Analytical Methods

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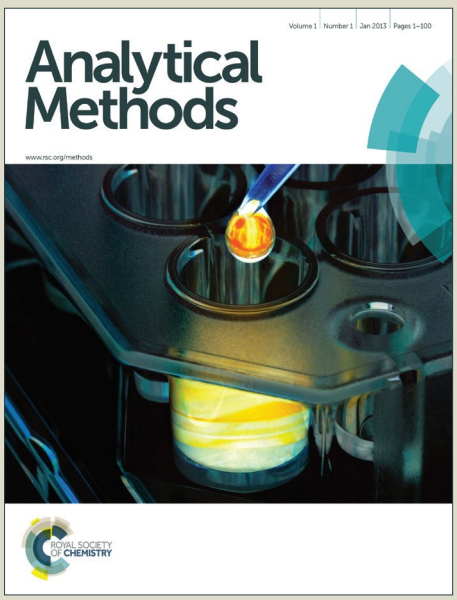

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ARTICLE

Ultrasensitive aptamer biosensor for the detection of codeine based on Au nanoparticles/polyamidoamine dendrimer-modified screen-printed carbon electrode

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This paper reports a novel aptamer biosensor for the ultrasensitive detection of codeine in aqueous solutions by the special interactions between codeine-binding aptamer. Polyamidoamine dendrimer (PAMAM) was used to absorb gold nanoparticles (AuNPs) and modify the surface of screen-printed carbon electrodes (SPCE). The codeine aptamer was immobilized on the PAMAM/glutaraldehyde (GA)/Chitosan (CHIT)-modified electrode through Au-SH affinity. The specific combination between aptamer and codeine can obstruct the electron transfer of electrochemical probe $K_3Fe(CN)_6/K_4Fe(CN)_6$, which can be used to detect codeine. The linear range covered from 1×10^{-12} mol/L to 1×10^{-7} mol/L, and the detection limit was 3×10^{-13} mol/L. The new aptamer biosensor is sensitive and selective enough to detect codeine directly in blood serum and other aqueous solutions. Compared with other conventional methods, it has several advantages, such as lower detection limit, broader detection range and fast detection process.

Introduction

Codeine (3-methylmorphine) is an alkaloid separated from opium. It has been widely used to relieve neuropathic pain for patients in clinics[1, 2]. Codeine inhibits spinothalamic projection neurons directly, alleviating pain[3]. It is not stronger than morphine for analgesic effects, but it has a diminished adverse reaction. Thus, it has been validated by World Health Organization (WHO) guidelines for cancer pain relief[4]. However, amounts of codeine in the body can also create a health risk. For that reason, the development of a highly sensitive method for detection of codeine is particularly critical.

In recent years, several methods for the detection of codeine have been developed, such as high-performance liquid chromatography (HPLC)[5, 6], gas chromatography-mass spectrometry (GC-MS)[7-9], chemiluminescence[10] and capillary electrophoresis (CE)[11]. However, these methods require expensive instruments and complex operational processes, and the detection limits are unsatisfactory. Therefore, to develop a simple, low-cost, portable and sensitive analytical method is of considerable interest.

Electrochemical biosensors have been drawing focus because of their potential utility as specific, simple and direct detection techniques with a reduction in size, cost and time of analysis compare with conventional assay techniques[12].

Aptamers are single-stranded DNA or RNA oligonucleotides evolved through an in vitro selection process termed SELEX (systematic evolution of ligands by exponential enrichment) that can bind to their targets with high affinity and specificity[13-15]. The targets include growth factors, antibodies, gene regulatory factors, cell adhesion molecules, plant lectins, intact viral particles, pathogens and so on. Aptamers are considered promising recognition elements for biosensor applications[16]. Compared to antibodies, aptamers offer several advantages, such as ease of manufacture, high stability under elevated temperatures and resistance to denaturation[17].

Since Tomalia et al. found the polyamidoamine (PAMAM) dendrimers in 1985, various polymers have been used in medicine, nanotechnology, catalysis and surface immobilization[18-20]. PAMAM contains internal amide groups, and its end branches have terminal primary amine for functionalization with suitable groups[21, 22], which can bind with nano- or microsized three-dimensional topology that allow the successful and stable

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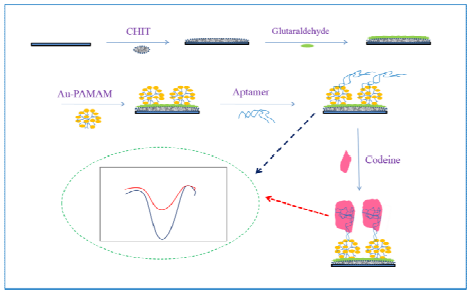
immobilization of the biomolecules and nanometer materials without affecting their biological function[23, 24].

At present, the sequence of the codeine aptamer has been successfully selected by Huang et al. in our group[25]. In this work, we utilized the codeine aptamer to construct a signal-off biosensor based on AuNPs/PAMAM-modified screen-printed carbon electrode (SPCE). The SPCE was first modified with chitosan (CHIT), which played an important role in immobilization of PAMAM according to the previous works on the use of CHIT and PAMAM [26-28]. CHIT was easy to form membrane on the surface of electrode but PAMAM was not easy to form membrane. Because both CHIT and PAMAM have amidogen, glutaraldehyde was used to cross-link PAMAM with CHIT on the surface of electrode and followed by absorption of AuNPs on the surface of bisaniline-cross-linked network[29]. Next, the codeine aptamer was immobilized on the AuNPs/PAMAM/CHIT-modified electrode through Au-SH affinity. The specific combination between aptamer and codeine can obstruct the electron transfer of electrochemical probe ($K_3Fe(CN)_6/K_4Fe(CN)_6$), which can be used to detect codeine. The stepwise fabrication procedure of aptamer biosensor was shown in Scheme 1.



Scheme 1 Stepwise procedure of the aptamer sensor to detection of codeine

Experimental

Chemicals and materials

Codeine aptamer (5'-SH-$(CH_2)_6$CCCCCTGGGTCGGGAGGGAAGGGGGTTGGGGGTG CGG-3')[25] was synthesized by Takara Biotechnology Co., Ltd. (Dalian, China); Screen-printed carbon electrode was purchased Zensor Corporation Ltd chitosan (CHIT) (MW ~ 1×10^6, 75%-80% deacetylation), PAMAM (dendrimer, generation 4, 10 wt.% solution in methanol) and phosphate buffer solution (PBS, pH 7.4) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Tetrachloroauric acid ($HAuCl_4$), mercaptoethanol, $K_3Fe(CN)_6/K_4Fe(CN)_6$, KCl and NaCl were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China); codeine, morphine, theophylline, caffeine and cocaine were purchased from the Material Evidence Identification Center of the Ministry of Public Security (Beijing, China).

Apparatus

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) was performed on a JOEl JEM-2100 (UHR) transmission electron microscope (Japan). The SPCEs were obtained from Chanpu Technology Co., Ltd. (Taiwan, R.O.C.)

The preparation of Au NPs/PAMAM

First, Au NPs were synthesized by using sodium citrate to reduce $HAuCl_4$, according to previous reports[30]. In brief, 1.085 mL of 2.346×10^{-2} mol/L $HAuCl_4$ was added to 98.915 mL of ultrapure water, and the solution was gently stirred at 110°C for 5 minutes. Then, 10 mL of 1.455×10^{-2} mol/L trisodium citrate solution was rapidly injected. The boiling solution was stirred rapidly and refluxed for 20 minutes. A few minutes later, the color of the solution gradually changed from colorless to wine-red. The solution was continuously stirred for 30 minutes at room temperature. The concentration of obtained Au NPs was about 2.53 mM with average size about 20 nm ± 2nm. PAMAM was diluted to 0.05% and added to gold colloidal at a 1:50 proportion. It combined very well with Au NPs. The color of the solution changed from wine-red to purple.

The fabrication of electrochemical aptamer sensor

The SPCE was cleaned with distilled water. The electrode was pipetted with 5 μL of 0.05% CHIT solution (dissolved in glacial acetic acid) and contained overnight. Subsequently, the electrode was gently washed with ultrapure water to remove CHIT that hadn't combined with the electrode. In order to activate the surface of electrode, 3 μL glutaraldehyde (0.5%) was added to the electrode for one hour. Then, 3 μL of Au NPs/PAMAM (PAMAM and gold colloidal at a 1:50 proportion) was placed on the electrode and left to dry for 12 hours at ambient temperature; 3 μL of 2 μmol/L aptamer was placed on the electrode and remained there for four hours to immobilize on the electrode by Au-SH affinity. Next, 2 μL of 3 mmol/L mercaptoethanol was added on

the electrode and left for one hour to block nonspecific site. Finally, the electrode was washed with PBS buffer solution, and the codeine biosensor was developed. Differential pulse voltammetry (DPV) was used to detect codeine in 10 mL of 1.0 mmol/L $K_3Fe(CN)_6/K_4Fe(CN)_6$ solution containing 1 mmol/L KCl. The DPV parameters were as following: potential interval from -0.3 to +0.5 V; increment potential, 4 mV; pulse amplitude, 50 mV; pulse width, 10 ms; scan rate, 20 mV s^{-1}.

Results and Discussion

Interaction of PAMAM and Au NPs

PAMAM dendrimers have special nanoscopic, spherical polymers and with primary amino surface groups to associate Au NPs. G4-polyamidoamine dendrimer (PAMAM) was used to connect with Au NPs, which have a great ability to integrate nanoparticles. From the TEM image shown in Fig. 1, it can be seen that the Au NPs were dispersed very well with wine-red color (Fig. 1A). After 0.05% PAMAM dendrimers were added, Au NPs gathered along the branch of PAMAM, and the structure became a large dendritic with purple color (Fig. 1B). The average size of the AuNPs was about 20 nm ± 2 nm from the size statistical analysis. The SEM image of Au NPs/PAMAM/GA/CHIT/SPCE was shown in Fig. 1C which display great dendritic structure due to AuNPs combineing with PMAM (Fig. 1 C).

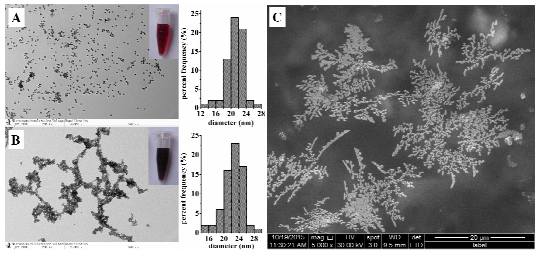


Fig. 1 TEM image of Au NPs (A). Au NPs/PAMAM (B) and SEM of Au NPs/PAMAM /GA/CHIT/SPCE (C).

Electrochemical characteristics of different modification processes

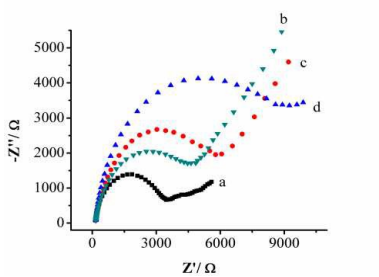


Fig. 2 Nyquist plots of impedance spectra using different modified electrodes. (a) CHIT/SPCE; (b) AuNPs/PAMAM/GA/CHIT/SPCE; (c) aptamer/Au NPs/PAMAM/GA/CHIT/SPCE; (d) codeine/aptamer/Au NPs /PAMAM/GA/CHIT/SPCE.

The electrochemical behavior of different modification processes was characterized by impedance spectra (Zim vs. Zre, Nyquist plot) in 5.0 mmol/L $K_3Fe(CN)_6/K_4Fe(CN)_6$ solution containing 0.1 mol/L KCl. As shown in Fig. 2, SPCE modified with CHIT exhibits a relative low impedance value of about 3100 Ω (Fig. 2a). After CHIT/SPCE was modified with Au-PAMAM through GA, the impedance value increased to 4500 Ω (Fig. 2b). Because the PAMAM isn't a good conductor comparing with Au nanoparticle, which can obstruct the electron transfer of electrochemical probe $K_3Fe(CN)_6/K_4Fe(CN)_6$. So, the impedance value increases upon functionalizing the surface with PAMAM/Au[31]. When thiolated codeine aptamer was immobilized on the electrode, the R_{et} increased more (Fig. 2c). After the biosensor was incubated with 50 nmol/L codeine, the interfacial resistance increased further (Fig. 2d) because of the obstruction of current by the specific combination of aptamer and codeine.

The influence of PAMAM to the impedance of electrode

The influence of PAMAM to electrode impedance is shown in Fig. 3. The electrode was modified respectively with codeine/aptamer/Au NPs/CHIT (Fig. 3a) and codeine/aptamer/AuNPs/PAMAM/CHIT (Fig. 3b). It can be clearly seen that curve b has a larger impedance value than curve a. The reason is that PAMAM absorbed much more AuNPs, which can combine more aptamer and codeine on the surface of electrode than monodispersed AuNPs to extend the range of detection and increase the sensitivity of electrode.

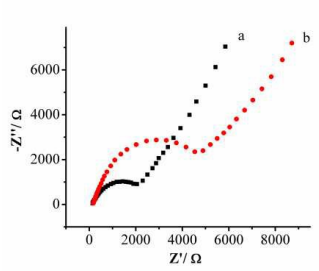


Fig. 3 The comparison of electrochemical impedance under different conditions. (a) codeine/aptamer/AuNPs/CHIT/SPCE; (b) codeine/aptamer/AuNPs/PAMAM/GA/CHIT/SPCE.

Optimal experimental condition of electrochemical aptamer biosensor

As an important factor influencing the performance of the biosensor in DPV test, the incubation time used for an assay was investigated, and shown in Fig. 4 A. With the incubation time of codeine increasing from 20 minutes to 140 minutes, the current response of aptamer biosensor decreased and reached the lowest point at an incubation time of 80 minutes, indicating that codeine combines with its aptamer at the surface of electrode and hinders electrochemical probe from reaching the surface of electrode. After 80 min, current response reaches a steady state because there is no new complex formed. SEMs of biosensor with 80 min, 140 min and 160 min of incubation time of codeine were shown in Fig. 4 B, from which one can see that the modified membrane on the surface of electrode appeared dehydrated state and began to break after 140 min, indicating that too long incubation time is not good for the stability of modified membrane. Therefore, the incubation time of 80 minutes was chosen as the optimal incubation condition for the codeine aptamer biosensor in DPV test.

The effect of assay solution pH in DPV test was also investigated and is shown in Fig. 4 C. $K_3Fe(CN)_6/K_4Fe(CN)_6$ solution was prepared with varying pH, from 6.8 to 8.0. As it can be seen, the lowest current value was at pH 7.2, which was selected as the best experimental condition in DPV test.

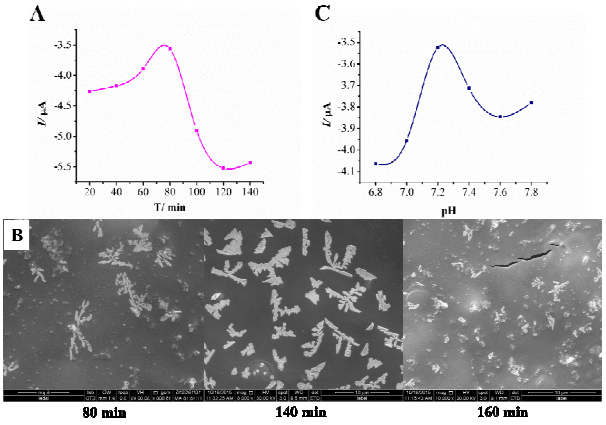


Fig. 4 A. Effect of the incubating time of codeine on the response of the sensor. B. SEM of codeine/aptamer/Au-PAMAM-GA/CHIT/SPCE with 80 min, 140 min and 160 min of incubation time of codeine. C. Effect of pH on the response of the aptamer sensor.

Characteristics of electrochemical aptamer biosensor

The electrochemical properties of the aptamer biosensor for codeine recognition were characterized under the optimal experimental conditions and studied by DPV. Taking the same concentration of codeine on three SPCEs and the results were shown in Fig. 5. With codeine concentration increasing from 1×10^{-12} mol/L to 1×10^{-7} mol/L, the current decreased and the linear regression coefficient was 0.9896. The linear equation was $I/10^{-6}$ A = -0.9148 lg C + 6.620. According to 3σ criteria, the detection limit was 3×10^{-13} mol/L. This occurred because PAMAM possesses a large amount of the amino group that could absorb abundant AuNPs, which can immobilize much more aptamer for combining with codeine.

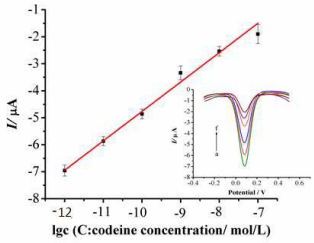


Fig. 5 The calibration curves of the aptamer sensor at different concentrations. Inset shows the DPV response of different concentration of codeine. (From curve a to f: 1 pmol/L, 10 pmol/L, 100 pmol/L, 1 nmol/L, 10 nmol/Land 100 nmol/L).

A comparison of performance and properties of this aptamer sensor with that of previous reports was given in Table 1. From Table 1 one can see that the detection limit of this aptamer sensor is lower than that of previous reports.

Table 1 Comparison of performance and properties of this aptamer sensor with that of previous reports

No	Method	Detection limit	Reference
1.	Colorimetric sensor	0.9 μmol/L	1
2.	Solid State Potentiometric Sensor	50 μmol/L	3
3	Chemiluminescence	500 nmol/L	10
4.	Aptamer biosensor based on Au-mesoporous silica nanoparticles	3 pmol/L	25
5	Electrospray ionization ion mobility spectrometry	8.5 nmol/L	32
6.	Aptamer biosensor based on AuNPs-polyamidoamine	0.3 pmol/L	This work

Selectivity of aptamer biosensor

To demonstrate the specificity of the aptamer sensor, 1 μmol/L morphine, 1 μmol/L theophylline, 1 μmol/L caffeine, 1 μmol/L cocaine and 10 nmol/L codeine were tested and shown in Fig. 6. The result showed that morphine, theophylline, caffeine and

cocaine have negligible response under the same conditions, illustrating that the selectivity of sensor is high.

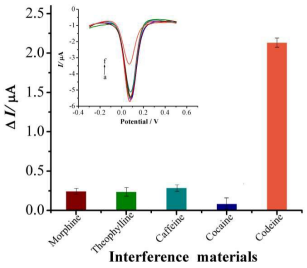


Fig. 6 Interference of different substances to response current of codeine aptamer sensor. From curve a to f: blank, 1 μM morphine, 1 μM theophylline, 1 μM caffeine, 1 μM cocaine and 10 nM codeine.

Recovery of the proposed biosensor

In order to investigate the practicability of aptamer sensor, the standard addition method was used to detect codeine spiked in real serum samples which was diluted 10 times. Table 2 shows that recovery of the biosensor was very good, which suggests that it probably can be applied to clinical detection.

Table 2 Recovery of the aptamer biosensor.

Sample added (nmol/L)	Found (nmol/L)	Recovery (%)	Average recovery (%)
0.1	0.094±0.001	94.00	
1	1.029±0.003	102.90	96.22
10	9.175±0.004	91.75	

Conclusions

In this work, a novel aptamer biosensor with excellent performance has been successfully developed for the detection of codeine based on the use of AuNPs/PAMAM- modified SPCE. The biosensor displayed many advantages, including a wide linear range from 1×10^{-12} mol/L to 1×10^{-7} mol/L, with a detection limit of 3×10^{-13} mol/L. Moreover, the results indicated that the biosensor could be used to detect codeine in real blood serum.

Acknowledgements

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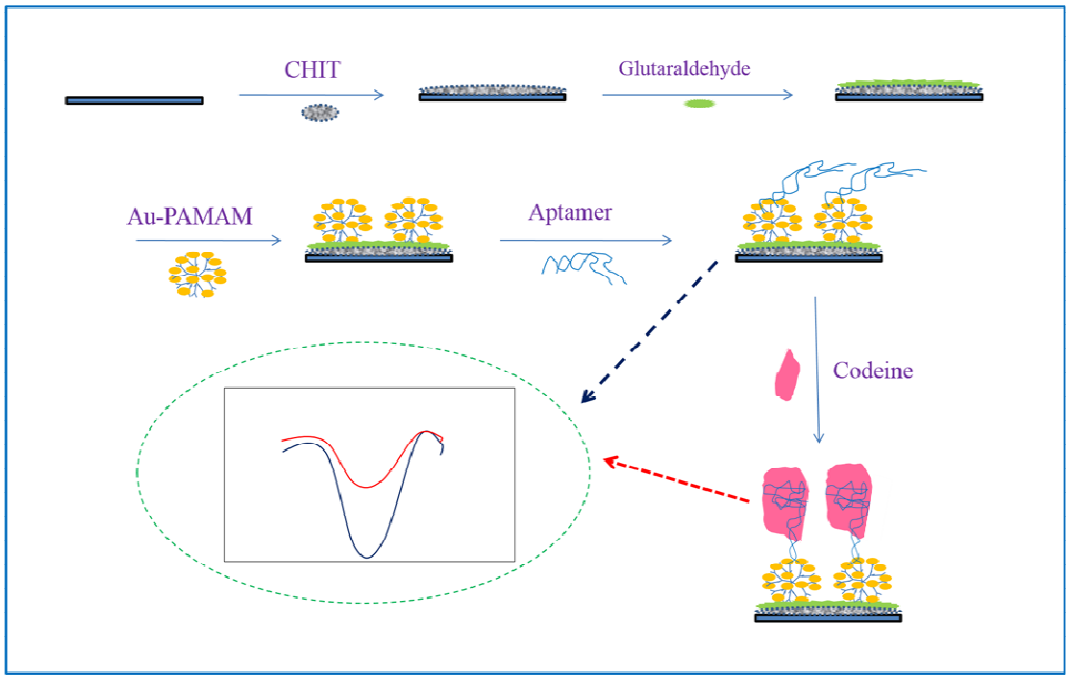
Graphical abstract



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