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A Highly Sensitive Electrochemical Immunosensor for the detection of CCR4 Biomarker with Star-Shaped Polymer Covered Disposable Electrode

Yıldız Şeklinde Polimer Kaplı Tek Kullanımlık Elektrot ile CCR4 Biyobelirtecinin Tespiti için Yüksek Hassasiyetli Elektrokimyasal İmmünosensör

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ABSTRACT

An electrochemical immunosensor prepared with the use of star-shaped polymer (StrPol(GMA)) as a biosensor matrix material was developed to quantify the CC chemokine receptor 4 (CCR4) molecules in human serum. Various characterization techniques, including electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV), and scanning electron microscopy (SEM), were used for the analysis of the fabrication steps of the proposed system. The impedance and CV analyses at each modification step of the fabrication process illustrated the charge transfer kinetics of the ferri-ferro redox solution. The binding of anti-CCR4 antibody and the related antibody-its CCR4 antigen biointeraction on the working electrode surface changed the interfacial electron transfer. The interactions of anti-CCR4 with different levels of CCR4 were further followed via variations in impedance responses. The results illustrated that the electron transfer resistance increased linearly with increasing amounts of CCR4 antigen. The dynamic range and detection limit (LOD) were determined as 0.115-23 pg/mL and 34.9 fg/mL, respectively. The regeneration, storage stability, and reproducibility tests were also performed. Furthermore, CCR4 detection in the presence of interfering biomarkers was accomplished with success using the biosensing approach. The results of these studies illustrate that the developed biosensor was a useful tool for monitoring low concentrations of CCR4 in human sera.

Key Words
CC chemokines receptor 4, prostate cancer, disposable biosensor, star-shaped polymer.

ÖZ

İnsan serumundaki CC kemokin reseptörü 4 (CCR4) moleküllerini ölçmek için, biyosensör matris malzemesi olarak yıldız şekilli polimer (StrPol(GMA)) kullanılarak hazırlanan bir elektrokimyasal immunosensor geliştirildi. Önerilen sistemin üretim aşamalarının analizi için elektrokimyasal empedans spektroskopisi (EIS), döngüsel voltametri (CV) ve taramalı elektron mikroskopisi (SEM) dahil olmak üzere çeşitli karakterizasyon teknikleri kullanılmıştır. Üretim sürecinin her bir modifikasyon adımındaki impedans ve CV analizleri, ferri-ferro redoks çözeltisinin yük taşıma kinetiğini göstermiştir. Anti-CCR4 antikorunun bağlanması ve ilgili antikor- CCR4 antijeni biyoetkileşiminin çalışma elektrodu yüzeyinde arayüzey elektron taşınımını değiştirmiştir. Anti-CCR4'ün farklı CCR4 seviyeleri ile etkileşimleri, empedans yanıtlarındaki değişimler yoluyla daha fazla takip edilmiştir. Sonuçlar, elektron taşıma direncinin artan CCR4 antijen miktarıyla doğrusal olarak arttığını göstermiştir. Dinamik aralık ve tespit limiti (LOD) sırasıyla 0.115-23 pg/mL ve 34.9 fg/mL olarak belirlenmiştir. Rejenerasyon, depolama stabilitesi ve tekrar üretilebilirlik testleri de gerçekleştirilmiştir. Ayrıca, biyoalgılama yaklaşımı kullanılarak müdahale eden biyobelirteçlerin varlığında CCR4 tespiti başarıyla gerçekleştirilmiştir. Bu çalışmaların sonuçları, geliştirilen biyosensörün insan serumlarındaki düşük CCR4 konsantrasyonlarını izlemek için yararlı bir araç olduğunu göstermektedir.

Anahtar Kelimeler
CC kemokin reseptörü 4, prostat kanseri, tek kullanımlık biyosensör, yıldız şekilli polimer.

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INTRODUCTION

Prostate cancer (PC) is one of the most frequently detected types of cancer worldwide that causes a death in almost 10% of all cancer cases [1]. Prostate cancer is a kind of malignant tumor in the male prostate gland. Currently, digital rectal examination, serum prostate-specific antigen (PSA) measurement, and biopsy are the main analysis techniques for the diagnosis of PC [2,3]. Among them, protein markers are yet known as a gold standard for PC diagnosis [4,5]. For measuring tumor markers, enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, and fluorescent immunoassay are general analysis methods. These techniques require long analysis times, high-cost devices, and special personnel. Hence, the development of ultrasensitive and economical sensors with high selectivity and sensitivity is very important [6,7].

PSA amounts in the blood biological fluid may rise not only in PC but also in benign growth of the prostate and inflammation in prostate tissue. The sensitivity of PSA in PC is about 80% [8,9]. As a result, there is an urgent need to find a new sensitive and specific biomarker for prostate cancer. Chemokines are a group of cytokines and are divided into subfamilies according to the domain found at the N-terminal. Chemokines and their receptors have a significant role in tumorigenicity. Both the cancer cells and the healthy cells that are close to the cancer cells introduce them. CCR4 is an activation-regulated cytokine that controls immune homeostasis. Also, it is a biomarker of prostate cancer [10,11].

Electrochemical biosensors have a major role in tumor biomarker detection because of their advantages, such as fast response, miniaturization, and simple operation [12,13]. The primary stage of the biosensor development is immobilization matrix material selection for biosensing element attachment. In recent years, the use of nanomaterials and polymers has improved the sensitivity of electrochemical biosensors. Polymers are inseparable from biosensor modification strategies and sensor construction platforms. They are helpful during the fabrication of electrochemical sensors/biosensors owing to their unique properties, such as biocompatibility, stability, and low cost [14,15]. Star-shaped polymers are branched molecules and bear the same or distinct linear polymer chains. They are soluble, globular, and cheap. In addition, they share several characteristics with dendrimers, including a large number of active end groups. They have the advantage of being less expensive than dendrimers [16,17]. Therefore, they are candidate materials for biosensor fabrication. Immunosensors are biosensors that utilize the interactions between an antibody and its antigen. The interaction between these molecules provides a stable complex. On account of the strong forces between these biological molecules, they show high selectivity and sensitivity. Immunosensors using electrochemical detection have been utilized in several analyses because of their specific, versatile, and simple properties [18,19]. EIS is an effective, nondestructive, and illuminating method and gives information about the electrical features of practically any biosensor and sensor interface. This technique can analyze electrode surface variations, determine diffusion kinetics, and obtain mass-transfer data. The impedimetric response of any biosensor is illustrated with Bode and Nyquist curves. The semicircle of the Nyquist curve illustrates capacitance and resistance; the linear portion displays diffusive influences [20,21].

In the proposed study, an electrochemical label-free immunosensor, which could detect the low amount of CCR4 antigen, was developed. Herein, a *StrPol(GMA))*-coated electrode was prepared through a spin-coating strategy, and anti-CCR4 antibody recognition molecules were cast on the *StrPol(GMA))*-coated single-use ITO electrode by covalent immobilization. The specific biointeraction between anti-CCR4 and CCR4 caused an electrochemical signal that was recorded with the use of the EIS technique. Furthermore, this immunosensor had appropriate reproducibility, great selectivity, and good stability during its storage. The application of the developed sensor for measuring CCR4 on serum samples was investigated. This work developed a simple and sensitive CCR4 detection route for real human sample analysis.

MATERIALS and METHODS

Reagents and Apparatus

Potassium hexacyanoferrate ($K_4Fe(CN)_6 \cdot 3H_2O$), potassium ferricyanide ($K_3Fe(CN)_6$), potassium chloride (KCl), potassium hydrogen phosphate (K_2HPO_4) potassium dihydrogen phosphate (KH_2PO_4), ITO-coated polyethylene terephthalate, 1,1,1-tris(hydroxyl methyl propane), N,N,N′,N″,N‴ -pentamethyldiethylenetriamine, copper(II) bromide, α-bromoisobutyryl bromide, tetrahydrofuran, and anisole, monoclonal anti-CCR4 antibody, CCR4, bovine serum albumin (BSA), human serum

(H4522), interleukin (IL-6), kallikrein-related peptidase 4 (KLK4), prostate specific membrane antigen (PSMA), and protein 53 (P53) were purchased from Sigma-Aldrich, and necessary dilutions were made with 0.05 M phosphate buffer (pH 7.4). Ultrapure water was employed in all electrochemical experiments.

All electrochemical analyses were recorded at a Gamry electrochemistry workstation with conventional three electrodes: the modified ITO working substrate, a platinum counter wire, and an Ag/AgCl reference electrode. SEM images were attained utilizing a QUANTA FEG SEM 250. FTIR spectra analysis was measured in an FTIR spectrometer (Bruker Company Vertex 70). Proton nuclear magnetic resonance spectra were taken by employing Bruker Avance II (400 MHz) via $CDCl_3$ deuterated solvents. The spin-coating operation was implemented with a classic spin-coater (MTI VTC-50).

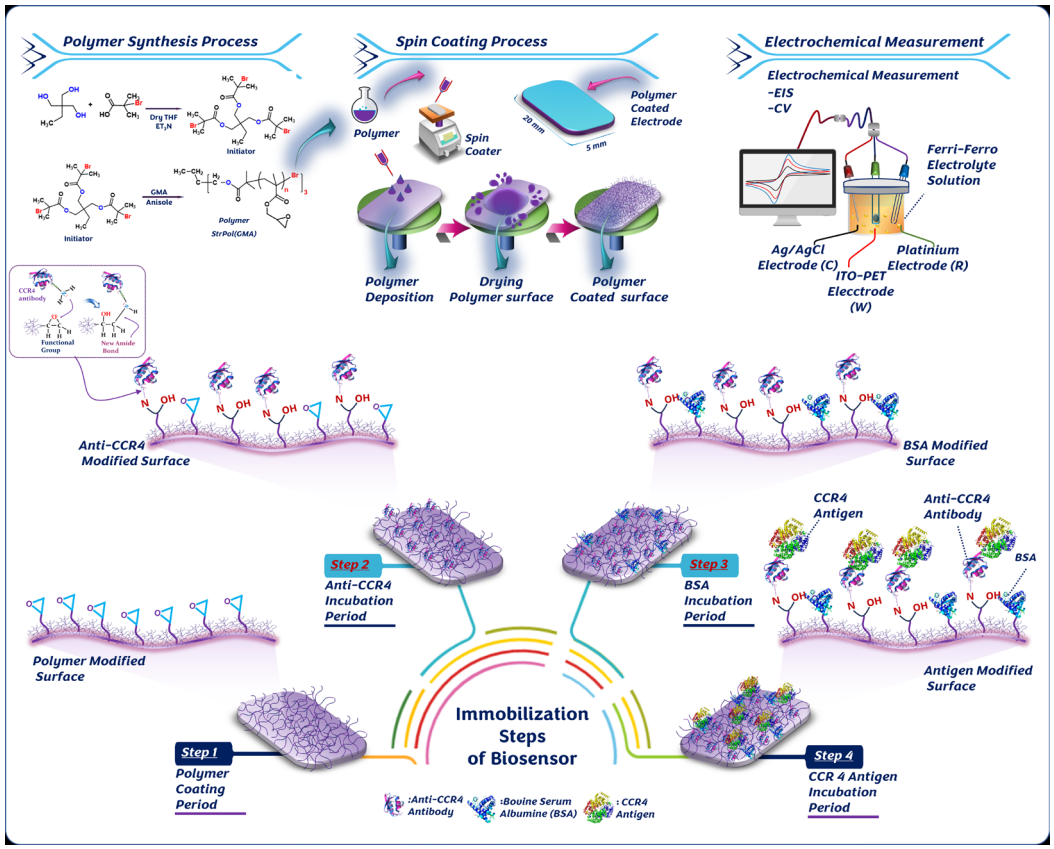
Electrochemical Measurements

CV, EIS, and single-frequency EIS (SFI) of the modified electrodes were recorded on a three-electrode cell employing a Reference 1000 (Gamry) electrochemical device. CV was recorded in a redox solution (5 mM $Fe(CN)_6^{3-/4-}$ and 0.1 M KCl) within a potential range from -500 to 1000 mV at a potential scan rate of 100 V/s. EIS was measured in the same electrolyte solution with a frequency rate from 0.5 Hz to 50 kHz. All electrochemical measurements were taken at 25 ± 2 °C.

Synthesis of StrPol(GMA) Polymer

Three armed initiators were prepared according to the previous report. [22]. FT-IR (ATR, cm^{-1}): 2969, 2926, 1729(C=O); 1467; 1392; 1275; 1161; 1106; 976; 932; 636 (-C-Br); 480. ^{1}H-NMR ($CDCl_3$, 400 MHz)δ: 0,97 ppm (3H), 1,63 ppm (2H), 1,93 ppm (18H), and 4,18 ppm (6H).

StrPol(GMA) polymer was synthesized (Scheme 1) with atom transfer radical polymerization (ATRP) and characterized according to the previous report [22]. FT-IR (ATR, cm^{-1}): 2966, 2939, 1726(C=O); 1451; 1392; 1256; 1144; 1068; 968; 906; 848; 756; 538; 454. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 0.8 - 1.8 ppm (Ha, Hb and He), 4,05 ppm (Hc), 1.9 ppm (Hd), 3.80 ppm and 4.30 ppm ($Hf_{1,2}$), 3.23 ppm (Hg), 2.84 ppm and 2.64 ppm ($Hh_{1,2}$).



Scheme 1. Schematic illustration for the analysis of CCR4 employing StrPol(GMA)-covered electrochemical biosensor.

Fabrication of *StrPol(GMA)*-Coated Electrochemical Biosensor

Scheme 1 illustrates the schematic diagram of the preparation of a *StrPol(GMA))*-coated electrochemical biosensor. Before the analysis, ITO electrodes were washed with the aid of ultrasonication for 10 min in acetone, soap solution, and distilled water. The first stage of the fabrication procedure was composed of a *StrPol(GMA))* layer coating on the ITO surface to enlarge the efficient surface area. The polymer solution prepared in acetone (0.5 mg/mL) was spread on the ITO surface (1000 rpm, 60 seconds). The *StrPol(GMA)*-covered ITO substrate was washed with ultrapure water and dried under nitrogen. Next, anti-CCR4 was covalently immobilized on the ITO-modified surface during the 30 min incubation period. Then, the modified surface was blocked with BSA to prevent nonspecific interactions. The last step was biointeraction between anti-CCR4 and CCR4 molecules.

Human Serum Sample Testing

To assess the feasibility of the presented procedure, electrochemical analysis was utilized to determine the CCR4 amount in the human sera. The sera samples were obtained from Sigma-Aldrich (H4522). Before the electrochemical analyses, they were diluted 100-fold with phosphate buffer (pH 7.4, 0.05 M). Additionally, to verify the biosensor's accuracy, standard CCR4 antigens were added to the human serum samples, and recoveries were calculated.

RESULTS and DISCUSSION

The development procedure of the disposable biosensor is shown in Scheme 1. This process was composed of 4 steps: *StrPol(GMA)* polymer coating (step 1), anti-CCR4 antibody attachment (step 2), BSA blockage to free epoxy ends (step 3), and CCR4 capturing (step 4).

Chemical Characterizations

Star polymer was synthesized by the ATRP method. These reaction stages are shown in Scheme 1. The chemical characterization of the *StrPol(GMA)* was performed with different spectral methods (FTIR, ^{1}H-NMR) to display the polymer synthesis procedure, and the spectroscopic results of this synthesis are illustrated in our previous report [22].

The FTIR spectrum of the *StrPol(GMA)* polymer is illustrated in Figure 1. When Figure 1 was investigated, the FTIR spectrum of the *StrPol(GMA)* polymer had the characteristic responses of epoxy ends as two sharp peaks at 907 and 848 cm^{-1} [16]. These sharp peaks proved the epoxy side groups present on the polymer structure. Thus, the sharp peak seen at 1726 cm^{-1} was illustrated as the C=O groups in the *StrPol(GMA)* polymer. In addition, the ester groups in the *StrPol(GMA)* polymer were viewed to have symmetric and asymmetric stretching vibration frequencies at 1256 cm^{-1} and 1144 cm^{-1}, respectively [23]. Since the spectrum of the anti-CCR4 immobilized electrode offered important information on protein immobilization, it was also assessed. The pre-

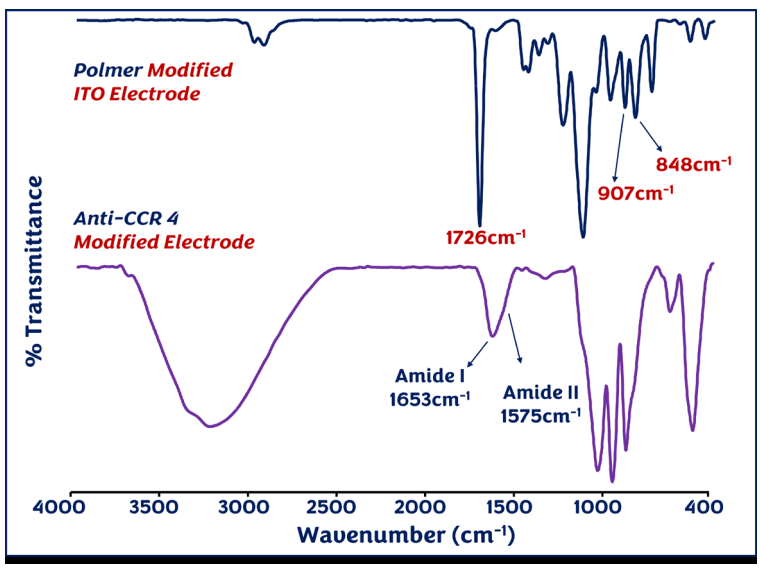


Figure 1. FTIR spectra of the StrPol(GMA) (A) and anti-CCR4 (B) coated electrode.

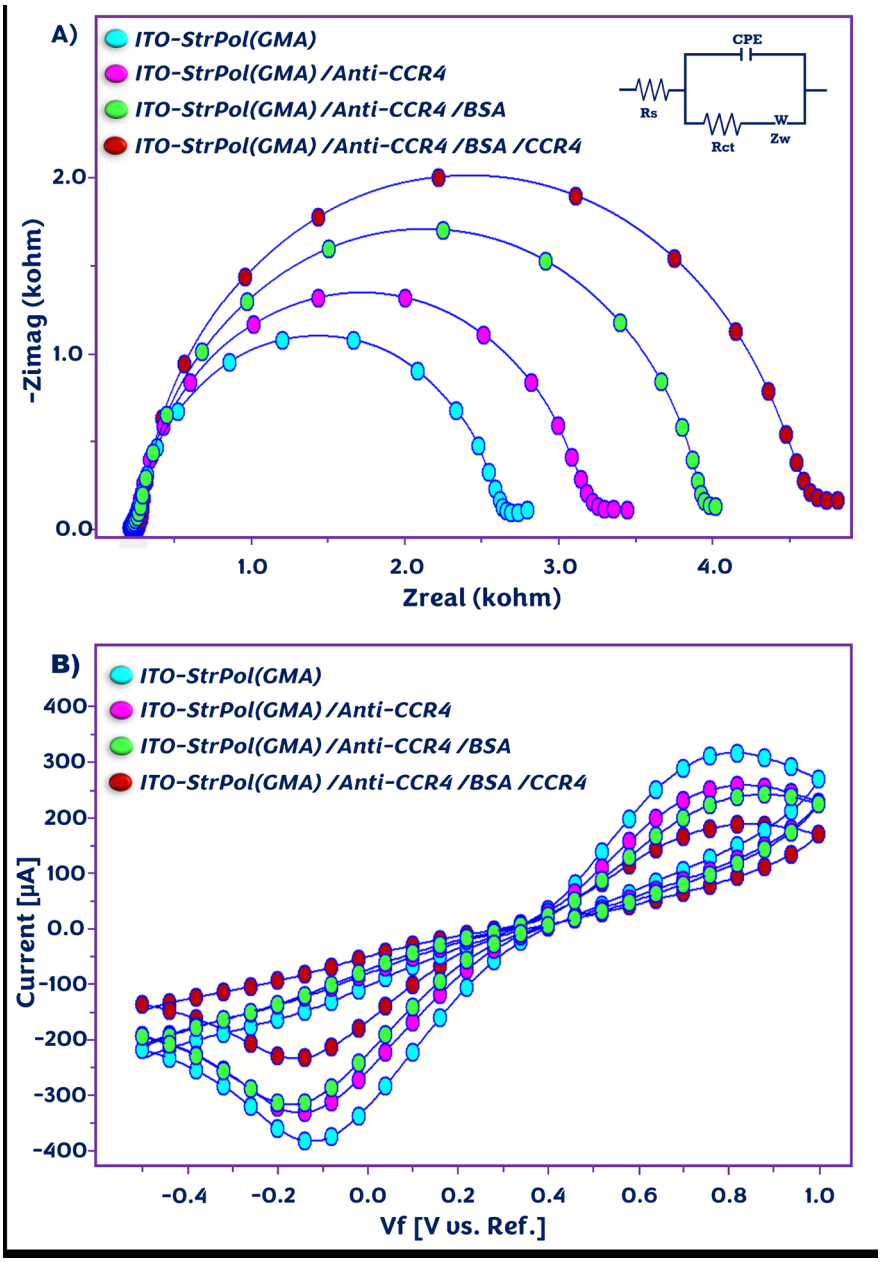


Figure 2. EIS (A) and CV (B) characterizations of different modified ITO electrodes.

sence of amide groups of the anti-CCR4 antibody immobilized on the electrode surface was shown by the broad and strong bands at 1653 cm^{-1} and 1575 cm^{-1} [24,25].

Electrochemical behavior of the sensor platform

EIS measurements were utilized to appreciate the interfacial features of the sensor. During the analysis, the potential was kept at 0 V versus the Ag/AgCl electrode. Figure 2A shows the EIS spectra recorded after modification steps.

Theoretically, the changes in impedimetric signal were viewed with charge transfer resistance (R_{ct}). R_{ct} controlled the charge transfer kinetics of the redox couple at the working electrode interface. R_{ct} varied with the modification of the working electrode, and the fitting of R_{ct} was performed via the Randles equivalent circuit [26,27]. The *StrPol(GMA)*-coated electrode had a small semicircle diameter and R_{ct} values. This impedimetric curve was found to be 2445 Ω. After attachment of anti-CCR4, R_{ct} dramatically increased to 3046 Ω, indicating successful assembly of anti-CCR4 at the polymer-coa-

ted surface. The further increment in R_{ct} (3804 Ω) was seen after the addition of BSA due to the blockage of any residual free sites on the surface. An increase in R_{ct} (4328 Ω) was also measured after the target CCR4 antigen was captured by anti-CCR4 molecules.

After EIS characterization, CV characterization was also performed. Figure 2B illustrates the CV changes on modification of the sensor. The kinetics of the redox couple were changed at different modified electrodes due to the conductivity feature of the electrode. As shown in Figure 2B, the anti-CCR4-immobilized electrode had a lower peak current compared to the polymer-coated electrode, probably due to the non-conductive features of proteins. In the next step, decreases were observed in peak currents because BSA molecules immobilized successfully on the electrode surface, and they prevented the electron transfer process. Finally, after binding the CCR4 antigen to the BSA-blocked surface, the peak current declined owing to the immobilization of non-conductive CCR4 molecules.

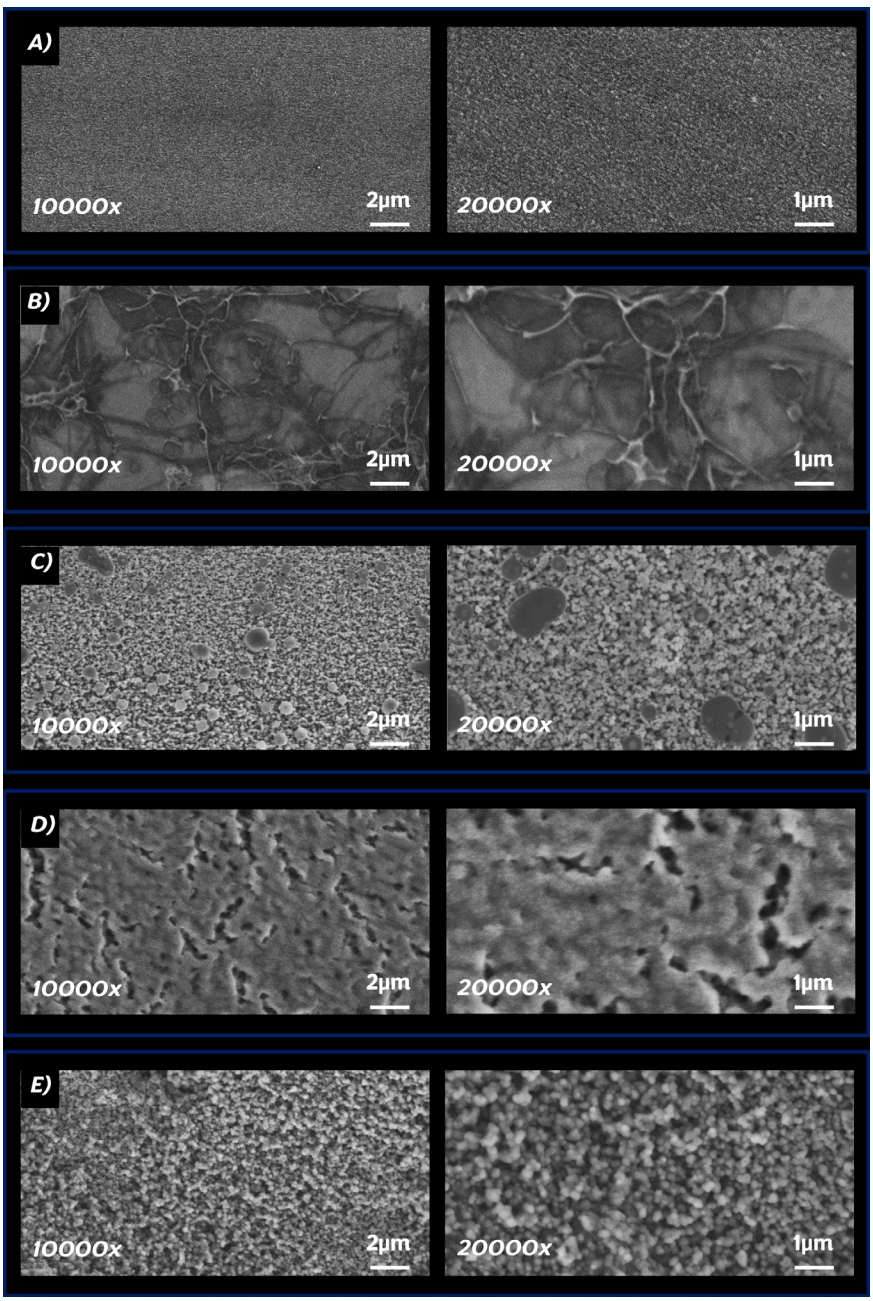


Figure 3. SEM characterizations of the electrode surfaces.

Morphological behavior of the biosensor platform

The morphological behavior of the modified electrode surface was investigated with SEM and AFM measurements.

Figures 3A and 4A depict the SEM and AFM images of a bare electrode that had a pure surface. The average roughness (R_a) was measured as 0.56 nm. After spin coating of ***StrPol(GMA)*** on the bare electrode surface, an apparent polymer layer was coated on the ITO electrode surface (Figure 3B). An increase in the R_a value was observed with the coating of the ***StrPol(GMA)*** (Figure 4B). The image of the ***StrPol(GMA)***-coated electrode surface was changed after anti-CCR4 attachment (Figure 3C), and large protein molecules could be seen in SEM and AFM images. A smooth surface was obtained after anti-CCR4 attachment, and the R_a value was measured as 24.3 nm (Figure 4C). Blockage of free ends with BSA caused a protein layer covering, as seen in figures 3D and 4D (R_a=18.3 nm). In the last image of morphological characterization, the electrode surface was changed due to CCR4 antigen capturing (Figure 3E). This capture caused a smooth surface and a low R_a (Figure 4E, 10.5 nm).

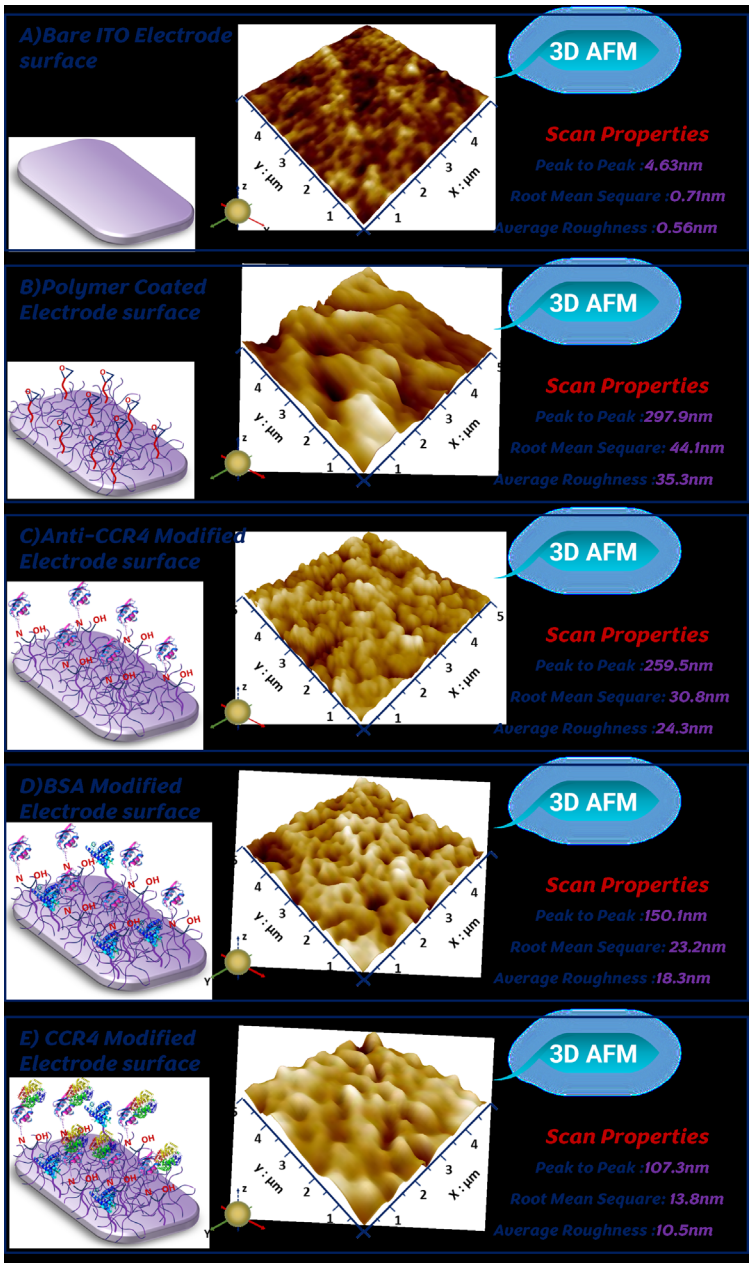


Figure 4. AFM characterizations of the electrode surfaces.

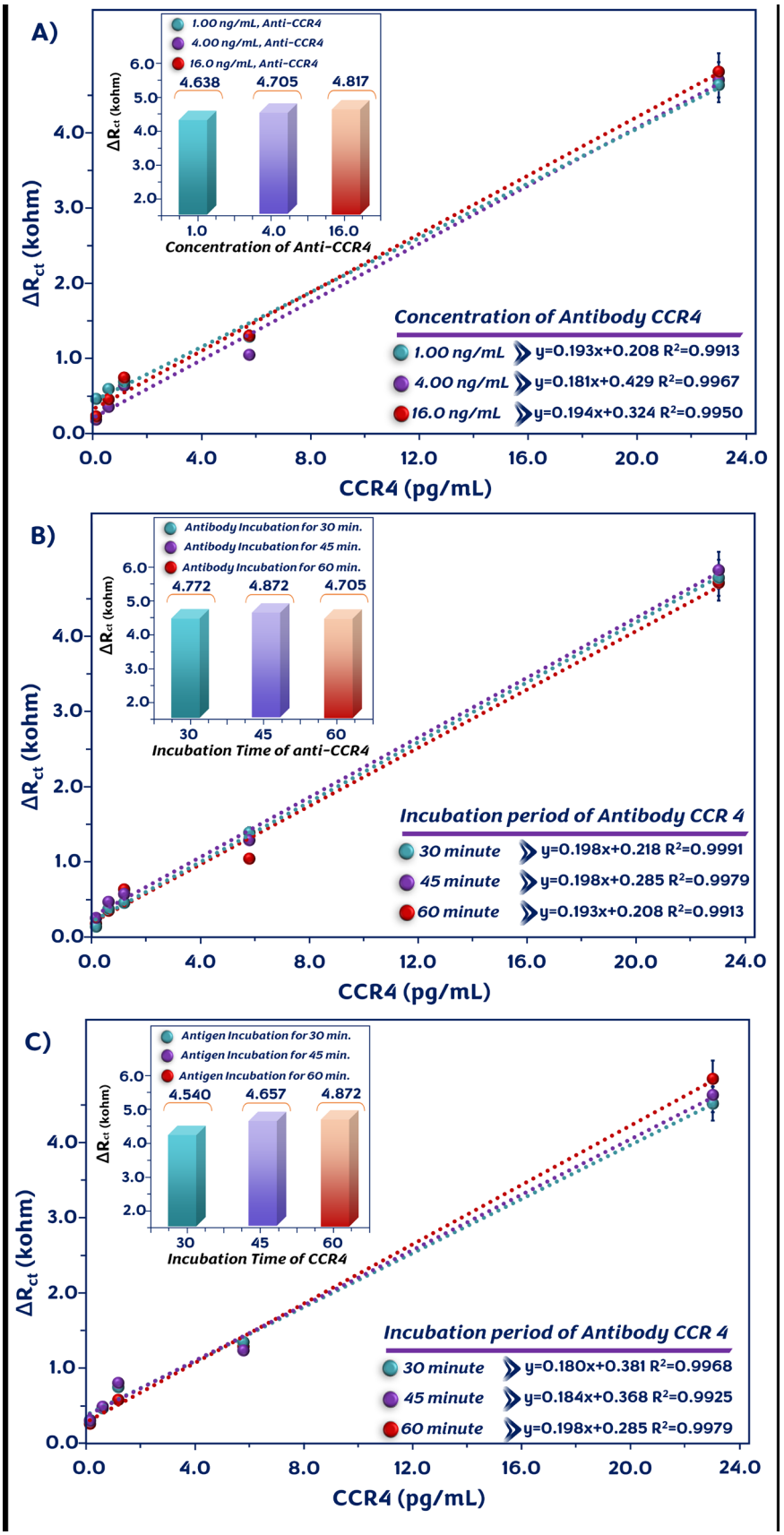


Figure 5. Optimization of anti-CCR4 concentration (A) and its incubation time (B), and the reaction time between the anti-CCR4 and CCR4 (C).

Optimization of analytical parameters

In order to determine optimum experimental parameters, different analytical conditions were studied. The first optimization parameter was the amount of anti-CCR4. The responses of utilized CCR4 to biosensor response in the range of 1-16 ng/mL were studied. Figure 5A illustrated that a 1 ng/mL amount was enough for CCR4 capturing. Next, the required time to immobilize anti-CCR4 was studied in the range of 30-60 min. After incubation, EIS spectra were recorded to determine the maximum ΔR_{ct} of these spectra. Figure 5B illustrated that 30 min was enough for anti-CCR4 binding. Lastly, the required time for CCR4 capturing was studied in the range of 30-60 min. The recorded EIS responses illustrated that, as the ΔR_{ct} at the electrode surface increased, the response enhanced, and 60 min of incubation caused the maximum response. Thus, 60 minutes was chosen as the optimal incubation time (Figure 5C).

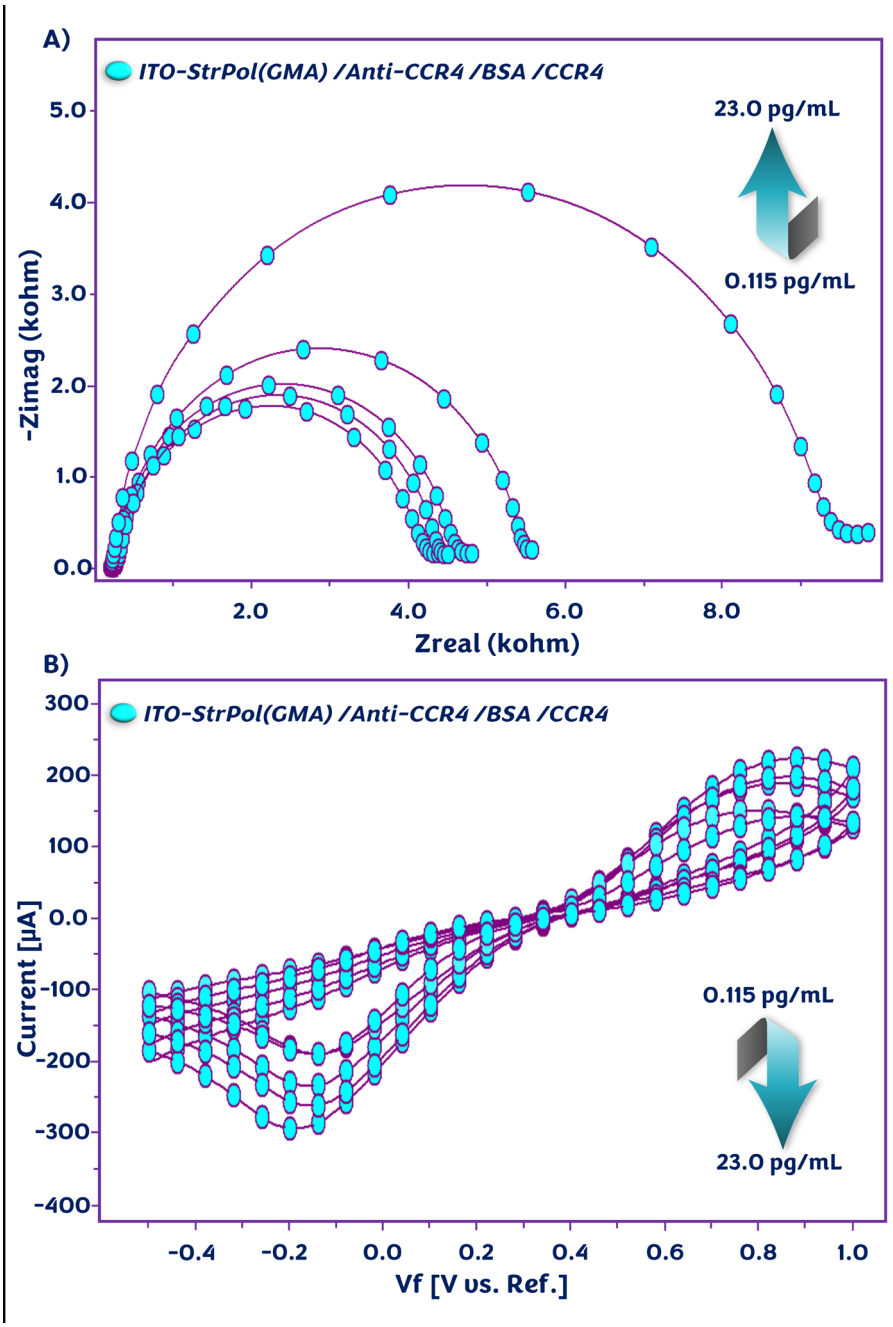


Figure 6. Electroanalytical performances of the developed CCR4 biosensor; EIS (A) and CVs (B) of different concentrations of CCR4-immobilized ITO electrodes.

Electrochemical responses of CCR4 antibody-antigen interactions

To appreciate the interaction between anti-CCR4 and CCR4, the antibody-attached electrodes were exposed to increasing concentrations of CCR4 (from 0.115 to 23 pg/mL). The Nyquist curves of impedance spectra are displayed in Figure 6A. The EIS results illustrated that the Nyquist curve diameter increased with the rising CCR4 amount. These increases proved that more CCR4 antigens were immobilized during 60 min of incubation. In other words, incubation in increasing concentrations of CCR4 antigen caused higher impedimetric responses. The increases in the ΔR_{ct} with increasing coverage showed the hindrance effects of proteins. To further investigate the effect of increasing concentrations of CCR4 antigen, CV measurements were also performed (Figure 6B). The peak currents of CV declined with the increasing of CCR4 amount. The immobilization of higher concentrations of CCR4 antigen to anti-CCR4 antibody hindered electron transfer significantly. In order to determine the dynamic range, impedimetric responses were utilized. The impedimetric signal increased with the level of CCR4 and showed a great linear relationship with CCR4 amo-

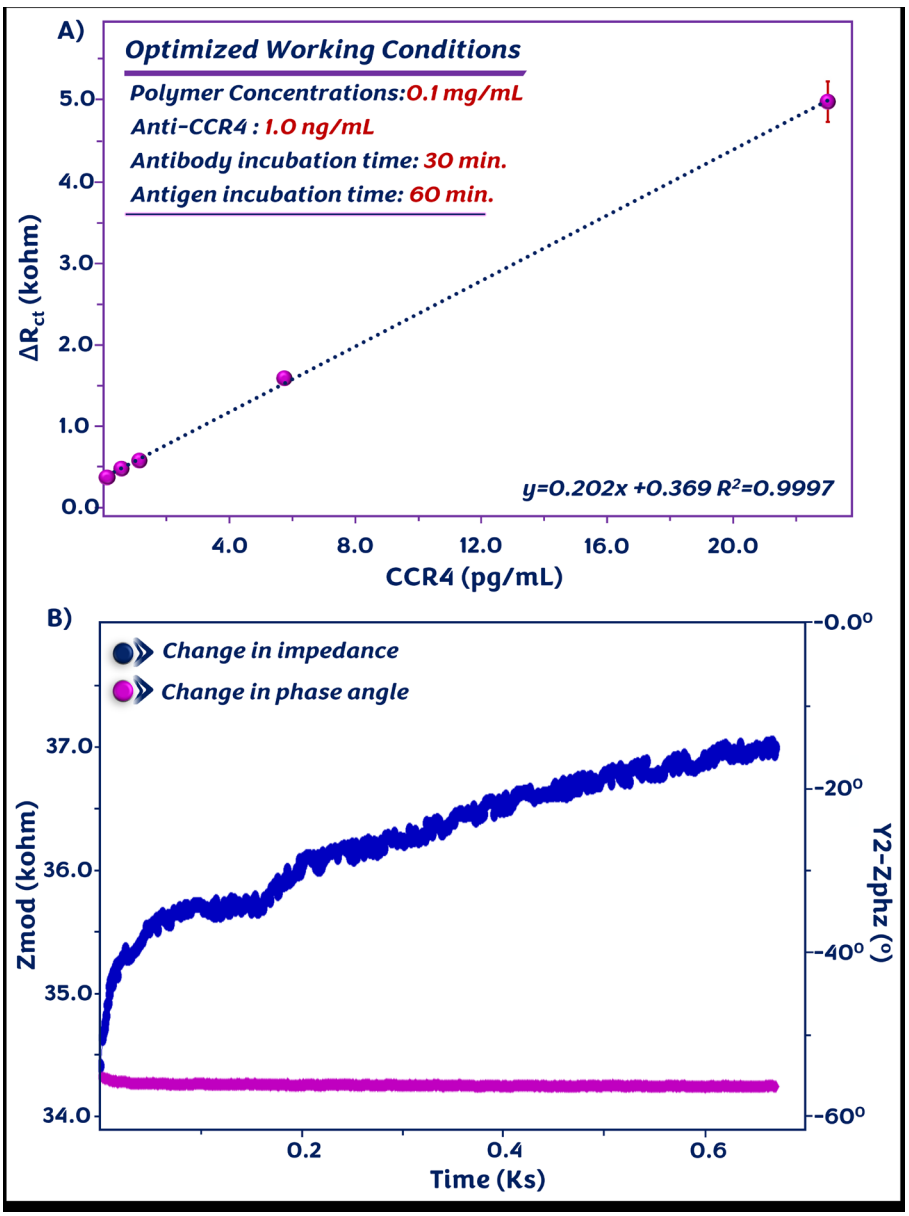


Figure 7. The calibration graph obtained between electrochemical signals of working electrodes and different concentrations of CCR4 (A). Impedance signal at a constant frequency (B).

unt in the linear range from 0.115 to 23 pg/mL (Figure 7A). The equation of the calibration plot was ΔR_{ct} = 0.202 [CCR4] + 0.369 (R^2=0.9997). The LOD, quantification limit, and sensitivity were calculated as 34.9 fg/mL, 116.4 fg/mL, and 0.886 kΩ $pg^{-1}mLcm^{-2}$, respectively.

The linear range, LOD, and detection technique of the CCR4 immunosensor are compared to other CCR4 analysis techniques as tabulated in Table 1. This immunosensor had a wide linear range and a low LOD. This low LOD level illustrated that suitable results of analysis could be performed utilizing the suggested biosensor. Apart from EIS measurements, the constant frequency impedance response of the biosensor was recorded. This technique was known as a single-frequency EIS, and it was utilized to pursue variations in electrochemical impedance versus time.

With this method, the interaction between antibody and antigen can be clearly observed. [28]. The impedance was measured in phosphate buffer solution containing 7.5 pg/mL of CCR4 antigen at a constant frequency of 8 Hz (determined from the Bode plot). Figure 7B illustrates the variations in impedance at constant frequency. The increment in impedance demonstrated specific biointeraction between the anti-CCR4 and CCR4 proteins.

The repeatability of the biosensor was investigated by using 15 independent electrodes under optimal conditions. These electrodes were utilized to measure impedimetric response to 3 different CCR4 amounts (0.115, 7.5, and 23 pg/mL). The relative standard deviations (RSDs) were 6.01%, 5.27%, and 1.16%. Further, the fabrication reproducibility was also investigated. On different days, 15 independent electrodes were utilized to monitor the biosensor impedimetric response to 3 different CCR4 amounts (0.115, 7.5, and 23 pg/mL). The RSDs of reproducibility were 5.78%, 5.87%, and 1.07%. The obtained low RSDs suggested good repeatability and reproducibility of the bioelectrode (Figure 8A).

Table 1. Comparison of CCR4 analysis methods.

Material	Detection Technique	Linear Range (pg/mL)	LOD (pg/mL)	References
*PPyr-CSsg Modified ITO	EIS	0.024-12	0.0073	[10]
**P(Pyr-Pac) Modified ITO	EIS	0.02-8	0.0064	[2]
StrPol(GMA) modified ITO	EIS	0.115-23	0.0349	This work
ELISA	Colorimetric	78.13 – 5000	46.88	Novus Biologicals
ELISA	Colorimetric	78.125-5000	46.875	FineTest
ELISA	Colorimetric	160-10000	56	ELK Biotechnology

*Succinimide groups of poly(pyrrole), **Acid-substituted poly(pyrrole)

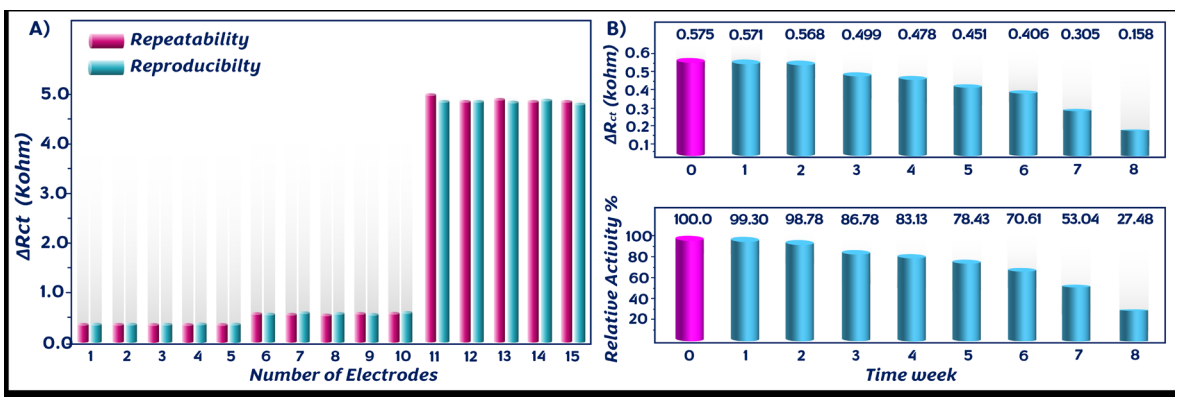


Figure 8. Results of repeatability and reproducibility (A) and storage stability (B) analyses.

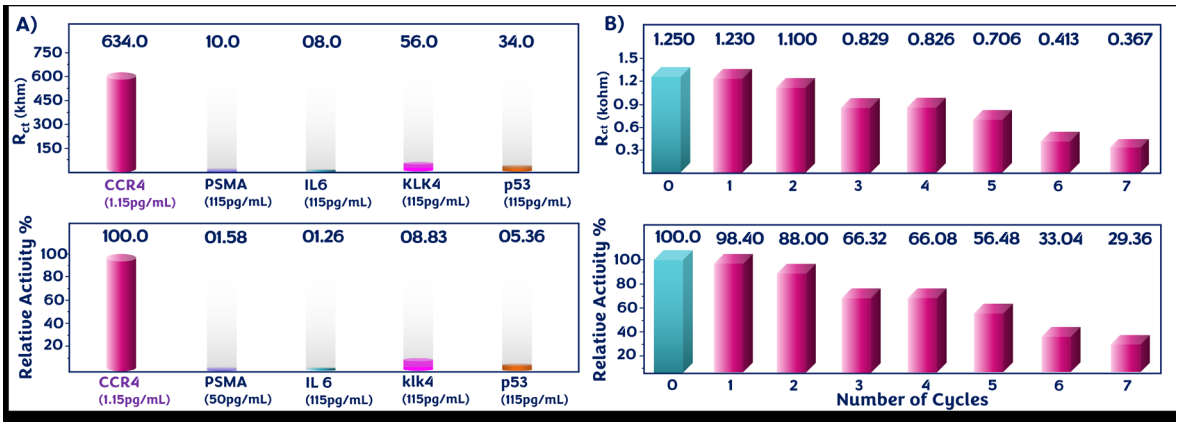


Figure 9. The selectivity (A) and regeneration (B) analyses results.

As a main factor, the storage stability of the proposed biosensor was also investigated. The sensor was kept at 4 °C after various times (from 0 to 10 weeks). The EIS signal declined gradually as storage time increased. After 7 weeks of storage, the impedimetric signal decreased 53.04%, illustrating that the sensor could hold its response for a long period (Figure 8B).

Specificity is a crucial sensor characteristic [29]. The sensitive specificity of the developed sensing system was examined by using different interferent biomarkers. The prepared electrodes were incubated in the interferent biomarker solutions and CCR4. As seen in Figure 9A, the biosensors incubated in the interferent biomarker solutions gave no obvious responses. Thus, the developed biosensor had a good performance in capturing CCR4 molecules.

The regeneration of the constructed bioelectrode was also studied. The ITO/***StrPol(GMA)***/anti-CCR4/BSA/CCR4 electrode was held in acid solution to damage the specific interaction between anti-CCR4 and CCR4. After electrochemical measurement of the electrode, it was used to monitor the binding of 7.5 pg/mL CCR4 under optimum experimental conditions. After each binding event, the electrode was regenerated with an acidic solution. The impedimetric response of the immunosensor retained 56.48% of its initial activity after 5 regeneration cycles, demonstrating the immunosensor's long-term performance protection (Figure 9B).

Table 2. Recovery values for CCR4 in human serum samples.

Human Serum Number	CCR4 level	Added CCR4 amount	Total CCR4	% Recovery	% Relative
1	2.34 pg/mL	1.15 pg/mL	3.40	97.40	-2.60
2	3.15 pg/mL	1.15 pg/mL	4.16	96.80	-3.20
3	2.36 pg/mL	1.15 pg/mL	3.47	98.71	-1.29
4	3.57 pg/mL	1.15 pg/mL	4.74	100.32	0.32
5	3.16 pg/mL	1.15 pg/mL	4.02	93.71	-6.29
5	3.16 pg/mL	1.15 pg/mL	4.02	93.71	-6.29

Analysis in Serum Sample

The immunosensor was implemented to determine the CCR4 in 5 real samples (obtained from Sigma-Aldrich), and Table 2 provided a summary of the findings. To verify the biosensor method, standard CCR4 antigens were added to the serum samples. Then, electrochemical detection was performed under optimal conditions. Recoveries of the five spiked samples were between 93.71% and 100.32%. The recoveries in the CCR4 analysis were admissible, which means that the immunosensor ensured the right results for real sample analysis.

Conclusions

In this research, an electrochemical disposable immunosensor was introduced for highly sensitive detection of CCR4 with a low LOD and good reproducibility. ***StrPol(GMA)*** polymer was utilized as biosensor preparation material, and the development steps of the biosensor were monitored by several analytical techniques: FTIR, EIS, CV, SEM, and AFM. This polymer matrix provided a large surface for anti-CCR4 recognition probe immobilization, and they were immobilized without utilizing a crosslinking agent. The anti-CCR4 molecules were specific to CCR4 antigens, and the biosensor gave increasing electrochemical signals in a linear dynamic range of 0.115-23 pg/mL. The developed biosensor gave low signals to interferences due to the specific recognition sites. The usefulness of the constructed sensor was successfully evaluated by the analysis of CCR4 in real samples with satisfactory recovery results (93.71-100.32%). Consequently, the electrochemical analyses illustrated that the developed biosensor displayed sensitive and accurate determination for CCR4 in biological samples. In addition, the immunosensor illustrated that it was a very attractive alternative to quantify the CCR4 antigens because of its sensitivity, stability, and low cost.

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