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Electrochemical Determination of Cancer and Cardiovascular Biomarkers Based on Advanced Screen-printed Immunosensors

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Screen-printed electrodes (SPE), previously widely used in the design of disposable sensors, has recently attracted considerable interest for the application in electrochemical immunosensors for field-clinical analysis. For the sake of increasing the sensitivity of SPE for the determination of cardiac troponin T (cTnT) which is a specific biomarker for the acute myocardial infarction diagnosis, graphene was incorporated to the SPEs in this study. Differential pulse voltammetry (DPV) with ferrocyanide/ferricyanide redox probe was employed for the determination of cTnT after the electrode was incubated in the cTnT solution. The current response was related linearly with the concentration of cTnT ranging from 0.01 ng/mL to 1 ng/mL with correlation coefficient of 0.995. The obtained limit of detection (LOD) of 0.005 ng/mL was lower than previously reported immunosensors and comparable with conventional analytical techniques. The proposed immunosensor exhibited high sensitivity, good reproducibility, high stability and clinical range, demonstrating the potential application in point-of-care diagnose of acute myocardial infarction.

Keywords: Electrochemistry; Immunosensor; SPE; cTnT; Graphene

1. INTRODUCTION

In the past few years, point-of-care (POC) systems have gained intense attention owing to the requirement of decentralization of analytical operations stimulated by time and money saving [1-3]. POC testing is an ancillary technology with representative characteristics including near-patient, remote and decentralized [4]. The most attractive advantage of POC testing is that it can be performed near the site of the patient, in other words, it can be used even at home. In addition, the POC testing can also be successfully applied in resource-limited areas [5].

In order to realize the POC testing, a miniaturized and inexpensive device with fast, reliable and sensitive selective detection capacity is highly demanded. Electrochemical immunosensors (EIs) have proven to be a promising instrument owing to its low cost, high sensitivity and most importantly the potential of automatization and miniaturization. As one type of biosensor, an immunosensor is generally composed of an antibody or antigen specie interconnected to a signal transducer, through which the binding of complementary species can be detected [6].

As an important technique that has achieved widespread use in electronics, screen-printing technology can also be employed for the production of solid and planar electrodes with reliability and low cost in large quantities. Besides, it is also found to be a promising technique for on-site monitoring [7, 8]. The fabrication of screen-printed electrodes (SPEs) includes a large number of basic steps such as the selection of inks, substrate and screen that will affect the size and geometry of SPE, printing, drying and curing. Specifically speaking, ink firstly permeates through the screen, and then it was deposited onto a substrate. SPE can be obtained through layer-by-layer deposition of ink sequentially. The thickness of film on the final electrode can be controlled by times of deposition [9, 10]. SPEs can be easily modified to achieve versatile properties, which give the electrode the capacity to meet diverse applications.

Cardiovascular diseases (CVDs) are the general term of a series of diseases such as congenital heart disease, peripheral arterial disease, pulmonary embolism, coronary heart disease and rheumatic heart disease featuring the disorder of blood vessels and heart. On the basis of World Health Organization (WHO), about 31 % (17.5 million) of all global deaths in 2012 were found to be caused by CVDs, making CVDs the leading cause human death in both developed and developing countries [11]. Various factors including genetic, hypertension, obesity, age, cholesterol and stress can lead to the incidence of CVDs [12, 13]. Early detection and diagnosis of CVDs is of great importance for both life rescue and time-saving in patient treatment [14]. As to the diagnosis of acute myocardial infarction (AMI), human cardiac troponin T (cTnT) is considered to be the gold standard marker of cardiac injury owing to its specific and sensitive. In the early stage of cardiac disease, the detection of cTnT at ultra-low concentration counts a great deal for the prognosis and classification of the heart damage. The concentration of cTnT in blood increases rapidly within 3-4 h upon the onset of AMI. The well-established enzymatic immunoassay that is based on the reaction between chemiluminescent substrates and cTnT can be employed for the determination of cTnT in laboratory [15-18]. In comparision with other determination methods for cTnT, point-of-care testing is a more promising analytical tool to reduce assay time. Therefore, immunosensor with immunoassay is a simple and effective approach for the detection of cTnT in the emergency departments.

In this study, an immunosensor was constructed by immobilizating antibodies on the graphene modified SPE was developed. The proposed sensor can detect cTnT rapidly owing to the electrochemical activity of the graphene. No labels were required during the measurement of antigen-antibody interactions with DPV. The successful determination of cTnT by immunosensor demonstrates the promising potential of SPEs in the application of POC testing.

2. EXPERIMENTAL

2.1. Chemical

Native cTnT extracted from human cardiac muscle tissue was supplied by Chenxinbio. Graphene powder was supplied by MoxiTech. Potassium ferrocyanide ($K_4[Fe(CN)_6]$), Potassium ferricyanide ($K_3[Fe(CN)_6]$), dimethylformamide (DMF), glycine, *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide (EDC), mouse monoclonal anti-cTnT and *N*-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. Phosphate-buffered saline (PBS) (10 mM) with pH of 7.4 was synthesized by completely dissolving NaCl (8.0 g), Na_2HPO_4 (1.44 g), KCl (0.2 g) and KH_2PO_4 (0.24 g) in 1 L of deionized Milli-*Q* water. All chemicals were analytical reagents.

Blood serum samples were collected from patients that suffer from AMI in Shenyang People Hostiptal. Samples were stored at -20 °C when not in use. The determination of cTnT was performed via electrochemical chemiluminescence immunoassay (ECLIA) on the automatic Elecsys 2010 Immunoassay Analyzer (Roche Diagnostics).

2.2. Screen-printed electrode fabrication

The screen-printing ink was prepared based on the following steps. Firstly, cellulose acetate (0.02 g) was added into the solvent mixture of acetone (1 mL) and cyclohexanone (1 mL), and the obtained solution was treated with sonication for 10 min to make cellulose acetate completely dissolved. Ionic liquid n-butylpyridinium hexafluorophosphate (0.20 g) and graphene powder (0.78 g) was added into the solution in order. Finally, the homogeneous and viscous ink was successfully prepared by dealing with the obtained composite under ultrasonic sound for 30 min. A manual screen printer (MT-45A, Ming Tai Screen Printing Machine Co., Ltd.,) was employed for the printed process. Prior to the printing, the poly(vinyl chloride) (PVC) substrate was rinsed with ethanol and deionized Milli-*Q* water, and then dried at 25 ^oC. The ink was printed onto the PVC substrate through screen mesh with size of 200 μm by a squeegee. Subsequently, the obtained electrode was heated at 70 °C for 30 min in order to evaporate the solvents. At last, the surface of carbon film was decorated with an insulating layer and irradiation with 254 nm ultraviolet was used to solidify the layer. The electrode composed of commercial conductive graphite ink (denoted as SPE*) was also synthesized for comparision. The prepared SPEs were cut into 4 mm × 2 mm strip with working area 0.08 cm^2. The size of connecting strip was 8 mm × 4 mm.

2.3. Preparation of electrochemical immunosensor

In order to activate the carboxylic groups, anti-cTnT antibodies (10 μg/mL) were firstly treated in PBS (10 mM, pH 7.4) that composed of NHS (10 mM) and EDC (5 mM) for 90 min. Subsequently, the immobilization of activated anti-cTnT antibodies on SPE was carried out as follows: 5 μL of activated anti-cTnT antibodies was pipetted onto the SPE and incubated in a moist chamber at 25 ^oC

for 1h, and then the PBS (10 mM, pH 7.4) composed of glycine (50 mM) was added to the surface of SPE for 30 min in order to block non-specific bindings.

2.4. Electrochemical immunoassay

DPV was applied for real-time monitoring of the Antigen-antibody interactions on the surface of SPE. The current signal in DPV measurements were recorded with potential varying from 0 V to 0.8 V. Obviously, the difference between the peak current (ΔI) obtained on SPE with or without cTnT was exactly the analytical response of cTnT. It was found that the current signals were occurred at unchangeable potential of +0.25 V.

The performance of as-prepared immunosensor was investigated by cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS). The potential range involved in CVs were from 1.0 V to -0.6 V with scan rate of 50 mV/s. The frequency range from 1×10^{-2} Hz to 6.5×10^{4} Hz was used in AC impedance measurements with amplitude of open circuit voltage being 10 mV. $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (5 mM) in PBS (10 mM, pH 7.4) was used as the probe in the entire experiments.

3. RESULT AND DISCUSSION

As can be seen from the obtained cyclic voltammetry curves (Fig. 1A), a ΔEp of 0.52 V obtained on SPE was higher than that obtained on graphene modified SPE (0.48 V), suggesting the decrease of electron transfer resistance with the incorporation of graphene into electrode. In contrast, the redox peaks decreased with the join of anti-cTnT to SPE, which could be ascribed to the hindrance of electron-transfer by antibody with insulating property. Moreover, the redox peak was found to be slightly reduced after the adhibition of negatively charged glycine, demonstrating that the electron transfer between electrode surface and anionic species in electrolyte was further hindered by glycine through blocking non-specific binding.

Electrochemical impedance spectroscopy (EIS) was also employed for evaluate the performance of SPE electrode toward the reduction of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (5 mM) in an electrochemical cell. As shown by the Nyquist plot (Fig. 1B), charge-transfer resistance (*Rct*) calculated from the diameter of semicircle decreased from 4850 Ω to 2235 Ω after the incorporation of graphene to SPE. Besides, *Rct* increased with the addition of anti-cTnTs owing to its insulating property. In other words, the successful immobilization of anti-cTnTs was confirmed with increased *Rct*. Similarily, the observed increase of *Rct* to 2957 Ω also confirmed the successful decoration of the blocking agent on the electrode surface. All results obtained with EIS were in consistent with that obtained with CV. For the sake of investigating the electron diffusion at the sensor interface, voltammograms obtained on glycine/anti-cTnT/graphene/SPEs with different scan rates were recorded. Both anodic peak current (*Ipa*) and cathodic peak (*Ipc*) current related linearly with the square root of

scan rate, indicating that the electrochemical reaction was diffusion-controlled. According to Randles–Sevcik equation, the calculated electroactive surface area of glycine/anti-cTnT/SPE was 0.051 cm^2.

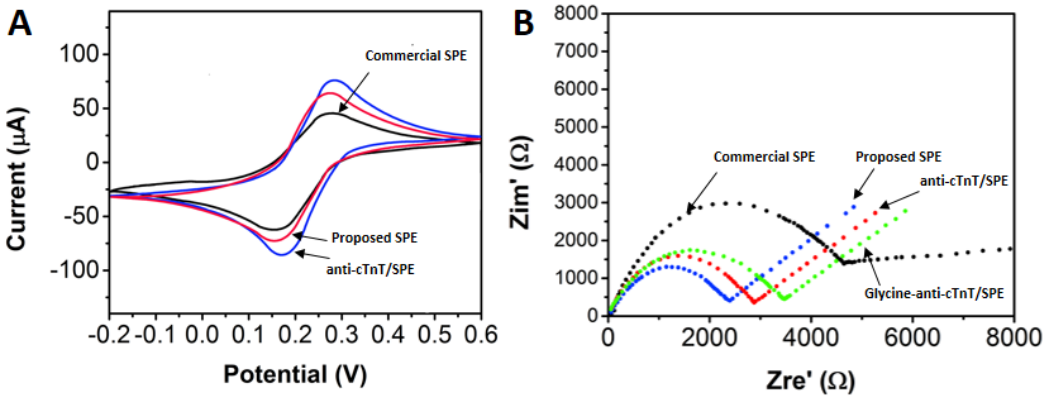

Figure 1. (A) CVs and (B) Nyquist plots toward the electrochemical reaction of $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ that obtained on SPEs, graphene/SPEs, anti-cTnT/graphene/SPEs and glycine/anti-cTnT/graphene/SPEs.

The effect of the pH value of the buffer (PBS) on the performance of proposed immunosensor was investigated since the electron transfer can be influenced by the electrostatic intermolecular forces in electrolyte [19]. As shown in the Fig. 2A, the current firstly increased with increasing pH and then decreased in the tested pH range of 5.5-8. The maximal current was achieved at pH of 6.5. Thus, pH of 6.5 was used for all measurements.

Except the pH value of PBS, the immobilized amount of anti-cTnT antibody that depends on the concentration and incubation time of antibody can affects the performance of immunosensor as well. When the concentration of cTnT in PBS was fixed at 0.05 ng/mL, the current response was proportional to the concentration of anti-cTnT antibody in the tested concentration ranging from 0.01 μg/mL to 1 μg/mL. A plateau was achieved at the anti-cTnT antibody concentration of approximately 1 μg/mL (Fig. 2B). The optimal incubation time at the plateau of curve was used (Fig. 2C). It was found that the current showed a remarkable decrease as the incubation time increased to 60 min, indicating maximal antigen-antibody interaction.

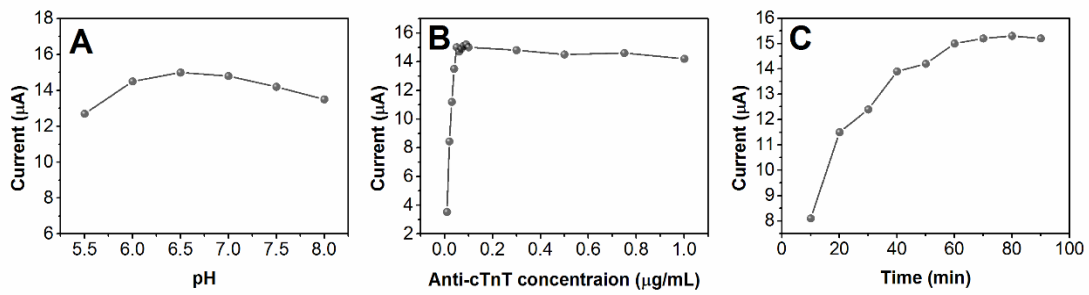

Figure 2. Effects of (A) the pH value of PBS, (B) the concentration and (C) incubation time of anti-cTnT antibody on the performance of immunosensor.

Fig. 3 showed the DPV curves obtained on the proposed immunosensor for the determination of cTnT with different concentrations ranging from 0.01 to 1 ng/mL. Specific experimental procedures are as follows: the electrodes were firstly incubated in the cTnT solution with various concentrations for 1 h under above-obtained optimum conditions, and then tested by DPV measurements with 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ in PBS (5 mM, pH 6.5) as the probe. As can be seen from Fig. 3, the current decreased with the increasing concentration of cTnT and a linear regression equation $\Delta I=3.25C_{cTnT}+4.2$ was obtained with correlation coefficient of 0.995. The limit of detection (LOD) calculated according to the slope of calibration curve and RSD of the blank sample was found to be 0.005 ng/mL, which was lower than previously obtained on various electrochemical immunosensors for cTnT determination [20-23]. The sensitivity of the proposed sensor was compared with that of other reported cTnT sensors and the results were presented in Table 1. Many earlier labeled immunosensors are speculated to suffer from the dependency of electrode status (interaction or passivation) on the chemical mediator or enzyme substrate [24]. In addition, the increasing analyses time with worse selectivity generally occurs when Michaelis-Menten kinetic dominated the reaction.

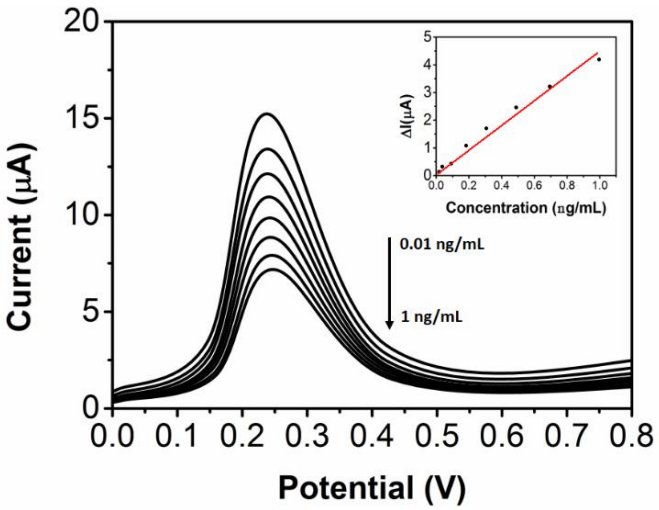

Figure 3. DPV curves obtained on the proposed immunosensor with different cTnT concentrations (0.01 to 1 ng/mL). Inset: linear fit of the calibration curve.

Table 1. Comparison of the present immunosensor with other cTnT sensors.

Electrode	Linear detection range	Detection limit	Reference
AuNPs co-immobilized on a dithiol-modified surface	0.003 to 0.5 ng/mL	0.0015 ng/mL	[25]
Carbon nanotube-based electrochemical immunosensor	0.1 to 10 ng/mL	0.033 ng/mL	[26]
SPR immunosensor	0.03 to 6.5 ng/mL	0.01 ng/mL	[27]
Silicon nanowire clusters-metal-oxide	—	1 fg/mL	[28]
Sandwich immunoassay with AuNPs as fluorescence quenchers	60 to 660 ng/mL	0.7 ng/mL	[29]
Glycine/anti-cTnT/graphene/SPE	0.01 to 1 ng/mL	0.005 ng/mL	This work

The reproducibility and stability of immunosensors are of great importance when considering their practical applications [30]. As shown in Fig. 4A, acceptable reproducibility with a relative standard deviation (RSD) of 3.2% was obtained for the proposed immunosensors by detecting cTnT samples (0.5 ng/mL) with 10 different electrodes that were prepared under the exactly same conditions. Besides, the repeatability of the immunosensor with the same electrode was also investigated. As can be found from Fig. 4B, the immunosensor demonstrated a remarkable stability with low RSD of 2.44% by 20 times test with each 1 min interval. The excellent stability obtained might be resulted from the strong interaction between immobilized cTnT and the surface of electrode modified with graphene.

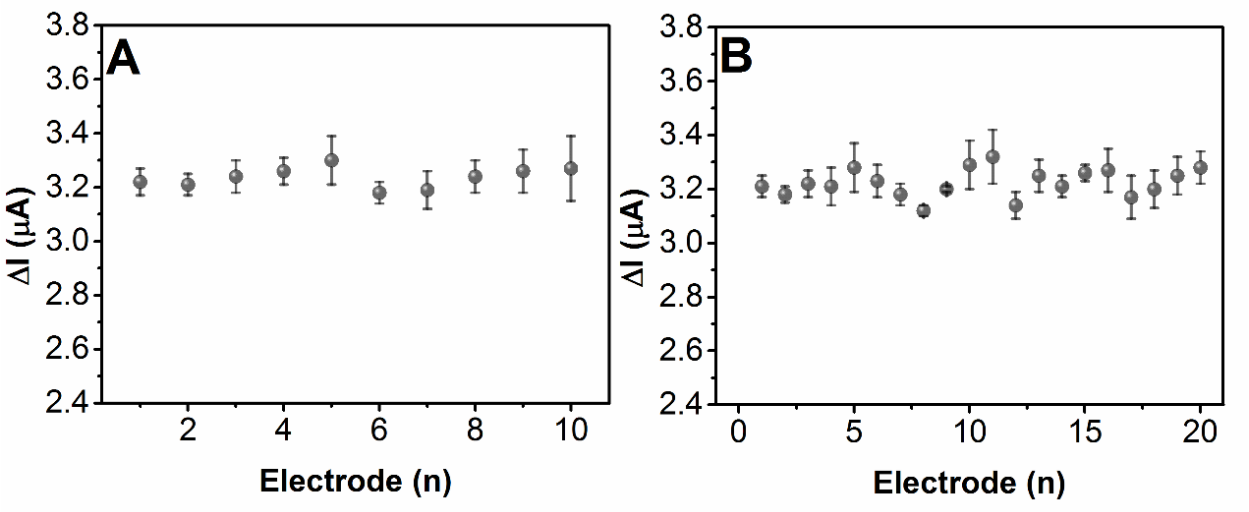

Figure 4. (A) Reproducibility and (B) repeatability of the immunosensors.

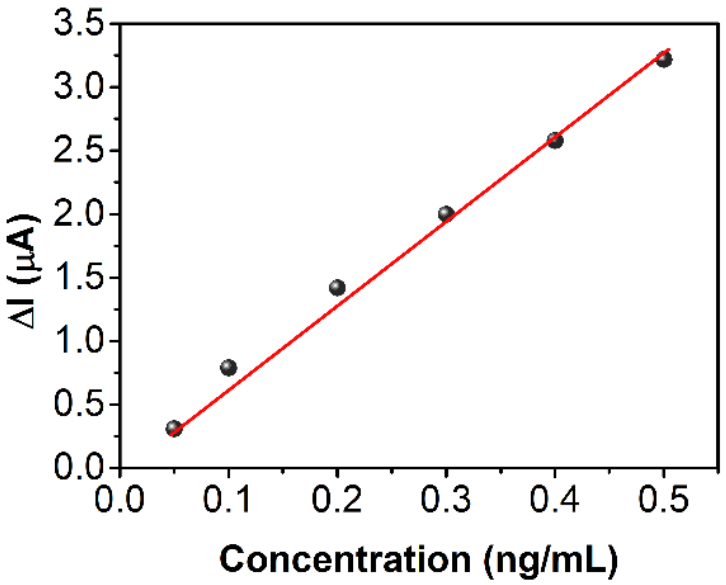

Figure 5. Calibration curve of the proposed immunosensor for the determination of cTnT in serum samples.

DPV was also performed for the determination of cTnT in human serum samples. As can be seen from Fig. 5, the current was related linearly with the concentration of cTnT ranging from 0.02 to 0.5 ng/mL^{-1}.Besides, the concentration of cTnT tested with immunosensor was in consistent with that measured with electrochemical chemiluminescence immunoassay (ECLIA) at 95% confident level. High sensitivity with the limit of detection (LOD) was found to be 0.015 ng/mL which was lower than the cutoff 0.02 ng/mL in the AMI diagnosis, indicating the promising application of immunosensor for cTnT determination in clinical routine.

4. CONCLUSION

An immunosensor with disposable screen printed electrode (SPE) was developed for the determination of cTnT in human serum. The proposed SPE exhibited excellent stability and sensitivity owing to the incorporation of graphene and anti-cTnT immobilization. Low LOD of 0.005 ng/mL and 0.015 ng/mL were obtained on the proposed SPE for the determination of cTnT in the standard solution and human serum samples, respectively. Owing to the wide linear range and low LOD, the proposed immunosensor demonstrated higher performance than previously reported electrochemical immunosensors. The linear range for the determination of cTnT in human serum was 0.02-0.5 ng/mL^{-1}, which was within cTnT clinical levels for AMI diagnostics.

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