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A label-free Electrochemical Immunosensor Based on Polythionine-nanogold Nanocomposite for Detection of Trypsin Using screen-printed Electrode

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Trypsin is a digestive enzyme specifically expressed in pancreatic exocrine function, and plays a key role in the genesis, development and invasion of pancreatic cancer. In this work, we mainly developed a label-free electrochemical immunosensor based on polythionine-nanogold nanocomposite (PTh-NG) as signal indicator system modifying screen-printed electrode (carbon). In phosphate buffer solution with pH=5.5, the developed label-free electrochemical immunosensor could accurately determine trypsin by differential pulse voltammetry based on the principle of specific capturing target antigen by antigen-antibody immune reaction. PTh-NG nanocomposites were characterized by UV-vis spectrum and transmission electron microscopy. Under the optimal experimental conditions, the linear range of trypsin was 1~200 ng/mL, and the detection limit was 0.58 ng/mL. The label-free electrochemical immunosensor was used to measure trypsin in serum of patients with pancreatic disease, and the relative deviation between the results and ELISA results was less than 8.72%, which was satisfactory, suggesting that it can be used for the determination of trypsin in serum.

Keywords: Label-free electrochemical immunosensor, polythionine-nanogold, nanocomposite, trypsin, screen-printed electrode

1. INTRODUCTION

Pancreatic cancer (PC) is a common gastrointestinal malignancy that has a high fatality rate. Currently, it is difficult to diagnose and treat pancreatic cancer. Since the clinical symptoms of early

pancreatic cancer are not obvious, most patients have been diagnosed with intermediate and advanced pancreatic cancer [1]. The 5-year relative survivals of pancreatic cancer were only 7.2% in China and the lowest level in all cancers [2]. Globally, the incidence of pancreatic cancer is projected to increase to 18.6 per 100000 in 2050, with the average annual growth of 1.1%, meaning that pancreatic cancer will pose a significant public health burden [3]. Considering the high fatality rate of pancreatic cancer, early diagnosis of pancreatic cancer could significantly reduce the fatality rate.

Recent studies have found hot spot mutations of trypsinogen gene (PRSS1) in peripheral blood of patients with pancreatic cancer (P-A121T and P-C139S) [4-5], and trypsin has been proved to affect the occurrence, development and clinical outcome of pancreatic cancer. Trypsin can be involved in the occurrence, invasion and metastasis of pancreatic cancer by degrading extracellular matrix and basement membrane, and the level of trypsin in peripheral blood of 80% of patients with pancreatic cancer is significantly increased [6-10]. The mechanism is as follows: normal pancreatic tissue contains rich trypsinogen storing within the pancreatic acini; 80%~90% of pancreatic cancers are mostly ductal epithelial canceration, in which pancreatic acinus is destroyed, and trypsin is released into peripheral blood [6, 10]. Therefore, abnormal increase of serum trypsin level indicates the risk of pancreatic cancer [10]. The serum level of trypsin in healthy people is relatively low, which provides a good reference for the medical detection of trypsin level. Moreover, trypsin is specifically expressed in the pancreas, so the detection of trypsin has a good specificity for the early diagnosis of pancreatic cancer.

Electrochemical immunosensor is a new type of biosensing technology, which mainly combines antigen-antibody specific reaction (immune response) with electrochemical method with high sensitivity to achieve accurately and sensitively rapid detection of the target [11]. In recent years, the label-free electrochemical immunosensors being developed rapidly has been widely concerned and favored by researchers from all over the world, and can be used to detect different types of antigens. The label-free electrochemical immunosensors generate the signal mainly through the following two ways: (1) The modified electrode generates electrochemical signal in electrolyte solution containing reversible electric pair (such as potassium ferricyanide electric pair), and the response signal difference before and after the antigen-antibody reaction on modified electrode was used to indicate the concentration of the target in the detection solution [12]; (2) Signal indicators (such as thionine, methylene blue, potassium ferricyanide) were used to modify the electrode interface to obtain electrochemical signal in buffer solution, and the concentration of the target was measured by the response signal difference before and after the immune reaction on modified electrode [13-15]. Compared with the traditional "sandwich" electrochemical immunosensors [16], the label-free electrochemical immunosensors has the advantages of high sensitivity, simple operation, fast response, easy miniaturization and low preparation cost.

According to the actual clinical needs for trypsin detection and the advantages of label-free electrochemical immunosensor, this study designed a label-free electrochemical immunosensor using polythionine-nanogold (PTh-NG) nanocomposite as signal indicator system for the accurate determination of trypsin. The design and application process of the electrochemical immunosensor was shown in Figure 1. The design principle of the label-free electrochemical immunosensor was as follows: Firstly, PTh-NG composite was synthesized by chemical method and used to modify screen-printed electrode (carbon), and then gold nanoparticles (AuNPs) synthesized by sodium citrate reduction were dropped onto the surface of the modified electrode; Secondly, the anti-trypsin antibody was chemically

bonded to the surface of the modified electrode, and then bovine serum albumin was used to seal the blank sites of the modified interface to reduce the non-specific adsorption of antigen on the modified interface; After that, immune reaction was used to capture trypsin in the solution, and then the modified electrode was placed into buffer solution to detect the target by DPV. The variable quantity of electrochemical signal before and after the antigen-antibody reaction on PTh-NG modifying electrode interface was proportional to the trypsin concentration in the detection solution in a certain concentration range, which just met the needs of trypsin detection. AuNPs dripping on the modified electrode surface played the following three roles in the immune sensor: (1) AuNPs has good conductivity, promoting the electron transfer between the modified electrode interface and the detection solution [17]; (2) AuNPs has good biocompatibility and can fix the anti-trypsin antibody on the surface of the modified electrode by chemical bonding without affecting the antibody activity [18]; (3) AuNPs are nanomaterials that can increase the surface area of the modified interface and fix more antibodies on the electrode surface [19]. The experimental results showed that the label-free electrochemical immunosensor had high sensitivity and selectivity, and could be used for the detection of clinical serum samples.

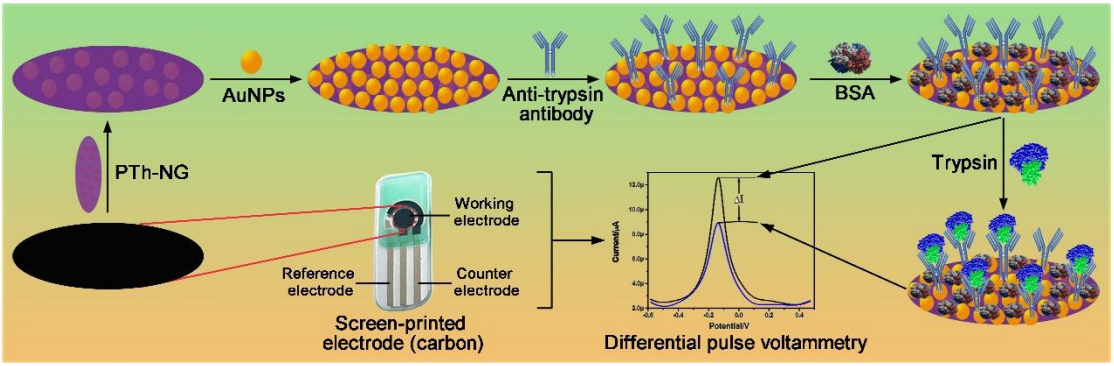

Figure 1. Schematic diagram of preparing label-free electrochemical immunosensor based on polythionine-nanogold nanocomposite for detection of trypsin on screen-printed electrode

2. EXPERIMENTAL

2.1. Reagents and serum samples

Trypsin, lyophilized bovine serum albumin (BSA; 99%), and thionine were purchased from Sigma-Aldrich Company (China). Chloroauric acid ($HAuCl_4$), 30% aqueous solution of hydrogen peroxide (H_2O_2), iron trichloride hexahydrate ($FeCl_3$•$6H_2O$), trisodium citrate dihydrate ($C_6H_5Na_3O_7$•$2H_2O$), disodium hydrogen phosphate dodecahydrate (Na_2HPO_4•$12H_2O$), sodium dihydrogen phosphate dihydrate (NaH_2PO_4•$2H_2O$), sodium chloride (NaCl), potassium ferricyanide ($K_3[Fe(CN)_6]$) and potassium ferrocyanide trihydrate ($K_4[Fe(CN)_6]$•$3H_2O$) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Anti-trypsin rabbit polyclonal antibody (Ab) was purchased from Shanghai Sangon Biotechnology Co., Ltd. (Shanghai, China).

A mixture of Na_2HPO_4•$12H_2O$ (14.5046 g), NaH_2PO_4•$2H_2O$ (1.4821 g) and NaCl (2.922 g) in 500 mL of ultrapure water was use prepared phosphate buffered saline (PBS) with pH of 7.4 or 5.5. The pH values were adjusted with HCl or NaOH solutions. PBS with pH of 7.4 was used as the diluents and reaction solution of antibody and trypsin. PBS with pH of 5.5 was used as the detection solution.

A mixture of $K_3[Fe(CN)_6]$ (0.1646 g), $K_4[Fe(CN)_6]$•$3H_2O$ (0.2112 g) and KCl (0.3728 g) in 50 mL of ultrapure water was use prepared 10 mmol/L $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 mol/L KCl. $[Fe(CN)_6]^{3-/4-}$ solution was used for impedance measurements.

Human Trypsin ELISA Kit was purchased from Xiamen Promont Biotechnology Co., LTD (authorized dealer of Abcam in Fujian Province). Human serum trypsin was detected by the ELISA kit according to the Assay Procedure. After obtaining informed consent, clinical human serum samples were collected from Affiliated Hospital of Putian University as real samples. All of the other reagents used in this study were analytical grade and used as received without any purification.

2.2. Preparation of gold nanoparticles (AuNPs) [20]

The glass instrument used in the preparation of AuNPs was soaked in the newly configured aqua regia for 30 min, and then was thoroughly cleaned with double steam water. The volume ratio of HCl to HNO_3 in aqua regia is 3:1. After drying, 250 mL of $HAuCl_4$ solution (0.01%) was added to 500 mL cleaned round bottom flask. After boiling, 4 mL of sodium citrate solution (1%) was added quickly. The color of the solution in the round bottom flask gradually changed from yellow to wine red. Under the condition of continuous agitation, the solution was boiled for 10 min to obtain AuNPs sol liquid with particle size of about 13 nm. The as-prepared AuNPs sol liquid should be stored at 4 ℃ when not in use.

2.3. Preparation of polythionine-nanogold (PTh-NG) composite nanomaterial [21]

Firstly, 50 mL double distilled water and 0.03 g solid $FeCl_3$ were successively added into a 250 mL round-bottom flask and heated to 50 ℃ with continuous agitation. And then, 0.08 g thionine monomer, 2 mL $HAuCl_4$ solution (1%) and 200 μL H_2O_2 solution (30%) were successively added to react for 24 h with continuous agitation. After that, the liquid in the round-bottom flask was centrifuged 5000 RPM for 20 min to remove the supernatant, and then the precipitation was washed with HCl solution (0.1 mol/L) and centrifuged to remove the supernatant, which was repeated 3 times to remove $FeCl_3$ adsorbed on the polymer surface. Finally, dry PTh-NG composite nanomaterial was obtained in a vacuum drying oven at 40 ℃.

In order to explore the effect of the drying degree of PTh-NG nanocomposite on their own electrochemical signals, the nanocomposite were dried for different times, including 15, 18, 21, 24, 27, and 30 h, and then used for subsequent experimental studies.

2.4. Preparation of label-free electrochemical immunosensor based on PTh-NG signal system

The 0.01 g of dried PTh-NG was dissolved in 1 mL of DMF and treated with ultrasound for 10 min to obtain PTh-NG dispersion solution with a concentration of 10 mg/mL. A 4 μL of the above dispersion solution was dropped on the surface of working electrode in SPE and dried at room temperature for 4 h to obtain PTh-NG modified SPE, namely PTh-NG/SPE. And then 5 μL of AuNPs solution was dropped on the surface of the modified electrode and placed overnight at room temperature to obtain Au/PTh-NG/SPE. After that, 8 μL of anti-trypsin antibody solution (100 ng/mL) was added onto the Au/PTh-NG/SPE surface and incubated at 37 ℃ for 2 h, which is sufficient amount of anti-trypsin antibody was fixed on the surface of the modified electrode. The modified electrode surface was successively rinsed with PBST and PBS to remove free antibodies on the electrode surface, and nitrogen gently blew away the residual liquid on the electrode surface to obtain Antibody/Au/PTh-NG/SPE. Then, 100 μL of BSA solution (0.1%) was added onto the surface of the modified electrode, and incubated at 37 ℃ for 2 h. The blank sites of unfixed antibodies on the interface of the modified electrode were closed, which reduced the non-specific adsorption of target antigen on the interface of the modified electrode. Once again, the modified electrode surface was successively rinsed with PBST and PBS to remove the free BSA, and nitrogen gently blew away the residual liquid on the electrode surface to obtain BSA/Antibody/Au/PTh-NG/SPE. Finally, the target antigen solution was dropped onto the surface of the closed modified electrode with reacting for 1 h at 37 ℃ for electrochemical detection.

2.5. Electrochemical measurements

The electrochemical data of the as-prepared label-free electrochemical immunosensor in this study were carried out at CHI 660C electrochemical workstation (Shanghai ChenHua Instruments Co., China). Differential pulse voltammetry (DPV) was measured in PBS with pH of 5.5, and was used to detect trypsin. The parameters of DPV were an initial potential of -0.5 V, a stop potential of 0.4 V, a potential increment of 0.004 V, an amplitude of 0.05 V, a pulse width of 0.05 s, a sampling width of 0.0167 s, a pulse period of 0.2 s and a stationary time of 2 s. Electrochemical impedance spectroscopy (EIS) was measured in 2.5 mmol/L $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 mol/L KCl, and the parameters were a frequency range of 0.1−100 kHz with applied voltage of 0.2 V and an amplitude of 5 mV.

3. RESULTS AND DISCUSSION

3.1. UV-vis absorption spectrum of PTh-NG, thionine and DMF

UV-vis absorption spectrum of PTh-NG, thionine and DMF was shown in Figure 2. DMF as a solvent, its UV-vis absorption spectrum was shown in curve a. Curve b was UV-vis absorption spectrum of thionine dissolved in DMF, and there were three obvious absorption peaks at 267 nm, 508 nm and 607 nm, and obvious shoulder peak at about 317 nm. UV-vis absorption spectrum of PTh-NG dissolved in DMF was shown as curve c, and there were an obvious absorption peak at 285 nm and a blue shifted absorption peak at 264 nm compared to that at 267 nm of thionine. In curve c, a shoulder peaks at about

365 nm and an absorption peak at 611 nm showed red shift, which may be the reason that the large conjugated structure of PTh-NG makes n-π* electron transition in the amine ring relatively easy. The absorption peak at 508 nm in thionine disappeared in PTh-NG. By comparison, the π-π* absorption at 267 nm in thionine was blue shifted to 264 nm in PTh-NG, and the absorption peak at 611 nm was a polymer spin bipolar absorption. These characteristics of UV-vis absorption indicated that PTh-NG nanocomposites were successfully prepared and exhibited the properties of conductive polymers.

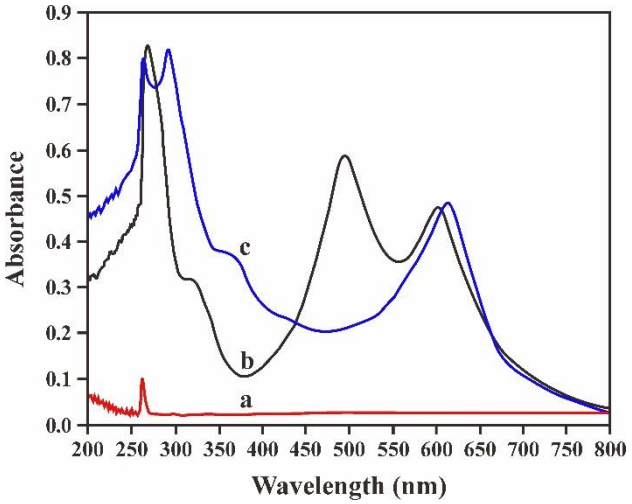

Figure 2. UV-vis absorption spectrum of DMF (a), thionine (b) and PTh-NG (c)

3.2. TEM characterization of PTh and PTh-NG

TEM characterization of PTh and PTh-NG was shown in Figure 3. PTh in Figure 3A showed an incomplete sheet structure, lacking obvious integrity. PTh-NG in Figure 3B not only showed a complete sheet structure, but also gold nanoparticles with uniform particle size embedded into its interior. The particle size of gold nanoparticles was about 80 nm. In addition, it can be clearly seen from Figure 3B that the gold nanoparticles were wrapped in polymers, and were evenly distributed, which was conducive to electron transfer inside PTh-NG. The results showed that PTh-NG nanocomposite formed the structure of PTh-wrapped gold nanoparticles.

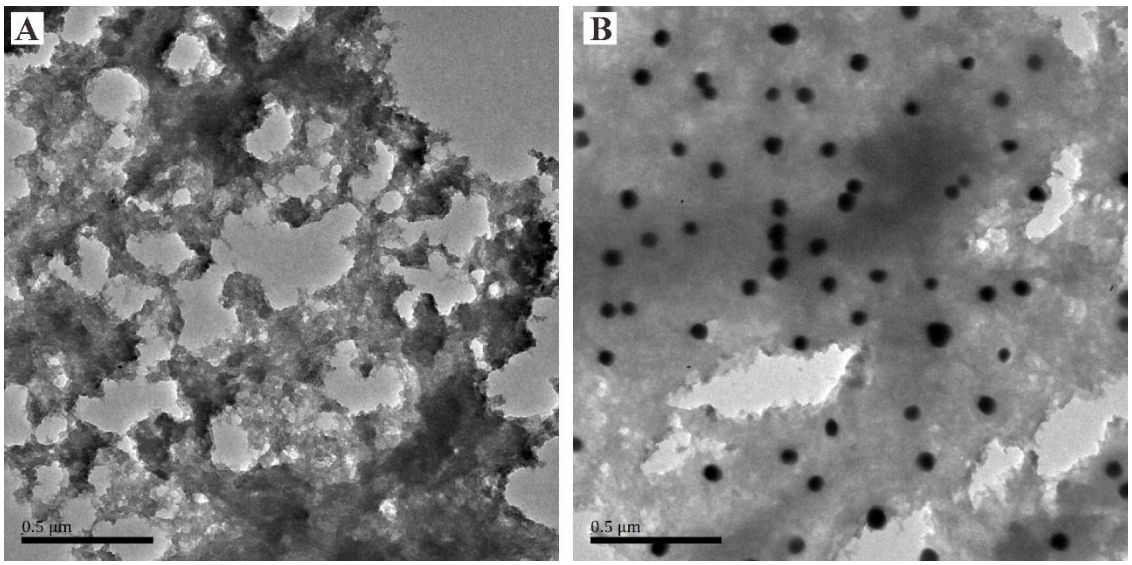

Figure 3. TEM images of PTh (A) and PTh-NG (B)

3.3. Effect of drying time of PTh and PTh-NG on intensity of DPV peak current

The DPV peak current of PTh and PTh-NG on SPE surface was related to the drying time. As shown in Figure 4, within the range of 15-30 h, with the extension of drying time of PTh and PTh-NG, the peak current intensity of DPV first showed an increasing trend. When the drying time was extended to 21 h, the peak current value reached the maximum. After the drying time was further extended, the peak current decreased gradually.

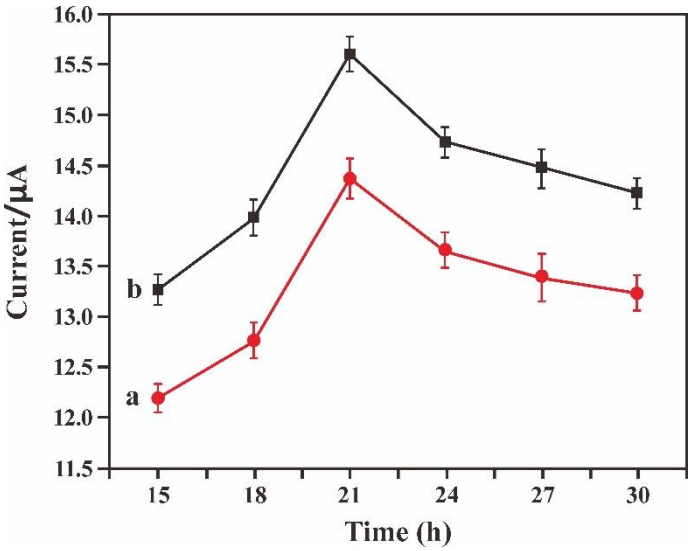

Figure 4. Broken line graph of DPV peak current of PTh (a) and PTh-NG (b) with different drying time on SPE surface; the error bars were relative standard deviation of three parallel experiments.

The reason might be due to the decrease of water content in PTh and PTh-NG with the extension of drying time, which enhanced the conductivity of the polymer and increased the peak current value of

DPV on the electrode surface. After the drying time was further prolonged, the structure of PTh and PTh-NG might be affected to weaken the conductivity of the polymer, and the peak current value of DPV on the electrode surface was decreased. It can be seen from Figure 4 that the peak current value of DPV generated by PTh-NG (curve b) was always larger than that generated by PTh (curve a). The reason should be that gold nanoparticles in PTh-NG can promote electron transfer inside the polymer, enhance the conductivity of the polymer, and generate a larger peak current of DPV on the electrode surface.

3.4. Electrochemical characterization (electrochemical impedance spectroscopy) of label-free electrochemical immunosensor

The preparation process of label-free electrochemical immunosensor based on SPE was characterized by electrochemical impedance spectroscopy (EIS). The changes of electron transfer resistance (Ret) on bare SPE, PTh-NG/SPE, Au/PTh-NG/SPE, Antibody/Au/PTh-NG/SPE, BSA/Antibody/Au/PTh-NG/SPE and Trypsin/BSA/Antibody/Au/PTh-NG/SPE were detected in 10 mmol/L $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 mol/L KCl, as shown in Figure 5. The impedance diagram of bare SPE in $[Fe(CN)_6]^{3-/4-}$ solution was curve a, and almost a straight line, indicating that the electron transfer speed was fast on the surface of bare SPE. After bare SPE surface was modified with PTh-NG, the impedance curve b of PTh-NG/SPE showed a significant increase in Ret, and the reason was mainly that PTh-NG modifying on SPE surface prevented $[Fe(CN)_6]^{3-/4-}$ from reaching the electrode surface for redox reaction and restricted electron transfer on the surface of the modified electrode. After PTh-NG/SPE was further modified with AuNPs, the impedance curve c of Au/PTh-NG/SPE was obtained, and the Ret value increased slightly, that was because AuNPs on the electrode surface further hindered electron transfer on the modified electrode surface. After immobilizing antitrypsin antibodies on Au/PTh-NG/SPE surfaces, the impedance curve d of Antibody/Au/PTh-NG/SPE showed a significant increase in Ret, that was because the negative charge of the antibody immobilized on the modified electrode prevented $[Fe(CN)_6]^{3-/4-}$ from reaching the modified electrode for redox reaction and restricted electron transport across the modified electrode. After Antibody/Au/PTh-NG/SPE was sealed with 0.5% BSA, BSA attached to the surface of BSA/Antibody/Au/PTh-NG/SPE was a non-conductive protein molecule that impeded electron transfer on the electrode surface, increasing Ret, as showed curve e. After trypsin reacted with the antibodies on the surface of BSA/Antibody/Au/PTh-NG/SPE, trypsin/BSA/Antibody/Au/PTh-NG/SPE was obtained, and its impedance curve f showed a significant increase in Ret, that was because trypsin captured on the surface of the modified electrode had a large amount of negative charge to further repel $[Fe(CN)_6]^{3-/4-}$ and impede redox reaction of $[Fe(CN)_6]^{3-/4-}$ on the surface of the electrode, resulting in a significant increase in Ret. The above experimental results indicated that the preparation process of the label-free electrochemical immunosensor could be carried out successfully.

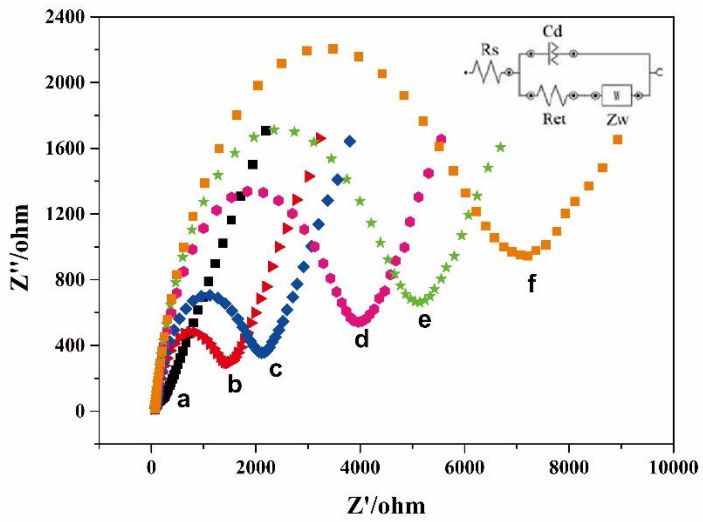

Figure 5. EIS of bare SPE (a), PTh-NG/SPE (b), Au/PTh-NG/SPE (c), Antibody/Au/PTh-NG/SPE (d), BSA/Antibody/Au/PTh-NG/SPE (e) and Trypsin/BSA/Antibody/Au/PTh-NG/SPE (f) were detected in 10 mmol/L $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 mol/L KCl; the inset was the equivalent circuit diagram.

3.5. Electrochemical characterization (differential pulse voltammetry) of label-free electrochemical immunosensor

In this study, differential pulse voltammetry (DPV) was performed to characterize the preparation process of label-free electrochemical immunosensor, as shown in Figure 6. The bare SPE without any modification was measured in PBS with pH of 5.5 and had no any peak current (curve a). After bare SPE was successively modified with PTh-NPs and AuNPs, obtained PTh-NG/SPE and Au/PTh-NG/SPE had a significant peak current at -0.120 V in PBS with pH of 5.5 (curve b and c), suggesting that PTh-NG and AuNPs could be successfully modified on SPE. AuNPs only slightly reduced the peak current of Au/PTh-NG/SPE. After antibodies were immobilized on Au/PTh-NG/SPE, the peak current of Antibody/Au/PTh-NG/SPE decreased in PBS with pH of 5.5 (curve d), but its value was still high up to about 15 μA. Next, BSA modified Antibody/Au/PTh-NG/SPE to obtain BSA/Antibody/Au/PTh-NG/SPE, and there was a slightly reduced peak current in PBS with pH of 5.5 (curve e). BSA was mainly used to occupy blank sites of unimmobilized antibodies on the modified electrode surface, which could avoid the non-specific adsorption of detecting target protein and reduce the occurrence of false positive results. And then, the target antigen (trypsin) reacted with the antibody on BSA/Antibody/Au/PTh-NG/SPE by antigen-antibody immune response to obtain Trypsin/BSA/Antibody/Au/PTh-NG/SPE, and the peak current obviously reduced (curve f), indicating that the combination of trypsin and antibody was successful on modified electrode. The peak current of DPV curves from b to f gradually decreased, the reason was that each modification affected the transfer of electrons across the surface of SPE. After antigen-antibody immune response, the non-conductive trypsin impeding electron transfer on the modified electrode resulted in an obviously decrease of peak current in DPV, similar to that reported in the literature [22]. At the same time, the changing value of peak curren between curve e and f could be used to indicate the concentration of antigen.

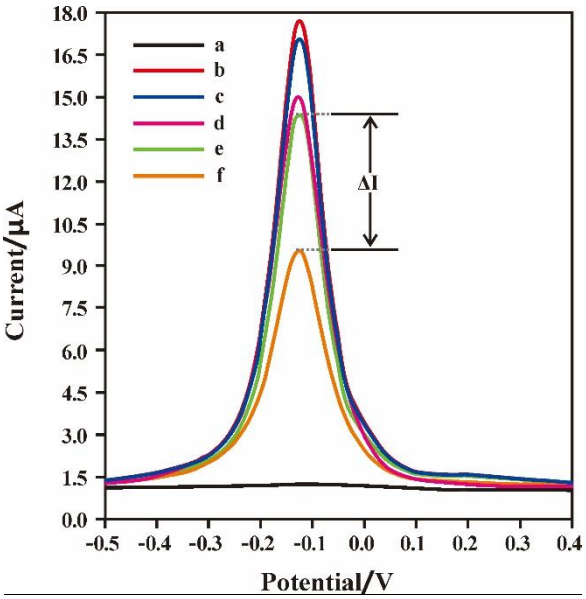

Figure 6. DPV curves of bare SPE (a), PTh-NG/SPE (b), Au/PTh-NG/SPE (c), Antibody/Au/PTh-NG/SPE (d), BSA/Antibody/Au/PTh-NG/SPE (e) and Trypsin/BSA/Antibody/Au/PTh-NG/SPE (f) were measured in PBS with pH of 5.5.

3.6. Specificity of label-free electrochemical immunosensor

In this study, the specificity of label-free electrochemical immunosensor was investigated by detecting common typical protein antigens and digestive enzymes in serum, including carbohydrate antigen 19-9 (CA19-9), alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), amylase (AMS), chymotrypsin (CTP), pepsinogen 1 (PG 1), and pepsinogen 2 (PG 2). Under the same experimental conditions, the as-prepared immunosensor was used to detect the above six typical serum markers at a concentration of 1 ng/mL, and the results were compared with that of trypsin at a concentration of 1 ng/mL. As shown in Figure 7, the determination of the above typical markers in serum at the concentration of 1 ng/mL were similar to those of the blank test, and much smaller than the determination of trypsin at the same concentration. In addition, the six markers in serum were mixed with trypsin, and the detecting results were still similar to those of trypsin alone, suggesting that the as-prepared label-free electrochemical immunosensor had good specificity for trypsin under the same experimental conditions.

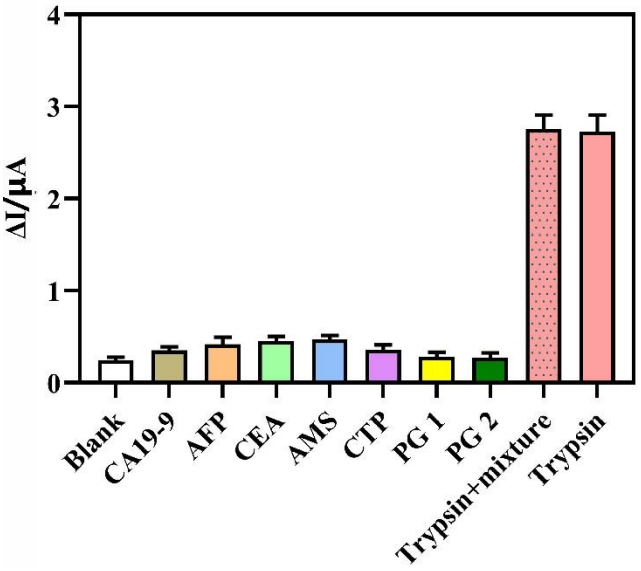

Figure 7. Histogram of detecting result of six clinical common typical serum markers (including CA19-9, AFP, CEA, AMS, CTP, PG 1 and PG 2), trypsin+mixture and trypsin; Concentrations were all 1 ng/mL. The error bars were relative standard deviation of six parallel experiments.

3.7. Quantitative determination of trypsin at different concentrations by label-free electrochemical immunosensor

Under the optimal experimental conditions, a series of different concentrations of trypsin were determined by the as-prepared label-free electrochemical immunosensor, and the concentrations were 1, 2, 5, 8, 10, 20, 50, 80, 100 and 200 ng/mL, respectively. As shown in Figure 8A, the peak current value of DPV curves decreased gradually with the increase of trypsin concentration.

In the range from 1 to 200 ng/mL, as shown in Figure 8B, the variation (ΔI) of DPV peak current had a good linear relationship with the logarithm of trypsin concentration. The corresponding linear equation of calibration curve was ΔI (μA)=1.86logC(ng/mL)+0.395. The correlation coefficient R^2 was 0.979, and the limit of detection (LOD) was 0.58 ng/mL.

The label-free electrochemical immunosensor was used to measure trypsin in human serum, and the results were compared with those determined by ELISA, as shown in Table 1. Six serum specimens were collected from patients with different pancreatic diseases from Affiliated Hospital of Putian University. The absolute deviation of the two methods was less than 8.72%, which was similar to that of ELISA. Therefore, the label-free electrochemical immunosensor can be used for the analysis and determination of trypsin content in serum.

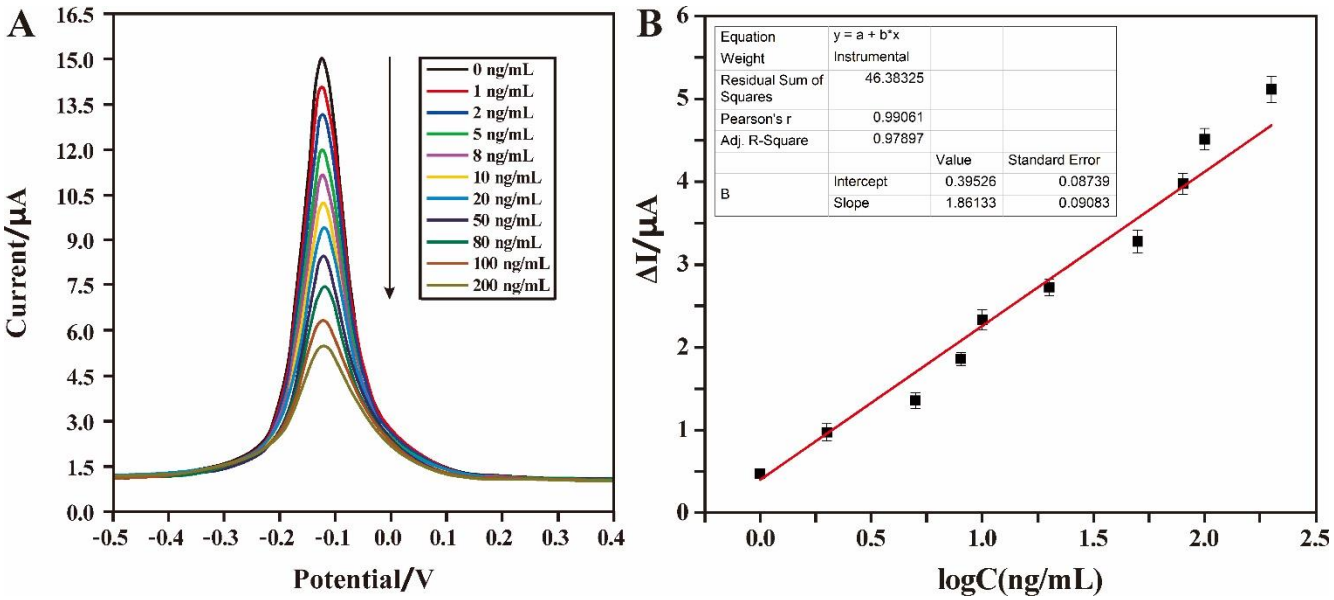

Figure 8. (A) DPV curves of different concentrations of trypsin; the concentrations of trypsin were successively 0, 1, 2, 5, 8, 10, 20, 50, 80, 100 and 200 ng/mL from top to bottom. (B) Calibration curve between logarithms of trypsin concentrations and variation of DPV peak current; the error bars were relative standard deviation of three parallel experiments.

Table 1. Comparison of detecting results between as-prepared label-free electrochemical immunosensor and ELISA.

Number★	Found by the immunosensor# (ng/mL)	Found by the ELISA# (ng/mL)	Relative deviation (%)
1	6.94	7.51	8.21
2	25.09	26.75	6.62
3	36.37	39.54	8.72
4	82.55	75.78	-8.20
5	134.33	124.43	-7.37
6	164.32	175.81	6.99

★: 1-3 were pancreatitis; 4-6 were pancreatic cancer.

#: The data showed the average value of three independent determinations.

3.8. Comparison between the as-prepared label-free electrochemical immunosensor and the reported detection method of trypsin

A variety of electrochemical technologies for detecting trypsin have been reported in the literatures, mainly including electrochemical sensing technology based on nanomaterial signal system and electrochemical detection technology dependent on solution signal system [23]. Therefore, we performed a detailed comparison of reported techniques of detecting trypsin with our work, as shown in Table 2. We can clearly find that each detection for trypsin had its own characteristics and advantages.

However, the best method to detect trypsin is to meet the detection requirements of actual samples. According to the current development trend of biochemical detection instruments, the detection of trypsin is mainly carried out on a screen printing electrode in this work, so as to miniaturize the detection of trypsin, lay a research foundation for the development of a portable instrument for the detection of trypsin, and also open a new starting point for pancreatic cancer screening. The biggest advantage of screen printing electrode is that the sample detection is carried out on a small platform, so this work also provides research experience for the development of miniaturized protein detector.

Table 2. Comparison of electrochemical biosensor systems for detecting trypsin.

Signal substance	Auxiliary substance	Biosensing platform	Linearity range	LOD	Clinical samples	Ref.
Polythionine-Au nanocomposites	Au nanoparticles	Glassy carbon electrode	20~20 000 pg/mL	1.2 pg/mL	Human serum	[21]
Thionine (Thi)	Peptide substrates/Au nanoparticles/DNA	Gold electrode	0.005~0.15 μg/mL	1.8 ng/mL	No	[24]
$[Fe(CN)_6]^{3-/4-}$	Electropolymerized poly(o-phenylenediamine)	Gold-coated quartz crystal electrode	0.24~48 μg/mL	0.07 μg/mL	Human serum	[25]
$[Fe(CN)_6]^{3-/4-}$	Nickel oxide nanoparticles/molecularly imprinted polymers	Glassy carbon electrode	1~90 pg/mL	0.75 pg/mL	Human serum/urine	[26]
$[Fe(CN)_6]^{3-/4-}$	Nitrogen-doped carbon nanoboxes shelled with nanoporous copper hydroxide	Glassy carbon electrode	10~80 pg/mL and 10-80 ng/mL	3 pg/mL	Human serum/urine	[27]
Polyethylenimine-sensitized TiO_2 mesocrystal	Boron-doped carbon quantum dots labeled peptide	Glassy carbon electrode	1×10^{-7}~1.0 mg/mL	0.32×10^{-7} mg/mL	Human serum	[28]
Copper oxide nanoparticles (CuO NPs)	Molecularly imprinted polymer	Glassy carbon electrode	0.0005~0.5 μg/mL	No	Fetal bovine serum	[29]
$[Ru(NH_3)_6]^{3+}$	Heptapeptide (CRRRRRR)	Gold electrode	0.0047~0.052 U/mL	0.0012 U/mL	No	[30]
Thionine-loaded graphene oxide	Multi-functional substrate peptide (HHHAKSSATGGC-HS)	Gold electrode	1×10^{-4}~1 U	3.3×10^{-5} U	No	[31]
Polythionine-nanogold nanocomposite	Gold nanoparticles	Screen-printed electrode (carbon)	1~200 ng/mL	0.58 ng/mL	Human serum	This work

4. CONCLUSION

In this study, a label-free electrochemical immunosensor based on PTh-NG as signal system was developed for the detection of trypsin. The preparation process of the immunosensor was simple and feasible, and easier to operate than traditional "sandwich" electrochemical immunosensor. In the concentration range of 1~200 ng/ml, the immunosensor showed good linearity and high sensitivity for

the detection of trypsin, and could be used to detect trypsin in serum of patients with pancreatic disease. The results were similar to those of ELISA, and the absolute deviation was less than 8.72%, which was satisfactory. The experimental results showed that the label-free electrochemical immunosensor had high accuracy and good practicability, and could be used for the detection of trypsin in clinical serum specimens under certain conditions.

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CONFLICT-OF-INTEREST STATEMENT
All the authors in this paper declare there is no conflict of interest.

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