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DNAzyme-based magneto-controlled electronic switch for picomolar detection of lead (II) coupling with DNA-based hybridization chain reaction

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ABSTRACT

A novel magneto-controlled electrochemical DNA biosensor is designed for the ultrasensitive detection of lead coupling a lead-specific DNAzyme with DNA-based hybridization chain reaction (HCR). To construct such a magnetic lead sensor, DNAzyme-based molecular beacons, selective to cleavage in the presence of Pb^{2+}, are initially immobilized onto magnetic beads, which were used as the recognition elements. Upon addition of target lead, catalytic cleavage of substrate DNA segments in the double-stranded DNAzymes results in the capture of the initiator strands via the conjugated catalytic strands on magnetic beads. The captured DNA initiator strands trigger the hybridization chain reaction between two alternating hairpin DNA structures labeled with ferrocene to form a nicked double-helix on the magnetic beads. Numerous ferrocene molecules are formed on the neighboring probes, each of which produces an electrochemical signal within the applied potential. Under optimal conditions, the electrochemical signal of the magnetic lead sensor increases with the increasing lead level in the sample, and exhibits a linear response over a Pb^{2+} concentration range of 0.1–75 nM with a detection limit of 37 pM. Quantitative measurement of Pb^{2+} in the complex sample demonstrates the selectivity of the sensor scheme and points favorably to the application of such technologies to the analysis of environmental samples. The unique combination of a DNAzyme with hybridization chain reaction makes it possible to change the DNAzyme to select for other compounds of interest. This work represents the initial steps toward the creation of a robust field sensor for lead in groundwater or drinking water.

1. Introduction

DNAzymes that can catalyze a broad range of chemical and biological reactions such as RNA cleavage have been isolated in test tubes (Yang et al., 2010). Most require certain metal ions as the cofactors for structure and function, and many DNAzymes show high metal-binding affinity and specificity (Tian et al., 2012; Dong et al., 2012; Zhang et al., 2012a). For practical applications, DNAzymes are cost-effective to produce, much more stable than protein- or RNA-based enzymes and can be denatured and renatured many times without losing activity or binding ability (Kim et al., 2007; Wen et al., 2012). DNAzymes were first used in lead-ion dependent RNA cleaving, and their catalytic action was a hundred fold that of the noncatalyzed process (Kruger et al., 1982; Guerrier-Takada et al., 1983; Breaker and Joyce, 1994). These intrinsic advantages of DNAzymes make them a potential candidate for applications as components for DNA logic gates and nanomachines, DNA computing circuits, proofreading units in nanomaterial assembly, and high-efficiency tools for metal ion monitoring (Li et al., 2009; Tian et al., 2005; Elbaz et al., 2010; Liu and Lu, 2006; Hollenstein et al., 2008).

Lead ion (Pb^{2+}) is a common environmental pollutant accompanied by severe health risks (Lu et al., 2013). A very small amount of Pb^{2+} can cause serious damage to the brain and central nervous system (Needleman, 2004; Schneider and Clark, 2013). For the sake of human health care and environmental protection, it is essential to devise techniques for routine and effective monitoring of lead (Vasudev et al., 2013). While spectroscopic techniques, such as atomic absorption spectroscopy (AAS) and inductively coupled plasma atomic emission spectroscopy (ICP-AES), can offer high sensitivity for lead detection (Kunkel and Manahan, 1973; Ma et al., 1994; Asfaw et al., 2012), the development of on-site Pb^{2+} analysis is still required. Recently, the Pb^{2+}-dependent DNAzyme has turned out to be a promising

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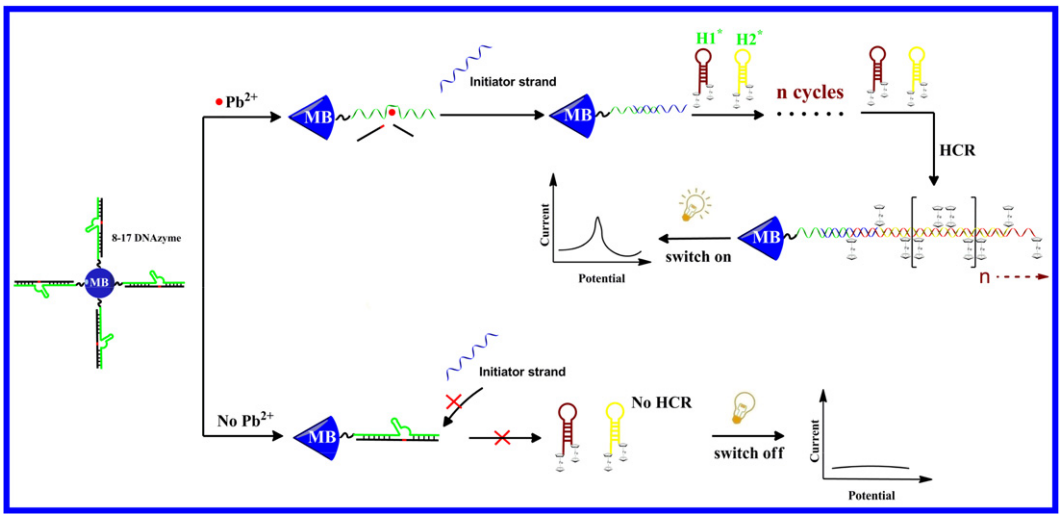

Scheme 1. Measurement protocol of the DNAzyme-based electronic switch for the detection of Pb^{2+} ions coupling with HCR reaction.

molecule for lead-sensor design, and has been customized for use in various signaling transduction strategies, including fluorescence Pb^{2+} ion sensors that lead to the activation of fluorescence through the cleavage of a fluorophore-quencher-modified substrate, colorimetric sensors based on the catalyzed deaggregation of gold nanoparticles or DNAzymes yielding the horseradish peroxidase-mimicking catalytic nucleic acids, and electrochemical sensors based on the catalyzed removal or capture of redox-active groups (Zhang et al., 2012a; Chen et al., 2012; Guo et al., 2012; Pelossof et al., 2012; Li et al., 2012; Nie et al., 2012). Also, more sophisticated analytical techniques, such as surface-enhanced Raman spectroscopy (SERS) and surface plasmon resonance (SPR), have been used to obtain the transduction signals for the reported DNAzyme-based Pb^{2+} determination (Liu and Lu, 2004; Elbaz et al., 2008; Xiao et al., 2007; Shen et al., 2008; Wang and Irudayaraj, 2011). Among these methods, the electrochemical biosensors can achieve ultrasensitive detection for Pb^{2+} with the introduction of amplification strategies, and possess many advantages, e.g. good portability, low cost, and simple instrumentation (Yang et al., 2010). Despite many advances in this field, there is still a quest for new schemes and protocols for the improvement and enhancement of sensitivity of Pb^{2+}-based sensing strategy.

For the successful development of electrochemical sensing system, high sensitivity with signal amplification and good selectivity are crucial. DNA has been extensively used as a very versatile material for programmed construction of biosensors due to the simplicity and predictability of its secondary structure (Brackmann, 2004; Seeman, 2003; Yuan et al., 2011). Wang's group designed a label-free sensor for the sensitive detection of lead(II), combining high selectivity of a Pb^{2+}-dependent DNAzyme with signal amplification of quantitative polymerase chain reaction (PCR) (Wang et al., 2010). The increase in the amount of product was achieved by repeated thermal cycling in an exponential way. Therefore, the PCR-based thermal cycling methods were time-consuming, sometimes, nonspecific and limited to a thermostable enzyme and a laboratory setting (Zhang et al., 2012a). In contrast, hybridization chain reaction (HCR) driven by the free energy of base pair formation without an enzyme is a straightforward method for constructing one-dimensional DNA assemblies created by the triggered self-assembly of two stable species of hairpins for signal amplification in nucleic acid analysis (Dirks and Pierce, 2004; Huang et al., 2011). Recently, a new electrochemical immunoassay protocol with signal amplification was constructed for the determination of human IgG at an ultralow concentration using DNA-based hybridization chain reaction (Zhang et al., 2012b). The signal was greatly amplified by the HCR-based reaction with hairpin DNA structures in the presence of the initiator strands.

Herein we combine the merits of DNAzymes with the signal amplification properties of DNA-based HCR, and design a novel magneto-controlled electronic switch for the sensitive detection of lead ions by using a lead-specific DNAzyme and two ferrocene-labeled DNA hairpins (Scheme 1). Each DNAzyme consists of a catalytic strand and a substrate strand. To construct such a DNAzyme-based sensor, DNAzyme-based molecular beacons, selective to cleavage in the presence of Pb^{2+}, are initially immobilized onto magnetic beads (MB) by using 3-glycidyloxypropyl trimethoxysilane as the cross-linking reagent, which were used as recognition elements. Upon addition of Pb^{2+} ions, catalytic cleavage of substrate DNA segments in the DNAzymes result in the capture of the initiator strands (S0) *via* the conjugated catalytic strands on the magnetic beads. The captured S0 strands trigger the hybridization chain reaction between two alternating hairpin DNA structures labeled with ferrocene (H1 and H2) to form a nicked double-helix on the magnetic beads (*Note*: The procedure and principle of hybridization chain reaction between H1 and H2 are introduced in detail in our previous paper (Zhang et al., 2012b)). Numerous ferrocene molecules are formed on the neighboring probes, each of which can produce an electrochemical signal within the applied potential. By monitoring the change of the electrochemical signal, the concentration of Pb^{2+} ions can be indirectly determined with high sensitivity. Importantly, in the absence of Pb^{2+} ions, the substrate strands on the DNAzymes cannot be cleaved, and the molecular beacons are switched off. At these conditions, the initiator strands cannot be captured on the MB, thus both hairpin DNA molecules (H1 and H2) are in the closed form, resulting in no HCR.

2. Experimental

2.1. Materials and chemicals

The synthesized oligonucleotides containing the initiator strands (S0), Pb^{2+}-specific DNAzyme (catalytic strands) and H1/H2 probes were obtained from Sangon Biotech. Co., Ltd. (Shanghai, China). The chimeric DNA substrate (substrate strand) was purchased from TaKaRa Biotech. Co., Ltd. (Dalian, China), and purified

by RNase Free HPLC by the company. The sequences of oligonucleotides are listed as follows:

S0: 5'-AGTCT AGGAT TCGGC GTGGG TTAAA CTCAC TATTT C-3'

Catalytic strand: 5'-NH_2-$(CH_2)_6$-TTTCA TCTCT TCTCC GAGCC GGTCG AAATA GTGAG T-3'

Substrate strand: 5'-ACTCACTAT**rA**GGAAGAGATG-3'

H1: 5'-NH_2-$(CH_2)_6$-TTAACCCACGCCGAATCCTAGACTCAAAG-TAGTCTAGGATTCGGCGTG-$(CH_2)_6$-NH_2-3'

H2: 5'-NH_2-$(CH_2)_6$-AGTCTAGGATTCGGCGTGGGTTAACACGCC-GAATCCTAGACTACTTTG-$(CH_2)_6$-NH_2-3'

Ferrocenecarboxylic acid, 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC), and N-hydroxysulfosuccinimide (NHS) were purchased from Alfa Aesar®. 3-Glycidyloxypropyl trimethoxysilane ($C_9H_{20}O_5Si$, GPOS) was obtained from Sigma-Aldrich. Magnetic Fe_3O_4 beads (MB, particle size: ~100 nm) in an aqueous suspension with a concentration of 25 mg mL^{-1} were obtained from Chemicell GmbH (Berlin, Germany). $Pb(OAc)_2 \cdot 3H_2O$ was purchased from Sinopharm Chem. Re. Co., Ltd. (China). All the other chemicals were of analytical grade, and used without further purification. Ultrapure water obtained from a Millipore water purification system ($\geq$ 18 MΩ, Milli-Q, Millipore) was used in all runs.

The stock solution of Pb^{2+} was prepared by dissolving $Pb(OAc)_2 \cdot 3H_2O$ in 10% HOAc; further dilution was performed with 50 mM Tris–acetate (0.5 M NaCl, pH 8.2).

2.2. Synthesis conjugation of DNAzyme-functionalized magnetic beads (DNAzyme-MB)

The catalytic strands were initially conjugated onto the MBs according to our previous report with a little modification (Tang et al., 2008). 60 mg of MB was initially dried at 100 °C for 1 h, and then allowed to react overnight with 5 mL of GPOS (5%, v/v) in dry toluene at room temperature (RT) under continuous stirring with a magnetic stirrer (~50rcf). GPOS-modified MBs were separated by an external magnetic field, and washed thoroughly with toluene and ethanol to remove the physically adsorbed GPOS. The functionalized nanoparticles were then dried under nitrogen atmosphere at 100 °C for 1 h to acquire active epoxy groups on the surface. Afterward, 50 μL of 10 μM catalytic strands was added into 1.0 mL of a suspension of 50 mg mL^{-1} MBs in distilled water. The mixture was incubated for 12 h at RT under gentle stirring. The surplus of catalytic strands was removed by magnetic separation. After the catalytic strand-MB were washed 3 times with 100 mM PBS, pH 7.4, the functionalized MBs were re-suspended in 1.0 mL of 100 mM PBS (1.0 M NaCl, pH 7.4) containing 2.0 μM substrate strands to make the catalytic strands hybridize with substrate strands in a water bath at 65 °C for 10 min. Afterwards, the hybridization process was further carried out at RT for 24 h. The obtained catalytic/substrate strands-functionalized MBs (designated as DNAzyme-MBs) were separated and dispersed in 1.0 mL of 50 mM Tris–acetate (1.0 M NaCl, pH 8.2) buffer for further use.

2.3. Preparation of ferrocene-functionalized H1 and H2 hairpins

Ferrocenecarboxylic acid was conjugated to DNA hairpins (H1 and H2) with the NH_2-moiety using the succinimide coupling (EDC-NHS) method (Wu et al., 2010). Briefly, 1.0 mg of ferrocenecarboxylic acid was initially added into 1.0 mL of 0.3 M Tris–HCl buffer (pH 7.4) containing 0.1 M EDC and 0.1 M NHS, and then the resulting mixture was incubated for 60 min at RT. Following that, 100 μL of H1 or H2 (5 μM) was injected, and incubated for 4 h at RT with gentle stirring. Removal of un-conjugated ferrocenecarboxylic acid was carried out by ultrafiltration for 12–15 times until the peak corresponding to ferrocene in the elutate disappeared.

2.4. Electrochemical measurement

All electrochemical measurements were carried out with a CHI 630D Electrochemical Analyzer (CH Instruments Inc., Shanghai, China). A conventional three-electrode system was used in a QCM detection cell comprising a gold-disk working electrode, a platinum wire auxiliary electrode, and a saturated Ag/AgCl reference electrode. The gold-disk electrode was installed at the bottom of the QCM detection cell, and an external permanent magnet with a pot shape was set under the cell. Scheme 1 represents the assay protocol and electrochemical measurement method. The HCR procedure was carried out as follows: 30 μL aliquot of Pb^{2+} standards with various concentrations was initially added into a 1.5 mL centrifugal tube. Then, a 50 μL aliquot of DNAzyme-MB suspension (10 mg mL^{-1}) was introduced into the tube, and incubated for 60 min at RT on an end-over-end shaker (MS, IKA GmbH, Staufen, Germany). The resulting mixture was separated simply with an external magnet and washed 3 times with 0.3 M Tris–HCl buffer (pH 7.4), to remove abundant Pb^{2+} and the split DNA fragment. Afterward, 30 μL of 1.0 μM initiator strands (S0) was added and incubated with the DNAzyme-MB for 50 min at RT. During this process, the initiator strands were captured by the catalytic strands which were released from the ds-DNA through the catalytic action. The abundant initiator strands were removed by magnetic separation and washed by Tris–HCl buffer for 3 times. Following that, 40 μL of H1+H2 with a concentration of 1.0 μM was added to the tube and incubated for 90 min at RT. After washing, 2 mL of 0.3 M Tris buffer (pH 7.4) with 500 mM NaCl and 1.0 mM $MgCl_2$ was added into the centrifugal tube, and the resultant solution was transferred into the QCM detection cell. With the aid of an external magnet, square wave voltammetry (SWV) from 250 to 600 mV (vs. Ag/AgCl) (amplitude: 25 mV; frequency: 15 Hz; increase *E*: 4 mV) was collected and registered as the sensor signals. All incubations and measurements were conducted at RT (25 ± 1.0 °C). Analyses are always made in triplicate.

3. Results and discussion

3.1. QCM characteristics of the DNAzyme-based Pb^{2+} sensors

Since the S0-triggered HCR between H1 and H2 hairpins was investigated in detail by using TEM, gel electrophoresis and UV–vis absorption spectroscopy in our most recent work (Zhang et al., 2012b), we utilized quartz crystal microbalance (QCM) to investigate the validity of the method herein. A large frequency shift (f=47.3 Hz, vs. time) was observed at the DNAzyme-modified MB (Fig. 1a). When the modified MB reacted with blank sample (i.e. zero Pb^{2+} analyte), the frequency was not nearly changed relative to curve '*a*' (Fig. 1b), indicating that S0, H1 and H2 could not be hybridized onto the DNAzyme-based MB in the absence of Pb^{2+}. The reason might be the fact that DNAzyme-based molecular beacons on MBs were switched off, thus the chain reaction of hybridization events could not be propagated. In the presence of target Pb^{2+}, however, the steady-state frequencies were increased (Fig. 1c and d). Significantly, the frequency shift in the simultaneous presence of H1 and H2 (f=72.1 Hz, Fig. 1d) was greatly larger than that of H1 alone (f=53.9 Hz, Fig. 1c), indicating the progression of HCR reaction. These results revealed that (i) DNAzyme-based molecular beacons on the MB could be regulated and controlled by the added Pb^{2+} ions, (ii) the initiator strands (S0) could be hybridized onto the MB when the molecular beacons were cleaved by Pb^{2+} ions thus resulting in the switch-on, and (iii) the initiator strands could promote the HCR reaction between H1

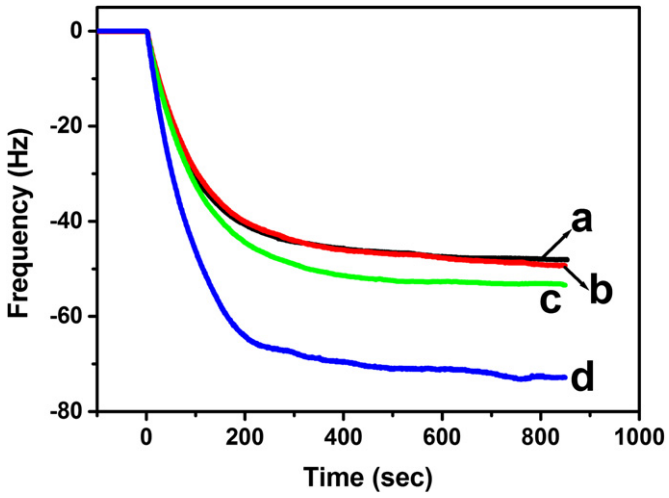

Fig. 1. QCM responses (vs. time) of variously modified probes: (a) the DNAzyme-modified MB, (b) probe '*a*' after successive treatment with 0.2 μM S0+1.0 μM (H1+H2), (c) probe '*a*' after sequential treatment with 10 nM Pb^{2+}+0.2 μM S0, and (d) probe '*a*' after sequential treatment with 10 nM Pb^{2+}+0.2 μM S0+1.0 μM (H1+H2).

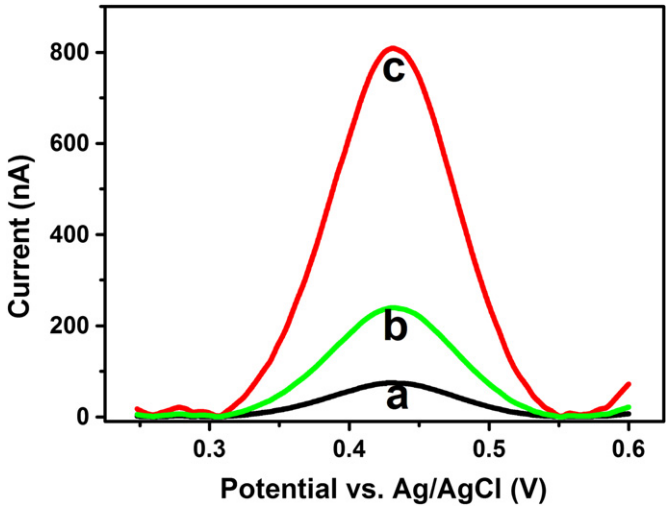

Fig. 2. SWV responses of the DNAzyme-based MB after sequential treatment with various substrates: (a) 0.2 μM S0+1.0 μM (H1+H2), (b) 10 nM Pb^{2+}+0.2 μM S0+1.0 μM H1, and (c) 10 nM Pb^{2+}+0.2 μM S0+1.0 μM (H1+H2), in 0.3 M Tris buffer (pH 7.4) containing 500 mM NaCl and 1.0 mM $MgCl_2$.

and H2. Hence, this strategy could be preliminarily applied for the detection of Pb^{2+} ions.

3.2. Signal amplification of HCR-based Pb^2 sensors

To further monitor the signal amplification efficiency of this method with the aid of DNA-based HCR, the same-batch DNAzyme-based MBs were employed for the detection of 10 nM Pb^{2+} (as an example) under different conditions using the square wave voltammetric (SWV) method. The SWV measurement was carried out in 0.3 M Tris buffer (pH 7.4) containing 500 mM NaCl and 1.0 mM $MgCl_2$. A low background current was obtained when the DNAzyme-modified MBs were reacted with 0.2 μM S0 and 1.0 μM (H1+H2) in the absence of Pb^{2+} (Fig. 2a), which might be derived from the immobilized DNAzyme biomolecules on the magnetic beads. After treatment with 10 nM Pb^{2+} and 0.2 μM S0, the resultant sensing probes (probe '*a*') were incubated again with H1 hairpins alone, the SWV peak current was increased (Fig. 2b, versus curve '*a*'). Inspiringly, the SWV peak current was further increased when H1 and H2 were simultaneously present in the incubation solution (Fig. 2c). This is most likely a consequence of the fact that the long nicked DNA polystrands could be formed in the presence of H1 and H2 with the guidance of the conjugated initiator strands on the MBs. Compared with pure H1 hairpins, the H1/H2-resulted polystrands were largely longer, thus could carry over more ferrocene molecules to participate in the redox reaction. Therefore, the electrochemical signal of the DNAzyme-based Pb^{2+} sensor could be amplified using H1/H2-based HCR strategy.

3.3. Optimization of experimental conditions

In this work, the electrochemical signal mainly derives from the carried ferrocene molecules through the triggered HCR reaction of S0 between H1 and H2. So, the hybridization time of the initiator strands (S0) with the cleaved Pb^{2+}-specific DNAzymes on the MBs should be optimized. During this process, 10 nM Pb^{2+} was used as an example. As shown in Fig. 3a, the SWV peak currents increased with the increasing hybridization time, and tended to level off after 50 min. Hence, 50 min was used for S0 hybridization with the catalytic strands.

Typically, the conjugated amount of H1 and H2 on the MB directly affects the detectable electrochemical signal. As seen from Fig. 3b, the peak current increased with the increasing incubation time. However, the increased ratio was relatively rapid at the initial stage. The reason might be the fact that it takes some time to open each hairpin DNA, thus remanding a long time to achieve the steady-state equilibrium. Considering the practical application, 90 min was chosen as the hybridization time of H1+H2 with S0 in this study.

3.4. Electrochemical response of the developed strategy toward Pb^{2+} ions

Under optimal conditions, the sensitivity and dynamic range of the developed Pb^{2+} sensor were evaluated by using DNAzyme-based MB as the sensing probe and the formed nicked double-helix of H1/H2-based HCR as the reporter. As shown in Fig. 4a, the SWV peak currents increased with the increase of Pb^{2+} concentrations in the samples. A linear dependence between the peak currents and the logarithm of Pb^{2+} concentrations was obtained in the range of 0.1 nM to 75 nM with a detection limit of 37 pM at a signal-to-noise ratio of 3σ (where σ is the standard deviation of a blank sample, $n=11$, i.e. IUPAC Recommended Method, 1978) (Fig. 4b), which was obviously lower than the DNAzyme-based electrochemical methods reported (Xiao et al., 2007; Shen et al., 2008) and polymerase chain reaction-based DNAzyme fluorescence sensor (Wang et al., 2010). Since the cutoff value of Pb^{2+} ions in the drinkable water is 72 nM (defined by the US Environmental Protection Agency) (Yang et al., 2010) (http://www.epa.gov/safewater/contaminants/index.html, 1/30/2010), the developed sensors could completely meet the requirement of water-quality monitoring. For comparison, the assay was carried out by using H1 alone (i.e. the incubation solution is without H2 hairpin probes), and the linear range and LOD were 5.0–120 nM and 2.5 nM Pb^{2+}, respectively. Although the system has not yet been optimized for maximum efficiency, the assay sensitivity of the use of H1+H2 was 125 orders of magnitude lower than that of the use of H1 alone.

3.5. Specificity and reproducibility

The specificity of our designed strategy was evaluated by challenging it against other metal ions including Hg^{2+}, Co^{2+}, Cu^{2+}, Ag^{+}, Ni^{2+}, Zn^{2+}, Fe^{2+}, Cd^{2+} and Mg^{2+}. Significantly, higher current was observed with the target Pb^{2+} than those of other metal ions (Fig. 5).

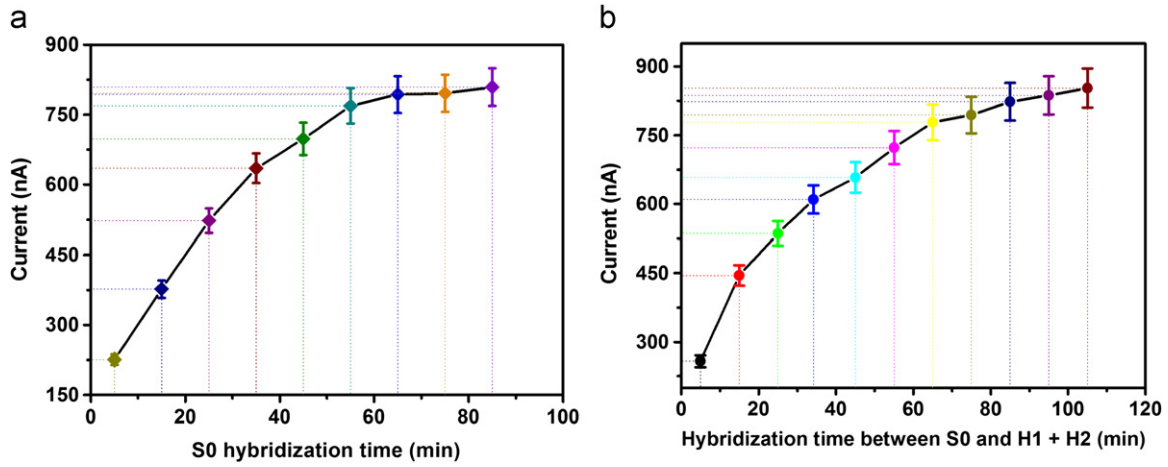

Fig. 3. Effect of (a) S0 hybridization time and (b) (H1+H2) hybridization time with S0 on SWV peak current of the DNAzyme-based sensors.

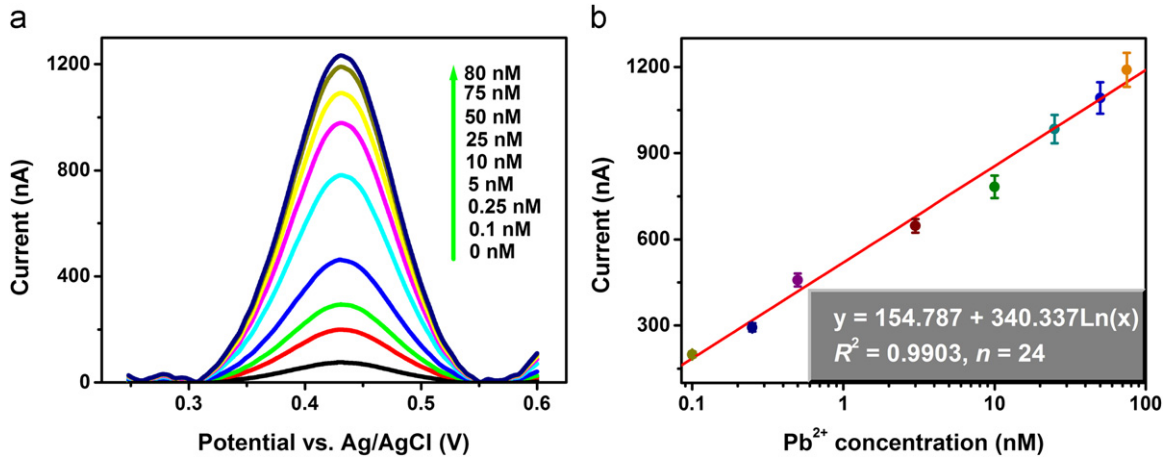

Fig. 4. (a) Typical SWV curves of the developed sensor relative to Pb^{2+} concentrations in 0.3 M Tris buffer (pH 7.4) containing 500 mM NaCl and 1.0 mM $MgCl_2$, and (b) calibration curve from 0.1 nM to 75 nM.

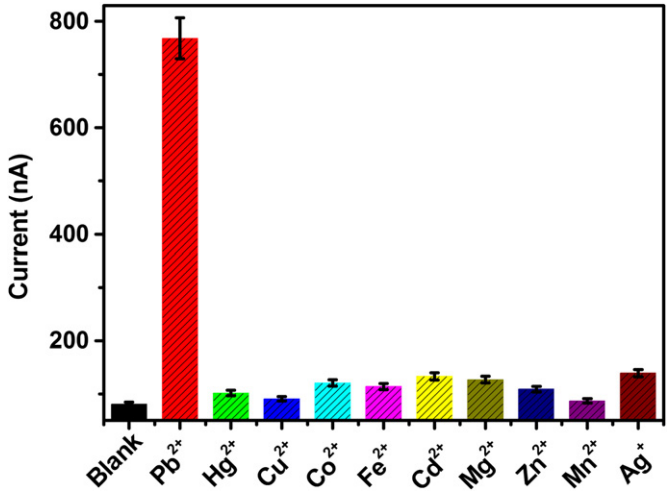

Fig. 5. The specificity of the Pb^{2+} sensor toward different metal ions (*Note*: 10 nM Pb^{2+} or 10 μM interfering ion was used as an example).

The results clearly revealed the high specificity of the developed Pb^{2+} sensors.

To further investigate the reproducibility of the as-prepared Pb^{2+} sensors, we repeatedly assayed the Pb^{2+} ions with 3 different levels, using identical batch DNAzyme-based MBs. Experimental results revealed that the coefficients of variation (CVs) of the intra-assay between 6 runs were 5.3%, 7.7% and 6.8% for 0.5 nM, 2.5 nM, and 50 nM Pb^{2+}, respectively, whereas the CVs of the inter-assay with various batches were 9.9%, 8.5% and 9.6% toward the above-mentioned analyte, respectively. The low CVs indicated that the developed strategy could be regenerated and used repeatedly, and further indicated the possibility of high-quality batch-to-batch preparation.

4. Conclusions

In summary, we have successfully developed a DNAzyme-based magneto-controlled electronic switch for highly sensitive and selective detection of Pb^{2+} coupling the DNA-based hybridization chain reaction with the Pb^{2+}-specific DNAzyme. The Pb^{2+}-specific DNAzyme as an electronic switch exhibits excellent specificity. The signal is amplified by the labeled ferrocene on two hairpin probes based on the HCR reaction protocol. Meanwhile, the methodology can pull DNA molecules bound to magnetic beads from one laminar flow path to another by applying a local magnetic field gradient, and selectively remove them from flowing biological fluids without any washing steps. Importantly, by controlling the target probes with various metal ions-specific DNAzymes (e.g. Cu^{2+}, Mg^{2+} and Hg^{2+}), the assay

can be easily extended for use with other metal ions and thus represents a versatile detection method.

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