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Selection and characterization of DNA aptamers for the development of light-up biosensor to detect Cd(II)

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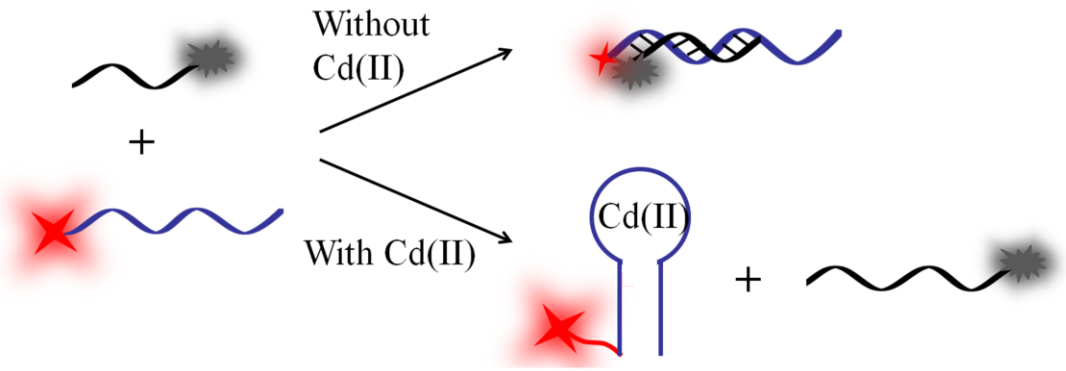
Abstract

In order to develop a facile, cost-effective and quick-testing light-up biosensor with excellent specificity for cadmium ions (Cd(II)) detection, a modified selection method based on target-induced release of strands was used to isolate aptamers of Cd (II) with high specificity. Circular Dichroism (CD) data confirmed that one of the selected aptamers underwent a distinct conformational change on addition of Cd (II). A biosensor for Cd(II) was developed based on the Cd(II)-induced release of fluorescence-labeled aptamer from complex with a quencher-labeled short complementary sequence. The sensing platform displayed a Cd(II) concentration-dependent increase of fluorescence intensity in the low micromolar range and had an excellent selectivity in the presence of various interfering metal ions. Such biosensor could potentially be used for the detection of Cd(II) in environmental samples.

Keywords: Aptamer; SELEX; Biosensor; Cadmium

Introduction

Cadmium is an extremely toxic heavy metal that could cause health issues and environment problems even in the presence of trace levels[1, 2]. As one of the transition metals, cadmium is usually used in industrial workplaces and has a long metabolic half-life[3, 4]. The US Environmental Protection Agency (EPA) recommended 5.0 μg/L standard for Cd(II) in drinking water. Environmental exposure to cadmium has caused several pollution incidents due to its high toxicity and bioaccumulation process along the food chain. Accumulation of Cd(II) may cause nephrotoxicity, flu-like symptoms, renal tu



bular dysfunction, bone demineralization and cancers[5-9]. Cadmium has been classified as a carcinogenic substance by the International Agency for Research on Cancer (IARC)[10]. For the above reasons, the monitoring and removing Cd(II) is of great importance[11-13]. Several analytical methods have been reported to detect heavy metal ions such as inductively coupled plasma mass spectrometry[14, 15],

inductively coupled plasma atomic emission spectrometry[16], atomic absorption spectrometry[17], atomic fluorescence spectrometry[18] and electrochemical methods[19]. Although these methods offer good precision and resolution, complicated sample pre-treatment steps and specialized instruments are required. Therefore, efforts are ongoing to develop simple, cost-effective and quick-testing methods to detect trace Cd(II) in the environment.

Fluorescent sensors have been widely used for metal ions detection due to their simplicity and sensitivity. Fluorescent chemosensors for Zn(II) and Cd(II) have been reported[20-23]. However, due to the lack of appropriate sensing elements, the development of high selective sensors for metal ions remains a challenge. DNAzyme and RNAzyme-catalyzed sensing platforms were then selected and developed to selectively detect ions like Cd(II), lanthanide ions, Zn(II), UO_2 (II), Hg(II), Pb(II), Cu(II) [24-30] etc. Their wide applications were impeded due to the easy degradation and high cost of RNA substrates. Recently, a “signal-on” sensing strategy was designed to detect Pb(II) based on Pb(II)-induced conformational conversion of G-rich sequence[31]. Upon addition of Pb(II), the structure of G-rich sequence was converted into G-quadruplex along with the enhanced fluorescent signal of NMM. However, the G-quadruplex structure is easy to be influenced by some metal ions, molecules and proteins, which prevented its wide applications[32]. Besides, most of the reported sensors required relatively specialized designing and long analysis time. It would be extremely useful if a versatile sensor could be developed in the presence of interfering ions.

Aptamers that have unique secondary structures, high affinity and selectivity to targets are being attractive candidates for designing biosensors. Aptamers are single-stranded oligonucleotides that are selected by Systematic Evolution of Ligands by Exponential Enrichment (SELEX)[33, 34]. Various aptamers for different targets have been isolated by SELEX including proteins, small molecules and cells, and their bioanalysis applications were extensively explored[35-37]. RNA and DNA aptamers for a few metal ions with high specificity and affinity have been selected, which could be used for the detection of metal ions[38-42]. However, RNA aptamers are not favorable for the construction of biosensors due to degradation and high cost, and the developed DNA aptamers had long sequences, which impeded the design of sensors. So the shorter DNA aptamers for heavy metal ions are waiting to be explored to develop the versatile sensing platforms.

In this paper, we designed a modified in vitro selection method based on the interaction between biotin and streptavidin[43, 44]. The principle of the method is the target-triggered release of the bound DNA sequences from ssDNA library-capture oligonucleotide complex on streptavidin column (scheme 1). The aptamers of metal ions could be selected efficiently by this method since the metal ions are in the free state in solution. This method enables the immobilization of the DNA library instead of the target molecules which are generally immobilized in common aptamer selection process. It is benificial to the target molecules which are not suitable for immboilization on solid surfaces, like metal ions. This method also allows us to apply aptamers in biosensors. So the selected aptamers could be further shortened and used

as probes to develop biosensors directly. A 76-mer DNA library including 30 random bases was designed for the selection process to isolate DNA aptamers of Cd(II). The result shows that one shorten aptamer (Cd-2-2) folds into specific structure to bind Cd(II) with high selectivity. By employing Cd-2-2, we engineered a simple, cost-effective and quick-testing light-up biosensor with excellent specificity for cadmium.

2.Experimental

2.1 Materials

Streptavidin-agarose beads and molecular probes SYBR® Green I were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Agarose was purchased from Biowest (Nuaillé, France). BL21 Escherichia coli was purchased from American Type Culture Collection (Manassas, VA, USA). 20bp DNA marker, 6×loading buffer, pMDTM18-T vector cloning kit, 10×PCR buffer, deoxynucleotide triphosphates (dNTPs), rTaq polymerase were purchased from TaKaRa Bio Inc. (Kusatsu, Japan). The "random" DNA library, biotinylated oligonucletide primers, capture oligonucleotide, quenching strand were synthesized by Sangon Biotech Company (Shanghai, China). The DNA samples were prepared in ultrapure water, and the DNA concentrations were accurately quantified according to UV absorbance at 260 nm. $Cd(NO_3)_2 \cdot 4H_2O$ and other chemicals used in this experiment were from Sinopharm Chemical Reagent Company (Shanghai, China). Milli-Q water was used to prepare solutions.

2.2 Selection protocol

A single-stranded DNA (ssDNA) pool (5'-GGAGGCTCTCGGGACGAC-$(N)_{30}$-GTCGTCCCGATGCTGCAATCGTAAGAAT-3') containing 30 randomized nucleotides was designed to have 5' terminal complemented to the capture oligonucleotide (5'-GTCGTCCCGAGAGCCATA-biotin-3'). The biotin-capture oligonucleotides could bind to the streptavidin-agarose beads as a library sample. The library sample (~1 nmol for the initial round of selection, ~200 pmol for the subsequent rounds) was subjected to multiple rounds of selection, amplification and purification.

The column filled with 200 μL streptavidin-agarose beads was pre-equilibrated by 600 μL selection buffer, 20 mM Tris-HAc (pH = 7.4, 140 mM NaCl, 5 mM KCl, 10 mM $MgCl_2$). For a given round of selection, ssDNA pool and 3'-biotin-labeled capture oligonuecleotide were denatured in elution buffer at 95°C for 5 min and then cooled down slowly to room temperature. The hybridized DNA sample was subsequently incubated and bound with streptavidin-agarose beads for 5 min at room temperature to bind onto the beads based on the biotin-streptavidin interaction. Unbounded DNA was washed away with 3 column volumes of selection buffer. Then the ssDNA library-capture oligonucleotide complex bound column was subjected to a positive selection process. The column was then washed 3 times with 200 uL selection buffer to remove the poor bound and unbound sequences. Then the column was treated with Cd(II) solution, the wash solution was collected for 3 times to obtain the eluted nucleic acid sequences that are supposed to have good binding with Cd(II). The column was washed 2 times with 200 uL selection buffer to remove the remaining

sequences. All the collected fractions were amplified and checked by electrophoresis to verify the efficiency of selection. The released DNA sequences eluted by the solution with $Cd(NO_3)_2 \cdot 4H_2O$ dissolved in selection buffer were concentrated by Microcon YM-10 column and amplified by Polymerase Chain Reaction (PCR). PCR products were first incubated with streptavidin agarose resin in selection buffer for 5 min with periodic shaking. The column was then washed 3 times with the selection buffer to remove the PCR reagents while the amplified double-stranded DNA (dsDNA) was retained on the column through the interaction between streptavidin and the biotinylated DNA. The desired DNA strands were subsequently eluted from the column by incubating with 0.15 M NaOH at room temperature and neutralized and concentrated by ethanol precipitation to generate a new more enriched DNA pool for the target. The new enriched DNA pool was used for the next round of selection.

2.3 PCR amplification and verification

In each selection round, the collected fractions were PCR-amplified and checked by electrophoresis to verify the efficiency of selection before concentrating. The PCR reaction master consists of 200 μM dNTPs, 50 μM forward primer (5'-GGAGGCTC TCGGGACGAC-3'), 50 μM biotinylated reverse primer (5'-biotin-ATTCTTACGAT TGCAGCATCGGGAC-3'), 0.025 unit/μL rTaq polymerase and 7.5 mM $MgCl_2$ in 10 × ThermoPol PCR buffer (20 mM Tris-HCl, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH = 8.8). About twelve cycles of denaturation (92 °C, 15 s), annealing (58 °C, 30 s), and extension (72 °C, 20 s) were performed, followed by a final 5 min extension period at 72 °C. The high magnesium concentration helps

to improve the PCR efficiency. The length and the yield of the PCR products were verified and estimated using electrophoresis on a 3% agarose gel stained with SYBR Green I and imaged using LAS4000 mini (FujiFilm, Tokyo, Japan). Due to the interaction of some sequences in the enriched pool to Cd(II), the fractions eluted by Cd(II) solutions have brighter electrophoresis band than fractions eluted by selection buffer (Fig. S1).

2.4 Cloning and sequencing

DNA sequences were cloned after fourteen rounds of selection using a PMD18-T vector cloning kit from TaKaRa (Kusatsu, Japan). The selected pool was PCR-amplified under the same condition described previously. The PCR products were ligated with PMD18-T vectors and transformed into BL21. Individual colonies were randomly picked and cultured in liquid LB broth after growing 16 h on an LB plate at 37 °C constant temperature incubator. Plasmid vectors containing selected sequences were extracted from the cells after overnight growth in LB broth at bed temperature incubator (37 °C, 200 rpm) using the plasmid miniprep kit from Thermo Fisher Scientific Inc. (Waltham, MA, USA). The vectors were sequenced by GENEWIZ, Inc. (Su Zhou, China) using the M13F(-47) promoter primer. 30 clones were sequenced, and the sequence alignments were carried out. The secondary structure analysis was performed using mfold.

2.5 **Fluorescence measurements**

Fluorescence spectra were obtained at room temperature by F-4600 fluorescence spectrometer (Hitachi, Tokyo, Japan). The selected aptamers labeled by 6-carboxyl

fluorescein (FAM) at its 5' terminal was mixed with its partially complemented quenching strand 5'-CCGTTGTCCTGAG-3' labeled by Dabcyl (4-[4-(dimethylamino) phenylazo]benzoic acid at its 3' terminal in the presence of different concentrations of Cd(II) solution. Excitation wavelength was set at 492 nm, and the emission spectra were collected in the range of 500~580 nm. For quenching efficiency measurement, the change of fluorescence ($I = F/F_0$) was determined by comparing the fluorescence intensity of different solutions at 518 nm. F_0 represents the fluorescence of 50 nM aptamer Cd-2-2, and F represents the fluorescence intensity of aptamer Cd-2-2-quenching oligonucleotide complex. In Cd(II) concentration-dependent assay and specificity measurements with various interfering metal ions, the quenching strand was present in a 7:1 (350 nM:50 nM) ratio to Cd-2-2. The fluorescence change ($I = F/F_0$) was determined by comparing the intensity of the fluorescence emission of different solutions. F_0 is the fluorescence intensity of the aptamer-quenching oligonucleotide complex in the absence of metal ions, and F is the fluorescence intensity of the aptamer-quenching oligonucleotide complex in the presence of different concentrations of Cd(II) or 10 μM different interfering metal ions.

2.6 Circular Dichroism

Circular dichroism (CD) spectra were collected by a Chirascan-plus circular dichroism spectrometer (Applied Photophysics Ltd, Surrey, UK). The CD spectrum was measured in the wavelength range of 200 ~ 340 nm at 1 nm intervals and a speed of 100 nm/min. Cuvette of 10 mm path length are used. CD spectra were recorded using various combinations of DNA and $Cd(NO_3)_2 \cdot 4H_2O$ in the selection buffer.

3. Results and Discussion

3.1 Selection of Aptamers for Cadmium

We developed a selection mechanism based on target-induced release of ssDNA to select aptamers of Cd(II). Scheme 1 illustrates the selection principle. In short, a random DNA pool containing 30 random bases was annealed with its partially complemented biotin-labeled capture oligonucleotide and was then fixed on streptavidin-coated beads-filled column. Upon addition of a low concentration of Cd(II), some library sequences that underwent metal-dependent conformational change would be subsequently released from the capture oligonucleotide immobilized beads. The released species were amplified and purified. The PCR products were then made single strands as the new library of next selection round. This process continued until the selection pool was extremely enriched to bind Cd(II). As zinc and cadmium belong to the second sub-group of the periodic table, zinc is a common interfering metal ion in cadmium detection. Therefore, an vital negative selection process was introduced at round 11 in order to remove some aptamers that also interact with Zn(II) to improve the aptamer selectivity.

All the sequences detached from streptavidin-coated beads were PCR-amplified and tested by agarose gel electrophoresis. As shown in Fig S1, the fractions eluted by Cd(II) solutions show much brighter bands than the fractions eluted by the selection buffers which intended to remove the unbound and poor bound species. After 14 rounds of positive and negative selection, we obtained the desired specific aptamers

for Cd(II).

3.2 Cloning and sequencing

After 14 rounds of selection, the DNA pool was amplified and cloned, and 30 individual aptamers were sequenced. Table S1 summarized the information of the sequences. The picked sequences could be divided into about 5 families. Family 1 possesses a common "GGGTTCACAGTCCGT TG" box in its N_{30} region and Family 2 has a common "GCAAGTCCATGGTTTGT GGCG" box, and the sequences of the two families had the base deletions in terms of the initial DNA library design. We hypothesize that it is due to the error of the initial library synthesis and the error during PCR amplification. Family 3~Family 5 have their own common boxes. We then analyzed the secondary structures of these sequences by NUPACK.

Some sequences were shortened based on their secondary structures. One sequence, named Cd-2 (Fig. 1b) was truncated and we hypothesize that the conserved region (Cd-2-1) is the vital binding site for Cd(II). CD measurements were performed to monitor the conformation change of the shortened aptamers in selection buffer. Fig. 1a shows the CD spectra of Cd-2-1 in the absence (black line) and presence (red line) of 3 μM Cd(II). By comparing all the simplified sequences, the CD spectra of Cd-2-1 changed most significantly. In the absence of Cd(II), the CD spectrum of Cd-2-1 has a negtive peak at 240 nm and a positive peak at 275 nm. Upon addition of 3 μM Cd(II), the positive peak at 275 nm increased apparently, and a new positive peak was observed at 256 nm instead of the negtive peak at 240 nm, which indicated that Cd(II) interacted strongly with Cd-2-1.

3.3 Biosensor designing

We then transformed Cd-2-1 into a biosensor probe. As shown in Scheme 2a, Cd-2-1 processes self-complementary sequence at its ends, and thus is expected to form a hairpin structure. The stem of Cd-2-1 was then lengthened and labeled by FAM (named Cd-2-2) to develop a Cd(II) biosensor. We designed a Dabcyl labeled DNA strand which is partially complemented with Cd-2-2. The sequence of Cd-2-2 is 5'-FAM- CTCAGGACGACGGGTTCACAGTCCGTTGTC-3', and the sequence of the quenching strand is 5'-CCGTTGTCCTGAG- Dabcyl-3'.

When the quenching strand was mixed with Cd-2-2, partial double strand helices formed at the 5' terminal of Cd-2-2, so Dabcyl got close to FAM and decreased the fluorescence intensity of FAM. On addition of Cd(II), Cd-2-2 was converted into a haripin structure, and the fluorophore and quencher were separated along with the restoration of fluorescence intensity. Scheme 2b shows the design principle of the biosensor to detect Cd(II), which is based on the enhancement of fluorescence intensity in the presence of Cd(II).

To determine the biosensor could indeed function to detect Cd(II), we firstly tested the quenching efficiency of the quenching strand when mixing with Cd-2-2 in different ratios. As expected, hybridization of Cd-2-2 with the quenching strand led to the quenching of the fluorescence signal. Fig. 2 shows the fluorescence quenching efficiency of different concentrations of quenching strand incubated with 50 nM of Cd-2-2. The results show that the fluorescence intensity decreases along with the increase of the concentration of quenching strands. When the concentration of the

quenching strand increased to 350 nM, the sensing system achieved a maximum decrease of fluorescence intensity. So we took the mixing ratio of Cd-2-2 to quenching strand as 1:7 in the following experiments.

Subsequently, the fluorescence enhancement of Cd-2-2-quenching strand complex upon addition of Cd(II) was measured. The hybridization of Cd-2-2 with quenching strand (blue line in Fig. 3) induced 28.6% decrease of fluorescence intensity (black line in Fig. 3) due to the proximity between FAM and Dabcyl. Upon addition of 20 μM of Cd(II), the fluorescence intensity (cyan line in Fig.3) of the Cd-2-2-quenching strand complex restored to 87% of the maximum possible fluorescence response (red line in Fig. 3). It should be noted that there was a slight quenching to Cd-2-2 alone due to the addition of cadmium alone (red line in Fig. 3), the signal was quenched by approximately 22%. The results indicate that Cd-2-2 could act as a probe to develop light-up sensor to detect Cd(II).

Cd-2-2-quenching strand complex exhibited a Cd(II) concentration-dependent increase in fluorescence intensity. As shown in Fig. 4, the quenching strand was incubated with Cd-2-2 in a ratio of 7:1. The fluorescence signal enhanced along with the increase of Cd(II) concentration. The signal reached a stable level when the concentration of Cd(II) increased to 25 μM and the calibration curve looked like a sigmoid shape. To improve the sensitivity of the biosensor, low concentration of probing strand Cd-2-2 (10 nM) was used to detect the fluorescence response to Cd(II) (Fig.S2). A linear range was determined to be 0~1000 nM. The limit of detection was 40 nM for Cd(II) based on a signal-to-noise ratio (S/N) of 3. Comparison with most of

the small-molecule organic probes, our method could be performed in the aqueous phase. The new aptamer used in this work is shorter, and it is easier to construct biosensors with comparable analytical performance. To determine the analytical performance of the method, we validated this method by comparison with a standard method. We took some lake water and filtered it by 0.22 μm membrane. The concentration of endogenous Cd(II) in the lake water was below the detection limit of Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES). Therefore we added some Cd(II) (4 μM) into the lake water. Then the sample was detected by both ICP-OES and our method. The results showed that the recovery of ICP-OES was 98.1% and 3680 ± 60 nM Cd(II) was found using this biosensor with a recovery rate of 92.6±1.5%.

To determine the specificity of Cd-2-2, the fluorescence intensity of Cd-2-2-quenching strand complex was measured when incubation with twelve different kinds of metal ions (Fig. 5). The fluorescence response in the presence of cadmium was compared with other metal ions including Ag(I), Hg(II), Ni(II), Ba(II), Ca(II), Co(II), Mn(II), Pb(II), Zn(II), Cu(II) and Al(III). The sensor platform displayed an increase in the fluorescence upon the Cd(II) binding, and showed excellent selectivity in the presence of various interfering metal ions. However, a moderate enhanced fluorescence response from the Cd-2-2-quenching strand was elicited when incubation with Cu(II), indicating that Cd-2-2 might somewhat bind with Cu(II). The relative change of fluorescence intensity of Cd-2-2-quenching strand complex when incubation with Cd(II) was 1.37-fold of incubation with Cu(II). The

binding of Cd-2-2 to Cu(II) may attribute to the negative selection in which the solution we used as elution buffer only contains Zn(II). As the solubility product constant (Ksp) of $Cd(OH)_2$ is 7.2×10^{-15} which is much higher than that of $Cu(OH)_2$ (2.2×10^{-20}), the detection of Cd(II) at pH 8.5 should eliminate the interference from Cu(II). As shown in Figure 5, the fluorescence response to Cd(II) at pH 8.5 was almost the same with that at pH 7.4, whereas the fluorescence response to Cu(II) at pH 8.5 was greatly suppressed. The Cu(II) interference was eliminated due to the formation of $Cu(OH)_2$ precipitation.

4. Conclusion

The DNA aptamers of Cd(II), were successfully isolated by a modified selection method based on target-induced release of strands. One of the selected aptamers was optimized to a 30-mer nucleic acid after 14 rounds of positive and negative selection process. Among all the shortened aptamers, Cd-2-1 exhibited the most obvious change on CD spectra. Our results showed that the modified Cd-2-2 aptamer folds into specific secondary structure to bind Cd(II) with high affinity and specificity. A biosensor with a limit of detection (LOD) of 40 nM was developed based on Cd-2-2. The sensor platform displayed a dose-dependent increase of fluorescence intensity on cadmium binding and showed excellent selectivity in the presence of various interfering metal ions. The light-up biosensor is simple, cost-effective with rapid and reliable response on cadmium detection. Moreover, this study demonstrated the feasibility of the aptamers of metal ions engineered as probes for continuous

environmental monitoring.

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Fig. 1 (a) CD spectra of the Cd-2-1 solutions in the absence and presence of 3 μM Cd(II). The concentration of Cd-2-1 was 1 μM. **(**b) The secondary structure of Cd-2.

Fig. 2 Fluorescence quenching efficiency of different concentration ratios of

quenching strand with 50 nM of Cd-2-2. Q represents Dabcyl, and F represents FAM.

Fig. 3 Fluorescence intensity of Cd-2-2 alone (black and red) and the aptamer-quenching strand complex (light and dark blue) in the absence and presence of Cd(II) (20 μM). The quenching strand was present in a 7:1 ratio to Cd-2-2 (50 nM).

Fig. 4 Cd(II) concentration-dependent increase in fluorescence intensity of the Cd-2-2-quenching strand complex in different concentrations of Cd(II). The quenching strand was present in a 7:1 (350 nM:50 nM) ratio to Cd-2-2.

Fig. 5 Specificity of the aptamer when interacting with different kinds of metal ions. The change in fluorescence of the Cd-2-2-quenching strand complex was measured in the presence of 10 μM metal ions with the incubation of 30 min. The quenching strand was present in a 7:1 (350 nM:50 nM) ratio to Cd-2-2.

Scheme 1 In vitro selection protocol of Cd(II) specific aptamers. The following illustration interpreted the binding principle of the biotin-labeled capture oligonucleotide-ssDNA library complex on the streptavidin coated column used in the selection experiment.

Scheme 2 (a) The secondary structure of Cd-2-1 and Cd-2-2 (b) The principle of the biosensor to detect Cd(II), which is based on the enhancement of fluorescence intensity in the presence of Cd(II).

Highlights

- The DNA aptamers of Cd(II), were successfully isolated by a modified

selection method based on target-induced release of strands, and one of the selected aptamers was optimized to a short 30-mer DNA.

- The aptamer-based sensor platform showed excellent selectivity in the presence of various interfering metal ions.
- The developed aptamer-based light-up biosensor is simple, cost-effective and quick-testing.

Fig. 1

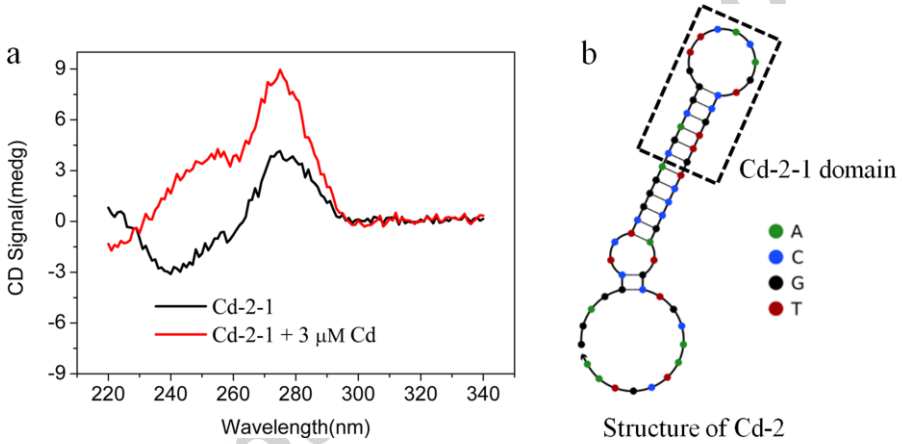


Fig. 2

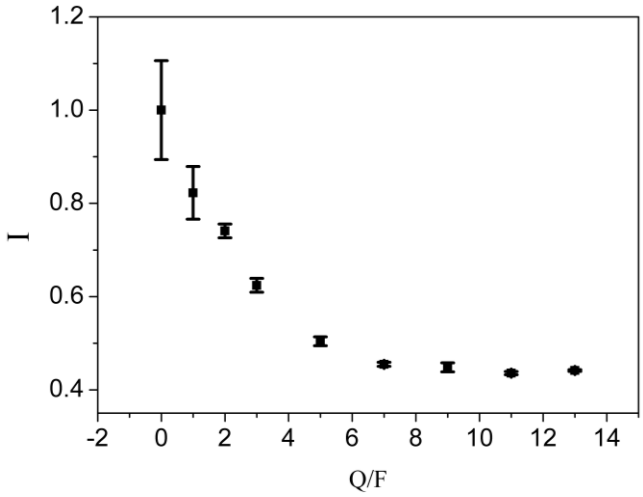


Fig. 3

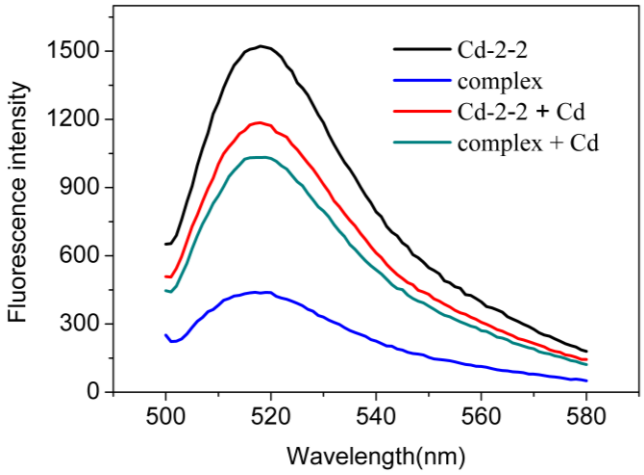


Fig. 4

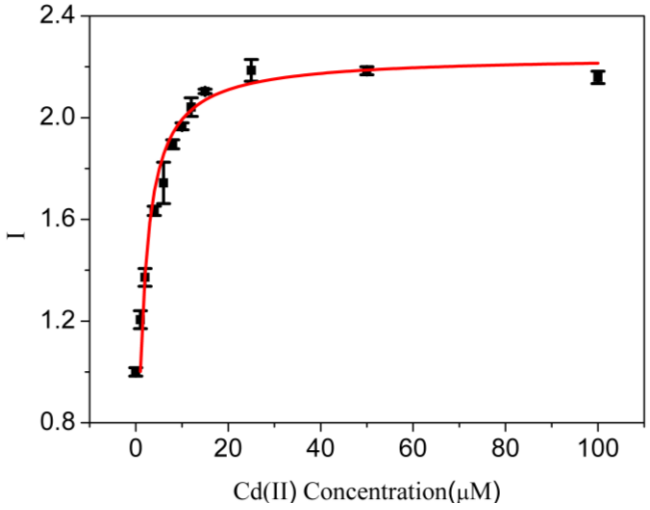
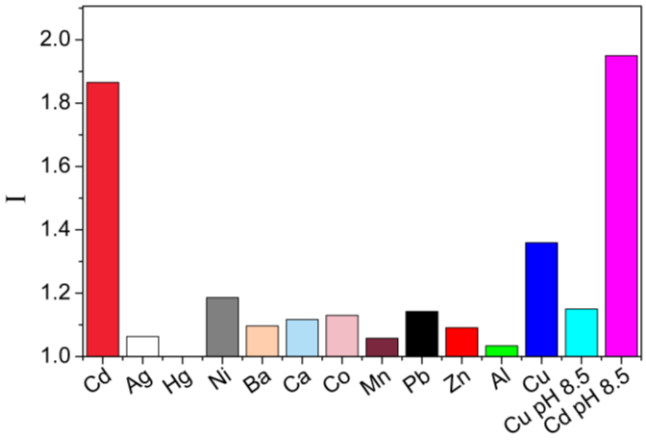
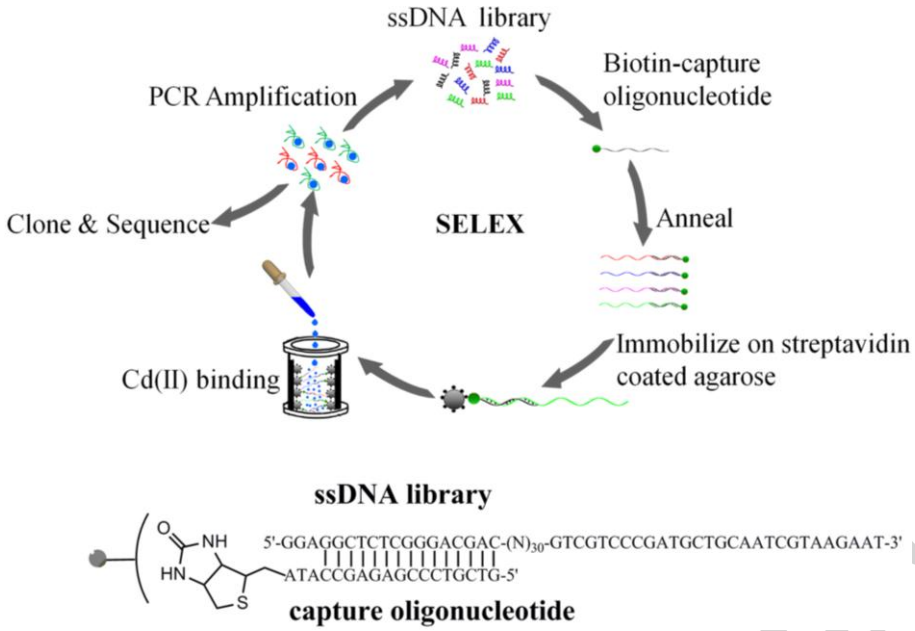


Fig. 5



Scheme 1



Scheme 2