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Joint enhancement strategy applied in ECL biosensor based on closed bipolar electrodes for the detection of PSA

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ABSTRACT

A highly sensitive electrogenerated chemiluminescence (ECL) biosensor was developed on the basis of a closed bipolar electrode (BPE) apparatus for the analysis of prostate specific antigen (PSA). Bipolar modifications bring up two different stages of enhancement on the same electrode. Anodic enhancement was conducted by modifying gold nanoparticles (Au NPs) to catalyze the anodic ECL reaction between luminol and hydrogen peroxide. Cathodic introduction of thionine tagged PSA antibody led to a further pertinently enhancement synchronized with the PSA amount variation, because the existence of thionine greatly increased the rate of electron gains on cathode, leading to the corresponding acceleration of anodic ECL reaction. The more thionine modified target molecules were introduced, the faster luminol was oxidized, the higher faraday current approached, and sensitive quantification was realized in correlation with the responsive ECL intensity differences. The quantification resulted in a good determination range between 0.1 pg/mL and 0.1 μg/mL. This strategy mainly took advantage of the special structure of closed BPE to realize a simultaneous amplification on both ends of BPE. Moreover, the platform had a potential of providing a multi-functional strategy for the realization of other bio-detections by simply substituting the PSA sandwich structure with other bio-structures.

1. Introduction

Biomarkers have emerged as important diagnostic tools for cancer and many other diseases. To date, a lot of methods have been developed for the detection of cancer biomarkers such as fluorescence [1–4], surface plasmon resonance [5,6], chemiluminescence (CL) [7], mass spectrometry as well as ECL [8–10]. In particular, ECL, a joint strategy of both optics and electrochemistry, perfectly combined the sensitivity of optics and the controllability of electrochemistry which in return offered a reliable means of bio-detection in the area of life analysis [9,11,12]. At current stage, most methods including ECL remained at laboratory phase. To develop practical device which are not only sensitive, but also efficient, low-cost and ease of use is highly demanded for clinical analysis.

Bipolar electrodes (BPEs), different from traditional three-electrode system, can be easily miniaturized to make lab on a chip device, since there is no need for a direct external connection to the BPE [13–15]. Considering it is not easy to directly measure the current through BPEs, Manz and co-workers firstly introduced ECL as an optical signal transduction method to detect targets participated in ECL reaction [16]. Crooks group furtherly developed a closed BPE system that decoupled the sensing and reporting poles using BPE as the only current flow path, which opened the door to BPE-ECL sensing to a wide variety of analytes [17]. In such closed BPE-ECL system, both anode and cathode poles determine the detection sensitivity. Therefore, efforts have been devoted to the amplification technologies at the poles. On one hand, the modification of electrochemical sensing pole focused on enhancing electron transfer [15,18,19]. On the other hand, at the ECL reporting pole, the amplification tags were introduced for enhancing or quenching the ECL emission [20,21]. Since most closed BPE-ECL systems use ITO as the BPE and the signal collection is accomplished using a photomultiplier tube (PMT) because of its high sensitivity, the transparency of the reporting pole must be maintained after modification. To our knowledge, most amplification strategies focused on modification at one end of the BPE [13]. Recently, our group reported a BPE-ECL biosensor for the measurement of cancer cell surface based on a dual-amplification strategy on both poles. Au was electrochemically deposited on the cathode to enhance the electron transfer rate, and ferrocene was

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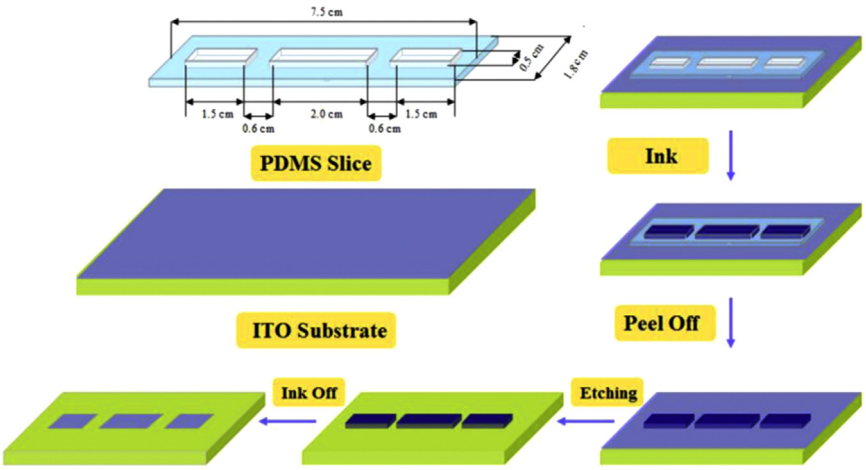

Fig. 1. Structure and preparation process of the microchip.

introduced to the anode for $Ru(bpy)_3^{2+}$signal quenching amplification. [22].

To develop more efficient amplification strategy for highly sensitive bioanalysis, in this paper, we report a BPE-ECL biosensor for the detection of PSA based on both anode and cathode amplifying technique. Similar to$Ru(bpy)_3^{2+}$, luminol is also an excellent luminescent material, which is usually utilized in ECL reaction with a co-reactant of H_2O_2 [23,24]. The luminol-H_2O_2 reaction is often catalyzed by introducing gold or platinum nanoparticles, which in a certain degree can increase the ECL intensity [21,25,26]. To avoid the decrease of transparency of ECL reporting pole, Au NPs were biologically connected on the anode instead of electrochemical deposition, to catalyze the luminol-H_2O_2 ECL reaction, which greatly enhanced the ECL signal. At the cathode end, Th@SiO_2 NPs were introduced as both recognition probes for PSA and signal amplification indicators. Though the enhancement on cathode is directly related to PSA, the simultaneous amplification also led to a more obvious difference in investigation. The jointed enhanced model exhibited a detection limit of 0.1 pg/mL, and the effective detection range is from 0.1 pg/mL to 0.1 μg/mL, which fulfilled the clinic requirements in PSA determination. And the reported sensor chip displayed excellent stability and reproducibility compared with traditional similar biosensors, which ensured highly sensitive detection and accurate analysis of biomarkers in human tissues or body fluids in early disease diagnosis as well as practical application [4].

2. Experimental section

2.1. Reagents and chemicals

3-aminopropyl triethoxysilane (APTES), bovine serum albumin (BSA), Tris(2-carboxyethyl) phosphine (TCEP), Triton X-100, cyclohexane, 1-hexanol, tetraethyl orthosilicate (TEOS) and thionine acetate were purchased from Sigma-Aldrich (USA). Glutaraldehyde (GA) was obtained from sinopharm chemical reagent company (Shanghai, China). Clone P27A10 (primary capture antibody (Ab1)) and clone P27B1 (secondary detection antibody (Ab2)), total prostate specific antigen (PSA) were obtained from Shuangliu Zhenglong Biochemical Lab (Chengdu, China). The clinical serum samples were from Affiliated Hospital of Nanjing University. Indium tin oxide (ITO) coated (thickness, ~100 nm; resistance, ~10 Ω/square) aluminosilicate glass slides were purchased from CSG (Shenzhen, China). Sylgard 184 (including PDMS monomer and curing agent) was from Dow Corning (Midland, MI, USA). The ECL detection solution was 0.10 M phosphate buffered saline (PBS, pH 7.4) containing 0.06 mM luminol and 0.2 M H_2O_2. All solutions were prepared using Millipore (model milli-Q) purified water and stored at 4 °C. All the other chemicals used in this experiment were of analytical grade.

2.2. Apparatus

The ECL signals were measured with MPI-E electrochemiluminescence analyzer from Xi'An Remax Electronic Science & Technology Co. Ltd. (Xi'An, China, 350–650 nm). Transmission electron microscopy (TEM) images were performed with a JEOL model 2000 instrument. Scanning electron microscopy (SEM) images were obtained using an S-3000 N scanning electron microscope (Tokyo, Japan).

2.3. Preparation of closed bipolar electrodes

The bipolar electrode was manufactured through wet etching on an ITO glass according to our former works [18]. ITO glass (7.5 cm × 1.8 cm) was used as the substrate for the microchip. The BPE system on the substrate consisted of three ITO microband electrodes, all of which were 0.5 cm in width but 1.5 cm, 2 cm, 1.5 cm respectively in length. The microband electrodes were horizontally arranged in the same line with a gap of 0.6 cm between adjacent bands as is shown in Fig. 1.

To get the pattern of the designed microchip, PDMS molds with ink reservoirs were employed to acquire the final closed BPEs. The ITO glass plate was first washed with boiled potassium hydroxide/2-propanol solution for 20 min. When cooled down, the plate was washed with ethanol, acetone and purified water three times successively. After drying, the ITO glass plate was covered with a slice of 2 mm PDMS sheet with reservoirs of the same size with designed electrode in the middle. A certain degree of diluted ink was added to the reservoirs with a syringe. After exposed in the air overnight, the ink dried and the PDMS mold was then peeled off, leaving the corresponding patterns on the ITO substrate. Finally, the pattern-protected ITO glass plate was put in the ITO removal solution ($FeCl_3$:HNO_3:HCl=0.5 M:1 M:1 M), and then the exposed ITO was removed with the solution. After getting rid of the dried ink, the bipolar electrodes were acquired.

2.4. Synthesis of Au NPs and Au NPs-DNA complex

Au NPs with an average diameter of 5 nm were synthesized through the reduction of $HAuCl_4$ by sodium borohydride ($NaBH_4$) according to the method in pervious literatures [27,28]. In brief,

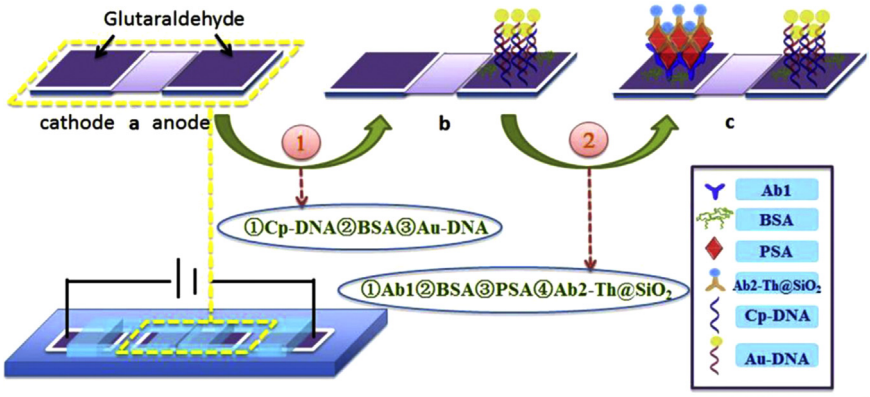

Fig. 2. Schematic process of the enhancing ECL model for the detection of PSA.

0.6 mL of ice-cooled 0.1 M $NaBH_4$ was added to 20 mL of 2.5×10^{-4} M $HAuCl_4$ aqueous solution under constant stirring. The solution was kept stirring in an ice bath for 10 min and then kept reacting at room temperature for another 3 h. Then the acquired Au NPs were stored at 4 °C for further use.

To obtain Au NPs-DNA complex hybridized to the anode surface, 9.8 μL thiol-DNA (10 μM) and 36.2 μL diluting DNA (100 μM) were first mixed under the existence of 1.5 μL TCEP (10 μM) for 30 min. The 1 mL synthesized Au NPs were then added to the mixture at room temperature and reacted for 12 h. After the linking reaction was done, 20 μL PBS (containing 3 M NaCl) was pipetted in every 60 min for 5 times to keep the stability of the product.

2.5. Synthesis of $Th@SiO_2$ and $Ab2\text{-}Th@SiO_2$ complex

$Th@SiO_2$ was synthesized according to former reports [18]. 1.77 mL of Triton X-100, 7.5 mL of cyclohexane, 1.8 mL of 1-hexanol and 340 μL of thionine acetate (50 mM) were mixed and reacted under constant magnetic stirring. After a water-in-oil microemulsion was formed, 100 μL of tetraethyl orthosilicate (TEOS) and 60 μL of $NH_3 \cdot H_2O$ were added, and the reaction was kept on for 24 h. Then, acetone was introduced to destroy the emulsion, followed by centrifuging and washing with ethanol and water respectively. The obtained nanoparticles were treated with 10% (v/v) APTES for 2 h at 30 °C and rinsed with ethanol and PBS to remove loosely bounded APTES. Thus, amino functionalized $Th@SiO_2$ NPs were acquired and dispersed in PBS. 100 μL 2.5% GA (v/v) was then added to 1 mL amino-functionalized $Th@SiO_2$ NPs dispersion and kept incubating for 3 h. After carefully washed with 0.1 M PBS (containing 0.1 M NaCl), the obtained GA modified $Th@SiO_2$ NPs were incubated with 25 μL of mouse monoclonal signal PSA antibody (2 mg/mL) and 25 μL of AFP antibody (2 mg/mL) at 4 °C for 12 h respectively. The precipitates were centrifuged and washed with 0.1 M PBS (containing 0.1 M NaCl). The resulting $Ab2\text{-}Th@SiO_2$ complex was dispersed in 5 mL 0.1 M PBS (containing 0.1 M NaCl) and stored at 4 °C.

2.6. Fabrication of microchip biosensor

After carefully cleaning, the resulted BPEs were immersed into 1 M NaOH for 4 h to ensure the presence of hydroxyl groups on the electrode surface. Then this hydroxyl-modified microchip was functionalized with $–NH_2$ by immersing in a 5% (v/v) ethanol solution of APTES at 4 °C for 12 h. Rinsed three times with ethanol and purified water successively, excess and loose bounded silane were removed. After that, two PDMS slices containing specific reservoirs (1.6 cm long, 0.6 cm wide) were affixed on the prepared ITO glass across two adjacent micro-electrodes respectively. Then, 30 μL 2.5% GA (v/v) aqueous solution was carefully added to both anode and cathode of the BPE, making sure that the two ends of BPE were immersed and kept at 37 °C for 1 h. After rinsed with 0.1 M PBS (pH 7.4) thoroughly, the anode and cathode began to be treated differently. For the cathode, 30 μL of 20 μg/mL primary anti-PSA antibody (Ab1) was introduced to the reservoir to immerse the cathode. After incubated at 37 °C for 3.5 h, the cathode was blocked by 30 μL 2 wt% BSA solution at 37 °C for 1 h and then carefully washed with PBS (pH 7.4). For the anode, 30 μL of 1 μM capture DNA (Cp-DNA) was added to the anodic reservoir. After 1.5 h incubation, the anode was similarly blocked by 2 wt% BSA solution at 37 °C for 1 h and still carefully washed with PBS (pH 7.4). Thereafter, 30 μL of 1 μM target DNA with Au NPs (Au-DNA) was introduced to the same reservoir to react with the Cp-DNA to form a double strand structure with Au NPs modified on the anode.

2.7. Operation of PSA determination

The measuring process and detection mechanism of the developed ECL immunoassay was illustrated in Fig. 2. Specifically, 30 μL of PSA with prepared concentrations was introduced to the cathodic reservoir and incubated at 37 °C for 40 min to ensure the complete reaction with Ab1. The reservoir was then washed with PBS (pH 7.4) for three times, after which 30 μL secondary detection antibody ($Ab2\text{-}Th@SiO_2$) conjugated with silica beads wrapped thionine was injected into the reservoir to react with the PSA on cathode. After washing with PBS twice and dried at room temperature, detection solutions were added to both reservoirs. 100 μL 0.1 M PBS (pH 7.4) containing 0.1 M NaCl was added to the cathodic reservoir while 98 μL 0.2 M H_2O_2 with 2 μL 3 mM luminol was added to the anodic reservoir. In later detection of PSA in real samples, the treatment for human blood centrifugal composition was the same with the procedure above.

ECL measurements were performed on a MPI-E system with the PMT set at −500 V and the driving electrodes were situated on the outer ends of the two side electrodes with two separate silver conductive tape.

3. Results and discussion

3.1. Principle of enhancing model BPE biosensor

The structure and fabrication process of the BPE-ECL biosensor are shown in Fig. 2. The anode and cathode were separated on a single conductor but treated individually, and the faraday current

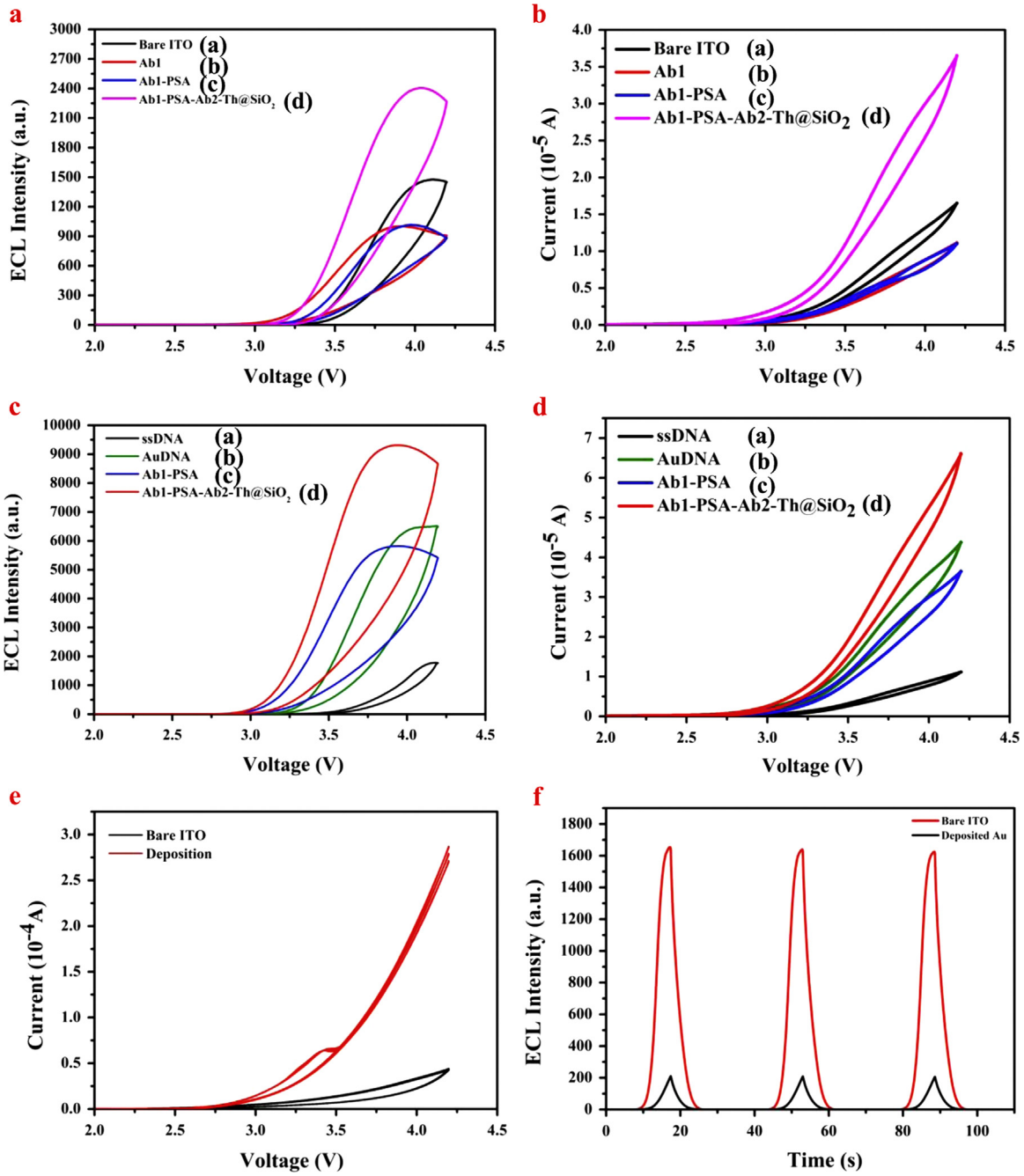

Fig. 3. (a) Typical ECL curves of anodic reaction after step by step modification on the cathode of BPE; (b) Corresponding current-potential curves of cathodic modification process; (c) Typical ECL curves of ssDNA without Ab2-Th@SiO_2 (black), with Au NPs (green) on anode and with both Au NPs on anode and Ab2-Th@SiO_2 on cathode. Thionine was introduced by immune reaction; (d) Corresponding current-potential curves of anodic modification process; (e) CV curves of bare BPE (black) and BPE with gold deposited on the anode (red) instead of assembling; (f) ECL curves of bare BPE (black) and BPE with gold deposited (10 s) on the anode (red) instead of assembling. The signals were measured in 0.2 M H_2O_2 with 0.06 mM luminol. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

became the only connection which directly defined the change of electrochemical reaction hence determined the ECL emission. Luminol-H_2O_2 was used in this sensor as ECL emission substance. AuNPs, which have been reported as an efficient catalyst of luminol-H_2O_2, were introduced to the anode by DNA double strand. Thionine, a special compound containing two primary amino groups that could covalently linking with biomolecules, was introduced as an electrochemical indicator to modulate the current flow through the BPE and then improve the ECL emission of luminol. At the cathode, a sandwich type Th@SiO_2 labeled Ab2/PSA/Ab1 was formed, resulting in an increased ECL intensity at the anode. Briefly, a quantitative standard for PSA with different concentrations and ECL signals was built up for the detection of this biomarker.

To qualify the signal amplification of this strategy, we investigated the ECL curves of the ITO electrode after each step of surface modification. The cathode modification was studied first without introducing AuNPs to the anode. Adding 0.2 M H_2O_2 and 0.06 mM luminol in the right reservoir, blank ITO showed ECL intensity of ca. 1500 a.u. (Fig. 3a, curve a) The modification of Ab1 at the cathode resulted in a slight decrease of ECL intensity to 1100 a.u. (Fig. 3a, curve b), while the further incubation in

0.1 μg/mL PSA solution made little difference in ECL intensity (Fig. 3a, curve c). After the immune reaction of PSA antigen with Ab2-Th@SiO_2, the anodic ECL emitting increased obviously to 2400 a.u (Fig. 3a, curve d). Such phenomenon could be ascribed to the lower reduction potential of thionine (−0.14 V vs. SCE) compared with dissolved oxygen (−0.5 V vs. SCE). The reduction of thionine at the cathode led to the faraday current change of BPE, which further affected the intensity of BPE-ECL. In order to acquire a lower detection limit as well as higher detection accuracy, Au NPs were introduced to catalyze the anodic ECL reaction. Corresponding CV curves were also displayed in Fig. 3b. ECL signals at different states of anodic modifications were measured. As shown in Fig. 3c, leaving the cathode unmodified, after introducing Au-DNA to the anode, the ECL signals (Fig. 3c, curve b) almost quadrupled compared with ssDNA modified anode (Fig. 3c, curve a), indicating the catalysis of Au NPs to ECL reaction. After Ab1-PSA was introduced to the surface of the cathode, the ECL signal slightly decreased (Fig. 3c, curve c), which was attributed to the hindrance of protein to the electron-transfer of the electrode. At last, when Ab2-Th@SiO_2 was linked to PSA, the anodic ECL emitting increased to 9500 a.u. (Fig. 3c, curve d) which was almost 4 times higher than that obtained from unmodified anode (Fig. 3a, curve d). For the detection of PSA, the ECL intensity difference between pre-thionine-modification state and post-thionine-modification state was more than 2 times after assembling Au NPs on the anode. While corresponding CV curves according to the anodic modification were also displayed in Fig. 3d.

To immobilize Au NPs on the electrode, electrochemical deposition is easier compared with the current method. However, when we applied a 10 s deposition under −0.5 V vs. SCE in 1% $HAuCl_4$ aqueous solution, the CV current increased obviously (Fig. 3e), but the ECL signal sharply decreased (Fig. 3f). We suppose although the deposition of Au improved the surface conductivity of ITO electrode, the transmittance of ITO electrode decreased too much. Therefore, electrochemical deposition has not been applied in this work.

3.2. Optimization of quantification conditions

The quantity of modified Au NPs on the anode depends on the hybridization reaction as well as the concentration of ssDNA, which closely determines the detection limit. Too many double DNA strands on anode may affect the electronic transfer of the BPE system. However, lack of Au NPs causes smaller catalytic surface, which also brings an obvious decrease on the ECL intensity. It did make sense if we maximized the difference in ECL intensity between dsDNA and ssDNA. Fig. 4a shows the trend of ECL signal increase of single DNA attached to the anode with the passage of time. The ordinate is the increase folds towards the blank intensity. The result indicates that at the modification time of 1.5 h, the ECL intensity reaches the maximum since larger density of DNA on electrode surface also hindered hybridization process due to the steric effect. Fig. 4b shows the influence of hybridization time on the ECL intensity. The ordinate is also the increase folds towards the blank signal. As hybridization time increases, the ECL intensity gradually increased and reached the maximum at 40 min. So in later experiment, 1.5 h and 40 min were chosen respectively as the capture DNA modification time and hybridization time.

3.3. Determination of PSA standard solutions

Quantification of PSA was achieved based on the relationship between ECL intensity differences and PSA concentrations. The results were shown in Fig. 5. Inset a is the ECL difference between pre-thionine-modification and post-thionine-modification with different PSA concentrations on the cathode. Fig. 5 inset b is the linear fitting of PSA (thionine dependent) concentrations and ECL intensity differences. The enhanced model exhibited a detection limit of 0.1 pg/mL, and the effective detection range is from 0.1 pg/mL to 0.1 μg/mL, which fulfills the clinic requirements in PSA determination.

Rigorously, in order to investigate the practicability of this method, we also applied it in the detection of real human serum samples. Four human serum samples (male) with different PSA concentrations were measured. Table 1 shows the detection results. The reference levels of PSA in real human serum samples here were detected with a chemiluminescent analyzer (Abbott i2000 system). The CL results offered by the hospital were in the same magnitude compared with our method, suggesting there was possibly no significant difference between this ECL biosensor and commercial instruments.

4. Conclusion

In summary, we proposed a highly sensitive ECL strategy for the determination of PSA on the basis of a closed BPE system. Benefiting from the two separated ends structure of closed BPE, we made a simultaneous amplification strategy towards the anodic emitting of luminol. This method could successfully realize the PSA determination at a critical level but even cut down to a lower

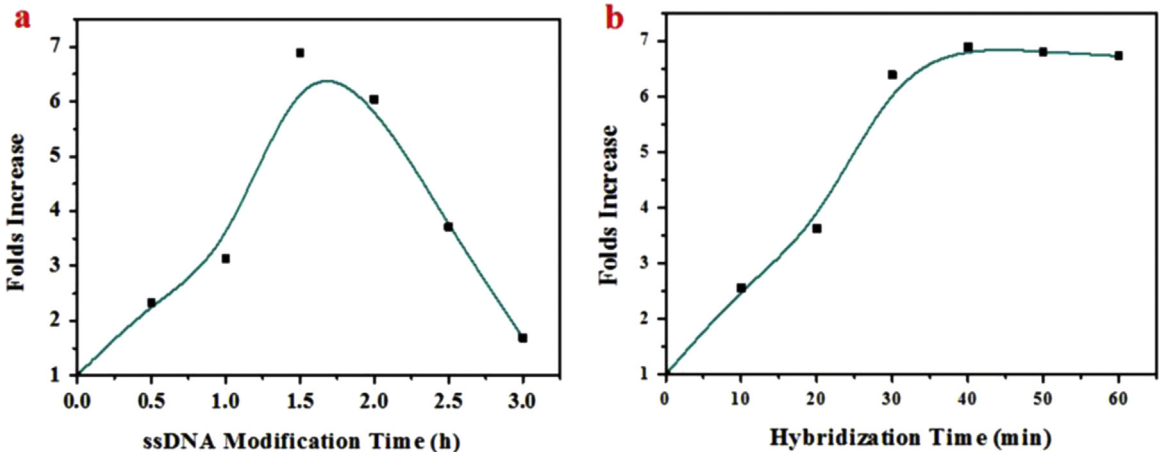

Fig. 4. (a) The folds increase of ECL intensity towards the original intensity versus different modification time of single strand DNA (ssDNA) from 0.5 to 3.0 h; (b) The folds increase versus different hybridization time from 10 min to 60 min. ECL curves were measured in 0.2 M H_2O_2 with 0.06 mM luminol.

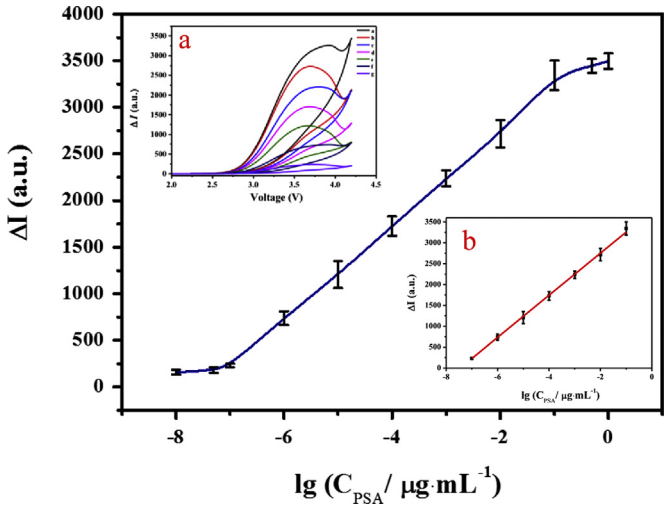

Fig. 5. Relationship between the logarithm of PSA concentration (from 10 fg/mL to 1 μg/mL) and the increment of ECL intensity. Inset (a) The ECL difference between pre-thionine-modification and post-thionine-modification curves with different PSA concentrations (from a to g respectively: 0.1 μg/mL, 0.01 μg/mL, 1 ng/mL, 0.1 ng/mL, 0.01 ng/mL, 1 pg/mL, 0.1 pg/mL; Inset (b) Linear relationship between the logarithm of PSA concentration and the increment of ECL intensity. ECL curves were measured in 0.2 M H_2O_2 with 0.06 mM luminol.

Table 1
Detection results of total PSA concentration in actual human serum samples.

Methods	Samples			
	1	2	3	4
CL (ng/mL)	0.66	0.83	1.24	16.54
ECL (ng/mL)	0.78 ± 0.15	0.96 ± 0.18	1.56 ± 0.23	18.22 ± 0.46

detection limit by four orders of magnitude except for a wider detection range. The method provided a good discrimination on prostate cancer and benign prostatic hyperplasia. In addition, the platform can be easily transplanted to other life analysis systems. The closed BPE system based device also offers good convenience in the process of modification and reduces the interference between the modified substances. This biosensor platform presented a compatible, rapid and efficient system that was attractive for sensitive analysis of cancer biomarkers.

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