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Nanoscaled aptasensors for multi-analyte sensing

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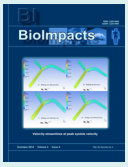

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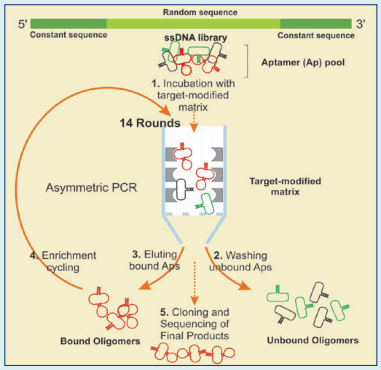
Abstract

Introduction: Nanoscaled aptamers (Aps), as short single-stranded DNA or RNA oligonucleotides, are able to bind to their specific targets with high affinity, upon which they are considered as powerful diagnostic and analytical sensing tools (the so-called "aptasensors"). Aptamers are selected from a random pool of oligonucleotides through a procedure known as "systematic evolution of ligands by exponential enrichment".



Methods: In this work, the most recent studies in the field of aptasensors are reviewed and discussed with a main focus on the potential of aptasensors for the multi-analyte detection(s).

Results: Due to the specific folding capability of aptamers in the presence of analyte, aptasensors have substantially successfully been exploited for the detection of a wide range of small and large molecules (e.g., drugs and their metabolites, toxins, and associated biomarkers in various diseases) at very low concentrations in the biological fluids/samples even in presence of interfering species.

Conclusion: Biological samples are generally considered as complexes in the real biological media. Hence, the development of aptasensors with capability to determine various targets simultaneously within a biological matrix seems to be our main challenge. To this end, integration of various key scientific dominions such as bioengineering and systems biology with biomedical researches are inevitable.

Introduction

Aptamers (Aps) are short single-stranded sequences of RNA or DNA, which have the capability of specific binding to given target(s) through electrostatic interactions, Van der Waals forces, hydrogen bonding, or combination of these interaction phenomena by forming a spatial structure.[1,2] Because of the specific three-dimensional (3D) architecture, aptamers display unique folded states with high affinity and specificity to a wide range of biotargets such as drugs,[3] toxins,[4] peptides,[5] proteins,[6,7] organic compounds[8,9] or even complete cells.[10] Aps have several advantages over the monoclonal antibodies (mAbs). Because of being smaller than mAbs, aptamers can be immobilized on the surface of electrodes in a denser pattern. Also, the physicochemical and biological stability of DNA Aps (but not the unmodified RNA Aps) are greater than that of mAbs. Further, the SELEX process on which Aps are selected is more cost-effective than the process used for the selection of mAbs.[11] Such unique properties make aptamers very attractive targeting entities that can be used for the sensitive detection of molecular markers involved in various diseases.[12-24]

Further, it has been reported that Aps are able to inhibit different kinds of functional proteins through competitive or non-competitive mechanisms or by hindering their regulatory regions. Aps, in addition to being candidates for therapeutic aims,[25] can be considered as suitable elements for the analysis of functional proteins as well as the validation of biotargets.[26] Nowadays, Aps have been used not only as homing devices for efficient delivery of therapeutics[27-29] and clear-cut sensing,[30] but also as therapeutic agents.[31-34]

This review provides an overview upon the sensing potential of aptamers towards simultaneous detection of multiple biotargets.

SELEX

As a powerful technique for the selection of specific aptamer(s) against a given target, SELEX exploits potential of chemically synthesized nucleic acid library (with

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approximately 10^{14}–10^{15} different random DNA or RNA sequences). This technology has widely been used for the selection of therapeutic Aps. It should be noted that the SELEX technology is a complex process with several iterative cycles consisting of (a) absorption of DNA or RNA sequences, (b) recovery of bound DNA or RNA, and (c) amplification of selected sequences via polymerase chain reaction (PCR) or reverse transcription-PCR (RT-PCR). Taken all, methodologically, the incubation of a designated library with an immobilized or free target in several rounds can result in the isolation of a number of oligomer sequences with high affinity and specificity to a given target.[35] Fig. 1 shows a schematic representation of in vitro SELEX process (panel A) and an aptasensor application (panel B) .

Aptasensors

Chemical sensors are defined as devices that are able to transform analogue data produced from the sensing of a specific chemical target into measurable digital signals. Technically, a chemical sensor includes a receptor and a transducer as the main components, in which the first part recognizes a particular analyte while the second receives information from the receptor and translates it into detectable signal(s). According to such description, biosensors epitomize the sensors that use biomolecules such as mAbs, Ab fragments, nucleic acids (DNA/RNA Aps), or other biological compartments as recognition probe to transform finally into electrical signals.[36] Among them, DNA/RNA aptasensors have attracted great deal of attention because of their ability to interact with a broad range of small molecules and macromolecules as well as higher stability, sensitivity and specificity. Aptasensors can sense the presence of a designated target analyte through the measurement of changes in mass, electrical, optical or electrochemical properties of the recognition

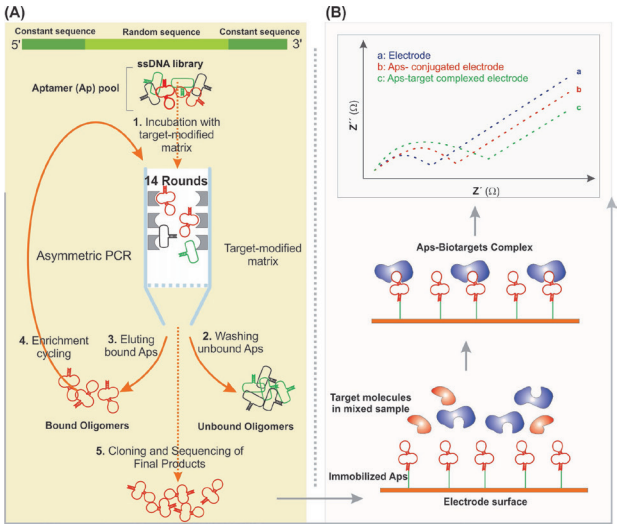

Fig. 1. Schematic representaion of SELEX process and aptasensor. A) Selection of aptamer against a target using systematic evolution of ligands by exponential enrichment (SELEX). B) Sensing of a given target by aptasensor. Note: not drawn to scale.

unit monitored by means of detectors.[37,38] It seems that optical and electrochemical aptasensors are much more common than those used for the analytical purposes, even though the development of electrical and mass based sensing tools will continue to grow. The emergence of aptamers in 1990 as novel and valuable sensing probes led to development of the first aptasensor which was engineered based on an optical method in 1996.[37] Photon transmission phenomenon and production of a detectable signal based on the complexation of Ap with its target is the central mechanism of optical aptasensors. While some aptasensors need to be labeled with chromophores, some approaches, e.g. surface plasmon resonance (SPR), do not require any label for the detection of target(s).[39,40] Further, Ap beacons, which have substantially been implemented as biomolecular recognition tools in biological studies, epitomize the labeled aptasensors. An aptamer beacon consists of an Ap sequence with an attached fluorophore at one end and a quencher at the other end. The nature of such beacons structure keeps the fluorophore and the quencher close to each other, and this proximity of fluorophore and quencher prevents the fluorescence signal to be created. However, the presence of target, which imposes a structural change in the 3D conformation of aptamer, increases the distance between the fluorophore and the quencher and causes a fluorescence signal. A large variety of Ap beacons have been designed for analytical applications, including double strand beacons, immobilized Ap beacons on a support, and signal off beacons developed based on the reduction in final intensity of fluorescence signals in the presence of target analyte.[41-50] As mentioned previously, SPR-based biosensors are powerful optical sensing tools that have successfully been used for the detection of a wide array of biotargets. Moreover, a number of aptasensors have been developed based on SPR including aptasensors designed for the detection of avian influenza virus H5N1,[51] human immunoglobulin E,[52] interferon-gamma[53], and mammalian eukaryotic initiation factor 4A.[54]

The application of optical aptasensors, which has been proposed since 1996, was introduced earlier than those of the electrochemical aptasensors that have been reported more recently in 2004. The electrochemical aptasensors are mainly classified into (a) amperometry, (b) potentiometry, (c) voltammetry, and (d) coulometry sensing techniques. Because of the simplicity of engineering together with high stability, the electrochemical aptasensors have been in the center of attention in the recent years.[55,56]

Classically, the electrochemical oxidation or reduction of the chemical electroactive species produces a measurable current that has been delineated as the basis of the amperometry technique. It has widely been used for the sensing of biological processes such as exocytotic events,[57,58] the status of metabolism and biophysical flux in the tissue and cellular or molecular domains,[59] and for the analysis of drugs.[60] The potentiometry technique as another electrochemical method is based on the measurement of potential difference between working and reference electrodes when no significant current flows between

them. It can be exploited for the analysis of a vast variety of target molecules because it offers a cost-effective portable platform with capability to measure continuously, even though its inability to detect small amount of analyte in some conditions is the major limitation of this method.[61,62] Further, the potentiometry sensing approach as a good paradigm of biosensing has been used for the detection of genomic targets. For example, the hybridization of probe DNA and target DNA can be monitored through the recording of potential changes during the reaction process and a significant light addressable potentiometric sensor (LAPS) signal, which is able to even detect trivial amount of target DNA.[63]

Moreover, the field-effect transistors (FETs), as a powerful subset means for the electrochemical method, have successfully been used for the development of biosensors for the detection of molecular markers such as lysozyme and thrombin.[64] Of these, the silicon nanowire FETs are deemed to provide a promising tool for label-free biosensing, in part due to their real-time detection capabilities with their ultrasensitivity and selectivity.

Multi-analyte detection by aptamers

Despite substantial progresses in development of aptasensors in the last decade, most of them have been shown to be capable of detecting single analyte in pure samples. However, the world of real samples deviates largely from the simplicity of the experimental conditions, and approximately all real samples are complex systems mixed with impurities that may interfere with the detection of single analyte. For example, in the biological body fluids (blood, serum or urine), biomarkers are found in complex fluidic system. Therefore, designing and engineering aptasensors with ability to target a particular analyte are of vital importance. This would be much more pivotal for the simultaneous detection of several biomarkers within the complex biological fluids. Taken all, improvement of methods for the detection of multi-analyte could save time and cost and also provide much more detailed information on array of biotargets. Unfortunately, up until now, few multi-analyte aptasensors have been introduced though there is a common consensus upon the impacts of highly sensitive, selective and easy to use biosensing methods for the concurrent detection of several biotargets. To the best of our knowledge, among the designed multi-analyte aptasensors, detection via optical and electrochemical methods appear to provide much faster and easier means as the next generation biosensing systems that are able to disclose the main pattern of the candidate biotargets in different diseases or even for biodefense purposes. Thus, we will discuss some important aspects of these methods.

Optical multi-analyte detection strategies

Among biosensing methods, great devotion has been given to the fabrication of multi-analyte aptasensors by means of optical detection devices. For example, in 2003, McCauley *et al.*[65] have developed a fluorescence-based aptasensor-chip for the simultaneous detection of thrombin, inosine monophosphate dehydrogenase (IMPDH), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in complex samples. In this study, all of the Aps were modified by a biotin group via a 12-carbon spacer at the 5′ position for immobilization on a streptavidin-glass surface and a fluorescein group at the 3' end as detecting element. It should be pinpointed that the main detection method for Ap-analyte binding assay was based on the fluorescence polarization change upon the binding of targets to the corresponding Aps. This phenomenon occurs because of an alteration in the rotation of Aps upon the formation of Ap-target complex, and hence the resultant fluorescent polarization could be measured as an indicative to the concentration of analyte in the samples. In the multi-analyte format, since the different aptamers were immobilized on specific position of chips, the analyte of interest could be detected via calculation of the exact position of the response signal on the surface of chips.

In another strategy, fluorescence Ap-based array was designed by the labeling of target(s) instead of the labeling of Aps, and successfully applied for the detection of lysozyme and ricin by RNA Aps and IgE and thrombin by DNA Aps.[66] In this sensor, the Aps were modified by biotin to be immobilized on the determined part of an avidin-coated glass slide, and then exposed to the related targets in pure/mixed solution. In both conditions, the aptasensor showed great ability towards detection of the analytes, while the concentration of target was measured through the intensity of fluorescence. Using this method, lysozyme was detected in picomolar concentration range, while ricin, IgE and thrombin were detected in nanomolar range. In fact, simplicity, sensitivity and time-saving and cost-effectiveness of the method make it very attractive strategy for the detection of multiple analytes. In a similar study, a bead-based aptasensor was devised for the detection of ricin and lysozyme using a single chip.[67] First, biotinylated Aps were immobilized on the streptavidin agarose beads, and then the beads were loaded into the wells of a chip (Fig. 2A). Labeled targets were added to the wells and the Aps-target complexation was detected via fluorescent labels (Fig. 2B). This method seems to be able to detect the targets specifically, and could be reactivated after exposure to the targets.

Since semiconductor quantum dots (QDs) as nonomaterials (NMs) with unique fluorescence properties have been able to emit different fluorescence wavelengths based on their sizes, they have attracted a lot of interest in medical and biological studies.[68] This unique optical feature makes them appropriate NMs to design nanobiosensors, in particular optical multi-analyte biosensors.[69-72] Having capitalized on the unique optical properties of QDs, Liu *et al.* have designed an aptasensor for the simultaneous detection of cocaine and adenosine.[73] In their study, thiolated DNA sequences were used as anchor part, while Aps specific to adenosine and cocaine were immobilized on the surface of gold nanoparticls (AuNPs). Similarly, some biotinylated DNA sequences fixed on the surface

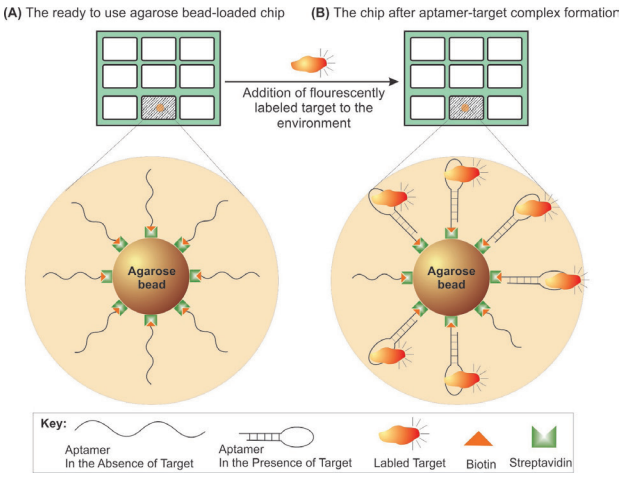

Fig. 2. Schematic representation of agarose bead aptasensors. A) The biotinylated aptemers are immobilized on streptavidin agarose beads, and loaded on a determined location of the chip. B) Presence of fluorescently labeled targets that can be detected upon aptamer-target complexation. For detailed information, reader is referred to the study performed by Ellington and coworkers.[67] Note: not drawn to scale.

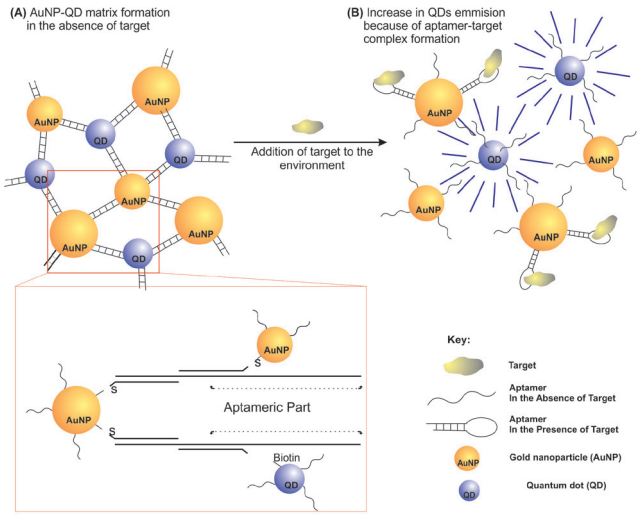

Fig. 3. Schematic representation of gold nanoparticles (AuNPs) and quantum dots (QDs) matrix. There are immobilized complementary strands on both surfaces of AuNPs and QDs. A) In the absence of target, complementary strands are coupled and form a matrix, resulting in the quenching of the QDs emission. B) Addition of target to the matrix-based biosensor induces the destruction of the matrix, leading to removal of the quenching effect and hence QDs emission. For detailed information, reader is referred to the study conducted by Yi Lu and coworkers.[73] Note: not drawn to scale.

of streptavidin-coated QDs were used as anchor for attaching QDs to the Aps (Fig. 3A). In the absence of the target molecules, the DNA anchors form double stranded DNA structures that cause aggregation of AuNPs with QDs, and lead to quenching the luminescence of QDs. In the presence of the target molecules, the spatial structures of Aps change, resulting in disassembly of the AuNPs-QDs complex (Fig. 3B). This phenomenon appears to intensify the emission that could simply be detected by the optical methods. The emission intensity is related to the target concentration, and since different QDs with different emission wavelengths were used in fabrication of this device, different types of analytes could be detected even in mixed samples. In this method, to design a double analyte biosensor, cocaine and adenosine aptasensors were prepared separately and mixed by 2:1 ratio for adenosine and cocaine, respectively. Such design makes the emission of two different QDs to be approximately the same in intensity. It has been claimed that even 10 distinct analytes could be detected simultaneously by this method, which could be used for either qualitative or quantitative detections.

Since developing specific Aps for different binding site of some targets is feasible (especially for proteins), it is possible to develop the sandwich arrays by capitalizing on the Aps alone or in combination with mAbs.[67,74] In fact, the Aps immobilized on beads can be introduced into micromachine chips on the electronic tongue sensor array, which can be utilized for the quantitative sensing of proteins in both capture and sandwich assay formats with possibility of multiple stripping and reusing steps. Hence, as a useful screening tool, sandwich Aps-based array can be exploited for a number of purposes. For example, Corn and coworkers have developed a methodology for the detection of protein biomarkers at picomolar concentrations using RNA Aps microarrays by means of the SPR imaging (SPRI) technique.[74] To fabricate such systems, a DNA anchor was immobilized on the gold-coated surface via its 3' thiolated terminal, and RNA Aps specific to thrombin and VEGF were then attached onto the immobilized anchors. When introduced to the target, thrombin attached not only to its specific Ap but also to its corresponding Ab that was added to the environment subsequently (Fig. 4A). Because of being tagged with the horseradish peroxidase (HRP), these attachments lead to Ap-Ab-HRP complex formation that makes a localized surface precipitation after addition of the HRP substrate (i.e., 3,3',5,5'-tetramethylbenzidine, TMB) into the environment. This surface precipitation could be detected by SPR method. In the presence of VEGF, the biosensor with a biotinylated VEGF Ab and an HRP-tagged anti-biotin Ab (Fig. 4A) resulted in the complexation of anti-VEGF Ap, VEGF, biotinylated VEGF Ab and HRP-tagged anti-biotin Ab. Such complexation was able to elicit a localized surface precipitation reaction in presence of TMB. The main advantage of this method seems to be the obtaining a very sharp signal with high sensitivity as compared to the common SPR signals.

An article, which describes bifunctional spectrophotometric-based aptasensor, was published in 2008 reporting the parallel detection of cocaine and adenosine 5'-monophosphate (AMP).[75] In this study, the specific Aps of AMP and cocaine were put in a unique sequence one after another, and hybridized with its complementary strand (Fig. 4B). The complementary strand consisted of complementary sequences of AMP, cocaine Aps and an additional sequence with

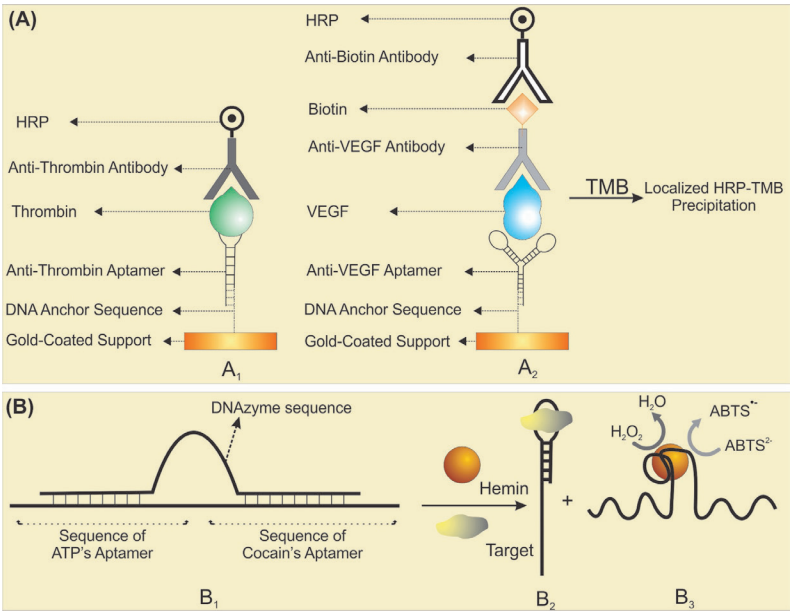

Fig. 4. Schematic representation of biosensing based on sandwich aptamer-based array system. A) Sandwich aptamer-based array system. Localized horseradish peroxidase (HRP)-3,3',5,5'-Tetramethylbenzidine (TMB) precipitation (A_2) occurs because of the formation of array. The HRP-TMB precipitation is detected by SPR technique. B) Parallel detection of cocaine and AMP by spectrophotometric method. The DNAzyme sequence (B_1) is inactivated by ATP and cocain aptamers. In the presence of targets, the DNAzyme sequence can be activated (B_2), in which presence of hemin elecits oxidative catalytic activity resulting in alteration of $ABTS^{2-}$ to its colored form $ABTS^{\bullet-}$ (B_3) that can be then detected by spectrophotometric method. For detailed information, reader is referred to a study accomplished by Willner and Corn research groups.[74,75] Note: not drawn to scale.

DNAzyme capability. In the presence of both AMP and cocaine, upon the Ap-target complexation, the stability of hybridized double strands appeared to be reduced resulting in the dissociation of the double strand structure. In this situation, the DNAzyme sequence can show HRP-mimicking activity in the presence of appropriate cofactor (hemin) in the environment (Fig. 4B). This event can catalyze the oxidation of 2,2'-azino-bis(3-ethylbenzothiazoline)-6-sulfonic acid ($ABTS^{2-}$), a substrate for peroxidase, to its colored form ($ABTS^{\bullet-}$), mediated by H_2O_2. Since $ABTS^{\bullet-}$ production is related to DNAzyme function, the amount of $ABTS^{\bullet-}$ can be considered as an indicative of the concentration of targets detectable by the spectrophotometric methods. For the confirmation of the results, an electrochemical measurement and faradic impedance spectroscopy were performed similarly by immobilizing the complementary sequences on the surface of a gold electrode. Then, the obtained data from electrochemical analyses confirmed the optical results.

Electrochemical multi-analyte detection strategies

Simplicity, high sensitivity, low cost, and high stability are some of the main advantages of electrochemical methods that make them versatile tools for the development of multi-analyte biosensing systems. In 2008, Erkang Wang and coworkers engineered an aptasensor specific to adenosine triphosphate (ATP) and α-thrombin detection by utilizing electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV).[76] In the fabrication of this aptasensor, a thiolated DNA sequence was attached on the surface of a gold electrode as an anchor (Fig. 5A). The main element of sensor (i.e., recognition sequence as a DNA sequence) consisted of three parts: (a) a short complementary sequence for hybridization with anchor sequence, (b) a DNA Ap sequence for ATP, and (c) a DNA Ap sequence for α-thrombin. Since some nucleotides in the ATP Ap and anchor sequence are complementary to each other, in the presence of ATP, the ATP Ap is affected by its corresponding target; hence the recognition sequence can be separated from the anchor part (Fig. 5A). This phenomenon causes a reduction in the negative charge of the electrode surface due to the scattering of recognition sequence from the electrode surface. The created distance between the recognition sequence and the electrode surface can reduce the EIS signal depending on the concentration of ATP in the samples. In another approach, in the presence of α-thrombin, the recognition sequence remained hybridized with the anchor, while the α-thrombin-Ap complex formation resulted in an increase in the EIS signal owing to the blockage of the electrode surface by α-thrombin volume (Fig. 5A). When the concentration of α-thrombin increases, the electron transfer resistance proportionally increases in part due to the accumulation of negative charges resulting from α-thrombin, hence the obtained EIS signals can be enhanced. It is worth to note that an excellent recovery of this biosensor is the outstanding characteristic of the established method.

As highlighted previously, given that QDs possess remarkable optical features that make them powerful tool for electrochemical sensing, a large number of researchers have used these NMs to engineer applicable biosensors. Wang and coworkers introduced an interesting QD-

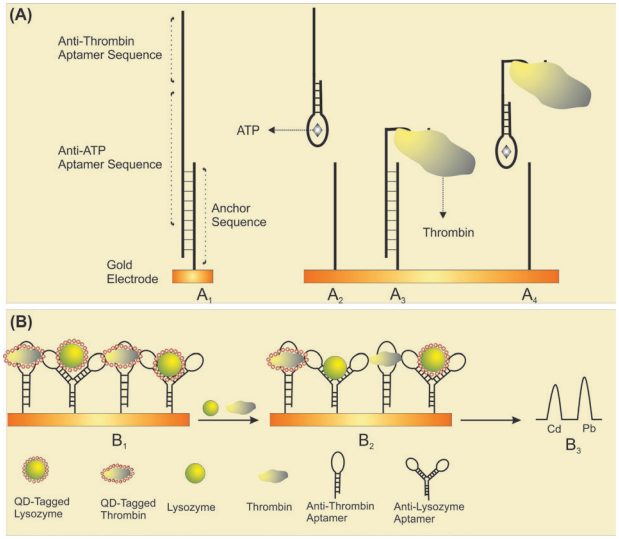

Fig. 5. Schematic representation of an electrochemical-based aptasensor. A) Anti-thrombin aptasensor as an anchor sequence immobilized on a gold electrode and a recognition sequence hybridized (A_1). In the presence of ATP, the recognition sequence separates and the EIS signal reduces (A_2). In contrast, in the presence of thrombin, the recognition sequence remains attached to the anchor and the thrombin causes an increase in EIS signal because of blocking the surface of electrode (A_3). In the presence of both targets, aptasensor behaves differently (A_4). B) A QD-based sandwich array. Aptamers specific to thrombin and lysozyme are immobilized on the electrode surface and conjugated by QD-tagged thrombin and lysozyme, respectively (B_1). In the presence of naked targets, QD-tagged targets are released to the environment (B_2) and can be measured via electrochemical methods (B_3). For detailed information, reader is referred to a study conducted by Wang and coworkers.[76] Note: not drawn to scale.

based aptasensor for the detection of thrombin and lysozyme.[77] In this study, cadmium and lead sulfide were successfully used as QD-tag for the detection of thrombin and lysozyme, respectively. The fabricated aptasensor was able to detect thrombin and lysozyme simultaneously in subpicomolar concentrations by means of the electrochemical method. Although this technique was devised based on a sandwich immunoassay technique, the method appeared to require only one step in comparison to the currently used sandwich assay methods which usually utilize two steps of analyses. This sensor showed high sensitivity and selectivity to thrombin and lysozyme in both single-pure and mixed samples. For the fabrication of this biosensor, thiolated Aps were immobilized on a gold electrode and conjugated with QD-tagged thrombin and lysozyme (Fig. 5B). In the presence of naked targets (i.e., thrombin or lysozyme), the QD-tagged targets were separated from their corresponding folded Aps and easily released into the environment due to their lower affinity compared to that of the naked targets (Fig. 5B). Therefore, the replaced amount can be straightforwardly measured using square-wave striping voltammetric method. The position of peaks on the voltammograms refers to the type of analyte while the height of the corresponding peak(s) is related to the concentration of each analyte (Fig. 5B). The developed sensor could simultaneously recognize two different types of analyte even in the mixed media with high sensitivity. Similarly, Zhang and coworkers set up an Ap-based sensor for the parallel detection of adenosine and thrombin via anodic striping voltammetry (ASV) using cadmium and lead sulfide as recognition elements.[78] The main part of this sensor (the so-called DNA linker sequence) was composed of (a) a complementary sequence for hybridization with DNA anchors sequences, (b) a specific Ap sequence for thrombin or adenosine, and (c) a sequence for DNA reporter sequences hybridization. In the basic form of biosensor, two different DNA sequences were immobilized on the surface of a gold electrode via their 3' thiolated end as an anchor, and hybridized with linker sequences subsequently. Then, the DNA reporters (i.e., sequences attached to QD-tagged AuNPs) were hybridized with DNA linker sequences (Fig. 6A). In the presence of targets, by changing in the structure of Aps (Fig. 6A), the reporter sequences seemed to be released into the environment, which could be detected by ASV method (Fig. 6A). This biosensing approach was reported to be a method with very low limit of detection (LOD) of adenosine in comparison with other studies.

Miscellaneous strategies for multi-analyte detection

Although optical and electrochemical methods are

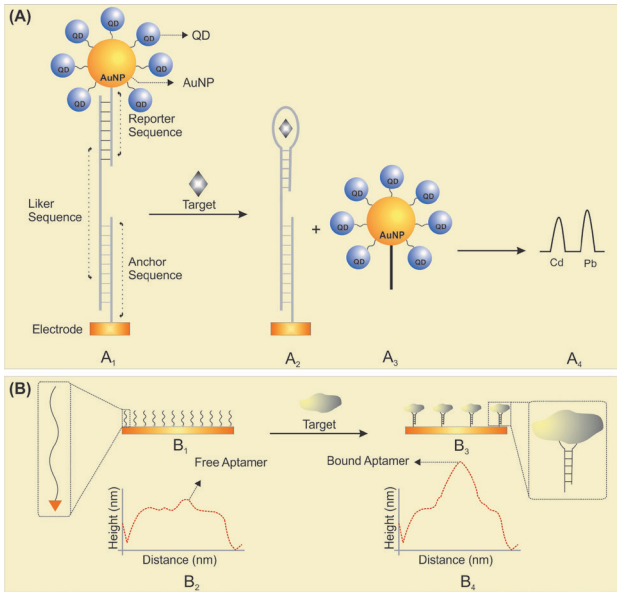

Fig. 6. Schematic representation of aptasensor conjugated with gold nanoparticles (AuNPs) and quantum dot (QDs). A) QD-based electrochemical aptasensor. The basic scheme of aptasensor includes anchor and reporter sequences (A_1). Aptamer-target complex formation causes the QD-tagged AuNPs to be released into the environment that can be detected by ASV method subsequently (A_2, A_3, A_4). B) Atomic force microscopy (AFM)-based array format. Tile shape arranged aptamers on a support (B_1). Line cross-section analysis of AFM images of unbound aptamers shows typical band for free aptamer (B_2). Change in spatial structure of aptamers occurs in the presence of targets (B_3). Line cross-section analysis of AFM images upon aptamer-target complex formation shows typical band for bound aptamer (B_4). For detailed information, reader is referred to a study conducted by Zhang and Yan research groups.[78,79] Note: not drawn to scale.

more common in designing biosensors, there exist few multi-analyte sensing strategies that use other methods. The DNA nanoarray device for α-thrombin and platelet derived growth factor (PDGF) is an interesting example.[79] Chhabra *et al.* engineered a specific Ap-based sensing system for the detection of α-thrombin and PDGF using a support in a tile shape (Fig. 6B). In the presence of targets, on account of the formation of Ap-target complex, the primary shape of Aps changes which can be detected by atomic force microscopy (AFM). This biosensor was shown to determine the presence of α-thrombin and PDGF either in pure or in mixed samples showing no/little interference with each other in an array format.

In 2003, Ellington and coworkers introduced a method which was able to detect different kinds of analytes concurrently through ribozyme ligases.[80] In this study, specific Aps for adenosine triphosphate (ATP), theophylline, and three other analytes were appended to a radiolabeled ribozyme ligase, engineering a biosensing system the so-called aptazyme (Fig. 7A). In this interesting system, the aptameric part acted as an effector for the aptazyme, which was able to cause a biotinylated substrate to ligate in the presence of the related target. The ligated biotinylated aptazymes were immobilized subsequently on a streptavidine-coated support. After thorough washing steps, the unbound aptazymes were eliminated from the environment and the radioactivity of the labeled aptazymes was detected. It should be noted that in this method, the location of aptazymes on the plate refers to a specific analyte and the intensity of radiation is proportional to the concentration of the analyte. Detection of different kinds of analytes even small molecules with low LOD seems to be the main advantage of this method.

Combination of molecular barcodes and double aptamer sandwich assay is another strategy used by Walton for simultaneous detection of thrombin and platelet-derived growth factor-BB (PDGF-BB).[81] This study was undertaken using (a) an Ap in capture sequence structure and (b) an Ap in sensing sequence structure. The capture sequences were comprised of an Ap specific for thrombin or PDGF-BB as well as a biotinylated sequence for immobilization on the streptavidin-coated magnetic beads (Fig. 7B). The sensing sequence, which also functioned as a detector element, was composed of three sequences (a) a sequence for PCR, (b) a sequence as molecular barcode, and (c) an Ap sequence specific for thrombin or PDGF-BB. In the presence of targets, every target can attach to its own capture Ap via its primary binding site and the sensing Ap can attach to the secondary binding site of the target. After sandwich complex formation, thorough washing is performed to eliminate the unbound sensing sequences from the environment. The attached sensing sequences can be then separated from the targets and amplified via PCR subsequently (Fig. 7B). The electrophoresis

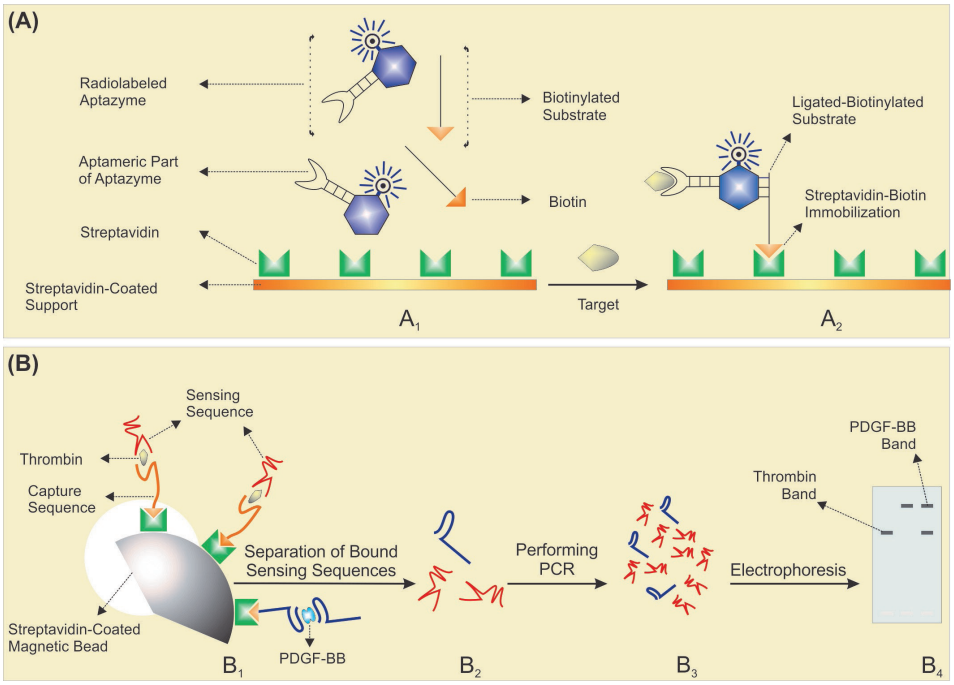

Fig. 7. Schematic representation of aptazyme array sensing system. A) An aptazyme ligase array. Radio-labeled aptazymes and biotinylated substrates are present in the environment separately (A_1). In the presence of target, upon target-aptamer complexation, the biotinylated substrates can be ligated to the aptazymes and immobilized on the support via biotin-streptavidin attachment subsequently (A_2). The radioactivity of immobilized aptazymes can be detected after appropriate washing steps. B) Molecular Barcode method. Capture sequences are immobilized on the streptavidin-coated magnetic beads, and in the presence of targets can form the sandwich complex as capture sequence-target-sensing sequence (B_1). By performing appropriate washing steps, the unbound sensing sequences are eliminated from the environment (B_2). The sensing sequences within the sandwich complex are separated and amplified by PCR method (B_3). The PCR products can be run into an electrophoresis gel and the spots on the gel represent the specific sensing sequences because of their different length (B_4). For detailed information, reader is referred to a study conducted by Ellington and Walton research groups.[80,81] Note: not drawn to scale.

analysis showed different spots on the gel for thrombin or PDGF-BB sensing sequences in part due to different sizes of sensing sequences (Fig. 7B). Considering all these features, the method seems to be a suitable biosensing paradigm for simultaneous detection of different analytes even in the mixed samples.

Aptamer-based multi-analyte detection challenges

So far, development of sensors with ability to simultaneously detect several analytes in the complex biological media has attracted lots of interests. Nonetheless, it seems that this scientific frontier needs to be reshaped towards multi-analyte aptamer array systems for concurrent detection of life and/or disease signals. Despite fascinating methods currently implemented for bioconjugation, hybridization and immobilization of oligonucleotides on the related surfaces (gold, glass, carbon, and other solid surfaces and supports), the dominion of biosensing demands miniaturized automated spotting robots for the development of seamless lab-on-chip systems. It seems we also need to improve the bioconjugation process which happens to be the main pitfall for the engineering of biosensors. In fact, robust concurrent and/or successive immobilization of different aptamers on a single support such as chip surfaces can significantly improve the multi-analyte detection strategies. The binding condition of aptamers could be affected by pH, temperature, and ionic changes, which may influence the binding efficacy of the aptamers to the surface.[82] It should be stated that although some methods introduce several procedures that are able to withstand the pH and ionic changes in experimental conditions, further developments for efficient immobilization seem to be inevitable. Difference in the length of aptamers' sequences, which have been immobilized on single supports, is another problem in the fabrication of multi-aptamer array chip systems. Since every aptamer has its own sequence and length, considerable difference in aptamers length may lead to interaction between adjacent immobilized Aps. This phenomenon may occur via excluding the aptamer-analyte complex formation of adjacent Aps by preventing spatial structure formation (e.g., through the electrical charge of the aptamers). The size of Aps could influence their attachment capability on surface, and accordingly long aptamers may be separated easily from the surface of the support than Aps with shorter length.[83]

It is note worthy that single chips with capability of multi-analyte detection appear to be (a) quicker in detection, (b) easier to use, and (c) cheaper to prepare in comparison with single-analyte chips.[84] The RNA-based aptasensors have an important problem, that is, they are highly sensitive to be degraded by the ribonuclease. Therefore, the RNA-based aptasensors are not re-generable in biological samples that contain endogenous ribonuclease. Hence, these biosensors are used solely for the single-use detection. This problem clearly influences cost-benefit of chips which contain RNA-aptamer sequences. However, in some cases, modified RNA aptamers could solve this problem.[65] Commonly, every aptamer needs its special environment and condition for the optimum action and target-aptamer complex formation, thus changes in pH and ionic concentration can affect the complex.[82]

It should be pinpointed that the aptamer-target complex formation is related to the affinity of Aps and biotargets, in which each aptamer-target complex has its own K_D that determines the affinity of this complex.[81] Therefore, in designing multiple analyte biosensors, the affinity factor must be taken into account while the density of each Ap on chips seem to be proportional to its K_D.

Of note, in the fabrication of multi-aptamer electrodes and chips, it is necessary to use Aps that work in the same condition (e.g., pH, temperature, ionic strength, etc.). Besides, they should also have similar sustainable washing and drying steps. This would be somehow problematic because each Ap per se needs to be separately optimized towards its best operational condition. Unlike multi-analyte aptasensors, handling the readout is less problematic in single-analyte biosensors. Depending on the sensing methodology, readout in multi-analyte systems need a careful design in terms of both hardware and software, since such system must process different detectable signals at the same time. Technologies involved in microarray or next generation sequencing may provide an important medium for devising biosensing arrays.

Final remarks and future prospects of aptasensors

Currently used hardwares in biosensing process seem to be bulky and sophisticated equipments, while biosensing systems need to be improved towards daily-used portable life-supporting devices. It could be imaginable that, in the future, our mobile phone may be equipped with a sensing system to monitor key biofunctions with capabilities to analyze the raw readouts, to normalize the resultant data and to report the endpoint outcomes. This results in real-time monitoring of the health or disease condition for each individual – an approach the so-called personalized life-monitoring system. This may sound "fiction" rather than "science". Nevertheless, personalized cell therapy has already been put in practice as reported for sipuleucel-T (APC8015/Provenge™) developed by Dendreon Corporation (Seattle, USA) as a cell-based cancer immunotherapy through customized programming of dendritic cells of prostate cancer patients.[85] Recently, Liu *et al.* introduced a promising method for the production of Ap-based dipsticks for sensing cocaine and lead.[86,87]

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Ethical issues

No ethical issues to be declared.

Competing interests

There is none to be disclosed.

Review Highlights

What is current knowledge?

√ Nanoscaled aptamers are short single-stranded DNA/RNA oligonucleotides capable of specific affinity to bind to a wide range of biotargets.

√ Systematic evolution of ligands by exponential enrichment (SELEX) is the main approach for the selection of aptamers.

√ Aptamers have been used as diagnostic and analytical sensing tools known as "aptasensors".

√ Multi-analyte detection of drugs, metabolites, toxins, microorganisms and biomarkers can be conducted by aptasensors.

What is new here?

√ DNA aptamers are stable probes to engineer biosensors.

√ Aptasensors can simultaneously determine various targets for the detection of biomarkers in complex biological samples.

√ Electrochemical impedance spectroscopic oriented aptasensors display a versatile method with low limit of detection.

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