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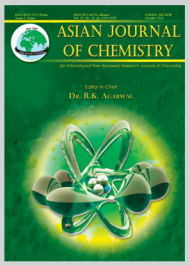

REVIEW

Aptamers as Recognition Elements for Analysis of Small Molecules

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Aptamers, which are functional oligonucleotides selected from random-sequence nucleic acids libraries *in vitro*, possess high recognition ability to specific targets. Owing to their inherent selectivity, affinity and multifarious advantages over the traditional recognition elements, aptamers can be considered as a valid alternative to antibodies or other biomimetic receptors and have been widely employed to design novel analytical methods. In this review, the evolving use of aptamers in analysis of small molecules by exploiting their molecular recognition properties is discussed.

Key Words: Aptamer, Small molecules, Biosensor, Analysis.

INTRODUCTION

For the highly sensitive analysis of small molecules, the selectivity of the recognition element is vital. As a new class of recognition elements, nucleic acid aptamers can recognize a large variety of small ligands, such as amino acids[1,2], nucleotides and derivatives[3,4], antibiotics[5-7] and other small inorganic/organic molecules[8-13]. Aptamers fold upon associating with their ligands into molecular architectures in which the ligand becomes an intrinsic part of the nucleic acid structure. Because the evolutionary pressure on aptamer sequences during selection is directed primarily toward the binding of the ligands, the three-dimensional structures of aptamer complexes reflect highly optimized scaffolds for specific ligand recognition[14]. Therefore, aptamers have high specificity and affinity for their ligands, which highlight the huge potential of aptamers for analytical applications.

Properties of aptamers: The term aptamer is derived from the Latin word "aptus" (meaning 'to fit') and the Greek word "meros" meaning particle[15]. Aptamers are typically single-stranded nucleic acids between 30 and 70 nucleotides in length. They are generated by a process called SELEX (systematic evolution of ligands by exponential enrichment, Fig. 1) which was first reported in 1990[15,16]. SELEX comprises an iterative process of *in vitro* selections using nucleic acid to target binding and partitioning events of unbound nucleic acids in order to isolate high-affinity nucleic acids from large pools of randomized sequence libraries. Starting libraries for SELEX usually contain more than 10^{15} different sequences. The initial library of random-sequence oligonucleotides, which is obtained through combinatorial chemical synthesis, is incubated with a target of interest. Oligonucleotides showing affinity for the target are partitioned by different methods, such as affinity chromatography[10,17], magnetic bead[18], capillary electrophoresis[19,20], microfluidics[21] and so on and then amplified by PCR (for DNA libraries) or reverse transcription PCR (for RNA libraries) to create a new pool enriched in those oligonucleotides having a higher affinity for the target. As this cycle of selection and amplification is repeated, the abundance of the high-affinity oligonucleotides increases exponentially. Negative selection and counter selection are often employed in order to remove aptamers which bind to supports and molecules similar to the target, respectively. Typically after 8 to 15 cycles, cloning and sequencing of the enriched library are carried out, revealing the sequence of oligonucleotides highly specific to the target. Once the sequence information has been obtained, the desired aptamer can be readily produced by chemical synthesis.

The multiple advantages of aptamers include their high specificity and affinity, their easy and highly reliable production by enzymatic or chemical synthesis, their regenerability by simple means and their storability. In addition, advantages over monoclonal antibodies are the higher inhibitory potential and the wider range of chemical modification possibilities because they can be synthesized enzymatically and chemically. Aptamers

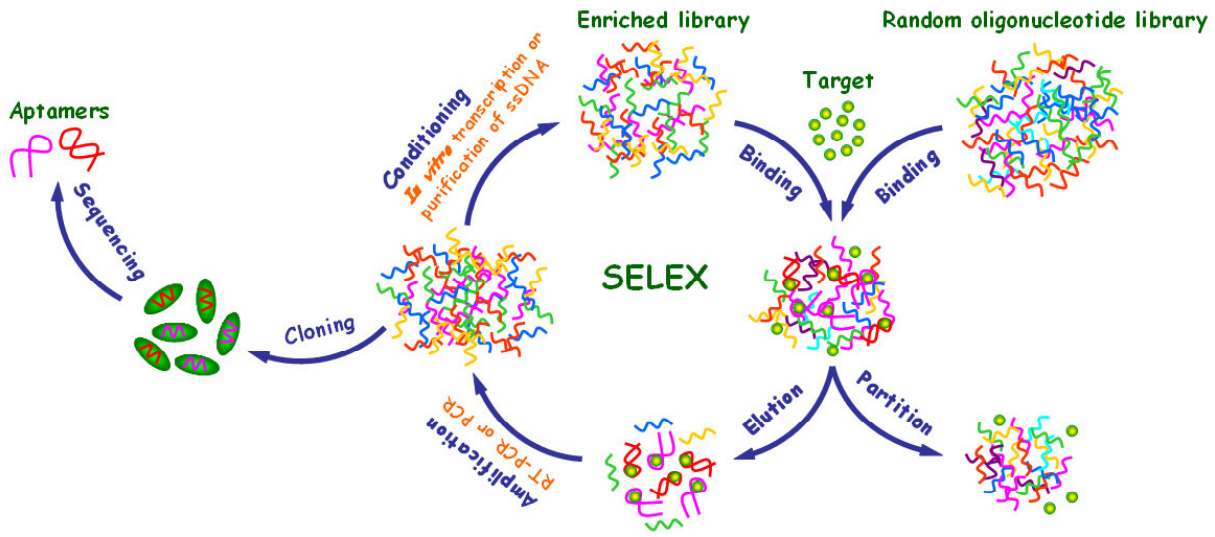

Fig. 1. Schematic representation of the SELEX process

are also stable, with no loss of activity under a wide range of buffer conditions and resistant to harsh treatments such as physical or chemical denaturation. Different chemical modification possibilities offer diverse immobilization properties using numerous additional molecules. Because it is possible to develop aptamers entirely *in vitro* without the need of cells or animal immune systems, aptamer generation offers the choice of a great diversity of binding conditions[22]. Interestingly, to date there has been no evidence for immunogenicity of aptamers when applied *in vivo*[23].

Aptamers that recognize small molecules: One of the biggest advantages of aptamers lies in their recognition for small molecules which can not stimulate the production of antibody molecules usually in bodies. In recent years, many aptamers have been isolated that recognize small molecules (Table-1). Meli *et al.*[24] isolated adenine-binding RNA aptamers and identified the adenine binding site which was composed of two independent secondary structure elements forming a bipartite binding site that interacts with adenine in a new mode of purine recognition. Vaish *et al.*[25] selected RNA aptamer to ATP. The RNA aptamer makes numerous contacts with ATP, including interactions with the triphosphate group. Recently, a DNA aptamer binding to 8-OHdG was selected by Miyachi *et al.*[26] using GMP-agarose as an analogue from a library of about 460 random ssDNA sources. This aptamer exhibited a high affinity for 8-OHdG with a Kc (the dissociation constant) of 0.1 μM.

Majerfeld *et al.*[27] selected histidine-binding RNA aptamers. The aptamers have a common internal loop-hairpin loop structure, based on a conserved $RAAGUGGGKKN_{0\text{-}36}$ $AUGUN_{0\text{-}2}AGKAACAG$. Notably, the repetitively isolated sequence contains two histidine anticodons, both implicated by conservation and chemical data in amino acid affinity, which strengthens experimental support for a "stereochemical" origin of the genetic code. Ravelet *et al.*[28] isolated an antityrosine RNA aptamer from a degenerate pool derived from a dopamine aptamer selected previously. The tyrosine-binding site of the aptamer would have some remembrance of the modalities of recognition of dopamine, which would explain its ability to bind with relative high affinity some structurally related analogs.

TABLE-1
SOME APTAMERS AND THEIR SMALL TARGET MOLECULES

Targets	Type of aptamer	Kd (μM)	References
Nucleotides and derivatives			
Adenine	RNA	10	[24]
ATP	RNA	4.8–11	[25]
8-OHdG	DNA	0.1	[26]
Amino acids			
Histidine	RNA	8–54	[27]
Tyrosine	RNA	25	[28]
Carbohydrates			
Sialyllactose	DNA	4.9	[29]
Antibiotics			
Daunomycin	DNA	0.02	[30]
Tetracyclines	DNA	0.063–0.483	[31]
Small organic molecules			
Ethanolamine	DNA	0.006–0.019	[32]
Thyroxine	RNA	50	[33]
Hemin	RNA	0.8	[34]
Diclofenac	DNA	0.042–0.166	[35]

The enantiomers of tyrosine and analogs (11 enantiomeric pairs) were separated using an immobilized antityrosine RNA aptamer.

As a carbohydrate, the sialyllactose is an essential receptor component of many viruses and its block could lead to the loss of viron binding and inhibition of the initial step of viral infection. Thus the sialyllactose-specific aptamer could be a useful tool for the study of cell adhesion and viral infection. To enhance binding of the aptamer, Masud *et al.*[29], used modified thymidine residues. These have a cationic protonated amino group at the C_5 position, which could enhance binding to sialyllactose, which has an anionic carboxyl group. The selected aptamer with the highest affinity to sialyllactose had a Kd of 5 μM. The aptamer was predicted to form a three-way junction structure with a stem region containing the modified thymidines. The aptamer with the modified bases was compared with its natural twin, comprising the same sequence but with no modified thymidines. The modified aptamer showed a stronger affinity to sialyllactose than the unmodified counterpart, suggesting that the positively charged amino group of

the modified thymidine residues did indeed enhance the binding ability.

Studies on the interaction of aptamers with antibiotic targets are valuable insights for the rational design of new and highly active antibiotic compounds against which no bacterial resistance has yet emerged. Wochner *et al*.[30] isolated antidaunomycin aptamers from a pool of random sequences using a semi-automated procedure for magnetic beads. All selected aptamers displayed high affinity for the target molecule daunomycin. One aptamer was further characterized and exhibited a Kd of 20 nM. To examine the aptamer's binding properties and clarify its applicability for diagnostic assays, its performance under various buffer conditions was evaluated by the researchers. The aptamer proved to be very robust and not dependent on the presence of specific ions. It also tolerated a wide pH range and immobilization *via* 50-biotinylation. Furthermore, a competition assay for sensitive daunomycin detection was established. Niazi *et al*.[31] identified the tetracycline group specific ssDNA aptamers by modified SELEX method by employing tosyl-activated magnetic beads coated with oxytetracycline, tetracycline and doxycycline, respectively, as targets and counter targets. Twenty tetracycline group specific aptamers were selected and a consensus sequence motif TGTGCT or its truncated terminal T-residue was found in most aptamers, which is predicted to be essential for high affinity and group specificity.

Other small organic molecules were also used for aptamer selection in recent years. For example, Mann *et al*.[32] selected aptamers that bind ethanolamine, the smallest organic molecule used for aptamer selection so far. Lévesque *et al*.[33] reported the isolation of thyroxine-specific aptamer, which bind to the iodine moieties of the thyroxine. In addition, the hemin RNA aptamers and diclofenac ssDNA aptamers were selected by Liu *et al*.[34] and Joeng *et al*.[35]. All of these aptamers can be turned into tools for the analysis of their targets.

Application of aptamers in analysis of small molecules

Aptamer sensors: Along with the rapid progress of modern analytical technologies and the application of novel analytical reagents, more and more aptamer-based assay formats have been developed to detect small molecules in recent years. Among these assay formats, aptamer-based electrochemical and optical sensors have attracted particular attention[36-39].

Typical electrochemical sensors operate by reacting with an analyte of interest and producing an electrical signal proportional to the analyte concentration. Baker *et al*.[40] designed an electrochemical aptamer sensor to detect cocaine, which is fabricated by self-assembly of the relevant methylene-blue (MB)-tagged aptamer on a *ca.* 1 mm^2 gold electrode *via* an alkanethiol group (Fig. 2A). In the absence of target, the aptamer is thought to remain partially unfolded, with only one of its three double-stranded stems intact. In the presence of target, the aptamer presumably folds into the cocaine-binding three-way junction, altering electron transfer and increasing the observed reduction peak. Zuo *et al*.[41] reported an electrochemical aptamer sensor that employed a DNA-duplex probe comprising an antiATP aptamer sequence labeled with ferrocene and its complementary sequence. In the presence of ATP, the complementary sequence was liberated while the aptamer sequence formed a rigid 3D structure with the aid of ATP. This transition brought the ferrocene tag to the proximity of the electrode surface, which turned on the electron transfer and produced electrochemical signals (Fig. 2B). This sensor could detect ATP at a wide concentration range (10 nM - 1 mM) with high sensitivity. Electrostatic interactions between a redox active molecule (such as $[Ru(NH_3)]^{3+}$) and the negatively-charged phosphate backbone of the immobilized aptamer can be used in combination with conventional and simple electrochemical techniques. A method for estimating the changes in electrode surface charges was used for developing a displacement chronocoulometric signal OFF assay to detect AMP[42]. The authors immobilized an anti-AMP aptamer and a complementary short ssDNA on gold electrodes (Fig. 2C). The binding of AMP provokes the release of the short ssDNA, so there was a decrease in the number of metal complex molecules electrostatically bound to the electrode surface. A linear range 0.1 nM - 1 mM indicated modest sensitivity.

Aptamers have also been widely used as recognition elements in optical sensor[43-45]. Of this assay format, fluorescence is widely employed due to the ease of labeling aptamers with fluorescent dyes. After covalently labeled by single fluorophore at a suitable position, aptamer can be direct employed as the signaling molecule for direct/indirect determination of targets or for demonstration of aptamer-target recognition by the means of fluorescence intensity, lifetime or anisotropy. The changes of fluorescence anisotropy of aptamers with or without targets can be adopted to investigate the binding behaviours between aptamers and targets and a series of small molecules have been analyzed by this way. Merino and Weeks[46] detected AMP, tyrosinamide and argininamide by using a 2'-ribose-linked fluorophore attached in a certain nucleoside, such as a deoxycytosine, close to the binding sites of an ssDNA aptamer (Fig. 3A). This 2'-ribose linked format has little influence on the affinity of aptamer to target and indicates three small

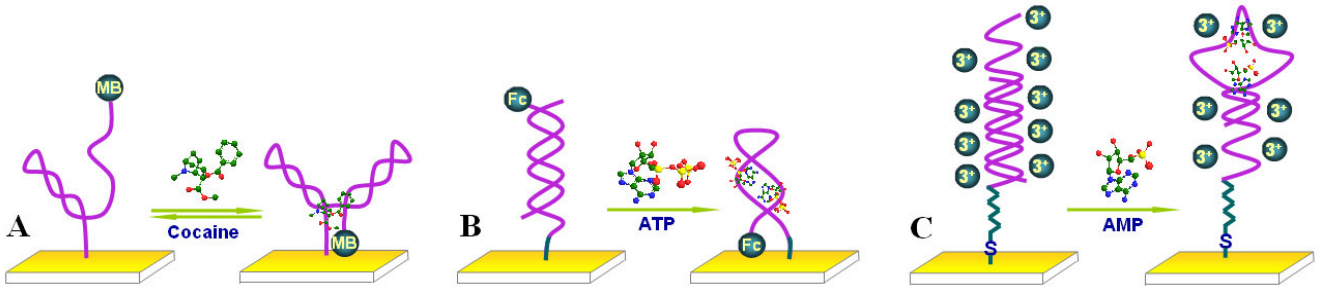

Fig. 2. Schematic representation of aptamer-based electrochemical sensors

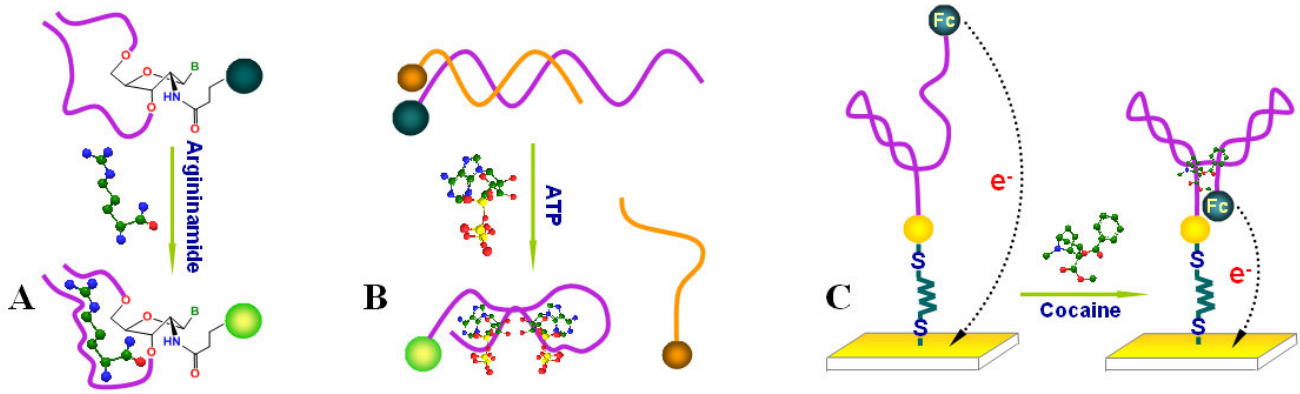

Fig. 3. Schematic representation of aptamer-based optical sensors

molecules present in simple buffers, human urine and bovine blood serum. Nowadays, double fluorescent probes labeled aptamer sensors based on fluorescence resonance energy transfer (FRET) or formation of pyrene excimer have more considerable applications (Fig. 3B). After proper truncation, fusion, or extension, aptamer was modified with a pair of donor/acceptor or pyrene molecules. The addition of target induces conformational change of aptamer and thus alters the distance between double fluorophores, leading to FRET or formation of pyrene excimer and a corresponding change of fluorescence. This configuration has been applied in analysis of small molecules, such as ATP[47] and cocaine[48].

Additionally, an electrogenerated chemiluminescent aptamer sensor has recently been developed for the detection of cocaine (Fig. 3C). The thiolated cocaine aptamer was labeled with $Ru(bpy)_2(dcbpy)NHS$ and immobilized onto a gold electrode by a thiol-Au interaction. In the absence of cocaine the aptamer was unfolded with only one double-stranded stem formed, such that the label was maintained far from the electrode surface and no chemiluminescent signal was generated. When cocaine was added, the aptamer adopted its characteristic structure with three double-stranded regions and in this folded configuration the $Ru(bpy)_2(dcbpy)NHS$ label was orientated close to the electrode and an chemiluminescent signal was generated. The signal was monitored at a constant potential of + 0.80 V in the presence of 0.10 M tripropylamine and cocaine levels were determined with a detection limit of 1 nM. This aptamer-based method is particularly important because it provides the possibility of selectively detecting small molecules with a high sensitivity[49].

Aptamer-functionalized nanoparticles: Much recent attention has been paid to the use of nanomaterials that make it possible to develop simple assay formats. Depending on their aggregation state, gold nanoparticles (AuNPs) have been reported to enable different colour emissions. Taking advantage of this observation, gold nanoparticles functionalized by aptamers can be used in analysis of small molecules[49-52]. Liu and Lu[54] have described a novel strategy for an aptamer-based colourimetric assay triggered by the disassembly of gold nanoparticles aggregate (Fig. 4A). Upon the addition of their respective small-molecule targets, the anti-adenosine aptamer was able to undergo a structure switching process that enabled tight binding to the small-molecule targets. This structural rearrangement resulted in the displacement of an gold nanoparticle-conjugated complementary strand, thus causing the overall gold nanoparticles deaggregation process. Consequently, a visible colour change ensued owing to shifts in the aggregation-dependent optical properties of the gold nanoparticles[53]. In another assay design[54], an adenosine-binding aptamer was first hybridized with a short complementary ssDNA self-assembled on gold nanoparticles, which were well dispersed in solution and looked red. Upon binding of the target, the aptamer strands underwent a structure-switching event that led to their dissociation from gold nanoparticles. The unhybridized gold nanoparticles were unstable at the same salt concentration and aggregated immediately, leading to a rapid, red-to-purple colour change (Fig. 4B).

In the assay designed by Zhang *et al.*[55], the cocaine-binding aptamers was, respectively engineered to be two pieces of random, coil-like single-stranded DNA, which reassembles into the intact aptamer tertiary structure in the presence of the specific target. Gold nanoparticles can effectively differentiate between these two states *via* their characteristic surface-plasmon resonance-based colour change. Using this method, cocaine in the low-micromolar range was selectively detected within minutes (Fig. 4C). In another similar design (Fig. 4D), an aptamer is engineered to consist of two pieces of random-coil like ssDNA which are, respectively attached to gold nanoparticles through their 5'-thiol-modified end. They can reassemble into the intact aptamer tertiary structure and induce nanoparticle aggregation in the presence of the specific target. Results have demonstrated that gold nanoparticles can effectively differentiate these two different DNA structures *via* their characteristic surface plasmon resonance-based colour change. With this method, adenosine can be selectively detected in the low micromolar range, which means that the strategy reported here can be applicable to the detection of several other small target molecules[56].

Affinity chromatography: Affinity chromatography is based on molecular recognition of the stationary phase for the analyte. Traditionally, antibodies have been incorporated into the stationary phase to provide molecular recognition. Aptamers are very useful in this field because they are capable of molecular recognition and have been applied in separation of many small molecules[29,57-59]. Deng *et al.*[60] 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. A biotin label was attached

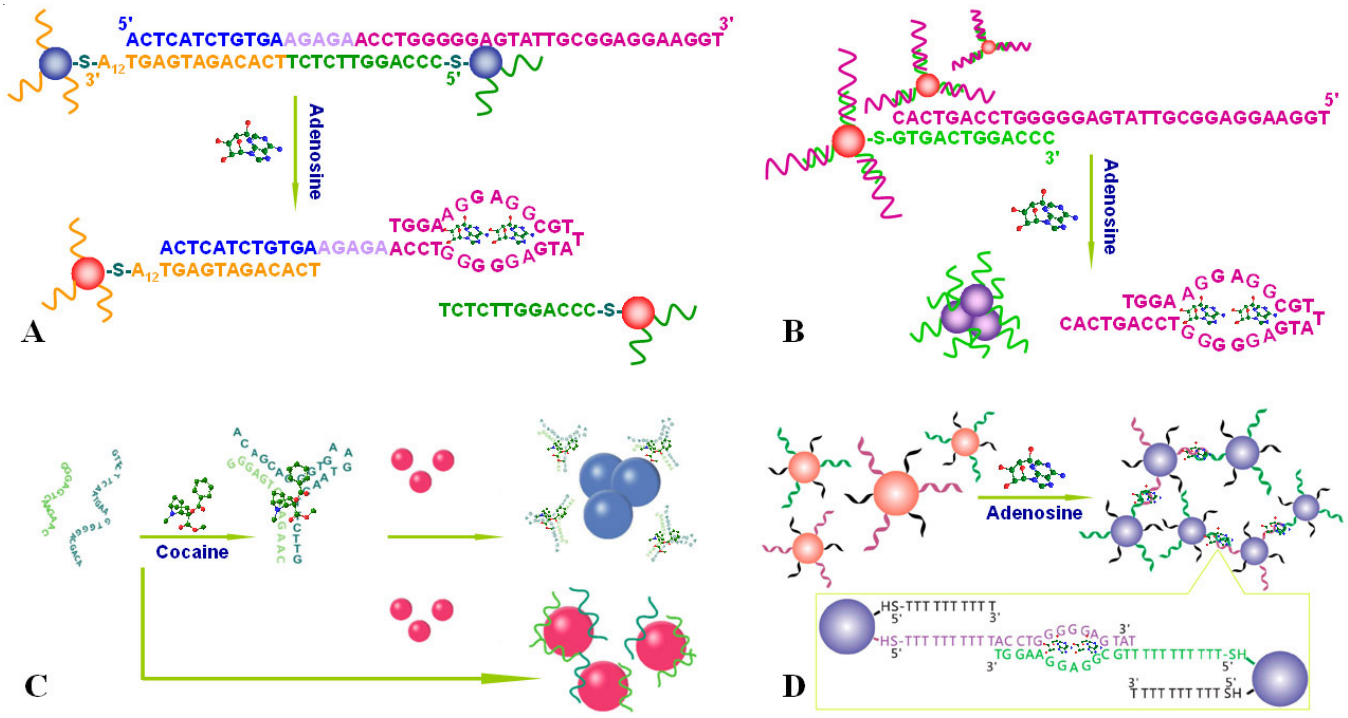

Fig. 4. Aptamer-functionalized nanoparticles

to the aptamer through a 15-carbon linker on the 3'-end. The biotinylated aptamer was then incubated with POROS (polystyrene porous particles) streptavidin media or streptavidin porous glass beads. The particles were then packed in fused-silica capillaries, resulting in affinity chromatography capillaries. The resulting particles contained 20 mmol binding sites per 100 µL of media. This value is 3.3 times larger than that reported for antibodies on similar media. Such aptamer affinity nano-column was further used to develop an efficient adenosine assay in microdialysis samples[61].

Michaud *et al.*[62] immobilized a 55-base biotinylated DNA aptamer (21 nmol of aptamer for a bed volume of 100 µL), specific for a D-peptide (arginine-vasopressin), on a streptavidin chromatographic support (polystyrene-divinyl-benzene). The influence of various parameters (such as column temperature, eluent pH and salt concentration) on the L- and D-peptide retention has been investigated in order to provide information about the binding mechanism and then to define the utilization conditions of the aptamer column. Very important apparent enantioselectivity was observed, the non-target enantiomer being roughly not retained by the column. Moreover, it has been shown by a complete thermodynamic analysis that both dehydration at the binding interface, charge-charge interactions and adaptive conformational transitions contributed to the specific D-peptide-aptamer complex formation. Furthermore, it was established that the aptamer column was stable during an extended period of time. As an extension of the work, such approach has been extended to the chiral resolution of small molecules of biological interest[63]. The DNA aptamers used have been selected against the D-adenosine and L-tyrosinamide enantiomers. An apparent enantioseparation factor of around 3.5 (at 20 ºC) was observed for the anti d-adenosine aptamer chiral stationary phase while a very high enantioselectivity was obtained with the immobilized anti L-tyrosinamide aptamer. This allowed obtaining baseline resolution even at a relative high column temperature. The results were of interest since they showed that, although the DNA aptamers were not selected for enantioselective binding *via* a counter selection with the non-target enantiomer, they were however able to discriminate the enantiomers.

Conclusion

Small molecules are involved in innumerable chemical processes in nature and can operate as substrate, catalyst or inhibitor in chemical reactions. Because of these properties, small molecules are valuable in chemistry, medicine, biology and so on. In all cases, the feasibility of quickly and reliably detecting them is essential. Aptamer-based assays have provided new applications in the field of analytical chemistry. In particular, the use of aptamers as capturing agents/receptors for small molecule represents an interesting approach and by coupling the aptamer-based assays to different amplification strategies analytical detection limits can be improved. In this regard, real improvements can be assured by an interdisciplinary approach that couples instrumental analytical chemistry (based, for example, on electrochemistry or spectroscopy) with molecular biology and nanotechnology. New combinations of aptamer-based assays bring innovative ideas that can be used to develop simple, sensitive, selective and fast analytical methods.

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