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A label-free electrochemical biosensor based on a DNA aptamer against codeine

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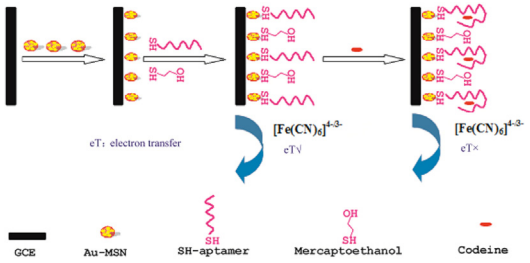
HIGHLIGHTS

- A novel DNA aptamers against codeine were generated by SELEX in this article.
- A novel electrochemical label-free codeine aptamer biosensor was developed.
- A very low detection limit was achieved.
- The optimal conditions for aptamer binding were studied by us.
- We studied the secondary structure of aptamer.

GRAPHICAL ABSTRACT

The stepwise procedure of electrochemical aptamer sensor.



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ABSTRACT

In order to develop a sensor for opium alkaloid codeine detection, DNA aptamers against codeine were generated by SELEX (systematic evolution of ligands by exponential enrichment) technique. An aptamer HL7-14, which is a 37-mer sequence with K_d values of 0.91 μM, was optimized by the truncation-mutation assay. The specificity investigation shows that HL7-14 exhibits high specificity to codeine over morphine, and almost cannot bind to other small molecule. With this new selected aptamer, a novel electrochemical label-free codeine aptamer biosensor based on Au-mesoporous silica nanoparticles (Au-MSN) as immobilized substrate has been proposed using $[Fe(CN)_6]^{3-/4-}$ as electroactive redox probe. The linear range covered from 10 pM to 100 nM with correlation coefficient of 0.9979 and the detection limit was 3 pM. Our study demonstrates that the biosensor has good specificity, stability and well regeneration. It can be used to detect codeine.

1. Introduction

Codeine (3-methylmorphine) is an opiate that is widely used to treat mild to moderate pain and cough suppression. It is the second-most predominant alkaloid in opium with a mild sedative effect [1]. Despite its extensive medical applications, codeine is often abused for its euphoric and depressant effects as well as to prevent opiate withdrawal [2]. It is very harmful to take codeine in large quantities and now it has been attributed to be controlled medicine in most countries. It is very meaningful to detect codeine in forensic analysis or clinic diagnostics. There are many methods, such as UV spectrophotometric techniques [3], gas chromatography–mass spectrometry [4], capillary electrophoresis [5] and high-performance liquid chromatography [6], have been applied in the determination of codeine. Although these above assay methods have accuracy

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and sensitive detection limit, it also has the obvious disadvantages of valuable apparatus, complicated manipulation and unapproachable in applying in field-tests. Therefore, it is highly desired to set up a sensitive method for detection of codeine.

Aptamers are factitious DNA or RNA ligands evolved by an in vitro selection technique named SELEX (systematic evolution of ligands by exponential enrichment) [7], which can specifically bind to a variety of targets [8]. Aptamers as a kind of novel molecules for the use of recognition are firstly suggested by Gold and Szostak [9,10]. Compared to antibodies, aptamers have more advantages, such as chemical synthesis, easy modification, high stability, target versatility, easy-to-stock, and resistant to denaturation and degradation [11]. Because of these advantages, aptamers have attracted more and more attentions. Up to date, more than 200 aptamers have been generated against various targets ranging from small molecules to live cells, such as metal ions [12–14], organic dyes [15], amino acids [16], nucleotides [17], and cells [18].

With the development of research, aptamers are being applied more broadly in the fields of diagnostics, therapeutics, research, separation and bioanalysis [19,20]. Aptamers for detection have been developed on the basis of different technologies, for example, fluorescence [21,22], surface plasmon resonance spectroscopy [23], microgravimetric [24], quartz crystal microbalance [25], and electrochemistry [26]. Amongst these technologies, electrochemical methods have attracted most attention because of its advantages such as simple instrumentation, rapidness, low production cost, high sensitivity and portability [27,28].

At present, RNA aptamers binding to codeine was confirmed [29]. A cyclic electrochemical voltammetry method based on this RNA aptamer has been reported to respond codeine in the concentration range of 0.1–1.0 μM [30]. However, it was still necessary for evolving DNA aptamers for specific recognition of codeine because DNA aptamers possess relatively high stability and low cost of chemical synthesis compared to RNA [16].

Due to the dual function of porous materials and nano materials of mesoporous silica nanoparticles (MSN), they have been applied in many fields, such as catalyst, drug release, sensors and separation [31,32]. Moreover, MSN possesses the properties of large surface area, high pore volume, controlled pore structure, high thermal and mechanical stability and easiness to be functionalized. And, the functionalized MSN can encapsulate or immobilize a large amount of payloads, such as mediators, enzymes, and antibodies [33]. Here, Au-MSN was synthesized by reduction method, which can immobilize thiolated DNA aptamers.

In this article, DNA aptamers that bind the opium alkaloid codeine have been evolved by a SELEX technique. The truncation-mutation assay identified a strong binding sequence (HL7-14) with a length of 37-mer. In order to find out the optimum binding condition of HL7-14, effects of ionic strength and pH on the binding ability and the specificity to codeine were investigated. A DNA aptamer biosensor based on the Au-MSN modified electrode for detection of codeine was fabricated and a very low detection limit of 3 pM was achieved.

2. Experimental

2.1. Chemicals and materials

All unlabeled oligonucleotides were synthesized by Sunbiotech (Beijing, China), carboxyfluorescein (FAM) labeled ssDNAs and biotinylated aptamers were synthesized by Sangon Biological Engineering Technology & Services Co. Ltd. (Shanghai, China). Thiolated aptamers were synthesized by Takara Biotechnology (Dalian, China). Epoxy-activated Sepharose 6B^{TM}, Streptavidin Sepharose High Performance and the NAP-5 column were purchased from GE Healthcare (GE Healthcare, Sweden). Codeine was purchased from the Material Evidence Identification Center of Ministry of Public Security (Beijing, China). 2-Mercaptoethanol (MCE, $HSCH_2CH_2OH$) was obtained from Lanji Biological Limited Corporation (Shanghai, China). Chitosan (CHIT) ($MW \sim 1 \times 10^6$, 75–80% deacetylation) and tris-(2-carbox-yethyl) phosphine hydrochloride (TCEP) were purchased from Sigma (St. Louis, MO, USA). Tetrachloroaurate(III) tetrahydrate ($HAuCl_4 \cdot 4H_2O$, 47.8% Au) were obtained from National Chemical Reagent Co. Ltd. (Shanghai, China). Taq polymerase, PCR buffer and dNTP were all obtained from Sunbiotech (Beijing, China). All other chemicals were of analytical grade and used without further purification. Deionized water (18.2 MΩ) was used throughout the experiment.

2.2. Apparatus

All the labeled oligonucleotides were purified by HPLC (FL2200, Wenling, China) with a C18 column (Agela, 5 μm, 100 Å, 4.6 mm × 250 mm, China). Electrochemical impedance spectroscopy (EIS) and differential pulse voltammetry (DPV) were carried out with a CHI 660D electrochemistry workstation (Shanghai CHI Apparatus Corporation, China). The transmission electron microscopy (TEM) measurements of the modified electrode surfaces were performed on a JOEl JEM-2100(UHR) transmission electron microscope (Japan)

2.3. Immobilization of codeine to epoxy-activated Sepharose

Epoxy-activated Sepharose 6B^{TM} was used for immobilization of codeine. According to the protocol from manufacturer, approximately 300 mg of epoxy-activated Sepharose 6B^{TM} was washed thoroughly with deionized water to obtain wet medium, and incubated with 2.5 mM codeine in coupling buffer [0.05 M Na_2HPO_4 (adjust the pH value to 13 with 1 M NaOH aqueous solution)] for 40 h at 37 °C with mild shaking. The coupled medium was washed for several times with 2 mL of coupling buffer to remove uncoupled codeine. Then, the medium was washed for several times with a solution containing 0.1 M NaAc (pH 4) and 0.5 M NaCl followed by a second solution containing 0.1 M Tris–HCl (pH 8) and 0.5 M NaCl. After washing, this obtained medium was suspended in 3.0 mL deionized water and stored at 4 °C before use. Cleaned epoxy-activated Sepharose 6B^{TM} was prepared for the counter-selection in order to reduce the non-specific sequences bound to beads.

2.4. In vitro selection of codeine-binding aptamers

The initial DNA random library consists of a central random region of 45 nucleotides (N45) flanked on either side by 20 nucleotides primer binding regions for amplification and cloning. The sequences of random library and primers were used as follows: ssDNA library 5′-AAGGAGCAGCGTGGAGGATA-(N)45-TTAGGGTGTGTCGTCGTGGT-3′; primer1:5′-FAM-AAGGAGCAGCGTGGAGGATA-3′; primer2: 5′-Biotin-ACCACGACGACACACCCTAA-3′.

For the aptamer selection process, codeine-immobilized beads were washed twice with 100 μL of binding buffer (140 mM NaCl, 2.5 mM $MgCl_2$, 2.5 mM KCl, 1.6 mM KH_2PO_4, 15 mM Na_2HPO_4 and 0.02% tween 20, pH 7.4) before each selection round. The ssDNA pool dissolved in 200 μL of binding buffer was denatured by heating at 95 °C for 5 min, immediately cooled on ice for 10 min and then kept at room temperature prior to use. In the initial selection round, the ssDNA pool (≈4.5 nmol) was incubated with 200 μL of codeine-immobilized beads at 25 °C for 60 min with mild shaking. After washing with 200 μL of binding buffer, the bound oligonucleotides were eluted by 200 μL of elution buffer (25 mM Tris–HCl,

10 mM EDTA, 3.5 M urea, pH 8.0) at 95 °C for 15 min and the fluorescence intensity of eluted ssDNAs was measured by SpectraMax M5 (Molecular Devices, USA) at 530 nm with the exciting wavelength at 485 nm. Then, the oligonucleotides were precipitated with ethanol in the presence of 20 mg mL^{-1} of glycogen at 20 °C. The ssDNA was then resuspended in deionized water for the PCR amplification with FAM-and Biotin-labeled primers (10–20 cycles of 30 s at 94 °C, 30 s at 56 °C, and 30 s at 72 °C). The sense ssDNA was separated from the biotinylated antisense ssDNA strand by streptavidin-coated Sepharose beads and used for next-round selection after being desalted with a NAP-5 column. To eliminate DNA molecules which non-specifically bind to beads, from the second round of selection, DNA pool was firstly incubated with unmodified beads. Then, the flow-through fraction was incubated with codeine-modified beads, and the subsequent procedure was the same as the initial round. After 19 rounds of selection, the selected ssDNA pool was PCR-amplified with unlabeled primers, then cloned into *Escherichia coli* using the TA cloning kit and sequenced by Beijing Sunbiotech Institute (Beijing, China).

The sequence similarities of the aptamers were analyzed by using web based Internet-tools (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The secondary structures of the ssDNA aptamers were analyzed by using an Internet-tool m-fold which is based on free energy minimization algorithm according to Zuker [34].

2.5. Characterization of aptamers

The affinity of these aptamer candidates binding to codeine was tested by the procedure similar to the SELEX. In short, synthetic ssDNA dissolved in binding buffer were denatured at 95 °C for 5 min, quickly cooled on ice for 10 min and then kept at room temperature. Then, 10 μL of codeine-immobilized beads were added into the above solution, incubated with the ssDNA for 30 min at room temperature. After washing with binding buffer, the bound ssDNA was eluted off by incubating the codeine-coated bead-ssDNA complex in elution buffer for 15 min at 95 °C. The absorbance of the eluted aptamers from the beads was measured by SpectraMax M5 at λ_{max} = 260 nm.

The equilibrium dissociation constant (K_d) of aptamer was determined by static adsorption method [16]. Different concentrations of FAM-labeled aptamers were incubated with 10 μL of codeine-beads in buffer (140 mM NaCl, 2.5 mM $MgCl_2$, 2.5 mM KCl, 1.6 mM KH_2PO_4, 15 mM Na_2HPO_4, 0.02% tween 20, pH 7.4) for 30 min, respectively. After centrifuging, the fluorescence intensities of the supernatants were measured by SpectraMax M5 at 530 nm with excitation at 485 nm. K_d was calculated by fitting the dependence of the amount of adsorbed HL7-14 on the concentration of free ssDNA to the equation ($Y = B_{max}X/(K_d + X) + N_sX$) using the Systat SigmaPlot version 11 (San Jose, USA).

2.6. The influence of ionic strength and pH on aptamer binding

The influence of Na^+, K^+ and pH on the binding of HL7-14 to codeine was investigated. For evaluating the influence of Na^+, binding experiments were performed in phosphate buffer (15 mM K_2HPO_4 and KH_2PO_4 buffer, 2.5 mM KCl, 2.5 mM $MgCl_2$, pH 7.4, 0.02% Tween 20) with different concentrations of Na^+. Phosphate buffer (15 mM Na_2HPO_4 and NaH_2PO_4 buffer, 140 mM NaCl, 2.5 mM MgCl, pH 7.4, 0.02% Tween 20) plus different concentrations of K^+ was used for evaluating the influence of K^+ on aptamer binding. The pH influence was tested in phosphate buffer (Na_2HPO_4/KH_2PO_4 buffer, 140 mM NaCl, 2.5 mM KCl, 2.5 mM $MgCl_2$ 0.02% Tween 20). The pH value was adjusted by adding 1 M NaOH or 2 M HCl to the buffer. The binding assay procedures in this section were identical to the binding assay experiments described above. The influence of Na^+, K^+ and pH on the binding of control sequence (5′-FAM-ACAGGCACGTTGGTTAGGTCAGGTTTGGGTTTCGTGCTTG) to codeine was also investigated.

2.7. Specificity assays

The specificity of HL7-14 to codeine was investigated by competitive binding assay using analogs of codeine as competitor 0.2 μM fluorescein-labeled aptamer HL7-14 and different kinds of competitor (binding buffer, 0.25 mM codeine, 0.25 mM morphine, 10 mM morphine, 10 mM L-phenylalanine, 10 mM glutamic acid, 10 mM L-valine, 10 mM L-histidine, 10 mM L-lysine, 10 mM L-arginine, all of the competitors were prepared with binding buffer) were incubated with codeine-immobilized beads in 200 μL binding buffer for 30 min at 25 °C with shaking, respectively. After washing with binding buffer, the bound aptamers were eluted with 200 μL of elution buffer. The fluorescence intensity of each elution fraction was measured by SpectraMax M5. All of the above experiments were performed in at least triplicates for error analysis.

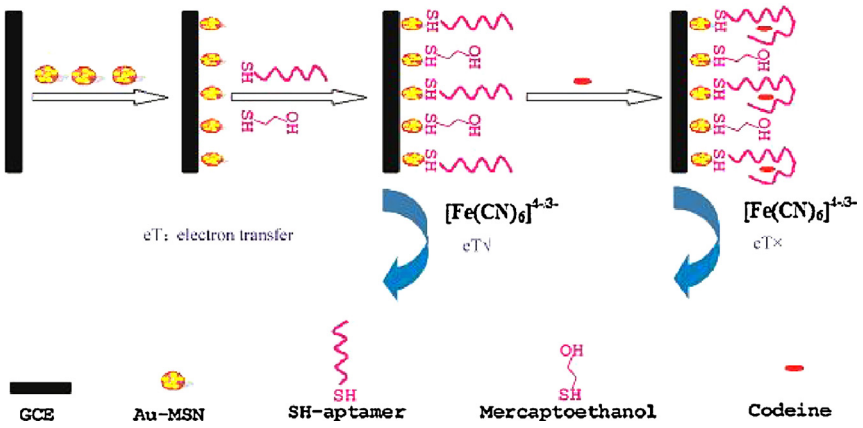
2.8. Synthesis of Au-MSN

Mesoporous silica nanoparticles (MSN) were synthesized according to following procedure reported by Lin [35]. Firstly, N-cetyltrimethylammonium bromide (CTAB, 1.00 g, 2.74 mmol) was dissolved in 480 mL of deionized water. Sodium hydroxide aqueous solution (2.00 M, 3.50 mL) was introduced to the CTAB solution and the temperature of the mixture was adjusted to 80 °C. Tetraethoxysilane (TEOS, 5.00 mL, 22.4 mmol) was added dropwise to the surfactant solution under vigorous stirring. The mixture was allowed to react for 2 h to give rise to a white precipitate. This solid crude product was filtered, washed with nanopure water and methanol, and dried under high vacuum to yield the as-synthesized MSN. To remove the surfactant template (CTAB), 1.50 g of the as-synthesized MSN was refluxed for 6 h in a solution of 1.50 mL HCl (37.2%) in 150 mL methanol. The resulting material was filtered and extensively washed with nanopure water and methanol. The surfactant-free MSN material was placed under high vacuum with heating at 60 °C to remove the remaining solvent from the mesopores. Subsequently, MSN (1.00 g) was refluxed for 20 h in 80.0 mL of anhydrous toluene with 1.00 mL of 3-amino-propyltrimethoxysilane (APTMS) to yield the 3-aminopropyl-functionalized MSN (NH_2-MSN) material.

On that basis, Au-MSN was prepared by the following method. 1.385 g of pretreated NH_2-MSN was dispersed in 22 mL of MeOH under ambient conditions, to which 2.77 mL of 1% $HAuCl_4 \cdot 4H_2O$ aqueous solution was added dropwise under vigorous stirring. The mixture was stirred for 2 h at room temperature. Then, 10 mL of 2 mM $NaBH_4$ (75.6 mg) MeOH solution was added into the mixed liquor and followed by adding 13.8 mL MeOH under vigorous stirring for the complete reduction of $AuCl_4^-$. The solid was recovered by filtration and thoroughly washed by MeOH to remove Cl^- after 30 min stirring. The sample was finally dried in a vacuum drying oven at 60 °C for further use.

2.9. The preparation of electrochemical aptamer sensor

The aptamer sensor was fabricated by placing a drop (10 μL) of a suspension of Au-MSN in 0.5% CHIT (W V^{-1}: 1/100) onto the surface of cleaned glassy carbon electrode and leaving it to dry for 12 h at ambient temperature. The electrode was dried with nitrogen after washing slowly with water. The immobilization of thiolated HL7-14 on Au-MSN modified electrode was performed with the immersion of electrode in 2 μM aptamer in phosphate buffer solution (PBS) solution (15 mM, pH 7.4) for 8 h. Then, the aptamer-immobilized electrode was immersed into the PBS buffer containing 1 mM MCH



Scheme 1. The stepwise procedure of electrochemical aptamer sensor.

for 1 h to eliminate the non-specific binding sites and to hold a good orientation of aptamer for its good recognition ability. Then, the electrodes were taken out and rinsed with PBS buffer solution and deionized water then dried with nitrogen.

2.10. Electrochemical detection of codeine with an electrochemical indicator of $[Fe(CN)_6]^{3-/4-}$

Under the optimal experimental condition, the modified aptamer biosensor was incubated with various concentrations of codeine at 37 °C for 1 h. The incubated aptamer biosensors were rinsed thoroughly with PBS to remove redundant codeine. The electrochemical properties of the modified biosensor were characterized by DPV and electrochemical impedances spectroscopy. DPV measurements were carried out in 10 mL of 15 mM PBS containing 1 mM $[Fe(CN)_6]^{3-/4-}$ at room temperature from −200 mV to 600 mV (versus SCE). The stepwise procedure of electrochemical aptamer sensor was shown in Scheme 1. Electrochemical impedances of the electrodes were performed in a background solution of 5.0 mM $K_3Fe(CN)_6$ and $K_4Fe(CN)_6$ containing 0.1 M KCl at room temperature and the frequency range was at 0.1–10,000 Hz at 220 mV (versus SCE). The used aptamer biosensor was reusable after thermal denaturizing by immersing in hot distilled water (80 °C) for 10 min, followed by a rapid cooling in an ice bath for 10 min. The regenerated transducer surface was finally rinsed with PBS and gradually cooled to room temperature before another experiment.

3. Results and discussion

3.1. Selection of DNA aptamers binding to codeine

DNA aptamers that bound to codeine were isolated from a library of DNA molecules containing a 45 mer random region flanked by constant primer-binding sequences by the affinity chromatographic SELEX protocol [16] using codeine-immobilized Sepharose beads. Codeine was coupled on beads through the reaction between its hydroxyl group and the epoxy group on the Sepharose beads as shown in Fig. S1. In order to acquire high affinity and specificity aptamers, the selection pressure was enhanced gradually by increasing the number of washing (from 2 to 4 times), the volume of washing buffer (from 200 to 600 μL), and the volume of counter-selection beads (from 0 to 200 μL), as well as decreasing the codeine-immobilized beads (from 200 to 10 μL). Not much increase was found in the subsequent 7 rounds of selection (Fig. S2). After 19 round of selection, the enriched pool was cloned and 39 colonies were sequenced. As shown in Table 1, the sequences of 39 clones are distributed in several groups based on their sequence similarity. Each of the 39 sequenced aptamer clones was tested for its ability to bind to codeine immobilized beads, according to SELEX conditions. Through experimental study, sequence HL7-1 showed strongest binding ability. So, it was further characterized.

3.2. Truncation-mutation experiments of HL7-1

The predicted secondary structure of HL7-1 was showed in Fig. 1A. In order to identify the binding motif of this aptamer, truncation-mutation assay of HL7-1 were inspected. Firstly, six bases CTGTTT was cut from the 3′ end to obtain HL7-11. Binding assay showed that HL7-11 had strong binding ability to codeine-immobilized beads as same as HL7-1, suggesting that the six bases had little contribution to the affinity of the aptamer. However, if further truncating two bases GG from the 3′ end of HL7-11, the affinity of the resulted sequence HL7-12, was greatly weakened, which suggests that this GG was very important for binding. Changing one C-G base pair in the middle of the long stem to A-G (HL7-13), did not much decrease the binding ability of aptamer. On the basis of the predicted secondary structure, there were two G-T wobble base pairs in the stem of HL7-11. When eliminating these two G-T wobble base pairs by removing two G bases from the 5′ end of HL7-11, the resulted aptamer (HL7-14) showed stronger binding ability to codeine-immobilized beads (Fig. 1B). Further removing bases from the 5′ end of HL7-14 or mutating the base pair in the stem decreased the binding ability of the obtained aptamers (HL7-15, HL7-17 and HL7-18). Changing a G base in the middle of the G5 tract at the 3′ end of HL7-14 to A slightly decreased the binding ability of HL7-16. Since HL7-14 possessed the strongest binding ability, it was chosen as the optimal aptamer for the further use.

3.3. The influence of ionic strength and pH on aptamer binding

The influence of ionic strength and pH on aptamer binding was discussed. In biological system, Na^+ and K^+ ions are the main ions to maintain the ionic strength. Additionally, Na^+ and K^+ ions might affect the structure of the aptamers that contain G-quadruplex structure [22]. In order to find an optimized condition for future application of HL7-14, the influence of Na^+, K^+ and pH value on the binding of HL7-14 to codeine was studied.

As shown in Fig. 2A, with the increase of the concentration of Na^+ from 1 mM to 500 mM, the binding ability of HL7-14 to codeine was greatly enhanced. If continue increasing the concentration of Na^+ to 1 M or 2 M, a slight decrease of the binding of HL7-14 to codeine was observed. The concentration of Na^+ did not affect the already-low binding of the control sequence.

Table 1
Core regions of the aptamer sequences (5′–3′ direction, without primers).

Name	Core region sequences	Copies
HL1-1	GACCAACCCTAAGACACTACAATCATGGATCGAGGACTCATCCGC	11
HL1-2	GACCAACCCTAAGACACTATAATCATGGATCGAGGACTCATCCGC	1
HL2-1	GAGGATTCGTCAACCTCACCCTAAGACTTAACCGCGGCGGCCTCA	1
HL2-2	- - - - - - - CTGCAACCACACCCTAAGACTTAACCGCGGCGGCATCA	2
HL3-1	GCTGCCCATGACACACACTTAAGGCATACCACACTACACTACCGC	1
HL3-2	CAGCACCA GACACATCCTTAAGACACAGTACGCGAACGACACTAG	6
HL3-3	CACAACAGCACACGTAACACCCTAACAGTCCCGATGCCACACC	1
HL4-1	CACGAGACACACACATCGATCGGCACACCTTTACATAGCTGCTCC	3
HL4-2	CACGAGACACACACATCGACCGGCACACCTTTACATAGCTGCTCC	1
HL5-1	CACAGCTCGTCGGCATCACCCTTAGACTCCACAGATTGACGCACC	3
HL5-2	CACAGCTCGTCGGCATCACCCTAAGACTCCACAGATTGACGCACC	1
HL6-1	CACAACCCACCGGCGTCACCTCATTACGAGACGGTTTGTCACCCC	1
HL7-1	GGCCCCCTGGGTCGGGAGGGAAGGGGGTTGGGGGTGCGGTTTGTC	5
HL7-2	GGCCCCCTGGGTCGGAAAGGGAAGGGGGTTGGGGGTGCGGTTTGTC	1
HL7-3	GGCCCCCTGGGTCGGG-AGGGAAGGGGGTGGGGGGTGCGGTTTGTC	2

Oppositely, stepwise increase of the concentration of K^+ from 0 M to 2 M greatly weakened the specific binding of HL7-14 to codeine but did not affect the already-low binding of the control sequence, as shown in (Fig. 2B). This result suggests that K^+ may be unfavorable for the maintaining of the tertiary structures of HL7-14.

The pH value may affect the affinity by varying the electrostatic interaction between the aptamer and its target. As shown in (Fig. 2C), the binding ability of HL7-14 to codeine rose with the increase of pH and reached a maximum at pH 7.4. Furthermore, binding affinity of the control sequence has no obvious change with the changes of pH.

Based on the above investigation, a buffer with the physiological pH (7.4), a high concentration of Na^+ (500 mM) and 0–10 mM K^+ was chosen as a the optimal conditions for HL7-14 binding because low concentration of K^+ did not affect the binding (as shown in Fig. 2), and in biological samples K^+ usually at this concentration. Under these conditions, the equilibrium dissociation constant (K_d) of HL7-14 is measured to be 0.91 ± 0.19 μM (Fig. S3).

3.4. The specificity of HL7-14

The specificity of HL7-14 to codeine was investigated by competitive binding assay as shown in Fig. 3, in which, low fluorescence intensity means the low amount of aptamers binding to beads in the presence of competitors. The target molecule, codeine showed the strongest binding ability to HL7-14. Morphine, an analog of codeine

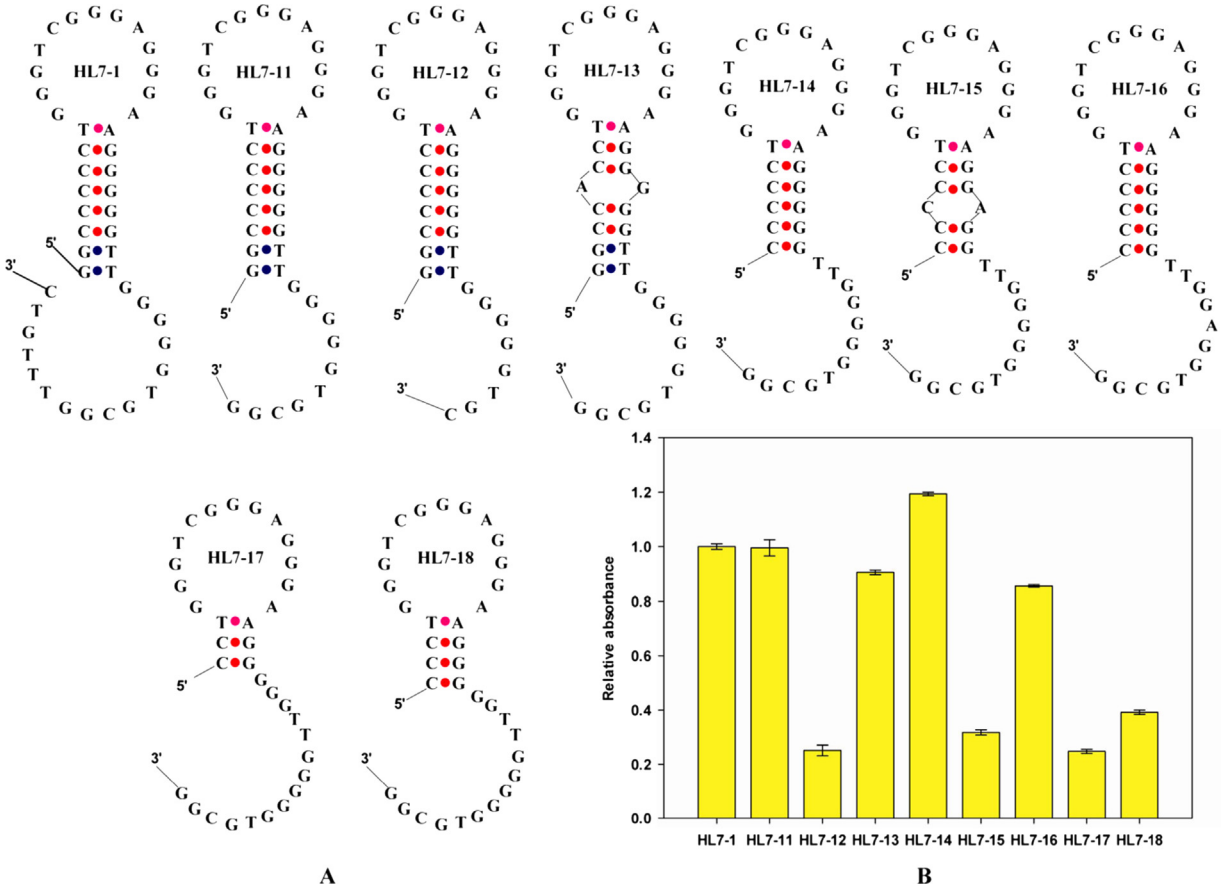


Fig. 1. Predicted secondary structure from HL7-1 to HL7-18 and (B) binding ability of aptamers to codeine-immobilized beads. Relative absorbance (%) = $A_x \times 100\%/A_0$, wherein, A_x is the absorbance of ssDNA bound on beads, A_0 is the absorbance of HL7-1.

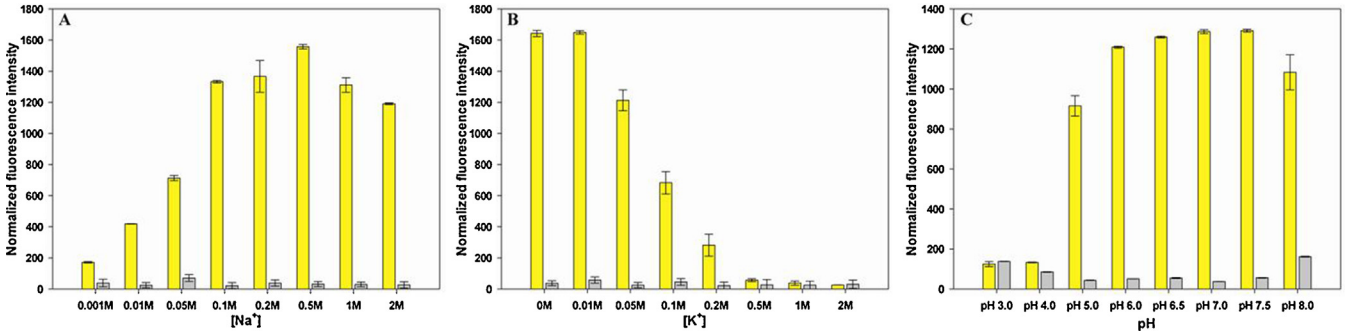


Fig. 2. (A) The influence of Na^+ on the binding of HL7-14, binding experiments were performed in phosphate buffer (15 mM K_2HPO_4 and KH_2PO_4 buffer, 2.5 mM KCl, 2.5 mM $MgCl_2$, pH 7.4, 0.02% Tween 20) plus different concentrations of Na^+. (B) The influence of K^+ on the binding of HL7-14, binding experiments were performed in phosphate buffer (15 mM Na_2HPO_4 and NaH_2PO_4 buffer, 140 mM NaCl, 2.5 mM $MgCl_2$, pH 7.4, 0.02% Tween 20) plus different concentrations of K^+. (C) The influence of pH on the binding of HL7-14, the experiments were carried out in phosphate buffer (Na_2HPO_4/KH_2PO_4 buffer, 140 mM NaCl, 2.5 mM KCl, 2.5 mM $MgCl_2$ and 0.02% Tween 20) with different pH value. Gray parts in figure are the data of the control sequences (5′-FAM-ACAGGCACGTTGGTTAGGTCAGGTTTGGGTTTCGTGCTTG).

showed much weaker binding ability than codeine. Amino acids did not show significant competing ability to the binding of HL7-14 to codeine. These results suggest that HL7-14 possesses high specificity to codeine.

3.5. Characterization of the Au-MSN nanoparticles

Because of the narrow size distribution, good biocompatibility, and ease of modification with thiol groups, AuNPs could serve as carriers for the immobilization of captured probes [36]. In our previous report, we have used Au NPs to immobilize thiolated DNA sequences by self-assembled, which verifies the signal enhancement and advancement of the performance after a modification of Au NPs due to increasing the surface area of the electrode [37]. In this work, the 5′-thiol-modified aptamer could be immobilized onto the Au-mesoporous silica nanoparticles (Au-MSN) by self-assemble. MSN displays a hollow structure. In order to increase the loading amount of AuNPs, the surfactant-free MSN was reacted with APTMS to yield the amino-group modified MSN (NH_2-MSN). $HAuCl_4$ in the mesoporous would be reduced to AuNPs by adding $NaBH_4$. Furthermore, the NH_2-MSN could chemically absorb more AuNPs onto the surface of NH_2-MSN, through Au–NH_2 binding [38]. Fig. 4 shows the TEM image of the Au-MSN, which shows a uniform particle size of approximately 100 nm.

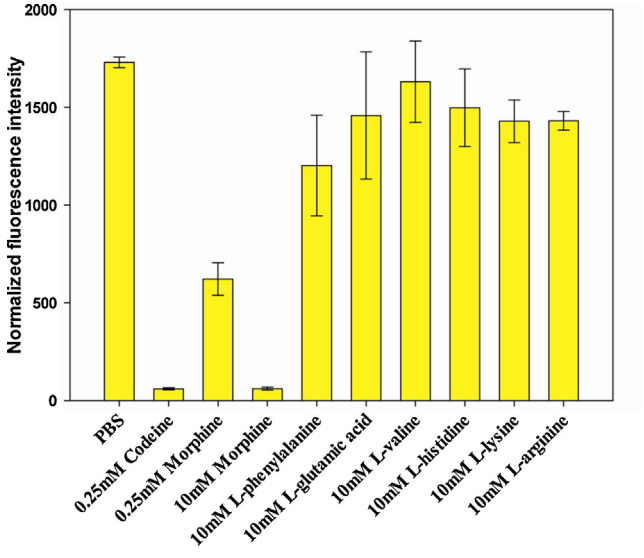


Fig. 3. Investigation of the specificity of HL7-14 by competitive binding assay.

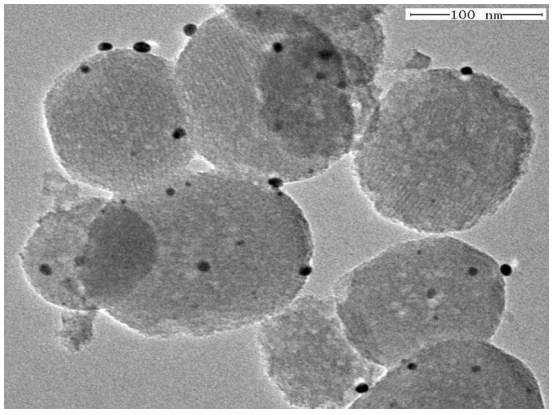


Fig. 4. TEM image of Au-MSN.

3.6. Electrochemical impedance spectroscopy of the modified electrode

Electrochemical impedance spectroscopy (EIS) could reflect the impedance changes of the electrode surface during the modification process. Fig. 5 shows the Nyquist plot of the differently modified electrodes in 5 mM $[Fe(CN)_6]^{3-/4-}$ and 0.1 M KCl at open circuit potential with the frequency varied from 0.1 Hz to

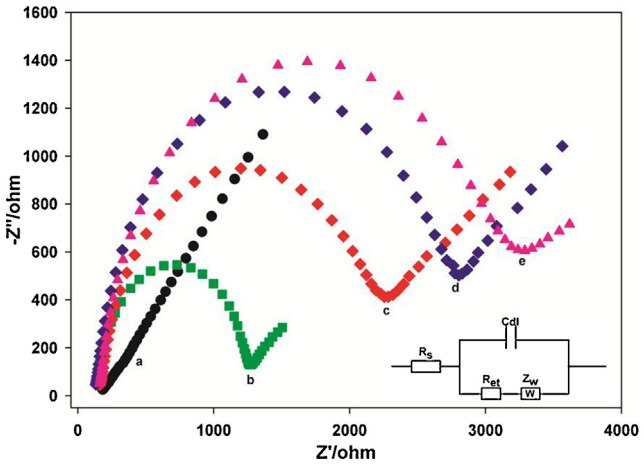


Fig. 5. Impedance characterization of different modified electrodes in 5 mM $[Fe(CN)_6]^{3-/4-}$ containing 0.1 M KCl. (a) Bare GCE, (b) Au-MSN/CHIT/GCE, (c) Aptamer/Au-MSN/CHIT/GCE, (d) MCH/Aptamer/Au-MSN/CHIT/GCE and (e) Codeine/MCH/Aptamers/Au-MSN/CHIT/GCE.

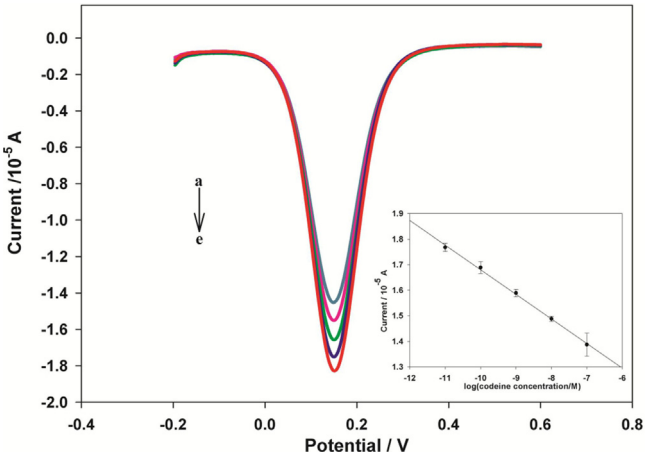


Fig. 6. The DPV responses for different concentration of codeine: (a) 100 nM, (b) 10 nM, (c) 1 nM, (d) 100 pM, and (e) 10 pM. Inset: linear relationship between the DPV peak current change and the logarithm of the codeine concentration.

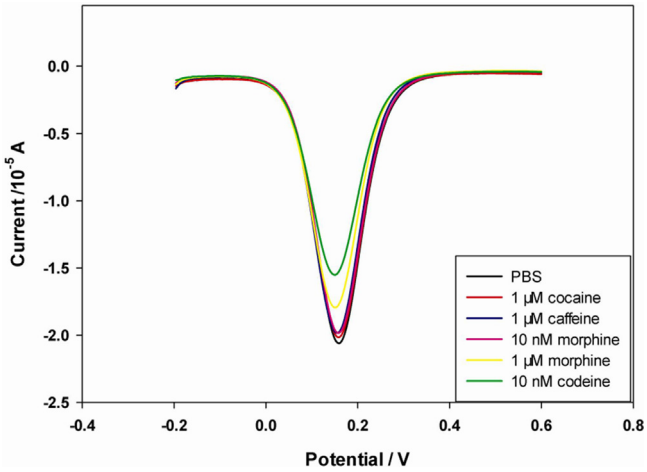


Fig. 7. The selectivity of electrochemical aptamer biosensor.

10,000 Hz. The bare glassy carbon electrode (GCE) exhibits a relative low impedance value and is almost a straight line. The R_{et} was increased to be about 1250 Ω after GCE was modified with Au-MSN. Impedance value continues to increases to 2800 Ω after incubating with aptamers and MCH. It may be attributed to the limited diffusion of $[Fe(CN)_6]^{3-/4-}$ toward electrode surface by the negative charged phosphate backbone of probe DNA. After incubating with 10 nM codeine, the Ret value was further increased to be about 3300 Ω, which could be well ascribed to the repellence of redox probe from approaching electrode surface by the specific combination of codeine and aptamers. The equivalent circuit was shown in Fig. 5 (inset).

3.7. The calibration of fabricated aptamer biosensor

Differential pulse voltammetry (DPV) was used to evaluate the performance of current aptamer sensor fabricated on the Au-MSN-modified electrode. It could be seen from Fig. 6 that the peak current decreases with the increasing of codeine concentration ranged from 10 pM to 100 nM. That is because the increased concentrations of codeine lead more switching of aptamer structure, which result in more anion exposed outsides and obstructed electron-transfer of $[Fe(CN)_6]^{3-/4-}$. The calibration curve was shown in Fig. 6 (inset). The peak current change of fabricated aptamer sensor exhibited a linear correlation to the logarithm of the codeine concentration ranged from 10 pM to 100 nM with a linear regression coefficient of 0.9979 and regression equation was $I(10^{-5}\,A) = -0.0963 \log C + 0.7175$. The detection limit of the aptamer sensor is 3 pM at 3σ, which is much lower than that of the previous report based on RNA aptamer [30]. This might attribute to the large loading amount of MSN-Au for the DNA aptamer.

3.8. The selectivity of fabricated aptamer sensor

A series of probable interferents were involved in order to investigate the selectivity of the aptamer sensor. Fig. 7 shows the DPV peak current of $[Fe(CN)_6]^{3-/4-}$ after incubating with PBS, 1 μM caffeine, 1 μM cocaine, 1 μM morphine, 10 nM morphine and 10 nM codeine respectively on the aptamer sensor. The peak current of caffeine, cocaine and 10 nM morphine are so approximate that their DPV profiles are almost overlapped. But, when incubating with 1 μM morphine, a decrease of the DPV peak current could been found. These indicated that morphine would have a little influence for the biosensor due to the similar molecular structure of morphine and codeine. Others who do not have the similar molecular structure with codeine would not produce interference.

3.9. Stability of the fabricated DNA biosensor

The stability of the fabricated DNA biosensor was investigated. Firstly, the biosensor was incubated with 10 nM codeine and its response was recorded via DPV. After regenerating, the biosensor was stored in the refrigerator at 4 °C over 10 days and then examined via DPV after it was incubated with 10 nM codeine. Experiments demonstrated that the DNA biosensor retained about 92% of its original response.

4. Conclusion

In this study, a group of DNA aptamers with high affinity and specificity to codeine were generated by an affinity chromatography-based SELEX strategy. A 37-mer sequence, HL7-14, exhibit strong affinity to codeine with the K_d of 0.91 ± 0.19 μM obtained by truncation-mutation assay. The investigation of the competitive binding assay suggested that HL7-14 exhibit high specificity to codeine over morphine and other molecules. Then a novel electrochemical aptamer biosensor based on Au-MSN as immobilized substrate had been proposed to determine codeine and a very low detection limit of 3 pM was achieved.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2013.05.024.

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