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Aptamer-based optical switch for biosensors

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압타머 광학 바이오센서

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Abstract: In this review, we will discuss aptamer technologies including *in vitro* selection, signal transduction mechanisms, and designing aptamers and aptazyme for label-free biosensors and catalysts. Dye-displacement, a typical label-less method, is described here which allows avoiding relatively complex labeling steps and extending this application to any aptamers without specific conformational changes, in a more simple, sensitive and cost effective way. We will also describe most recent and advanced technologies of signaling aptamer and aptazyme for the various analytical and clinical applications. Quantum dot biosensor (QDB) is explained in detail covering designing and adaptations for multiplexed protein detection. Application to aptamer array utilizing self-assembled signaling aptamer DNA tile and the novel methods that can directly select smart aptamer or aptazyme experimentally and computationally will also be finally discussed, respectively.

Key words: optical, sensor, aptamer, fluorescence

1. Introduction

Aptamers are single-stranded DNA or RNA (ssDNA or ssRNA) molecules that can bind to preselected targets including proteins and peptides with high affinity and specificity. Since 1990, numerous high-affinity and highly specific aptamers have been selected against a variety of analytes, including inorganic ions, small organics, metabolites, peptides, proteins, and even whole viruses or cells.[1-5] These molecules

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can assume a variety of shapes due to their propensity to form helices and single-stranded loops, explaining their versatility in binding to diverse targets. They are used as sensors, and therapeutic tools, and to regulate cellular processes, as well as to guide drugs to their specific cellular targets. Contrary to the actual genetic material, their specificity and characteristics are not directly determined by their primary sequence, but instead by their tertiary structure. Aptamers can be used for both basic research and clinical purposes as macromolecular drugs. Aptamers can be combined with ribozymes to self-cleave in the presence of their target molecule. These nucleic acid catalysts have additional research, industrial, and clinical applications.

Although aptamers have been extensively studied to expand the utility of nucleic acids as molecular recognition probes, standard DNA and RNA aptamers do not have inherent properties with which a convenient method can be devised to report an aptamer–target interaction. Therefore, to fulfill the capability of aptamers as affinity probes, it is necessary to develop methods to transduce the molecular recognition events to a signal generation process. The signal transduction should be simple (without a need of a complicated modification scheme to alter an aptamer for signal generation), universal (regardless of what characteristics an aptamer and its target exhibits), and convenient to use (with minimal sample manipulation during the signal acquiring process).[6] Many recent researches have focused on developing rational design strategies for designing nonfluorescent aptamers that can be turned into fluorescent-signaling probes, so called "signaling aptamer".[7]

2. Signal Transduction Mechanisms

2.1. Strategies for aptamer beacons

Optical signal transduction based on fluorescence resonance energy transfer (FRET) between dual-labeled fluorophores has become most attractive strategy; because wide variety of fluorophores and aptamer engineering are possible. Since the signal mechanism is originated from 'molecular beacons', this particular sensor is called as "aptamer beacon" or "molecular aptamer beacons". In this formation, FRET involves the transfer of energy from an excited donor fluorophore to a neighboring acceptor molecule. If the acceptor molecule is another fluorophore, this leads to fluorescence at the acceptor's emission wavelength. If the acceptor is a 'dark quencher' (for example, DABCYL), it does not subsequently fluoresce. There are several different schemes that can be imagined for converting aptamers to "aptamer beacons"; (A) signaling aptamer with engineered instability by altering its secondary structure, (B) signaling aptamer with engineered conformational change by altering its tertiary structure, (C) ligand mediated self-assembly of bipartite aptamers by altering its quarternary structure (ternary complex stabilization), and (D) signaling aptamers based on antisense strategy (ligand-mediated disassembly of tri- or bipartite systems).

First, the ligand-dependent rearrangement of aptamer secondary structure based on three-way junction has been first envisaged: (A) signaling aptamer with engineered instability by altering its secondary structure. Tang and colleagues have developed a new design that does not rely on three-way junction.[8] The new aptamer beacon was constructed with three parts; an aptamer, a short complementary strand to the part of aptamer, and a PEG linker that connects these two. A fluorophore and a quencher are covalently labeled to the two termini of conjugated DNA sequence. In the absence of target, the short DNS will hybridize with a part of aptamer, keeping fluorophore and quencher in close proximity, and resulting signal off. As target binds to aptamer, the conformation change aptamer disturbs the intramoleuclar hybridization, move away the quencher, and result signal on. The authors demonstrated the feasibility of this technique with anti-ATP and anti-thrombin aptamer by using Chlorin e6 (Ce6) and FAM as fluorophores and Black Hole Quencher (BHQ2) as a quencher. The anti-ATP signaling aptamer exhibits 30-fold intensity enhancement immediately upon addition of 3.5 mM of ATP (>90% response within 5s), while it did not respond to ATP analogues at 1 mM. The anti-thrombin signaling aptamer also showed the similar

performance; 17.6-fold signal enhancement in the presence of 300 nM thrombin but no response to negative controls such as IgG, IgM, and BSA at 200 nM.

Second, signaling aptamers that rely on tertiary structural transitions and thus form a particular “G-quartet structure” could also be engineered: (B) signaling aptamer with engineered conformational change by altering its tertiary structure. An advantage of this tertiary structural rearrangement strategy as opposed to the secondary structural rearrangement strategy described above is that the signal transduction mechanism is “signal-on” (signal increases in proportional to the analyte concentration) rather than “signal-off”. While there are numerous analytes in a complex mixture or biological sample that might lead inadvertently to fluorescence quenching, there should be relatively few compounds other than the target analyte that should lead to an increase in fluorescence intensity. Tan’s group[9] has noted that even in the absence of the protein target an equilibrium existed between the random coil state and the quadraplex states of the thrombin aptamer. Based on the fact that target binding shifts the equilibrium in favor of the quadraplex state, the researchers investigated the target recognition of anti-thrombin aptamer modified with various sets of fluorophores.

Third example of signaling aptamer in concert with beacon strategy was utilizing the capability of self-assembly in the presence of a cognate ligand. For example, aptamer sequence can be splitted in two pieces and fluorophores are used to label each of the aptamer pieces: (C) ligand mediated self-assembly of bipartite aptamers by altering its quarternary structure (ternary complex stabilization).[10]

Fourth, aptamer beacons can also be designed by using an “antisense strategy”[11] in which a complementary DNA sequence is used to intentionally denature the aptamer: (D) signaling aptamers based on antisense strategy (ligand-mediated disassembly of tri- or bipartite systems). This approach has become the dominant strategy in designing signaling aptamers because a number of variables can be further modified to improve the affinity such as sequence, fluorophores, and anti-sense oligos.

In addition to simple FRET-based signaling aptamer monitoring absolute intensity change, other strategies have been developed. Ligand-dependent conformational transitions can not only influence the optical properties of aptamer beacons with pendant dyes, but can also be exploited for the initial conjugation of fluorescent dyes. Weeks *et al.*[12] have made use of the differential nucleophilic reactivity of 2’ amine substituted nucleotides in flexible versus constrained nucleic acid structures to convert the anti-ATP DNA aptamer into an signaling aptamer. A 2’ amine moiety that was site-specifically incorporated into the aptamer could readily form an adduct with fluorescamine (FCM). However, in the presence of ATP, adduct formation is considerably reduced. Alternatively, the fluorescent adduct with FCM could in turn be detected via FRET to a fluorescence acceptor (Texas-Red) that was incorporated at the 3’ end of the molecule. This led to an interesting analytical method in which ATP was detected not only by FRET (K_d = 390 μM), but also by the ability to form the conjugate that leads to FRET in the first place.

However, FRET molecular probe used for protein studies could not solve two significant background-signal sources. One problem is that the probe always has some incomplete quenching, resulting in a significant probe background. The second source of background signal comes from the native fluorescence of the biological environment where the target protein resides. This emission wavelength switching (excimer and monomer switching of pyrene molecule results in a change of fluorescence wavelength from ~400 nm for excimer to 485 nm for monomer) solves the probe background signal problem that occurs with FRET molecular probes. One special feature of the pyrene excimer is that it has a very long fluorescence lifetime compared with other potential fluorescent species. The lifetime of the pyrene excimer can be 100 ns or longer, whereas that for most of the biological background species is 5 ns. With time-resolved fluorescence measurements,

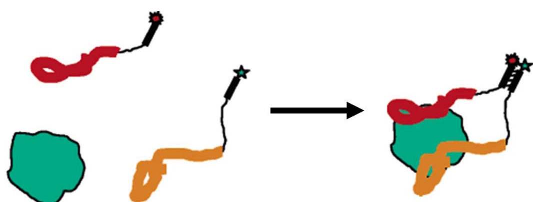


Fig. 1. Molecular beacons detecting proteins based on aptamers directed to two different epitopes of the protein.

target binding induced excimer signal can be separated from biological background interference. Combination of light-switching and time-resolved measurements was demonstrated for the detection of picomolar PDGF-BB in a few seconds.[13]

Aptamer beacon strategy can be further extended to the assay involves protein-induced co-association of two aptamers recognizing two distinct epitopes of the protein.[14] As a model system, two anti-thrombin aptamers were labeled with various FREP fluorophore pairs, respectively. As shown in *Fig*. 1, two fluorophores are located closed to the proximity upon binding to thrombin, resulting fluorescence quenching. This method was proven to detect thrombin in high selectivity in biological complex, picomolar sensitivity, and with high signal-to-noise ratio without sample manipulation. One of the advantages of these approaches as a whole is that they can potentially be applied to any aptamer, RNA or DNA. In addition, since aptamer beacon design relies upon knowledge of aptamer secondary structural features that can be readily predicted and engineered, their ligand-sensing and -signaling properties can likely be more finely-tuned than was the case for signaling aptamers, which are instead dependent upon small, hard-to-predict conformational changes.

Binding of an aptamer to its protein target can dramatically change the stacking of bases and lead to the alteration of a fluorescence signal if fluorescent nucleotide is incorporated at the position where conformational (base-stacking) change occurs. The sequences of several DNA aptamers (anti-thrombin, anti-IgE, and anti-PDGF aptamers) have been modified with 2-aminopurine (2AP), 4-amino-6-methylpteridone (6MAP) or 3-methylisoxanthopterin (3MI).[15] It is likely that this approach to producing fluorescent signaling aptamers is of general use for protein-binding aptamers because of their "induced fit" binding mechanism.

2.2. *In vitro* selection of signaling aptamers

The affinity of the selected aptamers can be controlled by varying the stringency of each round of selection, normally by varying the concentration of the target, the buffer conditions employed during the binding reaction, or the number and type of wash steps used during column or filter partitioning. Generally, the population is assayed after every several rounds of selection and amplification.

The binding affinities of aptamers are highly target-dependent and ranged from picomolar (1×10^{-12} M) to high nanomolar (1×10^{-7} M) for various protein targets. When small organics are targeted, the dissociation constants are higher, typically micromolar, as might be expected given the smaller number of interactions that will be formed. In either case, interactions tend to be extremely specific, and aptamers can discriminate between related analytes by over 10,000-fold on the basis of single amino acid changes or even single chemical moieties, such as hydroxyl or methyl groups. The specificities of aptamers can to some extent be controlled during selection; for example, negative selections against related analytes or the matrices used for target immobilization can remove cross-reactive aptamers from a population.[16] It is encouraging that this method could be adapted to the selection of signaling-aptamer probes that detect non-nucleic acid targets.

Ferguson *et al.* have reported a hybrid strategy that incorporated elements of both effector-binding[17,18] and activity-based[19] selection approaches typically used for generation of catalytic nucleic acids in developing allosteric ribozyme sensors that are specific for the small molecule analytes such as caffeine or aspartame.[20] Caffeine- or aspartame-responsive ribozymes were converted into fluorescence-based RiboReporter™ sensor systems that were able to detect caffeine or aspartame in solution over a

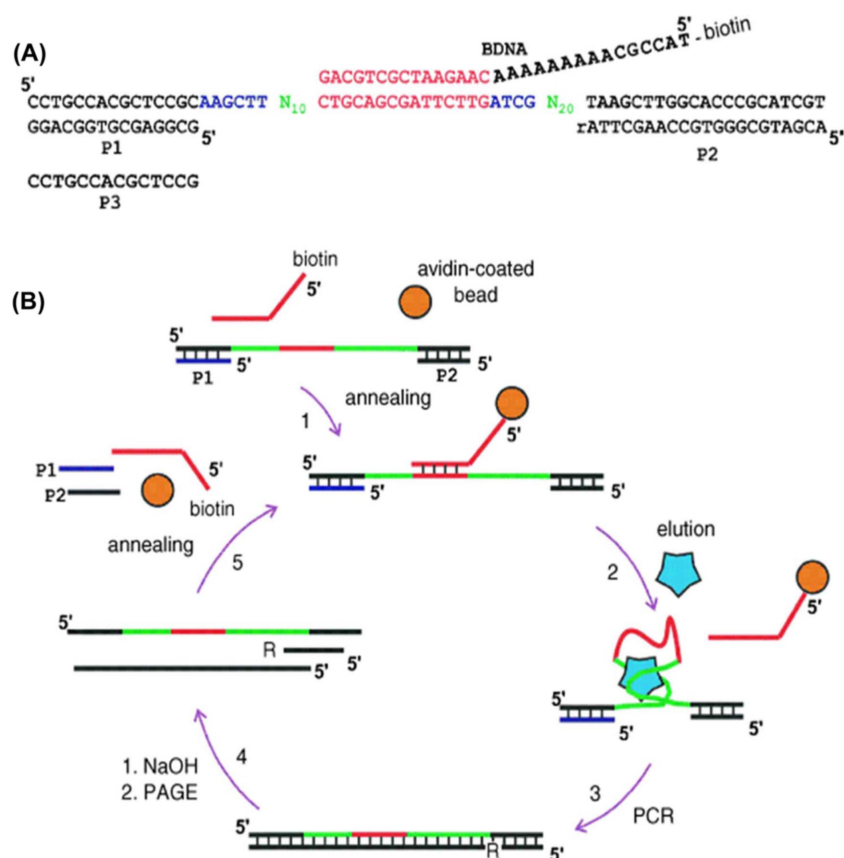


Fig. 2. *In vitro* selection of structure-switching aptamers. (A) DNA library design. (B) In vitro selection scheme.

concentration range from 0.5 to 5 mM.

Nutiu and Li have published a new *in vitro* selection approach based on structure-switching mechanism for generating unmodified DNA aptamers that can be immediately transformed into effective signaling probes without the need for further optimization.[21] The selection strategy is shown in *Fig*. 2. A special DNA library (*Fig*. 2A) was used that contained a central 15-nucleotide fixed-sequence domain (red) flanked by two random-sequence domains (green) of 10 and 20 nucleotides, each further flanked by primer-binding sequences (black). The central fixed-sequence domain was designed to be complementary to an antisense oligonucleotide biotinylated at its 5-end (denoted BDNA). This arrangement permitted immobilization of the DNA library onto an avidin-coated bead through DNA hybridization (step 1 of *Fig*. 2B). Two short oligonucleotides, P1 and P2, was added together to prevent the involvement of the primer-binding sequences in the tertiary folding of an eventual aptamer. A standard NTP (nucleoside triphosphate; blue star) was used as the potential aptamer target. The immobilized DNA assembly was then exposed to a solution of a mixture of NTP (step 2). An aptamer able to form the DNA/target complex should switch from the bead-bound state to the solution. This molecule was then collected and amplified by PCR (step 3). The reverse primer contained a ribonucleotide (R) at its 3-end to create a chimeric antisense strand prone to NaOH-mediated cleavage, which permitted the isolation of the sense strand by gel electrophoresis (step 4). The recovered sense DNA was re-annealed to BDNA, P1, and P2 and used for the next round of selection (step 5). It is

noteworthy that BDNA and P1 were intended as the eventual sequences for carrying a quencher and a fluorophore, respectively, so that upon completion of *in vitro* selection, the selected aptamer could be immediately converted into a signaling molecule.

2.3. Dye-displacement strategy

As known well, the key in the development of aptamer-based analytical methods and sensors is to transduce aptamer recognition events to detectable signals. "Signaling aptamers" having the ability to directly report target concentration are actively being sought, and most signaling aptamers developed thus far are for homogeneous or heterogeneous fluorescence assay via labeling with signaling probes. However, as the precise target binding sites and the conformational changes of the aptamers are generally unknown, it is not easy to design labeling strategies and could weaken the binding affinity between the target and aptamer. Additional steps such as fluorophore labeling or modifying also result complications and increase in analytical cost relatively high.

It has been explored that commercially available DNA-stain dyes, such as Cyanine dyes or ethidium bromide (EB) can be employed to design signaling aptamer. These so called "staining dyes" are non-fluorescent in solution but form a highly fluorescent complex with DNA by intercalating through the DNA double helix and thus have been widely used in dsDNA labeling, detecting, sizing, and DNA-protein study.[22-24] In this regard, it is worth noting that the non-covalent conjugation of a fluorophore to an aptamer may ultimately compromise the affinity of the aptamer to its ligand.[11] This new approach based on label-free aptamer and an intercalating dye would be useful for high-throughput screening in drug and environmental monitoring and further widely applicable for the analysis and study of proteins in biochemical and biomedical studies.[25]

In addition to utilizing fluorescence quenching/dequenching characteristics of cyanine dyes based upon DNA intercalation, the absorption wavelength change due to the equilibrium change between dimer and monomer formation was employed to design

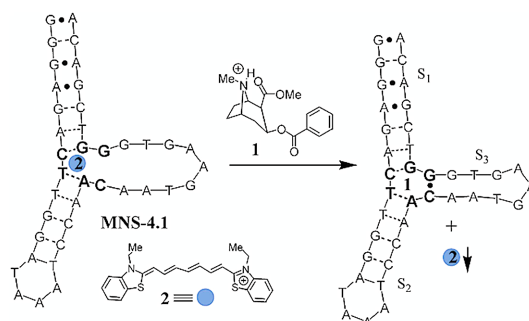


Fig. 3. Aptamer MNS-4.1 complexed with a diethylthiotricarbocyanine dye (2) and cocaine (1) displaces dye, causing an immediate attenuation of absorbance and eventual precipitation of dye.

signaling aptamer. Stojanovic and Landry have reported that intermolecular displacement of a cyanine dye in a complex with an anti-cocaine aptamer results the change of absorption maximum wavelength of monomer at 760 nm and could detect cocaine in the concentration range of 2-600 μM in *Fig.* 3.[26]

This concept above has been expanded for designing aptameric modular sensors composed of (a) an aptamer as a "reporting domain" that signals the binding event of an analyte through binding to a fluorophore with (b) other aptamers as "recognition domains" that bind the analyte.[27] They constructed a recognition region specific for flavin mononucleotide (FMN) in combinations with malachite green RNA aptamer (MGA)[28,29] as a signaling domain in *Fig.* 4.

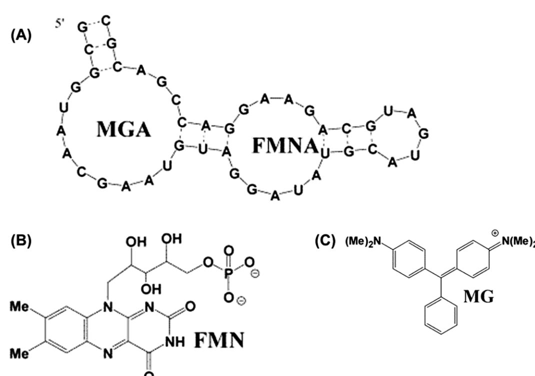


Fig. 4. Structure of FMN sensor (MGA-FMNA) (A), FMN as a target analyte (B), and MG (malachite green) as a fluorescent dye (C).

They were able to obtain a functional sensor capable of responding to an increase in FMN concentration with an increase in fluorescence.

Not only organic dye or inorganic complex but also water-soluble cationic polythiophene derivative has been demonstrated as a "polymeric stain" that can specifically transduce the binding of an aptamer to its target into an optical signal without any labeling of the probe or of the target.[30] When binding takes place between human a-thrombin and ss-DNA (a thrombin aptamer), for example, the aptamer undergoes a conformational transition from a single-stranded to a folded structure in *Fig.* 5. This conformational change of the negatively charged oligonucleotide can be detected by adding a cationic poly(3-alkoxy-4-methylthiophene) derivative, which transduces the new complex formation into an optical (colorimetric or fluorometric) signal. This biophotonic tool has been reported to detect as little as femtomole (2×10^{-15} mol) of human thrombin in few minutes so that it could be applicable for the detection of various other proteins as well as being useful in the high-throughput screening of new drugs.

Conclusively, dye-displacement method will allow to avoid relatively complex labeling and modifying steps, extending its application to any aptamers without specific conformational changes, in a more simple, sensitive and economical way.

In the following section, most recent and advanced technologies of signaling aptamer and aptazyme for the various analytical and clinical applications will be further described.

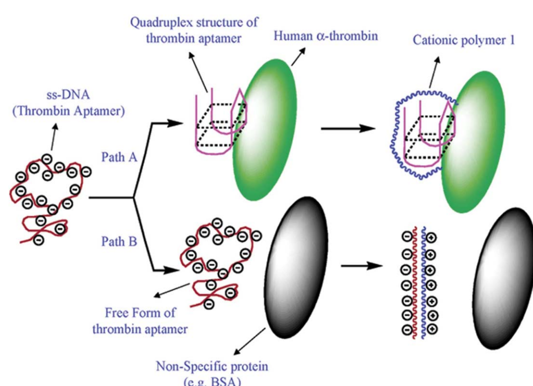


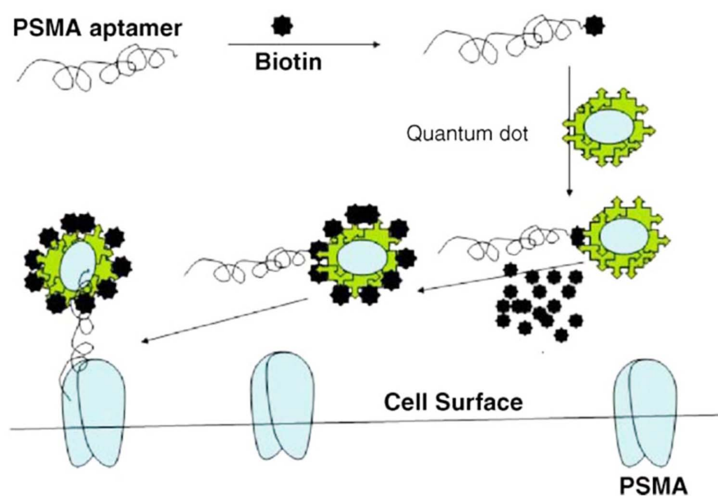
Fig. 5. Schematic description of the specific detection of human a-thrombin by use of ss-DNA thrombin aptamer and cationic polymer 1.

3. Advanced Application of Aptamers

3.1. Aptamer-quantum dot conjugate and application to cancer cell diagnostics

The fact that different QDBs can have finely tuned emission wavelengths should allow multiple different targets to be assayed in parallel in heterogeneous solution. The first QD-based protein biosensor that detects the small molecule maltose has been designed. In one configuration of this system, a maltose-binding protein was complexed to the ZnS shell of a CdSe QD through a 5-histidine tail appended to the protein. Binding of a dye-labeled cyclodextrin molecule to the protein resulted in a loss of photoluminescence, which could be restored by the displacement of the bound cyclodextrin by the addition of maltose.[31] These experiments pave the way for the expansion of QDB detection methods to other targets and analytes. By immobilizing different aptamer constructs onto different quantum dots, it should be possible to carry out multiplexed protein detection.

Early detection for the cause of cancer deaths offers best prospects for patient survival. For instance, prostate cancer screening currently relies on rectal examinations to detect anomalies in the prostate gland, along with blood tests for upregulated prostate specific antigen (PSA) levels.[32] In contrast to PSA, prostate-specific membrane antigen (PSMA), a membrane-bound glycoprotein, is overexpressed in many prostate cancers. This has therefore been identified as a good biomarker of cancer growth and metastases. In this regard, aptamers have been selected to target PSMA. Fluorescently labeled anti-PSMA aptamer, A10, has been shown to specifically bind PSMA-expressing prostate tumor cells (LNCaP cells).[33] Little or no labeling was observed with a non-PSMA-expressing prostate tumor cell line (PC3 cells). The same anti-PMSA aptamer has been also



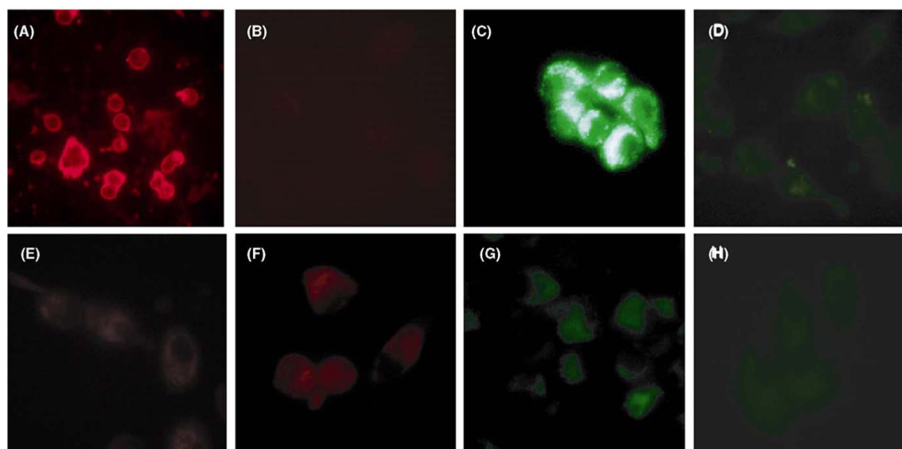


Fig. 6. Quantum dot conjugation and PSMA binding scheme. Aptamer:quantum dot labeling of *live* LNCaP (A–D) and PC3 (E–H) cells: (A and E) CdTe–PSMA aptamer; (B and F) CdTe nanoparticles only; (C and G) Qdot 525–PSMA aptamer; (D and H) Qdot 525 only. Deconvolution microscopy was performed using a Zeiss KS-400 with a TRITC optical filter with an excitation wavelength of 543 nm and a long bandpass emission filter of 590 nm.

used to localize polylactate/polyethylene glycol (PEG) nanoparticles to the surface of LNCaP cells.[34] Localization was associated with internalization of the nanoparticles, demonstrating a potentially novel therapeutic application for aptamer:nanoparticle conjugates.

CdSe and CdTe nanoparticles have been conjugated with a slightly different A9 anti-PSMA aptamer[33] and successfully demonstrated for *in vitro* labeling both LNCaP cells grown in tissue culture and LNCaP cells grown in a tissue phantom (a collagen matrix).[35] In all cases, binding was found to be sensitive and specific for cell lines overexpressing the PSMA antigen (*Fig.* 6). This approach has particular promise in animal model systems to dynamically visualize and understand tumorigenesis at the cellular and molecular level and to screen and study targeted therapeutics.

In addition, Herr *et al.* have reported a rapid collection and detection of leukemia cells using a unique two-nanoparticle assay with aptamers as the molecular recognition element.[36] Aptamer-conjugated magnetic nanoparticles were used to extract target cells, while aptamer-conjugated fluorescent nano-

particles were simultaneously added to amplify the signal intensity corresponding to a single aptamer binding event, improving sensitivity over methods based on individual dye-labeled probes. Combining two types of nanoparticles allowed for rapid, selective, and sensitive detection not possible by using either particle alone. This assay was possible only because there are sufficient aptamer binding sites for both types of particles on the target leukemia cells. Prolonged stability and facile synthesis make aptamers an ideal replacement for antibodies in cellular recognition studies.

3.2. High throughput drug screening

High throughput screening (HTS) of compound libraries to identify molecules that inhibit the catalytic activity of specific kinases helicases, proteases, and other enzymes is a major focus of pharmaceutical industry efforts. Strategies for the detection of enzyme activity in HTS formats that can readily translate to multiple targets have the potential greatly accelerated with therapeutic discovery. Previously, ADP-specific aptamer and allosteric ribozyme have proven to their capability to screen protein kinase inhibitors by utilizing scintillation proximity assay.[37]

Most recently, signaling aptamer technology has been demonstrated for the development and execution of a high-throughput screen for an otherwise problematic target, adenosine deaminase (ADA).[38] This approach employed a structure switching (i.e. anti-sense signaling) DNAaptamer that reports adenosine concentration over the course of the enzymatic reaction but no affinity for inosine. As shown in *Fig.* 7, as adenosine is transformed to inosine by ADA, the aptamer becomes available to complex with the quenching group and reports the progress of the reaction with decreasing fluorescence over time. Inhibition of ADA maintains high adenosine concentration and the highly fluorescent adenosine–aptamer complex are therefore formed. The assay was extremely robust in a screen of more than 44,000 molecules and revealed a new competitive inhibitor of the deaminase.

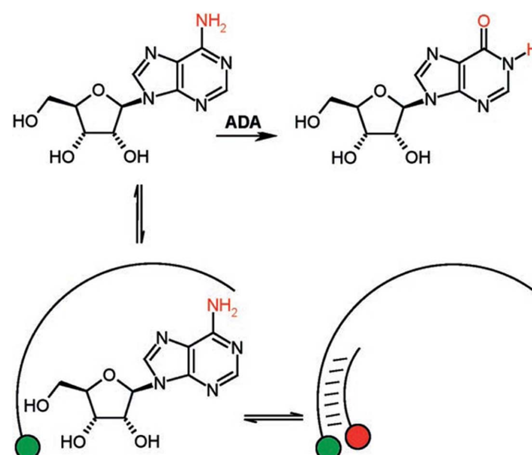


Fig. 7. Fluorescence-signaling aptamer assay for screening. The fluorescein-labeled aptamer, depicted as an arc with a green-filled circle (Fluorescein-5’-TCACTGACCTG-GGGGAGTATTGCGGAGGAAGGT), complexes with either adenosine or a quenching oligonucleotide (5’-CCCAGGTCAGTG-Dabcyl; arc with red-filled circle) resulting in the exclusion of the other.

3.3. Real time monitoring associated with signal amplification

Real-time amplification of nucleic acids is proving to be increasingly valuable for the quantitative detection of nucleic acids for many diagnostic applications.[39,40] While PCR is typically used for ligase-mediated sequence amplification, rolling circle amplification (RCA) methods have also been shown to detect nucleic acids with great sensitivity[41] and to be adapted to wide variety of analytical schemes and devices.[42-45] In RCA, a small nucleic acid circle hybridizes to a primer which is in turn extended around the circle, ultimately displacing the original primer and continuing to produce long concatameric nucleic acid products. The nucleic acid products can be detected by a variety of methods, including hybridization of fluorescent oligonucleotide probes. Interestingly, the concatamers are so massive that they can accumulate and be detected as discrete single molecules on surfaces.[41-43,46,47] It has recently proven possible to carry out real-time RCA reactions using strategies similar to those for real-time PCR detection, such as detection of newly synthesized

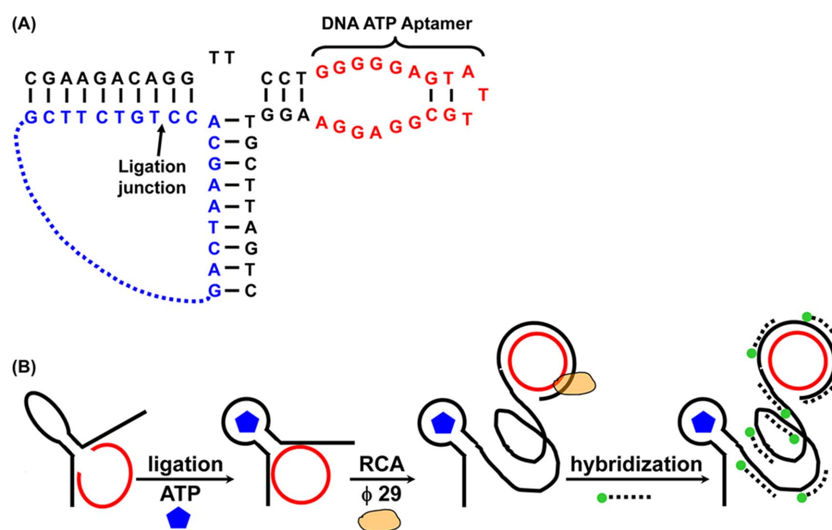


Fig. 8. (A) Deoxyribozyme ligase and substrate. The ligase is shown in black, the allosteric, ATP-binding domain is in red, and the padlock probe is in blue. An arrow indicates the ligation junction. (B) Schematic of deoxyribozyme-mediated RCA. The aptazyme labeled with biotin at its 5' end is immobilized on a streptavidin coated glass slide. The aptazyme is activated by ATP and ligates a padlock probe. RCA is initiated from the 3' end of the aptazyme, and the elongated aptazyme is visualized using fluorescent oligonucleotide probes labeled with Cy3(●).

products with molecular beacons, cleavable probes, or SYBR Green.[45,48-50] As described above, since aptazyme can uniquely convert the recognition of analytes into the production of amplicons, the most novel and robust detection schemes for aptazymes have involved sequence amplification.[51] In an effort to maximize the signal amplification effect, Cho *et al.* has meld the unique ability of aptazyme to transducer molecular recognition to sequence information and the capabilities of RCA to amplify nucleic acids. In this approach, DNA aptazyme that was previously generated by appending an anti-ATP DNA aptamer[52] to a selected deoxyribozyme ligase[53,54] and could activate autoligation[55] of a 3' phosphorothioate on a 5' iodine residue up to 460-fold by the cognate analyte, ATP was used as a model compound. This resultant deoxyribozyme could catalyze the ligation and circularization of a single-stranded DNA substrate upon ATP recognition, generating a probe that could be directly amplified via RCA (*Fig.* 8). ATP-dependent ligation followed by RCA in solution was first demonstrated in solution. The signal due to RCA increased approximately 1,500-fold after 10 min of amplification. The signal in the presence of ATP was about 19-fold higher than in the absence of ATP. The apparent activation by ATP is not as high as previously reported,[52] likely because the amplification provided by RCA overrepresented the background ligation reaction. Even without optimization of the ligation and RCA reactions, the positive signal was clearly detectable when compared to the negative control. Additionally these results suggested the potential for analyte quantitation in a multiplexed format.

RCA methods are now being adapted to the detection of proteins.[42,46-48,56] and thus combining RCA with signaling aptamer for the real time protein detection would be an promising alternative to overcome the difficulties caused from real-time PCR to the detection of protein (rather than nucleic acid) ligands; most schemes for immunoPCR do not directly couple protein-binding to PCR amplification, instead requiring wash steps and other processing prior to amplification. To this end, adapting RCA for the detection of proteins to real-time methods has been developed. Analyte-mediated protection of an aptamer from exonuclease digestion preceded oligonucleotide ligation and PCR, and could be used

Fig. 9. Design of the conformation-switching aptamer. (A) General design principles. The original aptamer is shown in red. Blue sequence is added to the 3' end of the aptamer in order to promote the formation of a duplex structure and denature the aptamer. Green sequence is added to the 5' end of the aptamer; there is also a 5' phosphate. In the presence of a protein analyte such as PDGF, the 3' extension (blue) is displaced and hybridizes with the 5' end (green) to form a ligation junction. This conformational change is assisted by the formation of a short hairpin structure involving the 3' extension. Following ligation with T4 DNA ligase, the circularized aptamer can serve as a template for RCA. (B) Sequence and secondary structure of the designed anti-PDGF, conformation-switching aptamer). Additional linker regions between the aptamer and the 3' and 5' extensions serve as primer and probe binding sites for RCA.

Fig. 10. Design of thrombin-mediated structure switching aptamer. (A) Anti-thrombin aptamer. (B) Schematic of structure switching ligation.

for the detection of a few hundred thrombin molecules. Proximity methods in which two aptamers bind adjacent to one another and are ligated have been harnessed to both RCA and PCR amplification technologies[48] and zeptomole amounts of platelet-derived growth factor (PDGF) were detected.[57]

To further facilitate the development of real-time RCA for protein targets in concert with aptamer technology, Litao *et al.*[58] has developed a novel conformation-switching aptamer that can be circularized upon interaction with its protein target, PDGF (*Fig.* 9). Using the structure-switching aptamer, real-time RCA can be used to specifically quantitate PDGF down to the low nanomolar range (limit of detection = 0.4 nM), even against a background of cellular lysate.

Litao and Ellington have also developed a method that directly couple protein detection to PCR amplification. In this method, antisense sequences (blue, in *Figs.* 10A and 10B) added to aptamer (red) termini to promote the formation of a non-binding conformation, similar to the design of other conformation-switching aptamers.[11,59,60] In the presence of analyte, the binding conformation is more favored, a short hairpin stem becomes available for hybridization to an oligonucleotide substrate, and subsequent ligation occurs to form a new amplicon. Additional sequences added to either end of the aptamer served as primer- and probe-binding sites for real-time PCR. Through the different optimization process for designed anti-thrombin and anti-PDGF BB aptamer, as low as 32 pM and 12.8 pM of thrombin and PDGF BB could be detected in 1 μg/mL of cell lysate, respectively.[61]

3.4. Aptamer array

Further extension of signaling aptamers for the applications such as multianalyte biosensing, metabolite profiling, reporting enzymatic activity[62] or affinity capture of specific analytes[63,64] often requires immobilization of the aptamer on a suitable surface.[65-67] Typically, covalent, affinity, or electrostatic interactions between the nucleic acid strands and a suitable surface have been used to immobilize single-stranded DNA on solid substrates.[68]

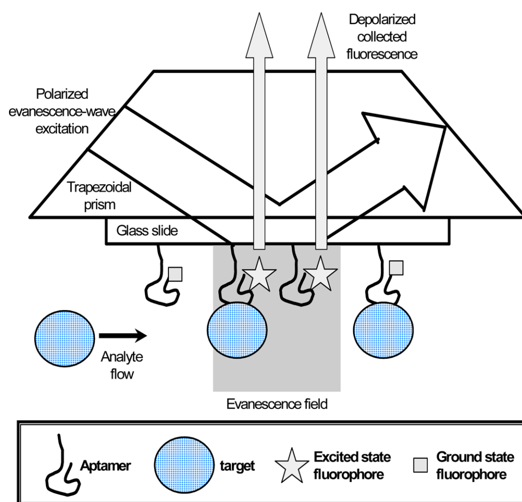


Fig. 11. Detecting fluorescence anisotropy changes in immobilized aptamer biosensors. A thin film of FITC-labeled anti-thrombin aptamer was immobilized on a glass slide and excited by a polarized evanescent wave. The two polarization components of the depolarized fluorescence emission were then monitored to detect and quantify target-dependent changes in fluorescence anisotropy.

Just as DNA arrays have been used to monitor gene expression patterns, aptamer-based arrays could potentially be used to monitor proteome or metabolome expression patterns. Toward this end, a number of strategies have been developed for adapting signaling aptamer to aptamer arrays. The first aptamer chip has been developed by merging the DNA microarray technology with the inherent advantage of fluorescence anisotropy.[67] As illustrated in *Fig.* 11, the anti-thrombin DNA aptamer labeled with FITC was immobilized on a microscope cover slip and the target was quantitated base on the fluorescence anisotropy change of FITC. The effect of covalent immobilization of aptamer onto a solid substrate was also investigated if this modification altered its sensitivity or selectivity for thrombin. The labeled aptamer revealed a dynamic range from nanomolar to micromolar concentration of thrombin; the detection limit calculated from the slope of the calibration curve was 5 nM. Despite the fact that the fluorescently-labeled aptamer in solution appeared to lose some sensitivity for thrombin, these latter results showed

that the labeled, immobilized aptamer had a higher sensitivity (apparent K_d = 47 nM) relative to the unlabeled aptamer ($K_d \approx$ 100 nM, as determined by filter-binding assay[69]).

As was the case for the aptamer functionalized microsphere, aptamer array was shown to be reusable by cycling between PBS buffer and guanidinium hydrochloride solution. However, one of the primary difficulties in using fluorescence anisotropy chips would be devising an optical set-up that could quickly move between different sectors on the chip. This problem has recently been tackled by researchers from the company, Archemix.[65] By incorporating a moveable mirror into their optical platform they could excite a number of aptamer sensor elements either selectively or altogether.

As DNA microarrays utilize nucleic acids to detect their complementary nucleic acids, the potential of aptamer-derived oligos for the detection of various ligands including proteins, small metabolites, and nucleotides have been explored by Yamamoto *et al.*. The principle of this strategy is to redesign aptamers based on ligand mediated self-assembly of bipartite aptamers as previously described, resulting two functional aptamer-deriven units. As illustrated in *Fig.* 12, anti-HIV-1 Tat aptamer has been redesigned as a model system; one of oligos labeled with a functional group is printed on the slide, while the other oligo labeled with fluorophore is incubated with a sample. In the presence of target molecule, Tat, the two oligos reconstituted the core binding region of Tat, resulting fluorescence signal. This so called "analyte-dependent-oligonucleotide modulated array" (ADONMA) has proved the potential for the use in nucleic acid microarrays for detecting various ligands with particular advantages such as; (1) shorter RNA oligonucleotides can be synthesized with higher efficiency than longer molecules at lower cost and (2) modifications to stabilize the nucleic acid are necessary for only a portion of the aptamer.

While glass or plastic substrates are very useful for high throughput screening, paper is extensively used as a barrier for protection from pathogens in applications such as medical face masks and protective clothing, reflecting the fact that paper is inexpensive, disposable, and autoclavable and can have well-defined porosity. In an effort to improve the functionality of paper as a "bioactive paper", incorporation of biorecongition elements coupled with signaling mechanism on the paper surfaces has recently been studied by a network of Canadian academic researchers. Initially, absorbing or immobilizing anti-ATP signaling aptamer[11] on to the cellulose membrane was attempted.[70] It was found that the physical absorption onto microcrystalline cellulose is weak and fully reversible, which confirms that physical absorption is not suitable method for imparting biologically specificity to the cellulose surface with aptamers. However, signaling aptamer covalently bound to the modified cellulose surface was proven

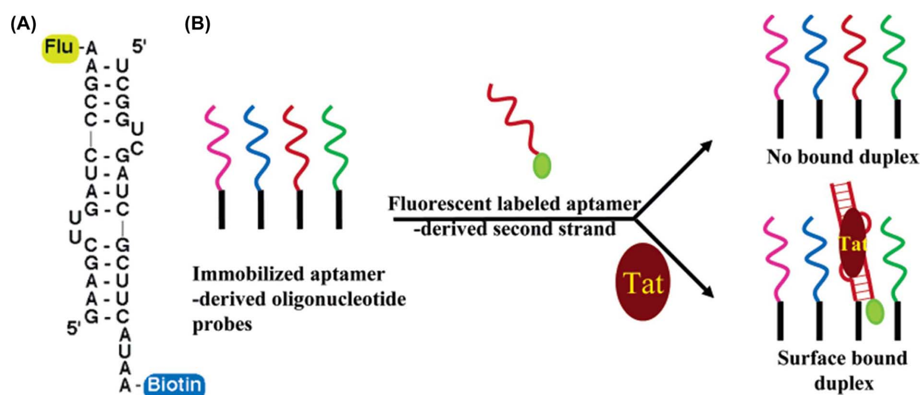


Fig. 12. (A) The aptamer-derived oligos DA-9 and DA-10 used in ADONMA. (B) Schematic representation of ADONMA.

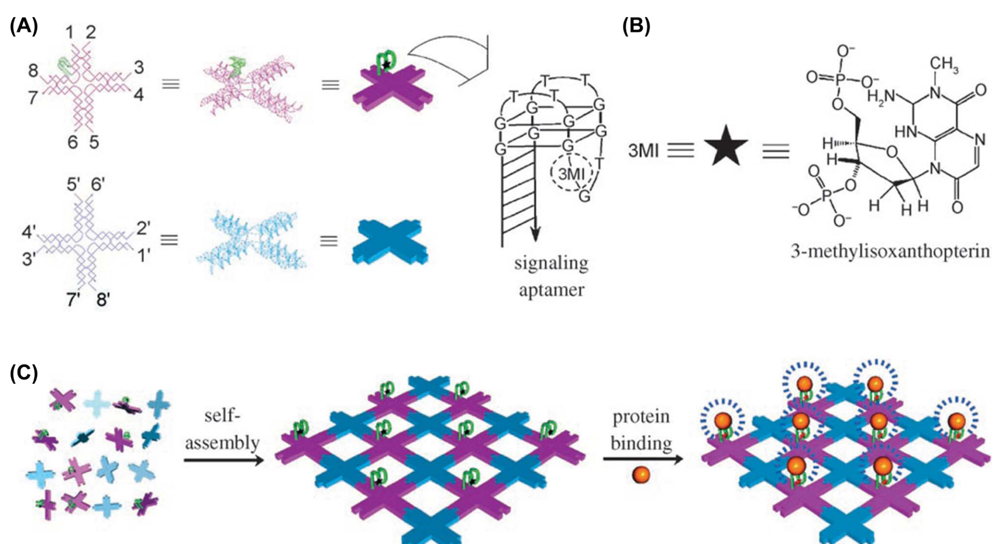


Fig. 13. Schematic drawing of the design and operation of the signaling aptamer array created by DNA-tile self-assembly. (A) The two tiles of the DNA nanogrid array. Complementary sticky ends are numbered as *n* and *n'*. The pink tile contains the thrombin aptamer sequence (a green loop) in a G-quadruplex structure. At the 7-position, the original dT is substituted by the fluorescent nucleic acid analogue 3 MI. (B) The molecular structure of 3 MI. It is also labeled as a black star in the tile without protein. (C) The self-assembly of the two-tile system into a 2D array that displays the thrombin-binding aptamer at every other DNA tile. Upon protein binding, the array containing the signaling aptamers “lights up”.

to maintain its activity and specificity upon ATP binding. It is apparent that amorphous hydrophilic nature of regenerated cellulose is particularly suited for maintaining signaling activity of DNA aptamer, in other words, aptamer activity can be regenerated via the re-wetting with buffer after drying.

As signal amplification methods are critical in improving sensitivity in heterogeneous system as well, label-free detection in concert with amplification methods described above could be adapted on array platform. The same technology combining signaling aptamer/aptazyme with RCA (described in above) was applied on surfaces by engineering signaling aptamer or anti-ATP aptazyme with biotin at its 5’ end to immobilize sensing probes to the straptavin coating on the glass substrate. For example, the RCA primer was conjugated to biotin either via a C3 spacer phosphoramidite or a dT residue at its 5’end. The modified primers were immobilized on streptavidin-coated glass slides. A mixture of analyte-mediated ligated circles and components of a RCA reaction (polymerase, dNTPs) was incubated with the primer array for 30 min at 37°C for the detection of a target (i.e., PDGF). The concatamer products were then hybridized with a Cy3-labeled probe, scanned, and then analyzed. Although the quantitation from the chip-based assay was proved to be more difficult, a real-time RCA array for the detection of proteins and ATP would not only be fast and quantitative, but could also potentially be carried out in heterogeneous solutions without having to wash away unbound affinity reagents or DNA templates. This method can be adapted to almost any aptamer or atpazyme and the real-time nature of the amplification reduces background, improving sensitivity. However, one disadvantage of such methods usually require external modification of the nucleic acid strands with functional groups for immobilization, whereas this modification can’t protect nucleic acids against degradation by species such as nucleases.

An alternative method for immobilization of biomolecules is entrapping into sol-gel derived silica materials. Although sol-gel derived xerogel has been widely employed for immobilization of proteins,[71]

reports on the entrapment of DNA into sol-gel-derived materials are few.[72] The first immobilization of DNA aptamer into sol-gel derived matrix has been demonstrated by utilizing structure-switching antisense DNA aptamer designed by Nutiu *et al.* targeting ATP.[11] Evaluating the sensitivity to ATP, they found that the signaling aptamer retained its structure-switching capabilities, selectivity, and sensitivity. Although the response was slow due to the diffusional barriers to mass transport of the analyte through the silica matrix, entrapment of aptamer results protection of nucleic acid against the degradation by nucleases. This work demonstrates that sol-gel derived materials can be used to successfully immobilize and protect DNA-based biorecognition elements and, in particular, DNA aptamers, opening new possibilities for the development of DNA aptamer-based devices, such as affinity columns, microarrays, and fiber-optic sensors.

On the other hand, synthetic nanoarchitectures based on DNA tile self-assembly have been recently adapted to immobilize signaling aptamers.[73,74] Nano-construct based on DNA tile self-assembly has rapidly progressed in the past few years[75-77] because DNA is an ideal structural material due to its innate ability to self-assemble into highly ordered structures with nanometer scale features based on the simple rules of Watson-Crick base pairing. DNA tile molecules can self-assemble into micro- to millimeter sized two dimensional (2D) lattice domains made from millions to trillions of individual building blocks.[78,79] A unique advantage of these self-assembled DNA tile arrays is the ability to assemble molecular probes with precisely controlled distances and relatively fixed spatial orientations.[80-82] Lin *et al.* first linked an anti-thrombin signaling aptamer (replacing a nucleotide at thrombin binding site with 3MI, a fluorescent analogue) to a DNA tile designed to self-assemble

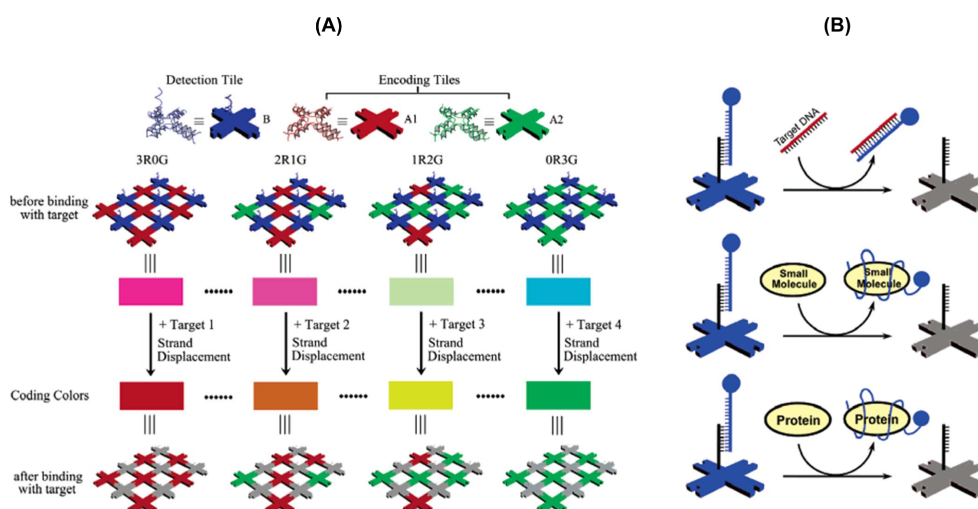


Fig. 14. (A) The design of self-assembled combinatorial encoding DNA arrays for multiplexed detection. Two subgroups of A tiles, A1 modified with a "red" dye (Cy5), and A2 modified with a "green" dye (Rhodamine Red-X), are used to perform the encoding. B tile carries a detection probe that is labeled with a "blue" dye (Alexa Fluor 488) and acts as the detection tile. Each subgroup of B tile carries a different probe but shares the same sticky ends, so that each subset of B tiles can bind to A tiles to form the 2D lattice. The probes are not involved in the DNA tile array formation but dangle out of the array plane through base paring with the anchor probes that are single stranded extensions of one of the strands within the DNA tile. By mixing the A1 and A2 tile at different ratios and then mixing the A tile and a certain B tile at a (A1+A2):Bi) 1:1 ratio, a series of different colored detection arrays can be generated. (B) The detection mechanism. A target binding with the probe displaces the dye-labeled probe (blue) causing a color change of the tile array from the blue-masked color to the two-color mixed encoding color as indicated by the arrows connecting the rectangles in the middle of (A). This mechanism can detect nucleic acid oligos with their complementary DNA probes and proteins or small molecules with their specific aptamers.

into 2D DNA arrays with ~27 nm periodic spacing between neighboring aptamers and then deposited on the surfaces.[73] As sensing schematics are illustrated in *Fig.* 13, this self-assembled signaling aptamer DNA arrays exhibited up to 2-fold fluorescence increase upon binding to thrombin. The K_d for 4 ± 2 nm obtained from this method was ~ 2.5-fold higher compared with the previously reported values, ca 10 nM[83,84] and the detection limit was ~ 20 nM based on the S/N level. Because this self-assembled DNA arrays are water soluble, the molecular recognition is occurred in solution, whereas the physical measurements of the sensing events is on the surface. As a result, water-soluble signaling aptamer can maintain its inherent binding affinity in compared to binding on solid surface. Overall, the advantages of nanotechnology are (1) no surface-chemistry steps involved which was required in microarrays and (2) no loss of binding efficiency compared with unmodified aptamers.

Self-assembled signaling aptamer DNA tile has been further extended to combinatorial arrays for multiplex biosensing.[74] In this approach, 2 sets of DNA tiles are encoded with green and red colored fluorescent probes and then mixed with a blue fluorophore labeled molecular probe, generating various colors depending on target molecules as shown in *Fig.* 14. As a model system, two partial DNA sequences of the viral genomes and two complementary sequences of anti-ATP aptamer and anti-thrombin aptamers were used to construct combinatorial encoding array. Upon addition of individual targets or mixture of different combination of targets, the presence of each target reveals a color specially coded to the array containing the probe for that target. Although the apparent K_d for the aptamer binding molecules are much weaker compared with other techniques (~ 400 nM for thrombin and ~ 600 μM for ATP), this new strategy provides unique advantages for parallel biosensing along with advantages such as no bioconjugation process and accurate control of spacial distance between neighboring probes and binding process to allow more efficient binding of targets to the probes.

4. Conclusions

Aptamers are the only biomolecules other than antibodies that have universal binding properties. The high sensitivity and specificity of aptamers towards target molecules, and the ease with which they can be chemically synthesized and engineered, make aptamers ideally suited for sensor applications. Fluorescently-labeled aptamers can be engineered either via rational design or *in vitro* selection to function as biosensors for the real time quantitation of targets in solution. A variety of fluorescent properties, including fluorescence intensity, FRET, or anisotropy changes can be monitored. Because of their exquisite specificity and the ease with which they can be engineered, aptamers should prove particularly useful for diagnostic applications. For example, aptamers can be used to distinguish between different isoforms of proteins that are differentially expressed in normal versus cancerous cells,[65] or to detect post-translational modifications such as phosphorylation.[85] Aptamers can also be immobilized on arrays and used to detect their cognate ligands. This has enabled the development of aptamer-based chip arrays for high-throughput screening applications.

Aptamers have been shown to undergo ligand-dependent conformational changes and can be joined to ribozymes to create allosteric ribozymes (aptazymes).[86,87] To date almost all of these aptamer and aptazyme biosensors have been generated by empirical design[88,89] or *in vitro* selection,[21,86,90] however, algorithms for the prediction of nucleic acid secondary structure have advanced to the point where nucleic acid secondary structures can be rapidly enumerated based on nucleic acid sequence.[91] Therefore, the computational design methods for the generation of biosensors would be extremely valuable for a variety of reasons. First, the ability to design nucleic acid biosensors further validates the utility of methods used for the computational prediction of nucleic acid secondary structures. Second, computational selection should be inherently faster than experimental selection methods, and thus the time and effort required for

the development of biosensors may be greatly decreased. Lastly, to the extent that computational models match experimental results, it should be possible to finely control the design process, generating biosensors with optimal sensitivities, signal-to-noise ratios, and dynamic ranges.

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