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Separation/Concentration-Signal-Amplification in-One Method Based on Electrochemical Conversion of Magnetic Nanoparticles for Electrochemical Biosensing

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Abstract: We propose a separation/concentration-signal-amplification in-one method based on electrochemical conversion (ECC) of magnetic nanoparticles (MNPs) to develop a facile and sensitive electrochemical biosensor for chloramphenicol (CAP) detection. Briefly, aptamer-modified magnetic nanoparticles (MNPs-Apt) was designed to capture CAP in sample, then the MNPs-Apt composite was conjugated to Au electrode through the DNA hybridization between the unoccupied aptamer and a strand of complementary DNA. The ECC method was applied to transfer MNPs labels to electrochemically active Prussian blue (PB). The anodic and cathodic currents of PB were taken for signal readout. Comparing with conventional methods that require electrochemically active labels and related sophisticated labelling procedures, this method explored and integrated the magnetic and electrochemical properties of MNPs into one system, in turn realized magnetic capturing of CAP and signal generation without any additional conventional labels. Taking advantages of the high abundance of iron content in MNPs and the refreshing effect deriving from ECC process, the method significantly promoted the signal amplification. Therefore, the proposed biosensors exhibited linear detection range from 1 to 1000 ng mL^{-1} and a limit of detection down to 1 ng mL^{-1}, which was better than or comparable with those of most analogues, as well as satisfactory specificity, storage stability and feasibility for real samples. The developed method may lead to new concept for rapid and facile biosensing in food safety, clinic diagnose/therapy and environmental monitoring fields.

Keywords: Magnetic nanoparticles · electrochemical conversion · signal amplification · antibiotics · electrochemical biosensor

1 Introduction

Detection is one of the most crucial issues in many fields, such as food safety, clinic diagnosis/therapy and environmental monitoring/treatment. Generally, for the detection in real samples, it involves two key procedures, namely, the separation/accumulation of targets from samples and the following quantification. However, in most cases, the two procedures are independent and separately conducted, which complicates the operations, increases costs of time and reagents, and may also lower the reliability due to the incorporation of different methods and reagents.

As well known, using magnetic materials, such as magnetic nanoparticles (MNPs), to separate and concentrate target has been one of the most common and promising methods for sample pretreatment due to their unique magnetism behavior and abundant surficial properties [1]. Magnetic materials also play important roles in concentrating the targets and facilitating the operations during the quantification procedures. Recently, Kalyoncu et al. reported novel electro-nano diagnostic platforms for simultaneous detection of multiple cancer biomarkers [2]. In this study, two types of nanocomposites of magnetic nanoparticle and bimetallic nanoparticles are synthesized and elaborated to simultaneously capture multiple targets and then to generate electrochemical signals based on voltammogram of metal nanoparticle labels. Besides the convenience deriving from magnetic operations, the simultaneous detection performance is also better than most analogues, which highlighted the promising prospect of MNPs for detection. Despites of above merits, magnetic materials are rarely involved in the signal readout part. Instead, it generally needs to introduce additional labels to realize signal readout and amplification, though few examples were reported solely based on relaxation time and nuclear magnetic resonance properties of magnetic materials [3]. Therefore, it remains a huge impetus and demand to explore a method that could integrate both two parts of the target separation/concentration and signal amplification, which is strongly expected to simply the

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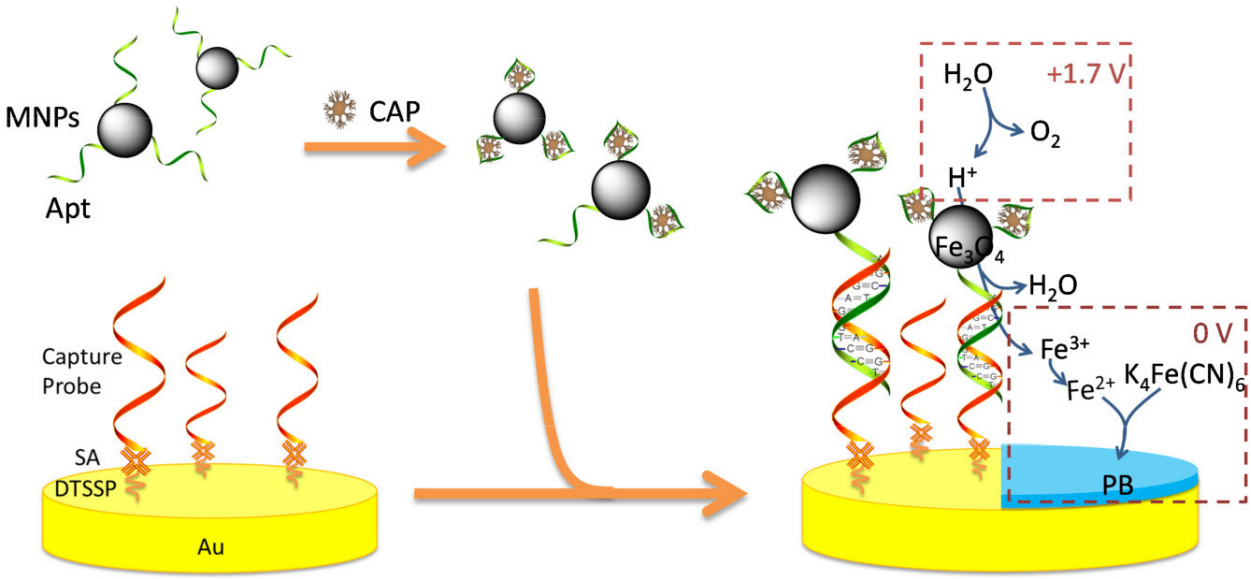

Scheme 1. Illustration of new method for electrochemical detection of CAP.

procedures and cut the usage of reagents, in turn promote the final detection efficiency and performance.

Electrochemical biosensor has attracted considerable attention for its high sensitivity and speed, simplicity, and low cost for signal output [4]. For small-molecule target, electrochemically active probes are generally required to produce and amplify signal. Currently, researches focus on the fabrication of signal composites to load more labels to enhance the sensitivity and/or more kinds of labels for simultaneous detection of multiple targets [5]. However, the procedures for the fabrication of these kinds of composites are generally complicated. Furthermore, the label composites of large-size unavoidably occupy large area of the binding interface and lower the binding amount due to the steric hindrance, in turn suppress the binding efficiency. Therefore, new method that could avoid the use of large-size composite is of high importance. Alternatively, the in-situ conversion to produce electrochemical labels might be promising.

Antibiotic residual in environment and animal foods has been concerned widely because of their serious threats to public health [6]. Chloramphenicol (CAP) is one of the most commonly used antibiotics in China and has been regulated as zero-tolerance in foods [7]. Therefore, it arises high importance and challenge to develop highly facile and sensitive detection method. Currently, there are various common methods for the detection of the antibiotics, such as HPLC [8], electrophoresis method [9], HPLC-MS [10], and immunoassay [11]. However, they either are accurate but require expensive instrument and skilled operators, or are facile but lack of sensitivity and accuracy. Therefore, developing new method that could facilely, sensitively and rapidly detect antibiotics still remains challenging.

Herein, we proposed a method that integrates the magnetism function and the chemical/electrochemical properties of MNPs to explore a separation/accumulation-signal-amplification in-one system for sensitive and facile detection of CAP. An aptamer of CAP (Apt) was adopted as the recognition element. As shown in Scheme 1, the Apt-modified MNPs (MNPs-Apt) captured and concentrated CAP from sample, followed by conjugating of MNPs composites onto electrode through the hybridization of the unoccupied Apt and the complementary capture probe DNA (ssDNA). Finally, an electrochemical conversion (ECC) method was adopted to amplify signal. Briefly, a high potential was applied to generate H^+ to react with Fe_3O_4 MNPs and make the latter release ferrous ions, followed by the application of a low potential to trigger the reaction of ferrous ions and co-existing $K_4Fe(CN)_6$ to produce Prussian blue (PB), which presented high electrochemical activity to output current signals through voltammetry. Comparing with conventional methods that require electrochemically active labels and related sophisticated labelling procedures, this method explored and integrated the magnetic and electrochemical properties of MNPs into one system, in turn realized magnetic capturing of CAP and signal generation without any additional conventional labels. Furthermore, the method elaborated the facile and efficient production of electrochemically active labels by a facile in-situ conversion of MNPs with abundant ferrous content. Therefore, the proposed biosensor presented the limit of detection down to $1\,ng\,mL^{-1}$ and feasibility for rapid detection in food samples.

2 Experimental

2.1 Materials and Apparatus

All electrochemical experiments were conducted on a CHI660C electrochemical workstation (CH Instrument Co.), and a conventional three-electrode electrolytic cell was used. The Au electrode with 3.0 mm diameter (area = 0.07 cm^2, Tianjin Incole Union Technology Co., Ltd) served as the working electrode, a KCl-saturated calomel electrode (SCE) as the reference electrode, and a carbon rod as the counter electrode. All potentials here are cited versus SCE (vs SCE). Scanning electron microscopy (SEM) images were collected on a JEM-6700F field-emission scanning electron microscope (JEOL, Japan). Fourier Transform Infrared (FT-IR) spectroscopy was collected on an Avatar370 Fourier Infrared Spectrometer (Thermo Nicolet, USA). Zeta-potential of nanoparticles was collected on a Zetasizer Nano ZS-90 (Malvern Instruments Ltd, UK). Atomic force microscopy (AFM) images were collected on a Bruker Multimode 8 AFM with nanoscope V controller (Bruker, USA). Magnetic separator (AIBIT Biotech Instrument, LLC, China) was used for magnetic separation.

Two kinds of phosphate buffer solutions, PBS (pH = 7.0, 0.1 M KH_2PO_4/K_2HPO_4 + 0.1 M K_2SO_4) and PB1 (pH = 6.8, 1 mM NaH_2PO_4 + 0.98 mM Na_2HPO_4), were used. Detection of PB was conducted in solution containing 0.1 M HCl and 0.1 M K_2SO_4. Aqueous solution containing 0.1 M K_2SO_4 and 0.4 mM $K_4[Fe(CN)_6]$ was used for ECC treatment. Streptavidin (SA), acetone, ethanolamine (ETA), CAP aptamer (Apt, 5′-NH_2-ACT TCA GTG AGT TGT CCC ACG GTC GGC GAG TCG GTG GTAG-3′) and its complementary strand (ssDNA, capture probe, 5′-biotin-CTACCACCGACTC-3′), CAP and other antibiotics were purchased from Sangon Co., Ltd. (Shanghai, China). 3,3′-Dithiobis (sulfosuccinimidyl-propionate) (DTSSP), *N*-ethyl-*N*-(3-(dimethylamino) propyl carbodiimide) (EDC), and *N*-hydroxysuccinimide sodium salt (NHSS) were purchased from Sigma-Aldrich (USA). All other chemicals were of analytical grade or better quality and used as received. Milli-Q (Millipore, ≥ 18 MΩ cm) ultra-pure water was used throughout.

2.2 Preparation of Aptamer-Conjugated MNPs

MNPs were synthesized using the classical chemical co-precipitation method with minor modification [12]. Briefly, under vigorous stirring, a mixture of $FeCl_2$ (50 mM, final concentration, same as below) and $FeCl_3$ (100 mM) dispersed in an oxygen-free HCl solution (1.2 mM) was mixed rapidly with equal volume of oxygen-free NaOH solution (1.25 M) by pumping N_2 gas. The reaction was kept in stirring and pumping N_2 for 2 hours. After the preparation, MNPs were magnetically separated and washed by numerous ultrapure water and absolute ethyl alcohol until the pH of MNPs suspension became neutral. Afterwards, MNPs were transferred into citric acid solution (0.2 M) and rotated at 120 rpm overnight at room temperature to prepare carboxyl-functionalized MNPs (MNPs-COOH). After three washing procedures with ultrapure water, the MNPs-COOH were dispersed in MES buffer solution (10 mg mL^{-1}) and stored at 4 °C.

In order to prepare MNPs-Apt, the MNPs-COOH suspension was added EDC (10 mM) and NHSS (15 mM) and kept stirring for 2 hours. Afterwards, amino CAP aptamer (10 μM) was added and kept for another time period of 1 hour in stirring, followed by adding ETA (1 mM) and keeping stirring for 30 min. The MNPs-Apt were stored at 4 °C. Before each addition of EDC/NHSS and ETA solutions, the MNPs suspensions were ultrasonically treated and then magnetically separated/washed three times.

2.3 Fabrication and Characterization of the Electrochemical Biosensor

Prior to modification, the bare gold electrode was cleaned according to the reported protocol [13]. Then, the modification and characterization of ssDNA modified electrode were as follows according reported methods [14]. Firstly, the clean Au electrode was immersed in the DTSSP solution (2 mM) for 4 h (DTSSP/Au). The DTSSP/Au electrode was then incubated with SA (10 μL, 1 mg mL^{-1}) in PBS for 1 h (SA/DTSSP/Au), then ETA (10 μL, 10 mM) was casted on the electrode for 1 h to block the nonspecific adsorption sites (ETA/SA/DTSSP/Au). Finally, the electrode was incubated with ssDNA (10 μL, 4 μM) for 30 min and the ssDNA modified electrochemical biosensor has been prepared (ssDNA/ETA/SA/DTSSP/Au). After each step, the electrode was thoroughly washed by PBS and characterized through the cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS).

2.4 Electrochemical Detection of CAP

In a typical experiment, CAP solutions with different concentrations were mixing with MNPs-Apt for 1 hour. After magnetically separated and washed with PB1 for three times, the MNPs composites (10 μL) were dropped cast onto the ssDNA/ETA/SA/DTSSP/Au electrode surface and kept for 45 min. After thoroughly washed using PBS, the MNPs-conjugated electrode was transferred to the ECC solution containing 0.1 M K_2SO_4 and 0.4 mM $K_4[Fe(CN)_6]$. Subsequently, the ECC method was applied, namely, a step applying high potential of 1.7 V for 450 s and then the other step applying 0 V for 300 s. Briefly, in the ECC solution containing 0.4 mM $K_4[Fe(CN)_6]$, H_2O was split to generate H^+ during the high potential; the generated H^+ reacted with MNPs on electrode surface and yielded Fe^{3+} ions. Afterwards, during the low potential step (0 V, 300 s), $K_4[Fe(CN)_6]$ reacted with Fe^{3+} to produce PB. Finally, PB on electrodes were further measured through CV from −0.1 to 0.5 V in detection solution for PB (0.1 M HCl + 0.1 M K_2SO_4) to obtain the

peak currents as signals. The higher the peak current, the lower the CAP concentration.

2.5 The Electrochemical Detection of CAP in Real Water Samples and Skim Milk Samples

CAP of given concentration was spiked into skim milk samples purchased from local supermarket and water samples from local river in campus. A simple pretreatment process of skim milk samples was taken as follows [15]. At first, skim milk samples were treated with Mcilvaine buffer in 1:1 volume ratio, shaken for 1 min, and centrifuged (20000 *g*, 10 min) to remove the proteins. Further, the samples were diluted with Milli-Q water (five times) prior to the analysis. The supernatants were collected and stored in 4 °C for further detection by the proposed method. Before detection process, the river water samples were filtered with a 0.22 μm membrane, and adjusted to neutral pH.

3 Results and Discussion

3.1 Characterization of MNPs-Apt Complex

We firstly investigated the ECC of MNPs to PB since it was crucial to the signal readout. MNPs-Apt were anchored onto the electrode surface by placing a magnet under the electrode. After the electrochemical conversion, the electrode was transferred to a solution containing 0.1 M K_2SO_4 and 0.1 M HCl to collect CV curves, as shown in Figure 1. Clearly, we observed characteristic peaks of PB located around 0.2 V [16]. In contrast, the electrode treated by ECC method in the absence of MNPs showed no peak. Above results should demonstrate that MNPs could be readily and efficiently conversed to electrochemically active PB.

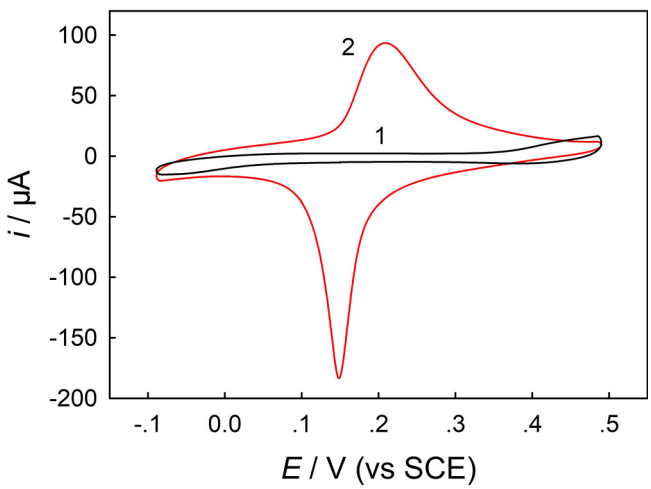

Fig. 1. CV curves of the Au electrode treated by the ECC method in the absence (1) and presence (2) of MNPs-Apt in solution containing 0.1 M K_2SO_4 and 0.1 M HCl. Scan rate: 0.05 V s^{-1}.

Furthermore, SEM was adopted to characterize the morphology of MNPs-modified electrodes before and after the ECC treatment, as shown in Figure 2. For the pristine MNPs-Apt, nanoparticles of ca. 20 nm in diameter were observed with smooth surface. However, after the ECC treatment, it appeared plenty of particles with cube-like shape, which is a characteristic shape of PB crystal [17]. Additionally, the surface of nanoparticles was rough, indicating new shell was covered onto the pristine surface. Therefore, both the CV and SEM results should solidly prove the successful preparation of PB.

To ensure the modification of aptamer on the MNPs, we adopted FT-IR and Zeta potential to investigate different processes of the modifications, including the pristine MNPs-COOH, EDC/NHSS activated MNPs-COOH and the MNPs-Apt. From the FT-IR curves as shown in Figure 3A, the pristine and activated MNPs-COOH showed minor difference, compared with which, the MNPs-Apt presented series of newly appeared absorbance peaks located from 1000 cm^{-1} to 1243 cm^{-1}, which are ascribed to the phosphodiester groups of nucleic acid [18]. For the Zeta potential measurements as shown in Figure 3B, the MNPS-COOH showed Zeta potential of −40 mV, indicating the presence of negatively charged COO^- groups on the MNPs. After the activation of MNPs-COOH with EDC/NHSS, the Zeta potential positively shifted to ca. −30 mV, which was ascribed to the transformation of COOH groups. The further conjugation of aptamer to the MNPs led to the Zeta potential recovered to −38 mV because the aptamer backbones were negatively charged [19]. Above characterizations should prove the successful modification of aptamer on the MNPs for further capture of CAP.

3.2 Fabrication of the Electrochemical Biosensor

The capture probe DNA-modified electrode is crucial to the conjugation of MNPs labels, therefore, its modification was carefully monitored by CV and EIS, as shown in Figure S1. The PBS solution containing 1 mM $K_3Fe(CN)_6$/$K_4Fe(CN)_6$ as the electrochemical probe was adopted. Along with the step-by-step modifications, the CV curves showed gradually decreased oxidation/reduction peak currents and enlarged peak-to-peak potentials. The EIS curves also presented gradually increased charge transfer resistance. Since all modified materials were insulating and in turn suppressed the redox electrochemistry of the electrochemical probe on the electrode, therefore, all CV and EIS results demonstrated the successful modifications.

Interestingly, we also conducted the ECC treatment of the final MNPs-conjugated electrode in the absence of $K_4Fe(CN)_6$ (no PB yielded), and found a refreshing effect through the monitoring by CV and EIS with electrochemical probe of 1 mM $K_3Fe(CN)_6$/$K_4Fe(CN)_6$, as shown in Figure 4. After the treatment, the redox peaks presented significantly higher peak current and smaller peak-to-peak potential, being close to that for the DTSSP

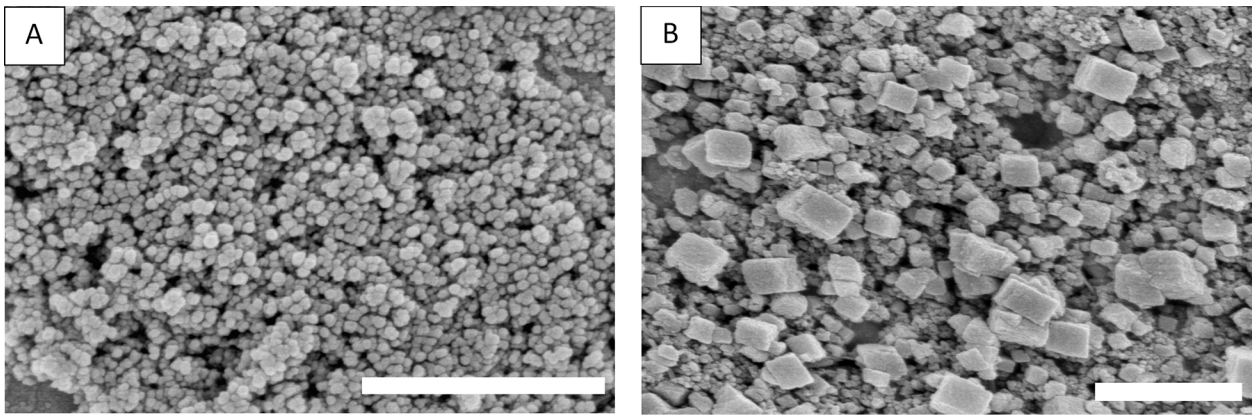

Fig. 2. SEM images of the MNPs-Apt modified electrode before (A) and after (B) the ECC treatment. Scale bar: 500 nm.

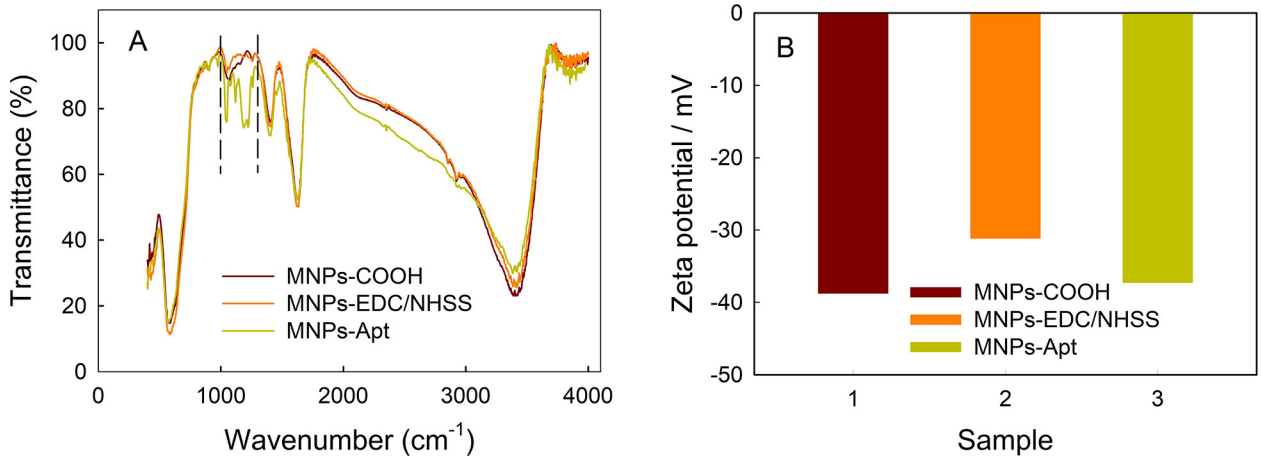

Fig. 3. FT-IR curves (A) and Zeta potentials (B) of MNPs-COOH, MNPs-EDC/NHSS and MNPs-Apt.

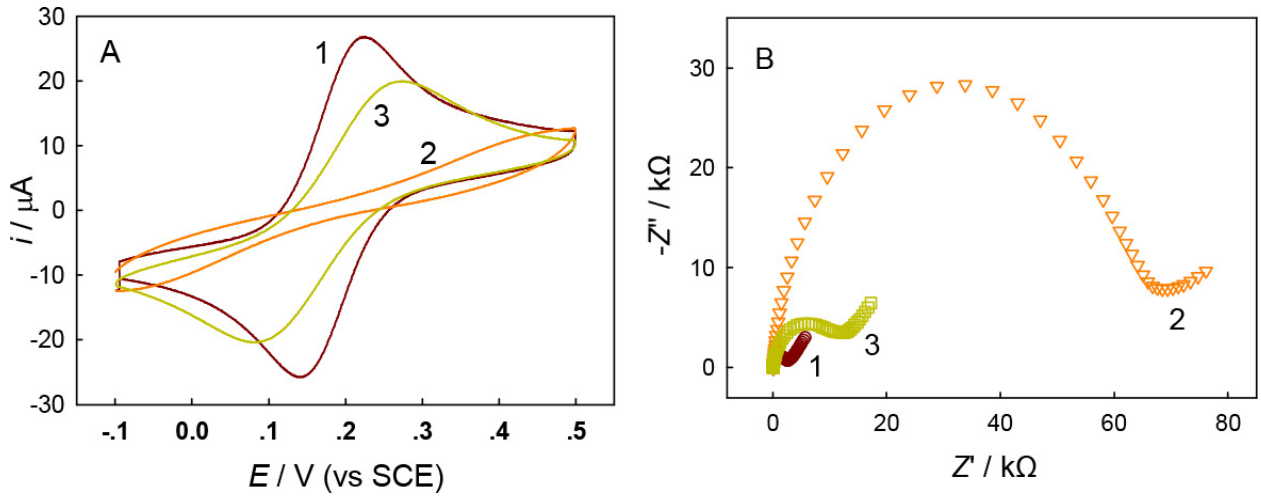

Fig. 4. CV (A) and EIS (B) of bare electrode, MNPs-Apt-conjugated electrodes before and after ECC process in the absence of $K_4Fe(CN)_6$. Characterization was conducted in a PBS solution containing 1.0 mM $Fe_3(CN)_6$/1.0 mM $K_4Fe(CN)_6$. Scan rate: 0.1 Vs^{-1}. EIS: 100 kHz–0.1 Hz, 100 mV rms, 0.21 V bias.

self-assembling monolayer-modified electrode. EIS also given large decreased charge transfer resistance from ca. 65 kΩ to 15 kΩ, which indicated significntly increased conductivity of the electrode (removal of insulating

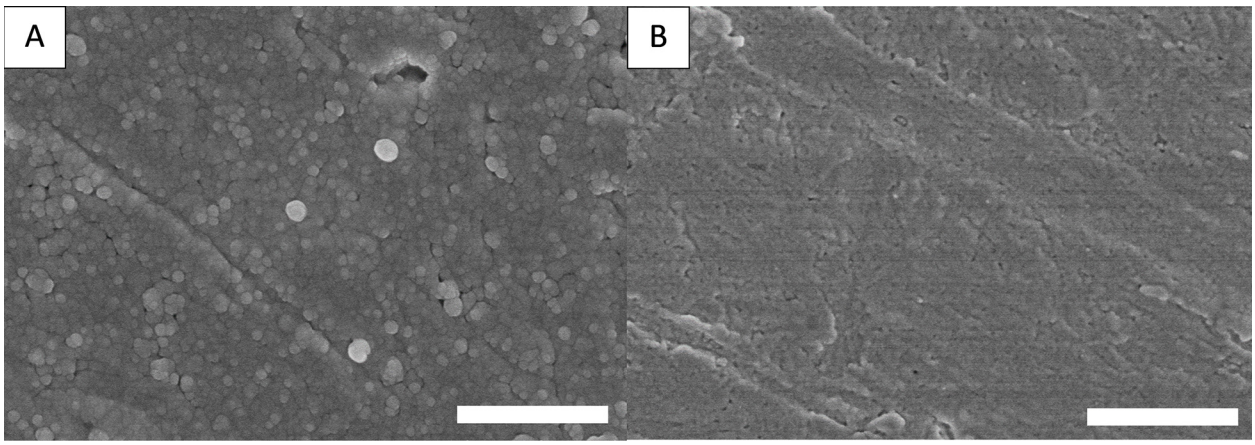

Fig. 5. SEM images of MNPs-Apt-conjugated electrode before (A) and after (B) the ECC treatment in the absence of $K_4Fe(CN)_6$. Scale bar: 500 nm.

species). All results pointed to electrochemically recovered electrode surface. Imaginably, the ECC treatment applied a high potential process to the electrode, during which the gold itself on the electrode surface was oxidized to oxide species; meanwhile, lots of oxygen bubbles also generated, in turn brought a significant refreshing effect to the electrode. This effect could also be proved by that the similar high-potential-treatment has well adopted for the electrode cleaning [13]. To further verify our speculation, the surfaces of the final MNPs-conjugated electrodes before and after the ECC treatment in the absence of $K_4Fe(CN)_6$ were visually inspected by SEM (Figure 5). Clearly, the electrode after the treatment presented much smoother surface and much less nanoparticles, which thus indicated the removal of surficial species from the electrode during the treatment. We have conducted the AFM characterizations to demonstrate the change of electrode surface before and after the ECC treatment, as shown in Figure S2. The Root-Mean-Square roughness of surface before and after the treatment were 22 nm and 5.1 nm, indicating much smoother surface after the ECC treatment. Much less particles were also observed. Above results agree well with the observation of SEM. As well known, low level of the charge transfer resistance of the modified layer is favored by the amperometry, which is especially important for the amperometric signal readout. Therefore, the ECC with refreshing effect should significantly benefit the performance of biosensor.

3.3 Optimization and Performance for CAP Detection

Because biosensor is based on the conjugation of MNPs labels to electrode through the unoccupied aptamer by CAP, the density of aptamer on the MNPs and the concentration of MNPs-Apt are key factors to determine the final performance. Therefore, above two parameters were carefully optimized to be 0.4 mg mL^{-1} (MNPs concentration) for MNPs-Apt and 1.0 μM for aptamer, as shown in Figure S3. Other experiment conditions including the modification of ssDNA, modification time and incubation time of MNPs labels with CAP were referred to the report papers [20].

Under the optimal conditions, different concentrations of CAP were determined using the developed biosensor. Both the reduction and oxidation peak currents of PB were collected and analyzed. As shown in Figure 6, along with the increase of CAP concentration, MNPs-Apt captured more CAP in the sample, leading to more aptamers occupied by CAP, and less MNPs-Apt conjugated to the electrode through the DNA hybridization. Subsequently, less PB could be transferred from the MNPs conjugated on electrode by the proposed electrochemical conversion method, which, finally, led to lower voltammogram peak intensity of the produced PB. When adopting both the cathodic and anodic peak currents as the signals, the biosensor exhibited a linear detection range (LDR) from 1 ng mL^{-1} to 1000 ng mL^{-1}, which is as wide as 3 orders of magnitude, as well as a limit of detection (LOD) down to 1 ng mL^{-1} ($S/N=3$). Above performance was better than or comparable with those of most analogues, as listed in Table S1. The satisfactory performance should be ascribed to (1) the proposed magnetic-separation/concentration-signal-amplification in-one strategy simplified the biosensor and enhanced the collection efficiency of MNPs labels, (2) the ECC explored the large abundance of iron content for PB generation and possessed refreshing effect, which also benefited the signal amplification.

In order to evaluate the specificity of the biosensor towards CAP, other different kinds of antibiotics were employed as interference reagents. The responses of tetracycline (TET), kanamycin (KAN), oxytetracycline (OTC), doxycycline (DOX) were measured. As shown in Figure 7, compared with the blank, the responses of interference antibiotics (100 μg mL^{-1}) showed negligible difference to that of the blank, while the CAP (1000 ng mL^{-1}) showed significant current decreases, indicating the method exhibited good specificity. The reason

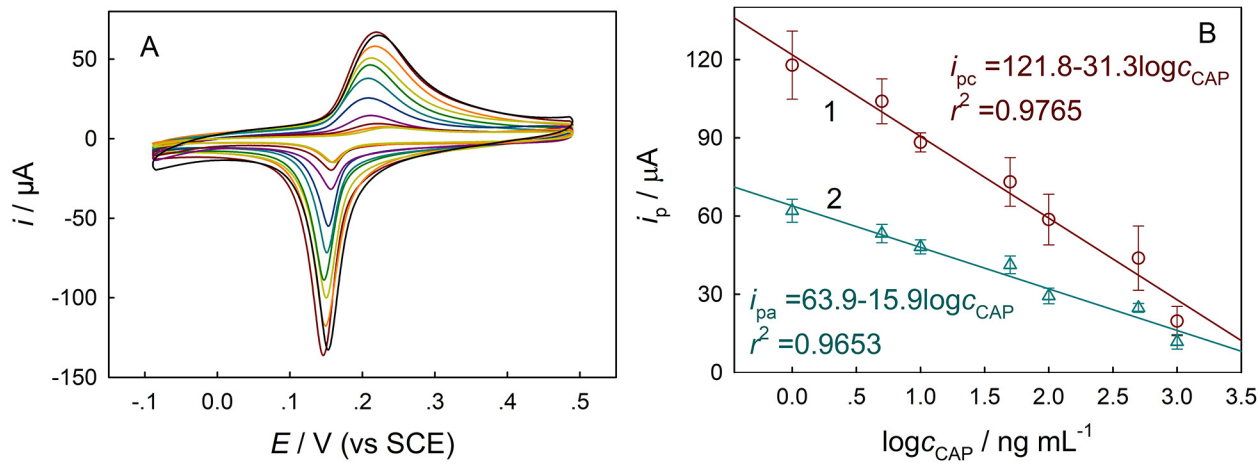

Fig. 6. (A) CV response curves of CAP at different concentrations (from bottom to top: control, 0.5, 1, 5, 10, 50, 100, 500, 1000, 5000, 10000 ng mL^{-1}). (B) The calibration plot of reductive (1) and oxidative (2) peaks currents to CAP concentrations.

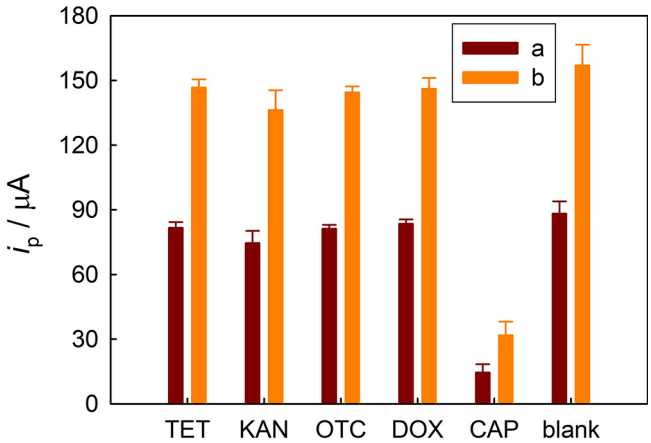

Fig. 7. The anodic (brown) and cathodic (orange) current responses of the biosensor to different kinds of species.

should be ascribed to the good specificity of the adopted aptamer [21]. The stability of the electrochemical biosensor was estimated by storing the modified electrode and the MNPs-Apt composites at 4 °C for given time. No apparent changes (less than 12 %) appeared after 2 weeks. Hence, the electrochemical biosensor possesses satisfactory long-term stability for CAP.

The feasibility of the biosensor for CAP detection in skim milk and water samples was evaluated by a standard addition method. Skim milk samples were purchased from local supermarket and the water samples were collected in a river in campus. As listed in Table S1, the recoveries were between 82 % and 113 % in skim milk samples, and between 89 % and 94 % in river water samples. Hence, this proposed rapid and sensitive electrochemical biosensor for CAP detection was applicable in quality assessment of real samples.

3 Conclusions

We have explored a magnetic-separation/concentration-signal-amplification in-one method to develop a facile and sensitive electrochemical biosensor for CAP detection. This method integrated the rapid separation and concentration function of MNPs, and signal readout/amplification ability by exploring the ECC process to obtain electrochemically active PB. Taking advantages of the high abundance of iron content in MNPs and the refreshing effect, the method significantly promotes the signal amplification without any additional labels. Therefore, the proposed biosensors exhibited satisfactory performance which was better than or comparable with those of most analogues. The developed method may lead to new concept for developing biosensor for rapid and facile detection in food safety, clinic diagnose/therapy and environmental monitoring fields.

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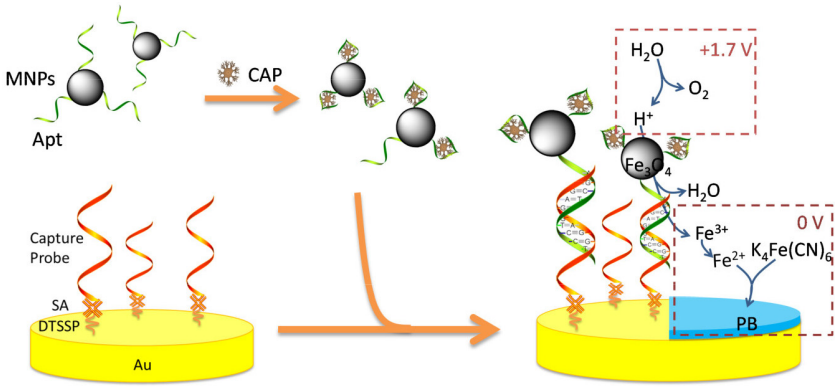

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Separation/Concentration-Signal-Amplification in-One Method Based on Electrochemical Conversion of Magnetic Nanoparticles for Electrochemical Biosensing