The Design and Improvement of Aptamer-based Fluorescent Probes

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Abstract: Fluorescent probes exhibit the characteristics of high sensitivity, diversified design, and strong quantitative analysis ability. Therefore, they have become one of the research hotspots in analytical chemistry and biological analysis. Aptamers are single-stranded DNAs or RNAs that are obtained in vitro and can selectively bind to the targets, making them ideal candidates as identifying units in fluorescent probes. However, most of the current aptamer probes are not regulatable because their fluorescence signals can’t be changed after the fluorescent probes are bound to the target, resulting in a high signal background and limited contrast. Actually, the structure of aptamers is flexible and can be combined with a variety of nanomaterials to achieve high sensitivity fluorescence for quantitative analysis, and can be used for a variety of target determinations, such as metal ions, small molecules, proteins and cells. In addition, with the development of research on DNA nanotechnology, researchers have designed many aptamer-based fluorescent probes with special properties, which have good application prospects in complex sample analyses and even in vivo cell imaging. In this review, the development of research in the field of aptamer-based fluorescent probes is surveyed and four mechanisms of aptamer fluorescent probes, including dye intercalation, conversion of DNA conformation, signal amplification, and mediation of nanomaterials, are comprehensively discussed.

Keywords: Aptamer, fluorescent dyes, fluorescent probe, nanomaterials, nucleic acid conformation, single-stranded DNAs.

1. INTRODUCTION

Aptamers are used as molecular recognition units in molecular probe design (Table 1). They are flexible in structure and can be changed or reconstructed after interacting with the target. It is an ideal choice for signal transduction [1]. At the same time, in molecular biology, nucleic acid quantitative methods have been relatively mature, and have various expansion methods and enable highly sensitive assays, including polymerase chain amplification, ligase chain amplification, and rolling circle amplification, etc. [2]. The fluorescence molecular probe has high sensitivity, fast response and simple technology. There are various forms of fluorescence analysis. Besides common methods based on fluorescence intensity, fluorescence anisotropy and fluorescence lifetime can also be used [3]. Due to the feasibility of synthesis, the diversity of fluorescent labels and the flexibility of switch design, the design of fluorescent probes based on aptamers has drawn extensive attention from researchers and has become one of the research hotspots in recent years [4]. At present, most of the aptamer fluorescent probes mainly use fluorescence intensity as a signal for quantitative analysis because of the high sensitivity of fluorescence measurement. At the same time, the design of such probes is easy and versatile, and the instrument requirements are also very simple [5]. According to the source of fluorescence signal and design strategy, in this review, the aptamer fluorescent probes are divided into the following categories: Dye intercalation, conversion of conformation, signal amplification, and mediation of nanomaterials.

2. DYE INTERCALATION

The dyes used in dye-based aptamer probes have different properties and act in different mechanisms (Table 2), which can be generally categorized into fluorescence attenuation and fluorescence enhancement, Fig. (1).

2.1. Fluorescence Attenuation Probes

Aptamer and target recognition processes lead to changes in the environment around aptamers, such as steric hindrance, charge and hydrophobicity, which is the molecular basis for designing label free fluorescent probes [1]. In the unmarked fluorescence analysis, the fluorescent dyes can usually interact with the probe by embedding, charge action and molecular accumulation. In aqueous solution, the fluo-
The design and improvement of aptamer-based fluorescent probes are described, focusing on the use of aptamers to modulate fluorescence intensity. The quenching effect of water molecules on dye excited electrons is reduced by hydrophobic interactions of aptamers, allowing for fluorescence restoration. Upon binding to a target, the protective effect of the aptamer on the dye is diminished, leading to decreased fluorescence. Joseph et al. showed that the fluorescence intensity of the YOYO dye significantly decreased when the aptamers were bound to thrombin [15]. Zhou et al. realized the quantitative determination of Platelet-Derived Growth Factor B chain (PDGF-BB) by using TOTO fluorescence signals. The detection limit is 0.1 nM and has high selectivity [13]. Owing to its spontaneous insertion into DNA duplex, [Ru(phen)2(dppz)]2+ were used as a metal coordination compound for fluorescence analysis. After it is embedded into DNA aptamer, the fluorescence would be significantly enhanced. When the target is introduced, leading to the change of the aptamer conformation, [Ru(phen)2(dppz)]2+ is displaced and fluorescence would be quenched. Through this strategy, 0.1, 1.0, 0.01, and 1 nM immunoglobulin (IgE), PDGF-BB, thrombin and ATP can be detected, respectively [16].

2.2. Fluorescence Enhancement Probes

The second type of intercalating agent is aggregative quenching dyes. When they bind to DNA strands, the dye molecules are close to each other and aggregative quenching occurs. Typical representatives are pyrazines and perylenes, with detection limits of 50 nM and 70 pM for thrombin and lysozyme, respectively [17].

In some aptamers with a G4 structure, G4-specific intercalating dyes can be used in label-free fluorescent probes, such as crystal violet, porphyrins, etc. [18]. A certain part of the aptamer is self-complementary, therefore, the formation of the G4 structure is inhibited and the dye can’t bind to G4, resulting in weak fluorescence. Upon binding to the target, the complementary sequence is dissociated and the intercalating dye has a chance to bind to G4 and the fluorescence is enhanced.
Label-free fluorescent probes can be achieved by the introduction of vacancy intercalators, as well as allosteric switches in aptamers. As a typical vacancy intercalator, naphthyridine can be paired with C bases, leading to the quenching of its own fluorescence. Xiang et al. adopted this feature of naphthyridine in the design of aptamer fluorescent probes, enabling enhanced fluorescence measurements of Pb²⁺ and ATP [19]. Furthermore, in the design of the allosteric switch of aptamers, the modulating effect of the aptamer on the fluorescence intensity of malachite green can also be applied to the design of fluorescent probes. For example, malachite green and ATP aptamer can be covalently linked. When a complementary sequence was introduced, the aptamer could not bind to malachite green and the fluorescence was weak. In the presence of ATP, the complementary sequence was replaced, and the aptamer could be combined with malachite green, resulting in enhanced fluorescence [20].

In the abovementioned design of dye-based aptamer probes, the fluorescent dye does not have to be covalently linked. Non-covalent presence of fluorescent dyes would reduce the cost of DNA synthesis and enable easy preparation. The use of an intercalating dye can be compatible with classical nucleic acid analysis methods and has versatility [21], Fig. (1).

3. CONVERSION OF CONFORMATION

The design of conformation-based aptamer probes can be divided into three categories, including intramolecular switch, intermolecular switch, and replacement of DNA strand [22-24] Fig. (2). In the cases of intramolecular switch and intermolecular switch, a complementary strand is generally required to regulate the aptamer conformation. Researchers have designed various types of connections between the aptamer and the complementary chain [25-30], Table 3.

3.1. Intramolecular Switch

Tyagi et al. proposed that molecular beacon is one of the typical representatives for intramolecular switches. Molecular beacon has been widely used in analytical chemistry research [31] and is usually composed of a neck and a ring. The ring serves to identify the target while the neck achieves complementary hybridization. The fluorophore and quencher are separately labeled at the ends of the neck. When the target is not present, the paired neck pulls the fluorophore and quencher close and the fluorescence is quenched. Once recognizing the target, the configuration of the ring would be
changed, leading to the opening of the neck structure as a result. The opening of the neck enables the separation of the fluorophore and the quencher, and the fluorescence is restored consequently.

In 2001, Hamaguchi et al. reported using an aptamer molecular beacon probe to determine the concentration of thrombin. In the absence of thrombin, the hairpin structure causes quenching of the fluorescence. After the target thrombin is added, thrombin binds to the aptamer molecular beacon and activates the fluorescence [25]. This kind of aptamer molecular beacon can also enable direct fluorescence detection of histidine recombinant proteins in cell lysates. Compared with traditional Western blotting, enzyme immunoassay and fluorescent protein labeling, molecular beacons are faster, simpler, and less expensive.

Tang et al. inserted polyethylene glycol between the aptamer and the complementary sequence to form a molecular beacon. Because a short complementary sequence was used in this design, the probe has the characteristics of low background, high affinity, fast response, and simple design, enabling quick and sensitive determination of small molecule target molecules, including: K⁺ [33], Hg²⁺ [34], antibiotics [35], ATP [26] and thrombin [25].

Table 3. Representative connections between aptamer and the complementary chain.

<table>
<thead>
<tr>
<th>Target</th>
<th>Type of Auxiliary Chain</th>
<th>Type of Conformation Conversion</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin</td>
<td>Directly add complementary strands</td>
<td>Intramolecular</td>
<td>[25]</td>
</tr>
<tr>
<td>ATP</td>
<td>PEG</td>
<td>Intramolecular</td>
<td>[26]</td>
</tr>
<tr>
<td>MCF-7 cells</td>
<td>Extend 3'- and 5'-terminus of aptamer with additional complementary six bases</td>
<td>Intramolecular</td>
<td>[27]</td>
</tr>
<tr>
<td>CCRF-CEM</td>
<td>X-shaped DNA core connector</td>
<td>Intramolecular</td>
<td>[28]</td>
</tr>
<tr>
<td>ATP</td>
<td>A short complimentary DNA strand as the output</td>
<td>Intermolecular</td>
<td>[29]</td>
</tr>
<tr>
<td>Tat protein of HIV-1</td>
<td>Split aptamer</td>
<td>Intermolecular</td>
<td>[30]</td>
</tr>
</tbody>
</table>

In the design of intramolecular switches, fluorophores and quenching groups are usually introduced into the single aptamer sequence. When the aptamer is combined with the target, the distance between them is changed to generate a fluorescent signal. As a result, the maximum distance between the fluorophore and quencher group is decided by the sequence and their separation is limited, which would limit the value of signal changes. In addition, the relatively stable structure of intramolecular hybridization tends to restrict the binding of the aptamer to the target.

3.2. Intermolecular Switch

In the design of intermolecular aptamer fluorescent probes, complementary sequences are not covalently linked to the aptamer sequence, but exist and hybridize with independent nucleic acid sequences [36]. This intermolecular design is flexible and can be combined with a variety of signal amplification strategies to improve the performance of the probes [37].

In 2000, Rika et al. split two halves of the RNA aptamer sequence of the Tat protein and successfully designed an enhanced fluorescent probe [30]. The two parts of the aptamer are isolated. One part is alone, while the second part is in the structure of a molecular beacon, with fluorescent group and quencher located at two ends of the molecular beacon and close to each other. In the presence of the target protein, the split two-part aptamers bind to the target and competitively open the molecular beacon to enhance its fluorescence. Alternatively, the sequence of the aptamer was cleaved into two segments and labeled with a fluorophore and a quencher, respectively. In the presence of the target, two free aptamer sequences hybridize to quench the fluorescent signal [38]. This strategy is suitable for the design of aptamers for most targets, including Pb²⁺ [19], ATP [23] and streptomycin [39], etc. Of course, it is generally necessary to optimize the design of sequences. Moreover, this split design generally significantly reduces the ability of the aptamer to bind to the target, which is detrimental to highly sensitive assays.

Nutiu et al. achieved the reversible regulation of ATP and adenosine deaminase-triggered fluorescence signals by labeling the fluorophore and the quencher on the aptamer and complementary sequences, respectively [29]. For stable hybridization, the aptamer sequence must have at least 12 bases that hybridize to the complementary sequence. This design will reduce the affinity of the aptamer to the target. Then this research group stretched several bases on the aptamer sequence as a template, and assembled two DNA
probes with a fluorophore and a quencher, respectively, to quench the fluorescence. In the presence of the target, one of the DNA probes is competitively displaced to enhance the fluorescence signal. The number of complementary bases of the aptamer sequence in this design is approximately 6-8, which minimizes the influence on the affinity of the aptamer, and the response speed is enhanced as well. This strategy can enable the detection of a variety of targets, with the detection limit of thrombin, ATP at 10 nM and 10 μM, respectively [40]. In order to achieve the separation of the recognition group and the signal group and enhance the versatility of the design, Zheng et al. designed a triple helix molecular beacon probe [41]. Pyrene is labeled at both ends of one strand of the triple helix, and the other aptamer is connected end to end. In the absence of the target, the rigidity of the triple helix causes the pyrene to separate and have only its monomeric fluorescence peak. After the target is bound to the aptamer, the triple helix is dissociated so that the pyrene-labeled sequence folds to form a molecular beacon structure, the pyrene fluorophore at both ends are close together, and the diploid fluorescence signal is significantly enhanced.

In the recent study, Chen et al. reported a novel type of bivalent aptasensor based on silver-enhanced Fluorescence Polarization (FP) for detection of lactoferrin (Lac) in milk powder with high sensitivity and specificity. The bivalent aptamers were modified to be linked with signal molecule fluorescein isothiocyanate (FITC) and enhancer silver decahedral nanoparticles (Ag10NPs). The split aptamers could bind to different sites of Lac and aggregate to form a split aptamers-target complex, narrowing the distance between Ag10NPs and FITC dye. As a result, Ag10NPs could produce a mass augmented and promote Metal-Enhanced Fluorescence (MEF) effect. In general, ternary amplification is based on Ag10NPs, split aptamers, and the MEF effect, all contributed to the significant increase of FP values. It was proved that the sensitivity of this assay was about 3 orders of magnitude over traditional aptamer-based homogeneous assays, with a detection limit of 1.25 pm [42].

In the intermolecular aptamer fluorescent probe, the design of the structural switch is flexible and has a strong universality. One of the most direct designs is to hybridize the complete aptamer sequence to the complementary sequences, and after the target binds to the aptamer, the distance between the fluorophore and the quencher was changed. However, the introduction of complementary sequences will attenuate aptamer’s affinity for the target.

### 3.3. Replacement of DNA Strand

In the process of DNA strand displacement, short DNA duplex is replaced by another DNA and converted into a longer double-stranded structure through a fulcrum-mediated triple-stranded transfer reaction. By controlling the activity of fulcrum, a series of chain replacement tandem processes can be precisely adjusted, and the construction of various DNA molecular devices and circuits can be realized, which has become a research hotspot of DNA nanotechnology [43]. The DNA strand displacement mechanism can be employed to construct an aptamer fluorescent probe. Xing et al. designed an ATP-responsive aptamer molecular switch. In the fulcrum region, some sequences of aptamers were introduced, and the hiding of the fulcrum inhibited the process of strand displacement. After the ATP was bound to the aptamer, the fulcrum region was exposed and the DNA strand replacement proceeded smoothly [44]. Although this study did not provide detailed information on quantitative ATP detection, a gradient change in the effects of ATP concentration on strand displacement between 10 μM and 1.5 mM was observed. Zhu et al. adopted the split-type fulcrum strategy and designed a fluorescent probe based on the dye-embedded G4 structure with a detection limit of 40 μM for ATP. In short, they proposed a new three-way DNA junction-driven strand replacement model, based on which they built an aptamer-based label-free fluorescent sensing platform. Aptamer played an important role in mediating the small molecule ATP to tune the DNA logic gate. By altering the aptamer sequence, this molecular platform will be sensitive to various stimuli and applied in a wide field [24]. Li et al. adopted this split-type fulcrum strategy to detect thrombin. In the presence of thrombin, the split fulcrum area smoothly assembles and induces the strand displacement process. The strategy contains a binding-induced DNA Three-Way Junction (DNA TWJ), which converts protein bindings to the formation of DNA TWJ. The binding-induced DNA TWJ utilizes two DNA motifs, each of which binds to an affinity ligand. The binding of two affinity ligands to the target molecule triggers assembly of the DNA motifs and triggers the subsequent displacement of DNA strand, resulting in a binding-induced TWJ. Real-time fluorescence monitoring of the binding-induced TWJ can detect a prostate-specific antigen limit of 2.8 ng/mL [45].

### 4. SIGNAL AMPLIFICATION

The signal amplification aptamer probes were designed using bioenzyme-catalyzed and enzyme-free strategies, respectively, and a wide variety of probes were successfully designed, Table 4. [46-55].

#### 4.1. Biological Enzyme Catalysis

The sequence of aptamer can be coded, which can be combined with nucleic acid amplification and detection method to realize the high sensitive determination of target. Among the commercial nucleic acid amplification and detection methods, the most commonly used is Polymerase Chain amplification (PCR) technology [56]. Ma et al. used it for the determination of thrombin [57]. As shown in Fig. (3), after the combination of two different aptamer sequences with thrombin, complementary ends and induced elongation of the chain to form a complete double-stranded DNA structure at normal temperature. The high signal of thrombin can be detected by means of real-time PCR, and the detection limit can be reached to 8 pM. However, in general, PCR requires repeated annealing at a temperature of 90 degrees, which is not suitable for the analysis system based on aptamers. Because at high temperature, aptamer and the target are no longer bound together, and the identification information cannot be converted into quantitative information of
nucleic acid. It is warranted to develop a nucleic acid detection method that can amplify DNA and amplify signals at room temperature.

Rolling Circle Amplification (RCA) technique is used to design nucleic acid amplification strategy. In the presence of polymerase, short primers amplified using circular DNA as a template to form a long sequence with repeated sequences at room temperature [3]. Yang et al. used RCA for the determination of Platelet-Derived Growth Factor (PDGF). In the presence of PDGF, the aptamer sequence folds to form a cyclizable structure. Polymerase uses the cyclizable structure as a circular template and it is amplified by RCA. Finally, fluorescent signal is emitted from the intercalating dyes for quantitative detection [58]. Wu et al. took another design: PDGF triggers the melting of the primer and complementary sequence, triggers subsequent RCA amplification and uses a molecular beacon to output the fluorescent signal. The detection limit of PDGF reached to 7 pM [59]. He et al. combined the ultrasensitive and versatile Surface Plasmon Resonance (SPR) and Quartz Crystal Microbalance (QCM) with a p-tamer-based RCA and bio-bar-coded AuNP enhancement were presented for human α-thrombin detection. The assay platform exhibited excellent selectivity and sensitivity with a detection limit as low as 0.78 aM [49]. Qiu et al. designed a system for sensitive detection of lysozyme that involves a short double-stranded DNA including the lysozyme aptamer, a circular template, QD-labeled DNA4, hemin/G-quadruplex-based DNA5 and the corresponding enzymes. The signal is amplified by fluorescence resonance energy transfer between QDs and hemin/G-quadruplex Dnzymes on the RCA products [60].

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Table 4. Representative signal amplification aptamer probes (ATP: Adenosine Triphosphate; LAMP: Loop-mediated isothermal amplification; RCA: Rolling Circle Amplification; SPIA: Single Primer Isothermal Amplification; CHA: Catalytic Hairpin Assembly; PSA: Prostate Specific Antigen; VEGF: Vascular Endothelial Growth Factor).

<table>
<thead>
<tr>
<th>Target</th>
<th>Design Type</th>
<th>LOD</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Enzyme catalysis (LAMP)</td>
<td>0.47 fg/µL</td>
<td>[46]</td>
</tr>
<tr>
<td>Mucin 1</td>
<td>Enzyme catalysis (LAMP)</td>
<td>120 MUC-1 molecules</td>
<td>[47]</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>Enzyme catalysis (LAMP)</td>
<td>0.3 pM</td>
<td>[48]</td>
</tr>
<tr>
<td>Thrombin</td>
<td>Enzyme catalysis (RCA)</td>
<td>0.78 aM</td>
<td>[49]</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>Enzyme catalysis (RCA)</td>
<td>0.02 pg/mL</td>
<td>[50]</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>Enzyme catalysis (RCA)</td>
<td>5 CFU/mL</td>
<td>[51]</td>
</tr>
<tr>
<td>VEGF</td>
<td>Enzyme catalysis (SPIA)</td>
<td>3.5 pg/mL</td>
<td>[52]</td>
</tr>
<tr>
<td>ATP</td>
<td>Non-enzymatic amplification (Entropy-driven catalysis)</td>
<td>20 nM</td>
<td>[53]</td>
</tr>
<tr>
<td>Antibiotics</td>
<td>Non-enzymatic amplification (CHA)</td>
<td>0.7pg/mL</td>
<td>[54]</td>
</tr>
<tr>
<td>Prostate specific antigen (PSA)</td>
<td>Non-enzymatic amplification (CHA)</td>
<td>2.3fg/mL</td>
<td>[55]</td>
</tr>
</tbody>
</table>

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Fig. (3). Schematic illustration of the thrombin sensing strategy using target-induced intermolecular hybridization. Probe A and Probe B contain aptamer sequences simultaneously bind thrombin, allowing the hybridization between dissociating ends of two probes. The resulting 5'-overhang end of probe A as a template, probe B is extended with polymerase and dNTPs to generate B1 strand. Followed by qPCR with P1 and P2 primers, thrombin detection is realized by PCR detection of B1 strand [57].
In addition, Endonuclease-induced Cycling signal Amplification (ECA) strategies are also ideal for aptamer fluorescence assays. It can be carried out at room temperature. Because of the high efficiency of enzyme digestion and the clear cleavage site, it is easy to design aptamer probes. In 2008, Li et al. combined endonucleases with molecular beacons for highly sensitive determination of target DNA, reducing the detection limit by 2 orders of magnitude [61]. Later, ECA strategies have been widely used in the study of nucleic acid probes. Zheng et al. combined the aptamer hairpin probe design with it to achieve a sensitive determination of thrombin, the detection limit is reduced by 3 orders of magnitude [62]. Liu et al. achieved sensitive detection of ATP, cocaine and thrombin by using Exonuclease III. In absence of target, aptamer sequence cannot be cut by Exonuclease III, because the aptamer probe sequence exists in single-stranded form. After the target molecule is bound to the aptamer probe, aptamer 3’ is modified to form a double-stranded structure, thereby inducing its enzymatic hydrolysis. As the aptamer probe degrades, the target molecule is released, recombines with the other new probe and induces enzymatic hydrolysis. Thus, A single target can repeatedly interact with multiple probe molecules, inducing its enzymatic hydrolysis and enhancing its fluorescence signal [63].

The proteolytic enzyme signal amplification strategy generally has a high signal derived from the energy released by the hydrolysis of multiple phosphodiester bonds, and the number of conversions of an exponential amplification strategy such as PCR or Ligase Chain Reaction (LCR) may even be up to about 8 orders of magnitude, high sensitivity or even single molecule determination can be achieved. However, due to the involvement of biological enzymes in these signal amplification approaches, these assay methods are commonly susceptible to reaction environments, and harsh reagent storage conditions are also required [64].

4.2. Non-enzymatic Amplification Strategies

The dynamic assembly of DNA nanostructures based on strand displacement cycles can amplify molecular recognition signals and improve the performance of aptamer fluorescent probes. Two of the typical representatives are amplifications of the Hybridization Chain Reaction (HCR) (refer to Fig. 4) [65] and the Catalytic Hairpin Assembly (CHA) [66]. They are driven based on a fulcrum-mediated strand displacement mechanism: The energy is first hidden in the DNA hairpin structure, and in the presence of catalytic DNA, hybridization of the DNA hairpins is initiated with the fulcrum hybridization as a driving force. It is worth noting that this switch probe is designed based on the difference in dynamics, prolonging the reaction time will significantly reduce the SBR. Zhang et al. introduced a design strategy that allows a specified input oligonucleotide to catalyze the release of a specified output oligonucleotide, which in turn can serve as a catalyst for other reactions. This reaction, which is driven forward by the configurational entropy of the released molecule, provides an amplifying circuit element that is simple, fast, modular, composable, and robust [67].

Allosteric DNases can also be used in the design of aptamer probes. The design strategy for such probes can be divided into two categories: One is the remodeling of DNase and the other is based on the regulation of the binding force between the DNase chain and the substrate chain. Achebach et al. introduced ATP aptamer sequences in the 8-17 DNase region and inhibited its hybridization to the substrate strand through the antisense strand [68]. After ATP binds to the aptamer, the antisense strand is displaced to induce hybridization of the enzyme strand to the substrate strand and activate its activity. Zhang et al. split the DNase strand and introduced the complete sequence of the ATP aptamer. ATP induces the secondary structure remodeling of the aptamer, assembles the enzyme chain into a complete structure, and activates its catalytic activity. In the absence of ATP, the enzyme chain cannot form a complete structure and catalyze the degradation of the substrate chain [69]. Zhang et al. designed an aptamer fluorescent probe based on strand displacement and named it “molecular translator”. In this probe, a part of the aptamer is linked to the surface of the nanogold via a double-stranded portion, and the fluorescent group is labeled in the double-stranded chain. After the target is bound to the aptamers, they are induced to form an intramo-

Fig. (4). Basic HCR system. (a-c) Secondary structure schematic of HCR function (*denote complementarity). (a) Hairpins H1 and H2 are stable in the absence of initiator I. (b) I nucleates at the sticky end of H1 and undergoes an unbiased strand displacement interaction to open the hairpin. (c) The newly exposed sticky end of H1 nucleates at the sticky end of H2 and opens the hairpin to expose a sticky end on H2 that is identical in sequence to I. Hence, each copy of I can propagate a chain reaction of hybridization events between alternating H1 and H2 hairpins to form a nicked double-helix, amplifying the signal of initiator binding [65].
The fluorescent probe on the surface of the gold nanoparticle was replaced, and the fluorescence signal was enhanced to achieve a quantitative measurement [70].

However, the driving force of non-enzymatic amplification strategies is generally small: HCR and CHA use base hybridization as a driving force for the reaction, while DNA proteolytic enzymes are driven by the hydrolysis energy of a single phosphodiester bond. Therefore, the signal conversion based on non-enzymatic amplification strategies is generally low.

5. MEDIATION OF NANOMATERIALS

Nanomaterials are widely investigated owing to their diverse functional domains [71]. In the design of aptamer probes, a series of nanomaterials have been applied, such as nanogolds, quantum dots, upconversion luminescent nanoparticles, silver nanoclusters, and carbon nanomaterials [72-80], Table 5.

5.1. Gold Nanoparticles

The size of Gold Nanoparticles (AuNPs) is precisely controllable on the nanoscale, and the morphology varies, such as spherical, rod-like, polyhedral, or even cage-like. Through the covalent attachment of gold-sulfur bonds, it is prone to surface-modification and can be assembled with small molecules, oligonucleotides, and proteins [81]. In addition, nanogold has a strong absorption of fluorescence of almost all dye molecules, with high quenching efficiency and a quenching constant (stern volmer) of 5 orders of magnitude higher than that of common organic quenchers. Based on the adsorption of DNA with positively charged dyes and the quenching of fluorescence with gold nanoparticles, researchers designed a label-free fluorescent probe for protein determination, Fig. (5). After the aptamer is covalently cross-linked with nanogold via Au-S bond, the fluorescent dye embedded in the DNA is close to the nanogold and the fluorescence is quenched. After the target protein PDGF was bound to the aptamer, the dye was replaced and the fluorescence recovered to achieve quantitative determination of PDGF [82]. Zheng et al. used aptamer-functionalized nanogold probes for ATP measurement and imaging studies and named them nanoflare. Firstly, the aptamer of ATP was covalently modified on the surface of nanogold as a recognition unit, and then a fluorescently-labeled partially complementary sequence was added as a signal unit. After the probe entered the cell and interacted with ATP, DNA strand melted and the fluorescence signal was enhanced [83].

It is also possible to assemble the aptamer on the nanogold surface with a thiol modification. The covalent linkage would enable relatively uniform nanoprobess with a low detection limit. Due to the wide range of fluorescence quenching of nanogold, it can be applied in multi-target assays. Using a covalent modification or physical adsorption method, the aptamer fluorescent probe is attached to the nanogold surface, and the presence of multiple targets can restore the respective fluorescence signal and achieve simultaneous determination of multiple targets [84].

In addition, Pei et al. developed a polyA-based aptamer nanobeacon with improved efficiency and speed for ATP analysis [85]. It was found that poly adenine (polyA) can serve as an effective anchoring block for preferential binding with the AuNP surface, and the appended recognition block adopts an upright conformation that favors DNA hybridization. Then they designed polyA diblock oligonucleotides contained a polyA block for anchoring onto the AuNP surface and a functional block for the ATP recognition, resulting in “turn on” fluorescence detection of ATP [86-88].

5.2. Quantum Dot

Quantum Dots (QD) have many excellent photophysical properties, including high quantum yield, good light stability, size-dependent fluorescence emission wavelength, and long fluorescence lifetime. These properties can improve the performance of the probe, making the quantum dots ideal fluorescent signal groups, and have attracted wide attention in the research of biological analysis. QD are generally used in conjunction with organic quenchers or dye molecules to

Table 5. Nanomaterials applied in the design of aptamer probes (CEM: Carcinoembryonic antigen; AgNCs: Silver Nanoclusters).

<table>
<thead>
<tr>
<th>Type of Materials</th>
<th>Target</th>
<th>LOD</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gold nanoparticles</td>
<td>PDGF and PDGFR</td>
<td>2.5-10nM and 3.2nM</td>
<td>[72]</td>
</tr>
<tr>
<td>Gold nanorods</td>
<td>PDGF AA</td>
<td>10 pM</td>
<td>[73]</td>
</tr>
<tr>
<td>Quantum dots</td>
<td>Thrombin</td>
<td>0.55 fM</td>
<td>[74]</td>
</tr>
<tr>
<td>Graphene/QD composite</td>
<td>Thrombin</td>
<td>100 nM</td>
<td>[75]</td>
</tr>
<tr>
<td>Fe3O4 graphene oxide</td>
<td>ATP</td>
<td>0.1 nM</td>
<td>[76]</td>
</tr>
<tr>
<td>Upconversion nanoparticle</td>
<td>E. coli ATCC 8739</td>
<td>3 cfu/mL</td>
<td>[77]</td>
</tr>
<tr>
<td>AgNCs@Apt@UiO-66 nanocomposite</td>
<td>CEA</td>
<td>4.93 pM</td>
<td>[78]</td>
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<tr>
<td>Graphene</td>
<td>Lysozyme</td>
<td>0.5 nM</td>
<td>[79]</td>
</tr>
<tr>
<td>SWCNTs</td>
<td>IgE</td>
<td>250 pM</td>
<td>[80]</td>
</tr>
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Fig. (5). Schematic representations of PDGF nanosensors that operate based on modulation of the FRET between DMDAP and Apt-AuNPs (derived from [82]).

Fig. (6). (a) Schematic illustration of QD-aptamer (Dox) Bi-FRET system. (b) Schematic illustration of specific uptake of QD-aptamer (Dox) conjugates into target cancer cell through PSMA mediate endocytosis [91].

design aptamer fluorescent probes [89]. Once the aptamer is bound to the target, the resultant nucleic acid structure changes and affects the energy transfer process between them. In order to achieve high output of the fluorescence signal, researchers have used QD probe designs for a variety of proteins and small molecules, including thrombin, epithelial mucin 1, adenosine, cocaine, and others [90]. Bagalkot et al. designed QD-aptamer conjugate that can deliver doxorubicin (Dox) to prostate cancer cells, as shown in Fig. (6). RNA aptamer covalently attached to the surface of QD, which serve a dual function as targeting molecules and as drug carrying vehicles. Due to the FRET between QD and Dox, the fluorescence of QD is quenched as a result of Dox absorbance, and the FRET between Dox and aptamer, where Dox is quenched by double-stranded aptamer. Therefore, both QD and Dox of the conjugate are in the fluorescence “OFF” state. Until the conjugate is taken up by prostate cancer cells, Dox is gradually released from the conjugate, which induces the activation of QD and Dox fluorescence to the “on” state [91]. In addition to organic molecules, quantum dots can also undergo energy transfer with nanomaterials such as gold and graphene, affecting their fluorescence behavior. This strategy is also used in aptamer probes. Liu et al. designed an aggregation-quenching quantum dot fluorescent probe that splits the aptamers and connects them to the same quantum dot. After the target has induced the assembly of the two parts of the aptamer, the quantum dots gather and their fluorescence quenches with the energy transfer of the excited state [92]. This new type of fluorescence signal transduction mechanism gives a very high sensitivity to the aptamer fluorescent probe, which is difficult to achieve in the prior split aptamer probes.
5.3. Upconversion Luminescent Nanoparticles

The lanthanide-doped upconversion nanoparticles can absorb the energy of photons in a plurality of near-infrared regions and emit photons in the visible region. Therefore, they have been widely used in biosensing and bioimaging. The upconversion of this unique anti-Stokes luminescence phenomenon has many advantages. It is suitable for quantitative detection in complex biological samples. It can reduce the self-light and scattered light of the sample and obtain a large Signal-to-Noise Ratio (SNR) and sensitivity. At the same time, near-infrared excitation can reduce the damage to the sample and also reduce the photobleaching of the probe [93]. In order to design activated fluorescent probes, quencher groups are generally introduced, but conventional organic quenchers do not effectively quench the fluorescence of the upconversion nanoparticles because most of the lanthanide metal ions are inside the nanoparticles. It cannot be close to the organic quenching group. Nano materials such as carbon nanomaterial and AuNPs can quench fluorescence remotely, so that the fluorescence of the upconversion nanomaterial can be effectively quenched [77, 94-99]. Hu et al. used a label-free aptamer-based upconversion nanosensor with Fluorescence Resonance Energy Transfer (FRET) between \( \text{NH}_2\text{-NaYF}_4\cdot\text{Yb, Ho@SiO}_2 \) (UCNPs) and AuNPs to quantify the acetamiprid in food [96]. As show in Fig. (7), with the special supramolecular interaction between the acetamiprid aptamer absorbed on the surface of AuNPs and different concentrations of acetylaminobutyl, the electrostatic repulsion between the AuNPs can be easily adjusted, resulting in different aggregation state of AuNPs in salt solution. Correspondingly, FRET is inhibited, and as acetamiprid concentration increases, the fluorescence intensity of UCNP recovers linearly.

The use of carbon nanomaterials as quenchers for upconversion nanoparticles has led to the design of fluorescent probes that have been used for the sensitive determination of a variety of target molecules, including toxins and thrombin [93]. In addition, the multi-spectral characteristics of upconversion nanomaterials can be utilized for multi-target simultaneous determination. The design of these fluorescent probes is mostly based on the ability of carbon nanomaterials to distinguish single and double chains.

Hao et al. reported aptamer-based Gold-Upconversion-Nanoparticle (Au-UCNP) pyramid that can detect thrombin and Prostate-Specific Antigen (PSA) using Surface-Enhanced Raman Scattering (SERS) and fluorescence, respectively. It was found that the higher the concentration of thrombin, the lower the intensity of SERS, and PSA connected with the PSA aptamer leads to an increase in fluorescence intensity [100].

5.4. Silver Nanoclusters

AgNCs are generally synthesized using C-rich DNA as a template, containing approximately 2-30 silver atoms. They exhibit many unique photophysical properties, such as adjustable emission wavelength, good water solubility, low cost, and good biocompatibility, making it an ideal choice for fluorescence detection and imaging signals. In addition, the fluorescence intensity of the silver nanoclusters is very sensitive to the environmental changes of the DNA template, which is very favorable for the design of the sensitive fluorescent probes.

Yeh et al. reported the design of an activated silver nanocluster fluorescent probe. In this probe, the as-synthesized silver nanoclusters have very weak fluorescence,
but after adding a G-rich complementary sequence, the fluorescence intensity is enhanced by more than two orders of magnitude, and this mechanism is used to design binary nucleic acid probes [101]. Based on this, Zhang et al. connected the two aptamers of thrombin to the template DNA and G-rich DNA respectively, the binding of thrombin will connect them into the internal structure and induce a strong fluorescence signal of the silver nanoclusters, thus realizing the label-free detection of thrombin [102]. They also found that the complex of G4 and heme can also quench the fluorescence of silver nanoclusters and use it for highly sensitive assays. The ATP-induced aptamer's hairpin structure opens to form G4 that binds to the heme, where the silver cluster undergoes a photoinduced electron transfer process with the iron ion in the heme, quenching the fluorescence [102]. They also found that the complex of G4 and heme can also quench the fluorescence of silver nanoclusters and use it for highly sensitive assays. The ATP-induced aptamer's hairpin structure opens to form G4 that binds to the heme, where the silver cluster undergoes a photoinduced electron transfer process with the iron ion in the heme, quenching the fluorescence [102].

Li et al. designed aptamer-based silver nanoparticle aggregation and realized a highly sensitive optical detection method based on localized surface plasmon, Fig. (8). Two aptamer-based silver nanoparticles, Aptamer/Oligomer-A/Cy3-modified AgNPs (Tag-A) and Aptamer/Oligomer-B/Cy3-modified AgNPs (Tag-B) hybridization form a silver nanoparticle aggregate that produced a red shift. The enhanced fluorescence results from increasing the content of the Cy3 molecules, and their emission resonances couple with the extended Local Surface Plasmons (LSPs) of AgNP aggregation [110].

5.5. Carbon Nanomaterials

Similar to nanogold, carbon nanomaterials can quench the fluorescence signal of a probe in the vicinity, as show in Fig. (9). Due to the π-π stacking action, there is a strong non-covalent interaction between carbon nanomaterials and aptamers. However, once the aptamer is combined with its target, the steric hindrance becomes large and the interaction with the carbon nanomaterial is significantly weakened. These properties have become an important basis for the design of unlabeled fluorescent probes, and researchers have carried out a large amount of research work in these areas to achieve a sensitive measurement of a variety of targets, including ATP, thrombin and lysozyme [111].
ed cellular delivery and in situ molecular probing in living cells by employing Graphene Oxide nanosheets (GO-nS) as aptamer cargo and sensing platforms. Due to the particular interaction between GO and DNA molecules, an aptamer/GO-nS nanocomplex was designed and used as a real-time biosensing platform in living cell systems [112]. Furthermore, the combination of DNA and carbon nanomaterials enhances their dispersibility in solution, and this property can also be used in the design of aptamer fluorescent probes. Ouyang et al. introduced a rare earth coordination fluorescent group as a signal and designed an unlabeled aptamer fluorescent probe for the quantitative determination of lysozyme [113]. After investigated the effect of the combination of carbon nanomaterials and DNA on enzymatic hydrolysis efficiency, the researchers found that both carbon nanotubes and graphene can inhibit the enzymatic cleavage process, and this property is also used in the design of signal amplification strategies. Lu et al. used graphene to inhibit the enzyme digestion and designed an aptamer fluorescent probe for high sensitivity determination of ATP and cocaine, reducing the lower limit of detection by more than two orders of magnitude, and significantly improved the performance of the aptamer fluorescent probes [114].

CONCLUSION

As a kind of chemical antibody, aptamer has many advantages such as wide range of target, low relative molecular mass, high chemical stability, low toxicity and low immunogenicity, in addition to its specificity and affinity similar to protein antibody. Moreover, as a nucleic acid molecule, the aptamer has the characteristics of simple synthesis, flexible design, easy modification of multiple functional groups or nanomaterials, and convenience for introduction of nucleic acid signal amplification means. The aptamer fluorescent probe combines the molecular recognition properties of aptamers with the excellent optical detection performance of fluorescent probes, and has broad application prospects in the field of biochemical analysis and bioimaging. This article reviews the design of a series of novel aptamer fluorescent probes, some of which can achieve highly sensitive quantitative assays, some can achieve simultaneous detection of multiple targets, and others can be used in cell imaging and in vivo imaging. According to the source of fluorescence signal and design strategy, we categorize aptamer fluorescent probes into four groups: dye intercalation, conversion of conformation, signal amplification, and mediation of nanomaterials.

As for the dye molecules introduced in the probes, they can be varied according to customized requirements. When it comes to in vivo applications, such as in vivo imaging of targets in animals, infrared or near-infrared dyes are preferable to green dyes as the former dyes improve tissue penetration depth and reduce auto fluorescence of tissues, which would strongly enhances the sensitivity of in vivo imaging.

For the fluorescent probes based on the conversion of conformation, the aptamer mainly functions to recognize the target molecule and does not take an important role in the endocytosis and transport of the probe. Since aptamers are negatively charged and easily degraded by intracellular hydrolases, such switch-type probes generally contain nanoparticles. The presence of nanomaterials, such as nanogolds and carbon nanomaterials can protect the aptamers from hydrolysis and extend their applications in biological medium.

The proteolytic enzyme signal amplification strategy generally uses the energy released from the hydrolysis of multiple phosphodiester bonds as a driving force. The enzyme-free signal amplification strategy does not require the use of proteolytic enzymes, is cost-effective and easy to store, and is suitable for the analytical determination of complex biological samples.

In addition to organic fluorescent dyes, fluorescent nanoparticles can also be used for the construction of aptamer imaging probes, such as silver nanoclusters, quantum dots, and upconverted particles. They have many advantages, such as the non-labeling of silver nanoclusters, the light stability of quantum dots, and the low background of the upconversion, which have become hot spots in the design of aptamer imaging probes. Based on the significant quenching effects and long-range quenching effects of nanogold and carbon nanomaterials on the fluorescence signal, a variety of fluorescent molecular switches can be designed, which realize a variety of logical relationships. Especially nanogold, it can give a high gain value to the fluorescence switch. Graphene has good biocompatibility, high loading capacity and can protect nucleic acid aptamers from degradation by external enzymes, and is an ideal choice for carrying intracellular molecules.

Although the aptamer fluorescent probes have the abovementioned advantages, their application research is still in its infancy, and there are still many problems to be solved [115]. The methods for screening aptamers are relatively tedious and time-consuming, and the number of aptamers currently screened out is still very limited, which limits the development of aptamer fluorescent probes to some extent [116]. Aptamers have poor biological stability because they tend to be degraded by nucleases that are abundantly present in biological systems. It is susceptible to the effects of complex biological systems and generates false positive signals. At present, most of the analytical applications of fluorescent probes based on aptamers remain at the methodological stage of research, and the target of analysis is mainly focused on a few kinds of target substances such as thrombin, and the practical application is far less than the popularization of antibodies. How to overcome these limitations and further expand the application of aptamer-based fluorescent probes in the field of life sciences still requires the constant efforts of scientific researchers.

In the future, the application of aptamer fluorescent probes in biochemical analysis and bioimaging is mainly directed at the analysis of biomarkers and the analysis of tumor imaging, so as to be used for early diagnosis of cancer and improve the efficiency of tumor healing. In addition, aptamer fluorescent probes can also be applied to stem cell tracking to study the fate of stem cells in vivo.
LIST OF ABBREVIATIONS

AAP = Activated Aptamer Probe
Ag10NPs = Silver Decahedral Nanoparticles
AgNCs = Silver Nanoclusters
AgNPs = Silver Nanoparticles
ATP = Adenosine Triphosphate
Au-UCNP = Gold-Upconversion-Nanoparticle
AuNPs = Gold Nanoparticles
CCRF-CEM = T-leukemia Cell Lines
CEM = Carcinoembryonic Antigen
CHA = Catalytic Hairpin Assembly
DAPI = 4′,6-diamidino-2-phenylindole
DNA = Deoxyribonucleic Acid
Dox = Doxorubicin
EB = Ethidium Bromide
ECA = Endonuclease-induced Cycling Signal Amplification
FITC = Fluorescein Isothiocyanate
FP = Fluorescence Polarization
FRET = Fluorescence Resonance Energy Transfer
GO-ns = Graphene Oxide Nanosheets
HCR = Hybridization Chain Reaction
HIV = The Human Immunodeficiency Virus
IgE = Immunoglobulin E
Lac = Lactoferrin
LAMP = Loop-Mediated Isothermal Amplification
LCR = Ligase Chain Reaction
LSPR = Local Surface Plasmon Resonance
LSPs = Local Surface Plasmons
MCF-7 Cells = Human Breast Adenocarcinoma Cell Line
MEF = Metal-Enhanced Fluorescence
MEF = Metal Enhanced Fluorescence
mM = Mmol per liter; mmol/l
nM = Nanomoles per liter; nmol/l
PCR = Polymerase Chain Amplification
PDGF = Platelet-Derived Growth Factor
PDGF-AA = Recombinant Human Platelet Derived Growth Factor-AA
PDGF-BB = Recombinant Human Platelet Derived Growth Factor-BB
PDGFR = Platelet-Derived Growth Factor Receptor
PEG = Polyethylene Glycol
pM = Pimore per liter; pmol/l
PSA = Prostate-specific Antigen
PTCDI = Perylenes
QCM = Quartz Crystal Microbalance
QD = Quantum Dots
RCA = Rolling Circle Amplification
RNA = Ribonucleic Acid
SBR = Signal-to-Back Ratio
SERS = Surface-Enhanced Raman Scattering
SNR = Signal-to-Noise Ratio
SPIA = Single Primer Isothermal Amplification
SPR = Surface Plasmon Resonance
TASPI = Pyrazines
TOTO = Cyanine Dimer TO-PRO
TWJ = Three-Way Junctions
uM = Micro mole per liter; umol/l
VEGF = Vascular Endothelial Growth Factor

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (No. 31670997, No. 21275043, and No. 51604104), Natural Science Foundation of Hunan Province (2017JJ3421), and the Basic Research Program of Shenzhen City (No. JCYJ20160530193417959).

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