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Fabrication of Electrochemical Immunosensor for Cardiac Biomarker Troponin I Determination and Its Potential for Acute Myocardial Infarction Diagnosis

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The detection of cardiac troponin I (cTnl) at extremely low concentration was highly demanded since it was identified as a principal diagnostic marker for acute myocardial infarction. In this study, a composite of poly(diallyldimethylammonium chloride) (PDDA) and reduced graphene oxide (RGO) was successfully prepared and then employed for constructing immunosensors. The proposed immunosensor demonstrated excellent performance for the determination of cTnl with low detection limit (0.024 ng/mL) and wide linear response range (0.1-10 ng/mL). The proposed immunosensor has demonstrated promising potential in diagnose of acute myocardial infarction owing to the great deal of advantages such as remarkable sensitivity and reproducibility.

Keywords: Immunosensor; Cardiac biomarker; Troponin I; Graphene; Acute myocardial infarction

1. INTRODUCTION

Early diagnosis is highly critical to the successful medical treatment of acute myocardial infarction (AMI) disease. Human cardiac troponin I (cTnI), a kind of cardiac muscle protein with the molecular mass of approximately 29 kDa, of which the concentration in the bloodstream begins to rise sharply within 3-4 h after the onset of acute myocardial infarction [1, 2]. Moreover, the concentration of cTnI has risen steadily over the following 4-10 days [3, 4], leading to a long diagnostic window of AMI. cTnI is a biochemical marker of myocardial injury with higher cardiospecificity in comparision with other biochemical markers such as myoglobin and creatine kinase-MB isoenzyme [5]. As a result,

cTnI has been identified as a principal diagnostic marker of myocardial damage [6, 7]. The cTnI concentration of patients with AMI normally rises sharply to 112 ng/ml at 18 h, which is much higher than that of normal patient (10 ng/ml) [8]. For the accurate diagnosis of AMI at early stage, developing methods for the determination of cTnI at extremely low concentration with excellent sensitive are highly demanded. Recently, the most commonly used detection approaches are conventional enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA) [8-11]. Nevertheless, the above-mentioned detection approaches are expensive and time consuming owing to the requirement of pricey workout equipment, professional operator and complex operation. According to previous research reports, the clinical borderline of cTnI concentration between the normal and the patient is around 0.5-2.0 ng/mL [12]. Whereas, when the patient suddenly caught a AMI, the concentration of cTnI increases rapidly to a range of 20-550 ng/ml which is far higher than the normal levels for patients (around 10 ng/ml) [8]. Thus, the development of sensitive method for detecting cTnI at low concentration is of great important for the diagnosis of AMI at early stage.

Chest pain is considered as typical symptom of cardiovascular disease. The electrocardiogram test is usually carried out for the patients with chest pain after a series of physical examination in the emergency department. The electrocardiogram cannot detect all myocardial injuries due to the lack of sensitivity despite that it is the recommended detection method to identify patients with AMI. Standard assays such as the enzyme-linked immunosorbent assays (ELISA) are very sensitive (LOD > 10 pg/mL) for the determination of cardiac biomarkers. However, standard assays which are usually carried out in central laboratories of clinics and hospitals are time-consuming with test time larger than 6 h, leading to the difficulty of rapid diagnosis and treatment of heart-attack patients. In addition, standard assays are very expensive owing to the large amount of samples and reagents required for large-scale studies. Therefore, the development of new techniques such as electrochemical immunosensors with rapid detection and high sensitivity are very important to the early and accurate diagnose of cardiac cellular necrosis. Recently, certain information about the cardiac markers in patient's blood can be acquired with qualitative alarm medical devices and quantitative point-of-care tests. Nevertheless, the prompt and reliable technique for predicting the potential of AMI disease by testing the plasma samples of patients is still in in research and development.

Until now, electrochemical immunosensors based on cardiac biomarkers have been widely used for the diagnosis of cardiovascular in time with excellent sensitivity and selectivity. Recently, a lot of researches about immunosensors such as electrochemical sensor and optical sensor have been reported [13]. Based on previous research, it is found that the integration of biomolecules into transducers is crucially important to gain outstanding performance for immunosensor. In our work, the poly(diallyldimethylammonium chloride) (PDDA) with a large number of amine groups was employed for the bind of graphene oxide and the obtained composite was marked as PDDA-RG. In order to obtain more sensitive response, a nanostructured surface was synthesized for increasing the area of immunoreactive electrode. The capture protein anti-troponin mAb which is able to capture target antigen cTnI through immune binding reaction was immobilized on the surface of PDDA-RG-modified electrode. Subsequently, the HRP labeled secondary mAb with specific binding site was employed to interact with another part of antigen cTnI in a sandwich way. The proposed

immunosensor demonstrates relevant clinical range for the detection of acute myocardial infarction and the detectable cTnl concentration meets the needs of AMI diagnosis.

2. EXPERIMENTS

2.1. Chemicals

cTnl and antibodies (anti-cTnl and anti-cTnl-HRP) were supplied by Ruixing Duotai Co., Ltd. Poly(diallyldimethylammonium chloride) solution (PDDA) (20 wt.% in H_2O) with MW of 5000-40000, *N*-hydroxysuccinimide (NHS), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and horseradish peroxidase (HRP) enzyme were purchased from Sigma-Aldrich. H_2O_2 with volume fraction of 30% was purchased from Shanghai Guoyao Co., Ltd. Phosphate buffer saline (PBS) with the concentration of 0.01 M and pH of 7.4 was prepared by dissolving KCl (0.2 g), KH_2PO_4 (0.24 g), NaCl (8.0 g) and Na_2HPO_4 (1.44 g) in Milli-Q water (1000 mL). All other chemicals were analytical reagents and used directly after received. The Milli-Q water purification system that was purchased from Millipore Inc. was used to prepare Milli-Q water for all experiments. The famous modified Hummers method was employed for the preparation of graphene oxide (GO) from graphite powder.

2.2. Apparatus and measurements

All electrochemical experiments were performed on the CHI 760 electrochemical workstation with three-electrode system containing gold electrode, platinum wire and Ag/AgCl electrode as working, auxiliary and reference electrode, respectively. Cyclic voltammetry experiments were performed in an electrochemical cell with the volume of 10 mL at 24 °C. The voltammetry measurements were carried out in 4 mM $K_3Fe(CN)_6$ solution with the potential ranging from -0.1 to 0.6 V.

2.3. Preparation of PDDA-RGO nanocomposite

Poly(diallyldimethylammonium chloride) (PDDA)-RGO nanocomposite was prepared through the reduction of GO by hydrazine in the presence of PDDA. Specifically, 100 mg of GO was added into 100 mL of PDDA solution with the concentration of 0.5 wt% in a flask (250 mL). The obtained inhomogeneous brown-yellow dispersion was treated with sonication until a clear solution with no visible particulate matter was achieved. Then the solution was stirred overnight. Subsequently, 1 mL of hydrazine hydrate (32 mmol) was added into the clear solution and the mixture was heated at 100 °C in an oil bath for 24 h. The homogeneous black suspension was treated with filtration and the obtained solid product was dried in a vacuum oven for 24 h. The final product was donated as PDDA-G.

2.4. Self-assembling of the immunosensor

A gold electrode with surface area of 0.2 mm^2 was cleaned before use. The electrode was firstly washed with freshly prepared nitric acid solution with the concentration of 4 mM for 10 min, then rinsed with ethanol and water thoroughly in the ultrasonic bath. Cyclic voltammograms was measured to confirm the neatness of electrode. Subsequently, 5 μL of PDDA ethanol solution with the volume fraction of 5% was poured onto the surface of clean electrode and then the electrode was dried at 45 °C for 10 min, leading to the formation of a uniform polymer film. Afterwards, the obtained polymer film was further modified with 5 μL of previously prepared PDDA-RGO solution and then dried at 50 °C for 20 min. After the solvent was evaporated, the PDDA-RGO modified electrode was washed in order to remove the unbound PDDA-RGO. Then, 5 μL anti-cTnl solution with the concentration of 1 μg/mL was pipetted on the nanostructured surface of PDDA-RGO/Au electrode and incubated at 4 °C for 60 min. Finally, 0.05 M glycine solution was employed for blocking the remaining active sites in order to avoid non-specific binding.

2.5. Amperometric signal measurement

Firstly, 5 μL of cTnl PBS solution with concentration of 0.01 M and pH of 7.4 was prepared and the anti-cTnl coated electrode was incubated with above-mentioned solution for 30 min. Subsequently, certain amount of anti-cTnT-HRP with the concentration of 1 μg/mL was pipetted onto the surface of modified electrode and incubated for 30 min. The surface of electrode was rinsed with PBS after each step of immunoassay. All amperometric signals were generated by the reaction between H_2O_2 PBS solution (5 mM, pH 7.0) with enzyme conjugated to anti-cTnl in an electrochemical cell (10 mL).

3. RESULTS AND DISCUSSION

The PDDA-RGO composite was highly stable owing to the strong covalent binding between the amine groups of PDDA and GO through amide linkage [14-18]. Cyclic voltammograms were carried out in $K_3Fe(CN)_6$ solution with the concentration of 4 mM in order to investigate the bond strength between PDDA-RGO strength and electrode surface. The variance ratio of redox peaks in five cycles obtained on GO modified electrode and PDDA-RGO modified electrode were 95.9% and 0.4%, respectively. The huge variance ratio for GO modified electrode was mainly resulted from the release of RGO from electrode surface. The XRD pattern of pure GO exhibits a characteristic (002) peak at 11.0° with a *d*-spacing value of 0.82 nm (not shown) [19]. After reduction process, the peak shifts to 23.2°, which is the reflection of removal of oxygen-containing functional groups [20, 21], indicating the successful occurrence of the reduction process.

Fig. 1 showed the cyclic voltammetry curves obtained on bare Au, PDDA/Au, PDDA-RGO/Au, and anti-cTnl/PDDA-RGO/Au electrodes, respectively. The electrochemical experiments were carried out in 4 mM $K_3Fe(CN)_6$ solution with scan rate of 100 mV/s. According to the obtained

voltammograms, Γ of the PDDA film on bare electrode surface can be calculated with the following equation:

$$\Gamma ibf = 1\text{-}Q_{clean}/Q_{PDDA}$$

where, Q_{PDDA}, Q_{clean} is obtained by integrating the area of redox peaks obtained on PDDA/Au electrode and bare electrode, respectively. The calculated coverage degree was 19%, indicating the good volume ration.

As can be seen form the cyclic voltammogram obtained on PDDA-RGO modified electrode, the reversible redox peaks at 0.16 V and 0.29 V exhibited an increase, suggesting the enhancement of conductivity and electron transfer by the attachment of RGO on the surface of bare Au electrode [22]. However, the amplitude of redox peaks decreased slightly after the immobilization of anti-cTnl, which might be resulted from the reduction of electron-transfer kinetics after the addition of biological insulating material on the electrode surface [23]. Chronocoulometry investigation was carried out for analyzing the electrochemically effective area of the electrodes. According to the literature, the n and D for $K_3Fe(CN)_6$ is 1 and 5.7×10^{-6} cm^{-1}/s [24]. The electroactive surface areas of the the bare PDDA/Au and PDDA-RGO/Au are calculated to be 0.2241 and 0.3988 cm^2, respectively.

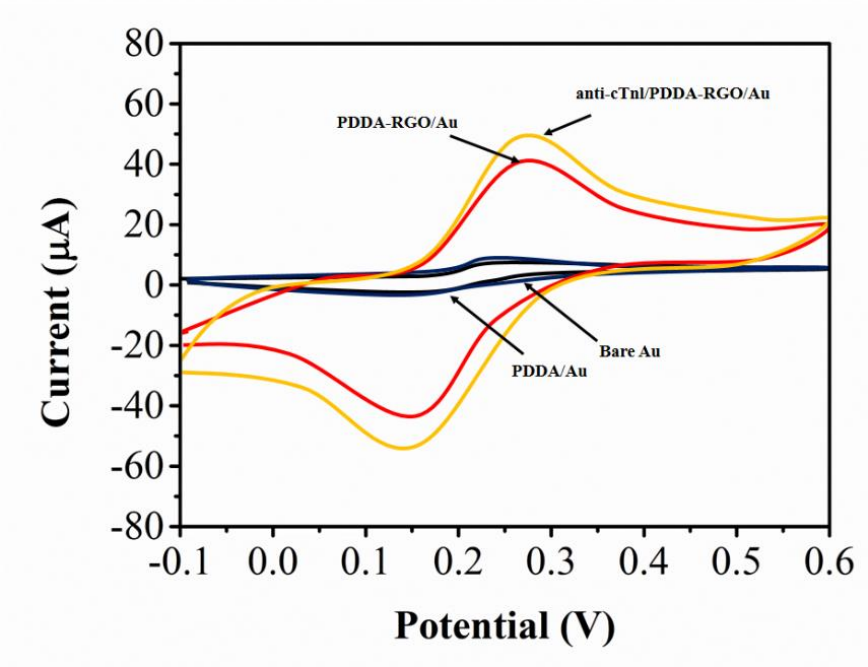

Figure 1. Cyclic voltammograms obtained on bare Au, PDDA/Au, PDDA-RGO/Au and anti-cTnl/PDDA-RGO/Au electrodes. Condition: 0.1 M PBS; Temperature: 25 °C; Scan rate: 50 mV/s.

PDDA solutions with different concentrations ranging from 0.5% to 10% (w/v) were used to investigate the effect of PDDA concentration on the electrochemical response of immunosensors and the results were shown in Fig. 2A. Cyclic voltammograms were measured in 5 mM H_2O_2 diluted in 10

mM PBS solution with pH of 7.0. As shown form Fig. 2A, the cathodic peak current firstly increased with increasing PDDA concentration, and then reached a plateau at PDDA concentration of 5.0% (v/v). Therefore, the PDDA solution with concentration of 5.0% (v/v) was selected for the remaining all experiments. pH of 7.0 also is favorable for many biomolecules survival [25, 26].

The attachment amount of PDDA-RGO on the surface of Au electrode was influenced by the size of electroactive area. Fig. 2B showed the effect of adhesion amount of PDDA-RGO on the cathodic peaks current. It was found that the cathodic peaks current has reached a plateau when the adhesion amount of PDDA-RGO reached 1.5 mg/mL. When the adhesion amount of PDDA-RGO was higher than 1.5 mg/mL, aggregation phenomena would occurred which lead to the release of PDDA-RGO from electrode surface. In addition, the electron transfer to the electrode surface would be hindered by the inhomogeneous solution formed with higher PDDA-RGO concentrations due to the longer penetration process [27, 28].

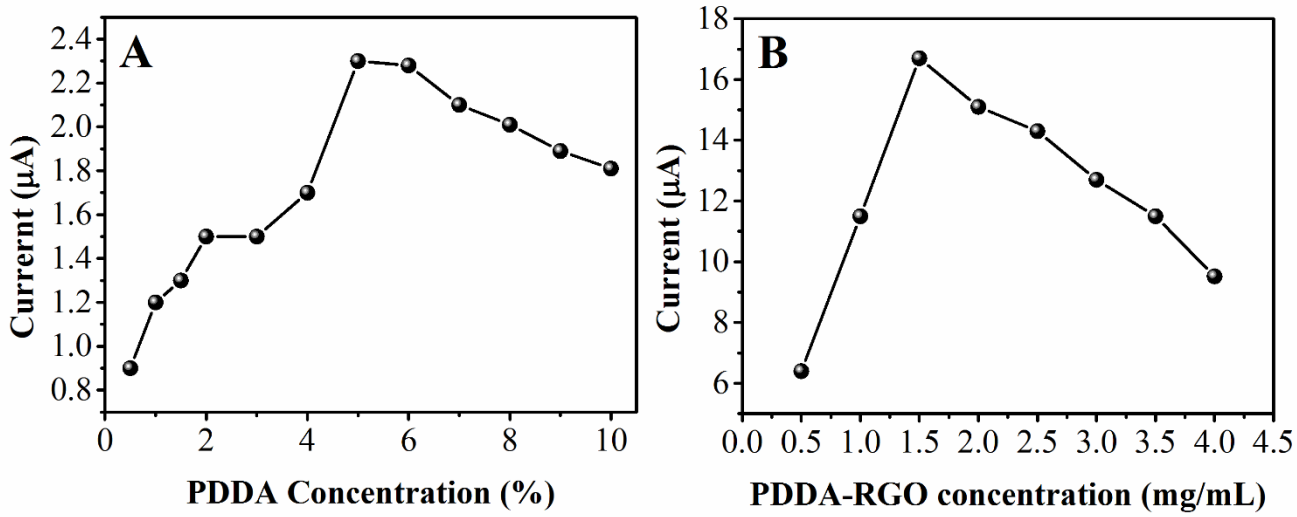

Figure 2. Effect of (A) PDDA concentration and (B) adhesion amount of PDDA-RGO on the cathodic peak current. Condition: 0.1 M PBS; Temperature: 25 °C; Scan rate: 50 mV/s.

The mechanism for the electrochemical process can often be confirmed by investigating the relationship between cathodic/anodic current peak and scan rate [29]. Therefore, cyclic voltammograms under different scan rates ranging from 5 to 250 mV/s were measured with anti-cTnI-HRP/cTnI/Glycine/anti-cTnT/PDDA-RGO/Au as electrode and 4 M $K_3Fe(CN)_6$ solution as redox probe, and the results were shown in Fig. 3A. Fig. 3B showed the peak currents in function of the square root of scan rate. It was found that both anodic and cathodic peak currents were related linearly with the square root of scan rate, suggesting the reversible charge transfer process and diffusion-controlled electrochemical process. Laviron equation below was employed for the calculation of electron transfer rate constant (*ks*) [30]:

$$ks=\alpha nFv/RT$$

where, α is electron transfer coefficient, n is the number of transferred electrons, F is Faraday constant, v is scan rate, R is gas constant and T the temperature. The calculated ks were 1.4×10^4 per second.

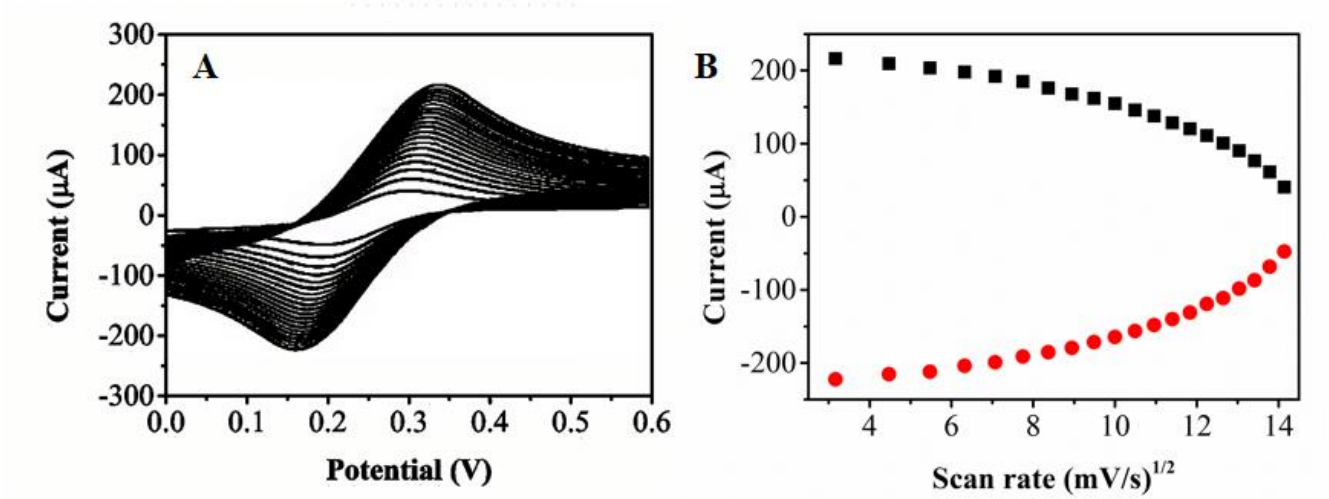

Figure 3. (A) Cyclic voltammograms obtained for immunosensor under different scan rates. (B) Plots of peak currents in function of square root of scan rate. Condition: 0.1 M PBS; Temperature: 25 °C.

The pH of medium will have great effect on the activity of enzyme that used as electroactive species in immunosensor. Therefore, the amperometry measurement was carried out in 5 mM H_2O_2 at constant potential of -0.2V and varying pH values. As can be seen from (Fig. 4A), the current peak showed a gradual increase firstly and then a gradual decrease with increasing pH value. The maximum current peak was obtained at pH of 7.0. Therefore, pH of 7.0 was selected as the optimized pH value and used in all following experiments. This immunosensor showed itself capable of evaluating cTnl in the range of the cardiac infarction myocardial based on the WHO criteria from the 1970s [31, 32].

The effect of PBS concentration on the electrochemical performance of anti-cTnl-HRP/cTnl/Glycine/anti-cTnl/PDDA-RGO/Au electrode was also investigated. The amperometry measurements were carried out in 5 mM H_2O_2 diluted in PBS solution with different concentrations under constant potential of -0.2 V. As shown from Fig. 4B, the maximum cathodic current was achieved at PBS concentration of 10 mM, and then PBS solution with concentration of 10 mM was used for all experiments.

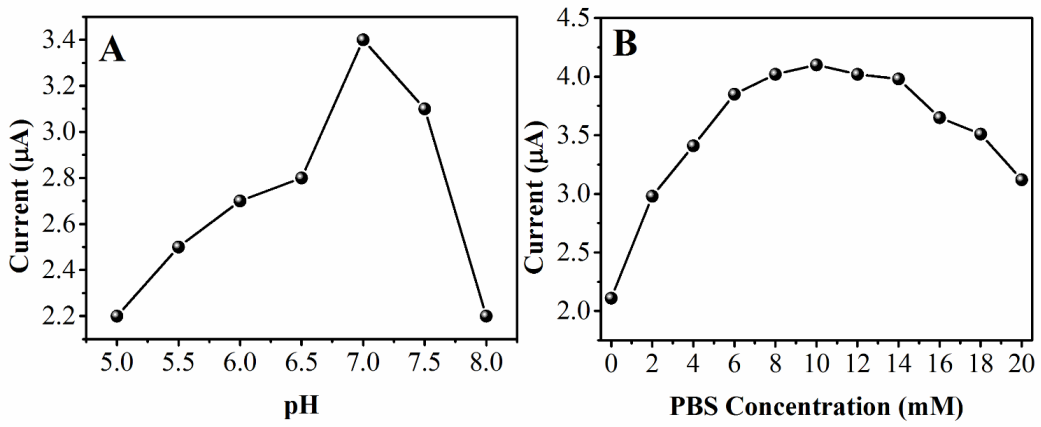

Figure 4. The effect of (A) pH and (B) PBS concentration on the electrochemical response of immunosensors. Condition: 0.1 M PBS; Temperature: 25 °C; Scan rate: 50 mV/s.

The reproducibility of the proposed immunosensor fabricated with anti-cTnl-HRP/cTnl/Glycine/anti-cTnl/PDDA-RGO/Au electrode was investigated. Specific experiment parameters were set as follows: 5 Mm H_2O_2 diluted in 10 mM PBS (pH 7.0) as electrolyte, 1 ng/mL as cTnl concentration and 100 mV/s as scan rate. As shown from Fig. 5, the variance ratio was calculated to be 4.1% for ten replicate measurements, indicating the excellent reproducibility of proposed immunosensors. The reproducibility of our proposed immunosensor was quite correspond to that of conventional laboratory tests such as enzyme immunoassays (ELISA and ECLIA) [33, 34]. Meanwhile, the reproducibility of immunosensor with same electrode was also investigated with 10 replicates experiments and the calculated coefficient of variation 2.6 % was quite satisfactory.

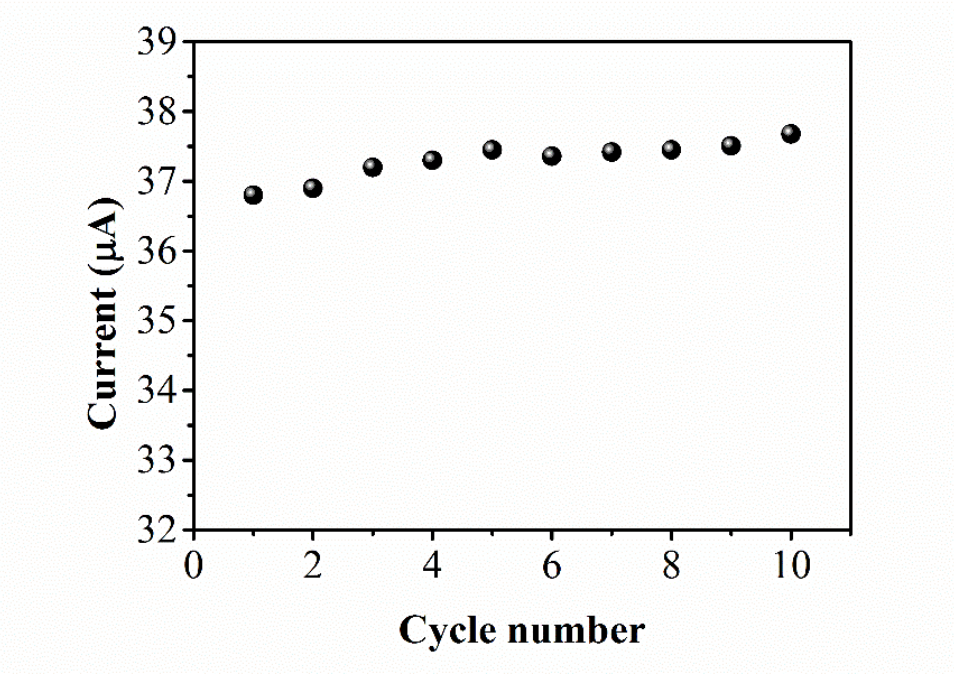

Figure 5. Reproducibility of the immunosensor by ten replicate measurements. Condition: 0.1 M PBS; Temperature: 25 °C; Scan rate: 50 mV/s.

As shown from the calibration plot of immunosensor measured in 5 mM H_2O_2 diluted in 10 mM PBS of pH 7.0 (Fig. 6A), the peak current was related linearly with cTnl concentration within the range from 0.1 to 10 ng/mL. The calculated detection limit of our proposed immunosensor for cTnl was 0.024 ng/mL, which was comparable to that of ECLIA method (0.015 ng/mL). In addition, the detection limit of immunosensors for cTnl was also lower than the cutoff value 0.1 ng/mL which was raised by WHO criteria in 1970s, indicating the successful application of immunosensors in the diagnose of cardiac infarction myocardial. Regarding the simplicity of this sensor, the PEI/CNT joint resulted in a highly sensitive immunosensor in which the use of chemicalmediators was not necessary in obtaining amperometric response [35, 36]. In addition, the immunosensor was also very simple owing to the unnecessary of chemical mediators for amperometric response, which was resulted from the excellent sensitivity by PDDA-RGO modified electrode. The sensitivity of the proposed sensor was compared with that of other reported cTnl sensors and the results were presented in Table 1.

The calibration plot for cTnl in real samples was also investigated. Firstly, 10 μL human serum was pipetted onto the surface of modified electrode, and then a moist chamber was covered above the electrode in order to prevent fluctuations of electrochemical responses. Then our proposed immunosensor was used for the determination of cTnl in serum samples after 1:100 dilution. As shown from the calibration plot of real sample (Fig. 6B), the peak current exhibited excellent linear correlation with cTnl concentration ranging from 0.01 to 0.30 ng/mL (r = 0.977). The results obtained with proposed immunosensors were similar to that obtained with ECLIA methods at 95% confident level when the paired t-test was applied. Our proposed immunosensor has demonstrated promising potential in particle application owing to the great deal of advantages such as flexibility, sensitivity and no-essential of chemical mediators. To test the accuracy of periostin, the results coming from five sera by using the well-defined method, was compared with the ELISA, which is a useful and powerful method for serum analysis. Data in Table 2 displayed the relative deviation between two method and the results.

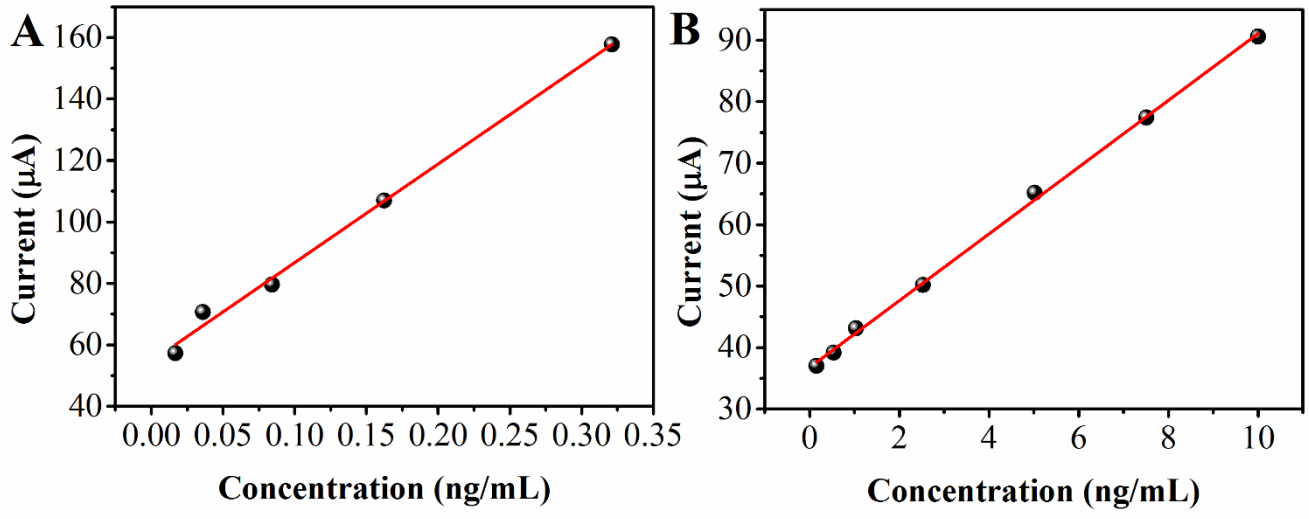

Figure 6. (A) Calibration curve of immunosensor. (B) Linear response of immunosensor with different cTnl concentrations in serum samples.

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

Electrode	Linear detection range	Detection limit	Reference
High-sensitivity assays	23 to 58 ng/L	—	[37]
anti-cTnI mAb	7-15 ng/mL	2.5 ng/mL	[38]
MCM-41 mesoporous/CPE	0.5-2 ng/mL	0.1 ng/mL	[39]
Spectral EIA	0.1-0.3 ng/mL	0.02 ng/mL	[40]
Electrogenerated chemiluminescence peptide-based method	0.02-0.5 ng/mL	0.005 ng/mL	[41]
PDDA-RGO	0.01 to 0.3 ng/mL	0.015 ng/mL	This work

Table 2. The comparison of results obtained in serum samples with different methods.

Serum samples	1	2	3	4	5
Immunosensor (ng/ml)	0.07	0.10	0.15	0.20	0.25
ELISA (ng/ml)	0.07	0.09	0.14	0.22	0.23
Relative deviation (%)	4.1	8.5	3.9	5.5	4.7

4. CONCLUSIONS

In conclusion, a nanostructured immunosensor fabricated with PDDA-RGO modified electrode was proposed for the detection of cTnl. The anti-cTnl monoclonal antibodies were combined firmly on the functionalized nanostructured surface. The proposed immunosensor demonstrated excellent performance for the determination of cTnl. The detection limit is 0.024 ng/mL and the wide linear response range is 0.1-10 ng/mL. In addition, the proposed immunosensor exhibited remarkable reproducibility with coefficient of variation being 2.6 % for ten replicate measurements. In general, the excellent sensitivity and reproducibility make the immunosensor promising technique for diagnose of acute myocardial infarction.

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