Developments in the production of biological and synthetic binders for immunoassay and sensor-based detection of small molecules

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The need for chemical and biological entities of predetermined selectivity and affinity towards target analytes is greater than ever, in applications such as environmental monitoring, bioterrorism detection and analysis of natural toxin contaminants in the food chain.

In this review, we focus on advances in the production of specific binders, in terms of both natural entities (e.g., antibodies) and synthetic binders (e.g., molecularly-imprinted polymers). We discuss the potential of emerging technologies for integration into immunoassay and sensing techniques. We place special emphasis on use of these technologies in bioanalytical applications.

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Abbreviations: AC-DON, Acetyl deoxynivalenol; ALP, Alkaline phosphatase; ASP, Amnesic shellfish poisoning; ATP, Adenosine triphosphate; AuNP, Gold nanoparticle; C domain, Constant domain; cDNA, Complementary deoxyribonucleic acid; CDR, Complementarity-determining region; DARPin, Designed ankyrin repeat protein; DC, Deoxy cortisol; DNA, Deoxyribonucleic acid; DON, Deoxynivalenol; E. coli, Escherichia coli; ELISA, Enzyme-linked immunosorbent assay; Fab, Fragment-antigen binding; Fc-PEI, Ferrocene-appended poly(ethyleneimine); fg, femtogram (10^{-15} g); FPA, Fluorescence-polarization assay; IC_{50}, Concentration of inhibitor that is required for 50% inhibition of its target; IEMA, Immunoenzymometric assay; Ig, Immunoglobulin; kDa, kiloDalton; LOD, Limit of detection; mAb, Monoclonal antibody; MC, Microcystin; MG, Malachite green; MIP, Molecularly-imprinted polymer; mRNA, Messenger ribonucleic acid; NECEEM, Non-equilibrium capillary electrophoresis of equilibrium mixtures; ng, nanogram (10^{-9} g); OBP, Odorant-binding protein; OS-IA, Open-sandwich immunoassay; OTA, Ochratoxin A; OTC, Oxytetracycline; PCR, Polymerase-chain reaction; pg, picogram (10^{-12} g); PMP, Pinacolyl methylphosphate; PVC, Polysynthetic chloride; QCM, Quartz-crystal microbalance; RNA, Ribonucleic acid; scFv, Single-chain variable fragment; sdAb, Single-domain antibody; SELEX, Systematic evolution of ligands by exponential enrichment; SPE, Solid-phase extraction; SPR, Surface-plasmon resonance; ssDNA, Single-stranded deoxyribonucleic acid; TNT, Trinitrotoluene; V domain, Variable domain; VH, Variable heavy chain; VL, Variable light chain; VOC, Volatile organic compound; ZEN, Zearalenone; μg, microgram (10^{-6})

1. Introduction

Biological and synthetic binders are at the heart of the majority of modern diagnostic screening assays. Traditionally, polyclonal antibodies and monoclonal antibodies (mAbs) have been the popular choice for small molecules. Subsequently, molecularly-imprinted polymers were developed as an alternative with applications mainly in the area of sample preparation. In the past decade, groups have begun to develop alternative biological and synthetic binders for use in detection systems for laboratory
and field-based applications. In the area of antibody production, alternative technologies have emerged; some are based on the modification of material that has been produced in vivo, with others relying on an in vitro approach eliminating the need to use animals.

In this article, we discuss three alternatives to conventional IgG antibodies – (i) nanobodies [1], (ii) aptamers [2,3] and (iii) protein scaffolds [4]. We investigate their current state of development and their application in assays designed to detect small molecules. We review recent developments in the area of synthetic binders, along with a range of applications including chemical warfare agents [5], toxins [6] and drugs of abuse [7]. We conclude with a short review on the applications of single-chain variable fragment (scFv) antibodies, which now comprise a more mature technology and are starting to be applied in working assays.

2. Evolution of antibodies as biorecognition elements for haptenks

Biomolecular recognition, based on non-covalent binding, is central to all biological interactions. In addition to ionic, hydrogen-bonding and hydrophobic interactions, shape complementarity plays a pivotal role in the process of biorecognition. Over a century ago, Paul Ehrlich first recognized the function of a special class of proteins and called them “antibodies”. With the discovery came the hypothesis that such substances, termed “magic bullets”, would seek their targets of their own accord [8]. Since their discovery, antibodies, and more recently non-immunoglobulin (non-Ig) scaffold proteins, have been exploited for scientific needs, such as analytical detection. To understand the fundamental principles of antibody-based detection systems, knowledge of antibody structure and function is required. This section describes the structure of antibodies, and antibody types and discusses the structural components of the antibody involved in binding to a target analyte.

Antibodies are highly-soluble serum glycoproteins involved in the defense mechanism of the immune system. All antibodies are constructed in the same way, from paired heavy and light polypeptide chains, and the generic term “immunoglobulin” (Ig) is used for all such protein molecules (Fig. 1). The discrete globular domains of Ig chains fall into two distinct structural categories, corresponding to V and C domains. Each Ig domain is connected in tandem and has a characteristic “Ig fold” composed of two roughly parallel layers of anti-parallel β-pleated sheets connected with an intra-chain disulfide bond. The positions of the two cysteine residues in the β-strands that form the disulfide bond are highly conserved [9]. The tertiary structure of the Ig domains promotes interaction between the faces of the β-sheets and subsequently allows the correct folding into a functional antibody molecule. Linking the β-strands are loops of amino acids that are more variable in sequence. Sequence flexibility in these hypervariable loops makes it possible to generate antibodies against a very broad range of antigens including conformationally intact or denatured proteins, short peptides, carbohydrates, drugs, hormones and low-molecular-weight compounds [10].

Antigens (any foreign entity or target for antibody production) used for immunization may be highly-purified preparations containing a single molecular species, a mixture of molecules, or an extremely complex “antigen” (e.g., microorganism or cell). Haptens are too small to elicit an immune response, so generation of anti-hapten antibodies requires conjugation to a larger molecule (e.g., a protein, termed a carrier protein). Irrespective of the antigen used for immunization, all of the antibodies that a given B-cell clone secretes have exactly the same specificity and affinity for antigen and are called mAbs. However, when the immune system is challenged with antigen, many different B cells respond and secrete antibodies. They can all potentially recognize the antigen, but in slightly different ways, with different specificities, affinities and cross-reactivities. This is called a polyclonal response and antibodies derived from the challenged immune system are known as polyclonal antibodies. While these antibodies are easier to produce, their batch-to-batch variation and tendency to cross-react with conformationally similar compounds limit their use for defined hapten-specific assays.

The discovery of hybridoma technology, first described by Milstein and Kohler in 1975, revolutionized the production of truly antigen-specific antibodies [11]. Primed B cells obtained from experimental animals (e.g., mice and rats) immunized with the desired antigen can be fused to myeloma cells, forming hybridoma cells capable of producing antibody of the desired specificity. Thus, mAbs provide reagents with single-epitope specificity and potentially limitless amounts of identical antibody. Even though, the production of mAbs of murine or rat origin is now a routine procedure, easily within the capabilities of most investigators, it can also be a time-consuming and expensive process, so serious thought should be given to the necessity of embarking upon a program of mAb production.

The emergence of recombinant antibody phage-display technology, developed during the past two decades, has transformed the way in which we generate antibodies for the specific detection of a chosen analyte. Recombinant antibodies can be generated by PCR from cDNA derived from hybridoma cells or from the spleens of immunized animals and assembling scFv or Fab fragments by genetic engineering or by a combinatorial approach. This is usually achieved by constructing an antibody-gene library by PCR amplification of the rearranged antibody genes from B lymphocytes. The combinatorial libraries can be generated from the B cells...
Figure 1. Structures of an antibody and antibody fragments. Immunoglobulin G (IgG) antibodies are made up of four polypeptide chains, comprising two identical light chains and two identical heavy chains, and can be thought of as forming a flexible Y-shaped structure. Each of the four chains has a variable (V) region at its amino terminus, which contributes to the antigen-binding site (ABS) and a constant (C) region, which, in the heavy chain, determines the isotype and hence the functional properties of the antibody. The light chains are bonded to the heavy chains by many non-covalent interactions and by disulphide bonds, and the paired V regions of the heavy-chain and light-chain domains generate two identical ABSs, which lie at the tips of the Y-shaped antibody structure. (A) Ribbon structure of an IgG antibody showing the various polypeptide domains, region of glycosylation, hinge region (flexible region) and the ABSs. β-Strands are coloured in yellow, α-helices in red and loops in solid grey. (B) Ribbon structure of an IgG antibody indicating the constant fragments (Fc), antigen-binding fragments (Fab), variable fragments (Fv) and the complementarity-determining regions (CDRs). The heavy-chain CDRs are coloured in purple and the light chain CDRs in green. (C) Ribbon structure of an enlarged view of the Fab fragment indicated in panel B. A Fab-antibody fragment comprises the constant light (CL) and variable light (VL) domains associated with variable heavy (VH) and constant heavy domain 1 (CH1). The β-strands of the light-chain constant domain are coloured in salmon for clarity. (D) Ribbon structure of an Fv-fragment antibody with CDR loops indicated. (E) The antibody and antibody fragments are presented in panels A–D. The fragments presented above may be produced by genetic, enzymatic or chemical manipulation. The whole antibody may be initially broken up into either Fab (antigen-binding fragment) or Fab’2 (two antigen-binding fragments linked) and Fc (constant-domain fragments) regions. Fab fragments may be further broken up into Fv (variable fragments) and scFv (single-chain Fv), which are all capable of antigen binding. A scFv antibody contains a synthetic 15–20 amino-acid-linker peptide (usually a glycine-serine repeat linker), which acts to stabilize the antibody fragment without interfering with domain association. The CDRs or hypervariable regions located on both the variable heavy and light chains are responsible for antigen binding.
of immunized animals (immune libraries) or from non-immunized animals (naïve libraries) [12]. Due to the random light-chain and heavy-chain combinations, combinatorial antibody libraries may contain antibody fragments not found in nature, so libraries can be used to isolate antibodies directed against antigens not suited to immunization (e.g., recognizing highly toxic substances or self-antigens). Having a direct link between the experimental phenotype and its encapsulated genotype, phage display allows the evolution of selected binders into optimized molecules [13]. The power of phage selection to choose those ligands having the desired biological properties permits us to mimic the immune system and synthesize “tailor-made” antibodies for use in diagnosis, immunotherapy or immunoassay development.

Affinity selection of scFv by panning (Fig. 2) and subsequent reinfection into E. coli greatly enhances the number of specific, strong-binding scFvs. During panning, the phage library is incubated in an immunotube coated with specific antigen, unbound phage particles are washed away and the bound phages are then removed under strict elution conditions. Successive rounds of panning are carried out to enrich antigen binders and to ensure that antibody fragments with the strongest binding affinities are isolated.

Antibodies are indisputably the most successful binding molecules in biomedical science with established analytical, diagnostic and therapeutic applications. However, limitations of antibodies have also been uncovered, based largely on their biophysical properties and their complicated molecular composition [14]. These limitations inspired a growing trend towards engineering alternative non-Ig-binding proteins and molecules. In the next section, we describe some of these emerging biorecognition elements in detail.

### 3. Nanobodies

The sera of camels (Camelus dromedaries and Camelus bactrianus) and llamas (Lama glama, Lama pacos, Lama guanicoe and Lama vicugna) not only contain the conventional IgG molecule comprising two heavy chains and two light chains but also IgG molecules comprising only two heavy chains that lack the CH1 domain [15]. The antigen-binding site of conventional four-chain antibodies is formed by combining the variable domains of a heavy chain (VH) and a light chain (VL). Heavy-chain-only antibodies possess a binding site comprising a single variable domain known as VHH (Fig. 3). The lack of the variable light chain and the variability that

![Figure 2](http://www.elsevier.com/locate/trac)
provides for antigen-binding capacity is compensated for by extension and increased exposure of one or more of the complementarity-determining region (CDR) loops on the VHH domain [16]. The VHH domain can be isolated as a fragment from the IgG of an immunized animal through filamentous phage display, and this individual fragment can also be called a single-domain antibody (sdAb) or nanobody.

It had been shown that heavy-chain antibodies produced in camelidae as a result of an immunogenic challenge are functional and possess substantial binding capacity to protein antigens [17]. It was thought that VHH domains may not be able to generate a crevice, such as that found in the cleft of the VL–VH interface, which conventional antibodies use to bind small haptens. Indeed, heavy-chain antibodies from llamas did not bind the low-molecular-weight hapten, clenbuterol, but did display affinity towards the BSA-carrier protein [18]. However, VHH domains were produced in llamas that were capable of binding the azo dyes RR6 [19] and RR1 [20]. Recombinant-antibody techniques were used to create phage-displayed libraries of variable-region fragments of anti-caffeine heavy-chain antibodies produced in a llama [21]. Caffeine-specific VHH fragments were selected and confirmed by a positive reaction in a caffeine ELISA that could be used to bind the stimulant at 70°C. The same fragment was able to recover its reactivity after exposure to temperatures up to 90°C. Antibodies were also produced in llamas to the explosive trinitrotoluene (TNT) and were fractionated into their three sub-classes [22]. The heavy-chain-only antibodies possessed titers 10 times lower than the conventional IgG1 but displayed similar selectivity and were more thermally stable. While the titers of IgG2 and IgG3 were too low to develop effective immunoassays, the IgG1 subclass was implemented in a successful competitive assay, although it did not perform as well as a commercially-available mAb. However the authors concluded that the production of heavy-chain-only antibodies able to bind a small hapten justifies efforts to produce an sdAb phage-display library from the mRNA of the animals.

The same workers produced single-domain antibodies (sdAbs) to the widely available toxin ricin [1]. The binders were selected from a phage-display library derived from the mRNA of heavy-chain antibodies obtained from lymphocytes of immunized llamas. The sdAbs were found to bind three different epitopes of ricin, allowing them to function as capture and tracer elements in a sandwich-assay format. The sdAbs were incorporated into fluid-array immunoassays, providing a limit of detection (LOD) of 1.6 ng/mL. One sdAb pair displayed better specificity to ricin than a polyclonal antibody, and the binders were able to regain their binding ability after being heated to 85°C for an hour. The introduction of a range of food matrices had little effect on detection ability compared to that of the buffer systems. The authors concluded that the sdAbs selected from the immune-derived library provided superior toxin detection compared to those that they had previously obtained from a semi-synthetic naive library but that both approaches showed potential for the production of specific, robust recognition elements.

Doyle et al. also used llamas to produce heavy-chain-only antibodies to the tricothecene mycotoxins, 15-acetyl-deoxynivalenol (15-AC-DON) [23]. A phagemid library was constructed from amplified cDNA and specific nanobodies were selected by panning against 15-acetyl-deoxynivalenol. The dominant clone (NAT-267) was expressed in E. coli and purified as an sdAb monomer (mNAT-267) and was also used to generate a pentamer (pNAT-267) version. A competitive inhibition fluorescence polarization assay (FPA) determined IC₅₀.
values of 426 ng/mL, 169 ng/mL and 480 ng/mL for the monomer, pentamer and polyclonal llama sera, respectively. No cross-reactivity was found with structurally similar tricothecenes 3-acetyl-deoxynivalenol and deoxynivalenol.

Anti-caffeine V_{HH} antibody [24] has been produced by grafting the complementarity-determining sequences of a previously generated V_{HH} fragment [21] onto an anti-RNase A antibody scaffold, followed by expression in E. coli. The antibody was found to bind other methylxanthines (theophylline, theobromine, and paraxanthine) with lower affinity but sufficient to perform affinity chromatographic separation of the group of compounds, albeit in buffer conditions.

It is not yet fully understood how sdAbs recognize small molecules compared to conventional antibodies that use two variable domains (V_{H} and V_{L}). Changes in heat capacity upon binding and size-exclusion chromatography were used to investigate the interaction between an anti-caffeine sdAbs and caffeine [25]. It was found that there is a non-conventional binding stoichiometry in which the final complex includes two V_{HH} domains for every caffeine molecule. It was suggested that dimerization of the sdAbs, induced by hapten binding, creates a relatively high affinity. The binding profiles of three caffeine metabolites, theophylline, theobromine, and paraxanthine, were also investigated. Each ligand maintained a 2:1 stoichiometry while displaying an approximate 50-fold range of observed binding affinities.

4. Aptamers

Aptamers are oligonucleotides (RNA or ssDNA), or short nucleic-acid strands, which bind to target molecules with high specificity and sensitivity, achieved by virtue of their three-dimensional shape. The systematic evolution of ligands by exponential enrichment (SELEX) is the in vitro method that has been developed over recent years for production and selection of aptamer molecules for targets ranging from small molecules to proteins [26]. The success of the SELEX method depends upon libraries containing large numbers (10^{13}–10^{15}) of random oligonucleotides that can be screened relatively quickly. Combinatorial chemistry techniques allow low-cost, straightforward manufacture of the random-sequence oligonucleotides by repeatedly duplicating the natural 3′–5′ linkage [3].

Berezovski et al. described an alternative selection process, whereby repetitive rounds of partitioning are performed without amplification [2]. The process is reportedly more rapid than SELEX with completion achieved in one week. The process, non-equilibrium capillary electrophoresis of equilibrium mixtures (NECEEM), also facilitates monitoring of bulk affinity of enriched libraries at every step of partitioning and screening of individual clones for their affinity to the target.

There have been many reports recently describing aptamer assays using adenosine or adenosine triphosphate (ATP) as a model-target compound to demonstrate the ability to detect small molecules. Many of these employ a structure-switching design, whereby the target aptamer initially forms a duplex structure with labeled complementary DNA; introduction and binding of the target breaks the duplex structure and causes a change in signal as the labeled DNA is released. This technique [27] was used in an electrochemical biosensor, and a similar approach was adopted by Deng et al. [28] in a bifunctional electrochemical sensor, where the aptamers comprising the duplex were able to detect adenosine and lysozyme. The sensitivity of both these assays was enhanced by labeling the aptamers with gold nanoparticles (AuNPs), which allowed increased loading of the [Ru(NH₃)₆]³⁺ electroactive complex.

Alternative formats to structure switching have been described. Wang and Zhou used a surface-plasmon resonance (SPR) biosensor with the amplifying effect of AuNPs to detect adenosine via surface-inhibition detection [29]. The adenosine aptamer was immobilized on a SPR gold film and then hybridized with complementary ss-DNA that had been tagged with AuNPs, resulting in a large change of SPR signal. However, the adenosine aptamer adopted a tertiary structure after adenosine was added to the SPR cell. In this formation, the aptamer could no longer hybridize with the tagged complementary ss-DNA, so the SPR signal decreased proportionally to the concentration of the small molecule (Fig. 4).

Successful integration of aptamers into a sensor has also been accomplished by using the two separated DNA strands of an adenosine aptamer individually immobilized on AuNPs [30]. Interaction with adenosine reassembles the two aptamers causing aggregation of the AuNPs and a subsequent measurable color change. Xu and Lu designed a sensor comprising unbound malachite green (MG) and a double aptamer comprising MG and adenosine aptamers partially hybridized with a bridging strand [31]. Without adenosine, the affinity of the aptamer for the MG is inhibited by the bridging strand. When adenosine is introduced, the aptamer binds to it, thereby dislodging the bridging strand. This allows the aptamer also to bind MG, leading to enhancement of fluorescence.

While several model assays have been described, few demonstrate the effect of a real sample matrix on the performance of the aptamers. A multi-functional, reusable and label-free electrochemical assay has been reported, along with an investigation of sample-matrix effects [32]. A mixed aptamer capable of binding ATP and z-thrombin formed a duplex with a partly complementary strand, which was immobilized on a gold-electrode surface. After binding ATP, the mixed aptamer
separated from the complementary strand, which could then be regenerated with fresh aptamer. If the sensor was used to bind α-thrombin first, the aptamer did not dissociate from the complementary strand, so the system could be used subsequently to bind ATP followed by regeneration. However, it was not possible to measure the two targets simultaneously. It was also found that, when the biological samples, human plasma and fetal calf serum, were applied to the system, strong, non-specific binding to the surface interfered with the assay. Human serum was therefore treated with ammonium sulfate to remove interfering proteins and the purified 1%-plasma sample allowed analysis to proceed.

A number of reports have described the application of aptamers with appropriate detection-platform technologies to develop assays capable of analyzing small molecules of interest (e.g., drugs of abuse and environmental or food contaminants). Du et al. constructed a label-free electrochemical aptasensor by repeating self-assembled multilayers of ferrocene-appended poly(ethyleneimine) (Fc-PEI) and AuNPs for detection of cocaine [33]. Primary aptamer fragments of cocaine were covalently coupled to the outermost AuNP layer. When the target cocaine and cocaine secondary aptamer were present simultaneously, the primary aptamer fragments of cocaine were covalently coupled to the outermost AuNP layer. When the target cocaine and cocaine secondary aptamer were present simultaneously, the primary aptamer hybridized partly with the secondary aptamer to bind the cocaine, and that led to a decreased differential pulse signal of Fc-PEI. This signal change could be used to detect cocaine sensitively with the lowest detectable concentration down to 0.1 μM and the detection range up to 38.8 μM, which falls in the expected range for medical use of detecting drug abuse involving cocaine. Meanwhile, the sensor was specific to cocaine in 25% solutions of biologic fluids (e.g., human plasma, serum, saliva and urine).

A sensor for female-steroid hormone 17β-oestradiol has been developed, employing a DNA aptamer-based electrochemical biosensor [34]. The aptamer was biotinylated and immobilized on gold-electrode chips with a streptavidin-modified surface. Cyclic voltammetry and square wave voltammetry were used to measure binding of 17β-oestradiol to the aptamer and an LOD of 0.1 nM was achieved. The author indicated that the electrochemical aptasensor is more suitable for small-molecule detection than other sensor techniques that rely on changes in mass [e.g., quartz-crystal microbalance (QCM) and cantilever methods], since small-molecule interaction with an immobilized aptamer will not generate sufficient signal. This could also be interpreted as an advantage of antibodies for these particular techniques, since they can bind an antigen immobilized on the surface to generate an adequate signal in a competitive inhibition format. In any case, aptamers have been used in this SPR format, as already described [29], by employing the amplifying effect achieved from AuNP labeling of the complementary aptamer.

Fluorescence polarization-based displacement has been used [35] to develop an assay for the mycotoxin, ochratoxin A (OTA). The LOD of 5 nM was adequate for the detection of the toxin at concentrations that exceed regulatory requirements, but the method was not as sensitive as others. No real samples were assayed, so the effect of matrix on aptamer performance was not evaluated.

Niazi et al. produced five aptamers for the antibiotic oxytetracycline (OTC); four of these displayed binding to OTC at the low-nM range (9–121 nM) [36]. These aptamers did not show significant binding to structurally similar antibiotics tetracycline and doxycycline. The
aptamers were not applied to a detection assay or evaluated for the effects of sample matrix.

Others have used an aptamer for neomycin B to develop a SPR-based biosensor assay [37]. The antibiotic was immobilized onto an activated gold-sensor surface and interaction with the aptamer created a measurable response. The competitive assay allowed quantification between 10 nM and 100 μM with the prediction that the assay would allow detection of neomycin B in animal samples, despite the fact that the effect of sample matrix was not investigated.

An RNA-aptamer-based fluorescence assay to detect MG and the leucocamachite metabolite in fish [38] has also been developed. The assay was capable of detecting MG residues to well below 2 μg/kg in test samples. Further advantages of the technique are the high tolerance of the aptamer to solvent and its stability when stored at 4°C.

5. Protein scaffolds

The idea to use protein scaffolds as molecular binders was to copy what nature had already designed but with improvements, where possible. The structure of IgG molecules allows antigen recognition due to the combination of a structurally-conserved framework supporting a spatially-defined binding site composed of peptide segments that are hypervariable in both sequence and conformation [39]. By selection of a very stable protein structure that can tolerate substitution, insertion or deletion of variable domains, specific binding regions can therefore be selected through recombinant-protein techniques. The protein scaffolds chosen are usually smaller than IgG, more robust, easily modified and less expensive to produce. Although over 50 scaffolds have been identified, their long-term applicability still needs to be proved.

Affibodies are binding proteins that make use of protein A as the scaffold; the 6-kDa structure comprises 58 amino acids with 13 randomized to create the binding domain [40]. While their ability to bind peptides has been demonstrated [41], they have yet to be shown to bind small molecules.

The same reservation applies to the designed ankyrin-repeat protein (DARPin) scaffold [42]. The 14-kDa binder has been shown to bind proteins [43] but not small molecules. DARPs have displayed some potential to rival and to surpass antibody-based approaches to drugs for therapeutic use [44].

By contrast, the protein lipocalin, which acts as a biological transporter of steroids, lipids and bilin, has been employed as a binder, termed anticalin, of small molecules [45]. The bilin-binding protein from the Large White butterfly, *Pieris brassicae*, was structurally adjusted to bind the steroid digoxigenin [46]. A total of 17 amino-acid substitutions within the binding site produced structural changes in the four loops that form the entrance to the ligand pocket on top of the structurally conserved β-barrel framework, allowing complexation with digoxigenin. Anticalins possess considerably more amino acids (160–180) than affibodies and DARPins. The binding domain comprises 16 randomized amino acids producing four loops with few disulfide bonds. It is the relative complexity of the binding domain that presumably allows small-molecule interaction.

Ramoni et al. investigated the binding capacities of four forms of lipocalin odorant-binding protein (OBP) for the detection of low-molecular-weight components of explosives (e.g., diphenylamine, dimethylphthalate, resorcinol and dinitrotoluene) [47]. The OBP bound these compounds with affinity constants in the range 80 nM–10.6 mM, indicating (according to the authors) that OBP could be used as a probe for the realization of a biosensor to sense explosive compounds.

Kim et al. [48] engineered human lipocalin, which naturally scavenges bacterial siderophores (iron chelating compounds), to bind rare-earth and related metal ions specifically as chelate complexes with [(R)-2-amino-3-(4-aminophenyl)propyl]-trans-(S,S)-cyclohexane-1,2-diaminopentaacetic acid (p-NH2-Bn-CHX-A0-DTPA). The anticalin produced was able to recognize benzyl-substituted cyclohexyl-DTPA-chelate complexes of yttrium (Y3+) and related lanthanide ions. The molecular docking module for chelated trivalent metal ions, including radioactive rare-earth elements, displays potential use in the field of nuclear medicine.

6. Synthetic alternatives to antibody-based molecular recognition

As well as the biological-based binders for small molecules described above, much research has focused on the potential for artificial molecules and materials to achieve selective recognition of molecules ranging in size from small organic compounds up to proteins. The advantages offered by selective artificial materials over their biological counterparts often include ease of production, greater chemical and thermal stability, and greater batch-to-batch reproducibility. Synthetic binders may also be suitable for specific recognition of smaller organic molecules, which may not provoke an adequate immunogenic response in order to obtain suitable antibodies. We discuss here some sensor applications wherein synthetic substitutes for antibodies are employed.

Careful design of synthetic molecules represents one means of generating species with a predetermined selectivity, which can then be incorporated into a sensing application. An excellent example of this is the array of chemoresponsive dyes, based on metalloporphyrins, developed by Suslick and co-workers [49]. This demonstrated μg/kg sensitivity towards a number of volatile organic compounds (VOCs), including amines, carboxylic
Figure 5. (A) Electropolymerization of a thin MIP film onto a gold electrode, followed by extraction of template molecule. (B) Shift in resonance angle of the SPR sensor, due to rebinding of analyte to imprinted film (Adapted with permission from [56]. Copyright 2009 American Chemical Society).
acids and thiols. The technique was applied to the analysis of VOCs in complex mixtures, including beers.

An azo-dye-reporter group for amines has also been integrated into a dendrimer to create a monomolecular imprinted species [50]. Changes in absorbance response of the dendrimer could then be used as a means of detecting the presence of individual amine compounds.

Dendritic polymers have also been used for detection of chemical-warfare agents, based on a fluorescent response to binding [51].

The sensing of heavy metals (e.g., mercury and silver) using calixarene-bridged binuclear phthalocyanines has shown promise [52].

Molecularly Imprinted Polymers (MIPs) offer an accessible method of creating substrate-specific materials. Their use as selective sorbents in chromatographic applications has been extensively exploited for a wide range of analytes, particularly in SPE [53]. Some of the more novel applications have included the development of MIP-based sensing protocols [54].

Piezoelectric sensing using MIPs has frequently been examined, and a review of this area has been published [55]. However, in general, transduction of the binding event into a detectable signal requires careful MIP design, which may account for the fewer examples in the literature of MIP-based sensing applications than applications of MIPs in separation methods. We here focus on three of the more salient areas of analyte detection – natural toxins, chemical warfare agents, and drugs of abuse.

SPR has proved to be one of the more adaptable techniques in the area of MIP-based sensing (Fig. 5), and has been used to determine the presence of the mycotoxin, zearalenone (ZEN) [56]. Electropolymerization of pyrrole onto the surface of a gold chip in the presence of ZEN produced a highly specific SPR-sensing assay, with a linear range of 0.3–3000 ng/mL. The imprinted film displayed selectivity efficiency of 100% towards ZEN and 15–27% for a number of structurally analogous compounds.

Alternatively, a photo-initiator can be immobilized on the gold surface of the chip, to produce a thin polymer layer (40 nm) suitable for SPR measurements. This approach was employed by Lotierzo and co-workers to produce a SPR assay specific for neurotoxin domoic acid, which can cause amnesic shellfish poisoning (ASP). The assay had a detection range of 5–1000 μg/L in buffer [57].

Detection of endocrine-disrupting compound bisphenol A was achieved using a piezoelectric approach. Self-assembled monolayers of 2-aminoethanethiol were used as a supporting substrate on the gold surface of a QCM chip for a methacrylate polymer selective for bisphenol A [58]. Novel synthetic binders to OTA have been reported for the preparation of affinity-based “clean-up” procedures. Giraudi et al. reported on the combinational synthesis of a hexapeptide binder for the isolation of OTA from different wines [59]. The limit of quantitation of the method was 0.1 μg/L.

MIPs are well-suited to selective recognition of small, structurally-defined molecules (e.g., organophosphate pesticides [60] and chemical-warfare agents [61]). Due to the safety implications of working directly with chemical-warfare agents, methods have typically focused on model compounds, namely less toxic analogues [62] or degradation products [63].

The MIPs technique has also proved amenable to detection of nitroaromatics [64], another significant set of analytes for counter-terrorism.

Prathish et al. developed a selective potentiometric sensor for methylphosphonic acid, a degradation product of various organophosphate-nerve agents [62]. A methacrylate polymer was imprinted with methylphosphonic acid, and the ground polymer was incorporated into a PVC-membrane sensor. The authors reported an LOD of 4.8 ng/mL. The sensor displayed a cross-reactivity of 0% up to 45% when exposed to compounds, including buffers and other organophosphates.

A different approach to generating an electrochemical response via a MIP-recognition element was developed by Xie and co-workers for organophosphate pesticide chlorpyrifos [60]. Glassy-carbon electrodes were modified with AuNPs, and, on this surface, electropolymerizable p-aminothiophenol molecules were assembled; the chlorpyrifos template was allowed to interact with the monolayer of aminothiophenol, which was then electropolymerized, creating the imprint. Sensitivity of the method was of the order of ~2000 ng/mL.

As an alternative to conventional methacrylate polymers, Taraneker et al. developed a dendrimer-based molecular recognition assay for detecting pinacolyl methylphosphonate (PMP), which is a breakdown product and analogue of toxic nerve agents [65]. Residues could be detected at nM and pM levels using SPR and electrochemical detection, respectively.

Prathish and co-workers developed a novel monolithic MIP membrane for the electrochemical detection of diethyl chlorophosphate, which is a less toxic analogue of nerve agents [66]. The LOD of the assay was 170 μg/L in aqueous solution.

A technical challenge in the use of conventional methacrylate-imprinted polymers is obtaining a suitable format for sensor application. Bulk polymers, as typically created for chromatographic use, are unsuitable, due to difficulty in immobilizing these polymers and poor mass-transfer properties for the rapid binding/transduction processes required in a sensing approach.

For piezoelectric sensing (e.g., the QCM work performed by Dickert and co-workers [67]), polystyrene and polyurethane layers have been spin-coated onto the QCM chip.

Suspensions of polymeric particles can also be sprayed onto the QCM chip, as described in a MIP-based TNT-sensing application [64].
Figure 6. (A) Multi-step immunoenzymometric assay (IEMA) for 11-deoxycortisol with attomolar detection (Adapted with permission from [93]. Copyright 2006 American Chemical Society). (B) Single-step non-competitive open-sandwich enzyme immunoassay for 11-deoxycortisol with femtomolar detection (Reprinted in part with permission from [94]. Copyright 2009 American Chemical Society).
Use of biological recognition elements is particularly prevalent in rapid screening assays for the detection of drugs in blood, including drugs of abuse [68]. However, the complex nature of the sample matrix in biological samples can pose a problem for the analyst, and selective clean up of samples using tailored stationary phases offers an attractive alternative to conventional analytical strategies.

One of the more commonly addressed analytical challenges in this field is the detection of morphine, an opioid. A means of colorimetric detection of morphine using a methacrylate MIP [69] has been reported. Rebinding of morphine to the polymer, in the presence of K\(^+\), Fe\(^{3+}\) and [Fe(CN)\(_6\)]\(^{3-}\), leads to the bound morphine acting as a reducing agent and thus causing precipitation of Prussian Blue and generating a colorimetric response. The technique permitted detection of morphine down to <3 ng/mL. An amperometric approach to designing a morphine sensor incorporating a MIP, by Yeh and Ho [7], offered an LOD of <6 ng/mL.

Detection of methamphetamine down to 1 mg/mL was achieved by Romero Guerra et al. by immobilizing a MIP film on a QCM chip, employing a monomer mixture designed computationally [70].

### 7. ScFvs and their selected applications

In this section, we discuss the application of scFvs in the area of trace analysis. Several scFvs have been developed

<table>
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<th>Binder</th>
<th>Performance examples</th>
<th>Means of production</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td>Polyclonal antibody</td>
<td>Biacore-based assay with LOD of 972 pg/mL for morphine-3-glucuronide [95], 3 ng/mL for aflatoxin B1 [96] and 5 ng/mL for benzimidazoles in milk [97].</td>
<td>Collection of serum from immunized host animal; low-molecular-weight molecule must be conjugated to carrier</td>
<td>Relatively inexpensive to produce</td>
<td>Requirement of conjugation may be complicated, depending on structure; production of binder is in vivo; no control over specificity;</td>
</tr>
<tr>
<td>Monoclonal antibody</td>
<td>Biosensor assay with LOD of 4 ng/mL warfarin in plasma ultrafiltrate [98].</td>
<td>Fusion of antibody-producing cells (spleen) from immunized animal with myeloma-cell line</td>
<td>Highly specific to antigen</td>
<td>in vivo production; expensive to produce</td>
</tr>
<tr>
<td>Nanobody</td>
<td>LOD for ricin = 1.6 ng/mL [1]; IC(_{50}) for 15-AC-DON = 1.24–0.5 (\mu)M [23].</td>
<td>Lymphocytes from immunized animals used to prepare sdAb library, binders selected by phage display</td>
<td>Robust to sample matrix effects [1]; thermally stable [21,22]</td>
<td>in vivo production (but only blood sample required); time-consuming production</td>
</tr>
<tr>
<td>MIP</td>
<td>LOD for zearalenone = 0.3 ng/g [56]; LOD for domoic acid = 5 (\mu)g/L [57].</td>
<td>Co-polymerization of monomeric species in presence of template or template analogue.</td>
<td>Inexpensive; chemically and thermally robust.</td>
<td>Materials can display a range of binding affinities towards target species; less compatible with water than with organic solvent.</td>
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<tr>
<td>Aptamer</td>
<td>LOD for cocaine = 0.1 (\mu)M [33]; LOD for oestradiol = 0.1 nM [34]; LOD for OTA = 5 nM [35].</td>
<td>Selected by SELEX using libraries of large numbers of random oligonucleotides</td>
<td>Production is in vitro; tolerance to solvent [37]</td>
<td>Problematic conversion of binding event into detectable signal; may be susceptible to sample matrix effects [32,33] (inconclusive evidence).</td>
</tr>
<tr>
<td>Protein scaffolds:</td>
<td>Anticalins for explosives: affinity constants 80 nM–10.6 mM [47]; Anticalins for lanthanide (III) chelates: affinity constants 2.77–95 nM [48].</td>
<td>Isolated from large protein libraries by phage display; yeast two-hybrid; rational design; ribosome display</td>
<td>Production is in vitro</td>
<td>Not many developed to bind small molecules, with notable exception of anticalins</td>
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<td>Affibody</td>
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<td>DARPin</td>
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<td>Anticalin</td>
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<tr>
<td>scFv</td>
<td>Biosensor assay with LOD of 195 pg/mL free morphine [100] and 390 pg/mL aflatoxin B1 [101].</td>
<td>Antibody genes cloned from immunized mice and scFv-antibody fragments selected by phage display</td>
<td>Prokaryotic production, high-level expression, affinity tags incorporated for purification and detection</td>
<td>Are usually less stable than full-length IgG and have a tendency to aggregate</td>
</tr>
</tbody>
</table>

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**Table 1. Overview of the characteristics of different binders and their performance in bioanalytical applications**

[Note: The table is not fully transcribed due to the limitations of the text representation tool. It is intended to provide a structured view of the data, and the excerpt focuses on the key aspects of the scFv section.]
to small molecules since the late 1990s. Some earlier successful applications were reported in recent reviews on immunoassays for aflatoxins [71] and sulfonamides [72]. As a result, these classes of molecules are not discussed in this section, which covers mycotoxins, marine toxins, hormones and agrochemicals.

The production of an scFv to deoxynivalenol (DON) and comparison with its parent mAb was reported [73]. The binding affinity of the scFv was found to be weaker than the mAb but dissociation from the chip surface of an SPR biosensor was relatively fast.

Wang et al. reported the development of an anti-DON scFv, which had IC50 values of 8.2 ng/mL, 98.5 ng/mL and 153 ng/mL for DON, 3-AC-DON and 15-AC-DON, respectively [6]. No cross-reactivity was seen towards nivalenol or T-2 toxin. The antibody showed improved sensitivity to 3-AC-DON (~12%) and 15-AC-DON (27%) when compared to the parent mAb.

A comprehensive evaluation of the anti-3-AC-DON scFv was carried out through application to spiked and naturally-contaminated samples. The results achieved correlated well against a commercial ELISA kit. Previously, the production of an anti-ZEN scFv had IC508 of 14 ng/mL and 17–54 ng/mL for ZEN and four ZEN analogs, respectively [74]. Sensitivity was improved by a factor of three to nine through production of the scFv. However, this improvement was offset by the lower tolerance of the scFv to methanol.

Other groups have subsequently produced anti-ZEN scFvs and optimized the process to increase the yield of binding protein [75,76]. Wang et al. recently developed a bispecific scFv antibody through linking anti-DON and anti-ZEN scFvs [77]. The resulting antibody allowed the simultaneous analysis of DON and ZEN residues after a single extraction. The bispecific scFv was compared to the original scFvs through application to a range of naturally-contaminated wheat samples. The production of an anti-fumonisin scFv was accomplished, with the fragments displaying affinity towards B1 and B2 analogs [78].

Min et al. recently reported the development of an anti-fumonisin scFv [79]. However, the scFv showed 12 times less binding affinity than its mAb and it was concluded that it required further optimization prior to practical application in working assays.

The development of binding proteins for detection of microcystin (MC) toxins [80] offers good examples of the use of phage-display libraries for the production of anti-MC scFvs [81,82]. Drake et al. recently reported the production of recombinant anti-MC binders using transgenic tobacco plants [83]. The anti-MC binding protein was suitable for the detection of microcystin-LR to <1 µg/L (WHO guideline level for water). An anti-domoic acid scFv was produced after pre-immunization of chicken [84]. The scFv was improved 10-fold through engineering to give an IC50 value of 150 ng/mL. The scFv was subsequently immobilized on a mesoporous silica support to further the development of a biosensor for environmental monitoring [85]. Garet and co-workers produced an scFv to the potent marine toxin, palytoxin, using phage-display libraries [86]. The IC50 value for the scFv was 100 ng/mL. A sensitive procedure was developed to allow detection of palytoxin at levels down to 2 µg/g in clams and mussels.

There have been a limited number of applications in agrochemical-residue analysis. Huet et al. reported the evaluation of different polyclonal antibodies and an engineered antibody to fluoroquinolone antibiotics [87]. The best results were achieved using a polyclonal antibody. Wang and co-workers produced a highly-specific anti-carbofuran scFv [88]. No cross-reactivity was shown to other carbamate residues, but sensitivity was excellent with an IC50 of 1 ng/mL. The generation of alternatives to costly mass spectrometry-based assays for growth-promoting agents continues with the development of novel binders for application in screening assays. Pan and co-workers produced an anti-clenbuterol scFv using phage-display libraries, showing that sensitivity of the scFv (IC50 = 0.78 ng/mL) could be significantly better than that of the mAb (IC50 = 1.34 ng/mL) [89].

A number of applications have been developed in the area of clinical chemistry. These also have potential for use in the areas of veterinary drug-residue analysis and sports forensics. An anti-17β-estradiol scFv was recently developed [90], as were artificial single-domain fragments (sdAbs) [91] through antibody engineering. The binding affinity of the scFv (Kd = 2.6 × 10^9/M) was found to be higher than the sdAb (Kd = 1 × 10^9/M). In the subsequent work, the affinity of the scFv (Kd = 6.3 × 10^8/M) was further improved [92]. Initial screening showed that 16-fold improvement in sensitivity could be demonstrated with the scFv (IC50 = 0.56 ng) compared to the mAb (IC50 = 9.0 ng). Working assays developed showed that higher sensitivities with IC50 of 21 pg and 0.47 pg could be achieved on radioimmunoassay (RIA) and competitive ELISA formats, respectively. In other work, the same group developed novel immunoassays for the detection of the corticosteroid 11-deoxycortisol (11-DC) using an scFv with high binding affinity (Kd = 1.3 × 10^10/M) [93]. The anti-11-DC binding protein was genetically linked with the enzyme alkaline phosphatase (ALP) to generate an enzyme-antibody fusion protein. This approach allowed development of a multi-step, non-competitive, immunoenzymometric assay (IEA) with an LOD of 20 attomol (Fig. 6a). The assay has a wide working range of 10 fg–100 ng per microwell. The suitability of the assay was evaluated through analysis of fortified serum samples. The same group subsequently reported the development of an simple one-step “open sandwich” immunoassay (OS-IA) for 11-DC (Fig. 6b) [94].
8. Summary

Different approaches are under investigation to produce alternatives to conventional IgG antibodies that can be used to detect small-molecule analytes. Some techniques have eliminated the need to use animals as host organisms for a specific immune response, but lower costs and shorter production times are other advantages. Table 1 gives an overview of different binders used in bioanalytical applications.

Polyclonal antibodies continue to be widely used in bioanalysis due to their low production cost [95–97]. As an alternative, mAbs can offer improved specificity but are more expensive to produce [98].

Researchers have demonstrated sdAbs or nanobodies from the heavy-chain-only antibodies produced by members of the camelidae family [1,16,24,99]. These binders display good stability and, when subjected to various denaturing conditions, it was observed that unfolding caused by chemicals or heat was reversible and biological activity was retained [37,38]. Their small size and good stability makes them ideal for fabrication of immunoaffinity columns with high capacity and enhanced stability [39]. It was observed that complex matrices do not affect their performance significantly [9] with respect to their use as bioanalytical tools in small-molecule-detection assays.

Protein scaffolds as alternatives to conventional IgG antibodies exploit pre-existing, natural binding proteins. These novel molecules provide favorable characteristics (e.g., robustness, ease of modification and cost-efficient production). The range of potential applications includes research, diagnostics and therapeutics. However, there is little evidence of the majority of these structures binding small molecules; one notable exception is the lipocalin protein scaffold [47] or anticalin [48]. While most research regarding these binders is directed towards medical applications, more investigation is required to realize their potential for use in small-molecule-detection assays.

MIPs continue to be developed as synthetic alternatives to antibodies. Very elegant chemistry is applied to design synthetic receptor molecules suitable for use in small-molecule-based sensing applications and MIPs represent an accessible, versatile means of creating specific synthetic binders. In addition to being easily adaptable to a variety of formats, these materials are typically stable, robust even to harsh chemical conditions and suited to small-molecule detection. These binders are ideal for field applications because of their chemical stability and ease of production and incorporation into portable formats.

Aptamer technology has been transferred from research to applications. A number of groups are now developing applications based on aptamers for detection of drug residues in complex biological matrices [35,36,38]. Various assay designs have been reported, many with adenosine as a model target, but, more recently, aptamers have been produced to bind drugs of abuse, mycotoxins, steroid hormones and antibiotics [22–26]. These are encouraging developments, but there needs to be further investigation with respect to the effects of sample matrices on aptamer performance in order for this promising binder to truly compete with immunoassays.

ScFv-binding proteins have become a mature technology in working assays for mycotoxins and drug residues in complex biological matrices [6,77,100,101]. Researchers have reported the production of enzyme-labeled scFv-binding proteins and their application in direct immunoassays to detect residues to the attomol level [93,94]. This has provided significant improvement in sensitivity compared to traditional assay formats (e.g., RIA and competitive ELISA).

Acknowledgements

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