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Engineered proteins as specific binding reagents

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Over the past 30 years, monoclonal antibodies have become the standard binding proteins and currently find applications in research, diagnostics and therapy. Yet, monoclonal antibodies now face strong competition from synthetic antibody libraries in combination with powerful library selection technologies. More recently, an increased understanding of other natural binding proteins together with advances in protein engineering, selection and evolution technologies has also triggered the exploration of numerous other protein architectures for the generation of designed binding molecules. Valuable protein-binding scaffolds have been obtained and represent promising alternatives to antibodies for biotechnological and, potentially, clinical applications.

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Introduction

Recombinant and engineered binding proteins have become powerful tools for therapy, *in vivo* and *in vitro* diagnostics, drug target analysis and laboratory research. Each of these applications has specific requirements for the binding molecule. These requirements translate to molecular criteria, such as the necessary target affinity and specificity, required protein size, thermodynamic and chemical protein stability, stability in serum, the presence or absence of disulfide bonds, protein domain composition, the presence or absence of post-translational modifications, concerns about immunogenicity, protein expression levels, solubility, and the presence of effector functions or moieties for labeling. Additionally, criteria such as manufacturing cost, shelf-life and intellectual property restrictions can determine whether potential binding molecules will become widely used.

For many applications, antibodies have traditionally been used. Almost all scientific, diagnostic and therapeutic applications require high specificity and a defined molecular composition, thus usually precluding the use of polyclonal antibodies. Monoclonal antibodies, by contrast, are expensive to manufacture, and for all uses except a fraction of therapeutic applications their Fc region is not really required. Antibody fragments in the form of single-chain Fv, Fab and multivalent fragments [1], which can be obtained from synthetic libraries [2] or recombinant libraries from B cells [3], have become important alternatives. They can be generated with greater control of specificity and can be manufactured relatively inexpensively in bacteria.

However, for several applications, such antibody fragments might not be ideal. For example, the stability of recombinant antibodies and antibody fragments relies on disulfide bonds and, despite significant progress [4], intracellular expression in the reducing milieu is only possible for a subset of antibodies [5]. High stability is also pivotal in other applications: for affinity chromatography, stability of the immobilized affinity ligand to very harsh cleaning conditions is essential, as are very low manufacturing costs. Finally, even for therapeutic uses, novel concepts may require fusion proteins and conjugates that would be much easier to manufacture with scaffolds other than antibodies: some antibody fusions are prone to aggregation, while a single cysteine residue that is convenient for conjugation is not as easily handled in a protein with disulfide bonds (such as an antibody fragment) as it would be in a protein without any other cysteine.

Advances in protein engineering and the availability of powerful library selection technologies have allowed the exploration of numerous alternative protein scaffolds for the generation of designed binding molecules throughout the past decade. In essence, the technologies first developed for antibody libraries to recreate the function of the immune system were extended to other protein scaffolds. In the 1990s, affinity maturation or changes of specificity of protease inhibitors using rational engineering and phage display provided the first examples of the use of scaffolds other than antibodies for selecting specific binders (see below). With increasing knowledge about protein–protein interactions, better understanding of protein engineering and the further development of selection technologies, several protein-binding scaffolds have now been explored and found suitable for binding virtually any protein target of choice. In these scaffolds, parts of the surface (typically loops, more rarely the exposed surface of α helices or β sheets) or a ligand-binding cleft of a protein framework are randomized to yield a protein library, which can then be selected towards new functions. In the beginning,

well-studied proteins that are naturally involved in protein–protein interactions were primarily chosen as scaffolds for library generation (Figure 1). The wealth of genomic sequences emerging towards the end of the last century, however, triggered the use of several other protein architectures that were revealed to be frequently used in nature for diverse protein–protein interactions.

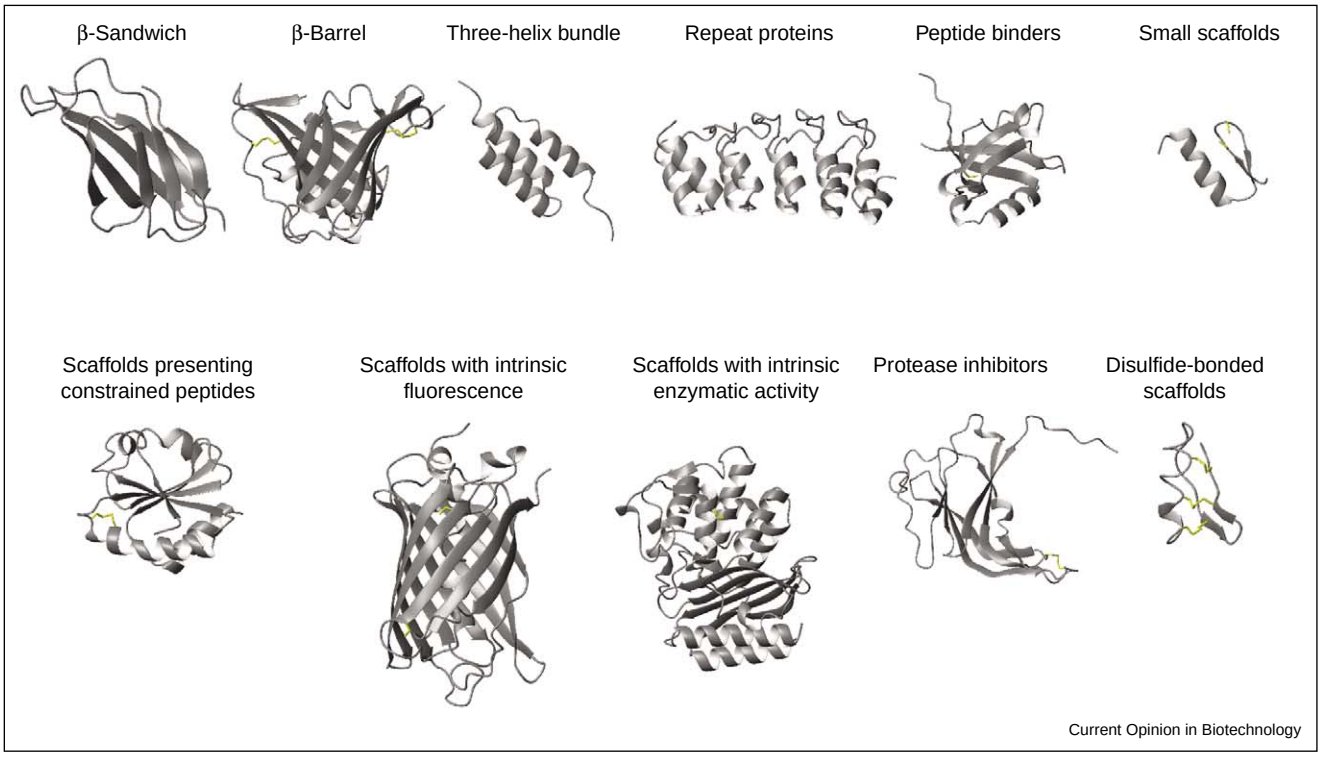
We give here an overview of the protein scaffolds that have been used as protein-binding alternatives to antibodies (Table 1). For the sake of clarity, we classify the different protein scaffolds in different groups (Figure 1). It should be noted that this classification is not absolute: scaffolds of one group often share features with scaffolds of other groups. Owing to length restrictions, we will focus on the most recent advances and publications in the field; older studies are mentioned for completeness only if they are not referenced by the more recent studies. For earlier references, the reader is directed to earlier reviews [6–8].

β-Sandwich and β-barrel proteins

In antibody variable domains, binding diversity is provided by variation of length and sequence in three loops that connect the strands of the immunoglobulin domain possessing a β-sandwich topology. Many attempts have been made to recreate this concept by using other proteins with β-sandwich or β-barrel topology as the recipients of the diversified loops. Tendamistat [9], fibronectin [10,11•], cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) [12], T-cell receptors [13,14••] and neocarzinostatin [15] are examples of β-sandwich scaffolds. CTLA-4, tendamistat, T-cell receptors and neocarzinostatin all contain disulfide bonds, and hence their use is essentially restricted to applications where antibodies are typically used (Table 1).

In the case of T-cell receptors, the aim was to generate specific binders for peptide–MHC complexes with improved affinity and/or stability, rather than to use this scaffold as a source for general protein binding ligands. Libraries of T-cell receptor mutants have successfully been used in combination with yeast surface display and fluorescence-activated cell sorting [13]. In several cases, the T-cell receptors were displayed as single-chain variants and yielded nanomolar affinity binders. For a long time, the display of T-cell receptors on bacteriophage was

Figure 1



Different protein backbones used as scaffolds for the generation of protein-binding agents, classified in groups. Typical representatives of each group are depicted. In total, over 30 different scaffolds with different folds have been used so far. The PDB IDs used to generate the figure are listed: β sandwich (1FNA, fibronectin); β barrel (A chain of 1BBP, lipocalin); thee-helix bundle (first model of 1Q2N, SpA domain); repeat proteins (1MJ0, AR protein); peptide binders (chain A of 1KWA, PDZ domain); small scaffolds (chain F of 1MEY, designed zinc-finger protein); scaffolds presenting constrained peptides (chain A of 2TrX, thioredoxin A); proteins with intrinsic fluorescence (chain A of 1GFL, GFP) or intrinsic enzyme activity (1M40, β-lactamase); protease inhibitors (1ECY, ecotin); and disulfide-bonded scaffolds (chain A of 1CMR, scorpion toxin). Cysteine residues and disulfide bonds are depicted in yellow. (This figure was generated using MolMol [80].)

Table 1

Scaffolds used for the generation of protein binders.

Scaffold Name[a]	Fold	Domain size (amino acids)	Cysteines/S–S bonds[b]
CTLA-4	β Sandwich	136	Yes/yes (2)
Tendamistat	β Sandwich	74	Yes/yes (2)
^{10}FN3	β Sandwich	94	No/no
Neocarzinostatin	β Sandwich	113	Yes/yes (2)
CBM4-2	β Sandwich	168	No/no
T-cell receptor	β Sandwich	~250	Yes/yes (2)
Lipocalins	β Barrel	160–180	Yes/yes (0–3)
Protein A domain	α_3	58	No/no
Im9	α_4	86	No/no
Designed AR proteins	α_2/β_2 Repeated	Variable (67 + n·33)[c]	No/no
Designed TPR proteins	α_3 Repeated	Variable (18 + n·34)[c]	No/no
Zinc finger	α/β (Zn^{2+})	26	Yes/no
pVIII	Mainly α	50	No/no
GCN4	α	33	No/no
WW domain	β_3	52 (WW motif 38)	No/no
SH3 domains	Mainly β	~60	Varying
SH2 domains	α/β	~100	Varying
PDZ domains	α/β	~100	Varying
TEM-1 β-lactamase	α/β	265	Yes/yes (1)
Green fluorescent protein	β Barrel	238	Yes/no
Thioredoxin	α/β	108	Yes/yes (1)
Staphylococcal nuclease	α/β	149	No/no
PHD finger	β/Loops	50–100	Yes/no
CI2	α/β	64	No/no
BPTI	α/β	58	Yes/yes (3)
APPI	α/β	58	Yes/yes (3)
hPSTI	α/β/Loops	56	Yes/yes (2)
Ecotin	β Sandwich	142	Yes/yes (1)
LACI-D1	α/β	58	Yes/yes (3)
LDTI	α/β	46	Yes/yes (3)
MTI II	α/β[d]	63	Yes/yes (4)[c]
Scorpion toxins	α/β_3	25–40	Yes/yes (3)
Insect defensin A	α/β_2	29	Yes/yes (3)
EETI II	Loops	28	Yes/yes (3)
CBD	β_3	36	Yes/yes (2)

[a] Abbreviations: APPI, Alzheimer's amyloid β-protein precursor inhibitor; AR, ankyrin repeat; BPTI, bovine pancreatic trypsin inhibitor; CBD, cellulose-binding domain; CBM4-2, carbohydrate-binding module 4 of family 2 of xylanase from *Rhodothermus marinus*; CI2, chymotrypsin inhibitor 2; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; EETI II, *Ecballium elaterium* trypsin inhibitor II; ^{10}FN3, tenth fibronectin type 3 domain; hPSTI, human pancreatic secretory trypsin inhibitor; Im9, immunity protein 9; LACI-D1, human lipoprotein-associated coagulation inhibitor domain 1; LDTI, leech-derived trypsin inhibitor; MTI II, mustard trypsin inhibitor 2; PDZ, domain present in the three proteins, post-synaptic density protein **P**SD-95, *Drosophila* **D**iscs-Large septate junction protein, and epithelial tight-junction protein **Z**O-1; PHD finger, plant homeodomain finger protein; pVIII, protein VIII of filamentous bacteriophage; SH2, src homology domain 2; SH3, src homology domain 3; TPR, tetratricopeptide repeat.
[b] The presence of cysteine residues and disulfide bonds is indicated as well as the number of disulfide bonds.
[c] The variable n denotes the number of consensus repeat modules in the molecule. The total length is that of the capping modules plus the consensus modules.
[d] Predicted by homology.

considered impossible, or at least very inefficient. However, Li *et al.* [14••] recently published a study, where T-cell receptors could efficiently be displayed on bacteriophage and peptide–MHC binders could be selected with affinities in the picomolar range. The display-enabling trick was to use a stabilizing nonnative interchain disulfide bond in the constant domains.

Li *et al.* [9] used the α-amylase inhibitor tendamistat in selections against different integrins. First, a loop library was inserted in a loop connecting two β strands of tendamistat and selected against $\alpha_v\beta_3$ integrin. The resulting sequences, mostly containing the known integrin-binding RGD motif, provided the basis for the construction of two further libraries that contained the RGD motif and seven flanking randomized positions. These two libraries were used in selections against different integrins. The selected sequences gave insight into the preferences of the different integrins for particular sequences flanking the RGD motif [9].

Among the β-sandwich scaffolds, neocarzinostatin is the most recently explored scaffold. It consists of 113 amino acids and has a bound chromophore. A lysozyme-binding neocarzinostatin variant has been engineered, proving the potential of this scaffold to adopt new binding

specificities [15]. In this variant, a CDR3 (complementarity determining region 3) loop of a VHH camel antibody domain was grafted at the place of an equivalent loop in neocarzinostatin. At 15 °C the engineered variant had an affinity to lysozyme of 0.5 μM, compared with 20 nM for the loop-donor VHH domain. The neocarzinostatin variant was well expressed with 30–35 mg protein per liter shake-flask culture, but a decrease in stability to 3 kcal/mol compared with 8.8 kcal/mol for neocarzinostatin was also observed. The phage-display selection of testosterone-binding variants from a library of neocarzinostatin, randomized in the chromophore-binding region, showed that this scaffold is also suited for the binding of small molecules [16].

In contrast to the other β-sandwich proteins, fibronectin (Figure 1) does not rely on disulfide bonds and hence might extend the range of uses of antibodies. The tenth domain of type 3 fibronectin (also named ^{10}FN3, FNfn10, trinectin, monobody or adnectin) [10,11•] is one of the best-characterized scaffolds of this type. This 94 amino acid protein is well expressed in soluble form in bacteria and is thermodynamically stable. Fibronectins with a novel binding specificity to ubiquitin could be generated with an affinity in the micromolar range from a library with two randomized loops using five rounds of phage display [17]. With a similar library, binders to the Src SH3 domain with micromolar affinities were recently selected [11•]. Clones with the typical SH3 domain 1 binding motif PXXP (in single-letter amino acid code, where X is any amino acid) were selected, as well as a sequence containing no PXXP motif. These fibronectins could be used in both western blotting and 'immuno'-precipitation experiments. In another approach with a slightly different and much more diverse library, binders in the nanomolar range were reported after nine selection rounds of messenger RNA display against tumor necrosis factor α (TNFα) [10]. From these nanomolar binders, picomolar binders could be evolved with further affinity maturation steps [10]. Fibronectin was also successfully used in a yeast two-hybrid approach, indicating that the framework could be interesting for intracellular applications [18].

Lipocalins comprise 160–180 amino acids and form conical β-barrel proteins with a ligand-binding pocket surrounded by four loops. Small hydrophobic compounds are the natural ligands of lipocalins, and different lipocalin variants with new compound specificities (also termed 'anticalins') could be isolated after randomizing residues in this binding pocket [19•]. The analogy of their loops to antibody CDRs is an indication that lipocalins might also be used as a source for protein binders. By randomizing these loops and selecting hemoglobin-binding lipocalin variants with micromolar affinities, Vogt and Skerra [20] recently showed that protein binding is indeed possible. More recently, preliminary data on a nanomolar affinity CTLA-4-binding lipocalin variant have been reported [19•], giving first indications that specific, high-affinity protein-binding anticalins can be generated. Lipocalins are usually disulfide-bonded scaffolds and could therefore be an alternative in those applications where recombinant disulfide-containing antibody fragments can also be used. The fatty-acid-binding protein (FABP), also a member of the lipocalin family, has been used as the carrier of an N-terminal peptide library by Lamla and Erdmann [21]. A library consisting of 15 random amino acids was used in ribosome display selections against streptavidin. In seven phage display selection cycles, a peptide–FABP fusion was isolated that had an affinity to streptavidin of 4 nM.

The carbohydrate-binding module CBM4-2 of a bacterial xylanase also has a β-sandwich-like architecture and has been used as a binding protein with novel specificity [22]. Similarly to fibronectin, CBM4-2 does not contain disulfide bonds, is thermodynamically stable and can be expressed at high levels in *Escherichia coli*. However, unlike the other β-sandwich and β-barrel proteins discussed, where loops were randomized in analogy to antibody loops, CBM4-2 was randomized in the carbohydrate-binding β-sheet surface. A phage display library with a diversity of 1.6×10^6 was used to select binders against different carbohydrates as well as against glycosylated human immunoglobulin G4 [22].

Protein Z and α-helical scaffolds

One of the first scaffolds investigated that did not belong to the β-barrel or β-sandwich family was protein Z (also named affibody; Figure 1), an engineered domain B of staphylococcal protein A (SpA) [23]. This 58 amino acid three α-helical bundle protein is rather stable (ΔG = 6.6 kcal/mol) and well-expressed in soluble form in *E. coli*. For protein Z, it was not the loops connecting the secondary structure elements that were randomized, but rather 13 residues on the surface of two α helices. These residues are naturally involved in binding the Fc part of antibodies. In recent years, libraries of protein Z variants have been used to generate binders against at least eight different targets by phage display. Usually, specific binders with micromolar affinities were rapidly obtained. Some of these binders could be evolved to nanomolar binders by a second randomization, followed by further phage display selection rounds. An affibody selected against human CD28 was shown to block the interaction between CD28 and CD80, hence being a therapeutic candidate [24]. Similarly, Wikman *et al.* [25•] selected protein Z variants that bound to the breast cancer target Her2 with nanomolar affinity, which were also active on Her2-expressing cells. The variant with the highest affinity does not bind to the same site as trastuzumab (Herceptin), which is clinically used in the therapy of breast cancer; nevertheless, these molecules could represent interesting candidates for the development of therapeutic and diagnostic agents.

Particularly interesting are the crystal and nuclear magnetic resonance (NMR) structures of the complex between an affibody and its target, the original protein Z (Protein Data Bank [PDB] IDs 1LP1 and 1H0T; Figure 2) [26••,27••]. The studies reveal the details of the selected interaction and show that most of the randomized surface of the 'anti-idiotypic' affibody was involved in the interaction, with a K_D of 6 μM. NMR studies further revealed that this particular affibody seems to be a molten globule that folds only upon binding to its target, protein Z [26••,28,29], thus possibly limiting the observed overall affinity by an entropy loss upon folding.

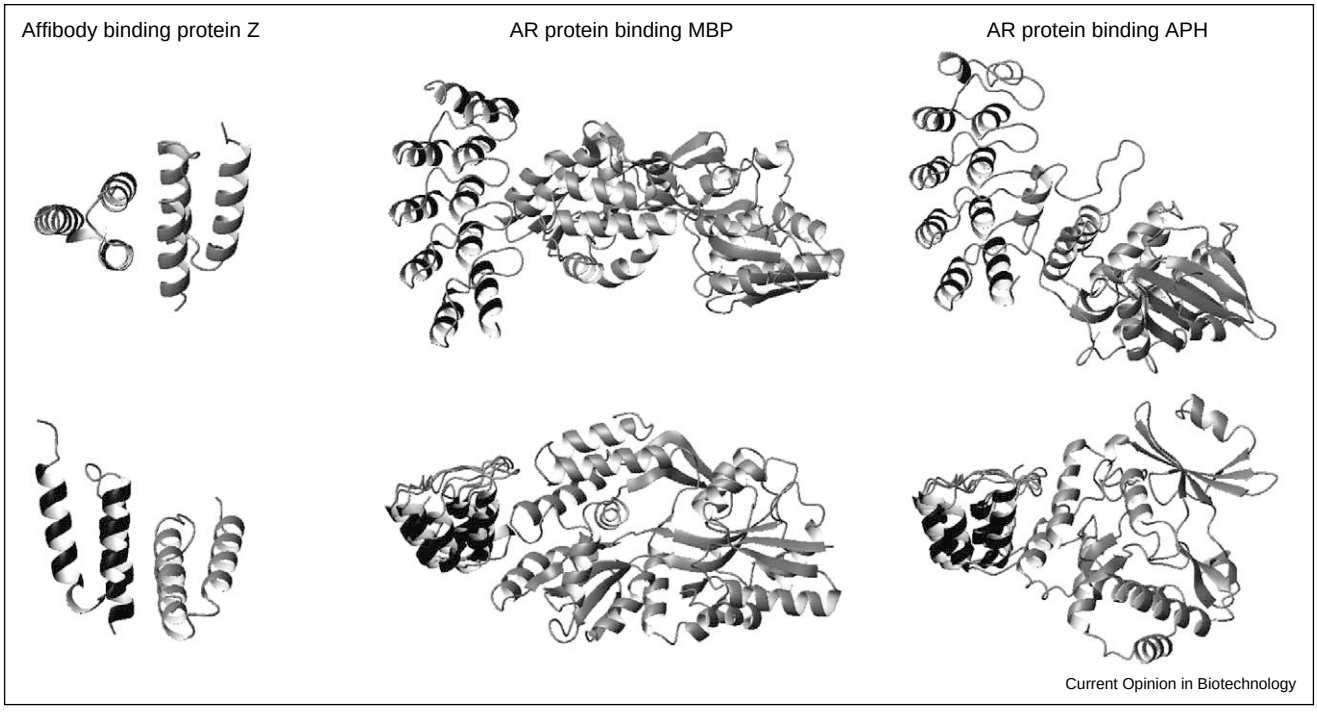
The bacterial nuclease inhibitors Im7 and Im9, naturally made by colicin-producing strains to protect themselves, are also α-helical proteins that could be used as alternative binding or inhibiting proteins. Indeed, by combining error-prone polymerase chain reaction (PCR) with an *in vitro* compartmentalization selection procedure, Bernath *et al*. [30] evolved Im9, the inhibitor of colicin E9, into a colicin E7 nuclease inhibitor that showed some features of Im7, the natural inhibitor of colicin E7 that is homologous to Im9. Besides the generation of nuclease inhibitors with new specificities, these well-characterized immunity protein scaffolds could also be considered for the generation of binding molecules with new binding specificities.

Repeat proteins

With the increasing availability of genomic sequencing data, it became obvious that nature has evolved repeat proteins as another important class of binding molecules, next to antibodies [31]. Ankyrin repeat (AR), armadillo repeat (ARM), leucine-rich repeat (LRR) and tetratricopeptide repeat (TPR) proteins are the most prominent members of this protein class (Figure 3). Repeat proteins are composed of homologous structural units (repeats) that stack to form elongated domains [31]. The binding interaction is usually mediated by several adjacent repeats, leading to large target interaction surfaces (Figure 3).

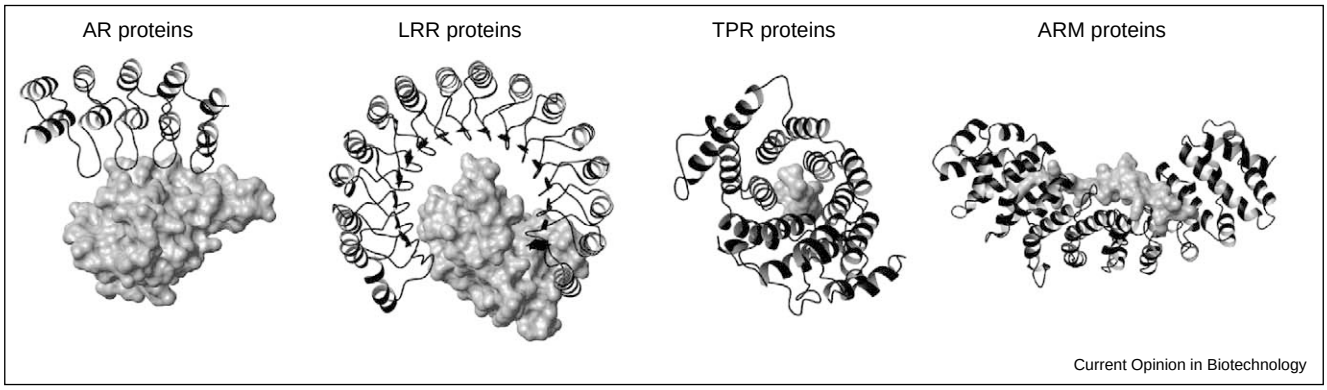
AR protein libraries have been used for the generation of binding molecules [32••]. In this case, the chosen

Figure 2



Crystal structures of designed protein-binding molecules with new binding specificities in complex with their targets. The affibody Z_{SPA-1} in complex with its target protein Z (PDB ID, 1LP1 [26••]), the AR protein off7 in complex with its target maltose-binding protein (MBP; PDB ID, 1SVX [32••]) and the AR protein AR_3a in complex with aminoglycoside phosphotransferase (APH; PDB ID, 2BKK [36••]) are shown in two different views (90° rotated with respect to each other). The crystal structures of the complexes reveal that all scaffolds interact with their target protein by means of the randomized positions, validating the randomization schemes. The affibody Z_{SPA-1} binds its target protein Z in an induced-fit manner [26••]. Although it appears to be in a molten globule state in free form, it adopts the typical affibody fold only upon binding protein Z. The AR protein off7 binds MBP in a rigid-body fashion with no structural alterations in off7 or MBP. The APH inhibitor AR_3a (also a designed AR protein) binds a conformation of APH that appears to be catalytically inactive. In this conformation, APH is unable to productively bind the substrate kanamycin, as several helices are distorted. (This figure was prepared using MolMol [80].)

Figure 3



Natural repeat proteins binding their target proteins or peptides. This illustration shows the variety of interactions repeat proteins can perform and, thus, the potential of repeat proteins as alternatives to antibodies. The repeat proteins are depicted as a black ribbon, while the target proteins or peptides are shown in a grey surface representation. Four repeat proteins were used to prepare this figure using MolMol [80]: the ankyrin repeat (AR) protein mouse guanosine-adenosine rich repeat binding protein β1 (GABP) binding to the α subunit (PDB ID, 1AWC); the porcine leucine-rich repeat (LRR) protein ribonuclease inhibitor binding bovine ribonuclease A (PDB ID, 1DFJ); the tetratricopeptide repeat (TPR) protein region of human Pex5 binding the peroxisomal targeting signal peptide PTS1 (PDB ID, 1FCH); and the mouse armadillo repeat (ARM) protein importin-α binding the nuclear localization peptide of the *Xenopus laevis* N1N2 phosphoprotein (PDB ID, 1PJN).

approach was fundamentally different from most other scaffold approaches in that no existing AR protein was used as scaffold. Instead, libraries of AR protein scaffolds of varying repeat numbers were generated using a consensus-designed AR module as a building block [33]. Individual members of these libraries are well expressed in soluble form in *E. coli*, are thermodynamically stable and have the AR protein fold (Figure 1) [33,34]. Designed Ankyrin Repeat Proteins (DARPins) with nanomolar to picomolar affinity binders against four different targets were isolated in only four ribosome-display selection rounds both from a four- and five-repeat library (N- and C-terminal capping repeats plus two or three internal, randomized repeat modules) [32••,35•]. The crystal structure of a complex between a five-repeat protein and its cognate target maltose-binding protein (MBP) (PDB ID 1SVX; Figure 2) revealed that a binding interface typical of protein–protein interactions was selected, where the number of tyrosine residues involved was very prominent, as seen with antibodies [32••]. In a combined *in vitro*/*in vivo* selection approach, intracellular inhibitors of the prokaryotic enzyme aminoglycoside 3′-phosphotransferase (APH(3′)-IIIa) could be selected that inhibited the enzyme both *in vivo* and *in vitro* [35•]. The crystal structure of one of the inhibitors selected in complex with APH(3′)-IIIa revealed details of the allosteric inhibition mechanism and also emphasized that the rigid AR domain scaffold can be used for co-crystallization (Figure 2) [36••].

As a first example of a synthetic TPR protein binding to a target peptide (Table 1), Cortajarena *et al.* [37] used consensus design to engineer a TPR protein that recognizes the C-terminal peptide of the eukaryotic chaperone Hsp90 with an affinity of 200 μM (compared with 5 μM for the wild-type TPR binding the same peptide). Although no libraries have been reported yet, libraries of TPR proteins might be especially interesting to generate peptide binders. Similarly, potential libraries of ARM proteins and the already existing LRR protein libraries of varying repeat numbers [38] could serve as a source for peptide- or protein-binding molecules.

Peptide-binding scaffolds

Many protein chip applications require peptide-binding reagents. Besides antibodies, several natural peptide-binding scaffolds have successfully been used for the generation of binders to peptides. Yet, most of these recognize only very short motifs and typically show only micro- to nanomolar affinities. These domains are all involved in cellular signaling and include SH3 [39–41], SH2 [42], PDZ [43,44,45••] and WW [46] domains (Table 1). These proteins usually recognize peptides in a specific context: SH3 domains bind peptides that have a polyproline II helix conformation and usually contain a proline-rich motif; PDZ domains (Figure 1) typically bind C-terminal peptides and thus recognize the terminal COO^- group; and SH2 domains are usually involved in binding of phosphorylated peptides. The example of PDZ variants, which can be used in western blotting, 'immuno'-precipitation and affinity chromatography [45••], shows the power of these peptide-binding proteins. Nevertheless, the applicability of these scaffolds is restricted to specific peptides close to the sequence they naturally recognize, leaving room for the development of domains that can be generically used for high-affinity peptide binding. Two such scaffolds could be TPR or ARM proteins (Figure 3).

Scaffolds presenting constrained peptides

The proteins mentioned so far were designed (with a few exceptions) to bind the target with more than one loop and sometimes with a surface provided by the domain fold. With a few exceptions [26••,27••,32••,36••,47,48], no crystal structures of selected complexes exist and it is therefore possible that in some cases the selected loops bind the target merely as constrained peptides. In this section, we will summarize the cases where this is the intended mode of binding. We will not discuss the many constrained peptide libraries that were used directly in phage or ribosome display or cases in which the peptide is typically restricted in conformation by a disulfide bond.

In several approaches peptides were inserted in constrained loop regions of other proteins. Fibronectin, green fluorescent protein (GFP) and lipocalins have been used to display constrained peptides, either as a loop insertion or as C-terminal fusions. Thioredoxin A (TrxA; Figure 1) [49] and staphylococcal nuclease [50] are two early examples of the display of constrained peptides. The main aim of these approaches is to protect the peptides from proteolytic degradation and/or to constrain their conformation [51]. In this manner, the integrity and thus the functional diversity of the peptide library is maintained, and the conformational restriction might allow the achievement of higher affinities. In a comparative study, Klevenz *et al.* [52] inserted two different peptides in TrxA, staphylococcal nuclease and GFP. While one peptide interacted with its target independently of the scaffold, the other peptide was only able to interact within the TrxA scaffold context, as revealed by yeast two-hybrid and glutathione S transferase (GST) precipitation experiments.

Recently, the cysteine-rich plant homeodomain (PHD) finger domain of the transcriptional cofactor Mi2β (second domain; Mi2β-P2) was investigated as a scaffold for the generation of novel binding molecules [53]. This PHD finger domain is stabilized by two zinc ions, which are complexed by seven cysteines and one histidine residue. Sequence alignments of different PHD domains and NMR analyses revealed that two loops (loops 1 and 3) are highly flexible both in terms of sequence and structural plasticity, suggesting that these loops could bear altered sequences. This loop-alteration tolerance was confirmed by mutagenesis and sequence insertion. A Mi2β-P2 variant with a PVDLS sequence inserted in loop 3 was made, creating a folded domain with affinity for the transcriptional corepressor CtBP2. This construct could efficiently be used in GST 'immuno'-precipitation experiments and in yeast two-hybrid experiments, the intracellular applicability of this scaffold was demonstrated.

Small scaffolds

Another way to circumvent the loss of entropy upon binding an unfolded flexible peptide to a target is to present the peptide in a conformationally frozen form. The introduction of a disulfide bond is often used to restrict the conformational flexibility of peptides. Another possibility is to use peptides that adopt a rigid conformation on their own. As in earlier approaches, where small domains such as zinc-finger domains [54], coiled-coil peptides or single helices [55] and pVIII of filamentous bacteriophage (Figure 1; Table 1) [56] were used to present conformationally uniform peptide libraries, Sia and Kim [57] used the GCN4 leucine-zipper for the rational construction of human immunodeficiency virus 1 (HIV-1) inhibitors with nanomolar affinity. They grafted 19 amino acids from a helical peptide derived from gp41 of HIV-1 onto GCN4, leading to a 34 amino acid peptide that can inhibit the HIV-1 envelope-mediated membrane fusion with IC_{50} (inhibition constant) values in the nanomolar range.

Scaffolds with intrinsic detection means

Protein chip applications, enzyme-linked immunosorbent assays (ELISAs) or localization studies require the binding of the target polypeptide to be easily detected. Traditionally, radioactive or fluorescently labeled detection agents, detectable fusion proteins, or strategies involving secondary detection reagents are used for this purpose. An alternative approach is to use binding proteins with intrinsic detection means, such as an enzymatic activity or fluorescence (Figure 1). GFP [58–61] and β-lactamase [62] are the most thoroughly tested examples. The β-barrel protein GFP was used both for library insertions in loops connecting the β strands [60,61] and as an N-terminal fusion protein for random peptide libraries [58,59]. In the latter, more recent approaches, individual library members could successfully be screened for either cellular localization (3.1% to 4.8% of library members showed some localization tendencies) or mediation of cell cycle arrest.

β-Lactamase (Figure 1) variants with new binding specificities have been isolated from libraries where one or two loops were randomized [62]. Altogether, seven different libraries were constructed and tested. Using phage display, binders could be isolated against monoclonal antibodies, streptavidin or ferritin. After affinity maturation, ferritin binders with low nanomolar affinities were isolated [62]. For some binders, the target interaction did indeed modulate the enzymatic activity. β-Lactamase therefore appears to be a sensitive detection probe.

Protease inhibitors

Owing to their importance in blood clotting and many other pharmaceutically relevant processes, protease inhibitors were among the first scaffolds to be chosen for protein engineering (Table 1). So far, protease inhibitors have always been adapted to novel protease targets and affinity and specificity can usually be improved. Also, the high affinity translated to extremely high inhibition

constants (low K_i), underlining the success of this approach in this clearly defined application. Among the inhibitors tested, libraries of bovine pancreatic trypsin inhibitor, Alzheimer's amyloid β-protein precursor inhibitor, human lipoprotein-associated coagulation inhibitor and human pancreatic secretory trypsin inhibitor were studied early on (summarized, e.g. in [6]). More recent examples include the leech-derived trypsin inhibitor (LDTI) [63,64], the mustard trypsin inhibitor II (MTI II) [65,66] and ecotin [67•].

Tanaka *et al.* [63] selected high-affinity LDTI-based thrombin binders and inhibitors in two rounds of phage display. Inhibition was restricted to thrombin and trypsin, while factor Xa, plasma kallikrein and neutrophil elastase were not inhibited. This study was further extended [64], and highly specific inhibitors to plasmin and neutrophil elastase were selected. To improve plant defense against aphids (soft-bodied insects), MTI II was subjected to phage display selection against trypsin and chymotrypsin [65,66]. Picomolar (trypsin) and nanomolar (chymotrypsin) inhibitors were obtained in four selection rounds. The authors suggest that such MTI II variants could be incorporated in transgenic crops to increase resistance against sucking insect pests.

The periplasmic *E. coli* protease inhibitor ecotin (Figure 1) was selected to bind urokinase-type plasminogen activator (uPA) in several different approaches. The knowledge gained from all these experiments was used to select picomolar uPA inhibitors by combining phage display and rational design [68]. The use of ecotin was recently investigated in even more detail [67•] and inhibitors against several proteases (plasma kallikrein, membrane-type serine protease 1 [MT-SP1] and factor XIIa) were selected from combinatorial ecotin libraries with up to 20 randomized amino acids, using six to seven rounds of phage display. The most potent inhibitor had an affinity of 11 pM to plasma kallikrein. Competition with soluble proteases of undesired specificity was used in phage display selections to increase the inhibitor specificity.

Small disulfide-bonded scaffolds

Small disulfide-bonded proteins usually exhibit a high thermodynamic stability and are known to bind a broad range of targets such as proteins, sugars and lipids. In this respect, the scorpion toxins charybdotoxin [69–71], scyllatoxin [72•,73] and α-conotoxin [74] (Figure 1; Table 1), the cellulose-binding domain of cellulases [75,76], the insect defensin A [77] (secreted by certain larvae to attack bacterial membranes), and the *Ecballium elaterium* trypsin inhibitor II [78] have been used as scaffolds for generating new binding molecules. While the cellulose-binding domain and charybdotoxin were used to generate novel binding specificities via surface residue randomization and selection, the charybdotoxin and other scaffolds were also used in loop grafting studies with loops of defined sequence. In an extension of preceding work, a scyllatoxin variant carrying a CD4 loop in its β-hairpin was optimized such that the affinity and inhibitory effect of the chimera equalled the potency of CD4, and an inhibitory effect of one designed variant on HIV-1 infection was shown in cell culture [72•]. A similar study has been performed with charybdotoxin [71]. As these CD4 mimetic proteins induce a conformational change in the HIV-1 protein gp120, leading to exposure of cryptic antigen parts, they were suggested as vaccine candidates, similar to what has been suggested for different α-conotoxin variants [74,79]. Different applications have also been reported for EETI-II. In addition to being used as a Sendai virus epitope carrier, EETI-II was used as a scaffold for the presentation of constrained peptides for selections against the parental target, bovine trypsin [78]. In the case of the insect defensin A [77], a phage library with a diversity of 3×10^8 members presenting constrained randomized peptides (seven amino acids) in defensin A, was prepared. This library was used in selections against TNFα, two TNF receptors and a monoclonal antibody, and phage enrichments could be observed for all four targets.

Conclusions

Well over 30 different protein scaffolds have been investigated as alternatives to antibodies. These proteins are of different topologies and folds and different structural elements mediate the target interactions, offering a large set of options. Proteins selected from libraries of such scaffolds can be used in manifold applications such as affinity chromatography, western blotting, tissue staining, and diagnostic applications. Some can also be used as intracellular inhibitors in target discovery and validation, as well as potentially in therapy. Unpublished work on, for example, γ-crystallins and ubiquitin (http://www.scilproteins.de), transferrin (http://www.biorexis.com), C-type lectin-like domains (http://www.borean.dk) and low-density lipoprotein receptor domain A (http://www.avidia.com) shows that the field of alternatives to antibodies is still dynamically developing. Particularly for peptide binding, generically applicable scaffolds are still sought. ARM and TPR proteins could represent solutions to this problem. To reach a state of maturity comparable to recombinant antibodies, where a wealth of data on the structure of antibody–antigen complexes, biophysical properties and both natural and biosynthetic affinity maturation strategies have helped to shape both libraries and selection technologies, similar studies will have to be carried out with alternative binding molecules. X-ray crystallography, NMR experiments or biophysical analyses have only been performed for a very limited number of synthetic binding molecules; however, with the first examples of atomic coordinates of binding molecules in complex with their protein targets, detailed insight of the mode of interaction of three scaffolds was gained [26••,27••,32••,36••,47,48]. This might stimulate future

design and experimental strategies to obtain such novel binding proteins.

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