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A Graphene Based–biomimetic Molecularly Imprinted Polyaniline Sensor for Ultrasensitive Detection of Human Cardiac Troponin T(cTnT)

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Abstract

In the present work, a biomimetic nano-molecularly imprinted polymer (N-MIP) electrode based on a graphene screen-printed electrode was developed for the ultrasensitive detection of cardiac troponin T (cTnT). The biomimetic cavities for targeted sensing for analyte were fabricated by the electropolymerization of conductive co-polymer matrix of aniline and carboxylated aniline on the graphene oxide (GO) electrode, in the presence template protein (cTnT for cardiac troponin T probe) by cyclic voltammetry. The surface characterization of the sensor was performed using cyclic voltammetry (CV) and differential pulse voltammetry (DPV), and scanning electron microscopy (SEM). The best biomimetic surface nanotexture was obtained at aniline/carboxylatedaniline ratio of 1:4. The linear range of cTnT probe was in the range of 0.02 to 0.09 ng/mL, with the detection limit of 0.008 ng/mL. The reliability of the N-MIP cTnT sensor was examined by comparing the results with those obtained from HPLC method, and it was observed that the results from N-MIP sensors and HPLC have a great correlation.

Keywords

Molecularly imprinted polymer, Graphene, Polyaniline, Sensor, cTnT

1. Introduction

Molecularly imprinted polymer (MIP) sensors, which have been developed in recent years by several groups, have gained a great attention due to their ability to provide ultrasensitive targeted detection of bioanalytes by specifically binding to the template molecule *via* their artificial biomimetic cavities [1]. The biomimetic MIP probes are ideal candidates for electrochemical sensor application because of their interesting advantages, including high sensitivity, high chemical and thermal stability, cost-effectiveness, facile synthesis and reusability [[2], [3], [4], [5]].

In MIP method, functional monomers are polymerized, followed by a template removal that is used to create biomimetic polymer cavities for capturing a specific target analyte. In the MIP surfaces, the functional groups of monomers create suitable sites within the cavities, which provide a promising rebinding condition for template analyte [6]. MIP is used for molecular diagnostic systems varying from small molecules to proteins [2]. The tailor-made implication of MIPs relative to protein is significant due to the influence of their molecular size and complex structure on the sensibility of imprinted sites to detect the target molecules [2].

MIP surfaces are usually obtained using bulk polymerization method [1][6]. However, this method is not ideal for preparing biomimetic surfaces because creating an MIP composite texture on the surface of probe is not easy and some active sites entrapped in the bulk of polymers and cannot expose to outer surface of sensor to detect analyte—it is difficult to maintain the active sites responsible for the target molecule absorption [7]. Also, it is a hard task to remove the target molecules into a composite, especially if the template is firmly attached to the sensor surface [8]. Biomimetic MIP probes can be fabricated *via* surface imprinting method [9].

The screen-printed electrodes (SPEs) have a great potential for use as disposable MIPs in point-of-care testing. SPE probes are prepared *via* printing the ink formulation on the solid substrates, which is a simple and low-cost method. Therefore, it is suitable for mass production of biomimetic sensors [10].

This new technique improves the sensitivity of the biomimetic sensor by facilitating the effective rebinding of template analyte. Also, the targeted cavities are near to the MIP probe surface, which improves the electron transfer.

The sensitivity of biomimetic MIP sensor could be compromised using conductive monomers [[11], [12], [13], [14], [15], [16]]. Among different conductive polymers that could be used in MIP sensor development, polyaniline is a more proper candidate because it is a biocompatible polymer with high conductivity and easy electrochemical synthesis. In order to improve the molecular interaction between MIP cavities and the target analyte, one of the best strategies is combining the conductive monomers with organic functional groups, such as carboxylic acid [17].

Graphene, an allotrope of carbon, is a two-dimensional molecule with excellent electrical properties, which makes it an ideal candidate for biosensor applications. In order to improve the conductivity and electronic transfer of graphene, reduced graphene oxide (RGO) is developed [18,19]. An alternative approach for enhancing the electronic transfer in graphene coated probes is using conductive polymers, which also improve the content of biomimetic sites in N-MIP electrodes.

Acute myocardial infarction (AMI) is the common life-threatening cardiovascular disease throughout the world, and cardiac troponin T (cTnT), a cardiac regulatory protein, is its specific biomarker [20]. AMI results in the rapid release of cTnT (37 kDa) from cardiac muscle cells into the bloodstream, which remains elevated up to 14 days after cardiac ischemia providing the possibility of prognosis of the disease [21].

In the literature, different efforts have been done for using N-MIP in detection of cTnT. This approach that employed in this research is fast, simple, cost effective, label free and not needed much sample pretreatment that make it an excellent and attractive method for cTnT sensor [21].

In this study, the aim was to develop a sensitive N-MIP sensor for the detection of cTnT. To do so, different ratios of aniline and carboxylated aniline monomers were used to obtain a probe with highest sensitivity. The N-MIP surface was obtained *via* electropolymerization of conductive monomer and target molecule followed by a template removal. Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) techniques were used to evaluate the sensitivity of probes against standard and serum samples.

2. Experimental procedures

2.1. Materials

Aniline (98%) and aniline-3-carboxylic acid were purchased from WorldChem Trading Corporation. Potassium ferricyanide ($K_3[Fe(CN)_6]$, 99%), potassium ferrocyanide ($K_4[Fe(CN)_6]$, 99%), oxalic acid (99%) and cardiac troponin T (cTnT, Mw =37 kDa) were obtained from Sigma-Aldrich (St. Louis, USA).

Graphite powder and carbon ink (Electrodag PF-407 C) were acquired from Fluka (St. Louis, USA) and Henkel (Germany), respectively. Blood serum samples were collected from volunteer patients and stored at −20 °C. The content of cTnT in blood samples was identified *via* electrochemical chemiluminescence immunoassay (Immunoassay Analyzer, Roche Diagnostics).

Ultrapure water (18 MΩ, Milli-Q, Millipore) and phosphate buffer (PB) were used in all assays for dilution. All chemicals were of analytical grade.

2.2. RGO-modified screen-printed electrode (SPE) fabrication

RGO was obtained as follows: first, a mixture of 6 g of graphite, 3 g of $NaNO_3$ and 14 g of $KMnO_4$ in 120 mL H_2SO_4 was prepared and stirred in an ice bath for 24 h. Then, this mixture was left to rest for 24 h. The mixture was washed with distilled deionized water repeatedly to reach a pH of 7 and obtain graphene oxide (GO). In order to reduce the GO, the powder was kept in an oven at 500 °C for 5 min, then cooled at room temperature. Finally, the synthesized RGO was dispersed in water (1 mg/mL) for further use [22].

The composition of screen-printed electrode (SPE) included a graphite modified (15%) carbon ink. The mixture of carbon and graphite was printed on the polyethylene-based surface, and a thin film electrode was formed. The circular working electrode had a diameter of 5.0 mm, which was connected to an electrical contact with dimensions of 2.0 mm*10.0 mm. Then, surface of SPE was polished 4 min and applied in an electrochemical pretreatment by recording 30 cyclic voltammograms among 1.5 V to −1.5 V (*vs*. Ag/AgCl (KCl sat.)) in a KCl solution (0.1 mol/L). Finally, 0.5 ml of RGO solution (1 mg/ml) was deposit on the prepared SPE surface and kept in 50 °C to evaporate solvent.

2.3. N-MIP senor construction

To fabricate the N-MIP surface, the solution of cTnT and carboxylated aniline monomers was deposited and electropolymerized on the graphite modified carbon electrode. To provide the electrostatic interactions between the monomers and the cTnT, the cTnT (0.02 mg/mL) was added to a mixture of aniline and carboxylated aniline monomers (0.02 mol/L) for 2 h at 4 °C before electropolymerization. The carboxylated aniline polymer provides an electrostatic bonding (−COOH) with molecules containing amine groups (cTnT) (under 100 °C) [17]. The preparation schematic of the N-MIP sensor in illustrated in Fig. 1.

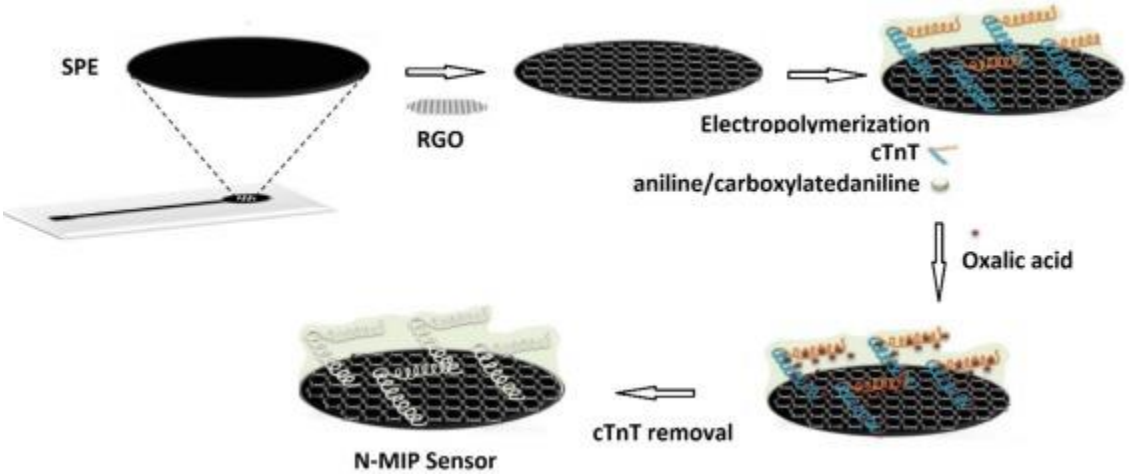

Fig. 1. The preparation schematic of the N-MIP sensor.

The mixture solution of aniline, carboxylated aniline monomers and cTnT was electropolymerized on the SPE surface by 10 cyclic voltammograms in the potential range from 1 to -0.2 V (*vs*.Ag/AgCl ($KCl_{sat.}$)) and scan rate of 0.02 vs^{-1} after addition of a $LiClO_4$ solution (0.008 mol/L) in PB (0.01 mol/L, pH 5.8).

Finally, the template molecule was removed by adding one drop of oxalic acid solution (0.005 mol/L) to the electropolymerized film and allowing it to rest for 10 h at 4 °C in a moist atmosphere. To prevent nonspecific binding from the blood sample, the N-MIP probe was immersed in 0.1% BSA for 1 h. Subsequently, the probe was washed with ultrapure water, then the electrochemical experiments were performed.

For control study, a non-molecularly imprinted polymer (N-NIP) modified electrode with nanotexture was prepared by the same approach of N-MIP fabrication without using cTnT during electropolymerization process. Finally, the two sensors were attached together face-to-face using a plastic adhesive spacer. The gap between two working electrodes is 0.7 cm.

2.4. Electrochemical analysis

All the electrode systems used in electrochemical analysis included a working electrode (SPE), a reference electrode (Ag/AgCl (KClsat.) and a counter electrode (a platinum wire). The calibration curves were prepared using different dilutions of stock solution of cTnT (10 M) standard solution in 0.1 M PBS (pH 7.4).

In order to remove the interfering proteins in blood samples, the serum was mixed with trichloroacetic acid (10%, w/v) and then centrifuged for 15 min. The samples were diluted 1:10 using PBS (pH 7.4). Then, N-MIP electrodes were incubated in the buffer solutions containing different concentrations of serum for 10 min. Finally, the probe was washed, and its electrochemical response was measured. The electrochemical measurements were performed through the cyclic voltammograms (CV) and differential pulse voltammetry (DPV) analyses in the presence of K3[Fe(CN)6]/K4[Fe(CN)6] (1:1) (5 mM) solution prepared in 0.1 M KCl.

CV of N-MIP probe was recorded in a potential range of -0.5 V to 1 V, at 50 mVs^{-1} scan rate. The DPV was employed from 0 V to 0.5 V with pulse amplitude of 50 mV, pulse time of 10 ms and 5 mV step potential for N-MIP and N-NIP sensor in different analyte concentrations.

The concentration of cTnT in blood serum samples was determined *via* monitoring the changes in electrocatalytic current (ΔI) due to rebinding of target analyte with N-MIP electrode using the base line signal at 5 s. To prepare the blood samples (1 µL in 0.1 M PBS containing 1% triton X-100), they were diluted 400-fold and hemolysed in a microwave oven for 5 s.

2.5. HPLC analysis

For HPLC test, a Waters 2695 HPLC system (Milford, MA) was used in this study including a photo-diode array detector, an auto sampler, a binary solvent manager and a column oven. The used analytical column was a Dikma Diamonsil C18 column (4.6 × 150 mm, 5 µm). The constant mobile phase (60% water containing 0.02 M potassium dihydrogen phosphate, 40% methanol) was applied at a flow rate of 1.2 mL/min. The temperature of the column was set at 25 °C and the detector was kept at 200 nm. The data processing and instrument control were conducted with Empower 2 software (Waters).

For the HPLC analysis, samples were prepared by pipetting 5 mL of serum sample into a polypropylene Microcon® YM-10 (10,000 molecular weight cutoff, MWCO) centrifugal filter tube. The tubes of sample were covered and centrifuged at 10,000 × g for 20 min at room temperature. The clear filtrates were transferred to deactivated HPLC auto sampler vials with 2 ml injected into the HPLC system for analysis.

2.6. Morphological and structural analysis

The surface morphology of the SPE electrode was observed by Scanning Electron Microscopy (SEM) using a Philips XL30 at 20 kV acceleration voltages.

3. Results and discussion

3.1. Surface morphology of the N-MIP SPE

The N-MIP probes were fabricated *via* electropolymerization of aniline in the presence of cTnT as a template molecule and then removal of template. The concentration of target analyte in blood samples was monitored electrochemically *via* measuring the reduction in redox current of a reaction probe.

The SEM micrographs show the presence of graphite aggregates (100 nm mixed in the carbon ink on the bare SPE surface (Fig. 2a)), while the RGO-modified SPE imposed a wrinkled surface due to the random distribution of the graphene sheets (Fig. 2b).

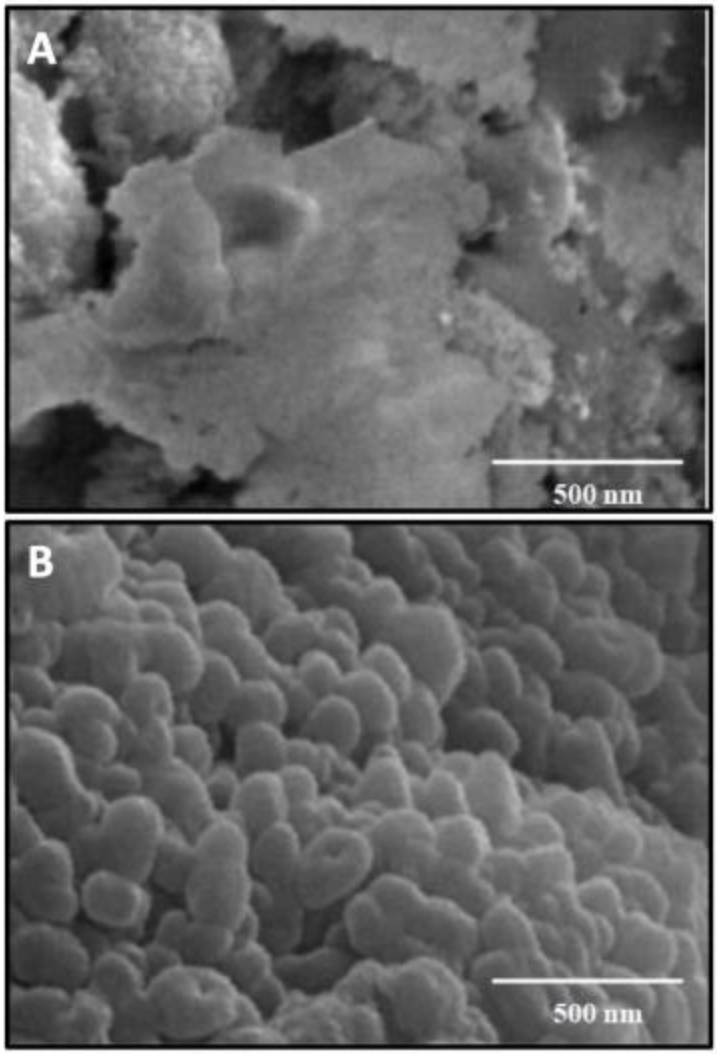

Fig. 2. Surface morphology of (a) bare and (b) RGO modified SPE.

In order to improve the selectivity and sensibility of biomimetic MIPs sensors, it is important to choose a proper conductive polymer to obtain maximal electron transfer. The biomimetic MIPs sensor could be improved by using a carboxylated polymer, which provides more reactive sites for cTnT bonding *via* −COOH groups. In this study, aniline was chosen as a conductive component and was copolymerized with COOH-3- aniline to obtain a MIP probe with high selectivity and sensibility. In order to find out the optimal aniline: COOH-3-aniline ratio for maximum cTnT binding, the current responses (IΔ) after rebinding with cTnT (0.5 ng/mL) were measured. The results show that at 1:4 ratio of aniline:COOH-3-aniline, a maximum value of current was achieved (Fig. 3). This result demonstrates that the carboxylic groups of aniline:COOH-3- aniline copolymer are mainly responsible for capturing the cTnT *via* non-covalent interactions, such as electrostatics interactions and hydrogen bonding [23].

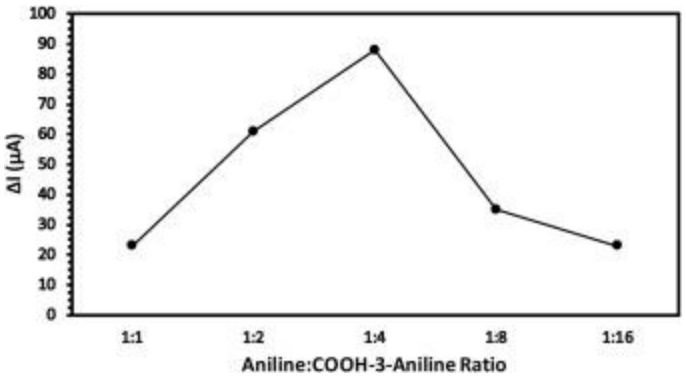

Fig. 3. Effect of aniline and COOH-3-aniline ratio in the N-MIP electropolymerization. ΔI values were obtained from CVs analyses in $K_3[Fe(CN)_6]$/ $K_4[Fe(CN)_6]$ solution (0.005 mol/L) prepared in KCl (0.1 mol/L).

Fig. 4 represents the stepwise of the N-MIP-modified electrodes by CV and DPV techniques. As shown in Fig. 4a, electropolymerization of the aniline and cTnT on the probe surface (Curve 2) increases the redox peaks in the CV compared with RGO-modified SPE (Curve 1). The reason of this slight increasing compared to RGO-modified SPE is related to deposition conductive polyaniline on the nanostructured surface of electrode [24].

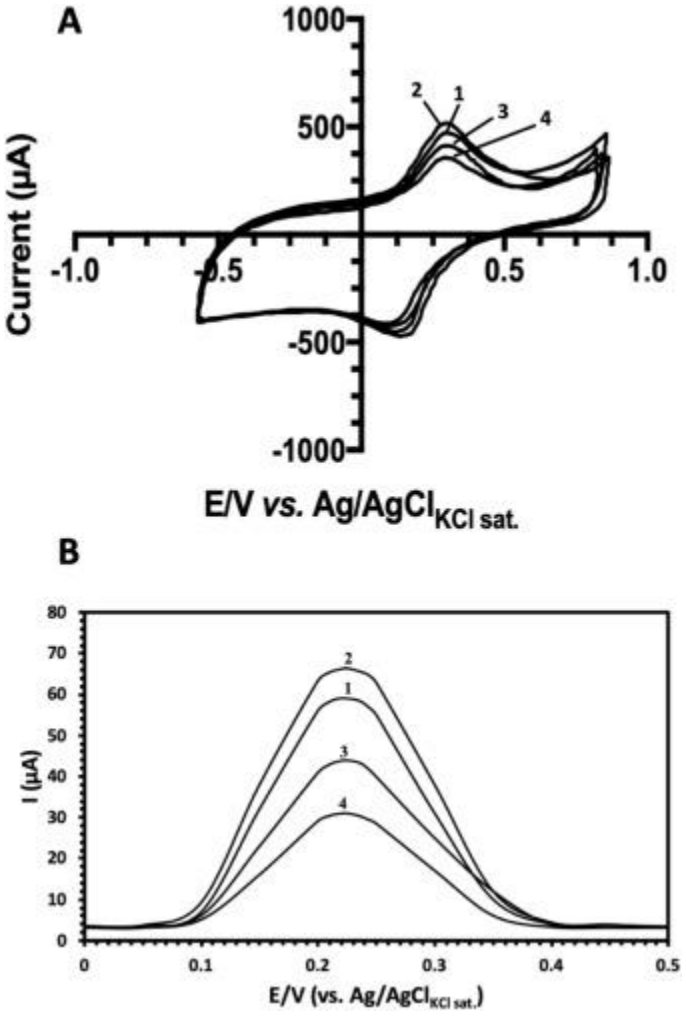

Fig. 4. Electrochemical profile CVs recorded for the stepwise of constructing of the N-MIP (a) cTnT sensor and (b) DPV profile of the probe; (1) RGO modified probe; (2) aniline, COOH-3-aniline and analyte electropolymerized probe; (3) electropolymerized probe after template molecule removal and (4) electropolymerized probe after

rebinding with analyte (0.5 ngm/L). Analysis was performed in $K_3[Fe(CN)_6]$/ $K_4[Fe(CN)_6]$ solution (0.005 mol/L) prepared in KCl (0.1 mol/L).

Template molecule removal results in the decrease of the redox peaks (Curve 3), which could be due to the hydrolysis of the amide and imine bonds between the template molecule and the carboxylated polyaniline matrix [25]. The decrease in the redox peaks at this stage also confirms the efficient removal of template molecule in the biomimetic cavities. These active sites serve as ideal site for rebinding of the analyte [26]. After rebinding of the target analyte (0.5 ng/mL), a further reduction in the peak current is observed (curve 4). When the active sites or cavities are filled with the target analyte, the electron transfer on electrode surface will be blocked, which results in the reduction of the peak current. The results of DPV also confirm those from CVs measurements (Fig. 4b).

3.2. Electrochemical evaluation of the N-MIP sensor probes

The performance N-MIP and N-NIP sensors were analyzed in different concentrations of cTnT diluted in buffer solution (pH 7.4, 0.01 mol/L) as it shown in Fig. 5. The N-MIP sensor response was achieved by using percentual decrease of current (ΔI%) of measurement of DPVs in K3[Fe(CN)6]/ K4[Fe(CN)6] (0.005 mol/L) during incubation by cTnT. The N-NIP sensor without cTnT in its polymeric matrix in its fabrication, was used as the control electrode. The N-MIP electrode was shown a hyperbolic response with a proportional increase of the ΔI% during contacting cTnT samples when it reached a plateau at 0.5 ng/mL. It demonstrated that rebinding of cTnT in printed sites on the surface of N-MIP electrode leaded to electron transfer blocking at the sensor interface. The linear correlation between ΔI% and cTnT concentrations was achieved among 0.02 to 0.09 ng/mL, which showed a linear regression equation: ΔI% = 130.93 (cTnT concentration) +6.4785 with a correlation coefficient of 0.9951 ($p < 0.0001$, $n = 5$) with a low relative error (<1%).

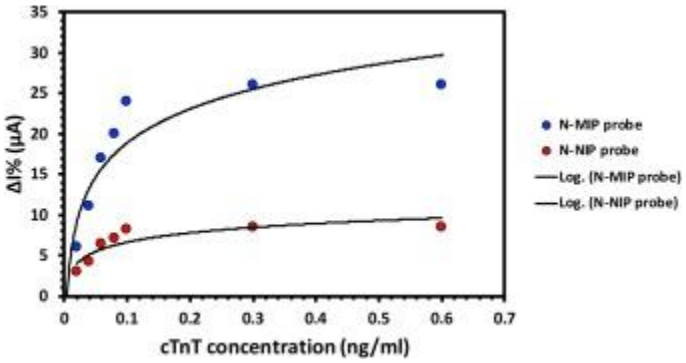

Fig. 5. Analytical curve of the (curve I) N-MIP and (curve II) N-NIP for different cTnT concentrations (0.01, 0.025, 0.05, 0.075, 0.1, 0.25 and 0.6 ng/mL). Analysis was performed in $K_3[Fe(CN)_6]$/ $K_4[Fe(CN)_6]$ solution (0.005 mol/L) prepared in KCl (0.1 mol/L).

(N-MIP equation (blue dots): ΔI% = 6.07 ln(cTnT concentration) + 32.831 Linear regression equation between 0.02-0.09 cTnT concentrations is: ΔI%=130.93 (cTnT concentration) +6.4785 With a correlation coefficient of 0.9951 ($p<0.0001$, $n=5$) with a low relative error (<1%). N-NIP equation (Red dots): ΔI% = 1.6452 ln(cTnT concentration) + 10.436 Linear regression equation between 0.02-0.09 cTnT concentrations is: ΔI%=35.53 (cTnT concentration) + 3.2891).

The N-NIP performance ascertained lower values of ΔI% in comparison to N-MIP. This behavior interpreted to cTnT lower affinity to N-NIP electrode, which confirmed the printed sites in polymeric matrix controlled cTnT interaction with aniline copolymeric film.

In order to examine the reproducibility of the sensor, N-MIP electrode underwent the procedure of template removal, followed by a subsequent re-plotting the calibration curve. After 8 times rebinding and template removal, the sensitivity of the regenerated probe changed about ±8-12%. This indicates that the reproducibility of N-MIP electrode was adequate up to 8 uses.

3.3. Analyte detection in serum sample

The performance of the N-MIP sensor in the presence of other blood components was evaluated using diluted human serum. The N-MIP sensor was incubated with different diluted serum samples for 30 min (0.01 mol/L, pH 7.4). The assays were carried out in triplicate.

The redox signal of probe during protein rebinding was measured *via* DPV (Fig. 6). The concentration of cTnT with the N-MIP electrode after rebinding is directly proportional to the value of decrease in probe response, while the probe signal was not affected by other serum protein. This indicates that the decrease in probe response was the result of specific rebinding of analyte with the N-MIP probe. For comparison, the N-NIP sensor was used showed a weaker response to cTnT in serum samples (Fig. 6)

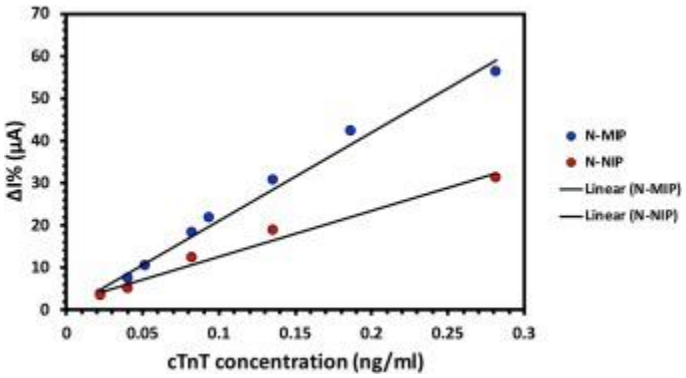

Fig. 6. cTnT concentrations in serum samples measured by N-MIP sensors.

In order to evaluate the reliability of cTnT N-MIP probe, the cTnT concentrations in blood samples (*n* = 8, normal persons and patients) measured using the N-MIP probes were compared with the results from the HPLC analysis. The correlation of the cTnT concentrations was measured using N-MIP sensors and HPLC is presented in Fig. 7.

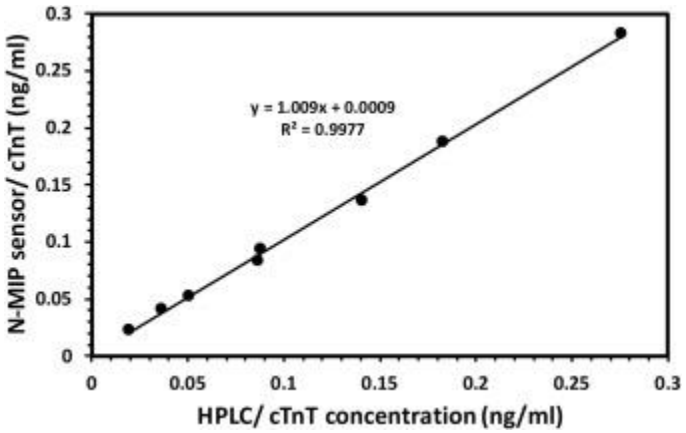

Fig. 7. Correlation between the cTnT concentrations in 8 blood samples obtained by the N-MIP sensor and HPLC.

The cTnT concentrations was obtained using the N-MIP sensors, which had high accuracy and showed small variations compared to the data from HPLC. The R^2 from regression curve for cTnT, was 0.9977. These results

demonstrate the high sensitivity and accuracy of N-MIP probes, which combined with the other advantages of these probes—like low-cost and ease of use—make those promising candidates for the point-of-care applications. Our study demonstrated that the fabricated N-MIP has an excellent sensibility to detect cTnT that is higher than the published paper by Moreira et al. [27] using carbon-nanotube-MIP. In other published paper, Karimian et al. [28] introduced a sensitive poly-o-phenylenediamine MIP for cTnT detection with same LOD. They utilized the conventional gold electrode that needs more progress to be point of care sensor.

4. Conclusions

In this study, a N-MIP graphene-based sensor was developed for the electrochemical detection of cTnT in buffer. The conductive N-MIP pattern was created on the grapheme probe surface *via* direct electropolymerization of aniline monomer in the presence of cTnT as a biomimetic template molecule. Finally, by removing the template molecule, the N-MIP probe surface was obtained. The linear sensor response for the TnT in buffer was in the range 0.02 to 0.09 ng/mL, with a detection limit of 0.008 ng/mL. Lastly, the obtained biomimetic sensor represents the advantages of good sensitivity, high stability, low cost, short response time and good reproducibility.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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