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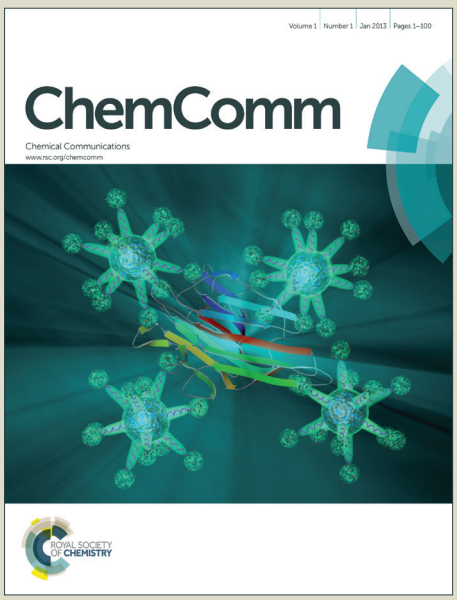

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DNAzyme-based Biosensors and Nanodevices

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DNAzymes, screened through in vitro selection, have shown great promise as molecular tools in the design of biosensors and nanodevices. The catalytic activities of DNAzymes depend specifically on cofactors and show multiple enzymatic turnover properties, which makes DNAzymes to be both versatile recognition elements and outstanding signal amplifiers. Combining nanomaterials with unique optical, magnetic and electronic properties, DNAzymes may yield novel fluorescent, colorimetric, surface-enhanced Raman scattering (SERS), electrochemical and chemiluminescent biosensors. Moreover, some DNAzymes have been utilized as functional components to perform arithmetic operations or as "walker" to move along DNA tracks. DNAzyme can also function as a promising therapeutics, when designed to complement with target mRNA or viral RNAs and consequently lead to down-regulation of protein expression. This feature article focuses on the most significant achievements in using DNAzymes as recognition elements and signal amplifier for biosensors, and highlights the applications of DNAzymes in logic gates, DNA walkers and nanotherapeutics.

1. Introduction

The developments in biology over the past 20 years have convincingly proved that nucleic acids are not only hereditary material for the storage of genetic information but also versatile biomacromolecules from which defined functions as ligand binding and catalysis can be derived, just like proteins. These functional nucleic acids (FNAs), including DNA/RNA aptamers, ribozymes/DNAzymes, have been widely explored as molecular tools for various applications.[1,2] Among them, DNA aptamers and DNAzymes, with the outstanding stability, were researched widely to construct robust recognition and catalytic units for bioanalysis and nanobiotechnology. This review is intended to focus on DNAzymes and their recent applications in the development of biosensors and nanodevices.

DNAzyme was a kind of catalytic nucleic acids, which could cleave specific substrates in the presence of cofactors. They can be created to recognize a wide range of analytes through in vitro selection, a test-tube evolution that does not need to use animals or cells. The synthesis of DNAzymes is relatively inexpensive and easy with excellent batch-to-batch consistency. Furthermore, they are easily modified and functionalized, which facilitate the versatile signal amplification and read out purposes.[3] The first kind of DNAzymes, which were capable of catalyzing a trans-esterification reaction in the presence of Pb^{2+}, was discovered in 1994 by Breaker and Joyce through a process called in vitro selection, demonstrating that single strand DNA could indeed act as catalyst similar to ribozymes and proteins.[4] Since then, several DNAzymes have been isolated to catalyze various biological reactions such as RNA cleaving[5], DNA cleaving[6], ligation[7], or phosphorylation reactions[8], et al. However, RNA-cleaving DNAzymes that cleaves a single RNA linkage embedded in a DNA sequence, remain the best characterized and likely the most widely used DNAzymes currently available. Generally, this type of DNAzyme is formed by a substrate strand and an enzyme strand (Fig. 1A). The substrate strand contains a single RNA linkage (rA) that serves as a cleavage site while the enzyme strand is composed of one catalytic core and two arms. The motifs of enzyme strand have distinct primary and secondary structures, unique cofactors, pH dependencies and specific substrates. In the presence of catalytic cofactors, the enzyme strand cut cleavage the substrate strand into two parts. The cofactors include different metal ions and amino acids, which makes it possible to design different kinds of cofactor responsive biosensors.[3]

Moreover, DNAzyme shows high specificity for its substrate strand and even one single base mismatch in the antisense arms will significantly decrease the cleaving activity. Therefore, the high flexibility in binding arm design, excellent specificity in substrate recognition and multiple enzymatic turnover properties make these DNAzymes not only versatile recognition elements and outstanding signal amplifiers for biosensing, but also promising mRNA-targeted DNAzyme therapeutics. Among all the DNAzymes, 10-23 and 8-17 are two well known DNAzymes that can cleave an all-RNA substrate under physiological conditions as long as its two binding arms formed stable duplexes with nucleotides flanking the cleaving junction. Taking 10-23 DNAzyme as an example, the name '10-23' come from the 23d clone from the 10th round of PCR during the selection. The 10-23 DNAzyme is reported to have catalytic efficiency of about 10^9 $M^{-1}min^{-1}$,[3] which is about 100-fold higher cleavage activities than that of the most active ribozymes and shows 10^5-fold higher stability under physiological condition.

Another kind of DNAzyme is named as G-quadruplex-DNAzyme. In such DNAzyme, the G-rich sequence or sequences will fold into parallel or antiparallel G-quadruplex in the presence of K^+, Pb^{2+}, or NH_4^+. The G-quadruplex DNAzyme could employ hemin to mimicking the peroxidase activity[9] and selectively catalyze luminol/H_2O_2 to produce chemiluminescence (CL),[10] or oxidize 2,2-azino-bis-(3-ethylben-zthiazoline-6-sulfonic acid (ABTS) to generate color change (Fig. 1B).[11] This kind of catalytic G-quadruplex-DNAzyme (G4-DNAzyme) is frequently utilized as recognition element in colorimetric biosensors for K^+, Pb^{2+}, or used as special label for signal amplification.

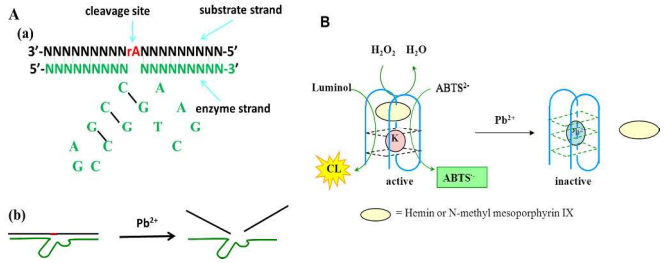

Fig.1 A. (a) Secondary structure of the DNAzyme (8-17). (b) Cleavage of substrate strand by enzyme strand in the presence of Pb^{2+}; B. The G-quadruplex bind to hemin will form a catalytic horseradish peroxidase-mimicking DNAzyme (HRP-mimicking DNAzyme or G4-DNAzyme). G4-DNAzyme can catalyze luminol/H_2O_2 to produce chemiluminescence(CL), or oxidize 2, 2 -azino-bis-(3-ethylben-zthiazoline-6-sulfonic acid (ABTS) to generate color change. The presence of Pb^{2+} ions will lead to a conformational change and the peroxidase-like activity will be inhibited.

Nanomaterials show unique optical, magnetic and electronic properties. With the rapid development in both nanomaterials and DNAzymes in recent years, it is possible to construct nano-bioconjugates by combining the unique physical properties of nanomaterials with the recognition and catalytic properties of DNAzymes. The integration of RNA-cleaving DNAzymes with metallic nanoparticles that combine the cleavage property of DNAzyme with the sensitive distance-dependent optical properties of nanoparticles provides novel sensing systems based on colorimetric[12], dynamic light scattering (DLS)[13], or surface-enhanced Raman scattering (SERS)[14]. Fluorophore/quencher (F/Q) pairs are commonly used to indicate the cleavage events of DNAzymes by judicious arrangement of F and Q so that they are close prior to the catalysis but separate from each other after the cleavage.[15,16] It is worth noting that quantum dots (QDs) are superior alternatives to traditional organic fluorophores[17], while gold nanoparticles (AuNPs)[12], graphene oxide (GO)[18] and carbon nanotubes (CNTs)[19] are long distance superquencher. The coupling of DNAzymes with QDs, AuNPs, GO or CNTs produces some new approaches in the designing of biosensors or nanodevices. Another important functional goal for combining DNAzymes with nanomaterials is to construct nanodevices for biological applications, such as logic gate operations, in vivo imaging or targeted drug delivery. Nanomaterial based delivery systems can afford a high loading efficiency and protect DNAzymes from nuclease digestion during the transfection process. By coupling with appropriate DNAzymes, the nanobioconjugates might also be promising nanotheropeutics for gene therapy.

This feature article will provide a survey of the most recent developments in lighting up DNAzymes as recognition elements and signal amplifiers for biosensing, as well as DNAzyme-based nanodevices for bioapplications.

2. DNAzyme-based biosensors

All biosensors are composed of two basic components, a molecular recognition element that can selectively bind to target analytes and a transducer component that can translate recognition events into a detectable signal. As an important kind of functional DNA, DNAzymes possess two unique properties: the recognition and cleavage ability that can be used to construct recognition elements mainly towards cofactors, and the multiple enzymatic turnover properties, which is well suited for signal amplification.

2.1 Biosensors employing DNAzymes as recognition elements

The DNAzymes with RNA-cleaving function always require specific cofactors including metal ions or amino acids to catalyze the cleaving process. The presence of cofactors helps the DNAzyme sequence folding into specific spatial structures in order to form catalytic core for the cleavage. Using this property, quite a number of highly specific DNAzymes-based sensors for cofactors have been constructed, including Pb^{2+},[15] Mg^{2+},[20] Zn^{2+},[5] UO_2^{2+},[21] Mn^{2+},[22] Co^{2+},[23] Cu^{2+},[24] and histidine[25].

2.1.1 Fluorescent DNAzyme biosensors

The most popular way of designing a RNA-cleaving DNAzyme based fluorescent probe is to report the analytes through the introducing of a fluorophore/quencher (F/Q) pair which would release the fluorophore and give a signal after substrate cleavage. The F and Q pairs are judiciously arranged, so that they are in close proximity prior to catalytic cleavage, but be separated from each other after the cleavage events. The accurate positions of the F and Q have many choices as shown in Fig. 2. In order to decrease background, the design of F and Q experienced several changes. At first, they were placed at the two ends of the substrate strands (Fig. 2a). Also, they could either be placed at the end of the binding arms or next to the cleavage site flanking the cleavage site (Fig. 2b). In order to shorten the distance of each other, they could be set on the same side but different strands (Fig. 2c). Other choices consist of using an extended enzyme part acting as a template to place the Q close to the F (Fig. 2d), introducing a second Q on the other end of the substrate (Fig. 2e) and using some nanoparticles like AuNPs and CNT as the quencher (Fig. 2f). For an instance, placing F and Q on the same side but different strands, Lu et al developed a Pb2+ sensor with low background at 4 ℃ due to the proximity between F and Q.14b However, high background fluorescence would be observed at higher temperature if the strands were not hybridized 100%. When a second Q was introduced on the other end of the substrate, even if the enzyme and substrate strands were dissociated, the fluorescent background was still very low.[26] Therefore, the detection showed no restriction on operation temperature, and this system was successfully applied in designing Cu^{2+} and UO_2^{2+} sensors.[27,28]

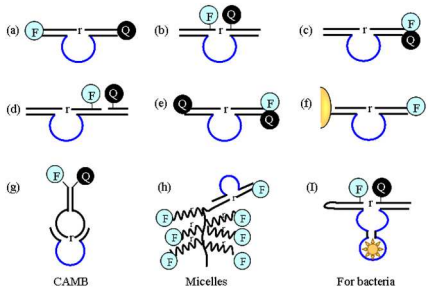

Fig. 2 Schematic illustration of the arrangement of fluorophore/quencher (F/Q) pairs on the DNAzyme. (a) F and Q were placed at the two ends of the substrate strands. (b) F and Q were placed next to the cleavage site flanking the cleavage site. (c) F and Q were placed on the same side but different strands. (d) Use an extended enzyme part acting as a template to place the Q close to the F. (e) A second Q was introduced on the other end of the

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substrate. (f) Nanoparticles as quencher. (g) F and Q were placed at the two ends of a hairpin-shaped molecular beacon, which called catalytic and molecular beacon (CAMB). (h) Using DNA–nanoparticle micelles as supramolecular fluorogenic substrates. (i) Fluorogenic DNAzyme probes for bacteria.

In these designs, however, the DNAzyme strand acted as both catalyst and quencher, and either equal or excess amount of enzyme strands were needed in order to maintain efficient quenching. Zhang and Lu suggested a general strategy of catalytic and molecular beacon (CAMB) that combined high quenching efficiency of the molecular beacons (MBs) with the catalytic beacon for multiple enzymatic turnover properties (Fig. 2g). The MBs based substrate presented lower background fluorescence and improved signal-to-noise ratios. The DNAzyme strands were liberated from the role of being a quencher and realized the true potential of multiple turnover enzymes for signal amplification. Therefore, the CAMB sensor for Pb^{2+}exhibited a detection limit of 600 pM, which was much lower than that of other catalytic beacon Pb^{2+} sensors.[29] Gianneschi and coworkers proposed DNA-nanoparticle micelles (Fig. 2h), which formed via the assembly of dye-labelled DNA-brush copolymer surfactants, as supramolecular fluorogenic substrates to overcome the product inhibition limiting of normal ssDNA substrates. The DNAzyme catalytic activity could be greatly enhanced because of the high effective substrate concentration and intraparticle strand invasion in the micelles. This study introduced a novel approach to substrate design and enabled a truly DNAzyme catalytic amplification detection assay.[30]

In addition to these DNAzymes, novel DNAzymes targeted other than cofactors can also be isolated from random-sequence DNA libraries to cleave a substrate predoposited with F/Q pair close to the cleavage sites. Li group carried out several in vitro selections to derive such fluorescent DNAzyme probes that were able to detect bacteria (Fig. 2i). They selected a highly sensitive and selective fluorogenic DNAzyme probes for Escherichia coli (E. coli) and demonstrated that this probe can detect a single live cell.[31]

The DNAzyme-based fluorescent sensors using organic molecules as quenchers frequently suffer from incomplete fluorescence quenching. Besides, it is unfavorable to remove the unhybridized substrates by annealing DNAzymes with substrates. In order to overcome this issue, gold nanoparticles (AuNPs), gold nanorods (GNRs), carbon nanotubes (CNTs) and graphene oxide (GO) are explored as fluorescence quenching materials in sensor designing. Chung and co-workers immobilized fluorescein labeled substrate on AuNPs through thiol linkages and acquired nearly 100% quenched fluorescence (Fig. 3A(a)).[32] This strategy could detect Pb^{2+} as low as 5 nM within 20min without the preliminary and the post treatments. Liu and co-workers[33] reported another similar turn-on fluorescent sensor based on GR-5 DNAzyme for the detection of Pb^{2+}, since GR-5 had been reported to offer a much higher selectivity towards Pb^{2+} ions than 8-17 DNAzyme over other competing metal ions.[34] Wang et al developed a sensitive gold nanorods-based FRET assay using 8-17 DNAzyme and got a 61.8 pM detection limit for Pb^{2+} (Fig. 3A(b)).[35]

Water-soluble graphene oxide (GO) and carbon nanotubes (CNTs) were reported to be fluorescence super quencher with the long-range nanoscale energy transfer property. So, they have attracted increasing interest in the development of biosensing systems. Many sensing systems were based on the fact that the ssDNA and dsDNA duplex exhibit different affinity toward GO and CNTs. Based on these properties, Zhang and co-workers[18] suggested a GO-DNAzyme based biosensor for amplified fluorescence "turn-on" detection of Pb^{2+} (Fig. 3 A(c)). In this design, a 5'-FAM-labeled substrate strand was hybridized with DNAzyme strand to form a DNAzyme-substrate hybrid, which contains a large ssDNA loop to bind with the GO through electrostatic interaction and π–π stacking. The binding arm near the FAM label had only 5 base pairs, which enhanced the quenching efficiency and also making the catalytic cleavage-induced FAM-linked oligonuleotide moiety difficult to bind to the GO to guarantee a high signal-to-background ratio (SBR) for the assay. This "turn-on" biosensor exhibits a high sensitivity toward Pb^{2+} with a detection limit of 300 pM. Fan and coworkers[36] utilized that the interaction difference between GO with ss- and ds- DNA would result in different fluorescent quenching efficiencies and developed a similar GO-DNAzyme nanoprobe for Pb^{2+} with tunable dynamic range.

Although DNAzyme-based sensors for metal ions have found wide extracellular applications, intracellular applications of DNAzyme sensors remain significant challenges. Another interesting application of the above mentioned AuNPs, CNTs and GO nanomaterials is to act as delivery carriers for intracellular sensing. In Dordick and coworker's study,[37] they concluded that DNAzymes were still highly active when conjugated to MWNTs through streptavidin-biotin interactions and exhibited multiple turnover enzyme behavior without the need of DNAzyme-substrate hybridization between each catalytic event.

Lu and co-workers (Fig. 3B)[38] demonstrated the first DNAzyme-AuNPs probe for uranyl ion in living cells. They chose AuNPs as carriers for cellular delivery of DNAzymes because the AuNP-DNAzyme conjugates have large DNA loading efficiency and increased resistance to enzymatic degradation in cell or serum. The 13 nm AuNPs were functionalized with DNAzyme whose enzyme strand was modified with a thiol at the 3' end to conjugate the gold surface and substrate strand contained a Cy3 fluorophore at the 5' end and a quencher at the 3' end. There was a poly-A spacer between the enzyme strand and AuNPs surface, which weakened the quenching efficiency of AuNPs. Thus, they added another BHQ-2 quencher at the 3' end of the substrate strand to ensure complete quenching. In the presence of UO_2^{2+}, the cleavage would release Cy3-labeld short oligonucletides from the gold surface and result in increased fluorescence. They demonstrated that this DNAzyme-AuNP conjugates could readily enter cells and serve as a metal ion sensor within a cellular environment. This simple and general platform could theoretically convert any DNAzymes into intracellular probes for metal ions.

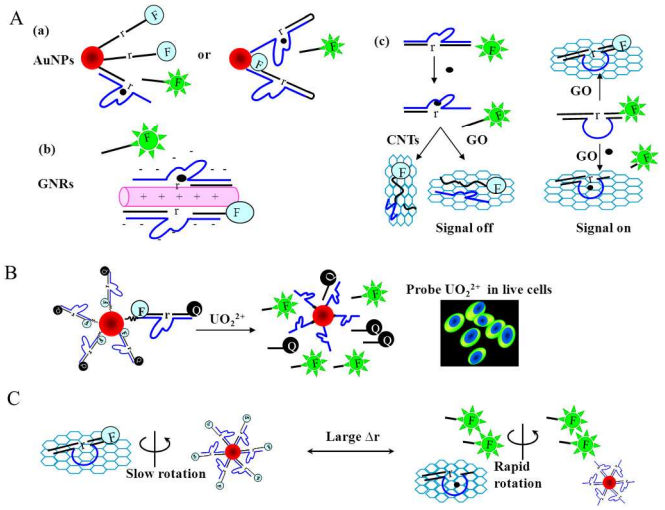

Fig. 3 A. DNAzyme-based fluorescent biosensors using (a) gold nanoparticles (AuNPs), (b) gold nanorods (GNRs), (c) carbon nanotubes (CNTs) or graphene oxide (GO) as fluorescence superquencher. In (c), a large ssDNA loop in the DNAzymes was used to bind with the GO through electrostatic interaction and π–π stacking. B. The application of DNAzyme-AuNPs probe for the detection of uranyl ions in living cells. C. DNAzyme-

based fluorescence anisotropy (FA) assay utilizing AuNPs and GO as FA amplifier.

Fluorescence anisotropy (FA) is a polarization-based attractive phenomenon which is sensitive to the mass and the size of the rotating body. Nanomaterials, especially AuNPs, CNTs and GO, could be used as promising FA amplifier (Fig. 3C). When fluorescence (F) labeled DNAzyme/substrate hybrids are installed on the surface of the nanoparticles through π–π stacking, or covalent linkage, or electrostatic adsorption, the rotation of the fluorescent labels could be coupled with the entire nanoconjugates to obtain high anisotropy according to the Perrin equation. Then, in the presence of cofactors, the cleaved short F-labeled DNA fragment is kept away from nanomaterials and rotate at a rate commensurate with its small size, providing dramatic decreases in FA. Yin et al proved the concept that AuNPs can enhance and improve the performance and sensitivity of the FA assay and their DNAzyme-AuNPs based FA assay can be used for Cu^{2+} and Pb^{2+} detection with a LOD of 1 nM.[39] Huang and co-workers demonstrated that the introducing of graphene oxide (GO) could effectively enhance the fluorescence anisotropy (FA) of TAMRA and established a GO-enhanced FA strategy for Cu^{2+} detection.[40]

2.1.2 Colorimetric DNAzyme Biosensors

Metal nanoparticles, especially gold nanoparticles (AuNPs), have some special distance-dependent surface plasmon properties. Besides, they are biocompatible, readily functionalized with a variety of ligands and relatively stable under physiological conditions. Recent years we have witnessed the explosion of interest in the use of RNA-cleaving DNAzymes-AuNPs platform to develop sensitive sensors based on colormetric.

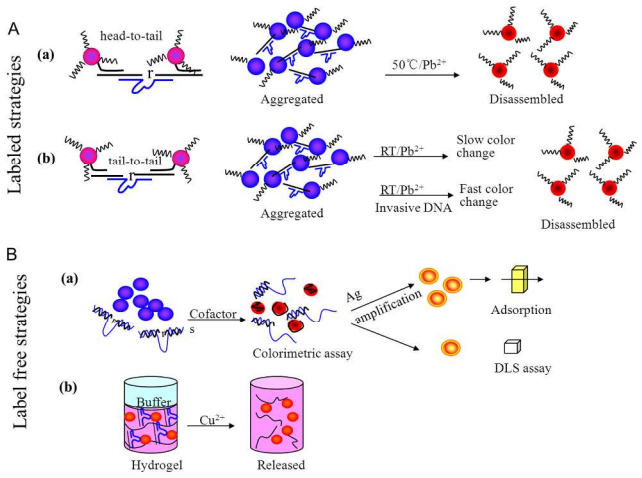

Fig. 4 Schematic illustration for the labeled and label free strategies of recognition-triggered aggregation state change of nanoparticles. A labeled strategies (a) Head-to-tail aligned AuNPs aggregates were disassembled in the presence of Pb^{2+} at 50 ℃. (b) Tail-to-tail aligned AuNPs aggregates were disassembled in the presence of Pb^{2+} at RT, the color change process could be accelerated with the help of invasive DNA. B. label free strategies (a) Utilizing the adsorption differences of single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) on gold nanoparticle surface to develop colorimetric, adsorption and DLS assay for cofactors. (b) DNAzyme cross linked hydrogel underwent gel–sol transition in response to Cu^{2+} ions and released the pre-trapped AuNPs.

The surface-plasmon resonance absorption of AuNPs is influenced by the particle size and the distance between particles. The color of the solution can change from red to purple (or even blue), in response to the dispersed and aggregated AuNPs, with the absorption peak change from about 520 nm to 650 nm, or even longer wavelength. According to this property, a series of colotrimetric sensors for metal ions detection based on RNA-cleaving DNAzyme-AuNPs platform have been constructed by the Lu group and other groups.[12, 41-44] In their first design, uncleaved substrates were used as linkers to assemble the AuNPs into aggregates, and in the presence of Pb^{2+}, the DNAzymes were activated and separated from substrates. The assembly state or color change of the RNA-cleaving DNAzymes-linked AuNPs in response to metal ions could be monitored either by a spectrophotometer using extinction ratio of E_{522}/E_{700} or by spotting on a thin layer chromatography (TLC) plate to see the color change. In this system, an annealing step of heating to 50 ℃ and cooling down to room temperature (RT) over 2 h was needed to form head-to-tail aligned aggregates and Pb^{2+} could be detected specifically with a detection sensitivity of 5×10^{-7} M (Fig 4A(a)).[45] The G-T wobble pair beside the cleavage site is essential to the activity of the enzyme (17E), and the activity would be abolished if the G-T pair changes into G-C pair (17Ec). If only a small fraction of active 17E and a large fraction of inactive 17Ec were mixed together for the detection, such as 5:95, the dynamic range of Pb^{2+} could shift about 1 order of magnitude to higher concentrations without signal saturation. However, a shortcoming of this design is the slow color change. In the following study, 42-nm AuNPs were used instead of 13-nm to decrease the assembling time needed to form an aggregate with defined size, and tail-to-tail format alignment was applied in order to decrease the steric effects related to nanoparticle alignment (Fig 4 A(b)). In this way, a clear color change was observed within 10 min, and an accelerated colorimetric RNA-cleaving DNAzymes-linked AuNPs system for fast Pb^{2+} detection was obtained.[41] These studies made them realize that there might be some methods that could help nanoparticle release after cleavage and consequently promote the disassembly process or color change. To further accelerate the disassembly process, two new strategies were developed. The first one was the introducing of invasive DNA, which was complementary to the cleaved substrate fragments.[43] The alternative way was designing asymmetric DNAzymes with one elongated and one shortened binding arms to facilitate the release of the nanoparticles after cleavage.[46] Both methods significantly accelerated the Pb^{2+}-induced disassembly of tail-to-tail aligned AuNPs aggregates.

The methods mentioned above belong to labeled strategies and labled-free strategies were also widely used. Lu's lab represented the systematic comparison of labeled and label-free method using DNAzyme-AuNPs system.[44]The labeled sensor was more versatile as DNA labeled AuNPs providing higher stability (Fig 4B(a)). However, the label-free method, with a detection limit of 1 nM after 6 min of reaction time at RT, showed better sensitivity, shorter operation time and lower costs compared with the labeled method. Other label-free methods, however, utilize the adsorption differences of single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) on gold nanoparticle surface, which influences the aggregation state of AuNPs in the presence of salt. The presence of target ions will result in cleavage, releasing cleaved ssDNA that can be adsorbed on AuNPs and prevent the aggregation. In the absence of target ions, the dsDNA could not protect AuNPs from aggregation under high salt condition. Taking advantage of this phenomenon, colormetric sensors for Cu^{2+}, Pb^{2+}, and UO_2^{2+} have been designed. Yang and coworkers proposed[47] a label free strategy using DNAzyme as crosslinker of hydrogel for visual detection of metal ions. The DNAzyme-crosslinked hydrogel would undergo gel–sol transition in response to Cu^{2+} ions and release the pre-trapped AuNPs (Fig 4 B(b)). They could selectively detect as low as 10 μM Cu^{2+} with the naked eye within 1.5 h. Jiang et al decreased the colorimetric detection limit of UO_2^{2+} to 40 pM by introducing nanogold-seeded nucleation amplification.[21] Besides, Ling et al used

DLS instead of colorimetric assay to construct label-free DNAzyme-AuNPs based sensors for the detection of Cu^{2+} and Pb^{2+}, and acquired pM-level detection limit, which seemed to be more sensitive than colorimetric methods.[13,48]

As mentioned above, G-rich bases could self-assemble into parallel or antiparallel G-quadruplex structure in the presence of K^+, Pb^{2+}, or NH_4^+. The G-quadruplex recruited hemin as cofactor to mimick the catalytic activity of horseradish peroxidase, and selectively catalyze luminol/H_2O_2 to produce chemiluminescence(CL),[10] or oxidize 2, 2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) to generate color change. These properties were utilized by Wang and Dong to develop label-free colorimetric approaches to detect K^+.[48] They proved another interesting phenomenon that Pb^{2+} could induce a conformational change of K-stabilized G-quadruplex-DNAzyme and inhibit the peroxidase-like activity. Inspired by this foundation, they developed a novel colorimetric and CL sensor based on Pb^{2+}-induced allosteric G4-DNAzyme. Using colorimetry, as low as 32 nM Pb^{2+} could be detected, while utilizing the CL, the detection limit could be 1nM.[49] Guo et al coupled G4-DNAzyme with N-methyl mesoporphyrin IX (NMM) instead of hemin and proposed a similar fluorescent biosensor for Pb^{2+} with a detection limit of 1nM.[50]

2.1.3 SERS DNAzyme Biosensors

The aggregation of AuNPs will greatly increase the coupling of surface plasmon resonance between adjacent particles, known as "hot spot". "Hot spot" could produce exponentially enhanced electromagnetic fields (EM), and as a result, induce significantly enhanced SERS signals. Taking advantages of the unique distance-dependent surface plasmon properties of AuNPs, high sensitivity of SERS and the metal ions-dependent RNA-cleaving DNAzyme, Wang et al. proposed a novel SERS and DNAzyme based biosensor for sensitive detection of Pb^{2+} (Fig. 5a) with a detection limit of 20 nM.[51] This sensing system was a "signal off" type assay, which restricted the sensitivity. In order to improve the sensitivity, our group[13] proposed a "signal on" type sensor using nanoscale DNA-Au dendrimer as efficient signal amplifier (Fig. 5b). In this design, the DNAzyme-substrate strand was immobilized on the gold surface. After cleavage, the DNAzyme strand released from gold surface. The remaining oligonucleotide moiety hybridized with the reporter DNA, and subsequently formed a nanoscale DNA-Au dendrimer through layer-by-layer assembly of reporter DNA and raman tag bifunctionalized AuNPs. The formation of the DNA-Au dendrimer would produce a strong SERS signal, which enabled a highly sensitive assay of Pb^{2+} with a detection limit of 100 pM. Zhang and coworkers demonstrated a sensitive SERS assay of L-histidine, another cofactor for DNAzymes, via a DNAzyme-activated target recycling cascade amplification strategy (Fig. 5c). This novel sensing system can be easily carried out by simple mixing and incubation and can afford high sensitivity for L-histidine with a detection limit of 0.56 nM.[52]

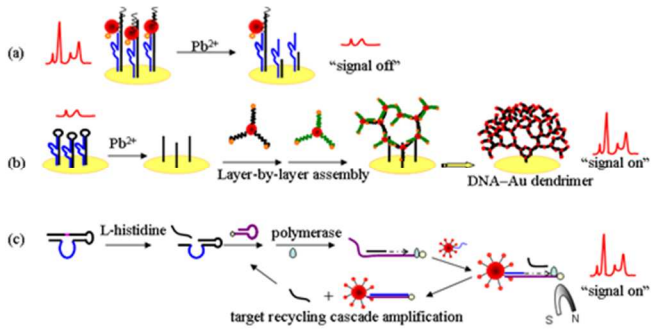

Fig. 5 Schematic design of SERS and DNAzyme based biosensor. (a) "Signal off" type biosensor for Pb^{2+} detection. (b) "Signal on" type biosensor using nanoscale DNA–Au dendrimer as efficient signal amplifier. (c) Sensitive SERS assay of L-histidine via a DNAzyme-activated target recycling cascade amplification strategy.

2.1.4 Electrochemical DNAzyme biosensors

Shao and coworkers ultilized the DNA-Au bioconjugates as bio-bar codes to amplify the electrochemical signals instead of as colorimetric reporters (Fig. 6 a). In this study, $Ru(NH_3)_6^{3+}$, which can bind to the anionic phosphate of DNA through electrostatic interactions, was served as signal transducer. The proposed DNAzyme-based "signal off" electrochemical sensor demonstrated a 1 nM detection limit for Pb^{2+}.[53] Yang and coworkers using DNAzyme-functionalized AuNPs/$Ru(NH_3)_6^{3+}$ constructed a "signal on" type electrochemical sensor for Pb^{2+} and got a detection limit of 0.028 nM (Fig. 6 b).[54] Recognition induced conformational state change is frequently used in the designing of DNA based electrochemical sensors, and this strategy is also suitable for the design of DNAzyme based biosensors. Plaxco and coworkers showed the first example of methylene-blue (MB) modified DNAzyme-based electrochemical sensor for Pb^{2+} detection. Cleavage would induce dissociation of substrates from the complex and facilitate the MB to approach the electrode surface and transfer electrons. This conformation switch electrochemical sensor reported a 300 nM detection limit (Fig.6c).[55] In order to improve sensitivity, Li et al adopted gold nanoparticles assembled gold electrode instead to construct sensing surface and got a 0.1 pM detection limit for L-histidine (Fig. 6d).[56]

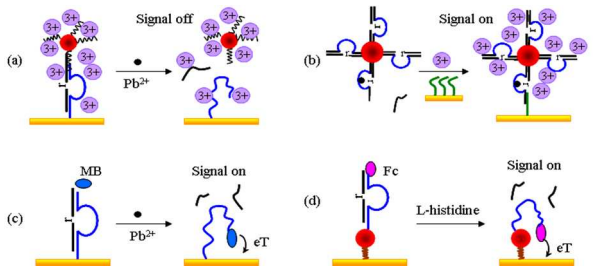

Fig. 6 (a) and (b) Schematic illustration of electrochemical biosensors for Pb^{2+} based on DNAzyme using DNA-Au bioconjugates as bio-bar codes to amplify the electrochemical signals from $Ru(NH_3)_6^{3+}$. The design was a "signal off" type biosensor in (a) and "signal on" type biosensor in (b). (c) and (d) Schematic illustration of conformation switch "signal on" electrochemical biosensors for (c) Pb^{2+} and (d) L-histidine.

2.1.5 Other DNAzyme biosensors

In these biosensing strategies, DNAzymes were used as cofactor recognition elements, the target-induced specific cleaving evens were usually transformed into colorimetric, fluorescent, electrochemical, or SERS signals, which were either semi-quantitative or required laboratory-based instruments that are not cost effective or commercially available to the general public. To meet this challenge, Lu and coworkers proposed an invasive DNA approach for portable quantification of metal ions using a personal glucose meter (PGM).[57] The invasive DNA yielded from the DNAzyme-based cleavage, would release the DNA-invertase bioconjugates from the magnetic beads, hydrolyzing sucrose into glucose for PGM measurement. The detection limits were reported to be 16 and 5.0 nM for Pb^{2+} and UO_2^{2+}, respectively, with excellent selectivity.

There are still some other explorations of enzyme- and nanoparticle-free signal amplification methods to indicate the DNAzyme based target-specific cleaving evens, such as hybridization chain reaction (HCR). By coupling DNA-based HCR with the DNAzyme based cleavage, Zhuang and coworkers reported a magneto-controlled electronic switch for highly sensitive and selective detection of Pb^{2+} (Fig. 7a). The HCR reaction was carried

out between two alternating hairpin DNA labeled with ferrocene to form a nicked double-helix on the magnetic beads. Through this method, a detection limit of 37 pM was reported.[58] Zeng and co-workers[59] developed an enzyme-free and label-free turn on biosensor for Cu^{2+} detection based on cleavage triggered formation of dsDNA concatamers and Sybr Green I (Fig 7b). The whole process does not need any protein enzyme and fluorescent-labeled DNA and they obtained a detection limit of 12.8 pM for Cu^{2+}. Lu and coworkers introduced a dSpacer into the duplex regions of DNAzyme to construct a label-free sensing strategy for Pb^{2+} (Fig 7c). The dSpacer can bind an extrinsic fluorescent compound and quench its fluorescence. Addition of Pb^{2+} enabled the DNAzyme to cleave its substrate and release the fluorescent compound from the duplex, recovering the fluorescence. This label-free method exhibits detection limits of 4 nM for Pb^{2+}.[60] Wang and coworkers developed a DNAzyme-based label-free fluorescing molecular switch sensor utilizing a double-strand-chelating dye Picogreen (PG) (Fig 7d).[61]

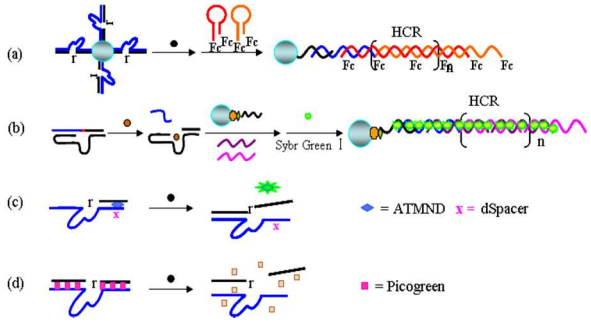

Fig. 7 Enzyme and nanoparticles free signal amplification methods for DNAzyme-based biosensors. (a) (b) Using hybridization chain reaction (HCR). (c) Using a dSpacer that can bind an extrinsic fluorescent compound and quench its fluorescence. (d) Using double-strand-chelating dye Picogreen (PG).

2.2 DNAzyme as catalytic signal amplifier to construct biosensors

In addition to be versatile recognition elements for sensor designing, DNAzymes are also excellent signal amplifiers. By rational design, the allosteric DNAzymes or simply aptazymes can recognize a broad range of targets besides cofactors. In these strategies, the binding events will activate the catalytic core, or trigger the synthesis of DNAzymes and the followed up cleavage will induce signal amplification. Therefore, DNAzymes are highly suitable signal transducer and amplifier for biosensors.

2.2.1 Cleavage-induced signal amplification

The concepts of allosteric aptamer and allosteric ribozymes have been adapted to the design of aptazymes, in which a DNA aptamegr is integrated with a DNAzyme in such a way that the DNAzyme can only be activated by the binding of the target analyte to the aptamer domain. These allosteric DNAzymes are well suited for construction of biosensors, because the target binding event of the aptamer domain could be translated into the activating of DNAzyme for signal generation and amplification.

Li and coworkers proposed a structure-switching DNAzyme based fluorescent probe for ATP detection, which composed of three separate oligonucleotides: an ATP aptamer-linked DNAzyme, a regulatory oligonucleotide and a substrate with F and Q placed right beside the cleavage site (Fig. 8a).[62] Willner and coworkers separated Mg^{2+}-dependent DNAzyme into two inactive subunits, one of which formed a hairpin structure that could be selectively opened by target binding (Fig. 8b).[63] By using a hairpin substrate that included a sequestered structure with the target sequence, an autocatalytic DNAzyme-mediated amplification process was achieved. Thus, very low concentrations of the target DNA would initiate the autocatalytic cascade and a significant improved detection limit of 1 pM was reported. Qi et al proved that using T-Hg-T interaction induced DNAzyme cascade with CAMB, sensitive and selective fluorescent sensor for Hg^{2+} could be developed with a detection limit of 0.2 nM.[64]

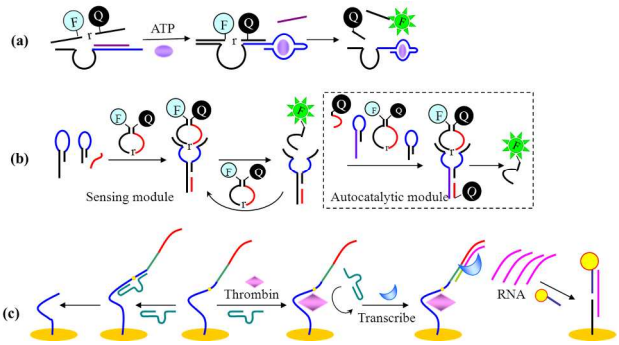

Fig. 8 (a) Structure-switching DNAzyme based fluorescent probe for ATP detection. (b) Amplified detection of DNA through an autocatalytic and catabolic DNAzyme-mediated process. (c) DNAzyme footprinting methodology using protein−aptamer complexes to block the DNAzyme cleavage activity. Subsequent surface RNA transcription and nanoparticle-enhanced SPR imaging was carried out for the detection.

In a novel DNAzyme footprinting methodology reported by Corn and coworkers (Fig.8c),[65] DNAzymes were used to recognize the specific adsorption of proteins onto aptamer monolayers. In the absence of protein targets, DNAzyme would cleave and remove the promoter-reporter sequence from the surface. While the formation of protein−aptamer complexes on the surfaces would block the DNAzyme cleavage site and a subsequent surface RNA transcription followed by nanoparticle-enhanced SPR imaging was utilized to enhance the sensitivity. A detection limit of 100 fM thrombin was obtained and the only limitation was the relatively slow DNAzyme cleavage reaction.

Lu and coworkers demonstrated using adenosine-activated aptazyme to assemble AuNPs responsive to adenosine (Fig. 9a).[42] In the absence of adenosine, aptazyme activity was inhibited due to the bulging aptamer motif, and the AuNPs were aggregated into blue color. Only the binding of adenosine to the aptazyme could activate the DNAzyme cutting the substrate, and show a red color. In this method, DNAzymes could be combined to aptamers as transducer to construct colorimetric sensors for a broad range of targets.

Lu and coworkers tried another T-Hg-T interaction to modulate DNAzyme activities through similar allosteric interactions (Fig. 9b).[66] The addition of Hg^{2+} would quickly fold the UO_2^{2+}-DNAzymes into active conformations without kinetic traps, and the optimal DNAzyme labeled with F and Q got a detection limit of 2.4 nM for Hg^{2+}.

Many aptazyme-based sensing systems reported for small molecules, such as ATP, only showed moderate sensitivity and could not discriminate them from analogues. In order to address this limitation, our group developed a ligation-triggered DNAzyme cascade by coupling a split DNAzyme-based background reduction strategy with CAMB-based amplification (Fig. 9c).[16] In this design, the DNAzyme was split into two building blocks for the DNA ligation reaction, which provided a zero-background in the absence of target, while CAMB could realize the true enzymatic multiple turnovers for signal amplification. A detection limit of 100 and 50 pM for ATP and NAD^+, respectively, was reported. Moreover, by taking advantage of the highly specific biomolecule-dependence of the DNA ligation reaction, the proposed strategy show significantly

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high selectivity toward ATP and NAD^+, and could distinguish them from their analogues.

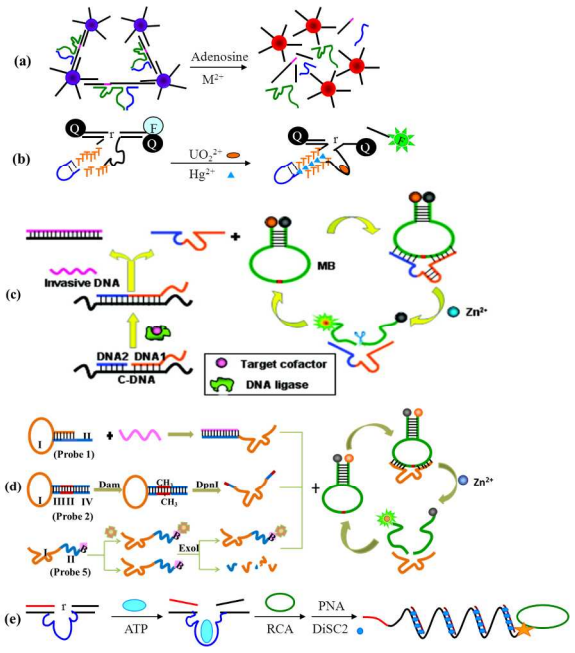

Fig. 9 (a) The principle of adenosine-dependent assembly of aptazyme-functionalized AuNPs for colorimetric biosensing. (b) Rational design of allosteric DNAzyme catalytic beacons for Hg^{2+}. (c). The principle of the ligation-triggered DNAzyme cascade for amplified fluorescence detection of biological small molecules with zero-background signal. Reproduced with permission from ref. 16. Copyright 2011 American Chemical Society. (d). Versatile DNAzyme-based amplified biosensing platforms for target DNA, Dam MTase, and streptavidin detection. Reproduced with permission from ref.67. Copyright 2013 American Chemical Society. (e) Colorimetric biosensor for ATP using allosteric-DNAzyme-coupled rolling circle amplification and a peptide nucleic acid-organic dye probe.

Using high catalytic cleavage activity like Zn^{2+}-dependent DNAzyme (with a catalytic rate of 1.35 min^{-1}), our group developed a more general sensing platform for amplified detection of nucleic acids, DNA methyltransferases (MTases) activity, and protein based on topological effect of 8−17 DNAzyme and terminal protection of small-molecule-linked DNA (Fig. 9d).[67] In these designs, no modification in the catalytic core was needed and one could employ any DNAzyme with high catalytic cleavage activity as catalytic core, which provides high amplified efficiency. Additionally, a CAMB was adopted to realize the true enzymatic multiple turnovers of catalytic beacons. These designs together allowed a high sensitivity and obtained a detection limit of 20 pM, 0.2 U/mL, and 1 ng/mL for target DNA, Dam MTase, and streptavidin, respectively.

One of the challenging topics in DNAzyme based amplification involves the development of new signal-triggered of isothermal amplification methods similar to the polymerase chain reaction (PCR), such as rolling circle amplification (RCA), autonomous RNA transcription and assembly of polymer DNAzyme wire. By coupling the aptazyme with rolling circle amplification (RCA) and a peptide nucleic acid (PNA)-organic dye probe, Li and coworkers developed a colorimetric sensing strategy (Fig. 9e).[68] In this strategy, the target ATP binding would release a cleaved fragment, which could be used as primer for the subsequent RCA reaction to produce long ssDNA molecules to enable the colorimetric sensing through the hybridization of complementary PNA in the presence of 3,3-diethylthiadicarbocyanine (DiSC2(5)), a duplex-binding dye.

Willner and coworkers introduced a novel paradigm based on the target-triggered autonomous cross-opening of functional DNA hairpin structures, which assembled into polymer wires composed of Mg^{2+}-dependent DNAzymes. In their design, a "helper" hairpin was introduced to recognize target BRCA1 oncogene. Upon opening, the cross-opening of two functional hairpins may be activated, and leading to the autonomous synthesis of the "two-sided" DNAzyme subunits polymer wires that could cleave F and Q labeled substrates. The proposed strategy provided a sensitivity of 1×10^{-4} M for the analysis of the BRCA1 oncogene.[69]

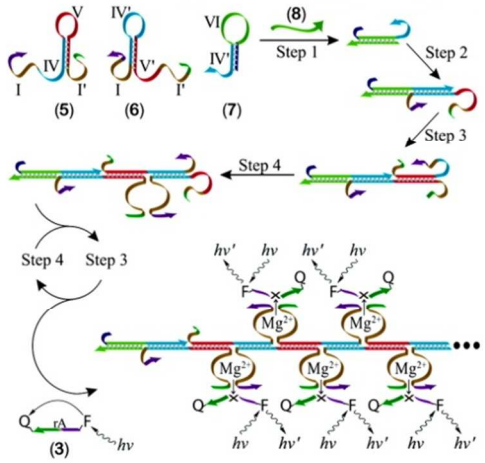

Fig. 10 Analysis of the BRCA1 oncogene (**8**) using probe hairpin (**7**) and the two functional hairpins (**5**) and (**6**) for the autonomous synthesis of the "two-sided" Mg^{2+}-dependent DNAzyme subunits polymer wires. Reproduced with permission from ref. 69. Copyright 2011 American Chemical Society.

They also demonstrated an enzyme-free amplified detection of DNA by an autonomous ligation DNAzyme machinery.[70] The Zn^{2+}-dependent ligation DNAzyme was applied as a versatile amplifying unit for the amplified detection of target DNA by the autonomous replication of a nucleic acid reporter unit that is generated by the catalyzed ligation process. The system was used to detect Tay-Sachs genetic disorder mutant, and gave a detection limit of 10 pM. Furthermore, they utilized Zn^{2+}-dependent ligation DNAzyme as amplifying biocatalyst to construct ligation DNAzyme-driven enzymatic and nonenzymatic cascades for the amplified detection of DNA. One strategy used a "helper" nucleic acid sequence to release the ligation product for opening of the hairpin and a nicking enzyme to cleave fluorescent labeled molecular beacon and recycle the free ligation product. The coupling of "helper" sequence and nicking enzyme enabled the detection of target DNA with a detection limit of 20 pM. The other strategy adopted Mg^{2+}-dependent DNAzyme subunits to displace the ligation product, leading to assembled DNAzyme that could cleave F and Q labeled substrates to yield fluorescence. This nonenzymatic cascades strategy could detect target DNA down to 10 pM.

Some of the metal sulfide nanocrystals, such as PbS, ZnS, CdS and CuS that contain the specific triggering elements for DNAzyme cleavage, could be coupled with DNAzymes to develop dual signal amplification strategies. Tang and coworkers reported a novel electrochemical immunoassay protocol for prostate-specific antigen (PSA) by coupling PbS nanolabels with the cleavage of the corresponding lead ion-induced DNAzymes. In the presence of target protein, the PbS-labeled antibody could be conjugated to the microplate surface through sandwiched immunocomplex. The nanolabels released numerous Pb^{2+} ions by the addition of acid, and induced the cleavage. The confirmation change of the DNAzyme strand resulted in an increased currents due to the approaching of

ferrocene labels to the electrode surface. The proposed immunoassay allowed detection of PSA as low as 0.1 pg mL^{-1}.[71] In another design, they coupled the PbS nanolabels with silver nanocluster (AgNC)-based rolling circle amplification (RCA) strategy, and constructed a electrochemical sensor for alpha-fetoprotein (AFP) with a detection limit of 0.8 pg mL^{-1}.[72] Chu and coworkers reported a more general fluorescent sensing strategy for human immunoglobulin G (IgG) by combining the PbS nanolabels with fluorescent labeled DNAzyme-substrate, and reported a detection limit of 0.8 ng mL^{-1}.[73]

2.2.2 G4-DNAzyme as amplification label

As we known, G-quadruplex DNAzyme could employ hemin to mimicking the peroxidase activity, and measured by adopting common substrates used in peroxidase activities assays, such as luminol/H_2O_2 and ABTS. Especially, this kind of G4-DNAzyme-hemin complex could enhance 250-fold peroxidase activity to free hemin, so the super catalytic properties enabled G4-DNAzyme excellent amplifier for biosensor designing. In addition, the HRP-mimicking DNAzyme also revealed bioelectrocatalytic properties and could electrocatalyze the reduction of H_2O_2. This function was used by Willner and coworkers to develop electrochemical sensors that followed the activity of glucose oxidase.[74] Willner and coworkers [75] discovered that instead of hemin, the integrating of zinc(II)-protoporphyrin IX (ZnPPIX) into the G-quadruplex nanostructure may enhanced fluorescence of fluorophore, which could be served as optical labels for direct fluorescent detection. However, we will focus on G4-DNAzyme-hemin for colorimetric/ chemiluminescent biosensor in this part.

G4-DNAzyme colorimetric biosensors have attracted a lot of attentions due to their advantages include easy-construction, rapid-reaction and low-cost. Willner and coworkers designed the first G4-DNAzyme containing hairpin structure, whose duplex structure at the stem prohibited the self-assembly of the DNAzyme due to its stability.[76] Followed target DNA hybridization, the loop region opened and led to the formation of the hemin/G-quadruplex structure that could oxidize $ABTS^{2-}$ to colored $ABTS^{\cdot-}$. This colorimetric strategy was further extended to the detection of telomerase activity in cancer cells. The binding-induced activating of G4-DNAzyme eliminates the nonspecific adsorption processes associated with protein-based labels, and presents very low background signals. In addition, they expanded the sensing targets to small molecules and proteins by applying blocked aptamer-DNAzyme conjugates. Amplified biosensors based on the generation of a G4-DNAzyme upon the formation of the respective aptamer-adenosine 5'-monophosphate (AMP) or aptamer-lysozyme complexes were demonstrated. A detection limit of 4×10^{-6} M and 1×10^{-13} M was reported for the analyzing of AMP and lysozyme, respectively.[77] In those designs, a blocker DNA together with an aptamer-DNAzyme sequence was needed. In their further studies, a single aptamerDNAzyme hairpin, which included a "caged" G4-DNAzyme sequence in the stem region and a part of the aptamer sequence in the loop regions, was designed and used for the colorimetric detection of AMP and lysozyme. The formation of the respective analyte-aptamer complex would open the hairpins and resulted in the self-assembly of the activated G4-DNAzyme. Though the detection sensitivity of this aptamer DNAzyme hairpin was not as high as that of the blocked aptamer-DNAzyme conjugates, they revealed significantly improved analytical performance as compared to analogous F and Q labeled haipins.[78]

Wang and coworkers found that thrombin-binding aptamer could bind hemin to form a G-quadruplex complex whose catalytic activity would be significantly promoted in the presence of thrombin. Based on this principle, a colorimetric sensor for thrombin was developed with high specificity and sensitivity in a facile way.[79] They designed another novel catalytic molecular beacon consisting of two hairpin structures and a split G-quadruplex-DNAzyme in the middle for the development of colorimetric sensor for separated analysis of DNA and thrombin (Fig. 11 A(a)).[80] Wang and coworkers designed a colorimetric sensor for cocaine by combing the advantages of magnetic nanoparticles (MNPs) with the G4-DNAzyme using 3, 3, 5, 5-tetramethylbenzidine sulfate (TMB) as substrates instead of ABTS. A detection limit of 50 nM cocaine in biologic fluid sample was reported (Fig. 11 A(b))[81] Zhu et al proposed an optical sensor for thrombin detection based on MNPs and thrombin aptamer, which employed split G4-DNAzyme halves as sensing element. The binding of thrombin to its aptamer led to the structure deformation of the DNA strands, causing the separation of the G-quadruplex structure. This signal off sensor reported a detection limit of 0.5 nM for thrombin (Fig. 11A(c)).[82]

G4-DNAzyme-based chemiluminescent methods have also been employed to design biosensors. Combining AuNPs with G4-DNAzyme, Yan et al demonstrated a turn on chemiluminescence assay in homogenous media for adenosine and target DNA detection (Fig. 11B(a)).[83] Also based on split G4-DNAzyme-hemin complex, Cui et al[84] designed a turn on homogeneous aptasensor for interferon-gamma (IFN-γ). The binding of IFN-γ to the aptamer would release the G4-DNAzyme halves from the luminol functionalized AuNPs surface, leading to the formation of G4-DNAzyme/hemin complex. This turn on chemiluminescence sensor enabled the facile detection of IFN-γ with a sensitivity of 0.4 nM (Fig. 11 B(b)).

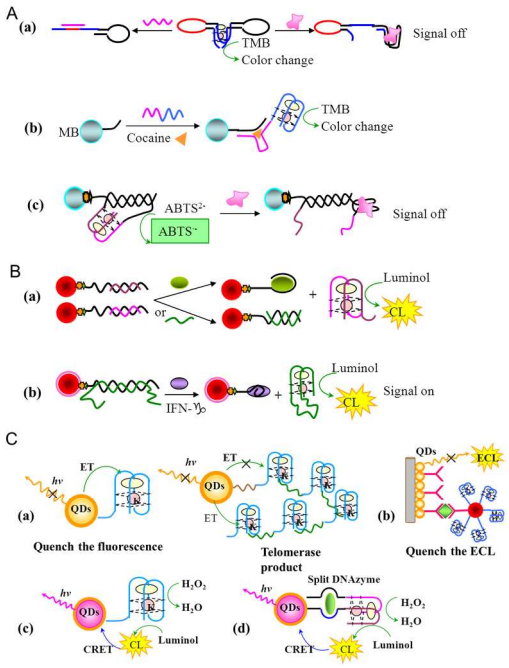

Fig. 11 A(a) Bifunctional oligonucleotide probe for colorimetric detection of DNA and thrombin. (b) G4-DNAzyme based colorimetric detection of cocaine using magnetic nanoparticles as the separation and amplification element. (c) An optical thrombin aptasensor based on magnetic nanoparticles and split DNAzyme. B (a) "Turn on" chemiluminescence assay for adenosine and target DNA detection. (b) A split G4-DNAzyme based chemiluminescence aptasensor for interferon-gamma (IFN-γ) detection. C (a) The electron transfer (ET) process between CdSe/ZnS QDs and G4-DNAzyme or telomerase product on the surface resulted in quenching of the luminescence from the QDs. This mechanism was adopted to design optical method to detect telomerase activity. (b) An immunosensor for α-fetoproteinbased on electrochemiluminescent (ECL) quenching of QDs by G4-DNAzyme-labeled-AuNPs tags. (c) The chemiluminescence generated by hemin/G4-DNAzyme act as an internal light source for the

chemiluminescence resonance energy transfer (CRET) to CdSe/ZnS QDs. (d) Target recognition induced split G4-DNAzyme-aptamer fragments assembly into active hemin-G4-DNAzyme structure to trigger the CRET process.

Willner and coworkers found that when CdSe/ZnS QDs were modified with the thiolated G4-DNAzyme, the addition of hemin would resulted in quenching of the luminescence from the QDs via an electron transfer mechanism,[85] since the photoexcited electrons from the conduction band would be transferred to the hemin complex. This phenomenon was utilized to construct fluorescent DNA sensors or aptasensors by functionalizing QDs with hairpin structure containing caged G-quadruplex, which would be deprotected upon the target binding, allowing the self-assembly of the G4-DNAzyme/hemin complex on the QDs. The telomerase-stimulated telomerization on the surface of QDs could similarly fold into hemin/G4-DNAzyme structure to induce the electron transfer quenching of the QDs, thus providing an optical method to detect telomerase activity (Fig. 11C(a)).[86]

Based on electrochemiluminescent (ECL) quenching of QDs by hemin-G4-DNAzyme bio-bar-code AuNPs tags, Ju and coworkers designed an ultrasensitive immunosensor for protein biomarker, α-fetoprotein, with a detection limit of 1.0 fg mL^{-1}(Fig. 11C(b)).[87] Xu and coworkers developed a DNA sensor based on the efficient quenching of ECL from K-doped graphene–CdS:Eu NCs by hemin/G4-DNAzyme and nicking endonuclease (NEase) assisted target strand-scission cycle. Their detection limit was found to be as low as 50 aM, which is superior to those obtained from other methods.[88] The chemiluminescence generated by hemin/G4-DNAzyme was also found to act as an internal light source for the chemiluminescence resonance energy transfer (CRET) to CdSe/ZnS QDs, triggering on the luminescence of the QDs (Fig. 11C(c)). By introducing split G4-DNAzyme and aptamer subunits into the modification of QDs, Willner and coworkers developed a CRET-based sensing platform for the detections of DNA, metal ions, and aptamer-substrate complexes. In the presence of target analytes, the split G4-DNAzyme-aptamer fragments self-assembled into active hemin-G4-DNAzyme structure, stimulating the CRET to QDs to provide optical read out (Fig. 11C(d)). Three different sized QDs functionalized with different haipins were successfully applied for multiplexed analysis.[89] If glucose oxidase-G4-DNAzyme conjugates were immobilized on CdSe/ZnS QDs, the presence of glucose would activate the CRET induced luminescence of QDs, providing an optical means to detect glucose.[90]

Actually, many of the aptamer sequences can form G-quadruplexes upon binding to the target.[91] The incorporation of hemin into the aptameric G4-target complex produces a catalytic chemiluminescent DNAzyme structure. The coupling of the nanostructures with CdSe/ZnS QDs yielded novel CRET-based sensors for thrombin and ATP with detection limits of 200 pM and 10 μM, respectively.

G4-DNAzyme-hemin complex its self has been proved to be excellent catalytic amplification label for signal read out in biosensor designing. When AuNPs or GO was used as carriers for the G4-DNAzymes labels, a single recognition event could be converted to multi-labels of the G4-DNAzymes, thus, the sensitivity of the sensor would be significantly improved. This kind of signal amplification also named "bio-bar-code" amplification as shown in Fig. 12A. G4-DNAzyme-functionalized AuNPs was used as biocatalytic conjugates by Willner and coworkers for construction of chemiluminescence sensor for the amplified detection of DNA and telomerase activity.[92] Their detection limit for DNA was 1×10^{-10} M and the telomerase activity originating from 1000 HeLa cells could be detected, which was 10^2-10^4-times lower than normal chemiluminescent assays for DNA analysis or telomerase assay. Meng et al[93] developed a microRNA (miRNA) electrochemical biosensor utilizing "bio-bar-code" amplification and hemin/G4-DNAzyme using hydroquinone as substrate. A detection limit of 0.006 pM for mi-RNA21 was reported. By coupling difunctionalAuNP–DNA dendrimers with G4-DNAzyme and methylene blue for signal amplification, Xu et al proposed an electrochemical biosensor for femtomolar detection of nucleic acids.[94] A quadruple electrochemical signal amplification was reported by Tang and coworkers using nanogold-functionalized DNAzyme concatamers and redox-active intercalators.[95] Some other researches also demonstrated that the integrating of hemin/ G-quadruplex with functionalized graphene-Pd nanoparticles composites (PdNPs-RGs), or dendrimer functionalized reduced graphene oxide (PAMMA-rGO), or Pt nanoparticles/carbon nanotubes (Pt NPs/CNTs) could be served as dual signal amplifier in construction of electrochemical or chemiluminescent sensors.[96]

Isothermal polymerase-stimulated synthesis, such as "DNA machines" or RCA reaction, for the substitution of PCR (Polymerase chain reaction) process to produce G4-DNAzymes was adopted by some researchers to develop analytical methods with high sensitivity. The polymerase-stimulated synthesis of G4-DNAzymes does not require thermal replication cycles, costly optical labels, or dedicated instrumentation. Upon recognition of the input analyte, the "DNA machines" or RCA is activated and numerous HRP-mimicking G4-DNAzymes were synthesized, which in turn, generates colorimetric or chemiluminescence readout signals. Thus, a single recognition event will be translated through the dual amplification steps into numerous transduction signals.

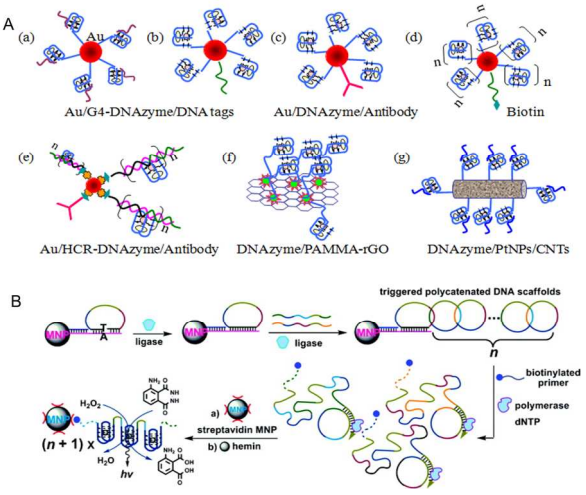

Fig.12 A. "Bio-bar-code" amplification. (a), (b), (c) and (d) Using G4-DNAzyme-functionalized AuNPs as trace tags. (e) Electrochemical quadruple signal amplification using AuNPs-functionalized DNAzymeconcatamers and redox-active intercalators (f) Using dendrimer functionalized reduced graphene oxide (PAMMA-rGO) to immobilize G4-DNAzyme as labels. (g) Using G4-DNAzyme-functionalized Pt nanoparticles/carbon nanotubes (Pt NPs/CNTs) as signal amplifier. B. Triggered polycatenated DNA scaffolds for the design of DNA and aptamer sensors using RCA reaction produced G4-DNAzymes for signal amplification. Reproduced with permission from ref. 91. Copyright 2010 American Chemical Society.

Jiang and coworkers developed a highly sensitive and selective label-free single nucleotide polymorphisms (SNP) genotyping technique on the basis of ligase reaction and an isothermal strand displacement amplification (SDA).[97] The use of DNA ligase enabled specific identification of SNP, and the application of two consecutive nicking enzyme based SDA allowed the synthesis of a great abundance of G4-DNAzymes, which could be detected by chemiluminscence. The proposed method displayed superb

selectivity in discriminating SNP, and a detection limit as low as 0.1 fM with a dynamic range from 1 fM to 1 nM was reported.

Zhang and coworkers combined the polycatenated DNA scaffold-mediated RCA reaction with G4-DNAzyme amplification for the identification of SNP and the detection of thrombin by employing streptavidin-MNPs to reduce background (Fig. 12B)[98] The single recognition events was amplified by RCA reaction to produce hundreds to thousands HRP-mimicking G4-DNAzymes, which generated greatly enhanced chemiluminscence signal. This biosensor exhibited a detection limit of 71 aM and 6.6 pM for SNPs and thrombin, respectively. Li and coworkers reported an aptamer-based protein detection assay that integrated RCA with G4-DNAzyme catalyzed colorimetric reaction for dual signal amplification.[99] The biomarker binding on the aptamer-functionalized microbeads was amplified by RCA reaction to create G4-DNAzymes to produce a blue-green colorimetric signal. This biosensor allowed the sensitive detection of PDGF-BB at a concentration as low as 0.2 pg/mL within 2 hours. By integrating RCA with nicking enzymecleavage and G4-DNAzyme catalytic amplification, Li and coworkers presented a highly sensitive colorimetric method for microRNA (miRNA) detection witha detection limit as low as 1 aM for miRNA.[100] In Zhang's study,[101] they employed a hairpin probe-induced primer generation rolling circle amplification (PG-RCA) for the G4-DNAzyme generation and developed a highly sensitive chemiluminescence method for the assay of DNA MTases. In the presence of MTase, methylation of the hairpin probe followed by cleavage would happen to create primers for initiating the PG-RCA reaction. The proposed method exhibited an extremely low detection limit of 1.29×10^{-4} U/mL for the assay of MTase.

In another interesting study, G4-DNAzyme was used as cross linker by Willner and coworkers to construct a cyclic on/off switchable catalytic acrylamide hydrogels.[102] The formation of the hydrogel in the presence of hemin results in a hemin/G-quadruplex-cross-linked catalytic hydrogel, which could generate chemiluminescence in the presence of luminol/H_2O_2. They demonstrated the reversible formation and dissociation of the hydrogel by the cyclic addition of K^+ ions and 18-crown-6 ether, respectively.

3. DNAzyme-derived nanodevices

DNAzymes are not only ideal candidates in biosenser construction, but also play increasingly important roles as building blocks of nanodevices. Recently, many researchers in nanoscience have used the unique, programmable molecular recognition properties of DNA to creat artificial, machine-like devices. As an important kind of functional nucleictide acids[103], DNAzyme got great development in deriving nanodevices.

3.1 DNAzymes-incorporated logic gates

With the feature of easy to code, DNA has been used for computational operations and logic gate which provides the functional units of computers.[104] Generally, logic gates could be activated by two electrical inputs that regulate the electrical output, and the combination of several gates permits computations for binary computing. So, in recent research, some molecular or biomolecular were used as functional components which could been triggered by electrical，chemical, light and pH inputs to generate logic operations. In addition, DNAzymes recognizing and reacting specifically with some metal ions made them be used to perform arithmetic operations.[105]

In the past decade, a variety of molecular Boolean logic gates based on supermolecular complexes have been reported and most of them, however, employed fluorescence as signal output, which needed precious and preciseness apparatus. Inspired by colorimetric detection methods of metal ions, Zhang and co-workers [106] depended on a series of DNAzyme-based circular substrates, constructed a complete set of two-input logic gates (OR, AND, INHIBIT, XOR, NOR, NAND, and XNOR) with AuNPs-induced colorimetric outputs (Fig. 13). In their design, supermolecular circular structures were formed by ion-dependent DNAzymes-based repetitive units and the cofactors like Pb^{2+} and Mg^{2+} ions were used as inputs for the activation of their corresponding DNAzymes. In the presence of either Mg^{2+} or Pb^{2+} ions, only one rA specific to Mg^{2+} or Pb^{2+} ions is cleaved and the substrate is opened from circular to strand form, hybridized with DNA-AuNP probes. After these, we can obtain a color change of AuNPs. This logic gate was simple and convenient and it was the first report about the application of colorimetric logic gate operations based on DNAzyme-catalyzed RNA cleavage. Wang and coworkers demonstrated a visible multi-digit DNA keypad lock system based on split G4-DNAzyme and silver microspheres (SMSs). When the DNA inputs were added one by one in the designed order, a G-quadruplex-hemin complex would be formed on the SMSs surface and catalyzed H_2O_2-mediated oxidation of TMB from colorless to blue. Based on this principle, they constructed a three-digit and a five-digit DNA keypad lock, which proved the excellent scalability and flexibility of this molecular sensing platform.[72, 107]

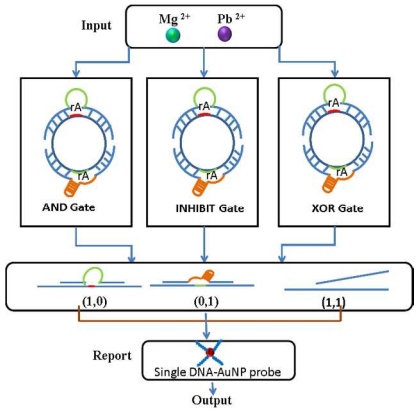

Fig. 13 AND, INHIBIT, and XOR logic gates connected to single DNAa-b–AuNP probes as colorimetric outputs. Each path is characterized by colorimetric and UV/Vis detection.Reproduced with permission from ref. 145. Copyright 2010 Wiley-VCH.

DNAzymes were also reported as a "walker" moving along a DNA track. This DNA device constantly extracts chemical energy from the substrate molecules of DNAzyme, and uses this energy to fuel the motion. For example, Tian et al integrated the DNAzyme catalytic cleavage activity with the strand-displacement strategy and designed a walking system.[108] They used 10-23 DNAzyme which can cleave RNA with sequence specificity as the walker, and a regular, linear array of RNA substrate as the track. In the present of cofactor, the catalytic core of DNAzyme cleaved the RNA substrate, one fragment, that can bind to recognition arm of DNAzyme, dissociates from the DNAzyme and the long fragment remains stably associated with the DNAzyme through Watson–Crick base-pairing. After the dissociation of short fragment, the free short arm of DNAzyme search for other complementary single strands of RNA substrate. The short duplex is so stable as a result of intracomplex hybridization that a strand replacement occurs through branch migration. In this case, the DNAzyme moves from one RNA substrate to the next RNA substrate. Later, Tan group reported a new light energy-powered DNA walker capable of regulated autonomous

movement along a nucleic acid track. They employed aromatic hydrocarbons photolysis the disulfide bonds artificial nucleic acid backbones as the energy sources for molecular-level switches.[109] This is an autonomous but controllable DNA walking device combining photosensitive moiety with DNAzyme. Semiconductor QDs could also be conjugated to functional DNA machines and provide an effective optical label for probing the dynamics and mechanical functions of the molecular devices. Liu group reported DNA hemin/G-quadruplex-DNAzyme-based "walkers" which making it possible for chemiluminescence, chemiluminescence resonance energy transfer (CRET), electrochemical, or photoelectrochemical transduction of the switchable states of the different DNA machines.[107]

3.2 DNAzyme based drug delivery and therapy

Herein, two different applications of DNAzymes in delivery and therapy will be discussed: Firstly, DNAzymes could act as novel stimuli-responsive caps for trapping drugs in the pores of nanocarriers, such as mesoporous SiO_2 nanoparticles, to develop drug delivery systems. In the presence of appropriate triggers, the pores would be unlocked, leading to the controlled release of drugs. DNAzymes, as a special kind of functional DNA, could be used to design nanocarriers for the delivery of drug or functional DNA. Secondly, DNAzyme itself is also a promising therapeutics, especially the 10-23 DNAzymes. They have the ability to cleave any purine-pyrimidine junction within an RNA chain under physiological conditions, and it can be used to targeting mRNA or viral RNAs and led to down-regulation of protein expression.

Willner and co-workers reported the first example of smart mesoporous SiO_2 nanoparticles for the DNAzyme-induced multiplexed release of substrates (Fig. 14a).[110] They adopted Mg^{2+} and Zn^{2+} -dependent DNAzyme as caps to trap fluorescent methylene blue (MB^+) and thionine (Th^+) in the pores of mesoporous silica. The presence of Mg^{2+} or Zn^{2+} ions would selectively cleave the certain caps and release the respective dyes. They also demonstrated the allosteric activation of the DNAzymes by aptamer−substrate complexes and the toxic Hg^{2+} ions, which had potential applications in targeted release of drugs. Zhang and Tan also demonstrated a non-canonical strategy to self-assemble multifunctional DNA nanoflowers (NFs) through rolling cycle replication (RCR) product.[111,112] These NFs exhibited versatile biomedical applications as selective cancer cell recognition, bioimaging, and targeted anticancer drug delivery. They designed a nanotrain for targeted anticancer drug delivery.[113]

Since Khachigian1, et al.[114] demonstrated the first delivery study of DNAzymes into animal model in 1999, the cleavage led to down-regulation of protein expression, and DNAzyme was proved to be potential gene therapeutics. Compared with other gene knockdown technologies reported, such as antisense oligonucleotide (ASO), ribozymes, and small interference RNA (siRNA), DNAzymes are more stable in serum, easy to synthesize and less expensive. ASO and SiRNA have to rely on the cellular machinery to achieve silencing, however, DNAzyme has self-possesses independent catalytic activity. Moreover, the 10-23 DNAzyme was reported to have favorable catalytic efficiency compared to the hammerhead and hairpin ribozymes.[115] DNAzymes have been widely explored as potential therapeutic agents in cancer therapy, treatment of cardiovascular diseases, nervous system diseases and antivirus for in-vitro or in-vivo studies. The DNAzymes were designed to target and cleave the specific mRNA sequences, and inhibiting the gene expression. Taking cancer research as an example, different DNAzymes designed toward different kinds of mRNA gene, including the *LMP1, β-integrin, Bcr-abl, uPAR, Survivin, Ezrin*and *k-ras* gene for in vitro studies have been reported.[116-121] The DNAzymes designed demonstrated inhibition of either proliferation and metastasis, or adhesion and invasion. These studies also indicated that different cancer gene could be successfully down-regulated by DNAzymes no matter they were cell-expressed or virus-expressed. Several in vivo investigations of DNAzymes have been done in animal disease models, which targeting the VEGFR-2, Egr-1 or c-Jun gene mRNA (Fig. 14b), and 60%-90% reduction in tumor size were observed.[117,122-125] These studies helped to pave the way for the clinical application of DNAzymes as promising therapeutic candidates.

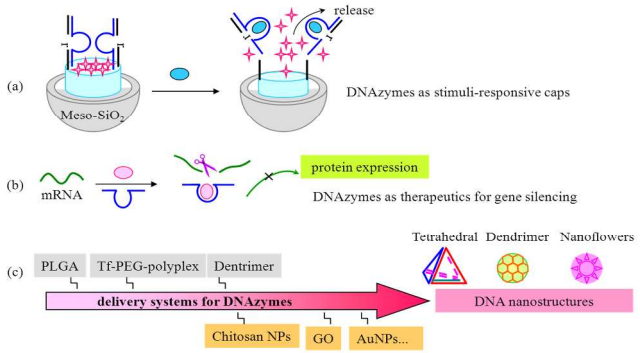

Fig. 14 (a) DNAzymes could act as novel stimuli-responsive caps for trapping drugs in the pores of nanocarriers to develop drug delivery systems. (b) DNAzyme could be designed as therapeutics to targeting mRNA and down-regulating protein expression. (c) The development of delivery systems for DNAzymes: from traditional polymeric microbeads, to GO and AuNPs, toward DNA nanostructures.

The cellular delivery and intracellular stability were the most important issues that therapeutic DNAzymes facing. In order to enhance the biostability of DNAzymes for in-vivo application, some structural modifications were applied, such as phosphorothioate modifications, the incorporation of a 3'-3' inverted nucleotide at the 3' terminus, and locked nucleic acid (LNA) incorporation.[126] Phosphorothioate linkages could enhance the stability of DNAzymes, however, some toxicity and immunologic responsiveness was reported. The inverted nucleotide at the 3'ends would prevent the DNAzymes from exonuclease degradation, and the DNAzymes can remain intact for at least 24 to 48 h in serum. The LNA incorporated DNAzymes appeared to have enhanced binding affinity toward complimentary sequences, enhanced resistance against nuclease degradation and higher thermal stability. Therefore, both of them were recommended for usage.

Effective delivery systems can help to enhance the stability of DNAzymes and transfect them into cells without clear toxicity.[127] In the early stage, poly (lactic acid) and poly (glycolic acid) co-polymeric microspheres (PLGA) and trans-ferrin-modified PEGylatedpolyplexes (Tf-PEG-polyplex) were tried as delivery systems to transport DNAzymes into cells. Later, dendrimer, a synthetic macromolecule with branched molecular structure, was used to bind DNAzymes for delivery. Dendrimers could bind the DNAzymes better and facilitate the release. Therefore, they showed higher transfection efficiency than that of the PLGA and Tf-PEG-polyplex. In recent years, nanoparticles have been widely used as delivery systems (Fig. 14c). Dass and coworkers demonstrated that chitosan-DNAzymes nanoparticles exhibited enhanced biological activities in cancer therapy via c-jun oncogene downregulation.[128] Yehl et al synthesized multivalent deoxyribozyme "10-23" gold nanoparticle (DzNP) conjugates for in vivo studies, which revealed less sensitive toward nuclease degradation. The DzNP could enter the mammalian cells and knockdown GDF15 gene expression in

breast cancer cells.[129] Min and coworkers loaded DNAzymes on nano-sized graphene oxide (nGO) surface and proved that they could be used for simultaneous sensing and silencing of the hepatitis C virus gene in liver cells.[130] These nanoparticulate systems could enhance the efficiency of DNAzymes with no toxicity and side effects and could significantly inhibit tumor growth.[131]

Though, inorganic nanocarriers are both biocompatible and chemically inert, they may retain in the body long after administration, and long term accumulation would lead to toxicity. DNAzymes are easy to synthesize, highly biocompatible and degradable in vivo. Using DNAzymes as biomaterial to design nanocarriers for delivery and target induced release would be a very interesting topic. Instead of using organic or inorganic nanoparticles for delivery, DNA nanostructures constructed through hybridization of short DNA building blocks have attracted wide attention in recent years. Among these, DNA tetrahedral is the most representative example, which has been utilized as delivery tool to transfect mRNA, si-RNA, aptamers or CpG oligodeoxynucleotides into cells for targeted gene silencing or immunostimulation. In the near future, using self-assembled DNA nanostructures for in-vivo delivery of DNAzymes for gene therapy will certainly arouse widely attention and extensively studies. Zhang and Tan designed a histidine-dependent DNAzyme based dentrimer scaffold as nanocarrier to deliver functional DNA into living cells. The DNAzymes embedded in the dendrimers maintained the catalytic activity in the cellular environment, without changing sensitivity or selectivity. These DNAzyme dendrimeric nanocarriers exhibited excellent biocompatibility and cell membrane permeability, with remarkably enhanced intracellular stability.[132] If these DNA nanoassemblies are coupled with the up mentioned mRNA targeting DNAzymes, biodegradable DNA nanoassemblies-DNAzyme therapeutic systems will be built with multiple biological functions for gene therapy.

4. Conclusion

DNAzymes created through in vitro selection possess the merits of easy to synthesize and functionalize, excellent specificity toward cofactors and multiple enzymatic turnover properties. These unique features make DNAzyme an attractive and versatile tool for the construction of both recognition element and promising signal amplifier in biosensor designing. Because of the highly selectivity toward cofactors, DNAzymes have been converted into biosensors for a broad range of cofactors, such as Pb^{2+}, UO_2^{2+}, Cu^{2+}, Zn^{2+}, and histidine based on either colorimetry, SERS, fluorescence, or electrochemistry. By rational designing, biosensors for a broad range of targets besides cofactors could be constructed. Target binding event of the aptamer domain could be translated into the activating of DNAzyme for signal generation and amplification.

In order to improve the sensitivities, nanomaterials assisted enhancement, like AuNPs, GO, CNT, QDs etc, or biological based signal amplification strategies were introduced. The unique distance-dependent optical properties of AuNPs coupled with RNA-cleaving DNAzymes provide novel sensing systems based on colorimetric, DLS, or SERS. Special ET, FRET or CRET process between nanomaterials and functionalized DNAzymes have been utilized to design novel fluorescent, chemiluminscence or electrochemical biosensors. The high loading efficiency of nanomaterials makes them excellent nanocarriers for catalytic G4-DNAzymes to build signal amplification tags. Substantial efforts have been directed lately to the development of ultrasensitive enzymatic and enzymatic-free biosensors. Researches have proved that the introducing of split-DNAzyme fragments could greatly reduce the background signal while polymerase or ligase mediated synthesis of multi-catalytic DNAzymes through ligation, RCA or SDA would significantly enhance the overall sensitivity. Some enzyme and nanoparticles free explorations such as cleavage triggered DNA-based HCR, or target recognition induced autonomous assembly of DNAzyme polymer wires provides alternative ways of signal amplification. Through ingenious design these specific recognition and transuding processes could be applied for the construction of robust molecular logic gates and DNA walking devices. DNAzymes have been proven to be highly useful building blocks to perform arithmetic operations. When combined with a DNA or RNA track, DNAzymes were reported as "walker" moving along the designed track and this walking device could even be powered by light energy.

Another important application of DNAzymes, especially 10-23 DNAzymes that could cleave an all-RNA substrate under physiological conditions, was mRNAs-targeted gene silencing. Quite a lot of in-vitro and in-vivo studies have demonstrated that DNAzyme was able to targeting and cleaving the specific mRNA sequences and inhibit the gene expression, and was a potential gene therapeutic agent in treatment of many diseases. The seeking of appropriate delivery system for DNAzymes for in-vivo application is another interesting topic. Gold nanoparticle and GO delivery systems exhibited enhanced biological activities.

At present, DNAzyme has usually been used for construting biosensors to detect biological analytes. Although other applications of DNAzymes are still at the preliminary research stage, we believe they can be achieved a wide range of applications in many research fields, such as materials science, biology, high throughput drug screening, and nanotechnology. We also have faith in that the coupling of certain DNA nanostructures, such as DNA tetrahedral, DNA dendrimer, and DNA nanoflowers, with mRNA targeting DNAzymes might yield biodegradable nanotheropeutics with great promising for gene manipulation. But we have to know it clearly that the screening of new DNAzymes would prompt the application and development of DNAzymes.

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