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Label-free Electrochemical Immunosensor for Ultrasensitive Detection of KLK4 Using Conjugated Polymer Modified Sensing Platform

Konjuge Polimer Modifiye Algılama Platformu Kullanılarak KLK4'ün Ultra Hassas Tespiti İçin Etiketsiz Elektrokimyasal İmmünosensör

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ABSTRACT

In the present research, a label-free impedimetric kallikrein 4 (KLK4) biosensor was fabricated by using a low-cost and disposable indium tin oxide (ITO) electrode and utilized for KLK4 detection in human serum. The ITO electrode was modified first by spin-coating of epoxy-substituted poly(thiophene) (Poly(TEpx)) conjugated polymer and thence anti-KLK4 antibody immobilization via the epoxy ends on the polymer. The specific interaction between the anti-KLK4 antibody present on the working electrode surface and the KLK4 antigen caused increases in the impedimetric response, and the KLK4 antigen was determined in a linear concentration range from 0.025 to 12.5 pg/mL with a detection limit of 7.78 fg/mL. Furthermore, the developed biosensor had acceptable repeatability and reproducibility, long storage stability, and high selectivity to KLK4 antigen. The applicability of the immunosensor was tested in human serum samples and satisfactory results were obtained.

Key Words
Kallikrein 4, electrochemical impedance spectroscopy, prostate cancer, conjugated polymer.

ÖZ

Bu araştırmada, düşük maliyetli ve tek kullanımlık indiyum kalay oksit (ITO) elektrot kullanılarak etiketsiz bir impedimetrik kallikrein 4 (KLK4) biyosensörü üretilmiş ve insan serumunda KLK4 tespiti için kullanılmıştır. ITO elektrot önce epoksi sübstitüe poli(tiyofen) (Poly(TEpx)) konjuge polimerin döndürülerek kaplamasıyla modifiye edildi ve ardından polimer üzerindeki epoksi uçları aracılığıyla anti-KLK4 antikoru immobilize edildi. Çalışma elektrodu yüzeyinde bulunan anti-KLK4 antikoru ile KLK4 antijeni arasındaki spesifik etkileşim, impedimetrik yanıtta artışa neden olmuş ve KLK4 antijeni, 7.78 fg/mL tespit limiti ile 0.025 ile 12.5 pg/mL arasında doğrusal bir konsantrasyon aralığında belirlenmiştir. Ayrıca, geliştirilen biyosensör kabul edilebilir tekrarlanabilirlik ve tekrar üretilebilirliğe, uzun depolama stabilitesine ve KLK4 antijenine karşı yüksek seçiciliğe sahipti. İmmünosensörün uygulanabilirliği insan serum örneklerinde test edilmiş ve tatmin edici sonuçlar elde edilmiştir.

Anahtar Kelimeler
Kallikrein 4, elektrokimyasal impedans spektroskopisi, prostat kanseri, konjuge polimer.

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INTRODUCTION

Prostate cancer (PCa) is the most commonly diagnosed cancer type among men, and 1 out of 9 men are diagnosed with prostate cancer in their lifetime [1]. Early detection of prostate cancer is important in the treatment of this disease. Recognition of cancer biomarkers in human serum is a strong technique for early diagnosis, prognosis, and monitoring of prostate cancer. The most commonly used PCa oncomarker is prostate-specific antigen (PSA) [2]. PSA levels increase in many noncancerous conditions, such as inflammation, infection, trauma, prostatic hyperplasia, and prostatitis [3]. Therefore, there is a need for new PCa biomarkers for PCa diagnosis.

Tissue kallikrein is a member of the serine proteases family and serine protease genes localized near KLK-encoding genes [4]. Kallikrein-related peptidase 4 (KLK4) is a highly expressed prostate gene, under the control of steroid hormones. KLK4 is upregulated by androgens with an androgen receptor [5]. Higher KLK4 levels of prostate tissue are correlated with higher Gleason scores and stages [6]. In addition, KLK4 expression is increased in malignant prostate tissue versus benign or normal tissue. Furthermore, KLK4 might be a proliferative factor acting directly or indirectly on cell cycle regulators [7].

In order to detect prostate cancer biomarkers, different old-style methods such as enzyme-linked immunosorbent assay (ELISA) [8], polymerase chain reaction (PCR) [9], radio [10], and chemiluminescent assay [11] have been utilized for PCa biomarker detection. These methods are high-cost, difficult, and require complex tools and a lot of time [12]. Therefore, the development of a highly sensitive and economical biosensor is needed for KLK4 biomarker detection. Highly sensitive electrochemical immunosensors are suitable alternatives due to their high selectivity and sensitivity, low cost, and rapid response [13]. Electrochemical impedance spectroscopy (EIS) is a technique that is used to determine quantitative parameters of the electrode process as well as to provide a finger point of the interfacial region. This technique is a label-free, sensitive, and cost-effective detection of the target antigen [14-16]. EIS-based immunosensors have gained considerable interest in different areas of bioanalytical chemistry due to their superiorities over other electrochemical immunosensors [17]. These types of immunosensors are label-free with direct detection of specific binding events, less destructive to the processes, and easy to operate. EIS gives information about molecular interaction as well as specific recognition of proteins, lectins, antibodies, and nucleic acids [18-20]. Different EIS-based biosensors have been developed for cancer biomarker detection [21].

In the fabrication of a label-free electrochemical immunosensor, antibody immobilization is the crucial step [22]. Conducting polymers have become attractive as suitable matrices due to good conductivity and easy-to-prepare properties [23,24]. In addition, they provide a large surface area, which allows for a higher loading of immobilized elements such as enzymes and antibodies. Poly(thiophene) conjugated polymers and their composites have been employed as an immobilization matrix for biomolecules because of their good conductivity, suitable biocompatibility, and proper stability [25,26].

In this work, we described the development and characterization of a label-free immunosensor for KLK4 prostate cancer biomarker detection. The ITO platform was modified first by spin coating of *Poly(TEpx)* polymer, and then anti-KLK4 antibodies were immobilized to the epoxy ends without utilizing a crosslinking agent. These epoxy ends served as a linker for covalent immobilization of anti-KLK4 antibodies. This simple and user-friendly fabrication protocol provided a new way for sensitive and label-free immunosensor development. This biosensor was successfully applied to human serum samples, and good recovery rates were found.

Experimental

Reagents and Apparatus

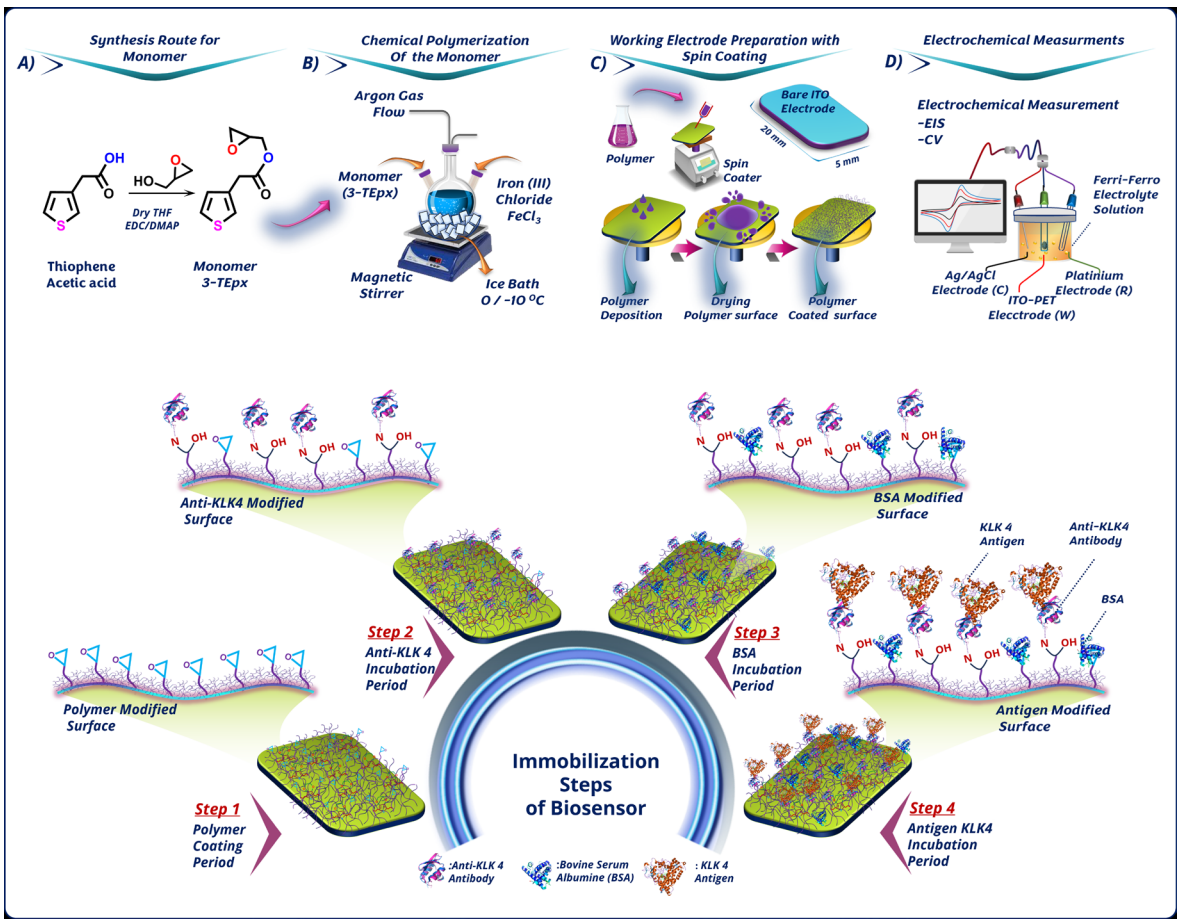
Anti-KLK4 human antibodies, KLK4 human antigens, bovine serum albumin (BSA), prostate-specific membrane human antigen (PSMA), interleukin 6 human antigen (IL6), CC chemokine receptor 4 human antigen (CCR4), tumor necrosis factor alpha antigen (TNFα), potassium ferricyanide ($K_3Fe(CN)_6$), potassium ferrocyanide ($K_4Fe(CN)_6$), disodium hydrogen phosphate (K_2HPO_4), sodium dihydrogen phosphate (KH_2PO_4), potassium chloride (KCl), 3-thiopheneacetic acid, glycidol, N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride, 4-dimethylamino pyridine, anhydrous iron (III) chloride, nitromethane, tetrahydrofuran, and ITO conductive PET were obtained from Sigma-Aldrich. Phosphate buffer solution (PBS) was prepared by mixing K_2HPO_4 and KH_2PO_4.

Electrochemical analyses such as EIS, cyclic voltammetry (CV), and single frequency impedance (SFI) were performed with a Gamry Reference 1000 electrochemical workstation. Modified ITO electrode, Ag/AgCl, and platinum wire were utilized as working, reference, and auxiliary electrodes, respectively. EIS and CV measurements and SFI measurement were performed in ferri-ferro solution (5 mM $K_3[Fe(CN)_6]$ / $K_4[Fe(CN)_6]$ (1:1) and PBS (50 mM, pH 7.4), respectively. EIS spectra were recorded from 0.5 Hz to 50 kHz frequency, and CVs were recorded from -0.5 V to 1 V at a scan rate of 100 mV/s. Fourier transform infrared spectroscopy (FTIR) analyses of the polymer and antibody modified electrodes were performed using a Bruker Vertex 70. The morphological investigations of the modified working electrodes were performed by scanning electron microscopy (SEM, FEG Quanta 250) and atomic force microscopy (AFM, Nano-Magnetics AFM PLUS).

Synthesis of Poly(TEpx) Polymer

Before the polymer synthesis, thiophene monomer bearing epoxy ends (*3-TEpx*) was prepared with the Steglich esterification method summarized in the previous report [16]. FT-IR (ATR, cm^{-1}): 3103; 3058; 2978; 1735; 1537; 1410; 1257; 1102; 1008; 947; 857; 762; 733; 612; 490. Raman ($\lambda_{laser=780\ nm}$): 3110; 2997; 2934; 1738; 1540; 1412; 1259; 1153; 1085; 994; 948; 861; 837; 764; 678; 535; 461. ^{1}H-NMR ($_{400\ MHz,\ CDCl3}$): 7.38 ppm (Ha), 7.14 ppm (Hb), 7.35 ppm (Hc), 3.80 ppm (Hd), 4.56 and 4.05 ppm ($He_{1,2}$), 3.30 ppm (Hf) and 2.92 and 2.71 ppm ($Hg_{1,2}$).

To synthesize the *Poly(TEpx)* polymer, oxidative polymerization of monomer was carried out with ferric chloride (Scheme 1). Chemical characterization of the *Poly(TEpx)* polymer was performed according to the previous report [26]. FT-IR (ATR, cm^{-1}): 2950; 2886; 1726; 1405; 1321; 1248; 1157; 1038; 942; 846; 744; 620; 558; 485. Raman ($\lambda_{laser=780\ nm}$): 1739; 1487; 1343; 1237; 1036; 918; 853.



Scheme 1. KLK4 impedimetric biosensor construction process.

Fabrication of the KLK4 Biosensor

Before modification, the ITO electrodes were successively sonicated with acetone, soap solution, and ultrapure water. The preparation procedure for the immunosensor is shown in Scheme 1. To generate a polymer matrix on the ITO electrode, 10 µl of the *Poly(TEpx)* polymer solution was spin-coated on the ITO electrode. After washing with ultrapure water to remove any unbound chemicals and drying under a steam of argon, the ITO electrodes were immersed in an anti-KLK4 antibody solution for coupling of antibodies on the polymer film-coated electrode. After this step, the electrodes were washed with ultrapure water, and they were immersed in a 0.5% BSA solution for one hour to occupy the non-reacted epoxy ends. The prepared ITO/*Poly(TEpx)*/anti-KLK4/BSA electrodes were immersed in KLK4 solution to analyze the level of KLK4 antigens.

Impedimetric Detection of KLK4

The measurement of the biosensor was performed in a 10 mL ferro-ferri solution after modifications. The ITO/*Poly(TEpx)*/anti-KLK4/BSA electrodes were utilized as a detection platform for KLK4 antigens. These electrodes were immersed into PBS solutions containing increasing concentrations of KLK4 antigens. The impedimetric responses of these electrodes were recorded in the frequency range of 50 kHz-0.5 Hz. The signals were utilized to draw a calibration of a biosensing system.

RESULTS and DISCUSSION

The biosensing platform was prepared in four steps. In the first step, spin-coating of *Poly(TEpx)* was performed on the ITO electrode (Step 1). In the second step, the *Poly(TEpx)* coated electrode was modified with anti-KLK4 antibodies (Step 2). The free epoxy ends were blocked by using BSA protein (Step 3). In the last step of the biosensing system was the detection step of the KLK4 antigen (Step 4). Scheme 1 illustrates the schematic diagram for the fabrication of the bioelectrode.

Chemical Behavior of the Biosensor

The *Poly(TEpx)* polymer was prepared successfully using a classical chemical oxidative polymerization procedure, and this polymer was coated on the working electrode by using a spin coater. The polymer-coated and anti-KLK4 immobilized electrodes were characterized chemically by the FTIR technique.

The peaks at 948 cm^{-1} and 846 cm^{-1} were attributed to oxirane rings in the side groups of the *Poly(TEpx)* polymer and prove the presence of the polymer on the ITO electrode [27,28]. The two bands observed around 1159 cm^{-1} and 626 cm^{-1} were attributed C-S-C asymmetric and symmetric stretching vibrations in thiophene ring [29]. The strong and sharp peak at 1729 cm^{-1} marked the C=O stretching vibration of carbonyl groups in polymer (Fig. 1A) [30]. The spectrum of the anti-KLK4 immobilized electrode (Fig. 1B) was also evaluated because it provided significant insight for protein immobilization. The broad and intense bands at 1651 cm^{-1} and 1547 cm^{-1} suggested the presence of amide groups of the anti-KLK4 antibody immobilized on the electrode surface.

In order to investigate the electrode surface coating with *Poly(TEpx)* polymer, Energy-dispersive X-ray spectroscopy (EDX) measurements were also performed. Figs. 1C and 1D illustrate the EDX analysis results

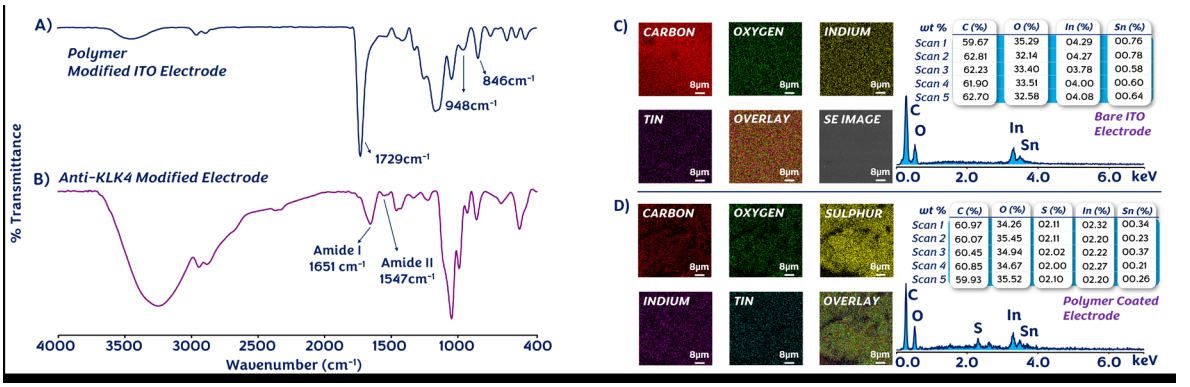


Figure 1. FTIR spectra of poly(TEpx) polymer-coated (A) and anti-KLK4 immobilized (B) electrodes.

of bare and polymer-coated electrodes. The primary distinction between the bare and *Poly(TEpx)* polymer-coated electrodes was the S element. This change was evidence that the polymer was successfully coated on the electrode surface.

Electrochemical Behavior of the Biosensor

EIS uses a small-amplitude perturbation signal, which makes it an excellent tool for monitoring modified surfaces [31]. Thus, EIS is utilized to monitor the changes in the charge transfer resistance (R_{ct}) and/or capacitance at the electrode surface. In order to calculate the R_{ct} of this system, an equivalent circuit was utilized for fitting EIS responses. This circuit includes 4 elements: R_{ct}, a constant phase element (CPE), an electrolyte resistance (R_s), and a Warburg impedance (W) [32].

The electrochemical characteristics of different modified electrodes were measured by EIS and CV in ferri-ferro redox probe solution, as shown in figure 2. The Nyquist plots of EIS studies are illustrated in Fig. 2A. The Nyquist plot of the ITO/*Poly(TEpx)* electrode was smaller than the other modification steps. After anti-KLK4 antibody biomolecule immobilization, there was a significant increase in the diameter of the Nyquist plot due to the presence of non-conductive protein. The BSA blocking of free epoxy ends caused a larger Nyquist plot due to the hindrance effect in the electron flow. When KLK4 antigen was immobilized on the immunoelectrode, the diameter was further increased, and a large R_{ct} was calculated because of the interference in electron flow.

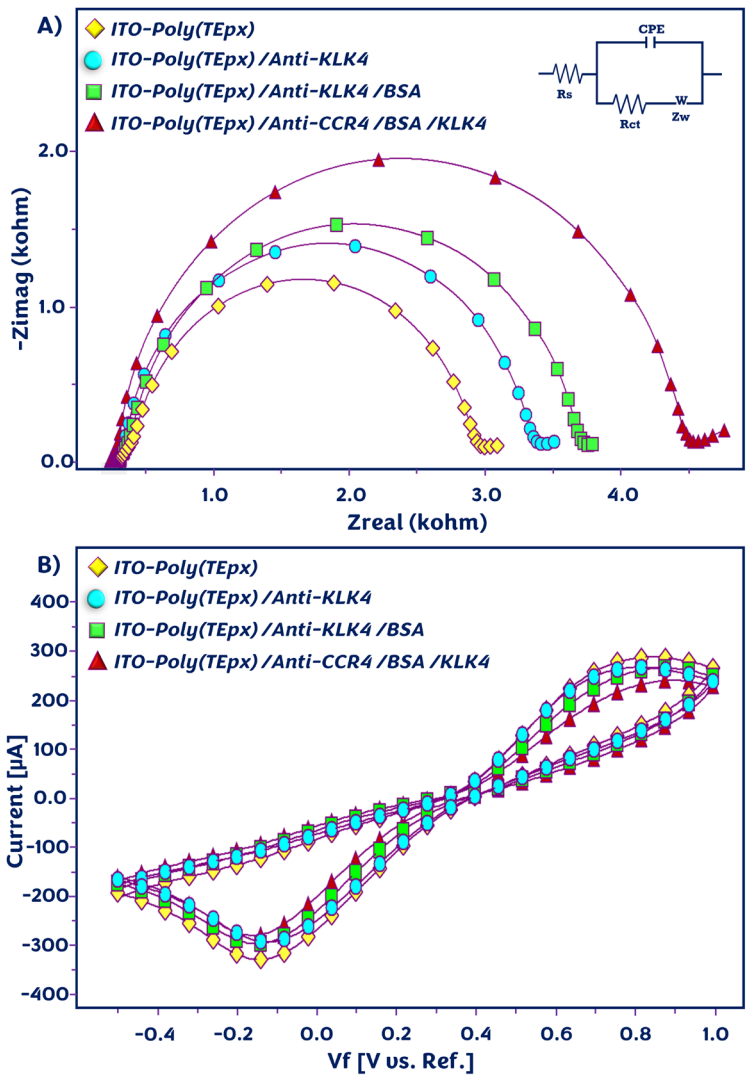


Figure 2. Nyquist plot (A) and CV (B) of sequential immobilization steps on ITO electrodes.

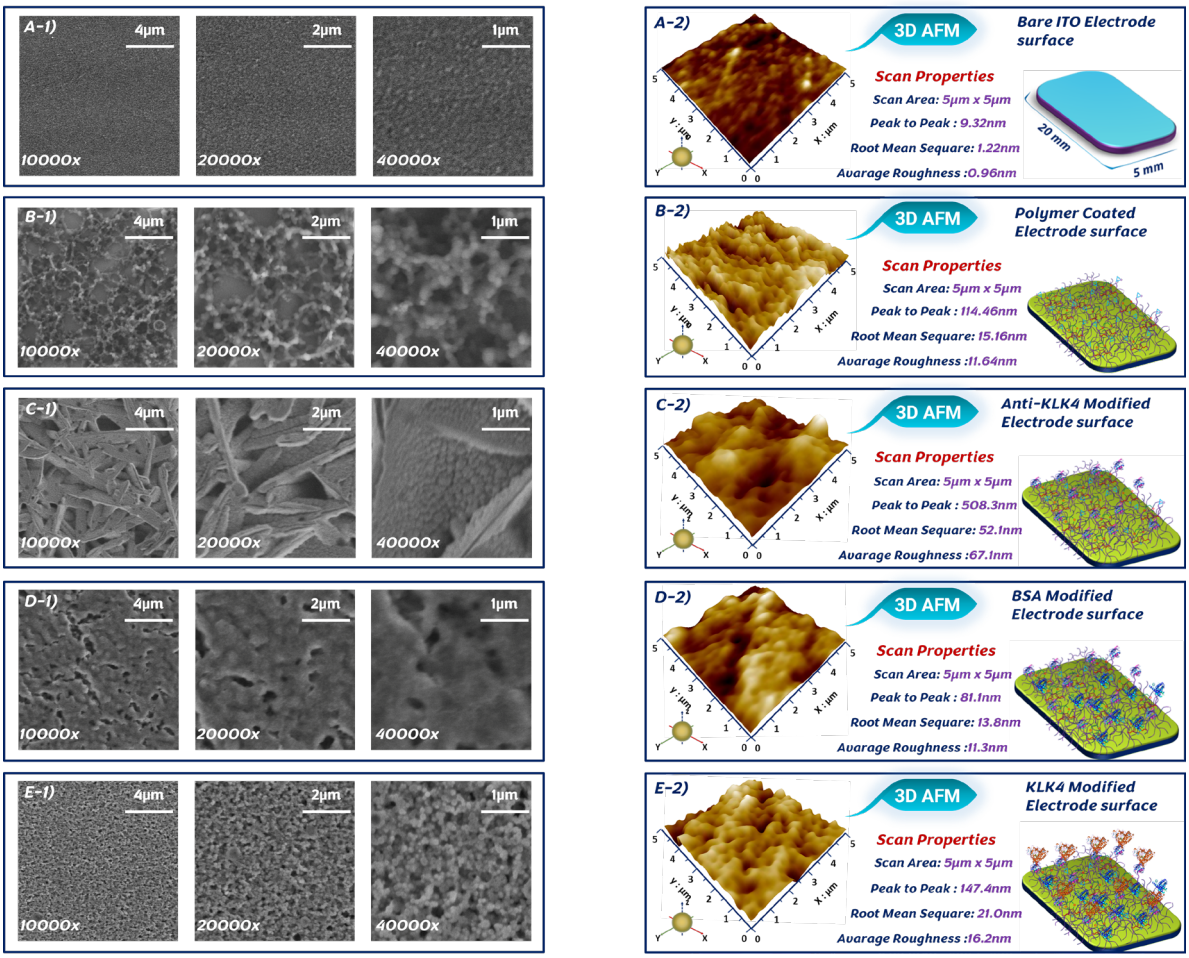


Figure 3. SEM characterizations of the electrode surface.

Fig. 2B illustrates the results of the CV measurements performed on ITO/*Poly(TEpx)*, ITO/*Poly(TEpx)*/anti-KLK4, ITO/ *Poly(TEpx)*/anti-KLK4/BSA, and ITO/*Poly(TEpx)*/ anti-KLK4/BSA/KLK4 electrodes. The CV curve of ITO/ *Poly(TEpx)* electrode had the highest peak current due to the good conductivity of the polymer matrix. The presence of anti-KLK4 antibodies on the electrode surface resulted in low peak currents due to the prevention of electron transfer and low electrical conductivity. With the binding of BSA molecules on the free epoxy ends, decreases in peak currents were caused by the inhibition of the electron transfer. The formation of an immunocomplex between anti-KLK4 antibodies and KLK4 antigens resulted in decreases in peak currents and hindered the diffusion of $[Fe(CN)_6]^{3-/4}$ towards the electron surface.

Morphological Behavior of the Biosensor Platform

The morphology of different electrode surfaces was investigated by SEM and AFM analyses. AFM analysis provided the surface roughness information of polymer coating and immobilized biomolecules. The SEM image of the bare electrode (Fig. 3A-1) pointed out that this electrode had a pure surface. The average roughness (R_a) was 0.96 nm (Fig. 3A-2). SEM analysis of ITO/ *Poly(TEpx)* in Fig. 3B-1 revealed that the polymer film was uniformly dispersed on the ITO electrode surface. With the polymer coating on the electrode surface, R_a increased to 11.64 nm (Fig. 3B-2). Besides, as shown in Fig. 3C-1, the surface morphology of ITO/*Poly(TEpx)*/ anti-KLK4 was different from the previous step. This change in morphology proved the successful immobilization of anti-KLK4 antibodies. When the anti-KLK4 protein was immobilized, the AFM morphology was totally changed with large biomolecule aggregates. The R_a was measured as 67.1 nm (Fig. 3C-2). The SEM and AFM images of ITO/*Poly(TEpx)* /anti-KLK4/BSA are illustrated in Fig. 3D-1. The change in the image illustrated the blockage of free epoxy ends, and the R_a was found as 11.3 nm (Fig. 3D-2).

Meanwhile, the SEM image of ITO/*Poly(TEpx)*/anti-KLK4/BSA/KLK4 showed large protein molecules, implying that KLK4 antigens had been successfully captured by anti-KLK4 antibodies on the electrode surface (Fig. 3E-1). With the coupling KLK4 (Fig. 3E-2), the surface roughness was changed (Ra, 16.2 nm).

Optimization of Analysis Conditions for the Biosensor

The analysis conditions, such as anti-KLK4 concentration, anti-KLK4, and KLK4 incubation duration, were optimized. First of all, the utilized anti-KLK4 amount was optimized. In this experiment, three different amounts of anti-KLK4 (1, 4, and 16 ng/mL) were utilized. A low amount of anti-KLK4 utilization caused a low signal. The large amounts of anti-KLK4 utilization caused larger signals, and the signals after 4 and 16 ng/mL antibody usage were similar. Therefore, a 4 ng/mL anti-KLK4 concentration was utilized (Fig. 4). Then, the effects of anti-KLK4 incubation times on the biosensor response were studied.

As viewed in Fig. 5A, similar responses were recorded after 45 and 60 min, and 45 min was enough for anti-KLK4 immobilization. Therefore, 45 min was utilized in further experiments. Lastly, the incubation time of KLK4 antigen was optimized. The increase in the incubation period increased the impedimetric signal, and 45 minutes were chosen as optimum periods for immobilization (Fig. 5B).

Sensing Performance of the KLK4 Biosensor

Figure 6 illustrates the EIS responses of the immunosensor after interaction with the KLK4 concentration ranging from 0.025 to 12.5 pg/mL. As the concentration of the KLK4 increased from 0.025 to 12.5 pg/mL, the Nyquist plot diameter of the immune-sensor increased due to the formation of an immune reaction between antibody and antigen. This reaction caused inhibition in electron transfer. More KLK4 concentration caused more interference and more increase in R_{ct} (Figure 6A). Moreover, Fig. 6B shows the CV responses of the immunosensor after interaction with KLK4 concentrations ranging from 0.025 to 12.5 pg/mL. In the same way, the

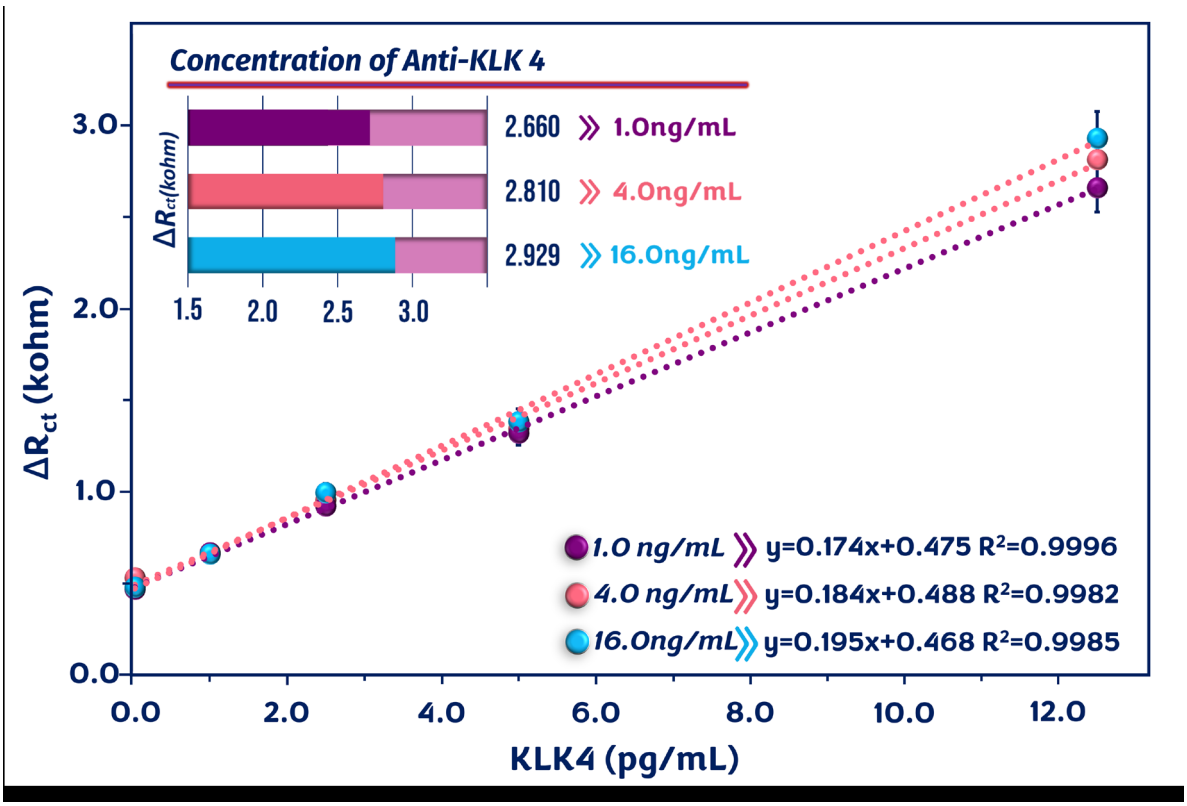


Figure 4. Effects of anti-KLK4 amount on the biosensor response.

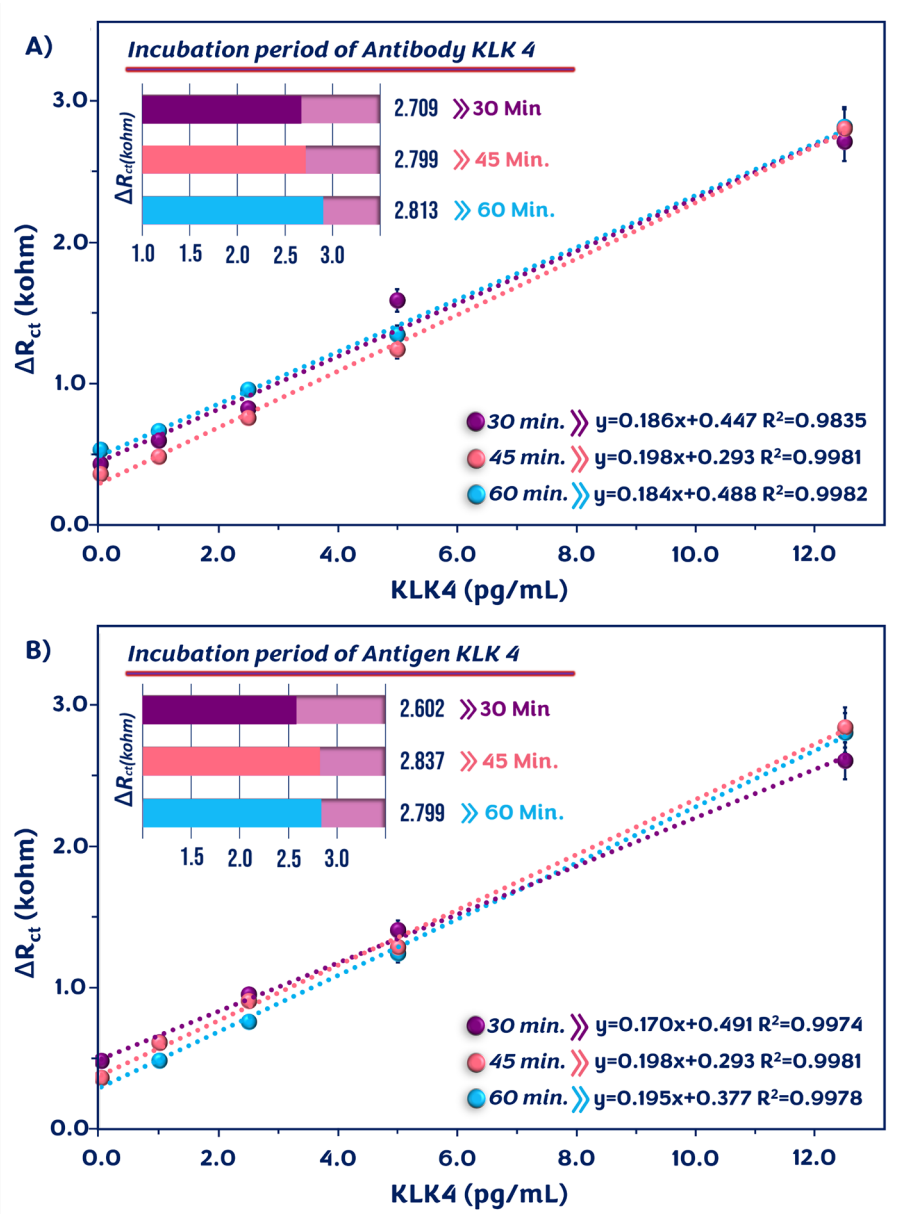


Figure 5. Effects of anti-KLK4 (A) and KLK4 incubation time (B) on the biosensor response.

increase in KLK4 concentration decreased the peak currents of CV because of more KLK4 capturing. The decreases in peak currents with increasing protein coverage illustrated the hindrance effect of KLK4 proteins. A linear calibration curve in the range of 0.025-12.5 pg/mL illustrated the linear relationship between ΔR_{ct} and KLK4 antigen concentrations (Fig. 7A).

Based on the linear detection range obtained from 0.025 to 12.5 pg/mL with the linear equation of ΔR_{ct} = 0.196 [KLK4] + 0.335, the detection limit (LOD), quantification limit, and sensitivity were calculated as 7.78 fg/mL, 25.92 fg/mL, and 0.935 kohm pg^{-1} $mLcm^{-2}$, respectively. Single frequency impedance analysis is an EIS technique that reduces the complexity of signal acquisition. This technique is an appropriate, straightforward, and affordable analysis method for investigating biomolecular interactions [33,34]. In this system, this technique was utilized to characterize the binding of KLK4 to anti-KLK4. The SFI analysis was done at 12 Hz (from the Bode plot, inset Fig. 7B), and the impedance was a function of time. The significant change in the R_{ct} proved the binding between the antibody and antigen (Fig. 7B).

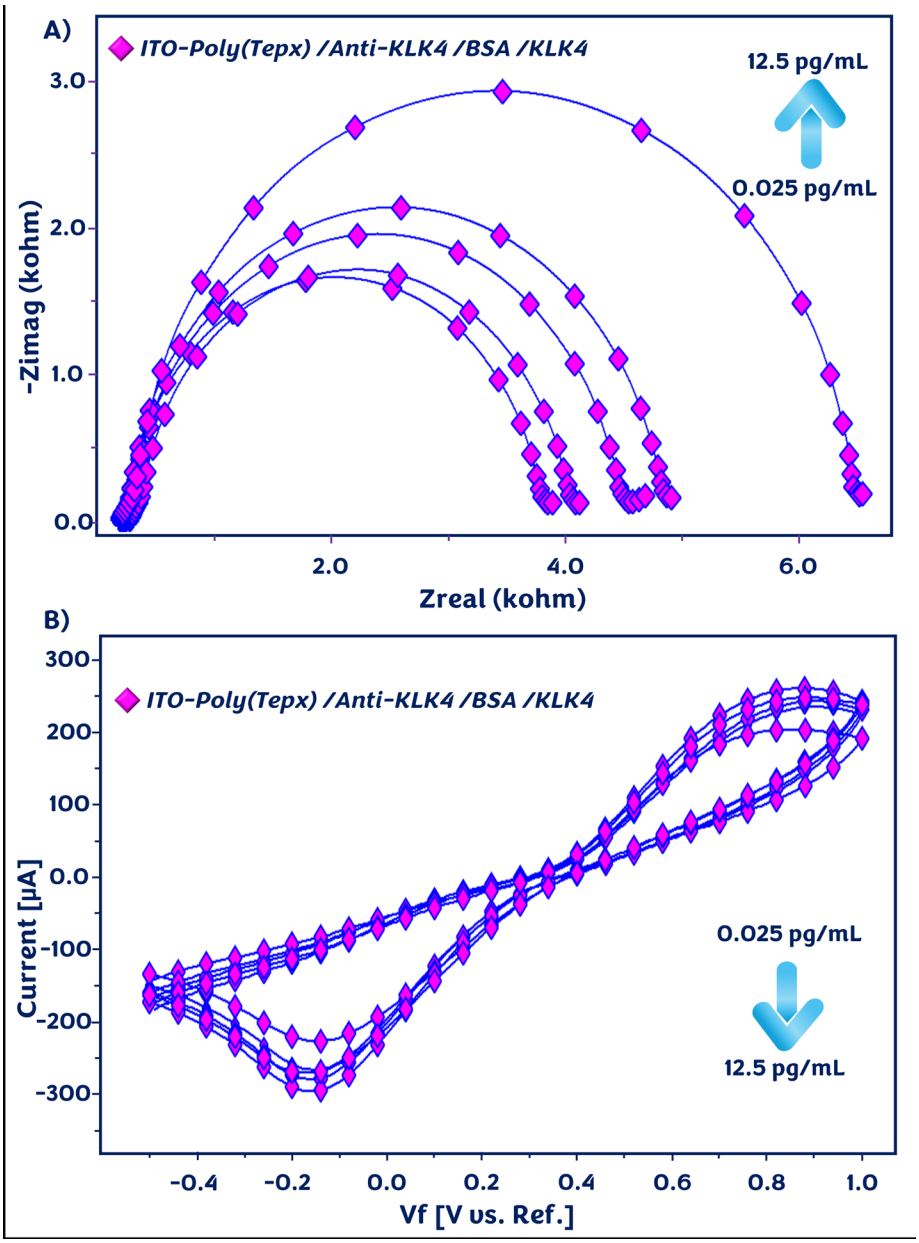


Figure 6. EIS (A) and CV (B) responses in 5.0 mM ferri-ferro solution.

The analytical performance of the developed biosensor using the *Poly(TEpx)/ITO* with other techniques reported in the literature for the analysis of KLK4 is displayed in Table 1A. This table shows that the proposed biosensor outperforms ELISA kits for KLK4 measurement by providing a lower detection limit. For the electrochemical study of KLK4, this biosensor was a great choice because of its affordability, ease of use, and quick reaction time.

Repeatability of the proposed immunosensor was tested by using 15 different electrodes prepared under the identical experimental conditions. In this experiment, 3 different concentrations (0.025, 2.5, and 12.5 pg/mL) were measured 5 times. The RSDs of the measurements were found to be 5.13%, 5.25%, and 1.64%, respectively. Reproducibility of the biosensor was also tested by measuring 3 different concentrations (0.025, 2.5, and 12.5 pg/mL) of KLK4 antigen on different days. The RSDs of the measurements were found to be 5.11%, 5.23%, and 2.90%, respectively. These results indicated acceptable repeatability and reproducibility (Fig. 8A).

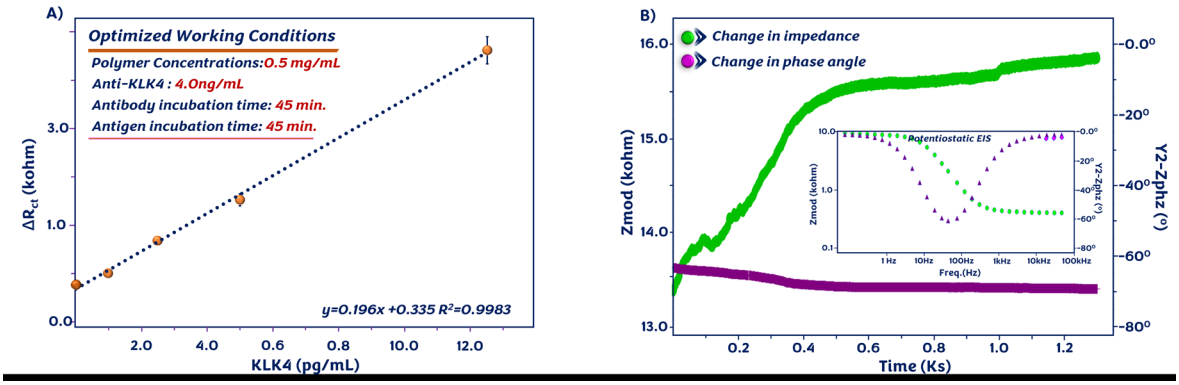


Figure 8. The test results of repeatability/reproducibility (A) and storage stability (B).

As a key factor, the storage stability of the proposed biosensor was also tested. The biosensor was stored at 4 °C for different periods (from 0 to 10 weeks). The EIS response decreased with the storage time increased. After 8 weeks of storage, the EIS response of the immunosensor decreased 79.64%, indicating that the biosensor could keep its performance for a long time (Fig. 8B).

In addition, EIS measurements were also performed to evaluate the specificity of the designed biosensor by detecting the electrochemical responses to different cancer biomarkers and KLK4. The impedimetric response was high in the presence of KLK4 antigen, and there was no significant response in the presence of other biomarkers. The experiment results are illustrated in Figure 9A. Another key factor, the regeneration ability of the proposed immunosensor, was also tested. The regeneration process included the incubation of the *ITO/Poly(TEpx)/anti-KLK4/BSA/KLK4* electrode in an acidic solution (10 mM HCl) for 4 min. The regenerated electrode was then incubated in KLK4 solution. After each regeneration and incubation step, the impedimetric responses of the electrode were recorded. The regenerated ITO electrode retained 72.91% of its original EIS response after a 4-cycle regeneration process. This result illustrated that this biosensor could be regenerated successfully and reused with good electrochemical characteristics (Figure 9B).

Biosensor Response to Actual Serum Samples

Actual serum samples were obtained from Sigma-Aldrich. In order to test the accuracy of the system, the standard addition method was utilized. Before the KLK4 measurement, the non-spiked and spiked actual serum samples were diluted (1:10) with PBS, and EIS measurements were performed. This biosensor exhibited good recycling rates from 96.45% to 107.14%. These results demonstrated the enormous potential of this biosensor for further analytical applications of other biomarkers (Table 1B).

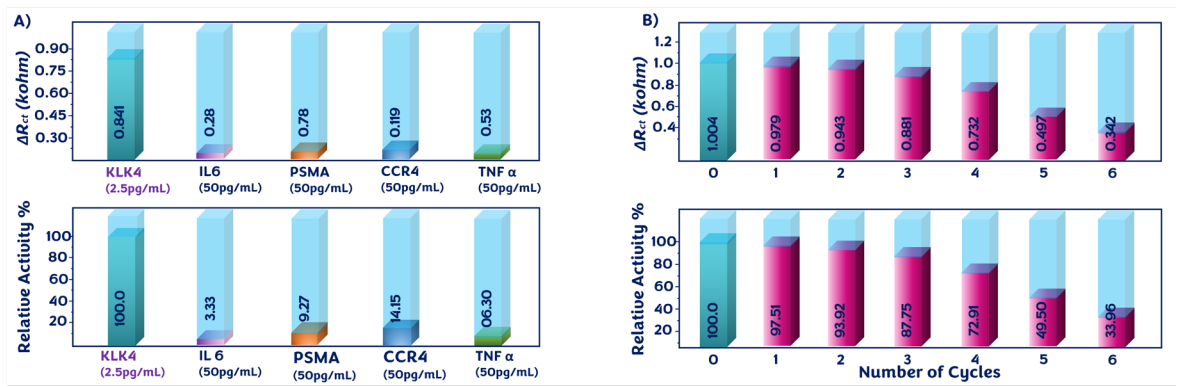


Figure 9. The test results of specificity (A) and regeneration (B).

Table 1. Comparison between KLK4 analysis methods (A), and determination of KLK4 in actual serum (B).

A) Technique	Linear range (pg/mL)	Detection limit (pg/mL)	References
ITO/Zinc(II) phthalocyanine tetracarboxylic acid/antibody	0.02–15	0.007	35
ITO/Polythiophene/antibody	0.04-30	0.012	36
ELISA	156-104	39	MyBiosource
ELISA	156-104	39	Cusabio
ELISA	156-104	49	Aviva Systems
ELISA	312-2x104	65	ReddotBiotech
ELISA	312-2x104	65	Bioss Inc.
ITO/Poly(TEpx)/antibody	0.025-12.5	0.008	This work

B) Sample	Found by biosensor (pg/mL)	Added KLK4 (pg/mL)	Total KLK4	% Recovery	% Relative Difference
1	1.64	1.0	2.62	99.15	-0.85
2	1.96	1.0	3.08	104.06	4.06
3	2.36	1.0	3.24	96.45	-3.55
4	1.80	1.0	2.98	106.48	6.48
5	1.47	1.0	2.64	107.14	7.14

Conclusions

A label-free, highly sensitive, accurate, and fast impedimetric immunosensor was developed to determine KLK4 antigen in human serum. The results obtained in the present study illustrated that *Poly(TEpx)* polymer-coated electrodes provided a large and biocompatible surface for the immobilization of anti-KLK4 antibodies. Thus, a wide linear detection range (0.025-12.5 pg/mL) and a low LOD (7.78 fg/mL) were obtained. In addition, the developed immunosensor exploited a remarkable specificity to KLK4 antigens in the presence of other biomarkers. The applicability of the immunosensor was successfully evaluated by determining KLK4 antigen in actual serum samples with satisfactory recovery (96.45 - 107.14%) results. Furthermore, the fabrication cost of the developed biosensor was cheaper than that of a traditional ELISA kit. Therefore, due to its low cost, storage stability, and sensitivity, the immunosensor may be a very appealing substitute for measuring KLK4 levels in human serum.

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