G-Quadruplex as Signal Transducer for Biorecognition Events

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Abstract: G-rich nucleic acid oligomers can form G-quadruplexes built by G-tetrads stacked upon each other. The basic building block of the G-quadruplexes is similar, but the formation of different quadruplex structures is highly responsive to the strand stoichiometry, strand orientation, guanine glycosidic torsion angle, connecting loops, and the metal coordination. Because of its structural variations and different functions, G-quadruplex applied in biorecognition events can function as a versatile signaling component. A variety of strategies that incorporate G-quadruplex have also been reported. In this review, we mainly discuss G-quadruplex as signal transducer from the following aspects for biorecognition events: analyte-induced G-quadruplex reconfiguration and fluorescence enhancement of small ligand; analyte-induced G-quadruplex reconstruction and formation of DNAzyme; Stimulus-responsive G-quadruplex refolding and manipulation of electron transfer; Stimulus-responsive G-quadruplex and its combination with nanopore systems; Small ligand-responsive G-quadruplex stabilization for drug screening; Nanomaterial-reponsive G-quadruplex reformation; Target-triggered continuous formation of G-quadruplex by DNA nanomachine. We have comprehensively described the recent progress in our labs and others. Undoubtedly, bioanalytical technology and nanotechnology based on G-quadruplex will continue to grow, leading to develop new diagnostics, therapeutics and drug development.

Keywords: G-quadruplex, drug, biosensor, signal transducer, fluorescence, DNAzyme, electrochemistry.

1. INTRODUCTION

Since 1980's, there have been many literature discussing about G-quadruplex, and the detailed structure information and important biological roles have been comprehensively studied and reviewed as well as the stability and kinetics of G-quadruplex [1-6]. The driving force for investigating these specific DNA structures is to find out what kind of small ligands are suitable to selectively kill the cancer cell and manipulate the gene expression [7-12]. Although these studies have been very attractive to biologists in the earlier days, nowadays, more scientists outside of biological field devote themselves into G-quadruplex study, based on which a series of bioanalytical methods were established [13-15]. More recently, we and other labs have extensively investigated the application of G-quadruplex in biosensor field.

G-quadruplex which has been proven to be highly successful signal transducer is due to the fact that the folding and unfolding of G-quadruplex is responsive to environmental stimulus. The first category of stimulus is those small metal ions such as K^+, Cs^+ and Pb^{2+} etc., which have been proven to stabilize the structure of G-quadruplex, therefore, biosensors involving G-quadruplex can be specifically designed for selective detection of certain ions as well as in the complex real sample. The second category of stimulus is the small molecules such as ATP and cocaine, etc.. The third category of stimulus is the biomolecules such as DNA and protein, etc.. All these above-mentioned stimulus can be the target of interest in certain circumstances, which will directly affect the mechanism how the G-quadruplex being involved in the biosensor is responsive to the target.

In order to detect the biorecognition event, the consequent folding (or unfolding) of G-quadruplex has to transduce the binding events into the recognizable signal which can be measured by different kinds of instruments or can be directly observed by naked-eyes. Until now, several techniques such as fluorescence spectrometry, electrochemistry and colorimetric visualization, etc., have been adopted to monitor the target-driven reconfiguration of G-quadruplex. In this review, we have comprehensively introduced how the G-quadruplex in combination with different readout technologies to detect a series of biorecognition events. Future challenging tasks were also briefly summarized. We believe more and more interesting works will come out in this field.

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2. G-QUADRUPLEX-BASED BIOSENSOR VIA FLUORESCENT READOUT

Fluorescent readout of biorecognition-induced reconfiguration of G-quadruplex can be monitored via two approaches. The first approach is to directly label the end of G-quadruplex with fluorescent tags. The fluorescent tags act as transducers that transform biorecognition (hybridization, ligand binding) into a fluorescence signal. Fluorescent labeling has its own unique advantage such as high sensitivity and multiple transduction approaches including fluorescence quenching or enhancement, fluorescence anisotropy, fluorescence lifetime, fluorescence resonance energy transfer (FRET), and excimer-monomer light switching [16, 17]. The second approach was based on the interaction of G-quadruplex and fluorescent ligand, which lead to significant fluorescence enhancement of small dye. A series of fluorescent ligands such as crystal violet, Thiazole orange, Oregon green, coumarin, pyrene, Benzothiazole, Triphenylmethane and others [16, 18-23] have been reported to be highly fluorescent in the presence of G-quadruplex [18, 19]. Among various detection methods for bio-recognition events, the homogeneous fluorescence measurement has become the subject of intense research. The development of sensitive and selective sensors based on G-quadruplex enhanced fluorescence intensity of small dye molecules has become a very active research field in recent years [24-26], since they have a great potential for their applications in detection and visualization of DNA and RNA in genomic analyses, and detection of nonnucleic acid analytes such as proteins, small molecules and metal ions.

2.1. Detection of Metal Ions

Fluorescence enhancement based on G-quadruplex has excellent advantages, including higher stability and reproducibility. For practical quantitative measurement, the fluorescence based detection approach is much more desirable. Since the structure of

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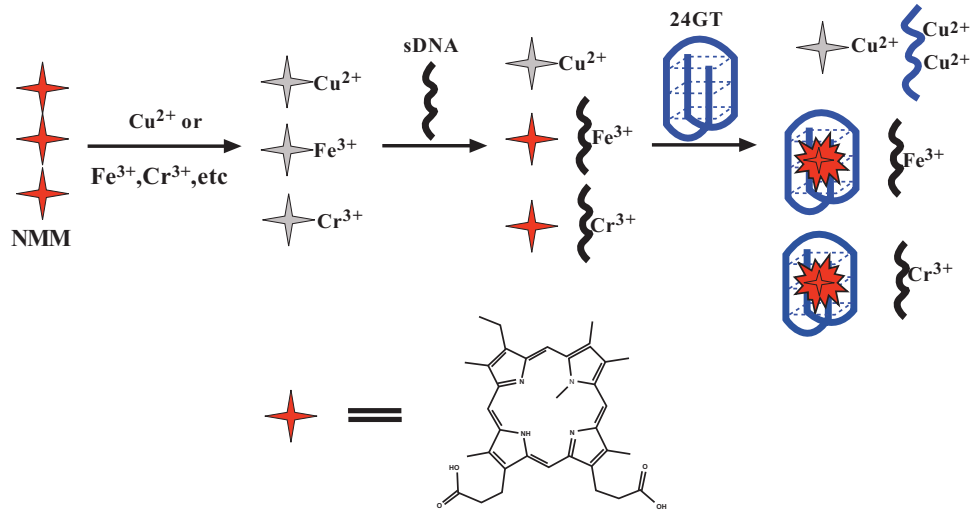


Fig. (1). Illustration of turn-off fluorescence response toward copper ion. The molecular structure of NMM containing two proximal carboxylate groups which can function as a copper ion receptor.

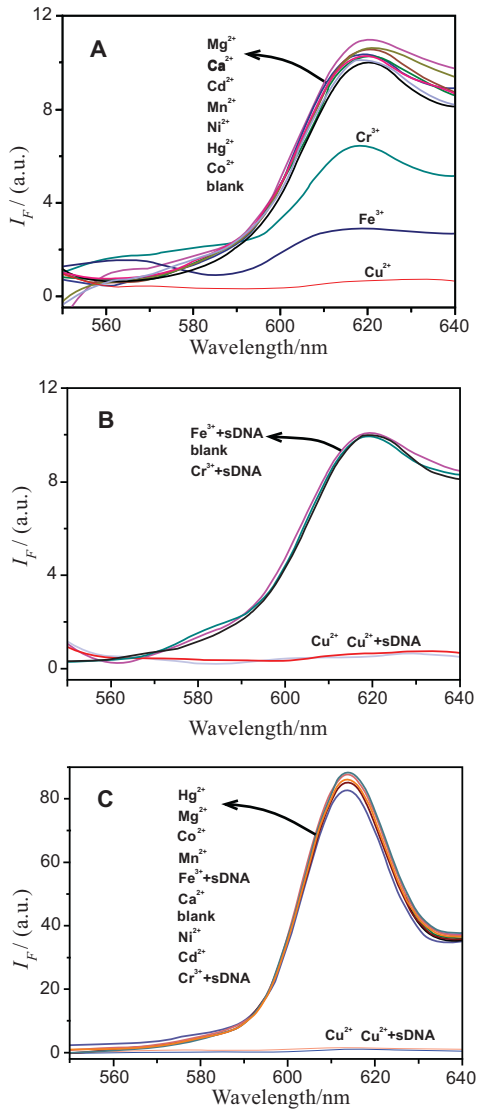


Fig. (2). Fluorescent sensor for selective detection of cupric ion using G-quadruplex. (**A**) Fluorescence responses of NMM toward different metal ions. (**B**) Emission spectrum of NMM in presence of different metal ions upon addition of sDNA used as masking agent to eliminate the interference from $Cr^{3+}$and Fe^{3+} (**C**) Fluorescence responses of NMM+24GT toward different metal ions. (**D**) Fluorescence responses of NMM+24GT in the presence of various concentrations of Cu^{2+}. Experimental conditions: 50 mM each ion, 2 mM NMM, 0.5 mM 24GT, 20 mM HEPES buffer (pH 7.0) containing 140 mM NaCl and 5 mM KCl, excitation of NMM at 399 nm.

G-quadruplex is highly cation-dependent [27], it can be employed as a fluorescent biosensor for detection of metal ions such as copper and potassium [27-30]. After careful design, biosensors for other heavy-metal ions such as silver ion and lead ion can also be fabricated. The detailed information can be referred to the following literatures [31-33]. For K^{+} and Pb^{2+}, Sr^{2+} ion detection. The sensor can be designed based on the direct interaction between G-quadruplex and each metal ion, since G-quadruplex is highly responsive to these metal ions. For silver and mercury ion, the sensors have to be carefully designed in terms of competition between two DNA structures: one is metal ion-induced specific conformation; the other is G-quadruplex. More interestingly, biosensor for some metal ions such as copper ion can be designed based on the interaction of metal ion and G-quadruplex binding ligand. In our lab, we have successfully used G-quadruplex in combination with fluorescent ligands N-methyl mesoporphyrin IX (NMM) to detect copper ions [27].

As it showed in Fig. (**1**), the fluorescence intensity of aromatic ligands like NMM can be directly quenched by copper ion. The free ligand without binding with copper ion can still have the chance to switch on its fluorescence after binding with G-quadruplex. As shown in Fig. (**2**), NMM alone, which suffers from low quantum yield was not sufficient to distinguish the copper ion from other heavy-metal ions, such as Cr^{3+} and Fe^{3+} etc. Upon the addition of 1 mM sDNA into the solution containing Cr^{3+} or Fe^{3+} Fig. (**2B**), the interference from the two metal ions can be totally removed. Fig. (**2C**) showed that further addition of 0.5 mM 24GT into the solution dramatically magnified the discrimination between copper and other metal ions. Fluorescence responses of NMM+24GT in the presence of various concentrations of Cu^{2+} as showed in Fig. (**2D**). By this way, quantitative and qualitative detection of copper ion can be realized. As we all know, there are a lot of G-quadruplex binding ligand, for each metal sensor, careful chose of small ligand is a very important step for success. For example, the aromatic ligand for detection of potassium is tetrakis (diisopropylguanidinio) zinc phthalocyanine (Zn-DIGP) [34], which possess the high capability to detect potassium with excellent selectivity as compared to previous biosensors Fig. (**3**).

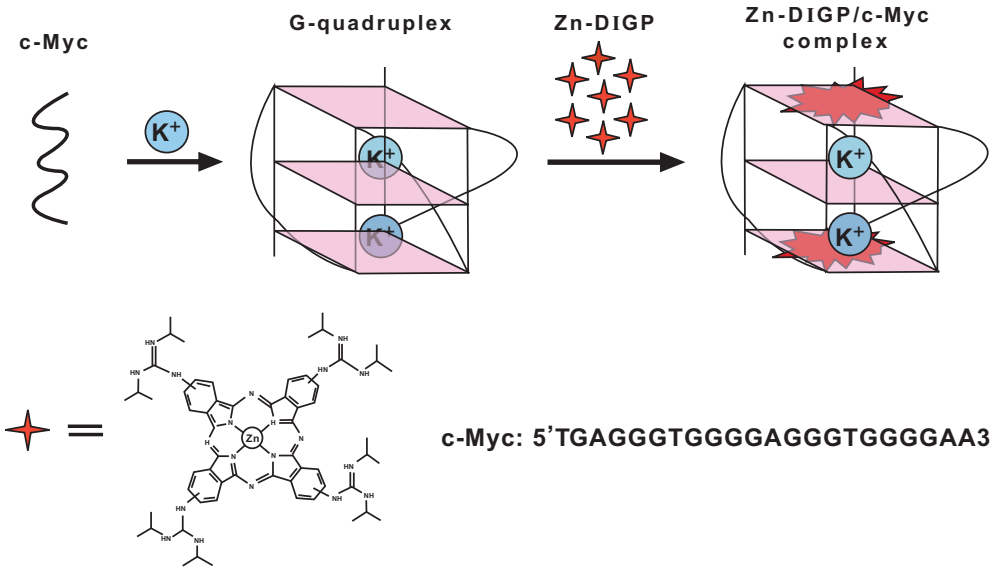


Fig. (3). Schematic illustration of the label-free assay for K^+ ions.

2.2. Detection of Protein

Proteins are ubiquitous in nature and essential for life. In the proteomics era, numerous disease related protein biomarkers are expected to be discovered, and methods of protein detection are indispensable tools for the understanding, diagnosis, treatment, and prevention of many diseases [35]. A G-quadruplex-based biosensor for immunoglobulin E (IgE) was first reported by Yu and coworkers [36] because IgE can trigger the most powerful immune reactions and the level of IgE in human serum is significantly raised in patients, including allergic asthma, atopic dermatitis, AIDS and other immune deficiency-related diseases. Yu and coworker adopted pyrene-labeled G-rich DNA probe and applied it in the IgE detection. This is the first example of intermolecular G-quadruplex-based signaling probe for protein analysis by labeling G-rich nucleotides with single pyrene. In the presence of IgE, the aptamer for IgE wrapped around IgE without hybridizing with the G-rich nucleotides, leading to the self-association of G-rich nucleotides and formation of pyrene excimer. The big advantage of this sensing strategy lies in that it can be applied in complex real sample. Except detection of protein biomarker, the G-quadruplex based probes can also be applied to analyze the enzyme activities. Considerable attention has been directed towards enzyme due to their importance in the human. Leung and coworkers [37] used crystal violet as a G-quadruplex-binding probe to detect 3′-5′ exonuclease Fig. (**4**). It was the first selective, label-free, high-throughput G-quadruplex-based turn-on fluorescence assay for 3′-5′ exonuclease activity.

In another work, Qu and coworkers demonstrated a unique quadruplex-based fluorescence assay for detection of RNase H activity and inhibition by using a G-quadruplex formation strategy [38]. As compared to the above probes used for detection exonuclease, the probe proposed by Qu and coworker is composed of duplex instead of hairpin structure. Nonetheless, both methods provide excellent results.

It has to be noted that the above two approaches are based on fluorescence enhancement of one dye by one G-quadruplex, providing ideal systems for real-time detection of enzymes and enzyme inhibitors. However, signal amplification still has not been incorporated into these detection systems. Recently, as a typical example, the detection and quantification of thrombin via aptamer-based fluorescence assay which is reported by Zhou and coworkers [39] has reflected this interesting trend Fig. (**5**). Zhou's group has synthesized multi-branched dyes which highly bind single strand DNA via electrostatic interaction. Thrombin as a key enzyme in pathologic processes such as leukemia, arterial thrombosis and liver disease, is clinically relevant in biological serum or other complex samples. In the absence of thrombin, dye molecules (TASPI) will aggregate along with the DNA polymer chain, resulting in quenching of multiple dyes. In the presence of thrombin, aptamer (TBA) (5'-GGTTGGTGTGGTTGG-3') forms an intramolecular quadruplex with two G-tetrads that strongly binds to thrombin. All the dye molecules are released due to folding of TBA, leading to the fluorescent recovery. It is expected that this novel sensing platform can be applied in real plasma samples and also can be extended to detect other proteins.

Other ligands instead of TASPI can also be designed and synthesized in combination with folding of G-quadruplex to detect a series of targets of interest [40]. Notwithstanding that the above mentioned aptasensors are highly sensitive to protein, we have to realize that the big challenge for application in the real complex sample still exists due to its highly charged character and hydrophobicity of DNA binding ligands, which will lead to unknown unspecific interaction.

2.3. Detection of Small Molecules

A variety of strategies that use G-quadruplex/fluorescent ligands complexes to detect small molecules have also been reported [41, 42]. Sintim and coworkers [41] discovered that bacterial biofilm regulator, c-di-GMP can form G-quadruplex at physiological conditions in the presence of certain aromatic compounds, such as acriflavine and proflavine Fig. (**6**). Small nucleotides, do not readily form different aggregates at low micromolar concentrations due to the high tropic cost but low enthalpic gain associated with forming aggregates of small. Fluorescence measurement indicates that the fluorescence of these compounds is quenched upon binding of c-di-GMP. This work provides new evidence that small nucleotides such as C-di-GMP can form very stable G-quadruplex at physiological conditions in the presence of these compounds.

Recently, Li and coworkers [42] developed a label-free method for detection of hemin utilizing graphene oxide (rGO)-based fluorescent sensor. They utilized hemin aptamer, a G-rich oligonucleotide sequence that can form G-quadruplex structure in presence of Na(I) or K(I), to capture acridine orange (AO) from reduced graphene oxide (rGO). In the absence of hemin, the fluorescence is in "on" state, when the AO–hemin aptamer /rGO mixture is incubated with hemin, the fluorescence is turned off due to competitive inter-

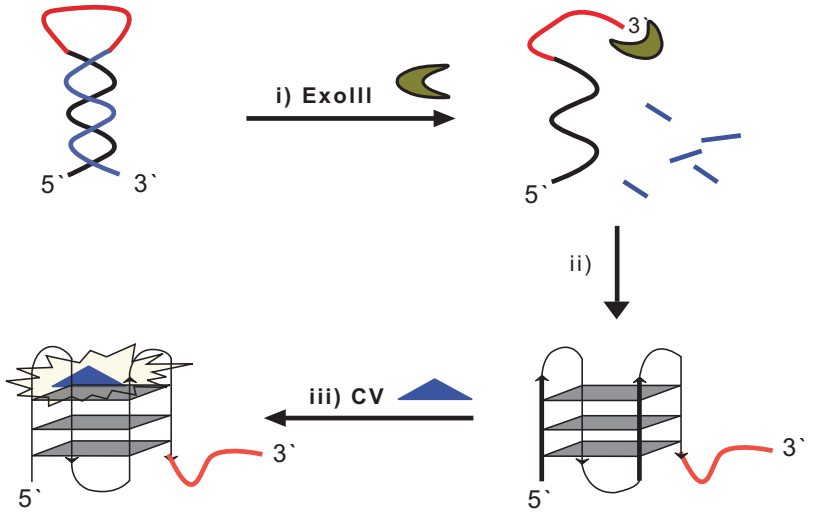


Fig. (4). Detection of 3'- 5'exonuclease activity by G-quadruplex-binding probe.

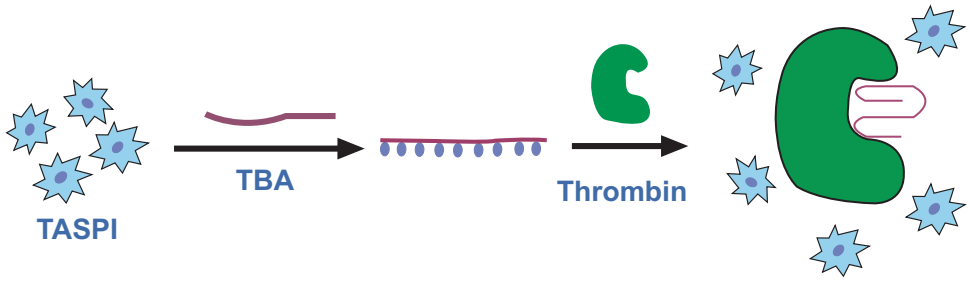


Fig. (5). Thrombin detection using aptamer-based fluorescence assay.

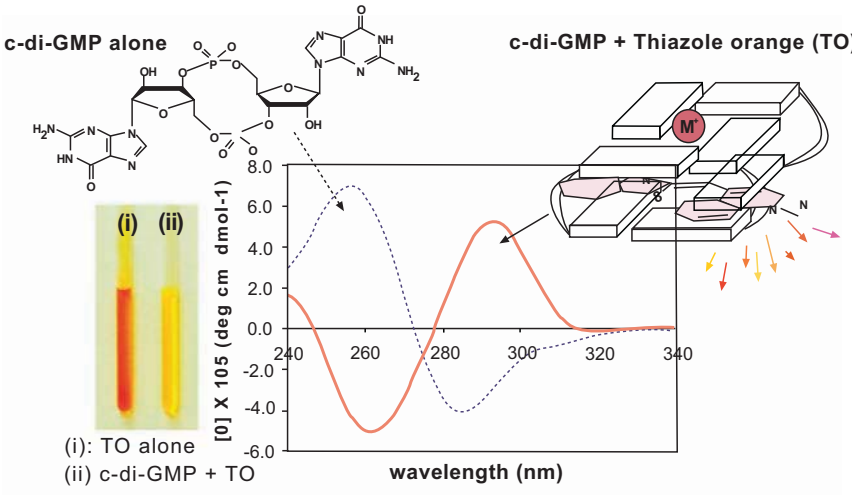


Fig. (6). A simple fluorescent detection of c-di-GMP.

action between hemin and acridine orange (AO) Fig. (**7**). Based on quenching of fluorescence, the target hemin was detected sensitively and selectively.

2.4. Detection of Nucleic Acid

Detection of nucleic acid is important due to the important role of DNA in modern molecular biology [43]. Sensitive and selective detection of nucleic acid is important for gene therapy, mutation analysis and clinical diagnosis [44]. A variety of strategies for detection of nucleic acid have also been reported. Molecules with G-quadruplex-mediated fluorescence properties provide interesting new opportunities for label-free detection of nucleic acids due to the advantage of stability and reproducibility [19, 45-48]. In our lab, it is for the first time reported that target DNA can induce the reassembly of G-quadruplex which can be monitored by fluorescent readout [49].

The interaction between G-quadruplex and dye molecules is quite different. The G-quadruplex is split into two probes each of which is appended with a fragment DNA complementary to half of the target DNA. Coexistence of target and dual probes will favor the assembly of duplex-quadruplex three-way junction that binds to fluorescent dyes. Compared with samples lacking any target, the

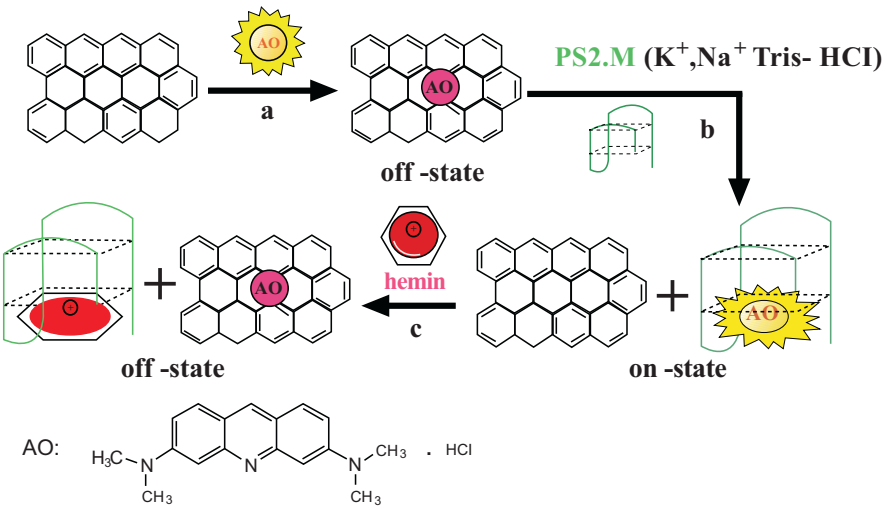


Fig. (7). The method of the fluorescent sensor for the determination of hemin.

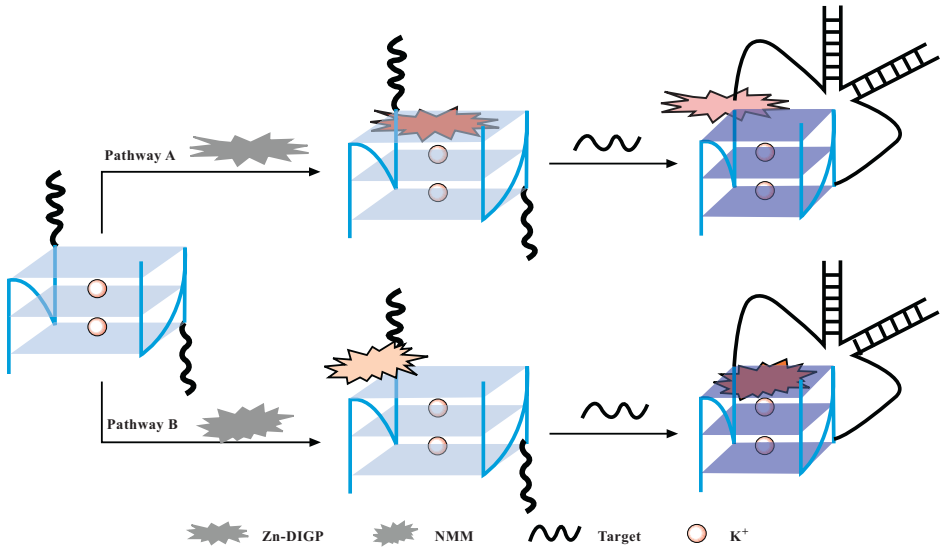


Fig. (8). Schematic illustration of DNA sensors based on turn-off (pathway **A**) and turn-on (pathway **B**) fluorescence changes, utilizing split G-quadruplex probes.

fluorescence intensities of tetrakis (diisoprogylguanidino)-zinc-phthalocyanine (Zn-DIGP) containing sample were lower, and the fluorescence intensities of N-methylmesoporphyrin IX (NMM) containing samples were higher on addition of the target DNA. The resulting biosensors based on Zn-DIGP are therefore termed "turn-off" whereas the biosensors containing NMM are defined as "turn-on" Fig. (**8**). Both of these biosensors can detect target DNAs with a limit of detection in the nanomolar range, and can discriminate mismatched from perfectly matched target DNAs. Our approach is direct, easy to conduct, and fully compatible with the detection of specific DNA sequences in biological fluids.

Qu and Ren reported label-free, quadruplex-based functional molecular beacon for fluorescence turn-on detection of DNA and nuclease. Detailed information can be referred to the literature [50]. Recently, this same group investigated a label-free fluorescent turn-on enzymatic amplification assay for DNA detection using ligand-responsive G-quadruplex formation [44]. They used exonuclease III (Exo III), which does not require a specific recognition site and cleave duplex from 3'ends irrespective of the sequence present at the blunt terminus, to design a duplex DNA probe with one DNA strand designed as a quadruplex-forming oligomer. When the probe was challenged with complementary target DNA which was hybridized with the ssDNA at 5'end of the duplex probe, the probe-target DNA duplex was subsequently digested by Exo III, releasing the G-rich oligomer to fold into quadruplex structure and allowing target DNA into the second catalytic circle. Thus, the fluorescence of the probe was turned ''on'' Fig. (**9**). The result of DNA detection in Fig. (**10**) showed the amplification provided by Exo III leads to a 8.2-fold increase by adding perfectly matched target DNA. This detection platform is also selective to differentiate mismatched DNA, which makes it promising for biomedical application.

3. G-QUADRUPLEXES DNAZYME

A few of G-quadruplex–hemin complexes exhibit superior peroxidase-like activity and effectively catalyze the H_2O_2-mediated oxidation of 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonicacid) diammonium salt (ABTS) or luminal [51-55]. As compared to traditional peroxidase, the G-quadruplex-based DNAzyme can be facilely tethered to DNA sequences as probes, serving as a novel kind of catalytic label or beacon via colorimetric or chemilumines-

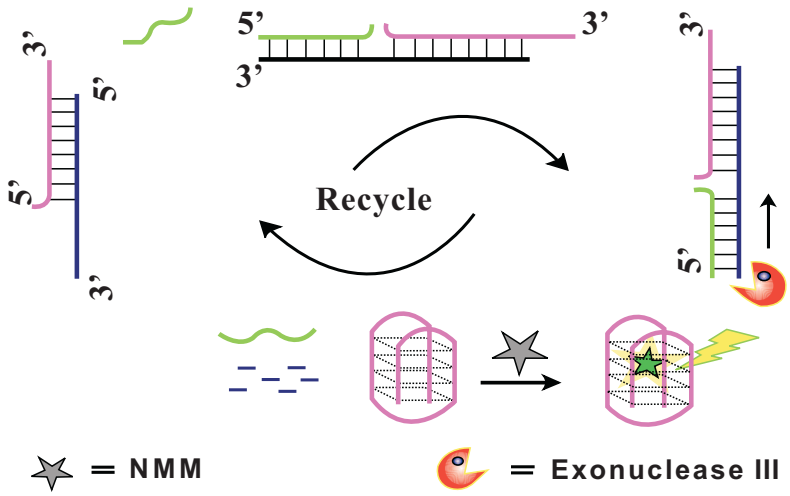


Fig. (9). Enzymatic amplified DNA detection platform based on Exo III assisted ligand-responsive quadruplex formation.

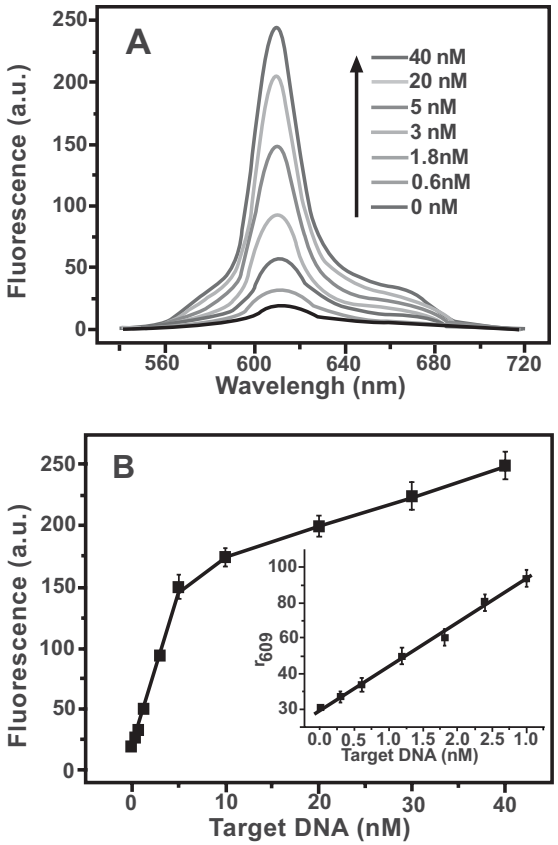


Fig. (10). The result of enzymatic amplification assay for DNA detection. (**A**) The amplification provided by Exo III leads to a 8.2-fold increase in the final fluorescence intensity. (**B**) specificity of the DNA assay detecting different target: no Target: no Target DNA was added; T: perfectly matched target DNA; T1: one-base mismatched DNA; T2: two-base mismatched DNA; T3: three-base mismatched DNA.

cence's (CL) change [56, 57]. A serial of biosensors based on G-quadruplex DNAzyme have indicated their great potential in various applications territory, including disease diagnosis, drug screening, environmental and food-safety monitoring, pathogen identification, and forensic science.

3.1. Detection of Metal Ion, Protein and Cancer Cell

Some metal ions, for example, lead and mercury, are harmful environmental contaminants with a health risk to humans. Sensors for easy detection of these metals are therefore highly desirable. Advances in many different design principles have led to development of DNAzyme-based sensors for metal ions detection including Cu^{2+}, Ag^{+}, Pb^{2+} and Hg^{2+} [58-62]. In Chang's lab, they have developed a fluorescence approach for the highly selective and sensitive detection of Pb^{2+} ions using AGRO100, a G-quadruplex DNAzyme [63]. The sensing strategy was based on Pb^{2+} inducing increased DNAzyme activity of AGRO100 in the presence of hemin, which acts as a cofactor to catalyze H_2O_2 mediated oxidation of Amplex UltraRed (AUR). They also validated the practicality of the use of the AGRO100 (AUR) probe for the determination of the concentrations. In the presence of sodium ion (100 mM), the detection limit of 1 nM can be obtained.

Except the above biosensor based on G-qauderuplex/hemin as DNAzyme for Pb^{2+}, several other biosensors based on G-quadruplex/hemin for Pb^{2+} have also been reported [62]. As far as we know, the biosensor as shown in Fig. (**11**) offers the best selectivity and sensitivity.

Thrombin as an important protein, has been detected using DNAzyme-based sensors [52]. In our lab, we have applied multifunctional G-quadruplex aptamers to detect protein [64]. Since thrombin has two binding sites, thrombin can be sandwiched by two aptamers. One aptamer (TBA) is immobilized on the gold substrate to capture the target protein, second aptamer linked with DNAzyme can occupy the second binding site, and therefore, the protein can be quantitatively detected via luminal-H_2O_2 method Fig. (**12**). This interesting detection method can be extended to detect any protein which has two aptamer binding sites.

We also applied AGRO100 for detection of nucleolin expressed at the surface of HeLa cells via the chemiluminescence's method [65]. Based on the specific AGRO100–nucleolin interaction, the surface-expressed nucleolin of HeLa cells was labeled in situ with the hemin–AGRO100 DNAzyme, and then determined in the luminol–H_2O_2 system. Through this approach, the sensitive detection of total nucleolin expressed at the surface of about 6000 HeLa cells was accomplished.

3.2. Detection of Small Biomolecules and Nucleic Acid

In addition, small molecule's detection utilizing DNAzyme derived from G-quadruplexes have also been reported. Deng and coworkers developed a new method to detect adenosine triphos-

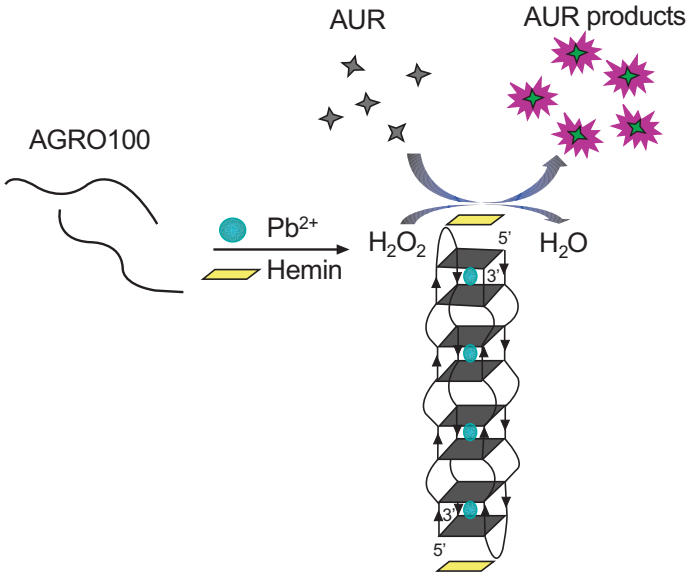
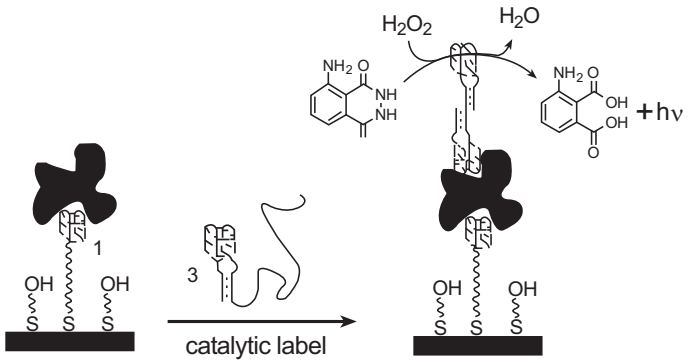


Fig. (11). Schematic Representation of a Pb^{2+} sensor that operate based on modulation of the catalytic activity of Hemin/AGRO100 compelxes.



1 = 5'-HS(CH_2)$_6$ TTTTTTTTTTTTTTTTTTTTGGTTGGTGTGGTTGG-3'
3 = 5'-AGTCCGTGGGTAGGGCGGGTTGGGGGTGACTGTCCGTGG
TAGGGCAGGTTGGGGTGAC-3'

Fig. (12). Construction of CL thrombin aptasensor by using the DNAzyme G31 tethered to 27-mer TBA as the catalytic label.

phate (ATP) by using a DNAzyme aptamer sensor [66]. They designed two DNA sequences. One was a functional chain (chain A) consisting of two parts, the anti-ATP aptamer (recognition part) and the DNAzyme (signal transduction part). The other was a blocker chain (Chain B), which could partially hybridize with Chain A. By addition of ATP and hemin, the hybridized chains A and B were unfolded. The DNAzyme in the functional chain formed a G-quadruplex with hemin and then catalyze the oxidation by H_2O_2 of ABTS, resulting in a color change. The detection limit was $1X10^{-6}$ M. In our lab, we reported a simple and sensitive DNAzyme-based colorimetric sensor for cocaine detection in a 3,3,5,5-tetramethylbenzidine sulfate (TMB)–reaction system by combining the advantages of magnetic nanoparticles (MNPs) with colorimetric method using hemin-G-quadruplex complex as the sensing element Fig. (**13**).

This is the first colorimetric method for the detection of drug cocaine based on the catalytic activity of the DNAzyme in the TMB–H_2O_2 reaction system. Fig. (**14B**) showed with increasing concentration of cocaine, A_{450} signals were obviously increased. A linear range from 100 nM to 20 mM and a detection limit of 50 nM were obtained. As shown in the photographs of Fig. (**14C**), it was observed that 10 mM cocaine led to an evident color change. While other drugs did not cause an observable color change compared with the background. This proved the cocaine aptamer was highly specific to cocaine.

The designed sensor enlarged the applications of both G-quadruplex DNAzymes based TMB oxidation and colorimetric sensors for real time drug detection [67].

Sequence-specific detection of nucleic acid sequences is central to genetic analysis. It is important in disease diagnosis, drug screening and food safety monitoring domain [68]. Until now, there are several examples which have been shown to detect nucleic acid based on the G-quadruplex DNAzyme. There is a common feature shared by these experiments: DNA template–directed G-quadruplex reconfiguration.

Kong reported specific nucleic acid and single nucleotide polymorphism detection [69]. Differ from previous report, they developed a new sequence-specific DNA sensor based on the amplified formation of G-quadruplex DNAzymes using polymerase chain reaction (PCR)-like temperature cycles. If the target had a three consecutive cytosines downstream of the primer binding site, three corresponding guanosines could be added to the 3'-end of the probe. Thus, the continuous formation of G-quadruplex can be achieved Fig. (**15**). Using this method, tedious labeling of oligonucleotides was not needed and the test results could be easily judged

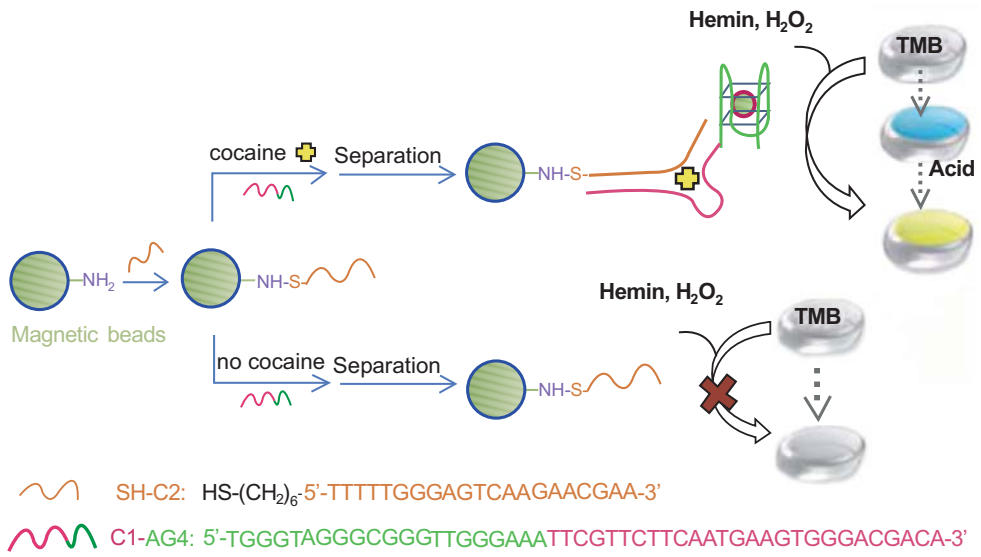


Fig. (13). The sensing strategy for colorimetric analysis of cocaine by using MNPs as separation and amplification element.

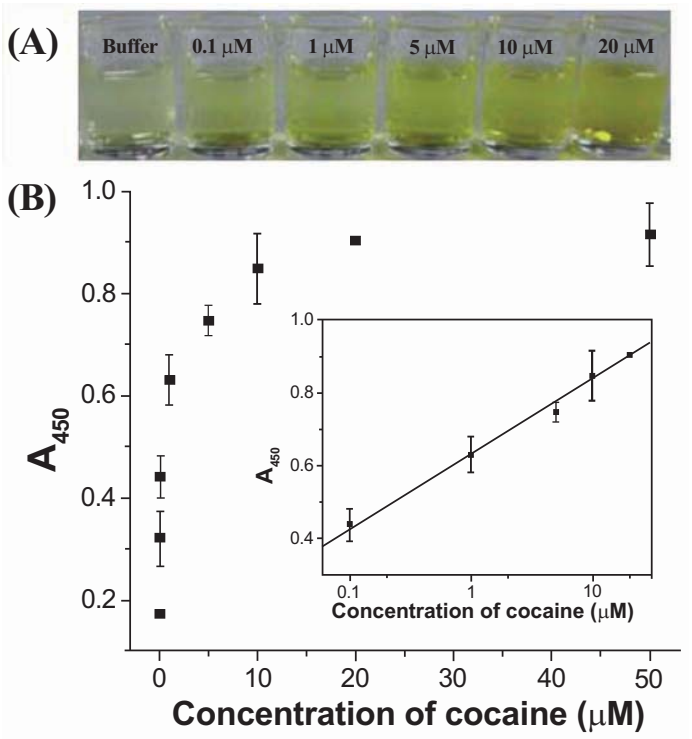


Fig. (14). Sensitivity of the sensing strategy based on the MNPs as amplification elements. (**A**) The photograph of the detection of cocaine of utilizing the color change caused by the catalytic activity of DNAzyme in the TMB–H_2O_2 reaction system. (**B**) Dependence of the absorbance at 450 nm on the concentration of cocaine (from 50 nM to 50 mM). Inset: A liner detection range was from 0.1 mM to 20 mM.

by simple colorimetric detection, eliminating the need for expensive instruments.

Since the first discovery of G-quadruplex-incorporated split probes by Kolpashchikov [70], several interesting works have being published related to the split G-quadruplex probes. There are two kinds of split G-quadruplex probes, including symmetrical split probes and asymmetrical split probes, all of which have been shown to selectively detect single nucleotide polymorphism. Comparatively, asymmetrical split probes have the best sensing performance.

In 2008, Zhou's group investigated colorimetric and visual detection of nucleic acids using an asymmetrically split peroxidase DNAzyme [71]. The G-quadruplex containing peroxidase DNAzyme utilizes the 3:1 split mode and can be directly used for the detection of SNPs with a detection limit in the nM range Fig. (**16**).

Motivated by the above interested results, Sintim and others have systematically investigated the salient features and reaction conditions that facilitate proficient enzymes [72]. Several important findings were disclosed: (1) the loops that connect the G3-tracts in a G-quadruplex structure can be replaced with a stem-loop or loop-

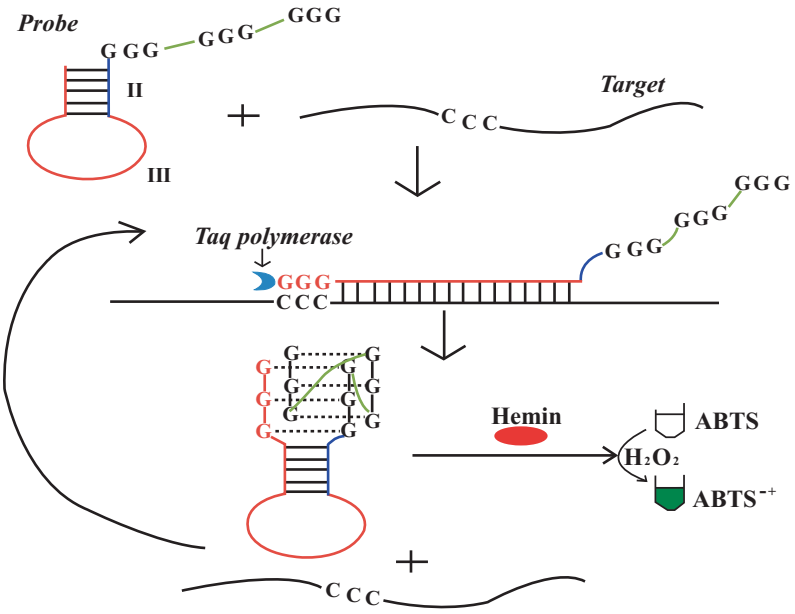


Fig. (15). Schematic representation of the specific nucleic acid detection.

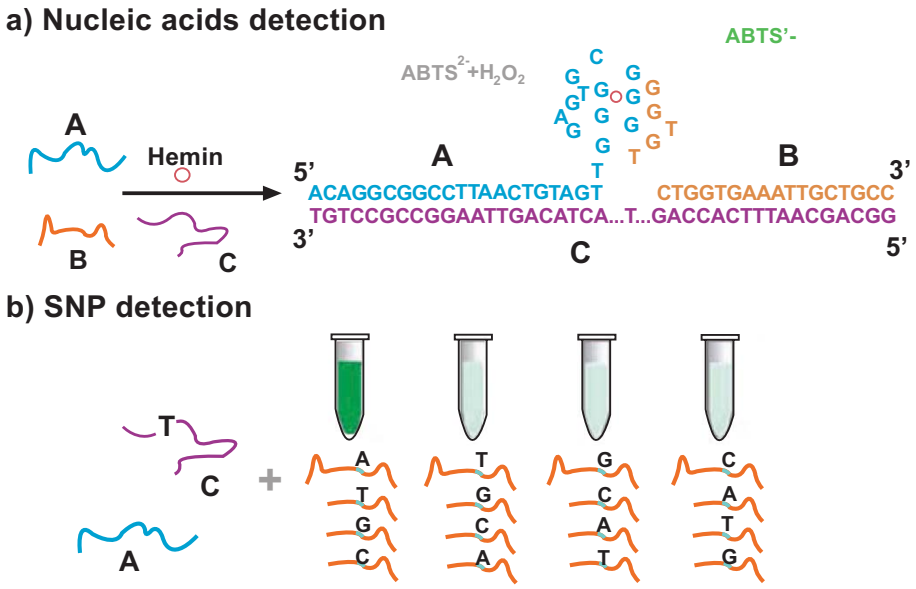


Fig. (16). Detection of nucleic acids split peroxidase DNAzyme.

stem-loop motif with endowing the split G-rich probes with high proficient enzymes. (2) The binding of hemin with G-quadruplex induces the blue-shift of CD spetrum of G-quadruplex. (3) The DNA base sequence adjacent to the G-quadruplex influence both the stability and the enzymatic proficiency of the reassembled enzyme. (4) The characteristic of monovalent ions influences the enzyme activities.

Without any labeling in the above experiments, the sensing probes after target-induced reassembly can be used to catalyze ABTS, leading to colorimetric change, which is also can be detected by UV-vis absorption spectrum [73]. As we all know, fluorescence detection is much more sensitive than UV-vis absorption detection. Chen's group has go one more step further by designing a new sensing platform based on G-quadruplex DNAzyme which can catalyze the nonfluorescent substrate to the fluorescent substrate Fig. (**17**).

4. G-QUADRUPLEX BASED ELECTROCHEMICAL BIOSENSOR

Recently, the electrochemical methods due to their rapid response, miniaturization of the instruments, low cost, high selectivity and sensitivity, have been received more and more attention [74]. The strategy based on G-quadruplex in combination with electrochemistry was promising for application including drug screening, DNA circuit design, protein detection, and various analytes [75-77].

4.1. Drug Screening Based on Electrochemical Aptasensor

By integrating G-quadruplex with electrochemistry, G-quadruplex-binding drugs can be selectively screened. In 2010, Jin and coworkers reported selection of G-quadruplex-binding ligands based on structure switching using the electrochemical method [75]. This group used $[Ru(NH_3)_6]^{3+}$ (RuHex) as signal transducer, and exploited the electrostatic interactions between RuHex and anionic

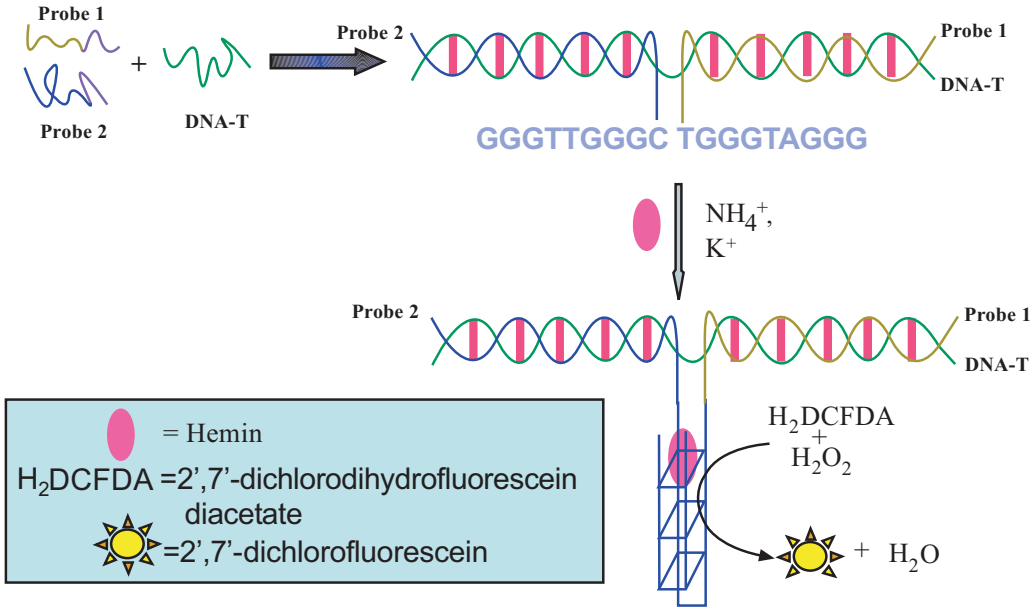


Fig. (17). Working principle of this sensor based on G-quadruplex DNAzyme.

phosphate backbones of DNA strands. In the presence of quadruplex-binding ligand, the oligonucleotide of the human telomeric sequence folded into a G-quadruplex structure, as a result, the adsorption amount of RuHex reduced and the peak current decreased. Six traditional Chinese medicine monomers were investigated as potential ligands using the above-proposed method. They confirmed this electrochemical method could induce the GDNA folding into G-quadruplex. So this electrochemistry method offers a simple and effective approach to identify ligands with potential anticancer activity.

4.2. DNA-Based Nano-Circuitry Characterized by Electrochemistry

DNA-based nano-circuitry can be designed based on G-quadruplex and electrochemical characterization methods. In 2010, Ge immobilized the DNA nanoswitch on the gold electrode, it is able to switch repeatedly between a structurally extended electronic "off" state in the absence of G-quadruplex and "on" state in the presence of G-quadruplex [76]. The formation of G4-DNA in the contracted mode of the nanoswitch may bring the ferrocence closer to the gold surface and therefore enhance the current signal.

4.3. Detection of Protein and Enzyme

Electrochemical aptasensor based on G-quadruplex can be used to detect enzyme activities. In 2010, Li and coworkers reported electrochemical aptasensor to apply in the assay of adenosine deaminase activity [77]. The group designed oligonucleotide containing three functional regions (an adenosine aptamer region, a G-quadruplex halves region, and a linker region). The enzymatic reaction of adenosine catalyzed by adenosine deaminase played a key role in the regulation of the binding of the G-quadruplex halves with hemin. The electroactive probe reflected the activity of the enzyme indirectly but accurately. The detection limit of the fabrication biosensor can be lowered to 0.2 U mL^{-1} of adenosine deaminase.

Except detection of deaminase activities based on electrochemical aptasensor, detection of other protein like thrombin has also been reported based on integration of G-quadruplex and electrochemistry. Plaxco and coworkers reported thrombin detection using electronic and aptamer-based sensors [78]. In the presence of thrombin, binding of protein drove the equilibrium from duplex to the G-quadruplex, liberating the 5'end of the methylene blue-tagged oligonucleotide as a flexible, single-stranded element and producing a detectable electronic signal. Subsequently, different detection methods of thrombin utilizing electrochemical/quadruplexes have been reported [79-82]. In 2010, He and coworkers [80] developed another G-quadruplex-based DNAzymes aptasensor for the amplified electrochemical detection of thrombin. In the presence of thrombin, the enzyme activity could be extensively promoted, resulting in amplified electrochemical readout signals for detecting thrombin Fig. (**18**).

Fig. (**19A**) showed that bovine serum albumin (BSA) and lysozyme had no influences on thrombin detection. Therefore, the proposed aptasensor was highly selective. As showed in Fig. (**19B**), the catalytic currents were logarithmically related to the thrombin concentration across the range of 0.1 nM to 1mM, spanning a response region of at least 4 orders of magnitude. This demonstrated that the aptasensor was responsive to its target protein. Inset in Fig. (**19B**) outlined the relationship between current and the Log concentration of thrombin. As a result, a good linear relationship ($R=0.997$) equation for analyzing thrombin could be obtained with a detection limit of 0.06 nM, which reflected the high signal amplification of the DNAzyme.

This aptasensor exhibited high sensitivity and selectivity for thrombin determination with a detection limit of 6×10^{-11} M. Protein detection can also be accomplished based on integration of G-quadruplex and electrochemluminescence. In 2011, Fu and coworkers [79] reported electrochemiluminescence method for the detection of ultratrace thrombin. This group developed an aptamer biosensor in combination with electrogenerated chemiluminescence (ECL) for detection of ultratrace thrombin based on a structure-switching ECL-quenching mechanism. In the absence of thrombin, capture probe, aptamer probe and ferrocene-labeled probe will hybridize to form a ternary "Y" junction structure and resulted in a quenching of ECL of $Ru(bpy)_3^{2+}$. Whereas, in the presence of thrombin, the capture probe prefers to form the G-quadruplex aptamer–thrombin complex and lead to an obvious recovery of ECL of $Ru(bpy)_3^{2+}$ Fig. (**20**). Detection limit of thrombin was 8.0×10^{-15} M. The assay provided a significant step towards the development of method for monitoring ultratrace thrombin in clinical detection.

Wang and coworkers [81] detected thrombin using amplified electrochemical signal based on the mechanism that the presence of

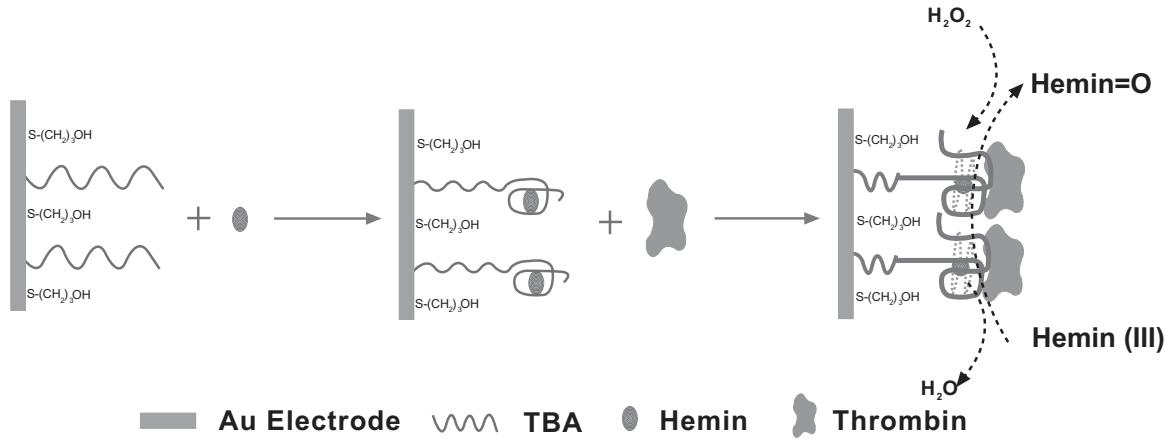


Fig. (18). Schematic of the thrombin detection based on DNAzymes.

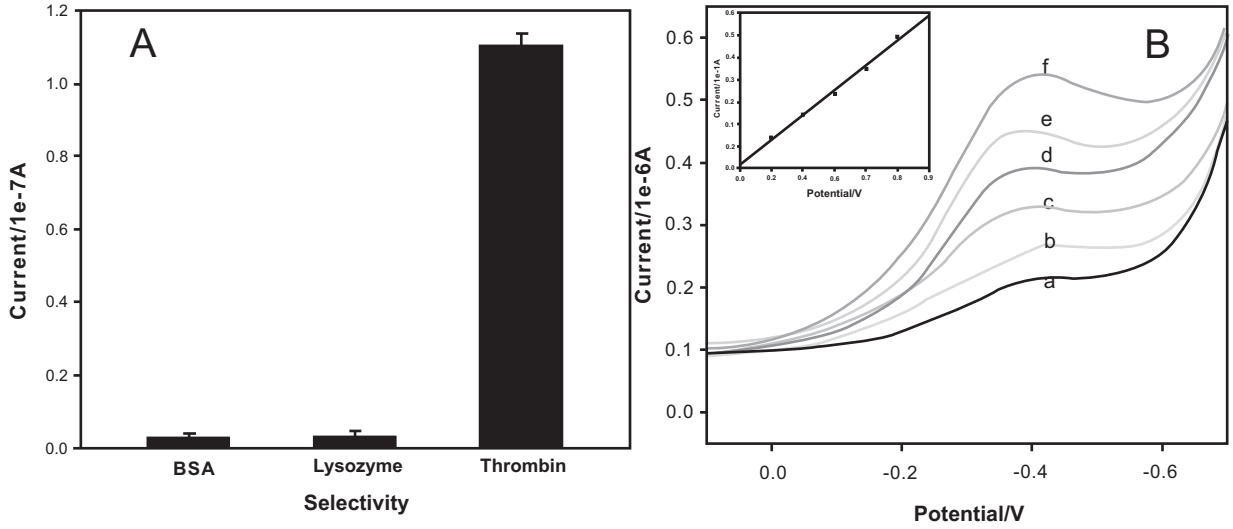


Fig. (19). Amplified electrochemical detection of thrombin based on G-quadruplex DNAzymes aptasensor **A**) The variations of the peak currents of the aptasensors after incubation with 100 mM BSA, Lysozyme and 1 nM thrombin respectively for 30 min. **B**) Cyclic voltammograms of aptasensor corresponding to analysis of different concentrations of thrombin. Calibration plot of catalytic currents response as a function of logarithm of thrombin concentrations derived from the cyclic voltammograms: (**a**) 0 M, (**b**) 1x 10^{-10} M, (**c**) 1 x 10^{-9} M, (**d**) 1 x 10^{-8} M, (**e**) 1 x 10^{-7} M, (**f**) 1 x 10^{-6} M.

thrombin induced the conformation change of aptamer. They designed an aptamer modified with CdS hollow nanospheres to amplify the electrochemical signal because nanoparticles promoted the electron transfer between the gold electrode and $K_3[Fe(CN)_6]$ and facilitated the conformation conversion of the aptamer from hairpin to G-quadruplex after the aptamer interacted with thrombin Fig. (**21**). Under optimal conditions, the modified electrode could be used for the determination of thrombin from 0 to 33μg mL^{-1} and the sensitivity was 1.34 μA mL $μg^{-1}cm^{-2}$, while the linear range of the modified electrode without the immobilization of CdSHNs was from 2.75 to 27.5 μg mL^{-1} and the sensitivity was 0.062 μA mL $μg^{-1}cm^{-2}$.

4.4. Detection of Metal Ion

In 2009, Fu and coworkers [83] reported a new electrochemical biosensor for the monitoring of ultratrace terbium. The biosensor was fabricated by immobilizing a thiolated G-rich DNA on the gold surface as probes. Based on the conformational change of the G-rich DNA probe from flexible single stranded to rigid tetramolecular G-quadruplex in the presence of Tb^{3+}, it provided a switchable charge transport path for the oxidation of $[Fe(CN)_6]^{4-}$ Fig. (**22**). The method determined the Tb^{3+} with a detection limit of $6.0×10^{-11}$ M. Other metal ions such as Pb^{2+} has also been detected by electrochemical method [84]. Detection concentration of Pb^{2+} was from 50 mM to 0.5 nm.

4.5. Detection of DNA and Small Molecule

In 2010, Willner [85] reported the detection method of DNA and adenosine monoPhosphate (AMP). They used the horseradish peroxidase-mimicking DNAzyme as an electrocatalyst and immobilized hemin/G-quadruplex DNAzyme on Au-electrode surfaces. In the presence of the DNA analyte or AMP, the hairpin structures are opened, and the horseradish peroxidase-mimicking G-quadruplex DNAzymes are generated on the electrode surfaces. The cathodic currents generated by the functionalized electrodes, upon the electrochemical reduction of H_2O_2, provide a quantitative measure for the detection of the target analytes. The DNA target was analyzed with a detection limit of 1×10^{-12} M, while the detection limit for analyzing AMP was 1×10^{-6} M.

4.6. Detection of Cancer Cell

For label-free cancer cell detection, electrochemical technique is also an alternative to develop biosensors for cancer monitoring or impedimetric microtransducers for measuring the swelling behavior

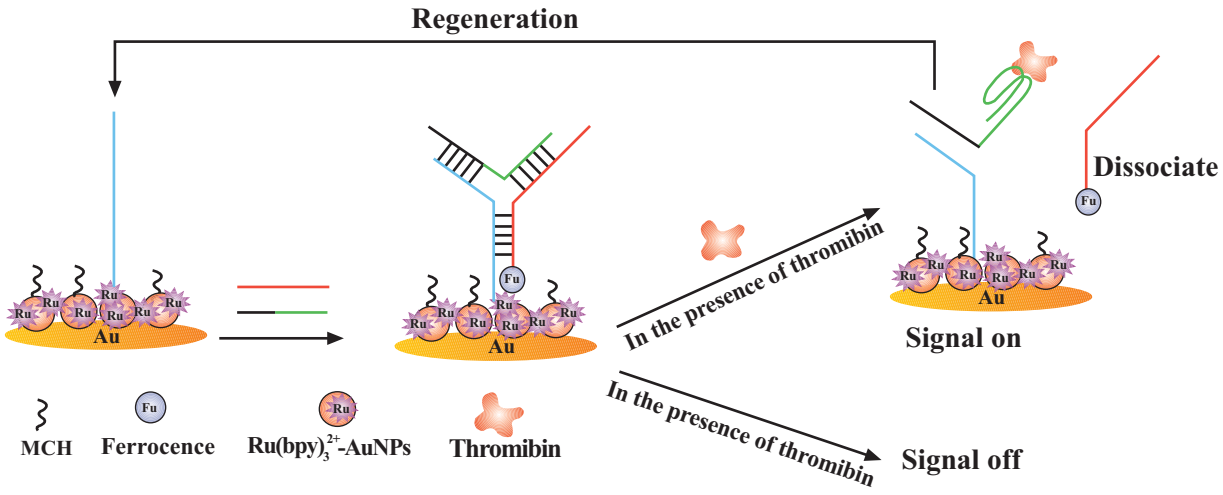


Fig. (20). Experimental principle of the junction-probe ECL aptamer biosensor for the detection of thrombin.

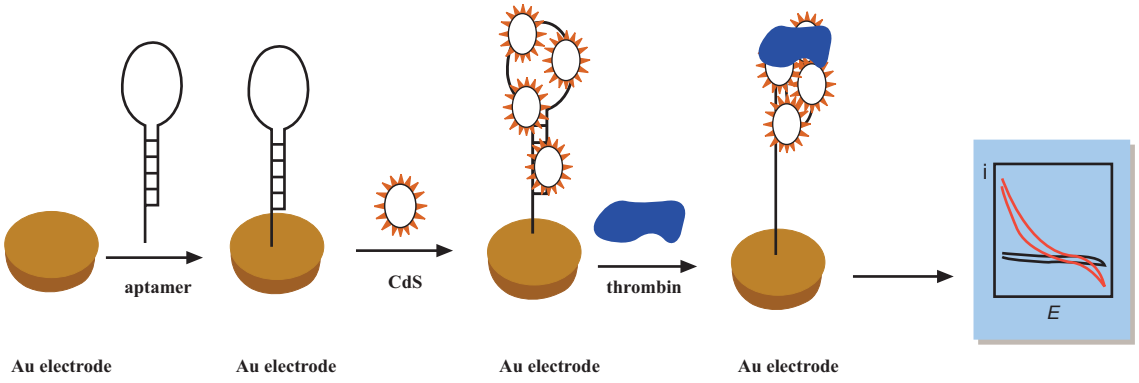


Fig. (21). Schematic representation of the modification process of the electrode and the detection of thrombin.

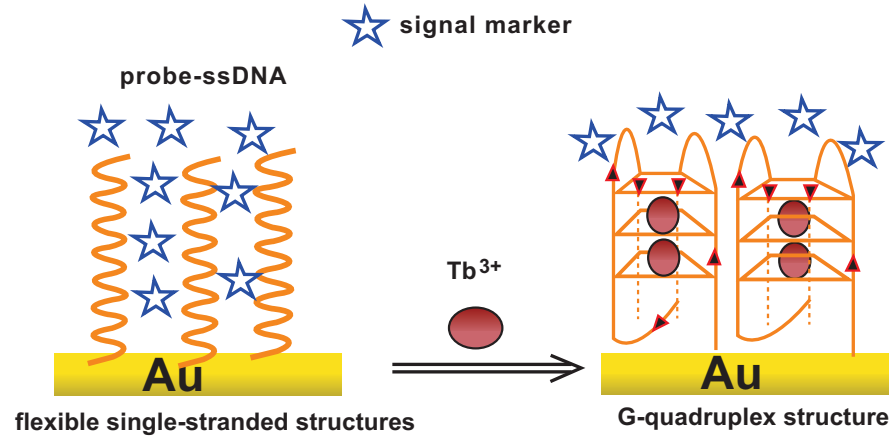


Fig. (22). Schematic representation of the modification process of the electrode and the detection of Tb^{3+}.

of different types of cancer cells. Recently, Qu and coworkers [86] reported a label-free method for detection of cancer cells utilizing graphene functionalized electrochemical aptasensor. The sensor is constructed based on graphene-modified electrode and the first clinical trials II used aptamer, AS1411. AS1411 and its complementary DNA are used as a nanoscale anchorage substrate to capture/release cells. When AS1411 form G-quadruplex and has high binding affinity and specificity to the over expressed nucleolins on the cancer cell surface as compared with normal cells, the designed electrochemical aptasensor has the ability to differentiate cancer cells and normal ones. The sensor can be regenerated using AS1411 cDNA and **was** reusable for cancer cell detection as shown in Fig. (**23**). This graphene/aptamer-based design can be adaptable for detection of protein, small molecules, and nucleic acid targets by using different DNA sequence and specificity.

5. INTEGRATING NANOTECHNOLOGY WITH G-QUADRUPLEX FOR BIOANALYSIS

Nanotechnology has opened up new avenues towards ultrasensitive, highly selective detection of biological molecules and toxic agents, as well as for therapeutic targeting and screening [13]. G-quadruplex-involved nanotechnology is interesting in this field to identify ligands with good selectivity which can discriminate quadruplex DNA from nucleus duplex DNA or single-stranded DNA, as their toxicity to healthy cells has to be as low as possible. Different nanostuffs in combination with G-quadruplex have been used to detect different proteins, metal ions, nucleic acid in recent years [87-91].

As the leading nanostuff candidate, single-walled carbon nanotubes (SWNTs) have potential therapeutic applications in gene therapy and novel drug delivery [92-95]. Single-walled carbon nanotubes (SWNTs) can selectively induce human telomeric i-motif DNA formation at pH 7.0. Using this property, Qu and coworkers [96] designed a DNA nanomachine induced by SWNTs on gold surface. Their work provided a new concept for designing an SWNT-induced DNA nanomachine and for the detection of i-motif DNA structure at pH 7.0.

Based on unique size and distance-dependent optical properties [97], gold nanoparticle (AuNPs) in combination with G-quadruplex has been employed as a signal transducer/amplifier on a variety of biosensing platforms. In 2009, Jin and coworkers [98] reported selection of quadruplex-binding ligand using gold nanoparticle as a quencher. They investigated two species Chinese medicine, flavonoids and alkaloids. When a dye-labeled single-stranded probe sticks to the gold nanoparticles, the attendant proximity of the dye to the gold leads to fluorescence quenching of the dye. However, in the presence of quadruplex-binding ligands, the formation of G-quadruplexes leads to release the probe DNA from AuNPs, and the fluorescence enhancement is observed. Chang and coworkers [99] have used aptamer–gold nanoparticles, PDGF aptamer, and Oligreen to detect adenosine. The presence of adenosine induced the conformational switch of the aptamer from coiled state to a G-quadruplex structure, leading to the less binding of aptamer onto the surface of AuNPs. As the more the adenosine was present, the less the amount of aptamer was adsorbed, resulting in the fluorescence change of the dye labeled aptamer. This sensor provides the limit of detection of 70.0 nM for adenosine. Using gold nanoparticle/quadruplex property may also detect other small molecule. In 2010, Yang and coworkers [100] reported the colorimetric detection of ochratoxin (OTA) utilizing OTA's aptamer and unmodified AuNPs. The assay method was based on the conformation change of OTA's aptamer in phosphate buffered saline (PBS), and the phenomenon of salt-induced AuNPs aggregation. The conformation of OTA's aptamer in PBS buffer changed from random coil structure to compact rigid antiparallel G-quadruplex structure. Because this compact rigid G-quadruplex structure could not protect AuNPs against salt-induced aggregation, as the conformation change of aptamer, the color change from red to blue could be observed by the naked eye and the detection limit of 20 nM was obtained. It has also been reported by us [101] that G-quadruplex which is induced by OTA is resistant to absorption onto graphene oxide Fig. (**24**).

Recently, Qu and coworkers [102] designed an ideal system for screening G-quadruplex ligands and evaluating selectivity using gold nanoparticles (AuNPs). This method is based on knowledge that DNA-modified AuNPs are stable at certain salt concentrations, and unmodified AuNPs tend to aggregate. In the presence of G-quadruplex binding ligand, the DNA grated onto gold nanoparticles will switch from linear state to G-quadruplex conformation, protecting the DNA from degradation by enzyme, however, in the absence of G-quadruplex binding ligand , the DNA can easily be cleaved by enzyme, leading to a red-to-purple color change Fig. (**25**).

These findings may have important implications for the future use of nanoparticle-based technologies in the discovery of potential therapeutic agents, and offer a new approach to the detection of a wide spectrum of analytes.

Gold nanoparticle and gold nanorods have been used to detect protein and nucleic acid in combination with G-quadruplex. In 2010, Jiang and coworkers [103] reported a method to detect

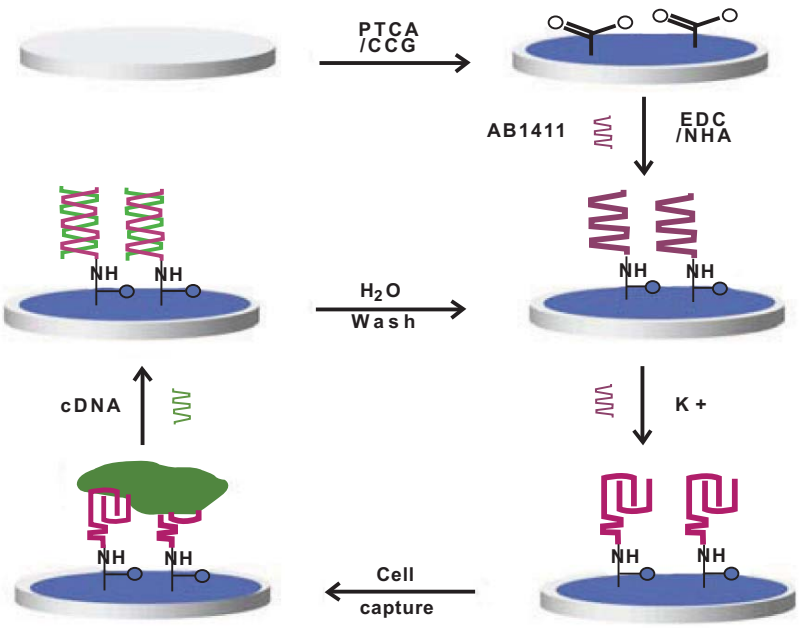


Fig. (23). Schematic representation of the reusable aptamer/graphene-based aptasensor.

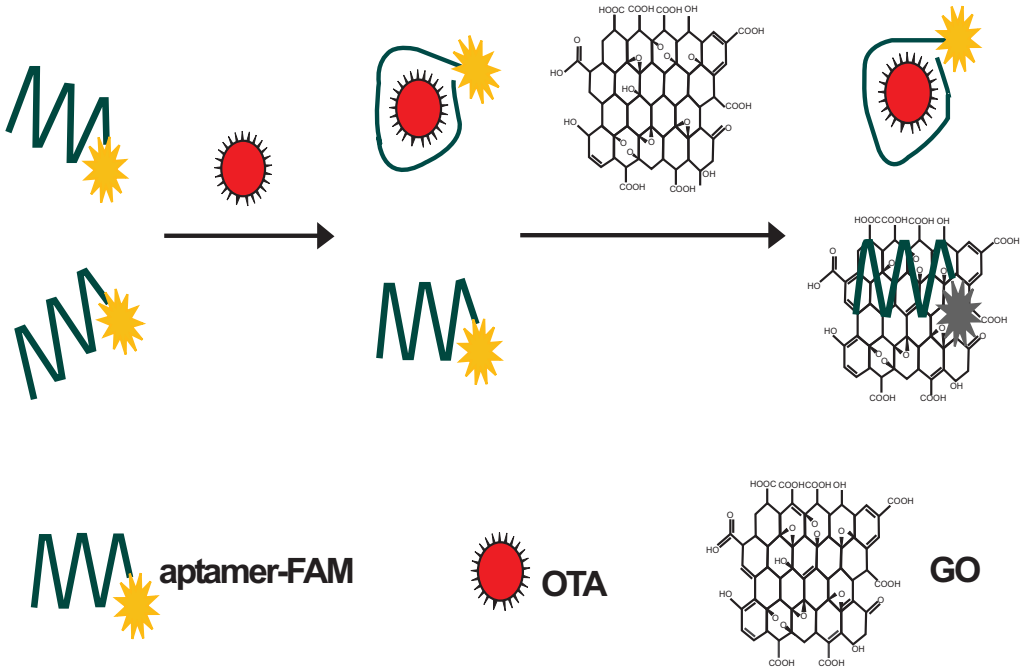


Fig. (24). Schematic illustration of graphene oxide sensing platform for detection of ochratoxin A.

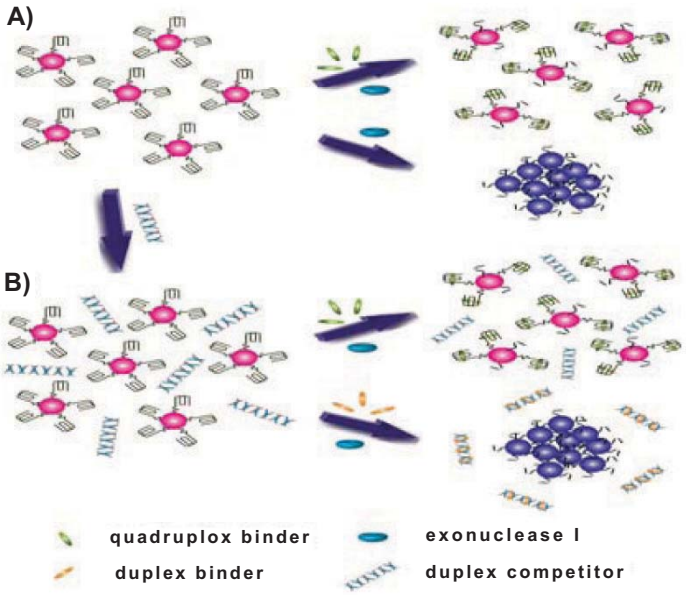


Fig. (25). Scheme of the assay for screening of quadruplex binders and evaluating quadruplex–duplex selectivity.

thrombin using aptamer-modified AuRe nanoprobe in combination with resonance scattering (RS) spectral method. In the presence of metal ion (K^+, Na^+), the conformation of the aptamer on AuRe nanoparticle changed from single strand to G-quadruplex structures. In the absence of thrombin, the resonance scattering signal of aptamer, thrombin, and AuRessDNA was very weak. However, in the presence of thrombin, AuRe nanoparticles modified with aptamer were interconnected to three dimensional networks due to the specific interaction between aptamer and thrombin. AuRe-aptamer (G-quadruplex structure)-thrombin cluster exhibited a RS peak at 560 nm. As the amount of thrombin increased, the amount of AuRe-aptamer-thrombin cluster also increased and the value of I_{560} nm increased linearly. Thus, thrombin concentration may be detected with a detection limit of 13 pmol/L. Zhang's group [104] reported an interesting method for detection of human telomere DNA hybridization and G-quadruplex formation using gold nanorods. The conformation change of the telomere DNA due to hybridization and G-quadruplex formation was transferred into multiple optical signals such as fluorescent emission, near infrared (NIR) absorption, plasma resonance light scattering and dynamic light scattering (DLS) response. Based on these characterization tools, human telomere DNA can be detected at a concentration as low as 58 nM.

Except the above-mentioned nanomaterials-based bioassays, DNA origami as recently emerging new nanoscaffold has also attracted extensive attentions [106]. As shown in Fig. (**26**), Yan's group [105] has successfully assembled patterned G-quadruplex onto DNA origami. Using DNA-tile directed self-assembly of signaling aptamers into high-density nanoarrays with～27 nm periodic spacing between neighboring aptamers, protein thrombin can be selectively captured. In the presence of thrombin, the fluorescence of signaling aptamer for human thrombin, which has been modified with a fluorescent nucleotide, significantly increased. Low concentrations of thrombin protein bound to the arrays may be detected by using confocal fluorescence microscope imaging.

More interesting, the G-quadruplex formation can also be directly imaged if the G-quadruplex was incorporated into the inside of the DNA origami as shown in Fig. (**27**). The introduced G-rich strands formed an interstrand (3+1) G-quadruplex structure in the presence of K^+, and the formed four-stranded structure can be disrupted by removal of K^+ [107]. The conformational changes can be visualized on the DNA origami in real time.

Finally, we would like to introduce stimulus-responsive nanochannels which can be constructed by immobilization of conformation-switchable G-quadruplex in a synthetic nanopore [108-110]. Jiang's group reported a novel biomimetic nanochannel system which can achieve a K^+ response within a certain ion concentration range [111]. This new phenomenon is caused by the G-quadruplex DNA conformational change with a positive correlation with ion concentration. As shown in Fig. (**28**), G-quadruplex DNA was immobilized onto a synthetic nanopore, which undergoes a potassium-responsive conformational change and then induces the change in the effective pore size. The responsive ability of this system can be regulated by the stability of G-quadruplex structure through adjusting potassium concentration. The situation of the grafting G-quadruplex DNA on a single nanopore can closely imitate the *in vivo* condition because the G-rich telomere overhang is attached to the chromosome. Therefore, this artificial system could promote a potential to conveniently study biomolecule conformational change in confined space by the current measurement, which is significantly different from the nanopore sequencing. Moreover, such a system may also potentially spark further experimental and theoretical efforts to simulate the process of ion transport in living organisms and can be further generalized to other more complicated functional molecules for the exploitation of novel bio-inspired intelligent nanopore machines.

6. INTEGRATING DNA NANOMACHINE WITH G-QUADRUPLEX FOR BIOANALYSIS

It is highly interesting to incorporate two amplification processes into one system, which has been reported by several highly novel papers [112-118]. The main feature of these researches is that formation of G-qaudruplex via target-activated DNA nanomachine has been realized in these sensors. Much lower detection limit and highly selective detection can be achieved without any labeling and modification. In this section, we will briefly introduce the recent progress in this aspect in three famous groups.

6.1. Small Molecule and Metal Ion Detection

Professor Willner has developed a nicking/replication machines [112] to detect Hg^{2+} ions in aqueous solution. This DNA machine provided a colorimetric detection method which involves two amplification steps and its advantage is highlighted by the astonishing sensitivity and selectivity Fig. (**29**). The nucleic acid track 3, includes three regions. Domain I includes Hg^{2+} recognition region which is composed of T-rich DNA sequence. The domain II represents the core part of the machine; the domain III includes the complementary sequence to the Horseradish peroxidase (HRP)-mimicking DNAzyme. The presence of Hg^{2+} will induce a serial of nicking and replication cycles.

In the same research group, a new phenomenon, where the G-quadruplex/hemin structure associated with CdSe/ZnS QDs quenches the luminescence of the nanoparticles, was developed Fig. (**30**) [116]. This enabled the development of new optical sensing platforms for the detection of DNA and for the analysis of aptamer-substrate complexes. Although a serials of drawbacks, the novelty and potential advantages of the systems have to be recognized.

6.2. DNA and Protein Detection

Professor Willner has also extended the above nicking/replication machines to detect protein [114]. As shown in Fig. (**31**), the

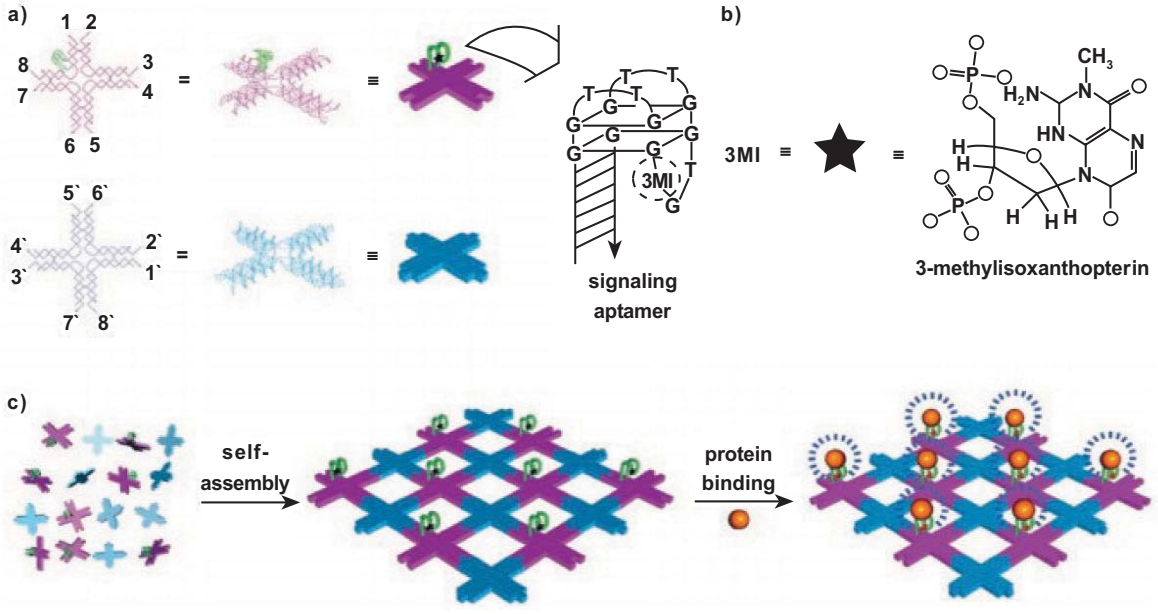


Fig. (26). Self-assembled signaling aptamer DNA arrays for protein detection.

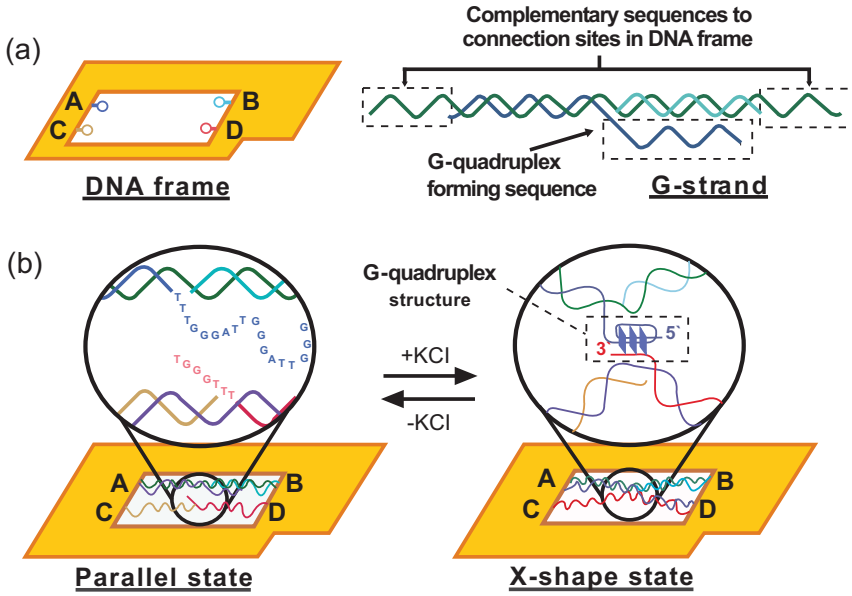


Fig. (27). Conformational switching of the G-quadruplex in a DNA nanostructure.

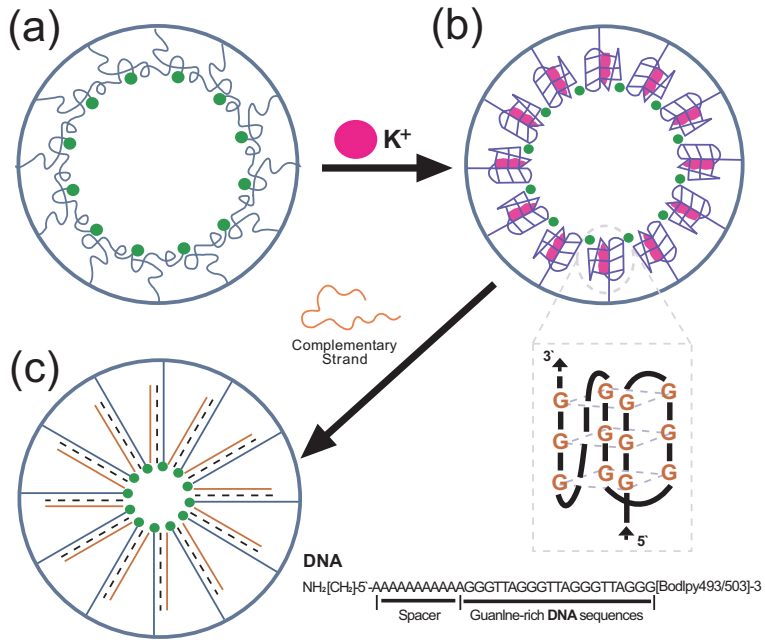


Fig. (28). G4 DNA was immobilized onto the inner surface of a single nanopore.
(**A**) There is no K^+; the G4 DNA relaxes to a loosely packed single-stranded structure. (**B**) Presence of K^+; the G4 DNA folds into densely packed rigid quadruplex structures that partially decrease the effective pore size of the nanopore. (**C**) After adding complementary DNA strands, G4 DNA forms a closely packed arrangement of double-stranded DNA on the single nanopore. The fluorescent group Bodipy493/503 (green circle) located at 3′-end of the attached DNA molecule. Before modification, the etched funnel-shaped single nanopores are around 20 nm wide at the narrowest point (drawing not to scale).

presence of protein thrombin can successfully inhibit the replication cycles. Considering the availability of a broad library of aptamers, the existence of various DNA/protein and DNA/small-molecule interactions, their results shows great promise in the near future.

For DNA detection with sensors involving G-qaudruplex, another original paper published by Professor Mao has to be mentioned [117]. He used rolling circle amplification (RCA) to produce a linear array of DNA peroxidases that catalyze chemical oxidation and generate a colorimetric output. The detection limit is 1 pM.

In our group, we have developed an extraordinary novel nanomachine to detect target DNA and bacterial toxin [118]. In this study as shown in Fig. (**32**), kinetically grafting G-qaudruplex onto one-dimensional DNA nanostructure with precise positioning was realized. The programs hold great promising for label-free and enzyme-free detection of various targets as result of signal amplification from G-qaudruplex and building DNA nanostructures as scaffolds due to molecular recognition capacity of G-quadruplex aptamers.

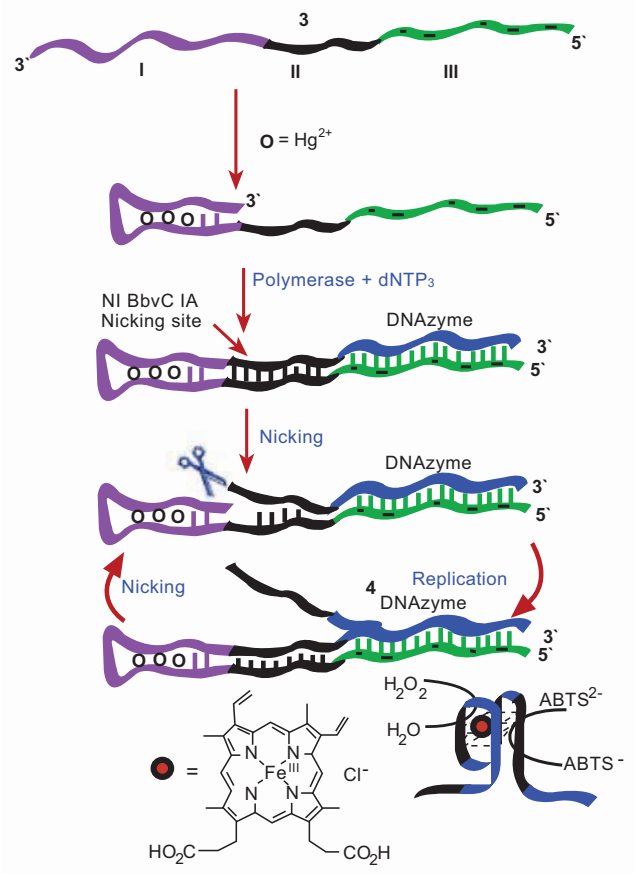


Fig. (29). The analysis of Hg^{2+} ions by a DNA-based machine utilizing 3 as a track, and the autonomous synthesis of the HRP mimicking DNAzyme units by repeated replication/nicking cycles, see text for details.

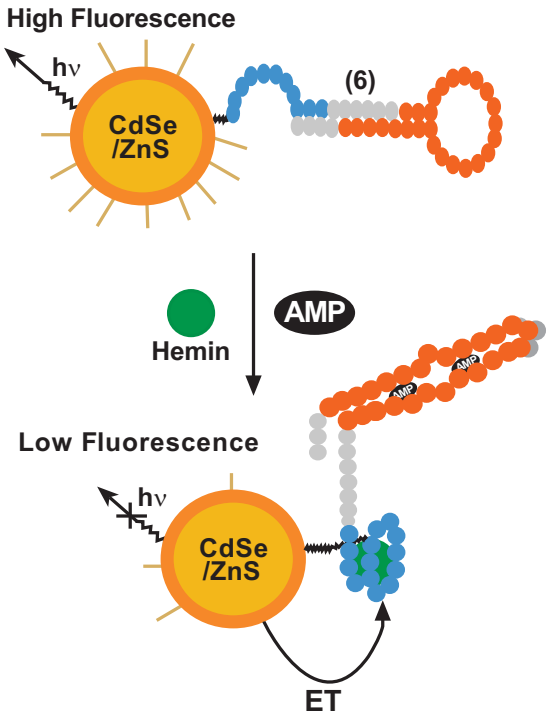


Fig. (30). Schematic Analysis of Adenosine Monophosphate (AMP) by the CdSe/ZnS QDs Modified with the G-Quadruplex/Hemin-Aptamer Hairpin.

7. CONCLUSION AND FUTURE PERSPECTIVE

This review mainly focuses on the bioassays which are constructed by integration of G-quadruplex with a variety of techniques such as fluorescence detection, nanotechnology and electrochemical

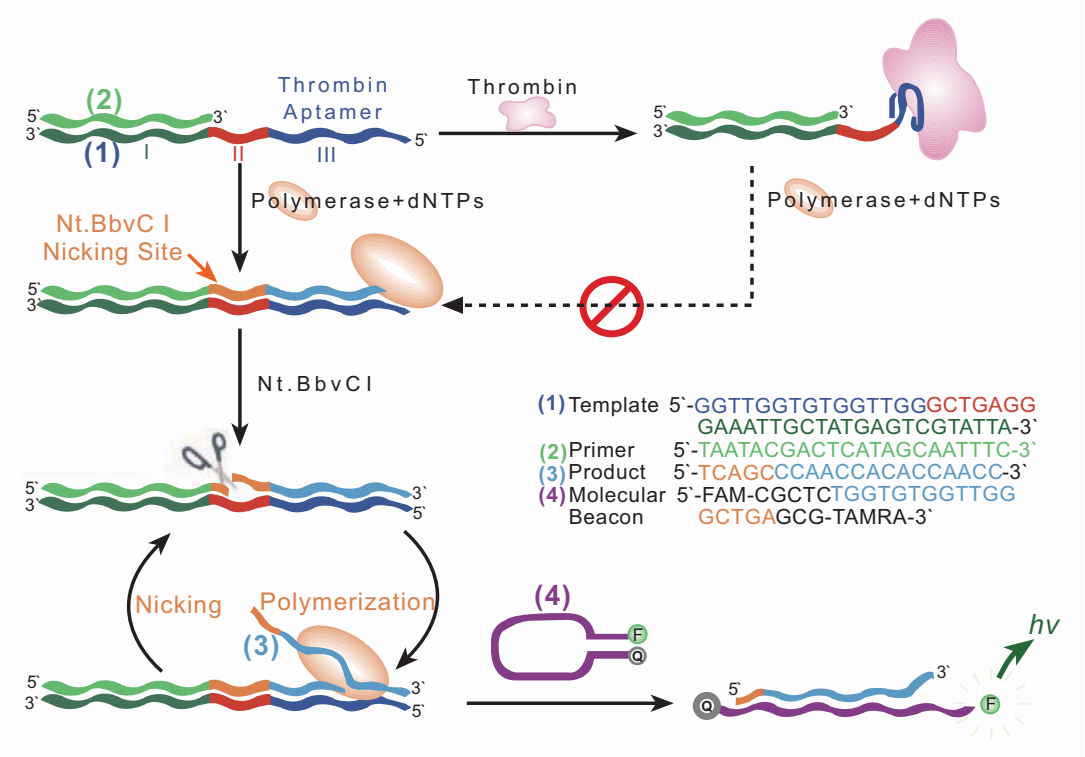


Fig. (31). Schematic illustration the DNA machine, thus demonstrating the inhibition of the polymerase reaction by aptamer-thrombin complexes.

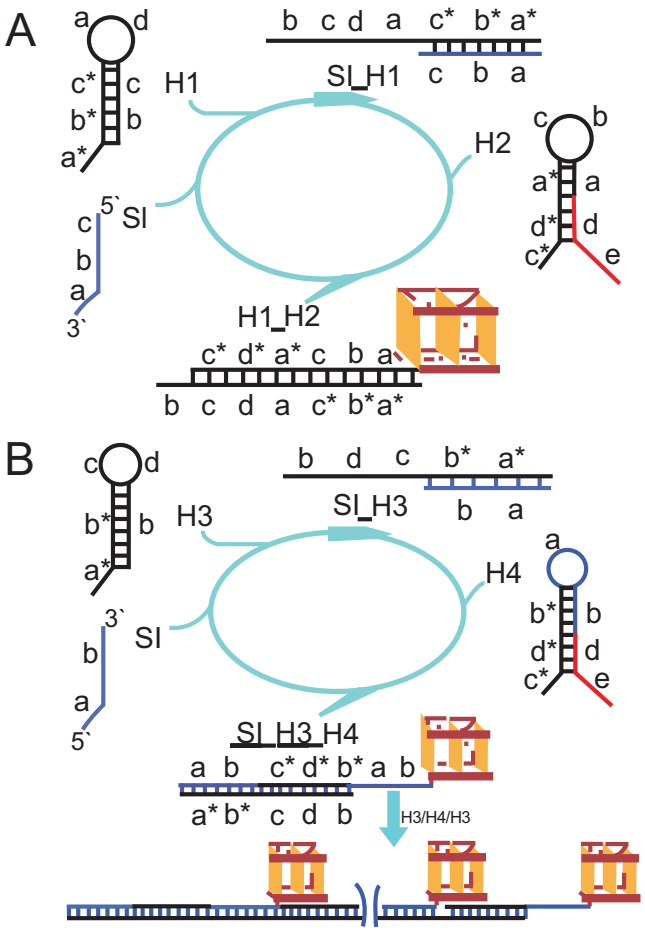


Fig. (32). (**A**) Catalytic circuit for autonomous formation of duplex ended with G-quadruplex. (**B**) Construction of one-dimensional DNA nanostructure grafted with a periodic array of G-quadruplexes via hybridization chain reaction.

method. In these detection methods, the G-quadruplex can transform the biorecogintion events into various readout signals. A serial of targets of interest such as drug candidates, small molecules, metal ion and biomolecules, etc., can be detected. Enormous progress in this field has been made. Various efficient and practical signaling strategies have been successfully developed to transform recognition event into physically detectable readout signals.

Meanwhile, we also have to realize the limitation of these bioassays based on G-quadruplex. First of all, not all the metal ions can be detected except a limited number of metal ions and proteins. In order to extend the G-quadruplex-based technology into bigger arena, other synthetically biochemical strategies have to be used to modify and improve the G-quaduplex. Secondly, the ratio of the signal to background noise in these sensing strategies still has very large space to improve. Finally, when using these detection platforms to screen drug candidates, these platforms have to be designed to specifically recognize the drug candidate. In this review, although we just provide a serial of detection platforms based on G-qaudruplex, the usage of G-quadruplex will certainly extend far beyond the above-mentioned applications. .

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