplification (Fig. 2). These cycles of mutation and selection can be carried out using either *in vitro* or *in vivo* strategies and have been far more effective than precisely designed alterations for affinity enhancement[39,48]. Even with the most detailed structural information, the techniques for design of precisely complementary surfaces through interface mutations remain in their infancy.

Using the cycles depicted in Figure 2, affinity enhancement can be restricted to mutations in the antigen-binding surface (CDR loops). Importantly, mutations in the underlying framework regions have frequently provided large increases in affinity, stability and expression[39,45,48]. Random mutations over the entire V-domain genes can be derived from *Escherichia coli* mutator cells, homologous gene rearrangements or error-prone PCR. Sequential 'chain shuffling' of the two V genes in the Fv module is also 'random' but offers the advantage that only one V domain is altered at a time, while the other domain is kept constant to provide a defined specificity[55]. Recent advances include the incorporation of highly mutagenic enzymes such as mRNA reverse transcriptase and DNA polymerase with no proofreading activity to achieve a high gene mutation rate[56]. The integration of such polymerases into the ribosome display and selection process could rapidly generate large libraries

Alternative scaffolds

Intact antibodies, Fab and scFv fragments provide an antigen-binding surface comprising six CDR loops; these can be mutated, sequentially or collectively, to bind to a vast array of target molecules. Some target molecules are refractory to the immune repertoire, however, particularly those with cavities or clefts that require a small penetrating loop for tight binding. The natural mammalian antibody repertoire simply does not encode penetrating loops, and only rarely has this type of antibody been selected[57]. Unexpectedly, both camelids (camels, llamas and related species) and sharks produce natural, single V-like domain repertoires displaying cavity-penetrating CDR loops that complement the repertoire of conventional antibodies[58–60]. This theory has led to a number of attempts to design single-domain display libraries *in vitro*, based on V domains[61] and other Ig-like scaffolds[60,62]. These small molecules complement both antibody and peptide libraries and are expected to have improved pharmacokinetics for several clinical applications, including those that require access to buried (immunosilent) sites or clefts in enzymes, receptors and viruses[62]. Many important diagnostic targets, notably prions[63], have also been refractory to conventional antibodies, and we expect that new molecular libraries and scaffolds will be required to provide the required binding reagents.

Clinical applications

Aside from radioimmunotherapy (discussed earlier), there are a variety of other clinical applications of engineered antibodies for viral infection, cancer, autoimmune disease, allograft rejection, asthma, stroke and glaucoma surgery. Specific clinical applications are discussed below.

Pathogen neutralization and antiviral therapy. Antibody binding can directly and effectively block the activity of many pathogens, often without requiring Fc-mediated cytotoxicity. Indeed, this has always been the promise of antibody-mediated viral neutralization. The first monoclonal antibody for the treatment of viral disease, Synagis, was approved by the FDA in 1998 (Table 1). Synagis is a humanized antibody used for the prevention of severe respiratory syncytial virus (RSV) disease[64]. Despite this success, and the wide range of antibodies available

plex virus (HSV), the use of recombinant antibodies as therapeutics for viral infection has been limited[65]. Only a few rare antibodies have exhibited potent neutralization *in vitro* and antiviral efficacy in animal models[57]. This is probably due to viral efficiency both in producing escape mutants and in evolving immunosilent receptor-binding surfaces[65]. For neutralization of other pathogenic molecules, monomeric Fab molecules have recently been approved by the FDA as antivenenes (CroFab; Table 1), and both scFv fragments and oligoclonal antibody mixtures have been effective against bacterial toxins[66,67].

Intracellular antibodies. Antibody fragments can be expressed as intracellular proteins, typically as scFvs termed intrabodies, and equipped with targeting signals either to neutralize intracellular gene products or to target cellular pathways. For example, expression of $p21^{ras}$, erbB2, huntingtin and MHC have all been individually downregulated using antibodies[68–71]. Intrabodies also have important antiviral potential, particularly through their targeting of intracellular action to mandatory viral proteins such as the Vif, Tat or Rev components of HIV[72]. Antibody frameworks have been adapted that substantially improve expression levels and solubility in the intracellular reducing environment[73]. Direct *in vivo* selection from large libraries will greatly facilitate the isolation of many previously unknown intracellular antibodies or 'intrabodies'[74,75]. Obviously, the expression of intrabodies *in vivo* can be encoded into gene therapy vectors, and this could ultimately be their most powerful clinical application.

Cancer therapy and cell recruitment strategies. The promise of engineered antibodies for effective cancer therapy, especially radioconjugates, has been described earlier. Blocking angiogenesis to prevent the establishment and growth of tumors is becoming an important strategy[76,77]. Cancer cells can be destroyed by cell recruitment of cytotoxic T cells, natural-killer (NK) cells or macrophages that can be targeted by encoding cell surface antibodies (usually scFv)[78]. Alternative cell recruitment strategies include bifunctional antibodies, fused to cytokines for T-cell stimulation and proliferation at the tumor site[79].

Innovative vaccine applications. Troybodies are engineered vaccine antibodies containing cryptic T-cell epitopes to enhance antigen presentation[80]. Troybodies effectively target antigen-presenting cells (APCs) and, after processing, expose cryptic T-cell epitopes to direct T-cell activation. In the preferred format, the Fv domain provides APC specificity and the C domains encode the cryptic T-cell epitopes. These new vaccines can be redesigned to target many different APCs and enhance immunity to many different T-cell epitopes. Alternative vaccine strategies include the use of engineered APC-targeted antibodies that direct adenoviruses to deliver vaccine-inducing epitopes as a gene therapy capsule[30] and B7-targeted scaffolds (scFv and V_L domains) that enable antigen-loading of dendritic cells[61].

Biosensors and microarrays: the future of diagnosis. A likely prediction is that biosensing devices and microarrays will dominate the *in vitro* diagnostic market by 2005. Antibodies currently provide high-sensitivity reagents for a huge range of diagnostic kits, accounting for approximating 30% of the $20 billion per year diagnostic industry. It is therefore not surprising that antibodies are the paradigm for proof-in-principle of new rays[81]. Already in 2002 we have seen more protein-friendly surfaces being developed as array platforms for antibody-based diagnosis (Triage from Biosite, San Diego, California; Protein Profiling Biochip from Zyomyx, Hayward, California; Hydrogel from Perkin-Elmer, Boston, Massachusetts). These platforms will become increasingly available over the next few years, driven by the demand for new reagents to diagnose the vast array of biomarkers stemming from proteomics discovery programs. These platforms will also be developed for robust *ex vivo* applications, including the detection of microbial contaminants, pesticides and biological (warfare) pathogens[82].

Conclusion

During the past few years, there has been growing excitement in both scientific and commercial communities in engineered antibodies. Scientific interest has stemmed from the elucidation of the key elements required for antibody fragment design, efficient expression and desired pharmacokinetics. Commercial interest is driven by increasing sales (Table 1). Many of the antibody fragments and fusion proteins discussed in this review are now undergoing scale-up production. It is likely that we will soon witness development of display and screening technologies incorporating nanoarray robotics. By providing a highly stable, protease-resistant scaffold, engineered recombinant antibody fragments will continue to be the model for selection of high-affinity clinical targeting reagents.

Note: Supplementary information is available on the Nature Medicine *website.*

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