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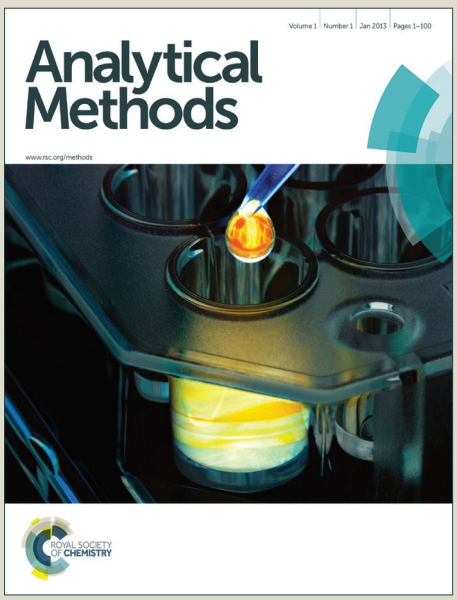

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PAPER

Designing a Biostable L-DNAzyme for Lead (II) Ion Detection in Practical Samples

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A promising biosensor for effectively lead (II) ion detection in practical applications was developed by constructing a Pb^{2+}-specific L-DNAzyme, the enantiomer of the natural nucleic acid-constructed D-DNAzyme. This fluorescent sensor contains the L-enzyme strand with a quencher at the 3' end, and the L-substrate strand with a fluorophore at the 5' and a quencher at the 3' ends that formed a complex. In the presence of Pb^{2+}, the L-substrate is cut into two fragments, leading to the recovery of fluorescence. The sensor shows high sensitivity and selectivity for Pb^{2+} detection with a linear response in the range of 5-100 nM and a detection limit of 3 nM in aqueous solution. Importantly, based on that L-DNAzyme consists of non-natural nucleic acids, which is insensitive to nuclease digestion, protein adsorption and D-DNA hybridization, our sensor shows specific response to Pb^{2+} in practical water and serum samples. Therefore, it is expected that our L-DNAzyme-based strategy may offer a new method for developing simple, rapid and sensitive sensors in complex systems.

1 Introduction

Certain heavy metal ions (e.g., manganese, copper, zinc, and iron) can have significant impact on living systems. Although small amount are necessary to maintain a healthy life, excess heavy metal ions in ecological systems, however, are difficult to be removed due to their non-biodegradability, which have a negative effect on health.[1-3] In particular, lead (II), a considerable contaminant, was reported to have an adverse impact on central nervous system, because such ions can change the molecular structure of proteins by binding with sulfhydryl groups and competing with calcium.[4] Besides, lead ions are also harmful to kidney and blood, causing related diseases (e.g., renal cancer and anemia).[4, 5] Conventional analytical methods, including inductively coupled plasma-mass spectrometry (ICP-MS), atomic absorption spectrometry (AAS), inductively coupled plasma-atomic emission spectrometry (ICP-AES) and anodic stripping voltammetry (ASV),[6-9] make it possible to achieve satisfied sensitivity and accuracy for lead ion detection, but expensive instruments, complicated pretreatment programs and trained personnel are highly required. As a result, simple and facile methods for Pb^{2+} detection need to be developed.

DNAzyme is a kind of functional nucleic acid molecules with catalytic capability in a series of biological and chemical reactions, such as nucleic acid substrates cleavage,[10, 11] porphyrin metalation[12] and ligation.[13] Most RNA-cleaving DNAzymes require specific metal ions as cofactors and can be used to generate DNAzyme-based sensors for metal ions detection with high specificity.[11, 14-19] These methods can be easily designed with high sensitivity (fluorescent sensors[20, 21]) as well as no need for expensive instruments (electrochemical sensors[22]). Some of them can even be read by naked eyes (visual measuring system based on gold nanoparticles[23, 24]). In spite of these advantages of DNAzyme-based sensors for Pb^{2+} detection, however, when used in complex practical samples (e.g., biological samples), enzymatic degradation poses a threat to the structural integrality of natural nucleic acid-constructed D-DNAzymes.[25] Meanwhile, regular DNAs (D-DNA) may also bind with some proteins or nucleic acids in cells or biological fluids.[26-31] To circumvent these problems, a stable DNAzyme for metal ion detection in biological and environmental samples is in urgent need, and the improvement of DNA molecules modification could be an immediate and simple method.

L-DNA, an artificial nucleic acid, stems from D-DNA via mirror design (Fig. 1A). L-DNA displays same thermodynamic properties with D-DNA and can bind to its complementary L-strand.[32, 33] but it is incapable of hybridizing with D-DNA. Therefore, L-DNA functions well in complex environment. Meanwhile, because of the stereoselectivity of degradative enzymes, the mirror form allows L-DNA to be insusceptible to nuclease degradation.[27, 34-37] Our previous study proved that L-DNAzyme possessed catalytic capability and high selectivity for metal ions, which is similar to D-DNAzyme.[38] More importantly, compared with D-DNAzyme, L-DNAzyme displayed excellent biostability with nucleases or nonspecific protein.

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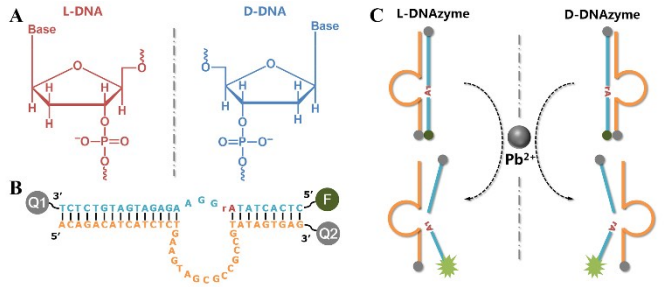

Fig. 1 (A) Mirror-image structure of L- and D-DNA. (B) Design of lead (II) ions sensor based on GR-5 L-DNAzyme. L-S (green) was modified with FAM (fluorophore) at 5' end and Dabcyl quencher at 3' end. L-E (yellow) with a modification of Dabcyl quencher at 3' end. The sensor was designed with all L-DNA and an L-adenosine ribonucleotide (L-rA) in the middle. (C) Schematics of the L-DNAzyme-based sensing mechanism.

Based on aforementioned discussions, we present an L-DNAzyme-based biosensor for the measurement of lead (II) ions in complex environments, e.g., river and serum. We proved that Pb^{2+}-specific L-enzyme could also catalyze the cleavage of the related L-substrate in the presence of Pb^{2+}. Meanwhile, L-enzymes were quite stable with DNase I and L-substrates stay at room temperature for over a month without much degradation. The advantages of L-DNAzyme fundamentally address some issues of D-DNAzyme based sensors for metal ions detection in biological samples, which may offer a suite of promising biosensors for various target measurements.

2 Experimental

2.1 Materials and Reagents

Both the L- and D-DNAzyme were synthesized on a PolyGen Column 12 DNA synthesizer, and the reagents for DNA synthesis were purchased from Chem Gene (Wilmington, MA, USA) or Glen Research (Sterling, VA, USA). The substrate of Pb^{2+}-dependent DNAzyme and DNase I were purchased from Takara Biotechnology Co., LTD. (Dalian, China). Lead (II) acetate and all other reagents were of analytical reagent grade, purchased from Sigma-Aldrich Chemical Co., and used without further purification. Milli-Q water (resistance >18 MΩ•cm) from a Millipore system was used throughout the experiments. Fetal bovine serum (FBS) was purchased from Invitrogen Ltd. (America).

2.2 DNA synthesis

Both the L- and D-DNAzyme were synthesized with the same procedure. The synthesis approach was set up on the basis of the requirements illustrated by the reagents' manufacturers. To synthesize Pd^{2+}-specific L-enzyme, an additional Dabcyl CPG was used. The complete DNA sequences were then deprotected in concentrated ammonia overnight at 65 ℃ and purified by high-pressure liquid chromatography (An Agilent 1200 HPLC with a Promosil C18 reversed-phase column). For Pb^{2+}-specific substrate which contains adenosine triphosphate, another 2'-TOM group was removed by incubating in 100 µL of DMSO plus 125 µL of triethylamine trihydrofluoride for 2.5 h at 65 °C after purification. Then, 1.5 mL of N-butyl alcohol and 25 µL of 3 M sodium acetate were added and the mixture was placed at -20 ℃ for 30 min to precipitate DNA. The DNA concentration was measured with a NanoDrop spectrometer (Thermo Scientific, Waltham, MA).

2.3 Fluorescence Measurements

Fluorescence measurements were recorded on a Fluoromax-2 (HORIBA Jobin Yvon Inc., Edison, NJ) fluorometer. In the time-scan experiment, excitation and emission wavelengths were set at 488 and 520 nm, with constant bandwidth. The emission spectra were acquired by exciting the samples at 488 nm and scanning the emission from 500 to 560 nm in steps of 1 nm. The water samples, simply filtered and showed that no Pb^{2+} was present, were collected from Xiang River (Changsha, China). A river water solutions was prepared by adding 50 mM HEPES, 50 mM NaCl, 50 mM KCl, and 5 mM $MgCl_2$ into Xiang River samples and the pH value was 7.2. Pb^{2+}-dependent L-DNAzyme experiments were tested in 100 µL Xiang River solutions containing 50 nM GR-5 L-substrate (L-S), 60 nM GR-5 L-enzyme (L-E) and different concentrations of Pb^{2+} (for sensitivity determination) or different metal ions (for selectivity determination), and the mixture was equilibrated at room temperature for 30 min before detecting. The Pb^{2+} titration experiment in 5% FBS solution (without acidolysis experiment) was carried out in the same buffer solution but with the pH was approximate 6.55 to maintain the form of lead ions in the FBS solution. (GR-5 D-enzyme was written instead of D-E and GR-5 D-substrate was written as D-S.)

2.4 Gel Electrophoresis

For Pb^{2+}-specific L-DNAzyme catalytic activity analysis, four tubes of reaction buffer mixture solution (50 mM HEPES, 50 mM NaCl, 50 mM KCl, and 5 mM $MgCl_2$ at pH 7.2, 10 µL) containing 1 µM D-S/L-S and 1.2 µM D-E/L-E were incubated with or without Pb^{2+} at room temperature. After 60 min, the cleavage reaction was quenched by introducing 10 µL of stop buffer (200 mM EDTA and 8 M urea) to the mixed solution. Afterwards, the reaction mixture were separated by 20% denaturing PAGE and analyzed by a fluorescence image scanner (FLA-3000G; Fuji, Tokyo, Japan). To monitor DNase I digestion of DNAzyme, four tubes of 10 µM L-E/D-E in solution with or without DNase I were prepared and incubated for 1 h at 37 ℃. A 15% denaturing PAGE was prepared using 1 × TBE buffer (pH 8.3). The gel was run at 120 V for about 1 h, stained for 15 min with Stains All solution (200 mg Stains All, 500 mL formamide, 100 mL 10 × TBE and 400 mL H_2O), and finally photographed with a camera.

3 Results and discussion

3.1 The principle of Pb^{2+} sensing system

In our approach, GR-5 DNAzyme was chosen to construct a sensitive and selective sensor.[39] The sequences of GR-5 L-E and corresponding L-S with a single ribo-adenosine (rA) were shown in Fig. 1B. Both 3' end of L-E (in yellow) and L-S (in green) were modified with quenchers Dabcyl (Q2, Q1) while a FAM fluorophore (F) was attached to the 5' end of L-S. This dual-quenching strategy was introduced to our detection system to achieve lower background signal. In absence of Pb^{2+}, L-E hybridized with L-S, which induced a close proximity of F to Q2, resulting in the quenched fluorescence. However, in the presence of Pb^{2+}, it triggered the cleavage reaction of L-DNAzyme-based system. As shown in Fig. 1C, L-S was cleaved into two small strands and unable to hybridize with L-E, leading the separation among F, Q1 and Q2. Consequently, the fluorescent signal could be restored.

3.2 The property of L- and D-DNAzyme

We first investigated the circular dichroism (CD) spectrum property of both L- and D-DNAzyme. In Fig. 2A, the CD spectrum of L-E showed a positive peak (around 248 nm) and a negative peak (around 275 nm), which were inverted compared to D-E, illustrating that L-E formed left-handed helical configuration. The melting temperature data (Fig. 2B) proved that L-E and D-E had similar thermodynamic property, at approximately 57 ℃. These results indicated L-DNAzyme was displayed as the mirror form of D-DNAzyme.

3.3 The catalytic activity of L- and D-DNAzyme

We further demonstrated the catalytic activity of L-DNAzyme in buffer solution. Denaturing polyacrylamide gel electrophoresis (PAGE) confirmed L-DNAzyme was capable of catalyzing cleavage reactions as similar as D-DNAzyme. As shown in Fig. 3A, in the absence of Pb^{2+}, enzyme strand and substrate strand remained hybridization form (lane 1 and 3). Whereas, the brightness of upper bands of lane 2 and 4

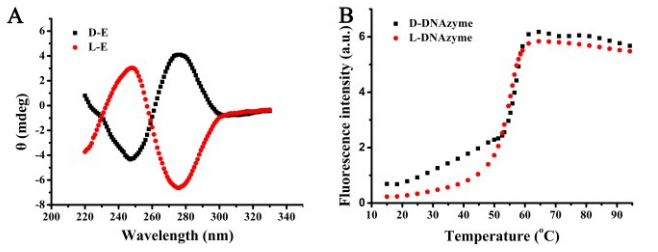

Fig. 2 (A) Circular dichroism spectra of L-E and D-E. A positive peak (around 275 nm) and a negative peak (around 248 nm) for D-E, while an inverted image for L-E. (B) Two enantiomers possessed similar DNA melting temperatures.

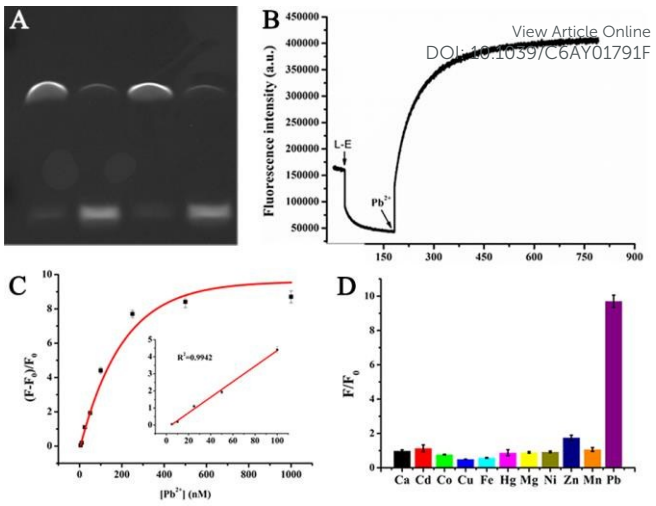

Fig. 3 (A) Denatured PAGE of the L-/D-DNAzyme with and without Pb^{2+}. D-DNAzyme without (Lane 1) or with (Lane 2) Pb^{2+}; L-DNAzyme without (Lane 3) or with (Lane 4) Pb^{2+}. (B) Time course investigation of Pb^{2+}-dependent L-DNAzyme-based sensing system in the presence of 5 μM Pb^{2+}. Response of Pb^{2+}-dependent L-DNAzyme-based sensor to various concentrations (0, 5, 10, 25, 50, 100, 250, 500 and 1000 nM) of Pb^{2+} in buffer (C) and control metal ions (D).

decreased, suggesting that both L-S and D-S was cleaved resulting from the present Pb^{2+}. Additionally, we conducted a real-time fluorescence experiment to further verify the catalytic capability of L-DNAzyme (Fig. 3B). The initial fluorescence value was obtained from 50 nM L-S in solution. While a remarkable decrease of fluorescence intensity (FI) was observed after addition of 60 nM L-E into the solution. Finally, an 11-fold increase of FI was achieved within 10 minutes after introducing 5 μM Pb^{2+} into the mixture. All these results demonstrated that L-DNAzyme can successfully facilitate the catalytic cleavage reaction via the same cofactor as D-DNAzyme.

To test the performance of the sensor, various Pb^{2+} concentrations within the range from 5 nM to 1 μM were detected in HEPES buffer (Fig. 3C), showing a linear response in the range of 5-100 nM, and a detection limit of 3 nM for $Pb^{2+}$was estimated based on the 3δ/slope rule. Meanwhile, a significantly high selectivity of this sensor was observed by recording the fluorescence responses against other divalent metal ions, including Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Ni^{2+}, Zn^{2+} and Mn^{2+}. As demonstrated in Fig. 3D, 1 μM of Pb^{2+} could induce a remarkably increasing fluorescence intensity in the sensing system. In contrast, negligible fluorescence enhancement was observed for all the other ions with 10-fold higher concentrations. These results illustrated the L-DNAzyme-based sensor displayed the similar performance with D-DNAzyme-based sensor in buffer (Fig. S1).

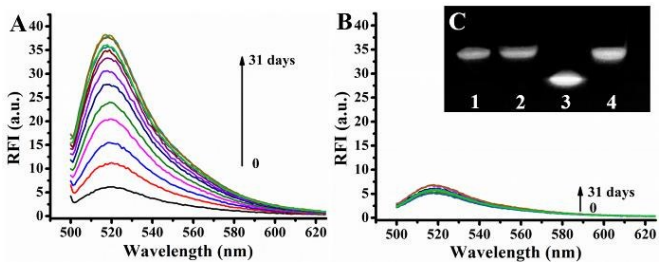

Fig. 4 Fluorescence spectra of D-S (A) and L-S (B) incubated in regular reaction buffer (50 mM HEPES, 50 mM NaCl and 5 mM $MgCl_2$ at pH 7.2) from 0 to 31 days. (C) Denatured PAGE of the Pb^{2+}-dependent L-/D-DNAzyme in the absence or presence of DNase I. L-E with (Lane 1) or without (Lane 2) DNase I; D-E with (Lane 3) or without (Lane 4) DNase I. RFI: relative fluorescence intensity.

3.4 The biostability of L-substrate and L-DNAzyme

Since "rA" was introduced in the middle of DNA substrate as a cleavage site, a formidable barrier was its long-term storage as a result of the ubiquitous nature of RNase.[40, 41] As shown in Fig. 4A, the fluorescence intensity of D-S gradually increased day-by-day over one-month period under regular reaction buffer and room temperature, indicating it was unstable and easy to be hydrolyzed. In contrast, almost no detectable change was observed in the FI of the L-S sample, even after incubation for one month (Fig. 4B). Next, to investigate the nuclease resistance of L-DNAzyme, DNase I was added into L-E and D-E respectively, followed by evaluation of the L-DNAzyme stability using gel electrophoresis. The band brightness of both L-E and D-E was measured in the presence (lane 1, 3) or absence (lane2, 4) of DNase I (Fig. 4C). L-E remained intact (lane 1, 2) with or without DNase I, whereas, D-E was cleaved completely with addition of DNase I (lane 3). All these results illustrated L- DNAzyme possessed preeminent biostability and inherent performance, which enable L-DNAzyme function for a long-term period under regular circumstances.

3.5 Real samples analysis

With the excellent biostability of L-DNAzyme, the sensing system consisted of L-E and L-S for Pb^{2+} analysis was tested in real water samples. The water samples, simply filtered and containing no Pb^{2+}, were collected from Xiang River (Changsha, China). To evaluate the performance of the sensor, a variety of Pb^{2+} concentrations were detected in the Xiang River mentioned above (Fig. 5A). The calibration curve for the sensor displayed a dynamic range from 5 nM to 1000 nM, and a good linear positive correlation ranging from 5 to 100 nM for Pb^{2+}. According to the 3δ/slope rule, the limit of detection was estimated to be 2 nM. Furthermore, the L-DNAzyme also exhibited excellent selectivity as well as the result of proposed sensor working in buffer. In contrast to Pb^{2+}, no obvious fluorescence change was observed in the respective presence of other metal ions with 10- fold higher concentrations (Fig. 5B). Therefore, L-E and L-S were able to well function in Xiang River samples, suggesting its potency for Pb^{2+} detection inside other complex samples. Compared to the results of D-DNAzyme-based sensor in Fig. S2, the L-DNAzyme-based sensor displayed similar performance when using Xiang River samples.

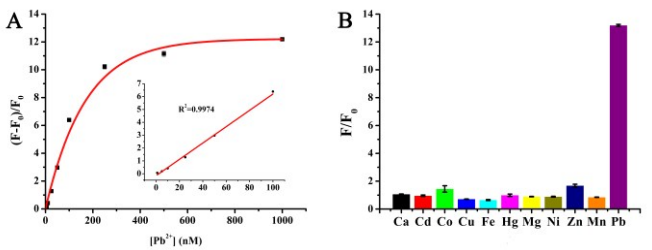

Fig. 5 Response of Pb^{2+}-dependent L-DNAzyme-based sensor to various concentrations (0, 1, 5, 10, 25, 50, 100, 250, 500 and 1000 nM) of Pb^{2+} in real water samples (A) and with control metal ions (B).

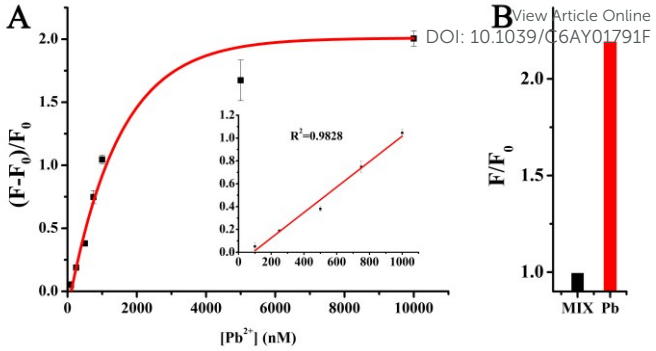

Fig. 6 Response of Pb^{2+}-dependent L-DNAzyme-based sensor to various concentrations (0, 100, 250, 500, 750, 1000, 5000 and 10000 nM) of Pb^{2+} in 5% FBS (A) and mixed control metal ions (B). The sensing system was on exposure to 1 μM Pb^{2+} (red) and the mixture containing 10 μM Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Ni^{2+}, Zn^{2+} and Mn^{2+} (black).

To further verify the performance of our sensor in practical samples, we detected Pb^{2+} in 5 % FBS which was tuned to weakly acidic (pH 6.55) to maintain free lead (II) ions in FBS. The FI of sensing system increased with elevated Pb^{2+} concentration (100 nM~10 μM) with a linear relationship ranged from 100 nM to 1 μM, as shown in Fig. 6A. It's worth to note the detection sensitivity of this sensor in biological fluids was slightly decreased in contrast to that in pure buffer solution, owing to the lower catalytic capacity of DNAzyme in the weakly acidic FBS solution that was studied by Lu's group[39]. Meanwhile, this sensor had a bit decrease resulting from the biological matrix and a high fluorescence background. In addition, the selectivity was another important issue for a sensing system. A mixture containing Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Ni^{2+}, Zn^{2+} and Mn^{2+} was tested by the proposed sensor, showing no observed fluorescence enhancement in contrast to the increasing FI with 1μM Pb^{2+}

(Fig. 6B). Compared with D-DNAzyme (Fig. S3), the sensor based on L-DNAzyme showed better sensitivity and selectivity. In FBS solution, D-DNAzymes lost their functions because of the nonspecific adsorption to proteins and nuclease degradation, while L-DNAzymes avoided these drawbacks and worked well. Thus, all assays above testified that the utilization of L-DNAzyme in a metal ion sensor possessed tremendous potentials for practical applications.

4 Conclusion

In summary, we have constructed a nuclease resistant sensor based on L-DNAzyme for lead (II) ion detection and confirmed its function in complex biological fluids. The sensing system was designed based on the concept of enantiomers properties. Due to the high selectivity of D-DNAzyme to Pb^{2+}, L-DNAzyme showed its excellent recognition capability and similar coordinative catalytic ability in contrast to D-DNAzyme. Furthermore, L-DNAzyme and its substrate had more merits: (1) resistance to degradation; (2) non-binding to D-nucleic acids or natural protein. Benefit from these characteristics, our sensor was employed for Pb^{2+} analysis in practical water samples and serum solutions. So far various DNAzymes have been selected to recognize different targets, which provide the potential of new L-DNAzyme-based sensing systems for sensitive and selective detection of numerous targets and extensive applications in the biomedical and environmental areas.

Acknowledgements

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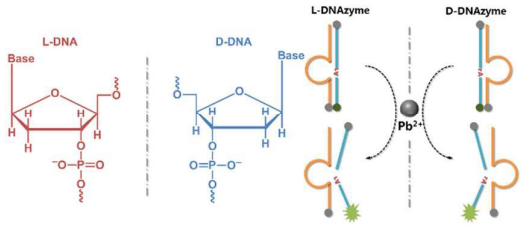

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