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Electrochemical Peptide Biosensor Based on in Situ Silver Deposition for Detection of Prostate Specific Antigen

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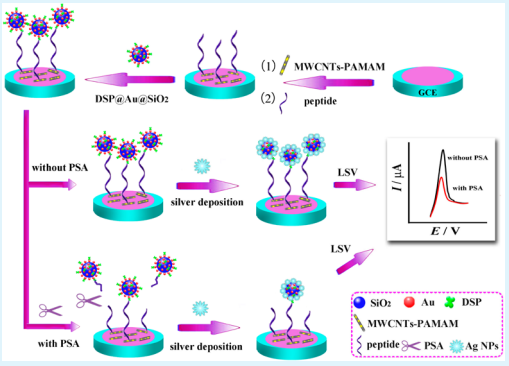
ABSTRACT: In this work, we have demonstrated a novel electrochemical method based on target-induced cleavage of a specific peptide for sensitive analysis of prostate specific antigen (PSA) by using silver enhancement. First, multiwalled carbon nanotubes/poly(amidoamine) dendrimers (MWCNTs−PAMAM) nanohybrids were assembled on the electrode to bind the peptide. Subsequently, dithiobis(succinimidylpropionate) (DSP)@Au@SiO_2 was prepared as a tracing tag and covalent bond with the peptides via the inherent interaction between DSP and the amino of peptide. In the presence of PSA, the peptide was specifically recognized and cleaved, resulting in the loss of the tracing tag in electrode surface. Thereafter, silver enhancement was performed on the left DSP@Au@SiO_2 nanohybrids. The electrochemical stripping signal of the deposited silver was used to monitor this process. Under optimal conditions, the proposed biosensor achieved a wide line from 0.001 to 30 ng mL^{-1} with a detection limit of 0.7 pg mL^{-1}. This work demonstrated the combination of the direct transduction of peptide cleavage events with the highly sensitive silver enhancement method, providing a promising effective strategy for PSA detection.



KEYWORDS: *prostate specific antigen, peptide, Ag deposition, electrochemical biosensor, multiwalled carbon nanotubes/poly(amidoamine) dendrimers, dithiobis(succinimidylpropionate) (DSP)@Au@SiO_2*

1. INTRODUCTION

Prostatic carcinoma is found to be the second most common cancer[1] and one man out of six is diagnosed with this deadly malignancy,[2] but up to now, there is still no effective treatment for it. Thus, early and precise detection of tumor markers provides a therapy hope for the treatment of prostate cancer.[3−6] Prostate specific antigen (PSA), which is secreted by prostatic epithelial cells, has been proved to be the best available tumor marker for early diagnosis of prostate cancer.[7] When the concentration of PSA is up to 2 ng mL^{-1}, it suggests that there is a risk of prostate cancer, even though the normal cutoff value of PSA is 4 ng mL^{-1}.[8,9] So far, many traditional immunoassay methods have been developed to detect PSA, such as colorimetric,[10,11] fluorescence,[12] surface-enhanced Raman scattering,[13] electrochemical,[14] chemiluminescence,[15] and electrogenerated chemluminescence (ECL),[16,17] which mainly involve special antibody−antigen recognition. However, antibodies are easily denatured with temperature changes and the assays usually require multiple steps and long incubation time.[18]

To overcome these shortcomings, a specific peptide with the amino acid sequence HSSKLQ, which can be selectively cleaved by PSA, is identified to be a promising alternative for detection of PSA. Compared to antibody, the peptides are more stable, easier to overcome harsh environments and more amenable to synthesize at the molecular level.[18] Since Denmeade et al. identified the specific peptide (HSSKLQ), which is used as a substrate to detect PSA in extracellular fluids,[19] there are several peptide-based methods for the measurement of PSA, including fluorescence,[20,21] ECL,[22] and the electrochemical method.[23,24] Among them, the electrochemical peptide-based methods have attracted considerable attention for PSA detection for its easy controllability. For example, Zhao et al. developed a ferrocene-functionalized helix peptide (CHSSKLQK)-based electrochemical biosensor using the direct transduction of peptide cleavage events into an electrochemical signal to simply and sensitively detect PSA.[23] Although this method is simple and easy to control, the sensitivity is not satisfactory due to the small current signal. So, it is an urgent task to develop a signal amplification method to achieve a high sensitivity for an electrochemical peptide sensor.

The signal amplifications and signal outputs are the two most important factors for an electrochemical method. The silver deposition is a widely used signal output way in electrochemistry due to the precision of the detection. In the meantime nanomaterial plays a crucial role in silver deposition for signal amplifications, Au nanoparticles (AuNPs)-catalyzed Ag deposition is reported to amplify the anodic stripping signal for the good biocompatibility and electrical conductivity.[25,26] By this method, silver nucleated onto the Au nanoparticles leads to a continuous deposition of silver, finally a whole

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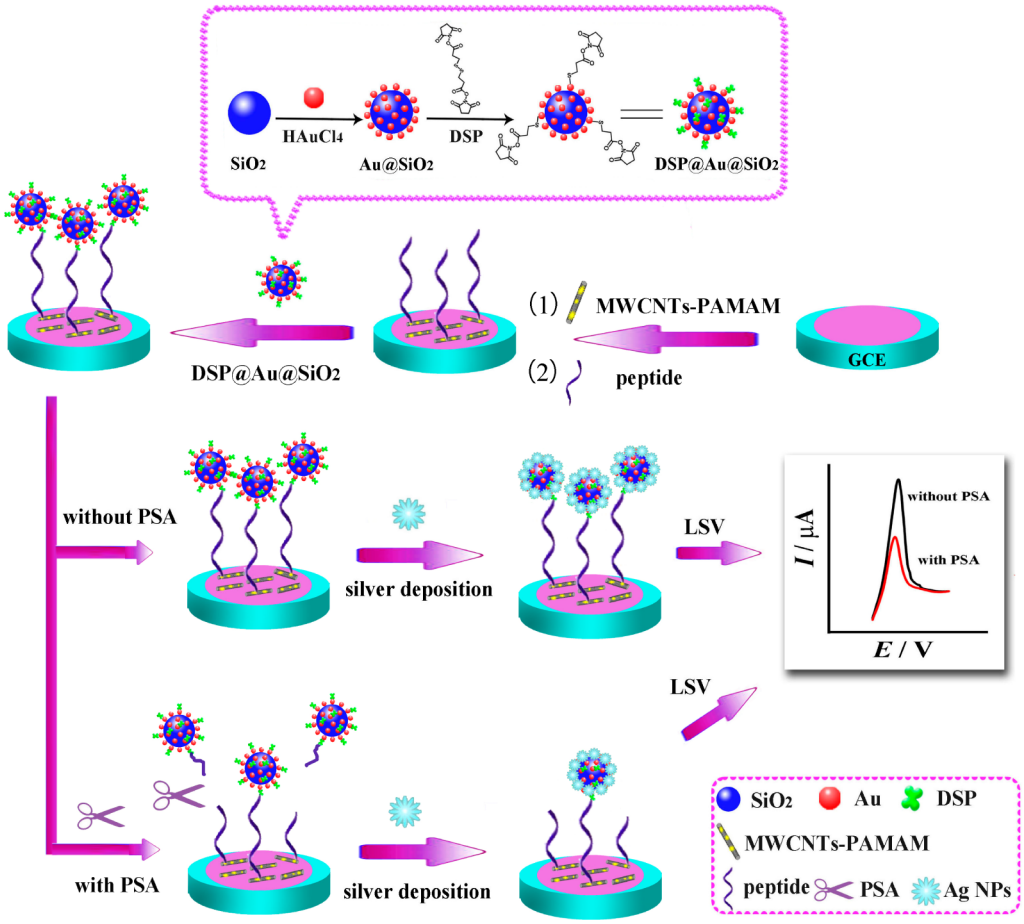
Scheme 1. Schematic Diagram of the Electrochemical-Peptide Biosensor for Detection of PSA



coverage formed on the entire structure.[27] In addition, SiO_2 nanospheres have obtained great interest for the application in biosensing for its nice chemical and mechanical stability and extensive large surface area. So in this work, AuNPs were in situ formed on the surface of SiO_2 nanospheres (Au@SiO_2), which catalyze the Ag deposition to amplify the signal for improving sensitivity of the biosensor.

Here we proposed an electrochemical peptide cleavage-based biosensor for the determination of PSA by coupling with Au@SiO_2 to amplify the anodic stripping signal of deposition Ag. In the presence of PSA, the peptide can be specifically recognized and cleaved resulting in release of the tracing tag from electrode surface. Thereafter, silver deposition was performed on the left dithiobis (succinimidylpropionate) (DSP)@Au@SiO_2 composites. The electrochemical stripping signal of the deposited silver was used to monitor this process. When the concentration of PSA increased, the signal of the deposited silver decreased accordingly. With MWCNTs−PAMAM nanohybrid as a sensor platform, the biosensor can immobilize a large amount of peptide to widen the linear range. Moreover, the nanohybrid can facilitate electron transfer to improve the signal response. With the above advantages, the peptide biosensor has achieved a wide linear range and high sensitivity.

2. MATERIALS AND METHODS

2.1. Materials and Reagents. MWCNTs were purchased from Chengdu Organic Chemicals Co., Ltd. of the Chinese Academy of Science (Chengdu, China). Poly(amino−amine) dendrimers (G3 PAMAM) were purchased from Weihai CY Dendrimer Technology Co., Ltd. (Weihai, China), dithiobis(succinimidylpropionate) (DSP) was obtained from Heowns (Tianjin, China). Chloroauric acid ($HAuCl_4$), *N*-succinimidyl-3-(2-pyridyldithiol)propionate (SPDP), trisodium citrate, silver nitrate ($AgNO_3$), hydroquinone (HQ) and Tris (2-carboxyethyl)phosphine (TCEP) were obtained from Sigma-Aldrich (St. Louis, MO). The peptide (CEHSSKLQLAK-NH_2) was synthesized and purified by Shanghai Science Peptide Biological Technology Co., Ltd (Shanghai, China). And the PSA from human semen was obtained from Biocell (Zhengzhou, China). 0.1 M phosphate buffered solution (PBS, pH 7.0) was prepared containing 0.1 M Na_2HPO_4, 0.1 M KH_2PO_4 and 0.1 M KCl. All other chemicals were of reagent grade and used as received. The solutions used in this experiment were prepared using ultrapure water (specific resistance of 18 MΩ·cm).

2.2. Apparatus. Cyclic voltammetry (CV) and linear sweep voltammetry (LSV) measurements were realized through a CHI 660D electrochemical workstation (Chenhua, Shanghai, China). All electrochemical experiments were performed in a conventional three-electrode system that consisted of a saturated calomel electrode (SCE) as the reference electrode, a platinum wire as the counter electrode and a bare or modified glass carbon electrode (GCE, Φ = 4 mm) as the working electrode. The measurement of LSV was performed in 1.0 M KCl solution with the scanning rate of 50 mV s^{-1}. The morphologies of nanoparticles were taken with scanning electron microscopy (SEM, S-4800, Hitachi, Japan).

2.3. Preparation of DSP@Au@SiO_2 Composites. The SiO_2 nanoparticles were prepared according to the literature,[28] 3.0 mL 28% (v/v) ammonia aqueous solution was added into 50.0 mL of absolute ethanol and the mixtures were stirred for 5 min. And then 1.5 mL of 6.7 mM tetraethylorthosilicate (TEOS) was added dropwise into above solution with continuous stirring until the color of mixtures changed from colorless to ivory.

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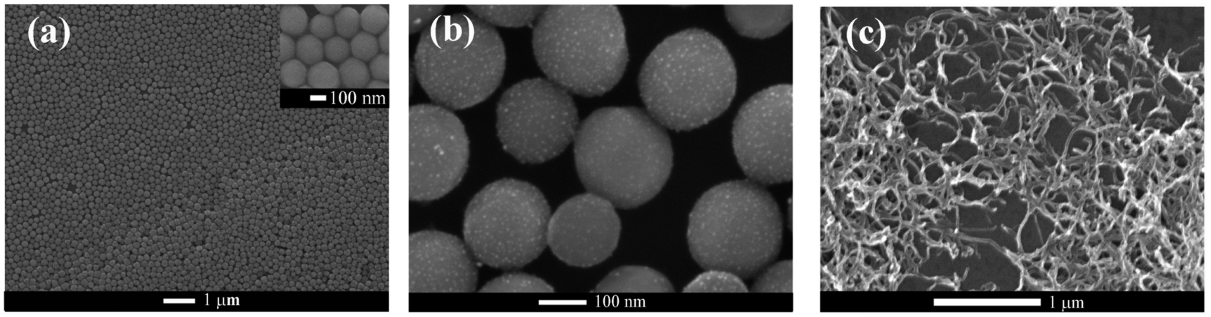

Figure 1. SEM images of (a) SiO_2. Inset: enlarged image of panel a; (b) Au@SiO_2; (c) MWCNTs−PAMAM.

A typical synthesis of Au@SiO_2 nanocomposites included three steps. First, 50 μL of 0.28 mM APTMS was added into the SiO_2 solution under stirring for 2 h to obtain NH_2-functionalized nano-SiO_2. Second, 1 mL of 1% $HAuCl_4$ was dropped into 2 mL of functionalized SiO_2 solution and the solution was stirred for 10 min. Third, 1 mL of 0.1 M $NaBH_4$ solution was added slowly under the condition of vigorous stirring, and the deposit was collected by centrifugation and then washed with ultrapure water several times.

The DSP@Au@SiO_2 was synthesized according to the following steps. 100 μL of 2 mM DSP was added into 2 mL of Au@SiO_2 suspension and then sonicated for 30 min to form a homogeneous suspension. To complete the reaction, the suspension was stirred at room temperature for 4 h. After that, the DSP@Au@SiO_2 suspension was subject to centrifugation and redispersed in 2 mL of ultrapure water for further use.

2.4. Preparation of MWCNTs−PAMAM. Preparation of MWCNTs modified by COOH groups was performed in following procedure.[29] 10 mg of MWCNTs was put into 10 mL of a mixture (H_2SO_4:HNO_3) in a volume ratio 3:1 and vigorously sonicated for 2 h. After this reaction, the sample of MWCNTs−COOH was centrifuged and washed several times with ultrapure water until the solution reached neutral in order to eliminate acid residues. Afterward, 1 mg of the functionalized MWCNTs and 100 μL of 0.01 M amination-PAMAM were suspended in 2 mL of ultrapure water and sonicated to obtain a homogeneous black solution (MWCNTs−PAMAM).

2.5. Fabrication of the Modified Electrodes. Prior to use, a bare GCE electrode was carefully polished to mirror with 0.3 and 0.05 μm alumina powder and sonicated with ethanol and ultrapure water for a few minutes. First, the amino groups of MWCNTs−PAMAM were activated by the heterobifunctional cross-linker SPDP, and then 5 μL of the amino activated MWCNTs−PAMAM solution was self-assembled onto the electrode surface to increase the effective area and conductivity of the electrode. Subsequently, 20 μL of thiol-terminated peptide was dropped onto the surface of the MWCNTs−PAMAM film and incubated for 1 h at room temperature to conjugate with MWCNTs−PAMAM through SPDP as a linker. Finally, the DSP@Au@SiO_2 could be immobilized stably through the inherent interaction between DSP and the amino of peptide. After each step, the modified electrode was entirely cleaned with ultrapure water. The finished biosensor was stored at 4 °C prior to electrochemical characterization. Scheme 1 shows the schematic illustration of the biosensor fabrication process.

2.6. Detection of PSA. To carry out the detection process, the biosensor was first incubated with 15 μL of PSA standard solution of different concentrations for 40 min at 37 °C. After the biosensor was washed with ultrapure water, silver-deposition enhancement was performed by dropping 10 μL mixtures of enhancer solutions (1.0 g HQ, 35 mg $AgNO_3$, 50 mL citrate buffer and 50 mL ultrapure water) on the biosensor surface for 3 min at room temperature in a dark incubator. The biosensor was finally washed with ultrapure water to perform the linear sweep voltammetry in 1.0 M KCl solution with the rate of 50 mV s^{-1} for recording the response.

3. RESULTS AND DISCUSSION

3.1. Characterization of Different Nanomaterials. Typical SEM images for the SiO_2 nanospheres shown in Figure 1a revealed that the nanospheres had good uniformity with an average size of about 160 nm. When AuNPs were in situ reduced onto SiO_2 (Figure 1b), the SEM image showed that many AuNPs are uniformly distributed on the surface of SiO_2 nanospheres. The SEM image of MWCNTs−PAMAM displayed a well-dispersed tubular structure (Figure 1c).

3.2. Electrochemical Characterization of the Stepwise Modified Electrodes. The fabrication process of the peptide biosensor was characterized by CV measurement in 5 mM $[Fe(CN)_6]^{3-/4-}$ solution with a scan rate of 100 mV s^{-1}. As is shown in Figure 2, the well-defined redox peaks of $[Fe(CN)_6]^{3-/4-}$ were obtained at a bare GCE (curve a). When MWCNTs−PAMAM was coated on the electrode, the peak current of the modified electrode increased (curve b), demonstrating that MWCNTs−PAMAM could accelerate the electron transfer efficiently. After peptide was assembled (peptide/MWCNTs−PAMAM/GCE), the peak current obviously decreased (curve c), which is due to the diffusion block of peptide. And the result illustrated that peptide had been successfully immobilized on the MWCNTs−PAMAM nanohybrid. The current response of $[Fe(CN)_6]^{3-/4-}$ on the DSP@Au@SiO_2/peptide/MWCNTs−PAMAM/GCE (curve d) was further decreased, indicating that the DSP@Au@SiO_2 was successfully cross-linked with peptide through the reaction between DSP and peptide. After incubation with a certain concentration of PSA, some of the DSP@Au@SiO_2 were released into the solution, leading to an increased peak current (curve e).

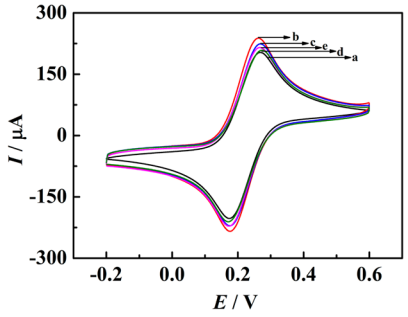

Figure 2. Electrochemical characterization of the different modified electrodes: (a) bare GCE, (b) MWCNTs−PAMAM/GCE, (c) peptide/MWCNTs−PAMAM/GCE, (d) DSP@Au@SiO_2/peptide/MWCNTs−PAMAM/GC and (e) after the biosensor incubated with PSA in 5 mM $[Fe(CN)_6]^{3-/4-}$ solution.

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3.3. Optimization of Assay Conditions. To increase the sensitivity and selectivity of the biosensor, the optimization of the incubation time of the peptide modified electrode in PSA solution was investigated. The PSA incubation time is an important parameter affecting the analytical performance of biosensor. At room temperature, the CV response signal for PSA (10 ng mL^{-1}) increased with the reaction time up to 40 min and then leveled off after 40 min (Figure 3), which showed the saturated bind between the peptide and PSA. Therefore, 40 min of incubation time was used for the detection of PSA.

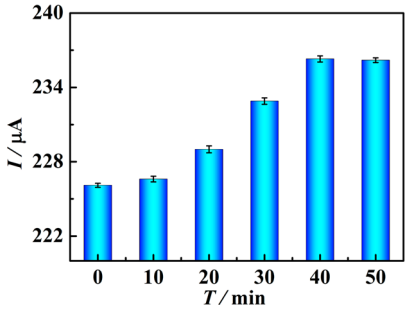

Figure 3. Optimization of the incubation time of target PSA.

3.4. Analytical Performance of the Biosensor for Detection of PSA. Under optimum conditions, the stripping peak current of AgNPs deposited on the DSP@Au@SiO_2 nanohybrid decreased with increasing concentration of PSA (Figure 4A). The calibration plot showed a good linear relationship between the stripping peak current and the logarithm of the PSA concentration in the range from 0.001 ng mL^{-1} to 30 ng mL^{-1} with a correlation coefficient of 0.9902 (Figure 4B). And the regression equation is $I = -1.5295 \lg c + 4.587\,29$ (where c is the concentration of PSA). The detection limit for PSA calculated according to IUPAC recommendation was 0.7 pg mL^{-1} at 3σ.[30,31] In addition, the analytical performance of the developed biosensor for PSA detection has been compared with other biosensors reported in the literatures, which were summarized in Table 1.[16,22,23,32−35] From Table 1, our proposed biosensor showed a higher sensitivity and wider linear range, which provided a powerful evidence of our strategy for highly sensitive detection of PSA. The good performance of this proposed biosensor may be caused by the follows: (1) The high sensitivity of silver enhancement and using Au@SiO_2 nanocomposites as tracing tags enhance the signal amplification. (2) MWCNTs−PAMAM has good conductivity to improve the electronic transport efficiency and large surface area to immobilize more peptide. In order to elucidate that, we quantitatively detect the electro-active surface area of MWCNTs−PAMAM modified electrode by recording CVs at different potential scan rates with $[Fe(CN)_6]^{3-/4-}$ serving as redox probes (Figure 5). The electro-active surface area (22.88 mm^2) for the MWCNTs−PAMAM electrode was calculated according to the Randles−Sevcik equation[36,37] $I_p = 2.69 \times 10^5 A D^{1/2} n^{3/2} v^{1/2} C$, in which A is the electrode area, D is the diffusion coefficient (at 25 °C, $D = 6.70 \times 10^{-6}$ cm^2 s^{-1}), n is the number of electrons transferred in the redox reaction ($n = 1$), C is the concentration of the reactant (5 mM $Fe(CN)_6^{3-/4-}$), I_p refers to the redox peak current and v is the scan rate of the CV measurement. This indicated that MWCNTs−PAMAM owns a larger electro-active

Table 1. Proposed Biosensor Performance Compared with Other Biosensors for PSA Detection

analytical method	detection method	linear range (ng mL^{-1})	refs
ELISA	8 pg mL^{-1}	2.1−10	32
ECL	0.33 ng mL^{-1}	1.0−40	22
SWV	0.2 ng mL^{-1}	0.5−40	23
ECL	0.8 pg mL^{-1}	0.005−0.5	33
EIS	1 pg mL^{-1}	0.001−1000	34
ECL	1 pg mL^{-1}	0.001−100	35
ECL	40 pg mL^{-1}	0.04−5	16
LSV	0.7 pg mL^{-1}	0.001−30	this work

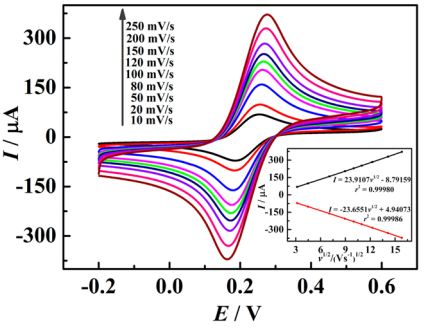

Figure 5. CVs of MWCNTs−PAMAM modified GCE in 5.0 mM $[Fe(CN)_6]^{3-/4-}$ at different scan rates from 10 to 250 mV s^{-1}. Insets show the linear relations of the MWCNTs−PAMAM modified GCEs with the anodic and cathodic peak current against the square root of scan rate.

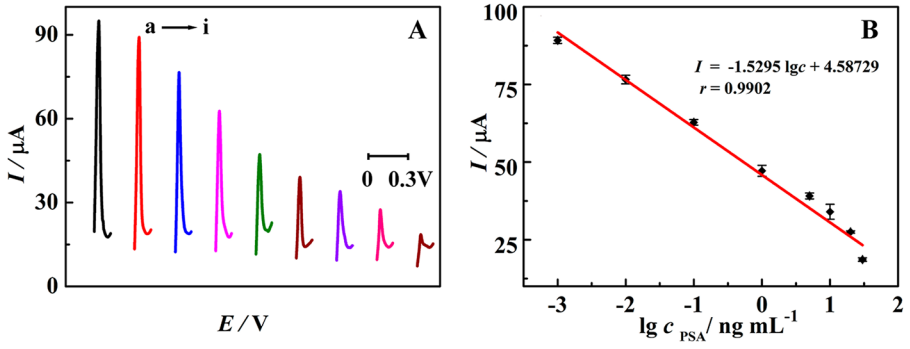

Figure 4. (A) Typical LSV of the biosensors with increasing concentration of PSA (ng mL^{-1}) from a to i: (a) 0, (b) 0.001, (c) 0.01, (d) 0.1, (e) 1, (f) 5, (g) 10, (h) 20 and (i) 30; (B) Calibration curve of the biosensor for detection of PSA in the range between 0.001 and 30 ng mL^{-1}.

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surface area compared with the surface area (12.56 mm^2) of bare glassy carbon electrode.

3.5. Selectivity, Reproducibility and Stability of the Biosensor. To evaluate the specificity of the electrochemical biosensor, we challenged the biosensor with other possible interferences such as bovine serum albumin (BSA), hemoglobin (HB), carcinoembryonic antigen (CEA) and human alphafetoprotein (AFP) in the same conditions. And the results are exhibited in Figure 6. When the biosensor was incubated with

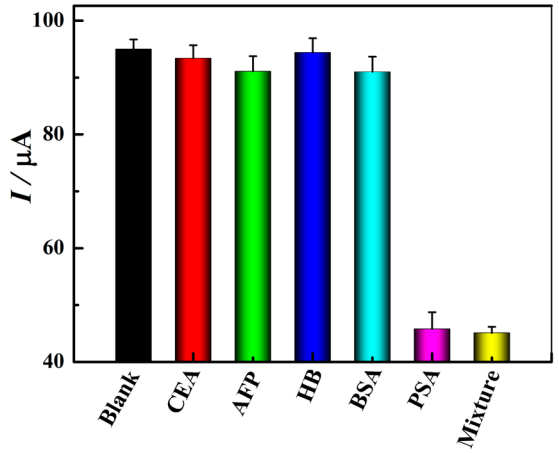

Figure 6. Selectivity of the proposed electrochemical biosensor. The concentrations of CEA, AFP, HB and BSA were 10 ng mL^{-1}. The mixture is containing CEA (20 ng mL^{-1}), AFP (20 ng mL^{-1}), BSA (20 ng mL^{-1}), HB (20 ng mL^{-1}) and PSA (1 ng mL^{-1}).

20 ng mL^{-1} BSA, HB, CEA and AFP solution, respectively, no apparent change of the current was observed compared to the blank test (no target molecular) in the same testing conditions. However, when PSA was coexisted with the interferences, the electrochemical response was almost the same as that with only PSA. All these results indicating that the proposed biosensor has a good specificity for PSA.

To evaluate the reproducibility of the biosensor, we investigated the biosensor through the coefficient of variation of the intra- and interassays. When the biosensor was investigated by using four electrodes to test the same concentration of PSA (10 ng mL^{-1}) at the same conditions, the relative standard deviation (RSD) was 5.7% (Figure 7A). When a same electrode was used to test repetitively for four times at a certain concentration of PSA (10 ng mL^{-1}), the RSD was 6.8% (Figure 7B). Furthermore, after storage at 4 °C for 15 days, the biosensor can still retain 90% of the initial response for a same concentration of PSA (Figure 7C). These results showed acceptable reproducibility and good stability of our proposed method.

3.6. Analysis of Clinical Serum Specimens. We evaluated the recovery in different concentrations of PSA solutions diluted in a healthy human real serum sample (acquired from Ninth People's Hospital of Chongqing, China) (Table 2). The obtained results showed satisfactory recoveries

Table 2. Determination of PSA Added in Human Blood Serum (n = 3) with the Proposed Biosensor

serum sample	concentration of PSA added (ng mL^{-1})	concentration obtained with immunosensor (ng mL^{-1})	recovery (%)	RSD (%)
1	0.01	0.01051	105.1	0.9
2	0.1	0.1051	105.1	3.6
3	1	1.072	107.2	4.1
4	5	5.012	100.2	4.6
5	10	10.14	101.4	2.4

in the range of 100.2% to 107.2% and the RSD values from 0.92% to 4.58%. The results suggested that the biosensor has a promising potential application for the detection of PSA in clinical analysis.

4. CONCLUSIONS

In summary, with the target-induced cleavage of peptide, a novel electrochemical biosensor for sensitive detection of PSA has been successfully constructed. At the same time, by employing silver and Au@SiO_2 enhancement, the biosensor can achieve a wide liner range and low detection limit. The good performance of the proposed biosensor should be attributed to the following reasons. First, a short peptide is used to replace traditional antibodies for PSA detection because of the good stability and the ability to overcome difficult environments. Second, Au@SiO_2-mediated silver enhancement improved the precision and sensitivity of the detection. Moreover, the MWCNTs−PAMAM nanohybrid modified electrode not only promoted the electron transfer but also provided a large surface area for immobilize specific peptide. In view of the above advantages, we anticipate that this high sensitive and selective method has potential to be applied in clinical applications.

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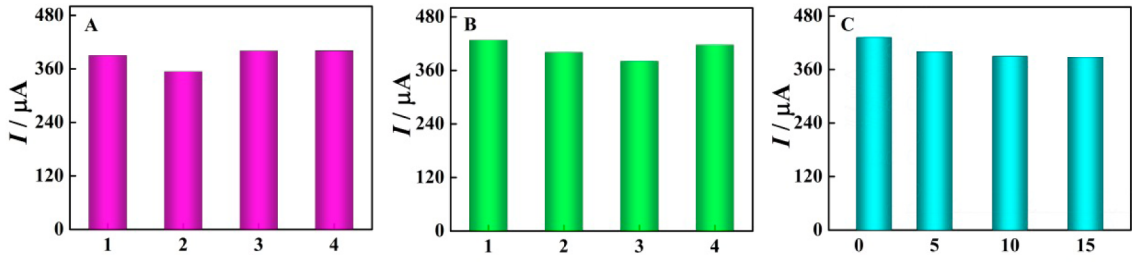

Figure 7. (A) Intra-assay: the four electrodes to test the same concentration of PSA (10 ng mL^{-1}). (B) Interassay: a same electrode was used to test repetitively for four times at a certain concentration of PSA (10 ng mL^{-1}). (C) Stability of the biosensor in the presence of PSA (10 ng mL^{-1}) was evaluated every 5 days.

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Notes

The authors declare no competing financial interest.

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