

Multivalent antibodies: when design surpasses evolution

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Evolutionary pressure has selected antibodies as key immune molecules acting against foreign pathogens. The development of monoclonal antibody technology has allowed their widespread use in research, real-time diagnosis and treatment of multiple diseases, including cancer. However, compared with hematologic malignancies, solid tumors have often proven to be relatively resistant to antibody-based therapies. In an attempt to improve the tumor-targeting efficacy of antibodies, new formats with modified, multivalent properties have been generated. Initially, these formats imitated the structure of native IgG, creating mostly monospecific, bivalent antibodies. Recently, novel trivalent antibodies have been developed to maximize tumor targeting capabilities through enhanced biodistribution and functional affinity. We review recent advances in the engineering of multivalent antibodies and further discuss their promise as agents for *in vivo* diagnostics and therapy.

Introduction

Antibodies are found in blood and other body fluids and are used by the immune system to identify and neutralize foreign pathogens, such as bacteria and viruses. The immune system has evolved unique genetic mechanisms that enable it to generate an almost unlimited number of antibody specificities. Antibodies act as molecular connectors, linking specific recognition of epitopes on their targets with nonspecific effector mechanisms, including antibody-dependent, cell-mediated cytotoxicity, complement-dependent cytotoxicity and antibody-dependent, cell-mediated phagocytosis. Intact antibody molecules are heavy (~150 kDa), globular glycoproteins. The basic unit of each antibody is a monomer [one immunoglobulin G (IgG) unit] composed of two identical light chains (L) and two identical heavy chains (H). The four chains are connected to one another by a combination of noncovalent and covalent (disulfide) bonds, which forms a Y-shaped molecule with the antigen-binding sites located at the tip of the two 'arms'. Each IgG is thus bivalent and able to attach to two identical antigenic sites, which increases antibody functional affinity and confers high retention times. The recruitment of effector functions is mediated by the Fc domain. Also, owing to molecular weights beyond the renal threshold limit of 70 kDa, as well as interaction with the neonatal Fc receptor (FcRn) – a major histocompatibility

Glossary

Affinity: biochemical parameter that represents the strength of a single antigen antibody interaction. Affinity is defined by the equilibrium between the association (k_{on} or k_a) and dissociation (k_{off} or k_d) from its antigen. It is sometimes called intrinsic affinity, to distinguish it from avidity.

Antibody fragment: the immunoglobulin molecule can be reduced in size by removing domains involved in recruitment of associated functions, thereby keeping the antigen recognition regions. The main fragments are Fab, scFv and 'single domain antibodies', which can be utilized as modular building blocks to make multimeric antibody constructs.

Area of influence: we have coined this concept to define the probability of a second binding event after the first interaction with an antigen occurs, and is applicable to multivalent interactions only. It results from the formula for area of a two-dimensional circle, $A=\pi r^2$, where the radius r is the length of the linker that connects the scFvs in multivalent antibodies.

Avidity: biochemical parameter used to describe the strength of the combination of multiple antigen antibody interactions. An increase in avidity is obtained through oligomerization of antibodies, which in turn leads to an interaction with increased stability as compared with monovalent antibodies. Thus, a tighter interaction, and greater retention on target, is achieved. It is often called 'functional affinity' to distinguish it from affinity.

Biodistribution: quantitative evaluation of the *in vivo* distribution of an antibody in different bodily organs over time following administration. For this, antibodies are typically conjugated with a fluorinated or radioactive tracer, by which their presence can be determined at a given time point and indicated as a percentage of the injected dose per gram of organ or blood (% ID/g).

Bispecific antibody: antibody that can bind to two different antigen epitopes (specificities). Although bispecific antibodies do not occur naturally, they have been generated through a number of strategies.

Blood clearance: rate at which an antibody is removed from the circulation, which is dependent on molecular mass and amino acid content.

Death domain: protein domain composed of six alpha-helices that mediate homotypic interactions. Activation of some of the proteins, such as the TNF receptor, provokes a downstream signal cascade through the intracellular death domains that leads to apoptosis and inflammation through activation of caspases and NF- κ B.

Fab (fragment antigen-binding): region of the antibody that interacts with the cognate antigen. It comprises the V_H and C_H1 domains of the immunoglobulin heavy chain and the V_L and C_L domains of the light chain.

Molecular trapping: therapeutic strategy used to capture and block the action of active, soluble agents.

Peptide linker: a sequence of aminoacids used to connect two protein domains, this sequence could be variable in length, composition, and flexibility. In the case of antibody engineering, one of the most commonly used linkers is the $(G_4S)_3$ sequence.

Single domain antibodies: antibody fragment consisting of a single monomeric variable domain. Single domain antibodies are engineered from variable heavy chains (V_H) derived from camelids, from variable novel antigen receptors (V_{NAR}) derived from cartilaginous fishes, and also from typical human and mice IgGs.

scFv (single chain variable fragment): fusion of the two variable domains of immunoglobulin V_H and V_L that comprise the antigen-binding site. Both domains are typically joined together using a flexible peptide linker of 10–25 residues.

Specificity: ability of an antibody to recognize a single antigen epitope and discriminate it from the rest. Monoclonal antibodies are monospecific because they bind with each of their two antigen-binding arms to only one epitope.

Tumor penetration: depth of infiltration of an antibody into tumor deposits. It is influenced by the affinity and avidity of the antibody, antigen metabolic turnover, antibody diffusion and vascularization of tumor.

Tumor retention: pharmacokinetic parameter that defines the residence time of an antibody within tumor deposits. It depends on the affinity, avidity and stability of the antibody, and antigen turnover.

Valency: the number of antigen-binding sites present per molecule of antibody construct.

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complex class I-related molecule known to protect IgG and albumin from intracellular catabolic degradation following pinocytic capture [1], IgG molecules show slow clearance from the circulation, thus explaining the long half-life of this antibody class in serum [2].

The advent of monoclonal antibody (mAb) technology has made it possible to raise antibodies against specific antigens, including tumor-associated antigens. MAbs are unique, versatile molecules that have found applications in research, diagnosis and in the treatment of multiple diseases, including cancer [3]. Currently, there are 26 mAbs approved by the US Food and Drug Administration (FDA) for therapeutic and diagnostic use. However, solid tumors have often proven to be relatively resistant to IgG or intact antibody-based therapies. Additionally, it has been shown that only a small amount of intravenously administered mAbs accumulates in the tumor tissue [4].

In an attempt to improve tumor penetration (see [Glossary](#)), recombinant antibodies with modified properties have been generated. Novel antibody formats, such as the single-chain Fv (scFv; 25–30 kDa), exhibit better pharmacokinetics than intact IgGs for tissue penetration, and enable binding specificity to be encoded by a single gene [2]. However, scFv antibody fragments exhibit rapid blood clearance as well as fast off-rates and poor retention times on the target due to their small size and their monovalent binding properties [2]. A wide range of strategies has therefore been developed to improve the pharmacokinetic properties of antibody fragments [5]. The covalent conjugation of small recombinant antibody molecules with poly(ethylene glycol) (PEG) – also known as ‘PEGylation’ – is a well-established procedure to improve serum half-life by increasing the molecular mass and the hydrodynamic radius [6]. Another approach is the genetic fusion of antibody fragments to albumin or albumin-binding proteins. This strategy is based on the observation that albumin has a half-life similar to that of IgG and also utilizes FcRn-mediated recycling processes [5]. Alternatively, the use of bispecific antibodies to recruit serum IgG provides the Fc-associated effector functions and prolongs residence time in serum. Multimerization is a particularly appealing approach to optimize antibody size for the desired pharmacokinetics, and to maximize tumor uptake by enhancing the functional affinity (avidity) of antibody fragments. Here, we review recent progress in the design of multimeric antibodies, and suggest that trivalent antibodies display the best combination of size and avidity for cancer diagnostics and therapy.

Strategies to multimerize antibodies: improving upon Mother Nature’s design

Conversion of monovalent recombinant antibodies (i.e. scFvs, Fabs or single-domains) into multivalent formats increases functional affinity, decreases dissociation rates when bound to cell-surface antigens, and enhances biodistribution. The most prevalent formats of engineered antibodies that have appeared in recent years are depicted in [Figure 1](#). Multimeric antibodies have been grouped according to structure into two categories: IgG-like (orthodox) and non-IgG-like (heterodox) formats.

Orthodox formatting strategies

Engineered antibodies that imitate the original structure of IgG, creating mostly monospecific bivalent antibodies, have been termed ‘orthodox’ formats in this review. The most common strategy to create orthodox formats has been the engineering of fusion proteins in which the antibody fragment makes a complex with homodimerization proteins. Based on the data available in the literature, the following orthodox, engineered antibodies are highlighted: the Fab₂ antibody and its single-chain counterpart, sc(Fab’)₂ (Fab₂/sc(Fab)₂) [7]; the ZIP miniantibody; the scFv-Fc antibody; and the minibody ([Figure 1](#)). The 64-kDa miniantibody is generated by the association of two scFv fragments through dimerization domains. Leucine zippers (Fos or Jun) are employed to mediate dimerization of scFv in a miniantibody molecule. Fos–Fos and Jun–Jun homodimer formation allow efficient production of stable, secreted, monospecific miniantibodies, and Fos–Jun heterodimer formation allows production of bispecific miniantibodies [8]. The scFv-Fc antibody [9] is a small IgG-like recombinant antibody (100–105 kDa) generated by fusion of a scFv with human IgG₁ hinge and Fc regions (C_H2 and C_H3 domains). The 80-kDa minibody results from the fusion of a scFv to the human IgG₁ C_H3 domain [10]. Other minibody-like molecules have been generated using the small immunoprotein technique [11], or by fusion to bacterial alkaline phosphatase [12]. Furthermore, the minibody has recently been modified to a 115-kDa trimeric bispecific format, the TriBi-minibody [13]. Another trimeric bispecific format is the tribody ([Figure 1](#)), which combines the heterodimerization of L (V_L-C_L) and Fd (V_H-C_H1) chains of a Fab fragment with the C-terminal fusion of two scFv domains to generate a trivalent molecule of intermediate size (115 kDa) [14,15].

Heterodox formatting strategies

The heterodox format in this review includes all engineered antibodies that do not imitate the original IgG structure. Here, different strategies to multimerize antibody fragments are utilized. For example, shortening the peptide linker between the V domains forces the scFv to self-associate into a dimer (diabody; 55 kDa) [16]. However, a tailored engineering protocol is frequently needed because the functionality and stability of diabodies depend on both their flexibility and orientation (V_H-V_L or V_L-V_H) [17]. Bispecific diabodies are formed by the noncovalent association of two V_HA-V_LB and V_HB-V_LA fragments expressed in the same cell. This leads to the formation of heterodimers with two different binding sites [18]. Single-chain diabodies (sc-diabodies) are bispecific molecules where the V_HA-V_LB and V_HB-V_LA fragments are linked together by an additional third linker. Tandem-diabodies (Tandabs) are tetravalent bispecific antibodies generated by two scDiabodies [19].

An orthodox format raised from a heterodox format is the di-diabody [20] ([Figure 1](#)). This 130-kDa molecule is formed by the fusion of a diabody to the N-terminus of the C_H3 domain of an IgG, resulting in an IgG-like structure. Further diabody derivatives are the tribody and the tetra-body ([Figure 1](#)), which fold into trimeric and tetrameric fragments by shortening the linker to <5 or 0–2 residues,

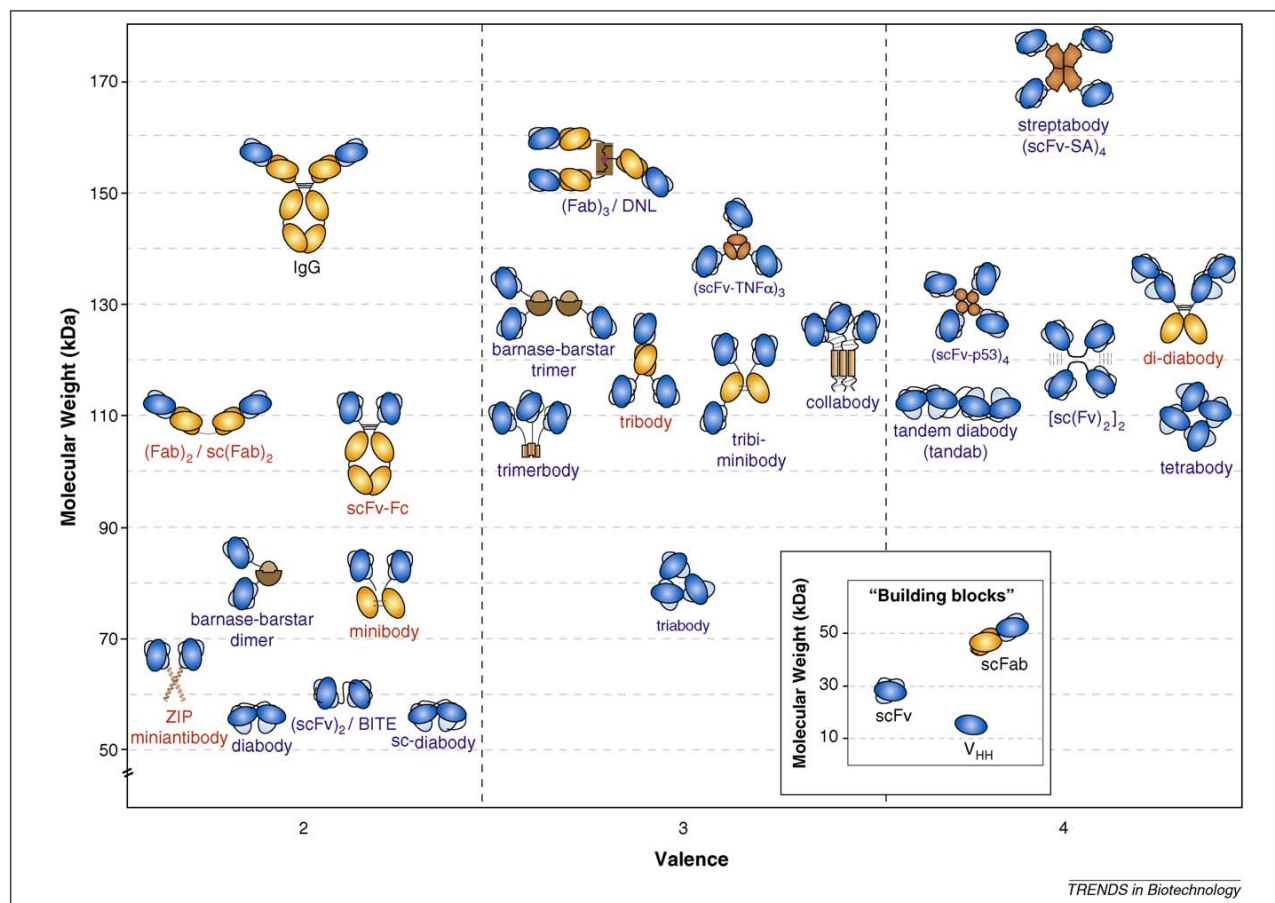


Figure 1. Recombinant multimeric antibodies classified as either 'orthodox' (IgG-like) or 'heterodox' (non-IgG-like) formats according to their similarity to the native IgG. Schematic diagram of the main recombinant antibody fragments (see Glossary) engineered to date distributed according to their molecular weight and valence. Only those antibody formats for which multivalency (structural and/or functional) or tumor targeting properties have been documented are included. Inset: schematic diagram of the monovalent 'building blocks' (scFv, V_{HH} , and scFab) arranged by molecular weight. IgG constant domains appear in yellow, and IgG variable domains in blue. Non-IgG derived oligomerization domains appear in brown. Antibody formats that belong to the 'orthodox' category are shown in red font; formats that belong to the 'heterodox' category are shown in blue font; names of 'building blocks' and native IgG are shown in black font.

respectively [21,22]. A different design for a $(scFv)_2$ is the 'bispecific T cell engager' (BITE). BITEs are bispecific single-chain antibodies consisting of two scFv antibody fragments, joined via a flexible linker, that are directed against a surface antigen on target cells and CD3 on T cells [23]. A similar strategy has been used to generate 120-kDa monospecific tetravalent antibodies by the fusion of two scFv with a linker, creating a dimeric $[sc(Fv)_2]$ that spontaneously associates as noncovalent $[sc(Fv)_2]_2$ dimers [4,24,25].

Oligomerization domains from other non-Ig proteins have been used to make other heterodox formats (i.e. molecular constructs based on the bacterial barnase-barstar module). The ribonuclease barnase (12 kDa) and its inhibitor barstar (10 kDa) form a very tight complex in which the N- and C-termini are accessible for fusion. This module, which has a dissociation constant of $\sim 10^{-14}$ M, has been exploited to generate stable, trimeric scFv (~ 130 kDa) [26] (Figure 1). Similar formats have been generated by fusion of a scFv to a cytokine that naturally exists in trimeric form, the tumor necrosis factor (TNF). TNF α genetically fused to the C-terminus of a scFv antibody

forms a homotrimeric structure that retains both TNF α activity and the ability to bind antigen recognized by the scFv [27,28]. Also, fusion proteins of scFv fragments with the C-terminal multimerization domain of the tumor-suppressor protein p53, or with a streptavidin subdomain, results in tetrameric antibodies $(scFv-SA)_4$ [29] (Figure 1).

Another successful approach for the generation of multimeric antibodies has been the use of extracellular matrix-derived oligomerization domains. Fusion of the trimerization region of the C-terminal noncollagenous domain (NC1) of collagen XVIII to the C-terminus of a scFv confers a natural trimeric state to the fused antibody (trimerbody; 110 kDa) [30]. Another trimeric antibody, collabody (125 kDa), is based on the use of triplex-forming collagen-like peptides $[(GPP)_{10}]$ [31].

Fab single domains have also been multimerized. The first attempts were based on covalent disulfide bonds, generating divalent (Fab_2) and trivalent (Fab_3) formats [21,32]. The dock-and-lock (DNL) method is an additional approach to develop Fab_3 -like molecules (Figure 1). The DNL strategy uses the interaction between the regulatory subunits of the cAMP-dependent protein kinase, which

form a dimer, and one anchoring domain of a kinase anchor protein. By fusing Fab fragments, bispecific, trivalent-binding complexes are produced with molecular masses of ~160 kDa. Additional stability of the complex is achieved by introducing cysteine residues in the interacting modules to prevent dissociation [33].

Optimization strategies for tumor-targeting antibodies

In recent years, experimental evidence has accumulated, revealing that there is a pharmacological window suitable for tumor targeting, which we have called the 'tumor target zone' (Box 1; Figure 2). Considering the defined structural requirements of intermediate size and multivalency that the antibodies need to accommodate [34], ideal tumor targeting antibodies largely comprise bivalent and trivalent constructs (Figure 1), thus excluding monovalent and 'high-end' multivalent antibodies. Although there are a few tetravalent constructs that would fit within the 'tumor target zone', they present molecular stability issues that might diminish their *in vivo* functionality, and are therefore not included in this selection. Additional experiments are needed for better characterization of these molecules.

In a detailed evaluation of well-characterized dimeric and trimeric (Table 1) antibodies, it becomes clear that not only size and valence, but also additional attributes – low immunogenicity, lack of associated functional effects, and serum stability, as well as a simple molecular design to facilitate large-scale manufacturing – are important in a tumor-targeting context. According to the criteria described above and the information accessible in the literature [10,18,30,35], it appears that the minibody and the trimerbody are among the best currently available antibody formats for tumor targeting.

Bivalent antibodies: minibody, the best-in-class for tumor targeting

The first minibodies were generated from the scFv T84.66/212 raised against carcinoembryonic antigen (CEA) [10]. The scFv was fused to the human IgG₁ C_H3 domain, either by a two-amino acid linker that resulted in a noncovalent, hingeless minibody (LD minibody), or by the IgG₁ hinge and a 10-amino acid linker peptide to produce a covalent,

Box 1. The 'tumor target zone'

Molecular targeting is of great interest for diagnosis and therapy. It is founded on the principle governing ligand binding to a specific macromolecule within a tissue-of-interest. Small monovalent antibodies show great tumor penetration, but possess fast off-rates (k_d) and blood clearance, resulting in insufficient tumor uptake and poor contrast images. By contrast, large, multivalent antibodies have high tumor retention and serum half-lives, but inefficiently penetrate the tumor. As a result, both heavy and light antibodies produce similar outcomes. In line with these concepts, we have termed the 'tumor target zone' as the area where recombinant antibodies that fulfill several features for optimal tumor-targeting are located. These properties include intermediate size, multivalency, stability and lack of undesired functional effects. Recent biodistribution studies [34,75] indicate that bivalent antibodies, such as diabodies (55 kDa) and minibodies (80 kDa) [10], and trivalent antibodies, such as trimerbodies (110 kDa) [30], might be best-suited for tumor targeting owing to a higher total tumor uptake and better tumor-to-blood ratios than native IgG molecules.

hinged minibody (Flex minibody). Both constructs demonstrated high affinity for CEA, each with a K_D of $3\text{--}5 \times 10^{-8}$ M. The minibody concept has been extended by the generation of analogous molecules with different specificities [36], and has been applied in preclinical and clinical studies [18]. In mice bearing human colon cancer xenografts, a radiolabeled anti-CEA minibody exhibited rapid tumor uptake (21.4–32.9% ID/g) [9,37,38] and a prolonged serum half-life compared with a scFv monovalent counterpart (0.9–22.5% ID g⁻¹) [39]. Furthermore, high-resolution microPET images of tumor xenografts were obtained with this fragment when labeled with positron emitters [40,41]. The encouraging results obtained with minibodies in diagnostic applications suggest that they could serve as therapeutic agents.

Trivalent antibodies

Trimerbody, three for the 'size' of two... and something else?

The trimerbody is a multivalent antibody comprising a scFv connected to the collagen XVIII NC1 trimerization subdomain through a 21-amino acid linker [35]. Recently, the trimerization subdomain of human collagen XVIII NC1 was crystallized, and the structure indicated its high

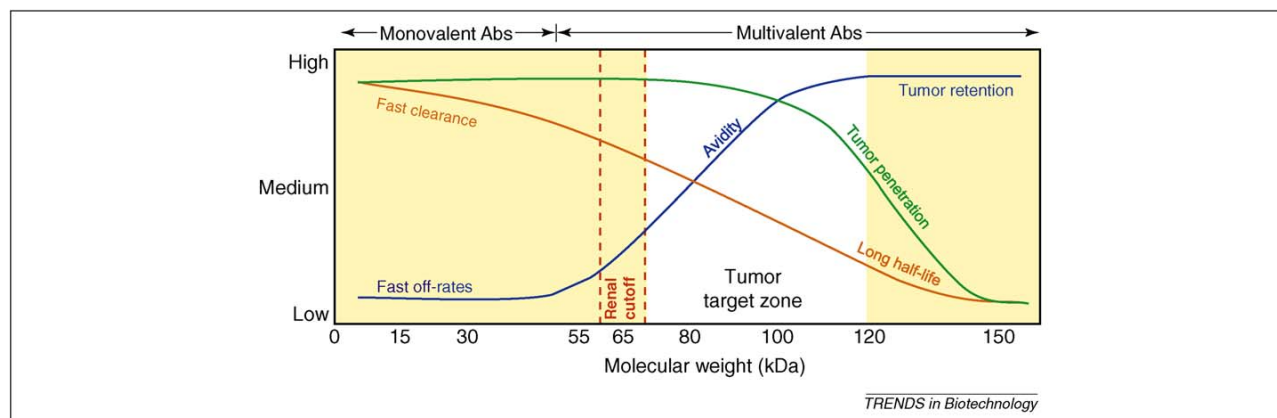


Figure 2. Definition of the concept 'tumor target zone' based on pharmacokinetic and antigen-binding properties of monovalent and multivalent antibodies. Schematic diagram displaying blood clearance (orange), tumor retention (blue) and tumor penetration (green) estimates of monovalent and multivalent antibodies. Small monovalent antibodies show great tumor penetration, but fast off-rates and blood clearance, resulting in insufficient tumor uptakes and poor-contrast images. By contrast, large multivalent antibodies show high tumor retention times, but long serum half-lives and poor tumor penetration, with a similar outcome.

Table 1. Dimeric and trimeric antibodies: important parameters determining *in vivo* tumor-targeting efficiency^a

Antibody	Molecular weight (kDa)	Molecular complexity ^b	Associated functional effect ^c	Immunogenicity ^d	Serum stability ^e	Tumor targeting ^f	Refs.
Dimeric							
Diabody	55	+	—	—	+/-	+	[16,59]
Single-chain (sc)-diabody	55	+	—	—	+	+	[19,55]
(scFv) ₂	55	+	—	—	+	+	[57,60,61]
Miniantibody	64	+	—	—	+/-	NP	[8,62,63]
Minibody	80	+	—	—	+	+	[10,13,57,64]
Barnase-barstar	85	++	+	+++	+	+	[26,29]
scFv-Fc	100–105	+	+	—	+	+	[9,65]
sc(Fab) ₂	110	+	—	—	+/-	+	[7,59,66]
Trimeric							
Triabody	80	+	—	—	—	+	[21,22,67,68]
Trimerbody	110	+	—	—	+	+	[30,35]
Tribody	115	++	—	—	NP	+	[14,15]
Collabody	125	++	—	NP	+	NP	[31]
Barnase-barstar	130	++	+	+++	+	+	[26,69]
(scFv-TNFα) ₃	150	+	+++	—	+	+	[27,28,70–72]
F(ab) ₃ /DNL	160	+++	—	—	+	+	[33,73,74]

Abbreviation: NP, not published.

^aKey: high (+++); intermediate (++); low (+); negative (—); inconclusive (+/-).^bNumber of genes or subunits required for production.^cFunctionality derived from the structural components.^dAbility to provoke an immune reaction predicted from the protein elements.^eProtein integrity following incubation in serum.^fDemonstrated and published ability to localize to tumor deposits.

potential for trimerization at picomolar concentrations [42]. The scFv L36, which recognizes an angiogenesis-associated laminin epitope [43] and inhibits tumor angiogenesis and growth [35,44], has been assembled in the trimerbody format. Secretion of the L36 trimerbody by genetically modified tumor cells was more effective in preventing tumor growth *in vivo* than the monomeric counterpart [35]. Based on the same design concept, trimerbodies with specificity for both CEA and the hapten 4-hydroxy-3-iodo-5-nitrophenylacetic acid (NIP) were constructed. The anti-NIP trimerbody demonstrated at least a 100-fold increase in apparent functional affinity ($K_D=1.5 \times 10^{-9}$ M) [30] for NIP-BSA conjugates compared with its source scFv. Multivalency was demonstrated by the generation of functional, bispecific trimerbodies [30].

The potential of trimerbodies for *in vivo* targeting was assessed in nude mice bearing human tumor xenografts. The anti-CEA trimerbody MFE23 labeled with the far-red cyanine fluorochrome Cy5 localized rapidly and specifically to CEA-positive tumor xenografts. The tumor uptake reached a maximum at 3 h post-injection and slowly cleared over time. Fluorescence was still detectable in the tumor at 48 h after trimerbody injection [30]. A Cy5-labeled anti-laminin L36 trimerbody localized in all the tumors studied, regardless of tumor type. The L36-Cy5 monovalent scFv also showed specific tumor accumulation, but at a much lower level than that of the L36-Cy5 trimerbody [30], most likely due to rapid clearance from the blood pool and its monovalent nature (see Figure S1 in the supplementary material online).

For full avidity of multivalent antibodies, all antigen-binding sites need to be occupied. When targeting surface-bound molecules, orientation of scFvs in the same direction could facilitate simultaneous binding of all surface domains. Analysis of the trimerbody model suggests a tripod-shaped structure with the scFv domains located at the tips of the arms (Figure 3). Flexibility among anti-

gen-binding sites is another important aspect in the design of multivalent antibodies required to crosslink surface receptors on either the same or adjacent cells [18,30]. A 21-amino acid linker with a fully extended length of 79.8 Å [30], for example, is very flexible linker that permits numerous binding geometries. When a first interaction takes place, the possibility of establishing a second effective antigen-antibody interaction depends on the valence, spatial orientation and flexibility of the antibody binding domain. According to our calculations, the remaining unbound scFvs in the trimerbody have an area of influence higher than that of the minibody [30], thus enhancing the probability of a second effective interaction (Figure 3). Multiple binding events reduce the off-rates of the interaction, thereby increasing the retention time of the antibody bound to its target.

Another advantage of the trimerbody format is stability. The trimerization subdomain of collagen XVIII NC1 is stable in the protease-rich tumor environment and also when incubated with human and mouse sera [30,35]. Notably, after incubation with a wide range of proteases from the cysteine protease, the matrix metalloprotease and the serine protease families, trimerbodies exhibited high stability, and only the lysosomal protease cathepsin L completely degraded the molecule [35]. However, similar studies necessary for comparison have not been conducted in the case of minibodies.

Achieving a clinical hat-trick

The increased avidity gained from the addition of a third arm coupled with the unique binding geometries derived from the different trimerization strategies (Figure 1; Table 1) and their tunable size are features inherent in trivalent antibodies. For these reasons, trivalent antibodies might be superior to bivalent ones in clinical applications, including tumor targeting, molecular trapping and as receptor agonists.

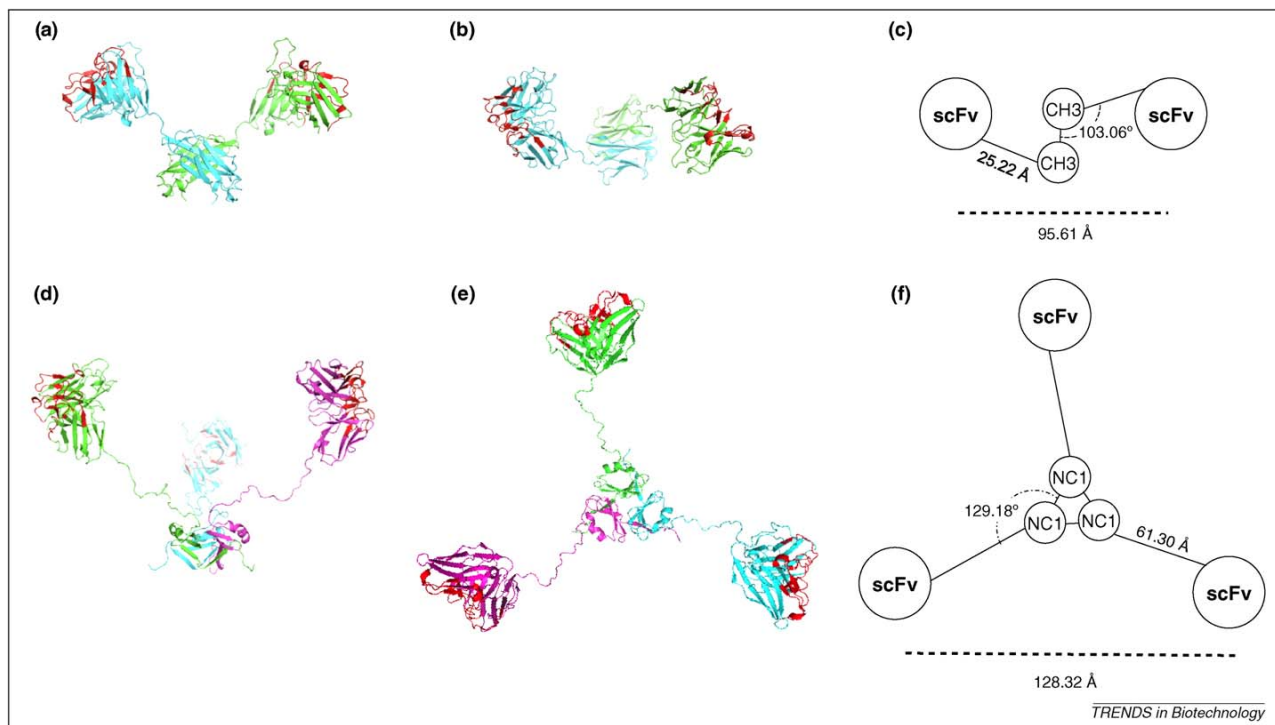


Figure 3. Structural models of the minibody and the trimerbody: minibody (a) lateral and (b) superior views; trimerbody (d) lateral and (e) superior views. The minibody model was constructed with the C α 3 domain of the human IgG $_1$ -Fc protein (PDB 3DO3) (Branden BC *et al.*, unpublished data) and the anti-sars scFv antibody, 80R (PDB 2GHW.B) [76]. The trimerbody model was constructed from the crystal structure of the NC1 trimerization domain of human collagen XVIII (PDB 3HSH) [42] and the anti-sars scFv antibody, 80R (PDB 2GHW.B) [76]. The minibody and trimerbody monomers were superimposed using Stamp to generate the multimeric model [77]. In the computer simulations, each monomer was colored differently (green and blue, or green, blue and purple). The complementarity-determining regions (CDRs) are colored in red. Schematic representations are also provided, illustrating differences in packing interactions within the minibody (c) and the trimerbody (f). The spheres represent the domains (scFv, C α 3 and NC1) with centers located at the centroids of the domains. The solid bars represent the linkers connecting the domains.

For tumor-targeting diagnostic or therapeutic applications, trivalent antibodies are most effective when their molecular parameters are within the limits of the tumor target zone (Figure 2; Box 1). In molecular trapping approaches, it is important to increase the antibody size above the renal threshold to retard clearance rates. Trivalent antibodies, or trivalent, soluble, truncated versions of membrane receptors (triple trap), would have an increased binding stoichiometry to soluble cytokines and growth factors. Coupled with slowed clearance rates, the greater avidity of trivalent antibodies might translate into a greater ability than their monovalent or bivalent counterparts to bind and sequester soluble molecules. This can also be applied to other pathogenic particles against which antibody antidotes have been generated. These include toxins, such as botulinum neurotoxin A [45] or anthrax toxin [46,47], and viruses, such as hepatitis [48,49] or varicella-zoster [50], in which multimerization has been demonstrated to be needed for effective neutralization.

As receptor agonists, trivalent antibodies could be well-suited for receptors that trimerize upon ligand binding, such as the TNF family of receptors. Members of the TNF family that lack death domains modulate T cell responses by providing co-stimulatory immune signals. Receptor crosslinking is essential for intracellular signaling [51,52], and *in vitro* activation of these receptors requires oligomerization of their ligands [53]. One of such receptors

is OX40, whose engagement potentially stimulates T cells and potentially inhibits Treg cells [54–56]. The therapeutic potential of OX40 immunostimulatory properties and its safety have been demonstrated in preclinical studies in primates, providing the grounds to pursue clinical trials [57,58]. Trivalent antibodies can have superior efficiency as agonists of such receptors because they display conformations that allow simultaneous binding and engagement of three receptors, thereby eliciting activation and signaling of a larger subset of receptors. By adjusting antibody size for decreased serum half-lives, short-lasting signaling can be ensured, thus reducing the risks of systemic activation. Consequently, trivalent antibodies might open a new avenue towards achieving superagonist therapeutics.

Concluding remarks

The field of tumor-targeted therapeutics and diagnostics has benefited immensely from the design of recombinant antibody constructs, which have overcome the initial shortfalls of intact IgG. Certain implemented modifications have been able to improve antibody pharmacokinetics; blood clearance, tumor penetration and tumor retention have been prolonged or shortened by modifying antibody size or valence, or through removal of Fc regions. Whereas most antibody constructs improve only certain properties, only a few multivalent, recombinant antibodies possess all of the necessary attributes to lie within the pharmacological

window we have termed the ‘tumor target zone’; these are bivalent and trivalent antibodies of intermediate size, which have risen from the diversification of formats with IgG-derived constant domains and non-IgG-derived oligomerization domains. Trivalent antibodies improve upon the properties of bivalent antibodies in terms of tumor retention and blood clearance, with the trimerbody – the only trivalent antibody that fits into the ‘tumor target zone’ – serving as a very promising scaffold for both diagnostic and therapeutic applications in oncology.

Acknowledgments

We thank Laura Sanz and David Sánchez-Martín for helpful discussion. This study was supported by grants from the Ministerio de Ciencia e Innovación (BIO2008-03233), the Comunidad Autónoma de Madrid (Angiobodies-S-BIO-0236-2006) and the European Union (Immunonet-SUDOE) to L.A.-V, and from the Ministerio de Ciencia e Innovación (BIO2008-00205) to B.O.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.tibtech.2010.03.007](https://doi.org/10.1016/j.tibtech.2010.03.007).

References

- Roopenian, D.C. and Akilesh, S. (2007) FcRn: the neonatal Fc receptor comes of age. *Nat. Rev. Immunol.* 7, 715–725
- Sanz, L. et al. (2004) Antibodies and gene therapy: teaching old ‘magic bullets’ new tricks. *Trends Immunol.* 25, 85–91
- Sanz, L. et al. (2005) Antibody engineering: facing new challenges in cancer therapy. *Acta Pharmacol. Sin.* 26, 641–648
- Goel, A. et al. (2001) Single-dose versus fractionated radioimmunotherapy of human colon carcinoma xenografts using 131I-labeled multivalent CC49 single-chain fvs. *Clin. Cancer Res.* 7, 175–184
- Kontermann, R.E. (2009) Strategies to extend plasma half-lives of recombinant antibodies. *BioDrugs* 23, 93–109
- Harris, J.M. and Chess, R.B. (2003) Effect of pegylation on pharmaceuticals. *Nat. Rev. Drug Discov.* 2, 214–221
- Beckman, R.A. et al. (2007) Antibody constructs in cancer therapy: protein engineering strategies to improve exposure in solid tumors. *Cancer* 109, 170–179
- Pack, P. and Pluckthun, A. (1992) Miniantibodies: use of amphipathic helices to produce functional, flexibly linked dimeric FV fragments with high avidity in *Escherichia coli*. *Biochemistry* 31, 1579–1584
- Li, S.L. et al. (2000) Single-chain antibodies against human insulin-like growth factor I receptor: expression, purification, and effect on tumor growth. *Cancer Immunol. Immunother.* 49, 243–252
- Hu, S. et al. (1996) Minibody: a novel engineered anti-carcinoembryonic antigen antibody fragment (single-chain Fv-CH3) which exhibits rapid, high-level targeting of xenografts. *Cancer Res.* 56, 3055–3061
- Li, E. et al. (1997) Mammalian cell expression of dimeric small immune proteins (SIP). *Protein Eng.* 10, 731–736
- Furuta, M. et al. (1998) Construction of mono- and bivalent human single-chain Fv fragments against the D antigen in the Rh blood group: multimerization effect on cell agglutination and application to blood typing. *Protein Eng.* 11, 233–241
- Shahied, L.S. et al. (2004) Bispecific minibodies targeting HER2/neu and CD16 exhibit improved tumor lysis when placed in a divalent tumor antigen binding format. *J. Biol. Chem.* 279, 53907–53914
- Schoonjans, R. et al. (2000) Fab chains as an efficient heterodimerization scaffold for the production of recombinant bispecific and trispecific antibody derivatives. *J. Immunol.* 165, 7050–7057
- Schoonjans, R. et al. (2001) A new model for intermediate molecular weight recombinant bispecific and trispecific antibodies by efficient heterodimerization of single chain variable domains through fusion to a Fab-chain. *Biomol. Eng.* 17, 193–202
- Holliger, P. et al. (1993) ‘Diabodies’: small bivalent and bispecific antibody fragments. *Proc. Natl. Acad. Sci. U. S. A.* 90, 6444–6448
- Carmichael, J.A. et al. (2003) The crystal structure of an anti-CEA scFv diabody assembled from T84.66 scFvs in V(L)-to-V(H) orientation: implications for diabody flexibility. *J. Mol. Biol.* 326, 341–351
- Holliger, P. and Hudson, P.J. (2005) Engineered antibody fragments and the rise of single domains. *Nat. Biotechnol.* 23, 1126–1136
- Kipriyanov, S.M. et al. (1999) Bispecific tandem diabody for tumor therapy with improved antigen binding and pharmacokinetics. *J. Mol. Biol.* 293, 41–56
- Lu, D. et al. (2003) Di-diabody: a novel tetravalent bispecific antibody molecule by design. *J. Immunol. Methods* 279, 219–232
- Todorovska, A. et al. (2001) Design and application of diabodies, triabodies and tetrabodies for cancer targeting. *J. Immunol. Methods* 248, 47–66
- Iliades, P. et al. (1997) Triabodies: single chain Fv fragments without a linker form trivalent trimers. *FEBS Lett.* 409, 437–441
- Wolf, E. et al. (2005) BiTEs: bispecific antibody constructs with unique anti-tumor activity. *Drug Discov. Today* 10, 1237–1244
- Goel, A. et al. (2000) Genetically engineered tetravalent single-chain Fv of the pancarcinoma monoclonal antibody CC49: improved biodistribution and potential for therapeutic application. *Cancer Res.* 60, 6964–6971
- Goel, A. et al. (2001) 99mTc-labeled divalent and tetravalent CC49 single-chain Fv’s: novel imaging agents for rapid in vivo localization of human colon carcinoma. *J. Nucl. Med.* 42, 1519–1527
- Deyev, S.M. et al. (2003) Design of multivalent complexes using the barnase*barstar module. *Nat. Biotechnol.* 21, 1486–1492
- Halin, C. et al. (2003) Synergistic therapeutic effects of a tumor targeting antibody fragment, fused to interleukin 12 and to tumor necrosis factor alpha. *Cancer Res.* 63, 3202–3210
- Borsi, L. et al. (2003) Selective targeted delivery of TNFalpha to tumor blood vessels. *Blood* 102, 4384–4392
- Deyev, S.M. and Lebedenko, E.N. (2008) Multivalency: the hallmark of antibodies used for optimization of tumor targeting by design. *Bioessays* 30, 904–918
- Cuesta, A.M. et al. (2009) In vivo tumor targeting and imaging with engineered trivalent antibody fragments containing collagen-derived sequences. *PLoS One* 4, e5381
- Fan, C.Y. et al. (2008) Production of multivalent protein binders using a self-trimerizing collagen-like peptide scaffold. *FASEB J.* 22, 3795–3804
- Hudson, P.J. and Souriau, C. (2003) Engineered antibodies. *Nat. Med.* 9, 129–134
- Rossi, E.A. et al. (2006) Stably tethered multifunctional structures of defined composition made by the dock and lock method for use in cancer targeting. *Proc. Natl. Acad. Sci. U. S. A.* 103, 6841–6846
- Schmidt, M.M. and Wittrup, K.D. (2009) A modeling analysis of the effects of molecular size and binding affinity on tumor targeting. *Mol. Cancer Ther.* 8, 2861–2871
- Sanchez-Arevalo, L.V. et al. (2006) Enhanced antiangiogenic therapy with antibody-collagen XVIII NC1 domain fusion proteins engineered to exploit matrix remodeling events. *Int. J. Cancer* 119, 455–462
- Leyton, J.V. et al. (2008) Humanized radioiodinated minibody for imaging of prostate stem cell antigen-expressing tumors. *Clin. Cancer Res.* 14, 7488–7496
- Yazaki, P.J. et al. (2001) Tumor targeting of radiometal labeled anti-CEA recombinant T84.66 diabody and t84.66 minibody: comparison to radioiodinated fragments. *Bioconjug. Chem.* 12, 220–228
- Wu, A.M. and Yazaki, P.J. (2000) Designer genes: recombinant antibody fragments for biological imaging. *Q. J. Nucl. Med.* 44, 268–283
- Schneider, D.W. et al. (2009) In vivo biodistribution, PET imaging, and tumor accumulation of 86Y- and 111In-antimindin/RG-1, engineered antibody fragments in LNCaP tumor-bearing nude mice. *J. Nucl. Med.* 50, 435–443
- Wu, A.M. et al. (2000) High-resolution microPET imaging of carcinoembryonic antigen-positive xenografts by using a copper-64-labeled engineered antibody fragment. *Proc. Natl. Acad. Sci. U. S. A.* 97, 8495–8500
- Sundaresan, G. et al. (2003) 124I-labeled engineered anti-CEA minibodies and diabodies allow high-contrast, antigen-specific small-animal PET imaging of xenografts in athymic mice. *J. Nucl. Med.* 44, 1962–1969
- Boudko, S.P. et al. (2009) Crystal structure of human collagen XVIII trimerization domain: a novel collagen trimerization fold. *J. Mol. Biol.* 392, 787–802

- 43 Sanz, L. *et al.* (2003) A novel cell binding site in the coiled-coil domain of laminin involved in capillary morphogenesis. *EMBO J.* 22, 1508–1517
- 44 Sanz, L. *et al.* (2002) Single-chain antibody-based gene therapy: inhibition of tumor growth by in situ production of phage-derived human antibody fragments blocking functionally active sites of cell-associated matrices. *Gene Ther.* 9, 1049–1053
- 45 Adekar, S.P. *et al.* (2008) Neutralization of botulinum neurotoxin by a human monoclonal antibody specific for the catalytic light chain. *PLoS One* 3, e3023
- 46 Maynard, J.A. *et al.* (2002) Protection against anthrax toxin by recombinant antibody fragments correlates with antigen affinity. *Nat. Biotechnol.* 20, 597–601
- 47 Pelat, T. *et al.* (2007) High-affinity, human antibody-like antibody fragment (single-chain variable fragment) neutralizing the lethal factor (LF) of *Bacillus anthracis* by inhibiting protective antigen-LF complex formation. *Antimicrob. Agents Chemother.* 51, 2758–2764
- 48 Bose, B. *et al.* (2003) Characterization and molecular modeling of a highly stable anti-hepatitis B surface antigen scFv. *Mol. Immunol.* 40, 617–631
- 49 Cao, J. *et al.* (2008) Efficient neutralizing activity of cocktailed recombinant human antibodies against hepatitis A virus infection in vitro and in vivo. *J. Med. Virol.* 80, 1171–1180
- 50 Drew, P.D. *et al.* (2001) Multimeric humanized varicella-zoster virus antibody fragments to gH neutralize virus while monomeric fragments do not. *J. Gen. Virol.* 82, 1959–1963
- 51 Watts, T.H. (2005) TNF/TNFR family members in costimulation of T cell responses. *Annu. Rev. Immunol.* 23, 23–68
- 52 Zhou, Z. *et al.* (2008) Human glucocorticoid-induced TNF receptor ligand regulates its signaling activity through multiple oligomerization states. *Proc. Natl. Acad. Sci. U. S. A.* 105, 5465–5470
- 53 Wyzgol, A. *et al.* (2009) Trimer stabilization, oligomerization, and antibody-mediated cell surface immobilization improve the activity of soluble trimers of CD27L, CD40L, 41BBL, and glucocorticoid-induced TNF receptor ligand. *J. Immunol.* 183, 1851–1861
- 54 Gough, M.J. *et al.* (2008) OX40 agonist therapy enhances CD8 infiltration and decreases immune suppression in the tumor. *Cancer Res.* 68, 5206–5215
- 55 Griffiths, G.L. *et al.* (2004) Reagents and methods for PET using bispecific antibody pretargeting and ⁶⁸Ga-radiolabeled bivalent hapten-peptide-chelate conjugates. *J. Nucl. Med.* 45, 30–39
- 56 Redmond, W.L. and Weinberg, A.D. (2007) Targeting OX40 and OX40L for the treatment of autoimmunity and cancer. *Crit. Rev. Immunol.* 27, 415–436
- 57 Kontermann, R.E. (2005) Recombinant bispecific antibodies for cancer therapy. *Acta Pharmacol. Sin.* 26, 1–9
- 58 Weinberg, A.D. *et al.* (2006) Anti-OX40 (CD134) administration to nonhuman primates: immunostimulatory effects and toxicokinetic study. *J. Immunother.* 29, 575–585
- 59 Tahtis, K. *et al.* (2001) Biodistribution properties of (111)indium-labeled C-functionalized trans-cyclohexyl diethylenetriaminepentaacetic acid humanized 3S193 diabody and F(ab')₂ constructs in a breast carcinoma xenograft model. *Clin. Cancer Res.* 7, 1061–1072
- 60 Adams, G.P. and Weiner, L.M. (2005) Monoclonal antibody therapy of cancer. *Nat. Biotechnol.* 23, 1147–1157
- 61 Wittel, U.A. *et al.* (2005) The in vivo characteristics of genetically engineered divalent and tetravalent single-chain antibody constructs. *Nucl. Med. Biol.* 32, 157–164
- 62 Huhlov, A. and Chester, K.A. (2004) Engineered single chain antibody fragments for radioimmunotherapy. *Q. J. Nucl. Med. Mol. Imaging* 48, 279–288
- 63 Willuda, J. *et al.* (2001) Tumor targeting of mono-, di-, and tetravalent anti-p185(HER-2) miniantibodies multimerized by self-associating peptides. *J. Biol. Chem.* 276, 14385–14392
- 64 Olafsen, T. *et al.* (2005) Optimizing radiolabeled engineered anti-p185HER2 antibody fragments for in vivo imaging. *Cancer Res.* 65, 5907–5916
- 65 Di, N.R. *et al.* (2007) Construction of miniantibodies for the in vivo study of human autoimmune diseases in animal models. *BMC Biotechnol.* 7, 46
- 66 Casey, J.L. *et al.* (2002) Tumour targeting of humanised cross-linked divalent-Fab' antibody fragments: a clinical phase I/II study. *Br. J. Cancer* 86, 1401–1410
- 67 Dolezal, O. *et al.* (2000) ScFv multimers of the anti-neuraminidase antibody NC10: shortening of the linker in single-chain Fv fragment assembled in V(L) to V(H) orientation drives the formation of dimers, trimers, tetramers and higher molecular mass multimers. *Protein Eng.* 13, 565–574
- 68 Khawli, L.A. *et al.* (2003) Comparison of recombinant derivatives of chimeric TNT-3 antibody for the radioimaging of solid tumors. *Hybrid. Hybridomics* 22, 1–9
- 69 Edelweiss, E. *et al.* (2008) Barnase as a new therapeutic agent triggering apoptosis in human cancer cells. *PLoS One* 3, e2434
- 70 Liu, Y. *et al.* (2004) Recombinant single-chain antibody fusion construct targeting human melanoma cells and containing tumor necrosis factor. *Int. J. Cancer* 108, 549–557
- 71 Huang, T.H. and Morrison, S.L. (2006) A trimeric anti-HER2/neu ScFv and tumor necrosis factor- α fusion protein induces HER2/neu signaling and facilitates repair of injured epithelia. *J. Pharmacol. Exp. Ther.* 316, 983–991
- 72 Bauer, S. *et al.* (2006) Structure-activity profiles of Ab-derived TNF fusion proteins. *J. Immunol.* 177, 2423–2430
- 73 Sharkey, R.M. *et al.* (2008) Improved therapeutic results by pretargeted radioimmunotherapy of non-Hodgkin's lymphoma with a new recombinant, trivalent, anti-CD20, bispecific antibody. *Cancer Res.* 68, 5282–5290
- 74 Goldenberg, D.M. *et al.* (2008) Multifunctional antibodies by the dock-and-lock method for improved cancer imaging and therapy by pretargeting. *J. Nucl. Med.* 49, 158–163
- 75 Williams, L.E. *et al.* (2001) Numerical selection of optimal tumor imaging agents with application to engineered antibodies. *Cancer Biother. Radiopharm.* 16, 25–35
- 76 Hwang, W.C. *et al.* (2006) Structural basis of neutralization by a human anti-severe acute respiratory syndrome spike protein antibody, 80R. *J. Biol. Chem.* 281, 34610–34616
- 77 Russell, R.B. and Barton, G.J. (1992) Multiple protein sequence alignment from tertiary structure comparison: assignment of global and residue confidence levels. *Proteins* 14, 309–323