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A Novel Electrochemical Immunosensor for Detection of AngiotensinII at a Glass Carbon Electrode Modified by Carbon Nanotubes/Chitosan Film

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A novel electrochemical competitive immunosensor was successfully developed for the detection AngiotensinII using the glass carbon electrode (GCE) modified with the single-walled carbon nanotubes/chitosan (SWNTs/CS) film. The electrochemical sensor was firstly prepared by covalently immobilizing AngII on SWNTs/CS modified GCE. Then the modified GCE was incubated with the mixture solution of Bio-Anti-AngII antibody and target AngII. After the competitive immunoreactions, the ST-AP was captured on the immunosensor surface to catalyze the hydrolysis of the substrate α-NP in the DEA solution. The whole fabrication processes of the immunosensor were characterized by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). Under the optimal conditions, the immunosensor showed a specific response to a small molecular target AngII and the DPV signals linearly decreased in the range of 0.05 to 10 μg mL^{-1} AngII with a detection limit of 0.03 μg mL^{-1}. Good specificity and reproducibility were obtained for this immunosensor and it was applied to assay the AngII in spiked serum. This facile electrochemical immunosensor strategy was firstly used to detect AngII and might be a promising method for detection of AngII for clinical diagnostic application in the future.

Keywords: AngII, electrochemical immunosensor, carbon nanotube, chitosan

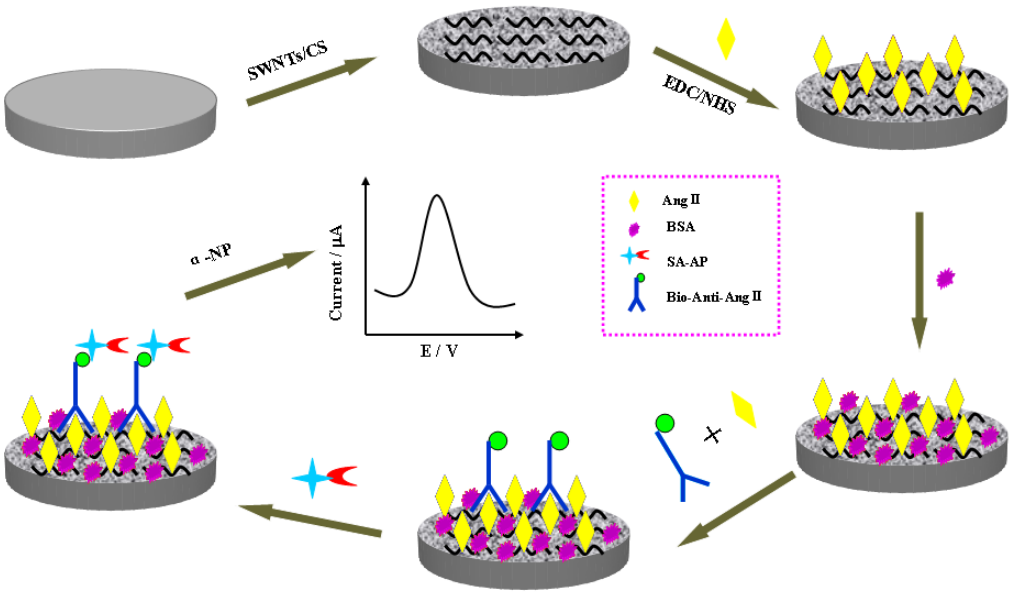
1. INTRODUCTION

AngiotensinII (AngII), a small molecular peptide contained eight amino acids, is from angiotensinogen by sequential enzymatic cleavages. AngII is considered to be the primary effecting

peptide of the rennin-angiotensin system (RAS) [1, 2] and is closely involved in the control of body fluid balance, electrolyte homeostasis and blood pressure regulation [3, 4]. By binding to the type 1 angiotensin receptor (AT1R), AngII is known to have important roles in the regulation of cell proliferation, angiogenesis, inflammation and cancer [5-7]. So it is important to measure the AngII concentration for exploring the various actions of AngII in multiple systems and drugs acting in human body [8]. Nowadays, various commonly available methods are developed for AngII assay, such as solid-phase immobilized epitope-immunoassay (SPIE-IA) [9], radio-immunoassay [10] and high performance liquid chromatography (HPLC) [11]. Although these methods are widely accepted and well proven, they are time consuming, labor-intensive, requiring relatively expensive equipment and high cost. Therefore, it is a challenge to develop a simple, cheap and fast method for the direct detection of AngII.

Recently electrochemical biosensors have been received considerable attention due to their inherent advantages, such as high sensitivity, fast response, low-cost, easy to use and suitability to miniaturization [12, 13]. Meanwhile, there are many signal amplification strategies for improving the sensitivity of the electrochemical biosensor by using gold nanoparticles (AuNPs) [14], magnetic nanoparticles (MNPs) [15], quantum dots [16] and carbon nanotubes [17, 18]. Among the above amplification strategies, carbon nanotubes (CNTs) have received particular attention in the fabrication of electrochemical biosensors because of their high electrical conductivity, chemical stability, extremely high mechanical strength, ultrahigh electrocatalysis capacity and surface area effects in electrochemical biosensing analysis [19-21]. On the other hand, Chitosan (CS), a natural biopolymer product, has been widely used in many practical fields, such as electrochemical biosensors, SPR biosensors and piezoelectric immunosensors. CS has many properties such as chemical inertness, biodegradability, high mechanical, good adhesion and good film-forming ability [22, 23]. Moreover, CS is nontoxic, biocompatible, abundant and cheap [24]. CS is one of the best materials to make the biological application of CNTs possible. Considering the above advantages of carbon nanotubes and chitosan, CNTs/CS composite is widely adopted in fabrication of electrochemical biosensors [25-27].

To the best of our knowledge, there is no study reported so far for the detection of small molecular peptide AngII by using electrochemical immunosensor. Herein, we firstly developed a novel electrochemical immunosensor for competitive detection of AngII at a glass carbon electrode modified with a functionalized single-walled carbon nanotubes/chitosan (SWNTs/CS) film. The process of this electrochemical biosensor was illustrated in Scheme 1. The electrochemical immunosensor was prepared by modifying the glass carbon electrode (GCE) with AngII covalently functionalized SWNTs/CS nanocomposite film. The excellent electrical conductivity of SWNTs/CS nanocomposite could greatly enhance electrochemical signal. In the competitive format, the more immobilized AngII amount on the SWNTs/CS film could extend the up-limit of the detectable range. Finally the electrochemical signal was achieved by highly catalytic activity of streptavidin-alkaline phosphatase (ST-AP) toward enzyme substrate α-naphthyl phosphate (α-NP). This fabricated platform might provide an alternative approach for the detection of AngII in the biological sample.



Scheme 1. Schematic illustration of the strategy for AngII assay using GCE modified with a single-walled carbon nanotubes/chitoasn film.

2. EXPERIMENTAL

2.1. Reagents

Rabbit anti-AngiotensinII/Biotin antibody (Bio-Anti-AngII antibody) was purchased from Beijing Biosynthesis Biotechnology Ltd. Co. (Beijing, China). AngII, Chitosan, N-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethyllaminopripyl) carbodiimide hydrochloride (EDC), bovine serum albumin (BSA), streptavidin-alkaline phosphatase (ST-AP) and α-naphthyl phosphate (α-NP) were obtained from Sigma-Aldrich (USA). Carboxylic group-functionalized SWNTs (< 5 nm diameter) were purchased from Shenzhen Nanotech Port Ltd. Co. (Shenzhen, China). Other reagents were of analytical grade. All aqueous solutions were prepared using Millipore-Q water (18 MΩ).

2.2. Apparatus

The electrochemical measurements were performed on a CHI 660D electrochemical workstation (Shanghai Chenhua Instruments Co. Ltd., China). The electrochemical system consisted of a conventional three-electrode system where platinum wire was used as an auxiliary electrode, the Ag/AgCl electrode was used as a reference electrode and a 3 mm diameter GCE modified by the SWNTs/CS film was used as a working electrode. Scanning electron microscopic (SEM) image was taken with the S-3000N scanning electron microscopic (Hitachi, Japan).

2.3. Preparation of SWNTs/CS nanocomposite modified electrode

5 mg carboxylic group-functionalized SWNTs was put into 5 mL of 1% acetic acid containing 10 mg CS , then sonicated for 2 h to obtain a uniform mixture. Before modification, the GCE was freshly polished to a mirror with 0.3 μm and 0.5 μm alumina slurry respectively and rinsed thoroughly with deionized water. Then, the GCE was rinsed ultrasonically in 1:1 nitric acid and acetone for 5 min, washed ultrasonically in water for 5 min and allowed to dry at room temperature. Afterwards, 2 μL of the SWNTs/CS suspension was dropped onto the pretreated GCE and dried in a desiccator. Thus, a glass carbon electrode modified with a SWNTs/CS film was obtained and used for the following experiment.

2.4. Preparation of electrochemical immunosensor

The SWNTs/CS GCE was rinsed thoroughly with deionized water, then immersed into 50 μL 400 mM EDC and 100 mM NHS mixed liquor for 1 h and incubated for activating the carboxylic group functionalized SWNTs. Subsequently, the activated GCE was washed with deionized water. 10 μL of 10 μg mL^{-1} AngII was immediately dropped on the activated GCE surface and incubated at 37 ℃ for 2 h. After washed with 0.01 M pH 7.4 phosphate-buffered saline (PBS, 136.7 mM NaCl, 2.7 mM KCl, 87 mM Na_2HPO_4 and 14 mM KH_2PO_4), the fabricated electrode was further treated in 1% BSA solution for 30 min to block the nonspecific binding on the surface. Finally, the GCE was washed with PBS again, and the immunosensor was successfully obtained.

2.5. Electrochemical measurements

The immunosensor was firstly incubated with 10 μL of the mixture of 10 μg mL^{-1} Bio-Anti-AngII antibody and AngII standard solutions or serum samples for 1 h at 37 ℃, and then washed with PBS. 10 μL of 1.25 μg mL^{-1} ST-AP was dropped onto the electrode surface and incubated for 30 min at 37 ℃, and then the GCE was rinsed with PBS. The electrochemical assay was performed in pH 9.5 DEA solution (1 mM L^{-1} $MgCl_2$, 0.1 M L^{-1} diethanolamine, 100 mM L^{-1} KCl) containing 1mg mL^{-1} α-NP. The differential pulse voltammetry (DPV) measurement was performed from 0 to 0.5 V, with step potential of 5 mV, the pulse amplitude of 50 mV and a pulse period of 0.2 s.

3. RESULTS AND DISCUSSION

3.1. Characterization of the SWNTs/CS composite

The scanning electron microscopic (SEM) image of the SWNTs/CS composite was shown in the Figure 1A. From seen in the Figure 1A, the SWNTs/CS composite was homogeneous and offered a large biocompatible film surface area. In the presence of CS, the strong attractive interaction among the nanotubes would be reduced and the aggregation of SWNTs could be prohibited [28, 29], which

resulted in a high dispersibility and long-term stability of SWNTs/CS composite in the solution. Meanwhile, the SWNTs/CS composite could be of better biocompatibility due to the properties of CS. Figure 1B, C and D showed different DPV peak currents at the GCE modified with different materials. As shown in the Figure 1B, the background of the base current was high due to the good electrical conductivity of the carbon tubes [30, 31]. Figure 1C showed the lower background current for the SWNTs/CS composite. As shown in the Figure 1B and Figure 1C, the $_{\Delta}C$ values ($_{\Delta}C$ is the difference between the $Current_a$ and the $Current_b$) were similar, which indicated the CS had no obvious effect on the signal detection. As shown in the Figure 1D, the current responses were smaller than the results obtained from the Figure 1B and Figure 1C. One reason would be that less AngII were immobilized at the GCE modified with SWNTs/AuNPs. AuNPs as an immobilized matrix could bind proteins through ionic interactions and other interactions between AuNPs and primary amine groups of proteins [32, 33]. But the interactions between AuNPs and AngII were weak because AngII is an eight amino acid peptide. Another reason would be that more AngII were bonded covalently with functionalized SWNTs and the covalent interactions were strong. These results showed that the SWNTs/CS composite not only reduced the background current and promoted the electron transfer, but also offered a biocompatible film with large surface area. Thus, the SWNTs/CS composite was chosen as the immobilized matrix for bonding covalently AngII.

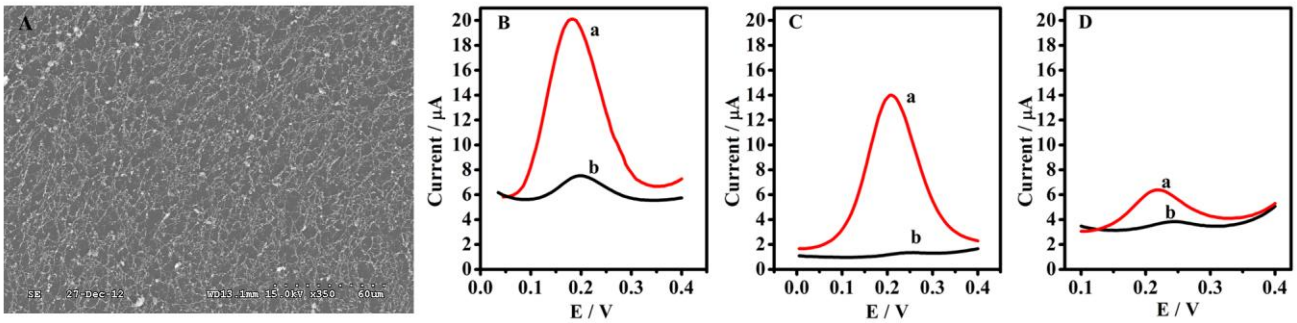


Figure 1. SEM image of the SWNTs/CS composite (A), DPV peak current of the immunosensor responding to 10 μg/mL AngII with (a) or without (b) 10 μg/mL Bio-Anti-AngII antibody at the GCE modified with a SWNTs film (B) , at the GCE modified with a SWNTs/CS film (C) and at the GCE modified with SWNTs/AuNPs (D).

3.2. Characterization of the immunosensor

The fabricated immunosensor was characterized by the cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). Figure 2A showed the different cyclic voltammograms of 5 mM $K_3Fe(CN)_6/K_4Fe(CN)_6$ in 0.5 M KCl at the bare, SWNTs/CS, AngII-SWNTs/CS, BSA-AngII-SWNTs/CS and Bio-Anti-AngII antibody/BSA-AngII-SWNTs/CS fabricated electrodes. The peak current increased obviously after the SWNTs/CS film was assembled on GCE surface (curve b). This result indicated that the fabricated SWNTs/CS film could promote the electron transfer of the $Fe(CN)_6^{3-}$ /$Fe(CN)_6^{4-}$ couple [34] and had a large specific surface area, enhancing the load amount of

AngII [35]. The presence of CS could clearly promote the film-forming and the dispersion performance of carbon nanotubes [36]. Subsequently, when AngII was dropped on the activated electrode, the peak current sharply decreased, indicating that AngII had been successfully immobilized at the surface of SWNTs/CS modified GCE (curve c). When 1% BSA was used to block the residual active carboxyl groups on the GCE surface, the peak current further decreased due to the steric hindrance of BSA to electron transfer (curve d). After the fabricated immunosensor incubated with Bio-Anti-AngII antibody, a decrease in peak current was observed, demonstrating that the constructed electrochemical biosensor was successful for recognition and binding of Bio-Anti-AngII antibody (curve e). The above results were in a good agreement with those obtained from EIS (Figure 2B), in which the surface electron transfer resistance (Ret) was used to indicate the fabrication of the electrochemical immunosensor. The results of the EIS proved the successful covalently immobilization of AngII on the GCE modified with SWNTs/CS.

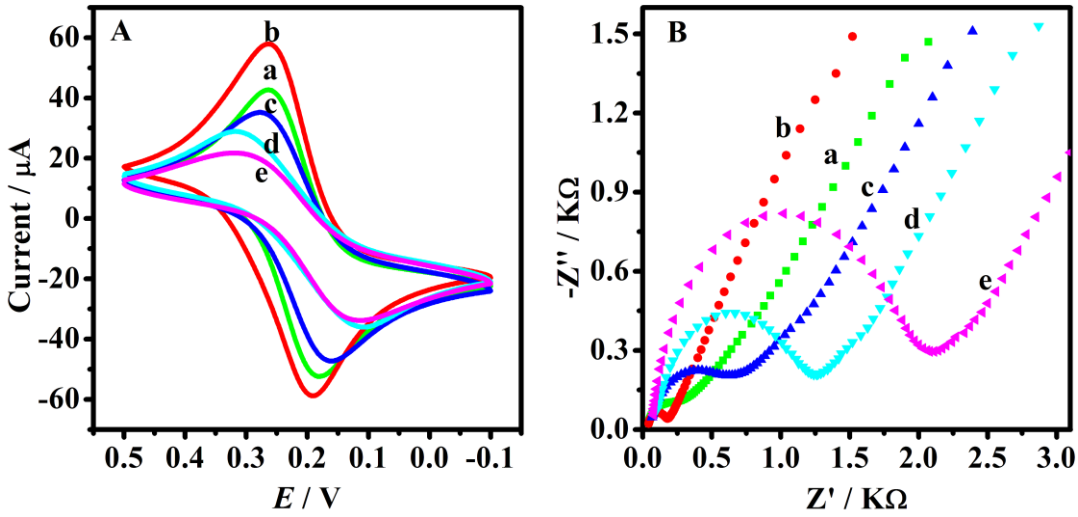


Figure 2. (A) Cyclic voltammograms and (B) EIS of bare GCE (a), SWNTs/CS modified GCE (b), AngII-SWNTs/CS modified GCE (c), BSA-AngII-SWNTs/CS modified GCE (d) and Bio-Anti-AngII antibody/BSA-AngII-SWNTs/CS modified GCE (e) in 0.5 M KCl containing 0.5 mM $[Fe(CN)_6]^{3-/4-}$ at 100 mV s^{-1}.

3.3. Optimization conditions for the electrochemical immunosensor

In the competitive immunoassay, the target AngII competed with the AngII molecules on the electrode to bind the Bio-Anti-AngII antibody. So, the concentration of Bio-Anti-AngII antibody in the incubation solution was a very important factor. In order to obtain the optimal concentration of Bio-Anti-AngII antibody, different concentrations of Bio-Anti-AngII antibody solutions were used to react with AngII bonded covalently on the SWNTs/CS modified GCE. Figure 3A showed that the peak current increased with the increasing Bio-Anti-AngII antibody concentration and tended to a platform at 10 μg mL^{-1}. This indicated that there were no available recognition sites of immobilized AngII to match with the antibody. Thus, 10 μg mL^{-1} Bio-Anti-AngII antibody was chosen as the optimal

concentration. As shown in Figure 3B, the current responses increased with the increasing incubation time of Bio-Anti-AngII antibody used in the competitive immunoassay and trended to level off at 1 h, which showed a saturated binding in the immunoreactions. Therefore, 1 h was selected as the optimized incubation time for the competitive immunoreactions.

The concentration of ST-AP was also a key parameter affecting the analytical performance of immunoassay. As shown in Figure 3C, when the concentration of ST-AP was greater than 1.25 μg mL^{-1}, the current response didn't increase and reached a plateau. Further increasing ST-AP concentration would lead to nonspecific adsorption on the sensor surface, so 1.25 μg mL^{-1} was chosen as the optimal concentration in electrochemical immunoassay. When the ST-AP concentration was constant, the concentration of substrate α-NP would be a critical parameter that influenced the enzyme-catalyzed reaction [37]. The DPV peak current of the electrochemical immunosensor in DEA solution increased with the increasing concentration of α-NP, and then tended to a steady value at 1.0 mg mL^{-1}. Therefore, 1.0 mg mL^{-1} α-NP was the optimal concentration for DPV detection in the enzyme-catalyzed reaction (Figure 3D).

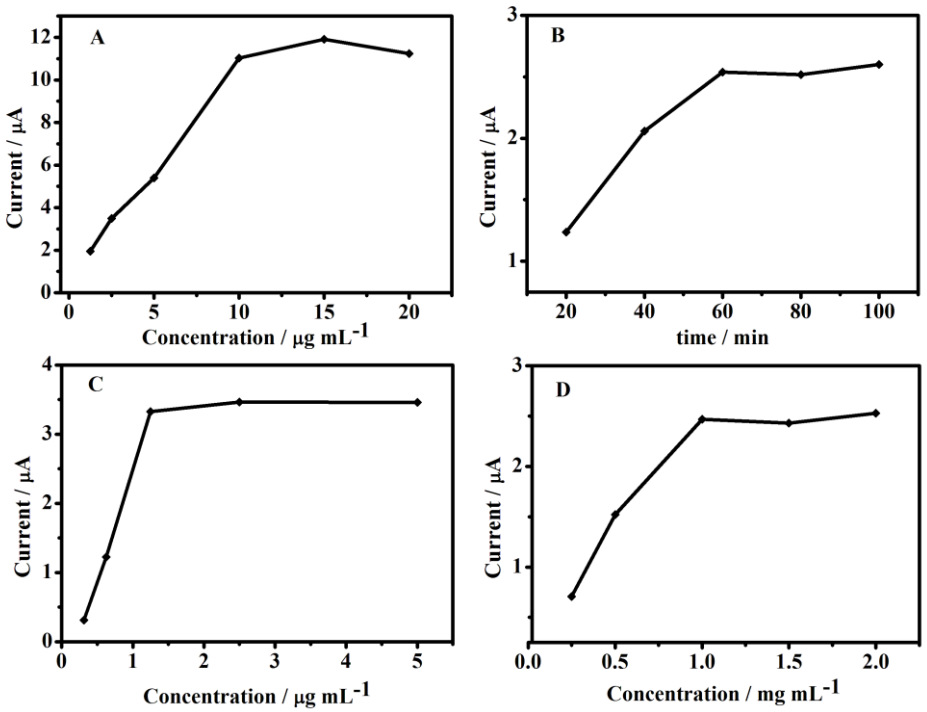


Figure 3. Dependences of DPV peak current on Bio-Anti-AngII antibody concentration (A), and incubation time of the immunoreactions (B), ST-AP concentration (C), and α-NP concentration (D) at 2 μg mL^{-1} Bio-Anti-AngII antibody, when one parameter changes while the others are constant.

3.4. Analytical performance of the electrochemical immunosensor

To prove the analytical performance of the designed electrochemical immunosensor, the competitive immunoassays were carried out by adding different concentrations of AngII to the

immunosensor. Under the optimum conditions, the DPV peak currents decreased proportionally with the increasing concentrations of the target AngII (Figure 4A). The calibration plots showed a good linear relationship between the DPV currents and the logarithm values of the AngII concentrations in the range from 50 ng mL^{-1} to10 μg mL^{-1} with a correlation coefficient of 0.9991 (Figure 4B). The limit of detection (LOD) for AngII was calculated to 30 ng mL^{-1} at a signal-to-noise ratio of 3. The reproducibility of the developed immunosensor was further investigated by measuring the target AngII peptides at 0.5 μg mL^{-1} and 5 μg mL^{-1} with five replicates, respectively. And the coefficients of variation for both concentrations were about 2% and 5%, respectively. Compared with the other methods for AngII assay, the reproducibility of this approach was acceptable [9, 11].

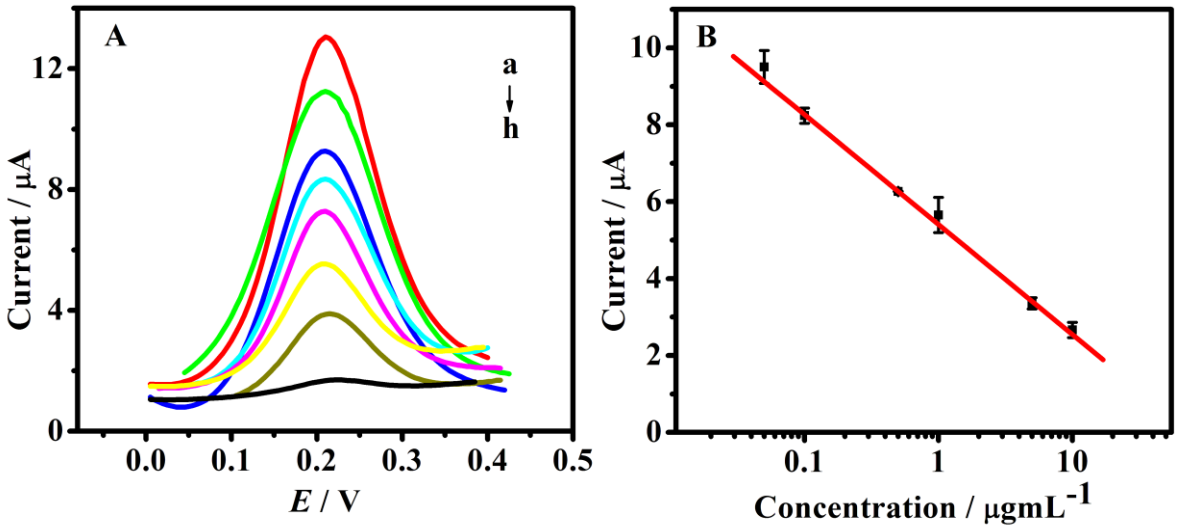


Figure 4. (A) DPV curves of the electrochemical immunosensor with target different AngII concentrations of 0, 0.05, 0.1, 0.5, 1, 5, 10 μg mL^{-1} (from a to h). (B) Plot of DPV peak current vs AngII concentration. The error bars represent the standard deviations calculated from three different assays.

3.5. Specificity of the immunosensor

To evaluate the selectivity of the sensor, 30 μg mL^{-1} bovine serum albumin (BSA), neuron specific enolase (NSE) and pregnancy-associated plasma protein (PAPP) were examined respectively. Meanwhile, 10 μg mL^{-1} AngII and the blank were also measured. The results were shown in the Figure 5. The peak currents responding to BSA, NSE and PAPP were similar to that of the blank (without AngII) and the signal to AngII decreased dramatically. The error bars represented average standard errors for three measurements. These results indicated that these nonspecific proteins had no significant influence on the immunosensor and the sensor was only specific to the target protein as expected.

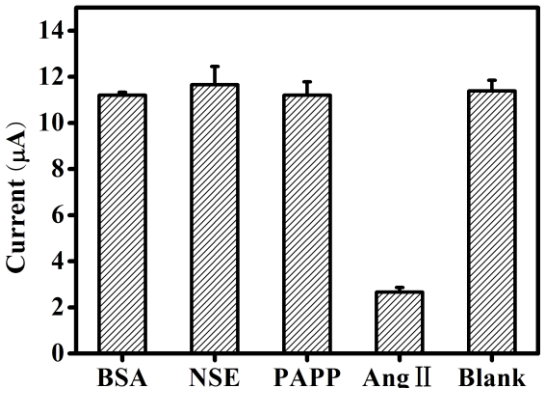


Figure 5. Comparison of the DPV peak currents by employing different proteins (AngII, 10 μg mL^{-1}; other proteins, 30 μg mL^{-1}). The error bars represent average standard errors for three assays.

3.6. Detection of AngII in the spiked serum samples

To further evaluate the analytical reliability and application potential of the electrochemical immunosensor, the sensor was applied to detect AngII in 50% diluted serum specimens. 10 μL different concentrations AngII standards were spiked into the 50% diluted serum samples, and the AngII contents were measured using the developed electrochemical sensor. As listed in Table 1, the recoveries were among 83.5% to 115.7%, indicating that the established electrochemical immunosensor obtained a good recovery [10]. Meanwhile, the fabricated immunosensor, having advantages of low assay cost, easy to construction and absence of sophisticated and expensive array instrument, showed more suitable for point-of-care testing. Thus, the developed immunosensor might be a potential tool for detection of AngII in serum samples in the future.

Table 1. The recoveries determined using the immunosensor via AngII standards into the blank serum samples.

Sample No.	Spiking value (μg/mL)	Assayed value (μg/mL)	Recovery (%)
1	0.2	0.18	90.0
2	0.5	0.53	106.0
3	1.2	1.21	100.8
4	2	1.67	83.5
5	2.5	2.59	103.6
6	4	4.41	110.3
7	5	4.76	95.2
8	7	6.95	99.3
9	8	8.51	106.4
10	10	11.57	115.7

4. CONCLUSIONS

The work presented here describes a new electrochemical immunosensor for the detection of AngII with the SWNTs/CS film modified GCE by using the high catalytic ability of ST-AP to α-NP. The SWNTs/CS film can offer a large surface area to make more antigens fabricated on the GCE and promote the electron transfer between the GCE and the measurement solution. Meanwhile, the ST-AP exhibits highly catalytic activity toward hydrolysis of α-NP. With a competitive immunoassay format, the fabricated immunosensor for AngII shows a good performance with a wide linear range, acceptable precision and good reproducibility. It is the first time to detect the small molecule AngII using the electrochemical competitive immunosensor. Although the LOD of this electrochemical method do not reach the level of physiological AngII concentration, this approach demonstrates a promising platform for the assay of AngII in the biological sample. By coupling with other signal amplification strategies, this biosensing method would become a pragmatic tool for convenient detection of AngII in clinical laboratory diagnosis in the future.

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