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Review

Aptamer binding assays for proteins: The thrombin example—A review

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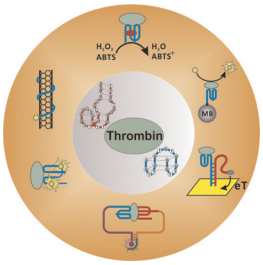
HIGHLIGHTS

- We review affinity assays that are based on aptamer binding to thrombin.
- Thrombin is most frequently used to demonstrate the proof-of-principle.
- The principles for thrombin assays are applicable to other molecular targets.
- The thrombin example describes key features of aptamer affinity assays.

GRAPHICAL ABSTRACT



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ABSTRACT

Experimentally selected single-stranded DNA and RNA aptamers are able to bind to specific target molecules with high affinity and specificity. Many analytical methods make use of affinity binding between the specific targets and their aptamers. In the development of these methods, thrombin is the most frequently used target molecule to demonstrate the proof-of-principle. This paper critically reviews more than one hundred assays that are based on aptamer binding to thrombin. This review focuses on homogeneous binding assays, electrochemical aptasensors, and affinity separation techniques. The emphasis of this review is placed on understanding the principles and unique features of the assays. The principles of most assays for thrombin are applicable to the determination of other molecular targets.

Contents

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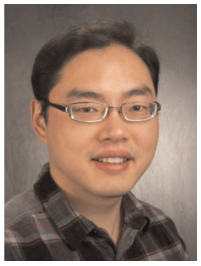

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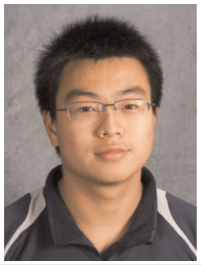

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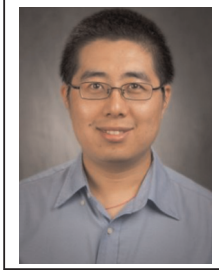

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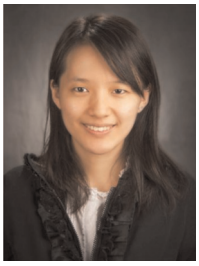

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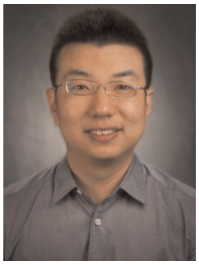

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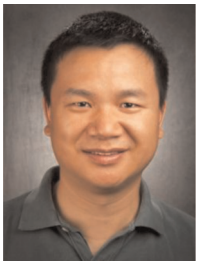

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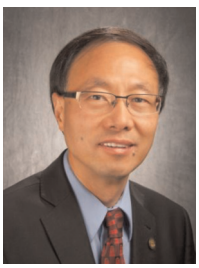

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1. Introduction

Since their introduction in 1990, single-stranded DNA and RNA aptamers [1,2] with high binding affinity and specificity have been used in the development of diverse bioanalytical assays [3]. Aptamers for specific targets can be artificially generated by an *in vitro* process, systematic evolution of ligands by exponential enrichment (SELEX), which consists of several repeated rounds of binding, partition, and amplification [4]. Many aptamers have been selected for various targets, ranging from proteins to small molecules and whole cells [5–9]. As complements to antibodies, aptamers exhibit several unique features, including ease of chemical modification, reversible folding, and excellent stability [7,10]. These features are beneficial to the development of affinity binding assays. Therefore, nearly 5000 papers published between 1990 and 2013 have made use of aptamers for analytical developments. Among these papers, ~20% deal with thrombin and its aptamers. Thrombin binding to its aptamer is the most commonly used model system to demonstrate the proof-of-concept of the aptamer-based affinity assays.

Thrombin (Fig. 1A), a Na^+-activated, allosteric serine protease, acts as the central protease in the coagulation cascade [11–14]. After vascular injury, thrombin is rapidly generated from inactive zymogen prothrombin by a series of enzyme cleavages. Activated thrombin cleaves fibrinogen into fibrin and clots are formed at the site of vascular injury to prevent bleeding. With crucial roles in physiological and pathological coagulation, thrombin and inactivated prothrombin are involved in various diseases, such as Alzheimer's disease and cancers [15,16]. Thrombin derived from bovine sources is also a U.S. Food and Drug Administration (FDA) approved hemostatic product to stop bleeding in surgery [17]. Thrombin is not present in blood under normal conditions, but its inactive form prothrombin is secreted into blood at a concentration of 1.2 μM. During coagulation, the concentration of thrombin may vary from pM to μM levels [18,19]. Accordingly, assays need to have sufficiently low detection limit and appropriate dynamic range. Clotting-based assays [20], enzymatic activity-based assays [21], and immunoassays [18,22], have been developed for the detection and quantification of thrombin in the blood.

Most of the assays for human thrombin deal with the intact α-thrombin (295 amino acids). Human α-thrombin can be proteolyzed to form β-thrombin and γ-thrombin [23]. Cleavage of the B chain of thrombin at the Arg106-Tyr107 bond generates β-thrombin (189 amino acids). Further cleavage of β-thrombin at the Lys190-Gly191 bond produces γ-thrombin. These proteolyzed forms of thrombin are much less active than α-thrombin. The ability of β-thrombin and γ-thrombin to act in the blood clotting process (to clot fibrinogen) [24] or to act as a protease (to cleave thrombospondin) [25] is markedly decreased. We will focus on α-thrombin because of its physiological significance.

The first aptamer binding to human α-thrombin was described by Bock et al. [26] in 1992. This 15-mer DNA oligonucleotide (5′-GGT TGG TGT GGT TGG-3′) can form a stable intramolecular G-quadruplex structure, which is in an antiparallel orientation with a chair-like conformation, as shown in Fig. 1B [27]. This 15-mer thrombin aptamer interacts with one of the two anion binding sites of thrombin, the fibrinogen-recognition exosite, with a dissociation constant (K_d) of ~100 nM. Another thrombin-binding aptamer is a 29-mer DNA oligonucleotide (5′-AGT CCG TGG TAG GGC AGG TTG GGG TGA CT-3′), which binds to the heparin-binding exosite of thrombin with a higher affinity (K_d = 0.5 nM) [28]. This 29-mer thrombin aptamer also contains a G-quadruplex structure (Fig. 1C). These aptamers do not bind to β-thrombin or γ-thrombin, because the binding sites, such as exosite I (including K36, H71, R73, R75, Y76, R77, and K109/110) and exosite II (including R93, K236, K240, R101, and R233), are partly or fully lost due to proteolytic cleavage of α-thrombin.

Several unique features of the thrombin-binding aptamers may contribute to the frequent choice of thrombin and its binding aptamers in demonstrating the concept of many assays. Firstly, both aptamers are short oligonucleotides, consisting of 15 n.t. and

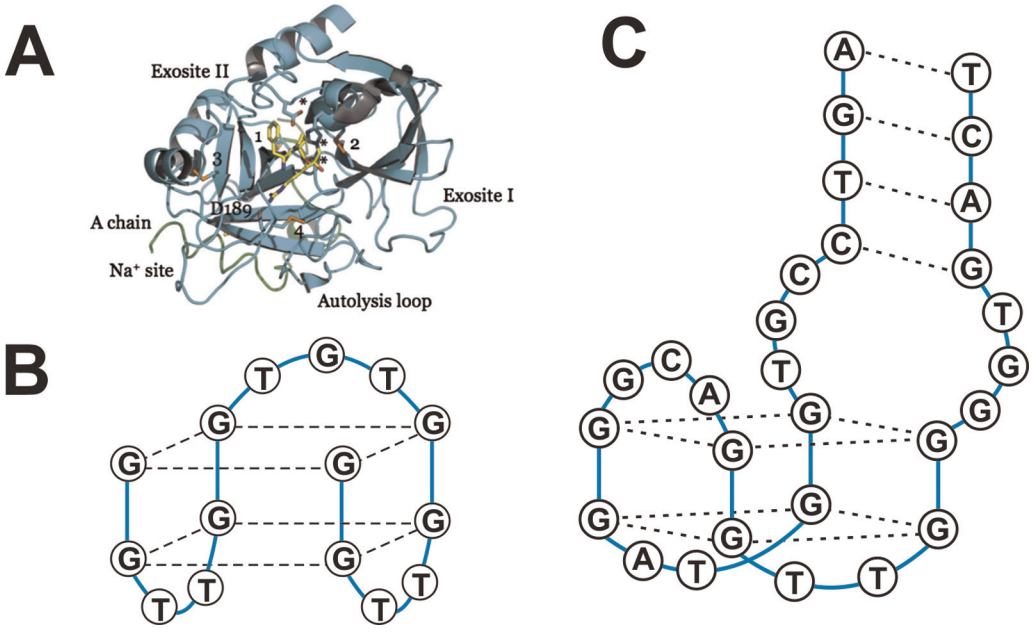

Fig. 1. Thrombin (A) and two frequently used aptamers (B and C) that bind to thrombin. (A) Thrombin is a protease critical to the blood coagulation process. The fibrinogen-recognition site and the heparin-binding site are highlighted [14]. (B) The 15-mer and (C) 29-mer DNA aptamers that can bind to thrombin. G-quadruplex structures are shown in the conformation of these aptamers.

29 n.t. It is relatively easy and inexpensive to synthesize these short aptamers and to add reporter and/or capture groups on them. Secondly, the K_d values of the two aptamers vary by two orders of magnitude, which is useful and suitable for design of assays that require different binding affinities. Thirdly, the two aptamers can bind on two distinct binding sites of thrombin without interfering with each other's binding. Thus, it is easy to establish detection methods using a "sandwich" format. Currently used immunoassays can also be converted to aptamer-based assays by replacing the antibodies with the aptamers. Fourthly, both aptamers fold into a G-quadruplex structure and their conformational switch is triggered by binding with thrombin. The G-quadruplex structure is also known as a DNAzyme, and the DNAzyme can catalyze H_2O_2-mediated oxidation reaction with hemin as a cofactor [29]. This reaction and the structure switch can be used for signal generation of detection method. Thus, the thrombin-binding aptamers can serve as both affinity reagents and reporter probes. Finally, thrombin is a protease enzyme that can specifically catalyze the cleavage of proteins and peptides. The binding sites of aptamers are different from the enzyme catalytic sites. With or without aptamer binding to thrombin, the enzymatic activity of thrombin can be maintained and utilized for the development of detection methods.

In this review, we illustrate the development of more than one hundred analytical techniques that rely on and take advantage of thrombin-binding aptamers. We focus on recent developments in three areas: homogeneous assays, electrochemical aptasensors, and affinity separation. The emphasis is placed on the principles of the techniques, with critical insights into the unique features and remaining challenges.

2. Homogeneous assays

Homogeneous assays do not require immobilization, separation, or washing procedures. This "mix and read" format can potentially deliver simplicity to end users, especially for on-site and resource-limited applications. Usually target recognition and signal output are two essential requirements for homogeneous assays. In this section we introduce some typical assays utilizing thrombin aptamer as the target recognition element (including single-recognition and dual-recognition) combined with various signal output methods, e.g., molecular beacon, catalytic beacon, enzymatic amplification, nanomaterials and proximity-based signal amplification. The first four types of assays involve a single-recognition event, and the last one involves a dual-recognition event.

2.1. Molecular beacons

Affinity binding of the aptamer to thrombin and the aptamer's structure switching upon binding were used for developing molecular beacon approaches. A molecular beacon was first introduced to detect a nucleic acid target with a specific sequence [30]. A single-stranded nucleic acid sequence (DNA or RNA) contained complementary bases at the 5′ and 3′ ends and each end was labeled with either a fluorophore or a quencher. A stem-loop structure was formed within the molecule and the resulting close proximity of the two ends led to fluorescence quenching (Fig. 2A). When a target with complementary sequences to the loop was present, hybridization between the target sequence and the loop separated the fluorophore and the quencher, altering the

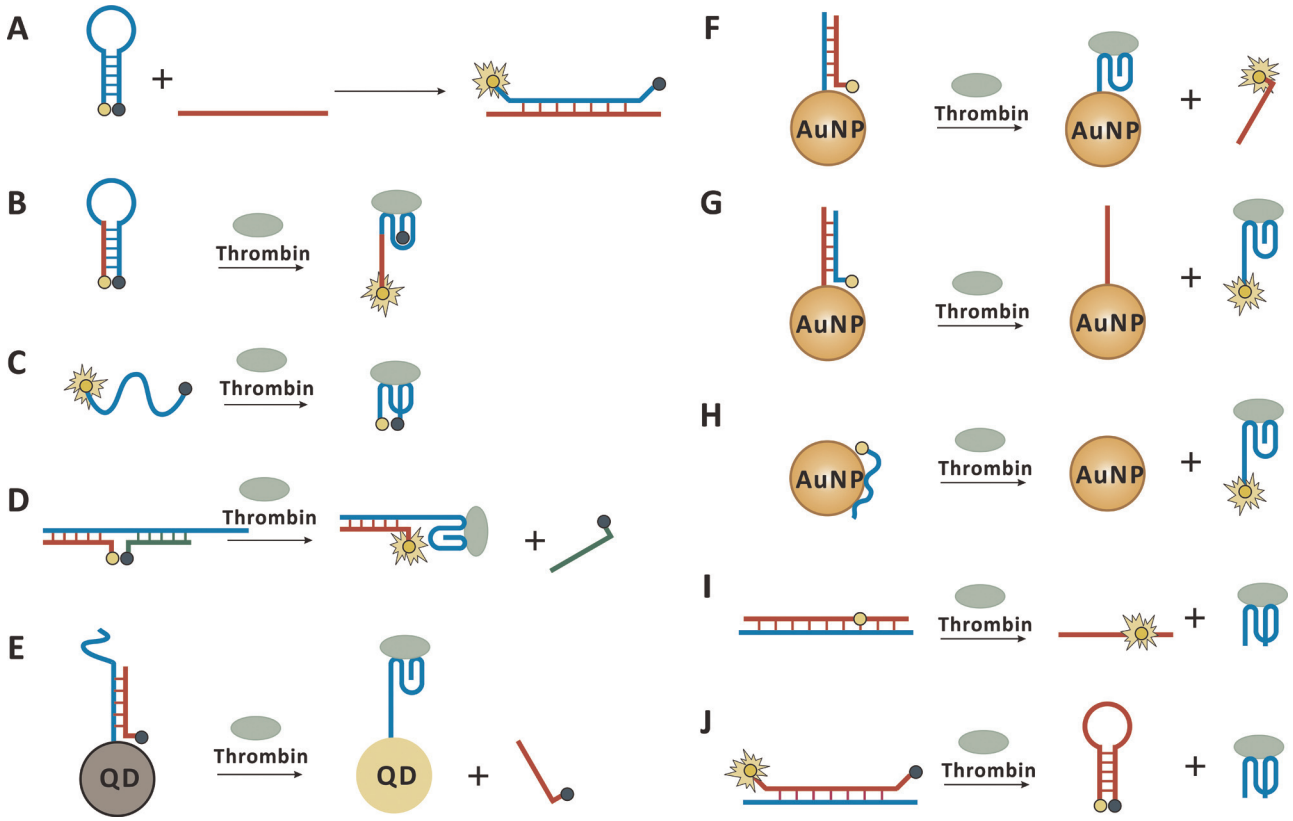

Fig. 2. Strategies for homogeneous assays based on molecular beacons. (A) Detection principle of molecular beacons; (B) "turn on" format thrombin aptamer beacon; (C) "turn off" format thrombin aptamer beacon; (D) "competitive" format thrombin aptamer beacon; (E) quantum dots duplex beacon; (F–H) gold nanoparticles thrombin aptamer beacon; and (I and J) competitor DNA molecular beacon, with both (I) "turn-on" and (J) "turn-off" strategies. The drawing is not to scale.

fluorescence resonance energy transfer (FRET) and generating fluorescence to be detected.

Inspired by the application of molecular beacons in DNA detection, Tan's group utilized molecular beacons to study protein–DNA interactions, using single-stranded DNA binding protein (SSB) to demonstrate the proof-of-principle [31]. Hamaguchi et al. [32] adapted this design for thrombin detection and termed it "aptamer beacon", broadening the applicability of the molecular beacon. The thrombin aptamer was first extended by a few nucleotides at the 5′ end which was complementary to the 3′ end, forming a stem-loop structure (or hairpin structure) (Fig. 2B). Binding of thrombin to the aptamer induced conformational change of the aptamer, increasing the distance between the fluorophore and the quencher and therefore reducing the quenching effect and producing fluorescence. This is a typical "turn-on" format of assay based on fluorescence detection. Li et al. [33] further reported a "turn-off" format, including both quench-type and FRET-type aptamer beacons for real-time detection of thrombin (Fig. 2C). Ratiometric imaging with a FRET-type aptamer beacon showed better quantitation results, compared to single wavelength fluorescence intensity detection. The authors noted severe interference and suggested the need to prevent non-specific interactions.

Designing the aptamer beacons requires some prior knowledge of the secondary or tertiary structures to prevent undesirable interactions and quenching of fluorescence by guanine in the aptamer sequence. To overcome this limitation, Nutiu and Li [34] devised a "structure switch signaling aptamer" (Fig. 2D). They constructed a duplex structure, using a fluorophore-labeled DNA and a quencher-labeled DNA to hybridize with the unmodified aptamer. The fluorophore was quenched at maximum efficiency when no thrombin was present. Following the addition of thrombin, structure switching of the aptamer due to its binding to thrombin released the quencher, restoring the fluorescence signal.

More competitive molecular beacons were reported based on the above strategy. Levy et al. [35] labeled an aptamer with quantum dots (QDs) and a short piece of competitor DNA with a quencher to construct a duplex beacon (Fig. 2E). When thrombin interacted with the duplex beacon, the conformational change disrupted the duplex structure (structure switching) and restored the fluorescence from QDs. Wang et al. presented an assay using gold nanoparticles (AuNPs) as the fluorescence quencher to design the competitive aptamer beacon [36]. The fluorophore was labeled either on the aptamer or on the competitive DNA to achieve a "turn-on" assay (Fig. 2F–H). Li et al. [37] proposed a generic design strategy for a simpler competitive molecular beacon that required only the competitor DNA to be modified, both "turn-on" (Fig. 2I) and "turn-off" (Fig. 2H) strategies were demonstrated.

2.2. Catalytic beacons

Catalytic beacons make use of specific DNA sequences that have catalytic activity. These DNA sequences, also known as DNAzymes, can catalyze series of reactions, such as H_2O_2-mediated oxidation, DNA self-modification, nucleic acid cleavage, and porphyrin metalation. Li et al. [38] (Fig. 3A) have developed a catalytic molecular beacon consisting of a thrombin-binding aptamer sequence that binds with a split G-quadruplex DNAzyme. The DNAzyme with hemin as a cofactor can catalyze the H_2O_2-mediated oxidation of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). The product can be detected by UV–vis absorption spectrometry. The method showed a detection limit of 20 nM for thrombin analysis.

Wang's group [39] further developed a catalytic molecular beacon consisting of two hairpin structures and a split G-quadruplex DNAzyme in the middle. The two loops of this molecular beacon consisted of the thrombin aptamer sequence and the complementary sequence of target DNA, which were utilized to sense thrombin and single-stranded DNA. The G-quadruplex DNAzyme effectively catalyzed the H_2O_2-mediated oxidation of 3,3′,5,5′-tetramethylbenzidine sulfate (TMB) to generate a colorimetric signal. When the thrombin concentration increased, thrombin bound to the aptamer sequence, and the structure of DNAzyme was dissociated, which caused decrease in catalytic activity and color change. The detection limit of thrombin reached 20.5 nM.

Zhang et al. [40] also reported a label-free and sensitive fluorescence method for detection of thrombin using a G-quadruplex-based DNAzyme. The aptamer sequence was able to bind hemin to form the G-quadruplex-based DNAzyme, and thrombin significantly enhanced the activity of the G-quadruplex-based DNAzyme. The G-quadruplex-based DNAzyme was found to effectively catalyze the H_2O_2-mediated oxidation of thiamine, giving rise to fluorescence emission. The limit of detection for thrombin was 1 pM. Another strategy was based on conjugation of the catalytic beacons to QDs; the DNAzyme catalyzed the oxidation of luminol in the presence of H_2O_2. A chemiluminescence resonance energy transfer (CRET) signal was produced when thrombin was present [41].

The binding of the RNA sequence that is complementary to thrombin aptamer and hairpin ribozyme catalysis gave rise to a different method of thrombin detection (Fig. 3B). The aptamer sequence completely abolished the catalytic activity of ribozyme. When thrombin was present, however, the aptamer bound to thrombin and the ribozyme was activated again; a positive signal was generated after cleavage of the RNA and release of the fluorescent label [42].

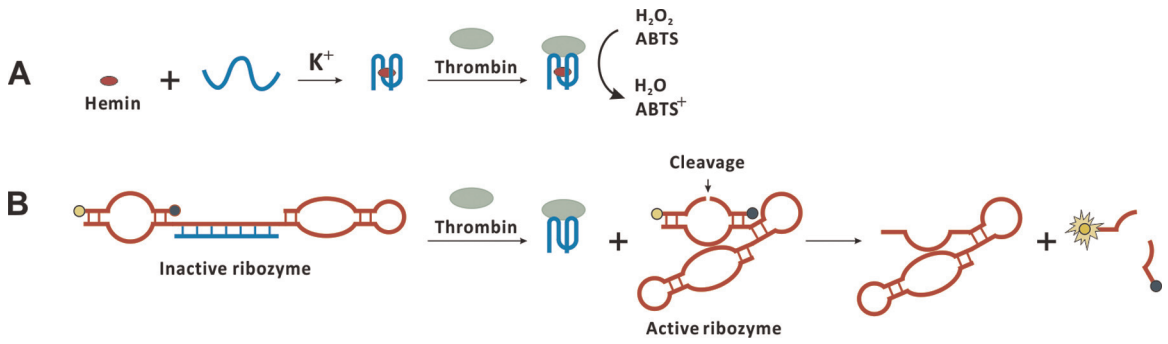

Fig. 3. Catalytic beacons. (A) Catalytic molecular beacon consists of a thrombin-binding aptamer that binds with a split G-quadruplex DNAzyme. The DNAzyme with hemin as a cofactor can catalyze the H_2O_2-mediated oxidation of 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) which can be detected by UV–vis absorption spectrometry. (B) The RNA sequence is complementary to a thrombin aptamer, which completely abolishes the catalytic activity of ribozyme. When thrombin is present, the aptamer binds to thrombin and the ribozyme is activated again and a positive signal is generated after cleavage of the RNA and release of the fluorescent label.

2.3. Enzyme-assisted assays

Enzymatic amplification of target molecules and enhancement of detection signals are two major approaches to improve the sensitivity. A typical example of target amplification is polymerase chain reaction (PCR) of target nucleic acid sequences. However proteins are not directly amplifiable by PCR.

Recently, the cyclic enzymatic signal amplification (CESA) method was developed for highly sensitive detection of nucleotides, DNA, and proteins [43]. This method utilizes enzymatic reactions or other signal amplification mechanisms to transduce target binding events to measurable signals. The advantages of this method are its simplicity, high sensitivity, low cost, and the ability to transform non-amplifiable proteins into amplifiable oligonucleotide signals. However, specific recognition sites are often required for the specific enzymes, which would limit the design of the oligonucleotides. Endonucleases and exonucleases are the most frequently used enzymes for CESA.

Yang's group [44] proposed a detection system consisting of a hairpin aptamer probe, a linker DNA, two sets of DNA-modified AuNPs, and nicking endonuclease (NEase) (Fig. 4A). In the absence of a target, such as thrombin, the hairpin aptamer probe and linker DNA can stably coexist in solution. Then, the linker DNA can assemble two sets of DNA-modified AuNPs, inducing the aggregation of AuNPs. However, in the presence of a target, the complex of the aptamer and the target is formed and the complex can hybridize to the linker DNA. The NEase recognizes a specific nucleotide sequence and cleaves the linker DNA into two fragments. After nicking, the aptamer–target complex is released, the cycle of binding and cleaving the linker DNA starts anew. The cleaved fragments of linker DNA are not able to assemble two sets of DNA-modified AuNPs, and thus the red color of the separated

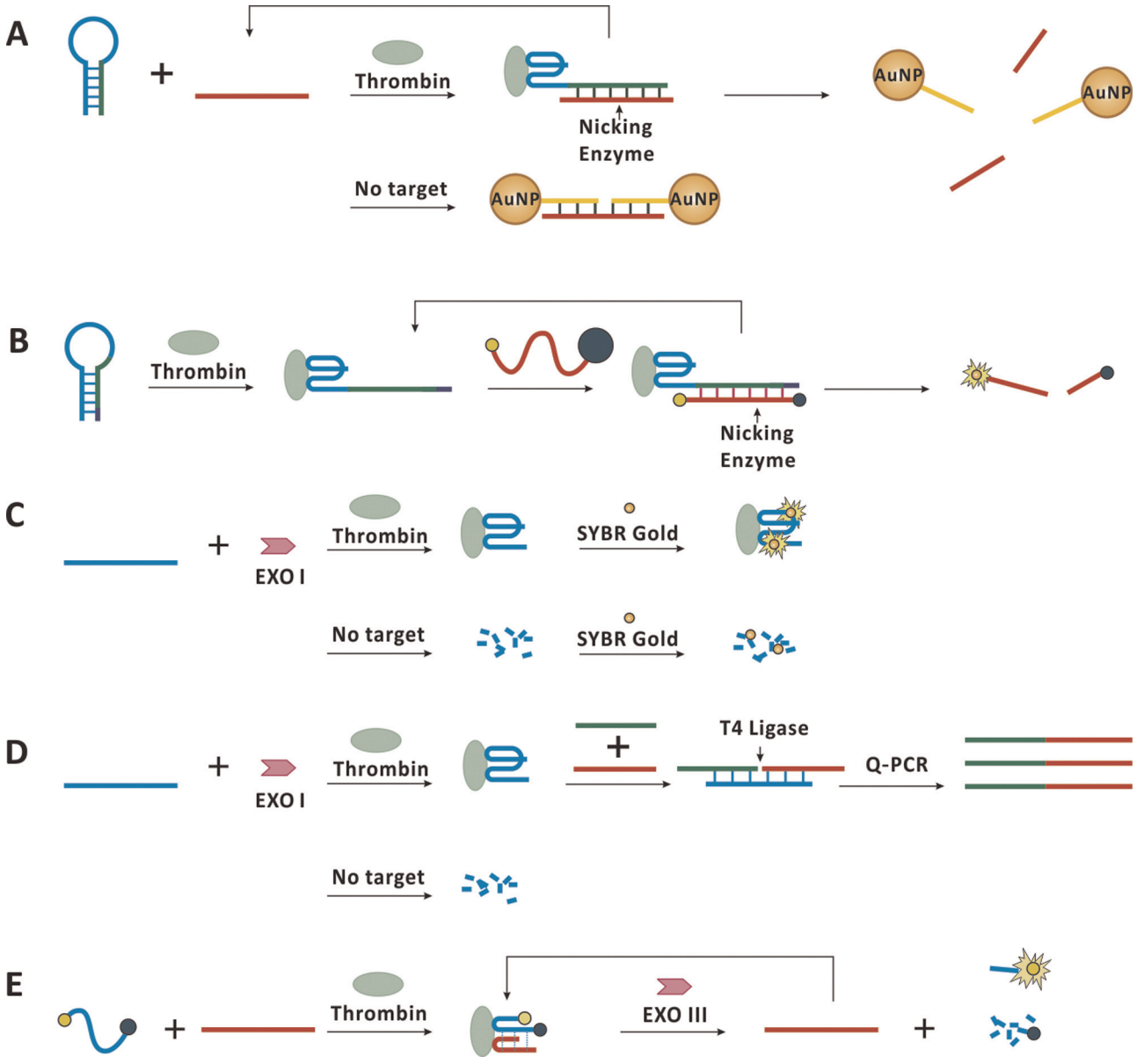

Fig. 4. Strategies for homogeneous assays based on cyclic enzymatic signal amplification (CESA). (A) AuNPs aggregation or dispersion by nicking endonuclease; (B) cleavage of black hole quencher-quenching fluorescence DNA probe by nicking endonuclease to regenerate fluorescence signals; (C) structure-switching aptamer after binding to thrombin is protected from Exo I digestion, and is subsequently detected with SYBR Gold. (D) Structure-switching aptamer after binding to thrombin is protected from Exo I digestion. The subsequent PCR amplification of the ligated products offers sensitive detection. (E) Binding-induced aptamer assembly and cleavage by Exo III generate fluorescence signals for detection.

AuNPs can be observed. With the naked eye, human thrombin can be detected with a detection limit of 50 pM.

Xue et al. [45] developed a protein assay based on a hairpin probe and NEase-assisted CESA (Fig. 4B). The hairpin probe is designed to contain an aptamer for target recognition. A short black hole quencher (BHQ)-quenching fluorescence DNA probe (BQF probe) contains the recognition sequence and cleavage site for the nicking enzyme. Binding to the target leads to the conformational change of the hairpin probe from hairpin shape to open form, facilitating the hybridization between the hairpin probe and the BQF probe. The fluorescence signal is amplified through continuous enzyme cleavage of the BQF probe to remove the BHQ. This method can detect thrombin with a detection limit of 100 pM. Using the same principle [46], the authors achieved thrombin detection using aptamer–protein–aptamer conjugates and Nb.BbvCI nicking enzyme amplification. The limit of detection of thrombin was improved to 40 pM.

Besides endonucleases, exonucleases are also used in the CESA method. Zheng et al. [47] demonstrated a sensing strategy employing structure-switching aptamers (SSAs), exonuclease I (Exo I) and SYBR Gold to detect a broad range of targets including thrombin (Fig. 4C). Once the structure switching aptamer binds with the target, it folds into a secondary structure, such as quadruplex or Y shape, and it is more resistant to nuclease digestion. SYBR Gold can be used to stain the aptamers for fluorescence quantification. In the absence of the target, the unbound aptamer is digested by Exo I, and the digested fragments cannot be stained by SYBR Gold. The assay was validated by detecting thrombin, ions, and small molecules. The authors were able to detect 680 nM thrombin spiked in buffer solution and in the human serum sample.

Wang et al. [48] incorporated Exo I, ligase and real-time PCR to achieve ultrasensitive detection (Fig. 4D). Thrombin binds to the aptamer and thereby protects the aptamer from degradation by Exo I, whereas the unbound aptamer is digested by Exo I. The protected aptamer serves as a linker to hybridize with two short oligonucleotides. The two oligonucleotides are joined together using T4 ligase, and the product is amplified with real-time PCR. Because of the PCR amplification, this exonuclease protection assay is extremely sensitive, and as few as a hundred molecules of thrombin can be detected.

One of the common limitations of the above enzyme amplification approaches is that a specific nucleotide sequence is often needed for the enzyme recognition. Sequence independent enzymes, such as exonuclease III (Exo III), can be used to overcome this problem [43]. Liu et al. [49] applied the analyte-induced self-assembly of the aptamer subunits and Exo III as an amplifying catalyst (Fig. 4E). The system consists of two aptamers that enable the self-assembly of the thrombin–G-quadruplex aptamer complex. One aptamer is modified at its 3′- and 5′- ends with the BHQ2 quencher and Cy3 fluorophore, respectively. In the absence of thrombin and the Exo III, the two aptamers exist as single-stranded configurations, and the fluorescence of Cy3 is quenched by BHQ2. The addition of thrombin results in the assembly of the two aptamer subunits into the thrombin/aptamer G-quadruplex structure, in which the 3′- and 5′- ends of the aptamers are cooperatively stabilized into a duplex domain that leads to Exo III-catalyzed digestion. The fluorescence of Cy3 is generated after the labeled aptamer is cleaved by Exo III. This reaction recycles the analyte, thereby achieving amplification. The detection limit for this analysis of thrombin corresponds to 89 pM.

2.4. Nanomaterial-assisted assays

New nanomaterials with extraordinary optical properties have been successfully applied in developing aptamer biosensors for thrombin detection. Examples of these nanomaterials include single-walled carbon nanotubes (SWCTs) [50], graphene [51], mesoporous carbon microparticles [52], poly(*m*-phenylenediamine) rods [53], and carboxylic carbon nanoparticles [54]. SWCTs [50] can non-covalently absorb single-stranded DNA, and possess high efficiency of fluorescence quenching. These properties have made SWCTs useful for developing a fluorescence quenching assay without the need of labeling a quencher. An aptamer was fluorescently labeled. In the absence of the target, the aptamer was adsorbed on the SWCTs and the fluorescence was quenched. In the presence of the target, the binding of the target with the aptamer restored the tertiary structure of the aptamer (Fig. 5). Increased distance between the fluorescence group on the aptamer and the SWCTs reduced fluorescence quenching and "turned on" fluorescence for quantification. The limit of detection for thrombin was estimated to be 1.8 nM.

Similarly, nano-C_{60} was used to develop an aptasensor for thrombin [55]. The fluorescence emission of the FAM-labeled aptamer was quenched by nano-C_{60}. However, with increasing thrombin concentrations, the fluorescence intensity also increased, giving rise to an assay for thrombin. A detection limit of 1 nM was achieved with this aptasensor.

Wang and co-workers [36] used a fluorescently labeled aptamer as the reporter and AuNPs as the quencher. Binding of thrombin changed the structure of the aptamer, releasing the aptamer from AuNPs. When the aptamer was released from the surface of AuNPs, the fluorescence of the aptamer was recovered. AuNPs are one of the most common nanomaterials used for developing thrombin assays, probably because AuNPs can serve as a fluorescence quencher and reporter.

Platinum nanoparticles (PtNPs) are also commonly used for protein detection after being functionalized with nucleic acids. The PtNPs have catalytic properties towards luminol and H_2O_2 for the generation of a chemiluminescence signal [56]. Using aptamer-functionalized PtNPs, Higuchi et al. [57] developed a colorimetric method on the basis of oxidizing 3,3′,5,5′-tetramethylbenzidine sulfate (TMB). Photoluminescence of QDs has also been used for developing an assay for thrombin. PbS QDs were functionalized with an aptamer for thrombin. Binding of thrombin to the aptamer quenched photoluminescence of the PbS QDs. Decreasing photoluminescence signal with increasing thrombin concentration gave rise to an assay for thrombin [58].

The peroxidase-mimicking Fe_3O_4 magnetic nanoparticles can be used to catalyze the H_2O_2-mediated oxidation of TMB, which leads to a color change [59]. After Fe_3O_4 nanoparticles were encapsulated by mesoporous silica and treated with hydrochloric acid, a modified aptamer was bound on the surface of the catalytic element [60]. Without the target, H_2O_2 and TMB could enter the silica layer and react directly with Fe_3O_4 nanoparticles inside the

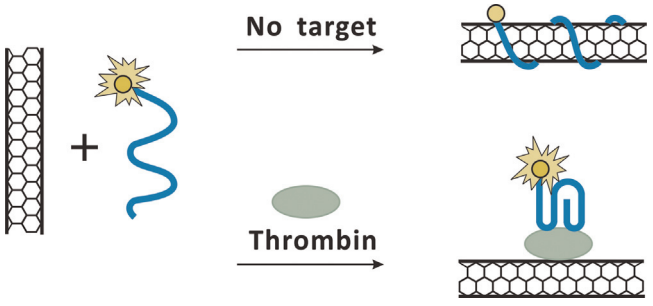

Fig. 5. Single-walled carbon nanotubes (SWCTs) appliedd to the development of aptamer biosensors for thrombin detection. (Top) Fluorescently labeled aptamer is adsorbed on SWCTs and the fluorescence is quenched. (Bottom) Thrombin binding to the aptamer forms G-quadruplex, increasing distance between the fluorescence group and SWCTs and restoring the fluorescence signal.

silica layer, resulting in a strong absorbance signal. Following the addition of thrombin, however, the aptamer could react with thrombin and form a block layer outside the nanoreactors, which made it difficult for substrates to diffuse inside the nanoparticles. This resulted in a decrease in absorbance signal. A detection limit of thrombin was 0.19 nM.

2.5. Assays based on binding to two aptamers and formation of DNA assembly

Binding of two aptamers to two distinct epitopes of the same target molecule is useful for developing highly sensitive assays. Assembly of new DNA motifs triggered by the dual-binding event can be detected with high sensitivity and specificity. Because the signal generation requires simultaneous binding of two aptamers to the same target molecule, the background commonly encountered in the single-recognition approaches is substantially reduced. Thus, assays based on detection of binding-induced assembly can achieve better detection limit (lower background) compared to assays involving a single binding event.

Typically, two DNA probes, each carrying an affinity ligand, are used to bind with the target. The two DNA probes are designed in such a way that they do not hybridize on their own or self-assemble. Only when both affinity ligands bind to the target molecule, the two DNA sequences are brought into close proximity, promoting the assembly of a new DNA motif. This newly assembled DNA motif is then detected as an indirect measure of the target. The background can be significantly reduced, because the incidence of two non-specific
binding events to the same target molecule is much lower than of one non-specific binding event to the target molecule. Therefore, a much lower detection limit can be achieved by using the dual-recognition and binding-induced assembly approach.

Fredriksson et al. [61] first reported a "proximity ligation assay" (PLA) utilizing the dual-recognition of two aptamers for thrombin. Two DNA probes were used and each was conjugated to an aptamer that recognized a different epitope of thrombin. After the incubation of the two probes with the target, two binding events brought the two DNA probes into close proximity. A "connector" oligonucleotide, with sequences complementary to the probes, was used to hybridize with the probes. A DNA ligase was then used to join the 5′-end of one probe with the 3′-end of the second probe, forming a longer piece of DNA. The ligated piece of DNA was finally detected by real-time PCR (Fig. 6A). Using this strategy, the authors could successfully improve the detection limit by up to three orders of magnitude, compared to the conventional enzyme linked immunosorbent assay (ELISA). The authors used more than 10,000-fold higher concentrations of the connector than the probes to drive the formation of the DNA assembly. Despite the high concentration of the connector oligonucleotide, the analytical background remained low because the ligation took place only when both affinity probes were bound to the same target molecule and were brought into close proximity.

Heyduk et al. developed a dual-recognition assay that did not require the ligation step [62]. They used two fluorescently labeled aptamers to comprise a FRET pair (Fig. 6B). A 29-mer aptamer was labeled with fluorescein as the fluorescence donor and a 15-mer aptamer was labeled with dabcyl as the fluorescence acceptor. Binding of the two aptamers to the same thrombin molecule brought the fluorescence donor and acceptor into close proximity, facilitating FRET. Detection of FRET signals provided quantification for thrombin. This assay eliminated the ligation step, but did not have amplification capacity. As a consequence, the detection limit was 50 pM, inferior to the proximity ligation assay.

Guisto et al. used rolling circle amplification (RCA) to achieve sensitive detection [63]. They linked one aptamer to a circular DNA sequence and added to another aptamer a short primer sequence that could hybridize with the circular DNA and initiate the RCA (Fig. 6C). The primer sequence was designed to be short enough to ensure minimum hybridization to the circular DNA. Binding of both aptamers to a thrombin molecule brought the primer to the close proximity with the circular DNA template, promoting the RCA reaction. The elongated RCA products were then labeled with intercalating fluorescent dye and fluorescence was detected. RCA increased signal intensity and enhanced sensitivity of the assay. A detection limit of 30 pM was achieved.

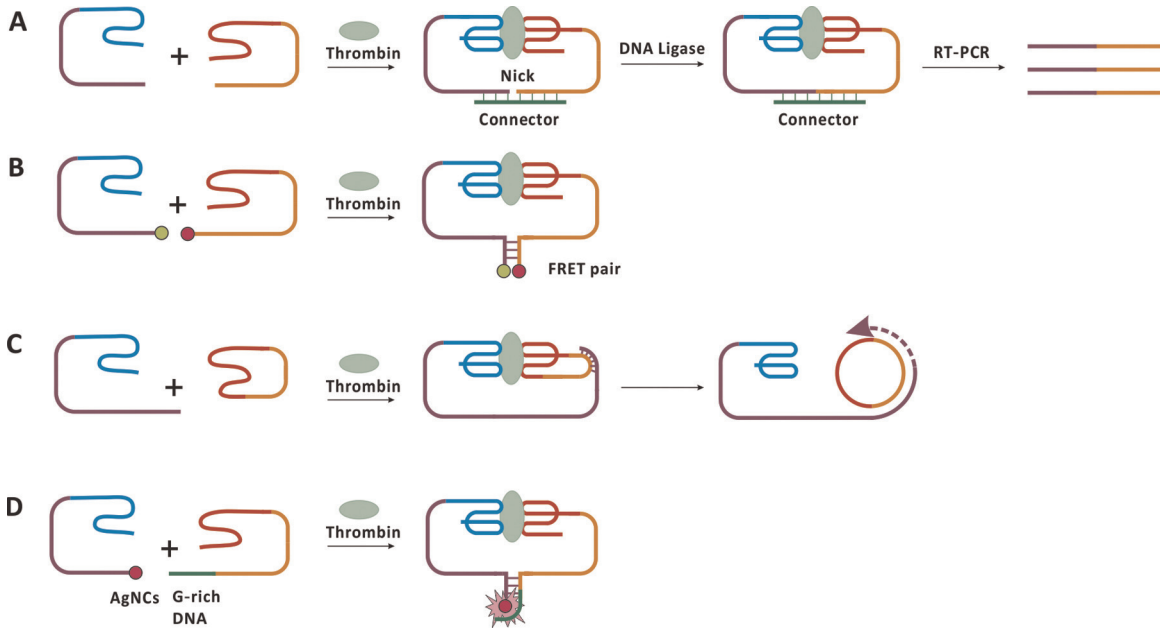

Fig. 6. Proximity based assays built on binding of two aptamers to the same thrombin molecule. (A) Two binding events bring DNA probes into close proximity which then hybridize with the connector and form a duplex, which is ligated and detected by real-time PCR. (B) Binding-induced proximity of two fluorophores generates FRET signal for thrombin quantitation. (C) Binding of both aptamers leads to the close proximity of primer and circular DNA, promoting rolling circle amplification. (D) Binding-induced DNA assembly of AgNCs nucleated aptamer and G-rich aptamer enhances the red fluorescence of AgNCs.

Because RCA is a linear amplification technique, the improvement of sensitivity by RCA is not as high as that by the exponential amplification of PCR.

Zhang et al. [64–66] developed the binding-induced DNA assembly (BINDA) strategy that benefited from more sophisticated DNA sequence design, achieving further reduced background and improved detection limit. The BINDA strategy has been used in conjunction with various detection and signal amplification approaches [67–69].

Li et al. incorporated silver nanoclusters (AgNCs) into this strategy for thrombin analysis [70] (Fig. 6D). The authors modified two aptamers by connecting an AgNCs nucleation sequence to one aptamer and a guanine-rich sequence to the second aptamer with linker complementary sequences. Binding of both aptamers to thrombin resulted in substantially increased local concentrations of the complementary sequences, facilitating the stable hybridization between complementary sequences. The formation of the DNA duplex structure ensured the close proximity of AgNCs with the G-rich sequence. The G-rich sequence could enhance the red fluorescence of AgNCs by up to 500-fold when in proximate distance with AgNCs [71]. Thus, the enhanced red fluorescence was used for thrombin quantification.

3. Electrochemical aptasensors

Over the last decade, tremendous efforts have been made in the development of electrochemical aptasensors for protein detection. The simplicity and low cost of electrochemical detectors make these aptasensors desirable as potential portable diagnostic devices for rapid protein analysis [72]. Generally, electrochemical signals are generated through three mechanisms: (1) introducing catalytic labels to the electrodes; (2) altering the proximity of

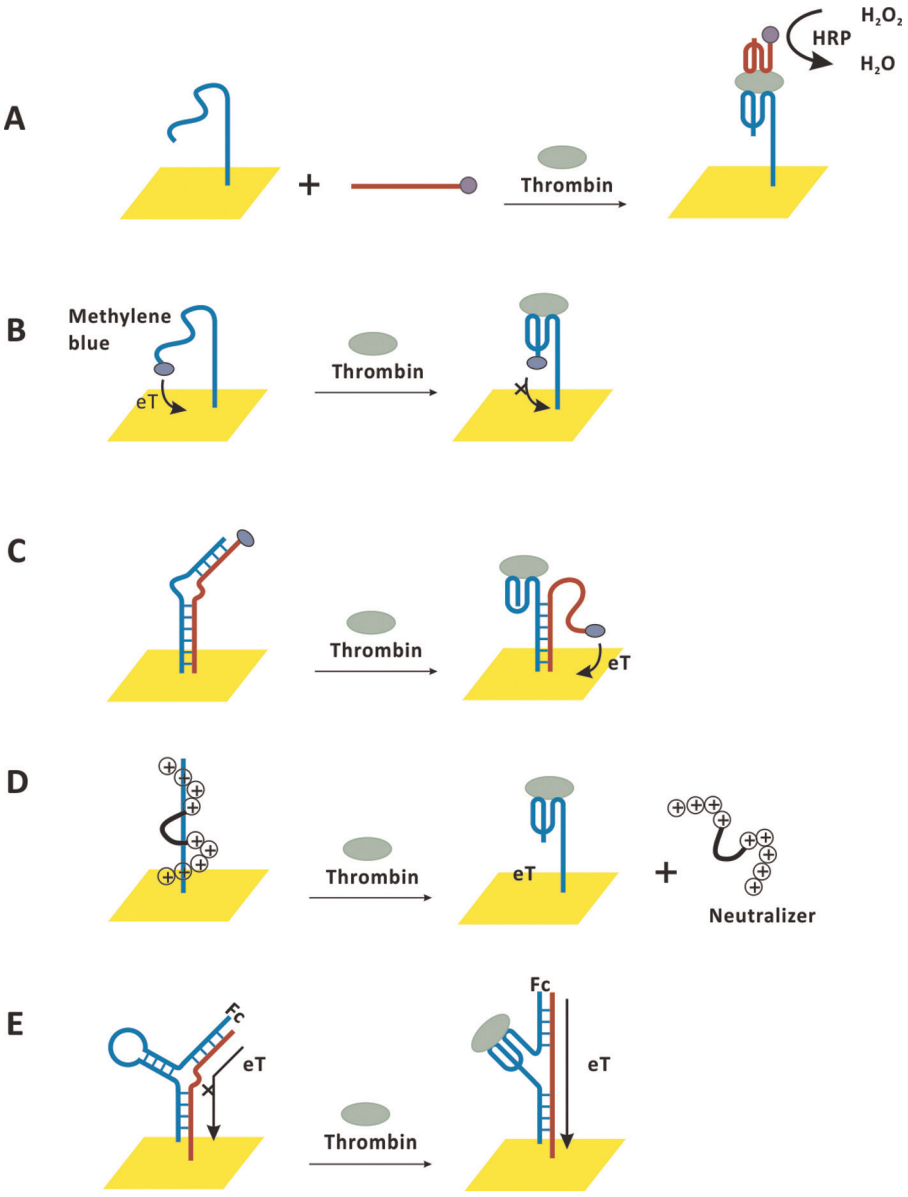

Fig. 7. Electrochemical aptasensors for protein detection. (A) Horseradish peroxidase (HRP) serves as a catalytic label for sandwich-type thrombin detection on gold electrode; (B) structure switching aptamers alter the proximity between the methylene blue label and the gold electrode, resulting in an attenuation of the electrochemical signal; (C) binding of thrombin to the assembly of DNA duplex on gold electrode changes the proximity between the labels and electrodes; (D) binding of thrombin releases the aptamer-tethered neutralizer, turning on the electrochemical signal; (E) binding of thrombin alters the conformation of the DNA three-way junction thus switching on the conductivity of the adjacent helical conduction path, leading to an increase in the measured electrical signal.

electrochemical labels to the electrodes; and (3) switching the conductivity of immobilized DNA on the electrodes.

3.1. Electrochemical aptasensors using catalytic labels

The use of catalytic labels, including redox enzymes and nanoparticles, for thrombin detection, often requires a sandwich assay format to produce thrombin-specific signals. The sandwich assay strategy takes advantage of the fact that thrombin has two different binding sites for two distinct aptamers. Usually, one aptamer is immobilized on the electrode as a capture probe and the second aptamer is modified with a catalytic label as a signal readout probe. The target thrombin can be captured on the surface of electrodes by binding to the capture aptamer and then forming a sandwich complex with the second aptamer. During this process, sandwiched binding complexes bring catalytic labels to the surfaces of electrodes to generate amperometric, voltammetric, impedimetric, or gravimetric signals for detection.

For example, Katakis and co-workers [73] described a strategy that used horseradish peroxidase (HRP) as a catalytic label (Fig. 7A). A thiolated aptamer was conjugated to a gold electrode through the gold–sulfur bond. The second aptamer was linked to HRP. The HRP catalyzed the relay-mediated reduction of H_2O_2, and the resulting amperometric response was measured. A detection limit of 80 nM thrombin was achieved. In addition to HRP, glucose dehydrogenase (GDH) has also been used in the same assay format to amplify amperometric detection of thrombin [74].

Enzyme-based electrochemical sensors have generally poor sensitivity partially due to the requirement of diffusional electron mediators [72]. To improve the sensitivity, many nanomaterials have been introduced as more efficient catalytic labels. For example, Willner and co-workers [75] described the use of PtNPs as a catalytic label to replace enzymes. PtNPs were able to catalyze the electrochemical reduction of H_2O_2 with high efficiency, and the resulting cathodic currents enabled the amplified detection of thrombin, with a detection limit of 1 nM. AuNPs have also been adopted to amplify the detection signals of voltammetric [76] and impedimetric aptasensors [77] for thrombin, achieving limits at sub-pM levels. Other materials, including QDs and graphene oxides (GO), have also been used to enhance the detection sensitivity for thrombin [78–80].

3.2. Electrochemical aptasensors based on proximity of labels

Electrochemical labels, such as methylene blue, require close proximity to the electrodes to produce electrical signals. Based on this principle, many strategies have been developed to generate or amplify electrochemical signals for thrombin detection without the need for any washing steps. Structure-switching aptamers are most frequently used to alter the proximity between the labels and electrodes upon binding of thrombin to the aptamer. For example, Plaxco and co-workers [81] have developed an electrochemical thrombin aptasensor by tethering a redox-active methylene blue label to the thrombin aptamer that was immobilized on an electrode (Fig. 7B). In the absence of the target thrombin, the flexible conformation of the aptamer chain enabled the close proximity between methylene blue and the electrode, generating a voltammetric response. However, upon binding to thrombin, the aptamer assembled into a G-quadruplex structure, distancing and shielding methylene blue from electron-transfer communication with the electrode. Therefore, an attenuation of voltammetric signal was used as a response for detection of thrombin. A detection limit of this assay was 20 nM. This sensor for thrombin detection has the advantage of being homogeneous, but one disadvantage is the negative signal readout format.

To overcome this disadvantage, the same group [82] (Fig. 7C) has improved their thrombin aptasensor by using a DNA duplex assembled on a gold electrode. The DNA duplex was composed of two double-stranded DNA (dsDNA) components linked with a non-complementary region. The upper dsDNA contained a thrombin aptamer, and its complementary sequence was labeled with methylene blue. The high rigidity of dsDNA prevented methylene blue from interacting with the gold electrode, thus turning off the amperometric signal. However, in the presence of the target thrombin, binding of thrombin to the aptamer trigged the dehybridization of the aptamer strand from the complementary strand of the upper dsDNA. Due to the increased flexibility of the single-strand DNA probe, the attached methylene blue label was able to get close to the gold electrode. The detection limit has been improved from 20 nm [81] to 3 nM [82].

To further push the detection limit, Das et al. [83] developed a neutralizer displacement strategy for thrombin (Fig. 7D). A thrombin aptamer was tethered to the surface of an electrode and was joined by a neutralizer that was composed of peptide DNA (PNA) and cationic amino acids. The neutralizer to the thrombin aptamer through the hybridization between the aptamer and PNA, and neutralized the charge of the aptamer. Upon binding to thrombin, the aptamer changed its structure and released the neutralizer, turning on the electrochemical signal. A detection limit of 10 fM was achieved using this design.

In addition to the use of a single structure-switching aptamer, sandwiched binding of thrombin with two distinct aptamers can also be used to alter the proximity between electrochemical labels and the electrodes. For example, Easley and co-workers [84] have developed an electrochemical proximity assay that was able to quantify thrombin at levels as low as 50 pM. They achieved this by combining the binding-induced DNA assembly with electrochemical detection.

3.3. Electrochemical aptasensors based on conductivity changes

The electrochemical aptasensors for thrombin can also be constructed by directly measuring changes in the conductivity of DNA probes immobilized on electrodes. Although a complete understanding of the DNA conductivity is still elusive, the molecular conductivity of DNA double helices has been shown experimentally to depend on their conformational state [85]. Based on this principle, Yu and co-workers [86] developed an electronic sensor for thrombin by incorporating a thrombin aptamer into a double-helical conduction path (Fig. 7E). The double-helical conduction path was formed by a DNA three-way junction. Ferrocene (Fc) served as an electrochemical label and was conjugated to the strand that encompassed an aptamer sequence. In the absence of the target thrombin, the three-way junction structure prevented conduction between ferrocene and the electrode. Thus, the conduction path was turned off. Upon binding to thrombin, the aptamer altered the conformation of the three-way junction, thus switching on the conductivity of the adjacent helical conduction path, leading to an increase in the measured electrical signal. Picomolar detection limits have been achieved for thrombin both in buffer and in diluted serum samples.

4. Affinity separations

Affinity separation is often achieved through specific interactions between a pair of binding partners, such as antigen and antibody, enzyme and substrate, receptor and ligand, or aptamer and protein. Affinity interactions can be incorporated into traditional separation methods, such as chromatography, capillary electrophoresis, microfluidics, and magnetic beads, to realize affinity separations.

4.1. Affinity chromatography

Through specific affinity interactions, affinity chromatography can provide unique separation selectivity of biomolecules, such as proteins. Usually, a biological ligand is immobilized on a column to capture or interact with the corresponding binding partner. For example, an aptamer can be used as the affinity stationary phase to capture a specific target. This affinity monolithic column took advantage of the specific affinity of the aptamer and the porous property of the monolith. Thrombin in dilute serum samples was selectively captured and pre-concentrated.

Zhao et al. [87] successfully immobilized the biotinylated DNA aptamer targeting thrombin on a streptavidin-coated polymer monolithic capillary column. The captured thrombin was subsequently eluted off rapidly from the aptamer affinity monolithic column with eluent of high ionic strength. Human serum albumin, hemoglobin, transferring, and immunoglobulin G did not interfere with the capture and assay for thrombin. On-line detection with a UV absorbance detector achieved a detection limit of 4 nM, after a pre-concentration factor of 120-fold.

Similarly, Deng et al. [88] conjugated aptamer to an organic–inorganic hybrid silica monolith, and developed an aptamer affinity monolithic capillary column. They covalently attached the 5′-NH_2-modified aptamer for human α-thrombin to the hybrid silica monolith. With selective capture of thrombin on the affinity capillary column and UV absorbance detection, they also achieved a limit of detection of 3.4 nM.

Zhao et al. [89] further improved the aptamer-based affinity chromatographic assay for thrombin by using a pair of aptamers for selective capture and laser-induced fluorescence (LIF) for sensitive detection. The detection limit of thrombin was further improved to 0.1 nM [89], after the pre-concentration. One aptamer, e.g. Apt15 that has a weaker binding, was immobilized on the monolithic column to capture thrombin, and the second aptamer (e.g. Apt29) was fluorescently labeled and used as the reporter probe. Thrombin was efficiently captured on the column by the first aptamer. Despite the relatively low affinity of the immobilized aptamer ($K_d \sim 100$ nM), the increased local concentration of the aptamer on the monolithic column enhanced the binding between the aptamer and thrombin. As a consequence, the aptamer affinity chromatography could utilize aptamers of weaker affinity. The fluorescently labeled second aptamer (Apt29) was introduced to bind with the captured thrombin, forming a sandwich complex. The sandwich complex was subsequently eluted off with 2 M $NaClO_4$ and was detected with LIF.

4.2. Affinity capillary electrophoresis

Affinity capillary electrophoresis making use of aptamer–target interactions has shown diverse analytical applications [90–94]. For protein detection using affinity capillary electrophoresis, the use of aptamers has benefited from two distinct features: modulation of electrophoretic mobility and introduction of detection probes. First, the mobility of proteins under the free-zone electrophoresis conditions can be modulated by binding the proteins to their respective aptamers. Under the pH conditions (pH 7–9) typically used for capillary electrophoresis separation, each nucleotide of an aptamer carries a negative charge due to the phosphate group. Most proteins have a very small net charge. Thus, binding of aptamers to proteins substantially alters the size-to-charge ratio, which is the basis of free-zone capillary electrophoresis separation. Zhang et al. [95] have shown that the mobility of proteins could be modulated by binding the proteins with DNA aptamers of varying lengths. Comparing a full-length aptamer (76 n.t. long) and a truncated aptamer (38 n.t.) for thrombin, they determined the mobility of the thrombin–aptamer complexes (-2.22×10^{-4} vs -1.68×10^{-4} $cm^2 V^{-1}s$), demonstrating the ability of tuning the mobility of different sizes. They applied this principle to the simultaneous determination of thrombin, HIV reverse transcriptase, platelet derived growth factor (PDGF), and human immunoglobulin E (IgE) in a single CE analysis [95]. Similarly, Janssen et al. [85] have achieved detection of multiple proteins at the pM levels.

The second important benefit of aptamer binding to proteins is the incorporation of labeled aptamers as detection probes for proteins that otherwise lack highly sensitive fluorophores or chromophores. Zhang et al. [95] labeled the aptamers at the 5′-end with highly fluorescent 6-carboxy fluorescence. The binding of fluorescent aptamers to the target proteins makes the proteins amenable to highly sensitive LIF detection (at 488 nm excitation and 515 nm emission). The detection limit for thrombin was 100 pM. Similarly, Li et al. [96] have achieved a detection limit of 2 nM thrombin both in the running buffer and in 5% (v/v) human serum. Zhang et al. [97] further improved the detection limit of thrombin to 56 pM.

In the event that the unbound species and the complex have similar electrophoretic mobility and cannot be separated by CE [98], laser-induced fluorescence polarization (LIFP) detection can be used to differentiate the complex from the unbound species [99]. Florescence from small unbound molecules is randomly polarized, giving negligible polarization values. When the small fluorescent molecule is bound to a large molecule, the motion of the larger molecule in solution is slowed down. The fluorescence is polarized, and polarization values can be measured with LIFP. Song et al. [100] have shown quantification of thrombin in human serum using aptamer binding, CE separation, and LIFP detection. The detection limit was 2.94×10^{-19} mol.

A potential problem of affinity CE is whether protein–aptamer complexes could be dissociated during separation. To solve this problem, Berezovski et al. [91] proposed a method based on non-equilibrium CE of the equilibrium mixture (NECEEM). Using a fluorescently labeled aptamer, they demonstrated that despite the dissociation, as few as 4×10^6 molecules of thrombin could be detected with NECEEM without sacrificing accuracy. Thus, the aptamer affinity CE method could be used for analysis of proteins even when protein–aptamer complexes are unstable.

4.3. Affinity separation using microfluidic devices

Since the 1990s, the development of micro-electro-mechanical systems (MEMS) has enabled "micro total analysis systems" (μTAS) and microfluidic analytical platforms. Similar to chromatography and CE, separation of amino acids, peptides, proteins, and small molecules, driven by pressure or electric force, can be carried out in the microchannels of microchips.

Obubuafo et al. [101] used fluorescently labeled aptamers to bind to thrombin, and separated the unbound aptamer from the thrombin–aptamer complex using affinity microchip gel electrophoresis. With LIF detection, they achieved detection limits on the order of 10–50 nM. They also successfully determined the thrombin concentration in a plasma sample of 543.5 nM. Gong et al. [102] also used microchip CE to separate the thrombin–aptamer complex from the unbound aptamer in less than 10 s. They achieved a detection limit of 5 nM.

Kim et al. [103] demonstrated a nanoporous membrane device integrated with an on-chip microfluidic platform for the electrokinetic separation of biomolecules. A thin (500 nm) film was assembled into a compact microfluidic device. When a voltage was applied, the FITC-tagged ssDNA in the bottom reservoir was transported to the top chamber and detected by fluorescence. The fluorescence intensity was quantified to estimate the amount of transported DNA. Size-exclusion separation of biomolecules driven by an electric field was verified with the free aptamer and the

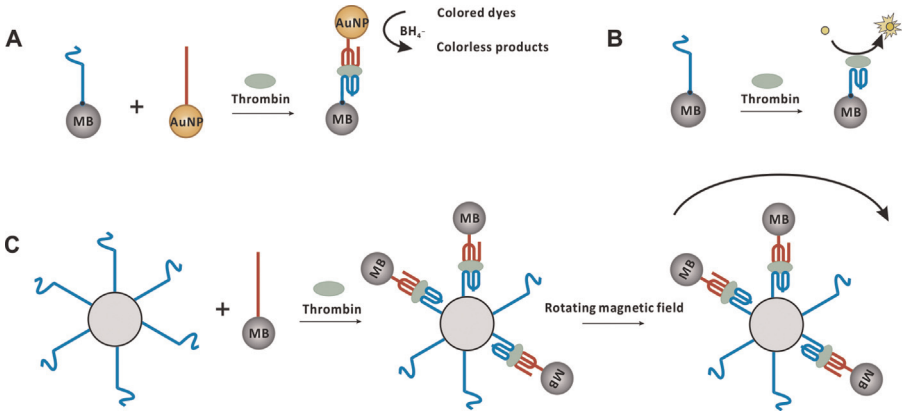

Fig. 8. Magnetic beads (MBs) used in affinity separations that make use of specific interactions between aptamer and thrombin. (A) Apt15 is conjugated to the MBs and Apt29 is conjugated to AuNPs. MBs are used for separation and AuNPs are used for colorimetric detection. (B) The captured thrombin on MBs catalyzes the subsequent conversion of fluorogenic substrate to fluorescent products. (C) Apt29 aptamer is coated on the non-magnetic "mother" spheres (10 μm diameter). Apt15 aptamer is coated on the magnetic "daughter" beads (1 μm diameter). Binding of thrombin to both aptamers forms a rotating sandwich complex. In a rotating magnetic field, the rotational frequency of the sandwich complex is proportional to the number of attached magnetic beads, which scales with the concentration of thrombin in the solution.

aptamer–thrombin complex. The results showed that the complex was not effectively transported through the pores while DNA aptamers easily passed through the pores due to their smaller size.

Aptamers could be immobilized on microfluidic chips to catch the specific target. Wang et al. [104] constructed thrombin functionalized aptamers in the microfluidic channels. The substrate solution containing 4-aminophenyl phosphate was introduced to the microchannels for the end-column electrochemical detection. The intensity increased linearly with the logarithm of the thrombin concentration from 1 to 100 pM. A detection limit was 1 pM. Aptamers could also be immobilized on small particles. Srinivas et al. [105] demonstrated a highly sensitive detection of human α-thrombin on encoded hydrogel microparticles functionalized with an aptamer. Using static imaging and microfluidic flow-through analysis techniques to evaluate the detection capabilities, they achieved three logs of dynamic range and a detection limit of 4 pM. Tennico et al. [106] utilized aptamer-functionalized magnetic beads to capture thrombin, and a second aptamer functionalized with quantum dots to serve as the reporter. Fluorescence microscopy was employed for on-chip detection. A detection limit for thrombin was 10 ng mL^{-1} (or ~0.3 nM).

Chen et al. [107] fabricated microarrays of RNA aptamers on thin gold films in a microfluidic format. With surface plasmon resonance imaging (SPRI) measurements, they monitored the affinity adsorption of human thrombin and vascular endothelial growth factor (VEGF) proteins. The microfluidic format permitted the feasible fabrication of aptamer microarrays for multiplexed protein biosensing. Through aptamer–protein interactions, femtomole amounts of thrombin were also detected by a four-chamber microfluidic biochip with the technique of SPRI. Jung et al. [108] constructed a surface acoustic wave (SAW) sensor chip by forming thrombin-sensitive biofunctionalized surfaces through immobilization of an RNA aptamer for thrombin. Binding of thrombin onto the aptamer-modified surface was the basis for the acoustic wave detection of this protein.

4.4. Affinity separation using magnetic beads

Magnetic beads (MBs) serve both as scaffold for aptamer immobilization and as unique material for rapid magnetic separation of the captured target from the sample matrix. Many techniques take advantage of two aptamers binding to two separate sites of thrombin, which allows for the formation of a sandwich complex. Li et al. [109] immobilized the 15-mer aptamer (Apt15) on magnetic beads to capture and pre-concentrate thrombin, separating thrombin from the sample matrix. They immobilized the 29-mer aptamer (Apt29) on AuNPs. Binding of thrombin to both aptamers resulted in the formation of the Apt15–thrombin–Apt29 sandwich complex (Fig. 8A). Using a magnet, they separated the sandwich complex from the unbound AuNPs. The bound AuNPs on sandwich complex catalyzed the reaction of methyl orange (MO), generating changes in colorimetric signals. By visualization with the naked eye, they were able to achieve a detection limit of 320 pM human thrombin. This detection limit was further decreased to 30 pM with UV–vis absorption detection.

Similarly, Szymanski et al. [110] used both aptamers to construct a sandwich assay on magnetic beads. But unlike Li et al. [109], Szymanski et al. [110] conjugated Apt29 on magnetic beads and Apt15 on AgNPs. Despite different binding affinities of the two aptamers for thrombin, both approaches worked. After magnetic separation of the Apt29–thrombin–Apt15 sandwich complex, Szymanski et al. used ionic silver amplification and anodic stripping voltammetry to generate electrochemical detection signals. The detection limit for α-thrombin was 164 pM.

Wang et al. [111] conjugated one aptamer (serving as the capture) on magnetic beads and the second aptamer (serving as the reporter) on quantum dots (QDs). After capturing of the thrombin sandwich complex, fluorescence of the QDs was measured for quantitation. They achieved a detection limit of 50 pM. Similarly, Tennico et al. [106] conjugated the two aptamers on magnetic beads and QDs respectively, and performed assays on microfluidic chips. With fluorescence microscopy detection, Tennico et al. achieved a detection limit of 10 ng mL^{-1} (~0.3 nM). With electrochemical detection, Centi et al. [112] also reached a detection limit of 0.45 nM. With further modification and by incorporating aptamer-labeled silica nanocapsules (SiNCs) for electrochemical detection, Wang et al. [113] improved the detection limit to 60 pM.

A second approach of detecting thrombin, after capturing of thrombin on magnetic beads, is based on the catalytic activity of thrombin. Typically thrombin catalyzes the conversion of a chromogenic/fluorogenic substrate to an optically measurable product (Fig. 8B). Zhao and Wang [114] used the aptamer-modified magnetic beads to capture and separate thrombin from the sample mixture. After 2 h of enzyme reaction and with absorption detection, they were able to detect as low as 400 fM thrombin. Zhao et al. [115] further improved the detection limit to 2 fM thrombin, by detecting fluorescent products generated from thrombin-catalyzed conversion of the fluorogenic substrate. With

electrochemical detection of the enzymatic product of thrombin, Centi et al. [116] reported a detection limit of 175 nM.

The third approach of using aptamers and magnetic beads has led to the development of a new signal transduction strategy (Fig. 8C). Hecht et al. [117] developed a label-acquired magneto-rotation (LAM) technique, which was able to detect thrombin at a concentration as low as 300 pM. They coated larger non-magnetic "mother" spheres (10 μm diameter) with one aptamer (Apt29). They immobilized smaller magnetic "daughter" beads (1 μm diameter) with the second aptamer (Apt15). Binding of thrombin to both aptamers formed a sandwich complex. In a rotating magnetic field, the rotational frequency of the sandwich complex was proportional to the number of attached magnetic beads, which depended on the concentration of thrombin in the sample solution. This principle gives rise to a new method of signal generation and can be applied to the detection of other target molecules.

5. Conclusions and outlook

Thrombin and its aptamers are widely used in the development and demonstration of analytical methods. The advantages of specific binding affinity, two binding sites, the availability of two aptamers each targeting a specific site, switching of aptamer structures triggered by binding to thrombin, and the catalytic activity of thrombin are the main reasons why thrombin is often selected as a test model. Assays for other molecular targets, based on the use of aptamers and assay design for thrombin, can be expected. Understanding the principle of the various assays for thrombin will help developing assays for diverse targets while recognizing limitations involved.

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