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New protein engineering approaches to multivalent and bispecific antibody fragments

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Abstract

Multivalency is one of the hallmarks of antibodies, by which enormous gains in functional affinity, and thereby improved performance in vivo and in a variety of in vitro assays are achieved. Improved in vivo targeting and more selective localization are another consequence of multivalency. We summarize recent progress in engineering multivalency from recombinant antibody fragments by using miniantibodies (scFv fragments linked with hinges and oligomerization domains), spontaneous scFv dimers with short linkers (diabodies), or chemically crosslinked antibody fragments. Directly related to this are efforts of bringing different binding sites together to create bispecific antibodies. For this purpose, chemically linked fragments, diabodies, scFv–scFv tandems and bispecific miniantibodies have been investigated. Progress in *E. coli* expression technology makes the amounts necessary for clinical studies now available for suitably engineered fragments. We foresee therapeutic advances from a modular, systematic approach to optimizing pharmacokinetics, stability and functional affinity, which should prove possible with the new recombinant molecular designs. © 1997 Elsevier Science B.V.

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1. Multivalency

1.1. An introduction to multivalency

Multivalency is a very general device that nature uses to increase the interaction energy be-

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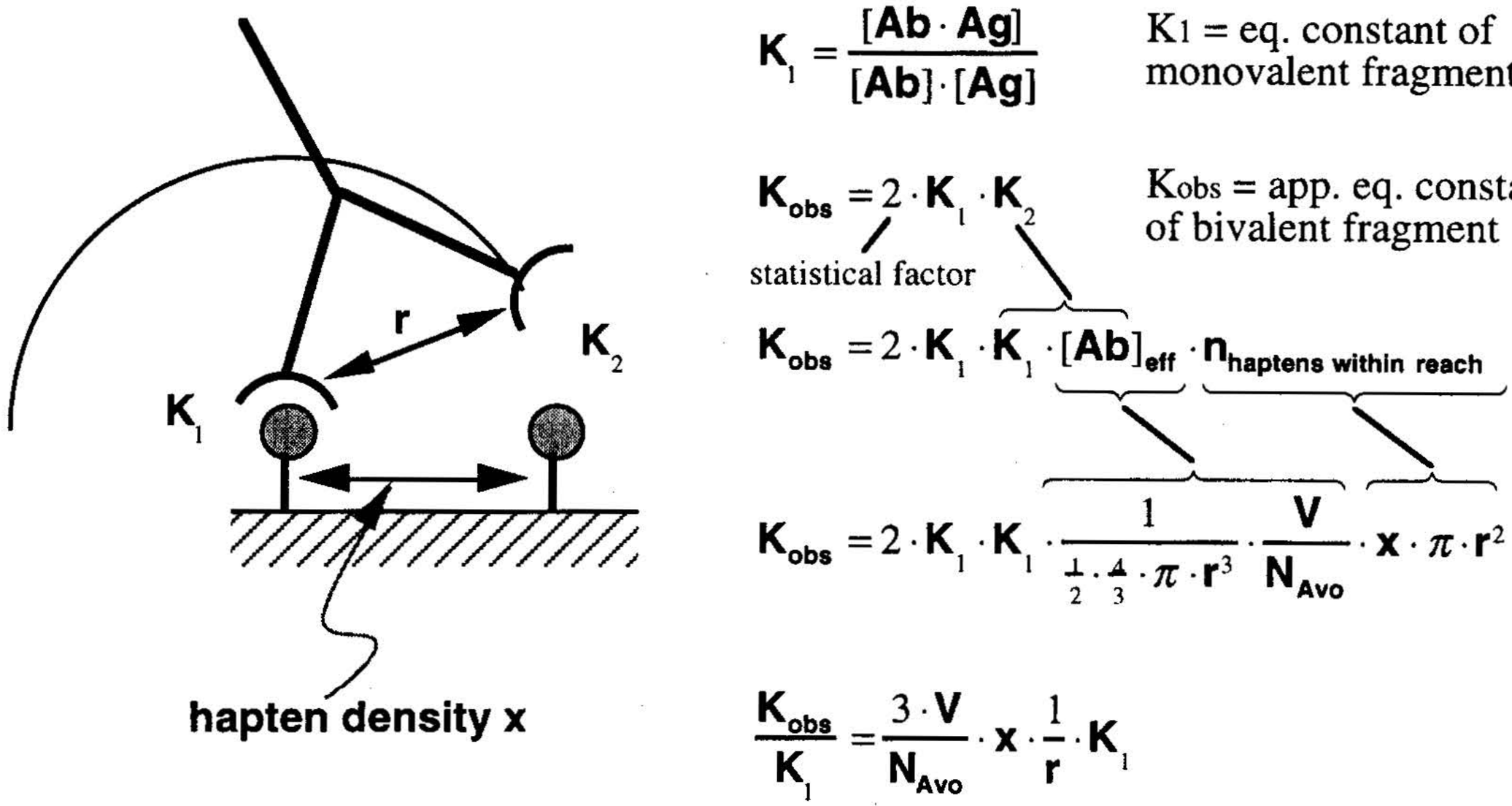
tween biomolecules. It makes use of the fact that biological structures are often multimeric. The surface of viruses consists of repetitive protein assemblies, cell surfaces contain multiple copies of the same surface proteins embedded in the cell membrane, and oligosaccharides consist of repeating sugar units. Even binding motifs on DNA can come in clusters, and are used as binding sites for oligomeric transcription factors in transcriptional control. Unsurprisingly, a multivalent protein (or peptide) which can make contact with more than one 'site' will bind with a greater free energy that one that can contact only one site. Furthermore, at low concentrations, such a multivalent protein will accumulate—at equilibrium—at those locations where the density of binding sites is highest.

The normal function of antibodies in the fight against foreign invaders is to bind to the surface of a virus, a bacterium or another pathogen and trigger biological effects with its Fc part. This principle of multivalency is used by the natural antibodies of all classes. This is most dramatically seen in the first molecule secreted in the immune response, IgM, which carries 10 or 12 binding sites [1] in a relatively stiff assembly, as IgM molecules do not have a flexible hinge peptide [2]. At this stage, somatic mutation has not yet taken place [3] and the individual binding interactions (the intrinsic affinity, see below) is rather weak. In the later course of the immune response, improvement in intrinsic affinity through somatic mutations is accompanied by class switching [4] and for most classes, the number of binding sites is reduced to two, probably because other design considerations of the molecule (such as Ig-class specific responses, segmental flexibility, tissue penetration, serum lifetime) take priority over the number of antigen binding sites. Nevertheless, bivalency is maintained at all times. A collection of bivalent antibodies recognizing different epitopes can also aggregate the antigen, e.g. a virus, and this may be of importance in the defense against certain viral diseases.

Over the last few years, a burst of reports on the construction and use of recombinant antibody fragments has appeared [reviewed in [5–9]], and there are good reasons to expect that the traditional approach to monoclonal antibodies will eventually be replaced by biotechnological approaches not relying on the immune system for antibody generation at any step. Initially, such recombinant molecules were either monovalent Fv, single-chain Fv (scFv) or Fab fragments of antibodies or reconstructed full antibodies. However, depending on the particular application, much better solutions may be available, such as combining small size and thus good tissue penetration with multivalency. It is the purpose of this article to review the various approaches to this problem, namely of engineering multivalency. It may be pointed out that very many typical in vitro applications of antibodies, even those directed against monomeric soluble proteins, can involve the binding to clustered antigens bound to a solid phase, such as the immobilized protein in a Western Blot, ELISA, or immunoelectron microscopy, as well as all in vivo or in vitro detection of cell bound antigens, from fluorescence activated cell sorting (FACS) to blood group analysis.

1.2. The quantitative aspects of multivalency: intrinsic and functional affinity

The specific energetic relationship between one antibody binding-site and its complementary antigenic determinant is described by the 'intrinsic' affinity, $K_1 = [AbAg]_{complex}/([Ab]_{free}[Ag]_{free})$. This is the affinity which is displayed by a monovalent fragment, and it also describes any binding of either monovalent or multivalent immunoglobulins to soluble, monomeric antigens, which is of course identical. In case of polymeric antigens or surface antigens, e.g. receptors distributed on a cell surface, the measured affinity can be considerably enhanced by the formation of multiple binding interactions within one antibody-antigen complex. The term 'functional affinity' was introduced by Karush [10,11] to replace the ill-defined term 'avidity' and to clearly distinguish between the specific monovalent interaction, the 'intrinsic affinity' and the enhancement of binding strength by multivalency [10,11]. In contrast to the intrinsic affinity, the measured enhancement factors strongly depends on the experimental conditions of solid phase assays, such as antigen density [12].



$$K_1 = \frac{[Ab \cdot Ag]}{[Ab] \cdot [Ag]}$$

$$K_{obs} = 2 \cdot K_1 \cdot K_2$$

$$K_{obs} = 2 \cdot K_1 \cdot K_1 \cdot [Ab]_{eff} \cdot n_{haptens\ within\ reach}$$

$$K_{obs} = 2 \cdot K_1 \cdot K_1 \cdot \frac{1}{\frac{1}{2} \cdot \frac{4}{3} \cdot \pi \cdot r^3} \cdot \frac{V}{N_{Avo}} \cdot x \cdot \pi \cdot r^2$$

$$\frac{K_{obs}}{K_1} = \frac{3 \cdot V}{N_{Avo}} \cdot x \cdot \frac{1}{r} \cdot K_1$$

Fig. 1. Enhancement of apparent equilibrium constant due to bivalency effects, according to the treatment of Crothers and Metzger [13]. K_1 is the observed binding constant for a monovalent fragment, K_{obs} for a bivalent fragment. [Ab], [Ag] and [Ab·Ag] are the molar concentrations of antibody, antigen and complex respectively (as if the number of molecules in the reaction well were evenly distributed). K_2 is the binding constant of the second binding site, made unitless by the constant effective antibody concentration $[Ab]_{eff}$ which results from constraining it within the hemisphere of radius r. The hapten density x is a two-dimensional concentration (molecules per area). N_{Avo} is Avogadro's number, used to convert molecular into molar concentrations, and V is the unit volume used for concentration units (l).

A number of quantitative approaches for multivalency have been developed (summarized in [10,11]) and perhaps the most intuitive is the one of Crothers and Metzger [13] (Fig. 1). It says that the gain contributed by the second binding site is only observed if the antigen is on a surface or is polymeric. In this case, the functional affinity K_{obs} is the product of the two binding constants of the two sites (Fig. 1) the first (K_1) being that which a monovalent Fab or scFv fragment would show. The second one (K_2) is the (dimensionless) binding constant of the second site, once the first is bound. This second binding is a monomolecular reaction, described by the second site being at a certain constant molar concentration, being restricted to the neighborhood of its epitope. The simplest way to view this concentration is to treat it as one binding site in the hemisphere which becomes accessible once the first binding site has docked. This concentration needs to be converted to molar units.

The above derivation is somewhat simplistic. First, it is valid only at very low occupancy of antigen, and this is not achieved in most experimental systems. Furthermore, it neglects a number of complications, such as any energy needed to 'bend' either antibody or antigen, steric inaccessibility, surface layer effects (different ion concentrations, inaccessibility) and uneven microscopic distributions of the antigen in the surface. Nevertheless, it makes clear that a minimum distance between the binding sites is needed to have a finite chance of binding two different antigen molecules simultaneously, and it illustrates the dependencies on a number of variables. Furthermore, additional factors arise in dynamic systems, such as the circulation velocity of the antibody in the (blood) flow and diffusion-driven movements of surface antigens in semi-liquid cell membranes. These problems have been summarized in the criticism that the whole concept of functional affinity is not a useful one [14], but we believe that the semiquantitative trends predicted are still instructive for molecular design.

The essence of the simplified treatment (Fig. 1) is that, by having a second binding site, the enhancement factor (K_{obs}/K_1) should be proportional to the true intrinsic association constant K_1, and inversely proportional to the distance r of the two binding sites (provided r does not become too short for reaching this second site). Furthermore, the enhancement factor is proportional to the epitope density x on the surface (Fig. 1). The introduction of three or more binding sites into predictions of multivalent binding events is even more difficult, because additional complications between the idealized situation to the actual situation in vitro or in vivo accumulate in the calculation. Nevertheless, the measurable effects by going from bivalency to tri- or tetravalency can be dramatic [12,15].

Therefore, this gain or enhancement factor K_{obs}/K_1 is not a constant, but depends on many variables of a particular molecular system, sometimes leading to differences in the literature data of several orders of magnitude of the measured functional affinity of identical antibody/antigen pairs [16,17]. Consequently, the prediction of in vivo delivery and localization of antibodies with the potential to bind at least bivalently to an antigen covered cell surface cannot be based on measurements in solution or monomeric antigens, but has to be analyzed on the basis of a bivalent model [18], ideally with cellular antigen.

This strong surface dependence of the multivalency effect can also be taken advantage of. It should be possible to strongly discriminate between cells having a high density of tumor markers from those having a low density, if stable multivalent binding is possible only in the latter case, e.g. if the intrinsic binding affinity is too low to lead to stable monovalent binding. At high epitope densities, the bivalent binding of monoclonal antibodies can enhance the functional affinity up to a 1000-fold compared with the intrinsic affinity of the monovalent antibody-antigen complex. In case of an anti-DNP IgM, the measured enhancement factor between intrinsic and functional affinity even appeared to be in the range of 10^6–10^7 under the chosen experimental conditions [19]. Thus, even antibodies of only moderate affinity can be made highly useful. An antibody which recognizes the his-tag [20] with an affinity of about $10^7\ M^{-1}$ can be used for affinity purification of proteins carrying the his-tag (Müller et al., manuscript in preparation), as binding is only monovalent and can be eluted under mild conditions, while highly sensitive detection on Western blots and ELISA is possible in a dimeric format, fused to alkaline phosphatase [20–22]. With protein antigens, the enhancement factor can be much smaller than with haptens, as even close epitopes often are not fully sterically accessible to unstrained dimers, or the distance between them may exceed the maximum reach of a bivalent antibody, which measures about 150 Å from the CDRs of one Fab to the other when fully stretched out.

How can the functional affinity be measured? In order to attribute the gain in functional affinity in going from monomers to higher oligomers to the multivalency effect, a number of other factors have first to be eliminated, such as a change in the intrinsic affinity or a different number of functional molecules in the preparation. Thus, the binding to monomeric antigen must be measured independently for all molecular species, e.g. by an inhibition ELISA according to Friguet et al. [23], and the percentage of active molecules must also be determined independently. A multivalency effect will be seen by a strong difference between species at different surface densities. Particularly informative can be BIAcore experiments, where the intrinsic affinity is available from inhibition of binding with soluble antigen [24,25] and the matrix density can be varied in a controlled way [12]. However, all quantitative treatments are subject to complications. The off-rates of multivalent species in BIAcore are usually not mono-exponential, since dissociation of monovalently and multivalently bound species occurs simultaneously with different rates. For similar reasons, Scatchard plots describing the binding to either cells or ELISA plates are expected to be curved upward [14,18], making the evaluation of the functional affinity semiquantitative at best. Nevertheless, we would like to stress that such experiments are still very useful in comparing different designed molecules on the same surface [12,15].

2. Recombinant antibodies

2.1. Brief overview on production and use of recombinant antibodies

Recombinant antibody technology has made many new formats and antibody fragments available, as well as fusion proteins, and has also been instrumental in the access to human antibodies for therapy [5–9]. Furthermore, through the generation of large libraries and phage screening technologies [9,26], antibodies can now be obtained without resorting to immunization of animals. Human antibodies to a wide variety of antigens will become routinely available within weeks, once the effort of library generation is completed. The display and selection of antibody fragments, especially with human sequences, will greatly enhance the development of new antibody-based substances for diagnostic and therapeutic purposes.

Antibodies or fragments thereof have been expressed in a wide variety of hosts, ranging from prokaryotes such as *E. coli* [27,28] or *B. subtilis* [29], to *Saccharomyces cerivisiae* [30–32], *Pichia pastoris* [33,34], *Trichoderma* [35], insect cells [36–39], plant cells or plants [40–46] and mammalian cells [47,48].

The use of *E. coli* is convenient since cloning and genetic manipulations are carried out with this host anyway, as are all operations with phage libraries. Compared with eukaryotes, fewer steps are needed for the manipulation of recombinant genes in *E. coli*, stable transformation of multiple copies into the host, inducible expression and characterization. Eukaryotic hosts are required, if whole antibodies are to be produced in good yields, since the Fc part carries oligosaccharide chains. For unglycosylated antibody constructs, however, different hosts have been considered, usually because of unsatisfactory yields in *E. coli* for the particular fragment tested. However, as will be discussed Section 2.2, the functional yield is highly dependent on the primary sequence of the antibody, and the use of engineered fragments and high-cell density fermentation appears to have given the highest molar yields of binding sites per culture volume of any expression host [49–51] (Section 6).

2.2. Expression of recombinant antibody fragments in E. coli

Antibody fragments can be produced in *E. coli* by refolding from inclusion bodies [52–58] or by functional expression by secretion to the bacterial periplasm [27,28]. The choice between secretion and refolding has to be guided by considerations such as expressibility of a certain sequence in functional form (see below), the effort to optimize a refolding procedure for a particular molecule, and the amounts or purity needed for subsequent use in scientific, diagnostic or therapeutic applications. Secretion is generally the faster strategy, ideal for rapid testing, and it can be scaled up to a true production method (Section 6). Refolding is especially useful for large amounts of a particular fragment, where the procedure can be individually optimized. It may be noted that there can be large differences in the in vitro refolding yields of different molecules as well. In the future, the majority of sequences may be derived from phage libraries [9]. Since only a successful secretion leads to a functional display on phage and subsequent enrichment, molecules of at least mediocre folding properties are selected, but 'good folders' can of course only be selected if the library actually contains such molecules.

Different monovalent fragments (Fig. 2), (Fv, Fab, scFv and disulfide-bonded Fv (dsFv)) fragments have been secreted [27,28,50,59,60] and those can serve as building blocks for multivalent fragments. Several chains can be co-expressed from the same plasmid using a dicistronic approach, which is useful not only for Fab, Fv and dsFv fragments, but also for the simultaneous expression of two chains which need to assemble in bispecific molecules (see below). While Fab or dsFv fragments may be more stable against thermal denaturation [61], only the scFv fragments [62,63] have the advantage of a single genetic chain, which simplifies the molecular assembly of multimeric fragments, and is a requirement to make the assembly of bispecific fragments unique.

To our knowledge, fully successful attempts to make functional whole antibodies in *E. coli* have not been reported. Part of the problem is that, at least in the Fc portion of IgG, for which a crystal

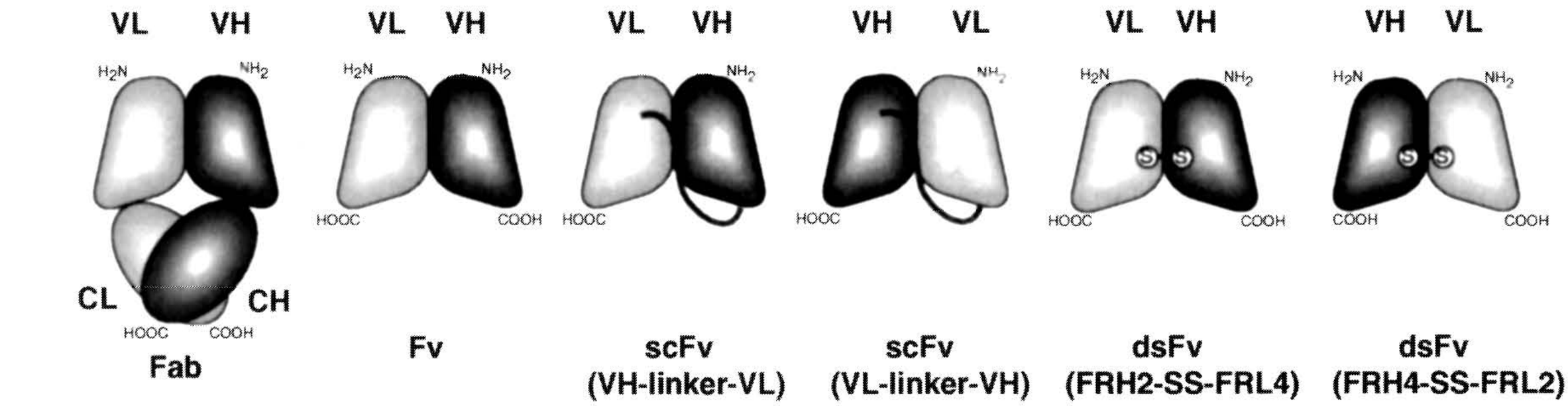


Fig. 2. Monovalent fragments of antibodies functionally expressed in *E. coli*. Fab fragment and Fv fragment consist of two separate chains, as do the disulfide-linked Fv fragments (dsFv), while the single-chain Fv (scFv) fragments with the orientation V_H-linker-V_L or V_L-linker-V_H are made from a single gene and are therefore particularly suitable for heterodimeric assembly. Monomeric scFv fragments with the domain orientation as shown are obtained, provided the linker is of sufficient length (see text and Fig. 5).

structure is known [64,65] the two C_H2 domains make no protein–protein contact at all, but their contact is entirely mediated by glycosylation [66] which of course does not take place in *E. coli*. Furthermore, even if such an unglycosylated molecule could be made, it would not be particularly useful, since the binding to Fc receptors and complement depends on glycosylation [67–70], probably because a certain distance and domain orientation must be maintained within the Fc portion. Thus, not all eukaryotic hosts [71] lead to antibodies with functional complement activation or antibody dependent cellular cytotoxicity (ADCC).

Even for an IgA, which is not glycosylated in C_H2 [72], the functional expression of a complete bivalent antibody (V_H-C_H1-hinge-C_H2-C_H3 co-expressed with V_L-C_L) results in severe proteolysis within the interdomain linker sequences as well as low yields of functional bivalent species, as was tested with the IgA McPC603 (Pack and Plückthun, unpublished results). Lo et al. [73] have investigated the assembly of a whole IgG antibody in the periplasm of *E. coli*, from which the C_H2 domain was deleted. A mixture of HL and H_2L_2 molecules was obtained, and no disulfide was formed between the two heavy chains.

While periplasmic folding has lead to functional molecules for a wide variety of fragments [6], the yields can differ significantly between fragments. There is a side reaction in periplasmic folding, in that a certain, sequence-dependent percentage of the antibody fragments tends to aggregate during folding [74–76]. This is observed in all types of fragments, and it appears to be more severe the more Ig domains are connected in a single chain.

It now appears that there are three strategies available to solve this aggregation problem. By a 'back-engineering' approach [75], single residues which determine the aggregation behavior of the molecule have been identified from comparison with well expressed antibodies. The fragments so obtained did not differ significantly in thermodynamic stability measured by urea unfolding, implying that it is not necessarily thermodynamic stability which limits folding. A more general solution to improving folding yield was then attempted in identifying exposed hydrophobic residues at the variable/constant interface [77]. Mutations in this interface can improve folding behavior of scFv fragments, again without changing thermodynamic stability. In a third approach, superior frameworks have been tested as recipients for diverse antibody specificities. Particularly favorable properties of the VH3/Vκ1 framework [50] were shown to still be present when different loops were used on this framework [50,78,79, Bothmann, Bauer, Knappik and Plückthun, unpublished data]. Recently, synthetic fragments based on consensus sequences of human germline families have shown surprisingly good secretion properties and a low tendency to form insoluble

aggregates, leading to functional yields of more than 10 mg/l in conventional shake-flask cultures (Knappik et al., in preparation). Thus, the folding problems appear to be related to the antibody sequence, but solvable by protein engineering. Interestingly, variable yields have been observed for different scFv fragments in COS cells as well [44], so that this engineering approach may be of more general utility than for bacterial expression. In general, it may be useful to keep the number of Ig domains per chain low, and use other oligomerization devices more compatible with *E. coli*.

3. Recombinant multivalent fragments

3.1. Overview of dimeric antibody fragments

For medical applications such as targeting to tumor-associated antigens, efficient tissue penetration must be combined with high functional affinity (see also Section 7), and fragments must be sufficiently stable against denaturation or proteolysis until they have reached the tumor site in the human body. An immunoglobulin-based structure should, ideally, combine small size and high functional affinity, whenever the possibility for multivalent interactions is given [80]. To confer the ability to heterologously expressed antibody fragments to bind at least bivalently, a variety of formats and protein designs have been investigated, such as miniantibodies [12,15,49,81–83], diabodies [84–89], disulfide-linked fragments [50,90–95]. We will discuss the properties of these fragments, and how well they mimic the rotational and segmental flexibility as well as the reach of the binding arms provided by the hinge regions.

3.2. Dimeric miniantibodies

The authors of this review have investigated the possibility to mimic the in vivo association of the poorly expressed and proteolytically sensitive C_H2-C_H3 domains with considerably smaller and well expressed 'association domains', which are C-terminally fused to scFv fragments (Fig. 3, Table 1). In general, association domains consist of a long and flexible hinge sequence followed by a self-associating secondary structure and an optional cysteine containing tail, with a molecular weight of only 2–5 kDa [12,15,49,81–83]. The association domains are encoded as modular gene cassettes and can easily be fused to scFv fragments. The various miniantibody formats can therefore be applied for every expressible scFv, regardless of the sequence, specificity or V_L-V_H interface of the fused fragment. In principle, they can be added to Fab fragments as well [96–98], but many of the advantages of small size and fixed V_H/V_L pairing for bispecific molecules (see below) would then be lost.

The term 'miniantibody' was chosen for the resulting fusion proteins, since the use of hinge regions creates a spacing, hinge bending and rotational freedom of the associated scFv fragments similar to the Fab-arms of a complete antibody [65,99] (Fig. 4), but with a fraction of its molecular weight. This was achieved by not adding the dimerization handle directly to the scFv fragment, but rather separated by the upper hinge from murine or human IgG3, known to lead to a flexible arrangements of domains as summarized in [99].

A variety of self-associating secondary structures such as helix bundles [100–103] or coiled-coils (leucine zippers) [104–106] were investigated to bring about in vivo dimerization of the scFv fragments [81] (Fig. 3 and Fig. 4).

Four-helix bundles are compact folding motifs of natural proteins. Eisenberg, deGrado and coworkers described a synthetic 4-helix bundle [100] made from either four single helices, two helix-turn-helix peptides or one continuous chain [101–103]. In this design, all four helices have the same sequence, and a surprisingly high thermodynamic stability has been measured with synthetic peptides [101]. Using this association principle, three different versions have been tested with scFv fragments. In the first, only one helix was fused to the scFv fragment, linked by the upper hinge region from mouse IgG3 (Table 1). No tetramers are formed, but rather a distribution between monomers and dimers is obtained, presumably because the association energy of single helices is

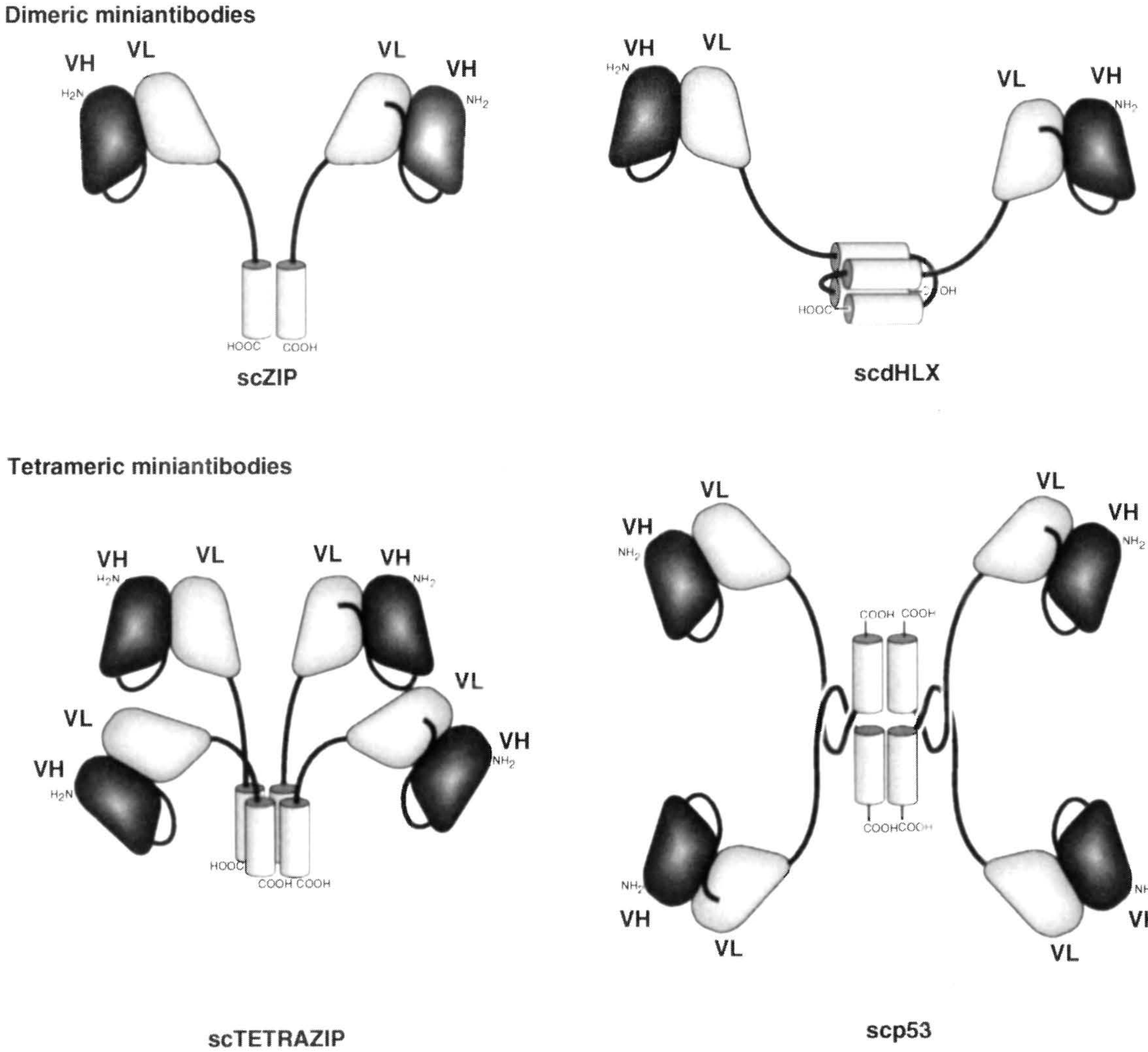


Fig. 3. Dimeric and tetrameric miniantibodies. The proline rich hinge region is indicated in black, as is the glycine rich spacer connecting V_H and V_L. The schematic orientation of the helices are indicated which mediate oligomerization. This orientation is derived from the published crystal structures of the coiled coil, tetrazipper and p53 domains, as well as from the unpublished NMR structure of the designed dHLX motif (W.F. deGrado, personal communication). For the exact sequences of the interchangeable modules, see Table 1. The detailed models are shown in Fig. 4.

too weak. This can be improved by extending the helix with a hydrophilic peptide ending in a cysteine to 'lock' the association by an interdomain disulfide-bridge [81]. Most preferable, however, is the non-covalent dimerization of the scdHLX construct carrying a helix-turn-helix motif. In this case, very little degradation is observed and functional affinities are obtained identical to a whole parent antibody [49]. It appears that these amphipathic helices are compatible with transport through the bacterial membrane and cause no significant problems in folding of most of the scFv fragments tested. For some scFvs with a relatively high expression yield as monomer (> 1 mg/l in shaking culture), a reduction of yields of the dimeric formats by around 20–50% has been observed, however (Pack and Knappik, unpublished).

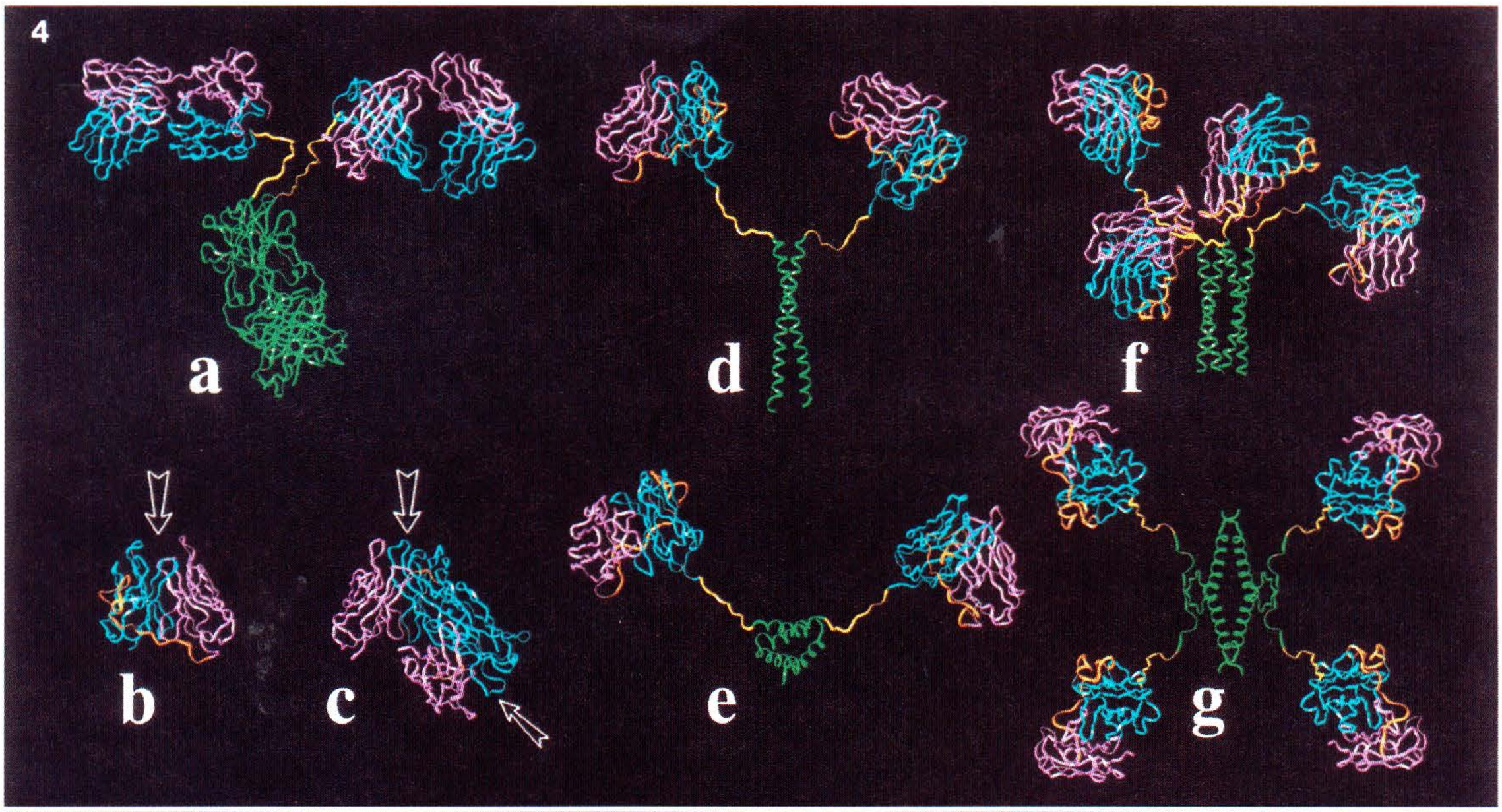
Table 1
Sequences of association domains for dimeric and tetrameric miniantibody constructs

Construct	Upper hinge	Self-associating peptide	Linker/tag
Bivalent			
scHLX	scFv-PKPSTPPGSS-	GELEELLKHLKELLKGef	
	Murine IgG3	Helix	
scHLXc	scFv-PKPSTPPGSS-	GELEELLKHLKELLKG-	PRKANSRNC
	Murine IgG3	Helix	Cys-tail
scdHLX	scFv-PKPSTPPGSS-	GELEELLKHLKELLKG-PRK-GELEELLKHLKELLKGef	
	Murine IgG3	Helix-1 turn helix-2	
scdHLX-His	scFv-PKPSTPPGSS-	GELEELLKHLKELLKG-PRK-GELEELLKHLKELLKG-	GSGGAP-HHHHH
	Murine IgG3	Helix-1 turn helix-2	spacer His-tail
scZIP	scFv-PKPSTPPGSS-	RMKQLEDKVEELLSKNYHLENEVARLKKLVGER	
	Murine IgG3	GCN4 leucine zipper	
scZIPc	scFv-PKPSTPPGSS-	RMKQLEDKVEELLSKNYHLENEVARLKKLVGER	GGCGG
	Murine IgG3	GCN4 leucine zipper	Cys-tail
Bispecific			
scJUN	scFv-PKPSTPPGSS-	RIARLEEKVKTLKAQNSELASTANMLREQVAQLKQKVMNY	
	Murine IgG3	JUN leucine zipper	
scFOS	scFv-PKPSTPPGSS-	LTDTLQAETDQLEDKKSALQTEIANLLKEKEKLEFILAAH	
	Murine IgG3	FOS leucine zipper	
Tetravalent			
scTETRAZIP	scFv-PKPSTPPGSS-	RLKQIEDKLEEILSKLYHIENELARIKKLLGER	
	Murine IgG3	GCN4 leucine zipper, modified	
scp53	scFv-TPLGDTTHTSG-	KPLDGEYFTLQIRGRERFEMFRELNEALELKDAQAGKEP	
	Human IgG3	oligomerization domain of human p53	
scp53-His	scFv-TPLGDTTHTSG-	KPLDGEYFTLQIRGRERFEMFRELNEALELKDAQAGKEP-	GGSGGAP-HHHHH
	Human IgG3	oligomerization domain of human p53	spacer His-tail

The sequences of various modules used for effecting oligomerization are shown. They are functionally separated into hinge, self-associating peptide and any tags. The whole module is cloned as a single restriction fragment, thereby converting any recombinant scFv to the desired format in a single step. Small letters signify amino acids introduced for creating restriction sites.

Coiled-coils [104–106] occur, for example, as dimerization devices in eukaryotic transcription factors. Because of their preference for leucine in every 7th position, they have been termed 'leucine zippers', a structurally slightly misleading name given before the coiled-coil structure was recognized. These peptides have also been used as dimerization devices in other proteins [107,108]. The zipper from the yeast transcription factor GCN4 was used and shown to be suitable as a dimerization device, although it does not give as high a signal in solid phase assays as 4-helix-bundles (see below). The reason for this difference is not quite clear, but it might have to do with the parallel arrangement of the helices, constraining the two hinge regions further. We cannot exclude that the antibody domains interact with each other during folding, since they are constrained in each others' neighborhood and, in a fraction of molecules, might form diabody-like dimers on the top of the coiled coil-based association domains with a significantly reduced flexibility and range (Arndt et al., manuscript in preparation).

In contrast, in the 4-helix bundle, which is antiparallel, the length of the helix itself contributes to the distance the miniantibody can span between two binding sites and it may therefore, increase the likelihood of two binding sites folding independently. These sites can then bind simultaneously to the same antigen-carrying surface. Optional cysteine tails at the C-terminus of zipper or bundle-helix based association domains result in a quantitative covalent linkage of the



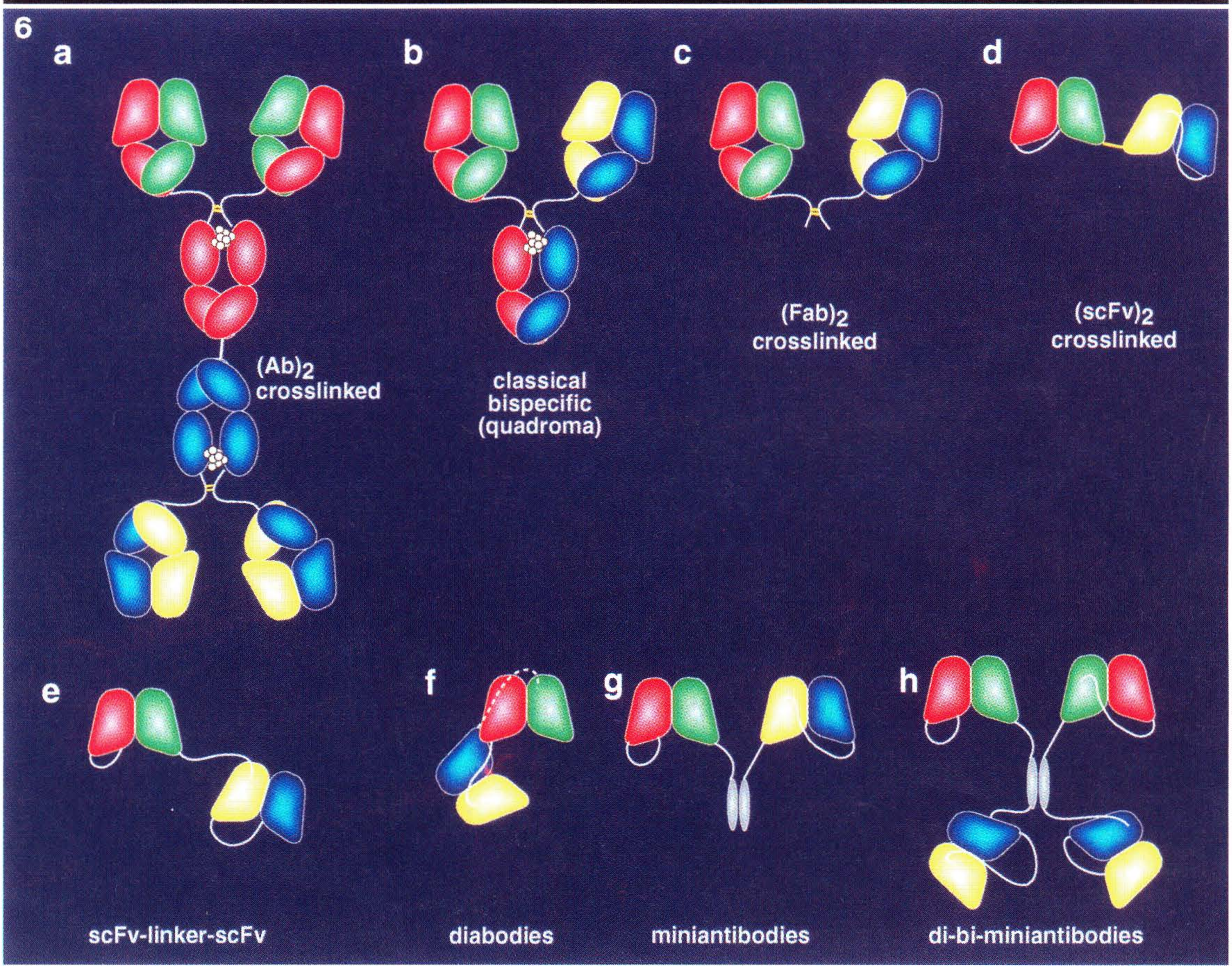


Fig. 4 and Fig. 6.

dimers, presumably due to the close proximity of free cysteines after dimerization [81]. The additional cysteine tails, however, may increase the risk of unwanted disulfide formation with cysteines within the variable domains or with other *E. coli* proteins (Pack and Plückthun, unpublished observations).

Beside these minimal motives based on helical structures, fusions to dimerizing enzymes and Ig domains as dimerization devices have also been investigated [see, e.g. 20–22], but will not be discussed in detail. McGregor et al. have reported the use of whole κ chain [109] in a V_H-linker-V_L-C_L molecule to achieve dimerization, while the C_H3 domain has been fused directly to a scFv antibody or via a hinge region as discussed above [110].

3.3. The monomer-dimer equilibrium of scFv fragments

It had been noticed in a number of research groups that certain single-chain Fv fragments, depending both on the V_H/V_L interface and the linker length, can spontaneously dimerize or even multimerize [84–86,111,112]. A more careful investigation of this phenomenon [86,87,89] then demonstrated that the percentage of monomer is directly proportional to the length of the linker, connecting the two variable domains. At short linker lengths, this is immediately understandable, since the linker is simply not long enough to span the distance to the other domain in the native monomeric scFv. Molecular modeling [86] and

Fig. 4. Structural models of various dimeric and tetrameric antibody constructs. The V_L and C_L domains are shown in purple, the V_H and C_H1 domains in blue, the dimerization domains (helices or constant domains) in green, hinge regions in yellow, linkers connecting V_H and V_L in orange. (a) Whole IgG2a, as observed in the crystal structure of the whole molecule [65]. Note that the two Fabs are rotated relative to each other, and that the Fc part is seen at an angle. This indicates considerable flexibility of the two antigen binding arms with respect to each other and the Fc part, guiding the design of molecules d, e, f and g. (b) Single-chain Fv fragment, for size comparison. The arrow points down the pseudo two-fold axis and indicates the direction of approach of antigen. (c) Diabody structure [88]. The two binding sites are identical (Fig. 5), and arrows pointing down the pseudo two-fold axis (relating V_H and V_L) indicate the approach of antigen. The arrow is drawn in the plane connecting the centers of mass of the two domains. This orientation of the binding sites also sets a relative orientation of the two epitopes required for simultaneous binding. (d) Bivalent miniantibody of the ZIP type (GCN4 [105]), with the arrangement V_H-linker-V_L-hinge-ZIP. (e) Bivalent miniantibody of the dHLX type, with the arrangement V_H-linker-V_L-hinge-helix-turn-helix. The helix-turn-helix motifs are interlocking like two clasping hands (see scheme in Fig. 3), as derived from the NMR structure of the corresponding peptide (W.F. DeGrado, personal communication). It can be seen that the distances of the binding sites reached by constructs of type d, e, f and g are similar to a whole antibody (a), and much larger than of the diabody (b). (f) Tetravalent miniantibody of tetrazipper type [121], with the arrangement V_H-linker-V_L-hinge-TETRAZIP. The four helices are parallel, thus bringing four binding sites close to each other ready to bind to a common surface. (g) Tetravalent miniantibody of p53 type, with the arrangement V_H-linker-V_L-hinge-p53. The tetramerization domain of p53 [122] leads to a more or less tetrahedral distribution of the four scFvs in space, which therefore span a very large distance and can reach epitopes far away from each other.

Models were built in INSIGHT II, using the modules with known 3D-structure referenced above, and mostly using extended poly-proline helices for the hinge region in (d)–(g). Secondary structures were automatically assigned with the Kabsch-Sander algorithm built into the INSIGHT II software. The atomic structure in (a) and (e) was regenerated from the Cα coordinates with the program MaxSprout [174].

Fig. 6. Summary of bispecific antibody formats. Different chains are given characteristic colors, and red/green or blue/yellow make up a correct binding site. (a) Two whole IgGs are chemically crosslinked, usually in a non-directional fashion and the crosslinking at the C_H3 domains is meant as an illustration only. (b) Bispecific antibody as obtained from heterohybridoma (quadroma). Note that this desired molecule is only one of ten species which may assemble from the random combination of heavy and light chains. (c) Crosslinked Fab' fragments, using the unpaired cysteines at the end of the constant domains. (d) Crosslinked scFv, using engineered cysteines at the end of either V_L or V_H. In (c) and (d), the crosslink can be effected by oxidation to a disulfide or by bis-maleimide crosslinking. (e) Genetic fusion scFv-linker-scFv. Very little is known about these molecules and it is not clear, whether the scFvs remain non-interacting, as schematically drawn here. (f) Heterobivalent diabodies, in analogy to Fig. 5, from using two different scFvs with short linkers. (g) Bispecific miniantibodies. The parallel helices illustrated here is only one of several heterodimerization motifs currently under investigation. (d) Di-Bi miniantibodies (Müller et al., manuscript in preparation). These molecules are derived from bivalent miniantibodies, fusing another scFv after the heterodimerization motif. They are bivalent for both antigens (top and bottom of molecule) and bispecific (left and right of molecule).

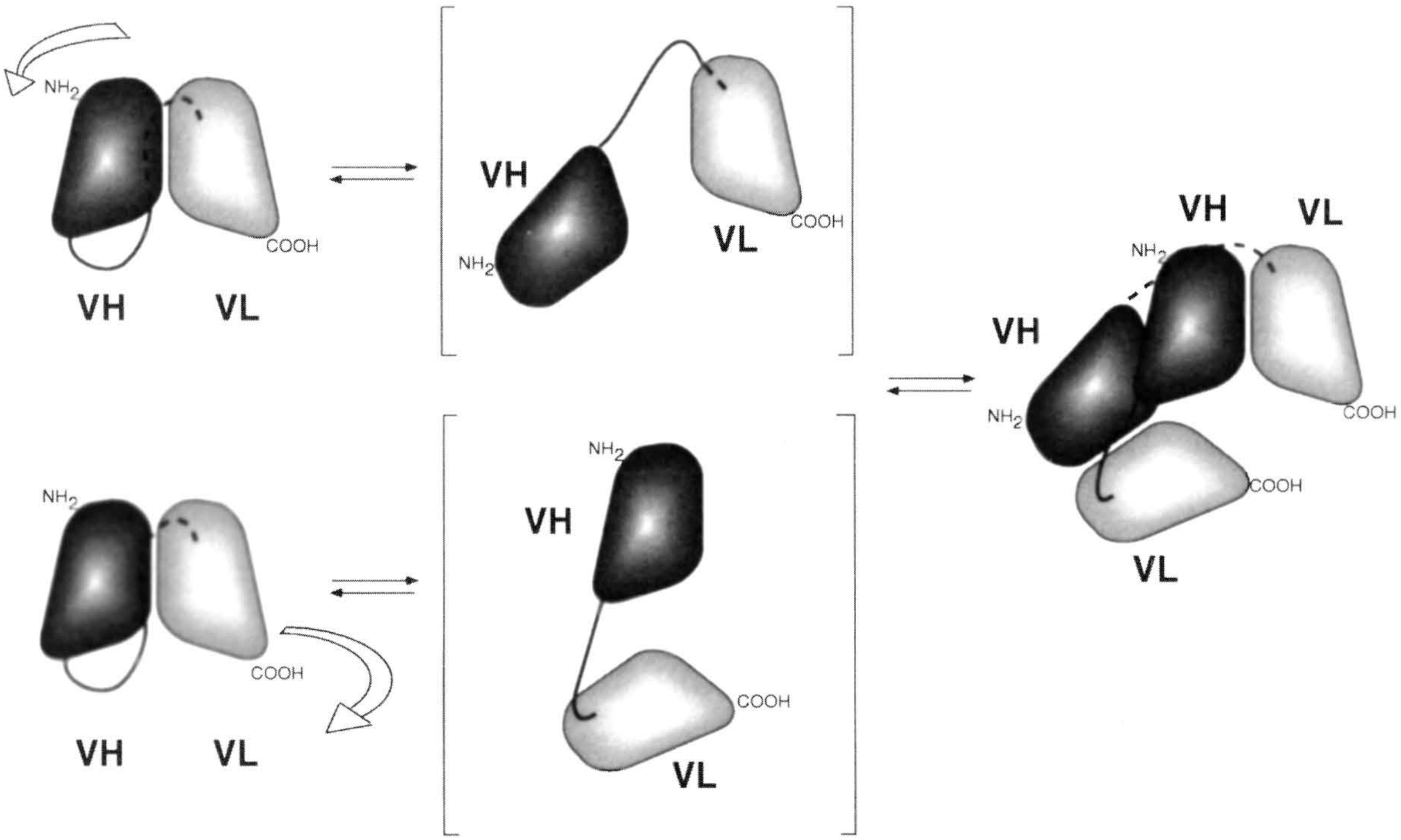


Fig. 5. Structural relationship between a monomeric scFv and a diabody. Using the structure in [88], it is schematically shown how the monomeric form and the dimeric form may interconvert. The monomer dimer equilibrium can be made to lie on the left (favoring monomers) by using long linkers (20 amino acids) and on the right by using short linkers (five amino acids). By using intermediate linkers, mixed populations are obtained, depending on the variable domain sequences. The rates and structural correlates of these equilibria are still only poorly understood.

subsequently the determination of the crystal structure of a dimeric scFv [88] clearly showed that the V_L and V_H domains can come apart and associate with a second scFv molecule, to create two V_L/V_H assemblies in one molecule, which has been termed 'diabody' [86] (Fig. 5). There is evidence that the two forms are in concentration dependent equilibrium ([113, Arndt et al., manuscript in preparation], and a detailed study of rates, equilibria and structural parameters is required.

In the crystal structure of a diabody, it was shown that V_H and V_L are rotated about an axis perpendicular to the V_H/V_L interface relative to the orientation in most Fab fragments [88]. However, the monomeric scFv of the same antibody has not been crystallized. The two 'bottoms' of the V_H domains do interact in the crystal, but not at the usual interface with the C_H1 domain. In two further X-ray structures of scFv fragments [113,114], the linker was invisible, and from the relative orientation of the variable domains, the existence of either a monomeric scFv or a diabody in the crystal appears to be compatible with the crystal packing.

It seems that in the diabody structure both binding sites can in principle bind the antigen, and it will depend on the geometry of the target antigen whether bivalent binding is sterically possible. We believe that this phenomenon of diabody formation is at least in part responsible for occasional reports that scFv in the V_L-$(gly_4ser)_3$-V_H orientation 'work better' than the corresponding V_H-$(gly_4ser)_3$-V_L molecules in ELISA. The two variable domains V_H and V_L are related not by a rotation of 180° about the pseudo twofold axis (parallel to the interface), but only by about 170° [54,115,116]. Thus, the *C*-terminus of

V_L is further away from the *N*-terminus of V_H (usually about 39–43 Å) than the *C*-terminus of V_L is from the *N*-terminus of V_H (usually about 32–34 Å) [54,116]. Consequently, the same linker $(gly_4ser)_3$ which leads to predominantly monomers in the V_H-$(gly_4ser)_3$-V_L case causes dimers in the V_L-$(gly_4ser)_3$-V_H case. A corollary of this finding is that for producing monomers, it is necessary to make the linker longer in the V_L-linker-V_H direction, and 20 amino acids have worked well [117].

3.4. Bivalent disulfide linked molecules

An alternative route to dimeric and thus bivalent fragments with enhanced functional affinity is the in vitro formation of an interdomain disulfide bridge by the oxidation of additional *C*-terminal cysteines. This strategy was shown to work for either scFv-cys, [91–94] or for Fab' fragments [50,90,94], using molecules obtained by secretion or after refolding from inclusion bodies.

The Fab' fragments can be assembled into homodimeric or heterodimeric $(Fab')_2$ by disulfide formation [90] or thioether formation (using bismaleimide derivatives) [50,79,94,118]. Using a homodimeric fragment as a test system, the thioether seems to lead to a longer serum residence time than a single interhinge disulfide [90], indicating disulfide exchange reactions occurring in serum, but a triple disulfide bond in a $(CPP)_3$ motif was found to behave identically to the thioether.

Interestingly, only the latter molecule gave rise to homodimeric $(Fab')_2$ molecules in the periplasm of *E. coli* [50,90], which reiterates the fact that disulfides form as a consequence of non-covalent interactions of sufficient residence time and are not the primary cause of crosslinking [119]. Intermolecular disulfide-formation has been successfully obtained in vivo in *E. coli*, e.g. between V_H and V_L [60], between C_H1 and C_L [28,76] or between two coiled-coil helices followed by a cysteine-carrying linker [81], and in the above-mentioned molecules with a $(CPP)_3$ motif [90]. Equilibrium dimer formation has been seen in vitro with a model peptide of the hinge region [120] and probably, this motif needs to have a certain length.

Bivalent scFv fragments have also been prepared from scFv fragments extended by a single cysteine [91], a gly_4cys tail [93], a hinge peptide [94] or a cys-ile-his_5 tail [92]. Dimers have been prepared by bis-maleimide crosslinking [91,93,94], and using a Tris–maleimide linker, even trimers could be made [94]. Kipriyanov et al. [92] isolated the insoluble periplasmic protein, refolded it without disulfide shuffling and found, among other species, $(scFv\text{-}cys)_2$ molecules. An interesting observation worth further investigations is that the $(scFv\text{-}cys)_2$ molecules reported by Cumber et al. [91] appeared to be more stable against thermal denaturation than the corresponding monomers and this may hint at a more intimate association of the two scFvs than just by the connected cysteines.

4. Tetrameric miniantibodies

The oligomerization state of a miniantibody is only governed by the properties of the self-associating part of the association domains and therefore not restricted to dimers. Small tetramerizing polypeptides have been described [121,122] and can be used as association domains, exploiting the potential of even higher valencies (Fig. 3 and Fig. 4).

A modified helix of the transcription factor GCN4, in which all hydrophobic positions of every heptad repeat making up the interface (the a and d position) have been modified (val → leu in a and leu → ile in d) results in the self-assembly of a stable tetrameric polypeptide [121]. Applied to a tetrameric miniantibody version (Table 1) of a phosphorylcholine binding scFv an additional gain of functional affinity compared with the corresponding dimeric GCN4 based association domain is clearly seen in functional ELISA as well as in BIAcore experiments [12].

A tetramerization domain entirely based on human sequences, using the human IgG3 hinge and part of the human p53 protein [122] (Fig. 3 and Fig. 4), has been developed recently [15] for therapeutic applications of miniantibodies, requiring reduced immunogenicity. The model of this 'humanized' miniantibody design suggests a

spider-like shape, in which the binding sites point into the directions of a tetrahedron and can therefore easily adapt to distant antigens, for example distributed on a cell surface. The tetrameric scp53 miniantibody format (Table 1) was applied to enhance the functional affinity of the anti-Le^Y scFv fragment MSL-5 [15].

The functional affinities of monomeric scFv, dimeric scdHLX and tetrameric scp53 were tested in ELISAs and with surface plasmon resonance (BIAcore) under conditions of high Le^Y density. The gain in functional affinities is most apparent when comparing the dissociation behavior of the different formats. The tetrameric scp53 prolongs the half life of dissociation ($t_{D1/2} = 485$ s) seven-fold compared with the dimeric anti-Le^Y scdHLX ($t_{D1/2} = 70$ s) and by two orders of magnitude compared to the monomeric anti-Le^Y scFv ($t_{D1/2} < 3$ s).

The fusion of scFv fragments to streptavidin, a tetramer, has been investigated [123]. These fusion molecules have also been shown to lead to slow off-rates in the BIAcore instrument. The fusion protein has to be prepared by refolding, however, and is probably highly immunogenic. However, there is interest in using antibody-streptavidin conjugates for pretargeting of radioactive biotin-conjugates ([124] and references therein) and the fusion molecules are a more elegant approach than the use of chemical conjugates.

5. Bispecific antibodies

5.1. Applications and non-recombinant approaches

Directly related to the problem of creating multivalency is the challenge of bringing two different antigen binding sites together. While the former approach strives to recreate the natural situation with means better adapted to biotechnology—and potentially molecules better suited for the task—in the latter approach unnatural molecules are needed, usually with particular therapeutic concepts in mind.

The most popular application of the concept of bispecific antibodies has been in tumor therapy, attempting to redirect effector cells to a tumor site [125–129]. This requires that the bispecific molecules can actually bridge two cells, overcome the repulsion between the cells, and trigger the desired biological response, ideally, exclusively close to the tumor site. Other applications of this concept have also been reported, including the delivery of a 'payload' (bound to one arm of the antibody), such as a toxin [130,131] or a radioactive hapten [132], while the other arm docks the bispecific molecule to the tumor site. Yet other applications may use neighboring epitopes of the same cell surface molecule, and thereby improve binding very much by the same principles as used for identical repeating epitopes for multivalent molecules. Potentially, the selectivity for particular cell types can be enhanced by this strategy [95,133,134].

For multivalent and bispecific antibodies, the spectrum of methods and molecules has been greatly extended through recombinant technology. Since murine antibodies elicit an immune response, humanized [135,136] or fully human antibodies [26] have to be used in all therapeutic settings requiring multiple doses. Thus, recombinant technology is already required in any event. Furthermore, it appears that only through recombinant technology is it feasible to produce the large amounts of homogeneous protein necessary for clinical testing (see below).

Before the advent of recombinant techniques, a first approach has been to chemically crosslink whole antibodies or Fab fragments [137–140] (Fig. 6), but this would not be expected to yield homogeneous preparations. Furthermore, the Fc portion may redirect the molecules to unwanted cell populations. Second, hybridomas have been fused again to give so-called hybrid hybridomas or 'quadromas' [141]. The same has been achieved by co-transfection of a hybridoma producing the first antibody with a plasmid encoding the second antibody [142]. Unfortunately, from the random combination of the two light and two heavy chains in either strategy, ten different species can result, making the purification of the desired molecules challenging and unattractive on a large scale [143]. However, recently, the reengineering of the C_H3 domain has

been investigated to provide two different variants, one with 'knobs', the other with 'holes' to achieve preferential pairing of the heterodimers [144]. This work would have to be extended for achieving a preferential association of the light chains to the appropriate heavy chain as well. Third, the preparation of $(Fab')_2$ fragments with subsequent reduction to Fab' fragments and reoxidation [145] or chemical crosslinking, e.g. by a bis-maleimide [146] to mixed $(Fab')_2$ has been described. While this approach employs well established techniques and is used now in several clinical trials [125] it is noteworthy that the proteolytic preparation of Fab' fragments can lead to secondary cleavages [147], the reduction of the hinge disulfides may not be completely selective [125,147], or the reoxidation of the hinge peptides may not be quantitative or involve the formation of intra-hinge disulfides [125,145]. Furthermore, molecules with a single inter-hinge disulfide bond are not as stable [90], while those with a thioether linkage might again be immunogenic [125]. Thus, it appears very difficult to routinely secure the very large amounts of homogeneous bispecific molecules required for therapy with any approach starting from whole antibodies. Bispecific molecules thus benefit from recombinant technology, which is used for obtaining human sequences anyway.

5.2. General considerations on bispecific recombinant molecules

The preferred format of a bispecific molecule obviously depends on the type of application and the amount needed. Since Fc parts are usually undesired because of concerns about Fc dependent targeting, no glycosylation needs to be present in the molecule, and bacterial expression would be the preferred production method. For in vivo applications, notably in tumor therapy, the physicochemical stability of the molecule at 37°C must be high enough to last until the molecules have reached the tumor site, which can last up to 10 h in a human being [148]. At the same time, just as for monospecific antibodies, the serum clearance rate determines the concentrations reachable in serum (arguing for large molecules) [94], the tumor penetration rate the buildup on the tumor (arguing for small molecules) [149–151], and the functional affinity the residence time on the antigen carrying cells (arguing for multivalent molecules), (see Section 7).

There is a choice about the fragments to be linked (Fab versus scFv) and there is a number of different linking technologies available. While Fab fragments may appear to be more stable [61] against thermal denaturation at 37°C than scFv-based constructs, there is much effort in elucidating the structural basis of antibody stability and improving the stability of scFv fragments by engineering [60,78,152,153]. Clearly, scFv-based designs are more versatile, smaller and offer the possibility of combining bivalency and bispecificity, such in the di-bi miniantibodies discussed below. In the scFv fragment, light/heavy assemblies are unique, because wrong V_H-V_L pairing (which occur during simultaneous in vivo expression or during simultaneous in vitro refolding of whole antibodies, Fab or Fv fragments) is not an issue. Thus, bispecific $(Fab')_2$ approaches must rely on two different hosts or will require further interface engineering [144] to make each H/L assembly unique.

5.3. $(Fab')_2$ and disulfide-linked $(scFv)_2$ fragments

While the linking of recombinant Fab' fragments to bispecific $(Fab')_2$ is chemically identical to the linking of proteolytic Fab' fragments, all clinical uses will require humanized or human fragments, and there is no obvious advantage in producing two whole antibodies which are later to be chopped up again. Bacterial secretion technology [27,28] has now reached very acceptable levels when combined with high cell density fermentation [49–51], even though the yield strongly depends on the variable domains and thus the sequence of the antibody [75,77,78]. It turns out that by grafting to a superior framework, which can be the same step as humanization, expression performances in *E. coli* can be dramatically enhanced [78], and the use of antibodies from a synthetic library of master-frameworks (Knappik et al., in preparation) also looks very robust and promising in this respect.

Heterodimeric recombinant $(Fab')_2$ fragments can of course also be assembled by disulfide formation or thioether linkage, using a $(CPP)_1$ motif or a $(CPP)_3$ motif or other hinge peptides at the *C*-terminus of the molecule [79,90,94,118]. Empirical testing of the advantages and disadvantages of these molecules will be required. For instance, molecules with the $(CPP)_3$ motif would require another reduction step of the homodimers forming in the periplasm, before heterodimers can be produced, while the $(CPP)_1$ motif may not lead to sufficiently stable disulfides, and the thioether would introduce again a foreign molecule. The main disadvantage of this technology, however, is that two hosts are required for the production of both molecules and chemical coupling adds additional production steps.

Heterospecific $(scFv\text{-}cys)_2$ fragments can also be prepared following the leads on homospecific fragments of this type (see above) using the technology developed for bispecific $(Fab')_2$ fragments.

5.4. Diabodies

The spontaneous dimerization of two scFv molecules (see Section 3.3) with short linkers has been elegantly exploited in creating heterodimeric 'diabodies' [86]. In this case, two different scFv molecules, either of the type V_{HA}-linker-V_{LB}/V_{HB}-linker-V_{LA} [86,154,155] or of the type V_{LA}-linker-V_{HB}/V_{LB}-linker-V_{HA} [156] were expressed in tandem, in a dicistronic operon [27,28], where A and B describe the specificity of the antibody and H and L describe the type of variable domain, and the linkers were usually around five amino acids in length.

It was recently demonstrated that diabodies can indeed crosslink cells [154] and the binding sites are facing away from each other at an obtuse angle, spanning a distance of about 50 Å [88] (Fig. 4 and Fig. 5). It will be interesting to compare their performance with miniantibodies spanning longer distances, and it is likely that the outcome depends on the accessibility of the epitope. Since the diabody appears to be fairly rigid, it will only be useful where the two epitopes are accessible from the angle inherent in the diabody molecule. As far as the in vivo performance is concerned relative to larger molecules, this will depend on the importance of long serum residence times versus tissue penetration properties [94].

The expression yields strongly depend on the variable domain under study, and require therefore the same attention to protein engineering as summarized in Section 2.2. Because the dimerization tendencies also vary with the antibody sequence given the same linker [111], there may be some concern about the generality of this technique. The use of disulfide bonds to favor the desired heterodimers at the expense of the homodimers has been investigated, albeit with an almost total loss of functional expression, while the use of knobs-in-the-holes approaches [144] appears more promising (Carter et al., unpublished). A different approach to optimizing diabodies has been taken by McGuinness et al. [155], who have displayed diabodies on phage, by fusing one of the scFv molecules to g3p, and secreting the other to the periplasm, in order to directly select molecules capable of binding both antigens in this format. While this approach is elegant, the amount of work needed to generate new comprehensive libraries in the required format should also not be underestimated.

5.5. scFv-scFv tandems

A number of groups have investigated the feasibility of fusing two scFv genes in tandem, separated by a flexible linker [133,157–162]. These molecules have been prepared either by refolding from inclusion bodies, periplasmic secretion in *E. coli*, or from CHO cells. It is likely that individual differences in the folding efficiency between different antibody variable domains will be enhanced in this one-chain assembly. An important but open question is whether both scFv molecules really fold independently, or whether domain swapping may occur in a fraction of the population.

An interesting extension of this theme has been developed recently [163], in which two scFv genes were interrupted by a domain of *Pseudomonas* exotoxin A. This approach may eventually be used with two neighboring epitopes [95,133,134] to improve functional affinity, and perhaps, selectivity for toxin mediated cell killing.

5.6. Leucine zipper and other fusions

Heterodimeric coiled coils can be used to extend the formation of multivalent miniantibodies to bispecific miniantibodies. This strategy was first applied to Fab fragments [96–98], which were extended either with a jun or a fos helix. Because Fab fragments have two unlinked chains, this strategy would lead to mixed HL assemblies in a single host. Two different hosts were therefore used and the homodimeric Fab fragments were dissociated from homodimers and reassociated to heterodimers. These fragments have been produced in eukaryotes, even though certain engineered Fab fragments can be produced in good yield in *E. coli* [50]. Recently, the same strategy has been used for scFv fragments [164], using the miniantibody format [81], with the two scFvs expressed individually and reassociated to heterodimers in vitro. The co-expression of fos- and jun-based miniantibodies (Table 1) in one cell leads mainly to separate jun–jun and fos–fos homodimers and partial degradation of the fos sequence [165]. Quantitative heterodimerization of association domains or peptides based on wild-type-fos and jun sequences requires prolonged incubation at temperatures greater than 37°C [166] and therefore creates problems with the folding and stability of the scFv part of the miniantibodies. Ongoing research (Müller et al., unpublished) is directed at obtaining quantitative heterodimeric in vivo assembly in a single host and temperatures suitable for *E. coli* growth. The improvement of such heterodimerization domains requires the careful modification of the coiled coil sequences, but will offer a very fast approach to producing heterodimers at high yield without further temperature treatments or in vitro manipulations.

A two-step approach to cell targeting has been tested, in which a scFv fragment with a peptide tag was used. The tag was recognized by a second bispecific antibody, which was to bind to an effector cell via the second arm [167]. It will be interesting to directly compare this approach with the use of bifunctional molecules, where the anti-tumor site and the anti-effector cell activity are directly linked.

5.7. Bispecific 'Di-Bi miniantibodies'

All the recombinant bispecific molecules described so far have only one binding domain of either type, and are therefore only monovalent for either epitope. However, the arguments made for bivalent binding as a means for increasing functional affinity, and perhaps selectivity for particular cell types carrying densely clustered antigens, are of course also valid in applications where two different cell types are to be crosslinked. Thus, molecules have been constructed which are both bispecific and bivalent (Müller et al., manuscript in preparation). This has been achieved by using miniantibodies of the scFv-hinge-helix-turn-helix type, to which another, different scFv was fused at the *C*-terminus. By dimerizing via the helix-turn-helix motif, the molecular assembly carries two scFvs of specificity A at the *N*-terminus and two scFvs of specificity B at the *C*-terminus.

6. Production of miniantibodies and diabodies by fermentation

The secretion and folding of many of the fragments described in this review has been demonstrated in *E. coli*. It is very difficult to judge the expression performance of a particular format in general terms, as it primarily depends on the antibody sequence. However, as pointed out above, various strategies are available to reengineer the fragments. Such favorable sequences (Section 2.2) have lead to excellent expression performances as Fab or Fab' fragments [50], bivalent miniantibodies [49,51] and diabodies [156], which have all been obtained in amounts approaching or exceeding 1 g/l of functional protein (see Section 3.1), not requiring any refolding.

These high yields are accessible by high cell density fermentation of *E. coli*. Several successful promoter/operator systems have been described [50,51,94] and it may be pointed out that *E. coli* appears currently to be the most powerful host of all in terms of volume yield, reaching up to 3–4 g/l of a fully functional miniantibody [51] which would correspond to 8–10 g IgG in molar yield. Using unlimited growth rates of *E. coli* and

regulating glucose levels by a feed-back control loop, these amounts are obtained in only 24–36 h [51] and this is a factor often overlooked in comparing different production hosts.

7. In vivo properties of bivalent antibody fragments

The pharmacokinetic properties of a number of bivalent fragments have been investigated up to now. The bivalent miniantibody formats scdHLX and scZIP (Fig. 3) where tested in mice and compared with those of the corresponding scFv fragment and complete, monoclonal antibody [83]. Both miniantibody formats, which are of the size of a monovalent Fab fragment, exhibit a serum half life intermediate between that of monoclonal antibodies and the well known rapid clearance of monovalent scFv fragments [149–151] (Table 2). They are almost quantitatively excreted via the kidneys and do not show proteolysis of the potentially sensitive hinge regions [83]. Similar results have been obtained by testing functionality of the miniantibodies as a function of time after injection into mice [82]. Qualitatively similar data have been obtained for other bivalent fragments. The clearance kinetics of a scFv-cys$_2$ fragment was found slightly slower than the monomer, most evident in the fast phase [91].

Tumor localization studies with a variety of dimeric forms of recombinant antibody fragments have been reported. They gave favorable results with respect to the monomers for scFv-cys$_2$ fragments [93], a scFv with short linker (monospecific diabody) [168] and a scFv-C_H3 fusion [110], especially when equipped with a hinge region as discussed above for the miniantibodies. Very good tumor to blood ratios were seen already at short times, making these molecules interesting for imaging. Adams et al. [93] also noted a better tumor localization of a scFv-cys$_2$, compared with a Fab fragment of the same MW. This comparison thus directly demonstrates the beneficial effect of bivalency, since both molecules gave very similar pharmacokinetics. This is a more meaningful comparison than the usual comparison to a scFv fragment which has a lower MW and somewhat faster clearance rates. Nevertheless, in a comparison of mono- bi- and trivalent scFvs and Fabs, King and co-workers [94] found better tumor to blood ratios for the multivalent Fab fragments. These discrepancies demonstrate that the question of optimal localization is very complicated and may require a modular approach to tuning the system empirically.

Table 2
Pharmacokinetic properties of miniantibodies

Antibody construct[a]	$t_{1/2}$ α (min)[b]	$t_{1/2}$ β (h)[b]
scFv[c]	8.1 ± 2.6	2.8 ± 0.3
scdHLX[c]	17.5 ± 3.8	3.4 ± 0.3
scZIP[c]	11.9 ± 4.3	4.1 ± 1.3
McPC603[d]	84.0 ± 7.3	21.1 ± 0.7

Data are from [83], where further details can be found, and serve to illustrate the differences between the various molecular species. Similar studies have been carried out with other bivalent molecules (see Section 7) and data can be found in references [91,93,94,110,168].

[a] Models of scFv, scdHLX and scZIP are shown in Fig. 4 b, d and e or schematically in Fig. 3; the whole antibody McPC603 is an IgA. All species carry the same variable domains, specific for phosphorylcholine.

[b] Mean $\pm$ S.D., mice ($n = 6$) were given intravenous injections of 2.5 mg of ^{125}I-MAb or MAb-fragments.

[c] $t_{1/2}$ α (time interval: 0–15 min) and $t_{1/2}$ β (time interval: 1–7 h).

[d] $t_{1/2}$ α (time interval: 0–120 min) and $t_{1/2}$ β (time interval: 4–120 h).

8. Potential applications of multimeric fragments

While many of the therapeutic applications have centered around tumor immunology, the advent of recombinant antibody technology may also bring a renaissance of passive immunotherapy with improved molecules of human origin. Such approaches have been taken by converting recombinant molecules, obtained as Fab fragments from phage display libraries, back to the whole antibodies [169,170].

More recently, it was investigated in a mouse model whether monomeric scFv and dimeric dHLX miniantibodies would be protective against vesicular stomatitis virus, a brain specific mouse pathogen related to rabies virus [82]. While monovalent scFv (preincubated with virus before injection) was not able to protect against the virus, bivalent miniantibody was protective. Using highly virus-sensitive, interferon $\alpha\beta$-receptor deficient knockout mice, both species were protective against a low virus titer, demonstrating that neither the Fc part nor bivalency are necessary for protection, but most likely the functional affinity has to be high enough that no virus particle escapes into the central nervous system. These monomeric and dimeric species are not yet protective in passive immunotherapy, probably because of the short in vivo half-life (see above). However, second generation miniantibodies with higher oligomerization states and improved in vivo stabilities may be able to solve this problem.

At the present time it may be difficult to draw definitive conclusions about the general merits of the various formats for applications such as passive immunization, screening and targeting of inflammation and cancer. The use of a certain design may depend on the available intrinsic affinity as well as on the location, vascularization, accessibility and density of the antigens on the targeted cells. The future will lie in a modular approach to size, combining an optimal functional affinity with the best achievable compromise between small molecular weight (for efficient penetration) and a tunable serum lifetime. It has been suggested that during penetration of the tumor, the antibodies arriving first bind to the target, but by doing so may create a barrier preventing further molecules from entering [171]. It is possible that this effect is also related to molecular size, but it may require a fine-tuning of affinities as well. One has to find the right compromise between long enough serum lifetime, which also translates into a high enough serum steady state, to actually reach the antigen in vivo and deliver sufficient amounts, but short enough to clear unbound molecules. It appears that this goal can only be reached by systematic, comparative studies.

In addition, future designs will not be restricted to the combination of one or two specificities in a single multimeric complex. Immunogenicity will be reduced by the exclusive use of human sequences and the avoidance of chemical linkers and unnatural peptidic linker sequences. The combination of bispecific and/or multivalent molecules with additional effector functions [172,173] such as cytokines, toxins, enzymes or metal binding sites in a multifunctional complex may bring us closer to the 'magic bullet'.

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