Reprinted from:
A. Plückthun, Alternative Scaffolds: Expanding the Options of Antibodies.
In: Recombinant Antibodies for Immunotherapy, Melvyn Little, Cambridge

CHAPTER EIGHTEEN

Alternative Scaffolds: Expanding the Options of Antibodies

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In the language of modern biotechnology, monoclonal antibodies (Köhler & Milstein,
1975) were the first “library” of proteins that was available, and the immune system
was the first “selection” technology by which a specific binder could be obtained.
However, only the subsequent introduction of molecular biology into this field
allowed a true control over the molecules (reviewed, e.g., in Plückthun & Moroney,
2005; Weiner & Carter, 2003). This development of technologies was largely driven by
the desire to use antibodies therapeutically, since the extraordinarily strong immune
response to a nonhuman antibody in humans had put an end to essentially all of these
endeavors. As will be illustrated in the following paragraphs, technological develop­
ments intended to solve this problem made not only the use of an animal immune
system, but, ironically, also the antibody molecule itself dispensable.

Three fundamental approaches have been developed to arrive at antibody mole­
cules that are able to evade the human immune surveillance and which, at least from
this perspective, may become potential therapeutics. The first approach, termed
“humanization” (Jones et al., 1986), converts an existing murine antibody obtained
by immunization into an analogous one with as much human sequence as possible.
Another approach, a technical tour de force, was to introduce human antibody genes
into a mouse and inactivate or delete the murine loci, such that an immunized mouse
would then produce antibodies after immunization that essentially consisted of
human sequences (Fishwild et al., 1996; Mendez et al., 1997). Finally, a third approach
made the antibody completely independent of an animal’s immune system: it con­
sisted of establishing methods for rapidly producing recombinant antibodies in vari­
ous formats, creating first a repertoire of the antibody genes outside the animal, and
second a selection technology with which the antibodies (and their genes) could be
enriched from the library (Burton et al., 1991; Hoogenboom & Winter, 1992; Knappik et
al., 2000; Marks et al., 1991; Mondon et al., 2008; Skerra & Plückthun, 1988; Vaughan et
al., 1996). For these antibody repertoires, either the natural immune repertoire is
polymerase chain reaction (PCR) amplified, or fully synthetic repertoires are created.
For the selection technology, display technologies such as phage display, ribosome
display, and surface display on bacteria and yeast (Bass et al., 1990; Boder & Wittrup,
1997; Hanes & Plückthun, 1997; McCafferty et al., 1990; Smith, 1985) are most widely
used and have proven most successful. Over the years, many more selection technol­
gies (Levin & Weiss, 2006) have been developed, all of which cannot be reviewed here.
It is with this last approach, using a synthetic antibody repertoire and a selection technology, that an endeavor that originally set out to mimic the immune system "in the test tube" finally became independent of using antibodies at all. In other words, the same technology enabling the selection of antibodies from libraries can in principle be used to select any protein from any synthetic library for specific binding. It might be worth pointing out that the concept of selection from libraries with phage display had actually first been demonstrated with synthetic peptide libraries, before it was applied to antibodies (Cwirla et al., 1990; Devlin et al., 1990; Scott & Smith, 1990; Smith, 1985).

**STATUS QUO: SIX HALLMARKS OF ANTIBODIES THAT OTHER BINDING PROTEINS NEED TO ADDRESS**

While, technically, almost any protein can be subjected to library creation and selection, there are clear criteria that should be met by a useful binding molecule. It is probably useful to critically analyze the antibody molecule in this respect, especially from a standpoint of its use as a therapeutic entity. Other proteins will have to equalize or surpass these properties, but researchers are free to choose molecular means by which this can be achieved. Six properties of antibodies can be denoted that other molecules will have to address:

1. *Wide range of targets and epitopes:* Regarding the range of molecules and epitopes that can be bound, the antibody-combining site is extremely versatile. This has to do with the fact that the six loops that constitute the CDRs can vary greatly in length, and some in relative disposition, allowing the creation of a pocket (e.g., to engulf an amino acid side chain or any other molecular entity protruding from the target), a groove (e.g., to harbor a linear oligomer, such as a peptide or an unstructured part from a protein, or an oligosaccharide), a rather flat surface (to bind to another flat surface on a target protein), or even a protrusion (which sticks into a cavity in the target) (Almagro, 2004; Ramsland & Farrugia, 2002). Each of these binding modes has been achieved with different non-antibody scaffolds just as well (see below), but probably at this time, all these options are not possible with the use of only one molecular scaffold. It may well be that these particular binding modes lead to epitope preferences on the target. However, this is not really an impediment, since different libraries with different randomized parts can be created, as can different loop lengths, or even different specialized scaffolds.

2. *High affinity and specificity:* Antibodies can bind their targets with very high affinity. This is achieved by an iterative affinity maturation (Di Noia & Neuberger, 2007; Peled et al., 2008) in the B cells, but high affinity is certainly not a guaranteed outcome from immunization. The iterative evolution strategy of the immune system was the inspiration to create similar approaches in a cell-free system, as realized in the cell-free evolution technology of ribosome display (Hanes & Plückthun, 1997) or mRNA display (Lipovsek et al., 2007; Roberts & Szostak, 1997). These cell-free techniques can be applied to well-folding non-antibody scaffolds (Xu et al., 2002; Zahnd
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et al., 2007a) and single-chain antibody Fv fragments alike (Luginbühl et al., 2006; Zahnd et al., 2004). Of course, directed evolution can also be combined with other selection technologies, such as phage display (Pearce et al., 1999; Schier et al., 1996) or yeast display (Boder et al., 2000), but perhaps somewhat more laboriously, since in vitro randomization (e.g., error-prone PCR) and library transformation have to be alternated. In summary, with modern technology it is possible to recreate the generation of high affinity and high specificity in vitro, thus imitating affinity maturation of antibodies, and it can be applied to non-antibody proteins just as well.

3. Long half-life: Intact IgGs have a proverbially long serum half-life. There are two molecular features that play a decisive role for this property. First, antibodies (like all high-abundance serum proteins) are too large to be filtered through the glomerular filters of the kidneys. The pores are thought to be 60 nm fenestrations in the epithelial cell layer, which are, however, filled with negatively charged proteoglycans, and this cell layer is covered by a glycocalyx toward the blood side, further restricting the effective size and introducing charge selectivity (Haraldsson et al., 2008). The next layer, the glomerular basement membrane, followed by a layer of podocytes, may also contribute to the size restriction. There is no sharp cutoff molecular size, but molecules up to 25 kDa will largely be filtered, while molecules above 65 kDa will be almost completely retained. While IgG molecules are safely above this size, Fab fragments are just at the critical size and scFv molecules are clearly below this limit. Second, when the antibody is taken up by vascular endothelium, which engulfs all serum proteins by endocytosis, it is largely recycled, and not degraded. This is due to the interaction with the neonatal FcRn receptor (also termed Brambell receptor after its discoverer), which is expressed in hepatocytes, endothelial cells, and phagocytic cells of the reticuloendothelial system, the main locations of protein catabolism. By this mechanism, the half-life of IgG is increased by a factor of 10 compared to IgG half-life in transgenic animals lacking this receptor (Junghans, 1997; Telleman & Junghans, 2000). The other molecule that shows an unusually long half-life and uses an analogous mechanism is serum albumin (Chaudhury et al., 2003).

Most non-Ig binding proteins will be below the critical size limit and, almost certainly, will not have a built-in mechanism for half-life extension. To increase half-life, one will thus have to resort to one of the following measures: (1) dramatically increase the hydrodynamic radius by attachment of a tail, most commonly polyethylene glycol (Caliceti & Veronese, 2003; Chapman et al., 1999; Greenwald et al., 2003; Yang et al., 2003), (2) attach a binding region specific for a molecule which by itself has a long half-life, usually serum albumin or IgG (Dennis et al., 2002, 2007; Holt et al., 2008; Kawe et al., unpublished; Silverman et al., 2005; Tolmachev et al., 2007), or (3) fuse an Fc region or serum albumin. For the interaction with the FcRn, glycosylation of the Fc region is not needed (Ghetie & Ward, 2002). Nonetheless, the production of such a fusion protein will usually have to be carried out in mammalian cells for higher folding yields of the disulfide-containing Fc region, abrogating many of the advantages of alternative scaffolds.

It must be pointed out that a long half-life is by no means always desired. A typical case in point is in vivo diagnostics; another one may be the use of radio-isotopes or
toxin conjugates, which need to be cleared rapidly to limit off-target toxicity. In these applications, most non-Ig-scaffolds already have the right size.

4. Bivalency: Immunoglobulin molecules are bivalent, or of even higher valency. The physiological reason is, of course, that this leads to a gain of functional affinity (avidity) if the target epitope is arranged in multivalent form, as on the surface of viral or bacterial pathogens, which after all are the natural targets of the antibody molecule. Importantly, this has no consequence when the target is monomeric and can even create problems when, in a therapeutic setting, a surface receptor molecule is targeted, as an undesired agonist activity can be induced. A fascinating discovery, whose molecular basis became clear only very recently (van der Neut Kolfschoten et al., 2007), showed that the Fc part of the human IgG4 molecules are unstable in the presence of trace amounts of thiols, such as glutathione that occurs in traces in blood: they dissociate and re-equilibrate with each other such that all human IgG4 molecules appear to be bispecific and that a therapeutic IgG4 would equilibrate with unexpected partners – a scenario that must be carefully considered for this class of molecules.

Bivalency, when needed, can of course be engineered into other protein classes. There are several ways of achieving this. Conceptually the simplest is to covalently link the molecules by a flexible linker. This requires that they fold well in such an assembly and that the target epitopes are arranged in such a way that they can actually be reached by the bivalent molecules. The next strategy is to fuse the binding molecule of interest to a module that dimerizes by itself. Numerous modules have been described (see, e.g., Plückthun & Pack, 1997, for a review of some examples), and the use of the Fc part is just one particular example. Importantly, because of the many options, in principle, a far wider range of molecular arrangements is possible than in the IgG configuration of binding sites, from higher valency to multi-specificity, from head-to-head over head-to-tail to tail-to-tail linkage.

5. Effector functions: The antibody is an adapter molecule. It “connects” a variable binding site specific for the pathogen with a constant part that binds to immune effector cells, carrying different types of Fc receptors (Nimmerjahn & Ravetch, 2007a,b) to induce antibody-dependent cellular cytotoxicity (ADCC). In addition, it can bind to the complement component C1q to induce complement-dependent cytotoxicity (CDC) (Wang & Weiner, 2008).

The key assumption that drives the field of alternative binding molecules is that the Fc-mediated triggering of ADCC and CDC, while extremely powerful in some cases, will not be sufficient to combat all diseases. It follows that other – adapted or artificial – effector mechanisms can, and need to be, engineered for numerous applications in human health care. And if this is so, then the need to use antibodies for the sole purpose of targeting is not apparent. As will be discussed at length in the remainder of this chapter, other molecules can be used as the “variable” part, with engineering, expression, and manufacture being in many cases much more straightforward than with antibodies or their fragments.

6. A – generally – low immunogenicity of human antibodies: Very few aspects of therapeutic molecules have been as hotly debated as the issue of immunogenicity. This is mostly because there are comparatively few certain facts, inviting
speculations and alleging immunogenicity or the lack of it, depending on which side of the fence one is on with regard to a particular molecule.

What is clear is that any type of protein, including fully human antibodies in human patients, can be in principle immunogenic, as found, for example, in adalimumab (Humira™) (Bender et al., 2007), but each individual case is still almost impossible to predict. In an extremely simplified summary, the lack of an immune response can be thought to be due to one of two scenarios:

First, the protein is recognized as “self,” in that no MHC-presented peptide triggers a T cell, the thymus having eliminated those T cells that would recognize any peptide-MHC complex carrying a “self”-peptide. Second, not a single peptide of the foreign protein can be presented, by not fitting in any MHC molecule and/or a lack of appropriate processing. More likely is a composite scenario, where some peptides are presented but are recognized as “self” (being similar enough to those of the human proteome), while others are not presented. A great part of the antibody sequence is shared between all antibodies and thus “self,” but every individual molecule, depending on its sequence, can potentially bear T cell epitopes and thus potentially raise an immune response, as in the case of adalimumab (Humira) (Bender et al., 2007).

How serious the problem of an immune response against a therapeutic protein is in patients depends on the outcome of such an immune response. In some cases, nothing happens as a result of an immune response, at least in the absence of chronic application. In others, the therapeutic molecule is neutralized, preventing multiple applications but not acute treatments. For example, in the case of adalimumab (Humira), different studies reached different conclusions over whether there is a connection between an immune response and reduced clinical efficacy of this fully human antibody (summarized in Bender et al., 2007). Only the third case must be avoided at all costs: if the immune response, induced by the recombinant protein and/or stimulated by impurities acting as adjuvants, leads to cross-reactivity with the body’s own proteins, a very serious condition may result. A well-known example is the red cell aplasia resulting from an induced immune response against some preparations of recombinant human erythropoietin (Casadevall et al., 2005; Ryan et al., 2006), and this immune response then turns against the body’s own erythropoietin.

THE OPPORTUNITIES: THE SHORTCOMINGS OF ANTIBODIES IN THERAPY

Nature’s design of the immunoglobulin molecule appears to be close to perfect when it is used as originally intended, namely, as an adapter molecule in fighting infectious agents: bivalent binding to a surface (typically, a microbial cell or a virus) and recruiting effector cells for ADCC and/or activating the complement system for CDC, and at the same time exploiting a long half-life. (The intricate levels of spatio-temporal immune regulation, requiring different constant regions and different receptors, will not even be mentioned here). However, most recombinant antibodies currently considered for human therapy are not intended for infectious diseases. Therefore, the properties of the protein molecule must be individually
considered, and almost always a tailor-made collection of properties can be engineered to adapt it to the needs of a particular medical application.

Conversely, even in the realm of infectious diseases, there is nothing that could not be achieved with other molecules as well, if they have been properly engineered for specificity, affinity, valency, and desired effector function. It may not be so compelling, however, to compete with antibodies on their home turf. Furthermore, in most infectious diseases, vaccination is the holy grail – that is, to bring the body to produce precisely the required antibodies itself – and passive immunization will always have to be measured against this promise.

WHEN BINDING IS ENOUGH

In some cases of therapeutic applications, no other feature of the antibody is needed other than specific binding. This would be the case in blocking a monovalent target in solution, when often ADCC and CDC are even undesirable. In these cases, the only redeeming feature of the Fc region is its mediation of a long half-life, which may translate to less frequent dosing. However, this property of the Fc region can also be achieved by other half-life extension strategies (summarized above). In other words, there is no problem with using an antibody, but also no definite requirement. Some popular examples, such as titrating cytokines with recombinant alternative binders, will be discussed below.

EFFECTIVENESS VERSUS COST

However, in other disease settings the whole antibody function is required, such as in several anticancer applications, where the effector functions of the Fc part are utilized. Nonetheless, a shortcoming of several antibodies used in oncology, in the form of IgGs, appears to be their unfavorable balance between effectivity and cost. A case in point is Herceptin™, which unquestionably provides an improvement for patient health, but showed only 8 complete responses among 222 patients with metastatic breast cancer observed in the pivotal trial (Cobleigh et al., 1999), with objective response rates in monotherapy only between 12% and 34% (Nahta & Esteva, 2006), and even in combination with chemotherapy the median time to disease progression was only 7 months.

In addition to the rather moderate clinical benefit of some antitumor antibodies generated so far, the costs of production are rather high due to their intricate molecular composition. This could, in the long run, jeopardize the support of these treatments by the public health service. As an example, both Avastin™ and Erbitux™ are no longer made available by the National Health Service of the UK at this time, with other countries and antibodies likely to follow. Importantly, when the natural effector functions are required, the IgG molecule cannot be “simplified”: disulfide bonds and glycosylation are both essential for immune effector functions mediated by the binding
of the Fc region to the Fc receptor (Jefferis et al., 1998; Krapp et al., 2003), as is of course the 4-chain nature of the molecule for creating the binding site and bivalent structure.

It implicitly follows that the next-generation therapeutics will have to address both effectivity and cost. Almost certainly, therefore, the IgG format by itself will be insufficient for many applications, and alterations may be needed. At this point, however, it is no longer necessary to use an antibody as a starting point.

In the following, molecular features will be summarized that are not intrinsic to the IgG molecule but could provide additional biological activity to binding molecules and expand the range of possible applications that become possible. It will become apparent that for those applications, alternative binding molecules may provide a more convenient engineering platform than either IgGs or other antibody fragment derivatives. Several such constructs have, of course, already been realized with alternative scaffolds, and some will be mentioned in the section below. Before going into the details of the scaffolds, it may be more useful, however, to first conceptualize the approaches.

### FORMATS BEYOND THE IgG: AN OPPORTUNITY FOR NEW SCAFFOLDS

1. Bispecific Binding Molecules

Bispecific binding proteins can become attractive in several scenarios. First, they would bridge two cells, and thereby enforce an interaction. Probably one of the most widely studied applications is the recruitment of a cytotoxic cell (a cytotoxic T cell, or a natural killer cell) (Müller & Kontermann, 2007). Recently, encouraging results have been obtained for application of such bispecific molecules in non-Hodgkin’s lymphoma (Bargou et al., 2008), while clinical efficacy data for solid tumors have not been reported yet.

In a related approach, bispecific molecules could be used to increase specificity for a particular cell type. If it were possible for the binding epitopes of two adjacent receptors to be oriented in such a way that bridging by a specific molecule is geometrically possible, and if the binding to each were of low affinity, then the binding of such a bispecific molecule would be expected to be of high affinity (I. Tomlinson et al., various seminar discussions). It is clear that only a small subset of epitopes will be appropriate for this approach. Nonetheless, this approach might be able to increase the selectivity for particular cell types, when the antigen is expressed at low levels in other cells. The same approach can also be used if both epitopes are on the same protein, even though this will enhance only the functional affinity, not the selectivity for certain cell types.

Another application of bispecific molecules would be their use as alternatives to a cocktail (reviewed in Presta, 2008), where several functionally redundant proteins must be targeted. However, as defined antibody cocktails are gradually gaining acceptance (Wiberg et al., 2006), it will be interesting to see how cocktails will measure up to linked molecules on the regulatory front.

Many technical approaches have been taken to engineer bispecific antibodies in the IgG format (Fischer & Leger, 2007; Müller & Kontermann, 2007; Marvin & Zhu,
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2005; Presta, 2008; Ridgway et al., 1996), yet none of them appears particularly convenient to carry out. The challenge is that the antibody-combining site is again made up from two chains, which, when recombined with the wrong light chain, lead to nonfunctional molecules. Nonetheless, asymmetric IgG molecules (Marvin & Zhu, 2005) have been engineered, and it remains to be seen how facile these approaches will be when implemented in large-scale production systems.

Recombinant antibody fragments have been used to solve this problem of creating bispecific antibodies with new molecular formats (Fischer & Leger, 2007; Müller & Kontermann, 2007; Plückthun & Pack, 1997). Notably in the single-chain Fv format, the connection of both parts of the antibody-combining site is covalent, and thus the assembly problem is simplified. To generate bispecific molecules, a dimerization module can be fused to the C-terminus (Plückthun & Pack, 1997) to generate mini-antibodies. Alternatively, two scFv can be fused in series, but since the in vivo folding of many antibody domains is often accompanied by some aggregation, these molecules tend to also lead to illicit pairing of VH and VL domains that usually makes their expression in mammalian cells mandatory (Bargou et al., 2008). Finally, linkers between VL and VH can be chosen that are too short to allow monomeric assembly, creating so-called diabodies (Holliger et al., 1993) and their bispecific and higher valency derivatives (Hudson & Kortt, 1999; Kipriyanov, 2002).

Despite the conceptual elegance of these methods, because of the great variation between the biophysical properties of antibody variable domains (Ewert et al., 2003), it is not guaranteed that the approaches are generic for every combination of binding sites to be tested. It follows that robust scaffolds, which may result in high-yielding assemblies of essentially all combinations of binders to be tested for biological activity, would be particularly attractive and potentially allow further exploitation of these biological approaches.

2. Protein-radio-isotope Conjugates

Radio-immunotherapy, the delivery of radioactivity to the site of a tumor, has a long history (Dearling & Pedley, 2007; Jain et al., 2007) and has shown promise largely in the area of lymphomas and leukemias, while challenges remain in solid tumors. Two antibody-radio-isotope conjugates are on the market, both for the treatment of non-Hodgkin’s lymphoma, namely, ibritumomab tiuxetan (Zevalin) and tositumomab (Bexxar). Zevalin and Bexxar carry yttrium-90 and iodine-131, respectively, but both are mouse antibodies. In the radio-immunotherapy setting, one of the main challenges is maximizing the dosage of radioactivity reaching the targeted tumor cells without delivering dangerous levels of nonspecific radiation to vital organs and tissues, notably the bone marrow, the site where hematopoietic stem cells, the precursors of all blood cells, are produced. This balancing act requires that the antibody must have a relatively short half-life, which is the reason that, historically, murine antibodies have been favored in this setting. They do not interact with the human FcRn. Interestingly, large quantities of the unlabeled antibody must be administered prior to or concomitantly with the radioconjugate to improve targeting. The relatively low dose that is sufficient for treating hematopoietic malignancies
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reduces adverse side effects and may be the reason that for this disease a useful therapeutic window can be found.

While it is unclear at the present time whether a sufficient therapeutic window can also be found for solid tumors–that is, a dose with enough radioactivity delivered to the tumor while keeping bone marrow toxicity (and potentially other off-target toxicities) at bay—it is clear that no intrinsic feature of an antibody is needed to deliver the radio-isotope. This field is thus wide open for other protein molecules. Those scaffolds that can conveniently be engineered to be site-specifically equipped with a radioligand (typically a metal chelate that would be attached to a unique cysteine remote from the binding site) and that can still be produced efficiently would seem especially well suited for this approach, assuming that uptake and half-life can be engineered over wide ranges.

3. Small Molecule Toxin Conjugates

Similar to radio-immunotherapy, the idea of coupling a small molecule toxin to a targeting protein was first tested with whole antibodies (reviewed by Carter & Senter, 2008). A case in point is gemtuzumab ozogamicin (Mylotarg), the only antibody-based drug derived from this approach to reach the market. Mylotarg is a chimeric anti-CD33 antibody conjugated to the highly potent enediyne drug calicheamicin, and is approved in the United States but not the EU, for the treatment of acute myeloid leukemia (Voutsadakis, 2002). The target for such toxin conjugates should be an internalizing surface protein, as most small molecule drugs act as inhibitors of cell replication and therefore need to reach the cytoplasm or nucleus to exert their effect (Trail et al., 2003).

There are two reasons that IgGs may not be the preferred molecules for this approach. First, chemical consistency is nontrivial to achieve, neither with coupling of the drug to sugars, to lysines, or to the cysteines from the partially reduced hinge region (Carter & Senter, 2008). Second, the long half-life of whole IgGs may again increase toxicity to nontarget tissues and thereby create side effects that decrease the therapeutic window. It thus appears that other scaffolds can well take the place of the targeting moiety, as no particular features of the antibody (other than the binding site) are needed. As outlined above in the case of radiolabeled antibodies, the optimal targeting molecule will have to be tailored together with the toxin. Most importantly, the elimination pathway of the toxin conjugate, be it through the kidney or liver, may have a bearing on the dose-limiting toxicity. Whether a long half-life is desirable at all will depend on the exact targeting modalities. With the wide range of possibilities available, this seems to be an area of great promise for scaffold proteins.

4. Protein Toxin Fusions

A conceptually similar approach as the chemical coupling of a small molecule toxin is the conjugation of protein toxins to antibodies (Kreitman, 2006). Such toxins, typically from plants or bacteria, are enzymes that catalytically inactivate essential cellular processes such as translation. By covalently modifying a translation factor or the ribosome itself in an enzymatic process, a single enzyme molecule can be sufficient
to kill a cell (Falnes & Sandvig, 2000; Perentesis et al., 1992; Stirpe, 2004). The best clinically studied members of this group are *Pseudomonas* exotoxin A, a tripartite protein that enzymatically ADP-ribosylates translation elongation factor 2, and ricin, derived from the plant *Ricinus communis*, which modifies a critical nucleotide in eukaryotic ribosomal RNA. The natural toxins are produced with their own, unspecific uptake mechanism that allows them to infect any cell, exploiting receptor molecules ubiquitously expressed on mammalian cells. By deleting these cell-binding domains and replacing them by an internalizing binding protein, tumor-selective killing can be achieved. The antibody thus mediates uptake of the enzyme by tumor cells. As the targeting moiety is only required for specific binding, alternative binding proteins are again very well suited for this approach, and especially those scaffolds with superior production properties can give rise to alternative targeted toxins.

5. Other Fusion Proteins, Such as Immunocytokines

Over the last few years, the use of immunostimulatory cytokines has been investigated to enhance the immune response to a tumor. In order to localize the cytokine to the tumor, fusion proteins with antibodies have been made. Constructs investigated include interleukin-2, interleukin-12, granulocyte macrophage-colony stimulating factor (GM-CSF), and members of the TNF superfamily (Gillies et al., 2002a,b; Helguera et al., 2002; Osenga et al., 2006; Sondel et al., 2003). As is the case with bispecific antibodies, where so far encouraging data have only been reported for lymphoma (see earlier discussion), the main challenge in the use of immunocytokines in the treatment of solid tumors will be to prevent systemic engagement of the cytokine receptor by the cytokine part of the conjugate in the absence of the antibody binding to the tumor, as this is the most likely source of adverse side effects manifest as the uncontrolled release of cytokines by inflammatory cells. The severity of the problem will depend on the complex interplay of pharmacokinetics of the fusion protein, and on whether it preferentially localizes to the tumor or prematurely to the cytokine receptor on the "unwanted" target cells. Nonetheless, this is again an area where alternative targeting proteins can play an important role, as no function other than antigen binding would be used. In fact, many fusion proteins will be substantially easier to produce with well-behaving alternative scaffolds. The desired half-life will very much depend on the application and can be engineered accordingly.

6. Immunoliposomes

Nanoscale drug delivery systems, including liposomes, polymers, and other nanoparticles have been investigated for improved delivery of cancer therapeutics (Park et al., 2004). Of these drug delivery systems, liposome encapsulated agents, particularly liposomal anthracyclines, have been most widely used, but of course a host of other agents lend themselves to this kind of delivery. Most frequently, PEGylated (or STEALTH) liposomes have been developed, using whole antibodies or scFv fragments as targeting agents (Hussain et al., 2007; Noble et al., 2004). Whole antibodies are not preferred in this approach due to their ability to bind to Fc receptors on effector cells.
Well-folding, stable, and easily derivatized scaffolds (especially with single cysteines) would allow a much wider range of coupling conditions, including high temperature or solvent mixtures, thereby increasing the number of different types of nanocontainers and nanoparticles that can be used. Here, it appears that scaffolds with these properties might have an advantage over at least some antibody fragments, which do not withstand these conditions.

**THE NEED FOR FACILE ENGINEERING**

Importantly, many of the above applications, several of which have not progressed beyond preclinical work, are very demanding in terms of the epitope that needs to be targeted, the affinity window that needs to be reached, and specificity. This usually requires many different binders to be tested, often in constructs with multiple arrangements. This in turn necessitates a system in which the protein can be produced conveniently and variants are rapidly accessible in good yields. It seems that at the present time, *E. coli* is unbeatable for this purpose. For this reason alone, scaffolds that express well, where only small *E. coli* cultures are sufficient to obtain mg amounts of a large number of candidates in parallel for testing, are at a huge advantage. While there has been enormous progress with antibody fragments made in *E. coli* in this respect over the years (reviewed, e.g., in Monsellier & Bedouelle, 2006; Wörn & Plückthun, 2001), the very high levels obtained with some non-Ig scaffolds (see, e.g., Binz et al., 2004) do not appear to be generally reachable with antibodies or antibody fragments at present. Importantly, this difference is even magnified when it comes to more demanding fusion proteins and conjugates, because of aggregation. The examples summarized above should serve to illustrate only some of the potential applications of such more demanding constructs.

**SOME GENERAL CONSIDERATIONS FOR SCAFFOLD SELECTION**

As has been outlined in the introductory section, the technology development in the field of recombinant and, later, synthetic antibody libraries has made it possible, almost ironically, that the immunoglobulin molecule is no longer needed, since synthetic library design and selection can now be applied to any protein. This, of course, immediately leads to the question of which protein scaffold should be used. So far, all protein scaffolds have been derived from natural proteins. Nonetheless, it can be foreseen that once *de novo* design (Butterfoss & Kuhlman, 2006) has become more robust, *ab initio* designed scaffolds might also be used as the basis for libraries. In an even more distant future, the full rational design of a binding protein to a target is also conceivable (meaning the fold and the specific binding site), but it should be remembered that the structure of the great majority of interesting targets is simply not known. Even with known folds and known targets, protein flexibility and plasticity is an enormous challenge, such that work on designing complementary interfaces
that will actually fold in reality is only just beginning. At the time of writing, combinatorial and evolutionary methods based on protein libraries with an underlying known structure of the scaffold appear still to be the only practical way to generate a specific, high-affinity binder against a given target within a reasonable time. The choice of scaffold to be used for designing protein libraries has been inspired by the following considerations, which are not mutually exclusive:

**Similarity to antibodies** – The first group of scaffolds can be characterized as those where similarity to antibody variable domains was desired. The immunoglobulin domain is a β-sandwich structure with a conserved disulfide bond between the two β-sheets, which supports three hypervariable loops. In antibodies from most species, two of these domains come together, such that six loops make up the binding site. The loops differ not only in sequence but also in length, giving rise to a wider range of shapes in the antigen-contacting surface. It should be pointed out that a fully synthetic library with significant length diversity in several loops is somewhat more laborious to construct (Knappik et al., 2000; Koide et al., 2007; Lee et al., 2004), and therefore, this has usually not been implemented in scaffold libraries. Representatives of the first group of antibody structure–inspired scaffolds are the 10th domain of type 3 fibronectin (FN3, Fn10), (whose library members have been dubbed tri-nectin, adnectin, or monobody) or lipocalins (whose library members have been dubbed anticalins), which will both be discussed in more detail later in the chapter.

A completely unexpected development was that, after libraries of leucine-rich repeat proteins and ankyrin repeat proteins (see later in the chapter) had already been published (Binz et al., 2003; Stumpp et al., 2003), leucine-rich repeat proteins were discovered to be the basis of the adaptive immune system in jawless fish (Pancer et al., 2004; Pancer & Cooper, 2006). Repeat proteins have an extended, rather rigid structure and are built from closely packed repeating units of secondary structure (Kobe & Kajava, 2000). Thus, these repeat proteins have in essence been “validated” as a perfectly suitable basis of a diversified immune response. Perhaps one therefore needs to broaden the term “similarity to antibodies” beyond the IgG domain fold.

**Favorable biophysical properties** – Another consideration has been the search for superior biophysical properties. This mandates a search for a stable starting position for constructing the library. In the case of designed ankyrin repeat proteins (DARPins), there is actually no evidence that natural proteins with ankyrin repeats are particularly stable. However, by using a method termed consensus design (Forrer et al., 2004), the information contained in all the thousands of ankyrin repeat sequences can be exploited, and an “idealized” fold can be constructed (discussed later). Such designed proteins indeed turn out to have extremely favorable biophysical properties and express to very high levels in the cytoplasm of any host cell. Other scaffolds, such as the engineered domain of *Staphylococcus aureus* Protein A (Nord et al., 1997), β-crystallin (Ebersbach et al., 2007), fibronectin (Koide et al., 1998; Xu et al., 2002), and many others can also be expressed in soluble form in the cytoplasm.

The success of minimalist randomization strategies, where only binary codes are being used (discussed later) and loops are often not even varied in length, shows the enormous importance of library quality and biophysical properties. At the expense
of diversity, notably the absence of long destabilizing loops, the structure of proteins derived from such methods is maintained. Thus, different lines of investigation underline the key importance of stable starting structures.

**Avoidance of potential antigenicity** – There are at least two critical components to protein immunogenicity. The first is a lack of protein aggregation (being equivalent to superior biophysical properties, described earlier), in order to prevent a T cell independent activation of B cells, and the second is an absence of T cell epitopes. It is useful to stress that in any library of proteins, irrelevant of whether a scaffold is called “human” or not, each member of the library may potentially present new such linear T cell epitopes, due to the randomized regions. A related factor is the frequency of antigen presentation in the MHC molecule, which may also be related to protein stability due to the required proteolytic processing, but this is not yet well understood.

Very small domains with three disulfide bonds (so-called LDL-A modules or A-domains) that occur, for example, in the low-density lipoprotein receptor (Koduri & Blacklow, 2001), have been investigated in this respect (Silverman et al., 2005), as it has been proposed that their processing might be ineffective leading to low immunogenicity in the case tested. Alternatively, a scaffold with a rather limited number of different linear peptides, such as realized in designed repeat proteins (Binz et al., 2004), holds the same promise, as among the pool of diverse binders that represent the outcome of a typical selection usually a number of high-affinity binders free of T cell epitopes can be obtained.

**Scaffolds that already have a similar function as desired** – Some scaffolds are not meant as a generic engine to generate binders to any target but to a particular subset of proteins. It is reasonable, for example, when trying to inhibit a particular protease, to start from a protease inhibitor template (see, e.g., Dimasi et al., 1997; Markland et al., 1996; Röttgen & Collins, 1995; Tanaka et al., 1999) and to adapt this to the protease target under consideration, especially to inhibit plasma proteases in applications such as angiodema and in potential anti-inflammatory applications (Attucci et al., 2006; Williams & Baird, 2003). After all, evolution has provided a set of solutions to the problem of how a catalytic site of a protease can be blocked by a protein, which can avoid being catalytically cleaved like a substrate. Conversely, the specificity of particular peptide-binding modules can be exploited to detect other proteins carrying variants of the recognition sequence. SH2 domains have been used to find binders for phosphorylated peptides (Malabarba et al., 2001), SH3 domains have been used to detect proline-rich peptides containing a polyproline II helix conformation (Hiipakka & Saksela, 2002; Panni et al., 2002), and PDZ domains were used to select binders for peptides with a free C terminus (Junqueira et al., 2003; Reina et al., 2002; Schneider et al., 1999; Sidhu et al., unpublished).

**Scaffolds for displaying a constrained peptide** – Finally, those scaffolds should be mentioned whose only function is to display a loop, but in a conformation that this constrained peptide can bind to a pocket in the target protein. A case in point is thioredoxin (Borghouts et al., 2005; Klevenz et al., 2002), which has been used for this purpose as a well-expressed protein, where a peptide can be inserted into the fold without destabilizing the structure too much, even though this destabilization differs greatly between constructs. Clearly, there are many more proteins suitable for such an approach.
When choosing the scaffold, the randomization strategy must be considered at the same time. In many cases, structures of natural members of the protein family will be known, providing information on where the protein scaffold tends to interact with its target. In other cases, one can work by analogy: Fibronectin, for example, has an architecture related to immunoglobulins, and hence a randomization of residues in the CDR-like loops appears attractive (Koide et al., 1998; Xu et al., 2002). However, the transposition of the CDR-loop concept to scaffolds with unrelated architectures may be delicate, as the example of GFP shows, which appears not to tolerate highly diverse β-strand connecting loops (Abedi et al., 1998).

Depending on the scaffold, diversity can be introduced within one single protruding loop (Borghouts et al., 2005; Klevenz et al., 2002; Norman et al., 1999), which binds into cavities of the target. Alternatively, adjacent loops can be randomized. We may conceptually distinguish the cases that the loops are rather short, and in fact form a contiguous surface, or that they are long and open up a cavity in the binding protein, giving it a concave shape, as, for example, in fibronectin domains or lipocalins (Beste et al., 1999; Karatan et al., 2004; Vogt & Skerra, 2004; Xu et al., 2002). However, there is no sharp distinction between them and the transition is rather fluid.

Finally, a surface of a secondary structure element (e.g., a β-sheet or the surface of an α-helix bundle) may be randomized as, for example, in protein A (Nord et al., 1997) or β-crystallin (Ebersbach et al., 2007).

All of these sequence alterations almost invariably destabilize the scaffold, with the consequence that a certain fraction of the library, depending on the quality of the design, may become aggregation-prone. In the case of loops, the insertion of a longer loop than in the original framework will involve a higher entropic cost in folding the molecule. A shorter loop, on the other hand, may not reach the target or provide insufficient variety. In the case of secondary structure elements, introduction of a few amino acids with a low propensity for this secondary structure may be tolerated, but a higher fraction will destabilize the structure, or if an extended hydrophobic patch is generated, may lead to aggregation. The same is true, of course, if a hydrophobic patch is generated from adjacent loops.

To counteract these problems, it is essential that the stability of the "master" framework is as high as possible and that despite the stability losses incurred by (random) sequence alterations, very stable proteins can still be obtained. This can be illustrated in the case of the designed ankyrin repeat proteins (DARPins): to arrive at an optimal starting position, an engineering strategy that had already proven useful in antibody engineering approaches was used: consensus design (Forrer et al., 2003; Knappik et al., 2000). The underlying idea of consensus design is that structurally important residues are more conserved than other residues in families of homologous proteins (Steipe et al., 1994). In contrast, residues involved in the binding of a particular partner will be "averaged out" over the family, as every member of the family will bind to a different target. The design of a protein based on a protein family consensus sequence should hence lead to an "idealized" protein. As it is a statistical approach,
Alternative Scaffolds: Expanding the Options of Antibodies

Consensus design is particularly well suited to protein scaffolds derived from protein families with many homologous members. In the case of repeat proteins, this can be multiplied by the number of repeats in each protein. To illustrate the power of this approach, using an idealized "full-consensus" ankyrin repeat, proteins resistant to boiling and saturated guanidine hydrochloride were obtained when more than three repeats were present between capping repeats (Wetzel et al., 2008). To randomize the binding surface, which consists of adjacent repeats and loops (discussed later), a library can be used using trinucleotide building blocks (Vimekiis et al., 1994), which in the case of designed ankyrin repeat proteins was devoid of prolines, glycines, and cysteines. The randomization strategy in this example is thus a composite of those mentioned earlier: the surface of adjacent helices and short loops is randomized, resulting in a very extended (depending on repeat number), moderately concave surface.

While it has been found that binders to many, if not most, targets can be obtained using proteins such as those discussed in more detail later, it is not clear how diverse the epitopes recognized on these targets are, as this is something that cannot easily be determined in high throughput. If, for example, a flat surface is randomized, the binding to a flat epitope on a folded protein may clearly be favored over binding to extended peptide epitopes or small molecules, which require a pocket or groove to bind to (Beste et al., 1999) and vice versa.

**Examples of Scaffolds Investigated in Some Detail**

This chapter cannot make an effort to be comprehensive, and the author apologizes to those whose elegant work may be inadequately adumbrated. By necessity, this chapter relies on studies that have been published and may thus underrepresent important work for which this is not the case. In 2005, we made an attempt to provide a comprehensive listing of alternative scaffolds, concentrating on those not derived from immunoglobulins (of any species) (Binz et al., 2005). Because of the rapid development of the field, a comprehensive update would be out of date the minute this book is in print.

**Staphylococcus Protein A Domains ("Affibodies")**

One of the first scaffolds to be investigated was an engineered domain B of *Staphylococcus* protein A (SpA) (Nord et al., 1997). This three α-helical bundle protein of 58 amino acids can be expressed well in the cytoplasm of *E. coli*. The randomization of the 13 residues of this domain that are naturally involved in human Fc binding allowed the construction of combinatorial phage display libraries that have been used to generate binders to a variety of targets.

The crystal and NMR structures of the complex between an affibody and its target, another affibody, have been obtained (Högbom et al., 2003; Wahlberg et al., 2003) (Figure 18.1a). The studies show that most of the randomized surface of this "anti-idiotypic" affibody was involved in the 6 μM affinity interaction. NMR studies revealed that this particular affibody seems to be a molten globule that folds only
Figure 18.1. Representative structures of non-antibody binders in complex with their target. The figure attempts to emphasize the different secondary structures of the different scaffolds. Structures were obtained from the PDB. The selected binder is shown with its side chains, and helices as cylinders, the target without side chains and helices as ribbons. (a) Affibody in complex with its target, here another affibody (PDB ID 2887), (b) DARPin in complex with Maltose Binding Protein (PDB ID 1SVX), (c) Anticalin in complex with fluorescein (shown as space filling model in the center); the two disulfide bonds of the lipocalin are also shown in space filling representation on the top left and top right (PDB ID 1NOS), (d) Monobody in complex with the human estrogen receptor alpha ligand-binding domain (PDB ID 2OCF). [See color plate.]

upon binding to its target, protein Z (Dincbas-Renqvist et al., 2004; Lendel et al., 2004; Wahlberg et al., 2003), which may explain the comparatively low affinity, despite the extended interaction surface in this example. Another affibody selected against human CD28 was shown to block the co-stimulatory interaction between CD28 and CD80 expressing cells, hence being a candidate for immune suppressive intervention (Sandström et al., 2003). The micromolar affinity of the anti-CD28 affibody was improved for cell binding by fusion to an Fc region which provides bivalency.

Initially, affinities around micromolar were obtained that had to be improved by secondary affinity maturation projects. More recently, nanomolar affinities were reached directly, and an affibody with specificity to HER2 could be further affinity-matured to a $K_D$ of 22 pM (Orlova et al., 2006). These affibodies to HER2, because of their small size, hold promise as imaging reagents (Engfeldt et al., 2007; Orlova et al., 2007). Different radionuclides (e.g. $^{76}$Br, $^{125}$I, $^{111}$In, $^{114m}$I, $^{99m}$Tc, and $^{211}$At) have been attached via different principles (Nygren, 2008). In a first clinical study, microdoses (<100 μg) of both $^{68}$Ga-labeled and $^{111}$In-labeled DOTA-labeled anti-HER2 affibody material were injected into patients with recurrent breast cancer. Using SPECT, small HER2-positive metastases were reported to be detectable (Baum et al., 2006). Using different isotopes and half-life extension strategies, such molecules are also being evaluated for radiotherapy (Tolmachev et al., 2007). The affibody technology is commercialized by Affibody AB (www.affibody.com).

The three-helix bundle domain is a "benign" protein and can thus be fused to many other proteins. For example, a head-to-tail dimeric version of an anti-HER2
affibody protein has been inserted into the H1 loop of the knob structure in adenovirus type 5 (Ad5) fibers (Magnusson et al., 2007) or even as a replacement for the knob domain (Belousova et al., 2008). Virus particles containing such fibers were demonstrated to infect cells via HER2 receptors rather than via the normal Coxsackie B virus and Ad receptor (CAR) route. Perhaps such and similar vectors can be further developed into vehicles for gene therapy.

**DESIGNED ANKYRIN REPEAT PROTEINS ("DARPins")**

Repeat proteins are, besides antibodies, other natural scaffolds that are abundant and used for sets of diverse natural specific binding proteins, notably inside the cell. Ankyrin repeat (AR), armadillo repeat (ARM), leucine-rich repeat (LRR), and tetra-tricopeptide repeat (TPR) proteins are the most prominent members of this protein class. Repeat proteins are composed of homologous structural units (repeats) that stack to form elongated domains (Kobe & Kajava, 2000) leading to large target interaction surfaces. They lose very little entropy upon binding, as they are rigid and do not possess flexible loops that would only rigidify upon complex formation. This rigidity probably partially explains the high frequency with which binders with sub-nanomolar affinities have been selected.

Ankyrin repeat proteins (Li et al., 2006) are built from tightly joined repeats of (usually) 33 amino acid residues. Each repeat forms a structural unit consisting of a β-turn followed by two antiparallel α-helices. Libraries of designed ankyrin repeat proteins (DARPins) have been developed for the generation of binding molecules (Binz et al., 2003). In this case, the chosen approach was different from most other scaffold approaches in that no existing AR protein was used as scaffold, but DARPin libraries of varying repeat numbers, between capping repeats that provide a hydrophilic surface, were generated using a consensus-designed AR module as a building block (Forrer et al., 2003; Forrer et al., 2004). This consensus strategy led to remarkably stable proteins (Interlandi et al., 2008; Kohl et al., 2003; Wetzel et al., 2008). Because of the absence of cysteines and low aggregation tendencies, they seem very well suited not only for the generation of novel fusion proteins and conjugates for extracellular targeting but also for intracellular applications.

DARPins can be expressed in soluble form in the cytoplasm of *Escherichia coli* constituting up to 30% of total cellular protein (200 mg per liter of shake-flask culture, and over 10 grams per liter in a fermenter [U. Horn et al., unpublished results]), while to isolate 1 mg of pure protein in high throughput purification only a few ml of *E. coli* are needed (Steiner et al., 2008).

Binders have been mostly selected by ribosome display, a cell-free technology that allows a true evolution of the library (Zahnd et al., 2007a), and this can also be combined with protein fragment complementation (Amstutz et al., 2006). Alternatively, DARPin libraries have been selected by phage display, which is of interest when selecting under more stringent conditions or on whole cells. In this case, a special signal sequence is required to direct the protein to the signal recognition particle (SRP) translocation pathway of *E. coli* (Steiner et al., 2006) to efficiently present it on the phage particle, since DARPin proteins fold too fast for the Sec-dependent signal sequences normally
used on standard phagemid vectors. Without any affinity maturation, binders with sub-nanomolar affinities could be isolated directly from the library (Steiner et al., 2008).

Specific DARPin binders were isolated against a number of rather diverse targets—for example, maltose-binding protein (MBP) (Binz et al., 2004)–several MAP kinases (Amstutz et al., 2005) (P. Parizek, L. Kummer et al., unpublished), several G-protein coupled receptors (GPCRs) (Milovnik et al., 2009; Batyuk, Mohr et al., unpublished), Caspase-2 (Schweizer et al., 2007), telomeric repeats of DNA (O. Scholz et al., unpublished), and many therapeutic targets including EpCAM (P. Martin-Killias et al., unpublished), EGF-R (Steiner et al., 2008), HER2 (Steiner et al., 2008; Zahnd et al., 2006; Zahnd et al., 2007b), HER3 (Y. Boersma et al., unpublished), and HER4 (Steiner et al., 2008), or antibody Fc regions (Steiner et al., 2008), among others. All binders showed affinities in the sub-nanomolar or low nanomolar range, and possessed very favorable biophysical properties.

Several crystal structures of selected DARPin-target complexes (see, e.g., Binz et al., 2004; Kohl et al., 2005; Schweizer et al., 2007; Sennhauser et al., 2007) (Figure 18.1b) show that the selected binding interface forms highly specific interactions, very similar in size and number to those in high-affinity antibody-antigen interactions. Because of their rigidity, they also lend themselves to co-crystallization with membrane proteins (Huber et al., 2007; Sennhauser et al., 2007). Moreover, in some cases, enzyme inhibitors have been selected (Amstutz et al., 2005; Kawe et al., 2006; Kohl et al., 2005; Schweizer et al., 2007), and the mechanism could be deduced as one of induced allostery on the target.

Using HER2 as a target, tumor localization experiments of proteins labeled with $^{99m}$Tc(CO)$_3$ of the His tag (Waibel et al., 1999) showed excellent targeting, with very high tumor-to-blood ratios, which was apparently a function of the picomolar affinity and the small size of the protein (Zahnd, Stumpp, Kawe, Dreier, Nagy, Waibel et al., unpublished). Similarly, fusion proteins with, for example, Pseudomonas exotoxin A gave highly specific killing of only antigen-positive tumor cells, relative to normal or tumor cells not expressing the antigen, in models with EpCAM and with HER2-specific DARPin (Martin-Killias, Wyss, Stefan, Binz, Zangemeister-Wittke, Jost, Morrison, Tamaskovic et al., unpublished). The very low aggregation tendency of DARPin and the restricted diversity of the framework part of the sequence has another important consequence: the first property secures against T cell independent activation of the immune system; the second guarantees that in every selection, where normally a wide range of different sequences is obtained, there are always some molecules obtained devoid of T cell epitopes. This gives a good prognosis for applications of DARPin in human therapy. The DARPin technology is commercialized by Molecular Partners AG (www.molecularpartners.com).

FIBRONECTIN TYPE III DOMAINS ("TRINECTINS," "MONOBODIES")

In contrast to most other β-sandwich proteins, fibronectin type III domains do not have disulfide bonds and can, therefore, be used under oxidizing and reducing
conditions alike. The 10th type III domain of fibronectin (also named FN3, FNfn10) (Karatan et al., 2004; Xu et al., 2002) has been used as a scaffold by several groups ("Trinectins," "Monobodies"). This 94 amino acid protein is well expressed in soluble form in the cytoplasm of bacteria and thermodynamically stable.

In early work, fibronectins with a novel binding specificity to ubiquitin with an affinity in the micromolar range could be generated from a library with two randomized loops by phage display (Koide et al., 1998). With a similar library, binders to Src SH3 domain with micromolar affinities were also selected (Karatan et al., 2004). Clones with the typical SH3 domain binding motif PXXP were found, but also a sequence containing no PXXP motif. In another approach with a different library having the three loops fully randomized, and by using mRNA display as a selection technology, binders in the nanomolar range were reported after nine selection rounds against TNFα (Xu et al., 2002). From these nanomolar binders, picomolar binders could be evolved with further affinity maturation steps (Xu et al., 2002). With a similar approach, binders to VEGF-R2 were selected, but the increase in affinity during affinity maturation was associated with a significant loss of stability and solubility, which could be improved again by structure-based engineering (Parker et al., 2005).

The fibronectin scaffold was also successfully used in a yeast two-hybrid approach, indicating that the framework could be of interest for intracellular applications (Koide et al., 2002).

More recently, phage display libraries were constructed, with a minimal alphabet, following similar experiments with synthetic Fab fragment libraries (Fellouse et al., 2004; Fellouse et al., 2005). The potential binding site (i.e., 3 loops with length variation, with a total diversity of ca. 10^10) was randomized to allow either only Tyr and Ser, or Tyr, Ser, and one other amino acid (Gilbreth et al., 2008; Koide et al., 2007). Binders to MBP could be crystallized when fused to MBP and helped to define the binding interactions. High-affinity binders could thus be obtained from large libraries with all loops randomized, either completely or with a reduced set of amino acids (Figure 18.1d). High affinity binders could also be obtained from a much smaller library, but with a rather complete sampling akin to CDR walking by using yeast surface display (Lipovsek et al., 2007). Interestingly, the highest affinity variant selected a disulfide bond between adjacent loops, illustrating the importance of rigidity for very tight binding.

Using mRNA display, a VEGF binder was isolated that was reported as the first member of this family to enter Phase I clinical trial (www.adnexus.com) with a view to eventual applications in anti-angiogenesis tumor therapy.

LIPOCALINS ("ANTICALINS")

Lipocalins are conical β-barrel proteins with about 160–180 amino acids with a ligand binding pocket surrounded by four loops. These loops show structure divergence in natural lipocalins. Small hydrophobic compounds, such as vitamins,
hormones, and secondary metabolites, such as retinol, retinoic acid, or bilin, are the natural ligands of lipocalins. Because of the disulfide bonds present in most lipocalins, members of this family and their library derivatives are typically produced in the bacterial periplasm, similar to antibody scFv fragments.

Different lipocalin variants (also termed “anticalins”) with new compound specificities such as fluorescein (Beste et al., 1999), benzyl butyl phthalate (Mercader & Skerra, 2002), and the toxic digoxigenin (Schlehuber et al., 2000) for which the selected binder might represent a therapeutic antidote could be isolated from a phage display library. This was achieved by randomizing amino acids in contact with the ligands, pointing toward the inside of the cup-shaped protein (Figure 18.1c). In contrast, by randomizing amino acids in the loops exposed at the protein surface, binding to protein targets could be achieved (Vogt & Skerra, 2004). For example, binders to cytotoxic T-lymphocyte antigen-4 (CTLA-4) (CD152), which inhibits T cell-mediated immune response, have been isolated (Schlehuber & Skerra, 2005). Such binders might be tested as immunostimulatory molecules in cancer therapy. A binder to vascular endothelial growth factor, an angiogenesis factor, has also been reported (Hohlbaum & Skerra, 2007), which might be tested for treatment of age-related macular degeneration (AMD) or in cancer therapy. In summary, lipocalins have been shown to be useful for binding either small molecules or proteins, depending on where the sequence is randomized. The anticalin technology is commercialized by Pieris (www.pieris-ag.com).

**LDL-A-MODULES ("AVIMERS")**

A family of very small domains of about 40 amino acids, held together by three disulfide bonds and a fourfold coordinated Ca\(^{2+}\) ion, formed the basis for the library of scaffolds termed Avimers. The domains are the so-called LDL-A-modules (or A-domains), being derived from various receptors, such as, for example, the low-density lipoprotein receptor, where they occur in tandem arrangement of a number of these modules. In contrast to the repeat proteins, where repeats are rigidly connected, the modules are flexibly linked, like beads on a string. Libraries were constructed encoding a domain of about 40 amino acids, with 12 conserved and 28 variable positions, and were selected by phage display. In order to derive a higher functional affinity, several of these domains need to be strung together to achieve multivalent binding at several epitopes on the target.

The selected proteins described (Silverman et al., 2005) were expressed in soluble form in *E. coli*, and appear to spontaneously oxidize with air to form the required disulfides. In contrast, the natural LDL-A modules form inclusion bodies (North & Blacklow, 1999) and were reported to require refolding in the presence of Ca\(^{2+}\), and they appear to be sensitive to certain mutations. It will be interesting to see what range of sequences is commensurate with the LDL-A module fold.

A Phase I clinical trial was initiated with IL6 as a target (Avidia, acquired by Amgen [www.amgen.com]), where three linked modules chelated the cytokine molecule,
and multivalent binders to other targets were described. IL6 is part of the acute phase response leading to inflammation, and the anti-IL6 avimer might have possible uses in preventing symptoms of autoimmune diseases such as Crohn’s disease.

THE FUTURE

When therapeutic antibodies first arrived, they were compared to the well-established small molecule drugs, and questions about the persistence of this phenomenon and the size of this market were raised by the skeptics. Today, therapeutic antibodies have become a mainstay of the pharmaceutic industry. However, the overwhelming majority of the molecules that are on the market, and even those that are in clinical trials, are still of the IgG format. Nevertheless, non-antibody-binding proteins have made the transition to the clinic already. It is therefore a reasonable prediction that in the future, there will be three main classes of therapeutic entities: small molecules, classic antibodies, and other engineered binding proteins. The latter may carry small-molecule payloads or other tailor-made effector functions, thus creating a continuum between these molecular classes.

Protein engineering, creating complex proteins to specifications, may turn out to be one of the most powerful ways to tackle some complex diseases. It may not be easy to create molecules better than IgGs to fight infectious diseases. It seems almost certain, however, that for many other disease settings, proteins will eventually be created which combine predesigned specificity with novel tailor-made effector mechanisms.

Acknowledgments

The author is indebted to Drs. Peter Lindner, Patricia Martin-Killias, Daniel Scott, Rastislav Tamaskovic, and Prof. Uwe Zangemeister-Wittke for critical reading of the manuscript.

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