

Short Communication

Amperometric Immunosensor of Procalcitonin Based on Amplification Strategy of Ferrocene-Modified Gold nanoparticles

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In this article a novel electrochemical immunoassay for procalcitonin (PCT) detection based on ferrocene-modified Au nanoparticles was developed and ferrocene-modified gold nanoparticles provided the electrochemical amplification signal; the nanoparticles was labeled with PCT antibodies which could bind with PCT and another PCT-antibody-modified gold electrode to form a sandwich structure. The PCT immunosensor exhibited good linearity of oxidization current when the potential is 0.3 V and PCT concentration lies between 1.5 pg/mL and 50 ng/mL, and the limit of detection was 0.8 pg/mL. Moreover, the PCT immunosensor was successfully applied to detect PCT in clinical samples.

Keywords: Ferrocene; electrochemical; procalcitonin; immunosensor

1. INTRODUCTION

Procalcitonin (PCT) is a useful biomarker of septicemia. There is low concentration of PCT in plasma of healthy individuals, whereas its level increases dramatically as a result of bacterial infection. This characteristic makes PCT a useful marker of infection that differentiates between bacterial and viral causes[1,2]. It facilitates the diagnosis of many diseases including bacterial pneumonia and neonatal purulent meningitis.

There are various techniques for PCT determination. The immunogold chip is traditionally used in clinical settings and the method only provides semi-quantitative results. However, PCT concentration is low and undetectable in the earlier stages of illness, so a more sensitive method to detect PCT is necessary. Newer methods such as fluorescence immunoassay (IFA), chemiluminescence immunoassay (CLIA), and enzyme linked immunosorbent assay (ELISA) were

employed. Though those methods were sensitive, they require longer turnaround time and larger sample volume[3-6]. Thus it remains challenging to improve the sensitivity and specificity of the simpler PCT determinations.

Electrochemical method has high sensitivity, simple instrumentation and pretreatment, low cost, and short response time[7-12]. It was introduced to immunosensors, especially those based on nanoparticles such as gold nanoparticles, single walled carbon nanoborns (SWCNHS), and magnetic particles, attracting much attention[12-15]. Gold nanoparticles with their biocompatibility, large specific surface area, and low electrical resistance were used in various fields[15-18]. Ferrocene is a common electrochemical probe used to trace DNA, amino acid polymers, and ligands. With the attachment of ferrocene to those bioactive species, their detection will have electrochemical-level sensitivity and simplicity. The aim of our work is to introduce a sandwich structure in which the electrochemical sign probe is ferrocene-modified-Au-nanoparticles labeled with PCT antibody (ABI), the immunosensor is PCT-antibody (Ab II)-modified gold electrode, and the two react with PCT. The electrochemical behaviors of the structure were investigated. Furthermore, clinical serum samples were also tested and the results compared to ELISA to prove the clinical applicability of our method.

2. EXPERIMENTAL

2.1 Materials

11-Ferrocenyl-1-undecanethiol (Fc-C11SH) was ordered from Dojindo (Kumamoto, Japan). N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), 3-mercaptopropionic acid (MPA), and 11-mercaptopundecanoic acid (MUA) were acquired from Sigma-Aldrich Corp. (St. Louis, Missouri). PCT antibodies (I and II) were obtained from Hangzhou Qitai Biochemical. Co. Ltd. (Zhejiang, China). Millipore water (18 M Ω -cm, Simplicity Model, Billerica, MA) served as the solvent of all aqueous solutions.

2.2 PCT immunosensor electrode preparation

Electrochemical cleansing of aurous disk electrodes were performed in 0.1 mol/L sulfuric acid by cycling potential between 0.0 and 2.0 V vs. Ag/AgCl for 10 min, rinsing in water, and drying in nitrogen. The following step was one-hour immersion of the electrodes in 100 μ L 5.0 mmol/L MPA. Then the electrodes were immersed for 0.5 h in 60 μ L pH 5.5 phosphate buffered saline (PBS) containing 15 mmol/L NHS and 75 mmol/L EDC. They were then kept 0.5 h in PBS containing 10 mg/mL PCT antibody (Ab I). The prepared electrodes were washed by PBS, and immersed to 5 mmol/L glycine to block the unreacted carboxyl groups.

2.3 Ferrocene-modified-Au-nanoparticles-labeled PCT antibody

Gold nanoparticles was prepared following published procedure[18]. The gold nanoparticles(10mg/mL) was mixed with dimethyl sulfoxide (DMSO) containing 1.0 mmol/L

FcC₁₁SH and 0.2 mmol/L MUA for 0.5 h. The mixed solution was centrifuged at 12 000 rpm for 0.5 h, resuspended 0.5 h in 500 μ L solution containing 75 mmol/L EDC and 15 mmol/L NHS, and centrifuged again. Then PCT antibody (Ab II) was added, the system was left to stand 0.5 h, after which 5 mmol/L glycine was added, and the solution was subjected to centrifugation at 12 000 rpm for 0.5 h and resuspended in 2 mL PBS for future use.

2.4 Detection of PCT with immunosensor

The PCT stock solution (1 mg/mL) was diluted with PBS. For the immunoassay, the PCT immunosensor electrode was immersed into 100 μ L sample solution, then washed with PBS, and immersed into 50 μ L ferrocene-modified-gold-nanoparticles-labeled PCT antibody. Electrochemical detections were consistently performed with Electrochemical Workstation CHI600B (CH Instruments, Austin, Texas, US). In measurement of electrochemical properties of the PCT immunosensor, ferrocene-modified-Au-nanoparticles-labeled PCT immunosensor served as the working electrode, Ag/AgCl electrode was the reference electrode, platinum flag was the auxiliary electrode, and the supporting electrolyte was 10 mmol/L KClO₄ in PBS. In amperometric detection of PCT with the immunosensor a constant potential of 0.3 V was used. All experiments were performed at ambient temperature unless otherwise stated.

2.5 Reference method of PCT measurement (ELISA)

Enzyme-linked immunosorbent assay (ELISA) was the common clinical PCT assay. It was included in our experiment as comparison. The test kit used was manufactured by Wuhan EIAab Science Co., Ltd. and its manual was followed:

1. Add 100 μ L of Standard, Blank, or Sample per well. Cover with the Plate sealer. Incubate for 2 hours at 37°C.
2. Remove the liquid of each well, do not wash. Add 100 μ L of Detection Reagent A working solution to each well. Cover with the Plate sealer. Incubate for 1 hour at 37°C. Detection Reagent A working solution may appear cloudy. Warm to room temperature and mix gently until solution appears uniform [19].
3. Aspirate each well and wash, repeating the process three times for a total of three washes. Wash by filling each well with Wash Buffer (approximately 400 μ L) using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
4. Add 100 μ L of Detection Reagent B working solution to each well. Cover with a new Plate sealer. Incubate for 1 hour at 37°C [19].
5. Repeat the aspiration/wash as in step 4.
6. Add 90 μ L of Substrate Solution to each well. Cover with a new Plate sealer. Incubate within 15-30 minutes at 37°C. Protect from light [19].

7. Add 50 μL of Stop Solution to each well. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
8. Determine the optical density of each well at once, using a microplate reader set to 450 nm [19].

3. RESULTS AND DISCUSSION

3.1 Electrochemical analysis with PCT immunosensor

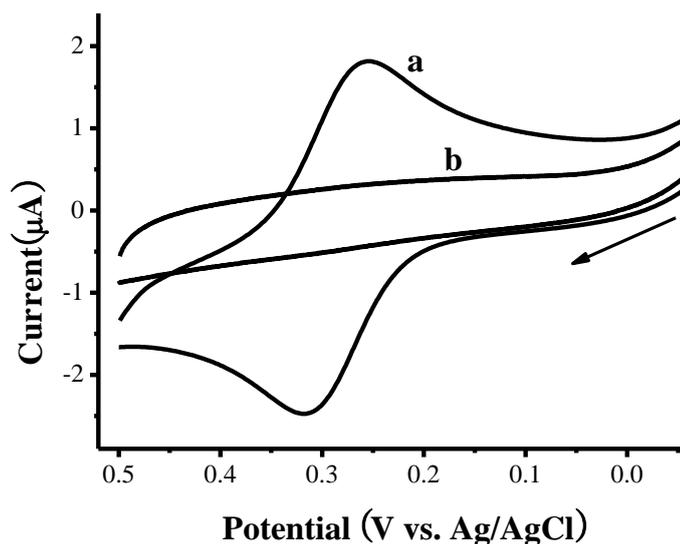


Figure 1. Cyclic voltammetry of Au nanoparticles modified with ferrocene (a) or ferrocene monocarboxylic acid (b) labeled PCT immunosensor. The scan rate was 50 mV/s.

Cyclic voltammetry was used to investigate the electro-chemical behaviors of PCT immunosensor. As shown in the upmost curve (labeled “a”) of Figure 1, there was two obvious voltammetric peaks. The peak potentials of the cathode and the anode, E_{pc} and E_{pa} , at 0.319 and 0.258 V vs. Ag/AgCl, respectively, were detected with ferrocene-modified-Au-nanoparticles-labeled PCT immunosensor. The peak current ratio was 1.05 and the peak currents were proportional to the scan rate’s root of degree 2. From those characteristics it could be inferred that the PCT immunosensor undergoes a reversible redox reaction and electrons can transfer between ferrocene group and electrode via the sandwich structure. When the ferrocene-modified gold nanoparticles were replaced with ferrocene-monocarboxylic-acid-labeled PCT antibody, the cathodic and anodic current were significantly lower (curve b in figure 1). Such a phenomenon was attributed to the Au nanoparticles which has large specific surface area and can load more ferrocene via the thionine group as an excellent electron mediator accelerates electron transfer between the electrochemical probe and the electrode.

3.2 Optimization of the immunosensor

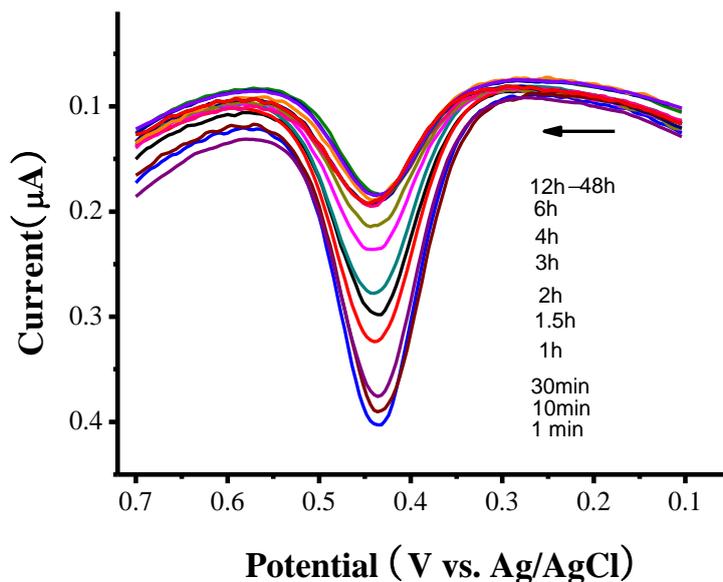


Figure 2. Differential pulse voltammetry of various ratio from 2:1 to 7:1(a-f) of Fc-C₁₁SH and MUA with the potential of 0.3 V.

The catalytic response of the PCT immunosensor was measured via differential pulse voltammetry (DPV) in pH 7.4 PBS. The concentration of Fc-C₁₁SH and MUA-modified Au nanoparticles was considered. As shown in figure 2, when the ratio of Fc-C₁₁SH and MUA was higher than 5:1 the oxidization current of the immunosensor did not increase with increased concentration of Fc-C₁₁SH. When the ratio was higher than 8:1 the current decreased, which was because MUA was too low and the ferrocene-modified Au nanoparticles could not react with PCT antibody, and thus could not form the sandwich structure with the PCT immunosensor. These results suggested that the current increased with increasing proportion of Fc-C₁₁SH and maximum oxidization current was obtained at a Fc-C₁₁SH-to-MUA ratio of 5:1, which indicated that 5:1 was the optimum ratio for ferrocene modified gold nanoparticles.

3.3 Linearity calibration of PCT immunosensor

Under the optimized condition, response of the oxidization current of the PCT immunosensor at potential of 0.3 V was observed by incubation in various PCT concentrations. A range of dilutions was made from the PCT stock solution, from 0.5 pg/mL to 100 ng/mL. Figure 3 shows that the oxidization current went up with increased concentration of PCT. Good linearity was observed between the oxidization current and PCT concentration from 1.5 pg/mL to 50 ng/mL. The calibration line was $I (\mu\text{A}) = 3.62 + 1.15 \times \lg c(\text{PCT}) (\text{mmol/L})$, the correlation coefficient being 0.98. According to the baseline signal variation, the limit of detection was determined as 0.8 pg/mL, which was similar with the latest literature and lower than traditional methods, as follows.

For example, Y. Fang, et al. conducted a research on PCT measurement. Using a special electrode coated by four layers of different nanocomposites, they got a limit of detection of 0.5 pg/mL. Their assay required optimal conditions including exact temperature, incubation time, and acidity[20]. F. Liu, et al. based their PCT assay on graphene-gold nanocomposite film sensor and carbon nanohorns/hollow platinum chains. Their assay yielded a detection limit of 0.43 pg/mL, owing to the complex materials and techniques used[21]. Our method achieved a comparable detection limit with simpler techniques and much less demanding experimental conditions. Such sensitivity is inseparable with the careful design of our antibody sandwich structure, and its optimal introduction to the electrochemical mechanism.

A comparable limit of detection has not been attained by non-electrochemical methods of PCT measurements. A surface plasmon resonance biosensor developed by Sener, Ozgur, et al. detected a lower limit of 9.9 ng/mL [22]. An immunoturbidimetric assay by Dipalo, et al. had a limit of detection of 0.26 ng/mL [23]. The detection limit of a chemiluminescence assay by Qi, et al. was 0.03 ng/mL [24]. Another chemiluminescent immunoassay had sensitivity of 0.025 ng/mL [25].

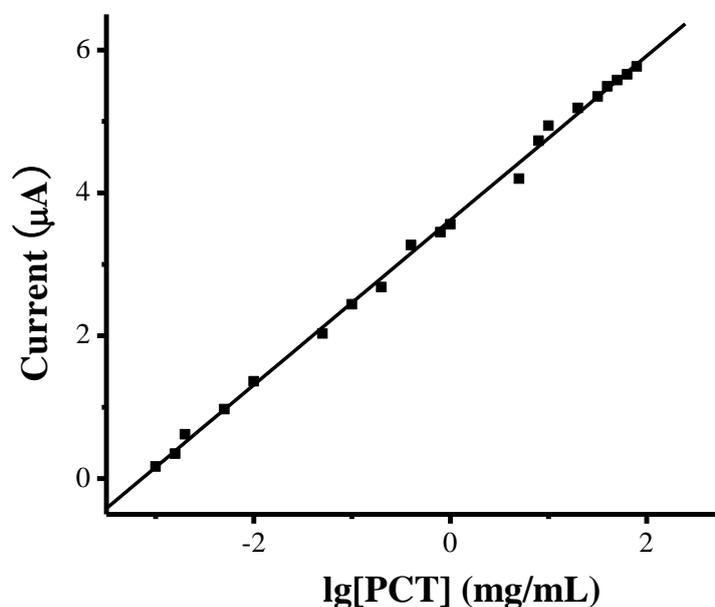


Figure 3. Linearity calibration of PCT immunosensor at various PCT concentrations from 1.5 pg/mL to 50 ng/mL.

3.4 Stability and repeatability of the PCT immunosensor

The stability and repeatability of the PCT immunosensor was estimated by assaying the response of 1.0 ng/mL PCT. As shown in figure 4, after 30-day storage the PCT immunosensor still retained a catalytical oxidization current of 96.4% the initial level (Figure 4), which indicated that the PCT immunosensor had good stability.

The PCT immunosensor electrodes were produced with the same procedure, and after 10 times continuous measurements with the PCT immunosensor, the results revealed the relative coefficient of

variation was 4.03 %, which suggested the PCT immunosensor could be used as a quantitative assay of PCT.

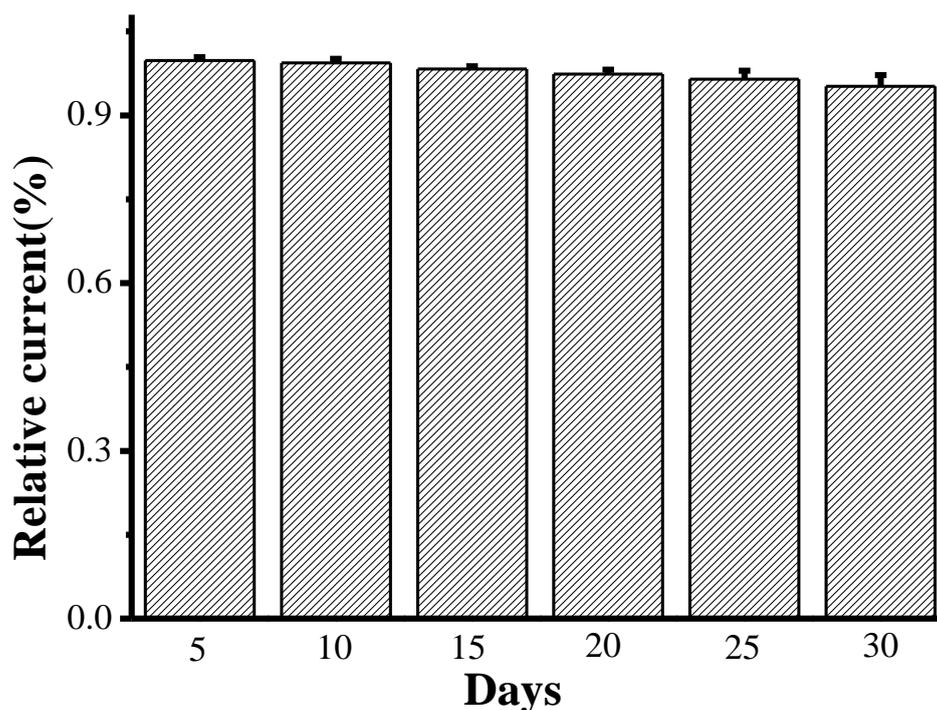


Figure 4. Stability of PCT immunosensor.

3.5 The anti-interference capacity of PCT immunosensor

The immunoassay of PCT is usually liable to interference by some nonspecific or specific interaction proteins, thus a selectivity assay was performed using BSA and AFP. With the potential of PCT immunosensor set at 0.3 V, no obvious signal change of the electrochemical response was observed after adding BSA or AFP. Therefore we concluded the PCT immunosensor had good anti-interference ability.

3.6 Clinical sample measurement

To further investigate the application of the PCT immunosensor, we tested six clinical serum samples with our assay and the reference method. The results revealed the relative deviation range was from -3.33% to 3.94% between the two methods (Table 1). These data showed no significant difference and demonstrated that such a method can be used to determine PCT in serum samples in clinical laboratory.

Table 1. Comparison of serum PCT determinations.

Samples	The PCT immunosensor (ng/mL)	The reference method (ng/mL)	Relative deviation (%)
1	1.32	1.27	3.94
2	2.56	2.49	2.81
3	0.87	0.90	-3.33
4	5.43	5.58	-2.69
5	1.74	1.66	4.82
6	10.21	10.03	-1.79

4. CONCLUSIONS

We developed a new electrochemical immunoassay of procalcitonin (PCT) based on ferrocene-modified gold nanoparticles. Because the gold particles have a large specific surface area and can load more ferrocene via the thionine group, ferrocene-modified gold nanoparticles were employed as an excellent electron mediator which accelerated electron transfer between the electrochemical probe and the electrode. They were labeled with PCT antibodies, which could bind with PCT and gold electrodes modified with other PCT antibodies, forming a sandwich structure. At the optimum proportion of ferrocene-modified gold nanoparticles, the PCT immunosensor exhibited good linear relationship between oxidization current at 0.3 V potential and PCT concentration from 1.5 pg/mL to 50 ng/mL. The detection limit was 0.8 pg/mL. Furthermore, the PCT immunosensor was employed to detect PCT in clinical samples in comparison with enzyme-linked immunosorbent assay (ELISA), and the two methods showed no significant difference, which indicated the PCT immunosensor could be applied to serum sample test in the clinical laboratory.

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