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REVIEW ARTICLE

Alternative affinity tools: more attractive than antibodies?

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Antibodies are the most successful affinity tools used today, in both fundamental and applied research (diagnostics, purification and therapeutics). Nonetheless, antibodies do have their limitations, including high production costs and low stability. Alternative affinity tools based on nucleic acids (aptamers), polypeptides (engineered binding proteins) and inorganic matrices (molecular imprinted polymers) have received considerable attention. A major advantage of these alternatives concerns the efficient (microbial) production and *in vitro* selection procedures. The latter approach allows for the high-throughput optimization of aptamers and engineered binding proteins, e.g. aiming at enhanced chemical and physical stability. This has resulted in a rapid development of the fields of nucleic acid- and protein-based affinity tools and, although they are certainly not as widely used as antibodies, the number of their applications has steadily increased in recent years. In the present review, we compare the properties of the more conventional antibodies with these innovative affinity tools. Recent advances of affinity tool developments are described, both in a medical setting (e.g. diagnostics, therapeutics and drug delivery) and in several niche areas for which antibodies appear to be less attractive. Furthermore, an outlook is provided on anticipated future developments.

Key words: affinity tool, antibody, aptamer, engineered binding protein, molecular imprinted polymer.

INTRODUCTION

Many of the current applications in life sciences and biotechnology require the use of highly selective binders for the detection, purification or removal of specific molecules. The tools that allow these processes to proceed are generally referred to as affinity tools. At present, antibodies of mammals are the best characterized and most widely used affinity tools. Natural antibody targets (antigens) are surface molecules of invading entities (bacteria and viruses), including (poly)peptides and carbohydrates. However, owing to their unlimited variability, an infinite number of organic molecules are potential antigens that can be captured with very high affinity and specificity. Nowadays, antibodies can easily be produced using a range of techniques by either the immune system or synthetic libraries [1]. Nevertheless, antibodies do have their limitations [2]. They are reasonably sensitive to harsh conditions and are rapidly inactivated under acidic conditions, in the presence of proteases or at elevated temperatures. In addition, antibodies are large multidomain protein complexes with specific disulfide bonds and glycosylations. This implies that producing antibodies is generally difficult and expensive. Moreover, the extensive use of antibodies and their applications (e.g. as therapeutic agents) has resulted in a complicated patent situation [3]. Therefore, in recent years, other affinity tools, such as engineered binding proteins, aptamers and MIPs (molecular imprinted polymers), have gained a lot of interest as potential alternatives for antibodies in many applications. An overview of selected companies that are currently developing new affinity tools as alternative to antibodies is provided in Supplementary Table S1 (at http://www.BiochemJ.org/bj/436/bj4360001add.htm).

The new affinity tools have several advantages compared with antibodies. Attractive features include a reduced molecular mass, an enhanced stability, a more efficient selection and screening procedure, and cost-effective production methods. In the present review, we aim to provide an overview of the current state of antibodies, engineered binding proteins, aptamers and MIPs, as affinity tools for different applications. We compare the properties and production methods of each tool and provide information on screening and binder-selection procedures. Furthermore, we elaborate on the potential of each affinity tool and relate this to various applications in a medical setting (diagnostics, therapeutics and drug delivery) and several niche applications for which antibodies appear to be less suitable.

ANTIBODIES

Antibodies recognize their target with high specificity, and they generally bind it with a very high affinity, typically in the nano/pico-molar range. Depending on their origin, antibodies are polyclonal or monoclonal. A pAb (polyclonal antibody) is purified from blood serum of immunized mammals; it is a set of antibody variants that bind different epitopes of a target antigen, and are therefore not very specific. A mAb (monoclonal antibody) is a single antibody variant that is derived from a single cell line (hybridoma) [4]; mAbs bind only one epitope on a single antigen, making them more specific than pAbs [5].

Because of their high specificity and affinity, antibodies are useful for a wide variety of applications. Antibodies, especially mAbs, are nowadays the most widely used tool for diagnostic applications in fundamental and applied research.

Abbreviations used: CDR, complementarity-determining region; CHO, Chinese-hamster ovary; DARPin, designed ankyrin repeat protein; FDA, Food and Drug Administration; FN3, fibronectin type III domain; IP, intellectual property; LDC, limiting dilution cloning; mAb, monoclonal antibody; MIP, molecular imprinted polymer; pAb, polyclonal antibody; SELEX, systematic evolution of ligands by exponential enrichment; siRNA, small interfering RNA; SPE, solid-phase extraction; ssDNA, single-stranded DNA; VEGF, vascular endothelial growth factor.

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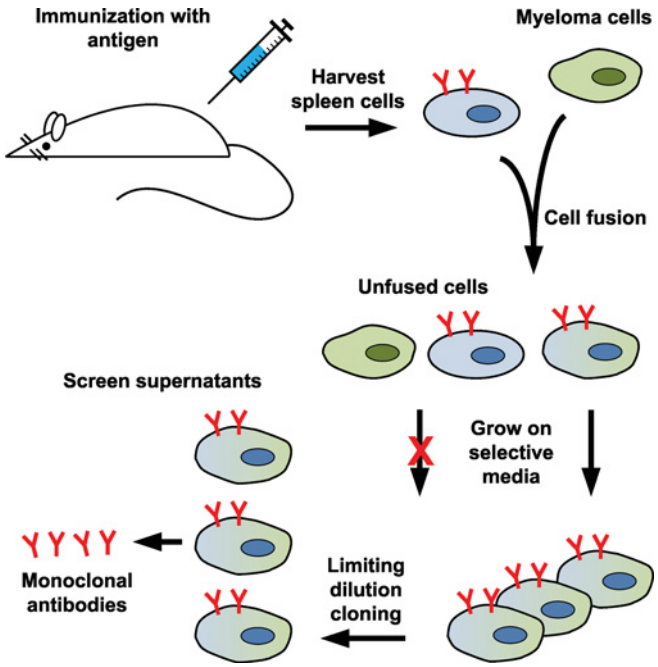

Figure 1 Hybridoma selection

When a mouse is injected with a certain antigen and detection of an immune response is confirmed, spleen cells are harvested and fused with myeloma cells. Fusion products are grown in selective media to select for fused cells. After LDC of fused cells, cell supernatants are screened for mAbs with the desired antigen specificity.

Furthermore, because of their potential to specifically interact with (neutralize) a target molecule, several FDA (U.S. Food and Drug Administration)-approved mAbs are used in the clinic as therapeutics in the treatment of diseases ranging from inflammation to cancer [6,7].

Hybridoma: an endless antibody supply

Once an antibody-producing hybridoma cell line is established, it provides an endless supply of mAbs, because hybridomas are fusions of antibody-producing spleen cells and immortal myeloma cells [4] (Figure 1). Immunization of mice with antigen is the first step to establish a hybridoma. An appropriate immunization protocol is critical for provoking an adequate immune response. Several strategies have been developed to increase throughput and success rate of immunization and to decrease the number of animals needed, including single-step immunization [8], genetic immunization [9] and multiplex immunization [10]. Only when an immune response is confirmed, can mouse spleen cells be harvested and fused with myeloma cells to continue hybridoma selection. After cell fusion, the mixture of hybridomas is generally diluted in such a way that each resulting microculture is derived from a single cell. This LDC (limiting dilution cloning) procedure is simple, but it is time-consuming and has a low throughput. Several other selection methods with higher throughput, based on flow cytometry and cell sorting, have been established [11]. The next step is screening of supernatants, to identify hybridomas that produce antibodies with the desired properties. To this end, the antigen microarray assay, basically a miniaturized ELISA, reduces the amount of time and reagents required compared with conventional ELISA screening [12]. Once appropriate hybridomas are selected, they can be preserved and used for antibody production.

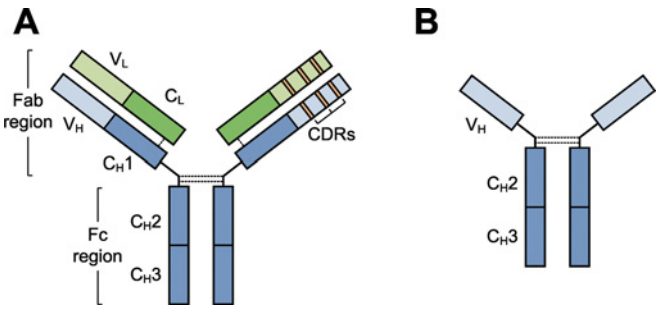

Figure 2 Schematic structures of a human IgG and heavy chain antibody

(**A**) Human IgG. Two heavy and light chains are linked together by disulfide bonds. The Fab region consists of constant (C) and variable (V) domains of the heavy (H) and light (L) chain. CDRs are located in the variable domains (V_H and V_L) in the Fab region. The Fc region consists of the constant heavy chain domains. (**B**) Heavy chain antibody. Two heavy chains, consisting of two constant regions (C) and one variable region (V), are linked together by disulfide bonds.

Regardless of whether antibodies are polyclonal or monoclonal, the most commonly used antibodies are of the IgG type. IgGs are Y-shaped and consist of two longer 'heavy' chains and two shorter 'light' chains bound together by disulfide bonds (Figure 2A). The Fab region consists of constant and variable domains of the heavy and light chain. Antigen binding takes place at the CDRs (complementarity-determining regions), which are located in the variable domains (V_H and V_L) in the Fab region. The Fc region consists of the constant heavy chain domains; it maintains stability and is involved in other interactions of the immune system.

Medical applications

Antibodies are often used in biochemical laboratories for numerous routine diagnostic tests such as ELISA and Western blotting. In a medical setting, these tests are generally used for the detection of infectious and parasitic diseases. In addition, ELISA is also often used for routine screening of food and environmental samples for microbial and chemical contamination. It is difficult to multiplex ELISAs because of cross-reactivity of secondary antibodies, which are required for enhanced sensitivity [13]. The pregnancy test is a well-known example of a cheap and easy to use diagnostic test that relies on antibodies [detection of the pregnancy-associated hormone hCG (human chorionic gonadotropin)]. Although this pregnancy test has been on the market for over 35 years [14], development of similar tests for other targets has been slow. However, increased interest in point-of-care diagnostics has rapidly increased the number of diagnostic tests available within the last 10 years [15], e.g. in 2004, the FDA approved the first rapid test for HIV in oral fluids [16]. The number of point-of-care applications is expected to increase steadily in the near future.

One of the most advanced applications of antibodies to date, however, is their use as therapeutic agents. Muromonab-CD3 (Orthoclone OKT3), an anti-CD3 antibody, became the first murine mAb to be approved by the FDA in 1986 as a therapeutic agent to prevent organ rejection after transplantation [17]. Administration of these murine antibodies, however, carried the risk of an undesired immune response (immunogenicity) and other side effects [18]. It took until 1994 before the second mAb [Abciximab (ReoPro), a chimaeric anti-GPIIb/IIIa antibody] received FDA approval, partly because the side effects of these murine mAbs had to be reduced; to date, over 30 mAbs are FDA-approved [19]. Technological advances have enabled production

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of chimaeric and later humanized antibodies (a human IgG scaffold with only the CDRs of murine origin), which have fewer side effects than their murine counterparts; the current state-of-the-art is a fully human antibody, produced by transgenic mice [20].

Therapeutic mAbs can operate through different mechanisms. Some mAbs act independently of the Fc domain of the antibody by blocking the interaction between a receptor and the receptor molecule (by binding to either one of them), or by inducing a signal transduction cascade upon receptor binding. Other mechanisms do require the Fc domain to recruit components of the immune system to lyse target cells by either antibody-dependent cell-mediated cytotoxicity or complement-dependent cytotoxicity [21].

Antibodies are also used for targeted drug delivery. For this purpose, mAbs are labelled with radioisotopes or toxic drugs, combining antibody specificity with enhanced toxicity because of their label. Two radioimmunoconjugates [Ibritumomab tiuxetan (Zevalin), anti-CD20, and Tositumomab-131I (Bexxar), anti-CD20] are FDA-approved [22], whereas the only FDA-approved conjugate with a toxin [Gemtuzumab ozogamicin (Mylotarg), anti-CD33] has recently been withdrawn from the market because of concerns about the product's safety and clinical benefits to patients [23]. Radioimmunoconjugates can also be used for *in vivo* imaging of tumours, to evaluate targeting or success of therapy. Specific binding of antibodies to their target allows for a very good signal-to-noise ratio. This imaging is Fc-independent, therefore conjugates of antibody fragments could be used for imaging as well.

Drawbacks of antibodies

High selectivity and affinity make antibodies especially suitable for diagnostic and therapeutic applications. Antibodies have a large and complex structure, which allows them to bind antigens and simultaneously recruit components of the immune system and, as we have seen before, this is an essential property in some forms of immunotherapy. However, owing to their complex structure, antibodies are susceptible to degradation, aggregation, modification (e.g. oxidation or deamidation) and denaturation. Moreover, the bulky nature of antibodies also limits their potential for some other applications; in affinity chromatography, smaller binders are preferred because of enhanced efficiency, whereas smaller binders exhibit an improved signal-to-noise ratio when applied to molecular imaging.

Antibodies have not yet been successfully produced in simple microbial hosts, because of their large complex structure and the required specific post-translational glycosylation. Hence they have to be produced in mammalian cell lines instead [e.g. NS0, Per.C6®, CHO (Chinese-hamster ovary) and HEK (human embryonic kidney)-293 cells], the cultivation of which is complex and costly (expensive media, long fermentation lead times, scaling issues, use of gases). Although nowadays mAbs are well produced and purified under mild conditions, they nonetheless remain sensitive to aggregation, deamination and oxidation. The use of antibodies is therefore often restricted to conditions resembling their physiological environment. In the presence of organic solvents or in other non-physiological conditions (e.g. elevated temperature, high pH and high salt concentration), they generally lose their function.

When administered intravenously, antibodies can have half-lives of several weeks [24]. A long half-life is desirable for some applications, because it increases efficacy and reduces the required dose. On the other hand, for certain applications, short half-lives are preferred, for instance when rapid clearance of toxic immunoconjugates is required to reduce whole-body exposure.

Size-related limitations of antibodies can be alleviated by using specific parts of antibodies that can be produced in microbial hosts (e.g. Fab regions or variable domains). These parts are smaller and, as such, they can reach deeper into tumours; in addition, half-lives can be tailored [24]. Removing fragments of the antibody that are not directly involved in target binding, however, can negatively affect affinity. In addition, antibody fragments tend to aggregate, making it difficult to purify them [25].

An alternative for antibody fragments are heavy chain antibodies (Figure 2B). Heavy chain antibodies are natural variants that lack a light chain; they are isolated from camelid species [26] and cartilaginous fish [27]. Heavy chain antibodies from camelid species are much smaller than classic antibodies, and, like artificially truncated antibody fragments, they can be produced in microbial hosts. As such, they are being optimized for certain specificities [28–30], by using high-throughput analyses such as phage display (see below).

ENGINEERED BINDING PROTEINS

In parallel to the development of antibody-based binding proteins, such as the antibody fragments and single-domain antibodies, independent studies have focused on modifying non-antibody proteins into binding proteins, with the goal of developing binding proteins with improved features [19]. The concept of engineering new binding functions is based on the molecular composition of antibodies (Figure 2). The typical structural element of an antibody is a well-conserved rigid scaffold on which highly variable loops (CDRs) are positioned. Similarly, binding proteins can be isolated from large libraries of protein variants with a constant framework and randomized variable regions that can, in principle, interact with any target molecule. These new binding proteins have great potential as affinity tools in various biotechnological applications and as therapeutic agents.

The generation of novel binding proteins

An important aspect of successful design and engineering of a binding protein scaffold is the ability to generate and analyse a large number of mutated derivatives. A powerful high-throughput technology for evolution-driven engineering is molecular display: the generation of large (poly)peptide libraries and subsequent selection for variants with desired biological and physicochemical properties. Display technologies are based on a physical link between a protein and its encoding gene, hence coupling phenotype and genotype. The most commonly used display technology is phage display [31]. However, other methods such as bacterial and yeast cell-surface display [32,33], ribosome display [34,35] and mRNA display [35] have also been successfully applied. In the present review, we will restrict ourselves to describing phage display for the selection of engineered binding proteins.

In phage display technology, nucleotide sequences encoding variants of peptides, antibodies or proteins are fused to a gene that encodes a phage coat protein. After correct assembly, phage particles display the encoded (poly)peptide on their surface [36]. The most widely used vectors for library construction are based on the filamentous phages fd, M13 and related phagemids [37–39]. However, display systems based on other phages and viruses have also been developed, including the lytic phages λ and T7 [40]. A current shortcoming of phage display is that only a

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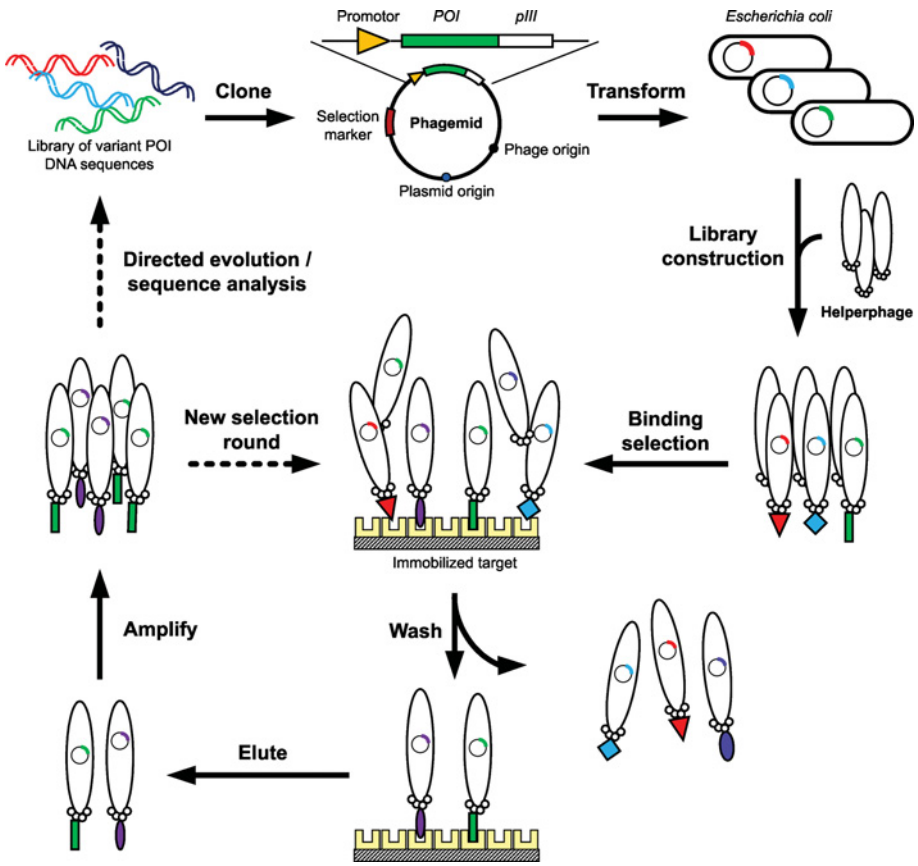

Figure 3 M13 phage display cycle

A library of DNA fragments encoding random variants of the protein of interest (POI) is created (fused to gene encoding M13 coat protein pIII) and cloned into a phagemid vector. *E. coli* cells are transformed with the obtained constructs and subsequently infected with helper-phage to create a library of phages, each displaying a variant of the protein of interest. The library is exposed to an immobilized target molecule. Non-binding phages are washed away. Bound phages are eluted and then amplified by infecting *E. coli* cells. This selection and amplification process can be repeated as necessary using more stringent washing conditions in order to obtain phages with the displayed protein of interest variants with the highest target-binding affinity. Finally, the DNA of high-affinity binding phages can be sequenced or subjected to another round of evolution.

limited number of vectors, libraries and complete systems are commercially available.

In general, when a phage display procedure with an M13 phagemid vector is used, a library of variant DNA sequences encoding a protein of interest is created using *in vitro* evolution techniques [41]. These variants are subsequently cloned into the phagemid vector as fusion to a coat protein gene (frequently the pIII-gene) (Figure 3). *Escherichia coli* cells are transformed with the phagemids and then infected with a helper-phage, generating a library of phages displaying the variant proteins. The phage library is exposed to an immobilized target molecule, and the phages with appropriate specificity and affinity are captured. The non-binding phages are washed off, although some non-specific binding can occur. Bound phages are eluted by conditions that disrupt the interaction between the displayed protein and the target. Eluted phages are then used to re-infect *E. coli* cells. The resulting amplified phage population is a secondary library that is highly enriched in phages displaying proteins that bind to the target. In an iterative process, these steps are repeated using washing steps with increasing stringency (bio-panning), resulting in a phage population enriched in a limited number of variants with the desired binding affinity and specificity. After several rounds of bio-panning (generally three to five), monoclonal phages may be selected and analysed individually. Target-binding (poly)peptides are identified by DNA sequence analysis of the phage and may subsequently be used as input material for another series of *in vitro* evolution in order to obtain a (poly)peptide with even higher binding affinity.

The diversity of protein scaffolds

In the last two decades, over 50 new alternative non-Ig protein scaffolds have been reported as potential affinity tools. These protein scaffolds comprise an extremely diverse group of binding molecules, which differ in many aspects such as origin, size, structural topology, engineering strategies, mode of interaction and applicability. The classification of these protein scaffolds is most often based on their structures and the strategies applied for engineering the binding affinity. Two examples of protein scaffolds used successfully are discussed in some more detail below; these and other protein scaffolds have been the focus of several excellent reviews [2,42–49].

FN3 (fibronectin type III domain) belongs to the immunoglobulin-like β-sandwich class of scaffold proteins. FN3 is a small 10-kDa domain occurring in many animal proteins involved in ligand binding (e.g. cell-surface receptors), and is one of the most widely used scaffolds today [50,51]. The β-sandwich structure consists of seven β-strands with three connecting loops on one end of the sheet, closely resembling the structure of an Ig variable domain (Figure 4A). Initially, two of the surface loops

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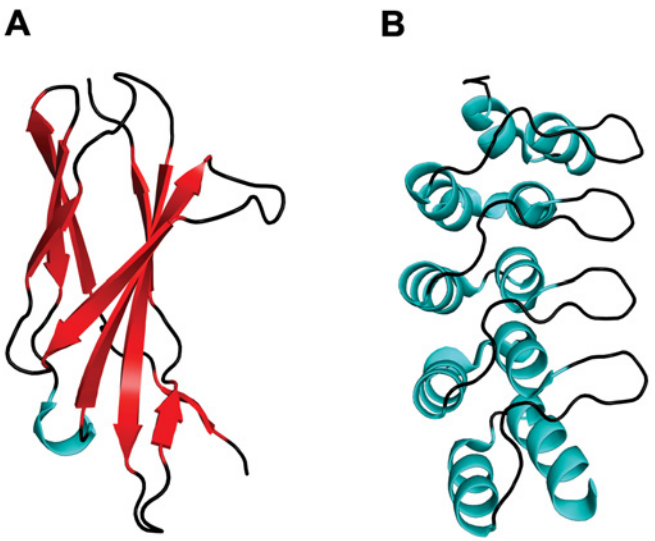

Figure 4 Three-dimensional structures of engineered binding proteins

The overall structures of an FN3 monobody (**A**) (PDB code 3K2M [131]) and a DARPin (**B**) (PDB code 1SVX [55]) are shown. The helices are displayed in cyan and the strands are in red.

were randomized, and binders were selected using phage display [51]. In a more recent study, all three surface loops were randomly varied, after which the best binders were selected successfully using mRNA display [52]. At AdNexus Therapeutics, FN3-based scaffold proteins (AdNectins) are being used to develop novel cancer therapeutics (see Table S1). The first AdNectin product concerns a protein designed to block the VEGFR2 [VEGF (vascular endothelial growth factor) receptor 2] signal transduction pathway, inhibiting the growth of new blood vessels in tumours.

DARPins (designed ankyrin repeat proteins) belong to the repeat protein class of scaffold proteins. DARPins are small (14–21 kDa) modular proteins derived from natural ankyrin repeat proteins, which are versatile binding proteins, the corresponding genes of which are highly abundant in the human genome [53,54]. DARPins have a modular architecture consisting of a single structural motif that occurs several times, and, after stacking, they form an elongated repetitive domain that is shielded by two capping repeats (Figure 4B). Libraries have been constructed in which residues residing in a loop and on a helix were randomized in each repeat; subsequently multiple repeats were randomly assembled [55]. The selection method most frequently used with DARPin libraries is ribosome display. High-affinity binders have been selected against targets such as maltose-binding protein [55] and an anti-IgE antibody [56].

Considerations for choosing the right scaffold

The wide variety of potential applications, such as therapeutics, diagnostics, affinity purification and molecular imaging, has led to the exploration of many different protein scaffolds. However, depending on the intended application, potential selection criteria include origin, size and structure of the protein to be used as a scaffold. For eventual commercial application of a selected scaffold protein, desirable features are their efficient production, high solubility, stability, specificity and a favourable IP (intellectual property) situation.

It is very important that the protein selected has a compact and structurally rigid core framework that has a relatively high intrinsic stability. This is an obvious requirement to generate a library by randomization of solvent-exposed amino acid residues, without affecting the scaffold's fold and half-life. The interface topography is a major determinant of protein–protein interactions. Therefore, if protein binding is intended, the shape of the selected protein's binding site (convex, flat or concave) could influence epitope selection and the binding mode [49].

In case the binding proteins are intended for therapeutic use, a potential problem of novel scaffolds may be their immunogenicity. Therefore considerations should be made about the origin of the protein used as a scaffold; compared with a protein from a distinct origin, a human protein is less likely to cause an immune response. Another disadvantage of using therapeutic proteins of non-human origin is the fact that they may face additional problems from regulatory authorities with respect to immunogenicity issues. However, it should be mentioned that human scaffold proteins may become immunogenic when multiple surface-exposed amino acids are randomized or when their binding site is altered.

Small size is often a desired feature of the selected protein used as a scaffold. For instance, in molecular imaging of tumour cells, the binding protein should rapidly find its target molecule in the patient, whereas unbound binding proteins should be quickly excreted; this will result in high-contrast tumour imaging and a reduced time between injection and imaging. Because small binding proteins tend to require less time for target recognition or for excretion, bigger scaffold proteins (e.g. antibodies) are less attractive for this particular application.

Stability of the selected protein scaffold is of great importance when used for affinity-purification applications. The protein should be sufficiently resistant to elevated temperatures, organic solvents, detergents and pH changes; in addition, the column material should preferably be cleanable and reusable. Most of the above-described conditions are detrimental to proteins, especially antibodies, and it might therefore be interesting to investigate proteins from thermophilic micro-organisms as candidate scaffolds. Proteins from these thermophiles generally display a high intrinsic thermal and chemical stability [57,58].

The above considerations indicate that non-antibody protein scaffolds do not constitute a homogenous group with similar properties. It is difficult to predict which of these diverse proteins are most suitable for a certain application. Although several of these binding proteins may have a lot of potential for biotechnological applications, the development of these alternatives to antibodies is still in an early phase of proof-of-principle evaluation. With respect to medical applications, only a few have progressed to clinical trials. Furthermore, expertise is often limited to single laboratories, and only a few libraries of protein variants are commercially available [59].

APTAMERS

Aptamers are oligonucleotide ligands that are selected for high-affinity binding to molecular targets. Both RNA- and ssDNA (single-stranded DNA)-based aptamers have been described, generally with a size of 15–60 nt. Reported aptamer targets range from small organic molecules such as ethanolamine and acetylcholine [60,61] to large protein complexes [62,63] and even cells [64,65]. The term 'aptamer' is derived from the Latin word 'aptus' (to fit) and the Greek word 'meros' (part or piece). Aptamers can have affinities in the nanomolar range, which is comparable with that of mAbs [66]. Target-binding aptamers are generally selected using a so-called SELEX (systematic

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evolution of ligands by exponential enrichment) procedure, reported independently by two groups in 1990 [62,67].

The choice for either DNA or RNA as a basis for aptamer development depends on practical considerations and partly on the final application. RNA has a relatively flexible backbone compared with DNA, and as such it has a broader range of potential target molecules. However, an obvious practical drawback of RNA is the fact that it is more prone to chemical and enzymatic degradation. In addition, selection of RNA aptamers is more laborious as its processing requires more enzymatic steps. Although RNA stability issues can partly be overcome by using modified nucleotides (e.g. spiegelmers, see below), it might be desirable for some applications (e.g. biosensors or affinity chromatography) to use the naturally more robust DNA aptamers. To date, however, the only FDA-approved therapeutic aptamer (Pegaptanib, see below), is an RNA aptamer.

Aptamers are selected *in vitro*

In contrast with the classical production of antibodies in animals, all steps of a SELEX procedure take place in a test tube. The *in vitro* SELEX procedure consists of multiple selection rounds, enriching proper binders in each subsequent round. The principle of iterative enrichment of binding oligonucleotides resembles the selection of binding proteins by phage display. A large pool of synthetic oligonucleotides (10^{14}–10^{15} molecules) is used for selection. The pool typically has a random region of 30–80 nucleotides and is flanked by two fixed primer sequences. After selection, the resulting aptamers can be shortened in additional work to find the minimal sequence required for target binding, which is usually 15–60 nt (Figure 5).

During each selection round, target-binding oligonucleotides are separated from those that bind the target with lower affinity or not at all. Efficient enrichment of the best binding sequences is essential, and several SELEX strategies have been developed to achieve this, including affinity chromatography [68], capillary electrophoresis [69] and target-coated magnetic beads [70] (for a more complete overview of SELEX strategies, see [71]). Selection strategies depend on the desired affinity and specificity of the aptamer, the properties of the nucleic acid (RNA or DNA) used during selection, and also the properties of the target (e.g. size, charge or presence of functional groups). In addition, aptamers with different features can be selected: e.g. aptamers that are very specific for a given target and do not bind any similar molecules [66], aptamers that self-report target binding [72,73], or aptamers that catalyse a chemical reaction (ribozymes) [68]. However, incorporation of such features requires a well-designed selection strategy.

Sequences are mixed with target and those bound to the target are recovered and subsequently PCR-amplified. For selection of DNA aptamers, the two strands of the PCR products are separated and one strand is recovered. For selection of RNA aptamers, transcription of the DNA is required before selection, and reverse transcription of the bound fraction is carried out before PCR amplification. In both cases, the resulting enriched pool is the starting material for the next round of selection.

After eight to fifteen selection rounds, remaining sequences are cloned and sequenced. In some rare cases, the pool of potential aptamers is dominated by just one sequence [68]; in most cases, however, a number of different sequences that all fold in similar ways are present [74,75]. After sequencing, further characterization of several individual sequences is needed to determine the binding kinetics and specificity of binding, to select the aptamer that is most suitable for a predefined application.

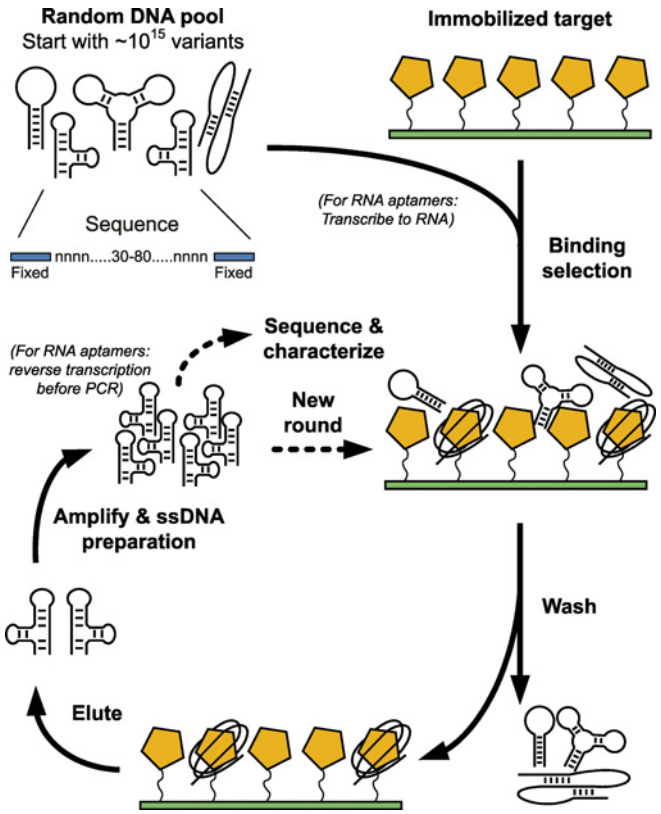

Figure 5 Selection of DNA aptamers

A library of random DNA fragments, with a random and two fixed regions, is exposed to an immobilized target molecule. Non-binding DNA molecules are washed away. Bound DNA molecules are eluted, PCR-amplified and made single-stranded. For selection of RNA aptamers, the DNA pool is transcribed to RNA before target binding, and the bound RNA sequences are reverse-transcribed before PCR. The selection and amplification process can be repeated as necessary using more stringent washing conditions in order to obtain higher-affinity molecules. Finally, the DNA molecules can be sequenced and characterized further.

The versatility of aptamers

Like antibodies and engineered binding proteins, aptamers can specifically bind targets of interest. Unique features of aptamers, however, are their small size (15–60 nt equals 5–20 kDa; antibodies are 150–160 kDa) and the relative ease of chemical modification (e.g. biotinylation or addition of fluorescent labels). These properties make them useful for a wide variety of applications. Aptamers can, for instance, substitute for antibodies in the frequently used ELISA assays [76], and they have potential for generating different types of biosensors (see below).

One feature of aptamers is of particular interest for the development of new types of biosensors: upon target binding, the structure of an aptamer changes to some extent. In contrast with antibodies, this structural change can be used to design aptamer sensors that are similar to molecular beacons [77], using a fluorophore–quencher pair at the ends of the molecule, or by incorporation of fluorescent base pair analogues in the sequence [72,78]. As a result of the structural changes induced by target binding, the fluorophore moves away from the quencher. Consequently, fluorescence is no longer absorbed by the quencher and this can be measured. Following the same principle, electrochemical detection of target binding is possible [79]. Measuring target binding as a result of structural changes is impossible for antibodies, because any structural changes are very

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small and it is difficult to incorporate a fluorophore–quencher pair within an antibody [80].

Unlike antibodies, aptamers are easily regenerated, at high temperatures or with high salt concentrations, and because of their small size, they can be efficiently immobilized on surfaces with high densities. This allows the design of small and reusable biosensors. Regeneration is also of interest for affinity-purification processes. Aptamers conjugated to resins can, for instance, be used to purify proteins, without a tag, from crude cell extracts [81], or they can be used for the separation of chiral compounds, provided that the aptamer is specific enough for one of the enantiomers [82].

In 2004, Pegaptanib (Macugen) [83] became the first, and currently the only, aptamer-based therapeutic to be approved by the FDA. This RNA aptamer has an antagonistic function, by binding all isoforms, except the smallest ($VEGF_{121}$), of human VEGF, a major regulator of aberrant and excessive blood vessel growth and permeability in the eye. The aptamer does not provoke any undesired immune response because of its nucleic acid origin [84]. However, 2 years later, an antibody-based treatment, Lucentis (antibody fragment), was approved and took over the market, because it can bind all VEGF isoforms, including the smallest. Despite this setback for the aptamer field, Pegaptanib clearly demonstrated the potential of aptamers as therapeutics, and currently several aptamer-based therapeutics are in clinical trials [85]. In addition to therapeutics, aptamers can also be used for targeted drug delivery by coupling them to liposomes [86] or micelles [87] that are loaded with drugs. A recent exciting development in targeted drug delivery is the usage of aptamer–siRNA (small interfering RNA) chimaeras [88–90]. The aptamer domain of the chimaera specifically binds to a receptor at the surface of a target cell, after which the RNA molecule is imported by endocytosis, and subsequently released from the endosome to enter the RNAi (RNA interference) pathway, via which the siRNA domain ensures silencing of selected target genes.

In the field of biomarker discovery, a new aptamer-based strategy has been described recently [91,92]. A protein sample is incubated with a mixture of aptamers that contain biotin and a photocleavable group. Aptamer–protein complexes are then captured on streptavidin beads. Once bound, proteins in the complexes are biotinylated and, after washing, the complexes are cleaved from the streptavidin bead. Biotinylated proteins in the complexes are again captured on streptavidin beads; after washing, bound aptamers are eluted and used for hybridization on a microarray. This is a multiplex proteomic approach allowing for high-throughput comparison of proteome profiles, whereas multiplexing in antibody-based methods is very difficult, as described above. This method can potentially be used to select new biomarkers that are specifically related to certain diseases, and to identify potential targets for treatment.

Drawbacks of aptamers

Numerous aptamer sequences for a wide variety of targets have been published, and aptamers have found their way into several applications [85,93]. SELEX, however, is currently rather labour-intensive, because optimal selection conditions (e.g. selection buffer, number of selection rounds) are target- and application-dependent and need to be optimized for each target. Efforts have been made to automate the SELEX procedure [94], but because optimal selection conditions can vary per target, it is still not straightforward to select a functional aptamer. It is, however, possible to develop aptamers that function under non-physiological conditions. For example, an aptamer against ochratoxin-A that was selected in aqueous buffer, also performed well in the presence of 20 % methanol [95].

Potential commercial developments using aptamers are slowed down because only a few companies possess the IP pertaining to aptamers and SELEX technologies. In 1999, Gilead Science Inc. acquired many patents related to general aptamer-selection strategies and of many individual aptamers. Archemix is now exclusively licensed to use the IP for therapeutic applications, and SomaLogic for diagnostic applications. This centralized IP position does not leave room for many competitors, but it should be noted that Archemix has issued commercial therapeutic licences to several companies. In the coming years, however, some patents will expire, allowing more companies to develop aptamer-based applications, possibly resulting in a boost of commercial applications in the near future [96,97].

In contrast with antibodies, aptamers are small and very stable; the latter feature is especially true for those made of DNA. Nevertheless, aptamers made of the naturally occurring four bases are not fully resistant to degradation; especially in blood serum, unmodified aptamers are rapidly degraded or filtered out of the bloodstream. Modifications in the DNA or RNA backbone or modified nucleobases are often used to make aptamers less susceptible to degradation by nucleases [98]. Modifications can be incorporated either chemically after selection or enzymatically during selection. The most widely used modifications are those that replace the 2′-OH of the ribose sugar, for instance by $-NH_2$, -F and $-OCH_3$; these modified nucleotides are all compatible with the enzymatic steps in the SELEX procedure [98,99]. Frequently used post-selection modifications include: (i) addition of 3′-end caps that reverse the polarity of the oligonucleotide (avoiding 3′ exonuclease activity), and (ii) addition of PEG [poly(ethylene glycol)] molecules at the 5′-end of the aptamer, to fine-tune their *in vivo* half-lives [85]. A completely different approach to prevent degradation makes use of non-naturally occurring L-RNA aptamers, or Spiegelmers [100]; owing to their non-natural origin, they are not recognized by RNases and hence they are very resistant to degradation. In addition, modified nucleotides are used to increase the chemical diversity of the random pool, which is otherwise limited to four bases. This has recently allowed for the selection of aptamers for targets against which no aptamers could be selected with an unmodified pool [101].

MOLECULAR IMPRINTED POLYMERS

A MIP is a polymer that is made in the presence of a template molecule, which is extracted afterwards, thus leaving complementary cavities behind. The procedure to produce these cavities by molecular imprinting was originally published in 1949 [102]. Unlike the above-described affinity tools, MIPs are entirely synthetic scaffolds that have no biological origin. The imprinted polymer material is able to recognize a single target molecule, varying from small organic molecules to proteins, or a group of structurally related molecules based on their shape, size and chemical functionality.

The process of molecular imprinting

Molecular imprinting takes place in several steps (Figure 6). It starts with selection of a template molecule that resembles the final target, and of functional monomers based on their ability to interact with functional groups of the template molecule. Functional monomers bind to the template molecule during a pre-polymerization step and are, subsequently, cross-linked to form the imprinted polymer. Finally, the template molecule is removed

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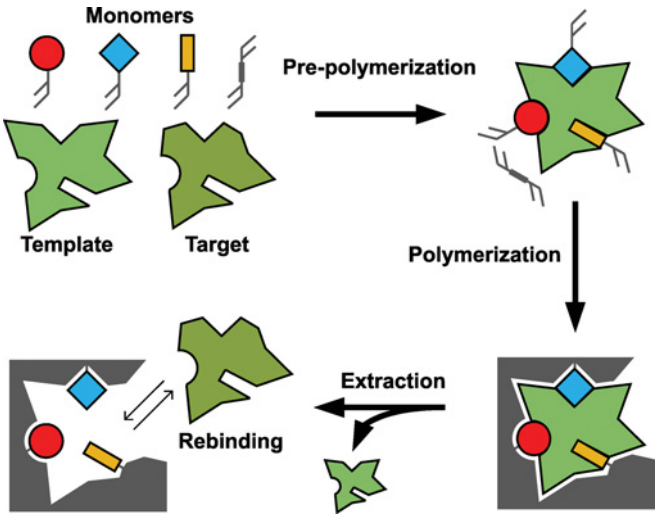

Figure 6 Molecular imprinting

Suitable monomers, and a template molecule that resembles the target molecule, are selected and mixed together for pre-polymerization. After polymerization, the template molecule is extracted, and the resulting cavity can be used for target binding.

from the matrix, leaving behind a cavity complementary in size and shape to the template. The cavity obtained can work as a selective binding site for a specific molecule.

Two major imprinting approaches can be distinguished on the basis of the interaction between the functional monomer and the template molecule. During imprinting, the template and monomers are linked either by covalent or by non-covalent interactions, such as hydrogen-bonding and Van der Waals interactions. Covalent imprinting generally produces more homogeneous binding sites (analogous to mAbs); however, template removal is often difficult. Non-covalent imprinting generally produces more heterogeneous binding sites, with a rather wide affinity range for the target (analogous to pAbs). Non-covalent imprinting is used most often nowadays, mainly because the process is simple and a wide variety of monomers are commercially available. Design of better monomers and optimized polymerization conditions for the non-covalent imprinting approach has resulted in the production of more homogeneous binding sites [103]. Selectivity and binding kinetics of MIPs need to be determined after imprinting, using the MIP as stationary phase in HPLC columns, or by performing batch rebinding studies [104].

MIPs can be made for small organic molecules as well as for proteins. Conditions for protein imprinting, however, are more restricted. Polymerization can only take place in the aqueous phase, because of limited protein stability in organic solvents. Protein imprinting has been studied intensively, and several imprinting strategies have been developed [105,106]. The different protein-imprinting strategies can be distinguished into three categories on the basis of the parts of the protein that are imprinted [105,107]: (i) bulk imprinting, in which the whole protein is imprinted; (ii) surface imprinting, in which only a part of the surface of the protein is imprinted; and (iii) epitope imprinting, where only a small, but typical, part of the protein is used for imprinting. MIPs produced by epitope imprinting can recognize the whole protein by binding a specific part of it, analogous to the way a mAb recognizes an antigen.

MIP composition and shape are important

Molecular imprinting looks deceptively easy, but, in fact, many aspects can influence the final properties of the MIP. Choice of template and functional monomers [108] obviously influence the end product, but the ratio of these molecules is also important. Porosity of MIPs depends on the solvent and temperature used for polymerization. Porosity is important, because pores that are too small limit flowthrough or make cavities inaccessible, whereas pores that are too large limit the overall binding capacity of the MIP. It is difficult to screen a large number of different polymerization conditions, by conventional techniques, to find the optimal conditions. Several routine conditions for molecular imprinting have therefore been developed over the years, although these might not be the best conditions to make a MIP for any specific target [109]. A semi-automated combinatorial imprinting strategy for preparation of MIPs under different conditions, and subsequent evaluation of these MIPS, has been developed and may shorten the time required to determine optimal polymerization conditions [110].

Conventional methods for MIP production result in large polymer blocks; subsequent grinding and sieving yield similarly sized, but irregularly shaped, particles. Irregular particles are not a problem for certain applications [e.g. SPEs (solid-phase extractions)], but some other applications require size- and shape-defined particles (e.g. *in vivo* applications). Size and shape-defined MIPs can nowadays be produced in several different formats such as bead, membrane, monolayer, nanowire or dendrimer [111].

MIPs work well in non-aqueous environments

MIPs are very stable because of their synthetic nature; they can resist high temperatures and a wide pH range, and can be stored for several years without losing affinity [112]. Unlike the affinity tools described before, MIPs work especially well in organic solvents instead of aqueous solutions. Production of MIPs is cheap and fast, once appropriate functional monomers and cross-linkers have been chosen. Numerous imprinted polymers, capable of binding toxins (e.g. Bisphenol A), pesticides (e.g. catechol), antibiotics (e.g. penicillin G) are now commercially available (see companies in Supplementary Table S1 at http://www.BiochemJ.org/bj/436/bj4360001add.htm), and some companies even offer to develop custom-made MIPs.

Imprinted polymers are most frequently used in SPE, a separation process that is used to concentrate target molecules or to remove contaminants during downstream processing. SPE is often used for sample preparation before analysis [113], for instance for the separation of enantiomers, or concentration of analytes from waste water or food samples. To ensure effectiveness of SPE, however, conditions should closely resemble those originally used to prepare the MIP. Even when using these conditions, extensive optimization of the SPE procedure is often required [113]. Most MIPs are prepared in organic solvents, hence it is difficult to use aqueous solutions directly [114]; conventional SPE can be used in this case to exchange aqueous solutions for those that more closely resemble polymerization conditions.

Application of MIPs in a molecular-imprinted sorbent assay, analogous to ELISA, for detection of theophylline and diazepam was reported for the first time in 1993 [115]. Binding affinity of target molecules to MIPs was deduced from a competition assay with non-labelled and radiolabelled target. Results and reproducibility were good compared with existing assays, but the procedure is more time-consuming and requires the synthesis of radiolabelled target compounds. Although this assay does not proceed well in aqueous solutions, it does work very well in

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organic solvents. In addition to these molecular-imprinted sorbent assays, MIPs are currently also used in chemical sensors; with different modes of detection, e.g. electrochemical, optical and mass-sensitive [116].

The application of MIPs in the drug discovery process was described previously [117]. Molecular imprints of receptor-binding molecules are made and, subsequently, used for screening compound libraries to identify molecules that have agonistic or antagonistic properties [118]. Imprinted cavities can alternatively be used as template for synthesis of compounds that fit within the cavity, mimicking the template molecule [119,120].

Few reports about *in vivo* applications of MIPs have been published, but efforts have been made to use MIPs for adsorption of cholesterol from body fluids [121]. MIPs have only recently been successfully used for absorption of melittin, which induces cell lysis at high concentrations in the bloodstream of living mice [122]. However, as the majority of MIP applications are restricted to non-aqueous environments, *in vitro* applications will be the main area of use for MIP technology.

THE MOST ATTRACTIVE AFFINITY TOOL

In the previous sections, the production, selection and relevant characteristics have been described for antibodies, engineered binding proteins, aptamers and MIPs. However, an important question remains unanswered: which tool has the most attractive features? This question is quite difficult to answer, because there is not a single affinity tool that fits all of the requirements for all of the applications. Each affinity tool has its own specific characteristics (see Table 1) and, depending on the intended application, one tool has more potential than another. In this section, we discuss the applicability of each affinity tool and relate this to various applications.

Affinity chromatography

The purification of proteins or small molecules by affinity chromatography is widely applied in all areas of life sciences and biotechnology. The process relies on purifying the target protein or molecule of interest from a complex mixture of molecules, on the basis of the target's specific and high-affinity binding to an immobilized ligand, resulting, in the ideal case, in a pure target in a single step.

Antibodies are currently used for antigen purification, immunoprecipitation and pull-down assays; however, they are rarely used for downstream processing of proteins or other molecules on an industrial scale. In general, large quantities of the affinity ligand are required to construct such columns. In addition, antibodies have a limited stability, a large size and high production costs, and are therefore not the first choice for this application. Recently, however, a new development in immunoaffinity chromatography was reported, where an anti-caffeine camelid antibody was used for the separation of caffeine and related methylxanthines [123].

Aptamers are very promising in the field of affinity chromatography [124]. Human L-selectin was purified from CHO cell-conditioned medium using a DNA-aptamer immobilized as an affinity chromatography matrix [125]. More recently, Taq DNA polymerase was purified using a DNA-aptamer immobilized on magnetic beads [126]. However, despite their promise as tools in affinity chromatography, there have been relatively few examples of the use of aptamers [127]. This may, at least in part, be due to their variable stability and their sensitivity to nuclease activity. However, it can be expected that stabilized non-natural nucleotides will circumvent the instability problem of natural oligonucleotides, and that aptamers will find a wider application as ligands for protein or small-molecule purification in the near future.

MIPs have specifically received interest for the extraction of small molecules from complex food and other biological samples. There are various examples of successful MIPs for relatively low-molecular-mass molecules, such as antibiotics and toxins [128]. MIPs are very well suited for SPE of small molecules; for example, a range of highly selective SPE phases based on MIPs have recently become commercially available (Biotage AB). However, MIPs, are at this moment, not suitable for protein purification purposes because of their typically harsh operational conditions.

Engineered binding proteins are potentially the most attractive alternative to antibodies in affinity chromatography. They are more likely to withstand the harsh column washing and regeneration conditions and are cheaper to produce due to their smaller size and simple scaffolds. At this moment, the most frequent use of affinity chromatography using an engineered binding protein is the purification of antibodies using recombinant Protein A or Protein G [129]. Recently, a number of Affibody proteins coupled to affinity resins were demonstrated to specifically capture their target molecule from complex mixtures [47]. In addition, Affibody proteins have been included in commercially available depletion columns from Agilent Technologies in order to remove interfering high-abundant proteins from human samples (e.g. plasma, serum). These results strongly suggest great potential for the development of engineered binding protein resins for the purification of specific proteins and other molecules.

Diagnostics

Antibodies are at this moment the best and most frequently applied tool for routine diagnostic tasks. ELISA and Western blot analyses are used for analyses in a range of medical applications, but also for analysing food and environmental samples. In addition, despite their relatively high costs and limited stability, antibodies are increasingly used as point-of-care diagnostics, such as pregnancy and HIV tests [15,16]. Commercial availability is an important contributor to the widespread use of antibodies; it compensates for their relatively high price and limited stability to some extent. Development of cheaper or more stable alternatives is to be expected in the future. However, actual implementation of an alternative affinity tool for an existing application has often proven to be difficult. Once a procedure has been established and validated, the advantages of using an alternative affinity tool must be worth the effort of repeating all of the laborious and expensive testing to validate the procedure. Alternatives for antibodies, e.g. MIPs [115] and aptamers [130], have indeed been developed for ELISA assays; however, because of the above-mentioned reasons, they are not yet widely applied. Antibodies remain the most attractive tool for routine diagnostic tests. However, in case an assay should be performed at non-physiological conditions, or in organic solvents, aptamers, MIPs or engineered binding proteins are certainly worthy of consideration as a binding tool.

Therapeutics

When an affinity tool has to be developed from scratch, the characteristics of each tool and prerequisites for the intended application should be evaluated carefully in order to select

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Table 1 Affinity tool characteristics

n.a., not applicable.

Characteristic	Antibody	Binding protein	Aptamers (ssDNA and RNA)	MIPs
Size (kDa)	~150–160	<30	5–20 (15–60 nt)	n.a.
Selection	*In vivo*	*In vitro*	*In vitro*	*In vitro*
Production	Animal or recombinant	Recombinant	Synthetic	Synthetic (large scale, low costs)
Post-selection modifications	Possible, but heterogeneous products	Possible, can be designed for homogeneous products	Wide variety of options (sugar, base or phosphate; 5′, 3′ or internal)	Possible, but should be taken into account during imprinting
Stability	Several weeks at 4°C	Variable	DNA: years at room temperature; RNA: several months at − 80°C	Years at room temperature
Binding site	Monoclonal: homogeneous; polyclonal: heterogeneous	Homogeneous	Homogeneous	Depending on imprinting strategy, heterogeneous or homogeneous
Target molecules	Mainly immunogenic macromolecules	Macromolecules and low-molecular-mass molecules	Low-molecular-mass molecules, macromolecules and cells	Mainly low-molecular-mass molecules (<1000)
In vivo half-lives	Days to weeks	n.a.	Untreated: seconds to minutes; treated: days	n.a.
Application conditions	Physiological	Physiological and non-physiological	Physiological, non-physiological and organic solvents (to some extent)	Mainly organic solvents, aqueous solutions to some extent

the most appropriate tool. Requirements for human *in vivo* applications, for example, are the most demanding, in terms of legislation and complexity of the conditions in which the tool should operate. This is probably why antibodies have received most attention, because they can be selected and produced in an environment that closely resembles human *in vivo* conditions. Over the years, antibodies have come a long way from their initial *in vivo* selection and production, and have become very successful especially as therapeutics; many problems concerning immunogenicity have recently been solved [18,19]. However, as antibodies also have their limitations, alternative tools have certainly gained interest; one aptamer has been FDA-approved, and several aptamers and several engineered binding proteins are currently in clinical trials [19,99]. In this respect, MIPs receive less attention, despite the recent development of using them for detection and binding of melittin *in vivo* [122]. Before MIPs can enter clinical trials, several key issues need to be addressed, such as compatibility of MIPs with aqueous solutions.

Although the potential for *in vivo* application of aptamers and engineered binding proteins is clearly demonstrated, it will take many years before they can compete with the success of antibodies. For antibodies, however, it also took years before they became successful, perhaps best illustrated by the 8 years it took before a second antibody therapeutic was FDA-approved, in 1994, after approval of the first antibody therapeutic, in 1986.

OUTLOOK

At present, antibodies are the most popular and best established affinity tools, especially in diagnostics. It appears very unlikely that alternative affinity tools will play a significant role in the field of diagnostics soon, simply because of the wealth of antibody-based assays that are readily available. Still, there may be exceptions. The alternative affinity tools are, at this moment, more attractive for applications in which antibodies are not well suited, for reasons that relate to size, physical and chemical stability, production costs and requirement for high-throughput analysis. For instance, affinity chromatography and SPE generally require cheap and stable affinity tools. Antibodies are too expensive and too unstable for most of these applications; hence application of engineered binding proteins, aptamers or MIPs might be more attractive in these cases. Moreover, aptamers and engineered binding proteins are both very promising for *in vivo* imaging, because they are much smaller than full-length antibodies, and hence can better penetrate tissues, resulting in increased signal to noise ratios. It is important to note, however, that, although diverse applications may require different affinity tools (Table 2), the most attractive tool for a certain application may not necessarily be the one with the best biochemical and physical features. Obviously, economic properties, such as the commercial availability and ease of selecting improved variants, are also very important considerations.

Table 2 Suitability of affinity tools for various applications

+, reported in literature; ++, commercially available.

Application	Antibodies	Engineered binding proteins	Aptamers	MIPs
Therapeutics/treatment	++	+	++	
Targeted drug delivery	++	+	+	
Molecular imaging	++	+	+	
Drug discovery				+
Diagnostics	++	+	++	+
Affinity purification	+	++	+	++
Biosensors		+	+	++

As research on aptamers, engineered binding proteins and MIPs continues, it is anticipated that, in the cases of the aforementioned niche applications in which antibodies perform poorly, they will either progressively replace antibodies or enable completely novel applications. It is tempting to speculate that the types of affinity ligands discussed will eventually become readily available for routine diagnostic and industrial applications, as a useful complement of the currently available antibodies.

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online data

SUPPLEMENTARY ONLINE DATA

Alternative affinity tools: more attractive than antibodies?

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Table S1 Selected companies developing affinity tools based on novel scaffolds

For a more extensive list of therapeutic antibodies, see [6,7] of the main paper.

Affinity tool	Company	Acronym	Scaffold	www address
Engineered affinity proteins	Adnexus (acquired by Bristol-Myers Squibb)	AdNectins	Human tenth FN3	http://www.adnexustx.com
	Affibody	Affibodies	Protein-binding domain of *Staphylococcus aureus* protein A	http://www.affibody.com http://www.affibody.com
	Amgen (formerly Avidia)	Avimer	LDL (low-density lipoprotein) cell-surface receptor domain A	http://wwwext.amgen.com
	Arana (formerly EvoGenix Therapeutics)	Evibodies	Human cytotoxic-associated antigen (CTLA-4)	http://www.arana.com
	Pieris AG	Anticalins	Lipocalins	http://www.pieris-ag.com
	BioTech Studio	Telobodies	Human PDZ domains	http://www.biotechstudio.com
	Bionexis	AFIM	Phospholipid interaction domain of annexin	http://www.bionexis.com
	Borean Pharma	Tetranectins	Human C-type lectin domain (CTLD)	http://www.boreanpharma.com
	Covagen	Fynomer	Fyn SH3 (Src homology 3) domain	http://covagen.ch
	Dyax		Kunitz domain of trypsin inhibitors	http://www.dyax.com
	Immunocore (formerly Avidex)	mTCR	Monoclonal T-cell receptors	http://www.immunocore.com
	Molecular Partners	DARPins	Ankyrin repeat protein	http://www.molecularpartners.com
	NascaCell (formerly Selecore)	Microbodies	Cysteine knots or knottins	
	Pacific Northwest National Laboratory, Department of Energy		Top7	http://www.pnl.gov
	Scil Proteins	Affilin	Human ubiquitin/human γ-crystallin	http://www.scilproteins.com
	Emergent Biosolutions (formerly Trubion Pharmaceuticals)	SMIPs	Binding domain from annexin	http://www.truemergent.com
Aptamers	AdAlta		Peptide aptamers	http://www.adalta.com.au
	Aptagen	Aptabody	Nucleic acid aptamers	http://www.aptagen.com
	AptaRes	MonoLex	Nucleic acid aptamers	http://www.aptares.net
	Archemix		Nucleic acid aptamers	http://www.archemix.com
	Imaxio		Peptide aptamers	http://www.imaxio.com
	NascaCell (formerly Selecore)		Nucleic acid aptamers	http://www.nascacell.de
	NOXXON Pharma AG		Nucleic acid spiegelmers	http://www.noxxon.com
	SomaLogic	SOMAmers	Nucleic acid aptamers	http://www.somalogic.com
MIPs	Biotage AB (formerly MIP Technologies)	AFFINILUTE	Molecular imprinted polymers	http://www.biotage.com
	PolyIntell	AFFINIMIP	Molecular imprinted polymers	http://www.polyintell.com
	Raptor Detection Inc.		Molecular imprinted polymers	http://www.raptordetection.com
	Semorex		Molecular imprinted polymers	http://www.semorex.com

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