Chapter 13
Nucleic Acid Amplification Strategies for In Vitro and In Vivo Metal Ion Detection

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Abstract Metal ions are critical to many biological, chemical, and environmental processes. In recent years, many nucleic acid-based biosensors have emerged for in vitro and in vivo metal ion detection. The structure of DNA is ideal for metal ion binding by both the nucleobases and phosphate backbone, although the essential biological role of DNA is to store genetic information. Three main groups of functional DNA were demonstrated for metal ion sensing: DNAzymes, G-quadruplex (also known as G4 structure), and mismatched DNA base pair. Combining with nucleic acid amplification strategies, many important metal ions can be sensitive determined down to the low parts-per-billion level. Here, we detailed the interaction between nucleic acid amplifications and metal ions, mainly including exonuclease-assisted circling, hybridization chain reaction, and rolling circle amplification. We also offer classic examples from the literature to reveal their impact in practical applications. Finally, we highlight the essential limitations that need to be addressed, and future research opportunities are discussed.

13.1 Introduction

Metal ions play critical roles in chemical environmental and biological systems (Zhou et al. 2017b). As most metal ions cannot be biodegraded and cause persistent pollution, they are easy to be ingested by the human body and cause serious harm to the human body (Jarup 2003; Saidur et al. 2017). Therefore, the in vitro and in vivo detection of metal ions is of great significance in the research fields of biomedicine, environment, and industry.

Traditional methods for the detection of metal ion, such as inductively coupled plasma mass spectrometry (Ashoka et al. 2009) and atomic absorption spectroscopy

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(Giokas et al. 2004), need costly and bulky instrumentation and significant training to use properly, making it very difficult for in vitro and in vivo biosensing. Significant progress, to overcome these limitations, based on functional nucleic acid isothermal amplification techniques has been made in developing sensors and imaging agents for the detection of metal ions.

13.2 Functional Nucleic Acids for Metal Ion Detection

To detect metal ions, functional nucleic acids are mainly categorized into three groups: DNAzymes, mismatched DNA base pairs, and G-quadruplexes related to guanine (G)-rich DNA probes. To achieve sensitive detection, signal amplification strategies such as peroxidase-mimicking DNAzymes, cascade DNAzyme, protein-based enzymes (e.g., rolling circle amplification (RCA), nicking enzymes, exonuclease III, polymerase/DNase I, and so on), enzyme-free amplification (e.g., hybridization chain reaction (HCR)) are particularly used.

13.2.1 DNAzyme-Based Sensing Strategies

DNAzymes, screened via in vitro selections, have been commonly employed as highly selective recognition elements for a broad range of cofactors, such as Cu^{2+}, Pb^{2+}, Zn^{2+}, Ca^{2+}, Mg^{2+}, $UO_2{}^{2+}$, and Hg^{2+} (Liu et al. 2009; Peng et al. 2018). DNAzymes can bind with substrates associated with target ions. For example, Fig. 13.1a, b exhibits the main structure of DNAzymes that are highly selective to Pb^{2+} and Cu^{2+}, respectively. With the addition of certain ions, the activities of DNAzymes can be activated and cut into two components from the specific positions marked by the triangles. Then, the dsDNA dissociates from each other and produces several short ssDNA fragments. DNAzymes have significant efficient catalytic activities and recognition abilities, which are especially dependent on the target ions being detected. DNAzymes have been extensively used in electrochemical and optical sensors for the detection of metal ions in a simple, rapid, and stable manner (Huang et al. 2017; Zhou et al. 2017b).

13.2.2 G-quadruplex-Enabled Detection Platforms

G-quadruplexes, also known as G4 structure, in which four guanines form a square planar tetrad, have attracted extensive interest in recent years (Mohanty et al. 2013; Vummidi et al. 2013; Yang et al. 2009). Since metal ions play a key role in the preparation of G4 structure (Fig. 13.1c), G4 structures have been widely employed for the sensitive determination of various metal ions, including Na^+ and K^+. An

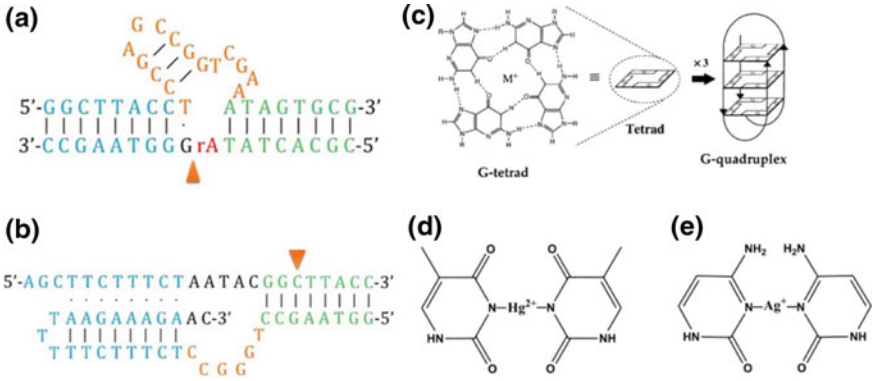

Fig. 13.1 Common functional nucleic acids used for metal ion detection: DNAzyme-specific complexes that are sensitive to **a** Pb^{2+} and **b** Cu^{2+}. **c** Illustration of the metal ion-mediated G-quadruplex structure. **d** T–Hg^{2+}–T and **e** C–Ag^{+}–C. Reprinted from Huang et al. (2017), Copyright 2017, with permission from Elsevier

interesting property of G4 structures is that its conformation can transform from single-strand structures to quadruplex states, which enables G4 structures compatible with diverse signaling mechanisms.

Traditionally, a ssDNA probe is designed with the two terminals that linked with a quencher and a fluorophore. It adopts a random coil structure and retains strong fluorescence intensity (Ueyama et al. 2002). However, it rearranges its configuration and forms a G-quadruplex structure upon the addition of K^{+}, which brings the quencher and fluorophore close to each other, thus allowing FRET process. The decrease of fluorescence reveals the presence of K^{+} selectively and accurately. Unlike the dual-labeled ssDNA probe, label-free ssDNA probe is also used, which provides the straightforward and cost-effective detection of K^{+}. The ssDNA probe is enabled to switch its random coil structure to a G-quadruplex structure in the presence of K^{+}. Riboflavin, a special ligand that has strong fluorescent signal by binding with G-quadruplexes, could be accommodated with G-quadruplex, producing a significant fluorescent signal.

13.2.3 Mismatched DNA Base Pair-Mediated Assays

Hybridization events between thymine (T) bases can be effectively induced to generate T–Hg^{2+}–T complexes in the presence of Hg^{2+} (Chiang et al. 2008; Li et al. 2009; Xue et al. 2008). The imino proton of T residues, as shown in Fig. 13.1d, can be replaced by Hg^{2+} to form T–Hg^{2+}–T complexes, which is much stable than the natural T-A base pair. Thus, T–Hg^{2+}–T coordination is a powerful protocol for Hg^{2+} detections. Similarly, Ag^{+} can link with cytosine (C) bases to induce the formation of C–Ag^{+}–C base pairs (Fig. 13.1e) (Hao et al. 2012; Yan et al. 2012; Zhao et al.

2010). Many efforts have already been initiated to establishing methods for effective and reliable detection of mercury and silver ions, which enable good use of metal ion-aided coordination chemistry: T–Hg^{2+}–T and C–Ag^{+}–C.

13.2.4 Nanomaterials for Signal Transduction and Amplification

With the development of nanomaterial and technology, functional nanomaterials have attracted extensive attention in nucleic acid amplification for in vitro and in vivo metal ion detection. Nanomaterials have significant electrical, optical, thermal, mechanical, and chemical properties, which can greatly improve the performance of biosensors. Notably, nanomaterials can serve as remarkable colorimetric indicators or fluorescence quenchers. Also, they can be used as efficient nanocarriers for superb signal enhancers or signaling probes to effectively amplify the readout signals, such as SERS and fluorescence polarization. The combination of nanomaterials with functional nucleic acid indicates high recognition affinity toward metal ions, enabling innovative biosensing nanoplatforms with advanced selectivity and sensitivity. In this chapter, the examples of metal ion biosensors on the basis of both nucleic acid amplification and advanced nanomaterials, such as carbon nanotubes (CNTs), quantum dots (QDs), gold nanoparticles (AuNPs), magnetic nanoparticles (MNPs), graphene oxide (GO), and so on, are illustrated.

13.3 Detection of Alkali Metal Ions

Alkali metal ions contain lithium ion (Li^{+}), sodium ion (Na^{+}), potassium ion (K^{+}), rubidium ion (Rb^{+}), and cesium ion (Cs^{+}). As general buffer salts, these metals have long been employed to screen charge repulsion in nucleic acid. Notably, Li^{+}, Na^{+}, and K^{+} play a critical role in biology process. Rb^{+} has not been demonstrated on biological functions yet. Cs^{+} is toxic to some degree. Until now, DNA-based biosensors were mainly established for Na^{+} and K^{+}.

Na^{+} is very important in organism. The increased concentration of Na^{+} might result in high blood pressure, water retention, and other physiological disorders (Grant et al. 2002; Jaitovich and Bertorello 2010). Twenty years ago, Geyer and Sen have in vitro selected DNAzyme using Na^{+} alone, as shown in Fig. 13.2a, when they tried to examine whether DNAzymes could be active without addition of divalent metals (Geyer and Sen 1997). In the presence of Na^{+}, the isolated DNAzyme was active; however, the secondary structure is not satisfied and the selectivity for other metals was not revealed, which might explain its lack of biosensing performances. In recent years, a few Na^{+}-specific DNAzymes have been demonstrated, and Lu et al., for example, selected a DNAzyme called NaA43 with a rate of 0.1 min^{-1} in 400 mM

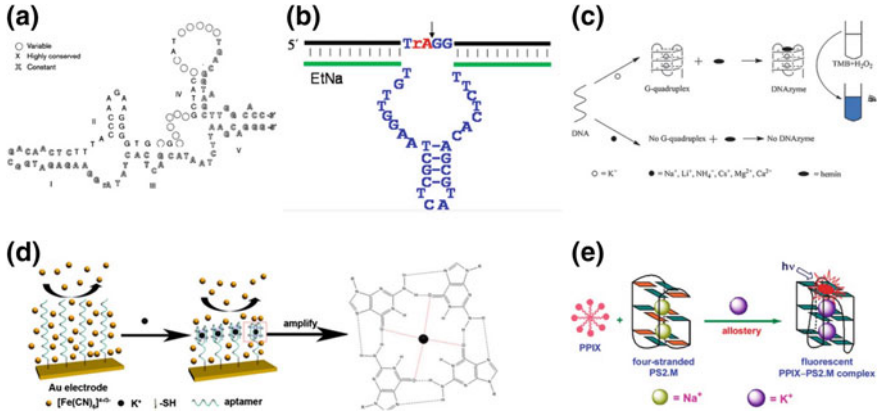

Fig. 13.2 **a** Proposed secondary structure of the Na^+ family DNAzymes. Reprinted from Geyer and Sen (1997), Copyright 1997, with permission from Elsevier and **b** secondary structures of the EtNa DNAzymes for Na^+ detection. Reproduced from Zhou et al. (2016) by permission of John Wiley & Sons Ltd. **c** The colorimetric detection of K^+. Reproduced from Yang et al. (2010) by permission of The Royal Society of Chemistry. **d** Illustration of the label-free electrochemical detection of K^+. Reprinted from Chen et al. (2013), Copyright 2013, with permission from Elsevier. **e** Turn-On fluorescent sensor for K^+ detection with G4 structure as the sensing element. Reprinted with the permission from Li et al. (2010). Copyright 2010 American Chemical Society

Na^+ specifically, while other mono-, di-, and trivalent metals are inactive (Torabi et al. 2015). Liu and coworkers isolated another DNAzyme called EtNa (Fig. 13.2b). Although the rate was slightly slower, about 0.06 min^{-1}, with much weaker Na^+ binding affinity in water, it showed excellent selectivity for Na^+ (Zhou et al. 2016).

Na^+ is able to promote the formation of G-quadruplex; however, it is generally less efficient than K^+. However, G-quadruplex with Na^+ specificity was developed by Tang et al. and it could be used for the detection of Na^+ (Sun et al. 2016). The authors chose a DNA sequence named p25, producing an antiparallel conformation with Na^+ but a hybrid-type conformation with K^+. Toward hemin, the binding affinities using these two conformations are different, and thus, the resulting hemin-p25 DNAzymes with peroxidase-like activity were employed for the detection of Na^+. However, to detect Na^+ using such method in complex biological samples has yet to be reported because of the high background ionic strength generating a high intracellular K^+ concentration for competition and nonspecific DNA condensation.

Using DNA for the detection of K^+ mainly relies on G4 structures. K^+ is coordinated by O6 groups of guanines from two consecutive stacked G-tetrads (Bhattacharyya et al. 2016; Mekmaysy et al. 2008). Many efforts have been conducted to improve selectivity to achieve its biological applications. It has been shown that K^+ sensitivity at nanomolar level with about 10^4-fold selectivity over Na^+ (Huang and Chang 2008; Qin et al. 2010). As shown in Fig. 13.2c–e, utilizing the K^+-sensitive G4 structures, researchers have achieved selective and sensitive determination of K^+ by colorimetric (Yang et al. 2010), electrochemical (Chen et al. 2013; Jarczewska et al.

2016), and fluorescent strategies (Lee et al. 2014; Li et al. 2010). Given these research progresses, using G4 structures for K^+ detection in biological samples, such as human serum and urine, has been achieved (Huang and Chang 2008; Yang et al. 2016). However, since many other metals could bind with G4 structures, new DNA sequences that can better selectively detect K^+ from Na^+ and other metal ions are still to be developed.

13.4 Detection of Alkaline Earth Metal Ions

Alkaline earth metals refer to beryllium ion (Be^{2+}), magnesium ion (Mg^{2+}), calcium ion (Ca^{2+}), strontium ion (Sr^{2+}), barium ion (Ba^{2+}), and radium (Ra^{2+}). Here, Be^{2+} is slightly toxic, Sr^{2+} usually acts as beta-ray source, Ba^{2+} is employed to help X-ray imaging, and Ra^{2+} shows radioactive property. Only Mg^{2+} and Ca^{2+} are the most important in biology.

13.4.1 Magnesium Ion Sensors

Magnesium ion, one of the most important, major intracellular divalent cation, is related to all nucleic acid in biological processes (Pechlaner and Sigel 2012; Anastassopoulou and Theophanides 2002; Auffinger et al. 2011). Mg^{2+} is demonstrated to be a critical cofactor for most ribozymes (Bowman et al. 2012). A recent molecular dynamics simulation reported by Li et al. indicates that Mg^{2+} specifically attaches to the negatively charged phosphate backbone and the major groove of G/C bases (Li et al. 2011).

Ju and coworkers reported a dual-potential ratiometric electrochemiluminescence (ECL) biosensing strategy on the basis of Mg^{2+}-specific DNAzyme-controlled ECL signals of CdS QDs and luminol (Cheng et al. 2014). Without addition of Mg^{2+}, the cathode ECL of the QDs is significantly quenched by electrochemiluminescence resonance energy transfer between Cy5 molecule and CdS QDs, while the anode ECL from Au@luminol is presented into the system (Fig. 13.3a). Conversely, in the presence of Mg^{2+}, the DNAzyme cleaves the substrate, releasing the Au@luminol and Cy5, which leads to the decrease of the anode ECL. Based on the ECL intensities, this method yields a detection limit as low as 2.8 μM of Mg^{2+} and achieves sensitive detection of Mg^{2+} in Hela cell extract.

Generally, Mg^{2+}-dependent DNAzyme is more commonly used as a universal tool for target detections, such as nucleic acid, protein, enzymatic activity, and other metal ions. For example, a new type of intracellular nanoprobe, named AuNP-based hairpin-locked-DNAzyme, was established to detect miRNA in living cells (Yang et al. 2017). Briefly, it contains hairpin-locked-DNAzyme strands and an AuNP (Fig. 13.3b). The hairpin-locked DNAzyme strand induces a hairpin structure via intramolecular hybridization in the absence of target, which could suppress the catalytic activity of DNAzyme and the fluorescence is quenched using the AuNP.

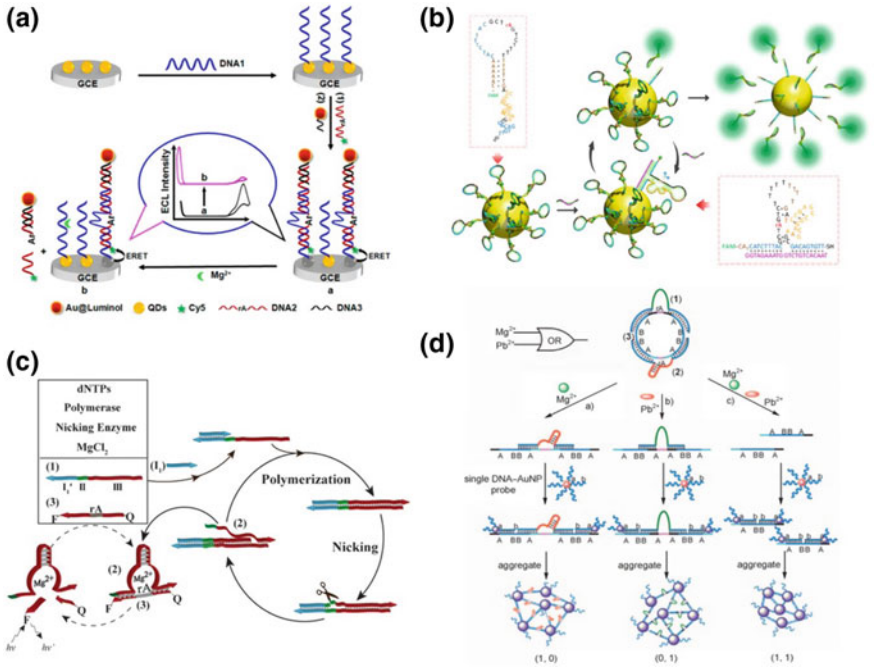

Fig. 13.3 **a** Illustration of ratiometric ECL approach initiated by Mg^{2+}-dependent DNAzyme for biosensing. Reprinted with the permission from Cheng et al. (2014). Copyright 2014 American Chemical Society. **b** Working mechanism of the AuNP-based hairpin-locked DNAzyme probe for miRNA detection. Reprinted with the permission from Yang et al. (2017). Copyright 2017 American Chemical Society. **c** Nucleic acid-controlled DNA machineries synthesizing Mg^{2+}-dependent DNAzymes for the logic-gate operations. Reproduced from Orbach et al. (2012) by permission of John Wiley & Sons Ltd. **d** Colorimetric logic gates based on DNA machineries structures and AuNPs. Reproduced from Bi et al. (2010) by permission of John Wiley & Sons Ltd.

However, the target–probe hybridization, in the presence of target, can open the hairpin and form the active secondary structure to yield an active DNAzyme, which then cleaves the self-strand with the aid of Mg^{2+}. The cleaved two shorter DNA fragments can be separated with the miRNA. In this case, the fluorophores are released from the AuNP and the fluorescence is increased. At the same time, the target is also released and attaches to another hairpin-locked DNAzyme strand to trigger another cycle of activation.

Due to its unique advantages, including site-specific, programmable, and reliable properties, it has been recently used to construct DNA machineries and logic gates. Willner's group used polymerase, nicking enzymes, and Mg^{2+}-dependent DNAzyme subunits to prepare DNA machines for the design of logic gates and for the development of amplified DNA detection schemes (Fig. 13.3c). They revealed the assembly of the OR, AND, and AND gates and discussed the interplay of the systems between biosensing and logic-gate functionalities (Orbach et al. 2012). We previ-

ously developed a complete set of two-input logic gates, including AND, OR, XOR, NOR, INHIBIT, XNOR, and NAND, based on the employment of ion-dependent DNAzymes as functional elements and the cofactor ions as inputs (Fig. 13.3d). The outputs of the gates perform the gate functions through AuNP-based colorimetric detection (Bi et al. 2010).

13.4.2 Calcium Ion Sensors

Ca^{2+} is an essential metal ion in biology process and in the environment, resulting in extensive research in developing biosensors for the detection of Ca^{2+}. While many proteins that are specific to Ca^{2+} are demonstrated, very few DNA or RNA can specifically bind to Ca^{2+}. Biosensors based on nucleic acid are interesting and attractive for its excellent programmability and high stability. Liu et al. reported an RNA-cleaving DNAzyme, termed EtNa, cooperatively binding with two Ca^{2+} but only one Mg^{2+} (Zhou et al. 2017a). Compared with four DNAzymes with known Ca^{2+}-dependent activity, the selectivity of EtNa for Ca^{2+} was the best. The EtNa was used for Ca^{2+} detection with excellent selectivity and a detection limit of 17 μM Ca^{2+}. The Ca^{2+} sensing in tap water was conducted, and the obtained result was comparable with that from standard ICP-MS method.

13.5 Detection of Lanthanide and Actinide Ions

Lanthanides represent an especially challenging classification of analytes, as these ions are very similar to each other. Lanthanides are belonging to hard Lewis acids with a high density of charge. These ions have strong affinity to the phosphate backbone of DNA and thus tend to coordinate with DNA molecules. For example, Ln^{3+} only binds to the negatively charged phosphate backbone of dsDNA by the loss of at least one hydration water (Gross and Simpkins 1981). Tb^{3+} binds with the phosphate backbone of the nucleotide monophosphates (Barry et al. 1971; Raj and Rao 1969). The resonance energy levels of Eu^{3+}, Ce^{3+}, and Tb^{3+} overlap with the triplet energy states of the nucleotide, leading to higher lanthanide luminescence by energy transfer (Yonuschot and Mushrush 1975). Thus, Tb^{3+} has been widely employed as an enhanced luminescent probe for nucleic acids (Chatterji 1988; Gross and Simpkins 1981). Ye et al. reported that GT-rich DNA sequence is highly effective in improving sensitized Tb^{3+} luminescence (Lin et al. 2014; Zhang et al. 2013).

In the early 1990s, Ln^{3+} has been reported for cleaving nucleic acids. Free Ln^{3+} ions at millimolar level cleave RNA nonspecifically through binding to the phosphate backbone and producing multinuclear complexes under alkaline conditions (Komiyama and Shigekawa 1999). In addition, Ce^{4+} and its complexes have been demonstrated to have more efficient in hydrolyzing DNA (Aiba et al. 2011). With a ribozyme or DNAzyme, site-specific cleavage was realized. For instance, in the

presence of Eu^{3+}, Yb^{3+}, or Ce^{3+} together with Zn^{2+}, DNAzymes for DNA cleavage were isolated (Dokukin and Silverman 2012). Introducing the lanthanide ions to the DNAzyme chemistry has generated several new signal amplifications for its detection.

Previous literature selected a Gd^{3+} aptamer (Edogun et al. 2016). By constructing a fluorescent strategy, the optimized conformation was characterized with a K_d of 330 nM Gd^{3+}, which could be transformed into a fluorescent biosensor with a detection limit of 80 nM. The biosensor was found to have excellent selectivity against several other ions, including divalent and trivalent metals.

Yttrium (Y^{3+}), a trivalent rare earth metal, has a similar size with holmium (Ho^{3+}), and its catalytic activity with the above-mentioned lanthanide-specific DNAzymes is also similar to that of Ho^{3+} (Huang et al. 2014, 2015, 2016). However, in vitro selection with Y^{3+} has not been reported yet. Adsorption of DNA by Y_2O_3 was investigated, indicating DNA phosphate backbone has essential for this adsorption reaction (Liu and Liu 2015). Also, Sc^{3+} is a trivalent rare earth metal; however, it is inactive with all known Ln^{3+}-dependent DNAzymes because it is much smaller than Y^{3+}.

Uranium is often employed for preparing power generation and nuclear weapons. Sensitive determination of uranium is a practical analytical need as a nuclear waste. In water, the most stable form of uranium is UO_2^{2+}. The Lu's group isolated a DNAzyme for UO_2^{2+} called 39E showing million-fold selectivity against other examined metals (Xiao et al. 2007). The 39E for the detection of UO_2^{2+} is down to 45 pM.

13.6 Detection of Post-transition Metal Ions

Post-transition metals are a set of metallic elements located after the transition metals in the periodic table, such as lead, bismuth, gallium, indium, thallium, and tin. Most of them are toxic, and their interactions with nucleic acid are barely reported except for lead. Here, we mainly review the nucleic acid amplification methods for lead ion detection.

Lead, one of the most toxic heavy metals, can pass through skin, respiratory organs, and digestive system into the blood. Lead is always accumulated in vital tissues and organs of the human body and causes reproductive, cardiovascular, neurological, and developmental disorders, especially in children (Wang et al. 2013). On the basis of Environmental Protection Agency (EPA), the safety limit is 15 ppb (0.07 μM) in drinking water, while the International Agency for Research on Cancer (IARC) defines a lower threshold of 10 ppb (48.26 nM) in water and food (Lu et al. 2015). Thus, it is very essential to develop the biosensing strategies for effective and routine monitoring of Pb^{2+}.

Plaxco et al. used the Pb^{2+}-dependent DNAzyme for electrochemical Pb^{2+} detection (Xiao et al. 2007) (Fig. 13.4a). The sensor contains a methylene-blue (MB)-modified catalytic DNA strand (1), which is used to hybridize with its complementary sequence (2). This complex is relatively rigid, preventing the MB from approaching the gold electrode to transfer electrons. With the addition of target Pb^{2+}, the catalytic

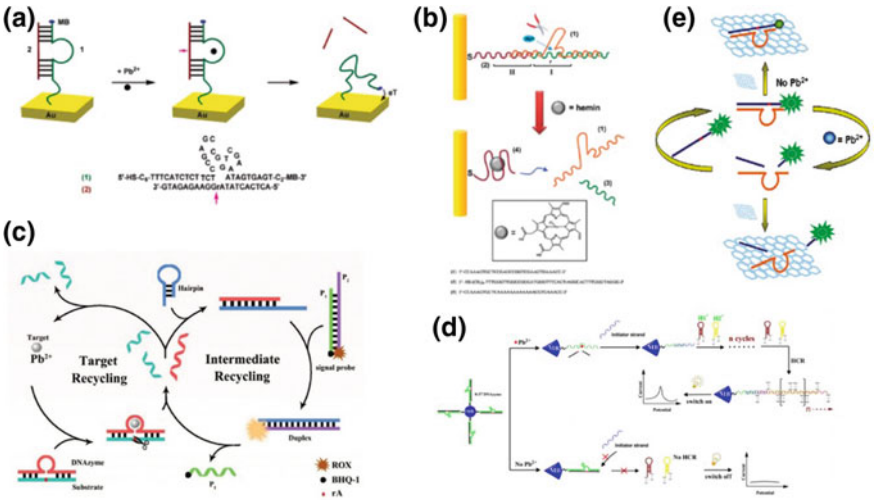

Fig. 13.4 **a** Schematic of electrochemical Pb^{2+} sensor based on the DNAzyme. Reprinted with the permission from Xiao et al. (2007). Copyright 2007 American Chemical Society. **b** Pb^{2+}-dependent DNAzyme-catalyzed reaction for Pb^{2+} detection based on a hemin/G-quadruplex structure. Reprinted with the permission from Pelossof et al. (2012). Copyright 2012 American Chemical Society. **c** Illustration of target–intermediate recycling amplification. Reproduced from Wen et al. (2017) by permission of The Royal Society of Chemistry. **d** Working mechanism of the DNAzyme-based electronic switch for Pb^{2+} detection based on HCR reaction. Reprinted from Zhuang et al. (2013), Copyright 2013, with permission from Elsevier. **e** Schematic of the DNAzyme-GO based fluorescence method for the detection of Pb^{2+}. Reprinted with the permission from Zhao et al. (2011). Copyright 2011 American Chemical Society

strand cleaves the substrate into two fragments, which dissociate from the complex, allowing the MB molecule to transfer electrons to the gold electrode. The directly measured detection limit of the proposed biosensor is 0.3 μM.

Similarly, Willner et al. used Pb^{2+}-dependent DNAzyme for electrochemical detection (Fig. 13.4b). With the addition of target Pb^{2+}, cleavage of the DNAzyme substrate proceeds, leading to the self-assembly of the hemin/G-quadruplex nanostructure label (Pelossof et al. 2012). The proposed sensing platform realizes sensitive detection of Pb^{2+} with a low detection limit of 5 fM.

Cai et al. proposed an ultrasensitive fluorescence method for intracellular determination of Pb^{2+} with a detection limit down to 0.3 nM by target–intermediate recycling amplification based on a strand displacement reaction and metal-aided DNAzyme catalysis (Fig. 13.4c). With the addition of Pb^{2+}, the DNAzyme cleaves the ribonucleotide in the substrate with excellent specificity, releasing the Pb^{2+} and DNAzyme. The released Pb^{2+} circularly activates another DNAzyme to realize the signal amplification. At the same time, the released DNAzyme could open the hairpin probe and trigger the intermediate recycling with the signal probe. In the intermediate recycling, the opened hairpin probe hybridizes with the P2 strand to form a completely

complementary DNA duplex, recovering the fluorescent signal. Thus, the DNAzyme was released and generated another intermediate recycling (Wen et al. 2017).

An enzyme-free strategy that couples a Pb^{2+}-specific DNAzyme with HCR was developed by Tang's group (Fig. 13.4d). Upon addition of Pb^{2+}, catalytic cleavage of substrate in the DNAzymes leads to the capture of the initiator strands by the conjugated catalytic strands that modified on MNPs. The captured DNA initiator strands induced the HCR process between two alternating hairpin probes modified with Fc to produce a nicked dsDNA on the MNPs. Massive Fc molecules were formed on the neighboring probes and thus amplified the electrochemical signal within the applied potential. This sensor displayed a detection limit of 37 pM and robust applications in groundwater or drinking water (Zhuang et al. 2013).

Yu et al. developed a GO-DNAzyme-based strategy for amplified turn-on fluorescent detection of Pb^{2+} (Fig. 13.4e). The DNAzyme strand is hybridized with the substrate strand by only five base pairs, allowing fluorophore attached to the GO surface to afford a high quenching efficiency and a low background signal. The FAM-labeled hybrid acts as both signal reporter and a molecular recognition module and GO as a quencher. Upon addition of Pb^{2+}, the substrate strand can be digested into two short segments, releasing the FAM-labeled portion and recovering the fluorescence. The biosensor shows a high sensitivity toward the Pb^{2+} with a low detection limit of 300 pM (Zhao et al. 2011).

Fan and coworkers proposed a novel detection platform using enzyme synergetic isothermal quadratic DNA machine (Xu et al. 2013). This platform integrates an NEase-aided signal amplification module and strand displacement amplification (SDA) module into a one-step system to realize ultrasensitive analysis of Pb^{2+} (detection limit down to 30 fM) within a short sensing time (40 min). The obtained results for the detection of Pb^{2+} in complex environmental water samples using the proposed sensing system are consistent with those obtained by ICP-MS (Zhang et al. 2014).

With the help of RCA and DNAzyme, Zhang et al. developed highly selective and sensitive electrochemical sensing system for the detection of Pb^{2+} (Tang et al. 2013). The DNAzyme could be activated in the presence of Pb^{2+} to digest the DNAzyme into two short DNA fragments. A long ssDNA probe with repeating sequence was generated after RCA reaction. Subsequently, CdS QD-labeled ssDNA were employed as signaling probes to hybridize with the long ssDNA product. Owing to the significant signal amplification by the massive QDs and the low background signal by magnetic separation, a low concentration of 7.8 pM Pb^{2+} could be detected.

Exonuclease-aided DNA recycling is a powerful strategy for signal-amplified detection of Pb^{2+}. Zuo et al. proposed a Pb^{2+} induced exonuclease III-assisted DNA recycling system to enhance the sensitivity of detection (Xu et al. 2013). With the addition of Pb^{2+}, the substrate DNA segments were digested and released from the dsDNA. The disassociated DNAzyme was employed as a primer of the exonuclease-assisted recycling system which can significantly amplify the fluorescent intensity by continuously recycling the DNAzyme. The detection limit of Pb^{2+} was down to 5 pM with excellent selectivity.

Similarly, Yu et al. proposed an isothermal signal-amplified DNAzyme method for the specific and sensitive quantification of Pb^{2+} (Li et al. 2014). In the presence

of Pb^{2+}, an RNA probe containing DNA substrate was digested by the DNAzyme, efficiently removing the 2′,3′-cyclic phosphate of the digested substrate by exonuclease III. The remaining portion of the ssDNA was subsequently employed as the primer for SDA reaction. The proposed assay could sensitively detect 200 pM Pb^{2+}.

13.7 Detection of Transition Metals

In recent years, nonspecific interactions between transition metal ions and DNA have been investigated by diverse methods, which collectively imply transition metals prefer nucleic acid bases to the phosphate with the following binding affinity: $Hg^{2+} > Cu^{2+} > Cd^{2+} > Zn^{2+} > Mn^{2+} > Ni^{2+}$, $Co^{2+} > Fe^{2+}$ (Duguid et al. 1993). Many reports have been shown to select their DNAzymes and aptamers. Although DNAzymes have been developed for Cd^{2+} detection, none of the DNAzymes were highly selective for Cd^{2+}. Furthermore, no nucleic acid-based high-performance sensors for manganese, cobalt, iron, molybdenum, nickel, and tungsten are reported yet. Noble metals, including gold, silver, platinum, and palladium, are transition metals as well. These metals are not endogenous ions in living organisms and barely participate in native biological processes. Silver and platinum are often used to form stable metal nanoparticles for DNA adsorption. Palladium usually coexists with platinum, and no DNA-based biosensors have been reported for palladium detection yet. Thus, the sensitive detection of Cu^{2+}, Hg^{2+}, Zn^{2+}, and Ag^{+} is mainly discussed.

13.7.1 Copper Ion Sensors

Copper, a broadly used metal worldwide, can leak into the environment by numerous ways. Copper with low concentration is a critical nutrient for human body. But gastrointestinal disturbance might occur when exposed to high level of copper even for a short period of time, while long-term exposure causes the damage of kidney or liver (Georgopoulos et al. 2001). The US EPA has set the limit of copper to be 1.3 ppm (~20 μM) in drinking water (Georgopoulos et al. 2001). Hence, it is very important to detect the concentration of Cu^{2+} in the environment.

Breaker et al. isolated DNA-cleaving DNAzymes by using Cu^{2+} as a metal cofactor (Carmi et al. 1996, 1998). Based on the discovery, Lu et al. developed a Cu^{2+}-dependent DNA-cleaving DNAzyme sequences for a Cu^{2+} detection (Liu and Lu 2007a) (Fig. 13.5a). The DNAzyme contained two DNA strands that formed a complex. A FAM fluorophore at the 3′-end and a quencher at the 5′-end are labeled on the substrate, while the enzyme modified a 5′-quencher. In the presence of Cu^{2+}, the substrate could be cleaved irreversibly at the cleavage site (the guanine in red). After that, the digested segments were released because of the low affinities to the enzyme, resulting in increased fluorescence. They obtained a low detection limit of 35 nM with excellent practicability for detecting Cu^{2+} in drinking water.

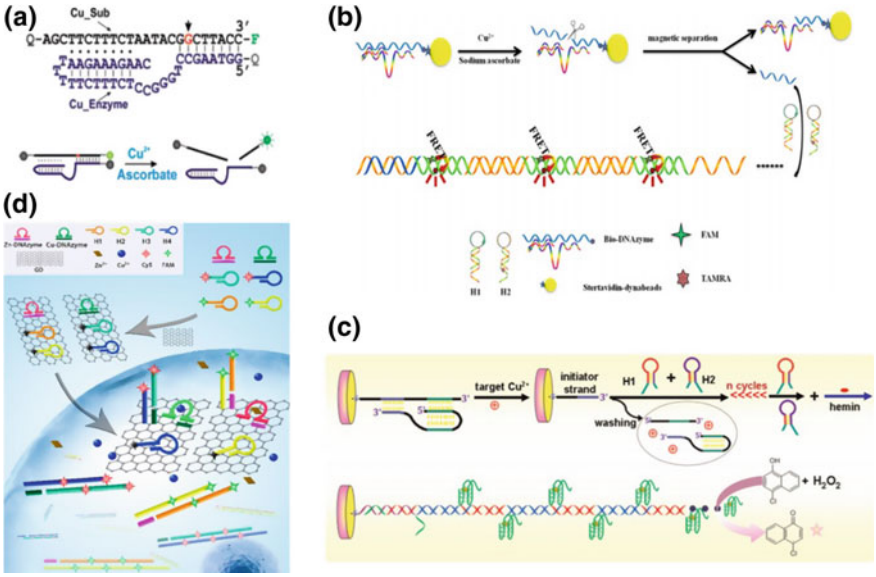

Fig. 13.5 **a** Illustration of the Cu^{2+}-specific DNAzyme for fluorescence detection. Reprinted with the permission from Liu and Lu (2007a). Copyright 2007 American Chemical Society. **b** Schematic illustration of DNAzyme-based fluorescent assay using HCR with FRET technique. Reprinted from Chen et al. (2016), Copyright 2016, with permission from Elsevier. **c** Schematic illustration of Cu^{2+}-specific DNAzyme-based electrochemical biosensor based on target-induced HCR for sensing of Cu^{2+}. Reprinted from Xu et al. (2015), Copyright 2015, with permission from Elsevier. **d** Signal amplification method for the sensitive fluorescence imaging of Cu^{2+} and Zn^{2+} in living cells. Reprinted with the permission from Si et al. (2018). Copyright 2018 American Chemical Society

Combining Cu^{2+}-dependent DNAzyme with HCR signal amplification, Guo and coworkers demonstrated the amplified detection of Cu^{2+} using fluorescence resonance energy transfer (FRET) method (Chen et al. 2016) (Fig. 13.5b). With the addition of Cu^{2+} ion, the DNAzyme attached on MNPs was selectively digested and released, initiating the HCR process of hairpin H1 and H2 modified with TAMRA as the acceptor and FAM as the donor, respectively. Long nicked duplexes were self-assembled to provide the acceptor and the donor in close proximity, leading to a FRET process. They achieved a limit of detection of 0.5 nM Cu^{2+} and successfully applied for detection in tap water with satisfactory results. Similarly, Tang et al. designed an impedimetric biosensor by coupling Cu^{2+}-induced hemin/G-quadruplex-based DNAzyme with enzymatic catalytic precipitation technique (Fig. 13.5c). Under the optimal conditions, the electrochemical resistance increased with raising Cu^{2+} concentration and exhibited a detection limit down to 60 fM (Xu et al. 2015).

Using polymerase/endonuclease reaction, He et al. realized amplified fluorescent detection of Cu^{2+} (He et al. 2014). With the addition of Cu^{2+}, the enzyme strand induces catalytic processes to cleave substrate strand, releasing DNAzyme substrates acting as primers to initiate the Klenow fragment polymerization. The

double-stranded niking site is cut by Nb.BbvCI endonuclease, opening a new site for a new replication. Thus, the complete dsDNA is regenerated to trigger another cycle of nicking, polymerization, and displacement. With the assistance of GO, Quan et al. designed a "turn-on" fluorescent biosensor for Cu^{2+} detection based on graphene–DNAzyme catalytic beacon (Liu et al. 2011). Owing to the excellent surface quenching property, GO acts as both "quencher" and "scaffold" of the Cu^{2+}-dependent DNAzyme, promoting the formation of self-assembled graphene-quenched DNAzyme complex. However, with the addition of Cu^{2+}, the graphene–DNAzyme conformation is disturbed, producing internal DNA cleavage-dependent effect. Thus, the quenched fluorescence in graphene–DNAzyme is rapidly recovered. Based on the change of fluorescence, they achieved a detection limit as low as 0.365 nM. Recently, Tang and coworkers used GO as intracellular transport carrier to load DNAzyme and hairpin probe into the cell (Fig. 13.5d). The sensor enabled the imaging of Cu^{2+} and Zn^{2+} by signal amplification based on DNA self-assembly under DNAzyme catalysis and reached low detection limits of 80 pM and 100 pM, respectively (Si et al. 2018).

13.7.2 Mercury Ion Sensors

As T-T mismatched base pairs revealed, Ono and coworkers first established a biosensor for the detection of Hg^{2+} in 2004 (Ono and Togashi 2004), as shown in Fig. 13.6a. They designed an ssDNA probe comprising T-rich bases with a quencher and a fluorophore labeled at the ends. Without addition of Hg^{2+}, the DNA probe tends to adopt a random coil conformation with the quencher and fluorophore being away from each other. In the presence of Hg^{2+}, the DNA probe formed a hairpin structure owing to the T–Hg^{2+}–T complexes at the ends, which bring the quencher and fluorophore into close proximity, resulting in significant fluorescence quenching. The decrease of fluorescence is greatly influenced by the concentration of Hg^{2+}. Although this turn-off strategy provides an unsatisfactory sensitivity (about 40 nM), it brings a new and potential method for Hg^{2+} detection by T–Hg^{2+}–T coordination as the basic recognition elements.

Hong et al. developed a signal-on electrochemical method for sensitive and selective detection of Hg^{2+} based on nicking endonuclease-assisted (NEase) target recycling and HCR amplification tactics (Fig. 13.6b). Upon addition of Hg^{2+}, PB probe hybridized with PA probe to form dsDNA by T–Hg^{2+}–T coordination, which automatically induced NEase to efficiently digest duplex region from the recognition sites, spontaneously releasing Hg^{2+} and PB and leaving the remnant initiators. The dissociated Hg^{2+} and PB could be reused to trigger the next cycle, and more initiators were produced. The HCR events occurred on the electrode surface triggered by the initiators, resulting in a significant signal increase. They obtained a linear range from 10 pM to 50 nM and a detection limit down to 1.6 pM (Hong et al. 2017).

Li's group developed a novel guanine nanowire (G-wire)-based approach for signal amplification (Gao et al. 2016). On the basis of Hg^{2+}-induced Exo III-aided

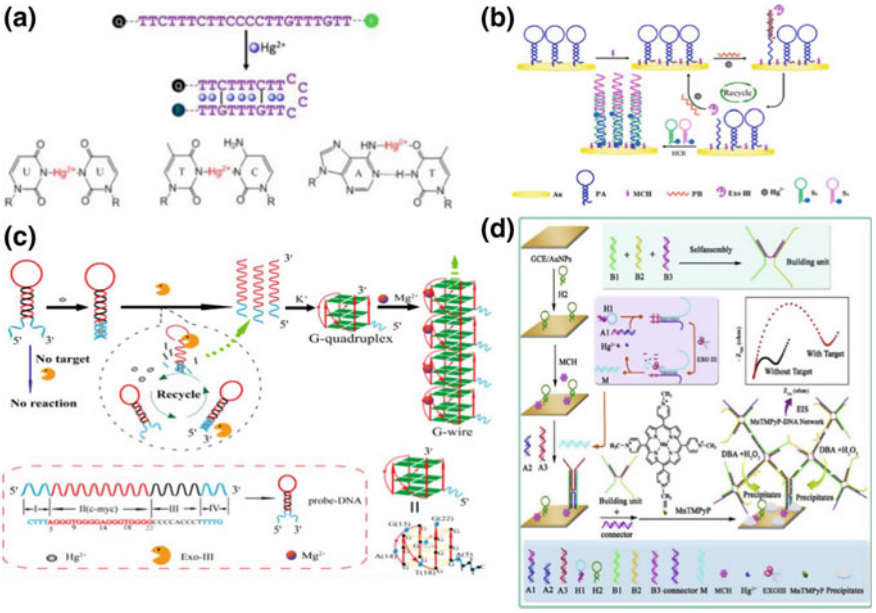

Fig. 13.6 **a** Schematic diagram of T–Hg^{2+}–T coordination. Reproduced from Ono and Togashi (2004) by permission of John Wiley & Sons Ltd. **b** Illustration of electrochemical sensor based on NEase assisted recycling and HCR strategy for the detection of Hg^{2+}. Reprinted from Hong et al. (2017), Copyright 2017, with permission from Elsevier. **c** Illustration of the label-free RRS aptasensor based on Exo III-assisted recycling and G-wire amplification for Hg^{2+} detection. Reprinted with the permission from Ren et al. (2016). Copyright 2016 American Chemical Society. **d** Schematic diagram of the impedimetric biosensor based on DNA network for Hg^{2+} detection. Reprinted from Xie et al. (2018), Copyright 2018, with permission from Elsevier

recycling and growth of G-wires for signal amplification, they designed a label-free and signal-on resonance Rayleigh scattering (RRS) aptasensor for detection of Hg^{2+} (Fig. 13.6c). The hairpin DNA probe was designed as a signal probe to recognize Hg^{2+} by T–Hg^{2+}–T structure, which automatically induced Exo III digestion to recycle the target and liberate the G-quadruplex sequence. The free G-quadruplex sequences can be self-assembled into G-wire superstructure with addition of Mg^{2+}, resulting in the dramatically amplification of RRS intensity. The limit of detection was down to 20.0 pM, and it successfully applied to test tap water and river water samples (Ren et al. 2016).

Xie et al. constructed a highly sensitive impedimetric biosensor to detect Hg^{2+} (Fig. 13.6d). With the addition of Hg^{2+}, T–Hg^{2+}–T structures initiated the Exo III-catalyzed target recycling and generated free ssDNA to promote the information of DNA networks on electrode surface, which could efficiently immobilize the porphyrin manganese (MnTmPyP). The formed MnTMPyP-dsDNA complex produced an insoluble precipitate on the electrode surface, significantly increasing the resis-

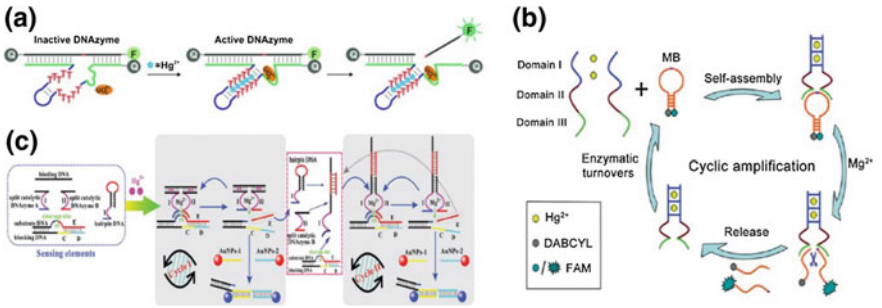

Fig. 13.7 **a** Schematic presentation of the DNAzyme sensor for the detection of Hg^{2+}. Reproduced from Liu and Lu (2007b) by permission of John Wiley & Sons Ltd. **b** Schematic of target-induced DNAzyme cascade for the amplified fluorescence detection of Hg^{2+}. Reproduced from Qi et al. (2012) by permission of The Royal Society of Chemistry. **c** Schematic presentation of the DNAzyme cascade for the amplified colorimetric detection of Hg^{2+}. Reproduced from Chen et al. (2017) by permission of The Royal Society of Chemistry

tance signal for the quantitative determination of Hg^{2+}. This platform obtained a detection limit of 1.47 pM (Xie et al. 2018).

DNAzyme is also a powerful tool to design sensitive strategies for Hg^{2+} detection. Liu and Lu first designed a turn-on allosteric DNAzyme catalytic beacons triggered by T–Hg^{2+}–T structure for ultrahigh sensitive and selective detection of aqueous mercury ions (Liu and Lu 2007b) (Fig. 13.7a). Zhao et al. reported that T–Hg^{2+}–T structure could activate Mg^{2+}-dependent DNAzyme in the presence of Hg^{2+} (Fig. 13.7b), leading to hybridize with a hairpin-structured substrate to release the fluorescent signal (Qi et al. 2012). Chen et al. designed a sensitive colorimetric method for visual detection of Hg^{2+} based on T–Hg^{2+}–T triggered DNAzyme cyclic amplification (Chen et al. 2017) (Fig. 13.7c).

13.7.3 Zinc Ion Sensors

Shen et al. reported a sensitive biosensor based on G-quadruplex for fluorescent determination of Zn^{2+} with a detection limit down to 0.91 μM. The thioflavin T was bound to the G-quadruplex in the absence of Zn^{2+}, leading to the significant enhancement of fluorescent signal, while with the addition of Zn^{2+}, thioflavin T was released by displacement of Zn^{2+}, resulting in a remarkable decrease of fluorescence (Guo et al. 2015).

Cellular fluorescent detection of Zn^{2+} has been reported. Fan and coworkers presented a dual-purpose fluorescent strategy to simultaneously monitor cellular $Cu^{2+}$and Zn^{2+} (Fig. 13.8a). As is shown, the probe comprised Cu^{2+}-specific DNAzymes, Zn^{2+}-specific DNAzymes, and AuNPs. To realize the synchronous imaging of cellular $Cu^{2+}$and Zn^{2+}, dual-purpose AuNPs were designed labeled

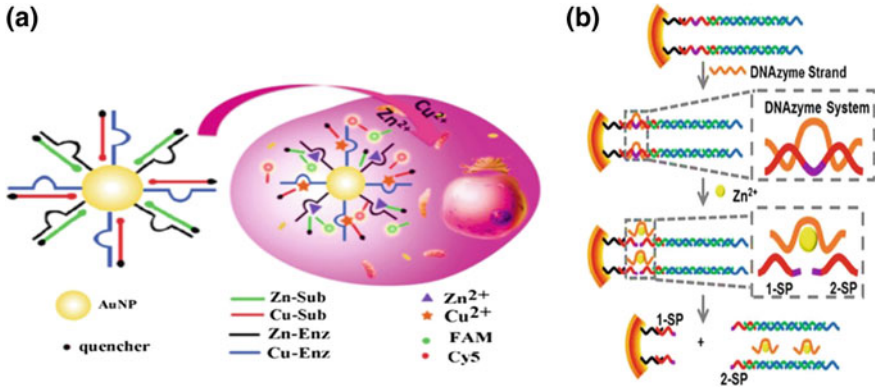

Fig. 13.8 **a** two DNAzyme-modified AuNPs were used to fabricate fluorescent biosensor for synchronous imaging of Zn^{2+} and Cu^{2+} in living cells. Reprinted with the permission from Li et al. (2015). Copyright 2015 American Chemical Society. **b** Electrochemical nanopore biosensor based on DNA supersandwich amplification for Zn^{2+} detection. Reproduced from Liu et al. (2016) by permission of The Royal Society of Chemistry

with two-color fluorophore-based DNAzyme probes. The fluorophores were greatly quenched by the AuNPs and quencher. In the presence of $Cu^{2+}$and Zn^{2+}, the substrate strands could be digested into two short fragments, when the nanoprobes were transported into the living cells, resulting in the separation of the fluorophore-modified DNA fragments, which generated significant fluorescent signals corresponding to the concentration and location of Cu^{2+} and Zn^{2+} (Li et al. 2015).

Traditional electrochemical biosensor contains immobilized nanomaterials and DNA probes on the surface. Nevertheless, Xia and colleagues developed an electrochemical nanopore biosensor for Zn^{2+} detection based on HCR amplification and Zn^{2+}-specific DNAzyme (Fig. 13.8b). As is shown, the HCR structures were formed by the multiple hybridization of DNA, leading to heavy blockage of nanopores and a significant current decrease in an I-V plot. The DNA supersandwich contains the SP probe, which could hybridize with the Zn^{2+}-specific DNAzyme. With the addition of Zn^{2+}, the SP was digested into two short segments, releasing the DNA supersandwich structures. In this case, the ionic pathway was smooth, leading to a rapid and complete rehabilitation of the current with a detection limit of 1 nM (Liu et al. 2016).

13.7.4 Silver Ion Sensors

For Ag^{+} detection, cytosine-rich DNA probes have been commonly employed. Similar to the specific interaction between thymine and Hg^{2+}, Ag^{+} was demonstrated to selectively stabilize cytosine–cytosine mismatches.

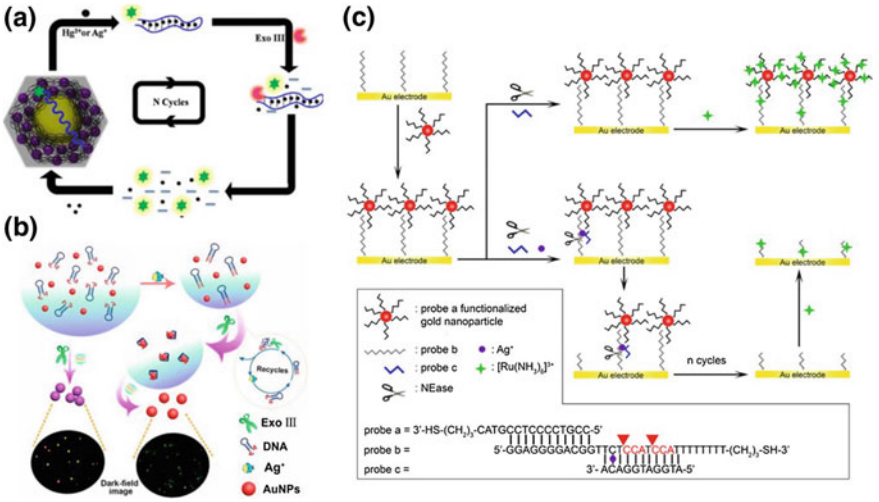

Fig. 13.9 Schematic illustration of **a** Exo III amplified fluorescent sensing system for the detection of Hg^{2+} and Ag^{+} based on metal-polydopamine framework. Reprinted with the permission from Ravikumar et al. (2018). Copyright 2018 American Chemical Society. **b** Detecting Ag^{+} based on C–Ag^{+}–C coordination chemistry by dark-field microscopy. Reprinted with the permission from Li et al. (2018). Copyright 2018 American Chemical Society. **c** Electrochemical strategy based on AuNPs and cleavage-mediated dual signal amplification for Ag^{+} detection. Reprinted with the permission from Miao et al. (2013). Copyright 2013 American Chemical Society

Exo III is frequently used to catalyze the stepwise removal of mononucleotides of dsDNA. The addition of Ag^{+} will trigger the formation of C–Ag^{+}–C hybrid, enabling Exo III to digest the generated hybrid duplex. Based on the principle, scientists have developed numerous strategies for colorimetric, electrochemical, fluorescent assays, and so on. Combining Exo III-aided strategy with nanomaterials, the sensing performance might be improved due to the unique superiority of nanomaterials. For example, Panneerselvam et al. proposed a metal–polydopamine framework with specific molecular probe as an effective fluorescent quencher (Fig. 13.9a) (Ravikumar et al. 2018). They achieved simultaneous detection of Hg^{2+} and Ag^{+} with the aid of Exo III. Integrating with AuNPs, colorimetric signal amplification strategy was developed for Ag^{+} detection at the femtomolar level using dark-field microscope (Fig. 13.9b) (Li et al. 2018).

Polymerization, DNAzyme, and nuclease-assisted amplification techniques have also been employed. Bi et al. studied Ag^{+}- and Hg^{2+}-triggered ligase activity and then established an RCA-based molecular logic gate to detect these two metal ions (Fig. 13.3d) (Bi et al. 2010). Miao et al. developed a gold nanoparticles and nicking endonuclease cleavage-based dual signal amplification method for the detection of Ag^{+} by electrochemical readout (Fig. 13.9c). They achieve a low detection limit of 470 fM with satisfactory selectivity and practicability in lake water and drinking water samples (Miao et al. 2013).

13.8 Conclusion

Heavy metal ions have a great influence on human health. How to detect the metal ions rapidly in vitro and in vivo with low cost and real time has become a challenge that needs to be addressed urgently. Nucleic acid amplifications have been extensively used in the development of biosensors to detect metal ions because they are highly sensitive, programmable, and stable. At present, the biosensors mainly include four detection strategies, DNAzyme, G4 structure, mismatched DNA base pair, and nanomaterial-based amplifications by fluorescent, colorimetric, and electrochemistry readout. Although nucleic acid biosensors demonstrate many merits, several limits are still existed. First, the reported biosensors are mainly focused on the detection of K^+, Mg^{2+}, Hg^{2+}, Pb^{2+}, Cu^{2+}, Zn^{2+}, and Ag^+, and other metal ions are less investigated. Second, the current studies mostly stay in the theoretical and laboratory level, and the relevant practical applications are few. Thus, nucleic acid amplifications urgently need to be improved in many areas, e.g., discover new signal amplification methods, promote binding nucleophilicity, enhance the sensitivity by combining nucleic acid amplifications with nanomaterials, such as carbon nanotubes, graphene oxide, gold nanoparticles, quantum dots, etc., extend the detection range of metal ions, improve detection approaches for metal ions detection in vivo, complete high-throughput assays, explore multiple metal ion detections, and develop easy to operate, simple, repeatable, real-time, and stable quantitative biosensors for in situ detection.

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