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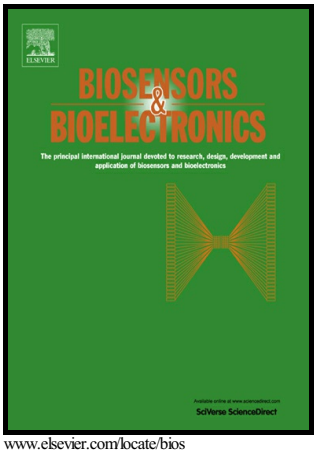

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Bovine serum albumin as an effective sensitivity enhancer for peptide-based amperometric biosensor for ultrasensitive detection of prostate specific antigen

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Abstract

Bovine serum albumin (BSA) was firstly implemented as an effective sensitivity enhancer for a peptide-based amperometric biosensor for the ultrasensitive detection of prostate specific antigen (PSA). A porous and conductive substrate of chitosan-lead ferrocyanide-(poly(diallyldimethylammonium chloride)-graphene oxide) was in-situ generated on a glassy carbon electrode (GCE), in which $Pb_2[Fe(CN)_6]$ served as a novel redox species with strong current signal at -0.46 V (vs. Ag/AgCl). Poly(diallyldimethylammonium chloride)-graphene oxide was applied to improve conductivity of the substrate. After adsorbing Pb^{2+} for signal amplification, chitosan provided active sites to simultaneously immobilize peptides and 1-aminopropyl-3-methylimidazolium chloride by glutaraldehyde. To enhance the sensitivity, BSA was chemically linked to the immobilized peptide, behaving as a serious decrease of current signal for BSA hindering the electron transfer. The dramatic increase of current signal of the biosensor was obtaine d by PSA cleaving

the immobilized BSA-peptide. The proposed biosensor exhibited a detection limit of 1 fg mL^{-1} for PSA and its sensitivity was seven-fold higher than previous works.

Keywords

Bovine serum albumin, Peptides, PDDA-GO, Chitosan, $Pb_2[Fe(CN)_6]$, Cancer marker.

1. Introduction

Recently, peptides as promising candidates for sensitive and early detection of cancer have attracted much attention (Xie et al. 2015; Kaspar and Reichert 2013). Peptide is easy to acquire in comparison to monoclonal antibody for the relatively simple, mature synthesis and purification technique (Jung and Beck-Sickinger 1992; Lewandowski et al. 2013). Owing to its specific amino acid sequences, the peptide-based biosensors have proven their excellent specificity for detecting tumor markers (Liu et al. 2015; Lalmahomed et al. 2016). Hence, this kind of biosensor is of great significance in clinical diagnosis. Up to now, many peptide-based biosensors for biomarkers were developed, including fluorescent, colorimetric, electrochemiluminescent, impedimetric and amperometric assays (Han et al., 2015; Liang et al. 2016; Wei et al. 2015; Wang et al. 2015; Tang et al. 2016). Among these methods, the amperometric assay is highly attractive owing to its advantages of relatively high sensitivity, low cost, time-saving procedure, and miniaturization (Hun et al. 2015; Tang et al. 2016).

The assembly of the sensing interface can significantly influence the analytical performance of the biosensor (Xia et al. 2017, Tang et al. 2015). Compared with antibody-based biosensor (Shan et al. 2015), the peptide-based sensing interface still possesses good conductivity after the immobilization of peptide (Yin et al. 2015; Tang

et al. 2016), indicating that the peptide-based biosensor is promising in the ultrasensitive detection of tumor marker. Although conductive nanomaterials are widely applied, the current responses of the obtained biosensor are generally at several microampere levels, indicating that new methods for sensitivity amplification are urgent.

Considering that the sensitivity highly relied on the redox species labeled peptide (Li et al. 2013), some effective strategies were developed by synthesizing peptides modified with redox nanocomposites (He et al. 2015), preparing conductive substrate with redox nanocomposites (Tang et al. 2016), and employing enzymes and exonucleases (Wang et al. 2016; Zhu et al. 2016). Although these biosensors exhibited relatively high sensitivities with low detection limits, the complicated experimental procedures, expensive nanomaterials and enzymes were commonly involved, which limited their application in clinical diagnosis to some extent. In this case, a simple and effective sensitivity amplification strategy for peptide-based biosensor for the ultrasensitive detection of tumor marker will be of great significance.

Herein, a porous and conductive substrate with strong current signal at -0.46 V (vs. Ag/AgCl) was constructed by in-situ generation of chitosan-$Pb_2[Fe(CN)_6]$-(poly(diallyldimethylammonium chloride)-graphene oxide) (chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO), in which $Pb_2[Fe(CN)_6]$ was used as a novel redox species. A simple and ultrasensitive amperometric peptide-based biosensor for prostate specific antigen (PSA) was developed using BSA as an effective sensitivity enhancer.

2. Experimental

2.1. *Materials*

Chitosan was purchased from Tokyo Chemical Industry (Japan). GO was purchased

from JCNANO (Nanjing, China). The specific peptide (CEHSSKLQLAK-NH_2) and 1-Aminopropyl-3-methylimidazolium chloride (99%) were purchased from Shanghai Sangon (shanghai, China) and Chengjie Chemical Reagents Company (shanghai, China), respectively. PDDA (35%, in water), $Pb(NO_3)_2$, $K_4Fe(CN)_6$, acetic acid (HAc) and sodium acetate were purchased from Beijing Chemical Reagents Company (Beijing, China). BSA and immunoglobulin G (IgG) were purchased from Xinjinke (Beijing, China). PSA, alpha-fetoprotein (AFP) and carcinoembryonic antigen (CEA) were purchased from Shanghai Linc-Bio Science Co. Ltd. (Shanghai, China). Dopamine hydrochloride (DA), urea acid (UA), ascorbic acid (AA), L-glutamic acid (Glu) and D-(+)-glucose were commercially obtained from Alfa Aesar (Beijing, China). Clinical human serum samples were obtained from the Capital Normal University Hospital (Beijing, China). Ultrapure water was purified through an Olst ultrapure K8 apparatus (Olst, Ltd., resistivity >18 MΩ cm).

2.2. Apparatus

Transmission electron microscopy (TEM) measurements were performed on a JEOL-100CX electron microscope (Hitachi, Japan) under 80 kV accelerating voltage (H7650, Hitachi, Japan). Zeta potential measurements were performed on a Zetasizer Nano ZS (Malvern Instruments Co., UK). Scanning electron microscopy (SEM) measurements were performed on a Hitachi S-4800 scanning electron microscope. Ultraviolet-visible (UV-vis) spectrum was recorded on a UV-2550 UV-vis spectrophotometer (Shimadzu, Japan). X-ray photoelectron spectroscopy (XPS) analysis was performed on an Escalab 250 X-ray photoelectron spectroscope (Thermofisher, American). Electrochemical impedance spectroscopy (EIS) measurements were conducted with a Princeton PARSTAT 2273 (America). Square wave voltammetry (SWV) measurements were conducted with a CHI 832

electrochemical workstation (Shanghai, China).

2.3 Preparation of chitosan-$K_4[Fe(CN)_6]$-PDDA-GO

GO-PDDA aqueous solution was synthesized according to the literature with a little modification (Cui et al. 2008; Suo et al. 2016). In brief, 10 mg GO was dissolved in 20 mL water. Then 114 μL PDDA (wt. 35%) aqueous solution and 1.169 g NaCl were added into the mixture with vigorous stirring for 6 h. After that, PDDA-GO was obtained by centrifugation, washed with water and dissolved to10 mL with water.

A homogeneous chitosan aqueous solution was prepared by mixing 98.8 mL water, 0.2 g chitosan and 1mL HAc and then was diluted with an equal volume of water. Ten microliters of 0.5% $K_4[Fe(CN)_6]$ aqueous solution was injected into 100 μL chitosan aqueous solution with ultrasonic treatment for 10 s. After injecting 60 μL PDDA-GO and 1120 μL water into the mixture, chitosan-$K_4Fe(CN)_6$]-PDDA-GO was obtained.

2.4 Fabrication of chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE

A glassy carbon electrode (φ = 4 mm) (GCE) was carefully polished with 0.05 μm alumina slurry with ultrasonic treatment. Then 10 μL chitosan-$K_4[Fe(CN)_6]$-PDDA-GO was coated on GCE and dried at 37°C. After being soaked in 0.1 M $Pb(NO_3)_2$ aqueous solution containing 0.05% glutaraldehyde for 30 min, chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE was obtained and then washed with water.

2.4 Fabrication of the proposed biosensor

The mixture of peptide and ionic liquid (peptide-IL) was prepared by mixing 1 mL 0.25 mg mL^{-1} peptide with 10 μL 1-aminopropyl-3-methylimidazolium (wt. 5%) aqueous solution. Chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE treated with 10 μL 1% glutaraldehyde for 30 min at room temperature, and then incubated with 50 μL peptide-IL for 1 h. For sensitivity amplification,

peptide-IL/chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE was further treated with 1% glutaraldehyde and then incubated with 50 μL 10 μg mL^{-1} BSA aqueous solution for 1 h. After being blocked with 0.1% BSA for 1 h at 37°C, the proposed biosensor for PSA was obtained. After each step the modified electrode was carefully washed with water. BSA/peptide-IL/chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE was stored at 4°C before use.

2.5 Electrochemical measurements

Electrochemical measurement was performed with a three-electrode system. Eighty microliters of PSA aqueous solution or human serum sample was incubated on BSA/peptide-IL/chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE for 1 h at 37°C followed by washing with water. Then SWV measurement was performed from -1.3 V to 0 V with an increasing potential of 0.025 V in 0.2 M acetate buffer solution (pH=5.5).

3. Results and discussion

3.1. Detection principle of the proposed biosensor

Fabrication procedure and principle of the biosensor are illustrated in the Scheme 1. Multiple amplification strategies for the proposed biosensor were conducted as follows. $Pb_2[Fe(CN)_6]$ as a conductive redox species was in situ generated on the porous chitosan-Pb^{2+}-PDDA-GO membrane (Zacher et al. 2009). Chitosan was used to link with peptide and adsorb Pb^{2+} ions through coordination effect for current signal amplification (Xu et al. 2014; Wang et al. 2014). GO-PDDA was employed to enlarge the specific area and conductivity of the sensing interface, resulting in further signal amplification. To improve the cleaving efficiency of PSA for peptide, the space between peptides was increased by the co-immobilization of 1-aminopropyl-3-methylimidazolium and peptide on the substrate, which was beneficial for sensitivity amplification. In order to effectively amplify the sensitivity,

BSA was used as a sensitivity enhancer. The current signal further decreased when BSA was chemically linked to the immobilized peptide resulting in the increase of resistance of the sensing interface. Then, a dramatic increase of current signal was obtained when the BSA-peptide was cleaved by PSA. Therefore, multiple amplification strategies were achieved and the proposed biosensor exhibited high sensitivity for PSA.

Scheme 1

3.2. Detailed composition of the synthesized chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO

Fabrication procedures of chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO were characterized by XPS (Table S1). C1s, N1s, and O1s belong to the C, N, and O elements in chitosan, GO, and PDDA, respectively (Fig. 1A). After mixing GO with PDDA, the Cl2p peak at 197.2 eV was assigned to Cl^- (Escard et al. 1974) (Fig. 1A), indicating that GO-PDDA was obtained. Fe2p peak at 708.4 eV was designated to $K_4[Fe(CN)_6]$ (Nemoshalenko et al. 1977) (Fig. 1A).The Pb4f, $Pb4d_5$, and $Pb4d_3$ peaks at 138.5 eV, 434.4 eV, and 412.1 eV were observed when chitosan-$K_4[Fe(CN)_6]$-PDDA-GO was incubated with $Pb(NO_3)_2$ solution containing glutaraldehyde (Fig. 1B), indicating chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO was obtained (Pederson, L.R. 1982; Ohno, Y. 1991). The content ratio value of Pb/Fe was 2.59 larger than 2 (GIL et al. 2010), indicating that some Pb^{2+} ions were adsorbed by chitosan.

Fig. 1

3.3. Stepwise fabrication procedures of the proposed biosensor

Compared with the morphology of chitosan-$K_4[Fe(CN)_6]$/GCE (Fig. S1), no obvious nanoparticles were observed on the surface of chitosan-$K_4[Fe(CN)_6]$-PDDA-GO/GCE, resulting from that GO-PDDA promoted the dispersion of chitosan-$K_4[Fe(CN)_6]$ (Fig. 2A). After chitosan-$K_4[Fe(CN)_6]$-PDDA-GO/GCE was incubated with $Pb(NO_3)_2$

solution containing glutaraldehyde, a porous surface was obtained and $Pb_2[Fe(CN)_6]$ nanoparticles were uniformly distributed on the surface of chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE (Fig. 2B).

EIS measurements were performed in 0.5 mM $[Fe(CN)_6]^{3-/4-}$ aqueous solution containing 0.1 M $NaNO_3$ (Fig. 3**C** and S2). The Nyquist plot of chitosan-$K_4[Fe(CN)_6]$-PDDA-GO/GCE was small (curves **a-b**), indicating that the chitosan-$K_4[Fe(CN)_6]$-PDDA-GO possessed good conductivity. Then diameter of the semicircle decreased after treatment of Pb^{2+}, suggesting that the conductive chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE was obtained (curve **c**). While the resistance of chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE gradually increased when peptides and BSA were incubated on chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE (curves **d-f**), indicating that the peptide and BSA were immobilized (Lin et al. 2016; Yan et al. 2013), since peptide and BSA hindered the electron transfer. In contrast, conductivity of BSA/peptide-IL/chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE was improved after being incubated with PSA (curve **g**), indicating that peptide was cleaved by PSA.

Further, SWV measurements were performed to characterize fabrication procedures of the proposed biosensor (Fig. 3**D**). A strong current signal at -0.45 V (vs. Ag/AgCl) was provided by chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE (curve **a**). The current signal gradually decreased when the peptide-IL and BSA were incubated on the substrate (curves **b-d**), indicating that peptide-IL and BSA were immobilized and the proposed biosensor was obtained. After incubating PSA on the biosensor, the current signal remarkably increased (curve **e**), indicating that peptide was cleaved by PSA.

Fig. 2

Fig. 3

3.4. Zeta potential of chitosan-$K_4[Fe(CN)_6]$-PDDA-GO/GCE

For the fabrication of sensing interface in this biosensor, it is necessary to obtain a positively charged chitosan-$K_4[Fe(CN)_6]$-PDDA-GO/GCE since Pb^{2+} ions could denature peptide and PSA. In this case, to avoid the adsorption of free lead ions on chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE, a positively charged chitosan-$K_4[Fe(CN)_6]$-PDDA-GO/GCE was obtained by regulating the content of $K_4[Fe(CN)_6]$. Zeta potentials of chitosan, chitosan-$K_4[Fe(CN)_6]$, GO and PDDA-GO were measured. The zeta potentials of GO (Fig. 4**A**) and chitosan (Fig. 4**B**) were -30 mV and 50 mV, respectively. After PDDA was mixed with GO, the zeta potential of mixture was 34 mV (Fig. 4**C**), indicating that the PDDA-GO composite was formed through electrostatic adsorption. After mixing chitosan with $K_4[Fe(CN)_6]$, the zeta potential of chitosan-$K_4[Fe(CN)_6]$ was 30 mV (Fig. 4**D**). In this case, the zeta potential of the mixture of chitosan-$K_4[Fe(CN)_6]$ and PDDA-GO was positive.

Fig. 4

3.5 Optimization experiments

The analytical performance of the proposed biosensor was significantly affected by the pH of the analytical solution, the concentration of peptide, and the incubation time of PSA sample. Related optimal experiments were performed. The current signal of chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE gradually decreased when the concentration of the peptide increased from 0.08 mg mL^{-1} to 0.25 mg mL^{-1}, and then the signal kept stable when the concentration of the peptide increased from 0.25 mg mL^{-1} to 0.4 mg mL^{-1} (Fig. S3**A**). Hence, 0.25 mg mL^{-1} peptide solution was chosen for the subsequent experiments. After that, the incubation time of PSA sample was investigated. The current signal was generally increasing when the incubation time of PSA increased from 30 min to 45 min, and then kept stable when the incubation time increased from 45 min to 1 h (Fig. S3**B**). In this case, the incubation time of PSA was chosen as 45

min. Further, a suitable pH of the analytical solution was essential for the bioactivity of PSA and peptide, and the affinity between the protein and the electrode surface. In this case, optimization experiments for the pH of the analytical solution were performed. The conductivity of the current signal of the proposed biosensor for PSA generally increased when the pH increased from pH 4.5 to pH 5.5. The current signal decreased with continuously increasing from pH 5.5 to 6.5 (Fig. S3**C**). The largest current response of the proposed biosensor for PSA in the pH 5.5 analytical solution was obtained, indicating that the highest sensitivity of the proposed biosensor was acquired. Therefore, pH 5.5 acetate buffer was chosen as the analytical solution for the subsequent experiments.

3.6. Analytical performance of the proposed biosensor for PSA

Analytical performance of the proposed biosensor was evaluated by determining different concentrations of PSA samples under the above optimal conditions. The current signal generally increased with the increase the concentration of PSA (Fig. 5 **A**). This biosensor exhibited a wide linear range from 1 fg mL^{-1} to 100 ng mL^{-1} with an ultralow detection limit of 1 fg mL^{-1}. The linear regression equation was I (μA) = $21.61\log_{10}c_{PSA}$ (ng mL^{-1}) + 147.0, with a correlation coefficient of 0.9959 (Fig. 5 **B**). Compared with some recent literatures for PSA, this method possessed a wider linear range with a lower detection limit, and its sensitivity was about seven-fold higher (Table S2).

Fig. 5

3.7. Evaluation the repeatability, specificity, stability and reliability of the biosensor

To evaluate the repeatability of the proposed biosensor, three parallel experiments were performed. Current variations for 0.1 ng mL^{-1} and 1pg mL^{-1} PSA were 3.51% and 4.35%, respectively, indicating that this method possessed good repeatability. To

investigate the specificity, 0.1 ng mL^{-1} PSA containing 100 ng mL^{-1} interference substances (such as AA, DA, UA, glucose, Glu, IgG, CEA and AFP) was determined by the proposed biosensor. The influence of interference substances in PSA sample on the current signal of the biosensor for PSA was negligible (Fig. S4), indicating the excellent specificity of the proposed biosensor. To investigate the stability, some freshly prepared biosensors were stored at 4°C. After 15 days, the current responses of these biosensors for PSA remained 97.2% in comparison to that of the fresh biosensor, indicating that the stability of the biosensor was acceptable. To evaluate the reliability, fifteen human serum samples were determined with the proposed method and the enzyme linked immunosorbent assay (ELISA). The relative error between the proposed method and ELISA was less than 10% (Table S3), indicating good reliability of the proposed biosensor.

4. Conclusion

In summary, $Pb_2[Fe(CN)_6]$ was synthesized and implemented as a novel redox species with strong current signal at -0.46 V (vs. Ag/AgCl). BSA was designed as a sensitivity enhancer for a peptide-based amperometric biosensor for the ultrasensitive detection of PSA with multiple amplification strategies. A porous substrate with multiple signal amplification was fabricated by chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO through drop coating method. The ionic liquid terminated with amino group was employed to promote the cleavage of the peptide. BSA immobilized on peptide was used as an effective sensitivity enhancer. The proposed biosensor for PSA exhibited a wider linear range (100 ng mL^{-1}~1 fg mL^{-1}) and a lower detection limit (0.01 fg mL^{-1}) in comparison to recent literatures. This peptide-based biosensor can be extended to the detection of other biomarker. BSA as sensitivity enhancer provided a new way to fabricate the ultrasensitive electrochemical sensor.

Acknowledgments

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Figure captions

Scheme 1. Fabrication procedures and detection principle of the proposed biosensor for PSA.

Fig. 1. (A) XPS spectra of $K_4[Fe(CN)_6]$, chitosan, GO and GO-PDDA. (B) XPS spectra of chitosan-$K_4[Fe(CN)_6]$-PDDA-GO and chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO.

Fig. 2. (A) SEM micrographs of chitosan-$K_4[Fe(CN)_6]$-PDDA-GO/GCE (A) and chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE (B). Insert: magnified SEM of chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE.

Fig. 3. (A) Nyquist plots of the modified electrodes: bare GCE (a), chitosan-$K_4[Fe(CN)_6]$-PDDA-GO/GCE (b), chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE (c), peptide-IL/chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE (d), BSA/peptide-IL/chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE (e), the proposed biosensor blocked by BSA(f) and then incubated with PSA (g). (B) SWV responses of the modified electrodes: chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE (a), peptide-IL/chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE (b), BSA/peptide-IL/chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO/GCE (c), the proposed biosensor blocked by BSA(d) and then incubated with PSA (e).

Fig. 4. Zeta potentials of GO (A), chitosan (B), PDDA-GO (C) and chitosan-$K_4[Fe(CN)_6]$.

Fig. 5. (A) SWV responses of the proposed biosensor for different concentrations of PSA. (B) Linear calibration curve of the current response and the logarithm of the concentration of PSA.

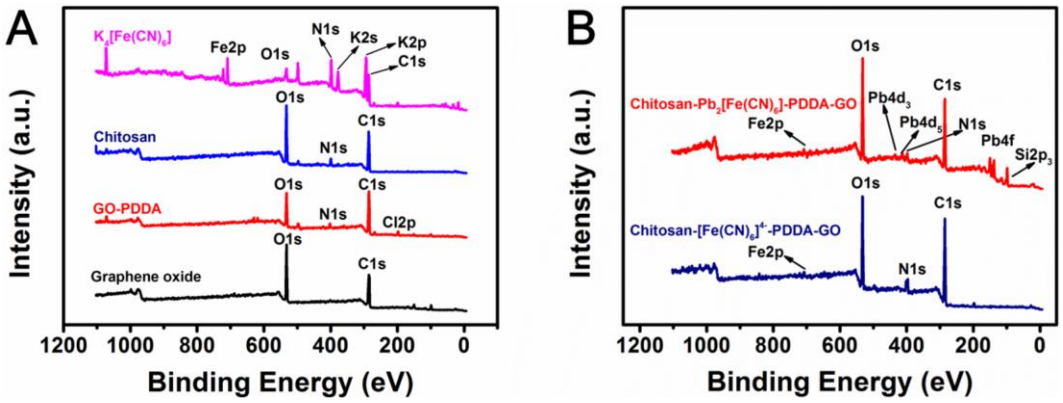


Fig. 1

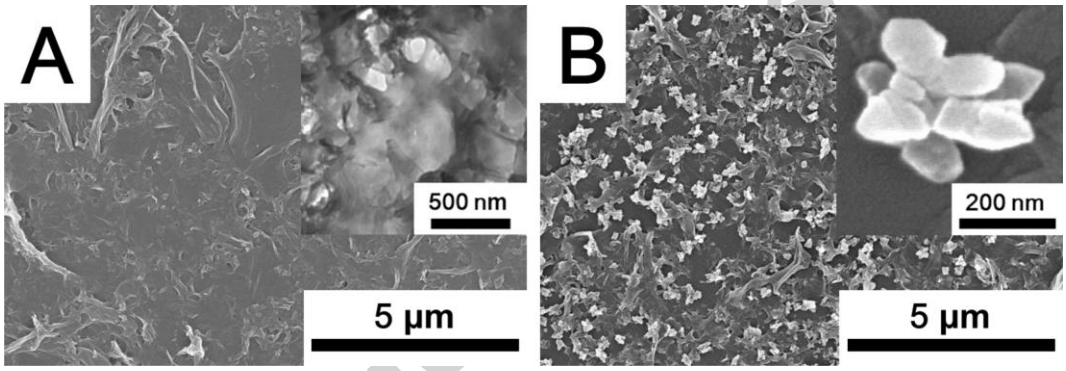


Fig. 2

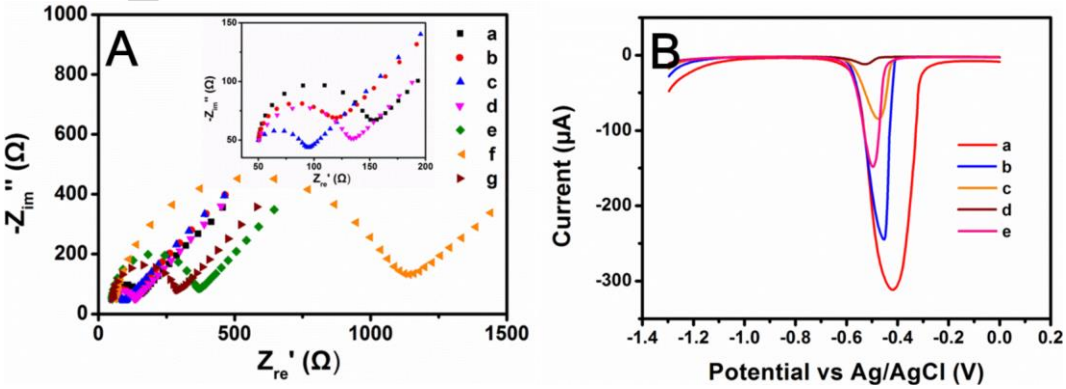


Fig. 3

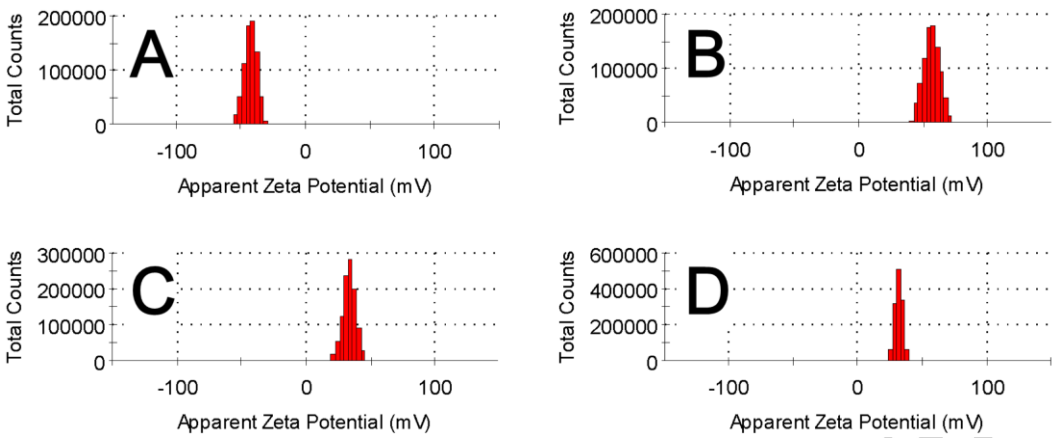


Fig. 4

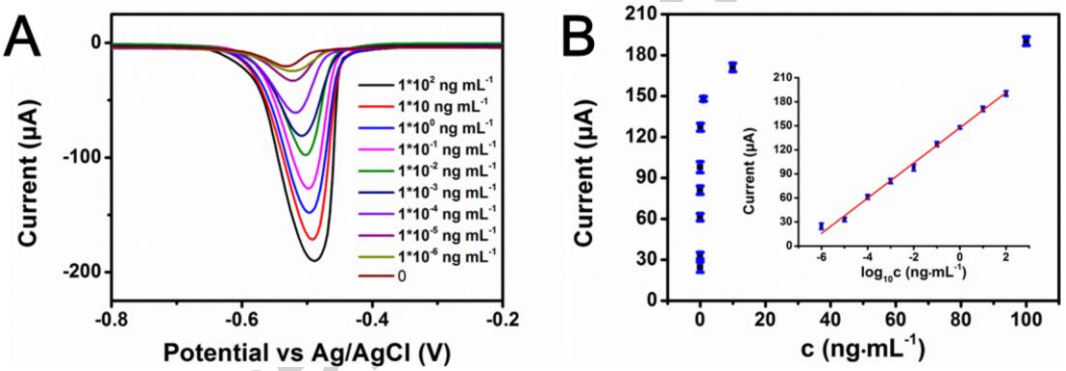
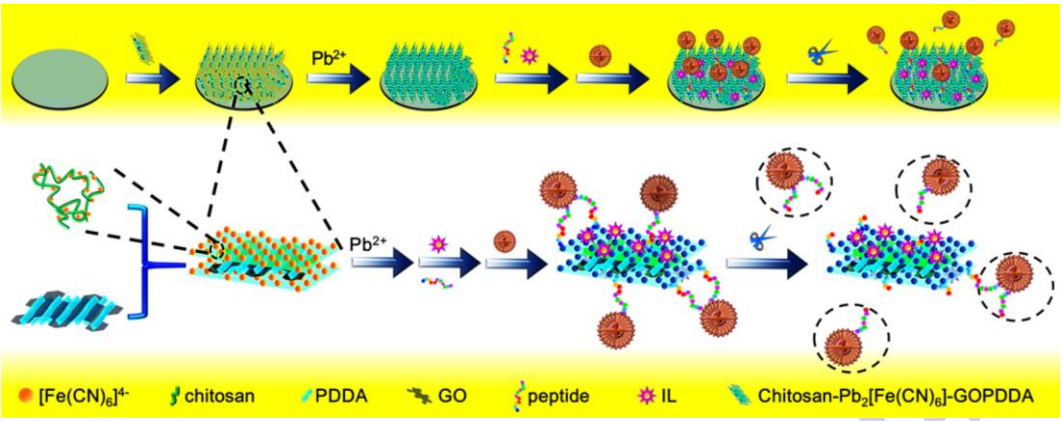


Fig. 5



Scheme

Highlights

- BSA was firstly implemented as an effective sensitivity enhancer for the peptide-based amperometric biosensor.
- Multiple amplification strategies were developed for the amperometric biosensor for PSA.
- Chitosan-$Pb_2[Fe(CN)_6]$-PDDA-GO as a new redox species was used as substrate.