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RESEARCH PAPER

Fabrication of an immunosensor for early and ultrasensitive determination of human tissue plasminogen activator (tPA) in myocardial infraction and breast cancer patients

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Abstract

Sensitive detection of biomarkers will mean accurate and early diagnosis of diseases. A tissue plasminogen activator (tPA) has a crucial role in many cardiovascular diseases and it is related to many processes such as angiogenesis in cancer cells. Therefore, sensitive determination of tPA is important in diagnosis and clinical research. tPA monoclonal antibody was covalently attached onto single-wall carbon nanotubes (SWCNTs) using diimide-activated imidation coupling. Functionalized SWCNTs were immobilized onto a glassy carbon electrode and the modification process was investigated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), SEM, cyclic voltammetry (CV), and electrochemical impedance spectroscopy (EIS). Cyclic voltammograms (CVs) in a scan rate of 100 mVs^{-1} was studied and comparisons were made between the modified glassy carbon electrodes (immobilized with antibodies) as a working electrode before and after the formation of tPA-antibody complex. Results of the SDS-PAGE demonstrated that the antibody was covalently and site directly attached to the SWCNTs. The fabricated biosensor provided a good linear response range from 0.1 to 1.0 ng mL^{-1} with a low detection limit of 0.026 ng mL^{-1}. The immunosensor showed selectivity, reproducibility, good sensitivity, and acceptable stability. Satisfactory results were observed for early and sensitive determination of tPA in human serum samples. For the first time, such specific biosensor is currently being fabricated for tPA in our laboratories and successfully could determine tPA in myocardial infraction and breast cancer patients.

Keywords Antibody functionalized SWCNTs · Biosensor · Cyclic voltammetry · Tissue plasminogen activator (tPA)

Abbreviations

CV	Cyclic voltammetry
Da	Dalton
EDC	*1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide*
DDW	Double-distilled water
GCE	Glassy carbon electrode
NHS	*N*-Hydroxy succinimide
EIS	Impedance spectroscopy
Ab	Monoclonal antibody
PBS	Phosphate buffer solution
MWCO	Por molecular weight cutoff
SEM	Scanning electron microscope
SWCNTs	Single-wall carbon nanotubes
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
tPA	Tissue plasminogen activator

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Introduction

Detection of most biomarkers that related to different stages of disorder and disease may facilitate early diagnosis and therapy. Determination of biomarkers has attracted increasing attention in biomedical fields, including disease diagnosis, monitoring therapy, and assessing the progress of the disease [1].

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Human tissue plasminogen activator (tPA) is a serine protease that plays an important role in cancers and cardiovascular diseases, because its concentration increases in the breast cancer and myocardial infarction diseases [2, 3]. Therefore, sensitive determination of tPA is necessary in early diagnosis of cancers and cardiovascular diseases. Successful utilizing of biomarkers in diagnostic and health care depends on sensitivity, selectivity, and accuracy of applied techniques that allow the early and rapid detection of biomarkers [4, 5]. Currently, biomarkers are detected by molecular techniques such as proteomics, glycomics and genomics, HPLC, and enzyme-linked immunosorbent assays (ELISA) [6–8]. While reliable, these techniques have limitations for clinical use, including cost, difficulty in multiplexing, low sensitivity, and time consuming. Advances in biomarker elucidation currently focus on several areas including developing new methods for quantitation of a specific interaction event through electrochemical measurements [7, 9] and surface plasmon resonance [10, 11].

Fabrication of nanobiosensors has been widely reported as an inexpensive and sensitive method to detect a variety of biological analytes [12, 13]. Carbon nanotube (CNT)-based electrodes have been commonly applied because of their biocompatibility, low cost, and good electron transfer kinetics [14, 15]. CNTs possess good properties such as the existence of unoccupied π orbitals at the surface for sensing applications and the high surface area. This makes CNTs extremely sensitive to any analyte [16, 17]. Single-wall carbon nanotubes (SWCNTs) are nanoscale wires showing good properties for miniature biosensor devices. Also, the SWCNT electrical characteristics are sensitive to changes in the electrostatic environment and surface charge transfer, undergoing drastic changes by simple modification [18–20]. SWCNT-based immunosensors have attracted attention due to the high selectivity and sensitivity, low price, extensive surface area, safety, excellent mechanical properties, and fast electron transfer capabilities in the clinical studies [21]. Currently, selective and specific recognition of proteins and other kinds of biomarkers in the clinical laboratory is often accomplished by kits and methods based on monoclonal antibodies. Usually, ultra-low concentration of cardiovascular and cancer biomarkers is present in the primary stages of the disease. Therefore, ultrasensitive monitoring of biomarker needs the development of new detection methods for signal detection and amplification [22].

Immunosensors are important analytical tools that combine the high sensitivity and selectivity of immunoreaction with convenience of diverse transducers [23]. Based on a transducer, there are four types of immunosensor: optical, electrochemical, thermometric, and microgravimetric devices. For fabrication, an immunosensor using SWCNTs and a bioreceptor, as well as a signal transducer, need to be introduced. To endow SWCNTs with a bioreceptor function, functionalization is done to immobilize proteins, antibodies, enzymes, or antigens onto the SWCNTs [24–26]. The attachment of an antibody to a carboxylate group on a SWCNTs via 1-ethyl-3-(3 dimethylaminopropyl) carbodiimide hydrochloride (EDC)/*N*-hydroxysuccinimide (NHS) coupling would likely result in predominantly horizontal or slanted orientation of an Ab and some Abs may even be attached via NH2 groups at the biorecognition sites of Ab rendering them inactive towards antigens. Hence, a better choice of the Ab attachment to carbon nanotubes may be by activating COOH groups which are present at the highest concentration at the bottom of the stem of an Ab and bind them to an amino-terminated modification of the CNT substrate [27]. Electrochemical-based immunosensors have been utilized as a major analytical tool in the clinical studies for detecting biomarkers such as transferrin, carcinoembryonic antigen, carcinoma antigen 125, IL-6, prostate-specific antigen (PSA), and alpha-fetoprotein [1, 17].

In this paper, for the first time, an electrochemical immunosensor for tPA was fabricated simply by immobilization of anti-tPA on SWCNTs/glassy carbon electrode (GCE) by EDC/NHS couple. Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were used in monitoring the modification process of the electrode. The fabricated biosensor has been successfully used in the quantitative detection of early myocardial infraction and cancer prediction (Fig. 1).

Experimental

Materials

Monoclonal antibody and tPA were purchased from Abcam and Cathflo companies, respectively. NHS, SWCNTs, EDC, and other chemicals were purchased from commercial sources like Merck. K_4 [Fe $(CN)_6$], K_3[Fe$(CN)_6$], phosphate buffer solution (PBS), and other chemicals used were of analytical grade. Solutions were freshly prepared with double-distilled water just prior to use, and all experiments were carried out at room temperature. tPA protein was dissolved in 0.1 M PBS. PBS, pH 7.4, containing 2.5 mM [Fe $(CN)_6$] and 0.1 M KCl (1:1) was used as the detection solution.

Apparatus

A potentiostat/galvanostat instrument (Autolab PGSTAT302 N) was used for measurements of EIS and cyclic voltammetry (CV), and data were recorded using the software Nova version 1.7. The employed three-electrode system consists of the working electrode (a modified glassy carbon electrode), the counter electrode (a platinum wire auxiliary electrode), and the reference electrode (Ag/AgCl electrode) in a 2.5-mM $[Fe(CN)_6]^{4-/3-}$ solution. Antibody attachment to SWCNTs and scanning electron micrographs (SEM) of the electrode surfaces were investigated with the sodium dodecyl

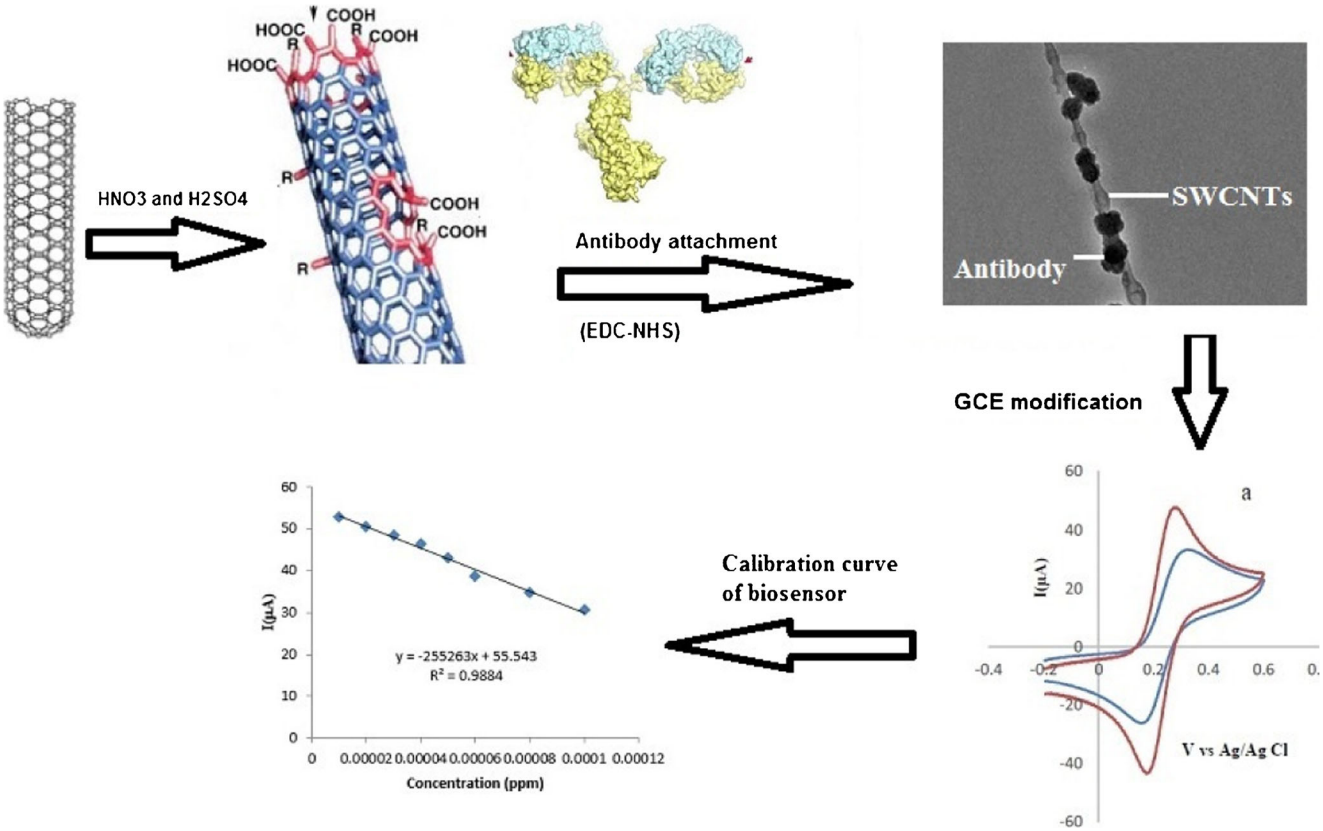

Fig. 1 Schematic illustration of the fabricated biosensor for determination of tPA

sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the VEGA TESCAN instrument, respectively.

Purification and activation of SWCNTs

SWCNTs were soaked in concentrated HCl for 24 h, centrifuged at 12000 rpm, and washed with double-distilled water (DDW) for five times to neutralize pH. The SWCNT sample was suspended in a mixture of concentrated HNO_3 and H_2SO_4 (*v*/*v*, 1:3), sonicated for 6 h, and followed by centrifugation at 12000 rpm for 30 min. The precipitated SWCNTs were extensively washed in DDW and dried in a vacuum.

SWCNT functionalization

Covalent and site-directed immobilization of the tPA monoclonal antibodies to the COOH groups of SWCNTs was performed using carbodiimide/succinimide reaction (EDC-NHS), as reported previously [17, 27, 28]. Basically, the purified SWCNTs (2 mg) in the last section were incubated in a solution containing a 4:1-M ratio of EDC and *N*-hydroxysuccinimide ester (NHS-ester). Afterwards, the SWCNT-activated carboxylic groups were left to react with the tPA monoclonal antibody (Abcam, 10 μg mL^{-1} solution prepared in 0.1 M phosphate buffer pH 7.2 (PB)) at 4 °C overnight. Thus, the activated carboxylic groups of SWCNTs reacted with terminal NH_2 groups of the antibody and form a stable covalent peptide bond.

Gel electrophoresis

SDS-PAGE was performed based on the Saify et al. method [2, 29] and run under reducing and non-reducing conditions (5% β-mercaptoethanol). The starting activated SWCNT material and the final Ab–SWCNTs were dialyzed using dialysis membranes (Breda, the Netherlands; molecular weight cutoff

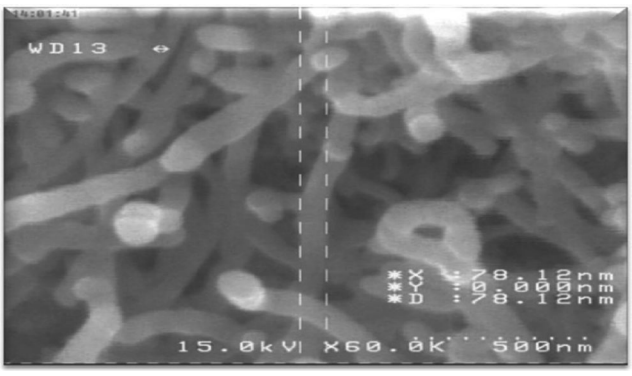

Fig. 2 The SEM image of SWCNTs on GCE

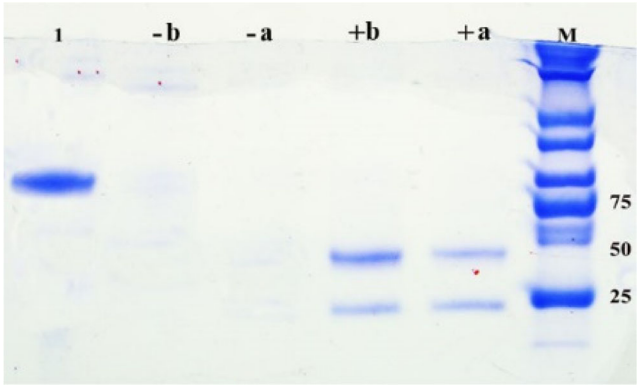

Fig. 3 SDS-PAGE of antibody–SWCNT conjugate under non-reducing conditions (−β Me, β-mercaptoethanol) and reducing conditions (+β Me). Lane 1: pure tPA antibody as a control; lanes b: antibody–SWCNT conjugate before dialysis; lanes a: antibody–SWCNT conjugate after dialysis. M is a protein molecular ladder

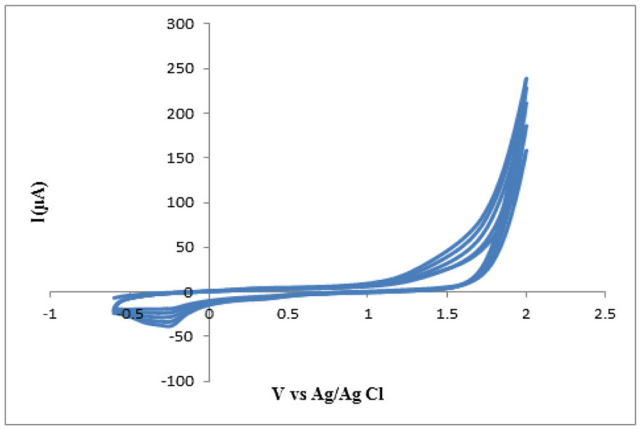

Fig. 4 Electrochemical activation of GCE surface using sulfuric acid

12–14,000 and 300,000 Da). The gel was stained with Coomassie Briliant Blue and photographed.

Immunosensor preparation and calibration

The working electrode (GCE) was completely polished using 1.0, 0.3, and 0.05 μm alumina slurries on pads, rinsed five times with DDW, and followed by sonication in DDW, ethanol, and DDW each for 5 min [28]. For preparation of the immunosensor, 15 μL of tPA-IgG-modified SWCNT solution was dropped onto the dried tip of the electrode, which then dried in the room temperature and rinsed with 2 mL washing buffer (20 mM phosphate buffer, pH 7.4) for 5 min to eliminate any weak or partially immobilized antibody SWCNTs. The fabricated immunosensor was carefully blocked with 1% BSA in PBS for 30 min to avoid any non-specific adsorption on the possible remaining free spaces and active sites between the antibody and SWCNTs. The prepared immunosensor was rinsed with DDW and incubated with different concentrations of standard tPA in a phosphate buffer solution (20 mM, pH 8.0); related CVs were recorded and, subsequently, calibration curve was drawn.

tPA assay in real samples

In order to test the applicability of the fabricated immunosensor for monitoring of real samples, serum samples were taken from the Bu-Ali Hospital (Hamadan, Iran); 1.0 mL of collected serum was diluted to 100 mL by PBS (pH 7.4). Initially, tPA was completely removed from the serum sample (to use as control experiment; a real sample with zero tPA) using specific designed affinity chromatography [2, 29]. Then, 20 mL of the tPA-free diluted serum was transferred to the electrode cell, different amounts of tPA were added, and the recovery percent was recorded by the CV technique. Moreover, in order to investigate the applicability of fabricated biosensor for monitoring tPA in myocardial infarction and breast cancer patients, five breast cancer patients at the age of 47–50 years (Khatam ol Anbia hospital, Iran) and five myocardial infarction patients at the age of 47–50 years old were identified by a physician (Besat Hospital, Iran). Blood samples were taken from a hand vein catheter and 3 mL blood was collected in EDTA-containing tubes. In addition to determination of tPA using a fabricated biosensor, similar determination in real samples was performed by HPLC and ELISA methods. The ELISA test was performed using the Human Tissue Type Plasminogen Activator ELISA Kit (Abcam, USA), according to the manufacturer's instructions. The HPLC method, consisted of a gradient system, was carried out according to the Hamidi et al. [30] protocol. The tPA detection was performed using a Symmetry 300 C_4 protein analysis column (pore size 300 Å; 50 × 4.6 mm; particle size 5 μm; Waters, USA) and a UV detector in a wavelength of 220 nm (model 746, Waters, USA).

Results and discussion

Characterization of the immunosensor

SEM

The morphology of SWCNTs on the working electrode was characterized with SEM. The SEM micrograph in Fig. 2 indicates that an appropriate layer of SWCNTs in nanoparticle size is deposited on the surface of the electrode. As seen, the SWCNTs were homogeneously dropped on the surface of electrode, and they were well dispersed and remained relatively stable without aggregation.

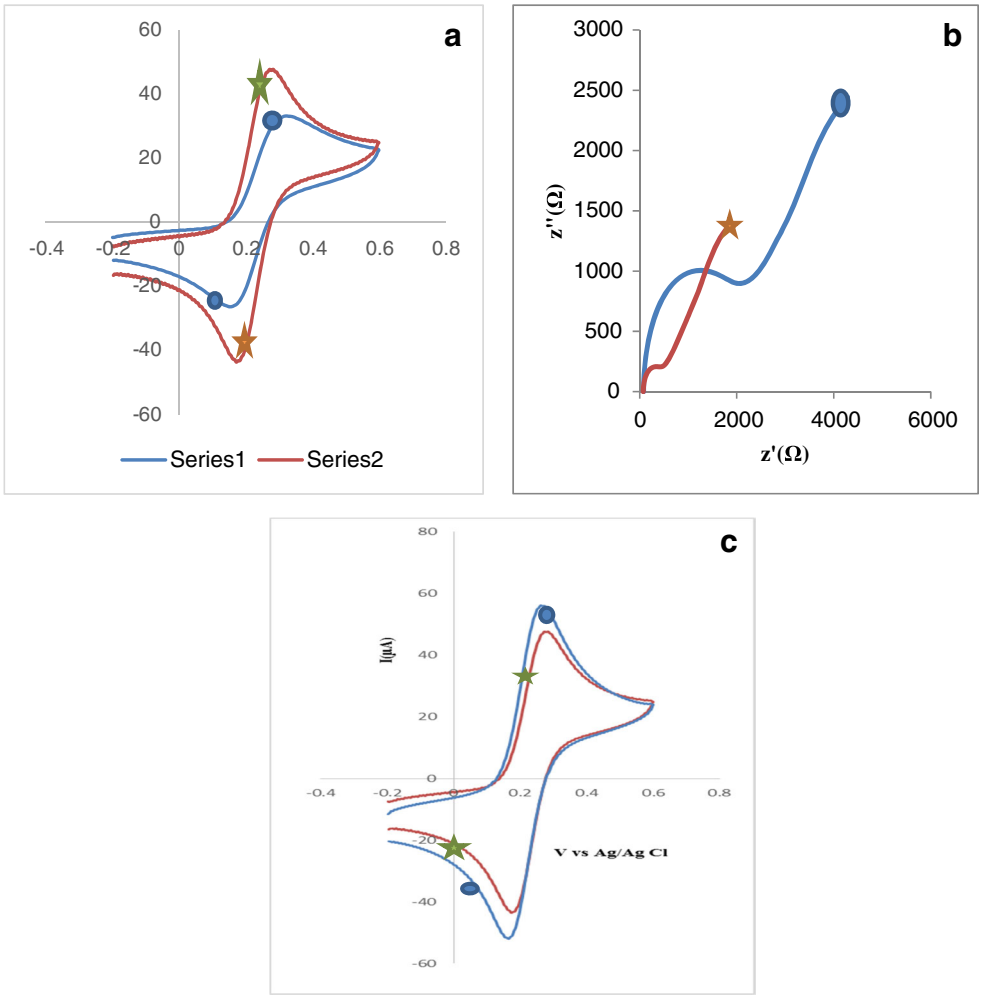

Fig. 5 Electrochemical characterization of the electrode modification process. **a** Cyclic voltammograms of bare GCE (curve blue/circle) and antibody/SWCNTs/GCE (curve red/star). **b** Nyquist plots of bare GCE (curve blue/circle) and antibody/SWCNTs/GCE (curve red/star). **c** Cyclic voltammograms of antibody/SWCNTs/GCE (curve blue/circle) and incubation in 0.04 ng mL^{-1} tPA (curve red/star) in 0.1 mol L^{-1} PBS (pH = 7.4) containing 0.1 mol L^{-1} KCl and 2.5 mmol L^{-1} $[Fe(CN)_6]^{3-/4-}$

Electrophoresis

SDS-PAGE electrophoresis was used to indicate covalent binding between SWCNTs and the tPA antibody. The free tPA antibody (≈ 75 kDa) was used as a control in the SDS-PAGE (Fig. 3, lane 1). Under non-reducing conditions, no SWCNTs entered the gel and no antibody was detected, which confirmed that the antibody was firmly immobilized on the SWCNTs, thus remaining in the loading well (Fig. 3, lanes −). Under reducing conditions, β-mercaptoethanol breaks disulfide bonds between cysteine residues of antibody fragments, so released antibody chains (heavy chain ≈ 49 kDa and the light chain ≈ 26 kDa) entered to the gel and a two-band pattern is appeared (Fig. 3, lanes +). Results suggest that the tPA antibody was covalently attached to SWCNTs and remains intact after conjugation during all experiment sections.

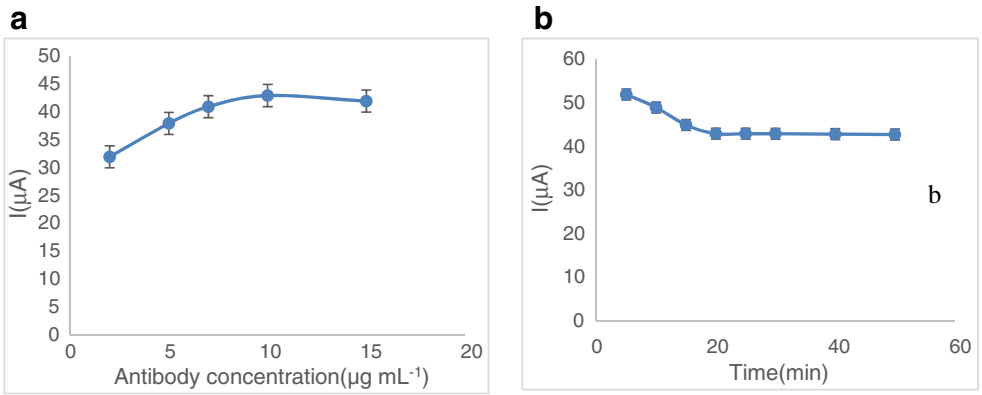

Fig. 6 Optimization of the experimental conditions for the fabricated biosensor. Effects of **a** SWCNTs modified with different concentrations of the antibody and **b** incubation time of 1.0 ng mL^{-1} tPA with modified electrode on the CV responses of antibody/SWCNTs/GCE

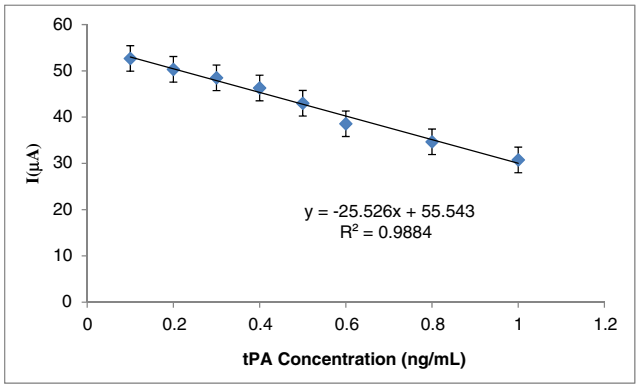

Fig. 7 CVs of modified electrode in 2.5 mM $[Fe(CN)_6]^{4-/3-}$ solution after incubation with different tPA concentrations (0.1 to 1.0 ng mL^{-1}) and obtained calibration curve for different concentrations of tPA

Electrochemical characterization of the modification process

Working electrode surface (GCE) was electrochemically activated using sulfuric acid (Fig. 4), the activation of the electrode depended on the properties of the examined GCE [31, 32], and then the antibody/SWCNT complex was dropped on the electrode surface.

The electrochemical modification process of GCE was performed in a 2.5-mM $[Fe(CN)_6]^{3-/4-}$ solution containing 0.1 M KCl (Fig. 5a). Recorded CVs of the modification process showed a couple of redox peaks for bare GCE with a peak-to-peak separation (ΔEp) of 0.117 V (Fig. 5a, curve blue/circle). When the GCE surface was coated with antibody/SWCNTs, a decrease of 0.078 V in ΔEp and an increase in peak current (Ip) were recorded (Fig. 5a, curve red/star). Comparing ΔEp of the bare electrode and modified electrode indicates that SWCNTs could boost the electron transfer rate between the electrode surface and $[Fe(CN)_6]^{3-/4-}$ solution.

Moreover, the modification process of the electrode surface was examined with the EIS, the frequency range 100 mHz to 100 kHz at 200 mV, in a probe solution of 2.5 mM $[Fe(CN)_6]^{4-/3-}$ containing 0.1 M KCl and 0.1 M PBS. Figure 5b displays the recorded Nyquist diagram of EIS corresponding to the GCE stepwise modification. There was a semicircle part at high frequencies and a linear part at low frequencies in the EIS of the bare GCE (Fig. 5b, curve blue/circle). The charge transfer resistance value for GCE was calculated according to Randles'equivalent circuit (R_{ct} = 418 Ω). When antibody/SWCNTs covered the GCE surface, an appropriate decrease was seen in the GCE resistance (Fig. 5b, curve red, R_{ct} = 76.8 Ω), implying that the SWCNT layer was an excellent electron conducting material and accelerated the electron transfer. Moreover, interaction and binding of tPA on the modified electrode indicated that a decrease in peak current (Ip) was recorded (Fig. 5c, curve red/star).

Table 1 Influence of interference molecules on selectivity of the fabricated biosensor

Interferences	Added (ng mL^{-1})	Found (ng mL^{-1})	RSD % (n = 5)
PAI	0.0	0.5	3.79
	3.125	0.6	3.77
	6.25	0.6	3.78
	12.5	0.6	3.73
	25	0.61	3.76
	50	0.63	4.30
	100	0.63	4.32
BSA	0.0	0.5	3.8
	3.125	0.5	3.7
	6.25	0.5	3.88
	12.5	0.6	4.01
	25	0.6	3.99
	50	0.65	4.22
	100	0.66	4.31
Plasminogen	0.0	0.5	3.64
	3.125	0.71	3.7
	6.25	0.71	3.62
	12.5	0.72	3.8
	25	0.73	3.82
	50	0.75	3.88
	100	0.75	4.01
Warfarin	0.0	0.5	3.82
	3.125	0.6	3.75
	6.25	0.6	3.85
	12.5	0.6	3.98
	25	0.64	4.3
	50	0.64	4.42
	100	0.66	4.6

Optimization of the experimental parameters of the biosensor

Optimization of antibody concentration

The influence of antibody concentration on the CV changes of SWCNTs/GCE was investigated, which was very effective on the sensitivity of the biosensor. Therefore, SWCNTs (2 mg) were incubated with different concentrations of antibody in the modification step and antibody/SWCNTs dropped on the electrode tip. As shown in Fig. 6a, with the increasing of antibody concentration, the CV of the SWCNTs/GCE decreased gradually and the CV value was inclined to best able when the antibody concentration was 10 μg mL^{-1}. Therefore, 10 μg mL^{-1} of antibody was applied as the optimal antibody concentration for the biosensor fabrication in this experiment.

Optimization of incubation time

The incubation time is an important factor for the formation of tPA-antibody complex, which affects the sensitivity of the fabricated biosensor. So, the incubation time of the fabricated biosensor was optimized using investigation of CV value change. For this purpose, the antibody/SWCNTs/GCE was incubated for different times (5–50 min) in the tPA solution

Table 2 Determination of tPA in serums and comparison with HPLC and ELISA methods ($n = 5$)

Method	Added (ng mL^{-1})	Found (ng mL^{-1})	RSD %	Recovery %	Detection limit (ng mL^{-1})	Analysis time
Biosensor	0.0	0.1	4.2	–	0.026	30–40 min
	0.5	0.48	3.8	96		
	1.0	1.01	4.3	101		
	10.0	10.04	4.01	100.04		
	50	49.21	3.9	98.42		
	100.0	98.01	4.1	98.01		
HPLC	0.0	NF	–	–	70	5 h
	0.5	NF	–	–		
	1.0	NF	–	–		
	10.0	NF	–	–		
	50	NF	–	–		
	100	94	11.2	94		
ELISA	–	0.09	4.3	–	0.163	4 h
	0.5	0.4	4.4	80		
	1.0	0.95	4.5	95		
	10	9.82	4.3	98.2		
	50	50.21	4.2	100.04		
	100	103.95	4.5	103.9		

NF data not found

(1.0 ng mL^{-1}). As shown in Fig. 6b, the biosensor response increased with increasing incubation time between 5 and 20 min and then CV response reached to a stable level after 20 min. Therefore, 20 min was considered as an optimized incubation time for later measurement of tPA using the fabricated biosensor.

Current response of immunosensor to tPA protein concentration

Binding of the analyte to the working electrode surface causes a change in the current response of the electrode [33]. Figure 7 shows the current changes of the immunosensor that incubated with various concentrations of tPA under the optimized conditions. Electrochemical characterization of the electrode indicated that the current response decreased with increasing tPA concentrations. Decreasing of current is due to binding of more tPA molecules to the biosensor surface at the higher tPA concentrations, which acts as a barrier against the electron transfer. A linear relationship between the ΔI and tPA concentrations was obtained in the range of 0.1 to 1.0 ng mL^{-1} (Fig. 7). The detection limit was 0.026 ng mL^{-1} at a signal/noise of 3 ($S/N = 3$) between the detection signal of low concentration samples and the noise of blank samples.

The efficiency of the fabricated immunsensor for the detection of tPA was compared with results of HPLC, ELISA, and other reported methods for tPA determination [32], which indicate that the fabricated immunosensor has advantages such as lower detection limit, more broad linear range, simplicity, and fast detection.

Reproducibility, repeatability, stability, and selectivity of the immunosensor

The repeatability of the immunosensor for determination of tPA was evaluated using five determinations with the same standard solutions of tPA and the same electrode. The relative standard deviation (RSD) for the response of electrode towards 0.5 ng mL^{-1} of tPA was 4.42%. The reproducibility of the electrochemical response of the immunosensor was also studied. Four electrodes were prepared and evaluated for detection of 0.5 ng mL^{-1} tPA solution. The RSD for the

Table 3 Comparing of tPA concentration in normal individuals and patients ($n = 5$)

Methods	Blood samples		
	Myocardial infarction (ng mL^{-1})	Breast cancer (ng mL^{-1})	Healthy (ng mL^{-1})
ELISA	15.69	41.31	9.49
RSD %	4.1	4.2	3.9
HPLC	14.79	42.13	8.23
RSD %	10.12	11.21	12.01
Biosensor	14.51	41.2	7.99
RSD %	3.73	3.82	3.61

responses between electrodes was 6.7%. The results show that the fabricated immunosensor has good repeatability and reproducibility for the determination of tPA. The fabricated immunosensor was incubated at 4 °C for 30 days (when not in use) and results demonstrated that the response of the stored biosensor retained over 95% of its initial value, indicating that the biosensor has an acceptable stability.

In order to study the selectivity of the fabricated immunosensor, tPA determination was performed in the presence of interference molecules. Interference molecules include enzymes that have the highest structural and functional similarity with tPA in the blood. Therefore, CV responses of the immunosensor, in a 0.5-ng mL^{-1} solution of tPA, to different concentrations of interfering substances were assayed. It was found that the biological concentration range of PAI, plasminogen, BSA, and warfarin did not affect the detection of tPA significantly (Table 1), so that the charge transfer resistance changes (R_{ct}) of biosensor unchanged after being incubated in the mixture of analyte and the interferences at equal concentrations.

Moreover, it was found that the 100 times of concentration of Na^{+}, Ca^{2+}, Zn^{2+}, Cu^{2+}, Cl^{-}, and SO_4^{-2} did not affect the detection of tPA (signal variation < 4%).

The tPA determination in real samples

In order to evaluate the possibility of the fabricated immunosensor for the serum sample analysis, tPA concentration was determined in human serum samples using the designed biosensor. After sample preparation and adequate dilution steps as described earlier, the CV method and a calibration curve by averaging five repeated measurements were applied for determination of tPA concentration in serum samples. The detection results showed that the recoveries were 96 to 101% and the RSD was 3.8 to 4.3% (Table 2). To evaluate the validity of the biosensor results, biosensor determination data were compared with results of ELISA and HPLC. Results demonstrated that in the present study, compared to the HPLC and ELISA methods, the fabricated immunosensor has a better ability such as detection limit, analysis time, and accuracy (Table 2).

Also, investigation applicability of the fabricated biosensor for monitoring tPA in myocardial infarction and breast cancer patients, which tPA is considered as an important related biomarker, showed that the fabricated biosensor ables to determinate tPA concentrations and differentiate the studied groups (Table 3).

Conclusions

In this study, a label-free immunosensor for the rapid and sensitive detection of low concentration of tPA (0.026 ng mL^{-1}) has been successfully developed and applied by modifying GCE by covalently conjugated antibodies to SWCNTs. Covalent attachment of antibodies to SWCNTs was characterized using SDS-PAGE electrophoresis, SEM, CV, and EIS changes. Decreased current density was observed on the formation of antibody-tPA complexes on the electrode surface, because of the insulating properties of the tPA protein. Results of the study demonstrate that the fabricated immunosensor can accurately and sensitively detect tPA in myocardial infarction and breast cancer blood (Table 3). This immunosensor protocol is ~ sevenfold more sensitive than the ELISA kit (Abcam, USA). The fabricated immunosensor, compared to ELISA and HPLC, showed appropriate reproducibility that is demonstrated by RSD %. In addition, validation of tPA determination was indicated by correlation of fabricated immunosensor results with ELISA and HPLC analysis for a range of conditioned blood samples. Selectivity was certified by precise detection of tPA in the serum that also contains thousands of other ions and proteins, especially in the presence of interference proteins, which have the highest similarity with tPA.

For the first time, such specific biosensor is currently being fabricated for tPA in our laboratories and have the potential to increase predictive power by detecting panels of tPA as a biomarker for a given myocardial infarction and breast cancer. In conclusion, we have represented a selective, ultrasensitive, and accurate immunosensor for detection of tPA representative of normal patients to high cancer patients and myocardial infarction patients. Therefore, early and sensitive detection of the tPA (as an important biomarker of cancers and cardiovascular diseases) using this biosensor is promising for early and rapid diagnosis and therapy of these diseases.

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Compliance with ethical standards

Ethical approval for the study was granted by the Research Ethical Committee of the Bu-Ali Sina University (ethical code: IR.BASU.BIO.1392.26), and all patients gave written, informed consent.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Rosi NL, Mirkin CA. Nanostructures in biodiagnostics. Chem Rev. 2005;105:1547–62.
2. Saify NH, Yaghoobi MM, Jalali JM, Hosseinkhani S. Expression analysis and purification of human recombinant tissue type plasminogen activator (rt-PA) from transgenic tobacco plants. Prep Biochem Biotechnol. 2011;41(2):175–86.
3. Teesalu T, Kulla A, Asser T, Koskiniemi M, Vaheri A. Tissue plasminogen activator as a key effector in neurobiology and neuropathology. Biochem Soc Trans. 2002;30(2):183–9.

4. Bansi DM, Saurabh K, Chandra MP. Nanomaterials based biosensors for cancer biomarker detection. J Phys Conf Ser. 2016;704:012011.
5. González M, Jacinto G, José AA, Luis M, González F. Genomics and proteomics approaches for biomarker discovery in sporadic colorectal cancer with metastasis. Cancer Genomics Proteomics. 2013;10:19–26.
6. Guest PC, Gottschalk M, Bahn S. Proteomics: improving biomarker translation to modern medicine? NPJ Genom Med. 2013;5:17.
7. Yamamoto K, Shi G, Zhou TS, Xu F, Xu JM, Kato T, et al. Study of carbon nanotubes–HRP modified electrode and its application for novel on-line biosensors. Analyst. 2003;128:249–54.
8. Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature. 2003;422:198–207.
9. Hawkridge AM, Muddiman DC. Mass spectrometry-based biomarker discovery: toward a global proteome index of individuality. Ann Rev Anal Chem. 2009;2:265–77.
10. Lee HJ, Wark AW, Corn RM. Microarray methods for protein biomarker detection. Analyst. 2008;133:975–83.
11. Sardesai NP, Barron JC, Rusling JF. Carbon nanotube microwell array for sensitive electrochemiluminescent detection of cancer biomarker proteins. Anal Chem. 2011;83(17):6698–703.
12. Bagheri F, Piri K, Mohsenifar A, Ghaderi S. FRET-based nanobiosensor for detection of scopolamine in hairy root extraction of Atropa belladonna. Talanta. 2017;164:593–600.
13. Topkaya SN, Azimzadeh M, Ozsoz M. Electrochemical biosensors for cancer biomarkers detection: recent advances and challenges. Electroanalysis. 2016;28:1–19.
14. Chengwei W, Candace KC. Carbon nanotube-based electrodes for detection of low-ppb level hexavalent chromium using amperometry. ECS J Solid State Sci Technol. 2016;5(8):3026–31.
15. Jacobs CB, Peairs MJ, Venton BJ. Carbon nanotube based electrochemical sensors for biomolecules. Anal Chim Acta. 2010;662:105–27.
16. Byoung CK, Inseon L, Seok-Joon K, Youngho W, Ki YK, Chulmin J, et al. Fabrication of enzyme-based coatings on intact multi-walled carbon nanotubes as highly effective electrodes in biofuel cells. Sci Rep. 2017;7:40202. https://doi.org/10.1038/srep40202OCUS.
17. Mendoza E, Orozco J, Enez-Jorquera C, Guerrero G, Calle A, Lechuga LM, et al. Scalable fabrication of immunosensors based on carbon nanotube polymer composites. Nanotechnology. 2008;19(7):075102. (6pp)
18. Li M, Xiyan L, Xiulan ZF, Xiao W, Yan L. Metallic catalysts for structure-controlled growth of single-walled carbon nanotubes. Top Curr Chem (Z). 2017;375:29.
19. Shim M, Wong Shi KN, Chen RJ, Li Y, Dai H. Functionalization of carbon nanotubes for biocompatibility and biomolecular recognition. Nano Lett. 2002;2(4):285–8.
20. Tavakkoli M, Nico H, Rasmus K, Hua J, Jani S, Esko I, et al. Electrochemical activation of single-walled carbon nanotubes with pseudo atomic-scale platinum for hydrogen evolution reaction. ACS Catal. 2017; Just Accepted Manuscript Publication Date (Web): 17 Mar; https://doi.org/10.1021/acscatal.7b00199.
21. Yang HC, Yuan R, Chai YQ, Su HL, Zhuo Y, Jiang W, et al. Electrochim Acta. 2011;56:1973–80.
22. Zhao J, Zhang Y, Li H, Wen Y, Fan X, Lin F, et al. Ultrasensitive electrochemical aptasensor for thrombin based on the amplification of aptamer–AuNPs–HRP conjugates. Biosens Bioelectron. 2011;26:2297–303.
23. Li H, Wei Q, He J, Li T, Zhao Y, Cai Y, et al. Electrochemical immunosensors for cancer biomarker with signal amplification based on ferrocene functionalized iron oxide nanoparticles. Biosens Bioelectron. 2011;26(8):3590–5.
24. Blondeau P, Rius-Ruiz F, Düzgün A, Riu J, Rius FX. Covalent functionalization of single-walled carbon nanotubes with adenosine monophosphate: towards the synthesis of SWCNT–aptamer hybrids. Mater Sci Eng C. 2011;31:1363–8.
25. Guo C, Venturelli E, Bianco A, Kostarelos K. Cellular internalisation of humanized IgG antibody changes by functionalization onto multi-walled carbon nanotubes. Drug Discov Today. 2010;15:1100–1.
26. Liu S, Shen Q, Cao Y, Gan L, Wang Z, et al. Chemical functionalization of single-walled carbon nanotube field-effect transistors as switches and sensors. Coord Chem Rev. 2010;254:1101–16.
27. Stobiecka M, Chalupa A, Dworakowska B. Piezometric biosensors for anti-apoptotic protein survivin based on buried positive-potential barrier and immobilized monoclonal antibodies. Biosens Bioelectron. 2016;84:37–43.
28. Asava E, Sezgintürk MK. A novel impedimetric disposable immunosensor for rapid detection of a potential cancer biomarker. Int J Biol Macromol. 2014;66:273–80.
29. Saify Nabiabad H, Piri K, Amini M. Expression of active chimeric-tissue plasminogen activator in tobacco hairy roots, identification of a DNA aptamer and purification by aptamer functionalized-MWCNTs chromatography. Protein Expr Purif. 2016; https://doi.org/10.1016/j.pep.2016.02.004.
30. Hamidi M, Zarei N, Shahbazi MA. A simple and sensitive HPLC-UV method for quantitation of lovastatin in human plasma: application to a bioequivalence study. Biol Pharm Bull. 2009;32(9):1600–3.
31. Madrakian T, Haghshenas E, Afkhami A. Simultaneous determination of tyrosine, acetaminophen and ascorbic acid using gold nanoparticles/multiwalled carbon nanotube/glassy carbon electrode by differe. Sensors Actuators B Chem. 2014;193:451–60.
32. Kafrashi F, Afkhami A, Saify Nabiabad H, Madrakian T, Piri K. Designing of a new label-free electrochemical impedimetric nanosensor based on selective interaction sequence of L-lysine with activase kringle domains for sensitive detection of activase protein. J Mol Liq. 2017;248:60–5.
33. Chikkaveeraiah BV, Bhirde AA, Morgan NY, Eden HS, Chen X. Electrochemical immunosensors for detection of cancer protein biomarkers. ACS Nano. 2012;6(8):6546–61.