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Engineered protein scaffolds as next-generation antibody therapeutics

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Antibodies have been the paradigm of binding proteins with desired specificities for more than one century and during the past decade their recombinant or humanized versions have entered clinical application with remarkable success. Meanwhile, a new generation of receptor proteins was born, which is derived from small and robust non-immunoglobulin ''scaffolds'' that can be equipped with prescribed binding functions using the methods of combinatorial protein design. Their ongoing development does not only provide valuable insights into the principles of molecular recognition and protein structure–function relationships but also yields novel reagents for medical use. This technology goes hand in hand with our expanding knowledge about the molecular pathologies of cancer, immunological, and infectious diseases. Currently, questions regarding the choice of suitable medically relevant targets with regard to a certain protein scaffold, the methodology for engineering high affinity, arming with effector functions, routes of administration, plasma half-life, and immunogenicity are in the focus. While many protein scaffolds have been proposed during the past years, the technology shows a trend toward consolidation with a smaller set of systems that are being applied against multiple targets and in different settings, with emphasis on the development of drug candidates for therapy or *in vivo* diagnostics: Adnectins, Affibodies, Anticalins, DARPins, and engineered Kunitz-type inhibitors, among others. Only few data from early clinical studies are available yet, but many more are likely to come in the near future, thus providing a growing basis for assessing the therapeutic potential – but possibly also some limitations – of this exciting new class of protein drugs.

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Introduction

Starting with the early work of Paul Ehrlich [1] the era of chemotherapy was tremendously successful for medicine in the 20th century, providing small molecule drugs for the treatment of many infectious diseases, metabolic disorders, cardiac diseases, neuromedicine, and cancer. However, the amount of approved new chemical entities (NCEs) per year has decreased lately, and a growing number of protein drugs, the so-called 'biologics', is entering the clinics, among those an increasing fraction of antibodies, especially during the past 10 years [2]. Today, more than 20 different antibodies have been approved in Europe and the USA, providing a considerable market potential for the pharmaceutical and biotech industry [3,4].

There are several reasons for the remarkable success of antibodies (immunoglobulins, Igs) as a class of biological drugs. First, they can rather quickly be generated against a wide range of target molecules (antigens or haptens), either by classical immunization of animals – followed by protocols for monoclonal antibody preparation – or, more recently, via *in vitro* selection from cloned or synthetic gene libraries [5]. Second, they usually possess extraordinary specificities for their targets, with affinities often in the low nanomolar to picomolar range, thus surpassing most chemical drugs. These beneficial properties were already noticed by Emil von Behring when he investigated the humoral immune response more than hundred years ago and, in fact, also by his colleague Ehrlich, who postulated the 'side-chain' theory in order to explain the formation of antigen-specific antibodies (originally termed 'antitoxins' by von Behring) [1].

Since then it took considerable time until gene technology permitted the heterologous production of recombinant antibodies as well as the 'humanization' of antibodies from rodents that, in combination with the methods for selection from cloned Ig libraries and also with the availability of transgenic animals carrying a human Ig locus, provides a mature technology today [5,6]. For antibodies, clinical safety and efficacy has been well established, including aspects of epitope specificity, immunogenicity (human anti-human antibodies, HAHA), pharmacokinetics, and immune-related effector functions, leading to wide acceptance by physicians and patients.

However, with the increasing application of antibodies several disadvantages have become apparent. For example, they have a large size and complicated composition, comprising four polypeptide chains, glycosylation of the heavy chains, and at least one structurally crucial disulphide bond in each of several Ig domains. Thus, full size antibodies require manufacturing in eukaryotic

expression systems, usually involving stably transfected mammalian cell lines, whose optimization and fermentation is laborious and costly [7]. Consequently, exploration of alternative protein reagents with the ability to specifically recognize and tightly bind 'antigens' has been stimulated, leading to a range of different antibody fragments – most prominently, Fab and single chain Fv, which may simply be prepared by shortening the reading frame of cloned Ig genes – and, ultimately, even to isolated Ig domains [8].

In parallel to the increasingly advanced manipulation of Ig fragments an independent development has focused on recruiting unrelated proteins for analogous applications. In fact, it was demonstrated that several protein families with non-Ig architecture can be equipped with novel binding sites by employing methods of combinatorial engineering, such as site-directed random mutagenesis in combination with phage display or other molecular selection techniques [9,10]. As result, novel biomolecular binding reagents have become available, thus triggering a paradigm shift in so far as antibodies are no longer considered as the unique and universal class of receptor proteins in biotechnology and medicine [10,11].

These novel alternative binding reagents are collectively called engineered protein scaffolds [12], illustrating the fact that a rigid natural protein structure is used to modify an existing – or to implement a new – binding site for a prescribed target. Usually, such a scaffold is derived from a robust and small soluble monomeric protein (such as the Kunitz inhibitors or the lipocalins) or from a stably folded extramembrane domain of a cell surface receptor (e.g. protein A, fibronectin or the ankyrin repeat). Compared with antibodies or their recombinant fragments, these protein scaffolds often provide practical advantages including elevated stability and high production yield in microbial expression systems, together with an independent intellectual property situation.

As these novel binding proteins are obtained by means of a biomolecular engineering process in order to achieve tight target-binding activity, they may also be subjected to further selection schemes focused at other desired properties (such as solubility, thermal stability, protease resistance etc.). Consequently, engineered protein scaffolds have become attractive for many applications in biotechnology and biomedical research. However, since the effort to generate such an alternative binding protein with beneficial properties still is higher than the preparation of a conventional antibody (or a recombinant Ig fragment), most of the ongoing activities in this area are directed toward therapeutic use, offering the chance of high return on investment. Here, we review the current state of the art in this field, with special emphasis on biomolecular structure and function as well as on approaches toward clinical application.

Old and new protein scaffolds

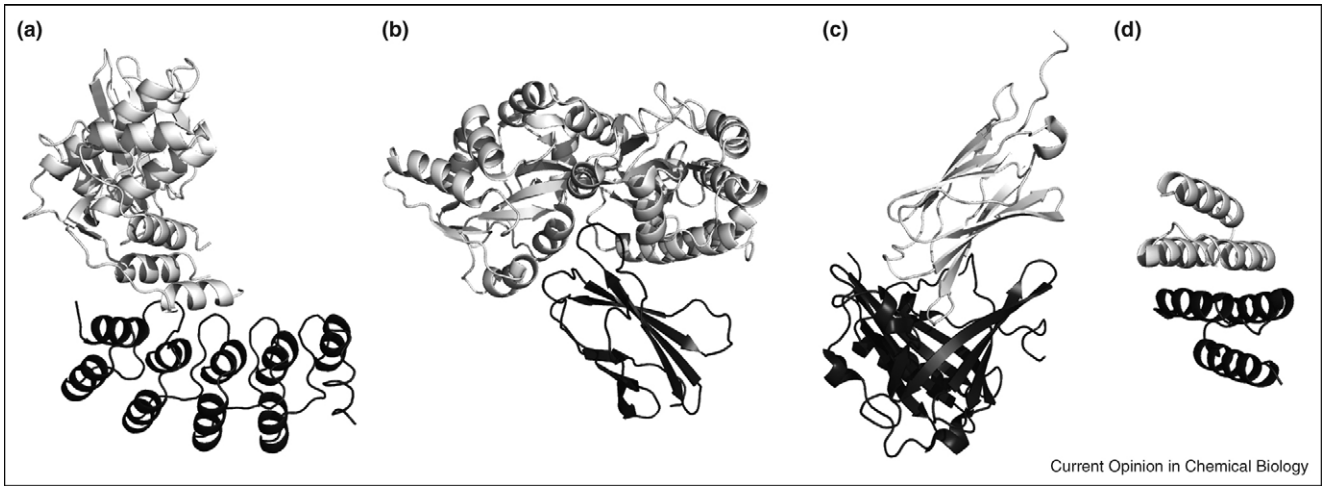
More than 50 different protein scaffolds have been proposed over the past 10–15 years and these numerous examples have been summarized in previous reviews (see e.g. [12–15]). The most advanced approaches in this field comprise the following protein classes:

- Affibodies based on the Z-domain of *staphylococcal* protein A, a three-helix bundle of 58 residues providing an interface on two of its α-helices (recently reviewed in [16]);
- engineered Kunitz domains based on a small (ca. 58 residues) and robust, disulphide-crosslinked serine protease inhibitor, typically of human origin (e.g. LACI-D1), which can be engineered for different protease specificities [17];
- Monobodies or Adnectins based on the 10th extracellular domain of human fibronectin III (^{10}Fn3), which adopts an Ig-like β-sandwich fold (94 residues) with 2–3 exposed loops, but lacks the central disulphide bridge [18];
- Anticalins derived from the lipocalins, a diverse family of eight-stranded β-barrel proteins (ca. 180 residues) that naturally form binding sites for small ligands by means of four structurally variable loops at the open end, which are abundant in humans, insects, and many other organisms (recently reviewed in [19]);
- DARPins, designed ankyrin repeat domains (166 residues), which provide a rigid interface arising from typically three repeated β-turns (recently reviewed in [20]);
- and, finally, a few other binding proteins based on more peculiar folds such as a multimerized LDLR-A module (Avimers [21]) or cysteine-rich knottin peptides (recently reviewed in [22]), for example.

In a wider context, a class of isolated Ig domains with high solubility, which are accessible via immunization of camels, llamas, sharks or by means of stringent selection from cloned human light or heavy chain variable gene segments, has also to be mentioned [8], even though these binding proteins are not truly derived from an 'alternative' scaffold.

Currently, the field of non-Ig binding proteins undergoes increasing consolidation, a trend that has been discussed before [15]. This means that many protein scaffolds that were once proposed have never been developed beyond an initial study, while just few approaches, in particular those listed above, have been expanded, leading to the engineering of high affinity binding proteins against several targets, sometimes even accompanied by structural analysis (Figure 1). Development of these latter protein scaffolds now primarily aims at medical applications, either for therapy [23] or for *in vivo* diagnostics [24], and some topical examples are discussed below. Just a few new protein scaffolds have been experimentally validated during the past 1–2 years:

Figure 1



Mode of target binding for some engineered protein scaffolds. **(A)** DARPin in complex with APH (PDB code 2BKK); **(B)** Monobody in complex with MBP (PDB code 2OBG; the two binding partners are from different symmetry-related protein chains); **(C)** Anticalin in complex with the extracellular domain of CTLA-4 (PDB code 3BX7); **(D)** Affibody in complex with the Z domain of protein A (PDB code 1LP1). Engineered protein scaffolds are always shown in dark gray at the bottom while their molecular targets are colored light gray and arranged above. For references see text.

The SH3 domain of the human Fyn tyrosine kinase was employed as an alternative binding protein for EDB, a disease-relevant domain of fibronectin associated with tumor growth and neovasculature. Randomization of two flexible loops of the SH3 fold resulted in a naive library from which the so-called Fynomers (Covagen) with low nanomolar affinity were selected via phage display [25]. In a subsequent study it was demonstrated that Fynomers are amenable to a covalent DNA display selection technique [26].

Sac7d from the hyperthermophilic archaeon *Sulfolobus acidocaldarius* provides a heat, pH and chemically stable SH3-like five-stranded incomplete β-barrel, which is capped at the opening by a C-terminal α-helix. The small protein (7 kDa) comprises just 66 residues, is devoid of disulphide bridges, and its natural function is the recognition of double-stranded DNA, yet without defined sequence specificity. A combinatorial library was prepared by randomizing 14 residues located in the β-sheet that are normally engaged in DNA-binding [27]. Using ribosomal display, high affinity (down to 140 pM) binding proteins (later dubbed Affitins [28]) specific for the bacterial secretin PulD were isolated and shown to inhibit PulD oligomerization, thereby blocking the type II secretion pathway.

Human γ-B-crystallin is a remarkably stable all-β-sheet protein expressed in the eye lens of vertebrates at high concentrations during embryogenesis and persisting at very high local protein concentration for the entire lifespan. Eight residues on the solvent-exposed face of one of the four β-sheets were randomized and variants (the so-called Affilins; SCIL proteins) with specific binding activities – albeit at moderate affinity – toward steroids, IgG-Fc, and proNGF were selected via phage display [29].

Interfaces for molecular recognition

The structural mechanism by which antibodies recognize their antigens is well understood. A set of six hypervariable loops (also known as complementarity-determining regions, CDRs), three in each variable domain of both the light and heavy Ig chain, come together at the tip of the Y-shaped molecule (the so-called Fab arm) und form there an extended contiguous combining site [10]. The extraordinary structural variability of this interface arises from the high sequence diversity of the CDRs in conjunction with a large set of different allowed backbone conformations (dubbed 'canonical' structures) while additional side-chain substitutions in the underlying framework of the Ig domains (the 'vernier' region), which are acquired via somatic hypermutation in the course of an immune response, effect fine-tuning of the shape complementarity with the antigen. On top of that, an induced fit – usually involving the mutual orientation of V_H and V_L as well as the conformation of at least one of the CDRs – may happen upon complex formation with the antigen.

For quite some time it was assumed that this intricate mechanism is a prerequisite for the successful generation of high affinity antibodies by the immune system, until the discovery of 'heavy chain only' antibodies in camels – followed by other species as mentioned above – revealed that similar affinities and specificities may also be realized with a simpler protein architecture. In fact, single domain

antibodies merely utilize three hypervariable loops, have fewer capabilities for induced fit, and they provide just about half the interface area for complex formation [8].

Not unexpectedly, alternative binding proteins even further deviate from the mode of antigen recognition seen for antibodies. Naturally, those protein scaffolds that are based on a β-sandwich and thus share a certain structural homology with the Igs, for example the Monobodies/Adnectins, show a binding mode most similar to the single Ig domains. Like binding proteins derived from the Kunitz scaffold, such wedge-shaped proteins, with the randomized loops gathering at the tip, tend to preferentially bind targets with concave surfaces such as clefts for enzyme substrates or pockets for natural ligands, as exemplified with *E. coli* maltose binding protein (MBP) [30], lysozyme [31], and others [32]. In these cases target recognition may benefit from variegation of loop length [33•] and also from introduction of inter-loop disulphide bridges to effect conformational rigidification [34].

Interestingly, however, in a yet unpublished structural study (PDB entry 2OCF; cf. [35]), one Monobody shows an unusual mode of binding in so far as the interface with the antigen (human estrogen receptor α ligand-binding domain [36]) is mainly formed by the surface of one of its rigid β-sheets (corresponding to the interface of an Ig domain when paired with the second variable domain), together with the loop corresponding to CDR-3, which in this case adopts two turns of an α-helix.

Recently, the use of combinatorial libraries with a restricted amino acid code and therefore much reduced diversity was applied to the ^{10}Fn3 scaffold. Originally proposed for synthetic libraries of Ig fragments, this approach exclusively allows the introduction of Ser and Tyr residues into the three randomized CDR-like loops [37•], resulting in Monobodies with affinity for MBP in the lower nanomolar range. One of them was shown to bind to the same pocket as β-cyclodextrin, whereby this natural substrate was mimicked by a cluster of Tyr side-chains. An increase in achievable affinity was observed when expanding the YS-code to an YSX-code (X is a mixture of A, L, R, H, D, N and G [30]). It will be interesting to see whether this or other minimalist approaches may be useful for a broader range of targets, especially in the light of a recent theoretical study suggesting that sparse sampling of a library based on a larger amino acid alphabet should promise higher success [38].

In contrast to these scaffolds, Anticalins are not homologous to the Ig superfamily. Nevertheless, they show a structural analogy in so far as a circularly closed β-sheet (the β-barrel) forms a highly conserved core unit, which supports a set of four loops showing hypervariability throughout the lipocalin protein family in terms of length, backbone conformation as well as side-chain composition [10,19]. On the basis of X-ray structural information available for Anticalins with engineered specificities for several haptens (e.g. fluorescein, digoxigenin, Y^{III}·DTPA) it appears that, while the loop conformations strongly respond to side-chain replacements, this scaffold can in some cases recognize the small molecule (e.g. digoxigenin or Y^{III}·DTPA [39•]) almost in a lock and key fashion, that is without extensive adaptation during complex formation. In contrast, a recent NMR study of a fluorescein-specific Anticalin clearly demonstrated rigidification of the loop region and also part of the β-barrel upon ligand binding [40].

A different picture appeared when the crystal structure of an Anticalin with high affinity (down to 240 pM) for a protein target, the extracellular domain of CTLA-4, was recently solved [41•]. This Anticalin, which was selected from a random library based on human Lcn2 (also known as NGAL), binds the protruding FG-loop of CTLA-4 – which otherwise interacts with the counter-receptors B7.1/2 – at the center of its calyx-like cavity, exhibiting tight structural complementarity and an interface area similar to antibodies. However, when the structure of this complex was compared with the one solved for the Anticalin in absence of its target, a pronounced induced fit became obvious for three of the four variable loops. Thus, engineered lipocalins show two features that so far have been considered typical for the binding sites of antibodies: (i) high structural plasticity as a consequence of sequence variation and (ii) elevated conformational flexibility, allowing induced fit to targets with differing shape and size.

Structural adaptation should play a lesser role for those protein scaffolds whose binding sites are built upon a rigid, regular secondary structural motif, as it is realized both with the purely α-helical Affibodies and with the ankyrin repeat proteins, which comprise several homologous segments of a β-turn and two anti-parallel α-helices. The DARPins usually carry three repeats corresponding to an artificial consensus sequence, whereby six positions per repeat are randomized. Consequently, DARPins lack structural flexibility and they have been proposed as reagents to facilitate the crystallization of other proteins after complex formation [42]. Indeed, several such co-crystal structures have been solved, for example with the human polo-like kinase 1 (Plk-1) [43] and the drug export efflux pump AcrB [44], a membrane protein of *E. coli*. All these proteins were also successfully crystallized in the absence of a DARPin and it is evident that most of the complexes obey a kind of lock and key mechanism, whereby observable structural variation always appears lower on the DARPin than on the target side [45]. In fact, in the example of the bacterial aminoglycoside 3′-phosphotransferase IIIa (APH) the DARPin pulls out an α-helix from APH, which is accompanied by a loss of

enzymatic activity, thus illustrating a kind of 'enforced' fit on the side of the target protein [46].

Affibodies are obtained by mutagenizing 13 exposed amino acid positions on the surface of two of the three α-helices in the Z-domain of protein A, again without involving flexible loop segments. So far, structural information is mainly available for Affibodies in complex with other Affibodies (in a kind of anti-idiotypic interaction). Z_{Taq}, originally selected against *Taq* DNA polymerase, was subjected to a solution NMR analysis in complex with anti-Z_{Taq}, an Affibody selected against it [47]. Both in their free states as well as in the mutual complex, Z_{Taq} and anti-Z_{Taq} form a three-helix bundle similar to the underlying Z-domain. Only minor differences in the orientation of the first helix and the side-chain conformations of three aromatic residues were seen upon complex formation, indicating that this scaffold possesses a rigid supersecondary structure. However, another extreme was observed in a similar anti-idiotypic pair comprising Z_{SPA-1}, an Affibody that had been selected against protein A and recognizes its own ancestor, the Z-domain [48]. In this case, uncomplexed Z_{SPA-1} behaved as an aggregation-prone molten globule, whose folding occurred upon binding to the Z-domain. The original three-helix bundle structure was fully regained in the resulting complex, thus providing an extreme example of 'induced fit', albeit actually leading to the original wild-type conformation.

Further beyond that, an event of 'neofolding' was observed for an Affibody that had been selected to bind the human amyloid-β peptide [49]. Surprisingly, the NMR structure of the complex showed the β-hairpin of the monomeric peptide embedded in a groove formed by a pair of Affibody proteins that were covalently linked via a disulphide bridge [50•]. Notably, the three-helix bundle was not preserved in this case. Instead, randomization of surface residues of the first helix resulted for each $Z_{a\beta3}$ in the formation of a short β-strand flanking the Aβ hairpin, leading to an intermolecular four-stranded antiparallel β-sheet. A kinetic and thermodynamic analysis of this system indicated that both Aβ and $Z_{a\beta3}$ fold upon binding, whereby a high-energy barrier is associated with the conversion of an initial $Z_{a\beta3}$:$A\beta_{1-40}$ recognition complex into the native assembly [51].

Targets and medical mode of action

In analogy to antibodies, targets of engineered binding proteins can be generally classified into (i) antigens, that is usually proteins, and (ii) haptens, that is (bio)chemical compounds of low molecular weight including small peptides or peptidomimetics. Up to now, most of the engineered protein scaffolds were directed against protein targets. One reason is their relevance as disease-related biomolecules and the second is that most of the successful non-Ig scaffolds provide extended interfaces for the recognition of macromolecular structures. Interestingly, other classes of biomacromolecules such as carbohydrates and nucleic acids, which also are inefficient antigens for the immune system, have so far not been in the focus of protein scaffold development either.

As for antibodies and their fragments, therapeutically useful targets for alternative binding proteins must be accessible from outside a cell via circulation and diffusion through the interstitial fluids. Therefore, almost all targets of protein scaffolds – including the single domain Ig fragments – in late preclinical or clinical development constitute either disease-related soluble protein factors or extracellular regions of membrane receptors (for detailed review see [23,24]). Recent examples for the first sort of targets are IL-6 [21], VEGF [52], plasmin [53], and kallikrein [54•], while those for the second class are VEGFR-2 [55•], HER2 [56•,57•], EGFR [58•], EDB [25], CD4 [59•], CTLA-4 [41•], IL-2Rα [60], $\alpha_v\beta_3$ integrin [61,62], and gp120 [63].

It appears that the overall number of these targets is rather small and cognate therapeutic antibodies are often already known, sometimes the same target is even addressed by several different scaffolds. This can be explained by the fact that the costly and risky Endeavour of developing a new type of therapeutic drug is only justified if (i) the disease-related target is already clinically validated and (ii) a rewarding market potential can be foreseen. Conversely, from the patients' perspective this situation may provide an advantage because alternative drugs with similar activities might be available in case an adverse reaction occurs.

However, with increasing progress in alternative protein scaffold technology and growing confidence after successful completion of the first couple of clinical trials this reservation will probably be overcome. In fact, although presumably not as huge as in the case of small molecule pharmaceuticals – with their many enzymes and ligand-dependent receptors as targets – the spectrum of excreted and cell surface proteins that constitute viable targets of non-Ig binding proteins should actually be much larger than the close to 100 targets that are addressed by approved biopharmaceuticals to date [2]. Many more 'druggable' targets are likely to be identified by means of modern proteome analysis [64]. In addition, the application of antibody (and probably soon also scaffold) selection technology itself will increasingly aid in the discovery of novel targets, in particular those overexpressed on malignant tissues [65].

Since non-Ig binding proteins – as well as the single domain Ig fragments – lack the immunological effector functions residing in the Fc region, corresponding biopharmaceutical reagents cannot rely on the ADCC and CDC mechanisms characteristic for the humoral immune

response. On the contrary, the attractiveness of engineered protein scaffolds as an alternative class of biological drugs largely arises from their distinct mechanisms of action, which are often made possible by their small size, monomeric nature, simple and robust fold as well as their accessibility from microbial biosynthesis, both as isolated entities and as part of multifunctional fusion proteins.

The simplest therapeutic mechanism is the action as an inhibitor or antagonist. Efficient enzyme inhibitors, especially for extracellular proteases, have been engineered on the basis of the Kunitz type scaffold, for example DX-88 (Ecallantide, Dyax) directed against kallikrein, which is subject to advanced clinical studies [54•]. Ecallantide has orphan drug designation for the treatment of hereditary angioedema (HAE). Treatment with DX-88 compensates insufficient amounts of the natural kallikrein inhibitor C1 and may replace conventional therapy with C1 isolated from human plasma. Furthermore, DX-88 is tested in conjunction with open heart surgery. In a similar approach DX-890 (EPI-hNE4) acts as a proteolysis-resistant inhibitor of human neutrophil elastase and is under consideration as anti-inflammatory drug for treating cystic fibrosis [66] while the plasmin inhibitor DX-1000 is in preclinical development for blocking breast cancer growth and metastasis [53]. Contrasting, in the case of intracellular enzymes DARPins have yielded viable inhibitors [43,67], even though their mechanism is more owing to peculiar conformational effects than to sterical blocking of the active site.

Antagonists directed against cellular receptors or soluble growth factors were successfully obtained from various protein scaffolds. The first Adnectin CT-*322* (Angiocept; Adnexus/Bristol-Myers Squibb) tested in humans to inhibit tumor angiogenesis is an antagonist of VEGFR-*2* [68]. After efficacy of CT-*322* was demonstrated *in vivo* using orthotopic pancreatic tumor models [55•], safety and pharmacological relevance were investigated in a phase I study [69••]. Stable disease in nearly half of the treated patients was achieved, however, also a dose-dependent toxicity was observed. A concomitant increase in the free receptor ligand VEGF-A was interpreted as indication of biological activity. Angiocept is about to enter phase II clinical trials with patients suffering from gliobastoma multiforme.

Another example for an antagonist that has been considered for clinical development is an Avimer that binds and neutralizes interleukin-6 (AMG-*220*; Amgen), an important pro-inflammatory cytokine [21]. AMG-*220* has been studied in Crohn's disease patients. An Anticalin that binds and neutralizes human and murine VEGF with picomolar affinity [52] is currently under preclinical development as inhibitor of tumor angiogenesis (PRS-050; Pieris). Another Anticalin (PRS-010) was engineered to tightly bind and block CTLA-4, a receptor with attenuating function expressed on activated T cells [41•]. This Anticalin stimulates the cellular immune response, which has been demonstrated in a mouse model of *Leishmania* infection, and also offers potential for immunotherapy of cancer.

Compared with antibodies, alternative binding proteins usually have the advantage of not being bivalent – which might lead to receptor activation via cell surface clustering – and of not forming immune complexes or otherwise eliciting an immunological response. Thus, corresponding applications are mainly in the fields of autoimmune diseases, hormone dysfunctions, and neoangiogenic pathologies. In the area of oncology, however, normally the goal is to specifically destroy a malignant tissue. To this end, approaches for 'arming' are needed, similar to those that are being discussed for antibodies in order to complement or supersede their natural effector functions [70].

Owing to their generally advantageous properties, most alternative protein scaffolds are particularly suited as tissue-targeting vehicles, by addressing toxic molecules, radioisotopes, cytokines or enzymes to disease-related cell surface receptors. So far, only a few attempts have been made in this direction, for example with an Affibody labeled with ^{177}Lu (via a conjugated DTPA group) for radioimmuno therapy (RIT) [71], but it is likely that this approach will quickly be expanded once the non-Ig binding proteins have been better accepted as a novel class of biological drugs.

In vivo imaging or radioimmuno diagnostics (RID) is a quickly growing area of medical application that is mechanistically related to protein-mediated drug targeting in cancer therapy. In both cases, a compound must be specifically addressed to a tissue and, in fact, when exchanging corresponding radionuclides the same protein reagent can serve both purposes. The HER2 specific Affibody ABY-025 (Affibody Technology) is currently under clinical investigation for *in vivo* imaging [72] and has shown benefits with respect to fast renal clearance and elevated tumor to blood ratio, as demonstrated for mice xenocrafts. Notably, the HER2-specific Affibody binds to an epitope that is not the target for trastuzumab (Herceptin; Genentech) and hence should also enable monitoring of receptor expression following tumor treatment with this therapeutic antibody [73].

Non-Ig binding proteins are particularly promising for *in vivo* imaging because their smaller size offers the advantages of better tissue penetration, lack of Fc-mediated non-specific adhesion and, most importantly, much faster excretion via the kidney, which leads to improved target/blood ratios and thus better contrast of tumor staining. An Affibody specific for EGFR is another reagent with

potential for *in vivo* diagnostics [74]. Apart from Affibodies, DARPins are currently under preclinical development for RID (for a topical review of see [24]).

For these applications protein scaffolds have to be covalently linked with chelating agents such as DTPA and DOTA, followed by charging with the radionuclide metal ion under rather harsh conditions (high temperature and acid pH, in particular for DOTA). Although the robust nature of many alternative binding proteins provides a benefit in this respect, the labeling chemistry often is incomplete, even for optimized Affibodies [75]. An alternative solution is offered by a recently developed Anticalin with picomolar affinity for DTPA-chelated lanthanides, especially Y^{III} [39•]. This Anticalin forms a tight non-covalent complex (with slow dissociation kinetics) under physiological conditions in the presence of the chelated metal ion and, after fusion with an appropriate targeting domain, it may provide an ideal tool for applications in 'pretargeting' RIT.

Notably, the only established non-Ig scaffold that intrinsically provides pockets and thus allows tight and specific complexation of small molecules is the one of the lipocalins. Anticalins with down to picomolar binding activities have been described for several haptens with therapeutic potential. For example, an Anticalin directed against digitalis shows prospects for the treatment of drug overdosing in cardiac therapy [52]. Thus, engineered lipocalins may provide a novel class of antidotes that can quickly scavenge toxic or otherwise irritating compounds from the body, for example siderophores that are produced in the course of a bacterial infection, similar to the natural function of human Lcn2 [39•].

Clinical aspects: delivery, half-life, and immunogenicity

The general experience with alternative scaffolds and also with small antibody fragments from the past few years has shown that one of the most crucial aspects for successful application *in vivo* is the affinity for the target. Owing to their monovalent nature, non-Ig binding proteins – as well as the single Ig domains (see e.g. [76]) – have a certain disadvantage compared with antibodies, whose antigen-binding activity is often boosted by an avidity effect. Attempts to mimic this physicochemical phenomenon have been made with the Avimers, where three individually engineered binding modules, which form part of the same polypeptide chain, bind to different epitopes on IL-6 [21]. Similarly, multivalent versions of Affibodies have been tested to bind with high avidity to HER2, which is expressed in multiple copies on the tumor cell surface [77]. In another approach an $\alpha_v\beta_3$-specific Monobody was fused with a pentamerization domain, which led to tighter binding with significantly slower off-rate when tested on purified integrins and on integrin-expressing cell lines [62].

To achieve similar efficacy with a truly monomeric protein scaffold, often target affinities in the picomolar range are needed. *In vivo* imaging experiments, which provide a particularly sensitive test system, have given clear indication that this high affinity range is essential for satisfactory tumor accumulation, as exemplified with an Affibody [78] and a DARPin (cf. [24]), both directed against HER2. However, engineering of high affinity is complicated by the fact that the interaction with a target *in vitro*, for example with a recombinant receptor domain used for ELISA or Biacore measurements, often appears better than in cell culture with the native membrane receptor or *in vivo*. Sterical accessibility on the cellular membrane, different glycosylation, and the presence of the glycocalyx as well as extracellular matrix have to be considered in this respect. Thus, ensuring the native structure and functional presentation of the target protein, which is usually obtained by recombinant DNA technology, during combinatorial selection of the binding protein is a crucial aspect. In addition, it must be kept in mind that all chemical modifications to the engineered binding protein, such as conjugation with chelator groups or with PEG (see below), can potentially hamper affinity.

The route of administration is another important factor for therapeutic application, especially with regard to patient compliance. Compared with small molecule drugs, biopharmaceuticals suffer from the general disadvantage that they must be injected or infused for systemic action. However, owing to their high specific activity compared with antibodies (resulting from the much lower molecular weight) and to their often remarkably robust nature, engineered protein scaffolds may also be amenable to alternative routes of administration, such as pulmonary delivery, in particular, if merely local activity is needed. Microproteins, which are based on the knottin scaffold, may even be stable enough to survive oral application [22], similarly as llama Nanobodies, both of which are crosslinked by several disulphide bonds [79].

A crucial parameter for the frequency of dosing is the plasma half-life of the engineered binding protein. Intact antibodies – and, similarly, human serum albumin (HSA) – exhibit extended circulation owing to their large size, which slows down kidney filtration, and to a peculiar endosomal recycling mechanism in the vascular endothelium and other tissues. As for most classical biopharmaceuticals (such as recombinant interferons and growth factors), the small size of alternative protein scaffolds, which is significantly below the renal threshold, typically leads to a much shorter circulation half-life in the range of minutes to hours. To allow dosing intervals of several days in humans, which are mandatory for therapy without hospitalization, several strategies are currently available (for review see [80]).

One approach is to fuse the engineered protein scaffold with another domain that has binding activity for HSA. For example, a human single Ig domain (dubbed Alb-Dab) was selected against HSA and subsequently used for fusion with the interleukin-1 receptor antagonist as a therapeutically active component, leading to prolongation of plasma half-life from a few min to around 4 h in mice [81]. Similarly, Nanobodies with specificity for EGFR were fused to an HSA-binding second Nanobody [76]. With the use of a naturally occurring albumin binding domain (ABD) derived from *streptococcal* protein G as fusion partner, a fluorescence-labeled Affibody specific for HER2 showed an extended half-life and improved tumor accumulation in mice, which was crucial for *in vivo* imaging [73] and, similarly, for therapeutic effect in an animal model [71]. The naturally moderate affinity of this ABD, which possesses a three-helix bundle structure closely related to the Z-domain scaffold of the Affibodies, was recently improved to the femtomolar range in a combinatorial protein engineering effort [82]. Owing to the bacterial origin of this protein, its suitability for the treatment of humans will have to be seen. A similar strategy for half-life extension was followed during the design of the IL-6 specific Avimer with the incorporation of a domain that binds to IgG [21].

Another established technique to retard rapid kidney clearance is the covalent attachment of polyethylene glycol (PEG) to biopharmaceuticals, taking advantage of the dramatic increase in molecular volume due to the expanded random conformation of the solvated chemical polymer. However, there are still challenges with respect to the high cost of activated PEG derivatives, low yield of *in vitro* coupling, additional down stream processing, heterogeneity of the final product, and tissue accumulation as a consequence of non-biodegradability [83]. Nevertheless, PEGylation of the Adnectin CT-322 was successfully employed to improve the pharmacokinetics of this small protein scaffold to meet clinical needs [84]. Also, the engineered Kunitz-type inhibitor DX-1000 is being developed as a PEG conjugate [53]. A beneficial alternative to chemical PEGylation could be the preparation of recombinant fusion proteins with biologically inert and intrinsically unstructured amino acid polymer sequences, which can adopt an expanded random chain conformation, too [80,85,86].

A still open question in the field of alternative protein scaffolds relates to their *in vivo* immunogenicity profile. In principle, any protein that enters the human body can elicit anti-drug antibodies (ADAs), which interfere with or neutralize the effect of the biopharmaceutical, especially if repeatedly administered [87,88]. This is even true for classical human therapeutic proteins such as insulin or erythropoietin. Meanwhile, it is known that protein aggregates and the presence of adjuvant substances raise the risk of immunogenicity, but these factors can be well controlled with the current methodology of biotechnological production and downstream processing. Unfortunately, intrinsic factors that contribute to immunogenicity are still poorly understood, as well as mechanisms to induce specific tolerance, and probably both depend on individual factors, given the genetic diversity of the HLA and also, to some extent, of the Ig locus. Furthermore, there are no valid *in vitro* tools available, and even regulatory authorities admit that 'the predictivity of animal models for evaluation of immunogenicity is considered low' [89].

For a long time it was assumed that the biochemical composition of a biopharmaceutical should be as close as possible to the endogenous counterpart, a concept that provided the theoretical basis for the 'humanization' of antibodies. A more advanced strategy that finds increasing acceptance is the 'deimmunization' of recombinant proteins via identification and elimination (using site-directed sequence variation) of potential T helper cell epitopes [87,90]. Today, however, there is growing concern (see the EMEA document quoted above) that biotechnological analogs to proteins of the human body may trigger an immune response that eventually may result in a loss of function for the natural biomolecule.

Considering those non-Ig binding proteins that are currently approaching the clinic, we may conclude that Adnectins, Anticalins, and the like, which are derived from human protein scaffolds, should be less immunogenic, while immune tolerance cannot be guaranteed and further assessment will have to await data collected from human patients. Conversely, protein scaffolds with a bacterial origin (e.g. Affibodies) are more likely to be immunogenic, whereas proteins with a designed background (e.g. DARPins) are difficult to predict at all.

Anticalins may provide a good compromise between the aspects mentioned above because the 10–12 lipocalins that are found in humans seem to exert partially redundant functions [91] and severe deficiencies arising from a functional loss are not to be expected [92]. Notably, lipocalins that occur in the saliva of blood-feeding ticks, which have undergone selection for low immunogenicity during host/parasite evolution, may provide suitable scaffolds, as well. At least two such natural lipocalins, one with histamine-scavenging function and another one inhibiting the complement component 5 (rEV131 and rEV576, respectively; Evolutec) have been subject to preclinical and clinical development [93,94].

Conclusions and outlook

The engineering and practical use of binding proteins derived from non-Ig scaffolds is an established methodology today that is going to boost biological chemistry both toward basic research and applied science. In contrast, antibody technology is about to reach its peak in the

biomedical area, with several hundred drug candidates directed against a broad range of targets currently awaiting clinical study and, finally, market approval. Consequently, under the pressure of ongoing innovation there is a need and also plenty of opportunity for alternative reagents. Yet, the true potential of the various protein scaffolds for human therapy – including *in vivo* diagnostics – also considering the different approaches to implement effector functions, will only become clear once a number of additional phase I/II trials will have been completed in the near future.

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