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Amperometric Immunosensor Based on Carbon Nanotubes/Chitosan Film Modified Electrodes for Detection of Human Leptin

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A novel sandwich electrochemical immunosensor was developed for the quantification of human leptin in serum using the glassy carbon electrodes modified with the single-walled carbon nanotubes/chitosan film. The preparation process of the biosensor involved covalently immobilization of a specific anti-human leptin antibody (capture antibody) on the modified glassy carbon electrodes, incubating with the target human leptin and reacting with the biotinylated anti-human leptin. After the well-known sandwich-type assembly, the streptavidin-alkaline phosphatase was attached to the immunosensor surface to catalyze the hydrolysis of the substrate α-naphthyl phosphate. The electrochemical impedance spectroscopy and square wave voltammetry were used to characterize biosensor. For detection of human leptin, the established immunosensor displayed a good amperometric response with a wide range of 0.05 ng mL^{-1} - 500 ng mL^{-1} and a detection limit of 30 pg mL^{-1}. Furthermore, good specificity and reproducibility were obtained, and this immunosensor was successfully applied to assay the leptin in spiked serum. The results demonstrated that the novel strategy might become a potential alternative for human leptin assay in the function study of human leptin and clinical diagnostic application.

Keywords: carbon nanotubes; chitosan; amperometric immunosensor; leptin.

1. INTRODUCTION

Leptin, as a product of obese (ob) gene, has made a stir in the field of obesity since its identification in 1994 [1]. This hormone is a 167-amino acid peptide that is primarily produced in adipose tissue. The level of leptin is directly proportional to the amount of body fat and fluctuates with acute changes in caloric intake [2]. By binding to specific leptin receptors expressed in the brain as well as in peripheral tissues, leptin plays a vital role in regulating energy homeostasis, neuroendocrine function and metabolism [3-5]. In humans, circulating leptin levels are closely associated with obesity, metabolic abnormalities, infertility and cancer [6-8]. Recently, some researches discovered that the concentration of leptin could also predict central sleep apnea in heart failure patients [9], and was independently and consistently associated with reduced cartilage thickness cross-sectionally and longitudinally. Meanwhile, other researches indicated that leptin may be involved in cognition, immune function, and bone metabolism [5, 10]. All these findings place leptin at a quite crucial position. So it is significant to detect leptin concentration for exploring not only the various function of leptin in multiple systems but also its association with diseases.

To date, various commonly available analytical methods have been reported for the detection of leptin, such as radioimmunoassay [11], immunofunctional assay [12], enzyme-linked immunosorbent assay (ELISA) [13], capillary electrophoresis [14], and localized surface plasma resonance [15]. However, these detection methods are expensive, tedious or time consuming. In contrast, electrochemical immunosensors [16, 17] with a sandwich-type structure are widely used due to their advantages including simple pre-treatment, low cost, rapid detection, and high sensitivity. Meanwhile, various types of elements have been applied for signal amplification, including gold nanoparticles (AuNPs) [18, 19], quantum dots [20, 21], graphenes [22, 23], and carbon nanotubes [24-26]. Among these elements, the use of carbon nanotubes (CNTs) has motivated great interest owing to their advantages, such as unique electrical, mechanical property, chemical stability, large specific surface area and possibility to be functionalized easily [27-29]. On the other hand, chitosan (CS), which possesses chemical inertness, high mechanical, good adhesion and good film-forming ability, has been widely used in many practical fields, such as electrochemical biosensor, piezoelectric immunosensor and SPR biosensor. CS is also an attractive biocompatible, biodegradable and non-toxic nature polysaccharide with abundant -NH_2 and -OH functional groups, and can be used to prepare CNTs/CS composite for enlarging the application of CNTs [30]. As a new biocomposite, CNTs/CS composite has excellent mechanical, biological and photoelectric properties for its unique structure and is widely applied in fabrication of electrochemical biosensors [31, 32].

To the best of our knowledge, there are a few reports available for electrochemical detection of leptin [33-35]. However, CNTs/CS modified electrode has not been used yet for electrochemical detection of human leptin. In this paper, we developed a novel sandwich-type amperometric immunosensor for sensitive detection of leptin based on functionalized single walled carbon nanotubes/chitosan (SWNTs/CS) composite and catalytic activated streptavidin-alkaline phosphatase (ST-AP) to remarkably dual amplify the response signal. This established sensing platform demonstrated an acceptable reproducibility, highly sensitivity and selectivity for leptin detection and might be a powerful tool for biomedical research and clinic diagnostic application in the future.

2. EXPERIMENTAL

2.1. Materials and reagents

Rabbit anti-human leptin (anti-leptin), human leptin and biotinylated anti-human leptin (bio-anti-leptin) were purchased from PeproTech (New Jersey, USA). 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 (St. Louis, USA). Angiotensin II (AngII), C-reactive protein (CRP) was purchased from Beijing Biosynthesis Biotechnology Co. Ltd. (Beijing, China). Carboxylic group-functionalized SWNTs (<5 nm diameter) were bought from Shenzhen Nanotech Port Co. Ltd. (Shenzhen, China). Other reagents were of analytical grade. All aqueous solutions were prepared using Millipore-Q water (18 MΩ). 0.01 M phosphate-buffered saline (PBS) containing 136.7 mM NaCl, 2.7 mM KCl, 87 mM Na_2HPO_4 and 14 mM KH_2PO_4 (pH 7.4) was used as washing buffer. Diethanolamine (DEA) buffer contained 0.1 M DEA, 1 M $MgCl_2$ and 100 mM KCl (pH 9.6).

2.2. Apparatus

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

2.3. Preparation of SWNTs/CS nanocomposite

SWNTs/CS nanocomposite was prepared according to the following method. 5 mg carboxylic group-functionalized SWNTs fine powder was added into 5 mL of the CS aqueous solutions (5 mg CS melts in 1% wt acetic acid). Then the solutions were sonicated for 2 h to obtain a uniform mixture of SWNTs/CS nanocomposite and stored at 4 ℃ for use.

2.4. Fabrication of the electrochemical immunosensor

The bare glassy carbon electrode (GCE) was evenly polished to a mirror using 0.05 μm alumina slurry and rinsed thoroughly with deionized water. Then the electrode was sonicated with 1:1 nitric acid and acetone, and deionized water for 3 min, respectively. For further removal of pollutants, the GCE was soaked in 0.5 M H_2SO_4 solution and scanned at the potential from -0.2 to +1.6 V with a scan rate of 100 mV s^{-1}. The GCE was again washed ultrasonically in water for 5 min and allowed to dry at room temperature. Afterwards, 2 μL of 1 mg mL^{-1} functionalized SWNTs/CS nanocomposite was coated on the pretreated GCE and dried in a desiccator. Thus, a glassy carbon electrode modified with a SWNTs/CS film was obtained and used for the following operation.

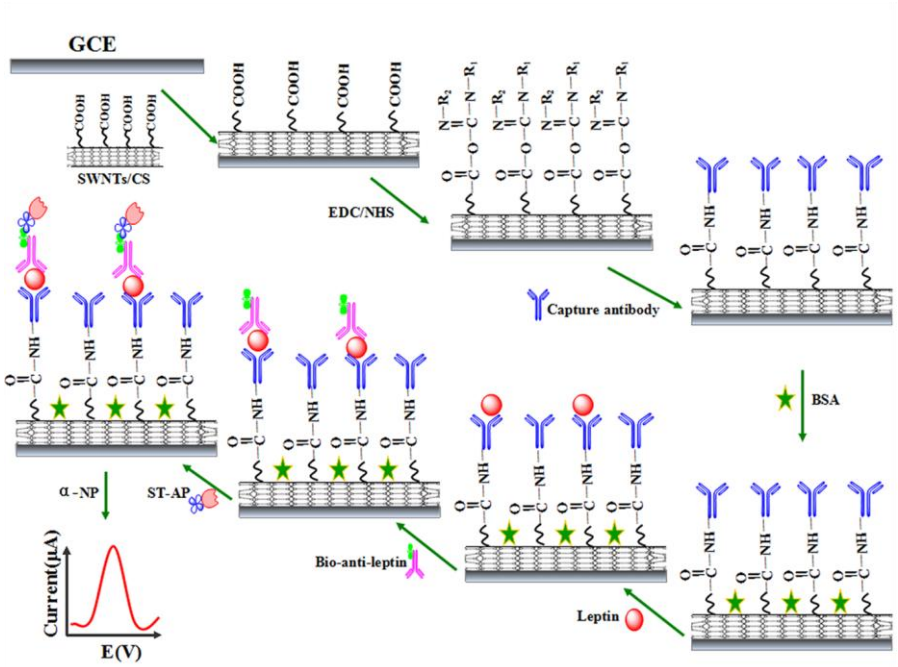
The above electrode was then washed thoroughly with the PBS, and incubated with the EDC-NHS mixed liquor (50 μL 40 mM EDC and 10 mM NHS) for 1 h to activate the carboxylic group of the functionalized SWNTs. Next, the activated GCE was rinsed with deionized water again, and 10 μL of 5 μg mL^{-1} anti-leptin was immediately introduced and incubated for 2 h at 37 ℃. Further, after rinsed thoroughly with PBS, the fabricated electrode was treated with 2% BSA solution for 1 h to block the nonspecific binding on the surface. Therefore, the leptin electrochemical immunosensor based on SWNTs/CS nanocomposite was successfully obtained and stored at 4 ℃ for the following experiment.

2.5. Electrochemical measurements

For the detection of leptin, the immunosensors were incubated with different concentrations of leptin solution and 1 μg mL^{-1} bio-anti-leptin solution for 2 h at 37 ℃, respectively. Then the electrode was washed carefully with PBS, and 10 μL of a 1.25 μg mL^{-1} ST-AP solution was dropped onto the fabricated electrode surface and incubated for 30 min at 37 ℃. Finally, after rinsed with 0.01 M PBS washing buffer, the electrochemical assay was performed in pH 9.5 DEA buffer containing 1 mg mL^{-1} α-NP. The DPV measurement was performed from 0 to 0.5 V with pulse amplitude of 50 mV.

3. RESULTS AND DISCUSSION

3.1. Electrochemical biosensor



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

The principle of the electrochemical biosensor was illustrated in Scheme 1. Firstly, the electrochemical immunosensor was prepared by modifying the glassy carbon electrode (GCE) with functionalized SWNTs/CS film. The SWNTs/CS composite could not only reduce the background current and promote the electron transfer, but also offer a biocompatible film with large surface area [24, 36]. Secondly, the SWNTs/CS modified electrode was covalently bound with capture antibody after activation by EDC/NHS. Then a sandwich type immunoassay was fabricated by successively incubating with human leptin and biotinylated anti-human leptin upon the specific recognition of antibody to antigen. Finally, the streptavidin-alkaline phosphatase (ST-AP) was captured on the immunosensor surface based on the affinity of biotin to ST-AP. Thus the quantification of leptin could be accomplished by differential pulse voltammetry (DPV) monitoring the electrochemical oxidation of the hydrolysis product generated in the AP enzyme reaction upon α-NP.

3.2. Characterization of SWNTs and SWNTs/CS nanocomposite

The scanning electron microscopic (SEM) images were used to investigate the morphology of SWNTs and SWNTs/CS composite. As shown in Figure 1A, the nanotubes were clustered in the absence of CS. Without CS, the nanotubes would dissolve incompletely and aggregate each other in water because of the Vande Waals action between nanotubes [37]. In contrast, Figure 1B showed a homogeneous distribution of SWNTs/CS composite. The strong attractive interaction among the nanotubes would be reduced and the aggregation of SWNTs could be banned in the presence of CS [38, 39], which resulted in a high dispersibility and long-term stability of SWNTs/CS composite in the solution. Seen from Figure 1C, an amplifying image of Figure 1B, we could observe that the SWNTs and CS formed a highly porous architecture by cross-linking with each other. The specific morphologies could provide a large biocompatible film surface area, and the presence of CS could clearly enhance the dispersion performance and film-forming of carbon nanotubes.

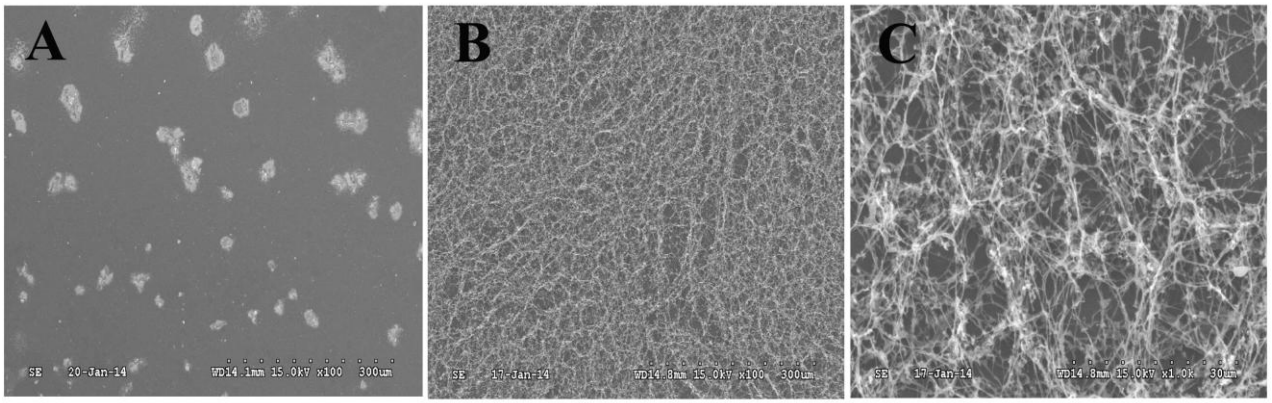


Figure 1. (A) The low magnification SEM image of SWNTs. (B) The low magnification SEM image of SWNTs/CS composite. (C) The high magnification SEM image of the SWNTs/CS composite.

3.3. Characterization of the immunosensor

The electrochemical behavior of the modified electrodes was described by cyclic voltammetry. Figure 2A showed the cyclic voltammograms of SWNTs/CS composite modified GCE electrode in 0.1 M KCl containing 5 mM $[Fe(CN)_6]^{3-/4-}$ at different scan rates. The dependences of peak currents on the scan rate were presented in Figure 2B. With the scan rate from 40 to 200 mV s^{-1}, the shapes of the voltammograms were only slightly affected, and both anodic and cathodic peak currents increased linearly on the scan rate with the correlation coefficients of 0.9953 and 0.9968, respectively. These results indicated that the reaction on the surface of SWNTs/CS modified electrode was a diffusion-controlled process [40].

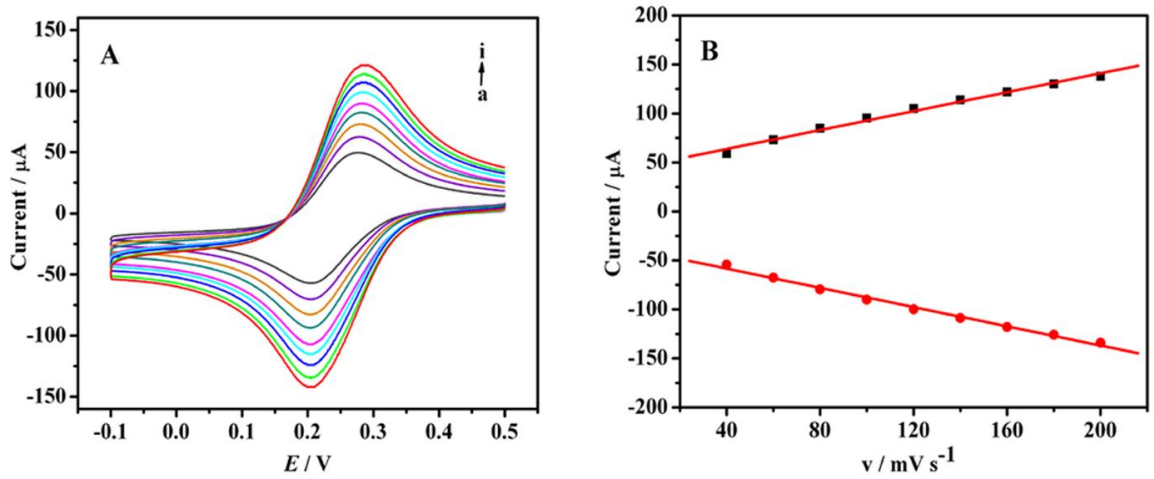


Figure 2. (A) Cyclic voltammograms of SWNTs/CS composite modified electrode in 0.1 M KCl containing 5 mM $[Fe(CN)_6]^{3-/4-}$ at various scan rates. (B) Relationship between the anodic and cathodic peak currents and scan rates.

Both electrochemical impedance spectroscopy (EIS) and square wave voltammetry (SWV) were used to characterize the fabrication process of the immunosensor via obtaining the electronic transfer properties [41]. EIS, composed of a semicircular part and a linear part, was the typical method to display the impedance changes of surface-modified electrodes. The semicircle diameter of EIS was equal to electron-transfer resistance (Ret) and used to indicate the fabrication of the electrochemical immunosensor. As shown in Figure 3A, When the SWNTs/CS film was assembled on bare GCE surface, the curve b exhibited an almost straight line in 0.1 M KCl containing 5 mM $[Fe(CN)_6]^{3-/4-}$, and showed the lower interfacial electron transfer resistance than the bare electrode (curve a). These results implied that the fabricated SWNTs/CS film could promote the electron transfer of the $[Fe(CN)_6]^{3-}$ /$[Fe(CN)_6]^{4-}$ couple effectively. Subsequently, when anti-leptin was added on the activated electrode, the Ret sharply increased, meaning that anti-leptin had been successfully immobilized on the surface of SWNTs/CS modified GCE (curve c). This showed that SWNTs/CS film had a large specific surface area and enhanced the loading amount of anti-leptin [36]. When 2% BSA was used to block the remaining active carboxyl groups on the GCE surface and the leptin was coated on it, the Ret further increased due to the steric hindrance of BSA to electron transfer and the binding of leptin to anti-leptin

(curve d). After the immunosensor was incubated with bio-anti-leptin, increasing electron transfer resistance was observed, demonstrating that the constructed sandwich electrochemical biosensor was successfully fabricated (curve e). The above results were well consistent with those obtained from SWV (Figure 3B), in which the peak current was used to indicate the fabrication of the electrochemical immunosensor. The results of SWV also proved the successful modification of each step on the SWNTs/CS modified GCE.

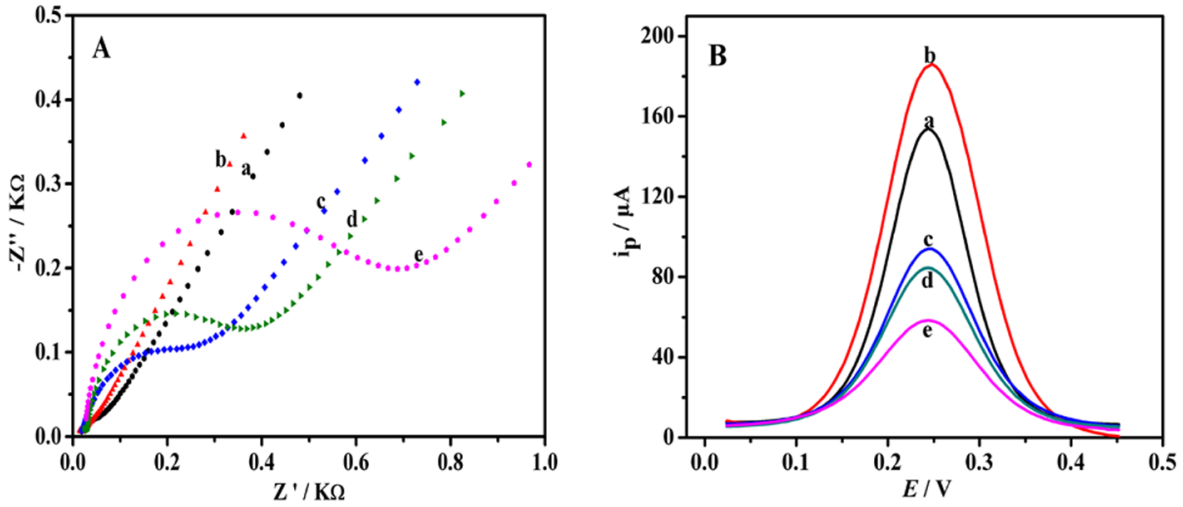


Figure 3. (A) EIS and (B) SWV of bare GCE (a), SWNTs/CS modified GCE (b), anti-leptin-SWNTs/CS modified GCE (c), leptin/BSA-anti-leptin-SWNTs/CS modified GCE (d) and bio-anti-leptin/leptin/BSA-anti-leptin-SWNTs/CS modified GCE (e) in 0.1 M KCl containing 5 mM $[Fe(CN)_6]^{3-/4-}$ at 100 mV s^{-1}.

3.4. Optimization of experimental conditions

It is necessary to optimize some parameters that could affect the performance of the developed biosensor. The incubation time for the sandwich immunoreaction (the specific binding of anti-leptin to leptin and leptin to bio-anti-leptin) was the important condition affecting the reaction of immunoassay. As shown in Figure 4A, with the increasing incubation time, the DPV peak current sharply increased and tended to a steady value after 120 min, and longer incubation time did not improve the response. So 120 min was chosen as the optimal incubation time.

In the sandwich immunoreaction, the concentrations of anti-leptin and bio-anti-leptin were also vital experimental factors [35]. Seen from Figure 4B, the peak current values for 500 ng mL^{-1} leptin increased with the increasing anti-leptin concentration and tended to a plateau at 5 μg mL^{-1}. When the concentration was greater than 10 μg mL^{-1}, the current response decreased slightly, indicating that more anti-leptin immobilized on the electrode might increase disorder of adsorbed antibody and steric hindrance of immunoreactions [42]. Thus, 5 μg mL^{-1} of anti-leptin was used for the following experiments. Meanwhile, when the concentration of bio-anti-leptin was greater than 1 μg mL^{-1}, the current response didn't increase and reached a platform (Figure 4C). Therefore, 1 μg mL^{-1} was adopted as the optimal bio-anti-leptin concentration.

The effect of the concentration of ST-AP on the performance of the electrochemical leptin immunosensor is demonstrated in Figure 4D. Electrochemical responses obtained at different concentrations of ST-AP were recorded. The responses reached to a steady value when the concentration of ST-AP was 1.25 μg mL^{-1}. Further increasing ST-AP concentration could not increase the current response but would lead to nonspecific adsorption on the sensor surface. Hence, 1.25 μg mL^{-1} was chosen as the optimal concentration in the electrochemical immunoassay.

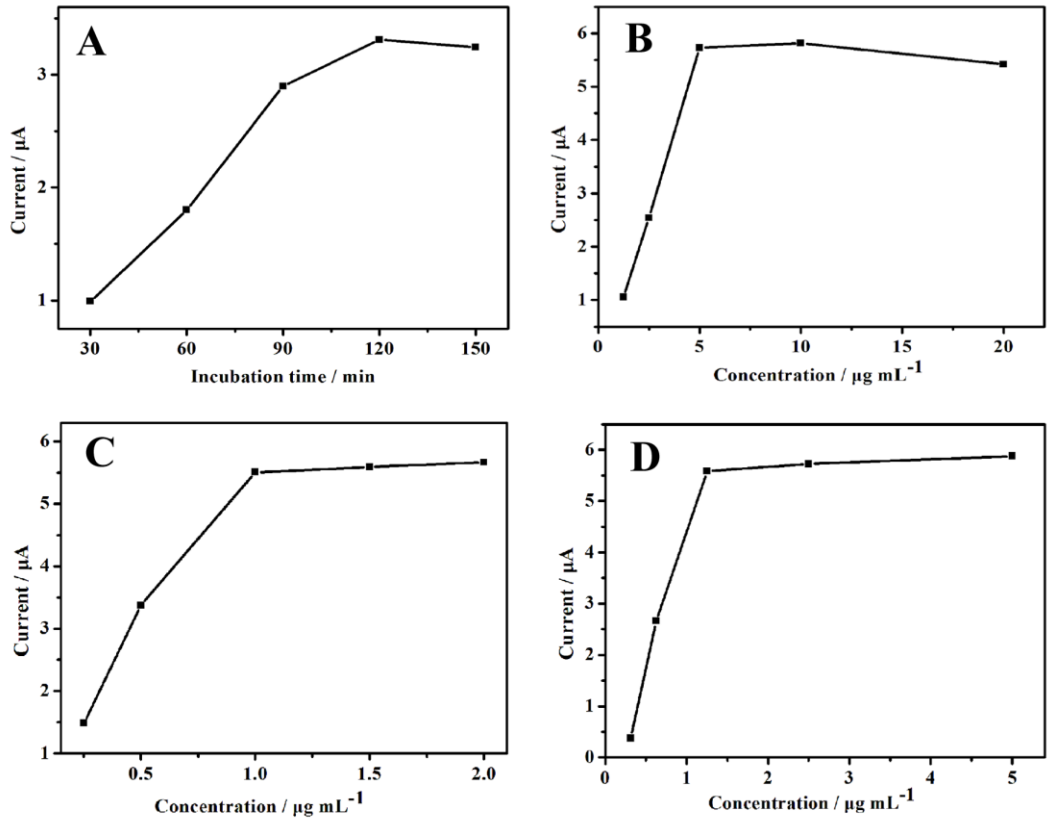


Figure 4. Dependences of DPV peak current on incubation time (A), anti-leptin concentration (B), bio-anti-leptin concentration (C), and ST-AP concentration (D), when one parameter changed while the others were under their optimal conditions.

3.5. Analytical Performance of Electrochemical Biosensor

Under the optimal conditions, the analytical performance of the proposed biosensor was assessed by monitoring the dependence of the DPV peak currents on the human leptin concentrations. Figure 5A showed that the DPV peak currents increased proportionally with the increasing concentrations of the target leptin. As shown in Figure 5B, the calibration plot for leptin was in a good linear relationship between the DPV currents and the logarithm values of the leptin concentrations in the range of 0.05 ng mL^{-1} to 500 ng mL^{-1} with a correlation coefficient of 0.9958, and the corresponding calibration equation was I (μA) = 2.30-1.03 log C (C is the concentration of leptin (ng mL^{-1})). The detection limit of this method was about 30 pg mL^{-1}, which was estimated as three times

the standard deviation of the blank sample measurements. To evaluate the reproducibility of this method, the target leptin at 0.5 ng mL^{-1} and 50 ng mL^{-1} were measured with five times. The relative standard deviation (RSD) values obtained for both concentrations were about 4.70% and 6.24%, respectively. These results indicated that the developed approach had a good precision and acceptable repeatability.

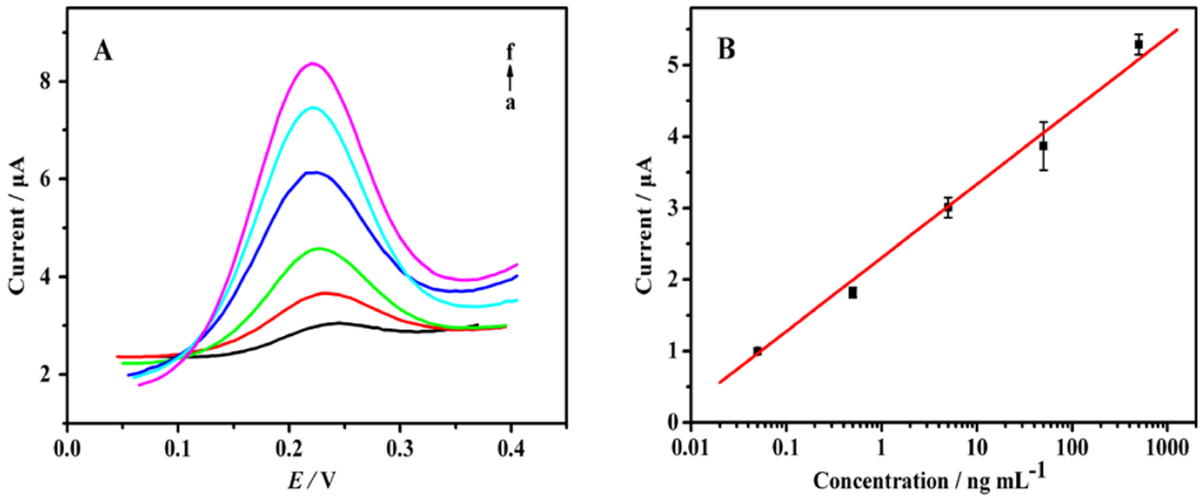


Figure 5. (A) Typical DPV curves of the designed immunosensor with different concentrations of leptin at 0, 0.05, 0.5, 5, 50, 500 ng mL^{-1} (from a to f). (B) Plots of peak current versus the logarithm of leptin concentration. The error bars represent the standard deviations calculated from three different assays.

The performance of the proposed immunosensor was also compared with other reported sensor methods for the detection of leptin. As shown in Table 1, compared with LSPR-based assay and Protein G/PPy/PPa/Au GCE-based assay for leptin [15, 33], the sensitivity of this proposed method was better. Meanwhile, compared with other three methods [34, 35, 43], the immunosensor had a wider dynamic linear range. Due to the features of relative wide dynamic linear range and acceptable detection limit, this method is more suitable for the detection of human leptin in real samples with no dilution (Normal levels of leptin are 3.7-11.1 ng mL^{-1} for women and 2.0-5.6 ng mL^{-1} for men, whereas obviously higher leptin levels have been observed in obese individuals).

Table 1. Comparison of analytical methods for the detection of leptin.

Analytical method	Linear Range	Detection Limit	Reference
LSPR	-	100 pg mL^{-1}	[15]
Protein G/PPy/PPa/Au GCE	10-100000 ng mL^{-1}	10 ng mL^{-1}	[33]
TEGCnSH/Au electrode	0.1-10 ng mL^{-1}	13.6 pg mL^{-1}	[34]
Magnetic beads SPCE	5-100 pg mL^{-1}	0.5 pg mL^{-1}	[35]
Chemiluminescent immunosensor	10-1000 pg mL^{-1}	1.9 pg mL^{-1}	[43]
SWNTs/CS GCE	0.05-500 ng mL^{-1}	30 pg mL^{-1}	This work

3.6. Specificity of the immunosensor

To investigate the selectivity of the fabricated sensor, different proteins and peptides including bovine serum albumin (BSA), Ang II and CRP were examined. The immunosensor was separately exposed to 5000 ng mL^{-1} Ang II, CRP and BSA, respectively. Meanwhile, 500 ng mL^{-1} leptin and the blank (without leptin) were also measured. As shown in the Figure 6, the DPV current responses of BSA, Ang II and CRP were similar to that of the blank (without leptin), while the signal to leptin increased dramatically. The error bars represented average standard errors for three measurements. These experimental results clearly indicated that the established immunosensor had a good selectivity for leptin assay.

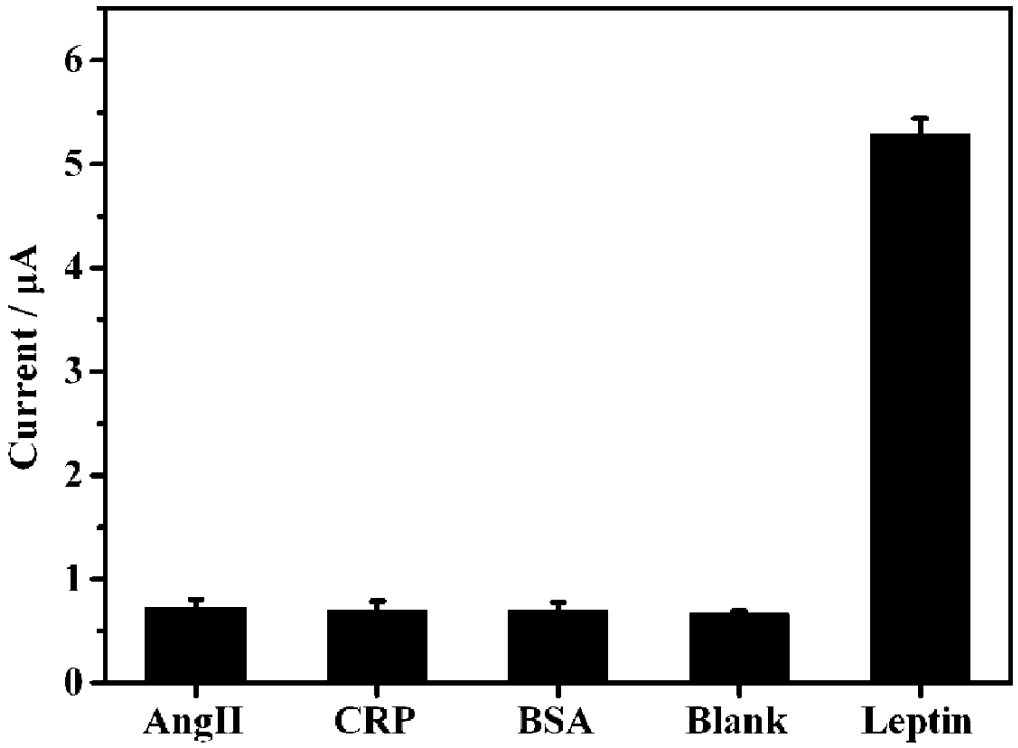


Figure 6. Specificity analysis of the amperometric immunsensor. The DPV peak currents of the immunosensor in the presence of 500 ng mL^{-1} of leptin, 5000 ng mL^{-1} of other proteins (Ang II, CRP, BSA) and the blank. The error bars represent average standard errors for three assays.

3.7. Detection of leptin in the spiked serum samples

In order to evaluate the applicability of the amperometric immunsensor, 50% diluted serum specimens spiked with different concentrations of target leptin were detected with biosensor. The results listed in Table 2 clearly indicated that the developed electrochemical immunosensor had a strong resistance to the complex matrix of serum, and can be used to detect leptin in real serum samples with a recovery of 92.13%-106.55% and a RSD＜6% (n=3). These results demonstrated that the designed biosensor could be of a potential clinical applicability for the detection of human leptin.

Table 2. Determination of leptin in blank serum samples (n =3).

Target leptin added (ng mL^{-1})	Detected results (ng mL^{-1})	Recovery (%)	RSD (%)
0.050	0.052	104.00	4.00
0.200	0.190	95.00	5.26
15.00	13.82	92.13	1.50
200.00	195.60	97.80	1.37
500.00	532.74	106.55	3.13

4. CONCLUSIONS

In this work, a novel electrochemical immunosensor for the determination of leptin was developed, involving the use of functionalized SWNT/CS composite, a sandwich-type immunoassay and ST-AP. The SWNTs/CS film on the GCE has superior features, which can not only offer a large surface area to immobilize more antibodies on the GCE but also enhance the electron transfer rate between the GCE and the measurement solution. With the sandwich immunoassay strategy, the fabricated immunosensor exhibited a good analysis performance in terms of the broad linearity range, good sensitivity and reproducibility. Moreover, this immunosensor was successfully applied to detect leptin in real serum. Therefore, it might become a promising platform for the assay of leptin.

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