

A Novel Gold Nanoprobe for a Simple Electrochemiluminescence Determination of a Prostate-specific Antigen Based on a Peptide Cleavage Reaction

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A novel gold nanoprobe for a sensitive and simple determination of a prostate-specific antigen (PSA) was designed on the basis of homogeneous detection and a peptide cleavage reaction. The gold nanoprobe (AuNPs-peptide-Ru1) consisted of a specific peptide tagged with a ruthenium(II) complex (Ru1) and gold nanoparticles (AuNPs) conjugated with the peptide *via* the strong Au-S bond between the AuNPs surface and the thiol group of the peptide. The electrochemiluminescence (ECL) enzymatic-cleavage-reaction-based bioanalytic system based on homogeneous detection has overcome shortcomings from a complicated fabrication process of traditional electrodes. In the presence of the target PSA, it specifically cleaved the peptide of the AuNPs-peptide-Ru1, and the ECL signal substance (Ru1) part dissociated from AuNPs-peptide-Ru1. This resulted in an increase in the ECL intensity. The ECL biosensor could detect PSA concentrations in the range from 1.0×10^{-12} to 1.0×10^{-9} g/mL, the detection limit was 4.0×10^{-13} g/mL. The assay with the advantages of a simple method for PSA was selective and fast. It is superior to the immunoassay, and is a promising strategy to develop biosensors based on enzymatic cleavage including electrochemistry and optics.

Keywords Electrochemiluminescence, Au nanoparticle, enzymatic cleavage reaction, prostate-specific antigen

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Introduction

A prostate-specific antigen (PSA) has been identified to be the most reliable tumor marker currently available for diagnosing prostate and breast cancer, and is a protease that cancerous and normal prostate cells secrete.^{1,2} Up to now, the current methods for the detection of PSA were reported, including enzyme-linked immunosorbent assays (ELISAs),^{3,4} a surface-enhanced raman-scattering immunoassay,⁵ a fluorescence immunoassay,⁶⁻⁸ a colorimetric immunoassay,^{9,10} electrochemistry,¹¹⁻¹³ chemiluminescence,¹⁴ a photoelectrochemical immunoassay,^{15,16} and electrochemiluminescence (ECL) analysis.^{17,18} The aforementioned methods are mostly dependent on an immune reaction between an antigen and an antibody, and can detect PSA with both high sensitivity and selectivity. However, these methods have problems such as complex test steps, high costs, high time consumption, poor stability, exposure of antigen-binding active sites, and variability of protein antibodies. In ELISA, each specific site must be bound to at least two antibodies. In addition, repeated washing steps may cause antibody loss and decrease the test efficiency. Therefore, it is necessary to identify new recognition factors and to develop more stable, faster, and simpler biosensor systems to detect PSA. Polypeptides are substitutes for antibodies and have advantages, such as easy synthesis, low costs, stability, less

likelihood of being inactivated, suitability for extreme conditions, and non-toxicity.^{19,20} In recent years, there have been reports of sensors in which proteases catalyze the cleavage of specific polypeptides. Zhao *et al.*²¹ reported an electrochemical biosensor for detecting the PSA concentration. A specific polypeptide (CHSSKLQK) labeled with a ferrocene reporter was used as an electrochemical proteolytic probe to detect PSA activities. Wang *et al.*²² has developed a new fluorescence proteolytic probe for detecting matrix metalloproteinase 2. A specific polypeptide (core sequence, PLGVR) labeled with a fluorescein isothiocyanate (FITC) reporter was used as a fluorescent probe to detect matrix metalloproteinase 2 activities.

Further development of new protease detection methods that have both high selectivity and sensitivity are significant for developing diagnostics and therapeutic drugs for protease-related diseases. Electrochemiluminescence (electrogenerated chemiluminescence, ECL) has been proven to be a powerful analytical technique due to its easy controllability, wide dynamic range, high sensitivity, and rapidity. It could be a sensitive alternative to amperometric detection.²³ To our best knowledge, there have been no reports on bioanalytical systems in which enzymes catalyze probe cleavage and the centrifugation and isolation of gold nanoparticles (AuNPs) for the ECL homogeneous detection of PSA. The aim of this study is to develop a novel gold nanoprobe for the sensitive and simple detection of PSA to overcome shortcomings from the complicated fabrication process of traditional electrodes.

Herein, we designed an ECL biosensor based on the biological

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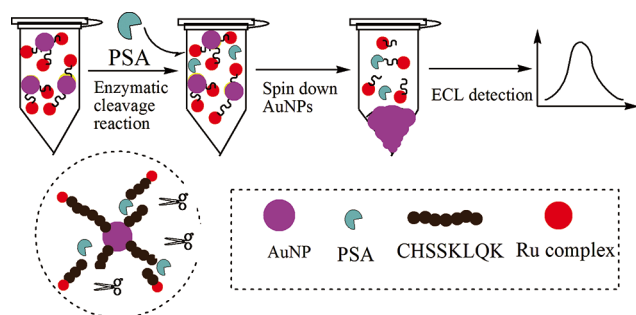


Fig. 1 Schematic diagram of the designed ECL biosensing for PSA based on homogeneous detection and peptide cleavage reaction.

cleavage of an ECL polypeptide probe and the homogeneous detection of PSA by centrifugation and the isolation of AuNPs (as shown in Fig. 1). A specific polypeptide (CHSSKLQK) labeled with a ruthenium(II) complex (Ru1) was self-assembled on the AuNPs surface through the strong Au-S bond, and AuNPs were used as carriers to form a new gold nanoprobe (AuNPs-peptide-Ru1). Since PSA can specifically catalyze the cleavage of this peptide, the signal substance will dissociate from AuNPs. After centrifugation and isolation, testing the electrochemiluminescence of the signalling substances in the solution can be used to achieve a highly sensitive detection of the target, PSA. In the present work, we carried out a study on the ECL biosensor and optimized the experimental conditions.

Experimental

Reagents and instruments

Chloroauric acid (HAuCl_4), human prostate-specific antigen, and the ruthenium complex $\text{Ru}(\text{bpy})_2(\text{mcbpy-}O\text{-Su-ester})(\text{PF}_6)_2$ (abbreviated as Ru1), were purchased from Sigma-Aldrich (USA). The specific peptides (sequence, CHSSKLQK, M_w , 929.48) purity is more than 95%, as supplied by Shanghai Apeptide Co., Ltd. (China). Bovine serum albumin (BSA, 96–99%), trypsin, thrombin, metalloproteinase-2 (MMP-2), MMP-7, were purchased from Beijing BioDee Biotechnology Co., China. Sodium citrate, N,N -dimethylformamide (DMF), and tri- n -propylamine (TPA) were purchased from Xi'an Wolsen Bio-technology Co., Ltd. Also, 10 mM phosphate buffer saline (10 mM PBS, pH 7.4), consisting of 10 mM $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ and 100 mM NaCl, was used to prepare solutions. The washing buffer was 10 mM PBS supplemented with 0.05% Tween 20. All other chemicals employed were of analytical grade, and Millipore Milli-Q water (18.2 $\text{M}\Omega\cdot\text{cm}$) was used to prepare all other solutions.

The device for ECL measurements consisted of a CHI600B Workstation (Shanghai CH Instruments, Inc.) and an Ultra-Sensitive Luminescence Analyzer (Institute of Biophysics, Chinese Academy of Sciences) with a three-electrode system. The three-electrode system consisted of a AuNPs/Nafion modified glassy carbon electrode (GCE, $\phi = 2.0$ mm) as the working electrode, a platinum wire as the counter electrode, and a Ag/AgCl electrode (in saturated KCl solution) as the reference electrode. The UV-Vis absorbance spectra in this study were obtained on a UV-3600 UV-Vis spectrometer (Shimadzu, Kyoto, Japan). The images of AuNPs were obtained with a Tecnai G2 F20 transmission electron microscope (TEM, FEI, USA).

Synthesis of AuNPs

AuNPs were synthesised according to the literature.^{24,25} A chloroauric acid solution (100 mL, 0.01%) was heated and being stirred. After the solution started boiling, 4 mL of a 1% trisodium citrate solution was rapidly added, and the color of the solution changed from pale yellow to purplish-red and eventually became red, which is a characteristic of AuNPs. Heating and stirring was continued for 15 min until the solution color changed to transparent orange-red, and then it was stopped. After cooling to room temperature, the solution was stored in a brown bottle at 4°C for future use.

Synthesis of gold nanoprobe

The Ru1-labeled peptide (peptide-Ru1) was synthesized according to a reported method,^{26,27} with some modifications. First, 1.0 mg of peptide (0.0010 mmol) was dissolved in 4.0 mL of 10 mM PBS solution. The peptide was added by 2.0 mL of 5.0×10^{-3} M Ru1 (dissolved in DMF), followed by incubation at 4°C for 12 h. The peptide-Ru1 was purified using gel filtration chromatography on a Sephadex G-15 by 10 mM PBS (pH 7.2) containing 0.10 M KCl. The peptide-Ru1 concentration solution was calculated to be 2.6×10^{-5} M ($\epsilon_{458 \text{ nm}} = 1.4 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$), based on the absorbance of Ru1 and peptide-Ru1 at 458, 457 nm, respectively.²⁸

After 1 mL of the peptide-Ru1 solution was added to 200 μL of 7.8 nM AuNPs, the mixture was slowly shaken on a thermostatic shaker for 4 h at 37°C so that the terminal cysteines were bound to the surface of AuNPs by gold-sulfur bonds. When the reaction was complete, 10 mmol/L of PBS was used to remove unreacted peptides. After centrifugation at 10000 rpm for 5 min was complete, AuNPs-peptide-Ru1 was obtained. The AuNPs-peptide-Ru1 obtained was resuspended in 0.1% BSA for 2 h at 37°C, so as to block the active sites on the non-covalent polypeptide on the surface of the AuNPs. After centrifugation and isolation, the Au nanoprobe was added to 600 μL of a 10 mmol/L PBS buffer and stored at 4°C for future use. Under the premise that there was no loss of AuNPs in the reaction, the mass concentration of the ECL probe obtained was calculated according to the amount of AuNPs added, and was found to be 7.8 nM.

Fabrication of the modified GCE

A AuNPs/Nafion modified GCE (AuNPs/Nafion/GCE) was prepared according to a reference.²⁹ Prior to modification, the GCE was polished with 0.05 mm $\alpha\text{-Al}_2\text{O}_3$ slurry, followed by thoroughly washing with absolute alcohol and pure water in an ultrasonic bath. The above AuNPs suspension was added to 0.5% Nafion (v/v = 2:1) and sonicated for 30 min. Then, 10 μL of the mixture (AuNPs/Nafion) was drop-coated onto the pretreated GCE surface with a microsyringe and air-dried to form AuNPs/Nafion/GCE.

ECL detection of PSA

AuNPs-peptide-Ru1 (1.6 mg/mL, 10 μL) was added to 90 μL of different concentrations of a PSA solution or serum and allowed to react for 40 min at 30°C. The reaction mixture was centrifuged at 13000 rpm for 5 min, and the separated signal Ru1 present in the supernatant was analyzed by the ECL technology. Homogenous detection of the cleaved signal substance was carried out in the presence of the co-reactant TPA.

After 100 μL of the supernatant was added to 100 μL PBS solution containing 0.1 M of TPA, the resulting mixture were transferred in an ECL cell for the ECL measurement. The measurement was performed with a scanning rate of 50 mV/s

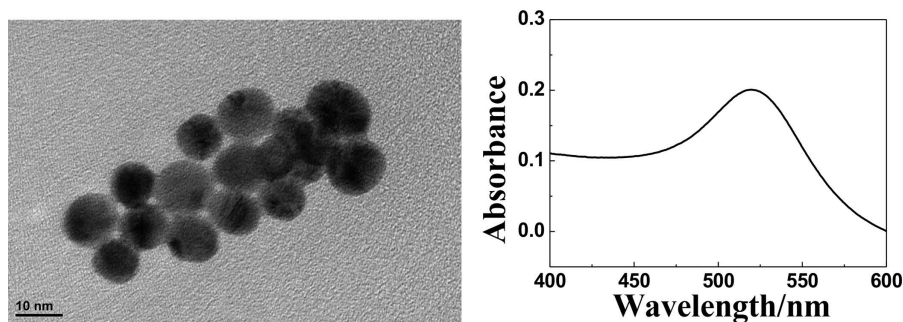


Fig. 2 TEM image (left), and UV-Vis absorption spectra (right) of the gold nanoparticles (7.8 nM) prepared.

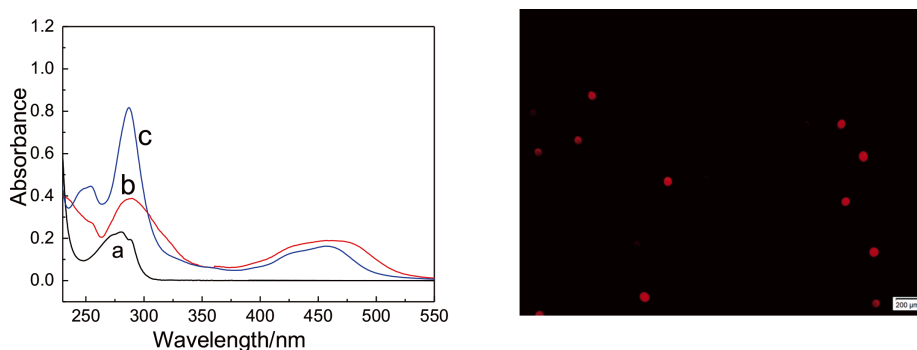


Fig. 3 UV-Vis absorption spectra (left) and fluorescence CCD imaging (right) of the peptide-Ru1. (a) Peptide; (b) Ru1; (c) peptide-Ru1.

and a range of 0 – 1.5 V. The negative voltage of the photomultiplier tube (PMT) was biased at –800 V throughout the detection process. The PSA concentration was quantified on the basis of the increased ECL peak intensity, $\Delta I = I - I_0$, where I_0 and I are ECL peak intensity before and after the AuNPs-peptide-Ru1 reacted with PSA, respectively. ECL detection was conducted in saturated and still air at ambient temperature.

Results and Discussion

Characterization of the AuNPs

In this study, AuNPs were employed as an ECL carrier platform. Transmission electron microscopy was used to observe AuNPs. An ultrasonic treatment (5 min) for AuNPs was carried out before an observation. Figure 2 (left) shows that the prepared AuNPs were of nanoscale size, spherical in shape, with smooth surfaces, and having a mean particle size of 10 nm. The synthesised AuNPs were also characterized by UV-Vis adsorption spectrophotometry. Figure 2 (right) shows broad absorption at 520 nm, with its absorbance value at 520 nm being 0.21. Also the AuNPs concentration obtained was calculated to be 7.8 nM ($\epsilon = 2.7 \times 10^8 \text{ M}^{-1} \text{ cm}^{-1}$).

Characterization of the peptide-Ru1

The Ru1-labeled peptide (peptide-Ru1) was characterized by UV-Vis absorption spectrophotometry and fluorescence imaging. Figure 3 (left) shows the UV-Vis spectra of peptide, Ru1, and peptide-Ru1. A characteristic peptide peak was observed at 280 nm for the peptide in Fig. 3 (left) (line a). Three

characteristic peaks were observed between 220 and 550 nm for Ru1 in Fig. 3 (left) (line b). The UV-Vis spectrum of the peptide-Ru1 is shown in Fig. 3 (left) (line c), where the characteristic peaks at 287 and 457 nm correspond to the absorption of Ru1 at 290 and 458 nm, respectively, with a slight shift towards blue light. The absorption of the peptide-Ru1 at 457 nm was characteristic of absorption of the metallic ruthenium, which indicates the conjugation of Ru1 into the peptide. Figure 3 (right) shows fluorescence imaging of the peptide-Ru1. These manifest that Ru1 has been conjugated with the peptide.

Optimization of experimental conditions

We investigated the effect of the reaction time between PSA and AuNPs-peptide-Ru1 on changes in the ECL intensity, ΔI . As is shown in Fig. 4A, the ΔI drastically increased when the reaction time was increased from 5 to 20 min. When the reaction time was in the range of 20 – 40 min, ΔI increased slowly. After 40 min, ΔI reached its maximum value and remained stable. This shows that 40 min is sufficient for this system to achieve equilibrium. Therefore, the reaction time for PSA to recognize and cleave AuNPs-peptide-Ru1 is 40 min.

Since PSA is a biologically active serine protease, the cleavage temperature will affect its biological activity. Therefore, we examined the effects of the reaction temperature on changes in the ECL intensity, ΔI . As is shown in Fig. 4B, ΔI drastically increased when the reaction temperature was increased from 5 to 20°C. Within the range of 20 – 30°C, the increase in ΔI is slow. At 30°C, ΔI reaches its maximum. When the temperature is higher than 35°C, ΔI decreases instead. This shows that the biological activity of PSA is disrupted at temperatures greater

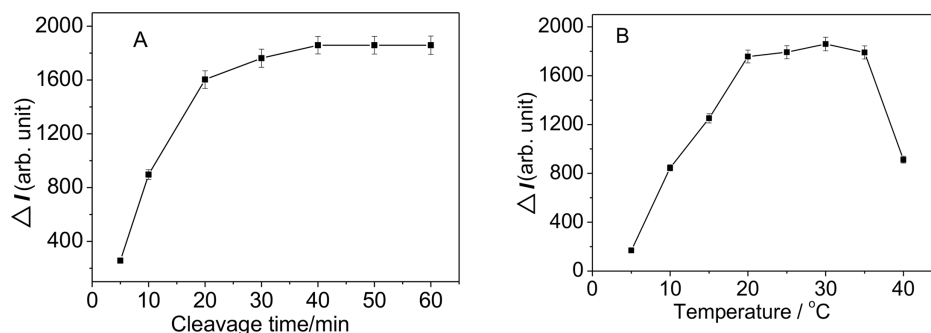


Fig. 4 Effects of the cleavage time (A) and cleavage temperature (B) of AuNPs-peptide-Ru1 for reaction with 3.0×10^{-10} g/mL PSA on the ECL intensity.

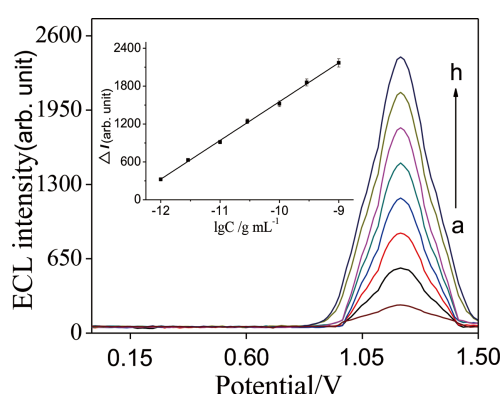


Fig. 5 ECL intensities vs. potential profiles of AuNPs-peptide-Ru1 after reaction with different concentrations of PSA in 0.10 mol/L PBS (pH 7.4) containing 50 mmol/L TPA, concentration of PSA (a - h): (a) 0; (b) 1.0 pg/mL; (c) 3.0 pg/mL; (d) 10 pg/mL; (e) 30 pg/mL; (f) 100 pg/mL; (g) 300 pg/mL; (h) 1000 pg/mL. Insert, calibration curve of PSA. The error bars represent the standard deviation of three repetitive measurements.

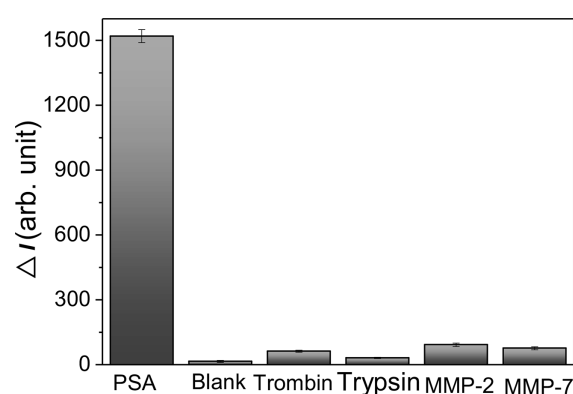


Fig. 6 ECL intensity for the AuNPs-peptide-Ru1 in the presence of 0.1 ng/mL PSA, and 2.0 ng/mL other proteases, including thrombin, thypsin, MMP-2, and MMP-7.

than 35 $^{\circ}\text{C}$. Therefore, the reaction temperature used in this experiment was 30 $^{\circ}\text{C}$.

Performance of the assay for PSA

We measured the detection performance of the constructed AuNPs-peptide-Ru1 on different PSA concentrations of under the optimized conditions. Figure 5 shows the ECL intensity curve of the AuNPs/Nafion/GCE in the solution before and after AuNPs-peptide-Ru1 reacted with different PSA concentrations. From Fig. 5, we can see that the ECL intensity increases with increasing PSA concentrations. In addition, ΔI and the PSA concentration exhibit a good linear relationship in the range from 1.0 pg/mL to 1.0 ng/mL. The regression formula was $\Delta I = 7692.2 + 613.8 \lg C$ (g/mL), with a regression coefficient of 0.9986, and the detection limit ($S/N = 3$) is 4.0×10^{-13} g/mL. This detection limit is lower than that (10 ng/mL and 1 pg/mL) reported in the literature.^{30,31} The low detection limit is attributed to the use of the modified GCE with Au nanoparticle and Nafion, which can enhance the ECL intensity of the cleaved Ru1 part based on the stronger electrostatic interactions between the positive charged Ru1 and the negative charged Nafion on the electrode. The threshold value when PSA is used as a marker for the diagnosis of prostate cancer (an abnormal value is greater than this value) is 4.0 ng/mL (most cases have values closer to

2.5 ng/mL). This shows that our analysis method can be used in the diagnosis of early prostate cancer.

To evaluate the selectivity of this sensor for the quantitative detection of PSA, we used different biologically relevant species including MMP-2, MMP-7, trypsin, and thrombin as interfering substances to be examined in an aqueous solution. Figure 6 shows the ECL reactions of this sensor using 0.1 ng/mL PSA or 2.0 ng/mL of other proteases under identical experimental conditions. As shown in Fig. 6, the biosensor showed a remarkable response to 0.1 ng/mL PSA, while a negligible response was observed in analogs with 20-fold concentration higher than that of PSA. This shows that PSA can specifically cleave the polypeptide with a specific sequence (CHSSKLQK), and the constructed sensor can be applied for the analysis of PSA.

In order to assess the application potential of this sensing method, we tested blood samples collected from six patients from the Xi'an Friendship Medical Laboratory. The collected blood samples were centrifuged and the supernatants were collected. PBS (10 mmol/L) was used to dilute the serum. The AuNPs-peptide-Ru1 designed and synthesised in this study was used to measure the PSA concentrations of blood samples. At the same time, the chemiluminescence immunoassay (CL) was used to compare the test results. From Table 1, we can see that the test results obtained using the biosensor constructed in this study are consistent with those of CL method used in clinical practice. This shows that the electrochemiluminescence sensor based on the enzymatic cleavage of polypeptide constructed in this study has both good accuracy and applicability.

Table 1 Analytical results of PSA in clinical serum samples

Sample number	CL method/ ng mL ⁻¹ ^a	This method/ng mL ⁻¹ (<i>n</i> = 3, <i>P</i> = 0.9)	Relative error, %
1	3.30	3.40 ± 0.36	3.0
2	3.53	3.55 ± 0.80	0.6
6	7.36	7.48 ± 1.00	1.6
4	11.73	11.71 ± 1.53	-0.2
5	13.83	13.07 ± 1.77	-5.4
6	58.92	58.56 ± 6.78	-0.6

a. The CL results of PSA in patients' serum sample from clinical reports provided by Xi'an Friendship Medical Inspection.

Conclusions

In conclusion, a novel ECL homogeneous detection method has been established based on enzyme-catalyzed cleavage of polypeptide and the separation of AuNPs centrifugation. The ECL method could transduce a signal corresponding to peptide biocleavage events into an ECL signal for the detection of PSA, which could reduce the complex fabrication process of electrodes and improve the reproducibility of biosensors. Compared with the traditional immunoassay method, the most significant advantage of the current approach is its high sensitivity, cost-effectiveness, and simplicity. More importantly, this principle could be further applied to the detection of other proteases.

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