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Assays for aptamer-based platforms

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ABSTRACT

Aptamers are single stranded DNA or RNA oligonucleotides that have high affinity and specificity towards a wide range of target molecules. Aptamers have low molecular weight, amenable to chemical modifications and exhibit stability undeterred by repetitive denaturation and renaturation. Owing to these indispensable advantages, aptamers have been implemented as molecular recognition element as alternative to antibodies in various assays for diagnostics. By amalgamating with a number of methods that can provide information on the aptamer–target complex formation, aptamers have become the elemental tool for numerous biosensor developments. In this review, administration of aptamers in applications involving assays of fluorescence, electrochemistry, nano-label and nano-constructs are discussed. Although detection strategies are different for various aptamer-based assays, the core of the design strategies is similar towards reporting the presence of specific target binding to the corresponding aptamers. It is prognosticated that aptamers will find even broader applications with the development of new methods of transducing aptamer target binding.

Contents

1. Introduction

Since the discovery of ribozyme, many cellular functions associated with RNA/DNA have been revealed, defining the essential roles of these molecules in replication, translational regulation, viral propagation and other functions. On the other hand, the discovery of nucleic acid-binding protein that plays important roles in cellular processes (such as translation and mRNA processing) infers the ability of these nucleic acids to bind virtually any target molecules to perform specific functions and roles (Jain and Belasco, 1996). These wide ranges of non-protein-coding RNA (npcRNAs) and DNA/RNA–protein interaction inspired the effort to develop artificial functional nucleic acids that can bind to target molecules. These artificial molecules known as aptamers, were discovered by three independent groups of scientists led by Joyce, Szostak, and Gold in the early 90s.

Aptamers are single-stranded RNA or DNA oligonucleotides which rely on hydrogen bonding, electrostatic and hydrophobic interactions rather than Watson–Crick base pairing for high affinity, specific recognition to their target. These molecules are extricated from a large random pool of nucleic acid through the process called Systematic Evolution of Ligands by Exponential Enrichment (SELEX) which mimics the natural selection (Ellington and Szostak,

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1990; Song et al., 2008). SELEX comprises three critical steps: (1) the incubation of the nucleic acid pool and the target, (2) separation of the bound from unbound molecules and (3) the amplification of the bound molecules. Successful generation of aptamers against target molecules is largely influenced by the type of separation or partitioning in the SELEX process (Gopinath, 2007). To date, aptamers are selected against a wide variety of target molecules such as dyes (Ellington and Szostak, 1990), ATP (Lato et al., 2002), metal ions (Kawakamia et al., 2000), proteins (Calik et al., 2009; Gopinath et al., 2006a,c; Niazi et al., 2008; Schurer et al., 2001; Sekiya et al., 2006; Stevenson et al., 2008; Wochner et al., 2008; Yoshida et al., 2008), toxin (Tang et al., 2007) and against whole organism (Boiziau et al., 1999; Cheng et al., 2008; Gopinath et al., 2006b).

Although antibodies have been used in several assays, these protein molecules are temperature sensitive and exhibit dissociation constant that is strongly influenced by the physiological condition. The conjugation of these antibodies to the surface amines are non-directed, which may result in the loss of function or affinity (Jayasena, 1999; Mairal et al., 2008). In contrast, aptamers are very stable, despite being subjected to the repetitive denaturation and renaturation along the process of SELEX. Stability of the aptamers can be further enhanced by adding base-pairs to the stem structures which attributes to the simple, stable secondary structural formation (Kirby et al., 2004). Moreover, the small molecular weight of aptamer (10–15 kDa) has the advantages of fast tissue, tumor penetration and rapid blood clearance (White et al., 2000). On the other hand, labeling of the aptamers with reporter groups did not alter the conformation and binding affinity. Aptamers can be generated to specific regions of targets (Shangguan et al., 2006). Therefore, an aptamer-based assay can be highly reliable and specific (Willner and Zayats, 2007).

It is a prerequisite to detect and measure the level of specific target in a sample, without compromising the convenience and the precision. The method used to analyze this target analyte should also be sensitive, cheap and robust. Undoubtedly, aptamers are able to fulfill these requirements of an assay, with leveraged efficiency. However, aptamers do not have the intrinsic property to provide information regarding aptamer–target complex. Therefore, in order to harness aptamers as affinity probes or tools to analyze the behavior of any molecule, it is of paramount importance to come up with a variety of methods of linking the molecular recognition capability of aptamers to signal generation process (Cho et al., 2009; Famulok and Mayer, 2011). These signaling can be achieved with the aid of labels such as fluorophores, luminophores, enzymes and nanoparticles to develop heterogeneous assays, biosensors and arrays (Sassolas et al., 2011). The variability of aptamer-based assays to transduce bio-recognition events is due to different recognition modes of aptamer–target complex (Hermann and Patel, 2000). The choice of aptamer-based assays is largely influenced by the downstream applications. This review presents the biosensing applications of aptamers utilizing different nano-labels and nano-constructs, suitable for different assay formats including fluorescence and electrochemistry.

2. Fluorescence

Fluorescence is one of the most prevalent signal transduction of aptamer–target complex in aptamer-based homogeneous and heterogeneous assays. This is due to its nondestructive, highly sensitive characteristic and the ease of modifying the aptamer with fluorescence tags (Iliuk et al., 2011; Mok and Li, 2008; Paige et al., 2011; Wang et al., 2011a,b,c,d). Transduction of aptamer–target binding event facilitated by fluorophores such as fluorescein isothiocyanate (FITC), rhodamine, coumarin, and cyanine enables real-time detection. Moreover, incorporation of these fluorophores into aptamers eliminates the need to separate target–probe complexes from unbound probes (Table 1) (Huang et al., 2010; Valeur, 2001). One of the flurophores was adapted in an assay employing anti-adenosine aptamer, which can identify nuance difference between adenosine 5′-monophosphate (AMP) and adenosine owing to the high specificity towards adenosine. The structure-switching property (the ability of aptamer to form complex structure upon target binding) of anti-adenosine aptamer imparts large change in fluorescence upon target binding. This assay enabled the monitoring of the enzymatic reaction of AMP to adenosine conversion by alkaline phosphatase (Nutiu et al., 2004).

Jhaveri et al. (2000) reported the development of aptamer based fluorescence assay using the aptamer selected from original RNA pool that consists of a few fluoresceinated uridine residues. They integrated the fluorophore groups directly into the starting nucleic acid pool of SELEX. One aptamer family, containing single fluoresceinated uridine residue, was able to detect ATP as low as 25 μM. This shows that aptamer conjugated with single flurophore uridine residue can convert molecular recognition event of aptamer-ATP complex into fluorescence signal. Fluorophore conjugated aptamers can also be immobilized on sensor surface without compromising their ability in binding target molecules. Moreover, the availability of various fluorophores with different excitation and emission wavelengths enables multiple assays of several targets concurrently. In a specific study, aptamers conjugated fluorophores were immobilized onto the surface of a chip (streptavidin derivatized glass substrate) to simultaneously analyze several cancer related proteins (Table 1). This assay was a plausible means of efficiently detecting multiple targets over a range of concentrations (McCauley et al., 2003).

Aptamers conjugated with fluorophores (immobilized on various platforms) are liable to reutilization. This is due to chemical stability of nucleic acids in maintaining three-dimensional structures towards binding the aptatopes on the surface of the target. Platforms such as fiber optics and ‘Electronic taste chip’ or ‘Electronic tongue’ were habituated for detection of thrombin and ricin toxin with corresponding limit of detection of 1 nM and 1 pM, respectively (Lee and Walt, 2000; Kirby et al., 2004). Fluorophore-labeled aptamer was used in vivo as a probe in real-time protein imaging in live cells, where spatiotemporal process of angiogenin interaction was visualized directly under confocal laser scanning microscope (Li et al., 2008a). Recently, an aptamer has been generated that bind fluorophores to form RNA–fluorophore complex which mimics the fluorophore in Green fluorescent protein. This complex termed Spinach is very resistant to photobleaching (Paige et al., 2011).

Fluorophores have limited signaling ranges, prevent lower limit of detection and often experience self-quenching (Anderson et al., 2008). Alternatively, Quantum dots (QD) are inorganic fluorescent materials that are more photostable than organic fluorophores (Bogomolova and Aldissi, 2011; Li et al., 2011). They are characterized by their long fluorescent lifetime, resulting in narrow emission spectra with broad excitation range (Algar et al., 2010; Michalet et al., 2005). In addition, QD exhibit high and near uniform fluorescence quantum yield, protected from environmental quenching. Thus, accurate measurement by fluorescent microscopes, even up to a few hours is possible (Nirmal and Brus, 1999). Ikanovic et al. (2007) developed a method for the detection of *Bacillus thuringiensis* (BT) spores based on a BT spore-specific QD conjugated aptamer. Up to 1000 colony-forming units can be detected. Choi et al. (2006) and Chu et al. (2006) have developed QD conjugated aptamers for the specific detection of thrombin and prostate specific membrane antigen (PSMA), respectively.

On the other hand, excimer is a heterodimeric molecule formed when one of the dimer components is in excitation state. Aptamer

Table 1
Summary of aptamer based assays and the applications.

Target and aptamer	Immobilization surface	Functional group attached to aptamer	Limit of detection	Application	References
Fluorescence					
ATP(RNA)	Solution	Fluorescein	25 μM	ATP detection	Jhaveri et al. (2000)
Thrombin (DNA)	Imaging fiber	Fluorescein phosphoramidite	1 nM	Thrombin detection	Lee and Walt (2000)
Cocaine	Solution	6-Carboxyfluorescein (6-FAM) 4-(Dimethylaminoazo)benzene-4-carboxylic acid (DABCYL quencher)	–	Cocaine detection in blood or urine	Stojanovic et al. (2000)
Tat protein of HIV-1 (RNA)	Solution	Fluorescein DABCYL	–	Binding of aptamer to Tat protein can potentially inhibit the transcription of viral messenger RNA	Yamamoto and Kumar (2005)
Thrombin (DNA)	Solution	Fluorescein DABCYL	–	Thrombin detection	Hamaguchi et al. (2001)
Plasma endothelial growth factor (PDGF) (DNA)	Solution	Fluorescein DABCYL	0.11 nM	PDGF detection, malignant tumor biomarker	Fang et al. (2003)
Inosine monophosphate dehydrogenase II (DNA) Angiogenic factor (DNA) Vascular endothelial growth factor (VEGF) (DNA) Basic fibroblast growth factor (bFGF) (DNA)	Streptavidin derivatized glass substrate	3′-FAM	–	Multiplex detection of cancer biomarkers	McCauley et al. (2003)
ATP (DNA)	Solution	Bis-pyrene labeled fluorophore	–	Detection of ATP	Yamana et al. (2003)
Ricin (DNA)	Electronic tongue/electronic taste chip	–	1 pmol	Sensitive detection of ricin, a terrorism threat tool	Kirby et al. (2004)
Adenosine (DNA)	Solution	–	–	Monitoring enzymatic reaction of AMP to adenosine conversion by alkaline phosphatase	Nutiu et al. (2004)
PDGF (DNA)	Solution	Fluorophore Quencher	10 ng	Sensitive detection of PDGF	Vicens et al. (2005)
Thrombin	Solution	Lead (II) sulfide (PbS) coated QD	1 nM	Quantification and detection of thrombin	Choi et al. (2006)
Prostate specific membrane antigen (PSMA)	Solution	Streptavidin conjugated CdSe and CdTe nanocrystals Biotin	–	Recognize and specifically label prostrate specific membrane antigen (PSMA)	Chu et al. (2006)
Bacillus thuringiensis (BT) spores	Solution	Quantum dot	1000 colony forming units	Sensitive detection of *Bacillus thuringiensis* (BT) spores	Ikanovic et al. (2007)
Angiogenin (DNA)	In vivo	Cy5	–	Visualization of spatiotemporal process of angiogenin interaction	Li et al. (2008a)
	Solution	6-FAM (reporter group) 6-carboxytetramethylrhodamine (TMR) (quencher group)	–	To quantify and monitor angiogenin, biomarker of tumor	Li et al. (2008b)
Sec7 domain of the cytohesin family of small guanine nucleotide exchange factors	Streptavidin coated donor beads Anti-fluorescein isothiocyanate-IgG coated acceptor beads	Photo sensitizer phthalocyanine Fluorescein	50 nM	Identification of small drug-like molecules that bind to the target protein Sec7 domain of the cytohesin family	Niebel et al. (2010)

Table 1 (*Continued*)

Target and aptamer	Immobilization surface	Functional group attached to aptamer	Limit of detection	Application	References
Cocaine (DNA)	Solution	Pyrene	1 μM	Sensitive detection of cocaine	Wu et al. (2010)
Aptamer linked immunosorbent assay (ALISA)					
Thrombin (DNA)	Microtiter plate	Biotin Horseradish Peroxidase (HRP)	1.8 nM 1.0 nM	Thrombin detection	Baldrich et al. (2004)
Thrombin (DNA)	Microtiter plate, lipid bilayer of liposomes	Cholesteryl–TEG Sulforhodamine B dye	6.4 fmol	Thrombin detection	Edwards et al. (2010)
Thrombin (DNA)	Magnetic beads coupled to microfluidic platform/chip	Quantum dot	–	Thrombin detection	Tennico et al. (2010)
Electrochemistry					
Thrombin (DNA)	Microfabricated thin film gold electrode	Thiol	0.1 nM	Thrombin detection	Cai et al. (2005)
Thrombin (DNA)	Gold electrode	Methylene blue	6.4 nM	Thrombin detection	Xiao et al. (2005)
Platelet-derived growth factor (PDGF)	Solution	Methylene blue	1 nM	Direct detection of PDGF in blood serum	Lai et al. (2007)
Thrombin (DNA)	Gold electrode	–	–	Thrombin detection	Lu et al. (2008)
Thrombin (DNA)	Gold substrate	Thiol CdS (Cadmium sulfide) nanoparticle	28 fmol	Detection of trace amounts of thrombin in blood	Numnuam et al. (2008)
C Reactive protein	Magnetic beads	–	0.02 mg/L	Detection of biomarker for inflammatory states in acute phase inflammation	Centi et al. (2010)
Nanomaterials and nano-constructs					
Thrombin (DNA)	Maleimide-functionalized siloxane monolayer	Gold nanoparticles	20 nM	Thrombin detection	Pavlov et al. (2004)
Thrombin	Single Walled Carbon Nanotubes Field Effect Transistor (SWNT-FET)	NH_2 modified 3′-end	10 nM	Sensitive detection of thrombin	So et al. (2005)
Thrombin (DNA) VEGF (RNA)	Carboxylic-acid-functionalized polypyrrole (CPPy) nanotubes	Covalent linkages	– 400 fM	Thrombin detection VEGF detection	Yoon et al. (2008)Kwon et al. (2010)
Hg^{2+}	Gold nanoparticles	–	0.6 nM	Detection of Hg^{2+}, a highly toxic ion contaminating food and water	Li et al. (2009)
Immunoglobulin E (IgE)	Carbon Nanotubes Field Effect Transistor Channels (CNTFET)	–	–	Sensitive detection of IgE	Maehashi et al. (2009)
IgG1 (RNA)	Gold-capped silica nanoparticles	2′-Fluoro Thiol	0.1 ng/mL	Sensitive detection of fibrinogen	Hiep et al. (2010)
Vasopressin (DNA)	Gold coated SERS active substrate	Thiol	5.2 μU/mL	Detection of vasopressin as biomarker for hemorrhagic shock	Huh and Erickson (2010)
ATP (DNA) Cocaine (DNA)	Graphene	Fluorophore	– 100 nM	ATP detection Cocaine detection	Lu et al. (2010)
Thrombin (DNA)	Nanogap capacitor	Thiol	–	Thrombin detection	Mannoor et al. (2010)
Nucleic acid amplification assay					
PSMA (RNA)	–	Extended sequences at 5′ and 3′ end 2′-Fluoropyrimidines	10–10,000 cells	Distinguish cells that differentially express PSMA, in the presence of non cognate cells	Pai and Ellington (2009)
Cytochrome-c (DNA)	Magnetic bead	Barcode markers	–	Detection of cytochrome-c (cyto-c), biomarker of apoptosis	Lau et al. (2010)

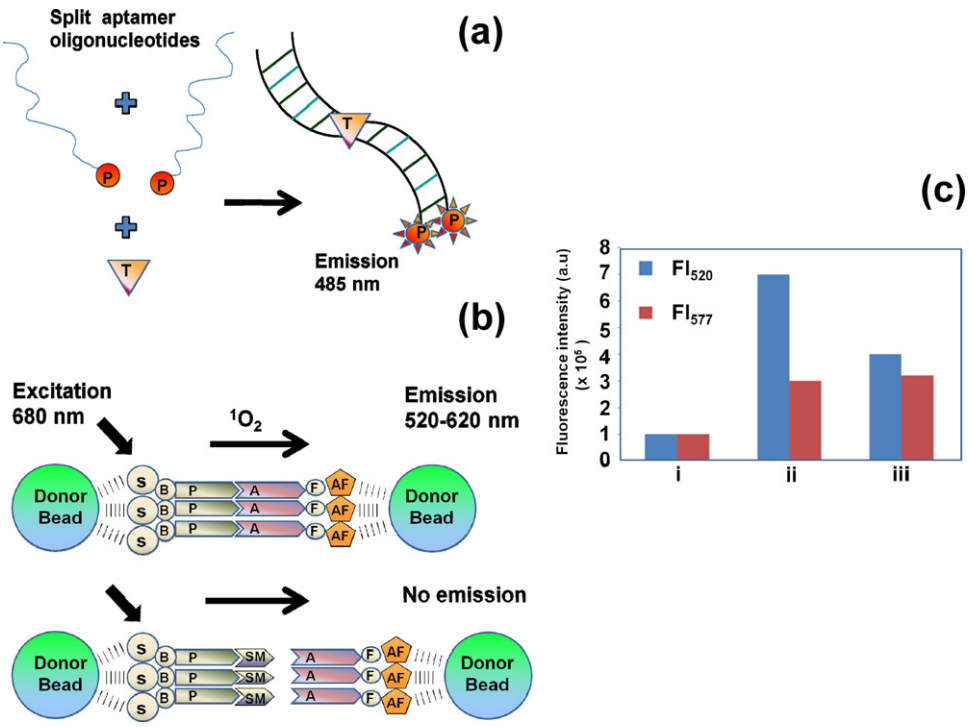

Fig. 1. (a) A schematic diagramme of excimer-based assay. This light switching excimer biosensor created by splitting ss aptamer sequence into 2 pieces labeled with 2 pyrenes at the extremities. Binding to the target molecule, these two fragments assemble to form aptamer–target complex, bringing the two pyrene molecules into close proximity to form excimer, switching the fluorescence emission from the wavelength of 400 nm to 485 nm. P – Pyrene Tagged Aptamer, T – Target (Wu et al., 2010). (b) Cartoon illustration of ADLOC (Aptamer Displacement detected by Luminescent Oxygen Channeling). Excitation of the donor bead containing photosensitive phthalocyanin at 680 nm, converts triplet oxygen to singlet oxygen, conveying the energy to the donor bead containing thioxene derivatives, emitting signal. Interference of small molecule interferes the complex of aptamer–target, resulting in the disappearance of the signal. S – Streptavidin, B – Biotin, P – Target protein, A – Aptamer, F – Fluorescein, AF – anti-fluorescein IgG, SM – Small Molecule (Niebel et al., 2010). (c) Fluorescence spectra of MBA-based angiogenin interaction. (i) 0.01 M Tris–Cl, pH 7.4 + 0.01 M NaCl (buffer), (ii) buffer + 0.2 nM aptamer probe and (iii) buffer + 0.2 nM aptamer probe + 0.4 nM angiogenin (Li et al., 2008b).

was split into two pieces labeled with 2 pyrenes at the extremities while the binding affinity to the target molecule is maintained. Upon binding to the target molecule, these two fragments known as light switching probes assemble to form aptamer–target complex. These two pyrene molecules are brought into close proximity to form excimer, resulting in the emission of fluorescence (Fig. 1a). These light-switching aptamer probes were used in cocaine and ATP detection, reducing background signal often conferred by single fluorophores. The detection limit was as low as 1 μM for cocaine (Wu et al., 2010; Yamana et al., 2003).

Based on another phenomenon known as Luminescent Oxygen Channeling (LOC) (Ullman et al., 1994), Aptamer Displacement detected by Luminescent Oxygen Channeling (ADLOC) was devised. ADLOC rests upon proximity-dependent energy transfer between donor and acceptor beads, containing photosensitizer phthalocyanine (which is an intensely blue-green coloured macrocyclic compound) and thioxene derivatives, respectively. Upon excitation at 680 nm, ambient triplet oxygen is converted to singlet oxygen by phthalocyanine and diffuses to the acceptor bead. This transfers energy to the thioxene derivatives in the acceptor bead, leading to signal detectable between 520 and 620 nm. Niebel et al. (2010) employed RNA aptamer M69 that binds to the Sec7 domain of the cytohesin family of small guanine nucleotide exchange factors. They created an assay based on ADLOC which detects the target protein Sec7 (up to 50 nM) using as low as 5 nM of aptamer (Fig. 1b). The availability of excimer and phthalocyanine provides alternatives to organic fluorophores, which are associated with non-uniform emission of the fluorescence from the fluorophores.

The utilization of fluorophores enables the design of a modified aptamer known as Molecular Beacon Aptamer (MBA) (Bruno et al., 2011; Wang et al., 2011a,b,c,d). MBA increases the sensitivity of an assay by reducing false signal in biological samples associated with the usage of single fluorophores. Binding of MBA to the target activates conformational changes of aptamer, reducing the distance between quencher and fluorophore. This quenches the fluorescence emission from the fluorophore (Wang et al., 2005). MBA was developed against angiogenin, a protein associated with growth and metastasis of numerous tumors. The quantification of angiogenin was carried out by monitoring fluorescence intensity of the reporter group (Li et al., 2008b). In this experiment, fluorescence intensity of acceptor fluorophore increased (FI_{577}) while that of donor fluorophore decreased (FI_{520}) upon the addition of angiogenin. Ratiometric nature of MBA has higher fluorescence sensitivity and signal stability compared to the utilization of single fluorophore (Fig. 1c). Equivalently, MBA were also developed against thrombin (Hamaguchi et al., 2001) and cocaine (Stojanovic et al., 2000). A similar approach was adopted to develop MBA labeled with fluorescein at 5′-end and quencher at 3′-end against platelet derived growth factor (PDGF), one of the oncoproteins over-expressed in malignant tumors. The detection limit was up to 0.11 nM (Fang et al., 2003).

In contrast to the approach embraced by Fang and colleagues, which involves the usage of single aptamer molecule, Vicens et al. (2005) have demonstrated aptamer-based assay using two different DNA aptamers, binding to two different aptatopes of platelet-derived growth factor (PDGF). These aptamers were labeled with fluorophore and quencher group. They are able to detect up to 10 ng of PDGF from biological specimen. Yamamoto and Kumar (2005) have developed a different approach known as Analyte Dependent OligoNucleotide Modulation based Assay (ADONMA) using anti-HIV-1 Tat RNA aptamer, which was generated against this aptamer. The Tat protein specifically inhibits

transcription of viral messenger RNA. In this investigation, rather than using 2 different aptamers (as exhibited by Vicens and colleagues) the anti-HIV-1 Tat RNA aptamer was split into 2 strands and attached with fluorophore and quencher at the 5′-end and 3′-end, respectively. One of these strands is biotinylated to enable immobilization on streptavidin coated microtiter plate. In the presence of target protein and the secondary strand, these two oligos will undergo conformational changes that move the fluorophore away from quencher, enhancing the fluorescence signal. In short, MBA enables the detection of target molecules in real-time without the need to separate target–probe complexes from unbound probes. Moreover, MBA also alleviates the problems associated with non-specific absorption of target onto solid substrates, which can otherwise lead to false positive or high background signal (Table 1).

3. Aptamer linked immunosorbent assay (ALISA)

Several protein targets have more than one binding sites, which enable binding to more than one recognition molecules, forming sandwich-like complex. These have been widely used in the design of ELISA. Similarly, aptamers are harnessed in the assay known as ALISA, analogous to the same format ELISA (Vivekananda and Kiel, 2006; Zhang et al., 2011). Sandwich ALISA assay, which relies on two different aptamers generated against one target, has been devised. One aptamer is attached to the surface (chip, microtiter plate, etc.) while the second aptamer is labeled with the reporter, which will give signal upon binding to the target. Edwards et al. (2010) have developed sandwich ALISA assay based on two different anti-thrombin aptamers on microtiter plate. One of the aptamers serves as decaptamer that was immobilized on the surface of microtiter plate while another DNA aptamer acts as detectamer that functionalize the lipid bilayer of liposomes. The aptamer serving as the detectamer was Cholesteryl–TEG-modified. The detectamer was implanted into the liposomal lipid bilayer, while the interior cavity of the liposomes encapsulated fluorescent sulforhodamine B dye. The instantaneous signaling through surfactant induces release of encapsulate, obviating the need for time dependant substrate conversion, as in ELISA. This assay is capable of tracking up to 6.4 fmol of thrombin. In another sandwich ALISA assay, two different aptamers against thrombin were functionalized, one being immobilized on magnetic beads coupled to microfluidic platform/chips, while another aptamer was conjugated with quantum dots (Tennico et al., 2010). Aptamer conjugated to magnetic beads captures the target protein thrombin while the addition of the secondary aptamer conjugated with QD crystal enables thrombin detection via fluorescence microscopy. This approach permits rapid and specific thrombin detection with reduced costs and material, offering brighter, prolonged fluorescence lifetimes.

Likewise, Baldrich et al. (2004) have performed direct Enzyme Linked Aptamer Assay (ELAA) and mixed ELISA–ELAA employing anti-thrombin DNA aptamer. In the ELAA method, biotinylated anti-thrombin DNA aptamer was immobilized on the surface of microtiter plate followed by incubation with Horseradish Peroxidase (HRP) labeled thrombin. On the other hand, in ELISA–ELAA format, the HRP labeled aptamer and thrombin of different concentrations were mixed and subsequently captured by anti-thrombin antibody on the surface of the microtiter plate. ELAA demonstrated a detection limit of 1.8 nM while the combination of ELISA–ELAA format showed a detection limit of 1 nM, implying the increased efficiency of this particular assay owing to its counterpart ELISA. ALISA assay is very reliable and cost effective due to the reversible denaturation property of the surface conjugated aptamer. These molecules can be reused several times without sensitivity reduction, with very minimal non-specific absorption onto the platform surfaces (Table 1). ALISA can be an alternative to ELISA owing to the unique properties of the aptamers that exceed that of antibody.

4. Electrochemistry

Electrochemical biosensor (electrochemistry based biosensor) couples biological recognition element to an electrode transducer. Nucleic acids are electrochemically active due to the presence of electrochemically oxidizable and reducible nucleobases. Aptamer as nucleic acid can definitely be an efficient biological recognition element to report the event of aptamer–target formation, on account of three-dimensional structure formation following binding to the target molecule. Subsequent to this, electrical signal produced is detectable by voltameter, potentiometry measurement and electrical impedance spectroscopy (Cho et al., 2009; Song et al., 2008) (Table 1). Compared to fluorescence, electrochemistry based aptamer assays are more economic, incorporates much simpler instruments, less viable to contaminants and non-specific bindings.

As the proof-of-concept, RNA aptamer against C-Reactive protein was generated and used for developing electrochemical sandwich assay. This RNA aptamer was conjugated with biotin and anchored to the streptavidin coated magnetic beads. Upon the addition of streptavidin–alkaline phosphatase, followed by the substrate α-naphthyl-phosphate, production of α-naphthol was initiated. This generated an electrochemical signal detectable by using Differential Pulse Voltammetry. The detection limit was as low as 0.02 mg/L (Centi et al., 2010). Lai et al. (2007) reported an electrochemical aptamer-based assay for the direct detection of PDGF in blood serum by employing methylene blue (MB)-modified PDGF binding aptamer. MB is an aromatic cationic dye that has electrochemical active properties, binding nucleic acid via interaction with two guanine residues in the stem structure. Upon binding to the target protein, this aptamer forms a stable G-quadruplex structure, driving the MB label to be in vicinity of the electrode, resulting in higher electron-transfer efficiency, detectable by voltameter. This assay which is termed signal-on configuration can detect up to 1 nM of PDGF directly in unprocessed blood serum. Xiao and co-workers demonstrated another scheme termed signal-off constructs using MB labeled anti-thrombin DNA aptamer (Xiao et al., 2005). In the signal-off construct, methylene blue was covalently attached to DNA aptamer and conjugated to the surface of gold electrode. In the absence of target, the aptamer remains unfolded, causing the MB label to collide with the electrode, transferring electron. In the presence of target, the aptamer undergoes conformational change that increases the distance between the MB label and electrode. Detection limit achieved was up to 6.4 nM. In contrast to the approach embraced by Lai et al. (2007) and Xiao et al. (2005), Lu et al. (2008) described an electrochemical aptasensor for thrombin detection independent of target binding-induced conformational change of the aptamer. In this study, cDNA probes were synthesized in such a way to have complementary bases at both 5′-end and 3′-end, whereby both thiol group and redox moiety were conjugated, respectively. This labeled cDNA probe were then confined onto gold electrode surface and hybridized with anti-thrombin aptamer to form Watson–Crick helix. In the presence of thrombin, label-free aptamer dissociates and binds to the target, leaving the cDNA probe on the electrode. The cDNA probe which forms a stable hairpin structure facilitates electron transfer of the redox moiety as the distance between redox moiety and electrode reduces.

In potentiometry assay, the target protein captured by thiolated aptamer (attached on the surface of gold substrate) interacts with secondary aptamer labeled with Cadmium sulfide (CdS)

nanoparticle. CdS, which has semiconductor characteristics result in more sensitive detection of target molecules by aptamer tagged CdS (large signal amplification upon target binding). When CdS dissolves in H_2O_2, a dilute electrolyte background suitable for the potentiometric detection of the released Cd^{2+} with a solid contact selective microelectrode was produced. Using two thrombin binding DNA aptamers designed as mentioned above, this assay represents the first aptamer-based potentiometric sandwich assay detecting up to 28 fmol of thrombin (Numnuam et al., 2008). The sensitivity of this assay to detect up to femtomolar level is useful to trace the amounts of thrombin in blood.

If label-free electrochemical assay is intended, electrical impedance can also be employed for aptamer-based assay. Electrical impedance is the measurement of the electricity flow through a given material, which depends on the degree to which an electric circuit resists electric-current flow when voltage is impressed across its terminals. In principle, negative and positive readout-signal following aptamer–target interaction can indeed be beneficial to monitor binding as the interaction of aptamer–target will alter the electrical impedance (McGuinness, 2007). This measurement is very sensitive, as small perturbation caused by target binding will be accurately measured due to linear characteristics of current–voltage relationship. Moreover, compared to voltammetry measurement, electrical impedance requires only small voltage application. In an assay utilizing anti-thrombin thiolated DNA aptamer immobilized on microfabricated thin film gold electrode, formation of thrombin–aptamer complex was monitored by tracking changes in electrochemical impedance spectroscopy which differs from that of the free aptamer. This is effective to track up to 0.1 nM of thrombin (Cai et al., 2005). Electrical impedance based sensor permits real-time monitoring of the sensor signal as well as analyzing kinetic aspects of the ligand–analyte interaction (Schlecht et al., 2006). In fact, aptamer based electrical impedance produces low background noise, decreases non-specific absorption and emits impedance signal with large difference in the absence or presence of target molecule (Xu et al., 2005). Electrochemical biosensors allow rapid detection of target molecules with low costs, fast response, with inherent miniaturization of modern electrical bioassays and low power requirement without compromising the accuracy (Hianik and Wang, 2009; Ikebukuro et al., 2004; Wang, 2006).

5. Nanomaterials and nano-constructs

The integration of the sensitivity rendered by nanomaterials and the specificity of aptamers have amassed great interest in the field of aptamer sensing applications such as molecular diagnostics and clinical applications (Kong et al., 2011; Yang et al., 2011) (Table 1). Nanomaterials can facilitate signal transduction just like fluorophores or electroactive tags (MB label and CdS nanoparticle). In addition, a single recognition event of target molecule can distort the balance between different nanoparticles, resulting in the change of the property of the whole assembly, greatly amplifying signal. Cooperative interactions of nanomaterials enable efficient recognition of target with multiple binding sites (Xu et al., 2009). Nanomaterials generally have wider surface areas and suitable to be made with unique size, shape and stability under varied physical and chemical conditions (Yang et al., 2011). Among several nanomaterials being developed for aptasensors, gold nanoparticles (AuNPs) are considered as one of the biologically non-reactive materials (Lim et al., 2011). AuNP-based colorimetric assay can be an efficient biosensor as the event of molecular binding can be transduced visually, obviating the need for any special equipment (Kim and Jurng, 2011). An aptamer-based colorimetric detection of Hg^{2+} using unmodified AuNP as colorimetric probe was created (Li et al., 2009). This assay is based on differential salt induced aggregation of unmodified AuNP in the presence or absence of target molecule Hg^{2+}. In the presence of Hg^{2+}, two T-rich single stranded oligonucleotide DNA aptamer form duplex through T–Hg^{2+}–T complex, thus unable to bind unmodified AuNP. This fails to stabilize the particles. The addition of salt (NaCl), will cap the repulsion among the unmodified negatively charged AuNPs, inducing the aggregation of these particles resulting in a blue solution. In the absence of Hg^{2+}, ssDNA oligonucleotide aptamers binds unmodified AuNP via nitrogen atoms of the bases. This stabilizes the nanoparticles, which exhibits resistances to salt-induced aggregation, giving red appearance in the solution as the AuNP particles dispersed in the solution (Fig. 2a). The corresponding changes in UV–vis spectra can be used to quantitatively analyze Hg^{2+}. In this study, at 5 min incubation, absorption ratio (A_{670}/A_{520}) of the assay demonstrated a linear correlation to the target concentration (from 1×10^{-4} M to 1×10^{-9} M). The sensitivity of AuNP is due to the high molar absorptivity in the visible region (as is evident with higher absorption ratio with increasing Hg^{2+} concentration) (Fig. 2b). This label-free approach is fast, robust and dependent only on common equipments, averting the additional steps such as modification of the aptamer and separation of bound and unbound aptamer–target complex. It was reported to have a detection limit of up to 0.6 nM. Similarly, Pavlov et al. (2004) reported the use of an aptamer functionalized with AuNP, in an assay termed biomaterial-metallic nanoparticle (NP) hybrid systems assay (Supplementary Fig. S1).

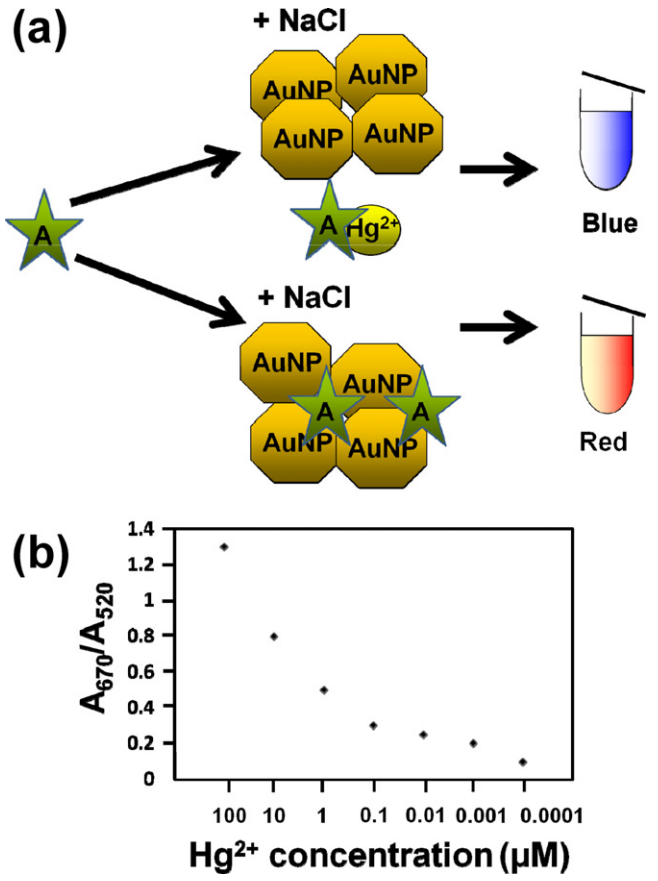

Fig. 2. (a) Aptamer-based colorimetric detection using unmodified gold nanoparticles. In the presence of Hg^{2+}, the aptamer will bind to this ion, causing the formation of AuNP aggregates, responsible for the formation of blue colour of the solution. In absence of the Hg^{2+}, aptamer will bind and stabilize the AuNP particles, giving the red colour appearance of the solution. AuNP – Gold Nanoparticle, A – Aptamer. (b) The absorption ratio (A_{670}/A_{520}) of the aptamer interaction with different concentrations of Hg^{2+} (Li et al., 2009). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

In the plasmon-based sensors, AuNPs can enhance the plasmon scattering light intensity when AuNPs aggregates. Moreover, AuNPs have a resonance light scattering peak close to the plasmon adsorption band wavelength (Wang et al., 2011a,b,c,d). These properties may pave ways to generate several optical-based aptasensors using AuNPs.

Developments of sensors based on silica nanoparticles have received enormous attention due to the associated high efficiency, sensitivity, and reproducibility (Lee et al., 2009). Silica nanoparticles are able to encapsulate large number of fluorophores (for large signal amplification of aptamer–target complex) as well as protecting fluorophores from photodamage (Wang et al., 2007). The capacity of this sensor is further enhanced by modification on the surface, changing the peak intensity and wavelength shift of absorbance spectra that are more efficiently detected. In this particular construct, immobilization of silica nanoparticles onto the surface of glass substrate deposited with gold layer is followed by coating of the gold material on the surface of silica nanoparticles. To evaluate the suitability of this construct in reporting aptamer–target interaction, aptamer raised against the Fc portion of human Immunoglobulin G subclass 1 (IgG1) was used. This aptamer is able to orientate the anti-fibrinogen antibody, a human IgG1 subclass, to properly capture the target protein fibrinogen of up to 0.1 ng/mL (Hiep et al., 2010).

Other nanomaterial such as graphene also garnered tremendous interest, due to the size and the ability to protect DNA from nuclease cleavage. Graphene impedes the nuclease from binding to the DNA on the surface of nanomaterial. Nanoscale size, low weight and inertness associated with this nanomaterial certainly favor graphene to be the kernel in the development of aptamer based applications (Loo et al., 2011; Wang et al., 2011a,b,c,d). Graphene has been utilized as the aptasensing platform for detecting ATP and cocaine, whereby the sensitivity limit of cocaine detection was reported up to 100 nM (Lu et al., 2010).

Of late, nano-constructs, materials constructed at nanoscale level, is increasingly finding niche in aptamer-based sensing applications (Table 1). Electroactive polymers such as polypyrrole (PPy) are widely used in a number of fields including sensing application due to its good electrical conductivity, environmental stability to air, water and can be synthesized easily through electrochemical and chemical routes (Brahim and Guiseppi-Elie, 2005). Functionality of the PPy is further enhanced by the incorporation of free carboxyl group into the polymer backbone via pyrrole-3-carboxylic acid (P3CA) to generate CPPy, whereby the chemical functionality was quantitatively and qualitatively controlled (Lee et al., 2006). A field-effect transistor (FET) sensor platform was constructed using CPPy nanotubes in detecting specific biological entities via a buffer solution as the liquid-ion gate. To investigate the suitability of this construct as aptasensor, anti-thrombin DNA aptamer was used. The binding of the aptamer with thrombin showed a decrease in current flow detectable by CPPy nanotube FET sensors (Yoon et al., 2008) (Fig. 3a). In another investigation, anti-VEGF RNA aptamer was covalently attached to the surface of CPPy nanotubes for the detection of VEGF. Their detection limit was 400 fM as measured by changes in electrical current in the presence of target protein (Kwon et al., 2010).

On the other hand, Surface enhanced Raman scattering (SERS) substrates involve analytical procedures based on vibrational spectroscopy, giving information on the characteristic spectrum of a molecule. Due to ultra sensitivity to a large number of analytes, having high specificity and low limit of biomolecular detection, SERS has become a choice for studies in biological systems. Consequently, SERS can be a tool to identify molecules in the vicinity of the metal through Raman spectrum. SERS is greatly enhanced by molecules absorbed onto the surface of nanoscopically rough metal (Abell et al., 2009). In this particular study, an aptamer based SERS was devised to detect vasopressin, biomarker for hemorrhagic shock using multilayer Au/Ag/Au structure nanotube arrays. This assay has a limit of detection of 5.2 μU/mL (Huh and Erickson, 2010) (Fig. 3b).

In addition, Nanogap capacitor consisting of two electrodes (separated by 20 nm with each other) fabricated by silicon dioxide sacrificial layers, can be used as an effective aptasensor. The positioning of the aptamer in-between the electrodes followed by target binding, altered the electrical impedance and reflected as a function of a frequency. Mannoor et al. (2010) have used anti-thrombin DNA aptamer as the experimental subject for this aptamer based sensor. Anti-thrombin DNA aptamer was linked with thiol group and immobilized on the gold surface electrode in-between the capacitance electrode. Binding of the thrombin to the aptamer affects the sensor capacitance, provides data regarding the binding between these two biomolecules (Fig. 4).

Due to the unique geometry, large surface to volume ratio and conductive properties, carbon nanotubes designed by rolling graphic sheets, are very effective candidates for highly sensitive label-free biosensor (Guo et al., 2011). By virtue of their cylindrical structure, unique mechanical, electrical and optical characteristics, carbon nanotubes enhance the molecular recognition capability of aptamer (Prato et al., 2008). An aptamer-based

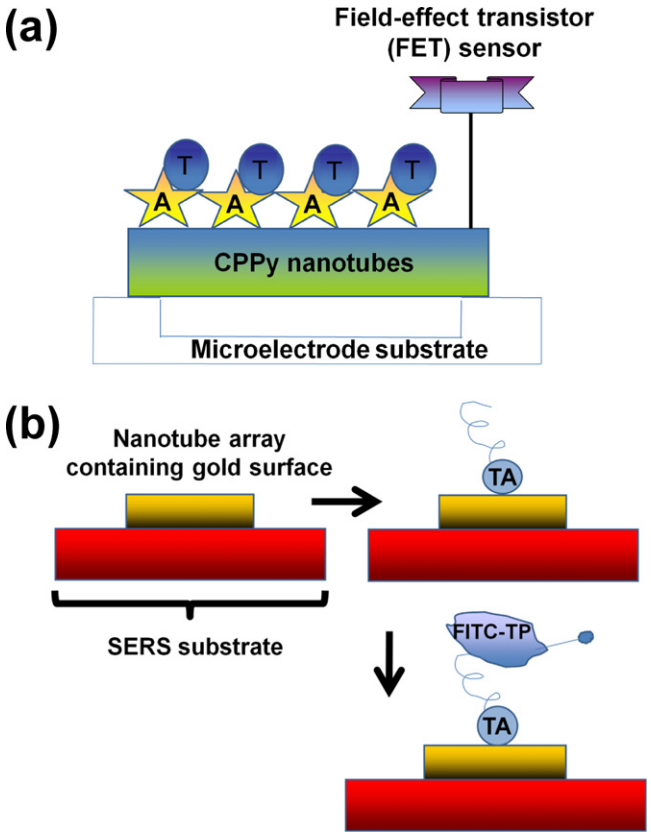

Fig. 3. (a) Cartoon illumination of Carboxylic-acid-functionalized polypyrrole (CPPy) nanotubes based aptamer assay. CPPys were deposited on the microelectrode substrate, forming FET sensor platform, with the buffer solution as a liquid-ion gate. Binding of the target protein to the aptamer immobilized on the surface of the nanotubes generates current flow detectable by FET sensor. T – Target, A – Aptamer (Yoon et al., 2008). (b) Schematic drawing of aptamer based assay based on Surface enhanced Raman scattering (SERS) principle using SERS substrate. Anti-vasopressin aptamer was immobilized on the surface of the multilayer Au/Ag/Au structure nanotube arrays. The target protein vasopressin labeled with fluorescein isothiocyanate (FITC) was injected through inlet port of the microfluidic device. The binding of vasopressin and the aptamer can be monitored by analyzing the Raman spectroscopy resulting from the excitation of the laser focused onto the nanotube substrate. TA – Thiolated Aptamer, FITC-TP – FITC Labeled Target Protein (Huh and Erickson, 2010).

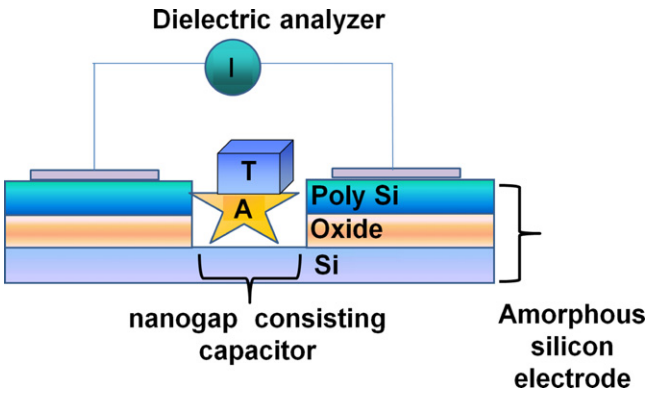

Fig. 4. Diagrammatical sketch of Nanogap dielectric spectroscopy based aptamer assay. Made up of capacitor between two parallel amorphous silicon electrodes, and connected to dielectric analyzer, aptamer placed in between the electrodes affects the electrical impedance, providing information on the interaction of the aptamer with the corresponding target. T – Target, A – Aptamer (Mannoor et al., 2010).

label free assay was designed to detect immunoglobulin E (IgE) using carbon nanotubes field effect transistor (CNTFET). After immobilization of anti-IgE aptamer on CNTFET channels, electrical properties were measured in real-time using semiconductor parameter analyzer following introduction of IgE at various concentrations. This aptamer modified CNTFET assay is sensitive as the aptamer–protein complex is smaller than Debye length (compared to antibody–protein complex). The interaction took place inside electrical double layer, enabling target protein to be detected with high sensitivity (Maehashi et al., 2009). Another biosensor termed single-walled carbon nanotubes field effect transistor (SWNT-FET) was constructed using standard chemical vapor deposition technique. A 15-mer ssDNA aptamer against anti-thrombin was chosen. This aptamer was proven to be efficient molecular recognition receptors using transducers (Potyrailo et al., 1998). Binding of thrombin causes decrease in the conductance, due to the thrombin molecules masking the negative charges of DNA aptamer upon the formation thrombin–aptamer complex. The lowest limit of detection of this sensor is approximately 10 nM (So et al., 2005). Instability of molecular recognition elements can be circumvented by the application of aptamers that increases the shelf-life of the nanoconstructs (high surface-to-volume ratio and unique size-dependant properties) towards sensitive detection of target molecules.

6. Nucleic acid amplification assays

One formats of aptamer-based assay is by amplifying aptamer via PCR technique. The magnificent ability conferred by PCR enables amplification of any nucleic acids to millions of molecules, greatly enhancing signal for detection. Aptamers that are specific for their respective target molecules, but present initially in a very minute amount, can be amplified by real-time PCR or conventional PCR for the convenient detection in biosensing application (such as Bio-barcode and Proximity Ligation Assay) (Table 1).

Bio-barcode assay involves two different particles, the first being a magnetic bead conjugated with polyclonal capturing antibodies, while the second is gold nanoparticles conjugated with monoclonal antibodies. Together they can form an immuno-sandwich. The gold nanoparticles also carry hundreds of thiolated ssDNA sequences which hybridize with its complementary DNA sequences, known as barcode markers. Following sandwich structure formation in the presence of target protein, the marker DNA sequences are released by melting and subsequently amplified by PCR and further detected on agarose gel or real-time PCR (Nam et al., 2003) (Supplementary Fig. S2). However in bio-barcode based aptamer assay, the gold nanoparticles are substituted with aptamers. In a particular experiment carried out by Lau et al. (2010), DNA aptamer specific against cytochrome-c (cyto-c) was utilized. It displayed a steady increase of the fluorescence in real-time PCR assay, as a function of cyto-c concentration, detecting up to pico-molar level of cyto-c in culture medium. This assay, integrating the specificities of antibody and aptamer, is very sensitive and can be expended into the development of multiple and automated platform for high-throughput analysis.

Proximity Ligation Assay (PLA) is an assay that associates the adjacent binding of two reagents, forming a sandwich, allowing nucleic acid tags on the reagents to be ligated and amplified by real-time PCR. This assay can be used to detect zeptomole amounts of protein as a result of very low probability of unique amplicon formation in the absence of target. Pai and Ellington (2009) have adopted PLA to develop an assay based on RNA aptamer selected against prostate-specific membrane antigen (PSMA). In this study, two aptamers modified with 2′ fluoropyrimidines were utilized, extended at 5′ and 3′-ends, to form a ligation junction with two different PLA DNA probes complementary to the extended sequences. As PSMA is a dimer, two different aptamer–probe ligands will bind adjacent to one another on the cell surface, resulting in ligation aided by splint oligonucleotides. The quaternary configuration is very much unlikely to form without the presence of target, which greatly eliminates the background signal. This is followed by real-time PCR amplification, where cells (10–10,000) can be detected in the presence of large amount of non-cognate cells (Fig. 5). These PCR based methods can greatly enhance the sensitivity of target molecule detection with very low amount of starting materials, due to the exponential amplification nature inherent in PCR to produce detectable signal.

7. Future perspectives

The widespread development and application of aptamer-based sensors have found ways in both basic research and biomedical diagnostics, eventually becoming tools that can alleviate the problems inherent in current antibody based technologies. Most of these aptamer-based assays have reached the detection limit to lower concentrations for the target thrombin (Fig. 6). Recently, nanotheranostics, the system or strategies in which disease diagnosis and therapy are combined, is coming of age. An array of actively targeted

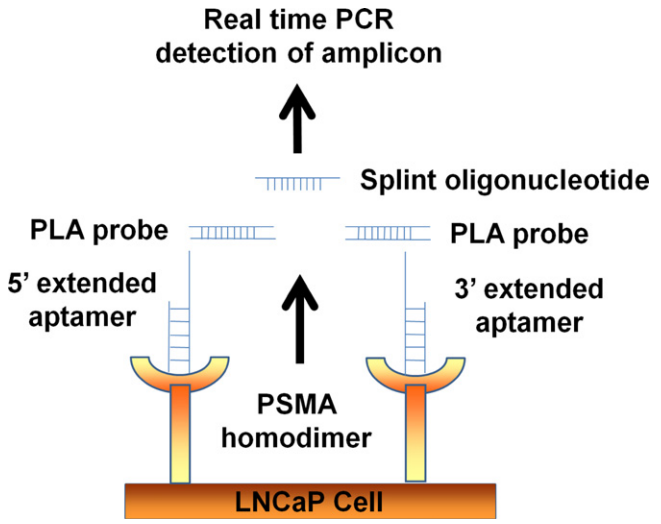

Fig. 5. Schematic representation of aptamer based Proximity Ligation Assay (PLA). Two aptamers binding to adjacent target on the cell surface containing nucleic acid tags at 5′-end and 3′-end, respectively will be ligated by splint oligonucleotide allowing amplification by Real-time PCR (Pai and Ellington, 2009).

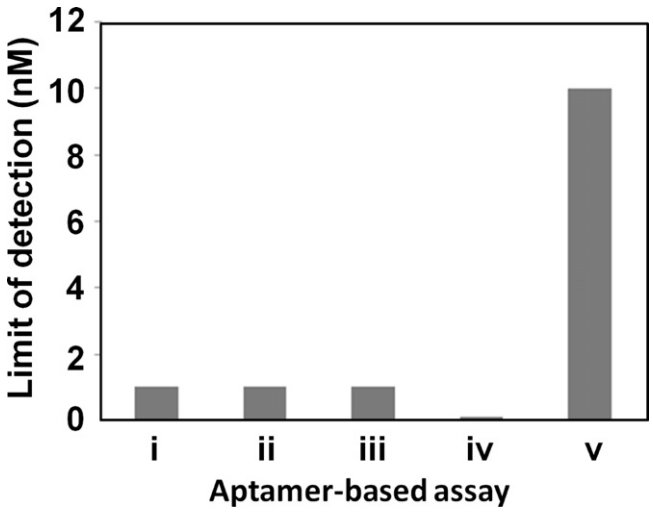

Fig. 6. The limit of detection of several aptamer-based assays using thrombin as the target. (i) Fluorescence aptamer-based assay on imaging fiber (Lee and Walt, 2000), (ii) QD-conjugated aptamer-based assay (Choi et al., 2006), (iii) ALISA-based assay (Baldrich et al., 2004), (iv) MB labeled-aptamer-based assay (electrochemistry) (Cai et al., 2005) and (iv) SWNT-FET aptamer-based assay (So et al., 2005).

nanomedicines have been investigated, with the clinical evidence proving that 5-200 nm of these nanosize carrier materials such as antibodies can render drastic improvement of therapeutic index of low-molecular-weight drugs. The supreme specificity and discerning ability of aptamers that can rival the antibodies capacitate them to a variety of aptamer-based applications. These 'miniaturized molecules' will soon find their way, carving niche in nanotheranostics applications (Lammers et al., 2010). In addition, the availability of nanomaterials such as gold nanoparticles conjugated aptamers enable these molecules to stand in lieu of the 'classic' drug targeting systems, such as antibodies, liposomes, polymer and micelles, to be increasingly implemented for combining disease diagnosis and therapy (Li et al., 2010). Future work also have to be carried out to enhance the detection limit of the target and to investigate the possibility of amalgamating more than one aptamer-based application into a single more effective aptasensor. Moreover, efforts to fine-tune particle size, shape, crystal structure, interface, and environment that influence the properties and functionalities of the nanomaterials will create a more efficient nanomaterial based aptasensor.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bios.2012.01.002.

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