# Chapter 7
# Alternative Scaffolds as Bispecific Antibody Mimetics

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## 7.1 Introduction

Alternative scaffold proteins are a class of antibody mimetics that share a number of essential properties with antibodies (Gebauer and Skerra 2009; Nygren and Skerra 2004), the most important being the ability to recognize molecular structures with high affinity and selectivity. In addition, alternative scaffolds often have improved properties in terms of stability, efficient recombinant production features, reduced size, few or no cysteines and an uncomplicated IP-situation. Due to their small size and ease of recombinant manipulation, they are well suited for generation of bispecific proteins. In fact, a number of alternative scaffolds are derived from natural precursors that are formatted as repeated domains, suggesting ability for function in a multimeric format, e.g., Affibody molecules (Löfblom et al. 2010; Nord et al. 1997), Adnectins (Koide et al. 1998), DARPins (Binz et al. 2004; Stumpp et al. 2008), and Avimers (Silverman et al. 2005).

## 7.2 Alternative Scaffolds as Antibody Mimetics

In combinatorial protein engineering, the term scaffold commonly defines a stable protein framework, harboring flexible loops or solvent accessible residues, which are randomized in order to construct a molecular library. Antibodies were the first,

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and are today still the most widely used, biomolecular scaffold for generation of affinity reagents. However, specific molecular recognition is not a unique property of immunoglobulins, and challenges associated with the antibody format have inspired researchers to investigate the possibilities of engineering specific affinity into other protein structures. In addition, in vitro display technologies (e.g., phage display) used for recombinant antibody generation, are readily modified for isolation of affinity proteins from non-Ig-based libraries, making isolation and identification of alternative affinity proteins relatively easy.

Alternative scaffolds can be designed to overcome several of the limitations associated with antibodies. The scaffold should preferably be small and composed of a single polypeptide chain in a fold that has an intrinsic biochemical and biophysical stability independent on post-translational modifications such as glycosylations. The small size and low complexity facilitates production using efficient prokaryotic systems and even chemical synthesis for the smallest scaffolds. To enable use in intracellular milieus and improve recombinant expression flexibility, the stability of the fold should if possible not be dependent on disulfide bonds. In addition, absence of natural cysteines allows for engineering of a cysteine into a suitable position for directed chemical modifications. It is also important that the protein scaffold demonstrates a high tolerance to randomizations in order to maintain high functionality of the resulting combinatorial library.

Non-antibody scaffolds can be classified in several different ways, e.g., based on origin, based on secondary structure composition, based on size, etc. One commonly used classification system is based on the site of diversification. The different types of scaffolds can be roughly divided into classes with a stable framework with one or several randomized loops (the immunoglobulin fold belongs to this class) and scaffolds in which randomizations are directed against surface-exposed residues on secondary structure elements. The antibody fold, with diversified loops displayed on a rigid framework, has been successfully employed for evolution of molecular recognition both in nature and in vitro. Several different alternative scaffolds are logically hence mimicking this molecular architecture. Diversifying loops generally require less information about the overall structure and the structure integrity and functionality might be less affected on average by the mutations. In addition, randomizing loops might result in additional diversity through the flexibility in the loops, i.e., one clone can adopt several structural forms and recognize several distinct target molecules. This may impact the specificity, something that has been demonstrated for antibodies where unmatured IgGs with relatively more flexible loops generally are more unspecific (James et al. 2003; Mouquet et al. 2010). In addition to scaffolds mimicking the antibody architecture, approaches in which randomizations are focused directly on secondary structure elements have also been described. Compared to flexible loops in antibodies and similar scaffolds, secondary structure surfaces are typically much more constrained, which might lead to less entropy losses upon binding and potentially a higher specificity.

A few examples of scaffolds from each group are listed in Table 7.1 and a more detailed description of the scaffolds that have been reported as bispecific agents is provided in the following section and in Table 7.2. Alternative scaffolds in general

**Table 7.1** Examples of non-Ig-based alternative protein scaffolds

| Name | Scaffold | Length (aa) | Disulfide stabilized | Bispecifics reported in literature | References | Commercialization |
|---|---|---|---|---|---|---|
| *Randomizations on secondary structure elements* | | | | | | |
| Affibody molecules | Protein A domain | 58 | No | Yes | Löfblom et al. (2010) | http://www.affibody.se |
| Affilins | γB-crystallin/ubiquitin | 198 | No | No | Ebersbach et al. (2007) | http://www.scilproteins.com |
| DARPins | Ankyrin repeat protein | ~133–199 | No | Yes | Stumpp et al. (2008) | http://www.molecularpartners.com |
| *Randomizations on loops* | | | | | | |
| Adnectins/monobodies | $^{10}$Fn3 | 94 | No | Yes | Koide et al. (1998) | http://www.adnexustx.com |
| Anticalins | Lipocalin | 170 | Yes | Yes | Skerra (2008) | http://www.pieris-ag.com |
| Avimers | A domain | ~105 | Yes | Yes | Silverman et al. (2005) | http://www.amgen.com |
| Fynomers | SH3 domain | 63 | No | No | Bertschinger et al. (2007) | http://www.covagen.com |
| Knottins/microproteins | Cysteine knot peptides | 30 | Yes | No | Kolmar (2009) | http://www.amunix.com |
| Kunitz domains | Tissue factor pathway inhibitor | 58 | Yes | No | Lehmann (2008) | http://www.dyax.com |

**Table 7.2** Overview of bispecific alternative scaffolds and selected results at different stages of development

| Scaffold | Target 1 | Target 2 | Biological mechanism | Biochemical data | Cell results | In vivo results | References |
|---|---|---|---|---|---|---|---|
| ABD | Albumin | TNF, Z-domain | Increased plasma half-life of binding function | Affinity capture on target column. High affinity binding against dual targets | – | – | Alm et al. (2010) Hober (personal communication) |
| Adnectins | EGFR | IGFR | Dual receptor blocking | $K_D$ EGFR: 7.7 nM $K_D$ IGFR: 0.4 nM Tm 57.5°C | Inhibition of pAKT. Degradation of EGFR and IGFR in cells. | Tumor xenograft growth retardation | Emanuel et al. (2011)[a] |
| Affibody molecules | EGFR | HER2 | Increased targeting selectivity | Simultaneous binding shown in SPR | Stronger binding to cells co-expressing targets as well as cell-cell ligation shown | – | Friedman et al. (2009) Wållberg et al. (2010)[a] |
| Affibody molecules-ABD | HER2 | Serum albumin | Increased plasma half life of targeting molecule | $K_D$ HER2 = 0.02 nM $K_D$ Albumin = 8 nM | Binding and retention on cells verified | 50-fold increased $t_{1/2}$ in mice (36 h). Targeted tumor radiotherapy | Tolmachev et al. (2007a, b) |
| Anticalins | Digoxin | Fluorescein | Dual hapten capture | Simultaneous binding shown in sandwich ELISA | – | – | Schlehuber and Skerra (2001) |
| Avimer | IL-6, BAFF, CD40L | IgG | Increased plasma half life of ligand blocking molecule | $K_D$ IL-6 < 0.2 nM | Inhibits IL6-stimulated TF-1 cell proliferation | Extended half life in monkeys (89 ± 15 h) | Silverman et al. (2005) |
| DARPins | FcεRI epitope "85" | FcεRI epitope "30" | More efficient receptor blocking | $K_D$ FcεRI = 0.07 nM Do not aggregate the receptor | Inhibition of degranulation in rat basophilic cells | – | Eggel et al. (2009) |
| VEGF | VEGFR-2 | avβ3 | Dual receptor blocking | Simultaneous binding of both receptors in SPR experiment | Inhibition of VEGF mediated phosphorylation and cell proliferation | – | Cochran (Personal communication) |

[a]Poster abstract

have been extensively reviewed elsewhere, where more comprehensive listings are found (Gebauer and Skerra 2009; Nygren and Skerra 2004). Below, we will focus on the relatively few cases described where alternative scaffolds have been used in bispecific formats to enhance the property of the resulting molecule.

## 7.3 Bispecific Antibody Mimetics

The concept of bispecific antibodies was postulated already in the beginning of the 1980s (Milstein and Cuello 1983), but the multi-chain structure of antibodies resulted in sample heterogeneity that hampered development of clinical products. Even though elaborate strategies for constructing defined homogenous antibody-based bispecific agents now exist, the task is still challenging.

Alternative scaffolds, however, are generally comprised of a short and single peptide chain, and can therefore be readily adapted into bispecific and multispecific formats. The possibility of facile construction of multispecific alternative scaffolds might potentially become a differentiating factor compared to traditional antibodies in the future. Applications of bispecific alternative scaffold proteins include increased binding selectivity, enhanced receptor blocking capacity, provision of one biomolecule that can block two different receptors and development toward recruitment of effector functions. Another general application for bispecific affinity molecules is to have one binding partner for prolonging serum half-life of the molecule, while the other partner is having a biological activity.

### *7.3.1 Bispecific Antibody Mimetics with Enhanced Biological Function*

Biological function can be enhanced in many different ways. One example is to increase the selectivity of a targeting moiety by the simultaneous targeting of two receptors using fine-tuned affinities. The idea is that targeted cells, expressing both receptors, will be recognized with higher functional affinity due to the avidity effect compared to normal cells expressing only one of the receptors, hence improving the selectivity. Another strategy is to block two receptors involved in the same signaling pathway. Here, simultaneous binding is not a requirement, as long as both receptors are blocked by either part of the bispecific molecule. The main advantage is instead two functions in one molecule, facilitating preclinical development, clinical practice, and potentially lowering treatment costs if blocking of two pathways is needed for full treatment effect. Yet another approach is to use bispecific molecules to increase the functional affinity and ability to block one single receptor. Here it is important to have molecules binding to epitopes adjacent to each other for a favorable effect. Another example is bispecific molecules where one moiety is

used to attract an effector function. Below, case studies using Adnectins, Affibody molecules, Anticalins, and DARPins are highlighted.

#### 7.3.1.1 Adnectins

An example of an immunoglobulin-like alternative scaffold is the tenth human fibronectin type III domain ($^{10}$Fn3, also called monobody or Adnectin) (Koide et al. 1998). $^{10}$Fn3 is a monomeric, 94 residue protein, based on a stable β-sandwich fold with seven strands connected by six loops (Fig. 7.1a). Similar to antibodies and antibody derivatives, Adnectins interact with the target molecule through the loops on the tip of the molecule. However, in contrast to antibodies and most antibody derivatives, $^{10}$Fn3 is not dependent on disulfide bonds for stable structure. Using phage display (Koide et al. 1998), mRNA display (Xu et al. 2002), and yeast display (Lipovsek et al. 2007), specific $^{10}$Fn3 molecules have been selected against a number of different targets including, ubiquitin, TNF-alpha, lysozyme, and Abelson (Abl) kinase SH2 domain. One of the most advanced Adnectins binds with high affinity and specificity to VEGFR2. It is currently in phase II clinical trials in patients with solid tumors or non-Hodgkin's lymphoma (by former Adnexus, now part of Bristol-Myers

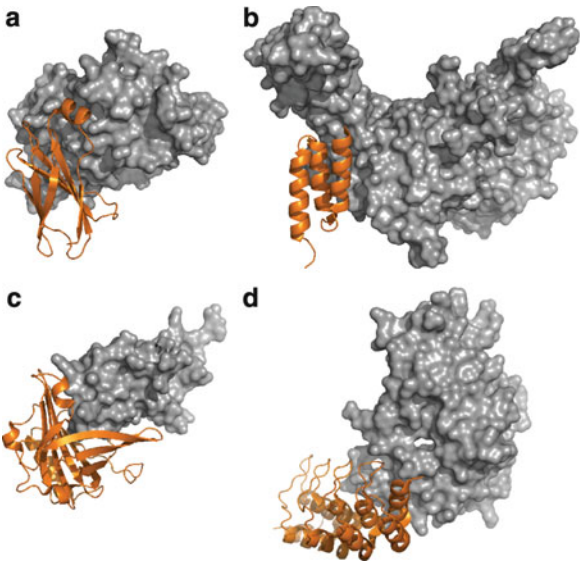


**Fig. 7.1** Structures of alternative scaffolds that have been reported as bispecific constructs with enhanced biological function. Scaffold proteins are visualized as *orange cartoon* representations and respective target as *gray surface* representation. (**a**) Engineered $^{10}$Fn3 in complex with human estrogen receptor alpha ligand-binding domain (PDB: 2OCF). (**b**) Affibody molecule in complex with HER2 extracelluar region (PDB: 3MZW). (**c**) Anticalin in complex with extracellular domain of human CTLA-4 (PDB: 3BX7). (**d**) DARPin in complex with aminoglycoside phosphotransferase (PDB: 2BKK)

Squibb). In addition to the commercial interest for this scaffold, the $^{10}$Fn3 domain has been studied extensively in academia. An example is the work by Koide and coworkers, in which $^{10}$Fn3 libraries have been constructed using a restricted diversity, in extreme cases using only serine and tyrosine in randomized positions (Koide et al. 2007), yielding high-affinity binders.

Recently, a bispecific Adnectin format targeting epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor 1 (IGFR) has been presented (Emanuel et al. 2011). The rational is that both EGFR and IGFR play important roles in tumorigenesis of several human cancers. Blocking of either receptor reduces tumor growth in mouse models and human clinical studies. However, blocking of one receptor can induce compensatory activation of the other to drive tumor growth as seen in preclinical models. It would therefore be attractive to develop one therapeutic molecule with the capacity to block both receptors. After several rounds of selection and optimization using mRNA-display and a cell-based receptor activation assay, Adnectins that could bind and block either the EGF- or IGF1-receptor were isolated. Next, several different EGFR-binders were fused to one IGFR-binder and all constructs were PEGylated, resulting in EI-tandem Adnectins. The best construct inhibited phosphorylation of either receptor equally well as the reference blocking antibody. There was also a synergistic inhibition on phosphorylation of AKT, which is a shared downstream signaling node. The bispecific Adnectin was furthermore shown to induce degradation of both the EGF- and IGF-receptor in cell cultures within 7 h after administration of the EI-tandem molecule. When tested in mice, the plasma half-life of the PEGylated EI-tandem was 19.5 h, suggesting suitable exposure of the drug in vivo. Indeed, plasma levels of the receptor ligands IGF1 and TGFα increased due to blockade of their receptors and the kinetics of the increase correlated with the pharmacokinetics of EI-tandem in plasma. When tested in EGFR-driven tumor-bearing mice, the EI-tandem Adnectin showed equal efficacy in tumor growth retardation as the registered anti-EGFR drug cetuximab, and in addition decreased the tumor growth rate of an IGFR-driven cetuximab-insensitive tumor. Although the molar ratios of EI-tandem were 10- to 20-fold higher compared to the antibody, this clearly shows that alternative scaffolds potently can inhibit a receptor–ligand interaction and the benefit of providing two receptor-blocking ligands in one molecule. It still remains to be seen if the receptor blocking is simultaneous or if the bispecific molecule uses either the EGFR blocking or the IGFR blocking capacity. Since the EGFR binding part of the EI-tandem does not compete with cetuximab or several other EGFR-specific antibodies, this drug could in principle be administered as an add-on therapy to existing α-EGFR-mab therapies.

#### 7.3.1.2 Affibody Molecules

Affibody molecules are based on small engineered three-helical proteins (Fig. 7.1b) (Löfblom et al. 2010). The basic scaffold was originally derived from the domain Z, a stabilized version of the B-domain of staphylococcal protein A, but has been

subjected to protein engineering to further improve its scaffold properties (Ahlgren et al. 2010; Feldwisch et al. 2010). The small size of the proteins allows for inexpensive production in prokaryotic hosts. However, due to the small size and rapid folding kinetics, most Affibody molecules are also efficiently produced by peptide synthesis, providing means for addition of non-biological groups in order to modify and enhance functionality (Engfeldt et al. 2005; Nord et al. 2001).

Diversity is created by randomization of 13 solvent accessible residues [nine of which also participate in the native interaction with Fc (Deisenhofer 1981)] in helix one and two of the Z domain in order to build a combinatorial Affibody library (Nord et al. 1995). In a pioneering article from 1997, Nygren and coworkers describe the isolation of several specific Affibody molecules from such a library displayed on phage (Nord et al. 1997). Additional Affibody molecules have later been selected against various targets, such as EGFR (Friedman et al. 2007), HER2 (Wikman et al. 2004), amyloid beta (Grönwall et al. 2007), HIV gp-120 (Wikman et al. 2006), and CD28 (Sandström et al. 2003) and detailed biophysical characterization of several isolated clones have demonstrated intact three-helical structure and typically maintained stability and folding properties compared to the parental Z domain (Dincbas-Renqvist et al. 2004; Dogan et al. 2006; Eigenbrot et al. 2010; Hogbom et al. 2003; Lendel et al. 2004, 2006; Wahlberg et al. 2003).

A high-affinity (20 pM) Affibody molecule (Eigenbrot et al. 2010; Orlova et al. 2006) directed against HER2, has been investigated thoroughly in several preclinical and pilot clinical studies and is an interesting candidate for development of diagnostic and perhaps even therapeutic agents (Engfeldt et al. 2007; Orlova et al. 2007a, b; Steffen et al. 2006; Tolmachev et al. 2006, 2007a, b). In one therapy study by Nilsson and coworkers (Tolmachev et al. 2007a, b), the HER2-specific Affibody binder was fused to another small and robust protein, an albumin binding domain (ABD), to increase the half-life and avoid uptake of the radiolabeled bispecific construct in the kidneys. The kidney had indeed a 25-fold decreased uptake, and in addition the total tumor uptake was increased by a factor 3. Another example of bispecific Affibody molecules is the engineering of HER2–EGFR-binding molecules to increase the selectivity in tumor targeting. Expression of both HER2 and EGFR has been reported in several studies of cancers of the bladder, colon, and prostate, and in several cases the prognosis is worse for patients with cancer that co-express the receptors, as opposed to tumors expressing either HER2 or EGFR (Osaki et al. 1992; Tsutsui et al. 2003). Therefore, Ståhl and colleagues set out to create a bispecific molecule that would bind better, or preferably only, at co-expression of the receptors on the cell surface. Such a binder could present a new tool for increased tumor selectivity in diagnostic and therapeutic tumor targeting. Binders for HER2 and EGFR were isolated and affinity maturated (Friedman et al. 2008; Orlova et al. 2006). In a subsequent study, they were dimerized and fused to each other via a $(G_4S)_3$ linker (Friedman et al. 2009). The binding ability of the bispecific construct was tested against the parental binders. Whereas the HER2-binding moiety showed no difference in binding, the C-terminal EGFR-binding moiety had a lower association rate than the parental binders. The dissociation rate was however comparable. In cell studies, fluorescently labeled

bispecific construct was shown to bind to both HER2-expressing SKOV-3 cells and to EGFR-expressing A431 cells. By using a device for real-time kinetics measurement of binding to live cells, it could be demonstrated that the bispecific HER2–EGFR-binder could mediate association of $^{14}$C-labeled A431 cells in solution to immobilized SKOV-3 cells, thereby suggesting ability to bind simultaneously with both moieties. Recently, a new set of bispecific constructs was presented, where Affibody molecules with different affinities to EGFR or HER2 have been combined to further investigate the possibility for synergistic and therefore more selective binding (Wållberg et al. 2010). In vitro, on a mixed Biacore surface containing both receptors, the apparent $K_D$ for the high-affinity bispecific construct was at least 30-fold higher compared to binding to surfaces containing only one of the two receptors. In real-time cell-binding experiments, using a lower affinity variant of the HER2-binding moiety, binding to HER2 high, EGFR low expressing SKBR-3 cells was blocked by addition of either monovalent HER2- or EGFR-binder. In addition, on A431 cells, the $K_D$ of the bispecific construct was increased 30-fold when the high-affinity HER2-binding variant ($K_D$ 22 pM) was used instead of the low affinity one ($K_D$ 50 nM), likely reflecting the very small but still present expression of HER2 molecules on the EGFR high expressing A431 cells (Kong et al. 2008). Although the results are promising in the in vitro setting, it still remains to be seen if an increased selectivity will indeed be possible to achieve also in vivo in suitable animal models.

#### 7.3.1.3 Anticalins

Anticalins are based on the protein architecture of natural lipocalins, demonstrating a rigid β-barrel fold displaying four loops targeted for diversification (Schlehuber and Skerra 2005; Skerra 2008) and specific binders have been isolated against both small molecules and proteins (Schlehuber and Skerra 2005; Skerra 2008) (Fig. 7.1c). Although structurally similar to antibodies in terms of displaying loops stabilized by a beta-sheet structure, Anticalins are smaller (around 180 amino acids), composed of only one polypeptide chain, are generally non-glycosylated and can be efficiently expressed in prokaryotic production systems. Natural lipocalins are generally secreted and often contain disulfide bridges. However, there are natural lipocalins lacking disulfide bonds and even examples where the disulfide bonds have been removed with retained functionality (Skerra 2008). Generally, the four loops of the lipocalin fold form a relatively deep pocket and specific Anticalins against small haptens and peptides have been successfully selected. However, as for antibodies, the loops demonstrate a relatively high flexibility, resulting in the ability to also interact with larger targets, such as proteins. An example is an anti-VEGF Anticalin with picomolar affinity to both the human and murine form of the growth factor. A pegylated variant (PRS-050) is currently in clinical trials for inhibition of angiogenesis in solid tumors by neutralization of VEGF (http://www.pieris-ag.com). The high stability of the lipocalin fold has motivated evaluation of Anticalins for local administration through alternative

routes, such as pulmonary delivery. An Anticalin, specific for IL-4 receptor alpha (IL-4Ra), is in preclinical development for severe asthma. IL-4Ra plays a central role in chronic inflammation through interaction with both IL-4 and IL-13 in the lungs and pulmonary delivery of the IL-4Ra-specific Anticalin offers an appealing alternative to invasive routes with potentially improved patient compliance as well as directed local administration to the inflamed tissue (http://www.pieris-ag.com). Another Anticalin has been generated against mesenchymal-epithelial transition factor (cMET), a tumor-associated growth factor receptor. An advantage of the Anticalin protein in this setting, compared to many antibodies targeting this receptor is the monovalent nature of Anticalins. Bivalent antibodies tend to crosslink the receptor, causing an undesired agonistic response, which is avoided with the monovalent Anticalin.

In a relatively early publication, a bispecific Anticalin was described and denoted "duocalin" (Schlehuber and Skerra 2001). Here, an anti-digoxin and an anti-fluorescein Anticalin were fused by a peptidic linker and shown to be functionally expressed in bacteria, although with a somewhat lower yield. The binders were shown to retain the binding activity of each molecule in a sandwich ELISA and in a fluorescence titration experiment, demonstrating that Anticalins in principle can be formatted for bispecific binding. This concept is currently exploited for several therapeutic applications (www.pieris-ag.com). In particular, it appears attractive for a bispecific pre-targeting approach using a recently described Me•DTPA-binding Anticalin (Kim et al. 2009) together with tumor targeting molecules, for example, by fusion to the cMet-binding Anticalin (Skerra personal communication). Here, the affinity matured Anticalin binding DTPA with 400 pM affinity could be targeted to cMet expressing tumors to yield high tumor uptake. After the non-bound fusion protein is cleared from the blood-stream, a DTPA-complexed radiometal such as $^{90}$Y or $^{177}$Lu could be administered, and would be trapped by the DTPA-binding Anticalin at the tumor site, thus resulting in high tumor to non-tumor ratio of therapeutic activity.

#### 7.3.1.4 DARPins

The DARPin scaffold is based on a class of proteins called ankyrin repeat proteins (Binz et al. 2004; Stumpp et al. 2008). Ankyrin repeat proteins are natural human proteins with a modular architecture, composed of repeats of a 33-residue domain. In protein engineering applications, the repeat approach has been kept and libraries are based on a protein composed of several randomized domains, hence denoted "designed ankyrin repeat proteins" (DARPins). Each domain is composed of a beta turn and two anti-parallel alpha helices. Two or three such domains generally constitute the binding surface with one capping domain on each side of the interacting domains, thus ranging in size from around 14 to 21 kDa (Fig. 7.1d). Randomizations are, as for Affibody molecules, to a large extent focused on secondary structure elements. Due to poor display level of DARPins on phages, specific binders were originally selected using ribosome display (Binz et al. 2004). However, in a recent study (Steiner et al. 2006), a modified phage display system was developed, in which

recombinant proteins were exported via the signal recognition particle (SRP) translocation pathway instead of the typical Sec pathway, increasing the display level of fast-folding proteins such as DARPins considerably. Specific DARPins have been selected to numerous different targets and the engineered variants typically display favorable properties, such as high stability, efficient production in microbial hosts as well as high affinity. The most advanced DARPin is currently in phase I clinical trials: An anti-Vascular Endothelial Growth Factor-A (VEGF-A) DARPin (MP01112) that is being developed as an anti-angiogenic compound for treatment of wet age-related macular degeneration and diabetic macular edema (www.molecularpartners.com).

An interesting bispecific application of DARPins was recently described by Eggel et al. (2009), addressing a shortcoming of antibodies, which is the difficulty to block a receptor without crosslinking it with another one, often activating the resulting receptor pair. Several attempts using antibodies to block the interaction of immunoglobulin E (IgE) with its high affinity receptor (FcεRI) have been made. Upon allergen-induced crosslinking of IgE, FcεRI is aggregated on sensitized mast cells or basophils, and proinflammatory mediators that induce allergy symptoms are released. Antibodies binding to FcεRI and blocking IgE-binding have been isolated, but due to their bivalent nature, they cross-link the receptor and activate cell degranulation, leading to anaphylactic symptoms. Thus, the antibody format is not suitable for blocking IgE-mediated allergic symptoms. Instead, the DARPin technology was explored for this purpose. Since two binding sites have been mapped to the extracellular part of FcεRI, it has been suggested that it is necessary to block both IgE-binding sites to obtain an efficient antagonist. Hence, Eggel and co-workers set out to explore if a bispecific combination of two DARPins binding to different sites of the FcεRI could constitute a potent inhibitor of allergic syndromes. First, a set of binders was generated against recombinant FcεRIα. After initial ELISA screening, two DARPin binders, B-A4-85 (85) and C-A3-30 (30), were selected and produced for further characterization. They were shown not to compete for the same binding surface on the IgE-receptor, and were therefore subsequently tested for binding in monovalent, bivalent or bispecific formats, including both N- or C-terminal position of the respective binder in the bispecific format. The highest affinity was obtained for the bispecific construct with the DARPin 30 in N-terminal position (30/85), suggesting that this orientation was favorable, in contrast to DARPin 85 at the N-terminus (85/30), where the affinity was no better than for either monomeric binder alone. In a competition study using surface plasmon resonance, the bispecific constructs or a mixture of the bivalent (monospecific) molecules inhibited binding of IgE to its receptor much better than either of the bivalent monospecific molecules, suggesting that blocking of both epitopes is necessary for efficient inhibition of the receptor. In an HPLC aggregation analysis, it was shown that bispecific binder 30/85 was the only one that did not induce aggregation, suggesting that this binder could bind the two adjacent epitopes on the same receptor domain simultaneously. Indeed, in a rat basophilic cell granulation assay measuring inhibition of mediator release, the DARPin 30/85 showed best effect, with an inhibitory effect similar to the registered IgE-blocking drug

Omalizumab. Finally, it was confirmed that the 30/85 DARPin did not trigger cell degranulation, in contrast to two receptor-binding antibodies or crosslinked IgE. This study clearly shows that alternative scaffolds can provide a viable and sometimes superior bispecific alternative compared to antibodies.

#### 7.3.1.5 Single-Domain Bispecific Protein Scaffolds

Most bispecific alternative scaffold molecules described to date are based on the use of several fused domains, each adding a binding specificity to the resulting fusion protein. A different approach is to engineer an additional binding specificity directly into a binding molecule. One example is the bispecific ABD molecule described below, taking advantage of the long half-life of ABD and adding onto it new specificities.

Another approach has been described by Cochran and co-workers at Stanford University, with their development of a bispecific agent antagonizing both the VEGFR-2 and αvβ3 integrin (Cochran, personal communication). Interaction between the VEGFR-2 and the αvβ3 integrin has been reported to increase VEGFR-2 phosphorylation and promote angiogenesis. In order to generate a molecule that would be able to target both of these receptors, a mutated single-chain format of VEGF (scVEGFmut), previously demonstrated to block VEGFR-2 signaling, was engineered for binding to αvβ3 integrin. A library of the mutated scVEGF protein was constructed by incorporating into one of the loops a peptide library containing the natural integrin binding RGD motif, flanked by randomized sequences. The library was displayed on yeast and integrin-binding variants were isolated using FACS. Several selected binders demonstrated retained affinity for VEGFR-2 as well as binding to αvβ3 integrin on cells, demonstrating a bispecific molecule. Using SPR technology and a sequential injection of the bispecific candidate followed by αvβ3 integrin over VEGFR-2 immobilized on the sensor chip surface, simultaneous binding to both receptors was verified. The bispecific variants were also able to inhibit VEGF-mediated VEGFR phosphorylation, cell proliferation as well as adhesion on vibronectin-coated surfaces.

In a relatively early study, Hufton et al. showed the suitability of the CTLA-4 protein for use as protein scaffold for engineering of new specificities (Hufton et al. 2000). As in the work described above, the authors first used the RGD motif flanked by a library of randomized amino acid residues to isolate the first binding specificity. They inserted the library in a CDR3 like loop on the CTLA-4 molecule, and then selected binders specific for the αvβ3 integrin. Using flow cytometry, it was showed that phage particles carrying the selected clone RGD-7B could bind to human umbilical vein endothelial cells. To investigate if the CTLA-4 protein scaffold could be developed toward bispecific applications, a short peptidic sequence was inserted in the loop formed by the A'B β-strands in the CTLA-4 framework opposite to the natural CDR-loops. Indeed, structural integrity was retained, and the CTLA-4 molecule could still bind its natural ligands B7-1 and B7-2, suggesting suitability for further development of bispecific ligands.

### 7.3.2 *Bispecific Antibody Mimetics with Enhanced Half-Life*

The small size of most alternative scaffolds results in good tissue penetration and rapid blood clearance, which in combination with strong and specific interaction enables high-contrast imaging of tumors for e.g., cancer diagnostics (Nilsson and Tolmachev 2007; Weissleder 2006) or therapeutic protocols where short exposure is key. In most therapeutic applications however, the short in vivo half-life may limit the therapeutic efficacy due to too low exposure, and if payloads are used, the high kidney uptake may result in toxic side effects. While a number of biologicals have been modified with PEG, or fused to a Fc-portion of Ig-molecules or to serum albumin for increased half-life, alternative scaffolds proteins can in addition be raised against proteins such as albumin or Fc to take advantage of the long circulation half-life of those serum proteins (Bertschinger et al. 2007; Jonsson et al. 2008; Tolmachev et al. 2007a, b). By fusing such a binder to another binder having a therapeutic function, a biotherapeutic with extended plasma half-life is created.

#### 7.3.2.1 Avimers

The Avimer scaffold is based on a family of small protein domains called A-domains (Silverman et al. 2005). A-domains are typically expressed as multimers with a short linker region between each domain and are reported to be involved in interactions with several different types of targets, such as haptens, proteins, and viruses (Silverman et al. 2005). The A-domain is approximately 35 residues long and is rapidly folded into a stable structure stabilized by disulfide bridges and a calcium ion. In a study by Silverman and colleagues, the natural multimeric architecture of A-domains was exploited using a sequential selection strategy. The selection process was started using a randomized monomeric library from which binders were isolated by phage display. In following steps, additional randomized domains were fused to the first binder and the panning was repeated, thereby constructing multimeric Avimers with potentially growing interaction surfaces. Using this approach, a multidomain Avimer protein with subnanomolar affinity was selected against interleukin-6 (IL-6). To render the IL-6 binding Avimer a kinetic profile suitable for development into a pharmaceutical drug, the half-life needed to be increased. Therefore, a monomeric Avimer with low affinity binding for human IgG was developed. After fusion to the trimeric IL-6 binding Avimer, the resulting heterotetrameric construct had a terminal phase of the profile of 89 $\pm$ 15 h in cynomolgus monkeys and an expected human serum half-life of 178 $\pm$ 30 h, based on allometric scaling. The IgG-binding domain was also fused to Avimers binding to BAFF and to CD40L to enhance their half-life, showing that the approach is general.

#### 7.3.2.2 Albumin-Binding Domain

Another approach to increase serum half-life is to use binding to serum albumin to confer the long half-life of albumin to the associated fusion protein of interest. This was recently used to increase the half-life of a radiolabeled HER2-binding Affibody molecule for therapeutic use in a murine xenograft model (Tolmachev et al. 2007a, b). It has also been used in conjunction with tandem scFvs, rendering a αCD3-αCEA-ABD bispecific antibody fragments a long half-life (Hopp et al. 2010; Stork et al. 2009). Although the ABD scaffold used for albumin binding originally bound to albumin, and as such is not a general alternative scaffold protein, it has been subject to extensive engineering, with an improved affinity to mid femtomolar $K_D$ (Jonsson et al. 2008), as well as a complete removal of T-cell epitopes. As described below, it has also been used as a single domain scaffold into which another target binding property has been engineered while preserving the original specificity.

#### 7.3.2.3 Bispecific Single-Domain ABD Molecules

As mentioned above, in vivo half-life extension by adding serum protein-binding capacity holds promise to offer an efficient and viable approach for non-Ig-based scaffolds to boost potency. A new strategy is to directly engineer the target-binding specificity into a serum protein-binding domain, hence reducing the overall size of the affinity protein even further. Hober and colleagues analyzed the albumin-binding properties of a natural albumin-binding domain (ABD) in order to identify positions suitable for such randomization (Alm et al. 2010). The ABD scaffold is merely 46 amino acids long and the task of engineering two high-affinity interactions, preferably onto two distinct surfaces on this domain, was indeed challenging. In a first proof-of-principle study, a phage-displayed library of randomized ABD molecules was constructed. Diversification was focused on residues on the opposite side of the albumin-binding site in the three-helical bundle in order to increase the probability of finding dual binding molecules. As a first step, the molecule was engineered into a small bispecific affinity handle for protein purification purposes. An engineered B-domain (the Z-domain) from staphylococcal protein A was used as target in the selections and ABD molecules demonstrating affinity for the Z-domain as well as having retained affinity for human serum albumin were isolated. By fusing the new bispecific ABD handle to different target proteins, excellent purification capacity could be demonstrated by affinity capture on a serially coupled protein A-based matrix column followed by an albumin-coupled column (Alm et al. 2010). The successful results inspired the group to investigate the approach for potential serum half-life extension of affinity proteins intended for therapy (JL, Sophia Hober personal communications). Using the same library, binders were selected against human tumor necrosis factor-alpha

(TNF-alpha). First-generation binders were isolated and identified, but although several were bispecific, a relatively high affinity for TNF-alpha seemed to limit the affinity for albumin and vice versa. To increase the affinity and to engineer binders with high affinity for both targets, a new library was designed based on the sequences from the first selection and the display format was changed from phage display technology to a bacterial-based cell display system. Since cell display enables the use of fluorescence-activated cell sorting (FACS), selections could be performed against both targets simultaneously by labeling TNF-alpha and HSA with different fluorophores followed by multiparameter sorting. The selection was successful and bispecific binders with high-affinity against both targets were isolated. Although challenging, the approach should be generally applicable, e.g., in selections against tumor-associated targets where a small overall size of the affinity protein is beneficial.

## 7.4 Conclusion

In this book chapter, the use of non-immunoglobulin-based protein scaffolds for engineering of bispecific recognition has been described. The use of alternative protein scaffolds as therapeutic antibody mimetics with improved properties over antibodies is now entering clinical validation. The promise of bispecific binding has initially been explored using the bivalent antibody format, but is increasingly expanded by adding new protein formats, both from immunoglobulin derivatives and completely unrelated scaffolds. Different problems can be addressed using bispecific alternative scaffolds, including half-life extension strategies, more efficient blocking of a receptor, better specificity for tumor cell targeting or blocking of two different receptors using only one biomolecule. A number of the alternative scaffolds in development holds promise for bispecific applications based on their ability to bind in the context of a multidomain format, the possibility to express them recombinantly as a single-chain polypeptide and their small size, allowing for multiepitope binding of surfaces too close to each other to be eligible for bispecific antibodies to access. An extension of engineering a scaffold protein for certain specificity and then to fuse it with another domain for e.g., half-life extension is to move the engineering of new specificity directly into an existing natural ligand, thus adding bispecificity to an already functional binding property. Alternative scaffolds are clearly useful and holds promise to further increase engineering possibilities to create new and optimized bispecific molecules. Many more examples of bispecific alternative scaffolds will likely be available in a near future, exploiting advantages scaffold proteins may hold over antibodies, and hopefully presenting new candidates for biotherapeutic drugs.

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