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ARTICLE TYPE

Label-free luminescent oligonucleotide-based probes

Dik-Lung Ma,*[a] Hong-Zhang He,[a] Ka-Ho Leung,[a] Hai-Jing Zhong,[b] Daniel Shiu-Hin Chan[a] and Chung-Hang Leung[b]

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Breakthrough advances in chemistry and biology over the last two decades have vastly expanded the repertoire of nucleic acid structure and function with potential application in multiple areas of science and technology, including sensing and analytical applications. DNA oligonucleotides represent popular tools for the development of sensing platforms due to their low cost, rich structural polymorphism, and their ability to bind to cognate ligands with sensitivity and specificity rivaling those for protein enzymes and antibodies. In this Review, we give an overview of the "label-free" approach that has been a particular focus of our group and others for the construction of luminescent DNA-based sensing platforms. The label-free strategy aims to overcome some of the drawbacks associated with the use of covalently-labeled oligonucleotides prevalent in electrochemical and optical platforms. Label-free DNA-based probes harness the selective interaction between luminescent dyes and functional oligonucleotides that exhibit a "structure-switching" response upon binding to analytes. Based on the numerous examples of label-free luminescent DNA-based probes reported recently, we envisage that this field would continue to thrive and mature in the years to come.

1. Introduction

The structure of the classical DNA double helix was first proposed by Watson and Crick nearly half a century ago. The role of DNA as a passive carrier for the transcription and replication of genetic material was cemented in the central dogma of molecular biology over the following years. However, breakthrough advances in chemistry and biology over the last two decades have vastly expanded the repertoire of nucleic acid structure and function with potential application in multiple areas of science and technology.[1] Seminal studies in the 1980s and 1990s led to the discovery of nucleic acid enzymes[2, 3] and aptamers[4, 5] capable of recognizing specific analytes with affinities rivaling those of protein enzymes and antibodies. Furthermore, a range of non-canonical nucleic acid structures such as triplex, G-quadruplex and i-motif DNA with putative roles in living organisms have been identified (Fig. 1).[6] These exciting discoveries have paved the way for the development of an extraordinary number and diversity of DNA-based probes.[7]

Luminescence is a widely-used technique for sensing and imaging applications.[8] A wide range of fluorescence or phosphorescence probes are available that exhibit changes in lifetime, anisotropy or emission intensity in response to variations in the local environment. Furthermore, conventional analytical methods generally require expensive instrumentation and/or

Dik-Lung Ma

Dik-Lung Ma completed his PhD in 2004 at the University of Hong Kong under the supervision of Prof. C.-M. Che. He spent the years 2005–09 at the University of Hong Kong, the Hong Kong Polytechnic University, and the Scripps Research Institute with Prof. C.-M. Che, Prof. K-Y. Wong, and Prof. R. Abagyan. In 2010, he was appointed as an Assistant Professor at the Hong Kong Baptist University. His research mainly focuses on luminescent sensing for biomolecules and metal ions, computer-aided drug discovery, and inorganic medicines.

Hong-Zhang He

Hong-Zhang He completed his MPhil in 2011 at Wenzhou Medical College, China. He is currently a PhD candidate at Hong Kong Baptist University under the supervision of Dr. Dik-Lung Ma and Dr. Chung-Hang Leung. His research interest centers on the development of label-free oligonucleotide-based luminescent detection assays for biomolecules.

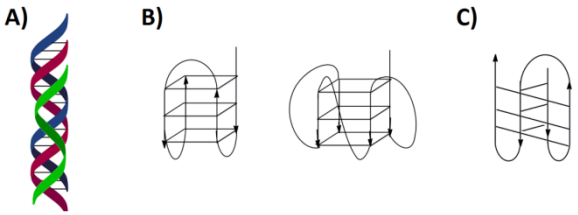


Fig. 1 A) Structure of the triple-helical DNA; B) Some common unimolecular G-quadruplex topologies; C) Structure of i-motif DNA.

tedious sample preparation for analysis. In contrast, luminescence can offer a convenient "mix-and-detect" methodology while retaining high sensitivity and specificity for the targeted analytes. Finally, luminescence is readily amenable to high-throughput analysis for the rapid quantitation of a large number of samples, while portable spectrofluorometers can facilitate in-field luminescence detection in a convenient and inexpensive manner. This review article aims to introduce the principles and recent developments of the field of luminescent oligonucleotide-based probes, with the aim of highlighting, in particular, the use of the "label-free" strategy for construction of simple and inexpensive sensing platforms. In the context of this review, the term "label-free" refers to unmodified oligonucleotides that lack covalently-labeled fluorophore and/or quencher units.

2. Oligonucleotide-based sensing platforms

Pioneering research conducted over the last two decades has uncovered the tremendous potential and versatility of nucleic acids in sensing applications. In contrast to organic chemosensors, which typically require the addition of a significant quantity of non-aqueous solvent for dissolution, nucleic acids often possess excellent solubility and stability in aqueous solution and biological media. Furthermore, functional oligonucleotides compare favourably with protein enzymes or antibodies due to their relatively small size, low cost, facile synthesis and modification, good thermal stability, reusability and low immunogenicity.[7] Consequently, oligonucleotides have received increasing attention for the construction of various sensing methodologies.

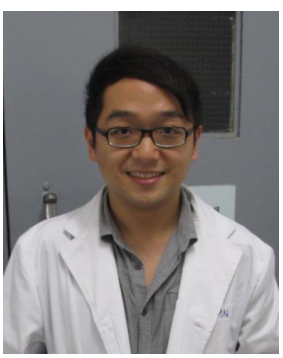
Functional oligonucleotides can bind either covalently or non-covalently to small molecules, metal ions, proteins and single stranded DNA/RNAs. Nucleic acid aptamers, developed through the systematic evolutions of ligands by exponential enrichment (SELEX),[4, 5] have been discovered for a myriad of targets such as proteins or small molecules. The target-binding specificity of aptamers has been reported to be comparable or even superior to that for protein enzymes or antibodies. For example, the aptamer for the platelet-derived growth factor B chain homodimer (PDGF-BB) binds to its cognate target with 370-fold higher affinity compare to platelet-derived growth factor A chain homodimer (PDGF-AA). Additionally, the theophylline aptamer exhibits 10,000-fold selectivity over closely related analogues such as caffeine, which differs only by a single methyl group.[9]

On the other hand, certain DNA bases can selectively bind to particular metal ions. For example, silver(I) ions can stabilize the cytosine-Ag^{+}-cytosine mismatch, while mercury(II) ions can take part in the thymine-Hg^{2+}-thymine interaction. In addition, G-quadruplexes that are selective for particular metal cations have been reported. Finally, certain enzymes or functional DNA-binding proteins are able to discriminate between different nucleic acid conformations or sequences. The strong specificities of particular nucleic acid-ligand interactions have enabled the development of highly selective oligonucleotide-based sensing platforms for various analytes.

An oligonucleotide-based sensing platform is generally constructed from three components: (1) a functional oligonucleotide which is able to interact specifically with its target accompanied by the presence or absence of a conformational change, (2) a signal transducer, which transforms the recognition event into a measurable signal, and (3) physical instrumentation which converts the signal into a readable output for quantitative analysis. For example, the target-responsive conformational change of oligonucleotides has been successfully coupled to both optical[10] and electrochemical[11] systems. In particular, luminescence detection offers a powerful combination of simplicity, low cost, rapid response and high sensitivity compared to colorimetric or electrochemical methods.

In general, DNA has been more widely used in oligonucleotide-based sensing systems compared to RNA. The latter is readily degraded by RNases present in the exogenous environment and is also more prone to hydrolytic cleavage due to its additional 2′-hydroxyl group. However, the rich structural diversity of RNAs has found application in riboswitches[12-14] or catalytic RNAs (ribozymes) sensitive to specific metal ion cofactors.[2, 15] A comprehensive discussion of nucleic acid enzyme sensors can be found in extensive reviews written by Lu and co-workers.[7, 16]

Early studies in the field of DNA-based sensing revolved around the molecular beacon (MB) strategy whereby the presence



Ka-Ho Leung

Ka-Ho Leung completed his BSc degree in chemistry at Hong Kong Baptist University. He is currently pursuing his PhD degree in Chemistry at the same university under the supervision of Dr. Dik-Lung Ma and Dr. Chung-Hang Leung. His research interest is in the development of metal based luminescent probe for detection of biomolecules and metal ions.

Hai-Jing Zhong

Hai-Jing Zhong completed her BSc degree in Pharmacy at Jinan University. She is currently undertaking her PhD degree at the University of Macau under the supervision of Dr. Chung-Hang Leung and Dr. Dik-Lung Ma. Her research interest lies in development of nucleic acid based platform for drug screening.

of the targeted analyte would influence the spatial arrangement of fluorophore and quencher in the labeled oligonucleotide. Subsequent studies have significantly expanded the range of oligonucleotide conformations and/or fluorophore/quencher combinations available for the construction of DNA-based detection platforms for various analytes. The labeled strategy has enjoyed consistent popularity due to its high target specificity, ease of operation, and the wide choice of fluorophore or quenchers available.[16, 17] Besides fluorescence labeling, a number of strategies based on nanoparticles or radioactive, electroactive or chemically-active agents have been devised.[18] Consequently, the development of new labeled methods for the luminescent detection of analytes still remains an active and important area of research.[10, 11, 17]

The use of covalently-modified nucleic acids, however, entails certain drawbacks. The covalent attachment of fluorophore or other labeling agents to the functional oligonucleotide may affect the recognition event, potentially interfering with the sensitive and selective detection of the targeted analyte.[19] Furthermore, the additional labeling or immobilization steps can be costly, tedious and time-consuming. The "label-free" concept aims to overcome some of the drawbacks associated with the use of covalently-labeled oligonucleotides prevalent in electrochemical and optical platforms, as will be discussed in the following section.

3. Label-free oligonucleotide-based sensing using luminescent molecules

In the label-free approach, luminophores are not covalently attached to the nucleic acid backbone but instead interact non-covalently with DNA through a number of binding modes such as intercalation, groove-binding, end-stacking or electrostatic interactions. The luminescent molecules used in label-free DNA probes are generally non-emissive or only slightly emissive in aqueous solution resulting from quenching of the excited state by solvent interactions. However, they show enhanced luminescence upon binding to defined DNA structures due to the protection of their excited states within the hydrophobic interior of the oligonucleotide.[20] Consequently, the analyte-induced structure-switching of the functional oligonucleotide may be transduced into an optical output using a suitable luminescent molecule. One of the potential advantages of the label-free DNA-based approach is that the detection can be carried out in homogenous solution without any pre-labeling or immobilization step, greatly reducing the time and cost required for performing the assay as well.

Luminescent molecules for DNA can be generally classified into two major types: organic dyes and transition metal complexes. Luminophores that can distinguish between various DNA topologies with a differential luminescence response can be employed to monitor the target-induced conformational transition of the functional oligonucleotide. While organic dyes have been most commonly used for both labeled and label-free DNA-based detection assays, luminescent metal complexes also display unique advantages that may be beneficial for sensing applications.[21, 22] These include their (i) high luminescence quantum yield, (ii) long phosphorescence lifetime that allows their emission to be distinguished from a fluorescent background, (iii) large Stokes shift for effective discrimination of excitation and emission wavelengths, (iv) sensitivity of their emissive properties to subtle changes in the local environment, and (v) modular synthesis that allows facile synthesis of analogues for fine-tuning of their chemical and/or photophysical properties.

Depending on the conformational switching event of the oligonucleotides, the luminescent response of the luminophores upon interaction with the oligonucleotides can be classified as either "switch-on", "switch-off" or "ratiometric". In general, switch-on or ratiometric modes are more desirable for sensing applications as the switch-off mode of detection may be more susceptible to quenching by biological interferences via a range of energy transfer mechanisms. Examples of luminescent probes that are able to specifically recognize certain types of DNA are shown in Table 1.

In recent years, a number of luminescent label-free detection methodologies have been developed for small molecules, metal ions, nucleic acids, and even proteins. In the following section, we describe interesting case studies of the label-free strategies that have been employed for the construction of luminescent DNA-based detection platforms by our group and researchers worldwide. Due to the breadth of this field, this survey aims not to be exhaustive but rather aims to highlight the advantages offered by the versatile combination of structure-specific luminescent probes and functional oligonucleotides.

3.1. Detection of DNA

The development of analytical tools for the specific detection of

Daniel Shiu-Hin Chan

Daniel Shiu-Hin Chan completed his BSc degree in Chemistry at the University of New South Wales, Sydney, Australia. He is currently appointed as a Research Assistant at the Department of Chemistry at the Hong Kong Baptist University under the supervision of Dr. Dik-Lung Ma and Dr. Chung-Hang Leung. His research interests include drug discovery and the development of luminescent oligonucleotide-based assays for biomolecules and metal ions.

Chung-Hang Leung

Chung-Hang Leung completed his PhD in 2002 at City University of Hong Kong. After completing a five year post-doctoral fellowship at the Department of Pharmacology, Yale University, he was appointed Research Assistant Professor at the University of Hong Kong and then at the Hong Kong Baptist University. He is currently appointed as an Assistant Professor at the University of Macau. His research interests are in anti-cancer and anti-inflammatory drug discovery and molecular pharmacology.

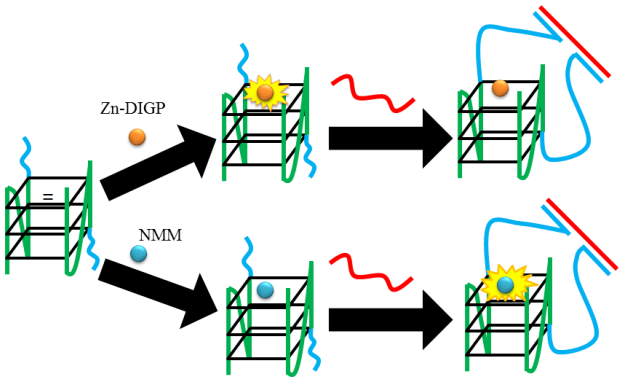


Fig. 2. G-quadruplex-based sensing platform for single strand DNA with switch-off (upper panel) and switch-on (low panel) detection modes.

DNA and RNA sequences has attracted widespread efforts aimed at rapid genetic profiling and disease diagnosis.[23] The classical "molecular beacon" strategy, first reported by Tyagi and Kramer in 1996, utilizes doubly-labeled oligonucleotides that become fluorescent upon hybridization to target sequences.[24] Other approaches such as hybrid-type and multi-component molecular probes for DNA sequence detection have also been developed for genetic screening, biochip construction and biosensor development, among other applications. Besides, luminescence sensing, electrochemical[25, 26] or colorimetric signal transduction mechanisms have also been employed for DNA sequence detection.[27] However, many of these approaches have required the covalent conjugation of oligonucleotides to a signaling component, such as organic dyes, quantum dots or gold nanoparticles. This has spurred the development of label-free approaches for the construction of luminescent detection platforms that have achieved comparable or even superior sensitivity and selectivity for target DNA sequences compared to their labeled counterparts.

A particular strategy that has been popular with the label-free approach is to use non-canonical DNA secondary structures such as the G-quadruplex in conjunction with a G-quadruplex-specific luminescent probe molecule. Wang and co-workers reported a G-quadruplex-based DNA detection method utilizing the anionic porphyrin *N*-methyl mesoporphyrin IX (NMM) and the cationic tetrakis(dicyclohexylguanidino)-zinc-phthalocyanine (Zn-DIGP) as G-quadruplex-selective probes (Fig. 2).[28] In this approach, a bimolecular c-*myc* G-quadruplex is formed from two fragment oligomers (5′- $GCAGACACATC_2AGT_5GAG_3TG_4$-3′ and 5′-$AG_3TG_4A_2T_4CGATAGC_2AG_2ACA_2$-3′) that each contain flanking segments complementary to a target DNA sequence (5′-$T_2GTC_2TG_2CTATCGCTG_2ATGTGTCTGC$-3′). The addition and hybridization of the complementary DNA to the system imposes additional conformational constraints to the complex, thereby changing the local environment and fluorescence properties of the dye molecule. Switch-on and switch-off modes of detection were demonstrated with NMM and Zn-DIGP, respectively. These assays had detection limits of 5.4 nM with NMM and 3.2 nM for Zn-DIGP, and could discriminate mismatched from perfectly matched target DNAs.

Recently, our group has reported an oligonucleotide-based detection platform for the highly selective recognition of gene deletion using a G-quadruplex selective luminescent iridium(III)

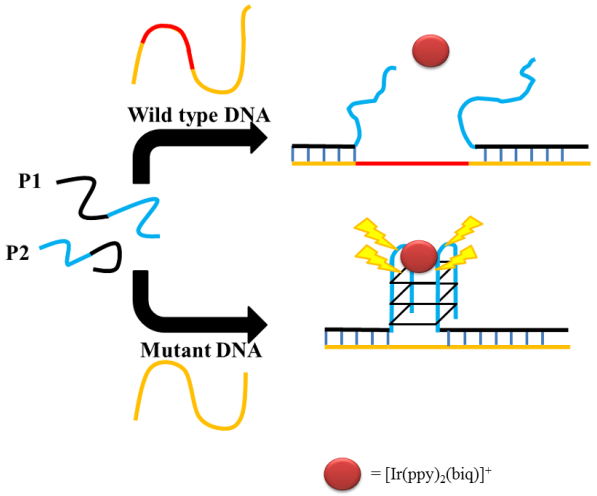


Fig. 3. Split G-quadruplex-based luminescent switch-on detection strategy for gene deletion detection.

complex $[Ir(ppy)_2(biq)]^+$ as a signal transducer (Fig. 3).[29] Two short oligonucleotides, P1 (5′-$ATGACTATCT_3A_2TG_3TAG_3$-3′) and P2 (5′-$G_3T_2G_3CGTAG_2A_4TGAG$-3′), both contain complementary regions that recognize the DNA sequences flanking the deletion site of the target gene (5′-$CTCAT_4C_2ATACAT_2A_3GATAGTCAT$-3′), as well as pendant guanine-rich overhangs that can form a split G-quadruplex when brought into close proximity. The wild-type DNA sequence (5′-$CTCAT_4C_2ATACAGTCAGTATCA_2T_2CTG_2A_2GA_2T_3C_2AGAC$ $AT_2A_3GATAGTCAT$-3′) is able to hybridize with P1 and P2, but the large distance between the overhangs prevents split G-quadruplex formation, resulting in a diminished luminescence signal. However, in the presence of the shorter mutant DNA sequence, the two G-quadruplex-forming sequences are brought into closer proximity, promoting the formation of the split G-quadruplex and producing a significant switch-on luminescence response. Importantly, specificity for a target sequence of any length could be readily achieved using this strategy by the judicious design of the split G-quadruplex oligonucleotides. This method was also capable of differentiating single-mismatched DNA sequences with a detection limit of 50 nM.

DNA-cleaving enzymes have been used to generate and/or amplify the luminescent signal resulting from the conformational change of the oligonucleotide probe sequence in response to the target DNA. To this end, Ren and co-workers described a label-free, quadruplex-based assay for sensitive DNA detection using NMM as a luminescent probe.[30] The initial duplex DNA consists of a probe strand (P4: 5′-$CTCGATCGCACT_2A_2GA_2T_3C$-3′) partially hybridized to a G-quadruplex-forming oligomer (P1: 5′-$G_3T_4G_3A_3T_2CT_2A_2GTGCG_3T_4G_3$-3′) (Fig. 4). The addition of the target strand (P5: 5′-$GA_3T_2CT_2A_2GTGCGATCGAG$-3′) forms a more stable P4–P5 duplex which releases P1. The G-quadruplex formed by the free P1 is subsequently detected by NMM with a switch-on fluorescence response. The detection limit for this system was reported to be 2.3 nM.

In an interesting variation of this strategy, Qu and co-workers developed a DNA detection system that utilizes Exonuclease III

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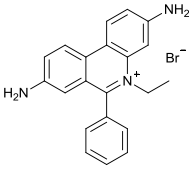
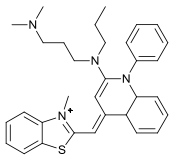
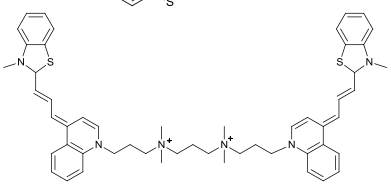
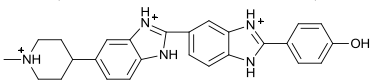
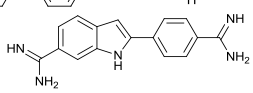
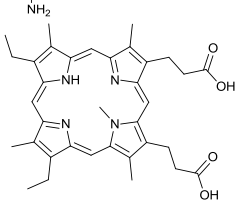
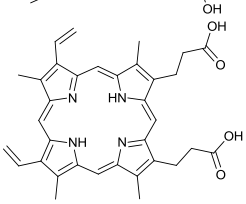
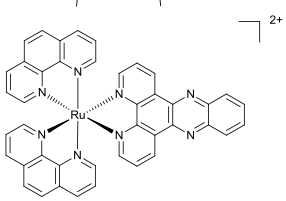
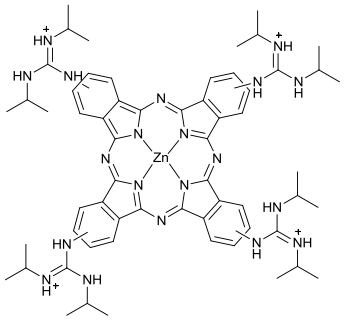
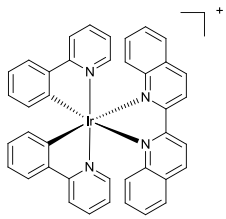
Table 1 Examples of luminescent organic dyes or metal complexes used as DNA probes for the construction of oligonucleotide-based detection methods and their corresponding target analyte(s) and detection limit(s).

Name	Structure	DNA target	Assay analyte(s)	Detection limit(s)	Reference(s)
Ethidium bromide		dsDNA	Thrombin	2.8 nM	82
SYBR green I		dsDNA	Hg^{2+} Ag^{+} EcoRI	0.5 nM 32 nM 8 U/mL	38 45 74
TOTO-3		dsDNA	Hg^{2+}	3 nM	37
Hoechst 33258		dsDNA	L-argininamide	3.8 μM	86
DAPI		dsDNA	L-argininamide	2.5 μM	86
N-methyl mesoporphyrin IX (NMM)		G-quadruplex	Nucleic acids RNase H	36 pM–5.4 nM 0.2 U/mL	28, 30, 31 70
Protoporphyrin IX (PPIX)		G-quadruplex	K^{+}	0.5 mM	52
$[Ru(phen)_2(dppz)]^{2+}$		dsDNA	Hg^{2+} K^{+} ATP	0.35–5 nM 50 nM 1 nM	40, 41 49 78, 79

Zn-DIGP		G-quadruplex	Nucleic acids K^+	3.2 nM 0.8 μM	28 53
$[Ir(ppy)_2(biq)]^+$		G-quadruplex	Nucleic acids Sr^{2+} Pb^{2+}	50 nM 13 nM 600 pM	29 58 59

[a] dsDNA = double strand DNA, dppz = dipyrido[3,2-a:2′,3′-c]phenazine, ppy = phenylpyridine, biq = biquinoline

(ExoIII) to generate a "strand-cleavage cycle" upon target binding with the release of a G-quadruplex structure at the end of each cycle that can be recognized by NMM.[31] A duplex DNA probe is designed to contain both a G-quadruplex-forming sequence and a complementary sequence to the target DNA at the 3′-overhang (Fig. 5). The addition and subsequent hybridization of the target DNA with the probe forms a blunt terminus that facilitates ExoIII digestion from the 3′-terminus, which ultimately releases both the target DNA and the G-quadruplex-forming oligomer. The nascent G-quadruplex is detected by NMM with a switch-on fluorescence response, while the released target sequence is free to bind to another DNA probe, resulting in significant signal amplification. This method exhibited very high sensitivity for the DNA target (detection limit = 36 pM), which compared favorably to other amplification methods using labeled oligonucleotides.

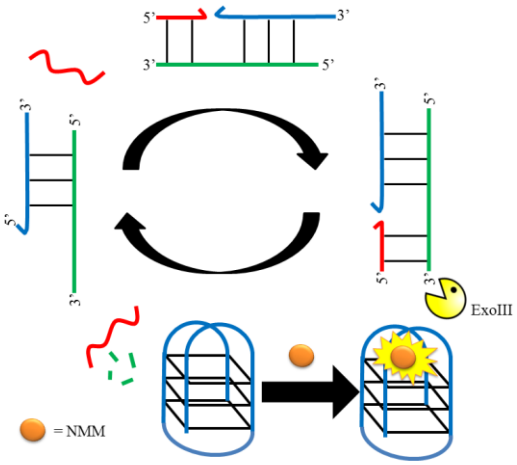


Fig. 5. Enzyme-assisted G-quadruplex based detection platform for DNA.

3.2. Detection of metal ions

Metal ions play vital roles in biological and environmental systems, and thus the development of metal ion detection systems has been an important goal over the past decades. Conventional analytical techniques such as atomic absorption spectrometry (AAS), inductively-coupled plasma mass spectrometry (ICP-MS), X-ray fluorescence spectrometry and capillary electrophoresis (CE) have been routinely employed for the detection of metal ions with high sensitivity (typically ppb).[32] However, such methods usually involve expensive and sophisticated instrumentation and/or complex sample pre-treatment procedures, and are not amenable for in-field analysis.[33] In recent years, the metal ion-binding properties of nucleic acids have attracted increasing attention for the fabrication of luminescent metal ion-sensing platforms. The stabilization of certain G-quadruplex topologies is highly dependent on the nature of the cation, such as K^+, Na^+, Sr^{2+} or Pb^{2+} ions. Furthermore, Ag^+ and Hg^{2+} ions are capable of mediating the formation of stable cytosine-cytosine and thymine-thymine mismatched base pairs, respectively. These phenomena have formed the basis for the construction of label-free metal ion assays utilizing unmodified DNA oligonucleotides and structure-specific luminescent probes. These detection platforms have often offered comparable sensitivities for metal ions compared to traditional analytical techniques.

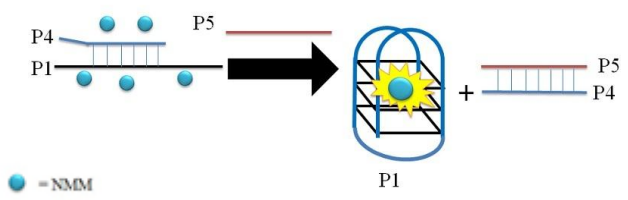


Fig. 4. Luminescent switch-on detection of nucleic acid based on analyte-induced strand dissociation and subsequent G-quadruplex formation.

The stabilization of thymine-thymine mismatches by Hg^{2+} ions (T-Hg^{2+}-T) has been well-documented in the literature,[34, 35] and has been exploited in a variety of both labeled and label-free luminescent probes for Hg^{2+} ions.[16, 36] A common mechanism of the label-free approach relies on the Hg^{2+}-induced conversion of single-stranded thymine-rich oligonucleotides into a duplex or hairpin conformation, which can be detected by intercalating

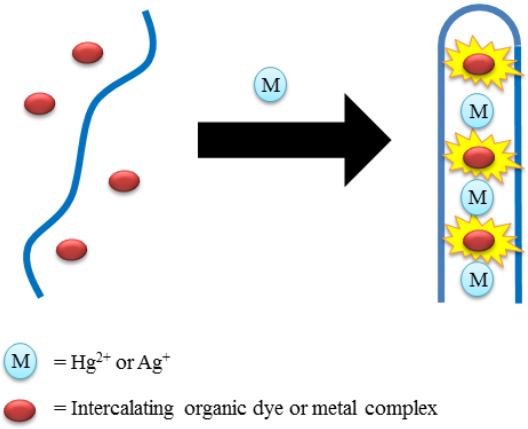


Fig. 6. Label-free luminescent switch-on detection assay for Hg^{2+} and Ag^{+} ions.

luminescent probes (Fig. 6). The groups of Chang[37] and Liu[38] utilized organic dyes such as TOTO-3 and SYBR Green I for Hg^{2+} ion detection. Soon afterwards, our group developed a platinum(II) metallointercalator as the first metal-based probe for the detection of Hg^{2+} ions,[39] while the group of Zhang later utilized the "molecular light switch" complex $[Ru(phen)_2(dppz)]^{2+}$.[40] The low nanomolar detection limits for these label-free assays for Hg^{2+} are comparable to those utilizing the fluorescent-labeled "molecular beacon" approach.

A variation on this theme employs the structural conversion of Watson-Crick base pairing into thymine-thymine mismatched base pairing induced by Hg^{2+} ions. The intercalating probes $[Ru(phen)_2(dppz)]^{2+}$ or SYBR Green I interact less strongly with the mismatched T–Hg^{2+}–T duplex compared to the Watson-Crick duplex, resulting in a luminescence switch-off response to nanomolar Hg^{2+} ions, as reported by the groups of Oh[41] and Liu,[38] respectively. Another strategy based on aggregation-induced emission of a tetraphenylethene derivative was reported by Ji, Tang and co-workers for the construction of a switch-on sensor for Hg^{2+} ion.[42] The group of Yang demonstrated that both switch-on and switch-off modes of Hg^{2+} ion detection can be achieved using the minor groove-binding dye 4′,6-diamidino-2-phenylindole (DAPI).[43] This assay successfully detected down to 5 and 1.5 nM of Hg^{2+} ions in the switch-off and switch-on modes of detection, respectively.

The mechanism for oligonucleotide-based assays for Ag^{+} ions is broadly similar to that for Hg^{2+} ions described previously (Fig. 6). In 2008, Ono and co-workers discovered the cytosine–Ag^{+}–cytosine mismatch,[44] which provided the foundation for a number of label-free oligonucleotide-based luminescent detection

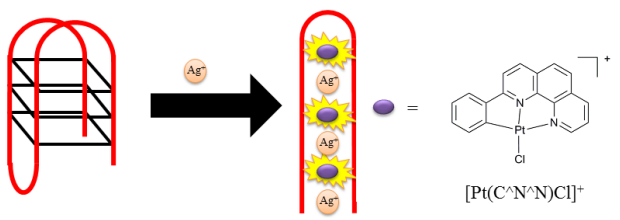


Fig. 7. G-quadruplex-to-duplex transition for the detection of Ag^{+} ions using a platinum(II) metallointercalator.

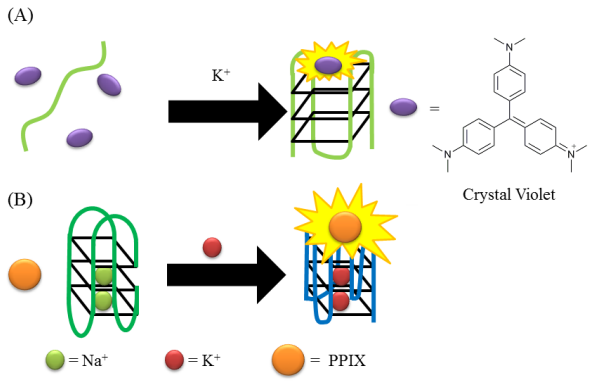


Fig. 8. G-quadruplex-based switch-on detection assays for K^{+} ions using G-quadruplex selective luminescent probes.

methods for Ag^{+} ions. For example, the presence of Ag^{+} ions transforms the polycytosine oligonucleotide C_{20} from a random coil conformation into hairpin structure, which was detected by SYBR Green I as reported by Tseng and co-workers.[45] Recently, the group of Xu and Xu utilized the monomer-excimer switch of the organic dye Thioflavine T (ThT) to probe the random coil-to-hairpin transition induced by Ag^{+} ions.[46] Both assays were able to detect nanomolar concentrations of Ag^{+} ions.

By rational design of the DNA structural transition event, the sensitivity of the detection platform may be further enhanced due to the differential binding affinity of the probe to different DNA structures. Our group developed a G-quadruplex-based system that harnessed the Ag^{+}-induced quadruplex-to-duplex transition using a specially designed guanine and cytosine-rich oligonucleotide (Fig. 7).[47] Since the platinum(II) complex interacted even more weakly with the G-quadruplex motif compared to single-stranded DNA, the background signal was reduced by a factor of 2 compared to the corresponding random coil-to-quadruplex transition-based system. This assay was able to detect down to 20 nM of Ag^{+}.

The examples described above have utilized a DNA duplex and a luminescent intercalator for monitoring of the metal ion-binding event. Alternatively, the metal ion specificity of the G-quadruplex motif can be harnessed in conjunction with structure-specific DNA probes. Chang and co-workers have reported a G-quadruplex-based switch-off probe for K^{+} ions using the probe Oligreen, which is selective for single-stranded DNA.[48] The K^{+}

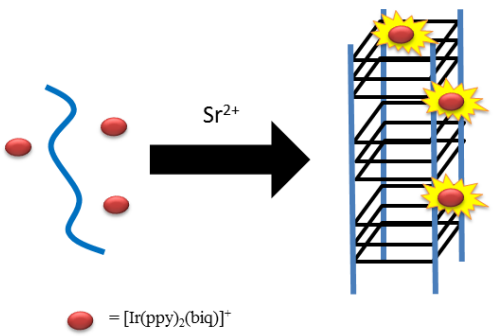


Fig. 9. Schematic representation of the G-quadruplex-selective probe for the detection of Sr^{2+} ions.

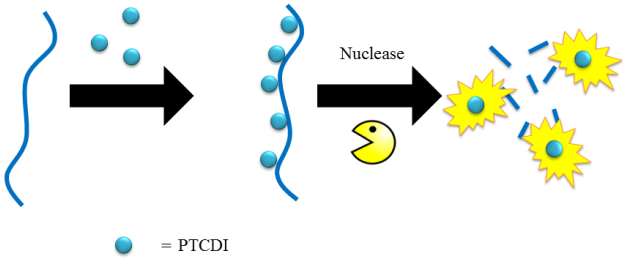


Fig. 10. Luminescent switch-on DNA based detection of nuclease activity using PTCDI as an aggregation induced quenching probe.

ion-induced conversion of the ATP-binding aptamer (5′-$AC_2TG_5AGTAT_2GCG_2AG_2A_2G_2T$-3′) from a random coil into a G-quadruplex conformation results in a decrease in fluorescence intensity. An alternative switch-off probe for K^+ ions was reported by Kim and co-workers by utilizing a DNA duplex and the metallointercalator $[Ru(phen)_2(dppz)]^{2+}$.[49] These assays had detection limits for K^+ ions of 75 and 50 nM, respectively.

To generate switch-on label-free detection platforms for K^+ ions, Kong and co-workers utilized the human telomeric G-quadruplex sequence (5′-$(G_3T_2A)_3G_3$-3′) and the triphenylmethane dye crystal violet (CV) to monitor the topological changes induced by the K^+ ion (Fig. 8A).[50] On the other hand, the use of other G-quadruplex-forming sequences in conjunction with CV generated switch-on detection modes for K^+ ion.[51] A variation of this strategy was reported by Wang, Dong and co-workers using the anionic porphyrin dye protoporphyrin IX (PPIX) as a parallel G-quadruplex fluorescent probe.[52] The oligonucleotide PS2.M (5′-$GTG_3TAG_3CG_3T_2G_2$-3′) adopts an anti-parallel quadruplex structure in Na^+ solution, but it gradually converts into a parallel G-quadruplex upon addition of K^+, which is recognized by PPIX (Fig. 8B). Soon afterwards, Wang and co-workers developed a luminescent switch-on probe for K^+ ions using the *c-myc* G-quadruplex sequence (5′-$TGAG_3TG_4AG_3TG_4A_2$-3′) and Zn-DIGP[53] that can detect K^+ ions in 3500 fold excess of Na^+, which is also a known G-quadruplex stabilizing cation.

Besides potassium, other metal ions including Pb^{2+} [54] and Sr^{2+} [55] ions have also been reported to stabilize the G-quadruplex. Thus, Pb^{2+} [56-58] or Sr^{2+} [59, 60]-mediated G-quadruplex structures have found use for the detection of these metal ions. For example, our research group has made use of the short oligonucleotide sequence T2 (5′-$G_4T_2G_4T_2G_4T_2G_4$-3′) and an iridium(III) complex $[Ir(ppy)_2(biq)]^+$ to fabricate a DNA-based detection platform for Sr^{2+}.[59] In the presence of Sr^{2+} ions, T2 folded into a G-quadruplex and the luminescence of the iridium(III) was significantly enhanced (Fig. 9). A similar mechanism was proposed by Qu and co-workers who employed single-walled carbon nanotubes (SWNTs) and the luminescent dye thiazole orange (TO) in conjunction with the Sr^{2+}-dependent human telomeric DNA sequence (HTS: 5′-$AG_3T_2AG_3T_2AG_3T_2AG_3$-3′).[60] In the absence of analyte, HTS is strongly adsorbed onto the SWNT surface and the luminescence of TO is low. The addition of Sr^{2+} ions promotes the dissociation of HTS from the SWNTs and subsequent G-quadruplex formation, thereby enhancing the luminescence signal of the system. Both assays were able to selectively recognize Sr^{2+} ions with detection limits of *ca.* 10 nM.

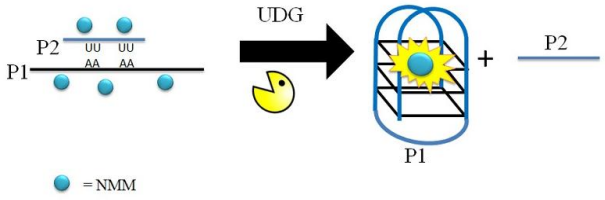


Fig. 11. Luminescent switch-on detection of UDG activity based on the analyte induced strand dissociation and subsequent G-quadruplex formation.

3.3. Detection of nuclease activity

DNA or RNA cleavage reactions catalyzed by nucleases are essential in a variety of fields ranging from biotechnology to pharmacology, as well as in biological processes involving replication, recombination, DNA repair, molecular cloning, genotyping, and mapping.[61] Currently used methods for the detection of nuclease activity including gel electrophoresis, high performance liquid chromatography (HPLC), and electrochemical assays[62-64] tend to be relatively time-consuming, laborious and may involve radioisotopic labeling. Consequently, DNA-based detection platforms based on fluorescence resonance energy transfer (FRET)[65-67] or label-free strategies have gained popularity in recent years. In the label-free oligonucleotide-based approach, the choice of the DNA backbone for nuclease activity detection is comparatively flexible. The design of probe sequence mainly depends on the function and specificity of the target cleavage enzymes.

Non-specific nuclease enzymes efficiently degrade the entire DNA or RNA strand in a non-selective manner. The use of structure-specific luminescent probes can transduce the enzyme digestion event into measurable luminescent output. Ren and co-workers reported a DNA-templated ensemble for the label-free and real-time fluorescence switch-on detection of S1 nuclease, a widespread multifunctional endonuclease which selectively digests single-stranded DNA or RNA.[68] The authors utilized a perylene derivative that is strongly fluorescent in aqueous solution but exhibits aggregation-induced quenching upon binding electrostatically to nucleic acids. The S1 nuclease-mediated degradation of ssDNA reduces the electrostatic interactions between the probe and DNA fragments, resulting in the dissociation and release of the fluorescent monomers into the solution (Fig. 10). This assay was able to sense S1 nuclease activity with a detection limit of 0.092 U/mL. Yam and co-

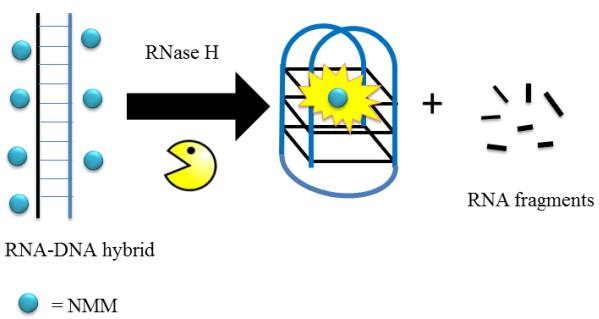


Fig. 12. Detection of RNase H activity based on a RNA-DNA hybrid and a G-quadruplex selective organic dye.

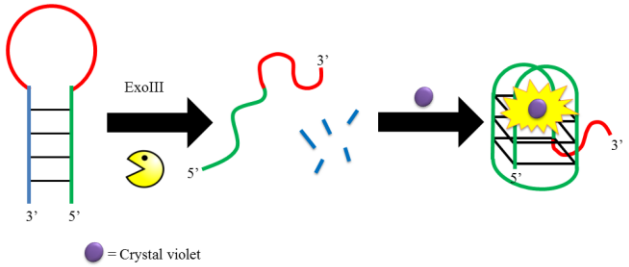


Fig. 13. Detection of 3′→5′ exonuclease activity exemplified using ExoIII as a model enzyme.

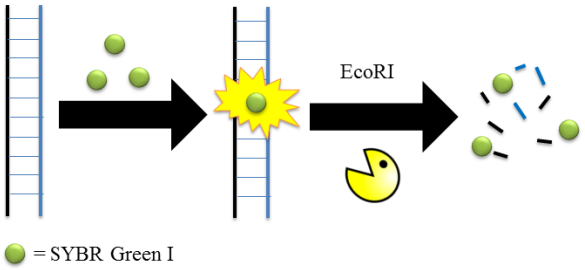


Fig. 14. Detection of EcoRI activity using SYBR Green I as double strand DNA selective luminescent probe

workers utilized a similar strategy to construct a switch-off platform for nuclease activity using the platinum(II) terpyridyl complex.[69]

However, one minor drawback to the use of non-specific, electrostatically-binding DNA probes in the label-free strategy is that the system becomes susceptible to interference by contaminating nucleic acids of any type. On the other hand, the use of a G-quadruplex motif and a quadruplex-selective probe represents a more robust approach as it is less likely for non-canonical structures to be present in the sample matrix. Consequently, many of the recent examples described for label-free sensing of enzyme activity have been G-quadruplex-based. For example, the label-free G-quadruplex DNA detection assay by Ren and co-workers described previously was also applied for the detection of uracil-DNA glycosylase (UDG) in the same work.[30] The G-quadruplex-forming oligomer P1 was partially hybridized with the uracil-containing strand P2 (5′-$CGCACU_2A_2GA_2U_2TC$-3′). The addition of UDG splices uracil from P2, which releases the quadruplex-forming strand P1. The G-quadruplex motif subsequently formed is detected by NMM with a switch-on fluorescence response (Fig. 11). The detection limit of this assay for UDG enzyme activity was 0.05 U/mL.

The same group have also reported a label-free G-quadruplex-based fluorescence assay for RNase H activity.[70] The DNA strand of the RNA–DNA hybrid duplex was designed as a quadruplex-forming oligomer. The addition of RNase H results in the cleavage of RNA strand and the subsequent release of the G-quadruplex-forming DNA oligomer, which is detected by NMM with a switch-on fluorescence response (Fig. 12).[71] A detection limit of 0.2 U/mL for RNase H activity was reported.

Our group developed a G-quadruplex-based switch-on fluorescence assay for 3′→5′ exonuclease activity.[72] We designed a hairpin DNA substrate containing a G-quadruplex-forming sequence at the 5′-terminus (5′-$AG_3(T_2AG_3)_3CAGA_2G_2AT_2A(C_3TA_2)_3C_3T$-3′). Enzymatic digestion by 3′→5′ exonucleases such as ExoIII occurs specifically from the 3′-terminus but is arrested at the loop region due to the inability of ExoIII to accept single-stranded DNA as substrate. Thus, the 5′ guanine-rich sequence is released and folds into a G-quadruplex, which is subsequently detected by CV with a switch-on fluorescence response (Fig. 13). The detection limit of the assay for ExoIII was determined to be 5 U/mL. We also demonstrated that this assay could be adapted for the real-time and high-throughput monitoring of 3′→5′ exonuclease activity, and further demonstrated the detection of the exonucleolytic activity of human TREX1, the major 3′→5′ exonuclease in mammalian cells. Importantly, the judicious design of the hairpin substrate allowed discrimination for 3′→5′ exonuclease activity over other nucleases such as T7 exonuclease, exonuclease I and DNase I. Subsequently, a variation of this enzyme digestion approach was successfully developed as a transcription factor inhibitor screening platform.[73]

In 2012, Jin and co-workers reported an assay for the real-time monitoring of DNA cleavage by EcoRI using SYBR Green I as a fluorescence probe.[74] In the absence of enzyme, the intercalation of SYBR Green I into the duplex substrate produces a strong fluorescence response. However, cleavage of the DNA substrate at the recognition site by EcoRI produces smaller, less stable fragments that dissociate at 37 °C, ultimately producing a switch-off fluorescence response (Fig. 14). This assay could sense EcoRI with a limit of detection of 8 U/mL and could potentially be adapted for the detection of any restriction enzyme by modification of the recognition sequence within the oligonucleotide substrate.

4. Luminescent aptamer-based probes

Aptamers are target-binding nucleic acid sequences generated *via* SELEX[4, 5] that have recently emerged as promising candidates in diagnostic and therapeutic applications.[75, 76] DNA aptamers are available for a wide variety of different targets such as organic dyes, nucleotides, biological cofactors, amino acids, peptides and enzymes.[77] Aptamers usually undergo a significant conformational change into well-defined three-dimensional structures upon binding to their cognate ligands. This conformational transition can be effectively monitored by the use of DNA structure-selective luminescent molecules that transduce the analyte binding event into a luminescence response. In this section, we highlight some interesting examples of luminescent aptamer-based probes. The ATP and thrombin aptamers have attracted the most attention as model systems for the development and validation of label-free aptamer-based assays.

In early work, the groups of Bai and Fang have employed $[Ru(phen)_2(dppz)]^{2+}$ for the switch-off detection of a variety of biomolecules such as immunoglobin E (IgE), PDGF-BB, thrombin and ATP using a ligand displacement approach (Fig. 15).[78, 79] In the absence of the target molecules, the aptamer existed in a specific three-dimensional conformation that interacted efficiently with the ruthenium(II) complex, resulting in a strong luminescence response. However, the binding of the analyte alters the conformation of the aptamer and displaces the metal complex from the DNA thereby producing a decreased

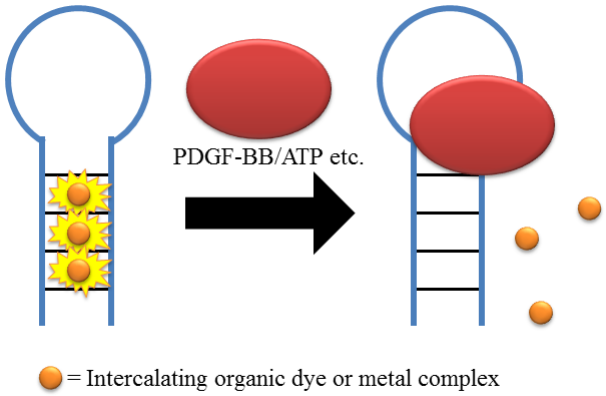


Fig. 15. Schematic representation for the luminescent switch-off detection of analytes using the ligand displacement approach.

luminescence signal. The detection limits for IgE, PDGF-BB, thrombin, and ATP were determined to be 0.1, 1.0, 0.01, and 1 nM, respectively.

Our group envisaged that switch-on aptamer-based detection method for ATP could be developed using a duplex-to-quadruplex conversion strategy.[80] In this assay, the ATP aptamer (5′-$A_2C_2TG_5AGTAT_2GCG_2AG_2A_2G_2T$-3′) is initially hybridized with its complementary sequence (5′-$AC_2T_2C_2TC_2GCA_2TACTC_5AG_2T_2$-3′) and the fluorescence of CV is weak. However, the addition of ATP promotes the dissociation of the duplex. The resulting ATP-aptamer complex folds into a G-quadruplex, which is detected by CV with a switch-on luminescence response (Fig. 16A). This assay was able to detect down to 5 μM of ATP, which was comparable to other aptamer-based ATP assays, and could further discriminate ATP from other nucleotide analogues and chemical species likely to be present in biological samples. Furthermore, the methodology was applied to the detection of ATP in HeLa cell extracts. A similar approach was employed by the group of Willner utilizing Zn-PPIX in conjunction with the free aptamer for the detection of low micromolar ATP.[81] Although these assays exhibited relatively high detection limits for ATP, their switch-on mode of detection may make them less susceptible to false positive signals arising from the presence of non-specific quenchers in the sample matrix.

The group of Dong have reported a switch-off detection platform for thrombin that utilized ethidium bromide (EB) as a duplex probe.[82] Initially, the thrombin aptamer (5′-$G_2T_2G_2TGTG_2T_2G_2$-3′) is locked into a duplex conformation by a complementary DNA sequence, facilitating intercalation and fluorescence emission of EB (Fig. 16B). The addition of thrombin promotes the dissociation of the thrombin aptamer from the DNA duplex due to the formation of the thrombin-aptamer G-quadruplex motif. The G-quadruplex structure is only weakly bound by EB, resulting in a switch-off fluorescence response. A detection limit of 2.8 nM was reported. Later, the group of Jin utilized the thrombin aptamer and CV for the label-free detection of thrombin through a single strand-to-quadruplex approach.[83] The assay was reported to have a detection limit of 8 pM and was selective for thrombin over immunoglobin G (IgG) and lysozyme.

To design a switch-on probe for thrombin, the group of Yan

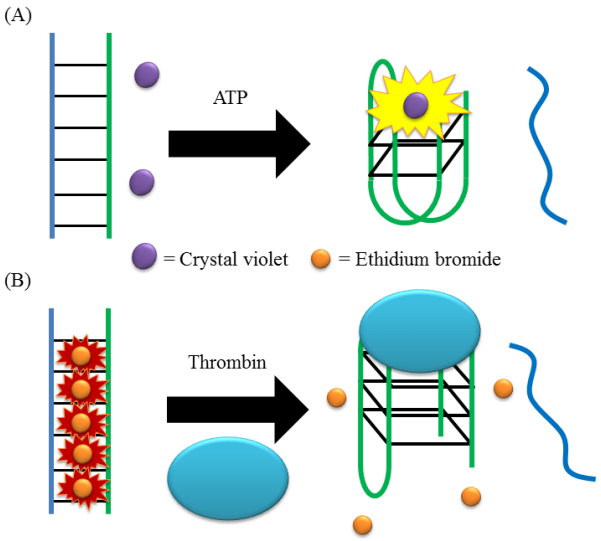


Fig. 16. G-quadruplex-based aptamer-based detection assay for (A) ATP and (B) thrombin.

have recently utilized the pyrazine derivative TASPI as a luminescent nucleic acid probe.[84] TASPI is emissive in aqueous solution but its fluorescence is reduced upon binding to DNA due to aggregation-induced quenching. However, the addition of thrombin weakens the binding between TASPI and the aptamer, resulting in restoration of the fluorescence signal (Fig. 17A). This method exhibited a detection limit for thrombin of 50 nM, and could selectively recognize thrombin over proteins such as trypsin, bovine serum albumin and tyrosinase. A similar strategy was employed by Yu and co-workers utilizing a cationic perylene tetracarboxylic acid diimide (PTCDI) for the switch-on detection of lysozyme. The addition of lysozyme displaces PTCDI from the lysozyme aptamer (5′-$ATCAG_3CTA_3GAGTGCAGAGT_2ACT_2AG$-3′), dissociating and restoring the fluorescence of the PTCDI monomers (Fig. 17B).[85] This method achieved a 70 pM detection limit for thrombin, which borders the femptomolar sensitivity of the "gold standard" ELISA.

Besides intercalators, minor groove-binding organic dyes such as DAPI and Hoechst 33258 can also exhibit a significant switch-on luminescent response upon DNA binding. These dyes were utilized by the group of Yang to develop a label-free luminescent assay for the detection of L-argininamide. In the presence of the target analyte, the L-argininamide aptamer (5′-$GA_2T_2G_3CA_3CG_2TAG_2TGAGA_2T_2C$-3′) forms a AATT-rich stem-loop region which facilitates DAPI or Hoechst 33258 binding, resulting in a switch-on luminescence response.[86] The detection limits for L-argininamide were reported to be 2.5 μM for Hoechst 33258 and 3.8 μM for DAPI, and the assays could selectively recognize L-argininamide over amino acids such as L-arginine and glycine.

5. Conclusion

The versatility and robustness of functional oligonucleotides have made them attractive tools in multiple areas of science and technology in recent years. Besides the sensing applications

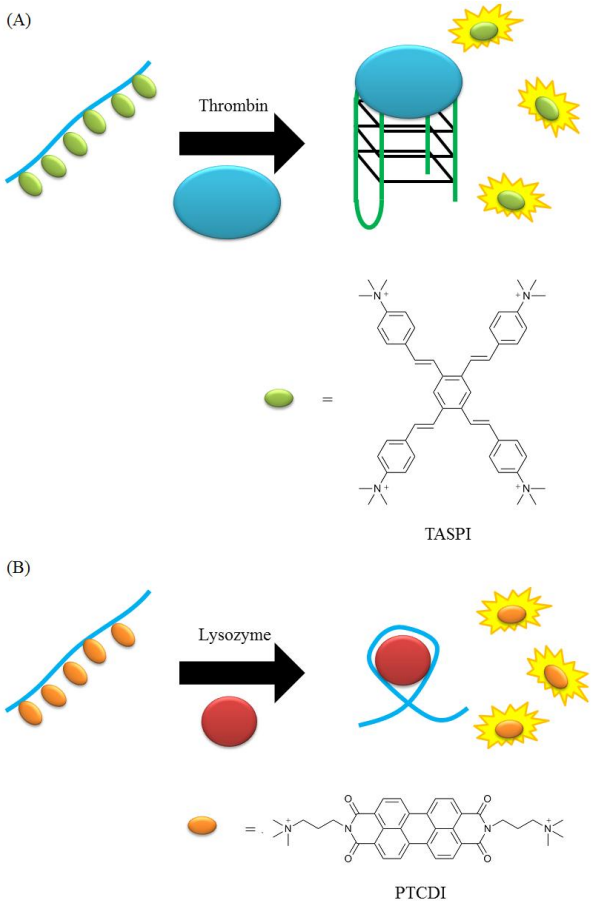


Fig. 17. (A) Switch-on detection of thrombin using TASPI and (B) switch-on detection of lysozyme using PTCDI.

described in this review, oligonucleotides have found use in the construction of DNA nanomachines[87] and DNA logic gates.[88, 89] These exciting discoveries may pave the way for the future development of DNA-driven molecular computers and machines.

In the context of sensing, oligonucleotides enjoy distinct advantages over protein antibodies or organic molecules that have historically received the majority of attention in the development of optical sensing platforms. These advantages include high thermostability, low cost, ease of production and modification, water solubility, and a rich structural polymorphism that can be responsive to a variety of ligands. In particular, the powerful conjunction of functional oligonucleotides with the label-free strategy has received growing popularity in the scientific community for the construction of simple and inexpensive luminescent sensing assays.

The sensitivity and selectivity of these luminescent label-free DNA-based probes in many cases rival those for optical or electrochemical assays utilizing covalently-labeled oligonucleotides. In addition, such systems are inexpensive, easy to operate, and do not require additional conjugation or purification steps for oligonucleotide modification. Such sensing platforms may warrant further investigation in a variety of fields such as point-of-care diagnostics in the clinical setting or the routine in-field monitoring of environmental pollutants. While the lack of true multiplex capability is a potential drawback of label-free systems, their cost-effectiveness and simplicity renders them as strong candidates for most single-analyte sensing applications. Another potential issue common to all DNA-based systems is the limited cell permeability and biostability of oligonucleotides in cellular systems. Researchers in chemical biology and biochemistry are persistently developing improved materials or techniques for the intracellular delivery of DNA, such as complexation of DNA with delivery agents such as nanoparticles, cationic lipids and hydrophilic polymers.[76, 90] Moreover, enzymatic degradation of the oligonucleotide probes can be deterred by chemical derivatization of the nucleic acid backbone,[91] though this may stray from the spirit of the label-free approach. The label-free approach may also suffer from potential interference due to non-specific quenchers in more complex sample matrices such as blood sera and cells. Although several oligonucleotide-based assays described here have functioned well in diluted biological samples, robust performance in unadulterated media is ultimately desirable.[29, 53, 80] We envision that a major future direction of oligonucleotide-based probes lies in continual advancements in nucleic acid delivery, biocompatibility and robustness with an eventual view towards application in the *in vivo* environment.

In this review article, we have described a large variety of oligonucleotide designs and luminophores that have been utilized for the construction of luminescent DNA-based probes. As the selectivity of these assays can be limited by the ability of the luminophore to distinguish between the various DNA conformations, we envisage that the further improvement could be achieved by the optimization of the specific DNA-dye interactions. We also promote the use of the G-quadruplex motif in conjunction with quadruplex-selective luminophores[92] for the construction of label-free DNA-based sensing platforms. The unique structural features exhibited by the diverse array of G-quadruplex topologies offers the potential for highly specific interactions between the luminophore and the functional oligonucleotide. Based on the multitude of examples of label-free luminescent DNA-based probes reported recently, we anticipate that this field would continue to thrive and mature in the years to come.

Acknowledgement

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Notes and references

[a] *Department of Chemistry, Hong Kong Baptist University, Kowloon Tong, Hong Kong, China. E-mail: edmondma@hkbu.edu.hk*
[b] *State Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macao, China.*

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