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"Fitting" Makes "Sensing" Simple: Label-Free Detection Strategies Based on Nucleic Acid Aptamers

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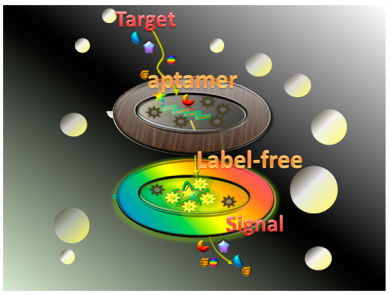
CONSPECTUS

Nucleic acid aptamers are small sequences of DNA made via in vitro selection techniques to bind targets with high affinity and specificity. The term aptamer derives from the Latin, aptus, meaning "to fit", emphasizing the lock-and-key relationship between aptamers and their binding targets. In 2004, aptamers began to attract researchers' attention as new binding elements for biosensors (i.e. aptasensors). Their advantages over other sensors include a diverse range of possible target molecules, high target affinity, simple synthesis, and ability to form Watson–Crick base pairs. These attributes create an enormous array of possible sensing applications and target molecules, spanning nearly all detection methods and readout techniques. In particular, aptamers provide an opportunity for designing "label-free" sensors, meaning sensors that do not require covalently labeling a signal probe to either the analyte or the recognition element (here, the aptamer). "Label-free" systems previously could only analyze large molecules using a few readout techniques, such as when employing the other recognition elements like antibodies. "Label-free" methods are one of the most effective and promising strategies for faster, simpler, and more convenient detection, since they avoid the expensive and tedious labeling process and challenging labeling reactions, while retaining the highest degree of activity and affinity for the recognition element. "Label-free" sensors are one of the most promising future biosensors.



In this Account, we describe our efforts exploring and constructing such label-free sensing strategies based on aptamers. Our methods have included using various readout techniques, employing novel nanomaterials, importing lab-on-a-chip platforms, and improving logical recognition. The resulting sensors demonstrate that aptamers are ideal tools for "label-free" sensors. We divide this Account into three main parts describing three strategies for designing "label-free" sensors: (1) Label-free, separation-free strategies. These include colorimetric sensors based on G-quadruplex-hemin complex, and fluorescent sensors based on fluorescent small molecules, novel conjugated polymers, and metal ion clusters. (2) Label-free, separation-required strategies. In this part, electrochemical sensors are introduced, including sensors with different subtechniques using an electrode array. (3) Logic sensors. Some logic recognition systems are introduced.

We emphasize that label-free aptasensors are not merely simple. We hope our introduction illustrates the powerful, flexible, and smart functions of aptamers in carrying out various detection tasks or playing various recognition games. Our work is only a start. We believe this field will bring additional knowledge on general designs, anti-interference, multianalysis, minimization, and auto-operation of aptamer biosensors.

1. Introduction

Aptamers are short single-stranded oligonucleotides that bind to specific targets similarly as antibodies, but have various advantages including wide targets, high affinity, small size, and ease of synthesis.[1,2] As its origin, "aptus" (means to fit), aptamers can adaptively bind targets by folding particular secondary structures. In a narrow sense, aptamers specially mean those nucleic acids that are generated from "systematic evolution of ligands by exponential enrichment" (SELEX) technique, which was first simultaneously reported by three independent laboratories.[3−5] Now, in a broad sense, metal ion specific oligos (such as oligos with T-T mismatch that can be selectively stabilized by forming T-Hg^{2+}-T base pair) or special structures stabilized/bound by small molecules (such as K^+ stabled G-quadruplexs) are also considered as aptamers in many literatures.

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Besides these advantages mentioned above, aptamers also possess comprehensive advantages such as wide target library, high affinity, stability, selectivity, and capability to form Watson–Crick base pairs. These unique properties make aptamers able to serve as new recognition elements in designing novel biosensors (i.e., aptasensors), with various sensing strategies compatible with almost all kinds of targets, detection requirements and readout techniques.[6−8] The sensing process of aptasensors is similar to other biosensors: aptamer recognizes its target, and the signal transducer transfers the recognizing information into measurable signals. Until now, multiple sensing strategies have been developed, combing with various transducers, such as quartz crystal microbalance (QCM), surface plasmon resonance (SPR), fluorescence, colorimetry, electrophoresis, electrochemistry, electrochemiluminescence (ECL), field-effect transistor, and so on.[9,10] These aptasensors can be generally categorized into two classes: "label" strategy and "label-free" strategy. "Label" describes a process to trace the target by directly monitoring the signal change of a probe covalently labeled on the analyte or recognition element (here, aptamer). As summarized in many outstanding reviews, this kind of strategy is specific, is easy to design, and possesses plenty of available signal probes. It promoted the appearance of aptasensors and now still dominates the whole field.[11] However, the label steps sometimes are challenged due to relatively high cost, limited probes, limited synthesis techniques, and operation/time-consuming. Additionally, the affinity of the aptamer and its target might have more chance to be affected, leading to reduced sensitivity. Therefore, to achieve lower-cost, faster, simpler, and more convenient detection, "label-free" strategy started to attract attention and has been developed into a very promising alternative in aptasensors. Under comparison, "label-free" does not require the probes to be covalently labeled, which avoids the expensive label process and the challenging label reaction/operation, and holds the highest activity and affinity of recognition elements. Also attractively, "label-free" depends more on careful and smart design, playing games with interactions between aptamers, its complementary strand, target, and signal probe. This property fully displays the advantage of aptamers as nucleic acids, which finally brings "label-free" sensors into wider targets, especially small molecules. This could hardly be realized when employing the other recognition elements, such as antibodies.

In this Account, we will review our years' efforts in exploring, constructing, and improving "label-free" aptasensors, inspired by their advantages. The works cover three most popular characterization techniques under "label-free" strategy, including colorimetry, fluorescence, and electrochemistry. According to whether a separation platform is required, the following description of "label-free" will be further divided into "separation-free" and "separation-required" sub-branches. The former one avoids both "label" and "separation" processes, which is absolutely the simplest mode to realize a "mix-to-signal" sensing, even though the separation-required ones (here, only including electrochemical readout) also contain great advantages (e.g., higher sensitivity and lower interference). It is hoped these works can serve as examples to provide a sense of how "label-free" works and how "fitting" makes "sensing" simple, effective, and interesting.

2. Separation-Free Strategies

2.1. Colorimetric Sensors. For colorimetric sensors, color change is observed when the analytes are present. They have a significant advantage: the need for analytical instruments may be minimized or even eliminated, and on-site and real-time detection are thus more manageable.[10,12] Until now, AuNPs[12] and G-quadruplex-hemin DNAzyme[10] are two most widely used signal reporters in "label-free" colorimetric aptasensors. Our interests were mainly put into hemin-G-quadruplex DNAzyme based ones.[13,14]

It is known that many G-rich ssDNAs can fold into single/multistrand-G-quadruplex structures, with parallel, antiparallel, or mixed conformations stabilized by different metal ions. Some of these G-quadruplexes under certain conformations can further bind hemin (a common peroxidase cofactor) as aptamers. Though in most cases the binding mechanism and contacts are still unclear, preliminary spectroscopic results indicate the hemin Fe(III) in the G-quadruplex–hemin complex displays a high-spin hexacoordinated condition, in which H_2O and a specific guanine(s) in the G-quadruplex act as the fifth and sixth axial coordination ligand, respectively. Compared with free hemin, this special structure will significantly accelerate the H_2O_2 leaving step (to form compound I in eq 2 in Figure 1A) in catalyzing H_2O_2 reduction. Therefore, the G-quadruplex–hemin complex can function as a kind of peroxidase-like DNAzyme, producing much higher (up to hundreds of fold) catalytic activity rather than hemin itself.[10,14,15] This phenomenon can be employed to realize aptasensing by either releasing or blocking the G-rich DNAs that could form effective hemin-DNAzyme after targeting.[10,14] Several peroxidase substrate systems can serve as the final readouts, including luminol, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and 3,3′,5,5′-tetramethylbenzidine sulfate (TMB) catalytically oxidized by H_2O_2.[10] Among the

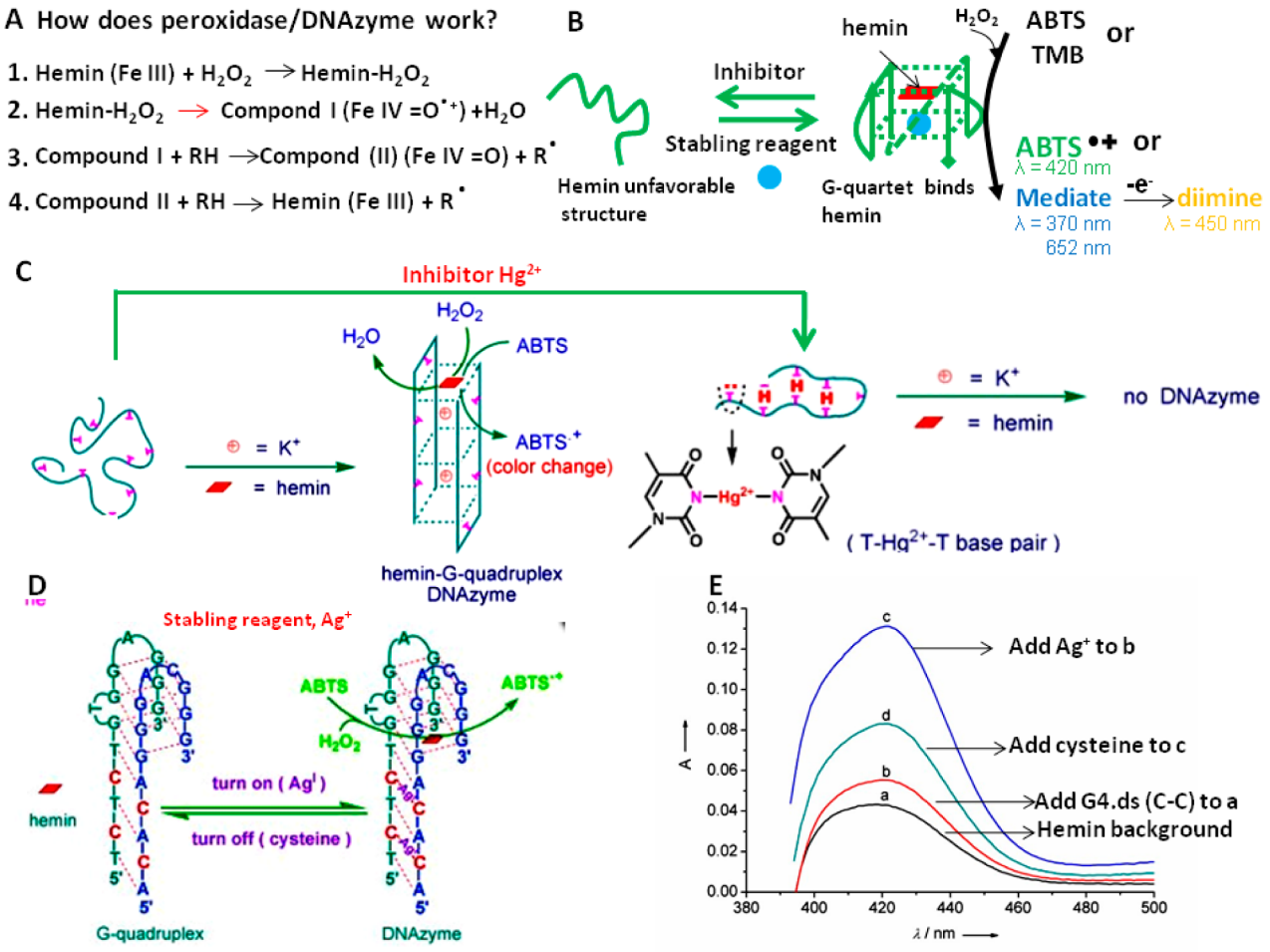


FIGURE 1. (A) Generally accepted working mechanism of hemin contained peroxidases. (B) Basic principle of the DNAzyme colorimetric aptasensors and ABTS/TMB substrate systems used. (C) Hg^{2+} detection based on its ability to inhibit formation of double strand AS1411 G-quadruplex. Adapted with permission from ref 19. Copyright 2009 American Chemical Society. (D) DNAzyme (hemin-G4.ds(C-C)) switch controlled by Ag^+ as stabilizing reagent and cysteine as inhibitor.[20] (E) Spectroscopic analysis of the hemin-G4.ds(C-C) switch.[20] Adapted with permission from ref 20. Copyright 2012 John Wiley and Sons.

three systems (Figure 1B), colorless ABTS can be oxidized to green ABTS$\cdot^+$ with maximum UV−visible absorption (MAbs) at 415 nm.[13] Colorless TMB can be oxidized to blue intermediate product (MAbs at 350 nm and 672 nm) and then to yellow final product (MAbs at 450 nm) after acid termination.[16,17] Therefore, both substrates allow the aptasensing results to be monitored by either naked eyes or spectra. For example, it was found the G-quadruplex binding structure (induced by thrombin interaction) of a 15-mer anti-thrombin aptamer could at the same time bind hemin and possessed hemin-DNAzyme activity, enabling the colorimetric detection of α-thrombin in a facile way. A linear detection range of 0.02−0.2 μM is with the limit of detection (LOD) of 20 nM, in the ABTS system.[13]

It was supposed that the catalytic activity was closely related to the stability of the G-quadruplex structure. This hypothesis was preliminarily confirmed by comparing the DNAzymes formed by hemin and five G-rich strands (T30695, PW17, PS2.M, AS1411, and PS5.M) selected from literature. Compared with the other four strands, AS1411 (a K^+ dependent double strand G-quadruplex), which binds hemin with highest affinity (K_d = 129 nM), also helped to produce highest initial catalytic rate (15.75 μM min^{-1}) to generate ABTS$\cdot^+$, about 1.5−2-fold over the other four strands and 10-fold over hemin itself.[18] A series of works were thus developed by using AS1411. For example, formation of Hg^{2+}-mediated T-Hg^2-T base pair (stronger than A-T pair) was utilized to hinder the proper folding of G-quadruplex structure of AS1411 and inhibit the DNAzyme activity (Figure 1C).[19] The sharp decrease of ABTS$\cdot^+$ absorbance in the presence of Hg^{2+} finally brought a 50 nM LOD of Hg^{2+}, with high selectivity. Similar strategies were gradually available to more targets (such as heavy metal ions[20,21] and amino acids[20]) using different designed G-quadruplex aptamers and different catalytic substrates (TMB or ABTS).

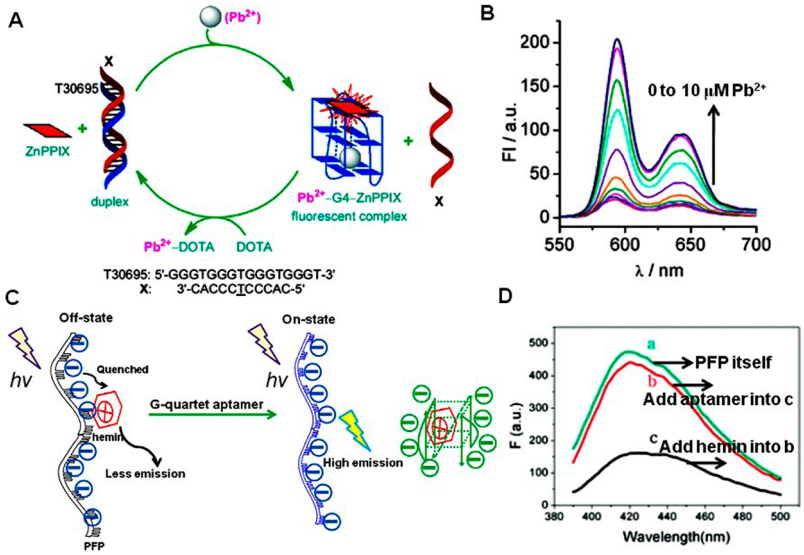


FIGURE 2. (A) Sequences and working cycle of the Pb^{2+}-driven DNA device based on T30695 and X. (Reprinted with permission from ref 27. Copyright 2010 American Chemical Society.) (B) Fluorescence spectra for analyzing different concentrations of Pb^{2+}. (Adapted with permission from ref 27. Copyright 2010 American Chemical Society.) (C) Fluorescent switch based on PFP, hemin G-quadruplex aptamer, and hemin. (D) Fluorescence spectra of the fluorescent switch. (C,D: Adapted with permission from ref 28. Copyright 2009 American Chemical Society.)

Shown in Figure 1D,E is an effective ABTS-H_2O_2 colorimetric Ag^+ and cysteine sensor using the quadruplex/duplex DNA structure G4.ds(C-C) as a DNAzyme switch.[20] Two aptamer parts were supposed to be able to self-assemble on the hemin site due to adding 5-mer duplex on terminals of the G-quadruplex. If there were two C-C mismatches instead of two G-C base pairs in the duplex, the resulting unstable quadruplex/duplex DNA structure (i.e., G4.ds(C-C)) would lose more than two-thirds of the hemin-DNAzyme activity. Upon incubation with Ag^+, formation of C-Ag^+-C base pairs (stronger than A-T base pair) could stabilize the mismatched duplex and recovered hemin-DNAzyme activity again, which enabled the G4.ds(C-C) to be a very simple Ag^+ detector. Additionally, thiols (here, cysteine) coordinating Ag^+ with higher affinity could compete Ag^+ with C-Ag^+-C base pair as inhibitors for the hemin-DNAzyme. Thus, despite in a nonselective way, cysteine could be sensed by measuring the decreased DNAzyme activity.

As described above, the formation of a stable G-quadruplex-hemin complex can be significantly dependent, inhibited or controlled by different metal ions, in which the G-quadruplex is switched to unfolded structure (e.g., Figure 1) or reshaped to other hemin unfavorable conformations (e.g., Figure 6A in section 4). And the final structure of the G-rich strand is decided by the binding equilibrium between the G-rich strand, its complementary strand, and different metal ions. That is why various "signal-on/off" metal ion colorimetric sensors/logic sensors can be fabricated.

2.2. Fluorescent Biosensors. Fluorescence is a traditionally sensitive, easy-use technique in bio/biochemical research. Therefore, it was not surprising that the earliest "label-free" and "separation-free" aptasensors originated from using fluorescence as readout. In these early works, target-triggered conformation change of aptamers (aptamer beacon)[22] could further affect the fluorescence intensity of traditional DNA binding dyes such as $[Ru(phen)_2(dppz)]^{2+}$, TOTO, OliGreen, and ethidium bromide.[23] But this introduction will focus on our interests in making use of novel fluorescent reporters.

Besides hemin, some fluorescent molecules are able to bind certain G-quadruplex aptamers, with significantly enhanced fluorescent properties, such as porphyrin-, phthalocyanine-, and triphenylmethane-based probes. The fluorescent enhancement may be due to that, like some proteins, DNA can serve as a hydrophobic and negative pocket to prevent the fluorescent ligands from approaching each other and thus avoid the aggregation-caused emission quenching in aqueous solution. Based on the nature of G-quadruplex DNAs, it is reasonable to believe that such enhanced fluorescence intensity is highly metal ion-dependent due to high selectivity. A series of works were developed based on this principle. For example, Cu^{2+} was proven to selectively quench the fluorescent dye molecule N-methyl mesoporphyrin IX (NMM). By adding G-quadruplex to bind NMM, significantly amplified fluorescence signal was obtained, moving the LOD for Cu^{2+} detection to as low as 83 nM.[24]

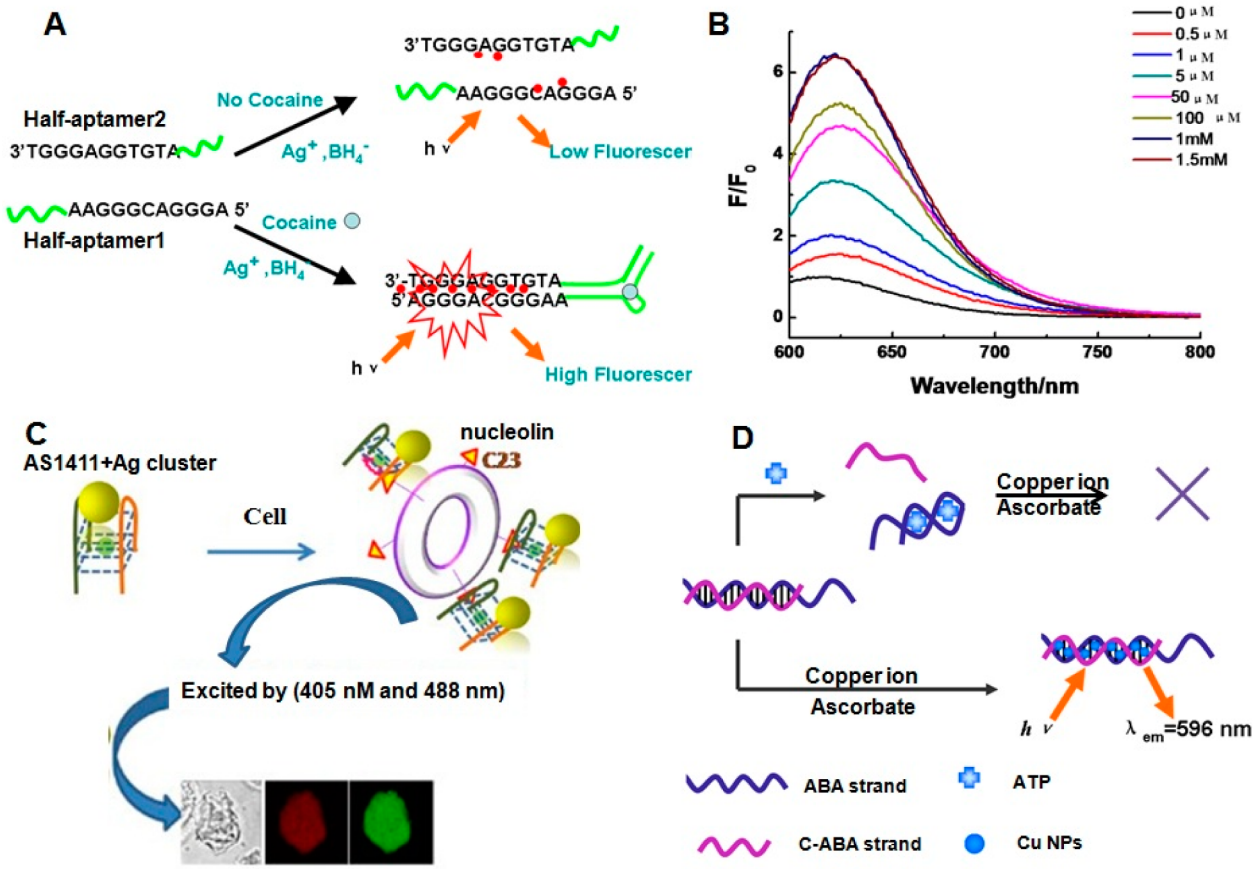


FIGURE 3. (A) Split aptamer based cocaine detection with Ag fluorescent clusters as readout.[31] (B) Concentration dependence of cocaine under fluorescence spectra.[31] Adapted with permission of ref 31. Copyright 2012 Elsevier. (C) AS1411 stabilized Ag clusters used as indicators in bioimaging of HeLa cells under confocal microscopy.[32] Adapted with permission of ref 32. Copyright 2012 Elsevier. (D) Novel fluorescent Cu clusters as readout in ATP sensing. (Reprinted with permission from ref 33. Copyright 2011 American Chemical Society.)

K^+ detection was also realized by making use of K^+ dependent G-quadruplex and its two fluorescent dye ligands zinc phthalocyanine (Zn-DIGP) and protoporphyrin IX (PPIX).[25,26] In another typical work, a Pb^{2+}-driven DNA molecular device was constructed based on DNA duplex–quadruplex exchange.[27] Shown in Figure 2A and B, G-quadruplex T30695 hybridized with its partly complementary strand to form a DNA duplex has no ability to interact with the fluorescent cofactor, zinc protoporphyrin-IX (ZnPPIX). In this case, the fluorescence signal was relatively low. It was found that Pb^{2+} was strong enough to kick out of the complementary strand, driving T30695 to fold into the quadruplex that could bind ZnPPIX. A 6-fold enhanced fluorescence signal was thus obtained. Similar to the principle described in Figure 1D, a reversed process and decreased fluorescence could be achieved by adding a stronger Pb^{2+} chelator, 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). The above working cycle could directly serve as a reusable fluorescent Pb^{2+} sensor with the LOD of 5 nM. Such a DNA device-based sensor indicated a new design concept: analysis will become smarter by making the sensitive and selective sensor also a reversible and programmable molecular device.

Recently, to solve the problems of broad emission, narrow absorption, and photobleaching of traditional organic dyes, new fluorescent materials have been extensively investigated. For example, our group built a fluorescent switch with G-quadruplex-hemin complex and an anionic conjugated polymer (poly(9,9-bis(6-phosphatehexyl) fluorenealt-1,4-phenylene) sodium salt, PFP), for the first time importing the anionic conjugated polymers into "label-free" DNA based sensing systems.[28] Shown in Figure 2C, D, in the "off-state", the fluorescence of PFP was sensitively quenched by slightly positive Fe^{3+} center in hemin through both fluorescent resonance energy transfer and light induced electron transfer. While in the "on-state", the formation of negatively charged aptamer/hemin complex forced hemin to be far away from the polymers, recovering the fluorescence intensity. The fluorescent switch was sensitive and selective to hemin, with low-nanomolar LOD. Thus, it could

serve as a general and sensitive signal transducer in both DNA sensors and ATP aptasensors.

Recently, the Ag nanoclusters, with outstanding spectral and photophysical properties, can be synthesized by using oligonucleotides as template, and reducing agent (e.g., $NaBH_4$). The photoluminescence emission could be tuned from visible to near-IR range by changing the sequence of oligonucleotides.[29] Importantly, our group found that the formation of fluorescent Ag nanoclusters in hybridized DNA duplex scaffolds can identify a typical single-nucleotide mutation, the sickle cell mutation.[30] Excited by these properties, Ag nanoclusters have become one of the current favorites in designing various aptasensors. We developed a new sensing strategy for detecting small molecule cocaine (LOD of 0.1 μM) and ATP (LOD of 0.2 μM) with high selectivity by using Ag nanoclusters as fluorescent reporters (Figure 3A,B).[31] Here, aptamer was split into two parts (either part is called half a ptamer), which could coassemble with each other promoted by the target, in optimized conditions. As shown, the assembly will bring the two G-rich tails on each part into proximity, which benefited the fluorescent synthesis of the DNA-Ag nanoclusters. Additionally, low toxicity and high biological compatibility of Ag nanoclusters offer big advantage and broad application prospect in bioimaging. In Figure 3C, the multifunctional aptamer AS1411 (not only for heimin and K^+, but also for nucleolin) were used as template to synthesize fluorescent Ag nanoclusters, which could serve as "label-free" signal reporters to recognize cancer cells based on the specific identification between aptamer and nucleolin on the surface of HeLa cells.[32]

Besides Ag nanoclusters, dsDNA can act as an efficient template for the formation of copper nanoparticles (CuNPs) with excellent fluorescence, whereas ssDNA template does not support CuNP formation. Based on this, an ATP-triggered duplex-to-complex structure switching was developed by destroying the duplex formed by ATP-binding-aptamer (ABA) and its partly complementary strand (PCS) (Figure 3D). The binding event of ATP and ABA could result in low fluorescence of CuNPs. The strategy indicated that novel fluorescent probe could also be used to determine ATP in 1% human serum and human adenocarcinoma HeLa cells.[33]

3. Separation-Required Strategies

Different from the "separation-free" strategies, the electrochemical aptasensor is a "separation-required" technique, because the electrode is the basic element in electroanalytical systems. The detection principle is that the changes of electrochemical features of a redox reporter upon target binding are correlated to target species or concentration. Although this strategy needs more sensing steps, it possesses many competitive advantages, including multitechniques, low-cost, relatively low interference, high reusability, and ease of minimizing/assaying. Importantly, the operation of washing as assistant "separation" process has changed the dependence of aptamer-target binding on homogeneous equilibrium, which can make the sensitivity much higher.

Though most developed electrochemical aptasensors required the redox reporters labeled on aptamers or other nucleic acids, such as the classical "E-AB" sensors,[34,35] "label-free" designs started to soar in 2005.[36] In most "label-free" electrochemical aptasensors, the redox probes interacted with nucleic acids through electrostatic adsorption/repletion, hydrogen bond and some other coordination interactions. Taking original electrochemical impedance spectroscopy (EIS) aptasensors as example, either slightly negatively charged protein IgE or α-thrombin could seriously block the anionic $[Fe(CN)_6]^{3-/4-}$couple from approaching aptamer-modified electrode surface, increasing the electron transfer resistance (Ret) between $[Fe(CN)_6]^{3-/4-}$ and electrode. Obviously, bulk proteins even cells are very suitable targets for "label-free" electrochemical aptasensing. Under comparison, small molecules with molecular mass or net charge are not easily detected with "label-free" strategies. Our lab contributed many efforts in solving this problem. In 2007, we developed a sensitive EIS aptasensor for adenosine based on duplex-to-complex design.[37] Shown in Figure 4A, a partly negatively charged DNA duplex which was comprised of PCS and adenosine-binding-aptamer (ABA) was fabricated on the Au electrode. Such negatively charged interface produced repulsive force against anionic $[Fe(CN)_6]^{3-/4-}$couple and deferred the electron-transfer process (high Ret). While the target binding took more ABA away from PCS, the decreased Ret could be monitored as a signal, with the LOD of 0.1 μM.The sensing surface would be directly regenerated by rehybridizing ABA with free-PCS on the electrode and no denaturing reagent treatment was required to destroy ABA/adenosine complex. As an extension, a multifunctional reusable EIS sensor based on an integrated aptamer (MBA) was further fabricated, which consisted of TBA grafted upon ABA, for parallel detection of ATP and α-thrombin.[38] α-Thrombin could be used to amplify the detection signal of ATP, and ATP could be used to regenerate the sensing surface after detection. Thus, the two targets could benefit each other.

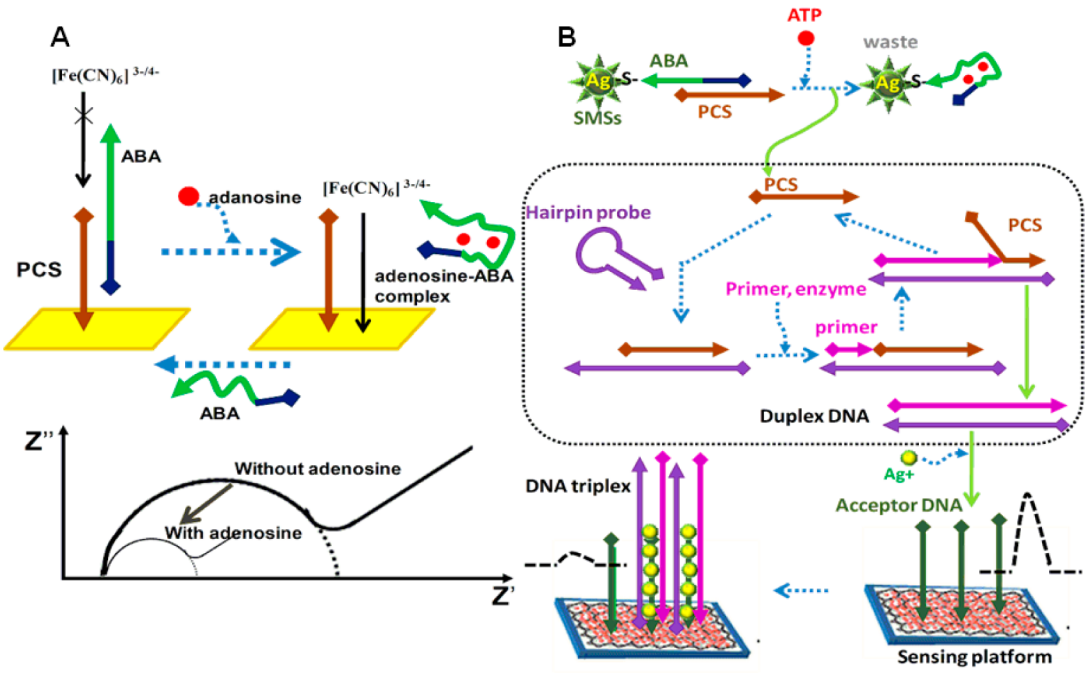


FIGURE 4. (A) EIS aptasensor for adenosine detection.[37] (B) GSGHs and Fc-PEI-based multilayer "solid-state" electrode as sensing surface in ATP sensing. (Adapted with permission from ref 44. Copyright 2011 American Chemical Society.) Note that ATP, AMP, ADP, and adenosine share the same aptamer.

Besides EIS aptasensors, our interest was also in importing ferrocene (Fc) into "label-free" strategies using differential pulse voltammetry (DPV) as detection techniques. Fc, as a perfect single electron transfer species with reversible and fast kinetics, has been widely used in labeled aptasensors.[8] However, since Fc has poor interaction with either nucleic acids or most targets, its excellent electrochemical activity is difficult to be used in "label-free" strategies. "Solid-state" technique is a reagentless method with probes premodified on the electrode surface stably, with advantages of saving expensive probes and making probes near to the electrode to get larger signals. Thus, we imported the integration of "solid-state probe" concept and layer-by-layer (LBL) technique into the Fc-based "label-free" electrochemical aptasensors. In these sensors, Fc was appended onto poly(ethyleneimine) (Fc-PEI) to make it positively charged. Such Fc-PEI complex was thus capable to attach on the negatively charged ITO electrode surface by forming LBL self-assembled multilayers with different opposite charged nanomaterials (CNTs, AuNPs, or graphene) as enhanced elements. These constructed "solid-state" systems were proven effective in detecting several different targets, such as protein,[39] drug,[40] peptide,[41] DNA,[42] and so on. The sensing principle is, when the sensing interface is covered or uncovered by poor conductors (e.g., aptamers, DNAs, and proteins), the electron transfer kinetics will be inhibited or accelerated, showing a decreased or increased electrochemical signal.[43] For example, taking advantage of strand-displacement DNA polymerization and parallel-motif DNA triplex system as dual amplifications, we fabricated a new electrochemical "label-free" integrated aptasensor based on silver microspheres (SMSs) as a separation element and graphene–mesoporous silica–AuNP hybrids (GSGHs) as an enhanced element of the sensing platform.[44] The sensing strategy contained three-step magnification process (Figure 4B): (1) SMSs with "clean" surface were first used to separate the undesirable ABA and ABA/ATP complex attached on SMSs surface after ABA-ATP interaction, leading to detachment of PCS into the solution. (2) Under the assistance of PCS, an amplified method was further introduced, based on the inherent signal-transduction mechanism of hairpin probe and strand-displacement property of DNA polymerase. (3) The obtained duplex DNA was used to hybridize with an acceptor DNA assembled on electrode to form triplex DNA, which could bring a more obvious detection signal compared with the duplex DNA without amplification. The electrochemical signal came from the Fc in $(Fc\text{-}PEI/GSGHs)_3$-acceptor DNA multilayer on ITO electrode array. By using above multiple effects, the sensitive analysis of ATP produced a LOD of 0.023 nM. Later, the electrode surface was further improved and functioned very well in discriminating D-vasopressin (D-VP) from the L-structure, by a split-aptamer sandwich mannered strategy.[41]

Besides the array–working-electrode system mentioned above, we further realized that the integration of the whole Au–Ag metal "three-electrode system" array, and achieved

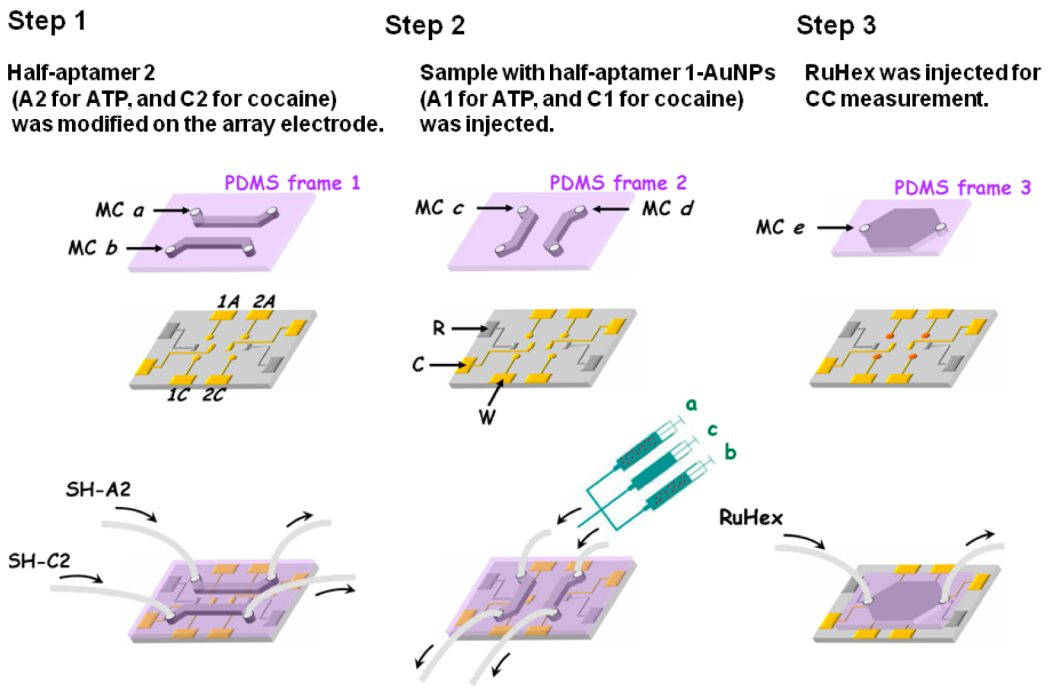


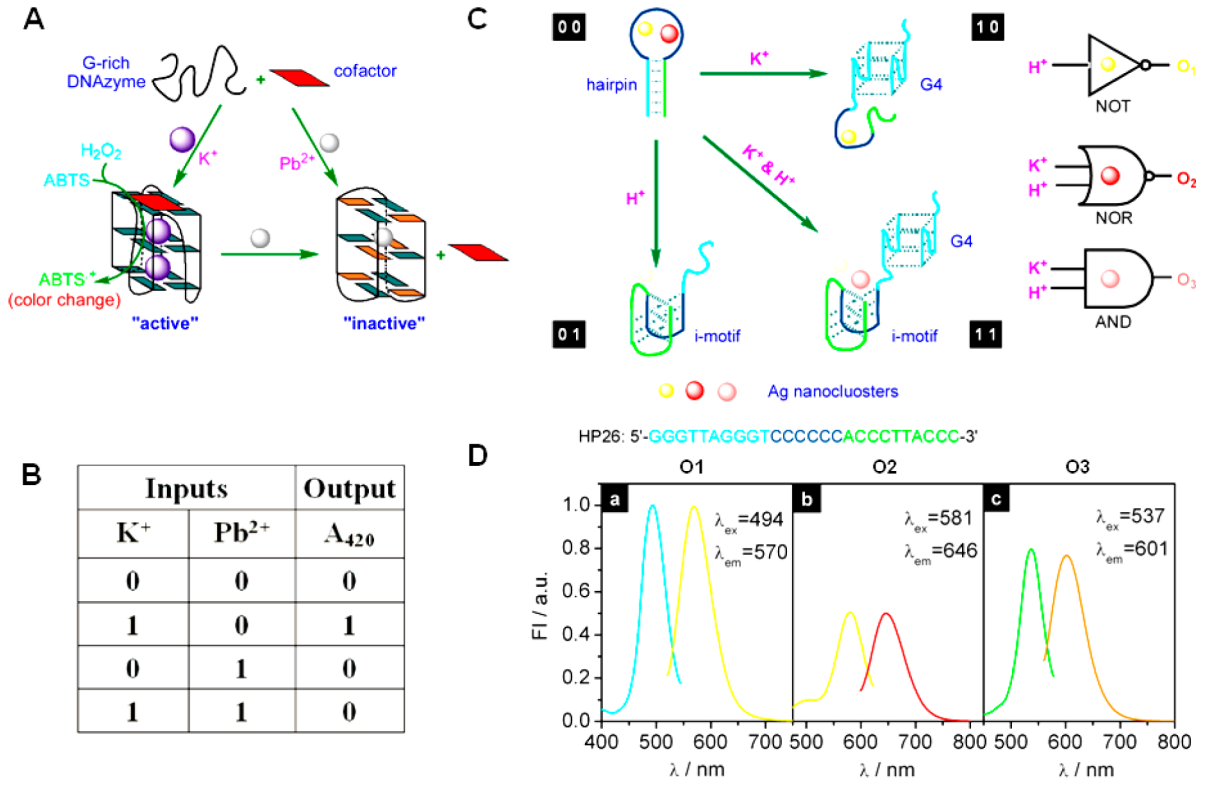
FIGURE 5. Microfluidic-based addressable multianalysis. Adapted with permission from ref 45. Copyright 2011 American Chemical Society.

addressable multianalysis of small molecules (here, ATP and cocaine) assisted by a microfluidic electrochemical aptamer-based sensor (MECAS, Figure 5).[45] In this work, $Ru[(NH_3)_6]^{3+}$ (RuHex), which is commonly used in chronocoulometric analysis to quantify surface-confined DNAs, was used as signal reporter.[34] Assisted by one of the designed microfluidic channel covering on the glass chip, different kinds of half aptamers (A2 and C2) were transported to the Au working electrodes to form different sensing interfaces. Then, by using another kind of channel, mixed targets were transported to the Au electrodes with different sensing interfaces which could selectively capture the corresponding target in the presence of another-half aptamer (A1 and C1, labeled with AuNPs to amplify signal). Such recognition process will be monitored by the increseased RuHex signal, since more DNAs were bound onto the electrode. As shown, this was an address-dependent sensing platform. Just using only one electrochemical probe, the multianalysis of two targets was achieved. The design was a development trend to release people out from hard work on polishing and cleaning electrodes manually, realizing more reproducible and high throughput sensing. It should be noticed that nonspecific adsorption is a frequently encountered problem in "label-free" electrochemical sensors. This concern should be minimized by doing more control and optimization, such as using blocking reagent and reasonable negative control molecules.

4. Logic Biosensors

After a series of basic works on "label-free" aptasensors, we started to transfer parts of our attention from improving sensing effectively to logic sensing. It is one of the possible ways to promote development of biocomputers that are still in early stages. These sensors followed the basic sensing principles introduced above, but played games with more than one group of aptamer/target complexes or binding inhibitors. Therefore, they were able to intelligently analyze the relationship between different targets in complex samples.[46]

Based on the Pb^{2+} sensor described above (Figure 2A), we designed a new class of DNA INHIBIT logic gate utilizing a K^+ and Pb^{2+} as two inputs, based on the phenomenon that Pb^{2+} could induce K^+-stabilized PW17 to undergo a parallel-to-antiparallel conformation transition, and then lose its traditional DNAzyme activity (in ABTS-H_2O_2 system) with hemin as cofactor (Figure 6A,B).[47] This work shows that, even for one G-rich strand, only certain subconformation can bind hemin to form active DNAzyme. Similar principle is also suitable to the G-rich strands that can enhance fluorescence of small molecules, as to be described in the following example of keypad (Figure 7). Then we built a new "label-free" molecular catalytic beacon (MCB) based on DNAzyme and established a series of "label-free" colorimetric DNA logic gates (NOT, NOR, IMPLICATION, AND, OR and INHIBIT) based on the formation and dissociation of G-quadruplex



Inputs		Output
K^+	Pb^{2+}	A_{420}
0	0	0
1	0	1
0	1	0
1	1	0

FIGURE 6. (A) DNA INHIBIT logic gate utilizing a K^+ and Pb^{2+}. (B) Table of true value of the INHIBIT gate. (A,B: Reprinted with permission from ref 47. Copyright 2009 American Chemical Society.) (C) Multichannel fluorescent logic system. (D) The multifluorescent spectra of Ag clusters synthesized by the HP26. (C,D: Reprinted with permission from ref 51. Copyright 2011 American Chemical Society.)

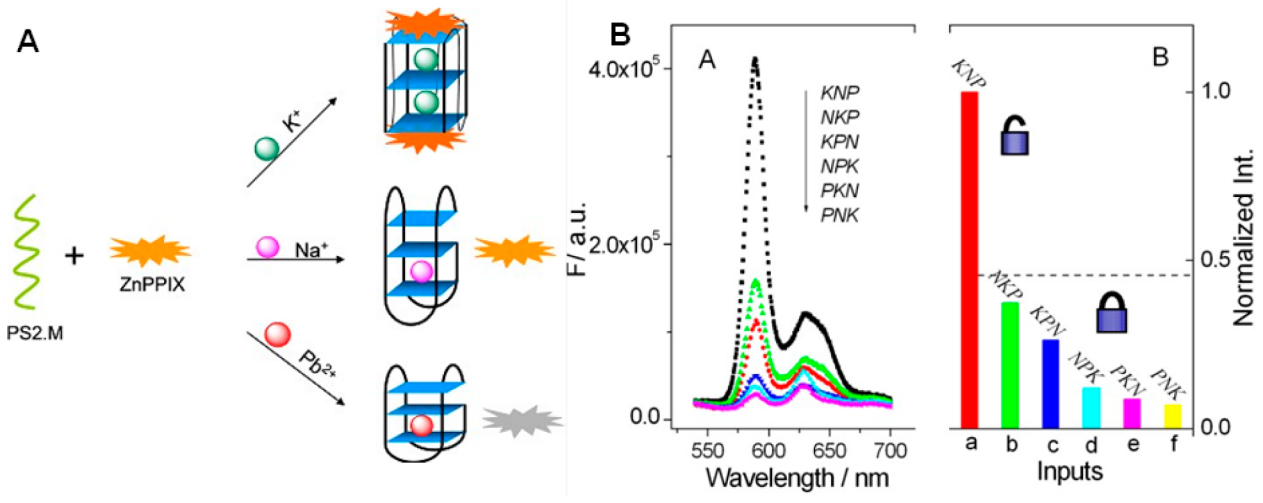


FIGURE 7. (A) Keypad lock systems controlled by K^+, Na^+, and Pb^{2+}.[52] (B) Output of the keypad with K^+-Na^+-Pb^{2+} (KNP) as the only unlock input.[52] Adapted with permission from ref 52. Copyright 2012 Royal Society of Chemistry.

DNAzyme.[48] In another electrochemical logic system, NAND gate was built on the biofuel cell, in which thrombin and lysozyme were used as two inputs to affect onset potential of bioanode and biocathode, respectively.[49] This work was further extended to an IMP-Reset logic operating one with thrombin and ATP as two inputs.[50]

It was recently found that a C-loop in a G-rich stem hairpin (HP26) could serve as a stabilizing reagent to synthesize a kind of multiexcitation/emission Ag nanoclusters.[51] In the presence of K^+ and H^+, the hairpin would switch to G-quadruplex/K^+-C strand and i-motif/H^+-G strand, respectively. In the presence of these ions, a G-quadruplex/K^+-i-motif/H^+

structure will be formed. As shown in Figure 6C,D, these structural changes were expected to remarkably influence the specific interaction between C residues and Ag nanoclusters, thereby modulating the fluorescence behaviors of Ag nanoclusters. A different feature of Ag nanoclusters (by changing the excitation wavelength) enabled multiple logic operations via multichannel fluorescence output, indicating the versatility as a molecular logic device.

An aptamer-based keypad lock system is also developed making use of the influences of metal ions stabilized G-quadruplex on the fluorescent intensity of ZnPPIX.[52] Shown in Figure 7, the system was turned ``ON'' with a strong fluorescent output signal only when the inputs were added according to the correct combination and exact output sequence (here K^+, Na^+, Pb^{2+} (KNP)). Otherwise, the system was kept "OFF" to prevent illegal access. This security system has important properties that may be useful in the analysis of DNA and the design of complex multiple input "label-free" DNA logic molecular devices in the future.

5. Conclusions and Outlook

Through above descriptions, we summarized our recent development in "label-free" aptasensors. As shown, "label-free" does not only mean "simple", but also can meet general sensing requirements, such as high sensitivity, selectivity, and multianalysis. It could also fit well with microfluidic chip or logic response. Therefore, it should be definitely one of the significant directions for aptasensors. Despite these advantages, disadvantages resulting from the nature of each readout method still cannot be ignored. The colorimetric method will be helpless when the sample is colored. Fluorescence is easily quenched or enhanced by some nonspecific additives in complex samples. Electrochemistry becomes picky when choosing from limited electrochemical probes and requiring more technical training. Also notably, compared with "labeled methods", "label-free" must face more challenges coming from its high potential to be interfered by nonspecific materials in complex biosamples. Therefore, reasonable control experiments and condition optimization should be very necessary once touching complex samples. For example, effective surface blocking in electrochemical systems could function well in nonspecific sensing. Several works mentioned above have tested the sensors in diluted serum, urea, or saliva, and satisfied recovery of the targets.[33,40] That also meant that the interference could be minimized or even eliminated by diluting the samples. Of course, this puts forward a harsh requirement for high sensitivity of both methods and instruments. Besides improving sensitivity, more work should be done to transfer the sensing strategies from traditional model targets (eg, α-thrombin, ATP, and cocaine) to more meaningful targets which urgently need good detection methods. This is a general problem that all kinds of aptasensors should face, and can get assistance from development of aptamer SELEX.

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FOOTNOTES

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