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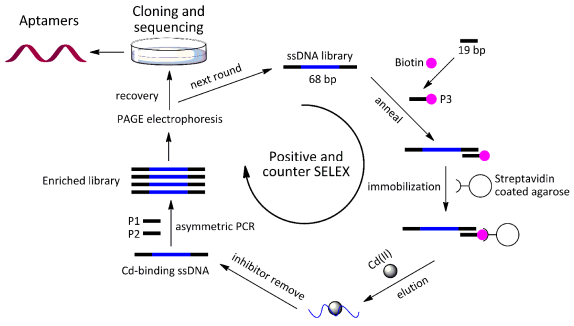

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PAPER

Selection of a DNA aptamer for cadmium detection based on cationic polymer mediated aggregation of gold nanoparticles†

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The demands for the selection of aptamers against various small chemical molecules have substantially increased in recent years. To incubate and separate target-specific aptamers, the conventional SELEX procedures generally need to immobilize target molecules on a matrix, which may be impotent to screen aptamers toward those small molecules without enough sites for immobilization. Herein we chose Cd(II) as a model of small molecule with less sites, and proposed a novel SELEX strategy that immobilizing ssDNA libraries rather than target molecules on a matrix, for the selection of aptamers with high affinity to Cd(II). After eleven rounds of positive and negative selection, twelve T and G-rich of nonrepeating ssDNA sequences were identified, of which the Cd-4 aptamer displayed highest binding affinity to Cd(II). The secondary structures of these sequences revealed that a stem-loop structure folded by the domain of their 30-random sequence is critical for aptamers to bind targets. Then the interaction between the selected Cd-4 aptamer and Cd(II) was confirmed by CD analysis, and the binding specificity toward other competitive metal ions was also investigated. The dissociation constants (K_d) of Cd-4 aptamer was determined as 34.5 nM for Cd(II). Moreover, the Cd-4 aptamer was considered as a recognition element for the colorimetric detection of Cd(II) based on the aggregation of AuNPs by cationic polymer. Through spectroscopic quantitative analysis, Cd(II) in aqueous solution can be detected as low as 4.6 nM. The selected Cd-4 aptamer will offer a new substitute for the detection of Cd(II) or other applications like the recovery of cadmium from polluted samples.

1. Introduction

Cadmium (Cd) is considered as one of the most extremely toxic metal for human beings, and the environmental exposure to cadmium can cause serious health issues involving in many serious diseases and even many types of cancers.[1] Recent works revealed that the cytotoxic and metabolic effects of cadmium may be ascribed to its interference with the normal functions of some essential metals like Zn(II) and Ca(II).[2] Due to a long metabolic half-life, cadmium tends to easily bio-accumulate in animals and human beings through the food chain system of the earth. The U.S. EPA (Environmental Protection Agency) sets a compulsory drinking water standard for Cd of 5 ppb to prevent kidney damage and other related diseases.[3] Such strict guideline value promotes researchers to develop more sensitive and reliable analytical techniques for detecting and monitoring Cd in drinking water or other environmental samples. However, the most popular methods for heavy metals detection generally require sophisticated equipment and a relatively long analysis time, so they are neither readily available in developing countries nor capable of on-site field detection. Recently, Kim and co-workers have summarized many fluorescent and colorimetric sensors for cadmium detection.[4] Most of these methods usually needed to design and synthesize some organic small molecules with multi-benzene ring structure,[1a, 5] in which the internal charge transfer (ICT) would occur in the presence of cadmium, thus caused the remarkable changes of color or fluorescent intensity. Besides small molecules, a variety of functionalized proteins [6] and nanoscale materials like quantum dots,[7] carbon nanotube and graphene,[8] were also employed to construct sensor for cadmium detection. Because these methods generally required some redundant reactions or complicated modification, they are not cost-effective and may be susceptible to the surrounding conditions. Therefore, it is still highly desirable to develop simpler and more sensitive methods to detect trace Cd in the environment.

Functional nucleic acids, including oligonucleotide ligands, aptamers and DNAzymes, have been widely used as the target recognition elements in the sensing applications due to their competitive advantages over other biological tools.[9] Up to now, several nucleic acids based methods have been developed for the detection of various heavy metal ions. For example, Lu and

co-workers adopted 8-17 DNAzyme and its variant for the fluorescent and colorimetric detection of Pb(II).[10] They also *in vitro* selected two specific DNAzymes toward uranium and Cu(II) based on the systematic evolution of ligands by exponential enrichment (SELEX) process,[11] and further tagged them with fluorophore and quencher for ultrasensitive detection of uranium and Cu(II). Combined with some signal outputs, the T-rich or C-rich of oligonucleotide ligands were widely employed as recognition elements for Hg(II) or Ag(I) detection *via* the special T-Hg(II)-T or C-Ag(I)-C mismatched base pairs.[12] Apart from DNAzymes and oligonucleotide ligands, aptamers may be the most promising nucleic acids tool in the field of analytical chemistry. The essence of an aptamer molecule is its three-dimensional structure arising from a folded single-stranded nucleic acid (usually 20~60 bases).[13] Recently, Kim and co-workers have *in vitro* selected the arsenic-binding DNA aptamer (named Ars-3aptamer) using an affinity column based on the SELEX procedure.[14] Our group have further adopted such aptamer to construct sensors for the colorimetric and Rayleigh Resonance scattering (RRS) assays of As(III).[15] To the best of our knowledge, there is still no report concerned in the selection of aptamer or DNAzyme for cadmium detection.

The aptamer isolation process (called SELEX) was firstly reported in 1990 almost simultaneously by the group of Ellington and Gold.[16] This technique consists of the repeated incubation, selection, and amplification of aptamers from the initial and enriched ssDNA libraries until one or more aptamer candidates displaying desired affinity to target have been isolated. The most important step for successful aptamer selection depends on the efficient incubation and separation of target-binding ssDNAs. For this purpose, some researchers coupled the SELEX procedure with powerful separation techniques such as capillary electrophoresis (CE),[17] flow cytometry (FC),[18] surface plasmon resonance (SPR),[19] magnetic bead,[20] microfluidic-based selection,[21] centrifugation,[22] *etc*. Besides, several modifications of the SELEX process were proposed for the rapid and simple selection of aptamers binding to various targets and their analogues.[23] The progress in SELEX technique has been extensively reviewed and the applications of selected aptamers were also introduced.[13, 24] So far, the targets for aptamers selection has been extended to inorganic and small organic molecules, peptides, proteins, carbohydrates, antibiotics, as well as complex targets like target mixtures or whole cells and organisms.[9, 24d] Compared to small molecules, those targets with high molecular weight will possess more sites for ssDNA binding and the bound aptamer candidates can be easily separated due to the obvious difference in their molecular weight. To incubate and separate target-specific aptamers, the conventional SELEX procedures generally need to immobilize target molecules on a matrix like magnetic beads or agarose beads, which may be impotent to screen aptamers toward those small molecules without enough sites for immobilization. It can be found that the amount of aptamers for small molecules is relatively rare. Thus, lots of efforts still need to be made to improve the SELEX process aim at the selection of aptamers especially for inorganic small molecules without enough sites.

In this paper, we chose Cd(II) as a model of small molecule with less sites for binding or immobilization, and proposed another strategy that immobilizing ssDNA libraries rather than target molecules on a matrix, for the selection of aptamers with high affinity to Cd(II). We characterized the selected aptamer and further employed it as a recognition element in an attempt to detect Cd(II), using cationic polymer mediated aggregation of gold nanoparticles (AuNPs) as colorimetric signal output. We believe this work will not only contribute to improve the SELEX process, but also extend the detection methods for cadmium.

2. Experimental

2.1. Chemicals and materials

Cadmium ion standard solution (1 mg/mL) was purchased from Merck (Germany). Poly(diallyldimethylammonium chloride) (PDDA), streptavidin-coated agarose and microspin column kit were obtained from Sigma-Aldrich (Milwaukee, WI, USA). Reagents for polymerase chain reaction (PCR) amplification, 3-(morpholino)propanesulfonic acid (MOPS), polyacrylamide gel, $HAuCl_4$, Chelex-100, TBE buffer, DNA Marker and N-(2-hydroxyethyl) piperazine-N-2-ethane sulfonic acid (HEPES) were purchased from Sangon Biotechnology (Shanghai, China). The initial ssDNA library contained 30 random nucleotides (5'-ACCGACCGTGCTGGACTCT-N30-AGTATGAGCGAGCGTTGCG-3', 68-mer) was used in SELEX. The forward primer P1 (5'-ACCGACCGTGCTGGACTCT-3') and reverse primer P2 (5'-CGCAACGCTCGCTCATACT-3') were used in PCR for the amplification of the selected DNA pool. The labeled primer P3 (Biotin-5'-CGCAACGCTCGCTCATACT-3') was used to immobilize ssDNA library on the surface of streptavidin-coated agarose beads. All of oligonucleotides were synthesized and HPLC-purified by Sangon Biotechnology (Shanghai, China). Metal salts for the competitive assay, nitrilotracetic acid (NTA) and 2,6-pyridinedicarboxylic acid (PDCA) were obtained from Sinopharm Chemical Reagent (Shanghai, China). The 96-well microplate was purchased from Thermo Fisher Scientific Inc. (Nunclon, Denmark). Unless otherwise mention, all other reagents were analytical grade and used without further purification or treatment. Ultrapure water (Milli-Q plus, Millipore Inc., MA) was used throughout.

2.2. Instrumentation

DNA amplification was performed in a Peltier thermal cycler (BIO-RAD, CA). Polyacrylamide gel electrophoresis (PAGE) was carried out in a cataphoresis apparatus (BIO-RAD, CA) to recover aptamer candidates. The amount of separated ssDNA was measured by UV-Vis spectroscopy (Shimadzu, Japan). The incubation of DNAs and target metal ions was performed in a thermomixer (Eppendorf, Germany). A model of F-4500 fluorescence spectrophotometer (Hitachi, Japan) was used to record the fluorescence intensity, with the excited slit of 20 nm and emission slit of 10 nm, and PMT voltage of 700 V. Circular dichroism (CD) spectra were collected with a J-815 Circular

Dichroism Spectropolarimeter (Jasco, Japan) in HEPES buffer (50 mM, pH 7.2) to characterize the structure change of aptamers at room temperature. The optical chamber (0.5mL volume) was utilized. The scanning speed was 100 nm per minute. The CD spectra were collected in the range of 200-400 nm. Analytical transmission electron microscope JEM-2010HT (Hitachi, Japan) was used to observe the images of AuNPs. Colorimetric assays were recorded on Microplate Spectrophotometer M200 Pro (Tecan Group Ltd, Switzerland). The average size of nanoparticles was determined by photon correlation spectroscopy (PCS) (Malvern Instruments, UK).

2.3. Aptamers selection

2.3.1. Immobilization of ssDNA library on beads

Prior to each round of SELEX selection, the initial or enriched ssDNA library need to be immobilized onto the surface of agarose beads. To ensure the aptamer candidates in ssDNA library could be eluted by Cd(II), an assistant P3 sequence with ability to bind 3'-terminal of ssDNA library was used. An appropriate volume of ssDNA was mixed with P3 sequence in a mole ratio of 1:2, and then the mixed solution annealed under following conditions: 60 s at 94 °C, 60 min at 59 °C, 5 min at 25 °C, with a cooling rate of 0.1 °C/s. Subsequently, the above mixed solution was transferred to the microspin column that filled with streptavidin-coated agarose beads and then incubated at room temperature for 30 min. Finally, the microspin column was washed for five times to remove free DNAs by washing buffer (50 mM HEPES, 50 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, and 0.01% Tween-20, pH 7.2). Through these procedures, P3 sequences were immobilized directly on the surface of agarose beads *via* the biotin-streptavidin affinity interaction, and thus ssDNA library was ultimately immobilized due to the base-pairing interaction between ssDNA library and P3 sequences.

2.3.2. Incubation and separation of specific ssDNA

In the first round of positive SELEX selection, an appropriate volume of Cd(II) standard solution was diluted in binding buffer (50 mM HEPES, 50 mM NaCl, 5 mM KCl, 5 mM $MgCl_2$, pH 7.2) to give the final concentration of 200 μM. To find out aptamer candidates from ssDNA library, about 500 μL of binding buffer with 200 μM Cd(II) was added into the above microspin column which had already filled with ssDNA-immobilized agarose beads. The subsequent microspin column was shook slightly for 5 min to mix agarose beads with Cd(II) and then incubated at 25 °C for 60 min. Thereafter, the microspin column was centrifugal separated at 8000 rpm for 3 min to collect the eluted ssDNAs solution, which were further treated by Chelex-100 to remove metal ions and precipitated using ethanol. Finally, the precipitated ssDNAs were then dissolved in 200 μL of distilled water for asymmetric PCR amplification. In the next several rounds of positive SELEX selection, the concentration of Cd(II) used in incubation had decreased from initial 200 μM to 20 nM to obtain aptamer candidates with highest affinity to Cd(II). To improve selectivity of the aptamer candidates, an additional round of negative (or counter) selection (NS) was performed using other competitive metal ions including Cu(II), Zn(II), Hg(II) and Ag(I) for incubation.

2.3.3. Asymmetric PCR amplification of ssDNA

To obtain large quantity of aptamer candidates, an asymmetric PCR amplification procedure was employed. The optimal conditions for asymmetric PCR amplification were based on our preliminary experiments. A PCR mixture containing 10 pmol aptamer candidates, 15 μL of 10 μM forward primer (P1), 3 μL of 1 μM reverse primer (P2), 2.5 μL of 25 mM dNTP, 1 μL of 5U/μL *Taq* DNA polymerase, 10 μL of 25 mM $MgCl_2$ and 20 μL of 10×PCR buffer. The above PCR mixture supplemented distilled water to 200 μL and then subdivided equally into five PCR tubes, and then put in a Peltier thermal cycler for PCR amplification. The PCR program used was as follow: 94 °C for 3 min (initial denaturation), followed by 20 cycles of a rapid three-step PCR (45 s at 94 °C, 30 s at 51 °C, 20 s at 72 °C), and a single final extension at 72 °C for 5 min.

2.3.4. Gel electrophoresis and ssDNA recovery

After asymmetric PCR amplification, the reaction products were separated on 16% non-denaturing PAGE in TBE buffer at 60~120 V. The gels were stained with silver nitrate and photographed under a common scanner, and the target bands were sliced by a sterile scalpel from gels and then put into several EP tubes for washing twice using distilled water. Subsequently, the target gels containing aptamer candidates were smashed thoroughly and dispersed in HEPES buffer (50 mM, pH 7.2). To recover aptamer candidates, the above EP tubes were put at -80 °C for 20 min, and then transferred immediately to a thermostatic bath at 80 °C for another 20 min, with continuous stirring. This operation was repeated for three times. Thereafter, the above EP tubes were incubated at 90 °C for 5 min and then centrifugal separated at 6000 rpm for 1 min. To obtain aptamer candidates, the supernatant was collected and precipitated using ethanol. Finally, the precipitated ssDNAs were dissolved in 200 μL of distilled water as the enriched ssDNA library for next round of SELEX selection.

2.4. Sequence analysis of selected aptamers

After eleven rounds of SELEX selection, the separated aptamer candidates were amplified by PCR using unmodified forward and reverse primers. The PCR mixtures were then cloned, purified and sequenced by Invitrogen (Life Technologies Corporation, NY). Secondary structure analyses of aptamers were performed using the *Mfold* program (http://mfold.rna.albany.edu/).

2.5. Binding assay for selected aptamers

The binding affinities of the selected aptamers for Cd(II) were analysed on the microspin column that filled with streptavidin-coated agarose beads. According to the sequencing results, the selected aptamers were synthesized and labeled with 6-carboxy-fluorescein (FAM) at the 5'-end, and then immobilized onto agarose beads in aid of biotin-modified P3 sequence based on the above procedure (section 2.3.1). After thoroughly washing for several times, the amounts of immobilized aptamers were determined. To investigate the affinity of

selected aptamers toward their target, 500 μL of binding buffer containing 200 μM Cd(II) was added into the microspin column and then incubated at 25 °C for 60 min. The eluted aptamers were collected by centrifugal separation and their amounts were quantified by fluorescence at 525 nm (λ_{ex} = 475 nm). The eluted rates (eluted aptamers/immobilized aptamers) were calculated to judge the binding affinities of the selected aptamers for Cd(II). For the selectivity evaluation, the aptamer with highest affinity to Cd(II) was immobilized on agarose beads to incubate with several typical classes of competitive metal ions, and accordingly their eluted rates were calculated.

2.6. Determination of dissociation constant

The aptamer with highest affinity to Cd(II) was used to determine the dissociation constant (K_d). In essence, 50 μL of 2 μM aptamer was mixed with 10 μL of 20 μM biotin-labeled P3 sequence and then annealed and immobilized on agarose beads according to the above procedure (section 2.3.1). After thoroughly washing for several times, the agarose beads containing aptamer were dispersed in 5 mL of binding buffer. For the K_d determination, varying volumes of above agarose beads were filled in several microspin columns and gave the final concentration of immobilized aptamer in the range of 10-160 nM. About 2 μM Cd(II) solution was added into each microspin column and then incubated at 25 °C for 60 min. The eluted aptamers were collected by centrifugal separation and the fluorescence intensities were measured at 525 nm (λ_{ex} = 475 nm). The K_d value was calculated by fitting the dependence of the fluorescence intensity of specific binding on the concentration of aptamer to the one-site saturation equation $Y=B_{max}X/(K_d+X)$ using SigmaPlot software.

2.7. Preparation of AuNPs

AuNPs were synthesized by sodium citrate reduction of $HAuCl_4$ following a literature procedure.[25] All glassware used in this procedure was cleaned in a bath of freshly prepared 3:1(v/v) HNO_3-HCl, then rinsed thoroughly in ultrapure water and dried in air. AuNPs were prepared by adding 3.5 mL of 1% (w/v) trisodium citrate to a boiling solution of $HAuCl_4$ (100 mL, 0.01%) and stirred for 30 min, within the time, the color of the solution changed from light grey, blue, purple, to wine red. The mixture continued to stir for 10 min after removal from the heater. Finally, the cooled solution was filtrated by 0.2 μm ultrafiltration membrane to remove aggregated particles, and then stored in dark glass bottles at 4 °C for further use.

2.8. Colorimetric detection of Cd(II) in aqueous solution

An appropriate volume of 500 nM Cd-4 aptamer solution and 5 μL of Cd(II) solution with varying concentration were mixed thoroughly in a 2 mL plastic tube, and then diluted to 390 μL with MOPS buffer (pH 7.0, 50 mM NaCl) and incubated at 25 °C for 30 min. The blank sample was added 5 μL ultrapure water instead Cd(II) solution. Subsequently, 10 μL of 76 nM PDDA was added into the above mixed solutions and incubated at 25 °C for another 30 min. Finally, 100 μL AuNPs stock solution was added to give a final volume of 500 μL. After incubation at 25 °C for 5 min, 200 μL of above samples were moved into a 96-well microplate for colorimetric assay. The absorption spectra (from 400 nm to 800 nm) and absorbance values at 520 nm (A520) and 650 nm (A650) were measured by Microplate Spectrophotometer M200 Pro. The ratio of A650/A520 in sample solutions and the blank solution without cadmium were recorded to indicate the aggregation of AuNPs. To test the selectivity of biosensor, other competitive metal salts including $Pb(NO_3)_2$, $Hg(NO_3)_2$, $AgNO_3$, KCl, $CaCl_2$, $ZnCl_2$, $BaCl_2$, $CrCl_2$, $CoCl_2$, $FeCl_3$, $MgSO_4$, $MnSO_4$, $NiSO_4$, $CuSO_4$ and $FeSO_4$ were used.

3. Results and discussion

3.1. Overview of current SELEX procedure

To obtain the desired specific aptamers, the incubation of target with random ssDNA libraries and separation of ssDNAs bound to target molecules are two essential steps for aptamer selection. The conventional SELEX procedures generally immobilized target molecules on a matrix as stationary phase, and the solution containing large quantity of ssDNA sequences as mobile phase to incubate with them, thus the desired specific ssDNAs would be absorbed on a matrix *via* the affinity interaction between the immobilized target molecules and ssDNAs. However, these strategies may be suffered from two major problems as follows. (1) They are incapable of selecting aptamers against those small molecules without enough sites for immobilization. (2) Because some sites of target molecules had already been occupied for immobilization, the affinity of selected aptamers may become weakly in the recognition of those free target molecules.

Herein we selected Cd(II) as a model of small molecule without enough sites, and proposed another strategy that immobilizing ssDNA libraries rather than target molecules on a matrix, for the selection of aptamers with high affinity to Cd(II). The current SELEX procedure is showed in **Fig. 1**, and its detail information is described in Materials and Methods. Before each round of SELEX selection, the starting or enriched ssDNA libraries consist of 10^{13}~10^{14} total molecules needed to be immobilized onto the surface of agarose beads. For this purpose, we firstly designed a short assistant ssDNA sequence (P3) with a label of biotin at its 5'-end, which contains 19 nucleotides that can be complementary pairing with the 3'-terminal of ssDNA library. Thus the ssDNA libraries could bind to P3 sequence and form ssDNA libraries/P3 complexes with partial duplex structure after some appropriate annealing treatment. Such complexes possess an active group of biotin at its terminal, so they were easy to absorb onto the surface of streptavidin-coated agarose beads *via* the strong affinity interaction between biotin and streptavidin. To facilitate the separation of ssDNAs bound to target molecules, the above agarose beads were filled into a microspin column. After thoroughly washing of microspin column by buffer solution, the ssDNA libraries were ultimately immobilized on agarose beads. The immobilized ssDNA libraries then incubated with a

certain amount of Cd(II) for 60 min, and the target-bound ssDNAs would elute along with Cd(II) from microspin column. The eluted ssDNAs displayed the binding ability to Cd(II), so they needed to be amplified by PCR to generate the enriched ssDNA libraries for next round of SELEX selection. To eliminate the potential adverse effect of Cd(II) on *Taq* DNA polymerase, Chelex-100 was employed to remove them before PCR amplification. Here we chose an asymmetric PCR amplification procedure to produce ssDNA. The key parameter for the successful asymmetric PCR amplification is the ratio of primer amounts. Based on our preliminary experiment, the mole ratio of forward primer (P1) to reverse primer (P2) with 50:1 was efficient for the amplification of desired ssDNA. Because asymmetric PCR is economic and less tedious, involves the same parameters of the PCR amplification step in SELEX (except for primer ratios), it has become the most prevalent procedure of generating ssDNA in DNA aptamer production.[26] The asymmetric PCR mixtures were purified by PAGE and the gels containing desired aptamer candidates were put into EP tube for several freeze-thaw cycles to obtain the enriched ssDNA libraries. In the next round of SELEX selection, the enriched ssDNA libraries were immobilized on the surface of agarose beads, and the rest of operations were complied with the above procedures. After several rounds of positive and counter SELEX selection, the separated ssDNA were amplified by PCR using unmodified forward and reverse primers. To confirm and characterize the selected aptamer candidates, the PCR mixtures were then cloned, purified and sequenced.

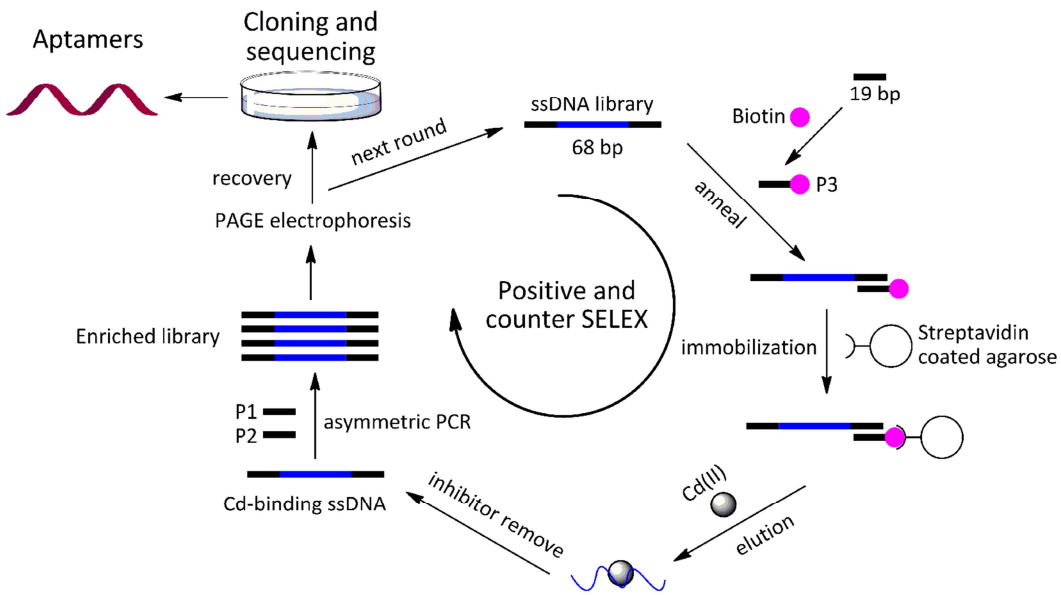

Fig. 1 Schematic protocol for the selection of cadmium-binding aptamers. The conventional SELEX procedures generally need to immobilize target molecules on a matrix for absorbing the target-specific ssDNAs. Conversely, the current approach adopted another strategy that immobilizing ssDNA libraries rather than target molecules on a matrix. For the subsequent separation of aptamer candidates, the starting or enriched ssDNA libraries consist of 10^{13}~10^{14} total molecules were firstly immobilized onto the surface of agarose beads before each round of SELEX selection. This approach is especially suitable for the selection of aptamers against those small molecules without enough sites for immobilization.

3.2. *In vitro* selection of ssDNA aptamers for Cd(II)

In the first round of SELEX selection, about 1 nmol of ssDNA library were mixed with P3 sequence in a mole ratio of 1:2 for subsequent annealing treatment, and then the mixed solution hatched with 0.2 mg of streptavidin-coated agarose at room temperature for 30 min. To remove the non-immobilized ssDNA sequences, the above agarose beads were filled into a microspin column and thereby washed thoroughly with washing buffer. The amount of eluted ssDNA was determined by recording the absorbance value at 260 nm. Through these procedures, the final amount of immobilized ssDNA library was calculated as about 870 pmol. Subsequently, 500 μL of binding buffer containing 200 μM Cd(II) was added into above microspin column, which was shook slightly for 5 min to mix agarose beads with Cd(II) and then incubated at 25 °C for 60 min. Finally, the ssDNA sequences bound to Cd(II) were collected by centrifugal separation, and the ratio of them to the immobilized ssDNA library was determined as 3.66% (**Fig. 2**). In the second round of SELEX selection, the quantity of immobilized ssDNA enriched library was about 200 pmol, and then incubated with the same concentration of Cd(II) as used in the first round of selection. On this occasion the ratio of eluted ssDNA increased up to 16.2%, which indicated that the affinity of enriched ssDNA library to Cd(II) had enhanced significantly. In the rest rounds of selection, the amounts of immobilized enriched libraries were fixed at the range of 100 pmol to 200 pmol, and the concentration of Cd(II) decreased gradually from 200 μM to 20 nM for screening aptamer candidates with higher affinity to Cd(II). As shown in **Fig. 2**, the ratio of eluted ssDNA increased under the incubation of same concentration of Cd(II), while their values would decrease if the amount of Cd(II)

reduced by an order of magnitude. In the negative selection, the immobilized enriched library was firstly incubated with 2 μM of other competitive metal ions (including Cu(II), Zn(II), Hg(II) and Ag(I)) to remove those non-specific aptamer candidates. Thereafter, 20 nM of Cd(II) was used to select Cd(II)-binding aptamers, and the final ratio of eluted ssDNA was determined as 10.6%.

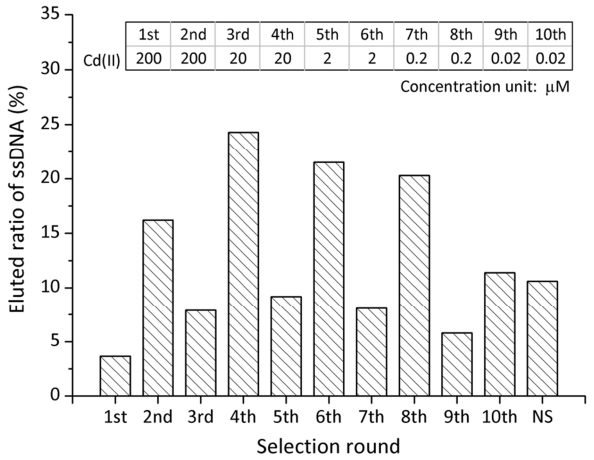

Fig. 2 The ratio of eluted ssDNA to the immobilized ssDNA libraries that incubated with varying concentration of Cd(II) in each selection round. To select the desired aptamers with high affinity to target, the concentrations of Cd(II) for incubation were decreased gradually from initial 200 μM to finial 20 nM. "NS" refer to negative (or counter) selection, where about 2 μM of other competitive metal ions (including Cu(II), Zn(II), Hg(II) and Ag(I)) were pre-incubated with the enriched ssDNA library after ten rounds of selection to remove those non-specific aptamer candidates.

After ten rounds of positive and an additional negative selection, the final enriched ssDNA library was confirmed by non-denaturing PAGE (**Fig. 3(b)**) and then used for cloning and sequencing. Twenty-eighth clones obtained from the cloning procedure were sequenced for the more detailed study of aptamer candidates, of which twelve nonrepeating sequences (named Cd-1 to Cd-12) were identified. **Table 1** listed some information about the selected Cd(II)-binding aptamers. Within this collection of sequences, Cd-4 aptamer was found as the dominant ssDNA, which makes up approximately a quarter of the total sequences. Cd-7 aptamer had the next highest number among all of 28 clones, and the rest of sequences were identically found as three or less clones. To find out the possible relationship between the sequences of selected aptamer candidates and their affinity to Cd(II), the base distribution of these sequences was also summarized in **Table 1**. Interestingly, thymine (T) and guanine (G) were found as the most two rich bases, which had almost occupied 90% of total bases in the domain of 30-random sequences. This phenomenon may be attributed to the special interactions between Cd(II) and such two bases. We speculated the special interactions and some information about the possible binding structure of them, just as shown in **Fig. S1 (ESI†)**. Cadmium holds a large quantity of electrons distributed in the outer shell, and some of them exist in the status of lone electron pairs, so it can bind to the adjacent T or G bases of the selected aptamer candidates through the coordination bonds between Cd(II) and the O or N of above bases. Conversely, adenine (A) and cytosine (C) lack enough sites for the formation of coordination bonds, so they may have weak affinity to Cd(II).

Table 1 Cloning sequences of the selected Cd(II)-binding aptamers and their base distribution.

Clone	Sequences of 30-random sequences (5' to 3')	Numbers of identical sequences	Base distribution			
			A	T	C	G
Cd-1	GGGGTTGTGTGGGGAGTTTGTCCTGTTGTG	3	1	12	2	15
Cd-2	GGTTTTGTGTTTGTGTTTTGTGGTTGTTGT	1	0	19	0	11
Cd-3	GGTGGATGGGGATGCTTTGTTAGTTTGTGC	2	3	12	2	13
Cd-4	GGACTGTTGTGGTATTATTTTTGGTTGTGC	6	3	15	2	10
Cd-5	GGAGTAGTGGGGATTGTGGTGTATGTTGTC	2	4	11	1	14
Cd-6	GGTGTGGAGTGGTGTGTGTTGTTGTTTGTC	2	1	14	1	14
Cd-7	GGAGCGATTAGTAGTGGTCTTGTCTTTTGC	4	4	12	4	10
Cd-8	GGTGTTGTTGTTGGCTAGTTTGTTGTGGGC	1	1	15	1	13
Cd-9	GGAGATGTCAGTTGTGTGTGTGGTTATGGC	2	4	11	2	13
Cd-10	AGTGTGAAGTGGTGTGTGTTGTTGTTTGTC	1	3	14	1	12
Cd-11	GGAGATGTATGAGCGGGTGTTTGTGTTTGC	2	4	11	2	13
Cd-12	GGTGGTTGCTGTTGTTTGTGGTTGGTGTGC	2	0	14	2	14

3.3. Binding measurement of selected aptamers

Cadmium binding assays were carried out for all of the selected aptamers. The complete sequences of aptamers (Cd-1 to Cd-12) containing 68 base pairs were synthesized and labeled with FAM at the 5'-terminal, and then immobilized onto agarose beads according to above procedure described in section 2.3.1. The amount of each immobilized aptamer was approximate to 400 pmol and with the fluorescence intensity of about 1400 (a.u.). For the binding measurement, the immobilized aptamers were incubated with 200 μM of Cd(II) and the eluted ssDNAs were quantified by fluorometric analysis. As shown in **Fig. 3(a)**, Cd-4 aptamer displayed the highest binding ability to Cd(II), where about 47% of them could be eluted in the presence of target. Cd-7, Cd-3, Cd-12 and Cd-11 aptamers have the next highest binding ability, with the eluted ratio of ssDNA exceeding 20%. The rest aptamers showed the moderate or lower ability to binding Cd(II). Accordingly, Cd-4 aptamer was used for further characterization and application in the subsequent experiments.

3.4. Characterization of Cd-4 aptamer

The binding ability of aptamers toward target may be associated with their folding structures in aqueous solution. To figure out this problem, the secondary structures of the twelve sequences were predicted at 25 °C, 100 mM NaCl, and 0.5 mM $MgCl_2$ using *Mfold* software. All the predicted secondary structures have the stem-loop architectures (**Fig. 3(c)** and **Fig. S2, ESI†**), but the length of stem and the size of loop are different each other. In the case of Cd-4 aptamer, the domain of 30-random sequence marked with red line has folded into a stem-loop structure (**Fig. 3(c)**). The loop may offer sufficient sites for target binding *via* the coordination bonds between Cd(II) and the adjacent short fragment rich in T or G (described in **Fig. S1, ESI†**), while the major role of stem is to maintain the stability of such a loop through hydrogen bonds among the base pairs of A-T or G-C. The sequences of Cd-7, Cd-3, Cd-12 and Cd-11 aptamers have folded the similar architecture as Cd-4 aptamer, but the domain of 30-random sequence among these aptamers did not form an integrate stem-loop structure, so the sites for target binding are limited, which in turn account for why they have lower binding activity to Cd(II) than that of Cd-4 aptamer. Surprisingly, the 30-random sequence domain of Cd-2 and Cd-10 aptamers does not fold into any stem-loop structure but distribute in a large loop (**Fig. S2, ESI†**), where the distance between adjacent bases of T or G may be too far to form coordination bonds, so they exhibit a relatively poor ability for binding Cd(II). The predicted secondary structures indicate that a stem-loop structure folded by the domain of 30-random sequence is critical for aptamers to bind their target.

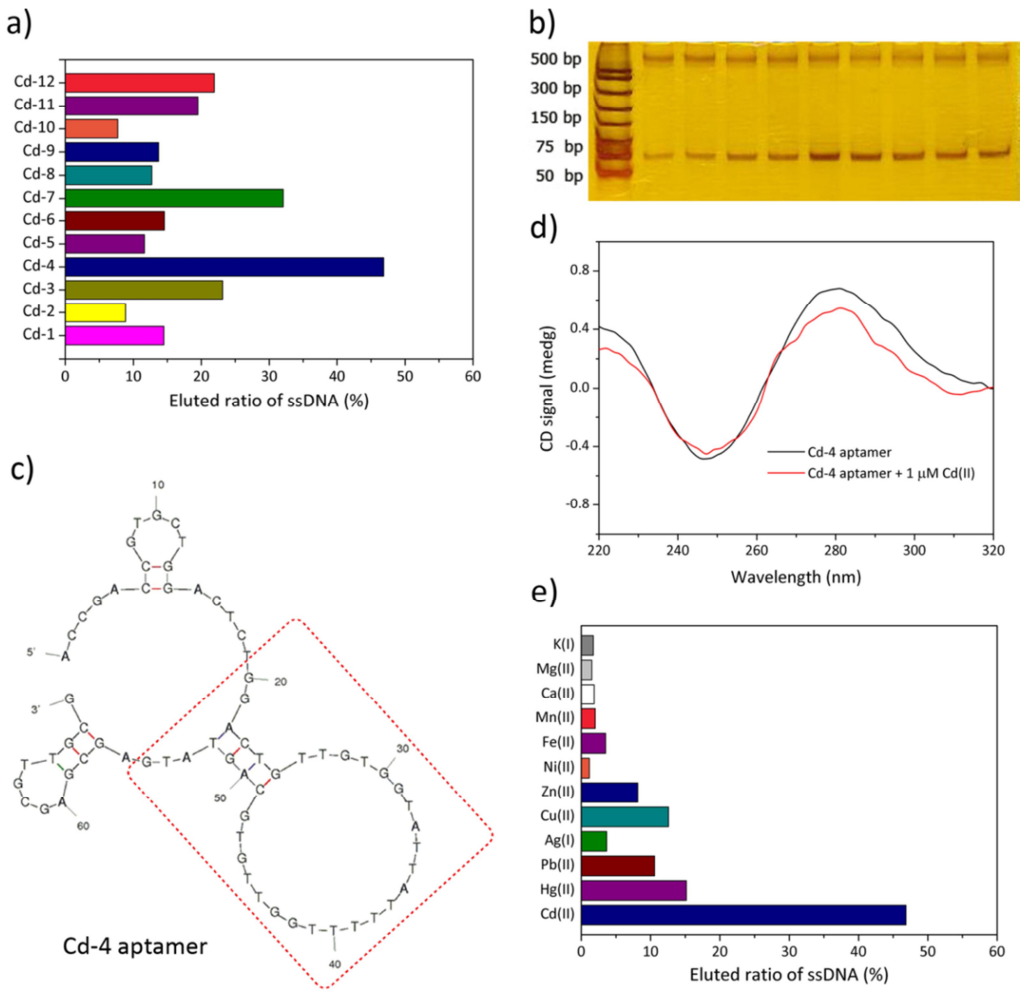

Fig. 3 (a) The eluted ssDNA from different immobilized aptamer candidates incubated with 200 μM Cd(II). The Cd-4 ssDNA showed the strongest binding affinity to Cd(II), whereas Cd-10 ssDNA showed the weakest such affinity. (b) Characterization of the enriched ssDNA libraries for cloning sequence after eleven rounds of SELEX selection by PAGE. (c) Secondary structure of Cd-4 aptamer predicted using *Mfold* software. The part of 30-random sequence marked with red line has folded into a stem-loop structure. (d) CD spectra of the Cd-4 aptamer solutions in the absence and presence of 1 μM Cd(II). The finial concentration of ssDNA used for CD analysis was about 500 nM. (e) The eluted ssDNA from the immobilized Cd-4 aptamer that incubated with 200 μM Cd(II) and other competitive metal ions.

The dissociation constant (K_d) is a key parameter for the potential application of aptamer, and its value is inversely associated with the binding affinity to target. Herein the nanomolar scaled K_d value of Cd-4 aptamer was determined as low as 34.5 nM (**Fig. S3, ESI†**), according to the fluorescence measurement described in section 2.6. To further understand the affinity interaction, the CD measurement was utilized to monitor the confirmation change of Cd-4 aptamer in the

solutions. **Fig. 3(d)** shows the CD spectra of aptamer solutions in the absence (black line) and presence (red line) of 1 μM Cd(II). Cd-4 aptamer (generally B-DNA) has a negative peak at 240-260 nm and a positive peak at 270-290 nm. On the addition of Cd(II), the values of negative and positive peaks were decreased, but the peaks position did not change. This result indicated that Cd(II) could bind to Cd-4 aptamer, which interfered bases from the transition of strong $\pi \rightarrow \pi^*$ with ribodesose, thus resulted in the decrease of CD peak.

The specificity of Cd-4 aptamer against other competitive metal ions was carried out. As shown in **Fig. 3(e)**, the ratio of eluted ssDNA is approximate to 47% in the presence of Cd(II), while most of other competitive metal ions except for Hg(II), Pb(II), Cu(II) and Zn(II), have displayed a negligible influence on the selectivity of Cd-4 aptamer. The addition of Hg(II) had caused the 15% elution ratio of immobilized Cd-4 aptamer, which may be attributed to the special T-Hg(II)-T mismatched base pair between Hg(II) and Cd-4 aptamer. The interference from Pb(II) may be ascribed to its special affinity toward the base of G, because Pb(II) will promote the G-rich sequences to form the structure of G-quadruplex under suitable conditions. Zn(II) and Cd(II) belong to the same family in the periodic table of chemical elements, so they share the similar electrons distribution and may form the same coordination bonds for binding Cd-4 aptamer. The non-specific binding of Cu(II) may be associate with the affinity to amino groups of bases or carboxyl groups of phosphate backbone in Cd-4 aptamer.

3.5. Colorimetric detection of Cd(II) using Cd-4 aptamer

According to above results, Cd-4 aptamer showed the highest affinity to Cd(II) with a K_d value of 34.5 nM, so it was further considered as a recognition element for the simple detection of Cd(II). Our previous report had proposed a novel colorimetric method for arsenic(III) detection based on the aggregation of AuNPs by the special interaction between aptamer and cationic polymer.[15c] Herein we adopted the similar strategy in an attempt to detect Cd(II), as shown in **Fig. 4(a)**. PDDA is a water-soluble cationic polymer, and serves dual functions involving in the aggregation of AuNPs and the non-specific binding to aptamer through the electrostatic interaction. In the absence of Cd(II), the Cd-4 aptamer is free and can hybridize with PDDA to form a "duplex" structure, thus the subsequent AuNPs cannot aggregate owing to the lack of PDDA. On adding Cd(II), the Cd-4 aptamer is exhausted firstly due to the formation of Cd(II)-aptamer complex, so that the subsequent PDDA can aggregate AuNPs, which lead to the remarkable change in color from wine red to blue. The optical property of solution depends on PDDA concentration, which is in turn conditioned directly by the amount of Cd(II). Therefore, this strategy makes it possible to detect Cd(II) by colorimetric assay.

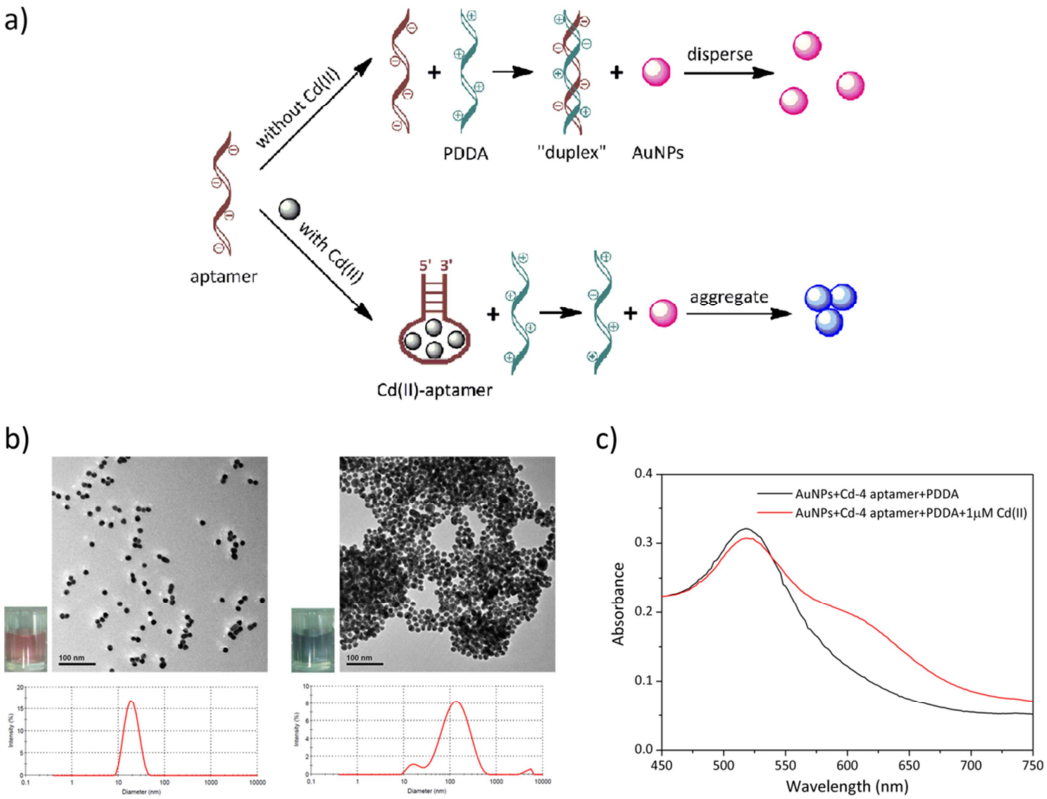

Fig. 4 (a) Schematic illustration of the colorimetric detection of Cd(II) based on cationic polymer mediated aggregation of AuNPs using Cd-4 aptamer as recognition element. (b) Colors, TEM images and size distribution (bottom) of AuNPs incubated with Cd-4 aptamer and PDDA in the absence (left) and presence (right) of 1 μM Cd(II). (c) The absorption spectra of AuNPs solutions treated with Cd-4 aptamer, PDDA and 1 μM of Cd(II). Experiment conditions: 1.52 nM PDDA and 10 nM aptamer.

To confirm the supposed principle of such strategy, the TEM and PCS analysis were employed to characterize the aggregation of AuNPs. As shown in **Fig. 4(b)**, the AuNPs solution incubated with Cd-4 aptamer and PDDA displayed wine red, in which AuNPs dispersed evenly with average particle diameter of about 30 nm. However, the introduction of 1 μM Cd(II) caused the color of above solution significantly changed to blue, and the average particle size of AuNPs had increased up to 110 nm. In addition, the absorption spectra of AuNPs solutions also changed in the presence of Cd(II) ions (**Fig. 4(c)**), where the absorbance values declined at 520 nm and enhanced at 650 nm, respectively. These data further indicate that the principle of such strategy is feasible to detect Cd(II) in aqueous solution.

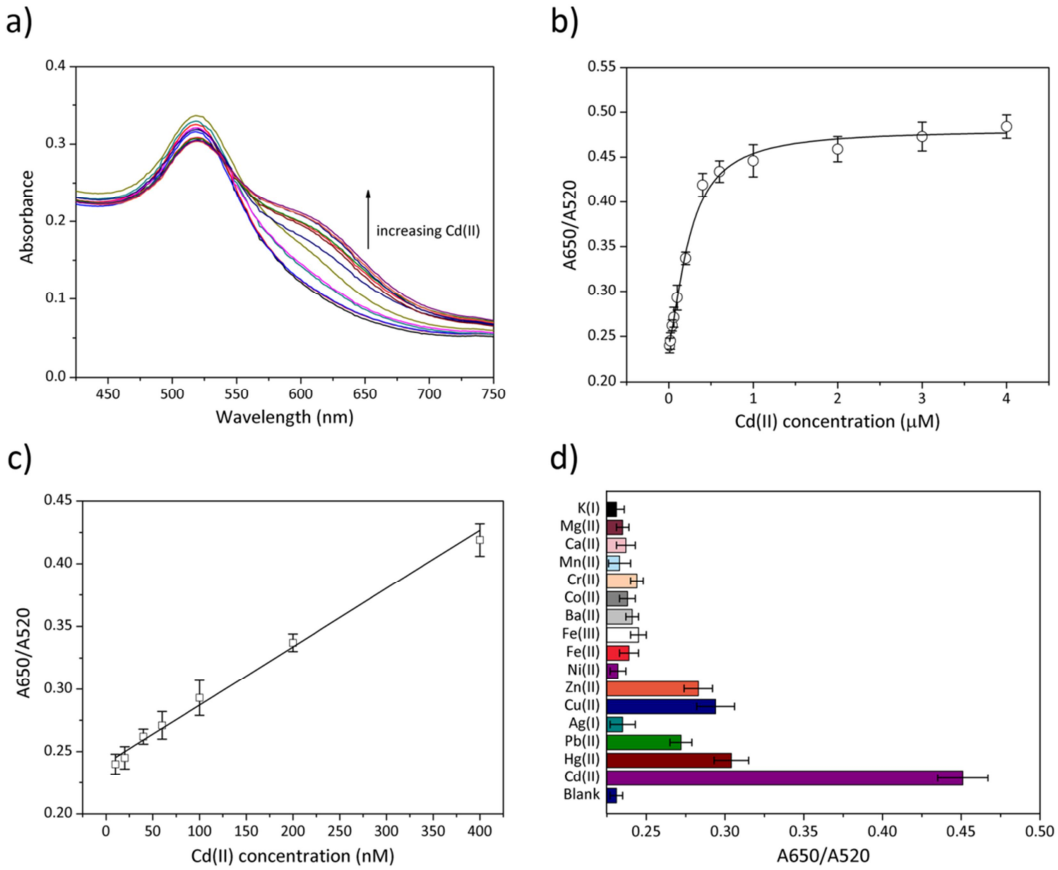

Fig. 5 Sensitivity and selectivity of the AuNPs-based colorimetric assay for Cd(II). (a) The absorption spectra of the sensing solutions treated with 0, 0.01, 0.02, 0.04, 0.06, 0.1, 0.2, 0.4, 0.6, 1, 2, 3 and 4 μM Cd(II). (b) Calibration curve of the absorbance values. The curve was fitted to Hill plot with a correlation coefficient of 0.992. (c) The values of A650/A520 at low Cd(II) concentrations, which was fitted to a Linear plot with a correlation coefficient of 0.991. (d) Selectivity of the colorimetric detection of Cd(II). The concentrations of metal ions were all 1 μM. Experiment conditions: 1.52 nM PDDA and 10 nM Cd-4 aptamer.

The aggregation of AuNPs was directly depended on the interaction between PDDA and aptamer, so the concentrations of them needed to be optimized prior to Cd(II) detection. Based on our previous study,[15c] 1.52 nM of PDDA was sufficient for AuNPs aggregation, thus such a concentration was used in the following experiments. To obtain the optimal condition for aptamer, the varying concentrations of Cd-4 aptamer in the range of 1 nM~50 nM were used. As observed in **Fig. S4 (ESI†)**, the maximum signal variation had achieved at 10 nM of Cd-4 aptamer in the presence of 1 μM Cd(II).

For the sensitivity study, the sensing solutions were treated with varying concentration of Cd(II) from 10 nM to 4 μM, and the absorption spectra and their values at 520 nm and 650 nm were recorded. As shown in **Fig. 5(a)**, the absorption spectra of solutions changed and the A650 values increased gradually with the increasing concentration of Cd(II). To quantify the detection limit of present method, the ratio of A650/A520 was plotted, and the calibration curve looked like a sigmoid shape (**Fig. 5(b)**), which was fitted to *Hill* plot with a correlation coefficient of 0.992. It can be observed that the ratio of A650/A520 had changed slightly when the concentration of Cd(II) was over than 1 μM in the sensing solution, which indicated that the binding interaction between Cd-4 aptamer and Cd(II) had reached a balance and saturation. Thus the saturated binding ratio of Cd(II) to aptamer is calculated as about 100:1 (mole ratio). Then the values of A650/A520 at low Cd(II) concentrations was fitted to linear (**Fig. 5(c)**) with the equation of $y=4.65\times10^{-4}x+0.024$ (R=0.991). Based on previous report,[27] 3σ/slope was used to determine the detection limit of present method as low as 4.6 nM (≈ 0.22 μg/L), which is one order of magnitude lower than U.S. EPA defined toxicity level of cadmium in drinking water (5 μg/L). The results indicate that

the present method can be potentially used to detect Cd(II) in aqueous solution with high sensitivity.

The selectivity of the method for Cd(II) detection was also investigated. A variety of competitive metal ions, including Hg(II), Pb(II), Ag(I), Cu(II), Zn(II), Ni(II), Fe(II), Fe(III), Ba(II), Cr(II), Co(II), Mn(II), Ca(II), Mg(II) and K(I), were individually added to the sensing solutions, and the values of A650/A520 were calculated. **Fig. 5(d)** shows the difference in absorbance values between blank and solutions containing 1 μM of Cd(II) and other metal ions. The results demonstrate that Hg(II), Pb(II), Cu(II) and Zn(II) display some interference on the present method for Cd(II) detection. To improve the selectivity of current method, some suitable chelating ligands were used for masking above interfering ions. Such strategy had been widely adopted elsewhere in the previous works.[1a, 28] Ethylene diamine tetraacetic acid (EDTA) is a common chelating reagent and has strong affinity to many metal ions. Ye and co-work recently used EDTA to eliminate the interference of Co(II) in the detection of Cd(II).[28a] But EDTA may be unsuitable for the present method because it has almost the same stability constant (log *K*) of 16.46 for Cd(II) and 16.50 for Zn(II). We found that other chelating reagents like PDCA and NTA could eliminate the interference of above four metal ions. As shown in **Fig. S5 (ESI†)**, the selectivity of current method toward Cd(II) has been improved to some extent. Especially, the interference from Hg(II) and Cu(II) has reduced significantly. The stability constants of metal ions with PDCA are log *K* = 20.2 (Hg), 17.1 (Cu), 13.0 (Zn), 11.8 (Pb) and 10.0 (Cd),[29] so it can effectively mask above four metal ions in the present assay. In solutions with excess of chelating ligand, heavy-metal ions form 1:2 complexes with the tridentate dianion of PDCA. Previous works also employed PDCA as an efficient chelating ligand to eliminate the interference of heavy-metal ions like Hg(II) and Pb(II).[28c-e] NTA has higher affinity for Zn(II) ion (log *K* = 10.66) than that for Cd(II) ion (log *K* = 9.78), which enable it to mask Zn(II) during the detection of Cd(II).[1a] Combined with these masking reagents, the selectivity of the method has been indeed improved to some extent.

Conclusions

In this paper, we have presented a novel SELEX strategy for the selection of DNA aptamers against those small molecules like cadmium ion that lack enough sites for immobilization. The principal character of such SELEX strategy is to immobilize ssDNA libraries rather than target molecules on a matrix for the incubation and separation of target-specific DNA aptamer. After ten rounds of positive and an additional round of negative selections, twelve nonrepeating T-rich and G-rich sequences (Cd-1 to Cd-12) were identified, of which the Cd-4 aptamer displayed highest binding affinity to Cd(II). The secondary structures of these sequences were then predicted and indicated that a stem-loop structure folded by the domain of their 30-random sequence is critical for aptamers to bind targets. The interaction between the selected Cd-4 aptamer and Cd(II) was confirmed by CD analysis, and the binding specificity toward other competitive metal ions was also investigated. Binding assays using FAM-modified aptamer reveal that the K_d value of the Cd-4 aptamer was determined as low as 34.5 nM for Cd(II). The Cd-4 aptamer was further considered as a recognition element for the colorimetric detection of Cd(II) based on the aggregation of AuNPs by cationic polymer. Using this approach, Cd(II) in aqueous solution can be detected as low as 4.6 nM, which is one order of magnitude lower than U.S. EPA defined toxicity level of cadmium in drinking water. Combined with some masking reagents to further improve the selectivity, the selected Cd-4 aptamer will play a potential role in the detection of Cd(II) or other applications like the recovery of polluted cadmium.

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