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Review

Small biomolecular scaffolds for improved biosensor performance

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Introduction

The fast and sensitive detection of pathogens and disease biomarkers is of great diagnostic importance. Biosensors are being used to an increasing extent and are aiming at being supplements to, or replacements for, standard established techniques. Biosensors are built up of a biological target recognition element that is associated with a transducer. The three most popular transducer types are currently surface plasmon resonance (SPR),[1] quartz crystal microbalance (QCM), and cantilever platforms [1]. All of these machines can be obtained from commercial suppliers, but the technologies are still being refined in academic settings. Common to the three types of transducers is that the capture ligands are being immobilized to a planar surface and the biomolecular interaction events that occur at the biofunctionalized surface are translated by the transducer into an electronic signal that can be used to quantify the analyte [1]. Biosensors can provide a rapid and low-cost alternative to conventional analytical methods such as enzyme-linked immunosorbent assay (ELISA) and real-time polymerase chain reaction (PCR). Current research is focused on the development of biosensors as high-throughput laboratory instruments and as small devices for challenging on-site analysis [1,2]. On-site analysis, also referred to as point-of-care (POC) analysis, could revolutionize medicine by enabling patients to make detailed diagnoses in the comfort of their own homes. In addition, the POC detection of plant/human/animal pathogenic microorganisms could help to secure food production because microorganisms could be detected in both pre- and postharvest settings. A further important use of POC testing is sampling for biothreats on the battlefield but also in subways and other places where pathogens could be unleashed [1]. In spite of the progress in laboratory-based biosensors, a lot of research and development still awaits before the promise of POC biosensor analysis can become a reality.

Complex matrices (e.g., blood, dirt, and homogenized foods) are often analyzed, and so it is extremely important that the ligand be specific and stable at the surface of transducers through multiple regenerations and in often varying chemical and physical conditions. Therefore, as transducers are being developed and validated, it is being realized that the quality of the biological recognition layer can be the limiting factor for optimal biosensor performance [3]. The stability of surface-immobilized ligands is also crucial in microarrays for protein expression level analysis. Therefore, the improvement of existing ligands and the identification of novel ligand scaffolds are of great importance to increase sensitivities of biosensors and microarrays and to further develop our understanding of surface binding events.

Polyclonal and monoclonal antibodies (pAbs and mAbs, respectively) are the most used ligands for solid phase recognition on immunomicroarrays and biosensors. To date, more than 10,000 specific antibodies are commercially available, and the number in academia is probably within the same range or higher [4]. Antibodies have been selected through evolution as the perfect scaffold for biomolecular interactions in solution and have a basically conserved Y-shaped scaffold with glycans attached to increase solubility. The paratope for antigen interaction consists of six loops of varying lengths that undergo extensive in vivo shuffling to accommodate binding sites for any antigen (see Fig. 2 below). The "active" paratope region is small compared with the total mAb scaffold and, seen from an analytical point of view, is the most relevant region.

pAbs and mAbs are the most important ligands used in clinics and research laboratories. pAbs are easily generated by animal immunization and isolation of immunoglobulin G (IgG) from serum, but batch-to-batch differences in affinity and possible unwanted cross-reactivity make these a less attractive choice for large-scale use in biosensors. Therefore, mAbs are the optimal choice because they can be produced by hybridoma technology and ligands can be developed toward any antigen [5]. The hybridoma technology also allows an infinite supply of valuable diagnostic reagents, a feature that is crucial for large-scale biosensor production.

Even though mAbs perform very well in solution, they have limitations when immobilized on sensor surfaces. These limitations include instability at changing temperature, humidity, and pH, and it is believed that the multiple polypeptide chain arrangement is what weakens the mAb integrity [6]. In addition, due to the large size of the antibody scaffold, steric effects limits the density of immobilized antibody layers that can be created and, thereby, the surface activity [7]. Therefore, a great deal of interest is focused on simplifying the antibody scaffold, and molecular engineering has pushed the concepts of antibody miniaturization to develop more stable binders that are less sterically hindered at surfaces

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[1] *Abbreviations used:* SPR, surface plasmon resonance; QCM, quartz crystal microbalance; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; POC, point-of-care; pAb, polyclonal antibody; mAb, monoclonal antibody; IgG, immunoglobulin G; scFv, single-chain variable fragment; VH, heavy-chain variable; VL, light-chain variable; VHH, single-domain camelid VH; IgNAR, immunoglobulin new antigen receptor; PSA, prostate-specific antigen; HIV, human immunodeficiency virus; ssDNA, single-stranded DNA; SELEX, systematic evolution of ligands by exponential enrichment; BBP, bilin binding protein; CTLA-4, cytotoxic T-lymphocyte-associated antigen 4; VEGF, vascular endothelial growth factor; DARPin, designed ankyrin repeat protein; APH, aminoglycoside phosphotransferase (3′)-IIIa; NTA, nitrilotriacetic acid.

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[8]. Clearly, Fab fragments (MW ~ 55 kDa) and single-chain variable fragments (scFvs, MW ~ 28 kDa) are relevant as surface-immobilized affinity ligands for some applications; however, other even smaller ligand scaffolds should preferably be pursued as alternatives or supplements to allow efficient biosensing in challenging environments.

During recent years, the extensive development of display techniques has opened up the opportunities to develop ligands independent of animal immunizations, and many different scaffold proteins that can rival antibodies in terms of specificity and affinity have emerged. The purpose of this review is to examine alternative small molecule protein scaffolds as useful ligands for integration in biosensor and microarray diagnostic applications. Currently, there are more than 50 non-antibody scaffolds suggested as affinity ligands, primarily as therapeutics [9,10]. Furthermore, synthetic chemical ligands, molecularly imprinted polymers or so-called "plastibodies," have started to make an impact in biosensor technology, and the reader is referred to current reviews on this subject [11]. Selected examples of small biology-derived ligands, all of which could represent the next generation of affinity ligands, are discussed. These ligands can be created entirely using in vitro techniques, thereby creating independence from animal experimentation. The scaffolds that are the focus of the current review are single-domain mAbs (nanobodies), aptamers, affibodies, anticalins, and designed ankyrin repeats. All of these scaffolds can be engineered for interaction with analytes by mimicking the way the immune system shuffles sequences to create diversity in loop structures. Indeed, all of these novel scaffolds (not aptamers and nanobodies) have surface-exposed loop structures that can be randomized without affecting the overall structure and stability of the protein. This makes it possible to engineer binding properties that are totally independent of their original biological function. These scaffolds are described in the context of their diagnostic potential.

Antibody fragments

Fab and scFv

The concept of mAb miniaturization is old, and Fab fragments have been made by papain digests of intact antibodies for many years [12]. Recently, however, the use of molecular cloning and display techniques has transformed the way antibody fragments are made and manipulated for diagnostic applications and therapeutics [8,13]. Display technologies physically link the genotype with the phenotype of the ligand. In phage display and ribosome display, large expression libraries (typically 10^7–10^{10} individual clones) can be handled and screened in a relatively short time. This makes the techniques very efficient and more appealing than the hybridoma technology that is more time-consuming and expensive.

Most popular are the murine Fab and scFv antibody formats (Fig. 1B), and these have been incorporated into numerous biosensors and arrays for detection of pathogens and biomarkers [1,14–16]. In spite of the relative success of Fabs and scFvs, these are still dependent on the random association of heavy- and light-chain variable (VH and VL, respectively) domain repertoires; therefore, the instability problems that are seen for intact antibodies at surfaces are still evident even though the stability can be enhanced by molecular engineering [17]. In addition, generally low expression levels (Fabs) and unwanted oligomerization issues (scFvs) can plague these antibody fragments during development [18].

Single-domain antibodies

The concept of single-domain antibodies, consisting of only the VH domain, has excited the antibody technology community because these opened up a whole new reductionistic concept in

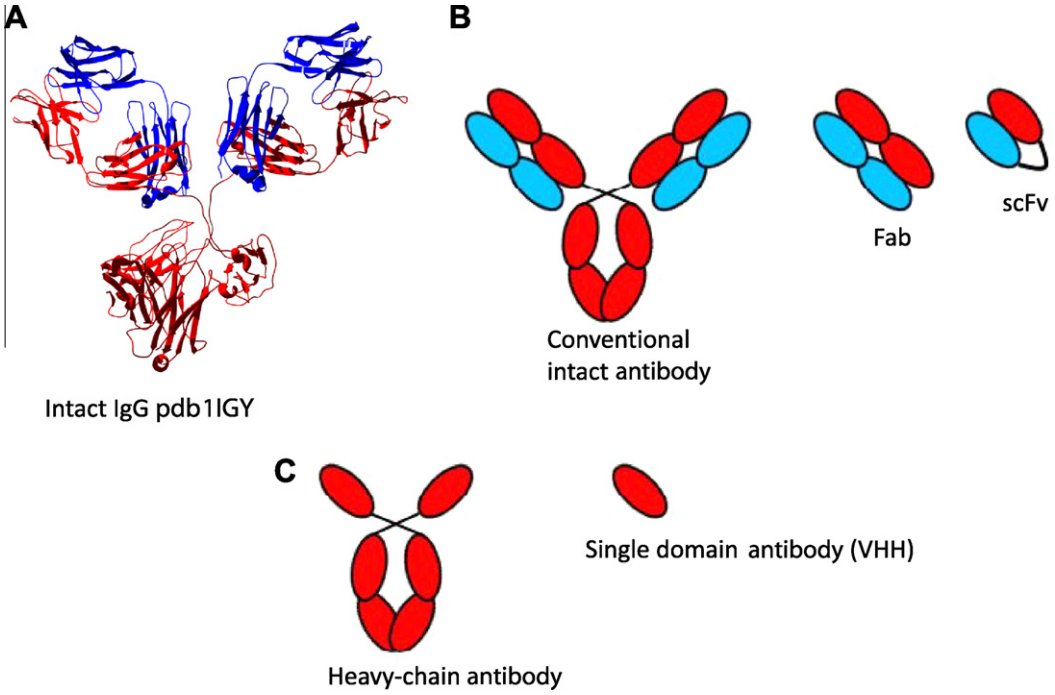

Fig. 1. Antibody structure and simplified cartoons. (A) Structure of intact IgG antibody made from pdb file 1IGY. IgG consists of two heavy chains (shown in red) and two light chains (shown in blue). The antigen binding sites are defined by three complementarity-determining region loops of the heavy and light chains (shown in yellow in Fig. 2A). (B) Carbon representation of the most popular antibody fragments used for therapy and diagnostics. Fab and scFv are currently made by cloning antibody genes from (non)-immunized material into libraries. These libraries are then screened for high-affinity binders by display techniques. (C) Cartoon illustrating the heavy-chain-only antibody found in the camelid family. From the heavy-chain-only antibody, it is possible to clone the single-domain antibodies that are highly useful for diagnostic applications. VHH, single-domain camelid VH.

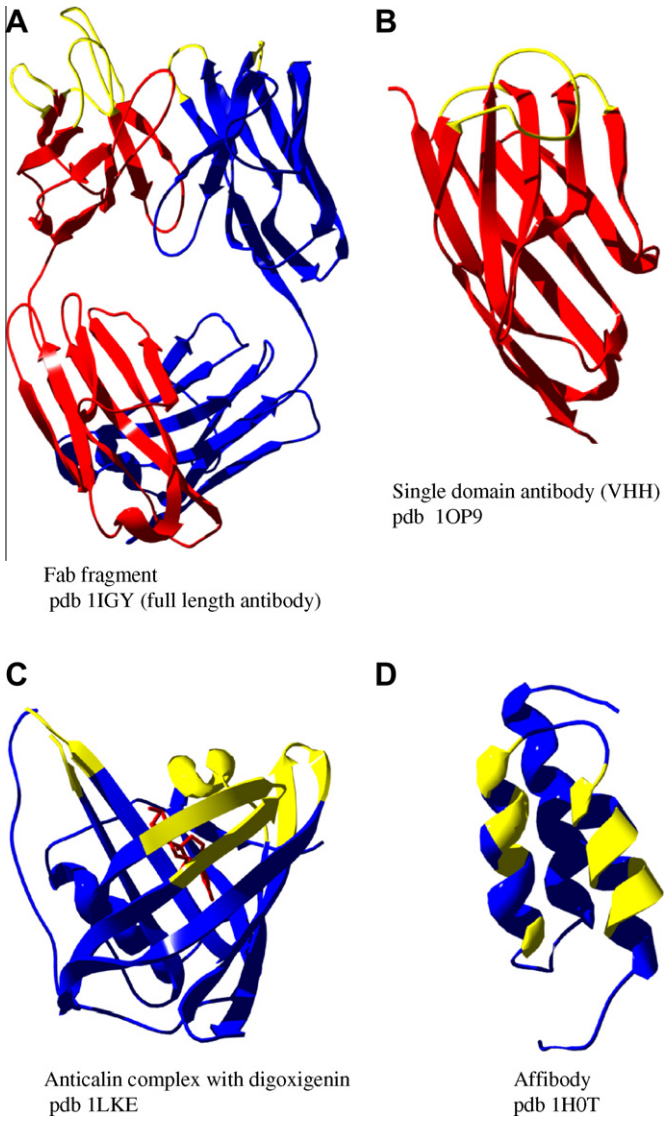

Fig. 2. Selected examples of in vitro selected scaffolds. (A) Fab fragment (derived from intact IgG structure pdb file 1IGY). The complementarity-determining region loops (CDRs) are shown in yellow, and the combined structure of these six loops makes up the antibody paratope. (B) Structure of a single-domain antibody that targets lysozyme. The three heavy-chain CDRs (shown in yellow) make up the simplified paratope that has unique binding characteristics (pdb file 1OP9). VHH, single-domain camelid VH. (C) Anticalin structure derived from a lipocalin of *Pieris brassicae*. The residues that have been randomized for construction of the anticalin library are shown in yellow at the edge of the molecule. The anticalin is shown in complex with digoxigenin (pdb file 1LKE). (D) Example of an affibody. Randomized residues are shown in yellow in the α-helices (pdb file 1H0T).

antigen–antibody interaction. VHs were first developed in 1989 from mouse spleens [19]. These VHs bind antigen, but the VH domain antibodies have a tendency to precipitate. This is because a hydrophobic stretch of seven amino acids that make up a hydrophobic core for interaction with VLs is exposed to the solvent when VHs are not stacking against VLs. The affinity constant of a mouse-derived VH was further found to be 250 times lower compared with the same antibody in the Fab format [20]. Therefore, VHs are not optimal alternatives to full-length antibodies and Fab fragments without further molecular stability engineering.

Blood from members of the camelid family (e.g., camel, dromedary, llama, alpaca) have been found to contain antibodies with no associated light chains [21] (seen in the cartoon in Fig. 1C). Therefore, these heavy-chain antibodies are dependent on the three loops of the VH domain only for interaction with antigen (see Fig. 2B). By molecular engineering, the stable single-domain camelid VH domain (denoted VHH to distinguish it from VH) has been isolated and used as a binder toward a wide variety of antigens [22]. These VHHs have been used in biosensors and simpler assays to identify viral pathogens, toxins, and biomarkers [23–25]. Unlike the murine VH, the VHH domains are highly stable in solution due to amino acid substitutions in four of the seven VH hydrophobic residues: V37F, G44E, L45R, and W47G (Kabat numbering) [21,26]. Interestingly, a similar form of heavy-chain-only antibody has been identified in some shark species [27,28]. These behave in a similar manner as VHH when isolated in the single-domain format (termed IgNAR [immunoglobulin new antigen receptor] variable domain) [29]. There is no evidence that sharks have ever had light chains associated with their antibodies, and it has been proposed that camelid heavy chains lost the ability to associate with light chains at some point through evolution [29,30].

VHHs are made from a single chain with molecular weights of approximately 15 kDa, which is only 1/10 the size of intact normal mouse mAbs and, therefore, can likely be used to create a denser biofunctionalized surface with limited steric hindrance (Fig. 1). Furthermore VHHs have been shown to be more resistant to temperature changes due to reversibility in their thermal unfolding process, and VHH antigen binding has been described at the extreme temperature of 90 °C [31,32]. Furthermore, VHHs can be expressed in very large amounts [21]. Therefore, the shelf life of VHHs is long compared with normal mAbs, and they would likely be more suited for immobilization on biosensor and microarray surfaces due to a higher ligand packing density and stability at challenging conditions. VHHs have been selected from both immunized libraries and naive libraries. There is still no consensus as to which approach is optimal, but VHH binders from naive libraries are selected with affinity constants in the nanomolar range [33], illustrating a technology that can eliminate the need for immunizations altogether.

The biomarker for prostate cancer, prostate-specific antigen (PSA), has been detected using an SPR biosensor based on a camel VHH as the specific binder. The VHH was immobilized to a surface of alkanethiols by carbodiimide coupling, and detection limits were in the low ng/ml range [24]. In a further development of the PSA VHH biosensor, several different immobilization chemistries were tested and it was demonstrated that two VHHs could withstand harsh regeneration conditions better than three PSA intact mAbs. However, it should be noted that 10 mM glycine–HCl was used for all regenerations, but it is widely accepted that individual antibodies require individual regeneration solutions for optimal performance. Therefore, the difference seen could reflect suboptimal regeneration conditions for the three mAbs. The study did, however, demonstrate a superior detection limit for the VHH biosensor compared with mAbs and scFv derivatives [34].

Llama VHHs have been selected against the ricin toxin from an immunized library. Using Luminex technology and a sandwich assay, ricin could be detected at 1.6 ng/ml and, furthermore, VHHs could eliminate the biological activity of ricin, thereby demonstrating both therapeutic and diagnostic applications [25]. The detection limit of the VHH assay could be further improved to 64 pg/ml by using VHH expressed directly on phage [35].

In two very interesting studies, a VHH was used for human immunodeficiency virus (HIV) detection. An existing rabbit scFv targeted against the HIV-1 Vif protein was engineered to the camel VHH format and maintained its antigen binding properties [36]. The VHH was immobilized on mixed self-assembled monolayers and evaluated as ligand in a QCM sensor. The VHH was found to exhibit excellent Vif protein binding properties and performed in a

comparable manner to the original scFv [37]. IgNAR variable domains have been selected from a naive library against cholera toxin and used for detection using Luminex technology and ELISA [38]. The authors found high-affinity binders and demonstrated that binders toward highly toxic antigens can be selected by the use of naive libraries.

From the consistent reports above, it is clear that the use of VHHs selected from phage display immunized and naive libraries is a powerful approach for ligand development and that VHHs are likely candidates for many different bioanalytical applications [13].

Aptamers

Affinity ligands have, until recently, been polypeptide based. In 1990, however, it was demonstrated that nucleic acid-based tertiary structures could be used as scaffolds for biomolecular interactions [39,40]. These scaffolds are termed aptamers and can be selected from random pools of oligonucleotides of RNA or single-stranded DNA (ssDNA) origin. Selection of analyte-specific binders is performed by an affinity enrichment process, SELEX (systematic evolution of ligands by exponential enrichment), analogous to biopanning in phage display [41]. The aptamer structures that can be obtained from short randomized nucleotide sequences mimic the binding of antibodies. Aptamers are especially useful alternatives to antibodies in therapeutics and diagnostics because they are structurally more robust than intact antibodies and can be denatured and still maintain activity [42]. Because the aptamer selection is performed in vitro, the target is more native-like in appearance than when injected into mammals for antibody production. Aptamers can be site-directed and modified to accommodate tags for improved surface immobilization, and they can be subjected to repeated cycles of regenerations with relatively limited activity loss compared with mAbs formats [41,42]. A major limitation of aptamers is their sensitivity to nucleases (especially RNA aptamers). However, this limitation can be solved by chemically modifying the RNA ribose structure [43]. Aptamers toward various viral pathogens and bacteria have been selected (for a review, see Ref. [44]), and examples of successful implementation of aptamer-based sensors are given below.

Aptamer-based HIV detection in which the specific target was the Tat protein has been described. The specific RNA aptamer was biotinylated and captured by streptavidin immobilized on a gold QCM surface via 11-mercaptoundecanol. The detection limit of the QCM aptasensor was comparable to that of a mAb-based QCM sensor [45]. The aptamer was used for development of the same assay on a Biacore X SPR system, and the authors found a broader linear range of the assay, but otherwise the sensors performed to the same standard [46]. However, it should be noted that the assays were not directly comparable because the immobilization chemistries were not identical; for the SPR assay, streptavidin was amine-coupled to carboxymethylated dextran, and this creates a different local environment for the biomolecular interaction than is the case for the QCM sensor. But the ability to transfer an assay from one sensor type to another clearly underlines the strength and quality of the isolated HIV Tat RNA aptamer. A very thorough study on the detection of C-reactive protein using an SPR aptasensor has been described [47]. In this article, many assay conditions such as binding buffer, aptamer linker type, and immobilization conditions were evaluated. The aptamer was modified with two different linkers capped with biotin (triethylene glycol or a 20-mer polyT) and immobilized on a streptavidin-carboxymethylated surface. Both immobilization strategies revealed a detection limit of 0.005 ppm, which is acceptable for clinical applications [47].

The relative ease of production of aptamers combined with the stability under challenging conditions clearly makes aptamers credible alternatives to antibody-based sensors.

Anticalins

Lipocalins are a group of small proteins found in many species (e.g., vertebrates, insects, bacteria, plants), and in human plasma/tissue fluids the current number of identified lipocalins is 12 [48]. The protein family members function as transporters of small molecules such as hormones, vitamins, steroids, and lipids [49]. Lipocalins share a conserved β-barrel with eight antiparallel sheets making up the structure (see Fig. 2C). The entrance to the ligand binding site is lined with four loops, giving the cup-like appearance seen in Fig. 2C with the loops shown in yellow. The sequence, length, and conformation of the four loops around the ligand binding site differ between individual lipocalins [50]. Therefore, the four loop regions have been the subject of intense protein engineering studies to explore the possibility of mimicking the six hypervariable loop regions of antibodies and, thereby, create novel binders for therapeutic and analytical applications [49]. These molecules created by in vitro mutagenesis from different lipocalin scaffolds have been termed anticalins [51]. Proposed advantages of anticalins over intact antibodies are their single polypeptide chain structure and their small size (160–180 amino acids).

By mutagenesis of the lipocalin bilin binding protein (BBP, a natural lipocalin from *Pieris brassicae*), anticalins against small molecules have been developed by mutagenesis of loop structures and adjoining residues of the β-barrel. The modified BBP libraries were screened for specific binders by phage display. The natural ligand for BBP is biliverdin Ixγ, and anticalins were selected for binding to the unrelated small analytes fluorescein and digoxigenin with nanomolar affinities [52,53]. This ability to alter the specificity of anticalins toward other small molecules suggests that this scaffold might be suitable for detection of small illicit drug molecules and mycotoxins [54,55]. However, anticalins have also been engineered for binding to target proteins of therapeutic value. To minimize the potential immunogenicity of the anticalins and, thereby, improve the value as therapeutic agent, these binders were selected from modified human lipocalin libraries. The engineering strategy focused on modifying 16–24 residues at the tip of the loops so that large protein targets would not be limited sterically by the lipocalin groove [48]. Cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) is a relevant target for cancer treatment, and an antagonistic anticalin toward CTLA-4 has been developed [48]. The CTLA-4 anticalin was able to block the interaction between CTLA-4 and the ligands B7.1 and B7.2 [56]. By taking the anticalin through two rounds of affinity maturation, the dissociation constant was improved from 80 nM to 240 pm. This is currently the only published report on an anticalin as protein binder, but in a recent review an antagonistic anticalin toward vascular endothelial growth factor (VEGF) was disclosed; however, no data are available on this yet [48]. The anticalin research field is currently under development; therefore, no real diagnostic applications of anticalins have surfaced as of yet, although one study has described the development of a fusion protein between a ligand-specific anticalin and alkaline phosphatase, thereby creating a novel approach to conjugate development that could find diagnostic applications [57].

Anticalins (and lipocalins) are very stable proteins, and melting curves above 70 °C have been reported [52]. This means that anticalins could be stable alternatives to antibodies, and their small size (~20 kDa), single-chain composition, and high expression levels in *Escherichia coli* make anticalins very promising

alternatives or supplements to antibody binders in biosensor applications and global proteome binder initiatives [58].

Affibody

Protein A is a protein located on the surface of *Staphylococcus aureus* that is designed for binding to the Fc part of immunoglobulins. Protein A has five homologous Fc binding domains, and one of them (domain B) is a Z domain composed of a three-α-helix bundle [59]. The 58-amino-acid domain (affibody) can be used as scaffold for binding to targets due to randomization of 13 crucial surface-exposed amino acids in helix 1 and helix 2 (shown in yellow in Fig. 2D). Therefore, a library of up to 3×10^9 affibody entities, with identical scaffolds and different analyte binding properties, has been constructed [60].

Affibodies are small (MW = 6 kDa) and, therefore suited to high-density microarray and biosensor surfaces [61]. In addition, affibodies contain no cysteines, meaning that they can be expressed in large amounts in *Escherichia coli* cytoplasm. Furthermore, affibodies are highly soluble and are stable in harsh conditions such as high pH and elevated temperatures [62]. A further important advantage of affibodies is the ability to make them directly on surfaces by peptide synthesis, thereby circumventing time-consuming bacterial expression and protein purification. Furthermore, direct synthesis on sensor surfaces can circumvent immobilization optimization, which can be a time-consuming process [59].

Affibodies have been produced against a wide variety of pharmaceutical targets and biomarkers, including cancer targets, amyloid peptides, cell surface receptors, and HIV proteins. The applications of affibodies are as diverse as those of antibodies and include ELISA, protein purification, and ligand-assisted target crystallization, to name a few (for an extensive review, see Ref. [9]). However, the affibodies have been used extensively for imaging purposes, and the rationale behind this lies in the small affibody size that makes it attractive for tissue penetration purposes. The small size is, however, also an affibody weakness for in vivo use because it will be cleared quickly from the system. However, innovative solutions to this problem have been to fuse cancer-specific affibodies with an albumin binding domain, thereby extending the plasma half-life [63]. In microarrays, affibodies have been used successfully [61]. In this study, an affibody specific to *Taq* DNA polymerase was immobilized in a random fashion or via an introduced cysteine residue to dextran array slides or to a CM5 SPR sensor chip. The results showed that oriented immobilization through the cysteine residue made the affibody more accessible for target binding than random immobilization, a general trend also seen for antibodies [1].

An affibody was used to replace a pAb in a sandwich ELISA for quantification of serum proteins. Tests showed that the affibody performed as good as the pAb even in the complex serum matrix [64]. This demonstrated that affibodies can rival antibodies in simpler assays, and even though no examples of affibody sensors for pathogen/biomarker detection and quantification have been demonstrated, affibodies are likely to be used as antibody supplements/replacements due to the advantages described above.

Repeat proteins

Designed ankyrin repeat proteins (DARPins) are ligands derived from naturally occurring ankyrin repeats that are found in binding proteins from many species, including humans [65]. In the human genome, ankyrin repeats are the most abundant binding proteins [65]. The number of repeats found in natural proteins differs from 2 to more than 30 [66]. As the name implies, the feature of repeat proteins in nature is a sequential number of identical sequences that stack to make up the target binding domain. The function of repeat proteins is very diverse, and for some organisms these proteins form the basis of the immune response [67,68]. Repeat motif proteins differ from the scaffolds described above in that the randomized libraries are designed with different numbers of repeats, leading to a more complex binding interface. DARPins are made from repetitive structural units of 33 residues that form a β-turn followed by two antiparallel α-helices. A loop links the structure with the turn of the next repeat. A preferred strategy for library construction consists of a randomization of selected residues in a fixed ankyrin framework. Varying numbers of repeats are then capped by N- and C-terminal sequence constant repeats. Repeats have two hydrophobic surfaces, but the capping repeats have one hydrophobic surface and one hydrophilic surface. Therefore, the function of the capping repeats is to provide a hydrophilic exterior to the molecule for improved solubility [69]. Because DARPins lack cysteine residues, they can be expressed in large amounts in the reducing cytoplasmic environment of *E. coli*. A further advantage of DARPins is remarkable thermostability even at extreme temperatures above 90 °C [69].

The DARPin technology is rather new, but binders with low nanomolar affinities toward different protein targets have been selected [70]. Functional DARPins that can block activity of target protein have also been selected. An allosteric inhibitor DARPin of aminoglycoside phosphotransferase (3′)-IIIa (APH), a bacterial mediator of antibiotic resistance, has been selected [71,72]. In this study, the binder was selected from libraries containing two and three diversified repeats with capping N- and C-terminal modules [71,72]. The kinase target mediates resistance to kanamycin; therefore, the DARPin is a promising therapeutic candidate.

Although the data are limited for this novel approach to in vitro affinity ligand development, the modular approach to library establishment is attractive and, combined with the ribosome display technique, many more binders will likely be selected from DARPin libraries in the future. The binders will likely find their use in diagnostic settings as antibody replacements or supplements in microarrays and biosensors.

Future outlook

The examples of novel antibody formats and non-immunoglobulin in vitro selected ligands given above represent only a small fraction of available scaffolds engineered for analyte interaction. However, the ones described are the most promising, and most of them have been used in diagnostic settings.

Full-length antibodies have proven their great potential when immobilized to varying kinds of sensor surfaces; however, it is clear that the ligand stability on sensor surfaces can be improved immensely. Often, 30–100 surface regenerations is the maximum number that can be performed [73–75], and this makes routine analysis extremely difficult because the sensor chip must be reliable with low variance before it can be used to accurately quantify samples. An alternative could be to prepare a new sensor surface before each analysis, but this would increase the need for antibody and sensor surfaces and, consequently, add to the expense of the assay. Therefore, the development of new ligands that are smaller and more stable and can be produced at a low cost is highly desirable.

The examples of small biomolecules reviewed here could prove to be useful as the next-generation biosensor ligands. These scaffolds are being studied intensively as drug candidates; therefore, the technologies have been developed and can be readily applied to the diagnostic field. One major advantage of recombinant ligands is the ability to engineer into the scaffold molecular tags that do not interfere with the analyte binding. It is recognized that one important negative factor when immobilizing intact antibodies is the random immobilization that occurs, meaning that the antigen binding sites may be inaccessible due to incorrect orientation [3].

Several attempts to improve the intact antibody orientation have been pursued with protein A/G or lectins, but by adding an extra protein layer at the sensor surface, this can lead to lesser packing density due to incorrect orientation of the capture molecules (protein A/G or lectins). Indeed, a recent study illustrated that detection limits did not change when an intact mAb was immobilized through protein A as compared with direct immobilization of the mAb at the sensor surface [76]. Therefore, by engineering a His tag into the scaffold of ligands, these can be efficiently immobilized on a Ni^{2+}–nitrilotriacetic acid (NTA) sensor surface and, thereby, correctly oriented toward the analyte-containing sample [77]. In this way, a standardized immobilization method can be achieved for any of the described scaffolds, and this can omit the time-consuming immobilization optimization.

Currently, the aptamer format has found the most biosensor applications, and clearly these scaffolds are very promising in terms of stability. One major advantage of aptamers is that they can be easily modified with biotin for oriented immobilization onto streptavidin/avidin sensor surfaces. Compared with the polypeptide-based scaffolds, large aptamer amounts can be produced by PCR at a relatively low cost, and this is also a highly desirable feature, especially for large-scale commercial biosensor development. Nanobodies have found fewer applications to date; however, due to the advantage of the very special paratope that binds primarily in target grooves, this kind of scaffold could prove to be useful for binding deeper into pathogen surfaces, thereby perhaps being able to discriminate between related pathogen species.

Affibodies, anticalins, and ankyrin repeats clearly are technologies that can rival both nanobodies and aptamers, and these will be implemented in biosensors over time.

Concluding remarks

Biosensors are the analytical tools of the future, but improvements in the biological recognition layer need to be made before the full potential can be reached. Small in vitro selected scaffolds may be the next-generation affinity ligands that can overcome the stability problems experienced with full-length antibodies.

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