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Graphical abstract

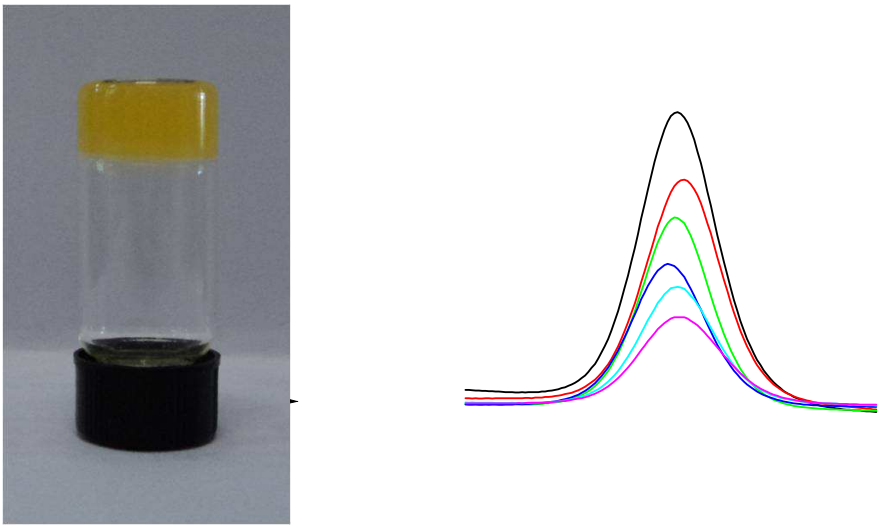

Image of the synthesized hydrogel and square wave voltammetry (SWV) response of the immunosensor to different concentrations of PSA

Redox hydrogel based immunosensing platform for label-free detection of cancer biomarker

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Abstract Redox hydrogel was prepared from ferrocene (Fc) modified amino acid phenylalanine (Phe, F) and utilized to construct an immunosensing platform for the label-free detection of a cancer biomarker prostate specific antigen (PSA). The synthesized hydrogel contains a great number of Fc moieties, which impart the hydrogel with redox properties. The hydrogel modified electrode displays a pair of reversible redox peaks, indicating the facile electron transfer of Fc in the hydrogel at the electrode surface. After the immobilization of the anti-PSA antibody onto the hydrogel modified electrode surface and the capture of PSA molecules, the redox current at the electrode was suppressed significantly due to the blocking of the electron transfer at the electrode surface by the formed immuno-complex. The current change was in proportional to the concentration of PSA detected in the range from 1 pg mL^{-1} to 10 ng mL^{-1} with a detection limit of 0.5 pg mL^{-1}. The proposed immunosensor is simple with low cost and high sensitivity, which could find wide clinical applications.

Keywords: Ferrocene; Label-free; Phenylalanine; Self-assemble; Redox hydrogel

1. Introduction

Immunosensors that are prepared based on the specific binding between antibody and corresponding antigen have found wide applications in clinical diagnosis, food safety testing and environmental monitoring.[1-3] For the successful preparation of immunosensors with good performance, it is crucial to construct the immunosensing platform that could facilitate the immobilization of antibodies and the transduction of immunosensor signals. Different kinds of immunosensing platforms have been reported, such as using nanomaterials (e.g., metal nanoparticles, carbon nantube, magnetic nanoparticles) to modify the electrode surface.[4-6] These nanomaterials have good biocompatibility and conductivity. While the biocompatibility of these nanomaterials could maintain the activity of the antibodies, the good conductivity of nanomaterials could enhance the electron transfer at the electrode surface. Other methods for the construction of the sensing platform include, but are not limited to, the modification of the electrode with various polymers (e.g., self-assembled monomers and molecularly imprinted polymers) and gels (e.g., silica based sol-gels and hydrogels).[7-10]

By functionalizing gels and hydrogels with different redox moieties, redox hydrogels can be formed.[11, 12] For example, a redox hydrogel is formed by cross-linking poly(N-vinylimidazole) (PVI) complexed to *Os*-(4,4‘-dimethylbpy)$_2$Cl (termed PVI$_{15}$-dmeOs) with poly-(ethylene glycol) diglycidyl ether (PEG).[13] However, the synthesis of such a redox hydrogel is rather complicated and time-consuming. Recently, we discovered stable redox hydrogels could be formed through the

self-assembly of ferrocene (Fc) modified phenylalanine (Phe, F) monomers (Fc-F) in water.[14, 15] Such prepared hydrogel holds a great number of water molecules, which means the hydorgel has good biocompatibility. The good biocompatibility, plus its facile preparation process and redox functionality enable many applications of the hydrogel in biosensing applications. Biomolecules can either be incorporated into the hydrogel or immobilized on the hydrogel surface.

In this work, we developed an electrochemical immunosensing platform based on the prepared redox hydrogel for label-free detection of cancer biomarker prostate specific antigen (PSA). PSA has been proved to be the most reliable and specific tumor marker of prostate cancer.[16] Electrochemical methods have the advantage of high sensitivity, low cost and simple instrumentation. The hydrogel modified electrode displays a pair of strong current peaks originated from the Fc moieties in the hydrogel. A chitosan layer was coated onto the hydrogel modified electrode surface to stabilize the hydrogel. Gold nanoparticles (AuNPs) were then adsorbed onto the chitosan layer for the immobilization of anti-PSA antibodies. The specific binding between antibody and PSA resulted in the formation of immuo-complex on the electrode, leading to the decrease of the peak current and quantitive detection of PSA.

2. Experimental methods

2.1 Apparatus and reagents

Prostate specific antigen (PSA) and anti-PSA antibody were obtained from Dingguo Biotechnology Co., Ltd. (Beijing, China). Chitosan (MW =140 000-220 000) was purchased from Sigma-Aldrich. Dichloromethane (DCM, ACS grade) used for

the synthesis was dried and distilled over CaH_2, and then stored over molecular sieves. Hydroxybenzotriazole (HOBt), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), and H-Phe-OMe·HCl were purchased from GL Biochem (Shanghai, China). Ferrocene monocarboxylic acid (Fc-COOH) was obtained from Xiya Reagent (Chengdu, China). For thin layer chromatography, glass plates coated with silica gel (60 GF_{254}) were used. For column chromatography, a column with a width of 2.7 cm and a length of 45 cm was packed 18-22 cm high with 200-300 mesh silica gel (Silicylcye, 230-240 mesh). Phosphate buffered saline (PBS, pH 7.4) solution was used as the supporting electrolyte during electrochemical measurements. All other reagents were of analytical grade and deionized water (MillQ, 18.2MΩ) was used throughout the study.

All electrochemical measurements were performed on a CHI 650D electrochemical workstation (Shanghai CH Instruments Co., China). A conventional three-electrode system was used for all electrochemical measurements: a glassy carbon electrode (3 mm in diameter) as the working electrode, a saturated calomel electrode as the reference electrode, and a platinum wire electrode as the counter electrode. Scanning electron microscope (SEM) images were obtained from Nova NanoSEM230 (FEI, USA).

2.2 Preparation of the hydrogel

The hydrogel was prepared according to our previously reported procedure.[15, 17].Fc-COOH (5 mM) and HBTU/HOBt (5.5 mM) were first dissolved in DCM (100 mL), and Et_3N was added dropwise to the solution to activate the carboxyl group.

After a 1-hour reaction at 0℃, H-Phe-OMe•HCl (5.5 mL) was then added and the reaction mixture was stirred overnight, followed by washing with saturated aqueous solutions of $NaHCO_3$, HCl (10%), and water. The resulting solution was then dried over Na_2SO_4 and evaporated to dryness under reduced pressure. The crude product was purified by flash column chromatography (DCM: EtOAc: PE=3:1:5,v/v/v), and evaporated under reduced pressure in a rotovap to an orange oil. The oil was dissolved in dimethyl sulfoxide (DMSO) and dried in a freeze dryer for overnight, resulting in an orange crystalline solid. The obtained crystalline solid (Fc-Phe-OMe, 3 mM) was dissolved in CH_3OH (30 mL) and mixed with NaOH (15 mL, 1 M). After being stirred for 2 h, the mixture was neutralized by HCl (10%). After removing the CH_3OH by evaporation under reduced pressure in a rotovap, an orange suspension was obtained. The suspension was dissolved in DCM followed by washing with HCl (10%) and water, and then dried over Na_2SO_4. The crude product was purified by flash column chromatography (DCM: EtOAc: MeOH=9:3:1,v/v/v), then evaporated under reduced pressure in a rotovap to an orange oil. The oil was dissolved in DMSO and dried in a freeze dryer overnight, resulting in an orange needle crystalline solid of Fc-Phe-OH.

For the preparation of the hydrogel, the lyophilized form of Fc-Phe-OH was dissolved in DMSO at a concentration of 100 mg mL^{-1} and used as a stock solution. Then the stock solutions were diluted to a final concentration of 4 mg mL^{-1} in PBS (10 mM, pH=7.4) followed by sonication for a few seconds. The suspension turned to clear yellowish hydrogel immediately.

2.3 Fabrication of the immunosensor

To fabricate the immunosensor, 3 μL of hydrogel solution was added onto an electrode surface. When the electrode was dried, another 3 μL of chitosan solution (1%, w/w) was applied to the electrode. After the chitosan solution was dried, the electrode was immersed in an AuNP solution for 1 h to immobilize the AuNPs on the electrode surface. Then, 3 μL of the antibody (10 μg mL^{-1}) solution was added onto the electrode and incubated for 1 h. After rinsing with PBS solution to remove non-physically immobilized antibodies, the modified electrode was blocked by immersing the electrode in 1% BSA solution for 0.5 h. Then, PSA solution of different concentrations were placed on the electrode surface and incubated for another 1 h. After rinsing with PBS again, the electrode was ready for measurement.

3. Results and discussion

To prepare the hydrogel, Fc was first coupled onto molecule F (Fc-F). After dissolving the resulted Fc-F into organic solvent and then the dilution of the stock solution with phosphate buffer, hydrogel was formed instantly that displays a yellow color (Figure 1A). The critical gelation concentration (w/w) of Fc-F is about 0.3 ∽ 0.4 % (3 - 4 mg mL^{-1}) at room temperature, which means the hydrogel constituted mainly of water. The synthesized hydrogel was characterized by SEM. As shown in Figure 1B, the hydrogel was composed of nanofibers. The diameter of the nanofibers ranged from 50 to 100 nm and the length reached as long as 1 mm. We speculate the mechanism for the formation of the nanofibers is that Fc-F monomers initially assembled into dimers, and then the dimers continue to assemble into fibers.[14] The

inter-fibril interaction contributed to the formation of the hydrogel, and the nanofibers act as an entangled matrix for holding a large amounts of water.[18]

Figure 1

The prepared hydrogel was utilized to modify the electrode by directly applying the hydrogel on an electrode surface. A chitosan layer was then coated on the electrode to help stabilize the hydrogel. Figure 2A displays 10 successive cyclic voltammetric scans of the modified electrode in PBS. In these voltammograms, a pair of well-defined redox peaks at approximately +0.43 and +0.52 V are attributed to the facile electron transfer of Fc was observed. The strong peak current indicated a significant number of Fc molecules were incorporated into the hydrogel. In addition, during the successive scans, no significant current decrease (< 5%) was observed, indicating good stability of the modified electrode.

After demonstrating the strong redox current at the modified electrode, the electrode was further modified with antibodies for the fabrication of immunosensors to detect PSA. AuNPs were immobilized on the electrode for the immobilization of antibodies. Each modification step of the electrode was characterized by cyclic voltammetry. After the immobilization of antibodies, it can be seen from Figure 2B (curve a) that the response of the current decreased significantly (55.4% compared to Figure 2A). When the electrode was further blocked with BSA to minimize non specific adsorption, the current decreased again (curve b, 73.9% of current decrease compared to curve a). Finally, when 10 ng mL^{-1} of PSA was captured onto the electrode, there is a continuous decrease of peak current (curve c, 42% of current

decrease compared to curve b). The electrochemical current was decreased as the formed non-conductive immuo-complex prevented electron transfer at the electrode surface. These data indicated the possibility of the prepared immunosensor for label free detection of PSA.[19, 20]

Figure 2

To test the linear range of the immuosensor towards PSA, a series of immunosensors were prepared for the detection of different concentrations of PSA. The responses of the immunosensors were measured by square wave voltammetry (SWV). As expected, the peak current at +0.45 V was decreased as the PSA concentration increased from 0.001 to 10 ng mL^{-1} (Figure 3A). Due to the increase of PSA concentration, more PSA molecules will be captured onto electrode surface, then the electrochemical current decreased due to the non-conductive nature of the PSA molecules. The calibration plot shows good linear relationships between the peak currents and the logarithm of the PSA concentrations in the range from 1 pg mL^{-1} to 10 ng mL^{-1} with a correlation coefficient of 0.993 (insert of Figure 3B). The detection limit, calculated based on S/N = 3, is 0.5 pg mL^{-1}. The detection limit is comparable or lower than literature report, such as the detection of PSA based on nanoporous silver@carbon dots as labels (0.5 pg mL^{-1})[21], based on graphene as label (1 pg mL^{-1})[22], based on ferrocene functionalized iron oxide nanoparticles (2 pg mL^{-1}) [23], and based on quantum dot functionalized graphene sheets as labels (3 pg mL^{-1}).[24] In addition, the developed method is much simpler, which does not require complicated labeling process.

The reproducibility of the immunosensor was studied. Five immunosensors were prepared independently for the detection of 1 ng mL^{-1} PSA. The relative standard deviation (RSD) of the response currents obtained was within 6%. This shows the reliability of the detection results, indicating the immunosensor can be applied for real-life sample analysis.

Good selectivity is very important for precise detection of analyte in real-life samples. The selectivity of the proposed immunosensor was investigated. The response of the immunosensor to different other proteins, such as human IgG and human serum albumin (HSA) were tested. As can be seen from Figure 3C, the presence of 100 ng mL^{-1} of IgG, HSA and lysozyme do not affect significantly the response of the immunosensor to 1 ng mL^{-1} of PSA, indicating good selectivity of the immunosensor.

Figure 3

Since the proposed immunosensor displays good selectivity, the immunosensor was then applied for the detection of PSA in serum samples. The diluted human serum samples (1%) were spiked with different concentrations of PSA, and these samples were then tested by the immunosensor. The detected PSA concentration was compared with the concentration of PSA added and the recovery results were obtained. Table 1 shows the recovery results were in the range from 96.9% to 108%, indicating the reliability of the immunosensor results.

4. Conclusions

In this work, we demonstrated an immunosensing platform for label-free detection

of PSA based on Fc functionalized redox hydrogel. The hydrogel preparation process that based on the self-assemble of Fc-coupled amino acid is simple and efficient. The hydrogel modified electrodes demonstrated strong redox current and good stability. The detection of PSA was achieved through the decrease of the electrode current after the capture of PSA. The resulting immunosensor displays a wide linear range and good selectivity towards PSA detection. The developed immunosensing platform can be easily adapted to develop other immunosensors and find wide clinical applications.

Acknowledgement

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Figure captions:

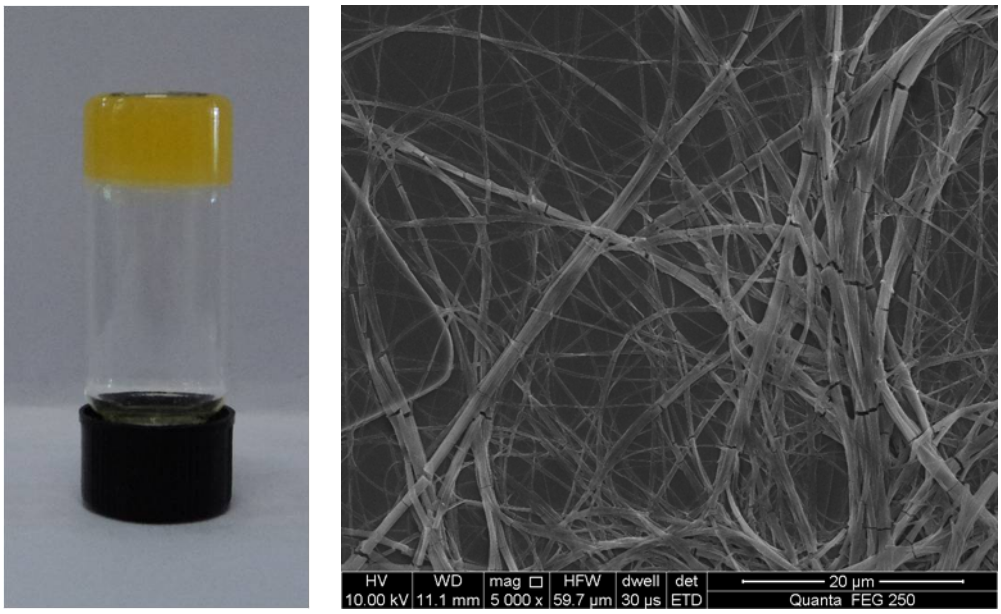
Figure 1. (A) Image of the synthesized hydrogel, (B) SEM characterization of the hydrogel.

Figure 2. (A) Ten successive cyclic voltammetry curve of the hydrogel modified electrode in buffer. (B) Cyclic voltammetry response of the immunosensor after different modification steps, (a) after antibody (10 μg mL^{-1}) immobilization, (b) after BSA (1%, w/w) blocking, (c) after the capture of PSA (10 ng mL^{-1}). Scan rate, 0.1 V/s.

Figure 3. (A) Square wave voltammetry (SWV) response of the immunosensor to different concentrations of PSA, from a to f, 0, 0.001, 0.01, 0.1, 1, 10 ng mL^{-1}. The parameters for SWV were as follows: potential increment of 4 mV, pulse amplitude of 25 mV and frequency of 15 Hz. (B) Calibration curve of the immunosensor to different concentrations of PSA. (C) Response of the immunosensor to different samples. The concentration of PSA is 1 ng mL^{-1} and the concentration of interfering proteins is 100 ng mL^{-1}. Error bar = SD, n=3.

Table 1 Recovery of the PSA concentration in human serum samples (n= 3)

Sample No.	1	2	3	4
Amount of PSA added(pg mL^{-1})	10	100	1000	10000
Amount of PSA detected(pg mL^{-1})	9.69±0.540	108±5.17	1026±43.2	10020±472
Recovery (%)	96.9±0.540	108±0.517	102±0.432	100±0.472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10.00 kV
WD
11.1 mm
mag
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ETD
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Quanta FEG 250

(A) **(B)**

Figure 1

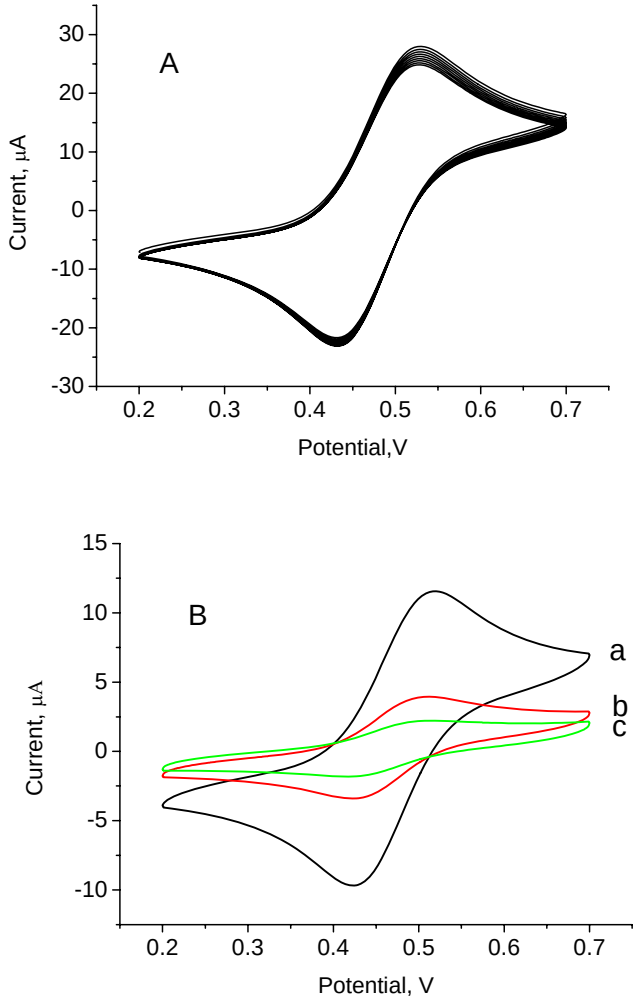

Figure 2

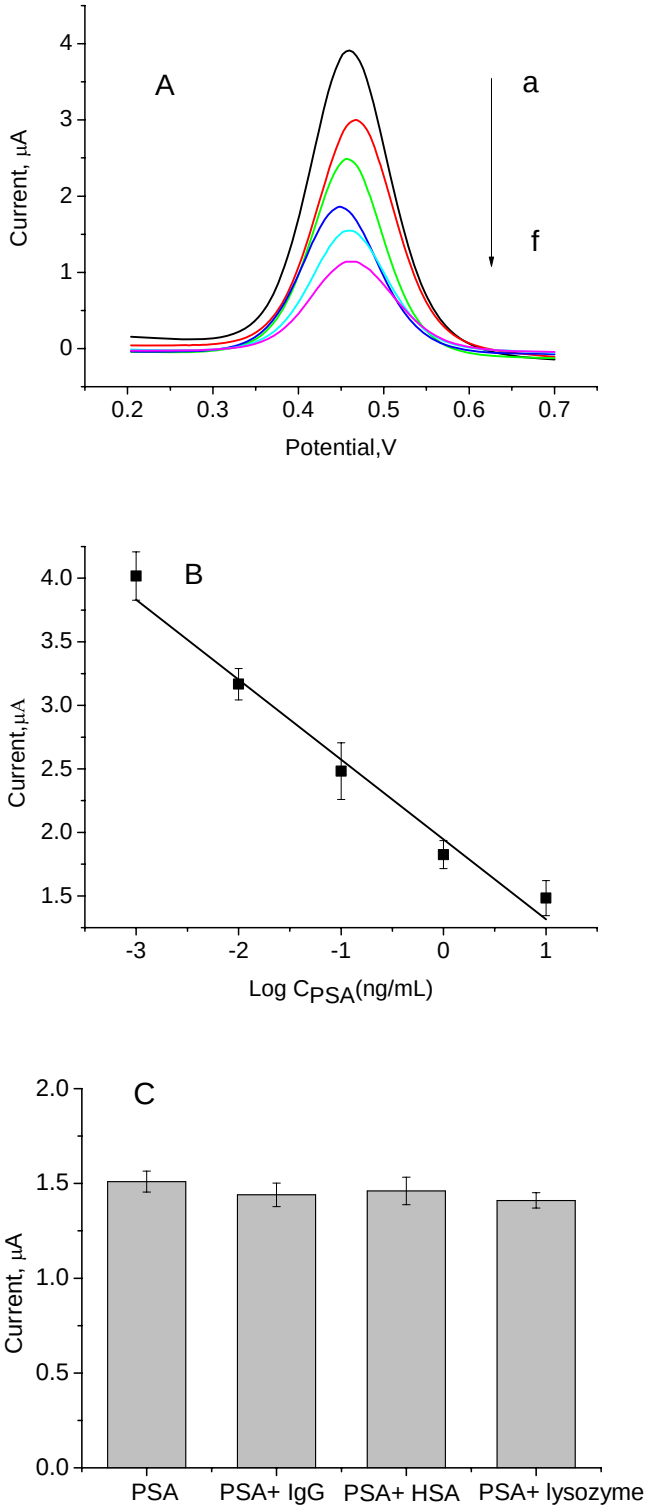
A
a
f
Current, μA
Potential,V
B
Current,μA
Log C_{PSA}(ng/mL)
C
Current, μA
PSA
PSA+ IgG
PSA+ HSA
PSA+ lysozyme

Figure 3