An Aptamer-based Biosensor for Troponin I Detection in Diagnosis of Myocardial Infarction

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ABSTRACT

Background: Acute myocardial infarction (MI) accounts for one third of deaths. Cardiac troponin I (TnI) is a reliable biomarker of cardiac muscle tissue injury and is employed in the early diagnosis of MI.

Objective: In this study, a molecular method is introduced to early diagnosis of MI by rapid detection of TnI.

Materials and Methods: The detection method was based on electrochemical aptasensing, being developed using different methods and evaluation steps. A gold electrode was used as a transducer to successful immobilize 76base aptamer to fabricate a TnI biosensor.

Results: The designed aptasensor could detect TnI in a range of 0.03 to 2.0 ng mL^{-1} without using any label, pre-concentration or amplification steps. The limit of detection was attained as 10 pg mL^{-1} without significant trouble of interfering species. The TnI biosensor demonestrated a stable, regenerative and reproducible function. 89 human samples were used to evaluate the performance of the TnI biosensor, and it represented 100% and 81%, diagnostic sensitivity and specificity, respectively.

Conclusion: This aptasensor may be used as an applicable tool in the future of early medical diagnosis of MI.

Keywords

Electrochemical Biosensor, Biomarker, Heart failure, Troponin, Molecular Detection

Introduction

Life expectancy has increased due to the advances in the principles of health, treatment and technologies; However, aging has led to more occurrence of cardiovascular diseases [1], none of these diseases may occur in all ages. Among cardiovascular diseases, myocardial infarction (MI) is known as the leading cause of death worldwide [2]. MI complications include congestive heart failure [3], myocardial rupture [4], arrhythmia [5], pericarditis [6], cardiogenic shock [7], etc. One of the most important challenges in these days is finding a way to predict MI quickly and find related risks [8]. For the patients with MI, the treatment needs a quick blood flow to be restored so as to prevent further from damaging to the myocytes, thus any early prediction is essential. After a heart attack, cardiac biomarkers are released from the damaged myocytes into the bloodstream [9]. Therefore, laboratory

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blood tests are intrigued to make a diagnosis rapidly, investigate of treatment status, and predict -the risk of re-attack in the future. The most important cardiac biomarkers related to MI which are measured through blood analysis are creatine kinase-MB (CK-MB) [10], aspartate aminotransferase [11], lactate dehydrogenase [12] and the different types of troponin protein [13, 14]. Except troponin, the non-specificity against MI is an important diagnostic challenge in using some of these cardiac biomarkers [13]. Troponin is a myofibril in the heart muscle that plays a key role in contraction of the heart muscle. Troponin I (TnI) with a molecular weight of 22.5 KD is a part of a triple complex comprising TnI, troponin C (TnC) and troponin T [15]. This triple complex along with tropomyosin regulates the activity of ATPas in actomyosin of the skeletal muscles and myocard through releasing calcium [16]. Several studies have shown that TnI is the preferred biomarker for rapid diagnosis of MI [13, 17] and its level is not affected by kidney and liver diseases (false positives) [18]. A level of TnI of higher than 0.1 ng mL^{-1} indicates damage to the myocardium [19, 20].

Nowadays, MI biomarkers have been detected by various methods, including immunosensing such as those based on the surface plasmon resonance [21], electrochemiluminescence [22], immunohistochemistry [23], enzyme-linked immunosorbent assay (ELISA) [24], fluorescence resonance energy transfer [25] and field-effect transistors [26], aptasensing [14], liquid chromatography [27], and chemiluminescence [28]. Electrochemical approaches in integration with nanotechnology have facilitated the diagnosis of MI through detection of biomarkers associated to the damage of the cardiac myocytes [14, 29].

Recently, a number of nucleotide sequences have been used in molecular diagnosis of pathogens and disease markers in the form of genosensors and aptasensors [14, 30-33]. Protein-binding nucleotide sequences (aptamers) due to high affinity against their targets and also high stability, hence they have been successfully used in clinical diagnostics and applied as an appropriate alternative for antibody-based immunological diagnostic methods [14, 34]. Recently, aptamer-based biosensors have been reported for detection of MI biomarkers [14, 29].

The accuracy and sensitivity of biomolecules detection using electrochemical methods have increased dramatically [34-36]. Changes in chemical signals due to interactions, the presence of an analyte in very small amounts converting to electrical signals are detectable by electrochemical methods [34-37]. High reproducibility and high stability in electrochemical methods, along with minimal consumption costs, have led to extensive use and integration of electrochemical science with biology and medicine in detection of analytes [14, 35-39]. Accuracy and high sensitivity of analyte detection using electrochemical methods have led to faster diagnosis and treatment of various diseases and an increase in the life quality of humans [14, 30-33].

Objective

In the present study, an aptasensor was employed to detect TnI with high sensitivity, specificity and stability for application in clinical samples and MI patients, and its utility was compared with the ELISA method.

Materials and Methods

All chemicals were purchased from Merck (Germany) or Scharlau (Spain), otherwise those stated. Deionized water was employed to prepare the solutions and rinsing. TnI from the human heart was prepared from Sigma (USA) and dissolved in deionized water. A lyophilized 76-mer aptamer was purchased from Bioneer (South Korea). The aptamer was thiolated at 5' end with a sequence of:

5'-(SH)-(CH_2)$_6$-AGTCTCCGCTGTCCTCCCGATGCACTTGACGTATGTCTCACTTTCTTTTTCATTGACATGGGATGACGCCGTGACTG-3'

Before immobilizing and self-assembling the aptamer on the surface of gold electrodes, its sulfhydryl group (-SH) was refreshed through 10 µL 1.0 mol L^{-1} dithiothreitol (DTT) from Sigma (USA) during 20 min. Then, it was purified by mixing and shaking with 150 µL ethyl acetate for four times, while the upper layer was discarding. Aptamer concentration throughout the work was 10.0 µmol L^{-1} dissolved in 10 mmol L^{-1} phosphate buffer saline containing 5.0 mmol L^{-1} NaCl and 2.0 mmol L^{-1} KCl and 1.0 mmol L^{-1} $MgCl_2$, pH 7.4. Screen printed electrodes were received from DropSens (Spain) and used in an experiment to find the best aptamer immobilization time on the gold surface, while a gold disk (Au) electrode was employed for other studies. Open circuit potential (OCP) was measured over time during the aptamer immobilization on the gold working surface of the screen printed electrodes by a Mastech MS8340B digital multimeter (China). The achieved optimized immobilization time (80 min) was applied in all aptamer immobilization steps at 4 C.

The Au electrode with a diameter of 2 mm was polished on a sand paper and an alumina powder-imbued pad, respectively, followed by ultrasonication in a 3:1 ethanol/water mixture in an ultrasound bath. After each immobilization period, to arrange the immobilized aptamers and block uncovered parts of the electrode surface, 10 µL 1.0 mmol L^{-1} of a 6-mercapto-1-hexanol solution was dropped on the surface, kept at room temperature for 40 min, and then rinsed. The obtained electrode was denoted as the TnI biosensor.

TnI binding into the TnI biosensor surface was done at 37 C for 30 min. This time was obtained by optimization at a TnI concentration of 1 ng mL^{-1}. A three-electrode system was used in voltammetric measurements using the TnI biosensor, a platinum wire and an Ag/AgCl, 3 mol L^{-1} KCl as the working, counter and reference electrodes, respectively. A redox marker solution of Tris-HCl buffer containing 0.5 mol L^{-1} KCl and 0.5 mmol L^{-1} potassium ferrocyanide was employed. All electrochemical signals were assigned to the anodic peak of oxidation of ferrocyanide. A µ-Autolab potentiostat/galvanostat (the Netherlands) was used and ran by GPES 4.9 software.

Differential pulse voltammograms (DPVs) were recorded with a pulse width of 25 mV, a pulse time of 50 ms, and a scan rate of 10 mV s^{-1}. 89 samples of human serum including 48 patients with MI and 41 healthy people were firstly analyzed using a TnI specified ELISA kit from Monobind (USA). Then evaluated by the TnI biosensor; the results were then compared.

Results

In order to find the best time for immobilization of the aptamer on the surface of gold, OCP changes were recorded during the aptamer immobilization, as shown in Figure 1. Changes in OCP occurred due to the immobilization of the (negatively) charged aptamer on the surface, and the self-assembling process of the aptamer was progressively continued until ~80 min. After this time, no significant change was observed. Consequently, this time was considered as the optimized time value for immobilization of the aptamer on the surface of the Au electrode for further studies.

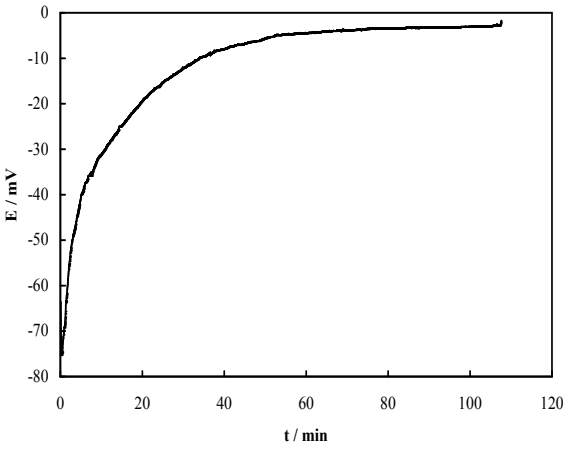

Figure 1: OCP changes during the aptamer immobilization on the gold surface

One of the important experimental parameters in designing a biosensors is finding the optimized binding time between the bioreceptor and the analyte. To find the best time of the aptamer capturing TnI, a concentration of 1 ng mL^{-1} of TnI was bound into the TnI biosensor at 37 C, and DPVs were recorded at different binding times. The data are presented in Figure 2. The peak current was continuously decreased upon prolonged binding and reached a stable value after 40 min. Therefore, this time was selected for TnI binding into the biosensor.

The proficiency of the TnI biosensor toward different TnI concentrations was evaluated by recording DPVs after binding the TnI biosensor into various concentrations of TnI; the data are presented in Figure 3A. A related calibration plot for the current TnI relationship is also shown in Figure 3B. The TnI biosensor could detect the target in a range of 0.03 to 2.0 ng mL^{-1}, with a regression equation of y=-(1.402±0.045)x + (2.4887±0.040), R2=0.9991. The limit of detection (LOD) of the TnI biosensor was obtained as a TnI concentration that generated a signal equal to 3SD, where SD is the standard deviation value of the blank signal.

Details of the TnI biosensor fabrication steps are shown in Figure 4.

In order to evaluate the preparation reproducibility of the TnI biosensor, it was fabricated six times and the related DPVs were recorded in the absence of TnI; the data are shown in Figure 5. A Change in the peak current of the TnI biosensor for the preparation reproducibility had a relative standard deviation (RSD) of about 8.3%.

To inspect the regeneration of the TnI biosensor, we firstly recorded a DPV.Then, we bound it with 1.0 ng mL^{-1} TnI and the related DPV was measured. After these steps, the TnI

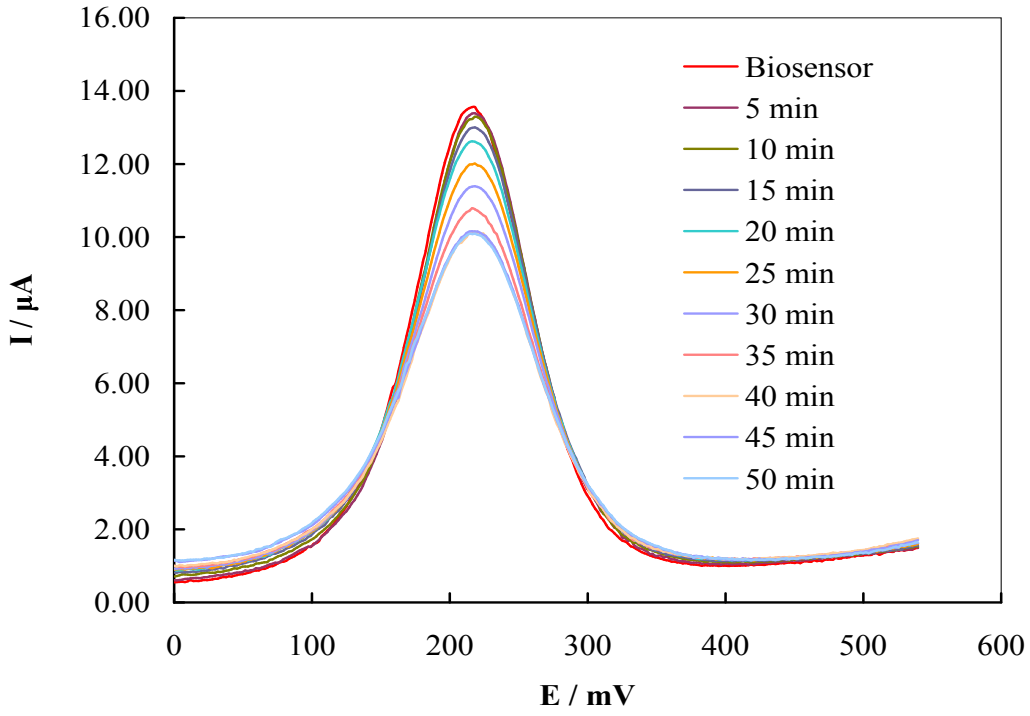

Figure 2: DPVs recorded using the TnI biosensor at 37 °C before (DPV with the highest peak current) and after binding with 1 ng mL^{-1} of TnI at different binding times

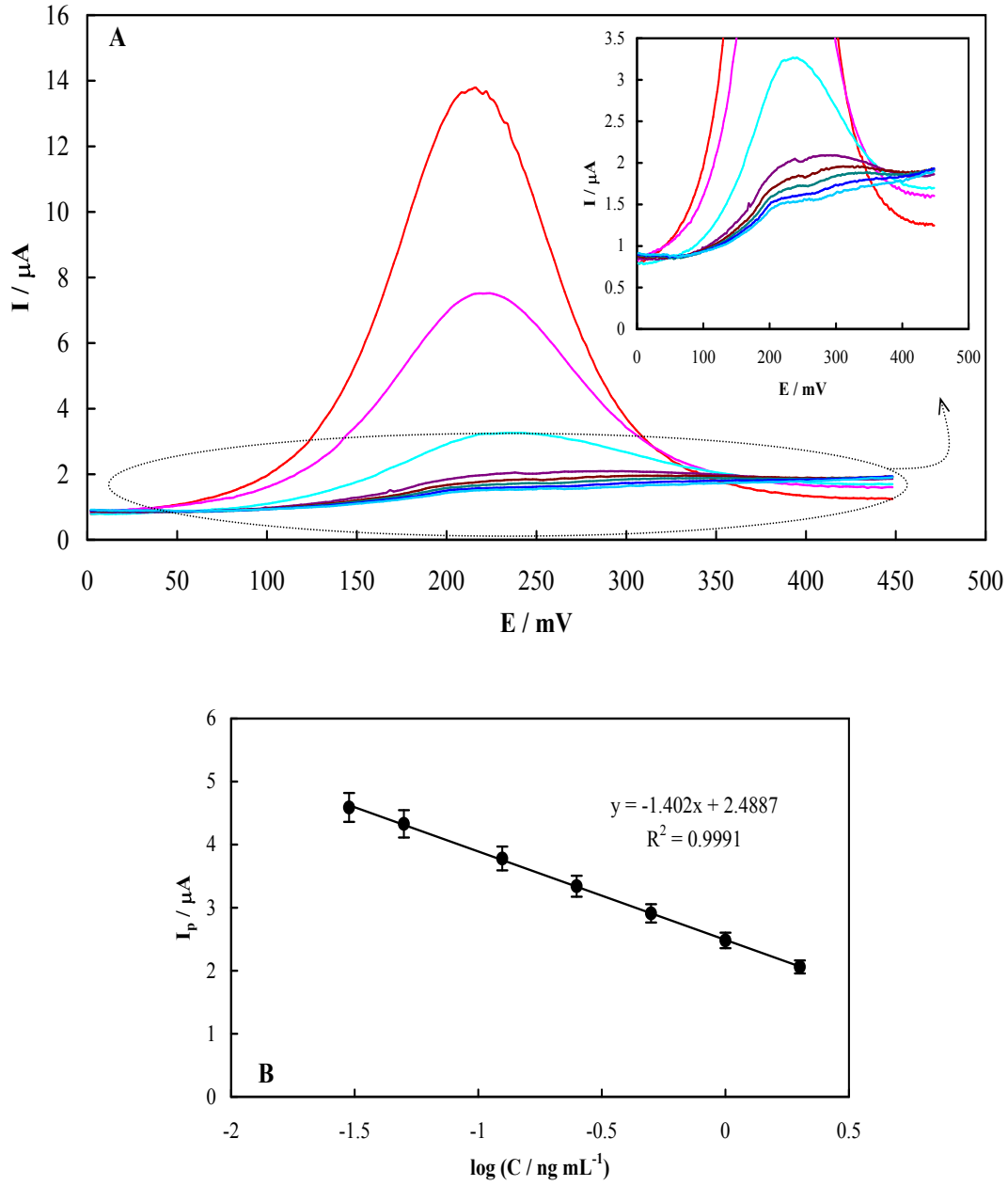

Figure 3: DPVs recorded using the TnI biosensor before (DPV with the highest peak current) and after binding with different concentrations of TnI. B) A calibration curve derived from DPVs presented in panel (A)

biosensor was kept at 95 C for 5 min to separate the bound TnI, and this procedure was repeated 6 times. The obtained data are presented in Figure 6. A RSD value of 7.8% was obtained, reflecting regeneration ability of the biosensor.

In order to evaluate the stability of the TnI biosensor, we bound it with 1.0 ng mL^{-1} TnI, and DPVs were measured within several consecutive days. The TnI biosensor signals are presented in Figure 7. It was revealed that they underwent irregular changes and remained

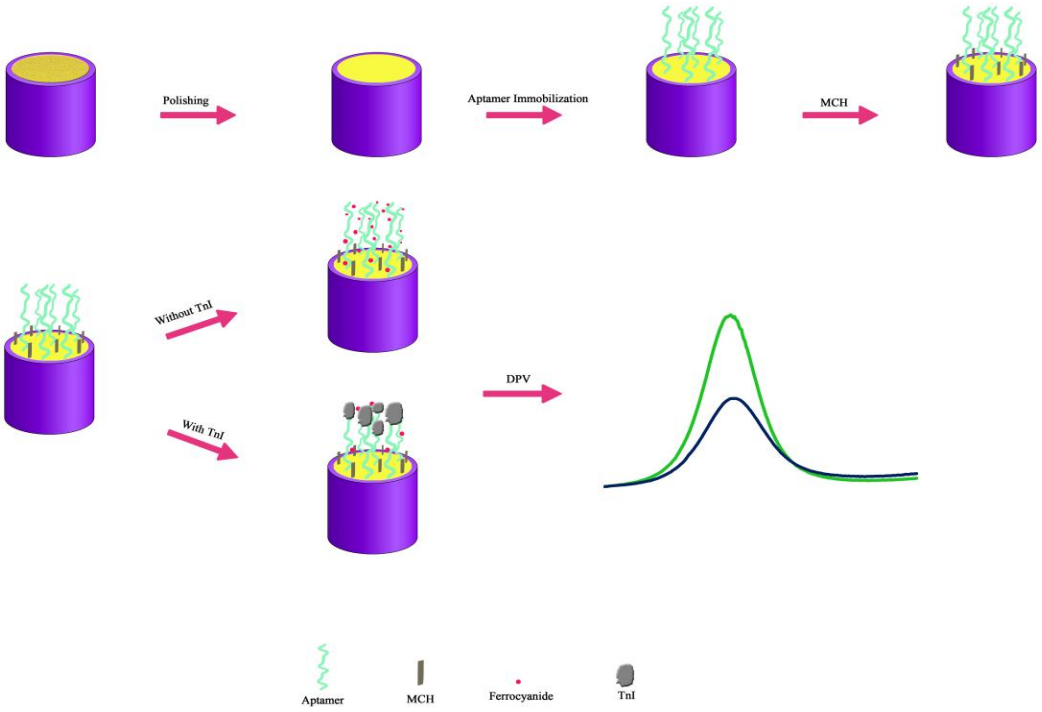

Figure 4: The fabrication and working principals of the TnI aptasensor

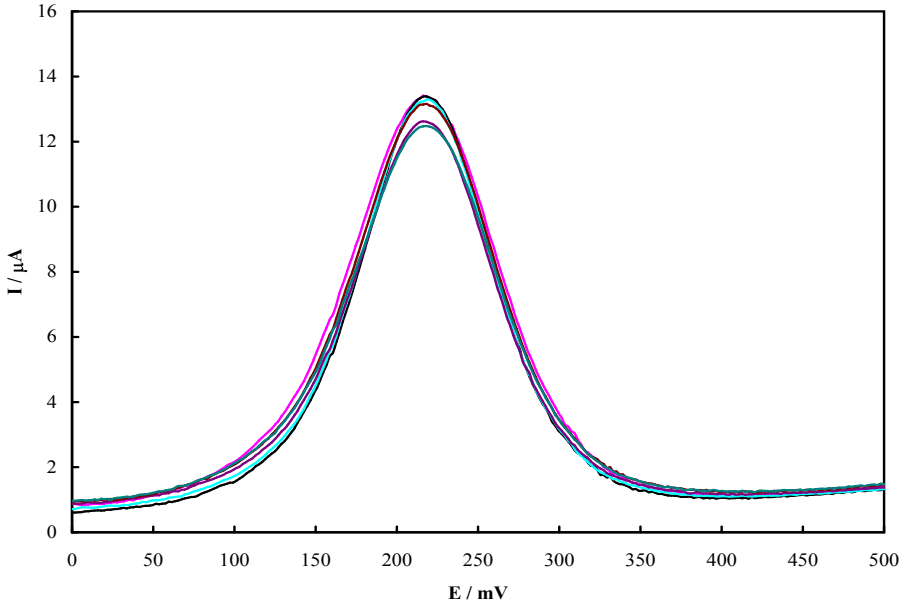

Figure 5: DPVs recorded using the TnI biosensor after six times of fabrication to evaluate the preparation reproducibility

stable at least for 25 days. Therefore, 25 days were the stability time of the TnI biosensor.

The selectivity of the TnI biosensor was followed by evaluation of interfering effects serum albumin of human, heparin, hemoglobin, EDTA and bilirubin in the presence of TnI. The interfering species were evaluated in three concentrations of 0.5, 5.0 and 20 ng mL^{-1}, or 0.5 ng mL^{-1} TnI. The obtained results are presented in Figure 8. according to the results, the interfering effects of these species on the TnI biosensor signal was negligible.

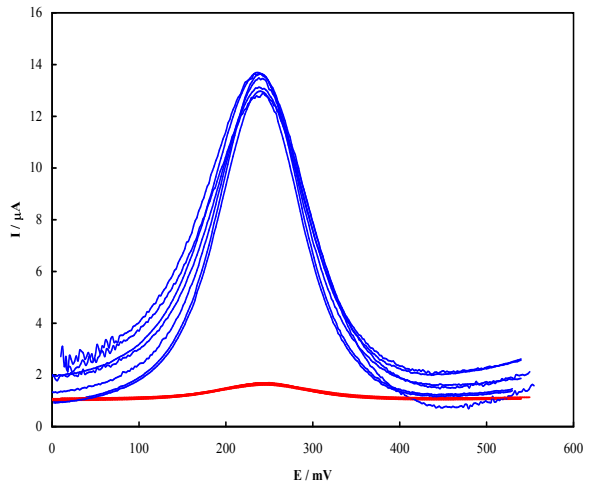

Figure 6: DPVs recorded using the TnI biosensor before (blue curves) and after binding with 1 ng mL^{-1} of TnI (red curves) upon 6 cycles of releasing the bound TnI and regeneration

89 serum samples from 48 MI patients and 41 healthy people were evaluated for the TnI level using the TnI biosensor, and the results were compared with the obtained by an ELISA method. The quantitative results of the experiment are displayed in Table 1, indicating that there were 5 false positive results without false negative results. Therefore, the TnI biosensor had a diagnostic specificity of 81% with a diagnostic sensitivity of 100%.

Discussion

Aptasensors with low design and low construction costs, high accuracy and rapid diagnostic performance and high reproducibility capabilities, can be reliable candidates for optimal medical diagnostics in the future. So far, several electrochemical biosensors have been designed to detect TnT that most of them have been based on immunological methods and the use of antibodies. In this research, a specific aptamer with a high affinity against TnI was used in the structure of an electrochemical biosenso; in order to understand the actual performance of the designed aptasensor, it was also evaluated by several groups of real samples. LOD was obtained as 10 pg mL^{-1} which is better or comparable with the technique already reported (Table 2).

In this study, the designed aptasensor could successfully detect TnI accurately with high specificity. Also, the real human serum samples evaluated with the aptasensor and the achieved results were successful. Interfering agents had very little effect on the performance of this aptasensor. Other advantages of it were the rapid and inexpensive early diagnosis of MI.

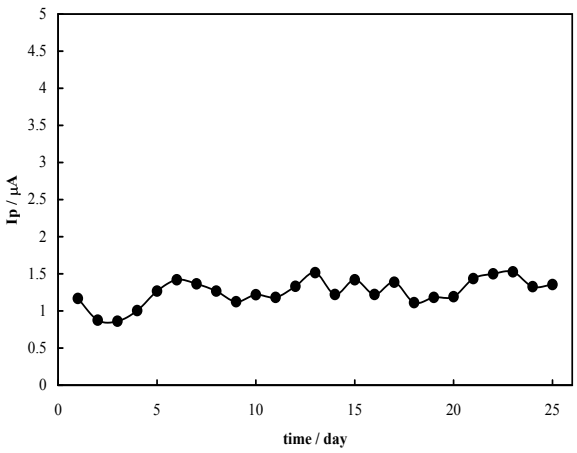

Figure 7: Changes in the peak currents in DPVs recorded within consecutive days using the TnI biosensor after binding with 1.0 ng mL^{-1} TnI

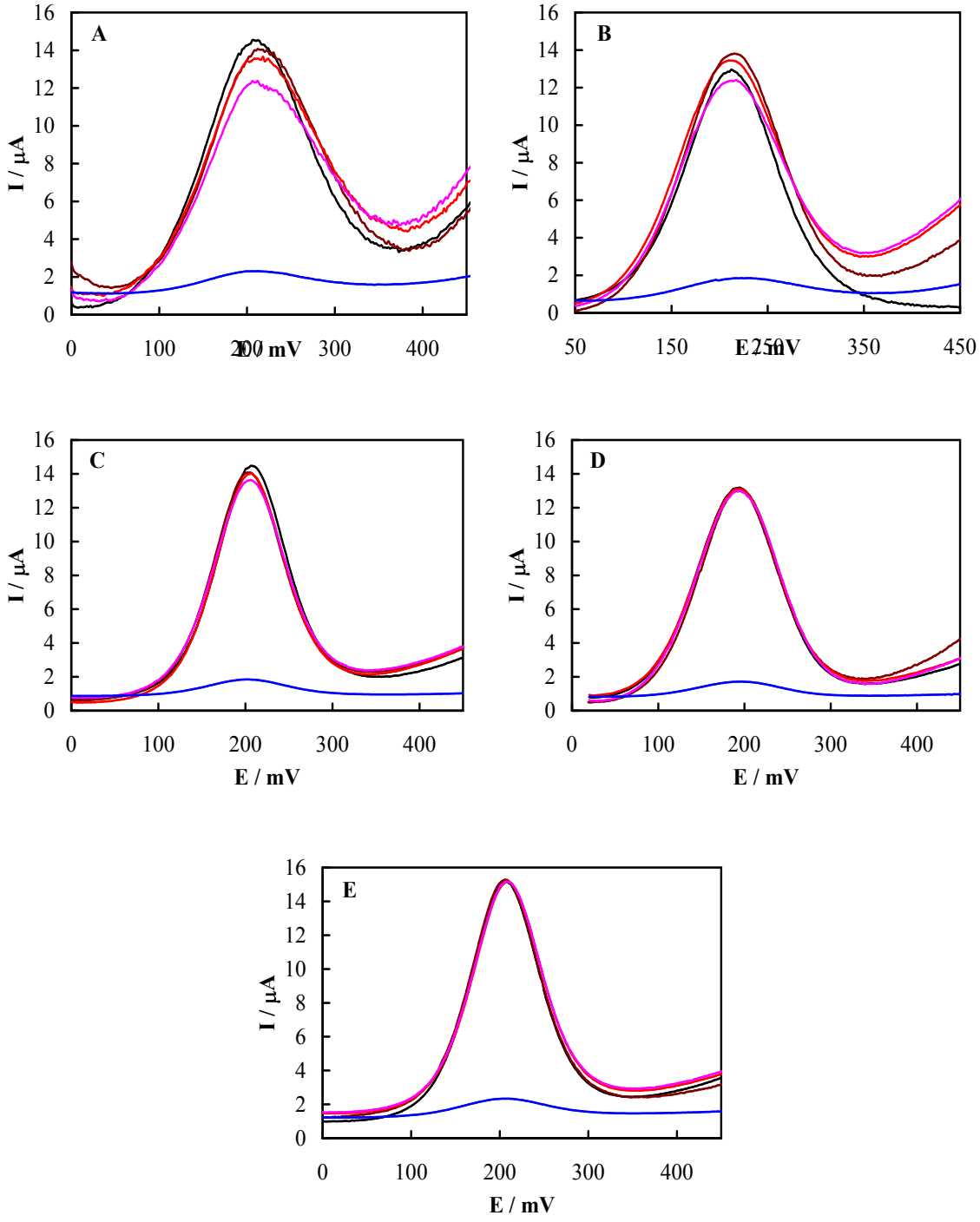

Figure 8: DPVs recorded using the TnI biosensor before (DPV with the highest peak current in all panels) and after binding with 0.5, 5.0 and 20 ng mL^{-1} of human serum albumin (A), hemoglobin (B), heparin (C), EDTA (D) and bilirubin (E), or 0.5 ng mL^{-1} TnI (DPV with the lowest peak current in all panels)

Table 1: TnI level (ng mL^{-1}) in human samples determined by ELISA and the TnI biosensor.

Sample number	TnI levels by ELISA	TnI levels by TnI biosensor
Negative samples-Negative results		
1	0	0.02
2	0	0.07
3	0	0.05
4	0	0.05
5	0	0.02
6	0.1	0.17
7	0.1	0.12
8	0.2	0.15
9	0.2	0.18
10	0.2	0.11
11	0.2	0.25
12	0.2	0.28
13	0.2	0.10
14	0.2	0.20
15	0.2	0.26
16	0.2	0.21
17	0.3	0.28
18	0.3	0.24
19	0.4	0.28
20	0.4	0.45
21	0.4	0.31
Negative samples-Positive results (false positive)		
22	0.1	0.61
23	0.3	0.58
24	0.3	0.66
25	0.4	0.50
26	0.48	0.55
Positive samples-Positive results		
27	0.5	0.55
28	0.5	0.79
29	0.5	0.66
30	0.5	0.60
31	0.6	0.67
32	0.7	0.69
33	0.8	0.66
34	0.8	0.85
35	0.9	0.84
36	1	1.10
37	1	1.00
38	1.1	1.15
39	1.1	1.08
40	1.1	1.23
41	1.2	1.23
42	1.3	1.37
43	1.4	1.25
44	2	2.11
45	2.1	2.66
46	2.9	2.44
47	3	3.04
48	3	3.02
49	3.2	3.43
50	3.2	3.40
51	4.3	4.39
52	4.6	4.58
53	5	4.19
54	5.1	5.28
55	5.6	5.73
56	6.0	5.83
57	6.5	6.92
58	8.4	8.10
59	8.6	8.33
60	9.4	9.93
61	9.5	9.16
62	9.6	9.72
63	9.8	9.98
64	10.7	13.1
65	11.7	11.9
66	11.8	11.3
67	12.6	12.9
68	12.9	12.4
69	14	14.4
70	14.9	14.9
71	15.4	14.0
72	16.3	15.3
73	21.3	22.2
74	24.7	21.5
75	28.7	26.6
76	29.2	27.9
77	31.8	31.4

78	32.1	34.2
79	35.4	35.2
80	38.9	37.6
81	41.4	45.0
82	49.8	45.5
83	53.9	54.6
84	55	54.9
85	68.7	68.6
86	68.7	68.3
87	73.7	80.9
88	84.4	78.4
89	84.8	83.5

Conclusion

Technological advances have led to development of miniaturized devices for the diagnosis of MI. TnI is a specific biomarker for MI which is immediately released into the bloodstream after any damages to the cardiac myocytes. In this research, a molecular detection method for TnI was provided which could successfully diagnose MI with high accuracy in a short time. The simple and rapid establishment principles, high repeatability, extreme molecular specificity against TnI and reasonable, and optimization of costs were some of the specific features of the designed aptamer-based biosensor. In the future of molecular

Table 2: A comparison between various TnI aptamer-based biosensors.

Aptamer sequence	Detection Techniques	Transducer	Detection Range	LOD	Ref
1	Electrochemiluminescence	Gold	8.0×10-13^{-1}.0×10^{-11} g mL^{-1}	0.3 pg mL^{-1}	(Liu et al., 2017)
2	DPV, Cyclic voltammetry	Gold	0.05-500 ng mL^{-1}	8.0 pg mL^{-1}	(Negahdary et al., 2017)
3, 4	Cyclic voltammetry, Impedance spectroscopy	Carbon	0.024-2.4 ng mL^{-1}	24 pg mL^{-1}	(Jo et al., 2017)
5	Impedance spectroscopy	Gold	0-10 µg mL^{-1}	0.34 µg mL-1	(Wu et al., 2010)
6-11	Square wave voltammetry	Gold	1-10 000 pmol L^{-1}	1.0 pM	(Jo et al., 2015)
12	DPV	Gold	0.03 to 2.0 ng mL^{-1}	10 pg mL^{-1}	This work

1. 5'-CGT GCA GTA CGC CAA CCT TTC TCA TGC GCT GCC CCTCTT A-NH_2-3'

2. 5'-(SH)-$(CH_2)_6$-AGTCTCCGCTGTCCTCCCGATGCACTTGACGTATGTCTCACTTTCTTTTCATTGACATGGGATGACGCCGTGACTG-3'

3. 5'-Amine-CGTGCAGTACGCCAACCTTTCTCATGCGCTGCCCCTCTTA-3'

4. 5'-Phosphate-CGCATGCCAAACGTTGCCTCATAGTTCCCTCCCCGTGTCC-3'

5. FYSHSFHENWPS-Cys

6. 5'TCACACCCTCCCTCCCACATACCGCATACACTTTC TGATT3'

7. 5'CCCGACCACGTCCCTGCCCTTTCCTAACCTGTTT GTTGAT3'

8. 5'ATGCGTTGAACCCTCCTGACCGTTTATCACATACT CCAGA3'

9. 5'CGTGCAGTACGCCAACCTTTCTCATGCGCTGCCC CTCTTA3'

10. 5'CAACTGTAATGTACCCTCCTCGATCACGCACCACT TGCAT3'

11. 5'CGCATGCCAAACGTTGCCTCATAGTTCCCTCCCC GTGTCC3'

12.5'-(SH)-$(CH_2)_6$-AGTCTCCGCTGTCCTCCCGATGCACTTGACGTATGTCTCACTTTCTTTTCATTGACATGGGATGACGCCGTGACTG-3'

diagnostics for MI, this TnI biosensor can be used as an alternative technique for diagnosis of myocardial infarction.

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Conflict of Interest

The authors declared no potential conflicts of interests with respect to the authorship and/or publication of this paper.

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