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Engineered proteins with desired specificity: DARPins, other alternative scaffolds and bispecific IgGs

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Specific binding proteins have become essential for diagnostic and therapeutic applications, and traditionally these have been antibodies. Nowadays an increasing number of alternative scaffolds have joined these ranks. These additional folds have raised a lot of interest and expectations within the last decade. It appears that they have come of age and caught up with antibodies in many fields of applications. The last years have seen an exploration of possibilities in research, diagnostics and therapy. Some scaffolds have received further improvements broadening their fields of application, while others have started to occupy their respective niche. Protein engineering, the prerequisite for the advent of all alternative scaffolds, remains the driving force in this process, for both non-immunoglobulins and immunoglobulins alike.

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Introduction

Antibodies, mainly of the isotype G, are the predominating class of binding proteins for applications where specific protein binders with high affinity are needed, and most of them — outside of therapy — are still derived from mouse immunizations. The advent of recombinant antibody technology, where the classical immunization was replaced with fully synthetic libraries, selection technologies, and built-in affinity maturation, finally made the IgG molecule itself dispensable. Devised to expand the range of applications of specific binding proteins, alternative scaffolds of non-immunoglobulin folds have increasingly gained attention during the last ~15 years.

In this review, recent developments in both of these main classes of binding proteins, Ig-derived molecules and non-Ig-derived scaffolds, will be discussed. The engineered target–binding interfaces of the non-Ig scaffolds have recently been discussed in a very pertinent review [1], comparing design of the topographies and variable residues in the designed paratopes with the actual usages found in X-ray structures. Also, the binding modes of classical immunoglobulins have been reviewed earlier (most recently in the context of computer-aided antibody design [2]). We will, therefore, focus here on general emerging principles in both fields that facilitate new applications.

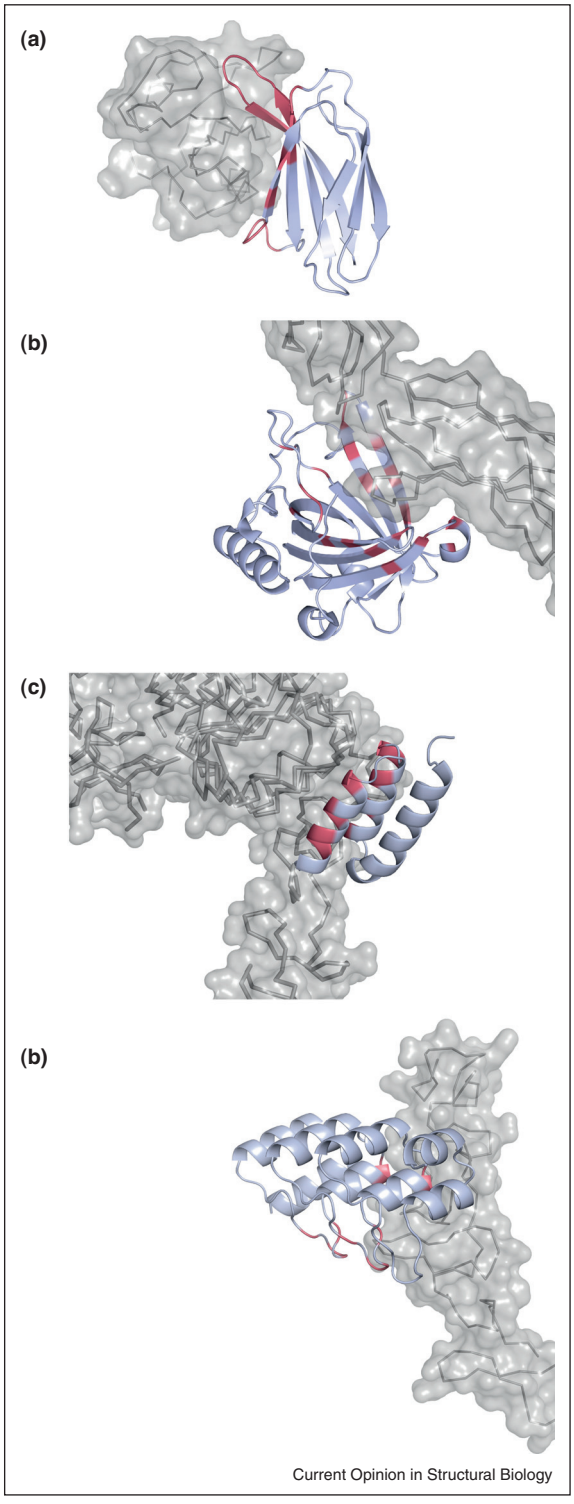
Binding proteins based on non-immunoglobulin folds

In principle, every protein can be converted to a library with a potential binding surface. The diversity of alternative scaffolds that has been developed and still is under development [3] can be brought down to less than a handful of different formats when focusing on those folds for which crystal structures of target/binder complexes have been reported: monobodies (derived from fibronectin type III (FN3)), anticalins (derived from lipocalins), affibodies (derived from the immunoglobulin binding protein A), and DARPins (based on the Ankyrin fold) can be regarded as the best established formats of alternative scaffolds [1] (see Figure 1 for examples of binder/target complex structures, illustrating the different binding modes taken from an increasing number of X-ray structures of binder/target complexes (Table 1)). Notably, these are also the classes where members have progressed to clinical trials. While we acknowledge progress in many other scaffold classes, space restrictions force us to mainly focus on the classes mentioned.

Recent developments in consensus design: improving the scaffolds

The fibronectin type III domain (FN3, monobody) has become one of the scaffolds for generating new binding proteins, where now many examples have been reported [4,5] (Figure 1a). The FN3-fold is similar to single Ig domains, but does not rely on the formation of an intradomain disulfide bond. Although initially developed to allow loop-mediated binding similar to the variable domains of antibodies, FN3 binders have in some cases been shown to have binding surfaces comprised by a single loop and the face of a β-sheet [6]. Since this 'side and loop' binding emerged frequently from directed evolution without being intended in this way, Koide *et al.* [7•] sought to facilitate it: by designing an alternative FN3-library diversifying additional positions on a β-sheet and surface loops that together form a concave surface, a

Figure 1



3D structures of alternative scaffolds (light blue with paratopes in raspberry) in complex with their targets (surface representation in grey). **(a)** Monobody in complex with SH2 domain (PDB ID 4JE4), **(b)** anticalin bound to Fn7B8 (PDB ID 4GH7), **(c)** affibody bound to HER2 domain III (PDB ID 3MZW), **(d)** DARPin in complex with HER2 domain IV (PDB ID 4HRN).

new side-and-loop monobody library was generated yielding high affinity binders. It appeared that the two classes of monobody libraries perform differently against different targets of different geometry.

Engaging the lipocalin-scaffold, Eggenstein *et al.* [8] reported on further improvements in affinity and structural insights into the binding of a previously generated anticalin, which specifically binds a chelated radionuclide (lanthanide(III) ions as chelated complex with p-NH_2-Bn-CHX-A″-DTPA) in a low nanomolar to picomolar affinity range [9]. From a structural point of view, anticalin libraries seem to be well suited for this type of target, since natural lipocalins typically use their basket-like binding pocket to bind small molecules. Previously, using scFv fragments as a basis, diabodies had been reported where one specificity was directed against DOTA, the other against the surface antigen of choice [10]. This 'indirect' labeling allows a pretargeting of the tissue of choice, to uncouple the pharmacokinetics of the targeting proteins from the requirements of the radionuclide, a strategy also lately pursued with various IgG–scFv fusions [11]. While the anticalin fold is well suited for the binding of haptens, it can still be employed to recognize bigger targets with affinities down to the single-digit nanomolar range [12] (Figure 1b).

In most cases reported for radio-immuno diagnostics (RID), small target–binding proteins have been directly labeled by covalently coupling the radionuclide, typically via a chelator or as a quasi-covalent adduct of technetium-(I) [13], to the protein. Originally developed for scFv fragments (see, e.g., [14,10]), this application has been recognized early on as well suited for small scaffold proteins. Probably most work has been done on affibodies, [15,16] (Figure 1c), which have progressed to clinical trials for *in vivo* diagnostics. For a summary, we would like to recommend a very recent review [17]. Other scaffolds have been under development as well for RID, and radiolabeling has also been used to quantify the uptake of the labeled proteins. It was found, both experimentally [18] and by elegant computational approaches [19], that there are actually *two* distinct optima for a labeled protein to localize to a given tissue, for example, a tumor: First, for very small proteins with extremely high affinity (picomolar), and second, for rather large proteins (e.g., PEGylated proteins), where the affinity is not as crucial.

During the last decade the class of non-Ig-derived scaffolds has increased especially due to the development of formats based on naturally occurring repeat proteins that have emerged as promising alternatives for both diagnostic and therapeutic applications and numerous research applications. The successful consensus design of, for example, leucine-rich repeat (LRR), Ankyrin repeat (AR), Armadillo repeat (ArmRP) and tetratricopeptide

Table 1

List of X-ray structures of binder/target complexes of alternative scaffolds in the PDB

Deposition	PDB ID	Target	Resolution (Å)	*R*-factor	R_{free}
DARPins					
2004	1SVX	MBP	2.24	19.5	24.9
2005	2BKK	Aminoglycoside phosphotransferase APH (3′)-IIIA	2.15	20.0	26.0
2006	2J8S	Acriflavine resistance protein B (AcrB)	2.54	22.9	27.1
2007	2P2C	Caspase-2	3.24	26.2	30.5
2007	2V5Q	Polo-like Kinase 1 (PLK-1)	2.30	18.4	22.4
2008	2V4H	NF-kappa-B essential modulator CC2-LZ domain	2.90	21.1	26.8
2009	3HG0	ORF49 from lactococcal phage TP901-1	2.10	20.9	24.3
2010	3NOG	Acriflavine resistance protein B (AcrB)	3.34	25.8	30.8
2010	3NOC	Acriflavine resistance protein B (AcrB)	2.70	24.3	26.8
2010	2XZD	Caspase-3	2.10	18.7	21.8
2010	2XZT	Caspase-3	2.70	19.9	23.1
2010	2Y0B	Caspase-3	2.10	19.3	21.7
2010	2Y1L	Caspase-8	1.80	18.2	21.8
2012	4ATZ	Ad5 knob	1.95	16.3	19.3
2012	4DRX	Tubulin	2.22	16.1	19.4
2012	4DX5	Acriflavine resistance protein B (AcrB)	1.90	20.2	23.1
2012	4DX6	Acriflavine resistance protein B (AcrB)	2.90	21.2	27.0
2012	4DX7	Acriflavine resistance protein B (AcrB)	2.25	18.7	22.7
2012	4HNA	Tubulin	3.19	17.8	21.1
2012	4HRL	HER2	2.55	20.3	25.3
2012	4HRM	HER2	3.20	31.5	33.8
2012	4HRN	HER2	2.65	21.9	24.9
2013	4JB8	Caspase-7	1.70	17.3	19.4
2014	4K5A	Bcl-2-like protein 2	1.50	15.8	19.0
2014	4K5B	Bcl-2-like protein 2	1.85	17.8	22.2
Monobodies					
2006	2OCF	Estrogen receptor a ligand binding domain	2.95	19.4	25.1
2008	3CSB	Maltose binding protein	2.00	19.9	23.6
2008	3CSG	Maltose binding protein	1.80	19.2	23.5
2009	3K2M	Src Homology 2 domain of Abelson Kinase 1	1.75	18.2	22.1
2011	3QHT	Yeast small ubiquitin-like modifier (ySUMO)	2.40	22.6	27.2
2011	3RZW	Human small ubiquitin-like modifier 1 (hSUMO1)	2.15	18.9	23.7
2011	3T04	Src Homology 2 domain of Abelson Kinase 1	2.10	19.3	25.1
2011	3UYO	Src Homology 2 domain of Abelson Kinase 1	1.83	19.0	23.7
2011	3QWQ	EGFR	2.75	20.4	24.6
2011	3QWR	Interleukin-12 subunit beta	3.25	23.5	26.4
2012	4HUK	NorM-MG	3.59	30.9	34.9
2012	4HUL	NorM-MG	3.81	31.4	37.6
2012	4HUM	NorM-MG	3.49	31.5	33.1
2012	4HUN	NorM-MG	3.59	30.7	32.7
2013	4JE4	SHP2	2.31	20.6	25.5
2013	4JEG	SHP2	2.30	18.9	22.7
Anticalins					
2002	1LKE	Digoxigenin	1.90	18.8	24.8
2002	1LNM	Digitoxigenin	1.90	20.6	24.5
2002	1N0S	Fluorescein	2.00	19.6	24.3
2008	3BX7	Cytotoxic T-lymphocyte antigen 4 (CTLA-4)	2.10	20.9	22.6
2008	3DSZ	p-NH(2)-Bn-CHX-A″-DTPA	2.00	21.3	23.0
2012	4GH7	Oncofetal fibronectin fragment Fn7B8	2.60	22.0	25.9
Affibodies					
2002	1LP1	Protein Z (Fc-binding domain from protein A)	2.30	22.5	25.6
2010	3MZW	HER2	2.90	20.8	27.8

repeat (TPR) proteins has been described and discussed in previous reviews [20–22]. Repeat proteins are very attractive as alternative binding scaffolds because they appear like a poster-child scaffold derived from biophysical considerations [23]: a large, easily extendable interaction surface is strengthened by a rigid backbone (precluding entropy loss upon binding to the target).

A consensus design approach for LRR proteins (LRRs) had been described for the first time by Stumpp *et al.* [24], who used the ribonuclease inhibitor (RI), which binds ribonuclease with femtomolar affinity, to derive their LRR-consensus sequence. Owing to the lack of other published RI-like LRR sequences at that time, not more than four mammalian RI-homologues could be used for

consensus design. Different repeat lengths are known in LRR and it is noteworthy that in each natural protein, all repeats typically have the same length [25]. After the first successful selections from synthetic AR-libraries had been published [26], the surprising discovery was reported that jawless vertebrates use an adaptive immune response based on LRRs. In mammals, LRRs are used as receptors of the innate immune system, and the adaptive immune system is based on the immunoglobulin-fold, while jawless vertebrates do not have Ig genes, and have created a repertoire out of the LRR fold, now termed the variable lymphocyte receptors (VLRs) [27–29].

The idea of harnessing VLRs as affinity reagents has lately been followed up [30•]. The described consensus design was based on a template derived from sequence alignments of more than 1000 LRR modules. For repeat proteins of many classes, the capping repeats have been recognized as a key liability. An important engineering step was therefore to replace the three natural N-terminal repeats of the VLR with three redesigned repeats based on the internalin-B N-cap, an alteration that strongly improved solubility and expression yield. However, no experimental structures have so far been reported to deepen our understanding of the molecular recognition by these binders.

Within the non-Ig scaffolds, DARPins might be regarded as the scaffold currently being studied most widely [31] (Figure 1d). DARPins with affinities down to the picomolar range have been selected against many targets [3,32,23] and proven useful for multiple different applications [31,33,34]. Nevertheless, their concave shape, rigidity and incompletely randomized binding surface might potentially limit the range of epitopes that can be targeted by this extremely stable scaffold.

Schilling *et al.* presented a strategy to overcome this limitation [35•]. Combining a conformationally diverse and convex paratope found in many immunoglobulins (in the form of an extended CDR–H3 loop) with the beneficial biophysical properties of DARPins, a next generation of DARPins ('LoopDARPins') with extended epitope binding properties was created through consensus design. Replacement of the central β-turn by an elongated loop with randomized positions did not decrease the stability of the scaffold. Key to success was the design of a stable stem of the loop which prevents its interference with neighboring β-turns in the scaffold. The introduced loop carries 10 variable positions, and the top of the loop has conformational flexibility, exposing several of the randomized positions. The LoopDARPin-library was biased for Tyr, Ser and Gly, as this combination is known to be enriched in binding sites from the analysis of natural antibodies [36], and has proven useful in libraries of antibodies [37,38] and monobodies [6,7]. Ribosome display selections against five different target proteins yielded LoopDARPins with affinities in the mid-picomolar to low nanomolar range against all targets tested. Interestingly, with the LoopDARPin scaffold, picomolar binders could be obtained with only one single round of ribosome display, an enrichment that has not been described previously for any scaffold.

Exploiting the versatility of repeat proteins

The modular structure of repeat proteins and their favorable biophysical properties, such as high thermodynamic stability and lack of aggregation, do not only allow the binding of a diverse set of targets in a specific manner and with high affinity, but also suggest the utilization of the rigid protein framework for generic engineering concepts, for example, for intracellular applications.

By taking advantage of a generalizable workflow Kummer *et al.* [39•] generated an ERK activity biosensor based on the DARPin scaffold. The most attractive feature of DARPins in this context is that these binders — per design — lack any cysteine, which allows for the introduction of a unique cysteine for facile chemical coupling of the fluorophore adjacent to the binding interface. Also, the binding surface is quite defined by the concave shape of the protein. By selecting a DARPin specific for phosphorylated ERK and coupling it with a solvatochromatic merocyanine dye, whose fluorescence increases in a more hydrophobic environment, that is, upon binding of phosphorylated ERK (pERK), a biosensor was created: it specifically responded to pERK2, but not to unphosphorylated ERK2 or other closely related mitogen-activated kinases tested [39•]. The activated state of the kinase was recognized by its conformation of the activation loop, which changes upon phosphorylation [40], and not by direct recognition of an exposed phosphate. Therefore, this approach would be applicable not only to kinases, but to any protein which changes its conformation and/or is covalently modified in the cell. The protein molecules in the desired state are recognized in a spatially and temporally resolved manner. In the particular case of ERK, it was discovered by this approach that there is a high activation of ERK in the nucleolus [39•], a finding that would been difficult to discover by other means. In the case of ERK, ultimately, a small conformational change is detected that reports on activation. In a related approach, the activation of Src kinase was monitored by a merocyanine-derivatized monobody, which recognizes the SH3 domain, which only becomes accessible upon kinase activation [41]. It should be noted that these approaches still require microinjection of the protein–dye conjugate.

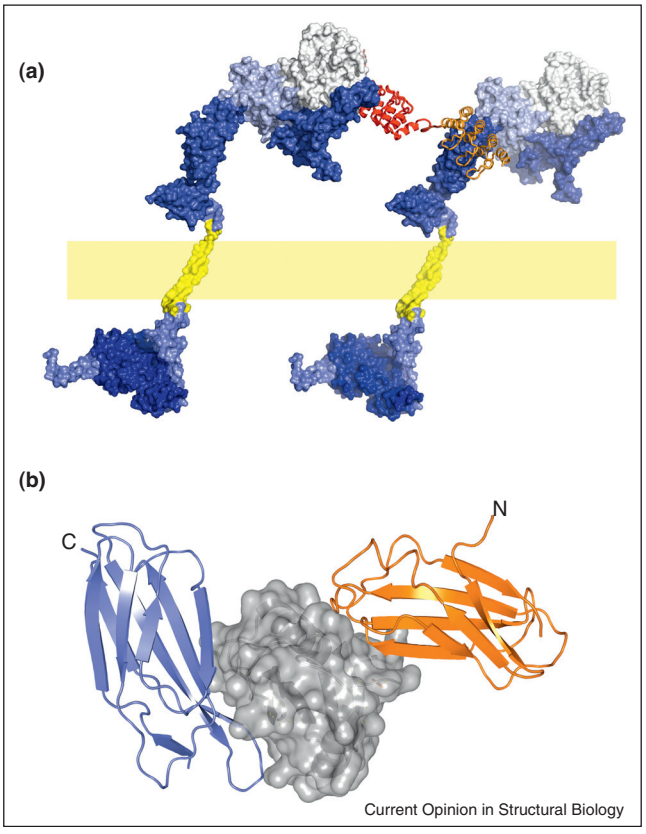
The need for an easy construction of multivalency and multispecificity was one of the main motivations when assembling the 'wish list' for properties of new scaffold proteins that could expand the range of applications of antibodies [31]. We limit our discussion here to those

cases backed up by structural information. Because of their favorable biophysical properties and lack of aggregation, DARPins can easily be fused to each other and different protein domains to, for example, generate multivalent or multispecific constructs. While DARPins (like scFvs and other scaffolds) have been used for targeting protein toxins to a tumor [42], it is of interest to investigate whether DARPins can also exert a biological function by themselves, for example, by causing an effect via binding to the extracellular part of a transmembrane protein.

Jost *et al.* [43•] took advantage of the facile generation of multispecific and multivalent DARPin constructs when creating biparatopic binders that would cause cytotoxic effects in certain breast cancer cell lines. Using DARPins that bind to the extracellular part of the human epidermal growth factor receptor 2 (HER2, hErbB2), overexpressed in these cells, constructs were tested that connect binders to different epitopes on the extracellular domain (ECD) of HER2. The construction and screening of these biparatopic constructs was strongly facilitated by one of the key advantages of the DARPin scaffold: As stable single-chain proteins, DARPins can easily be fused 'head to tail' without losing their high expression yield in *Escherichia coli* or creating any tendency to aggregate. Creating bispecificity — and a very particular geometric arrangement that had to be fine-tuned — was crucial to obtain the described cytotoxic effect: the simultaneous binding to epitopes on subdomains I and IV on different HER2 molecules was proven to lead to a completely new mechanism of receptor inactivation [43•]. On the basis of the crystal structures of the DARPins in complex with their target domains, it was deduced that HER2 becomes trapped in non-natural bent-over receptor pairs that are fully signaling-incompetent (Figure 2a). Meanwhile, the signaling mechanism leading to strong cytotoxicity with apoptosis induction has been elucidated, contrasting the bispecific DARPins to the more weakly acting therapeutic anti-HER2 antibodies, which do not cause the apoptotic response, and the bispecific anti-HER2 DARPins have also shown to elicit this effect *in vivo*, after site-specific PEGylation to extend the serum half-life (Tamaskovic *et al.*, submitted for publication).

Instead of bridging two target molecules, bispecific binders can also 'chelate' one molecule. For instance, the linking of two monobodies in tandem with a glycine–serine linker that bind different epitopes on the SH2 domain of the Bcr-Abl tyrosine kinase (Figure 2b) could be used to improve the inhibition of Bcr-Abl kinase activity [44], better than by either monobody alone. This kinase is constitutively active in chronic myelogenous leukemia (CML), and it would be important to work out how to bring such proteins to the cytoplasm efficiently, in order to eventually pave the way for proteinaceous inhibitors of kinases in clinical applications.

Figure 2



Targeting of tumor-relevant targets with bispecific binders. **(a)** Superposition (PDB entries 4HRL and 4HRN) and model of the structure of two HER2 molecules in complex with a bispecific DARPin. The domain I binder (red) and the domain IV binder (orange) are connected with a 5 aa Gly-Ser linker. Bivalent binding to the extracellular HER2 domains crosslinks two receptor molecules in a non-natural conformation, enforcing a large distance between the intracellular kinase domains and thereby inactivating the kinases that require dimerization for activation. **(b)** Superposition of two monobody-Abl SH2 complex crystal structures (PDB entries 3T04 and 3K2M). Monobody HA4 (blue) and monobody 7c12 (orange) can be connected with a flexible Gly-Ser linker (not shown) to gain avidity.

Engineering geometrically well-defined fusions is greatly facilitated by the fact that the DARPin scaffold ends with α-helices at both termini. Dreier *et al.* [45•] used computer modeling to design a trimeric clamp of DARPins, binding to adenovirus Ad5 at the fiber knob. The ΔTAYT knob was used, as this mutant of Ad5 does not bind to its own receptor [46]. The crystal structure of the knob-binding DARPin in complex with the trimeric knob protein was determined and it could be deduced that the stoichiometry was clearly defined, as three DARPins were binding to one trimeric knob. Because the C termini are exposed to the top, the capsid protein SHP from the lambdoid phage 21, a trimeric protein of extraordinary kinetic stability (which does not dissociate into monomers), could be fused to allow the trimerized DARPins to

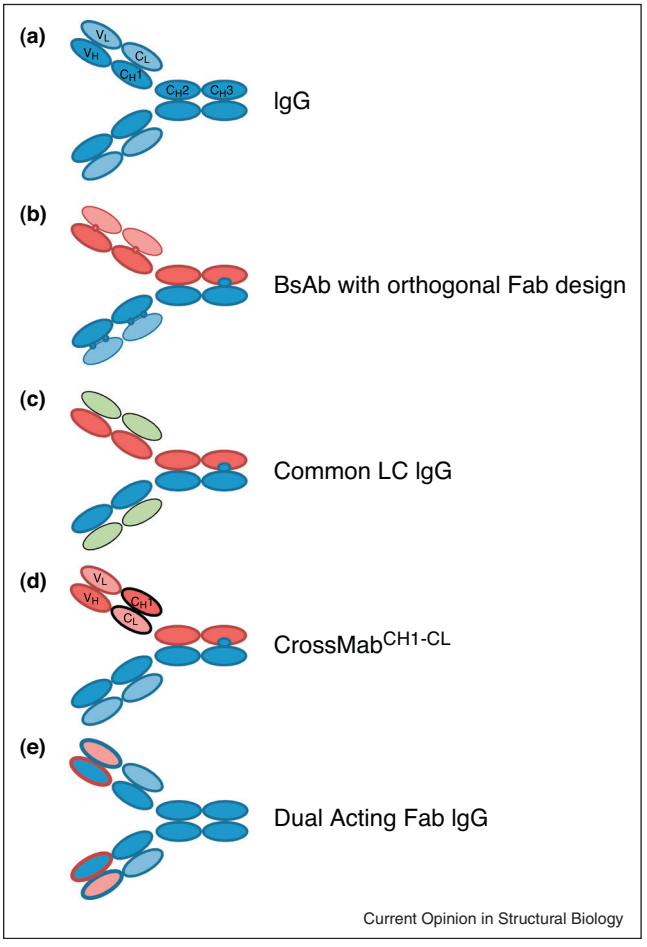
'clamp' the knob in an arrangement that binds to the knob virtually irreversibly over 10 days. This knob-binding 'clamp', which readily assembles by itself in *E. coli*, can be fused to any targeting molecule, for example, another DARPin, to retarget the bound adenovirus and thus form an 'adapter'. Importantly, the highly stable association of binding clamp and virion is fully compatible with virus disintegration during targeted infection, and rates of infectivity have been observed that equal or are even better than Ad5 with its native receptor.

Recent developments with immunoglobulins: bispecific antibodies through interface engineering

Ever since the early days of hybridoma technology, there has been a broad interest in the generation of bispecific antibodies that can bind two different targets simultaneously. Numerous applications have been suggested [47,48], from binding two neighboring epitopes on the same target (to either increase affinity or enforce a conformational change), binding two targets on a cell with low affinity (to obtain higher specificity), connecting two cells by engaging a target on either cell, or to use one specificity to deliver a cargo bound by one arm to a receptor bound by the other arm. Before the advent of recombinant technology, hetero-hybridomas (sometimes called quadromas) were created, but by doing so, 10 different molecular species from the two heavy and two light chains are formed which would have to be separated, rendering this method not very useful in practical terms. Over the last few years, a multitude of strategies to produce multispecific antibodies have been devised, recently reviewed by Chan and Carter [49]. We will not discuss the countless fusion strategies, where one specificity was simply *linked* to another (in a fusion protein), but we will focus on those approaches where protein engineering was used to change interfaces or binding surfaces, and we will concentrate on molecules resembling the IgG as closely as possible.

The molecular architecture of IgGs (schematically drawn in Figure 3a) poses basically two problems to be solved in order to produce the desired bispecific binders exclusively: First, the two different heavy chains need to heterodimerize exclusively. Secondly, a strict discrimination is required to favor the two desired light-chain/heavy-chain pairs among the four possible ones.

Figure 3



Schematic drawing of different IgG variants. The topology and domain arrangement of natural IgGs **(a)** is compared to bispecific formats (b–e) described in the text, which all have the normal H_2L_2 arrangement. For some bispecific assemblies, heavy chain heterodimerization needs to be ensured by the knobs-into-holes technology, indicated as knobs in the C_H3 domains. **(b)** Bispecific IgG (BsAb) with orthogonally designed Fab fragments, with little knobs depicting the mutant Fab interfaces. **(c)** Bispecific IgG with common light chains (light green). **(d)** Bispecific 'CrossMab' where C_H1 and C_L domain are swapped in one Fab. **(e)** IgG with dual acting Fab (DAF) which contains Fabs that were engineered and evolved to bind with dual specificity. DAF–IgGs do not require heavy chain heterodimerization and hence contain unmodified heavy chains. Domains of different specificities are indicated by different colors. V_L = variable region of the light chain, C_L = constant region of the light chain, V_H = variable region of the heavy chain, C_H1 = constant region 1 of the heavy chain, DAF = dual-acting Fab.

The task of joining the heavy chains in a directed manner was solved by Carter and coworkers early on, with their elegant 'knobs in holes' (KiH) strategy, which introduces large amino acid side chains into the C_H3 domain of one heavy chain (schematically drawn as knobs in Figure 3b–d) that fit into an appropriately designed cavity in the C_H3 domain of the other heavy chain [50,51], disfavoring both types of homodimers. The second problem, however, is more difficult to address, because only one out of four different putative IgGs is the desired one. In the KiH strategy, all possible isoforms will be composed of the same heavy chain heterodimers, yet contain different light chain compositions. Thus, the exclusive use of the KiH technology in the heavy chains either requires (unattractive) purification of the desired IgG fraction from four components, or it can only be applied if the light chains have been selected to be identical, and/or if they do not contribute significantly to antigen binding (the

'common light chain' approach [52] (Figure 3c)). While this solves the problem, it is not really a general recipe for creating heterospecific IgGs of any paratope, since the binding sites still have further constraints.

In a surprisingly simple and clever approach, correct association of the light chains and their cognate heavy chains was achieved by exchange of heavy-chain and light-chain domains (i.e., C_L by C_H1) within one of the Fab fragments of the IgG, while the other one had its native orientation [53••] (Figure 3d). This 'cross-over' IgG retains the antigen-binding affinity but makes the two arms so different that light-chain mispairing can no longer occur. Subsequently, the crystal structure of one of the described Fab (binding angiopoetin-2) was determined in its crossed and uncrossed form, showing that the cross-over does not induce significant perturbations of the structure [54]. Nonetheless, it will have to be tested how general the approach is and whether particular compositions of V_L and V_H domains might pose problems in terms of protein aggregation or interference with the desired selectivity.

Another approach to solve the same problem, but by other means, has very recently been developed by Lewis *et al.* [55••] (Figure 3b). The design goal was to stay as close to the IgG molecule as possible, but use designed point mutations to enforce specificity. On the basis of molecular modeling and feedback from X-ray crystallography, the full Fab-interface, including the V_H–V_L interactions, had to be considered to create a fully orthogonal heavy chain-light chain interface. Similar to the 'cross-over' strategy described above, the C_L/C_H1 interface was chosen as starting point for engineering. The two domains were not simply swapped, but the goal was to redesign the C_H1–C_L interface using the multistate design application in the modeling program Rosetta [56]. More than 40 initial proposed sequence pairs were constructed and experimentally screened for expression yield and proper assembly. In general, the designed proteins expressed better if the mutational load was lower — perhaps unsurprising. High-resolution crystal structures of three C_H1–C_L heterodimers were used as basis for a second round of multistate simulations to screen for the final design, a mutant C_L/C_H1 interface (comparable in expression and stability to the wildtype) that quantitatively assembled. However, testing the designed C_L/C_H1 interface in the IgG format with the variable domains being present revealed a loss in specific assembly. Therefore, the V_H–V_L interface had to be redesigned as well to add pairing specificity in that part of the Fab interface, further improving the observed specificity in interface formation. Considering the significantly lower thermal stability and lack of cooperative unfolding of the V_H and V_L domains compared with the C_H1 and C_L domains (cf. [57]), it is at first surprising that the variable domains dominated the specific assembly of heavy chains and light chains. If this variable domain dominance is a general phenomenon, it could present challenges for both this and the 'cross-over' approach described above.

Another approach for generating bispecificity in IgG molecules challenges the dogma of one Fab necessarily binding one target. The paratope with its six CDRs is generous in size, and if the affinity can be engineered well enough to be supported by only half of them, the others can be used for another specificity. Binding of both antigens simultaneously is sterically excluded, so it creates an 'either–or' situation. In other words, two 'subparatopes' for two different targets are created, thereby creating a so called dual-acting Fab (DAF) (Figure 3e).

Initial work of Bostrom *et al.* [58] demonstrated this concept, with variants of the therapeutic antibody trastuzumab (Herceptin) that bind HER2 and VEGF with the same antigen binding site. While technically elegant, the utility of this particular molecule over a simple bispecific (if it can be created, see above), or even a mixture of two IgGs, is not entirely compelling, since this heterospecific IgG targets a soluble antigen and a surface-bound one, which are not in any way connected.

Recently an IgG molecule was generated with the same approach with dual EGFR/HER3 specificity, in which the single antigen recognition surface binds two epitopes (alternatively) with high affinity [59••]. From a therapeutic perspective, these reports describe an appealing situation: Different from bispecific agents with two distinct monospecific binding functions (described above), the presence of two identical Fab arms raises the possibility that, independent of the density of the two targets EGFR and HER3 on the cell surface, any combination of EGFR and HER3 levels should be recognized with near-equivalent avidity, and thus all homodimers and heterodimers (EGFR–EGFR, HER3–HER3, and EGFR–HER3) should be addressable. To achieve the same would require a cocktail of three antibodies, two conventional ones (EGFR–EGFR, HER3–HER3) and a bispecific one (EGFR–HER3), the latter again requiring the solution of the perfect heterospecific IgG pairing (see above).

Yet another approach for creating a bispecific immunoglobulin was demonstrated when introducing additional binding sites into the constant region [60]. The choice was to use stretches of five amino acids in both the AB and the EF loop located in the C_H3 domain, that is, at the C-terminal tip of the Fc fragment, for randomization. In addition, five residues were inserted in the N-terminal part of the EF loop in a manner similar to the natural occurrence of CDR loop elongations. These loops are otherwise not involved in binding to effector molecules like Fc gamma receptors or to C1q of the complement system or to the neonatal Fc receptor (FcRn), the binding

of which endows antibodies with their long *in vivo* half-life. The described modification of the Fc scaffold's structural loops in the C_H3 domain were well tolerated by the overall protein fold.

Conceptually, this addition of a distant paratope to the IgG architecture does not only allow the construction of antibodies with two to three specificities (combining this with the bispecific approaches from above), but might in principle open the route to smaller antibody-like molecules, since the Fc is carrying all effector molecule binding sites as well as the binding site for FcRn that together make up the complete functionality of immunoglobulins.

Computational and evolutionary design of pH-dependent and metal-dependent binding

The advent of computational design has obviously an increasing influence in both fields of binding proteins, antibodies and non-antibodies. The recent report of Strauch *et al.* links both areas, since it describes the generation of an alternative binding molecule recognizing the Fc part of IgGs in a pH-dependent manner, designing the binding site around a critical exposed histidine residue in the Fc part [61••]. The computational *de novo* protein interface design was based on a hotspot-based design strategy [62]. After identifying the surface-exposed His-433 on the Fc as target site, idealized core interaction sites ('hotspots') that need to be present on the binding protein were computed. Then a set of 17 scaffold proteins was scanned for surfaces that could present these hotspots in order to form stabilizing interactions with the target site. Nine out of 17 designed proteins had detectable binding signals when screened in yeast surface display for binding of fluorescently labeled human IgG. The lead candidate, a scaffold based on pyrazinamidase from a hyperthermophilic archaeon [63], underwent one round of PCR mutagenesis followed by fluorescent-activated cell sorting (FACS) and next-generation sequencing, resulting in a high-resolution map of the sequence-function landscape [64]. Four additional rounds of selection from a library derived from the deep-sequencing data helped to further optimize the balance between affinity and the pH-dependence of binding. The obtained design binds IgG with a K_D of 4 nM at pH 8.2, and ~500-fold more weakly at pH 5.5.

Another approach to control binding made use of the power of directed evolution. Using a camelid antibody variable domain (VHH) as binding scaffold, histidine residues in the binding interface were introduced in a combinatorial manner which would not disturb antigen binding (in this case RNase A), unless they can bind a metal in a manner competitive to the antigen [65]. A phage display library with 22 residue positions within the binding interface, using stepwise selection of RNase A and metal binding over four rounds of selection, produced a VHH antibody that retained near wildtype affinity for its target antigen while acquiring a competitive metal ion binding site for nickel ions.

The two mentioned approaches, by the way, also very directly demonstrate the importance of combining computer-aided design with evolutionary fine-tuning — something that has been implicitly used in many of the projects discussed throughout this review.

Conclusions

The field of binding proteins has seen diverse advances over the last few years. The problem of generating binding activity in general can, for the most part, be considered a solved problem, at least from a pragmatic point of view: immunoglobulin formats and several of the various non-immunoglobulin folds can be evolved from synthetic libraries to picomolar affinities against a multitude of targets that is continuously growing. Nonetheless, the focusing of binding to desired epitopes, and the avoidance (or, on the contrary, the desired incorporation) of particular cross-reactivities is still a laborious undertaking, requiring extensive screening, without the guarantee for success.

Binding proteins use both loops and surfaces made from secondary structure elements for providing contact residues. Interestingly, one recent trend in further tuning and improving the different alternative scaffolds has been to implement *both* binding modes in the same molecule: a loop binder now also engaging other surfaces and *vice versa* [7•,35•].

With the novel engineering concepts for creating bispecific antibodies in the IgG format, both antibody engineering and scaffold engineering show some convergence. Without doubt, computational interface design has not only been valuable in contributing to these concepts, but is furthermore starting to enable *in silico* design of binding to defined epitopes in defined orientations. Nonetheless, evolutionary fine tuning is an integral part of current protein engineering. It has become very obvious that the use of a robust scaffold is a great advantage when it comes to creating more demanding assemblies. It will be exciting to explore the future synergies that will arise from the different fields.

Conflict of interest

AP is a co-founder and shareholder of Molecular Partners AG, which commercializes the DARPin technology.

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