Nucleic acid aptamers for biosensors and bio-analytical applications

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Oligonucleotides were once considered only functional as molecules for the storage of genetic information. However, the discovery of RNAzymes, and later, DNAzymes, unravelled the innate potential of oligonucleotides in many other biological applications. In the last two decades, these applications have been further expanded through the introduction of Systematic Evolution of Ligands by EXponential enrichment (SELEX) which has generated, by repeated rounds of *in vitro* selection, a type of molecular probe termed aptamers. Aptamers are oligonucleic acid (or peptide) molecules that can bind to various molecular targets and are viewed as complements to antibodies. Aptamers have found applications in many areas, such as bio-technology, medicine, pharmacology, microbiology, and analytical chemistry, including chromatographic separation and biosensors. In this review, we focus on the use of aptamers in the development of biosensors. Coupled with their ability to bind a variety of targets, the robust nature of oligonucleotides, in terms of synthesis, storage, and wide range of temperature stability and chemical manipulation, makes them highly suitable for biosensor design and engineering. Among the many design strategies, we discuss three general paradigms that have appeared most frequently in the literature: structure-switching, enzyme-based, and aptazyme-based designs.

1. Introduction

The ability to create and manipulate molecules with specific properties to be used as biosensors has been among the general goals of analytical chemistry and analytical biochemistry. The preferred method of creating these molecules is by rational design. That is, if we can envision a given specific molecular function, we should be able to use our knowledge about the basic properties of molecules and apply a systematic and logical process leading to a final design that fulfils the requirements of that function and further allows us to effectively monitor it in a measurable way. These properties might include selective recognition, catalytic ability, and conformational changes associated with the functional molecule. Once the molecules are designed, they have to be engineered to suit the conditions under which the molecules would be used, such as pH, salt concentrations, or temperature. The molecular engineering aspect is critically important because it requires the efficient manipulation and

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assembly of the individual components of the proposed device in order to achieve the required response.

Among the various potential candidate molecules for rational design, which include peptides or proteins[1–3] and organic polymers,[4] oligonucleotides are the easiest to design and engineer. The design is facilitated by the availability of structure prediction software and a vast repertoire of well-characterized oligonucleotide reaction-catalyzing enzymes. Molecular engineering of oligonucleotides is relatively easy because they are produced by chemical synthesis with extreme accuracy and reproducibility, and they can be purified to a very high degree. Thus, there is little or no batch-to-batch variation in their production. Added to this is the feature of chemical manipulation during and/or after synthesis. This allows for the incorporation of specific and desirable functional groups that permit subsequent derivatization with other molecules without compromising function. The properties of oligonucleotides are well understood at physiological conditions, but this additional flexibility of manipulating oligonucleotides makes them particularly suitable for use as molecular recognition elements in the development of biosensors.

To paraphrase a dictionary definition, a biosensor is a device consisting of two main components, a biological component, which reacts with a target substance, and a signal-generating component, which detects the resulting products. More specifically, we can consider a biosensor as a type of biomolecular probe that measures the presence or concentration of biological molecules or biological structures by translating a biochemical interaction at the probe surface into a quantifiable physical signal. Previously, molecular recognition elements were isolated from biological systems,[5] but now there are many recognition elements that are synthesized in the laboratory. These recognition elements can be classified into a variety of groups based on function. For instance, receptors include transmembrane (plasma and intracellular membranes) and soluble proteins that bind to specific molecules called ligands, with the binding event initiating specific cellular responses.[6,7] Enzyme-based recognition

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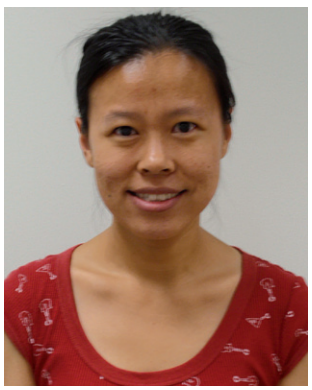

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is attractive for biosensor applications by virtue of the many measurable reaction products arising from the catalytic process.[8] Antibody-based recognition[9,10] and peptide nucleic acid-based recognition, or PNAs,[11] fall into another class of recognition elements that exhibit superior hybridization characteristics and improved chemical and enzymatic stability compared to nucleic acids.[12] Molecular imprinted polymer (MIP) recognition technology has proliferated as an inexpensive, accessible, and effective strategy for developing sorbent materials exhibiting high specificity for selected substrate materials. MIP is a method of making selective binding sites in synthetic polymers using molecular templates. This technology offers great promise for the development of stable artificial bio-sensing elements.[13] Finally, lectin-based recognition[14] and aptamer (oligonucleotide)-based recognition[15–17] involve conformational changes, catalytic ability, and ability of denaturation and renaturation, all producing an observable and/or measurable signal.

Aptamers can also offer a strong and reliable role as biological recognition elements in most analytical applications. The specificity and high affinity of aptamers to a wide variety of targets, coupled with the ease of design and molecular engineering, as outlined earlier, make aptamers highly suitable for development as molecular biosensors. By moving in this direction, investigators have made appreciable efforts in recent years to utilize these unique features of aptamers to devise appropriate strategies with which to effectively and efficiently apply aptamers for biological agent recognition, identification, characterization and quantification.

Aptamers are single-stranded (ss) DNA or RNA molecules, typically <100 mer, which have the ability to bind to other molecules with high affinity and specificity. They are selected from random oligonucleotide pools by a process called Systematic Evolution of Ligands by EXponential enrichment (SELEX).[18,19] Conceptually, the SELEX process is based on the ability of these small oligonucleotides to fold into unique three-dimensional (3-D) structures which can interact with a specific target with high specificity and affinity through such interactions as van der Waals surface contacts, hydrogen bonding and base stacking interactions. Aptamers have therefore been generated against a variety of targets, such as small molecules, including metal ions,[20,21,22] organic dyes and amino acids,[23–26] antibiotics,[27,28] and peptides,[29,30] as well as proteins of various sizes and functions,[19, 31–40] whole cells,[41–44] viruses[45] and bacteria.[46] The cell-SELEX strategy has been exploited even further, leading to the identification of specific molecular markers in various cancers.[47,48] The list of aptamer targets will continue to expand as the technology continues to improve and more research groups find interest in the versatile nature of aptamers as useful molecular probes.

Aptamers are identified through an *in vitro* process. Briefly, this involves the incubation of the oligonucleotide library pool (DNA or RNA) with the target of interest. After washing, the bound sequences are eluted and incubated with a control target (if negative selection is required) to remove sequences that exhibit recognition with the control as well. The remaining sequences are then amplified by PCR. The process is repeated until the pool is enriched for sequences that specifically recognize the target. The enriched pool is cloned and then sequenced to obtain the individual sequences. Since this is an *in vitro* process, the selection conditions can be manipulated to obtain aptamers with properties desirable for a particular purpose. That is, aptamers that bind to a target at different temperature and buffer compositions can be used for different purposes, or modified oligonucleotide bases can be introduced to enhance stability. For example, Biesecker *et al.*[49] generated 2′F-pyrimidine-modified nuclease resistance RNA aptamers that inhibit human complement C5. In addition, kinetic parameters, such as the on and off states of aptamers, can be changed on demand, and aptamers can undergo temperature-dependent cyclic denaturation and renaturation in minutes, a unique property for the development of aptamer sensors and motors.

These unique properties have led to the development of various strategies in implementing the technology for different fields, such as bio-technology, medicine (drug development), pharmacology (targeted therapy and gene delivery), cell biology (tracking and imaging), microbiology (virus and bacteria detection), and analytical chemistry (chromatographic separation, biosensors), to mention only a few.[50] In each of these cases, the aptamer can be uniquely designed to meet specific goals, showing the robust nature of these molecules. Although one important disadvantage of aptamers lies in the ease of hydrolysis (especially in the case of RNA aptamers), this is being addressed with the introduction of modified bases, such as locked nucleic acids (LNAs) and 2′-*O*-methyl nucleotide analogues,[51] to increase their hydrolysis time when adapted for *in vivo* assays.

Various novel biosensor engineering strategies and detection schemes that incorporate aptamers as a recognition element have emerged. The surge in this area will continue as aptamers exhibit desirable qualities such as engineering flexibility, target diversity, selectivity, and high affinity. For instance, the ability to use nucleic acids either in solution or immobilized on different supports without compromising activity has aided various design strategies.[52–57] In addition, the compatibility of aptamers with various detection schemes, such as electrochemical,[58] fluorescence,[59–61] chemiluminescence,[62] field effect transistors,[55,58] Surface Plasmon Resonance (SPR),[63] amperometry,[54] and potentiometry,[64] has enhanced the progress of this field.

We will review the three general paradigms that have appeared most frequently in the literature for the rational design of aptamer biosensors. We broadly classify these designs as

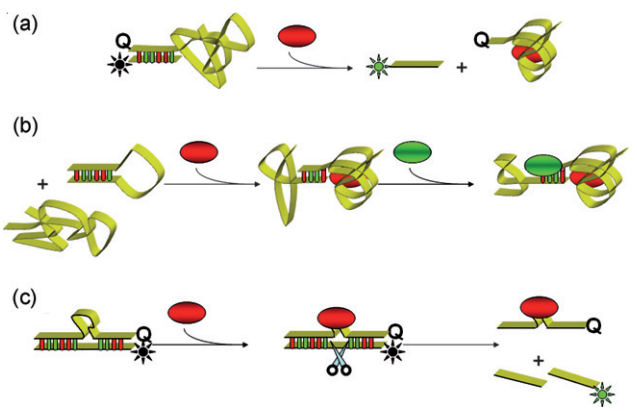

Fig. 1 General schematic of three basic paradigms frequently used in the design and engineering of aptamer-based biosensors: (a) cDNA structure-switching, (b) enzyme-mediated, and (c) DNAzyme-mediated.

structure-switching, enzyme-based, and aptazyme-based. Structure-switching designs take advantage of either duplex to single-stranded transitions or the inherent structural changes of the aptamer to signal ligand-binding events (Fig. 1(a)). On the other hand, enzyme-based designs incorporate DNA-acting enzymes, such as ligases, endonucleases, and polymerases, as mechanisms for signal transduction (Fig. 1(b)), while aptazyme-based designs are similar to enzyme-based designs, except that the molecular engineering principles are considerably different (Fig. 1(c)). Here, the catalytic property incorporated in the aptamer sequence and binding event activates the catalytic activity. This review will generally describe the rational design schemes and molecular engineering principles of each of these aptamer biosensor paradigms and then discuss in detail specific examples of each.

2. Structure-switching designs

The most common designs that are seen in the literature are structure-switching aptamer biosensors.[59,65–70] There are basically two design strategies that are often used for cDNA-based probes. The first class is bimolecular in nature, involving the addition of the cDNA and aptamer as separate molecules. The second class, considered unimolecular, involves linking the cDNA covalently to the aptamer. The easiest rational design for aptamer biosensors is to simply add the cDNA of an aptamer to act as a competitor to ligand binding. Many times the cDNA carries a signaling moiety that is either enhanced or suppressed when in complex with the aptamer. Thus, when the target binds the aptamer and pushes off the cDNA, a change in signal is observed. In this case, the binding affinity must be strong enough to dissociate the cDNA, and therefore the length of the cDNA must be optimized for the particular application. The optimization process is facilitated by the accuracy with which double-stranded (ds) DNA melting temperatures can be predicted. A classical example of this strategy is demonstrated by Nutiu and Li[71] in their report of structure-switching signaling aptamers. This strategy used the ability of aptamers to adopt different structures depending on the composition of the environment. They engineered solution-based signaling aptamers that function by a structure-switching/fluorescence-dequenching mechanism. This simple and generalized approach was adopted in order to offset some of the difficulties and uncertainties that are associated with designs in which the reporter molecule is directly linked to a location on the aptamer, with the assumption that the binding of the target will cause a conformational change that will substantially alter the electronic environment of the fluorophore, causing, in turn, significant change in the fluorescent properties. Such an approach requires a precise attachment location, as well as sufficient fluorescence enhancement, a task, which, in most instances, is challenging. Again, some sensors are designed as aptamer beacons where fluorophore and quencher are labeled at the 5′ and 3′ ends of the aptamer which form a stem of the aptamer-predicted structure and the loop acting as recognition of the target. This is analogous to the traditional molecular beacons, except that aptamer beacons have variable targets, whereas the targets for molecular beacons are nucleic acids. When the aptamer is in the unbound form, the fluorescence is quenched by the closeness of the fluorophore and quencher. The fluorescence is, however, restored when the target of the aptamer is introduced. In spite of advances in the design of molecular beacons, the fluorescence enhancement is often insufficient. Up to now, only a few aptamers have possessed a known molecular beacon structure, limiting their applications. The structure-switching/fluorescence-dequenching mechanism, however, can couple the fluorophores and quenchers to complements of key oligonucleotide sequences such that they are not directly involved in recognition of the detectable elements. In this case, the manipulation of sequence length for reversible renaturation and denaturation is done on the complement sequences.

In the Nutiu and Li strategy for the sensing of ATP and thrombin, a two-stem duplex assembly was constructed using fluorophore (F) containing DNA at the 5′ end (FDNA) and 3′ end quencher (Q) containing DNA (QDNA). The aptamer was linked at the 5′ end with a 15 mer sequence complementary to FDNA, and the entire structure was termed MAP. QDNA was complementary to the 5′ end of the aptamer. The length of QDNA was uniquely optimized to meet the requirements of fluorescence quenching and restoration. When all the sequences are in complex, the dabcyl quencher is brought into sufficient proximity to quench the fluorescence signal on FDNA. Upon introduction of the aptamer target, the aptamer–target complex causes QDNA to dissociate from the complex, thus restoring fluorescence (Fig. 2(a)). The structure-switching mechanism was tested with signals associated with differential temperatures as well as the introduction of aptamer targets, such as ATP and dATP, and non-binding molecules, such as CTP, UTP and GTP. The engineering strategy was further expanded to a variety of modifications, and all yielded similar results. The generality of this design was further assessed for protein detection. Here, the 15 mer thrombin aptamer was engineered to produce the required sensor similar to the ones obtained for ATP. By using both ATP and thrombin as targets for these sensors, the versatility of these designs to produce significant signals for real-time detection, irrespective of the binding affinity or size of the aptamer, was demonstrated.

The applicability of this strategy was further expanded by Yoshida and Yokobayashi,[72] as demonstrated in their communication entitled “Photonic Boolean logic gates based on DNA aptamers”. They constructed a pair of DNA-based logic gates that could sense the presence of ssDNA and targets of aptamers to generate a fluorescence signal according to the Boolean logic functions AND and OR. Molecular logic gates based on nucleic acids have many potential advantages. For instance, the straightforward hybridization rules enable convenient interfacing with other molecular computation devices based on DNA and RNA. Additionally, both DNA and RNA are known to possess catalytic and molecular recognition abilities which can be used to provide amplification and sensor functions.

Boolean logic gates have been constructed using organic molecules, peptides, proteins, and oligonucleotides. An AND Boolean logic gate is constructed in which there is an output only when all of the inputs are present, whereas an OR logic gate produces a signal when at least one of the inputs is present. The AND Photonic Boolean logic gate was engineered by fusing the adenosine and the thrombin DNA aptamers with 11 mer linkers which have a fluorescein conjugation in the middle (Fig. 2(b)(i)).

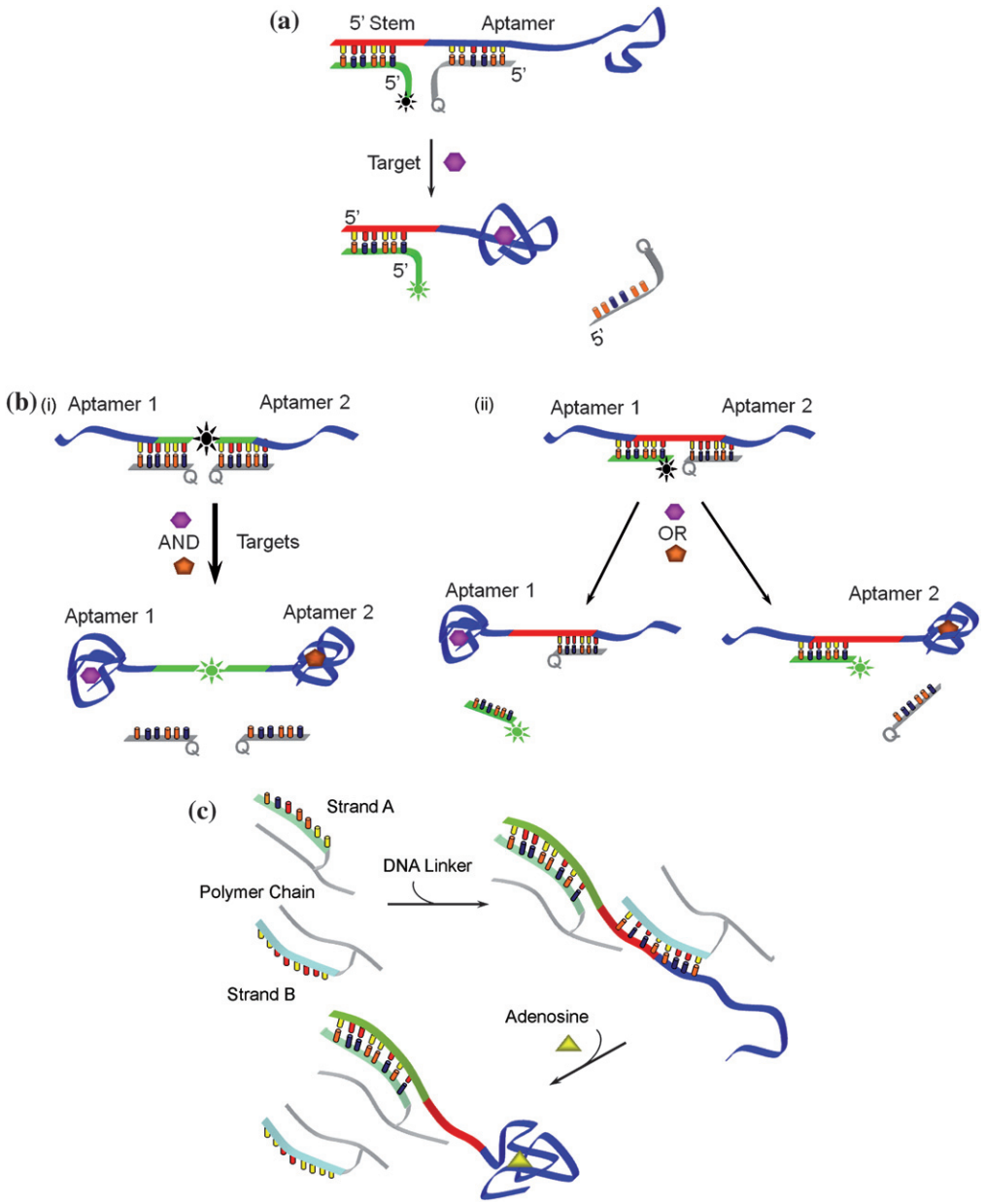

Fig. 2 (a) The general scheme showing the working principle of the structure-switching signaling aptamer. The aptamer (blue) is linked at the 5′ end with 15 mer sequence (orange) complementary to the fluorophore-labeled strand (green) FDNA, while the quencher strand (grey) QDNA, can hybridize with portions of the aptamer. When the two-stem duplex assembly hybridizes with the complex, the dabycl quencher and the fluorescein fluorophore are in close proximity such that the fluorescence signal is quenched. However, when the target of the aptamer (purple hexagon) is introduced, it causes the aptamer to fold into binding conformation resulting in the melting of the QDNA from the complex. This conformational change distances the quencher from the fluorophore, and the fluorescence signal is restored. (b) Depiction of the DNA logic gates. The AND logic gate (i) is activated only when both targets (purple hexagon and brown pentagon) of the two aptamers bind, causing the disruption of the quencher sequences from the fluorophore, resulting in fluorescence restoration. An OR logic gate (ii) can be activated with just one of the aptamer targets. (c) Representation of the DNA-induced formation and adenosine-induced dissolution of hydrogel. Addition of the DNA linker causes the formation of the gel structure as a result of the linking of strands A and B. In the presence of the aptamer target adenosine (yellow triangle), however, the binding disrupts the hybridization of the aptamer to strand B, and this leads to the formation of the sol state.

Two short DNA sequences complementary to part of the aptamers and the linkers were conjugated at the 5′ and 3′ ends with quenchers. The binding of these two sequences with the linked aptamers brings the two quenchers in close proximity to the fluorescein, thereby quenching the fluorescence signal. The introduction of either adenosine or thrombin, with the concomitant dissociation of one of the quencher sequences, did not restore the fluorescence signal since the remaining quencher is strong enough to exert its effect on the fluorescein. However, addition of both adenosine and thrombin led to the displacement of both quencher DNA sequences, resulting in enhanced fluorescence signal.

A similar construct was designed for the OR logic gate with the following modification. The 11 mer linker lacked the fluorescein conjugation, while one of the short DNA strands was conjugated with fluorescein at the 5′ and the other with quencher at the 3′ end (Fig. 2(b)(ii)). Here again, the annealing of the complex quenched the fluorescence signal. Unlike the AND gate, the addition of either adenosine or thrombin restored the fluorescence signal with the dissociation of either the QDNA or FDNA. Once again, we see a definitive demonstration of the flexibility of oligonucleotides in the construction of biological sensors.

Coupled with the ability of a well-defined cyclical renaturation and denaturation, the hybridization potential of nucleic acids makes their utility almost limitless for the rational design and engineering of various types of biosensors. Among such approaches, Yang *et al.*[73] reported highly selective target-responsive hydrogels based on DNA aptamers for controllable release. They constructed an adenosine-responsive polyacrylamide hydrogel using an adenosine aptamer. Two acrydite-modified oligonucleotides named strands A and B were copolymerized separately with acrylamide. Strand B was designed such that part of the sequence was complementary to seven bases of the adenosine aptamer (Fig. 2(c)). When the two were mixed together, they obtained a fluid system. They then designed a DNA linker consisting of three parts. One part consisted of bases that are complementary to strand A, followed by 5 bases complementary to the first 5 bases of strand B, and ending with the adenosine aptamer, which has seven bases of hydrogen-bonding with strand B as well. Once strand A, strand B, and the linker were in complex, the system gelled. On addition of adenosine, the aptamer binding caused the aptamer to be dissociated from strand B, leaving only 5 bases to hybridize with strand B. This was unstable at room temperature and led to complete dissociation of strand B, which, in turn, caused the dissolution of the hydrogel, and a liquid system resulted (Fig. 2(c)). This is analogous to a tightly woven rug where pulling out one fiber results in the others coming apart. The sol-to-gel and gel-to-sol transition was observed with an excess addition of the linker.

To go beyond the proof-of-principle, they loaded the hydrogel with high concentrations of water-soluble gold nanoparticles, which could be tracked with IR absorption. The nanoparticles were tightly trapped in the matrix so that no release of the nanoparticles was detected. However, after addition of 2 mM adenosine, an increase in absorbance in the solution was observed, indicating the release of the trapped gold nanoparticles. As a control to confirm that the release of the nanoparticles resulted from the addition of the adenosine, non-target compounds, such as cytidine, uridine, and guanosine, were added, and none of them caused the release of the nanoparticles.

The generality of the system was further tested by using the human thrombin aptamer in the construction of the hydrogel as the recognition element. Thrombin binds only if the aptamer is in the G-quartet structure; therefore, binding to thrombin disrupts the gel construct. As expected, the hydrogel behaved similarly, although the rate of release was slower than with the adenosine. The use of aptamers in this controllable release scheme is significant since it validates aptamers as having a wide variety of targets, leading, therefore, to the possibility of broadening the use of this system to potential drug release and other pharmacological and clinical applications. For example, it is envisaged that biocompatible hydrogels containing aptamers for specific tumors can be constructed. Such hydrogels could be loaded with anti-tumor agents, which are protected as the hydrogel is transported through the body in the unaffected parts. However, at the tumor site, the binding of the tumor cell to the aptamer would cause the dissolution of the gel and therefore the release of the payload to kill the cells, but only at the tumor site. Localized therapy is important, but it is still a challenge.

3. Enzyme-based designs

The previous section discussed the basic design elements and logic used for probes that involve only DNA or RNA in their design. This section will focus on the use of proteins and enzymes in the overall design of aptamer-based probes. To be successful, probe designs of this type require knowledge of aptamers and enzyme reactions. As such, there are relatively few examples in the literature in comparison to those illustrating structure-switching probe designs. Nonetheless, several benefits of enzyme-based designs can be cited.[74–76] First, the signal can usually be amplified by multiple catalytic cycles of the enzyme. Second, the aptamer binding interaction can be coupled to a wide variety of output signals. Third, since most commonly used enzymes have fast catalytic rates, the output signal can be rapidly obtained. Fourth, enzymes function well in complex biological fluids. Although the rational design of these probes is usually straightforward, the engineering of the probes can be quite complicated because most enzymes require strict control over temperature, salt, and pH to maintain their highly active forms.

Although specific requirements restrict the freedom with which such nucleotide sequences can be adjusted, realistic design schemes have emerged. Among these, Fredricksson *et al.*[77] demonstrated the detection of platelet-derived growth factor BB (PDGF-BB) by using a proximity ligation technique. This was made possible partly because the PDGF-BB is a homodimer, and two molecules of aptamer can bind to one molecule of PDGF-BB homodimer complex with high specificity and selectivity. They added different extensions to the two molecules of the aptamer such that the free ends of the extensions could hybridize with a connector sequence and then be ligated by an enzyme (Fig. 3(a)).

The binding of the aptamers to each of the dimers brings the free ends into close proximity suitable for the ligation. Of particular significance, the engineering principle was very flexible. For instance, the extension length made no overall significant difference, meaning that extensions could be custom designed for proteins of different sizes. The connector was optimized to hybridize with 10 bases of each extension since more base pairings led to false positive signals coming from the increased stability of the probes in the absence of target proteins. Moreover, the connector had 3 base non-hybridizing overhangs to prevent it from acting as a PCR primer. Molar concentrations were controlled in a way that ensures the binding of any free aptamer at 1 : 1, aptamer : connector, whereas only aptamers that are proximate bind at 2 : 1, aptamer : connector. Only the ligated probes could be amplified by PCR. The inclusion of PCR in the detection scheme is significant since PCR detection is one of the most sensitive systems. The amplification product was detected by a modified fluorescent detection system with

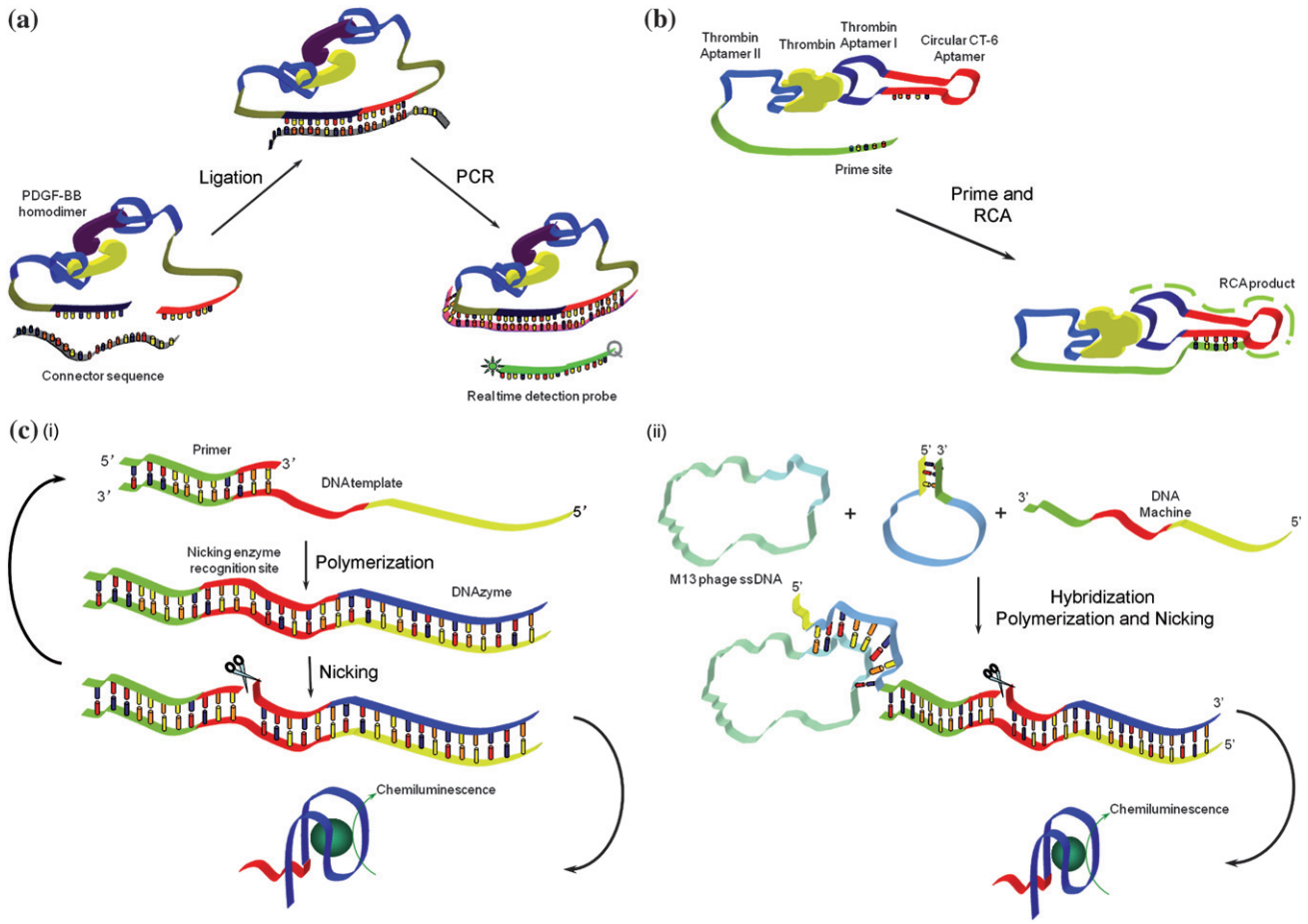

Fig. 3 (a) A demonstration of the proximity ligation technique to detect PDGF-BB. The homodimer PDGF (yellow and purple) is bound by two molecules of the PDGF aptamer (blue). This causes the extensions to orient such that they can be hybridized by the connector sequence and be ligated. The orientation is achieved only when the aptamers bind to the target, and only the ligated strands can be amplified by PCR and therefore be detected. (b) Detection of thrombin by the proximity extension strategy using the two aptamers of thrombin. Simultaneous binding of the two aptamers facilitates the hybridization of the complementary portions of the extensions to the aptamers, which primes the DNA polymerase-mediated RCA. (c) Coupling of DNAzyme activity to an autonomous primer-induced synthesis of the DNAzyme units on template DNA using polymerase/dNTPs and a nicking enzyme as biocatalyst (i), and (ii) the analysis of M13 phage ssDNA by DNA-based machine. The production of the DNAzyme is detected by the generation of chemiluminescence in the presence of luminol/H_2O_2 coupled to the DNAzyme mimicking peroxidase activity.

sensitivity in the zeptomolar range, better than ELISA. Since the design is suitable for macromolecules with two or more non-competing binding probes, they detected thrombin by using the two reported aptamers[34] in the construction. The significant limitation to this technique is that the macromolecule of interest must have at least two non-competing probes which must not differ significantly in binding conditions, such as buffer systems, pH, and temperature. Also dual-label designs have artifacts at high target concentration as a result of single labeling; thus, highly concentrated samples must be diluted.

This adoption of dual non-competing aptamers for a single molecule has become increasingly useful in many biological applications.[78] The advantage here is that these aptamers can be linked in a specific way during the construction in order to achieve the desired goal, such as increased affinity,[78,79] improved selectivity, and signal amplification.

Similar to the work reported by Fredricksson *et al.*[77], Di Giusto *et al.*,[51] demonstrated a scheme in which the binding of two aptamers to thrombin causes the priming of a rolling-circle amplification (RCA) reaction. The exosite II aptamer (Aptamer I) was designed to be part of a circular PCR template, while the exosite I aptamer (Aptamer II) had a primer extension built into its sequence. When both aptamers bind the target, the primer extension can bind to the circular aptamer and prime the RCA reaction, leading to increased production of the circular template, which is detected by polyacrylamide gel electrophoresis (PAGE) (Fig. 3(b)). RCA is an isothermal *in vitro* method for the hybridization-triggered enzymatic synthesis of hundreds to billions of linear copies of small, single-stranded, circular DNA probes. The amplification gives repeats of DNA sequences which can serve as signal amplifiers for ultra-sensitive detection of specific nucleic acids and other biologically important molecules in diagnostic genomics and proteomics.[74–76] In this scheme, unless both aptamers bind to the target, the short primer-loop interaction is not stable enough in solution to prime the RCA reaction. When both aptamers bind the target, a large molecular weight RCA product is observed.

To engineer these probes, the G-quartet structure of the linear aptamer I was first stabilized by adding nucleotides of complementary sequences to both 5′ and 3′ ends, which, upon hybridization, closed the aptamer into a hairpin loop structure. The number of nucleotides was optimized from 4 to 13 bases. An additional 48 poly dT was added to the 3′ end, providing a bridge to aptamer II when both bind to thrombin, and also containing the priming region for the RCA reaction. Different variants of this probe were designed to accommodate bridge length and the inclusion of LNA for the purpose of 3′-exonuclease resistance.

Aptamer II was circularized, which provided the target for the RCA reaction. This circular aptamer was created by ligating the aptamer to an unstructured loop, which contained the complementary sequence, to the primer region of aptamer I. The ligation reaction generated three different circular DNAs: aptamer–aptamer, aptamer–loop, and loop–loop. To obtain the correct heterodimeric products, these sequences were smartly designed such that aptamer–aptamer and loop–loop circular DNAs had a restriction enzyme recognition site and therefore were linearized by specific restriction enzymes and further degraded by exonuclease III. Simultaneous binding of constructs, *i.e.*, aptamer I and aptamer II to exosite I and II of thrombin, brings them into close proximity and primes the DNA polymerase-mediated RCA. The RCA reaction generated long ssDNA products of concatenated loop complementary sequences and therefore formed dsDNA complexes because of the self-complement loop design. This long ssDNA was further digested using another restriction enzyme, and the digestion fragments were verified using PAGE or monitored with real-time PCR. The coupling of this design to the real-time PCR detection system is significant because of the ability of PCR to amplify a given signal and therefore increase the sensitivity.

The coupling of the sensitivity and efficiency of PCR techniques with the selectivity of nucleic acids to various analytes is increasingly being exploited in various biosensor designs. Of significant interest is the DNA machine designed by Weizmann *et al.*,[80] which consisted of a DNA template, DNA polymerase, and DNA nicking enzyme acting in concert to produce a DNAzyme that intercalates hemin and mimics peroxidase activity. The general template consisted of the primer complementary site at the 3′ end, the nicking enzyme recognition site, followed by the complementary strand of the DNAzyme. The binding of the primer allows the DNA polymerase to synthesize the DNAzyme sequence. Although the DNAzyme sequence exists as a duplex and is non-functional in the synthesized state, it is recognized and nicked by the nicking enzyme. The nicking generates a new site for the DNA polymerase, and the next elongation cycle results in the displacement of the DNAzyme sequence. Upon release, the DNAzyme is activated in the presence of hemin by intercalating the hemin into the G-quartet structure. This series of chemical reactions, leading to the ultimate synthesis and activation of the DNAzyme, is observed by the oxidation of $ABTS^{2-}$ by H_2O_2 or the generation of chemiluminescence in the presence of luminol/H_2O_2 coupled to the DNAzyme mimicking peroxidase activity (Fig. 3(c)(i)). Since laboratory results cannot always be translated into actual biological applications, Weizmann *et al.* coupled this DNA machine to the detection of viral DNA to further validate the usability of their prototype designs. Here they used an intermediate nucleic acid hairpin structure that is opened by the analyte DNA (M13 phage DNA). The hairpin structure consisted of parts of the primer, a portion of the nicking sequence, and the recognition sequence for hybridization with the M13 phage DNA. The hybridization of the hairpin intermediate DNA with the M13 phage DNA causes the opening of the hairpin and the binding of the primer to the machine template (Fig. 3(c)(ii)). In the presence of dNTPs, DNA polymerase, and the nicking enzymes, the DNA machine is activated, leading to the synthesis of the DNAzyme and the subsequent displacement and detection by its peroxidase activity. Control experiment with calf thymus DNA, or the elimination of the intermediate hairpin structure, only yielded background signal, implying that these are important for the machine to operate.

4. Aptazyme-based designs

Intensive research into oligonucleotide biochemistry has unravelled the potential of nucleotides to store and transfer genetic information for protein expression and to act as recognition elements (aptamers) and catalytic molecules (DNAzymes and RNAzymes). These functional oligonucleotides can be rationally engineered to produce aptazymes with the capability of specific binding to analytes, and, as a result of their catalytic and/or binding ability, detectable signals can be generated.[81,82] Such systems are now being exploited to produce aptazyme biosensors.[83,84] In aptazyme designs for biosensors, the fundamental strategy calls for the binding of an analyte to the aptazyme complex which leads to the activation of the enzyme activity. This then causes the development of detectable and measurable signals, most frequently by fluorescence, colorimetry, and chemiluminescence.

Many design strategies have emerged. Lu *et al.*[85] demonstrated a selected catalytic DNA aptamer that binds metal ions with strong affinity and high selectivity. The ability to detect and quantify metal ions is important in many applications. These include environmental monitoring, waste management, household monitoring, developmental biology, and clinical toxicology. Proper and reliable assessment and management is critical as some of these metal ions have serious health and environmental complications, even in minute quantities. Traditionally, the methods for identification and quantification of metal ions have included atomic absorption spectrometry,[86,87] inductively coupled plasma spectrometry,[88,89] anodic stripping voltammetry,[90] X-ray fluorescence,[91] and microprobes.[92,93] Although these methods have been routinely used, their considerable drawbacks have limited their use. For instance, because of instrument sophistication, they require skilled labor for operation, and they also require sample pretreatment. In addition, while most of these techniques can detect total metal amount, only a specific oxidation state may be of interest to protecting human life and the environment. Consequently, there is the need for a rapid, risk-free, inexpensive and simple, but selective and sensitive, method that is capable of remote sensing and that permits real-time detection of bio-available metal ions in their different oxidative states.

Several innovative methods were previously designed to address these concerns. These were sensors based on different materials, such as organic,[94–96] organic polymers,[97] proteins,[98–101] or cells.[102,103] Remarkable progress has been made in developing

fluorosensors for metal ions such as Ca^{2+}, Zn^{2+}, and Pb^{2+}. However, designing and synthesizing sensitive and selective ion sensors is still a challenge, especially in the area of selectivity. For instance, the widely used Ca^{2+} sensor fura-2 binds Zn^{2+} and Cd^{2+} 10^2–10^5-fold more tightly than Ca^{2+}.[85] Consequently, design and synthesis of sensors capable of specific and strong binding of a metal of choice, but which do not bind to other competing metal ions, is important. The development of such a sensor will depend on the availability of probes that can specifically recognize a small amount of metal ion of choice in a soup of many highly concentrated competing ions. It is therefore not surprising that considerable efforts have been geared toward the development of reliable alternatives. Recently, an encouraging number of aptamer biosensors for detection of metal ions have emerged, and most of them are sensitive and selective for the metal ion of interest. [22,104–107]

In the work of Lu *et al.*,[85] fluorescent sensors were developed for the detection and quantification of Pb^{2+} in lake water. The design demonstrated a catalytic DNA fluorescent sensor that transferred metal ion-dependent conformational change or cleavage into an observable signal. This provided a significant signal amplification event at a low analyte level, and the fluorescent time scale was fast enough for real-time monitoring of concentration fluctuations. Being field-ready and compatible with fiber optic technology, the sensor was thought to be well suited for remote sensing technology. In this design, they used trans-cleavage catalytic DNA which consisted of the catalytic and substrate parts. The 5′ end of the substrate was tagged with TAMRA, a fluorophore, and the 3′ end of the catalytic DNA with dabcyl, a quencher. In the absence of the catalytic DNA, TAMRA fluoresces. However, upon hybridization with enzyme complex by Watson–Crick base pairing, dabcyl quenches the fluorescence of TAMRA due to the close proximity. On exposure of the system to Pb^{2+}, the substrate is cleaved, the short sequence containing TAMRA is released, and fluorescence emission of TAMRA is increased to about 400% (Fig. 4(a)). The sensor showed quantifiable detection range of 10 nM to 4 μM and could be used to detect Pb^{2+} ions in a real environmental sample taken from Lake Michigan which contained different concentrations of added Pb^{2+}. The selectivity was also demonstrated in samples where other divalent ions did not reduce sensitivity.

In terms of selective target recognition, the versatility of aptamers affords the flexibility required for various types of aptasensor designs and assembly. At the same time, the components can be changed to suit a specific need or target with the same detection strategy. In one such approach, Shlyahovsky and co-workers[108] detected low molecular weight analyte (AMP) or protein (lysozyme) by a DNAzyme–aptamer analyte hybrid complex. The DNAzyme provided the colorimetric or chemiluminescence signal. In this system, the biosensor consisted of three basic components, including, first, a portion of nucleic acid that contained the AMP or lysozyme aptamer sequence. Next, this portion was linked to a DNA base sequence which, in the presence of hemin, forms the horseradish peroxidase (HRPO)-mimicking DNAzyme. Finally, the ends of these two components could form Watson–Crick base pairing with a blocking nucleic acid sequence which acted as the control sequence. Addition of AMP or lysozyme caused the aptamer–blocker portion to be destabilized by the formation of aptamer–substrate complex. This also destabilizes the aptazyme–blocker base pairing and, in the presence of hemin, leads to the formation of the mimicking HRPO, bio-catalyzing the oxidation of $ABTS^{2+}$, or the generation of chemiluminescence in the presence of H_2O_2/luminol, depending on which detection system is employed (Fig. 4(b)). This system showed AMP- or lysozyme-dependent signal amplification with a detection limit of 4 μM for the AMP sensor, which was an improvement over known AMP sensors[109] and 100-fold improvement over a reported lysozyme electrochemical aptasensor.[110] What makes this system unique is the ability for a single design to be engineered for small molecule and protein detection. The modeling of a biomolecular assembly coupled to a biochemical reaction cascade would have a general

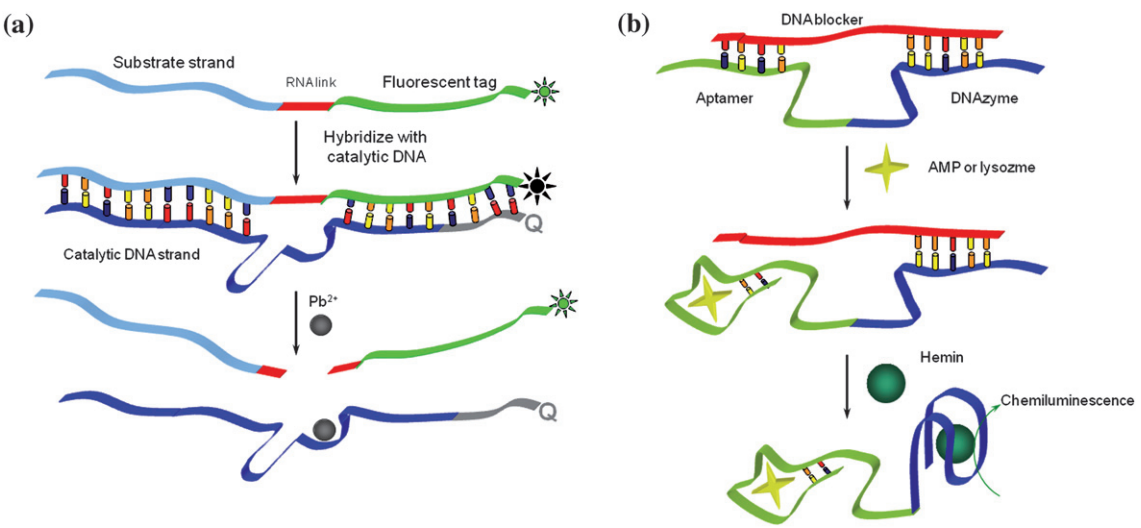

Fig. 4 (a) Pb^{2+}-facilitated catalytic DNAzyme for the detection and quantification of Pb^{2+}. The binding of Pb^{2+} activates the DNAzyme resulting in the hydrolysis of the RNA link (red), causing the release of the fluorescence tag strand from the quencher. (b) Detection of analyte (AMP or lysozyme) coupled to the generation of DNAyzme and its enzymatic mimicking of peroxidase activity by generating a chemiluminescence signal in the presence of hemin.

application for any analyte. This has been observed in a similar approach using glucose oxidase/DNAzyme complex with similar detection regimes.[111] In the same report, they developed micro-peroxidase-11/anti-thrombin aptamer conjugate for the detection of thrombin.

5. Concluding remarks

Aptamers have been shown to be versatile and effective as molecular probes for specific molecular recognition which is the critical issue in biosensors and bio-analytical chemistry. Many existing schemes have proven aptamers' easy incorporation with biosensors and bioassays. When integrated with current nanoparticle technology and other sophisticated bio-analytical devices, aptamers can be used to detect target molecules or even diseased cells in biological samples. Future work will involve integrating aptamers into analytical devices and biosensors in order to create inexpensive early detection schemes. Aptamers are generated at a much faster pace today than before with cell-SELEX[41–45] fully developed for target cell selection and the ability to generate many aptamers for many targets at one time. Exciting new research will focus on the study of interactions between aptamers and the cells they bind and the elucidation of the molecules these aptamers bind to. This will form a solid molecular foundation and will contribute to our understanding of specific types of cells and the associated diseases. Understanding aptamer–target binding interactions will also lead to an improved utilization of aptamers for biosensors and biochemical analysis for detection, diagnosis, treatment and basic studies of diseases.

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