nature biotechnology

Engineered antibody fragments and the rise of single domains

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With 18 monoclonal antibody (mAb) products currently on the market and more than 100 in clinical trials, it is clear that engineered antibodies have come of age as biopharmaceuticals. In fact, by 2008, engineered antibodies are predicted to account for >30% of all revenues in the biotechnology market. Smaller recombinant antibody fragments (for example, classic monovalent antibody fragments (Fab, scFv) and engineered variants (diabodies, triabodies, minibodies and single-domain antibodies) are now emerging as credible alternatives. Smaller recombinant antibody fragments (for example, classic monovalent antibody fragments (Fab, scFv)) and engineered variants (diabodies, triabodies, minibodies and single-domain antibodies) are now emerging as credible alternatives. These fragments retain the targeting specificity of whole mAbs but can be produced more economically and possess other unique and superior properties for a range of diagnostic and therapeutic applications. Antibody fragments have been forged into multivalent and multispecific reagents, linked to therapeutic payloads (such as radionuclides, toxins, enzymes, liposomes and viruses) and engineered for enhanced therapeutic efficacy. Recently, single antibody domains have been engineered and selected as targeting reagents against hitherto immunosilent cavities in enzymes, receptors and infectious agents. Single-domain antibodies are anticipated to significantly expand the repertoire of antibody-based reagents against the vast range of novel biomarkers being discovered through proteomics. As this review aims to show, there is tremendous potential for all antibody fragments either as robust diagnostic reagents (for example in biosensors), or as nonimmunogenic *in vivo* biopharmaceuticals with superior biodistribution and blood clearance properties.

Antibodies have proven to be an excellent paradigm for the design of high-affinity, protein-based binding reagents. Innovative recombinant DNA technologies, including chimerization and humanization, have enhanced the clinical efficiency of murine mAbs and, in the past decade, have led to regulatory approvals for immunoglobulin (Ig) and classic monovalent antibody fragment (Fab) molecules as treatments of cancer, infectious disease and inflammatory disease (see also p. 1147). Recently, recombinant mAbs have been dissected into minimal binding fragments, rebuilt into multivalent high-avidity reagents and fused with a range of molecules limited only by the imagination, including enzymes for prodrug therapy, toxins for cancer treatment, viruses for gene therapy, cationic tails for DNA delivery, liposomes for improved drug delivery and biosensors for real-time detection of target molecules. New structural designs have improved *in vivo* pharmacokinetics, expanded immune repertoires and enabled selection against refractory targets and complex proteome arrays. At the same time, new molecular evolution strategies have enhanced affinity, stability and expression levels. Redesigned mAb and antibody-based-like fragments[1–7] are poised to provide the next wave of antibody-based reagents for immunotherapy and *in vivo* medical imaging, with many already in late-phase clinical trials[8]. Recombinant antibody fragments are also expected to capture a significant share of the $6 billion (US) per year diagnostic market, from *in vitro* immunoassays to *in vivo* tumor- and clot-imaging applications.

This review provides an update of this fast-moving field, evaluates some of the emerging new technologies and describes the creation of a vast range of new, engineered, antibody-based fragments that specifically target biomarkers of human health and disease. We also describe the latest technologies used to deliver therapeutic 'payloads,' such as radionuclides, drugs, enzymes and vaccine-inducing epitopes.

Natural and synthetic fragment design

Intact antibodies (IgG, IgM, IgA, IgE) are highly specific targeting reagents and provide our key defense against pathogenic organisms and toxins. IgG, the main serum antibody and the intact format almost exclusively used in therapeutic antibodies, is a Y-shaped, multidomain protein with antigen-binding sites located on the two Fab tips and recruitment of effector functions mediated by the stem Fc domain (**Fig. 1**).

IgG antibodies are bivalent; the ability to bind to two antigens greatly increases their functional affinity and confers high retention times (also called avidity) on many cell-surface receptors and polyvalent antigens. The Fc domain recruits cytotoxic effector functions through complement and/or through interactions with γFc receptors (Fc receptors for gamma globulins) and can provide long serum half-lives (>10 days) through interaction with the neonatal Fc receptor (FcRn), which acts as a salvage receptor (binding and transporting IgGs in intact form both within and across cells and rescuing them from a default degradative pathway)[9,10].

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Published online 7 September 2005; doi:10.1038/nbt1142

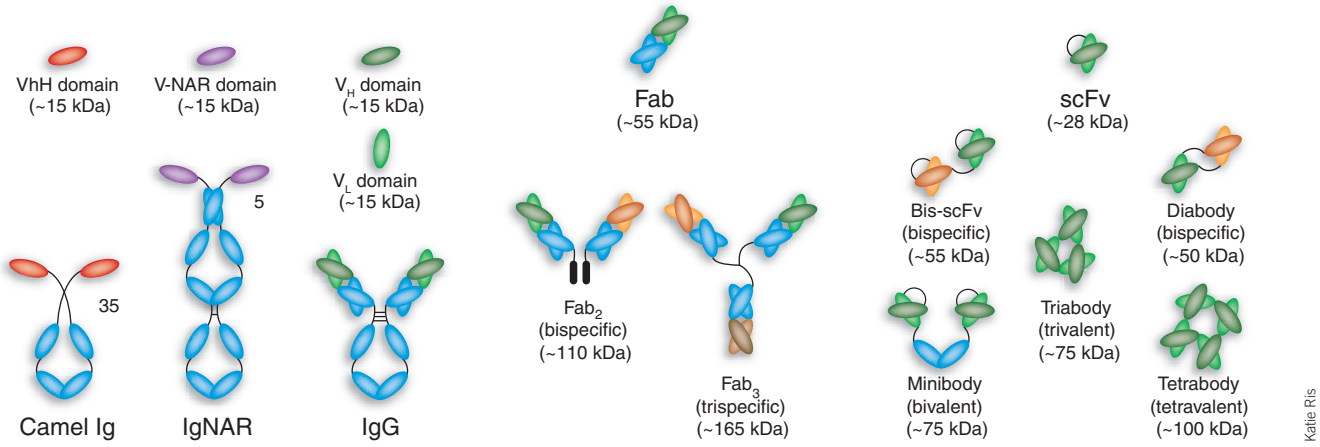

Figure 1 Schematic representation of different antibody formats, showing intact 'classic' IgG molecules alongside camelid VhH-Ig and shark Ig-NAR immunoglobulins. Camelid VhH-Ig and shark Ig-NARs are unusual immunoglobulin -like structures comprising a homodimeric pair of two chains of V-like and C-like domains (neither has a light chain), in which the displayed V domains bind target independently. Shark Ig-NARs comprise a homodimer of one variable domain (V-NAR) and five C-like constant domains (C-NAR). A variety of antibody fragments are depicted, including Fab, scFv, single-domain V_H, VhH and V-NAR and multimeric formats, such as minibodies, bis-scFv, diabodies, triabodies, tetrabodies and chemically conjugated Fab´ multimers (sizes given in kilodaltons are approximate).

There is a range of applications, however, in which the Fc-mediated effects are not required and are even undesirable. For example, a long serum half-life results in poor contrast in imaging applications, and inappropriate activation of Fc receptor–expressing cells can lead to massive cytokine release and associated toxic effects. To remove the Fc domain (and associated effects), IgGs have been dissected into constituent domains, initially through proteolysis (with such enzymes as papain and pepsin) and later genetically engineered into either monovalent (Fab, scFv, single variable V_H and V_L domains) or bivalent fragments (Fab′$_2$, diabodies, minibodies, etc.)(**Figs. 1** and **2**). Many of these products are now in clinical and preclinical trials, and a list of some leading candidates is presented in **Table 1**.

Fab, Fv and single V-type domains. Single-chain Fvs are a popular format in which the V_H and V_L domains are joined with a flexible polypeptide linker preventing dissociation (**Fig. 1**). Antibody Fab and scFv fragments, comprising both V_H and V_L domains, usually retain the specific, monovalent, antigen-binding affinity of the parent IgG, while showing improved pharmacokinetics for tissue penetration (see p. 1137).

In a seminal early publication[7], mouse single variable (V) domains were shown to be functional, and it was proposed that, because of their smaller size, they could potentially target cryptic epitopes. Indeed, to escape immunosurveillance, many pathogenic viruses have evolved narrow cavities (canyons) in their surface antigens, which bind their target receptors but are poorly accessible to intact antibodies and are thus largely immunosilent. This 'blind spot' of the antibody response is caused by the limited diversity of complementarity-determining region (CDR) loop lengths, which constrains the displayed antigen-binding surfaces to mostly flat or concave topologies[8,11] (**Fig. 2**). Only rarely are antibodies selected that provide penetrating loops into the target antigen, such as the anti- HIV mAbs 4E10, B12 and m14 (refs. 11,12). Indeed, there may only be a vestige of cavity-penetrating immunity in higher mammals, such as the V-like domains of 'neonatal' T-cell receptor gamma, which comprise an unusually long CDR3 loop[13].

Despite early excitement concerning the functional activity of single V domains[7], these fragments remained laboratory curiosities because they rarely retained the affinity of the parent antibody and were also poorly soluble and often prone to aggregation. Interest was recently revived when it was discovered that at least two types of organisms, the camelids (camels and llamas) and cartilaginous fish (wobbegong and nurse sharks), have evolved high-affinity single V-like domains (called VhH in camelids and V-NAR in sharks), mounted on an Fc-equivalent constant domain framework as an integral and crucial component of their immune system[14–18] (**Fig. 1**). Camelid VhH and shark V-NAR domains each display long surface loops, often larger than for conventional murine and human antibodies, and are able to penetrate cavities in target antigens, such as enzyme active sites (for example, lysozyme[19]) and canyons in viral and infectious disease biomarkers (including malaria apical membrane antigen-1 (refs. 18,20; **Fig. 2**)). Ig-NARs concentrate diversity in the elongated CDR3 regions, varying from 5 to 23 residues in length, although usually they are between 15 and 17 residues long[16]. The loops are typically stabilized by disulfide bonds and can extend over 20 Å from the immunoglobulin framework (**Fig. 2b–e**) or collapse over the V-domain surface and hide disadvantageous hydrophobic patches[15,19,21].

Unlike mouse V_H domains[7], camelid VhH and shark V-NAR domains are in general soluble and can be produced as stable *in vitro* targeting reagents for sensitive diagnostic platforms and nanosensors[20,22,23]. Compared to monoclonal antibodies, camelid single VhH domains have also demonstrated improved penetration against cryptic (immuno-evasive) target antigens such as trypanosome surface glycoproteins[24]. However, for *in vivo* administration, humanization (or deimmunization) may be crucial to reduce immunogenicity, although llama VhH domains have been claimed to be only minimally immunogenic[25]. Nevertheless, for *in vivo* applications, human single domains would be preferable, provided problems of poor stability and solubility can be solved. Very recently, these seem to have been overcome, or at least greatly reduced, for some V domains by the identification and design of mutations that minimize the hydrophobic interface[26–28] and by direct selection from phage libraries. As is frequently observed with display technologies, favorable properties (such as good expression, stability and solubility) are co-selected with binding activity. For example, human V_H- and variable κ light chain ($V_κ$)-domain antibodies

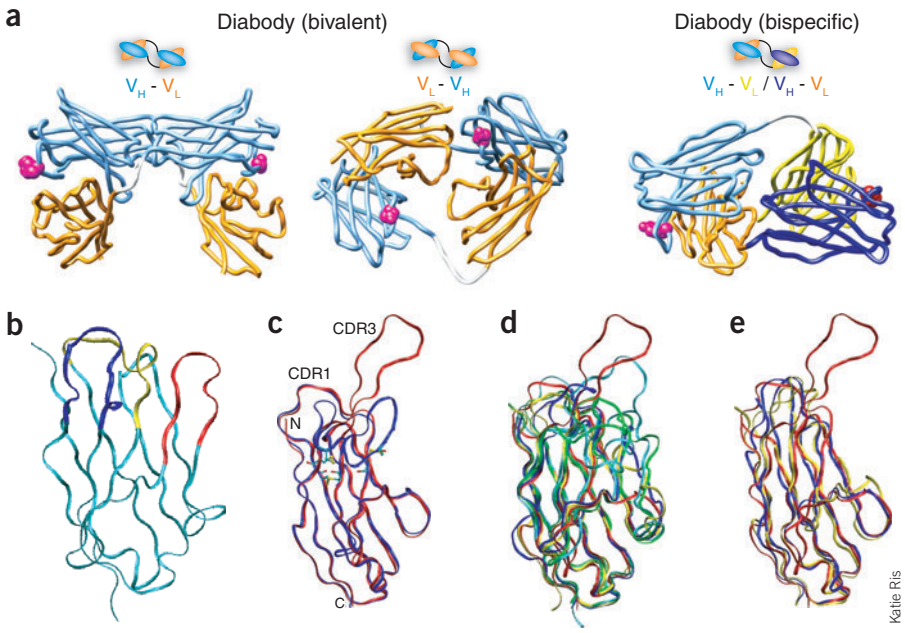

Figure 2 Structural comparison of antibody fragments and single domains. (**a**) Structural comparison of bivalent and bispecific diabodies showing the flexibility of the Fv heads. From left to right, a mouse antiphospholipase diabody (chain linkage V_H-V_L) (Protein Data Bank (PDB) identifier 1LMK), mouse anti-CEA diabody (chain linkage V_L-V_H) (PDB identifier 1MOE) and a bispecific anti-CEA (MFE-23)/anti-CD3 (OKT-3) diabody[120] (chain linkage V_H-V_L; X.Y. Pei, P.H. & R.L. Williams, unpublished data). To show the relative orientations of the 'Fv heads' in the diabody structures, the central residue in V_H CDR3 is shown (colored magenta). Worm representations were generated using the program UCSF Chimera[121]. Structural comparison CDR orientations of functionally active single domains depicted as visual molecular dynamics ribbon diagrams. (**b**) Human V_H domain (dAb) HEL4 (PDB identifier 1OHQ, chain A) with three CDR loops H1 (yellow), H2 (red) and H3 (blue). (**c**) Superimposed shark V-NARs: type 1 (PDB identifier 1SQ2) and type 2 (12Y-2; PDB identifier 1VES) in blue and red, respectively, showing CDR loops, cysteine residues and disulfide bonds. (**d**) Superimposed shark V-NAR (red) (12Y-2); human TCR V_α (blue) (PDB identifier 1A07); human V_H (green) and V_L (yellow) (PDB identifier 1IGM); and camel V_HH (cyan) (PDB identifier 1MEL). (**e**) Superimposed V-NAR (12Y-2) A chain (red); human telokin (blue) (PDB identifier 1FHG); and human NCAM domain 1 (yellow) (PDB identifier 1QZ1). Unlike the relatively 'flat' surface of mouse Fv and human V_H antigen-binding surfaces shown in **b**, both camelid VhH and shark V-NAR display CDR3 loop structures that can extend far (over 20 Å) beyond the V-domain scaffold shown in **c**,**d**,**e**, suggesting a distinct selective advantage in accessing cryptic antigenic epitopes. Although this is an attractive interpretation, alternative conformations for the long CDR3 loops in the camelid VhH and shark V-NAR repertoires have been observed (not shown) and appear overlaid across the V-domain surface, thus masking disadvantageous hydrophobic patches (particularly type 1 V-NAR). Shark V-NAR domains are also lacking the CDR2 loop of human V_H and camelid VhH domains, shown in **c**,**d**, and have two disulfide bonding configurations: type 1, two conserved cysteines within framework regions form disulfide bridges with matching residues within the CDR3, pulling the loop out laterally; and type 2, a disulfide bridge usually links the CDR1 and CDR3 regions, shown in **c**. Thus, camelids and sharks have evolved different structures that can either display extended CDR3 loops to access cleft-like epitope targets or provide a stable, soluble scaffold to display antibody-like nonprotruding CDR surfaces. Shark V-NARs structurally align and are presumably related to primordial versions of the I-set immunoglobulins, including members of the cell adhesion family NCAM, shown in **e**.

selected by phage display have been found to have good thermodynamic stability (26–44 kJ mol^{-1}) and solubility[28]. In addition, improved folding yield can be obtained by selecting V domains that resist aggregation when unfolded on phage[29]. Alternative scaffolds can be made from human V-like domains (N-CAM; telokin) that more closely match the shark V-NAR scaffold and thereby provide a similar underlying framework for displaying long penetrating loop structures[18] (**Fig. 2**).

The future of all small single domains is clearly to provide new binding specificities, particularly to targets inaccessible to conventional antigen-binding sites, such as G protein–coupled receptors, enzyme active sites and many viral surface canyons. We anticipate that the vast array of new targets generated by proteomic and metabolomic technologies will drive the development of new affinity reagents required to dissect both structure and function and yield new applications in diagnosis and therapy.

Indeed, there are competing scaffolds that access cryptic and other target sites, including a minimum protein fold of 23 residues described by P.J.H. and colleagues[30] as well as other highly stable engineered protein domains (reviewed in ref. 31). These will, however, have to compete with mAbs and antibody fragments, in which detailed structural analysis is beginning to yield increasingly sophisticated insights into critical parameters of antigen binding. In a striking example, a single designed point mutation increased hapten-binding affinity of an scFv fragment by 1,800-fold (ref. 32). Another example is the grafting of prion (PrPC) peptides into CDR loops to create a mimetic interaction surface recapitulating the interaction of PrPC with the elusive infectious prion form (PrPSc)[33]. Indeed, the engineered PrPC-mAb bound specifically to infective fractions of PrP in mouse, human and hamster prion-infected tissues, but not to PrPC, other cellular components or the HIV-1 envelope[33]. PrPSc-specific mAbs produced by the approach described may find widespread application in the detection of infectious prions in human and animal materials. However, even with the most detailed structural information, techniques for the design of precisely complementary surfaces via interface mutations remain in their infancy. The challenges in engineering antibody binding sites are underlined by a series of remarkable structures showing how an antibody binding site is capable of interacting with several unrelated antigens through side chain and main chain rearrangement[34].

Multivalent designs. In the nonequilibrium environment of the vasculature and tissues, even high-affinity monovalent interactions provide fast dissociation rates and only modest retention times on the target antigen. For many applications, it is therefore desirable to engineer the monovalent Fab, scFv fragments or V-domain molecules into multivalent molecules, which show significant increases in functional affinity (termed avidity) and significantly slower dissociation rates for cell-surface or multimeric antigens (**Fig. 1**).

Apart from increased avidity, multivalent engagement of cell-surface receptors (for example, CD20, epidermal growth factor receptor (EGFR) or CD28) can result in a desired activation and/or apoptosis through transmembrane signaling pathways, which may form an

Table 1 Antibody fragments in clinical and preclinical development

Fragment type/ format	Brand name (generic name)	Specificity/ target antigen	Stage	Indication	Reference or website
Fab/chimeric	ReoPro (abciximab)	GpIIb/gpIIa	FDA approved	Cardiovascular disease	http://www.lilly.com/
Fab/ovine	CroFab	Snake venom	FDA approved	Rattlesnake bite (antidote)	http://www.savagelabs.com/
Fab/ovine	DigiFab	Digoxin	FDA approved	Digoxin overdose	http://www.savagelabs.com/
Fab/ovine	Digibind	Digoxin	FDA approved	Digoxin overdose	http://www.gsk.com/
Fab/mouse	CEA-scan (arcitumomab)	CEA	FDA approved	Colorectal cancer imaging	http://www.immunomedics.com/
Fab/humanized	Lucentis (ranibizumab; Rhu-Fab)	VEGF	Phase 3	Macular degeneration	http://www.gene.com/
Fab/humanized	Thromboview	D-dimer	Phase 1	Deep vein thrombosis imaging	http://www.agenix.com/
Fab/PEGylated humanized	CDP791	VEGF	Phase 1	Cancer (antiangiogenesis)	http://www/nektar.com
Fab/PEGylated humanized	CDP870	TNF-α	Phase 3	Crohn disease	http://www.nektar.com
Fab/bispecific humanized	MDX-H210	Her2/Neu & CD64 (γFcR1)	Phase 2	Breast cancer	http://www.medarex.com/
Single-chain Fv (scFv)/humanized	Pexelizumab	Complement C5	Phase 2/3	Coronary artery bypass	http://www.alexionpharmaceuticals.com/
$(ScFv)_4$fused to streptavidin mouse	CC49	TAG-72 Pancarcinoma antigen	Phase 1	Pretargeting radioimmuno-therapy for gastrointestinal malignancies	123
ScFv fused to β-lactamase human	SGN-17	P97 antigen	Preclinical	Melanoma (ADEPT prodrug activation)	http://www.seagen.com/
ScFv fused to PEG human	F5 scFv-PEG Immunoliposome	Her2	Preclinical	Breast cancer as drug targeting	124
Diabody $(V_H\text{-}V_L)_2$ human	C6.5K-A	Her2/Neu	Preclinical	Ovarian and breast cancer	54
Diabody $(V_H\text{-}V_L)_2$ human	L19 L19–γIFN	EDB domain of fibronectin	Preclinical	Antiangiogenesis and atherosclerotic plaque imaging	125
Diabody $(V_L\text{-}V_H)_2$ human	T84.66	CEA	Preclinical	Colorectal cancer imaging	43
Minibody $(scFv\text{-}C_H3)_2$ murine-human chimera (minibody)	T84.66	CEA	Human Imaging Pilot Study	Colorectal cancer imaging pretherapy	126
Minibody murine-human chimera (minibody)	10H8	Her2	Preclinical	Ovarian and breast cancer	121
ScFv dimer Fc $(ScFv)_2$-Fc murine-human chimera (minibody)	T84.66	CEA	Preclinical	Colorectal cancer	48
Bispecific scFv $(V_L\text{-}V_H\text{-}V_H\text{-}V_L)$ mouse	r28M	CD28 and MAP	Preclinical	Melanoma (MAP antigen)	119
Bispecific scFv $(V_L\text{-}V_H\text{-}V_H\text{-}V_L)$ origin unknown	BiTE MT103	CD19 and CD3	Phase 1	B-cell tumors (non-Hodgkin lymphoma, acute and chronic lymphocytic leukemia)	http://www.micromet.de/
Bispecific scFv $(V_L\text{-}V_H\text{-}V_H\text{-}V_L)$ origin unknown	BiTE	Ep-CAM and CD3	Preclinical	Colorectal cancers	http://www.micromet.de/
Bispecific tandem diabody (VH-VL- VH -VL) (mouse)	Tandab	CD19 & CD3	Preclinical	B-cell tumors (non-Hodgkin lymphoma, acute and chronic lymphocytic leukemia)	www.affimed.de
VhH–β-lactamase fusion camelid	Nanobody	CEA	Preclinical	Cancer imaging	25
Dab/human	Anti-TNFα dAb	TNFα	Preclinical	Rheumatoid arthritis and Crohn disease	http://www.domantis.com/ http://www.peptech.com/
VhH/camelid	Nanobody	TNFα	Preclinical	Rheumatoid arthritis and Crohn disease	http://www.ablynx.com/
VhH/camelid	Nanobody	Von Willebrand factor	Preclinical	Antithrombotic	127

important aspect of the therapeutic efficiency of some antibodies[35,36]. Finally, some multivalent formats lend themselves to conversion into multispecific molecules (**Figs. 1** and **2**), which allow the direct association of two different targets with important applications in recruitment of effector functions, cytotoxic cells or gene delivery capsules and immunodiagnostics.

Fab and scFv fragments have also been engineered into dimeric, trimeric or tetrameric conjugates using either chemical or genetic cross-links (**Fig. 1**). For example, Fab fragments have been chemically cross-linked into di- and trivalent multimers, which have improved retention and internalization properties as compared with the parent IgG[37,38]. Of the various strategies to genetically encode multimeric scFv, the most successful design has been the reduction of scFv linker length to between zero and five residues, which directs self-assembly into either bivalent dimers (diabodies, 55 kDa)[4], trivalent trimers (triabodies[5,6] (80 kDa)[5,6], or tetravalent tetrabodies (110 kDa[39,40]; **Fig. 1**). Shortening the scFv linker to fewer than five residues affects both multimerization and stability, in a manner dependent on V-domain orientation and interface residue sequence. In one example, CD22-targeting diabodies with linkers zero residues in length were substantially more stable than the five-residue form when incubated in human serum at 37 °C[41]. As discussed later in the section on pharmacokinetics and biodistribution, these stable diabodies have outperformed chemically conjugated Fab and scFv dimers *in vivo*[42]. Notably, all these multimers are smaller than intact immunoglobulin molecules (150 kDa) and provide improved tumor penetration and faster blood clearance, resulting in enhanced cancer imaging and radiotherapy applications.

Bivalent diabodies can be stabilized by disulfide linkage[43]. Recently, stable Fv tetramers have been generated by noncovalent association in $(scFv_2)_2$ configuration[44] or as bispecific bis-tetrabodies[45]. For these V-domain chains and related multimeric designs, the initial preclinical results are encouraging (see later section and **Table 1**), although the additional linker residues can be exposed and thereby result in increased protease degradation[45].

The comparative avidity or effectiveness at triggering receptor signaling[35,36] of multivalent formats on different targets will depend on numerous factors, such as the density and accessibility of adjacent epitopes as well as the conformational flexibility of both target antigens and binding modules of the multimer (for example, between Fv head groups in a diabody; **Fig. 2a**). This has been accurately analyzed by single-molecule imaging using electron microscopy[39], cryoelectron microscopy showing diabody cross-linking (P.J.H., ref. 46) and crystallographic and modeling studies showing Fv angle flexibility diabodies[47] (**Fig. 2a**). Indeed, diabodies[39] and di-Fab designs[37] have been shown to be extremely flexible structures, showing potential cross-linking engagement angles from 60° to 180° between target molecules. Their high functional affinity, stability and favorable pharmacokinetics make multivalent mAb fragments prime candidates for clinical evaluation.

Optimization of fragment targeting

The efficiency of mAbs and their fragments *in vivo* for cancer therapy lies in their capacity to discriminate tumor-associated antigens at low levels. Antibody therapy has been more successful against circulating cancer cells than solid tumors because of the greater accessibility of lymphoma and leukemia cells to intact mAbs (see p. 1137).

Pharmacokinetics and biodistribution. Radiolabeled mAbs are important clinical reagents for both tumor imaging and therapy and also provide an ideal evaluation of pharmacokinetics[48,49]. Therapeutic administration requires a balance between prolonged retention at the target site and slow clearance—which can lead to liver accumulation and high radiation exposure of other tissues. The choice of radionuclide dictates both the application and the required pharmacokinetics: for example, ^{124}I for positron emission tomography (PET) imaging and ^{90}Y for radiotherapy[50] (see p. 1137 and p. 1147).

For solid tumors, there are the additional problems imposed by penetration through the vasculature and dispersion against an interstitial pressure, as first described by Jain and Baxter[51]. Recently, algorithms have been formulated that identify appropriate design criteria for cell targeting by inclusion of rates for diffusion, binding, internalization and systemic clearance[52]. Vascular penetration may be greatly enhanced by targeting fragments to surface molecules on endothelial caveolae, because these specialized plasmalemmal invaginations can transcytose across the normally restrictive endothelial cell barriers to reach underlying tumor cells[53]. This allows much greater penetration into, and accumulation throughout, solid tumors and should enable more effective delivery of imaging agents or therapeutic payloads.

Systematic *in vivo* studies have provided striking confirmation that size is an important parameter in pharmacokinetics and biodistribution of mAb molecules. Large IgG molecules (150 kDa) specific for tumor-surface molecules have been found to penetrate solid tumors only slowly, are nonuniform in their final distribution and have high serum levels and associated toxicities. Conversely, small scFv fragments (30 kDa) are cleared extremely rapidly and have poor tumor retention because of their monovalent binding properties (**Fig. 3**). The ideal tumor-targeting reagents are intermediate-sized multivalent molecules (for example, bivalent diabodies, 55 kDa), which provide rapid tissue penetration, high target retention and rapid blood clearance (**Fig. 3**). The most recent biodistribution studies[48,49] have independently confirmed that diabodies, because of their small size, are rapidly eliminated through the kidneys, thereby limiting the exposure to the bone marrow, which is most often the dose-limiting organ with intact radiolabeled mAbs. Diabodies possess an excellent combination of rapid tumor uptake and clearance for *in vivo* imaging when labeled with ^{123}I or ^{111}In and rapid xenograft visualization by PET[43,49,50] when labeled with positron emitters such as ^{64}Cu or ^{124}I. These studies set the stage for effective diabody radiotherapy. Indeed, a single intravenous dose of ^{90}Y-labeled diabody (150 μCi; $t_{1/2}$ = 64 h) inhibited growth rates of established Her-2 tumor xenografts in athymic nude mice[54]. Diabodies have also proven useful in imaging angiogenesis and atherosclerotic plaques[55,56].

Larger bivalent molecules, such as minibodies ($scFv\text{-}C_H3$ dimers), and $scFv_2$-Fc can accumulate to higher abundance in tumors (**Fig. 3**), and the latter can be designed with a spectrum of serum half-lives by modulating the interaction with the FcRn receptor. Minibodies may be ideal for tumor therapy because they achieve a higher total tumor uptake, substantially faster clearance and better tumor-to-blood ratios than either intact immunoglobulin (150 kDa) or Fab'_2 (110 kDa; ref. 57). Preclinical trials with Fv tetramers either as $(scFv_2)_2$ (ref. 44) or as bispecific bis-tetrabodies[45] have also been encouraging, although the additional linker residues can be exposed and thereby result in increased protease degradation.

Even so, tumor localization and uptake depends not only on size but also on other parameters such as interaction affinity. Notably, avidity seems to be more important than affinity, with diabodies generated from lower-affinity single-chain V_H- and V_L-domain fragments (scFvs) consistently achieving higher tumor uptake[58]. These results confirm an earlier hypothesis of Weinstein[59], who postulated that there would be an affinity threshold above which tumor penetration would be inhibited because of irreversible association with the first antigen encountered.

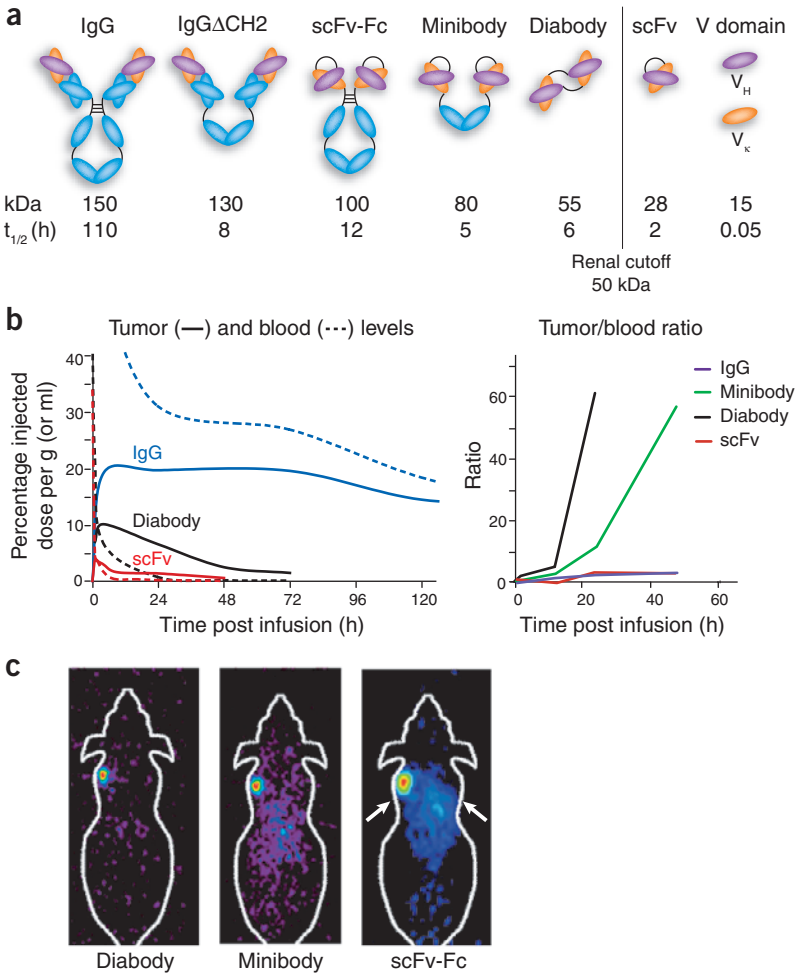

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Figure 3 Antibody formats used in imaging. (**a**) A cartoon representation of different antibody formats used for *in vivo* imaging, together with their respective molecular weights (kDa) and serum half-life (β phase). The scFv-Fc has been engineered for altered FcRn engagement[48,122]. (**b**) Biodistribution of different mAb formats in two xenograft models. Left graph: tumor (solid lines) and blood levels (dashed lines) plotted versus time after infusion (h) for anti-Her2 Mabs IgG 741F8 (blue), diabody C6.5 (black) and scFv C6.5 (red) in severe combined immune deficient (SCID) mice bearing solid subcutaneous SK-OV-3 tumors (expressing ~10^6 copies of Her2 per cell; G. Adams, personal communication). Right graph: tumor-to-blood ratios of anti-CEA T84.66 immunoglobulin, minibody, diabody and scFv in athymic mice with LS174 colon carcinoma xenografts plotted versus time after infusion (h)[43,48]. These biodistribution data, in two different murine (see **Table 1**) xenograft studies, show the superior tumor targeting of diabodies (tumor uptake and rapid blood clearance) as compared with immunoglobulin (higher blood pool) and scFv (poor tumor uptake). (**c**) MicroPET scan of athymic mice with LS174T colon carcinoma (left arrow) and C6 glioma (right arrow) tumors 18 h post infusion of ^{124}I-labeled anti-CEA T84.66 diabody, minibody or scFv-Fc[43,48,50] (see p. 1137; and A.M. Wu, personal communication), showing excellent tumor localization of all three fragment formats, but with substantially better contrast for diabodies because of rapid blood clearance as compared with the larger minibody and scFv-Fc fragments[48].

The pharmacokinetics of mAb fragments can also be modified using other strategies, such as linkage to polyethylene glycol (PEG). PEG linkage (PEGylation) has been very efficient for increasing half-life and scFv stability, conferring improved anti-tumor activity[60] and apparently also reducing immunogenicity. For example, conjugation of small human V domains (11–15 kDa) with a single PEG molecule extends the half-life from the normally rapid $t_{1/2}$ of 20 min to 39 h in mice (I.M. Tomlinson, Domantis, Cambridge, personal communication). Another strategy for increasing serum half-life of mAb fragments is through fusion or noncovalent interaction with long-lived serum proteins, such as albumin[61] or serum immunoglobulin (as shown in one of our laboratories (P.H.)[62]). Finally, if desired, systemic clearance can be accelerated by mannose glycosylation[63], which is provided by the use of yeasts, such as *Pichia pastoris*, as expression hosts.

***In situ* expression.** Efficient methods of gene delivery with localized functional expression of the encoded product are still in their infancy, but when successful, *in situ* expression will provide an attractive alternative to systemic administration of antibody fragments (reviewed in ref. 64). *In situ* expression potentially circumvents problems of tumor penetration, short serum half-life and even poor specificity. *In situ* secretion of therapeutic mAbs has the advantage that, in principle, expressor cells could be transformed *ex vivo* and reinfused into patients. Indeed, *in situ* expression and secretion of cytotoxic scFv fragments[65] or a bispecific diabody (P.H. and colleagues, ref. 66) *in vivo* from just a few expressor cells promoted significant growth inhibition of established tumor xenografts.

Perhaps the most important application will be to achieve functional expression of intracellular mAb fragments (termed intrabodies), which can be used to ablate or modify crucial transcriptional and translational regulators and controls, thereby enabling mAbs to be used in intracellular antiviral or antitumor therapies hitherto impossible. Intrabodies potentially provide a powerful way to ablate viral-encoded genes and products or influence cellular function in malignancies, for example by inhibiting the transcription of oncogenes, inducing apoptosis or restoring transcription of tumor-suppressor proteins, such as p53(ref. 67).

Great strides have recently been made both in framework designs[68] that achieve functional folding of the mAb scaffolds in the reducing intracellular environment and also in the isolation of functional intrabodies directly from repertoires. Together, these new molecular designs and selection technologies should facilitate and accelerate generation of potent intrabodies. For example, selection processes have been developed to evolve stable intrabody scaffolds without disulfide bonds and have further showed their effectiveness *in vivo* for reducing aggregation of the Huntington disease Htt protein[69]. Like their extracellular counterparts, intrabodies may be engineered into multivalent and multispecific forms to enhance their efficacy. For example, a bispecific intrabody (intradiabody) has recently been described that allows the simultaneous knockdown of two cell-surface receptors[70].

In the future, it may become possible to obviate the need for gene delivery and target intracellular antigens directly by fusion of the intrabody to a membrane translocator sequence[71], a naturally internalizing mAb[72] or by direct selection for internalization.

Engineering multiple specificity

Bispecific antibodies (bisAbs) comprise two different binding specificities fused into a single molecule. They can be designed to bind either two adjacent epitopes on a single antigen, thereby increasing both avidity and specificity, or to bind two different antigens for numerous applications, but particularly for recruitment of cytotoxic T- and natural killer (NK) cells or retargeting of toxins, radionuclides or cytotoxic drugs for cancer treatment). bisAbs can be produced by fusion of two hybridomas into a single 'quadroma' by chemical cross-linking or genetic fusion of two different Fab or scFv modules. Other promising formats (**Fig. 1**) are bispecific diabodies, formed by heterodimeric association of two domain-swapped scFvs of different specificity, and bis-scFvs, where two different scFvs are joined in tandem (**Figs. 1** and **2**).

Unwanted pairing of the heavy and light chains has been a major problem in some formats and has greatly hampered clinical-scale production and therapeutic application. Mispairing of noncognate heavy and light chains results in formation of inactive antigen-binding sites, which has been a major problem in some bisAb formats and has greatly hampered clinical-scale production and therapeutic application. However, elegant engineering of the interaction interface in antibody V and C_H3 domains can provide an efficient way to drive heterotypic pairing in whole antibodies[73] as well as minibodies[74] and diabodies[75].

A majority of both bispecific diabodies and bis-scFv are designed to bind to the CD3 T-cell coreceptor and thereby recruit cytotoxic T-cells (CTLs) to the tumor site. Preclinical testing of such diabodies and bis-scFv (which have been termed BiTEs for bispecific T-cell engagers)[76,77] in animal models of human tumors, such as lymphoma xenografts, demonstrated their effectiveness *in vivo* (reviewed in ref. 78). Others have successfully retargeted NK cells (via CD16; ref. 79) or T cells (via CD28) for tumor ablation. Recently, T-cell cytotoxicity has been directed to virally infected tumor cells using bis-scFv dimers targeted to the viral cell-surface protein and CD3 (ref. 80).

Diabody-Fc fusions can target two cell-surface receptors (for example, EGFR and insulin-like growth factor receptor, IGFR) simultaneously, eliciting rapid IGFR internalization and degradation and recruiting effective antibody-dependent cellular cytotoxicity (ADCC), leading to strong growth inhibition of two different human tumor xenografts *in vivo*[81]. A bispecific diabody has also been shown to allow the simultaneous engagement of vascular endothelial growth factor receptor 2 (VEGFR2) and receptor 3 (VEGFR3) on endothelial cells, leading to neutralization of both VEGF- and VEGF C–stimulated activation of VEGFR2 and VEGFR3 and p44/p42 mitogen-activated protein kinase[82]. Furthermore, the diabody was able to inhibit both VEGF- and VEGF C–induced cell migration, which suggests that a simultaneous blockade of both VEGFR2 and VEGFR3 may represent a potent approach to cancer therapy.

Other approaches that exploit multiple specificity binding include recruitment of gene delivery vehicles (such as adenoviruses) to new cell targets[83], epithelial cell adhesion molecules for specific brain-tumor targeting[84] and, in an innovative tetravalent design, the use of Fc-type scaffolds to display multispecific arms, such as two Her-2–targeting scFv modules and two CD86 domains for T-cell activation[85].

The ability to design multivalent specificity into mAb fragments is also enabling the re-emergence of pretargeting as a powerful and selective strategy for cancer therapy. In pretargeting, a mAb fragment bispecific for a tumor marker (for example, carcinoembryonic antigen, CEA) and a hapten (such as a peptide) is first infused to specifically localize the bisAb fragment at the tumor site; subsequently, a drug-hapten conjugate or radiolabeled hapten is introduced, which then binds to the bisAb. Results from preclinical studies have been encouraging. In one, bispecific diabodies targeting a ^{67}Ga-modified hapten were compared with chemically conjugated Fab_2 fragments, resulting in excellent diabody localization in the pretargeting stage[42]; in another, a bispecific diabody targeting a ^{99m}Tc-peptide showed five times greater uptake in tumors than a directly radiolabeled anti-CEA Fab[86]. For therapy, pretargeting can deliver to tumors nearly twice as high a radiation dose as can a directly radiolabeled IgG[86]. Other promising formats for pretargeting use, for example, a tetravalent streptavidin-scFv fusion protein[87]. Pretargeting represents a highly promising method for reducing toxicity of radionuclides in a clinical setting.

Engineering bifunctional fragments

Antibody fragments have been linked or fused genetically to a vast range of molecules that ascribe important ancillary functions following target binding. These include radionuclides (discussed above) and also cytotoxic drugs, toxins, peptides, proteins, enzymes, liposomes for improved drug delivery and even viruses for targeted gene therapy[88,89].

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 mAb-toxin conjugates (so-called immunotoxins) have been stabilized *in vivo* using *Pseudomonas aeruginosa* exotoxin and diphtheria toxin, respectively[90,91], but may still be limited by immunogenicity. The search for an effective, human cytotoxin has lead to the use of the human ribonuclease, angiogenin in mAb-toxin fusion proteins[92]. Potent receptor-mediated killing of target cells, expected lack of extracellular toxicity, low immunogenic potential and ease of production bode well for the application of this new immunoenzyme (scFv-angiogenin fusion) in immunotherapy.

Single-chain Fv–cytokine fusion proteins were expected to be highly effective cancer therapeutics. However, poor protein production, adverse immune reactions and vascular leak syndrome (see later section) have severely limited their preclinical evaluation. Indeed, chemokine fusions may be more promising than cytokines. Indeed, chemokine fusions may be more promising than cytokines in avoiding unwanted inflammatory side-effects. However, some scFv-cytokines have begun to show promise, such as scFv–interleukin 12 (IL-12) fusion proteins that were found to be superior to IL-12 alone in a lung-metastasis model[93].

Vascular leak syndrome (VLS), a dose-limiting side effect of systemic administration of both immunotoxins and cytokines, has recently been determined to be caused by a tripeptide motif mediating interaction and damage to endothelial cells. Elimination of the motif was shown to prevent VLS and increase efficacy of an immunotoxin[94] and might do so likewise for fusion proteins of cytokines such as IL-2, which share the tripeptide motif. Conversely, mild VLS might be desirable, as it might enhance penetration and localization in solid tumors.

An alternative strategy for promoting local immune activation is fusion to T-cell superantigens (for example, *Staphylococcus aureus* enterotoxin A, SEA) or B-cell superantigens (*Peptostreptococcus magnus* protein L, PpL). A Fab-SEA fusion is undergoing clinical evaluation[95] and a fusion of an scFv to an affinity-matured domain of PpL has shown efficient recruitment of IgG κ light chain (IgGκ)- and IgMκ-encoded effector functions, such as complement, mononuclear phagocyte respiratory burst and phagocytosis[96].

Antibody fragments fused to lipids can effectively retarget either cytotoxic drug-loaded liposomes for tumor ablation[84,97] or vaccine-loaded liposomes for dendritic cell targeting[98]. As immunoliposomes, mAb fragments can potentially deliver drugs to the brain, as they are able to cross the blood-brain barrier[84]. Antibody-like V domains (CTL-associated antigen 4, CTLA4) can effectively deliver fused antigens to dendritic cells for immunovaccine stimulation[99].

Another concept that has been galvanized by progress in mAb-fragment engineering is antibody-directed enzyme prodrug therapy

(ADEPT). In this strategy, mAb fragments (for example, a humanized scFv[100] or camelid VhH[25]) are fused to enzymes (such as β-lactamase and carboxypeptidase), which catalyze prodrug activation. *In vivo* studies have demonstrated that the conjugates have excellent biodistribution profiles and induced regressions and cures of established tumor xenografts. Humanized or deimmunized forms of the enzymes may be crucial to circumvent expected immunogenicity problems and may lead to higher clinical efficacy[101].

Overall, bifunctional mAbs have begun to realize their potential as promising agents for cancer diagnosis and therapy, with exciting future prospects for immune modulation and viral retargeting. Fusion proteins linking Fv-like single-chain T-cell receptors (scTCRs) to IgG1 heavy chains behave like mAbs but are able to recognize antigens derived from intracellular targets[102]. These fusion proteins represent a new group of immunotherapeutics that have the potential to expand the range of tumor targets beyond those currently addressed by conventional antibody designs.

New applications of fragments

Antibody fragments have a variety of uses, ranging from simple research tools as diagnostic reagents to highly refined biopharmaceutical drugs. Their exquisite selectivity and increasing ease of manipulation has facilitated more exotic applications, such as their uses in targeting quantum dots[103] and as delivery vehicles for genes and their unique crystallization properties, which can permit structural analysis of refractory and highly valuable target antigens[104]. Some of these and other applications are explored below.

Selection and screening for new properties. Library display has superseded hybridoma technology through the creation of large natural and synthetic *in vitro* repertoires of antibody fragments (see p. 1105). Library selection strategies generate high-affinity and high-specificity scFv and Fab fragments, and they have most recently been applied to the selection of diabodies and single-domain antibodies (dAbs; VhH and V-NAR), thus spawning many new biotechnology companies (including Ghent, Belgium–based Ablynx; Cambridge–based companies Domantis and Cambridge Antibody Technology; Melbourne, Australia–based EvoGenix; and Martinsreid, Germany–based Morphosys). Most recently, innovative methods have been devised to isolate mAb fragments with desirable properties directly from display repertoires. These methods have exploited three factors: the ability of phage to sustain thermal stress to isolate dAbs that unfold reversibly on the phage tip[29]; the avidity of bivalent diabodies or cell-surface display to isolate binders not found in monovalent scFv libraries[105]; and the incorporation of fluorescent probes into the antigen-binding site to generate immunodiagnostic sensors[106].

New selection and display technologies are becoming available and may further improve the speed with which binders can be generated and their properties improved. For example, an innovative strategy uses compartmentalization of *in vitro* transcription and translation in an emulsion to link peptides and the encoding DNA sequences on microbeads, which can then be selected for binding[107]. 'Bead display' may prove to be a rapid and versatile strategy for the selection of mAb fragments and also single domains. In another strategy from one of our laboratories (P.H.)[108], antibody-antigen interaction complexes are directly assembled on the phage tip, allowing the selection of matching antibody-antigen pairs.

Several selection strategies can improve the yield and stability of recombinant mAbs, most recently applied to single V domains. These include human V domains selected for high stability and resistance to aggregation[29], antigen-driven selection for highly stable camelid VhH mAb fragments (which bind antigen after a heat shock of 90 °C or after repeated exposure to denaturing detergents[109,110]), shark V-NARs that possess remarkable stability to urea and other denaturants[111], and new V-domain intrabody designs to circumvent the disulfide bonds usually required for scaffold stability[69,112]. Further refinements to improve protease resistance of phage-displayed proteins have also recently been described[113].

Recent structural studies have shown that specific targeted mutations in the V_H region (for example, Gly35) can also have dramatic effects on the stability of the scaffold and may therefore be preferred sites for engineering either more stable Fv modules or single V domains[28]. Other point mutations, such as Arg44 in camelid VhH, have been selected that provide high stability to harsh denaturants[110]. It should be noted, however, that these point mutations that improve stability and expression yields may not be generic solutions and can be applied to only some, not all, V_H-like domains.

New immunoassay formats. Conventional diagnostic immunoassays are limited to the analysis of a few hundred assays per day, whereas with antibody microarrays using individually addressable electrodes, many thousands of assays can be run in parallel[114]. Antibody fragments are providing valuable alternatives to full-length mAbs for new biosensing devices because they provide small, stable, highly specific reagents against the target antigen. Indeed, formats for antibody microarrays are already diversifying from immobilization of intact mAbs onto glass-surface microarrays[115] to other new, more protein-friendly surfaces. For example, in the past year, several such platforms have come on the market: San Diego–based Biosite's rapid enzyme immunoassay (Triage) system; Zyomyx's (Hayward, California) Protein Profiling Biochip for human and murine cytokines; PerkinElmer's (Boston) Hydrogel microarray substrate for optimizing protein activity and accessibility; and most recently, Pointilliste's (Mountain View, California) Molecular Canvas arrays, which comprise high-density spots of full-length mAbs for use in capturing small molecules, peptides or proteins.

We anticipate widespread use of recombinant mAb fragments to generate complex arrays for the expression and function analysis of candidate genes identified by genome projects. Such arrays will have an important impact in identifying genes involved in human diseases, including cancer. 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. New nonproteinaceous targets may also emerge from aberrant glycosylation patterns on tumor cells[116] or from innovative *in vivo* chemical labeling strategies of tumor-specific surface glycans[117].

Expression systems

Production of stable, high-affinity mAb fragments in high yield for preclinical and clinical trials can be a serious bottleneck in the product pipeline. Numerous expression systems, including bacteria, yeasts, plants, insect and mammalian cell lines and even cloned transgenic animals, have been evaluated and compared[118,119]. Bacteria are favored for expression of small, nonglycosylated Fab and scFv fragments, diabodies and V domains. Most studies have reported a preference for scFv over Fab due to their superior expression levels in bacteria (to over 1 g l^{-1} using fermentors), although yields vary for each mAb fragment. Human single-domain antibodies (V_H and V_L) also can be expressed at high yields (>50 mg l^{-1} in *Escherichia coli* and >0.5 g l^{-1} in *P. pastoris* shaker flask cultures)[18,109] (I.M. Tomlinson, Domantis, personal communication).

Nevertheless, several strategies have been developed to improve recombinant expression. Terminal polypeptides, such as c-Myc, histidine and the 'Flag' epitope (DYKDDDK in one-letter amino acid code), have been added for affinity purification after expression into the periplasm of *E. coli*. Whether these must be removed because of perceived

immunogenicity and related concerns at drug regulatory agencies is still under investigation. Heat-inducible systems allowed simple and inexpensive large-scale recombinant expression. Staphylococcal protein A affinity columns greatly assist purification for Fc-fusion constructs, as do protein L affinity columns for V_κ-domain expressions. In summary, mammalian or plant cells are favored hosts for high-yield expression of larger intact antibodies and minibodies, whereas bacterial and yeast systems are most useful for mAb fragments and V domains.

Conclusions

After a decade of intensive engineering followed by preclinical and finally clinical testing, antibody fragments seem set to join mAbs as powerful therapeutic and diagnostic agents, particularly for targeting cancer, inflammatory, autoimmune and viral diseases. On the basis of recent advances in scaffold design, repertoire construction and selection methodologies, there is now a rapid process for generation of specific, high-affinity mAb fragments against virtually any target.

Of particular note, V-like domains (for example, mammalian V_H and V_L, camelid VhH and shark V-NAR repertoires) now provide alternative, efficient scaffolds for the presentation of constrained polypeptides displayed as long surface-loop structures. Importantly, these V domains can penetrate antigen clefts (enzyme active sites, viral capsids and cell-surface receptors) and may therefore be able to target both refractory antigens and immunosilent 'canyon' epitopes.

Increasingly, mAb-fragment repertoires will be applied to proteomic discovery of new cancer biomarkers and their exploitation in the development of sensitive microarrays, point-of-care diagnostics and robust nanosensors. A great deal has been learned about the critical parameters for improving *in vivo* efficacy, and bacterial fermentation provides a cost-effective route to scale up production of many formats of engineered mAb fragments. Furthermore, the latest antibody designs for scFv multimers, such as diabodies and minibodies, have achieved impressive tumor-to-blood ratios and are now perfectly positioned to take full advantage of the new-generation positron-labeling chemistries and quantum-dot conjugations to open new opportunities in PET tomography and high-sensitivity (nonradioactive, noninvasive) laser technologies for medical imaging.

Together with the increasing ability to tailor both pharmacokinetics and functionality (for example, tumor-targeted toxicities) of engineered mAb fragments, these advances should provide for a fruitful dialog between clinicians and antibody engineers, leading to a burgeoning range of regulatory approvals for recombinant mAb fragments in diagnosis and therapy.

ACKNOWLEDGMENTS

We thank V. Streltsov, S. Nuttall and J. Atwell for their help in assembling **Figures 1** and **2** and A. Wu and G. Adams for allowing us to include their unpublished data in **Figure 3**. We also thank our colleagues L. Jespers and I. Tomlinson from Domantis Ltd. for sharing unpublished results and L. James (Medical Research Council) for helpful discussion.

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