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Aptamers as Functional Nucleic Acids: *In vitro* Selection and Biotechnological Applications

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Abstract Aptamers are functional nucleic acids that can specially bind to proteins, peptides, amino acids, nucleotides, drugs, vitamins and other organic and inorganic compounds. The aptamers are identified from random DNA or RNA libraries by a SELEX (systematic evolution of ligands by exponential amplification) process. As aptamers have the advantage, and potential ability to be released from the limitations of antibodies, they are attractive to a wide range of therapeutic and diagnostic applications. Aptamers, with a high-affinity and specificity, could fulfil molecular the recognition needs of various fields in biotechnology. In this work, we reviewed some aptamer selection techniques, properties, medical applications of their molecules and their biotechnological applications, such as ELONA (enzyme linked oligonucleotide assay), flow cytometry, biosensors, electrophoresis, chromatography and microarrays.

Keywords: aptamer, SELEX, ELONA, flow cytometry, biosensor, chromatography, microarray

INTRODUCTION

Aptamers are oligonucleotides (DNA or RNA molecules) that can bind with high affinity and specificity to a wide range of target molecules, such as proteins, peptides, amino acids, nucleotides, drugs, vitamins, and other organic and inorganic compounds.

Aptamers were discovered by the development of an *in vitro* selection and amplification technique, known as SELEX (Systematic Evolution of Ligands by EXponential enrichment) in 1990 [1-3], and was derived from a linguistic chimera composed of the Latin word "aptus" (meaning 'to fit') and the Greek suffix "–mer" [4]. As the name implies, aptamers have the capacity to distinguish targets from subtle structural differences, such as the existence of a methyl or hydroxyl group, in the target molecule [5]. The SELEX method has permitted the identification of unique oligonucleotide molecules, from random DNA and RNA libraries, which bind to the target molecules of interest. Using this technique many aptamers usually have affinity and specificity as high as an antigen-antibody complex [6,7].

In most medical applications, high affinity and specific molecular recognition are achieved by antibodies, but there are limitations, especially in their production, which requires animals or cell lines. Unlike antibodies, *in vitro* selected aptamers are reproducibly synthesized in a short time frame, and easily modified by chemical method to improve their affinity and stability. Moreover, as some aptamers have shown higher affinity and specificity toward target molecules than those of antibodies [8,9], they have many potential uses in many areas of research, and in applications based on molecular recognition, including diagnostic and therapeutic applications, such as an enzyme linked oligonucleotide assay (ELONA) [10], flow-cytometry [11,12] and biosensor [13].

Over the last twenty years, a large variety of aptamers have been isolated from combinatorial DNA and RNA libraries using the SELEX methodology [14-45]. As listed in Table 1, some oligonucleotide aptamers, and their target molecules, have been typically characterized. The continuous discovery of novel aptamers can further expand potential medicines and technologies based on molecular recognition.

APTAMER SELECTION TECHNIQUES

In 1990, the laboratories of G. F. Joyce (Research Institute of Scripps Clinic, La Jolla, USA) [1], J. W. Szostak (Massachusetts General Hospital, Boston, USA) [2] and L. Gold (University of Colorado, Boulder, USA) [3], independently discovered a technique allowing the rare functional nucleic acids to be isolated from pools of more than 10^{15} individual nucleic acid molecules for their particular functionality, such as the binding to target molecules of interest, or the *de novo* generation of ribozymes.

This technique of *in vitro* selection (also called *in vitro* evolution) is commonly known as "SELEX". This novel technique has been under development for a decade, and

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Table 1. Oligonucleotide aptamers and their target molecules

Target molecule	Type of aptamer	Reference
Proteins		
Syrian golden hamster prion protein	RNA	[14]
Escherichia coli SelB protein	RNA	[15]
L-Selectin protein	DNA	[16]
Tyrosine phosphatase	RNA	[17]
Ff gene 5 protein	DNA	[18]
Thrombin	RNA	[19]
Peptides		
Dopamine	RNA	[20]
Rex-binding element	RNA	[21]
CD4 antigen	RNA	[22]
Ras-binding domain	RNA	[23]
Amino acids		
L-Arginine	RNA	[24]
L-Argininamide	DNA	[25]
L-Valine	RNA	[26]
D-Tryptophan	RNA	[27]
Nucleotides		
Yeast phenylalanine tRNA	RNA	[28]
TAR RNA element of HIV-1	RNA	[29]
Oligo (dT)	RNA	[30]
5' UTR of RSETA mRNA	RNA	[31]
26S subgenomic RNA of Sindbis virus	RNA	[32]
Drugs		
Neomycin B	RNA	[33]
Streptomycin	RNA	[34]
Tobramycin	RNA	[35]
Tetracycline	RNA	[36]
Kanamycin A	RNA	[37]
Vitamins		
Cyanocobalamin	RNA	[38]
Biotin	RNA	[39]
Organic compounds		
ATP	RNA	[40]
FMN	RNA	[41]
Theophylline	RNA	[42]
Organic dyes	DNA	[43]
Inorganic compounds		
Malachite green	RNA	[44]
Mg^{2+}	RNA	[45]

is seen as an extremely useful method in the field of molecular biology. As a result, today, SELEX has become a general method for identification of oligonucleotide aptamers.

SELEX is an acronym for 'systematic evolution of ligands by exponential amplification', which uses large populations of random RNA or DNA (dsDNA or ssDNA) sequences as the raw material for the selection of aptamers. Aptamers are selected from the mainly random pool of RNA or DNA by column chromatography, or other selection techniques suitable for the enrichment of

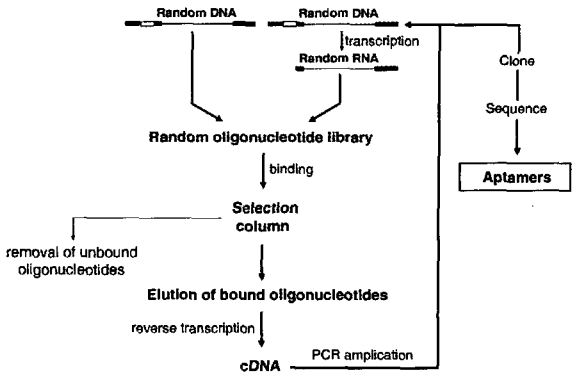

Fig. 1. Generalized schematic diagram of selection of aptamers in the SELEX process.

a desired property. As a result, the aptamers recognize target molecules with high affinity and specificity [46]. Combinatorial libraries, based on replicable nucleic acids, offer the convenience of iterative amplification of their members, making the screening process easy and fast [4]. As the aptamers isolated from the random pool using the SELEX process use iterative rounds of affinity-based enrichment alternating, with oligonucleotide amplification, the sequences of tighter binding with the targets are isolated and amplified [47]. The selection is repeated using the enriched pool derived from the first round selection. As several rounds of this process lead to the desired sequences, such as aptamers, this technology has been applied to a wide range of targets, including various enzymes.

A schematic diagram of the SELEX process is shown in Fig. 1. SELEX is a procedure which involves repeating cycles of a selection round, followed by an amplification round, and thus generating a large pool of random oligonucleotide sequences. The starting pool is generated by a standard DNA-oligonucleotide synthesizer. The machine synthesizes an oligonucleotide with a completely random pool, which is flanked by defined primer binding sites. The starting pool must be quite large to generate a high probability of producing an active aptamers. In this step, an immense pool of up to 10^{15} different DNA molecules can be synthesized at the same time. Following the generation of a sufficiently large pool of random sequences, they are exposed to a target molecule. The target molecule is bound to a supporting medium, such as an affinity chromatographic column. Sequences showing no affinity for the target molecule immediately elute, while those that bind to a target must later be washed from the column. Those sequences exhibiting affinity for the target molecule are amplified by a polymerase chain reaction (PCR). This is followed by several more cycles of the same treatment. Successive selection and amplification cycles result in an exponential increase in the abundance of aptamers, until they dominate the population. The end result is a final oligonucleotide population that is dominated by aptamers that bind to the target molecules.

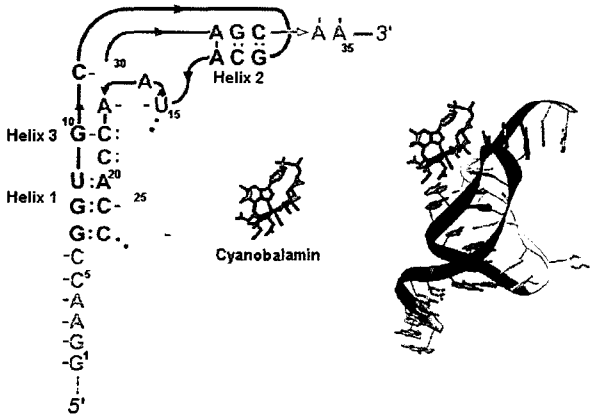

Fig. 2. The secondary and tertiary structures of the cyanocobalamin-binding RNA aptamer. Strands forming the triplex are colored dark blue (strand 1; nucleotides 7–11), cyan (strand 2; nucleotides 15–22) and yellow (strand 3; nucleotides 22–30). Strands forming the duplex are colored green (nucleotides 12–14) and magenta (nucleotides 31–33). Overhanging bases on the 5' and 3' ends are colored gray (the conformation of all of these nucleotides is defined by intermolecular contacts in the crystal lattice). Inset shows a stereo view of the tertiary structure using the same color scheme, with vitamin B_{12} colored red. Adapted from Reference 52.

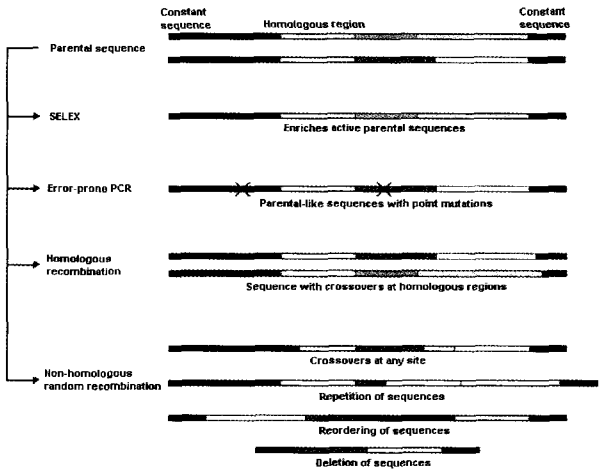

Fig. 3. Diversification methods for nucleic acid evolution. Starting with parental sequences, pure SELEX enriches active sequences. Error-prone PCR yields parental sequences with point mutations. Homologous recombination methods, such as DNA shuffling, allow crossovers between sequences only in regions of homology. Nonhomologous random recombination allows many other changes, including random crossovers between any sequences, repetition, reordering, reorienting, elimination of subsequences, or any combination of these changes. Adapted from Reference 53.

Also, the SELEX method has been applied to a number of different applications. For example, it has been used for the *de novo* isolation of catalytic RNAs and DNAs, such as a ribozyme, with polynucleotide kinase activity [40], and a deoxyribozyme, which cleaves DNA/RNA chimeric oligonucleotides [45]. Recently, ribozymes were modified to improve their catalytic activities, using a trans-splicing technique. These trans-splicing ribozymes contain altered flanking sequences to allow precise alignment of both 5'- and 3'-exon sequences during the trans-splicing, and contained an additional extended region complementarity to the chosen mRNA target [48, 49].

RNA aptamers have been actively identified using the SELEX method. The amplification step involved the reverse transcription of the RNA sequences, followed by PCR, which generated a large population of DNA encoding the desired aptamers. The double-stranded DNAs were then subjected to transcription, which regenerated the original RNA in large quantity. As a result, SELEX has permitted the identification of unique RNA aptamers from random RNA libraries that can bind target molecules, such as vitamins, antibiotics, cofactors, peptides or amino acids, with high affinities [50,51].

Crystallographic analysis of RNA aptamers, complexed with the small molecules they bind, can potentially provide important insights into the basic mechanisms by which RNAs fold into specific three dimensional structures, and the rules that define how they interact with small molecules. The structure of a cyanocobalamin-binding RNA aptamer is shown in Fig. 2. Lorsch and Szostak [38] isolated an RNA aptamer that specifically recognizes cyanocobalamin (vitamin B12). An RNA aptamer that binds the cyanocobalamin, with high affinity and specificity, was identified, and the 3 Å crystal structure of the cyanocobalamin-binding RNA aptamer determined. Central to this structure is a locally folding RNA triplex, stabilized by a novel three-stranded zipper. Perpendicular stacking of a duplex on this triplex creates a cleft that functions as the cyanocobalamin binding site [52].

Technological advances have already been made to eliminate the requirement for the fixed regions in random sequence libraries used in the SELEX process. This whole process is faster than the amount of time typically spent to generate a cell line that produces and purify a specific monoclonal antibody. The SELEX process has recently been automated to make aptamer discovery even faster and more economical. The automated platform carries out the iterative SELEX process with little or no human intervention.

Also, the development of SELEX methods, using modified nucleic acids, have been reported [53], and are shown in Fig. 3. In error-prone PCR techniques [54], point mutations are introduced at a frequency of approximately 1~10% per base per PCR reaction. In a pure SELEX method, repeated cycles of selection and amplification, without intentional mutagenesis between rounds, does not satisfy the exploration condition of DNA or RNA libraries in a purposeful and beneficial manner. In contrast, the SELEX method, with error-prone PCR, may enhance the desired binding or catalytic properties that arise at an accelerated rate, and can

Table 2. Comparison of aptamers with antibodies

Items	Antibodies	Aptamers
Identification	The identification of antibodies is restricted by *in vivo* parameters such as animals or cell lines.	Aptamers are identified through an *in vitro* process that does not required to animals or cell lines.
Production	The production of antibodies is laborious and can be produced costly for rare antibodies that requires a large scale of screening.	Aptamers are synthesized in short time frame at low cost and purified under denaturing conditions to very high degree of purity.
Limitation against target	Antibodies against target molecules that are inherently less immunogenic are difficult to generate.	Target molecules that do not elicit a good immune responses can be used to generated high-affinity aptamers.
Sensitivity to temperature	As antibodies are sensitive to temperature, they undergo irreversible denaturation.	Because functional aptamers could be regenerated easily within minutes after denaturation, aptamers undergo reversible denaturation.
Batch to batch variation	The performance of the same antibody tends to vary from batch to batch, requiring immunoassays to be reoptimized with each new batch of antibodies.	As aptamers are produced by chemical synthesis with extreme accuracy and reproducibility, they are little or no batch-to-batch variation.
Modification of kinetic parameter	The kinetic parameters of antibody-target molecule interactions cannot be changed on demand.	The kinetic parameters of aptamers antibody-target molecule complex can be changed for higher affinity, specificity, or stability.
Detective modification	Decrease or loss in affinity for antibody-target molecule can be caused by labeling of antibodies.	Fluorescein or biotin as reporter molecules can be attached to aptamers at precise locations not involved in binding.

compete with unmutated sequences. However, nucleic acids obtained by point mutagenesis, using an error-prone PCR technique, will still be quite similar to the nucleic acids in the starting pool. A nonhomologous random recombination (NRR) method was recently developed by Bittker *et al.* [53], which enables nucleic acid fragments to randomly recombine in a length-controlled manner, without the need for sequence homology, such as DNA shuffling [55], may offer substantial benefits over pure SELEX or error-prone PCR. In fact, starting with two parental sequences of modest streptavidin affinity, the evolution, using NRR for streptavidin-binding aptamers, resulted in aptamers with 15 to 20-fold higher affinities than the highest-affinity aptamers evolved using error-prone PCR, with a maximum 46-fold higher affinities than the parental sequences derived using SELEX [53]. Therefore, the NRR method can provide a powerful approach for the generation of aptamers as functional nucleic acids.

PROPERTIES OF APTAMER

For several decades, antibodies have been the most popular molecules in providing ligands as target molecules for recognition. Consequently, antibodies have contributed largely to the wide range of medical applications based on molecular recognition. However, because antibodies are produced in cultured cells, they have certain limitations.

Due to fast technological progress, the development of *in vitro* selection and amplification methodologies has allowed the facile identification of unique oligonuclotide ligands, referred to as aptamers, from random DNA and RNA libraries, which are released from the limitations of antibodies. Aptamers have the following properties:

- They are mostly the fragments of single stranded DNA or RNA.
- Are isolated from combinatorial nucleic acid libraries by *in vitro* selection method.
- Are usually less than 100 nucleotides in length.
- Possess a high affinity and specificity for a wide variety of target molecules.
- Are the nucleic acid equivalents of antibodies.
- Can be produced quickly by automation.

Aptamers are a new class of binding molecule presently competing with antibodies in the marketplace. For comparison, the advantages of aptamers, as a rival to antibodies, are summarized in Table 2. It is apparent that aptamers have superior characteristics, in that those binding to the targets molecules, with relatively high affinity and specificity, are generally small, easy to

manufacture and modify, and uniformly link to matrices. By preselecting the desired conditions for binding and eluting the target molecules, aptamers can be specifically designed to conveniently fit into an optimum purification scheme. Also, aptamers have the ability to discriminate between target and other closely related molecules [46]. The therapeutic and diagnostic potentials of aptamers may be applied to conventional pharmaceuticals. Even if aptamers must be able to, not only disrupt the function of a particular target, but also inhibit or modify the metabolism associated with that target, it is possible that they could specifically disrupt the function of their target molecules [56].

RNA aptamers are not as stable as their DNA counterparts, which has been enough to prompt researchers to try and overcome some of the daunting disadvantages, with some of the most obvious problems already having begun to be resolved [57]. While natural RNA and DNA molecules are relatively unstable in sera or within cells, chemically modified nucleic acids have been shown to be nuclease-resistant [57]. The development of stable nucleic acid aptamers has allowed researchers to proceed to the more difficult problems of delivery and bioavailability that still remain unknown. Even if the aptamers can be made bioavailable, their size and production cost remains problematic. Fortunately, many aptamers can form tight and specific interactions, and advances in synthetic methods may soon allow their economical synthesis. Finally, the specificity of aptamers for their targets may ward off the systemic side effects often associated with pharmaceuticals. This specificity may encourage the evolution of metabolic or viral resistance. A theoretical analysis indicates that high-affinity binding species fit snugly in the binding sites on their protein targets [56]

Aptamers and ribozymes are both functional nucleic acids. However, aptamers are distinguished from ribozymes by a functional feature. Aptamers are nucleic acids that act like an antibody in terms of the molecular recognition of target molecules, with high specificity and affinity, whereas ribozymes are RNA molecules capable of catalytic activity similar to that of enzymes.

APPLICATIONS OF APTAMER TECHNOLOGY

Therapeutics

There has been a continuing desire to develop new classes of specific ligands that could act as 'magic bullets', seeking out their molecular targets *in vivo* and destroying them. The possibility that aptamers might be candidate therapeutic agents has naturally received much attention [58]. The therapeutic potential of aptamers depends on many of the same issues that apply to more conventional pharmaceuticals. Firstly, they must interact tightly and specifically with their targets. Secondly, they must specifically disrupt the function of their targets. Finally, they must be able to, not only disrupt the function of a particular target, but also inhibit, or modify, the metabolism associated with that target [47]. For example, the aptamer antagonists of the toxin ricin have been isolated, with IC_{50} values in the nanomolar range [59]. Aptamers that inhibit the function of viral enzymes, such as HIV-1 reverse transcriptase [60], and the NS3 protease of hepatitis C virus, have also been isolated [61]. Other examples include the aptamers against viral surface proteins, such as human cytomegalovirus glycoproteins gB and gH, and can be used to neutralize cellular infectivity [62]. Similarly, aptamers against molecules such as Sialyl Lewis X, which is overexpressed on the surface of malignant cells, have antimetastatic potential, particularly in patients who are already receiving a continuous infusion of chemotherapeutic agents [63,64]. The completion of the aptamer selection process typically yields a high affinity and specific antagonist of the targeted protein. Several post selection optimization steps generally must be performed to translate a molecule from an *in vitro* antagonist to a molecule that can be tested for its pharmacological effects on animals, or used *in vivo* for target-validation studies. Once an aptamer suitable for chemical synthesis has been generated, additional modifications may be needed to enhance its bioavailability or delivery [65]. The bioavailability of an aptamer is determined by its molecular stability in biological fluids and systemic clearance. Because the stability of a nucleic acid in plasma is determined by its backbone composition the plasma stability of an RNA oligonucleotide can be increased by substitution of the ribonucleotides with 2'-amino, 2'-fluoro, or 2'-*O*-alkyl nucleotides [65,66]. For example, the stability of the modified anti-VEGF (vascular endothelial growth factor) aptamer in serum was increased at least 1000-fold relative to the unsubstituted RNAs, while the modified anti-VEGF aptamer in urine could survive for up to 17 h. Furthermore, because 2'-amino or 2'-fluoro CTP and UTP can be readily incorporated into RNA by *in vitro* transcription, these backbone modifications can be introduced into the combinatorial library at the outset of the selection process [67]. The resulting compounds are typically stable in human plasma for 15-24 h at 37°C. Typical of such aptamers is a 31-nucleotide 2'-fluorine/2'-oxymethyl mixed RNA aptamer (ADR58, GlaxoSmithKline), which binds to the proinflammatory cytokine oncostatin M with a low nanomolar affinity [68]. The ability of ADR58 to inhibit the binding of this cytokine to its receptor suggests such aptamers might have a useful role in the management of inflammatory diseases, such as rheumatoid arthritis [64]. An aptamer can be protected from exonuclease degradation by capping its 3' end [66]. Resistance to endonuclease degradation can be further increased by additional substitution of the ribose and deoxyribose nucleotides by modified nucleotides, or nonnucleotide linkers where possible [65]. A minimized aptamer, 25 to 40 nucleotides in length, corresponds to a molecular weight of about 8,250–13,200 Da. Due to their small size, aptamers are cleared from animals via the kidneys in minutes. Their clearance rates can be rationally altered by increasing their effective molecular size, such as by the site-specific addition of various

molecular weight polyethylene glycol (PEG) moieties or other hydrophobic groups, or by attachment of the aptamer to the surface of a liposome [69]. Conjugation of the aptamer to either lipids or polyethylene glycol has been reported to sufficiently improve the stability and distribution kinetics of DNA aptamers to produce therapeutic effects [58,70,71]. Coagulation is the normal process by which a fibrin clot is generated in response to a vascular injury. Conversely, a thrombosis is the pathological formation of a clot in response to injury, stasis or hypercoagulability. In these processes, thrombin is the most obvious target for the generation of both anticoagulant and antithrombotic compounds. Bock *et al.* [72] generated a 15 nucleotide DNA based thrombin aptamer that binds thrombin with moderate affinity (apparent $K_d = 10^{-7}$ M), and can prolong the clotting time of human plasma. As well as its ability to inhibit clot-bound thrombin and platelet thrombus formation *in* and *ex vivo* whole artery angioplasty models, this aptamer also exhibited potential as a novel antithrombotic [73]. However, for this thrombin aptamer to be successfully tested in animal arterial thrombosis models, modification would probably be necessary to improve its circulating half-life. Physiological coagulates are initiated by the enzymatic activity of protease FVIIa in complex with its requisite co factor and tissue factor. Recently, a 2'-amino modified-RNA aptamer has been generated to coagulate FVIIa. This aptamer binds FVIIa with high affinity, and has been shown to block its activity by preventing the formation of an active FVIIa–tissue factor complex. This aptamer is stable in plasma, with a half-life of approximately 15 h [74]. Angiogenesis, the growth of new blood vessels from pre-existing vessels, has been implicated in a variety of human diseases. VEGF is a central positive regulator of physiological angiogenesis. Inhibition of the function of VEGF, by means of VEGF neutralizing antibodies, has been shown to reduce tumor growth and vascularization in animal models [75]. Ruckman *et al.* [76] characterized a family of high affinity, nuclease-resistant aptamers against VEGF-165, which is the most abundant isoform. The association of these aptamers, with large inert entities, such as liposomes and PEG, has been shown to dramatically reduce aptamer clearance. The PEG-conjugated 2'F aptamer, also known as NX1838, has demonstrated activity against both vascular cell proliferation and permeability [76], and was the first to have reached phase I clinical trials. It has been shown to inhibit pathogenic angiogenesis in model systems, and is intended for the treatment of blindness induced by macular degeneration. It was found to be safe, and relatively long-lasting, following injection into the vitreous humor of rhesus macaques [77].

Diagnostics

Aptamers hold much promise as molecular recognition tools for their incorporation into analytical devices. Like antibodies, aptamers that react with a variety of targets can be selected, with high affinities and specificities for those targets. Aptamers are smaller and less complex than antibodies, and consequently may be easier to manufacture and modify. Aptamers can be raised against toxic, small or otherwise poorly immunogenic antigens. They may circumvent problems with biopolymer denaturation during storage, and are ultimately cheaper than antibodies. Thus, it is not unreasonable to speculate that the large markets associated with immunodiagnostics will see an increasing challenge from kits based on nucleic acid shape recognition. Aptamers have recently been used in flow cytometry, biosensors, affinity probe capillary electrophoresis, capillary electrochromatography and affinity chromatography [47]. One example of a new ligand showing potential in this area is an RNA aptamer that binds to S-adenosylhomocysteine (SAH), with an affinity and selectivity comparable to that of a monoclonal antibody. SAH is a potential diagnostic marker for cardiovascular disease, as it is the direct and only source of homocysteine, which is a marker known to be elevated in the plasma or serum with certain forms of cardiovascular disease [8]. Theophylline is a naturally occurring alkaloid that is used as a bronchodilator for the treatment of asthma, bronchitis and emphysema. A diagnostic agent must be able to recognise theophylline, but must not be closely related to other compounds also found in serum, such as caffeine, which differs from theophylline by one methyl group only. One of the aptamers was selected for competitive binding experiments, in which it was shown that the K_d for theophylline and caffeine were 0.32 and 3,500 mM, respectively. This impressive discrimination between two molecules of such similar structure suggests that this approach has potential for generating diagnostic reagents [78,79]. The first step in exploiting an aptamer is in its conjugation to a detectable moiety, an enzyme or a generic ligand. Nucleic acids can be radioactively 'body labelled', during transcription or replication, by the use of modified nucleotides [80]. In addition to body labelling, site-specific labelling with a radionuclide can also be useful. For example, a stannyl nucleotide was incorporated at the 5' end of a DNA aptamer during chemical synthesis, with the tin subsequently being replaced by ^{123}I in an oxidation reaction [81]. Aptamers can also be labelled using fluorescent or reactive groups. Here, the challenge is to introduce the detectable groups at positions that do not interfere with the ligand properties [58]. Labelling at the 3' end can be achieved by templated extension using Klenow polymerase, by T4 RNA ligase-mediated ligation and terminal deoxynucleotidyl transferase [82,83]. Labelling at the 5' end can be achieved by the supplementation of the *in vitro* transcription mix, with a 10-fold excess of guanosine-5'-*O*-(2-thiodiphosphate) over GTP, in which the thiol can then be used to attach biotin [84].

Enzyme Linked Oligonucleotide Assay (ELONA)

The two-site binding assay, also referred to as the sandwich assay, is one of the most commonly used diagnostic formats today. In this approach, the analyte is sandwiched between two ligands, one used as the capture, with the other used as the detector. Aptamers have also

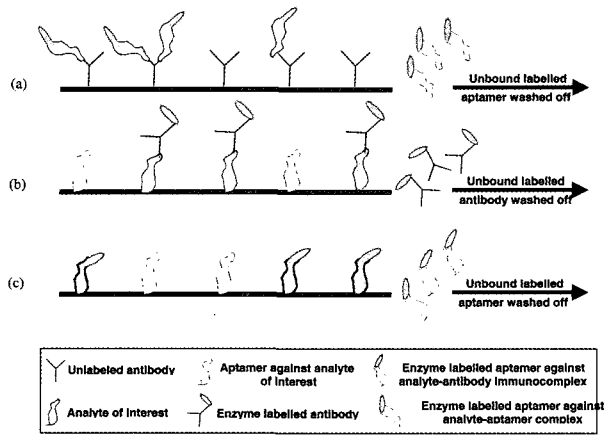

Fig. 4. Schematic of the possible assay formats for use in ELONAs and biosensors. (a) Aptamers as capture molecules, and antibodies as reporter molecules. (b) Antibodies as capture molecules, and labelled aptamers against the antibody-analyte immunocomplex as reporter molecules. (c) Aptamers as capture molecules and labelled aptamers against the aptamer-analyte complex as reporter molecules. Adapted from Reference 4.

been tested in two-site binding assays, as depicted in Fig. 4 [4]. Drolet *et al.* [85] synthesized an aptamer specific to VEGF, with a fluorescein group attached via a phosphodiester bond at the 5' end. They coated microtiter plates with a monoclonal antibody specific to VEGF, then reacted samples containing known amounts of VEGF to the bound antibodies. The fluoresceinated aptamers were bound to the VEGF, which were in turn bound to anti-fluorescein antibodies conjugated to alkaline phosphatase. The relative quantities of VEGF in each well were measured by the addition of a chemiluminescent alkaline phosphatase substrate [85]. In another example, the simultaneous binding of an aptamer and an antibody, to human CD4 expressed on cell surfaces, was demonstrated [84]. The two-site binding assays, based on the aptamers described above, involved an antibody as the second ligand. This was mainly due few aptamers sharing dissimilar, or non-overlapping, binding sites on the target of interest. Lochrie *et al.* [86] selected aptamers that bound either to nucleocapsid protein, or to the matrix protein, in the HIV type-1 gag polyprotein target. These two classes of aptamers did not compete in binding to the HIV-1 gag polyprotein. Tasset *et al.* [87] described the identification of DNA aptamers that bound human thrombin at a site different from those site bound by the aptamers isolated by Bock *et al.* [72]. The identification of two classes of aptamers with non-overlapping binding sites could have been the result of the two different partitioning methods used in the selections. The above aptamer examples could be applied to the format of a heterogeneous assay. In this method, the target is captured by the primary aptamer immobilized on a solid support, forming a target-aptamer complex, which is then specifically recognized by the secondary aptamer conjugated to a suitable reporter molecule [4]. The use of aptamers thus facilitates the detection of small and large molecular weight analytes in a sandwich-type format, overcoming the limitations of the competitive-type assays, allowing the development of robust and sensitive assays. The use of ELONA system was patented by NexStar (now Gilead Sciences, Foster City, California) (Fig. 4) [88].

Flow Cytometry

Flow cytometry is a powerful analytical tool that allows multiparameter analyses of cells and microsphere particles. Today, the technique is being used in basic research as well as in clinical diagnostics. Because particles can be distinguished on the basis of their color and size, flow cytometry is an attractive platform for multiplex analyses. Truncated DNA aptamers, labelled with fluorescein, have been chemically synthesized for staining cells that express L-selectin. The anti-L-selectin aptamers stained both leukocytes and neutrophils expressing L-selectin in human peripheral blood mononuclear cell preparations. This result was very similar to that obtained with an anti-L-selectin antibody [16,89]. Davis *et al.* modified a high affinity anti-HNE aptamer by attaching fluorescein groups to different residues via a variety of linkers. The fluoresceinated aptamers were incubated with polystyrene beads coated with HNE, and the resulting complexes passed through a flow cytometer; some of the modified aptamers proved to be more sensitive for detection than fluoresceinated antibodies [11,90]. This approach could be applied for the detection of cells expressing particular proteins on their surfaces; for example, aptamers specific for tumor antigens might be used to detect the growth or spread of tumors [47].

Biosensor

Certain applications demand analyte detection within a very short time period. Sensors based on molecular recognition, coupled to a transducer, have been developed to meet the needs of rapid detection. Therefore, it seems logical to explore the use of aptamers as the molecular recognition element in biosensors. The application of aptamers as biocomponents in biosensors offers a multitude of advantages over the 'state of the art' in affinity sensing [52]. One of the obvious limitations of immunosensors is their poor capacity to regenerate the antibody surface, however, several advantages are apparent in aptamer-based sensors. The ability to regenerate the function of immobilized aptamers would be their most attractive feature. Being nucleic acids, aptamers could be subjected to repeated cycles of denaturation and renaturation. Secondly, homogeneous preparations of chemically synthesized aptamers, with appropriate linkers, could be deposited precisely on a solid surface at a desired density. Finally, aptamers could be easily labelled with a wide range of reporters, enabling the design of a variety of detection methods [4]. Kleinjung *et al.* [91] immobilized a biotinylated RNA

aptamer, selective toward L-adenosine, on an optical fiber surface derivatized with streptavidin. The surface of the optical fiber was derivatized with avidin, after which the biotinylated RNA aptamers were added. Using the biosensor, and monitoring by the total internal reflection fluorescence, they were able to obtain the association and dissociation rates, resulting in the detection of L-adenosine in the submicromolar range. Potyrailo *et al.* [92] designed a biosensor that offered a one-step direct detection of the analyte. A DNA aptamer, specific for human thrombin, was used to detect the binding of the target protein by evanescent wave-induced fluorescence anisotropy. Here, the 5' end of the aptamer was labelled with fluorescein and its 3' end was modified with an alkyl amine attached to a glass surface. The assay was completed in 10 min, and as little as 5 nM of protein could be detected in an addressed volume of 5 nl. Lee and Walt [93] detected thrombin by displacement of fluoresceinated thrombin from DNA aptamers linked to silica microspheres in a fiber-optic biosensor system. The aptamer beads selectively bound to the target, and could be reused with no sensitivity change.

Unlabelled aptamers can also be used in certain analytical methods. For example, surface plasmon resonance (SPR) methods, such as those exploited by the BIAcore system (Biacore Inc., USA), have been used to detect activated 2',5'-oligoadenylate synthetase and CD4. In these cases, the aptamer was in the mobile phase, and the target immobilized. However, with appropriate derivatization and immobilization, aptamers could be used to detect specific molecules in complex mixtures using SPR [22]. An unexplored area of aptamers is in sensors based on electrochemical detection. Aptamers, being polyanionic, may be attractive for sensing the changes in conductance in the presence, or absence, of target binding. Although this research is still in its infancy, it has great potential in the area of molecular sensing [4].

A novel class of fluorogenic probes called molecular beacons was recently introduced for the homogeneous detection of nucleic acid sequences. Molecular beacons are simple hairpin-loop probes, in which a fluorophore is linked to the 3' end of an aptamer, and a quencher to the 5' end, but adjacent in the 3D structure. The nucleic acid sequence in the loop of the molecular beacon is designed to be complementary to the target of interest. When ligand binds, the conformational rearrangement of the aptamer removes the quencher from the fluorochrome, resulting in an easily detected signal (Fig. 5). Yamamoto and Kumar [94] reported the development of an immobilizable molecular beacon aptamer, which fluoresces specifically in the presence of TAT-1 protein, derived from HIV-1 or HIV-2, but not in the presence of RNA binding proteins, and they are working towards its incorporation into a biosensor [95].

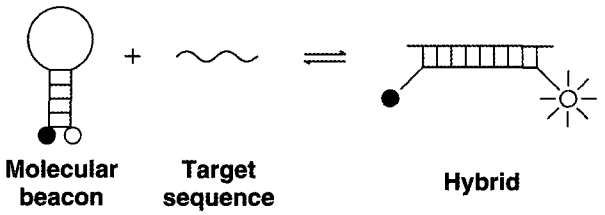

Fig. 5. Schematic representation of the light-generating mechanism for the molecular beacon that occurs upon binding to proteins.

Capillary Electrophoresis and Capillary Electrochromatography

Capillary electrophoresis (CE) is a separation technique traditionally used to separate analytes based on differences in their size and/or charge. Affinity probe CE and capillary electrochromatography (CEC), in contrast, are capable of separating uncharged species. German *et al.* [95] incorporated a DNA aptamer into an affinity probe for electrophoresis. Fluorescently labelled aptamers have been used to quantify IgE and thrombin, using a rapid method, based on capillary electrophoresis/laser induced fluorescence, in which detection down to 50 pM was reported. Kotia *et al.* [96] used two ssDNA aptamers as stationary phases, in capillary electrochromatography experiments, to separate nontarget molecules. One of the aptamers was earlier isolated based on its affinity for thrombin [72], the second having a similar oligonucleotide sequence. The aptamers were covalently bound to fused-silica capillaries using several immobilization schemes. Capillaries derivatized with aptamer, using each immobilization scheme, were used to successfully separate mixtures of amino acids, enantiomers and polycyclic aromatic hydrocarbons [96]. Rehder and McGown [97] also used aptamer-derivatized capillaries to separate nontarget molecules in CEC. The aptamer used was generated for its stability, and based on the structure of the aptamer specific for the human thrombin mentioned above. The aptamer was coated to the wall of a fused-silica capillary via the method used by O'Donnell *et al.* [98]. The capillaries were used to separate two forms of β-lactoglobulin, which differed by only two amino acid residues. The separation occurring with the linker-coated capillaries resulted in a much greater retention time. These results led to the conclusion that the aptamer-coated capillaries achieved a much gentler, less denaturing, separation than the hydrocarbon stationary phase, which may be important when considering the separation of proteins and peptides [97].

Affinity Chromatography

Affinity chromatography is based on the molecular recognition of the stationary phase for the analyte. Aptamers are very promising in this field due to their capability for molecular recognition, and theoretically, they can be generated specifically for a molecule [50]. Romig *et al.* [46] incorporated an aptamer specific for L-selectin into an affinity chromatography column. The aptamer was biotinylated at the 5'-end, and incubated with a streptavidin-linked resin. The resin was then packed into a chromatographic column, and the resulting

column used to purify the human L-selectin-Ig fusion protein produced in Chinese hamster ovary cells transfected to express the protein. When the aptamer column was used in the initial purification step, a 1500-fold purification, with an 83% single step recovery, was the result [46]. Deng *et al.* [99] used an aptameric stationary phase to separate cyclic-AMP, NAD, AMP, ADP, ATP and adenosine. The aptamer used had earlier been isolated due to its ability to bind adenosine/ATP [100,101]. The biotinylated aptamer was incubated with polystyrene porous particles or streptavidin porous glass beads. The particles were then packed in fused-silica capillaries, resulting in affinity chromatography capillaries. The resulting particles contained 20 nM binding sites per 100 μL of media. This value is 3.3 times larger than that reported for antibodies on similar media [99].

Microarray

Microarray technology provides a means of analyzing a large number of molecules simultaneously. These analyses, carried out at the DNA level, are facilitated by the high affinity and specificity of the interactions driven by the complementary base pairing. For proteomics, ligands that capture proteins, with high affinity and specificity, could provide the basis for an array technology. Although antibody-based microarrays are being developed for the analysis of a large number of proteins [102], microarrays based on high-affinity aptamers would be very attractive for the following merits: i) Aptamer identification is a rapid process that can be performed on an automated platform; ii) Aptamers can be immobilized at a defined density at precise locations on a solid surface; iii) Homogeneous preparations of aptamers are readily available from chemical synthesis; iv) Microarrays based on aptamers are robust, and expected to have a long shelf life. v) Aptamers containing 5-halo-uracil analogs could be used to lock the bound proteins irreversibly; and vi) The formation of irreversible cross-linking between aptamers and proteins is highly specific and provides another dimension of specificity in addition to the specificity provided by affinity [4].

The technology, being developed by SomaLogic Inc. (Boulder, Colorado, USA), could lead to the use of thousands of different photo-crosslinkable aptamers on microarrays, which would enable the sampled proteins to be detected by conventional staining following covalent cross-linking and rigorous washing [64,103]. In the near future, aptamer microarrays are expected to play an important role in the arena of proteomics, which will not only facilitate better disease management by analyzing the expression of proteins by patients, but will also help in the discovery of new therapeutics by target validation [4].

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