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A label-free immunosensor for sensitive detection of RACK 1 cancer biomarker based on conjugated polymer modified ITO electrode

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ABSTRACT

A new flexible biosensor based on conjugated polymer functionalized indium tin oxide (ITO) sheet was fabricated for Receptor for Activated C Kinase 1 (RACK 1) determination. Poly(3-thiophene acetic acid) (P(Thi-Ac)) was used as an immobilization matrix for construction of RACK 1 immunosensor. This polymer had a great number of carboxyl groups on its end site and these carboxyl ends provided anchoring points to the anti-RACK 1 antibodies. Anti-RACK 1 antibodies were covalently attached on the ITO electrode and recognized the RACK 1 antigens. Electrochemical characterizations were made by employing electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) techniques. Additionally, single frequency impedance method (SFI) was applied to follow the specific biointeraction between antibody and antigen. As a result of specific biointeraction, the designed immunosensor exhibited a wide linear detection range between 0.01 pg/mL and 2 pg/mL RACK 1 with a detection limit of 3.1 fg/mL. Scanning electron microscopy and atomic force microscopy analyses were employed for electrode surface morphology investigation. The designed RACK 1 biosensor had good repeatability (5.73 %, RSD), excellent reproducibility (2.5 %, RSD), long storage-stability and reusable property. In addition, the fabricated RACK 1 biosensor was applied to determine RACK 1 concentration in human serums and the recovery was ranging from 98.79%–100.22%. This work illustrated a new tool to construct a sensitive and low-cost disposable biosensor for applications in clinical monitoring.

1. Introduction

Cancer is an important threat to human health. Cancer biomarkers are present in blood, urine, saliva or tissue and they are related to the formation of cancer [1,2]. Biomarker concentration determination in human serum has a vital significance and the accurate and sensitive detection of cancer biomarkers gives sufficient information for clinical diagnosis and prognosis [3]. Several techniques have been suggested for cancer biomarker detection such as fluorescence immunoassay, radioimmunoassay, chemiluminescence immunoassay, and enzyme-linked immunoassay (ELISA) [4,5]. However, these techniques possess long, time-consuming and complex procedures; require high-cost devices and are inappropriate for real-time detection of biomarker [6,7]. On the other hand, electrochemical biosensors have been employed to detect cancer biomarker in human biological fluids because they have excellent specificity to their antigens [8]. Nowadays, electrochemical biosensors for cancer biomarkers detection have a significant contribution for diagnostic, control and treatment of cancers. Furthermore, they are successful tools for clinical diagnosis because they provide easy, cheap, accurate and reliable quantification of cancer biomarkers [9]. In order to determine biomarkers, biosensors demand a conductive substrate and a successful fabrication protocol. ITO sheets are the ideal candidates as transducers. It is well known that sensing materials possess a significant role in the electrochemical detection and a lot of ITO-based biosensors have been developed so far [10–12]. Receptor for Activated C Kinase 1 (RACK 1) is a scaffold protein with seven WD-40 repeats and it is a member of WD40 superfamily of proteins, containing the subunit of G-proteins. TheWD40 repeats of RACK 1 provides the complex protein–protein biointeractions formed between signaling biomolecules such as integrins, phosphodiesterase 4D5, and Src tyrosine kinase and protein kinase C [13]. RACK 1 has a role in signal transduction, immune defense, cell growth, migration, and differentiation [14]. In addition, RACK 1 is known as an adaptor protein in multiple intracellular signal transduction pathways [15]. These findings suggested that RACK 1 has role in carcinogenesis like oral squamous cell and hepatocellular carcinoma, melanoma,

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colon, non-small cell, lung [16], prostate [17] and breast cancer [18]. There were not many biosensing studies in the literature for the detection of RACK 1. Our group had two studies for the detection of RACK 1 biomarker. In both studies, the biosensors were developed by using silanization process. Aldehyde ended silane 11-(triethoxysilyl)undecanal (TESU) [9] and nitrile group ended silane 11-cyanoundecyltrimethoxysilane (CUTMS) [19] modified ITO electrodes were used as biosensing platform for fabrication of RACK 1 biosensor. These silane agents provided well-controlled layers for immobilization of biomolecules. When TESU was used as an immobilization matrix, the linear range and detection limit of this biosensor was found as 0.01425–0.7125 pg/mL and 30 fg/mL, respectively. The TESU modified biosensor retained 88.24 % of its initial activity after 8 weeks and it could be used 3 times after acidic regeneration [9]. The CUTMS modified biosensor had a linear range and a detection limit of 0.036–2.278 pg/mL and 10.8 fg/mL, respectively. The CUTMS modified biosensor retained 88.8 % of its initial activity after 8 weeks and it could be used 4 times after acidic regeneration [19]. In this study, we aimed to produce a more sensitive biosensor with wider linear detection range using conjugated P(Thi-Ac) polymer. Because of the promising features such as large surface area, good conductivity and carboxyl groups present its end site of the P(Thi-Ac) polymer, it was chosen as the interface matrix.

Electrochemical impedance spectroscopy (EIS) has been utilized in many fields, particularly corrosion, electrochemical sensors and biosensors. EIS is a suitable way for the analysis of bulk and interfacial electrical features of any kind of biosensing system. The impedance experiments are performed with modern computer-controlled instrumentation and this technique does not require high-cost devices and is easy to operate [20]. In this technique, redox couples such as $Fe(CN)_6^{3-/4}$-or $Ru(NH_3)_6^{2+/3+}$ are usually utilized and this technique measures the ability of these ions to become oxidized and reduced at the working electrode. Cyclic voltammetry is another electrochemical method and it gives information about the amount of oxidizable species on a working electrode and the number of electrons involved in the oxidation. Changes in impedance and peak currents illustrate the binding events [21].

Conductive polymers are important matrix materials in the development of electrochemical immunosensors. These types of polymers have some features such as good conductivity, high electron affinity and redox activity. They have unique properties which make them excellent matrices for immobilization of biorecognition molecules. In addition, they provide rapid electron transfer when they are used in the fabrication of immunosensors [22,23]. Different conjugated polymer-based biosensors have been constructed and used for different applications. A sensitive DNA biosensor using attached DNA on a pencil graphite electrode covered with polypyrrole/functionalized multiwalled carbon nanotubes was developed for 6-mercaptopurine determination [24]. Dong and Pires developed an optical microfluidic sensor with highly sensitive organic photodetectors (OPDs) for absorbance-based determination of salivary protein biomarkers including interleukin 8, interleukin 1β and matrix metalloproteinase 8. This optical microfluidic biosensor was integrated with polythiophene-C70 OPDs. The biosensor detected these biomarkers in spiked saliva with high sensitivity and selectivity in short duration [25]. An electrochemical immunosensor based on the three-dimensional (3D) macroporous polyaniline (PANI) doped with poly(sodium 4-styrene sulfonate) (PSS) was constructed for the detection of alpha-fetoprotein (AFP). This immobilization matrix material had large surface area, good conductivity and a lot of amino groups, and it offered a suitable substrate for the attachment of anti-AFP antibodies to fabricate an electrochemical immunosensor. In addition, the response sensitivity of this biosensor was 2 times higher than planar PANI-coated biosensor and this property could be originated from the 3D macroporous structure [26].

In this study, a new biosensor based on P(Thi-Ac) modified ITO sheet was developed to detect RACK 1 cancer biomarker. The developed immunosensor provided flexible and low-cost sensing system to determine RACK 1 antigen. P(Thi-Ac) polymer with good conductivity provided a biocompatible matrix with carboxyl side groups for binding of anti-RACK 1 antibodies. P(Thi-Ac) coated ITO surface was employed as an immobilization support for anti-RACK 1 antibody immobilization, and they bound to this platform covalently. In this work, ITO sheet was selected as a transducer due to its prominent characteristics such as good electrical conductivity and low cost. In previous researches, silane agents generally have been applied on the activated ITO electrode surface for the attachment of biomolecules [27,28]. The most important difference of the P(Thi-Ac) polymer from the silanization agents was that it was a semi-conductive material and therefore, it increased the conductivity of the ITO electrode. Modifying the electrode material surface with conductive material increased the sensitivity of the biosensor and made this developed biosensor superior to others. Electrochemical methods were utilized for investigation of the modification of the electrode surface. The modification of the ITO electrode with this P(Thi-Ac) polymer provided a cost-effective surface. Optimization experiments were performed to obtain the high signals of proposed biosensor. In addition, the proposed biosensor showed high sensitivity with a very low detection limit and a wide sensing linearity. The designed immunosensor had good performance in the quantification of RACK 1 antigens in human serum.

2. Experimental

2.1. Reagents and apparatus

Indium tin oxide coated polyethylene terephthalate (ITO-PET) sheet, anti-RACK 1 antibody, RACK 1 antigen, bovine serum albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), thiophene 3-acetic acid, anhydrous iron(III) chloride (98 %), potassium phosphate dibasic, potassium phosphate monobasic, potassium chloride, ammonium hydroxide, hydrogen peroxide (30 %) and 4-Dimethylaminopyridine (DMAP) were purchased from Sigma-Aldrich (Saint Louis, MO, USA). These protein solutions were prepared by utilizing phosphate buffer (50 mM pH 7.4). Ferricyanide/ferrocyanide solution was chosen as a redox couple and it was prepared by utilizing 5 mM $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$. Ultrapure water was employed throughout the study.

CV and EIS measurements were taken via a Gamry Potentiostat/Galvanostat (Gamry Reference 1000, Warminster, USA). All electrochemical measurements were taken by utilizing a conventional three-electrode system including an ITO-PET working electrode (0.5 cm–2 cm), a platinum wire auxiliary electrode, and an Ag/AgCl reference electrode (BASi, Warwickshire, UK). The electrochemical analyses were done in ferricyanide/ferrocyanide solution. EIS analyses were made within the frequency range from 0.5 Hz to 50 kHz. CV analyses were made at the range of −0.5 V - +1 V at a scan rate of 100 mVs^{-1}. The scanning electron microscopy (SEM) and atomic force microscopy (AFM) images were obtained on a FEI-Quanta FEG 250 Model microscope (Oregon, USA) and NanoMagnetics Instruments-AFM Plus Model (Ankara, Turkey). Average molecular weights and molecular weight distributions of the polymer poly(methyl 2-(thiophene-3-yl)acetate) was estimated on an Agilent 1260 Infinity GPC/SEC instrument (Santa Clara, USA).

2.2. Preparation of Poly(3-thiophene acetic acid) P(Thi-Ac) polymer

The conjugated polythiophene containing acid side groups (P(Thi-Ac)) was synthesized and characterized according to our previous report [22]. In brief, this polymer was synthesized in three steps. Firstly, methyl 2-(thiophene-3-yl)acetate monomer was synthesized by using 3-thiophene acetic acid and concentrated H_2SO_4. Then, poly(methyl 2-thiophene-3-yl) acetate was synthesized by chemical oxidation process of the monomer. Finally, the resulting polymer was hydrolyzed and thus, P(Thi-Ac) polymer was produced [22].

2.3. Preparation of P(Thi-Ac) modified ITO electrode

Briefly, the bare ITO electrodes were firstly ultrasonicated thoroughly in acetone, soap solution and ultrapure water. Then, the ITO sheet surfaces were gently dried by argon gas. After that, ITO electrodes dipped in a mixed solution of $H_2O_2/NH_4OH/H_2O$ (1:1:5) for 90 min at room temperature to form hydroxyl groups on the ITO electrode. Lastly, they were washed with ultrapure water and dried under argon flow. After that, the hydroxylated ITO electrodes were incubated overnight in P(Thi-Ac) polymer solution.

2.4. Fabrication of P(Thi-Ac) modified RACK 1 biosensor

In order to fabricate P(Thi-Ac) modified RACK 1 biosensor, the hydroxylated ITO electrode was dipped in P(Thi-Ac) solution to obtain a well-organized polymer monolayer and left there overnight. The carboxyl groups of P(Thi-Ac) polymer bond to hydroxyl groups via ester bond. Then, P(Thi-Ac) polymer modified electrode was immersed in EDC/NHS solution. NHS and EDC were used to activate the −COOH of P(Thi-Ac) polymer to better link with antibody through amide linkage. The immobilization of biorecognition molecule, anti-RACK 1 antibody, on P(Thi-Ac) polymer modified electrode was performed by immersion of P(Thi-Ac) modified ITO electrode in PBS solution containing anti-RACK 1 antibody. The anti-RACK 1 antibody immobilized ITO electrode was further immersed in BSA solution for 60 min to block free carboxyl ends of polymer, following by washing with ultrapure water to eliminate unspecific absorbed BSA molecules. For the specific interaction, ITO/P(Thi-Ac)/anti-RACK 1/BSA electrode was immersed into RACK 1 antigen solution containing different concentration of RACK 1. Then, the electrode was taken out and washed with ultrapure water and dried with argon.

2.5. Impedimetric biosensor measurements

Impedimetric measurements and CVs analyses were achieved in a reaction cell containing 5 mM $[Fe(CN)_6]^{3-/4-}$ solution containing 1 M KCl. After each step of immobilization, the electrochemical measurements were performed to characterize electrode surface in the presence of redox probe. All the measurements were performed at room temperature. In addition, RACK1 was detected using the ITO/P(Thi-AC)/anti-RACK 1/BSA immunosensor as a working electrode in the presence of various concentrations of RACK 1 antigen.

2.6. RACK 1 antigen measurements in real samples

The designed immunosensor was utilized for the determination of RACK1 in healthy human serum samples and patients with breast cancer. For this purpose, the serums obtained from healthy persons and patients with breast cancer were diluted 10,000-fold and 100,000-fold, respectively. Appropriate amounts of RACK 1 antigens were spiked to the human serum samples and they were analyzed using the modified ITO bioelectrodes.

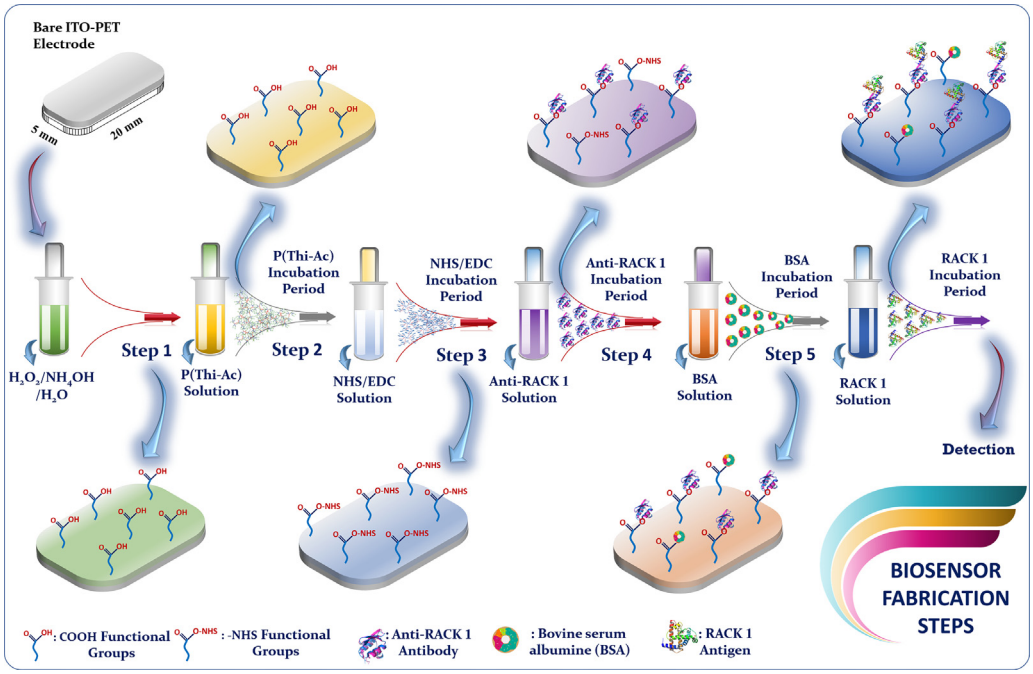
3. Results and discussion

Schematic illustration of the construction procedure of RACK 1 biosensor is displayed in Scheme 1. The P(Thi-Ac) modified ITO electrode was firstly prepared by a self-assembled monolayer strategy and then, employed as a platform for anti-RACK 1 antibody immobilization and specific interaction. The carboxyl groups of P(Thi-Ac) polymer bound covalently to hydroxyl group of the ITO electrode and ester bond was formed [29].

3.1. Electrochemical characterization of RACK 1 biosensor fabrication steps

The biosensor construction procedure was followed via the electron transfer of ferricyanide through the modified ITO electrode. EIS gives information about impedance changes formed on the electrode surface during the construction procedure. A Randles equivalent circuit was utilized to fit all the electrical parameters of the EIS results for each stage. As illustrated in the inset of Fig. 1A, the circuit contains the electrolyte resistance between the working and reference electrodes (R_s), the double layer capacitance of electrode and electrolyte (C), Warburg impedance (Z_w), and electron transfer resistance (R_{ct}) causing by the diffusion of ions from the electrolyte to the interface. In EIS, the semicircle diameter represents the electron transfer resistance, R_{ct}, which gives information about the electron transfer kinetics of the redox probe at the electrode interface [30]. Fig. 1A illustrates the Nyquist plots of differently modified ITO electrodes in ferrocyanide/ferricyanide solution. As observed in Fig. 1A, hydroxylated ITO electrode had minimum electron-transfer resistance when compared to other stages in biosensor fabrication. The low R_{ct} of the hydroxylated electrode illustrated the good electron transfer process between electrode surface and electrolyte solution. Additionally, P(Thi-Ac) polymer SAMs formation on the electrode surface increased Nyquist plot diameter because of successfully self-assembling process of polymer. The activation of carboxyl groups via NHS/EDC chemistry caused an increase in Nyquist plot diameter due to a repellence effect between redox probe and negatively charged carboxyl groups. After anti-RACK 1 antibody immobilization, a large Nyquist plot diameter was seen, illustrating the decrease in electron transfer property of redox probe. The blockage of carboxyl groups with BSA made an increase of R_{ct} because of formation of protein layer. After specific biointeraction between anti-RACK 1 antibody and RACK 1 antigen, the R_{ct} was further increased because of barrier effect of these proteins. The R_{ct} value of this system reached its maximum value while the RACK 1 antigen immobilized on the ITO surface.

Fig. 1B displays the CVs of differently modified ITO electrodes in 5 mM ferrocyanide/ferricyanide solution at the scan rate of 100 mV/s. A pair of well-defined redox peaks was seen at the hydroxylated ITO electrode. After self-assembling process of P(Thi-Ac) polymer on the hydroxylated ITO electrode the peak currents were decreased, and this decrease proved the formation of polymer SAMs. After the carboxyl groups activation step, decreases were seen in peak currents due to the repellence between negative charges present on the carboxyl groups and redox probe. The direct immobilization of anti-RACK 1 antibody on P(Thi-Ac) modified electrode surface caused decreases in peak currents, indicating that anti-RACK 1 antibody was successfully attached on the ITO electrode. The immobilization of BSA caused decreases in peak currents due to nonconductive feature of BSA. When the specific interaction was formed, the peak currents were decreased. The introduction of RACK 1 antigen increased the nonconductive prop-



Scheme 1. Schematic illustration of the construction procedure of RACK 1 biosensor.

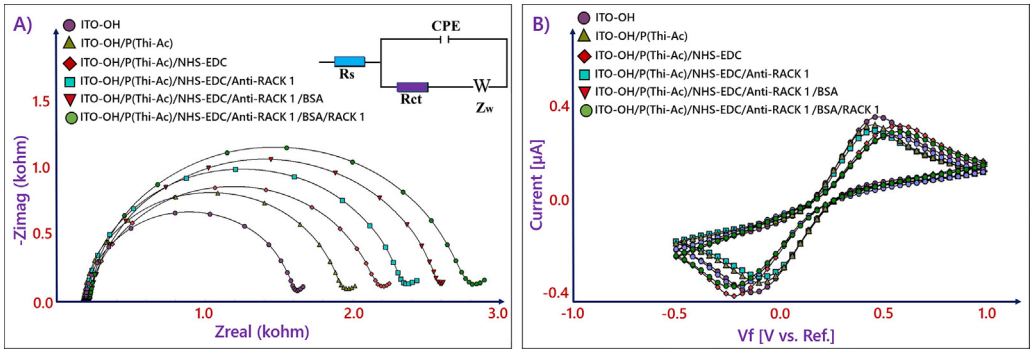


Fig. 1. EIS (A) and CV (B) responses of the RACK 1 biosensor during the fabrication procedure.

erty of the electrode surface. RACK 1 antigen attached ITO surface showed the lowest peak current as compared to other preparation steps of the electrode. The impedimetric results of the proposed immunosensor were in good agreement with the CV results.

3.2. Morphological characterization of RACK 1 biosensor fabrication steps

The RACK 1 biosensor fabrication stages were further characterized by SEM and AFM devices. Fig. 2 shows the SEM and AFM images taken after each fabrication step. It could be seen that, P(Thi-Ac) polymer coated ITO substrate had a homogenous and porous layer and the average roughness (R_a) was measured as 31.8 nm (Fig. 2A). At the anti-RACK 1 antibody immobilized electrode surface, the antibody molecules were seen clearly. The antibody immobilization caused an increase in R_a due to protein layer formation and R_a was found as 47.8 nm (Fig. 2B). The immobilization of anti-RACK 1 antibodies on the ITO surface caused a regular electrode surface because of covalent bonds formed between polymer and anti-RACK 1 antibodies. After BSA blockage, a relatively smooth surface was obtained, and the R_a was measured as 36.4 nm (Fig. 2C). The immobilization of RACK 1 antigen on the ITO electrode surface changed the electrode surface morphology and the RACK 1 antigens were clearly seen in Fig. 2D. The R_a was measured as 55.6 nm and this increase indicated that the RACK 1 antigens were specifically immobilized on the anti-RACK 1 antibodies. All morphological characterization results were consistent with other studies found in literature. In addition, these results supported the analyses results of electrochemical measurements.

3.3. Optimization of RACK 1 biosensor experimental conditions

To optimize the experimental conditions during the RACK 1 biosensor fabrication, several parameters were studied. Firstly, the used P(Thi-Ac) polymer concentration was optimized and therefore, three different (0.5, 1 and 2 mg/mL) polymer solutions were prepared. The RACK 1 biosensors signals were low, when 0.5 mg/mL and 2 mg/mL polymer solutions were utilized for electrode preparation. The maximum signal was measured after coating with 1 mg/mL P(Thi-Ac) polymer. Therefore, 1 mg/mL was selected optimal polymer level (Fig. SI-1A). Then, the used biorecognition molecule level was optimized and three different anti-RACK 1 antibody solutions (0.4, 2 and 10 ng/mL) were used. The low antibody level caused low signal because low anti-RACK 1 antibody amount did not sufficient to recognize RACK 1 antigen. The maximum biosensor signal was measured, when 2 ng/mL anti-RACK 1 anti-

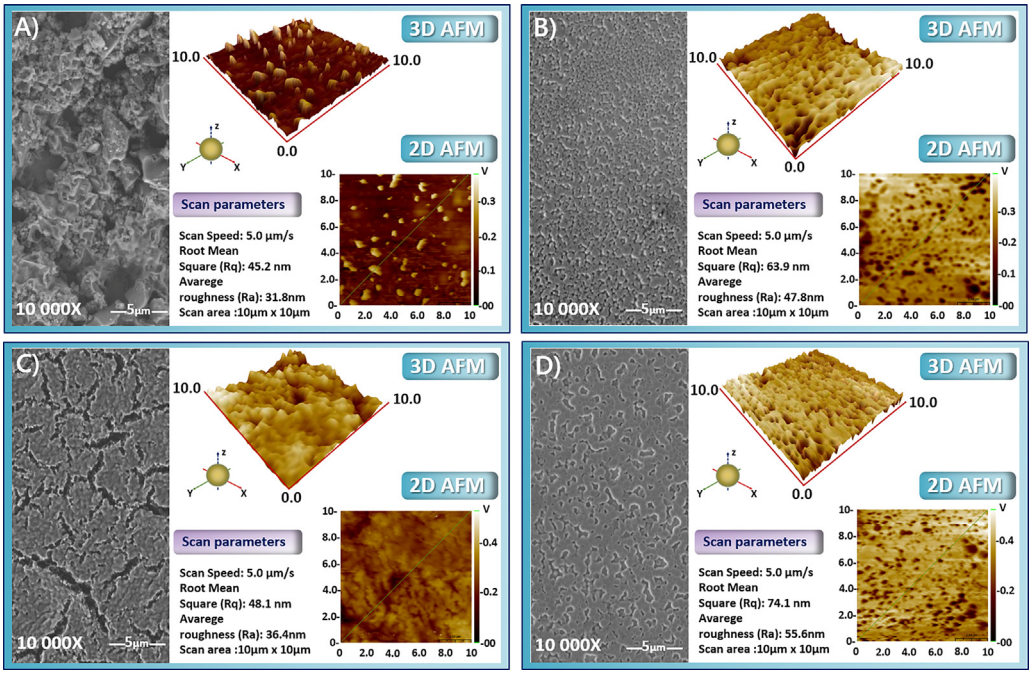


Fig. 2. SEM and AFM images of ITO/P(Thi-Ac) (A), ITO/P(Thi-Ac)/anti-RACK 1 (B), ITO/P(Thi-Ac)/anti-RACK 1/BSA (C), ITO/P(Thi-Ac)/anti-RACK 1/BSA/RACK 1 (D).

body was attached on the ITO surface. Therefore, 2 ng/mL was used as optimal antibody concentration (Fig. SI-1B).

After optimization of anti-RACK 1 antibody level, the incubation duration of anti-RACK 1 antibody was studied. The ITO/P(Thi-Ac) electrodes were dipped in anti-RACK 1 antibody solution for three different times (30 min, 45 min and 60 min). As observed in Fig. 3C, the maximum biosensor signal was found after 45 min incubation. The 30-min incubation caused a low biosensor signal and 30 min-incubation was not appropriate for anti-RACK 1 antibody immobilization (Fig. SI-1C). At least, the incubation duration of RACK 1 antigen was optimized, and three different duration was tried in this experiment. The signal after 30-min incubation was a bit lower than other incubation periods and therefore, 45 min was selected as optimum one (Fig. SI-1D).

3.4. Analytical characterization of the RACK 1 biosensor

The biomolecule immobilization and specific interaction between antibody-antigen onto different modified electrodes were characterized by EIS and CV. The analytical characterization experiments were performed under optimum conditions and used for the direct detection of RACK 1 antigen by EIS. The impedimetric responses of the ITO/P(Thi-Ac)/anti-RACK 1/BSA electrochemical biosensor with different levels of RACK 1 antigen are illustrated in Fig. 3A and the cyclic voltammograms are displayed in Fig. 3B. It can be seen that the current responses of the biosensor decreased and the impedimetric responses of the biosensor increased as the RACK 1 antigen concentration increased. When a higher concentration of RACK 1 antigen was added in phosphate buffer, more RACK 1 antigens were captured by anti-RACK 1 antibodies, resulting in a higher impedimetric response and lower current response. Fig. SI-2 illustrated the relationship between RACK 1 level and impedimetric response of the suggested immunosensor. The linear equation was ΔR_{ct} = 1.576[RACK 1] + 0.048 with a correction coefficient of 0.9994 (Fig. SI-2). The limit of detection (LOD) and the limit of determination (LOQ) were calculated as 3.1 fg/mL (signal-to-noise, S/N = 3) and 10.4 fg/mL (signal-to-noise, S/N = 10), respectively. This obtained LOD and LOQ were compared to previously reported immunosensors (Table 1). The good sensitivity together with the wide linear detection range of the proposed biosensor could be originated from the usage of conductive P(Thi-Ac) polymer.

Single frequency impedance (SFI) is a technique to follow impedance variations versus time. It is frequently employed for impedimetric biosensor development and for on-line monitoring of biological interactions. To follow the change in impedance, the measurements are performed at constant frequency obtained from Bode plot [7]. In order to monitor the change in impedance during the interaction between RACK 1 antibody and RACK 1 antigen, ITO/P(Thi-Ac)/anti-RACK 1/BSA electrode was immersed in RACK solution (PBS, 0.25 pg/mL RACK antigen). In this biosensing system, an increase in impedance value (pink) was observed due to binding between anti-RACK 1 antibody and RACK 1 antigen (Fig. 3C). This increase indicated successful binding between antibody and antigen.

3.5. Repeatability, reproducibility, regeneration and stability of the RACK 1 biosensor

Repeatability and reproducibility of this electrochemical method were examined by the proposed immunosensor. In order to test the repeatability, twenty different anti-RACK1 antigen immobilized ITO surfaces were incubated in PBS solution including RACK 1 antigen (0.25 pg/mL). As seen Fig. 3D, the bioelectrodes gave similar responses. The relative standard deviation (RSD) of repeatability test was calculated as 5.73 %. The low RSD indicated the good repeatability of the system.

In order to test reproducibility, ten different biosensors were prepared under the same conditions. The calibration curves of ten biosensors were plotted by using the change transfer resistance values and RACK 1 antigen concentrations. As seen in Fig. SI-3, the calibration curves of ten biosensors were overlapped. The RSD (%) value of the reproducibility test was calculated as 2.5 % and this low value demonstrated excellent reproducibility.

The reusability of RACK 1 immunosensor is very significant in practical applications. The regeneration of the suggested biosensor

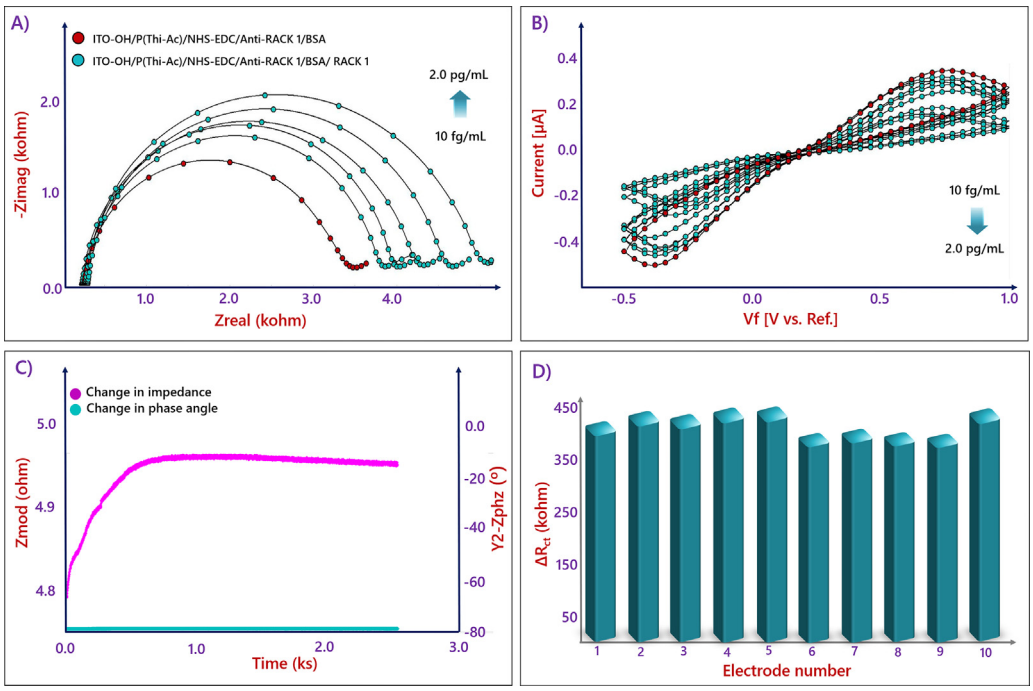


Fig. 3. EIS (A) and CV (B) responses of the RACK 1 biosensor after incubation in different levels of RACK 1 antigen, SFI experiment result (C) and repeatability test result (D).

Table 1
Different studies for detection of RACK 1 antigen.

Biosensor surface	Measurementprinciple	LinearRange (pg/mL)	Detection limit (fg/mL)	References
TESU modified ITO	EIS	0.01425–0.7125	30	[9]
CUTMS modified ITO	EIS	0.036–2.278	10.8	[19]
P(Thi-Ac) modified ITO	EIS	0.01–2	3.1	This study

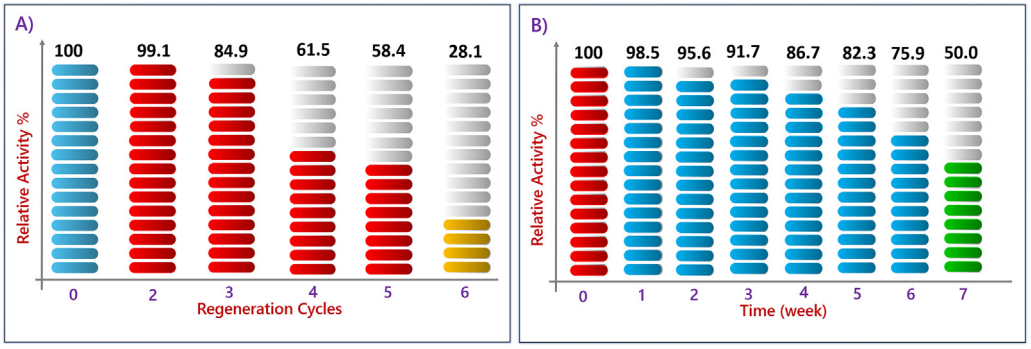


Fig. 4. Results of regeneration (A) and storage stability (B).

was examined by immersing in 10 mM HCl solution for 5 min to remove the RACK 1 antigens from the immunoelectrode surface. After each acid-immersion stage, the response of the electrode was measured. After re-exposure to RACK 1 antigen, the modified ITO electrode could again capture RACK 1 antigens. Finally, the constructed RACK 1 immunosensor could be regenerated 4 times with 38.50 % loss of the initial signal by immersing in acid solution (Fig. 4A). Lastly, the storage-stability of the RACK 1 immunosensor was also examined. The anti-RACK 1 antibody immobilized ITO electrode was stored at 4 °C for 6 weeks and the response of the electrode was measured at each week by EIS after biointeraction with RACK 1 antigen. Results illustrated that the RACK 1 immunosensor had 75.9 % of its initial signal after 6 weeks (Figure 6B).

3.6. Practical application of the designed immunosystem in human serum samples

In order to test the practical application of the designed immunosystem, human serum samples of healthy persons and patients with breast cancer were analyzed. The human serum samples taken from Tekirdağ Namık Kemal University (Ethic committee approval 2013/86/07/05). The samples of healthy persons and patients with breast cancer were diluted 10,000-fold and 100,000-fold, respectively. The analysis results of healthy persons and patients with breast cancer are listed in Table 2A and B, respectively. To evaluate the applicability of the designed immunosensor, the standard addition technique was used. A series of healthy human serum samples were prepared by adding RACK 1 antigen in human

Table 2
Human serum samples results of healthy persons (A) and patients with breast cancer (B) measured by the suggested RACK 1 biosensor.

(A) Sample	RACK 1 Biosensor (pg/mL)	Added RACK 1(pg/mL)	Total Found by Biosensor	Recovery%	Relative Difference %
1	1.49	0.2	1.67	99.17	−0.83
2	1.54	0.2	1.74	100.22	0.22
3	1.54	0.2	1.74	99.84	−0.16
4	1.54	0.2	1.73	99.43	−0.57
5	1.39	0.2	1.57	98.79	−1.21
(B) Sample	RACK 1 Biosensor (pg/mL)	Added RACK 1(pg/mL)	Total Found by Biosensor	Recovery%	Relative Difference %
1	0.83	0.5	1.31	98.13	−1.87
2	0.94	0.5	1.47	102.73	2.73
3	1.11	0.5	1.68	104.14	4.14
4	1.56	0.5	2.07	100.54	0.54
5	1.21	0.5	1.71	100.42	0.42
6	1.28	0.5	1.85	104.12	4.12

serum and the recovery was found between 98.79 % and 100.22 % (Table 2A). As seen in Table 2B, the recovery of the patients with breast cancer serum analysis was ranging from 98.13%–104.14%. The obtained results were consistent with the results of previous studies and they displayed that the proposed electrochemical RACK 1 biosensor had promising potential in human serum sample analyses.

4. Conclusion

In the present work, we developed a new strategy for electrochemical determination of RACK 1 antigen in human serum. The fabrication strategy of the proposed biosensor provided two main advantages. Firstly, the P(Thi-Ac) modified ITO electrode had good electronic conductivity and secondly, it had large surface area for loading more anti-RACK 1 antibodies. These properties provided more sensitive analysis of RACK 1 antigen and a wider linear detection range than other studies. The detection limit and linear range was found as 3.1 fg/mL and 0.01−2 pg/mL, respectively. Moreover, these important features made the suggested immunosensor a promising tool for biomarker detection. Furthermore, the use of cheap ITO thin film as immobilization platform provided the fabrication of a low-cost analytical platform. Additionally, the designed immunosensor could be utilized in the human serum samples to determine the level of RACK 1 antigen in patients with breast cancer. Because of high sensitivity, good selectivity, high stability and simple fabrication property, the proposed immunosensor exhibited the potential application in bioanalysis and clinical diagnostics

CRediT authorship contribution statement

Elif Burcu Aydın: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Muhammet Aydın:** Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft. **Mustafa Kemal Sezgintürk:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Funding acquisition.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2020.113517.

Declaration of Competing Interest

None.

References

[1] S. Prasad, A.K. Tyagi, B.B. Aggarwal, Detection of inflammatory biomarkers in saliva and urine: potential in diagnosis, prevention, and treatment for chronic diseases, Exp. Biol. Med. 241 (8) (2016) 783–799.
[2] Y. Wang, Z. Wei, X. Luo, Q. Wan, R. Qiu, S. Wang, An ultrasensitive homogeneous aptasensor for carcinoembryonic antigen based on upconversion fluorescence resonance energy transfer, Talanta 195 (2019) 33–39.
[3] M. Aydın, E.B. Aydın, M.K. Sezgintürk, A highly selective electrochemical immunosensor based on conductive carbon black and star PGMA polymer composite material for IL-8 biomarker detection in human serum and saliva, Biosens. Bioelectron. 117 (2018) 720–728.
[4] M. Aydın, E.B. Aydın, M.K. Sezgintürk, Electrochemical immunosensor for CDH22 biomarker based on benzaldehyde substituted poly (phosphazene) modified disposable ITO electrode: a new fabrication strategy for biosensors, Biosens. Bioelectron. 126 (2019) 230–239.
[5] V. Espina, E.C. Woodhouse, J. Wulfkuhle, H.D. Asmussen, E.F. Petricoin III, L.A. Liotta, Protein microarray detection strategies: focus on direct detection technologies, J. Immunol. Methods 290 (1-2) (2004) 121–133.
[6] M. Aydın, E.B. Aydın, M.K. Sezgintürk, A highly selective poly (thiophene)-graft-poly (methacrylamide) polymer modified ITO electrode for neuron specific enolase detection in human serum, Macromol. Biosci. 19 (8) (2019), 1900109.
[7] M. Aydın, A sensitive and selective approach for detection of IL 1α cancer biomarker using disposable ITO electrode modified with epoxy-substituted polythiophene polymer, Biosens. Bioelectron. 144 (2019) 111675.
[8] B.V. Chikkaveeraiah, A.A. Bhirde, N.Y. Morgan, H.S. Eden, X. Chen, Electrochemical immunosensors for detection of cancer protein biomarkers, ACS Nano 6 (8) (2012) 6546–6561.
[9] E. Bahadır, M. Sezgintürk, Label-free, ITO-based immunosensor for the detection of a cancer biomarker: Receptor for Activated C Kinase 1, Analyst 141 (19) (2016) 5618–5626.
[10] E.B. Aydın, M. Aydın, M.K. Sezgintürk, Selective and ultrasensitive electrochemical immunosensing of NSE cancer biomarker in human serum using epoxy-substituted poly (pyrrole) polymer modified disposable ITO electrode, Sens. Actuators B Chem. 306 (2020), 127613.
[11] E.B. Aydın, M.K. Sezgintürk, Indium tin oxide (ITO): a promising material in biosensing technology, TrAC, Trends Analyt. Chem. 97 (2017) 309–315.
[12] E. Burcu Aydın, A label-free and sensitive impedimetric immunosensor for TNF α biomarker detection based on epoxysilane-modified disposable ITO-PET electrode, Int. J. Environ. Anal. Chem. 100 (4) (2020) 363–377.
[13] R. Nagashio, Y. Sato, T. Matsumoto, T. Kageyama, Y. Satoh, R. Shinichiro, N. Masuda, N. Goshima, S.-X. Jiang, I. Okayasu, Expression of RACK1 is a novel biomarker in pulmonary adenocarcinomas, Lung cancer 69 (1) (2010) 54–59.
[14] Q. Ren, J. Zhou, X.-F. Zhao, J.-X. Wang, Molecular cloning and characterization of a receptor for activated protein kinase C1 (RACK1) from Chinese white shrimp; Fenneropenaeus chinensis, Dev. Comp. Immunol. 35 (6) (2011) 629–634.
[15] L. Hu, F. Lu, Y. Wang, Y. Liu, D. Liu, Z. Jiang, C. Wan, B. Zhu, L. Gan, Y. Wang, RACK1, a novel hPER1-interacting protein, J. Mol. Neurosci. 29 (1) (2006) 55–63.
[16] X.-X. Cao, J.-D. Xu, J.-W. Xu, X.-L. Liu, Y.-Y. Cheng, W.-J. Wang, Q.-Q. Li, Q. Chen, Z.-D. Xu, X.-P. Liu, RACK1 promotes breast carcinoma proliferation and invasion/metastasis in vitro and in vivo, Breast Cancer Res. Treat. 123 (2) (2010) 375–386.
[17] S. Kraus, D. Gioeli, T. Vomastek, V. Gordon, M.J. Weber, Receptor for activated C kinase 1 (RACK1) and Src regulate the tyrosine phosphorylation and function of the androgen receptor, Cancer Res. 66 (22) (2006) 11047–11054.
[18] X.X. Cao, J.D. Xu, X.L. Liu, J.W. Xu, W.J. Wang, Q.Q. Li, Q. Chen, Z.D. Xu, X.P. Liu, RACK1: A superior independent predictor for poor clinical outcome in breast cancer, Int. J. Cancer 127 (5) (2010) 1172–1179.

[19] H. Törer, E.B. Aydın, M.K. Sezgintürk, A label-free electrochemical biosensor for direct detection of RACK 1 by using disposable, low-cost and reproducible ITO based electrode, Anal. Chim. Acta 1024 (2018) 65–72.
[20] C. Fernández-Sánchez, C.J. McNeil, K. Rawson, Electrochemical impedance spectroscopy studies of polymer degradation: application to biosensor development, TrAC, Trends Anal. Chem. 97 (2017) 309–315.
[21] J. Halliwell, A.C. Savage, N. Buckley, C. Gwenin, Electrochemical Impedance Spectroscopy Biosensor for Detection of Active Botulinum Neurotoxin Sensing and Bio-Sensing Research, vol. 2, 2014, pp. 12–15.
[22] E.B. Aydın, M. Aydın, M.K. Sezgintürk, A highly sensitive immunosensor based on ITO thin films covered by a new semi-conductive conjugated polymer for the determination of TNFα in human saliva and serum samples, Biosens. Bioelectron. 97 (2017) 169–176.
[23] M. Singh, P.K. Kathuroju, N. Jampana, Polypyrrole based amperometric glucose biosensors, Sens. Actuators B Chem. 143 (1) (2009) 430–443.
[24] H. Karimi-Maleh, F. Tahernejad-Javazmi, N. Atar, M.Lt. Yola, V.K. Gupta, A.A. Ensafi, A novel DNA biosensor based on a pencil graphite electrode modified with polypyrrole/functionalized multiwalled carbon nanotubes for determination of 6-mercaptopurine anticancer drug, Ind. Eng. Chem. Res. 54 (14) (2015) 3634–3639.
[25] T. Dong, N.M.M. Pires, Immunodetection of salivary biomarkers by an optical microfluidic biosensor with polyethylenimine-modified polythiophene-C70 organic photodetectors, Biosens. Bioelectron. 94 (2017) 321–327.
[26] S. Liu, Y. Ma, M. Cui, X. Luo, Enhanced electrochemical biosensing of alpha-fetoprotein based on three-dimensional macroporous conducting polymer polyaniline, Sens. Actuators B Chem. 255 (2018) 2568–2574.
[27] E.B. Aydın, M.K. Sezgintürk, A sensitive and disposable electrochemical immunosensor for detection of SOX2, a biomarker of cancer, Talanta 172 (2017) 162–170.
[28] A. Gündoğdu, E.B. Aydın, M.K. Sezgintürk, A novel electrochemical immunosensor based on ITO modified by carboxyl-ended silane agent for ultrasensitive detection of MAGE-1 in human serum, Anal. Biochem. 537 (2017) 84–92.
[29] X. Deng, R. Nie, A. Li, H. Wei, S. Zheng, W. Huang, Y. Mo, Y. Su, Q. Wang, Y. Li, Ultra-low work function transparent electrodes achieved by naturally occurring biomaterials for organic optoelectronic devices, Adv. Mater. Interfaces 1 (7) (2014) 1400215.
[30] L. Fan, G. Zhao, H. Shi, M. Liu, Z. Li, A highly selective electrochemical impedance spectroscopy-based aptasensor for sensitive detection of acetamiprid, Biosens. Bioelectron. 43 (2013) 12–18.