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A novel electrochemical immunosensor based on acetylene black/epoxy-substituted-polypyrrole polymer composite for the highly sensitive and selective detection of interleukin 6

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ABSTRACT

An ultrasensitive immunosensor based on acetylene black (AB)/epoxy-substituted-poly(pyrrole) polymer (*EpxS-PPyr*) composite coated disposable indium tin oxide (ITO) electrode was fabricated for interleukin 6 (IL 6) detection. The *EpxS-PPyr* polymer was a promising matrix material to increase the loading capacity of immunosensor owing to its large surface area and abundance of epoxy groups. EpxS-PPyr polymer was synthesized by an esterification reaction and used to attach IL 6 receptor owing to its excellent biocompatibility and good conductivity. The electrochemical signals of the electrodes during the immunosensor fabrication were performed with electrochemical impedance spectroscopy and cyclic voltammetry techniques. Additionally, the changes formed on ITO electrode surfaces were followed by scanning electron microscopy and atomic force microscopy analyses. Under optimized conditions, the designed biosensor illustrated an ultra-sensitive signal towards IL 6 antigen at a broad concentration range from 0.01 pg/mL to 50 pg/mL. The detection limit and sensitivity were found as 3.2 fg/mL and 0.29 $pg^{-1}mLk\Omega\ cm^{-2}$, respectively. Acceptable reproducibility, good storage-stability and excellent selectivity were found for IL 6 determination. Moreover, the proposed biosensor was applied to human serums and the recovery rates were ranged from 99.5 to 100.5 indicating acceptable accuracy. The results illustrated that this immunosensor were suitable for detection of IL 6 in clinical samples.

1. Introduction

Interleukin 6 (IL 6) is a multifunctional cytokine which is a member of glycoprotein-130 cytokine family. It plays different roles including several biological events in immunoregulation, hematopoiesis, inflammation and oncogenesis. Numerous diseases containing osteoarthrosis, asthma, psoriasis, cardiovascular disease, diabetes and cancer are closely related to the IL 6 level and the increment of IL 6 level in human serum can illustrate the formation of these diseases [1,2]. Normal serum IL 6 antigen levels are around 6 pg/mL. Therefore, it is very significant to measure the accurate level of IL 6 for disease detection and diagnosis [3]. In order to determine IL 6 levels in human serum different techniques such as radioimmunoassay, enzyme-linked immunoassay (ELISA), immunoaffinity column assay and chemiluminescent immunoassay have been developed [4]. The drawbacks of these techniques are requiring large equipment, specialized personal, high-cost chemicals and high sample volumes, and having time-consuming complex operation procedure [2,3]. As a comparison, electrochemical immunosensors are successful techniques because of the merits of excellent sensitivity, high specificity, fast response, low cost and simple operation [5]. For the detection of IL6 biomarker, different biosensors have been developed and summarized in Table 1A. Yang et al. (2013) developed an electrochemical immunosensor based on gold nanoparticles (AuNPs)--single walled carbon nanotubes (SWCNTs) functionalized SiO_2/Si platform and employed for quantification of IL 6 antigen in human serum. The linear range and the detection limit were found as 0.01–100 fg/mL and 0.1 fg/mL, respectively. In addition, this biosensor was selective to IL 6 antigen in the presence of other interferent biomarkers [6]. An electrochemiluminescence (ECL) IL 6 biosensor was developed by employing graphene oxide nanosheets/polyaniline nanowires/CdSe quantum dots nanocomposite modified GCE. This nanocomposite increased the biosensor signal and it was a biocompatible substrate for anti-IL 6 antibodies. The linear detection range and detection limit of this study were found as 0.0005–10 ng/mL and 0.17 pg/mL,

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respectively. The designed ECL biosensor had long storage stability and excellent selectivity [7]. As summarized in Table 1, an immunosensor prepared by using acetylene black/*EpxS-PPyr* polymer composite has not been fabricated so far. In this context, this study will be a first. The combination of conjugated *EpxS-PPyr* polymer with AB generated composites with interesting properties, providing higher sensitivity and stability of the immobilized biomolecules. Furthermore, this composite improved the electron transfer between polymeric matrix modified electrode surface and electrolyte. In the literature, composites were formed by using poly(pyrrole) polymer and nanomaterials containing active groups and biomolecules were attached to these active groups. Since we created a composite using poly(pyrrole) polymer containing active groups, we got a new perspective.

In the designing of highly selective electrochemical immunosensor, efficient immobilization of biorecognition elements and production of amplified signals are the important stages [8]. Therefore, nanomaterial-based bioplatform fabrication has gained attention to improve the sensitivity [9,10]. Thus far, several nanomaterials such as metal oxide nanoparticles, noble metal nanoparticles and carbon-based nanomaterials have been utilized in the development of sensing platforms [11,12]. Acetylene black is a special kind of carbon black and it has gained attention because of its excellent features including high electrical conductivity, large surface area and high catalytic activity [13,14]. Acetylene black (AB) has been employed in the construction of electrochemical biosensors to increase the detection sensitivity and the signal response [15–17]. Shuai et al. (2010) constructed a DNA biosensor by using gold nanoparticles (AuNPs)-AB-tungsten disulfide composite modified glassy carbon electrode (GCE). The linear detection range and detection limit of this DNA biosensor were 0.002–100 pM and 0.12 fM, respectively. The developed biosensor was selective to the one-base mismatched DNA sequence [18]. A different DNA biosensor was fabricated by Huang et al. (2014) by using AB incorporated CuS nanosheet and AuNPs modified GCE [16]. CuS was a semiconductor material and had lower conductivity than AB. Therefore, CuS-AB composite were selected to facilitate the charge transfer. The linear detection range and detection limit of the developed DNA biosensor were 0.1 pM-1 nM and 20 fM, respectively [16]. Tung et al. (2018) used AuNPs/AB composite as a matrix material to rise the surface area and the conductivity of gold electrode. Thus, they increased the sensitivity of the proposed cytosensor. The linear detection range and detection limit of this study were 20-10^6 cells/mL and 3 cells/mL, respectively [19].

The stability of the biosensing platform depends on the binding affinity of biorecognition elements on the biosensing platform. Because of this, suitable surface chemistry has an important influence on the performance of biosensing platform [2]. Self-assembled monolayer formation [20,21], electropolymerization of monomers [22,23], molecular imprinted polymer formation [24,25], deposition of nanoparticles [26], polymer coating [27,28] are mostly utilized electrode modification techniques. Conjugated polymers have gained attention because of their usage potential in electrochemical biosensors. They have unusual electrochemical features such as good electrical conductivity and low ionization energy. The ability to be employed conjugated polymers as a suitable immobilization matrix for biorecognition element binding makes them important candidates for biosensor fabrication. In addition, conjugated polymers provide a suitable micro-environment for biorecognition element attachment and biochemical reactions formation [29]. Particularly, conjugated polypyrrole polymer shows promising potential in electrochemical biosensors because of their outstanding superiorities including good conductivity, excellent biocompatibility, redox activity, strong absorptive features towards proteins and DNA, and ability to form nanowires at room temperature [30]. Pyrrole monomer might be electrochemically generated and deposited on the electrode surface to form polypyrrole polymer [31]. Apart from this technique, spin coating of polypyrrole polymer on the electrode surface can be carried out to form matrix material for covalent binding of biorecognition element. The *EpxS-PPyr* polymer has epoxide groups and stable immobilization of biomolecule is achieved by covalently binding between epoxide groups of polymer and amino groups of biomolecules.

In this study, a novel impedimetric immunosensor decorated with *AB/EpxS-PPyr* composite was designed for the first time to detect IL 6 antigen. The *AB/EpxS-PPyr* composite not only illustrated large surface area to immobilize lots of IL 6 receptors, but also this composite improved the conductivity of the working electrode surface. The epoxy groups on the side groups of polymer were immobilized covalently to the amino groups of IL 6 receptor without requiring cross-linking agents. AB was chosen as a nanomaterial to increase the conductivity of the electrode surface due to its low cost. Thus, with usage of this composite, it was aimed to amplify electrochemical signal. AB could disperse uniformly in every matrix and based on this promising property, it was used for preparation of a homogeneous composite slurry consisted of *EpxS-*

Table 1
Comparison of the reported IL 6 biosensors and ELISA kits (A), human serum samples results obtained by the fabricated biosensor (B).

(A) Electrode Type	Modification Material	Technique	Detection technique	Medium	Linear Range (pg/mL)	Detection limit (pg/mL)	Ref.
Si/SiO_2 substrate	AuNPs/single walled carbon nanotubes (SWCNTs)	Antibody	EIS	PBS	10^{-5} – 10^{-1}	10^{-5}	[6]
ITO	AuNPs-polydopamine	Antibody	*i-t*	PBS	4–800	1	[45]
GCE	GO nanosheets/polyaniline/CdSe quantum dot	Antibody	ECL	PBS	0.5–10000	0.17	[7]
GCE	p-aminobenzoic acid/p-aminothiophenol/AuNPs	Aptamer	EIS	PBS	5–100000	1.6	[46]
Gold	AuNPs	Aptamer	EIS	PBS	0.02–20	0.02	[47]
Graphite SPE	Polypyrrole-AuNPs	Aptamer	EIS	PBS	1–15 × 10^6	0.33	[48]
Graphite SPE	SWCNTs	Antibody	*i-t*	PBS	0.5–30	0.5	[49]
ITO	***AB/EpxS-PPyr Composite***	**Receptor**	**EIS**	**PBS**	**0.01–50**	**$3.2x10^{-3}$**	**This study**
–	ELISA	Antibody	Colorimetric	PBS	10.24–400	1	Thermofisher
–	ELISA	Antibody	Colorimetric	PBS	15.6–1000	5	Mybiosource
–	ELISA	Antibody	Colorimetric	PBS	3–1000	3	Raybiotech
–	ELISA	Antibody	Colorimetric	PBS	7.81–500	4.69	Elapscience

(B) Sample	Found by proposed immunosensor (pg/mL)	Added IL 6 antigen (pg/mL)	Total found by proposed immunosensor	% Recovery	% Relative difference
1	1.05	0.5	1.55	99.97	−0.03
2	1.26	0.5	1.76	99.97	−0.03
3	1.41	0.5	1.98	103.72	3.72
4	0.91	0.5	1.48	105.04	5.04
5	1.83	0.5	2.35	100.59	0.59

PPyr polymer and AB. Furthermore, *AB/EpxS-PPyr* composite was an attractive material owing to their combined effects and better electrochemical performance than poly(pyrrole) polymer alone. The electrochemical signal formed after introduction of IL 6 antigen on the IL 6 receptor immobilized ITO electrode was used to determine IL 6 antigen concentration by using EIS technique. The stepwise development process of the proposed immunosensor was characterized by electrochemical (EIS and CV) and morphological techniques (SEM and AFM). As displayed in Scheme 1, the fabrication procedure of the designed immunosensor was easy and non-complex. The manufactured immunosensor illustrated high selectivity, good reproducibility and great potential for application in human serums.

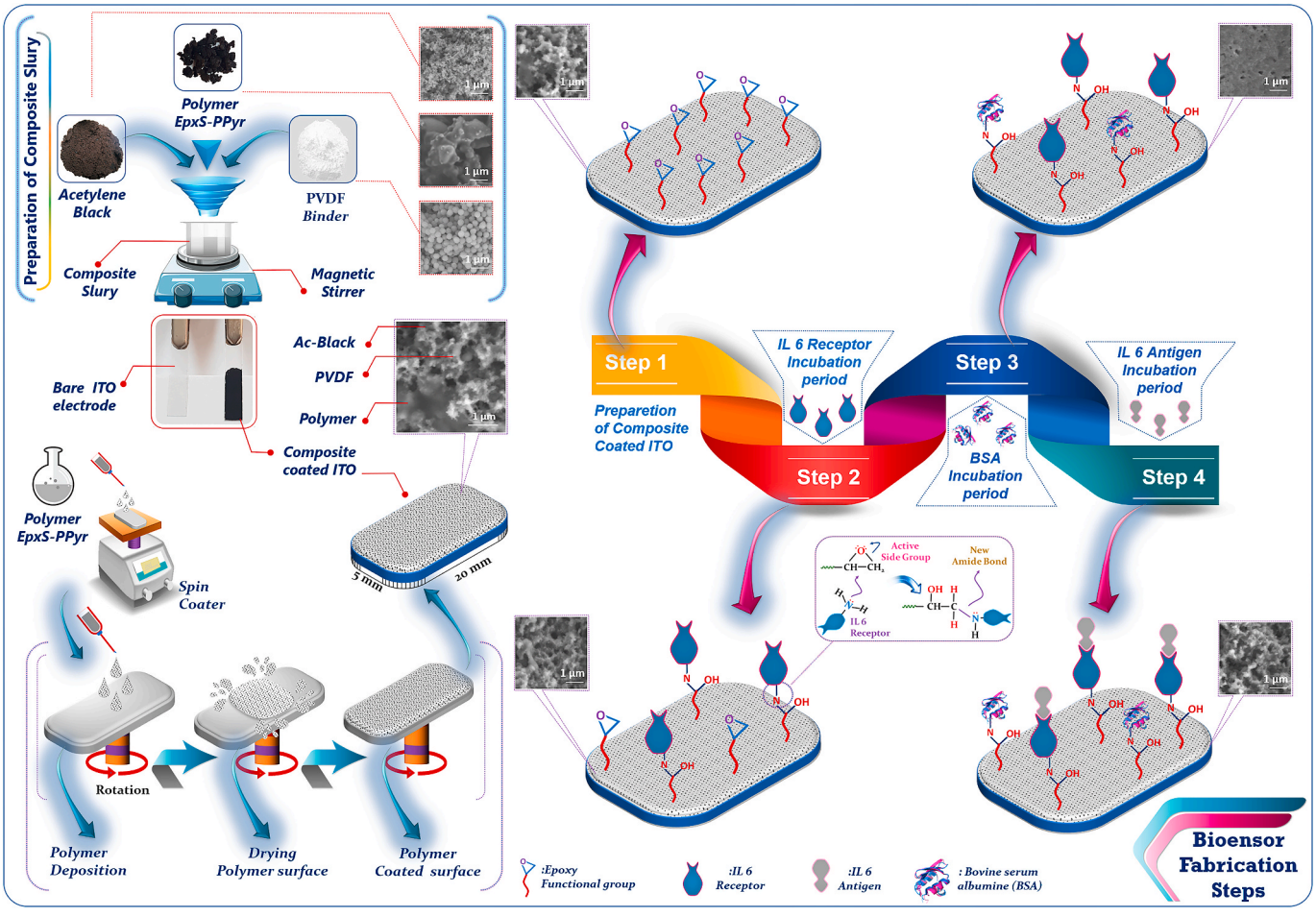
2. Experimental

2.1. Materials and reagents

Unless otherwise stated, all reagents were purchased from commercial sources and used without further purification. 1-(2-Cyanoethyl) pyrrole (99%), glycidol (96%), potassium hydroxide (99%), N-Ethyl-N′-(3-dimethylaminopropyl)carbodiimide hydrochloride (≥98.0%), 4-dimethylamino pyridine (≥99%), anhydrous iron(III) chloride (98%), nitromethane (98.5%), N-Methyl-2-pyrrolidone (99%), were purchased from Sigma-Aldrich. Conductive acetylene black (AB, particle Size 35–40 nm) as a conductive agent and polyvinylidene fluoride (*Mw: 600.000*) as a binder component in composite were purchased from MTI-XTL Corporation. Potassium ferrociyanide (K_4FeCN_6) and potassium ferricyanide (K_3FeCN_6), potassium dihydrogen phosphate (KH_2PO_4) and dipotassium hydrogen phosphate (K_2HPO_4), potassium chloride (KCl), acetone, soap solution, ITO substrate was provided by Sigma-Aldrich. IL 6 receptor (IL6R), IL 6 antigen, bovine serum albumin (BSA), tumor necrosis factor α (TNF α), p53 antigen, interleukin 1β (IL-1β), interleukin 1α, and interleukin 8 (IL 8) were purchased from Sigma-Aldrich. All solutions were prepared with ultra-pure water (18.2 MΩ cm). The solutions of BSA, IL 6R, IL 6 antigen and other antigens were prepared by using phosphate buffer (PBS, 50 mM, pH 7.0). Ferricyanide/ferrocyanide solution (5 mM) containing 1 M KCl was the redox probe of this system.

2.2. Instrumentations

Electrochemical experiments were performed on an electrochemical workstation (Gamry Reference 1000 Potentiostat/Galvanostat) with a standard three-electrode system. Pt wire, Ag/AgCl (3 M KCl) electrode, and modified ITO electrode were used as counter, reference and working electrode, respectively. CV was carried out in a 5 mM $Fe(CN_6)^{3-/4-}$ solution containing 1 M KCl between −0.5 V and 1 V potential at scan rate of 100 mV/s. The frequencies of EIS were between 0.5 Hz and 50,000 Hz. FTIR spectrum was recorded on Bruker Vertex 70 Fourier-transform spectrometer in the range of 4000–400 cm^{-1}. Raman spectra were recorded with a Thermo DXR Raman spectrometer. A diode laser of 785 nm was focused through an 100× objective of the microscope (Leica), which resulted in a laser power of approximately 25 mW on the sample. A traditional spin-coater (MTI-VTC-50) was used for electrode fabrication. The microstructures of modified electrode surfaces were investigated by scanning electron microscopy (SEM) and atomic force microscopy (AFM). SEM images were obtained with a high-resolution QUANTA FEG 250 FE-SEM microscope on 5 KV acceleration voltage



Scheme 1. Schematic representation of AB/EpxS-PPyr composite preparation and the immunosensor fabrication protocol for IL 6 biomarker biosensing.

and 3.5 spot size (FEI Company). Modified electrode surfaces were imaged in tapping mode with an ambient AFM (Nano-magnetic Instruments).

2.3. Construction of -AB/EpxS-PPyr composite modified ITO electrode

Prior to use, bare ITO substrates were successively sonicated in acetone, soap solution and ultra-pure water for 10 min. After drying under argon gas, conductive composite matrix was applied to the electrode surface. In order to prepare this composite, firstly, *EpxS-PPyr* polymer (10 mg), acetylene black (40 mg) and poly(vinylden floride) binder (10 mg) were mixed in tetrahydrofuran (1.7 mL) - N-Methyl-2-pyrrolidone (0.3 mL) solutions. Then, this mixture was dropped on the ITO surface and the spin-coater was accelerated to 1000 rpm for 1 min. After drying at room temperature, the conductive composite coated ITO electrode was ready to biomolecule immobilization. The preparation procedure of the conductive composite material is summarized in Scheme 1.

2.4. Construction of the IL 6 immunosensor

The prepared ITO electrode was dipped in IL 6R solution and incubated there at room temperature for 1 h. After being washed with ultra-pure water, it was immersed in BSA solution to eliminate non-specific binding points and after that, it washed with ultra-pure water. Thus, the suggested biosensor was obtained. The prepared ITO/*AB/EpxS-PPyr*/IL 6R/BSA stored at 4^{O}C until use.

2.5. Analysis of human serum samples

The human serum samples employed in the current study were obtained from Tekirdağ Namık Kemal Hospital Laboratory and approved by the Ethics committee. The obtained serum samples were diluted 5 times with PBS buffer. To evaluate the accuracy of the immunosensor, IL 6 antigens solution was added to the diluted serums.

3. Result and discussion

This work highlighted a new biosensor fabrication strategy for detection of a biomolecular signature like interleukin 6. Scheme 1 illustrates the biosensor matrix material preparation and immunosensor fabrication protocol for IL 6 detection. Firstly, *AB/EpxS-PPyr* composite was prepared and applied to disposable ITO electrode surface to form bioreceptor immobilization matrix (Step 1). Secondly, the IL 6 bioreceptor was immobilized covalently on the composite modified electrode surface (Step 2). Thirdly, free epoxy ends of *EpxS-PPyr* polymer were blocked by BSA (Step 3). Lastly, the immunoreaction was formed between IL 6R and IL 6 antigen (Step 4).

3.1. Chemical characterizations of AB/EpxS-PPyr modified electrodes

The epoxy group substituted pyrrole polymer were synthesized from three-step route sequence. The *EpxS-PPyr* polymer were synthesized and characterized according to our previous study [32]. The chemical characterizations of *AB/EpxS-PPyr* conductive composite interface material coated electrode and IL 6 receptor immobilized electrode were performed by FTIR and Raman Spectral techniques (Fig. 1). The FTIR spectra of composite coated electrode surface before IL 6 receptor immobilization (purple spectra) and after IL 6 receptor immobilization (red spectra) are displayed in Fig. 1A and B, respectively. The FTIR-ATR spectrum of composite modified electrode illustrated a broad band in the 2850–3000 cm^{-1} region because of the symmetric and asymmetric stretching vibrations of methylene groups (Fig. 1A) [33]. Characteristic absorption bands of ester group was observed at 1724 cm^{-1} [34]. The characteristic absorption bands at 924 and 853 cm^{-1} were attributed to carbonyl stretching vibration of epoxy ring in polymer which confirmed that the presence of polymer in the composite [35]. The amide bond formation between of IL 6 receptor and *EpxS-PPyr* polymer in composite coated electrode is shown in Fig. 1B [36]. The region of amide I and amide II gives information about the protein content and its secondary structure [37]. As seen in Fig. 1B, the Amide-I and Amide-II bands (as

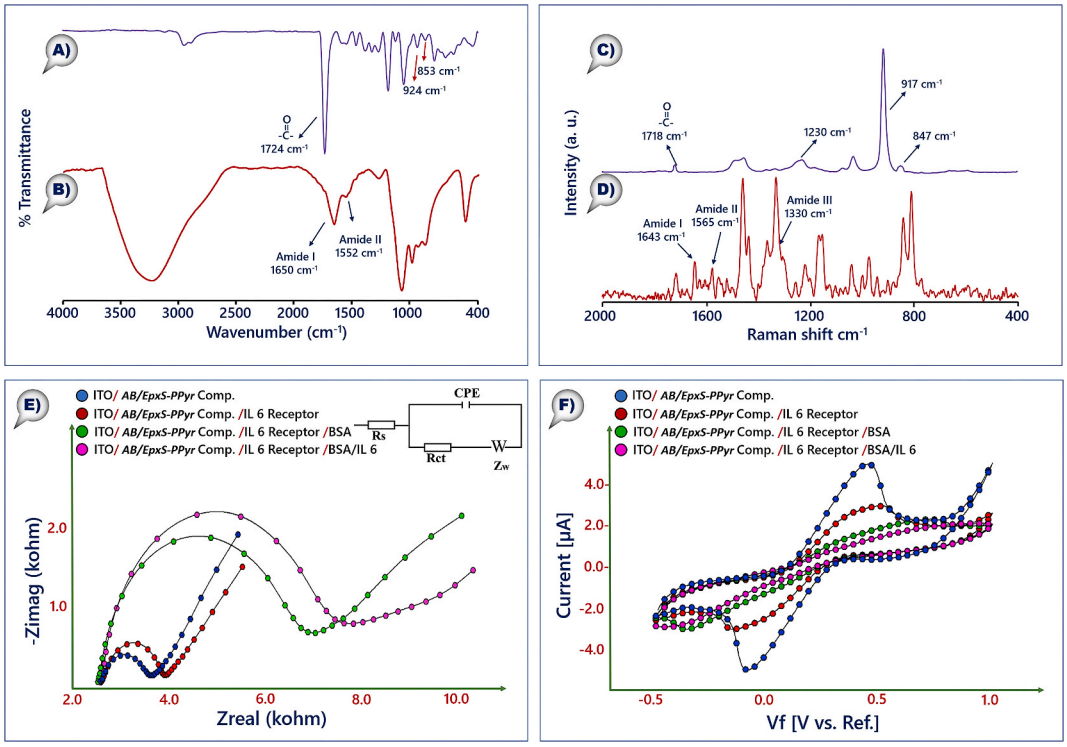


Fig. 1. FTIR and Raman spectra analyses of composite modified (A and C) and IL 6R immobilized (B and D) ITO electrode surface, EIS (E) and CV (F) characterizations of modified ITO electrode at each step in a 5 mM $[Fe(CN)_6]^{3-/4-}$ solution containing 1 M KCl.

observed region 1600–1700 cm^{-1}, and 1480–1600 cm^{-1}, respectively) were seen at 1650 cm^{-1}, and 1552 cm^{-1}, respectively [38,39]. These amide bands proved that the immobilization of IL 6R to epoxy side groups of *EpxS-PPyr* polymer in composite.

Raman spectroscopy is a significant and usually utilized spectral technique for identifying and understanding biological material (proteins, lipids and nucleic acids *etc.*) [40,41]. Fig. 1C and D shows Raman spectra of the *EpxS-PPyr-AB* composite modified electrode surface before and after IL 6 receptor immobilization stages, respectively. As seen in Fig. 1C, similar peaks found in FTIR spectrum were also monitored by the Raman spectrum. In general, the presence of proteins illustrates three main peaks, which can be noticed by amide I, amide II, and amide III bands (as monitored between 1650 and 1680 cm^{-1}, 1480–1570 cm^{-1} and 1235–1300 cm^{-1}, respectively) [40,42]. As illustrated in Fig. 1D, amide I, amide II and amide III bonds were found at 1643, 1565 and 1330 cm^{-1}, respectively [43].

3.2. Impedimetric characteristics of the suggested immunosensor

EIS was an effective method to evaluate the electrochemical feature of the modified electrodes during the immunosensor fabrication steps. The Nyquist plot contained a linear part corresponded to the diffusion-limited process and a semicircular part corresponded to electron transfer resistance (R_{ct}). Fig. 1E illustrated the Nyquist plots of the immunosensor step-by-step fabrication process. As seen in Fig. 1E, a smaller Nyquist plot was observed after the bare ITO electrode modified with *AB/EpxS-PPyr* composite. This small Nyquist plot proved the stronger electrical conductivity. After immobilization of IL 6R on the ITO electrode surface, an increase was seen in charge transfer resistance and therefore, a larger semicircle than composite modified ITO electrode was obtained. When the blocking agent (BSA) was applied to the electrode surface, an increment was obtained in R_{ct} value due to nonconductive property of BSA. When the IL 6 antigen successively immobilized on the ITO electrode surface, the semicircle diameter gradually increased. The reason of this increase was the blocking of electron transfer due to protein immunocomplex layers (between IL 6R and IL 6 antigen). The results illustrated that the ITO electrode was successfully modified and could be employed at the following experiments.

Apart from EIS technique, CV was used to characterize the modified electrodes during the immunosensor fabrication stages. Fig. 1F illustrates the CVs of the immunosensor step-by-step fabrication process. The CV of composite coated ITO electrode had larger peak currents than other immobilization stages. Decreases were seen in peak currents after IL 6R, BSA and IL 6 antigen immobilization. These macromolecules had nonconductive properties and they prevented the electron transfer. In addition, these dramatic decreases displayed that the successful immobilization process and they supported the impedimetric results.

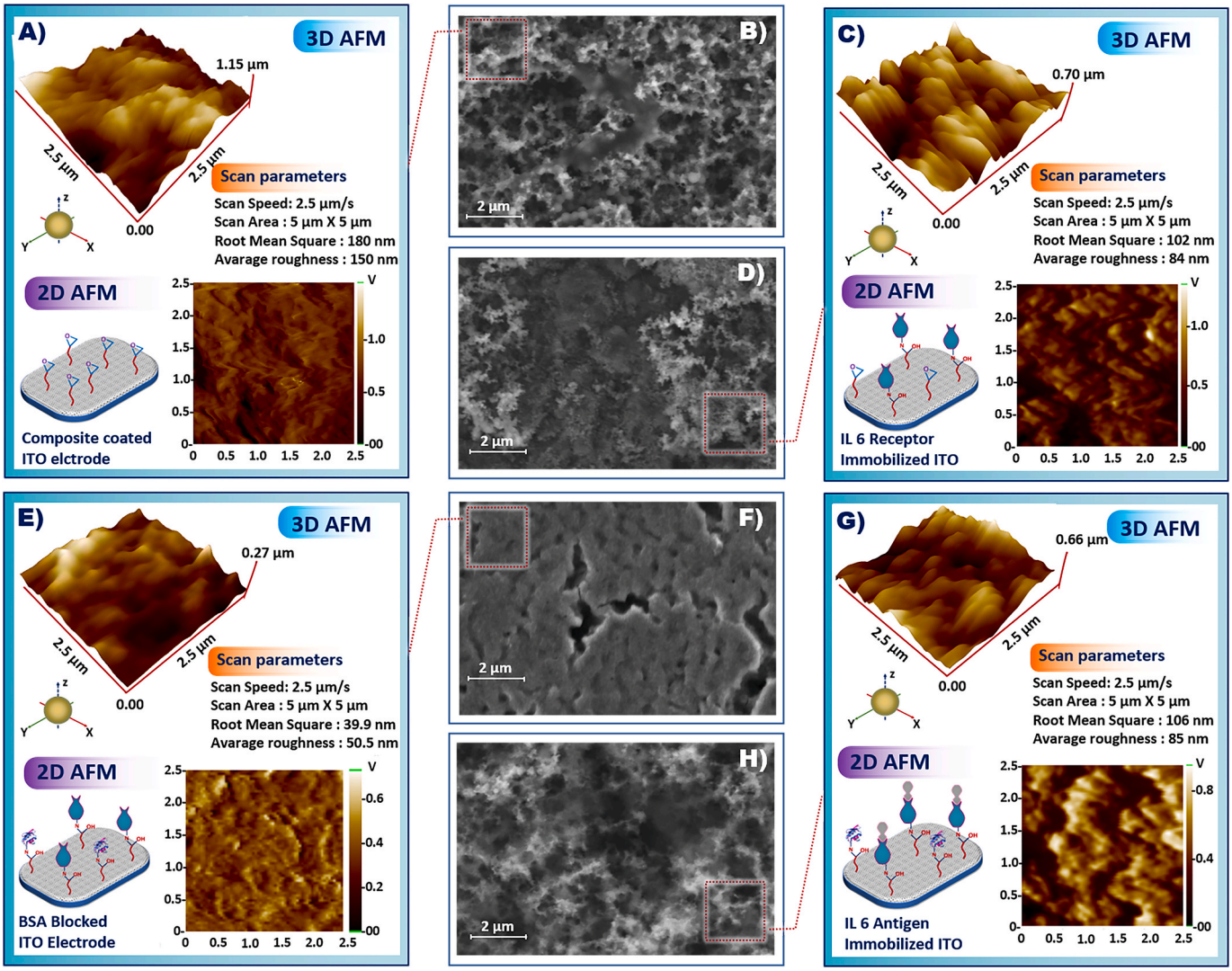


Fig. 2. The SEM and AFM images of AB/*EpxS-PPyr* composite coated (A), IL 6R immobilized (B), BSA blocked (C), IL 6 antigen immobilized ITO electrode.

3.3. Morphological characteristic of the modified ITO electrode surfaces

The microstructures of prepared electrode were investigated by SEM and AFM measurements. As displayed in Fig. 2B, composite modified ITO electrode had a rough surface and the average roughness (R_a) of this surface was measured as 150 nm (Fig. 2A). While the IL 6R functionalized ITO surface showed different morphology from composite modified ITO surface, which indicated the successful immobilization of IL 6R. The IL 6R molecules were clearly seen in Fig. 2D. The R_a of this stage was 84 nm (Fig. 2C). As seen in Fig. 2F, BSA macromolecules were clearly present on the electrode surface and blocked the free epoxide groups of polymers. The R_a of BSA modified ITO surface was found as 50.5 nm (Fig. 2E). After specific biointeraction between IL 6R and IL 6 antigen, the ITO electrode surface morphology was varied, and IL 6 antigen macromolecules were clearly seen in Fig. 2H. The specific interaction increased the R_a value and it was measured as 85 nm (Fig. 2G). The morphological analyses results illustrate that the proposed immunosensor was fabricated successfully and they supported the results of electrochemical characterization.

3.4. Optimization of the proposed biosensor fabrication procedure

Stepwise fabrication procedure of the proposed immunosensor was followed by EIS and CV analyses. During this fabrication process, same parameters that affect the immunosensor performance were optimized. First of all, the polymer concentration used for preparation of composite was optimized. To fabricate the proposed immunosensor 3 different amounts of polymer (5 mg, 10 mg and 20 mg) were utilized. As seen in Fig. 3A, the maximum immunosensor response was obtained after utilization of 10 mg polymer. Therefore, 10 mg *EpxS-PPyr* polymer was added to form composite. After that, the biorecognition element (IL 6R) level was optimized to obtain maximum immunosensor response. Three different IL 6R concentrations (0.5 ng/mL; 2.5 ng/mL; and 12.5 ng/mL) were utilized during the immunosensor fabrication. The low IL 6R concentration caused a low signal and increment of IL 6R concentration increased the signals. The maximum immunosensor signal was measured, when 2.5 ng/mL of IL 6R was utilized (Fig. 3B). Then, the incubation time of IL 6R was carefully optimized. Fig. 3C illustrated the effects of incubation duration of IL 6R on the analytical performance of the immunosensor. The immunosensor signal increased from 30 min to 45 min and, the signal of 45 min-incubation and 60 min-incubation were similar. Because of this, 45 min was selected as optimal incubation time for IL 6R. Fig. 3D displayed the effect of incubation duration of IL 6 antigen on the analytical performance of the immunosensor. As seen in Fig. 3D, the immunosensor had similar signals after incubation and thus, 30 min was the optimal incubation time for IL 6 antigen.

3.5. Analytical performance of the fabricated IL 6 biosensor

Under the optimized conditions, the prepared electrodes were incubated with different concentrations of IL 6 antigens in PBS. Fig. 4A and B shows the Nyquist plots and typical peak currents changes with different IL6 concentrations between 0.01 pg/mL and 50 pg/mL. As expected, the electron transfer resistances were increased, and peak currents were decreased with increasing concentration of IL 6 antigen. The variations illustrated that with the bound IL6 antigen amount was increased with increasing IL 6 concentration and formed a protein layer. This protein layer prevented electron transfer and acted as a nonconductive barrier. Fig. 4C displayed a linear relationship of the IL 6 antigen concentration with electron transfer resistance. The linear regression could be expressed as $\Delta R_{ct} = 0.072\ C_{IL\ 6} + 0.126$ ($R^2 = 0.9982$, n = 3). The linear detection range, limit of detection (LOD), limit of quantification (LOQ) and sensitivity of this proposed immunosensor were 0.01–50 pg/mL, 3.2 fg/mL, 10.9 fg/mL and 0.29 pg^{-1} mL kΩ cm^{-2}, respectively. The AB/*EpxS-PPyr* composite provided an excellent analytical performance owing to its good conductivity and large surface area. The developed immunosensor had lower detection limit than previous studies (Table 1A). The suitable biocompatibility and large surface area increased the immobilized IL 6R amount and provided more sensitive analysis.

Single frequency impedance technique (SFI) is a successful technique, which measures the impedance at a fixed frequency versus time.

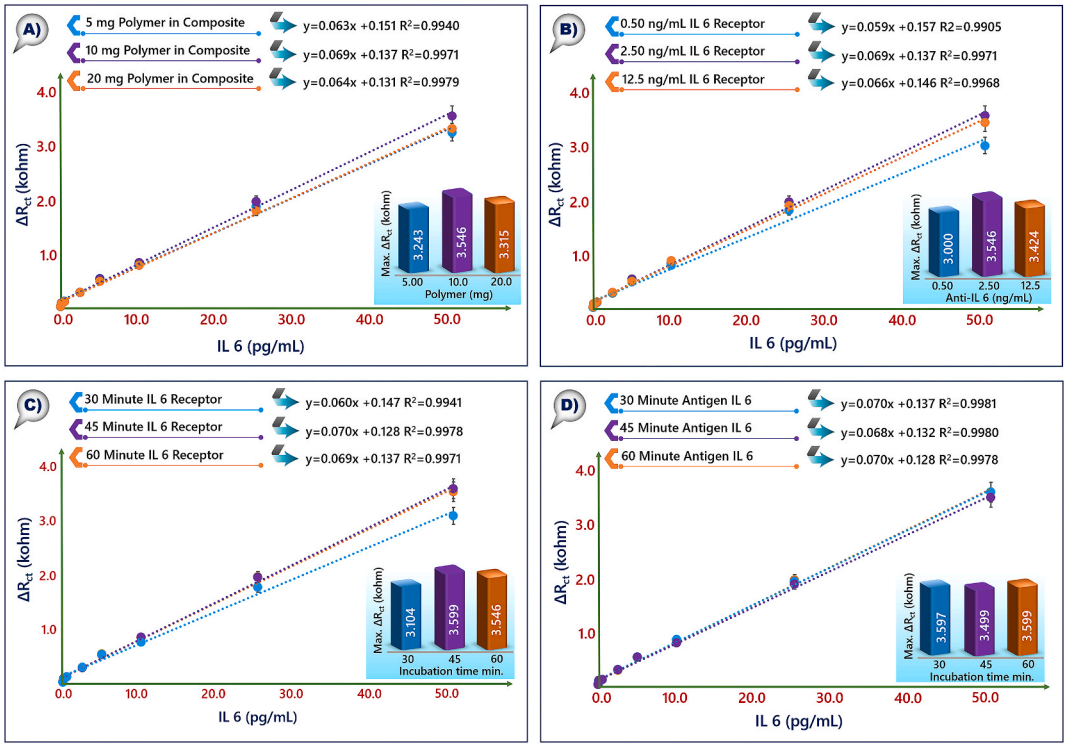


Fig. 3. Effect of different *EpxS-PPyr* concentrations (A), IL 6R concentrations (B), IL 6R (C) and IL 6 antigen incubation time on impedimetric response.

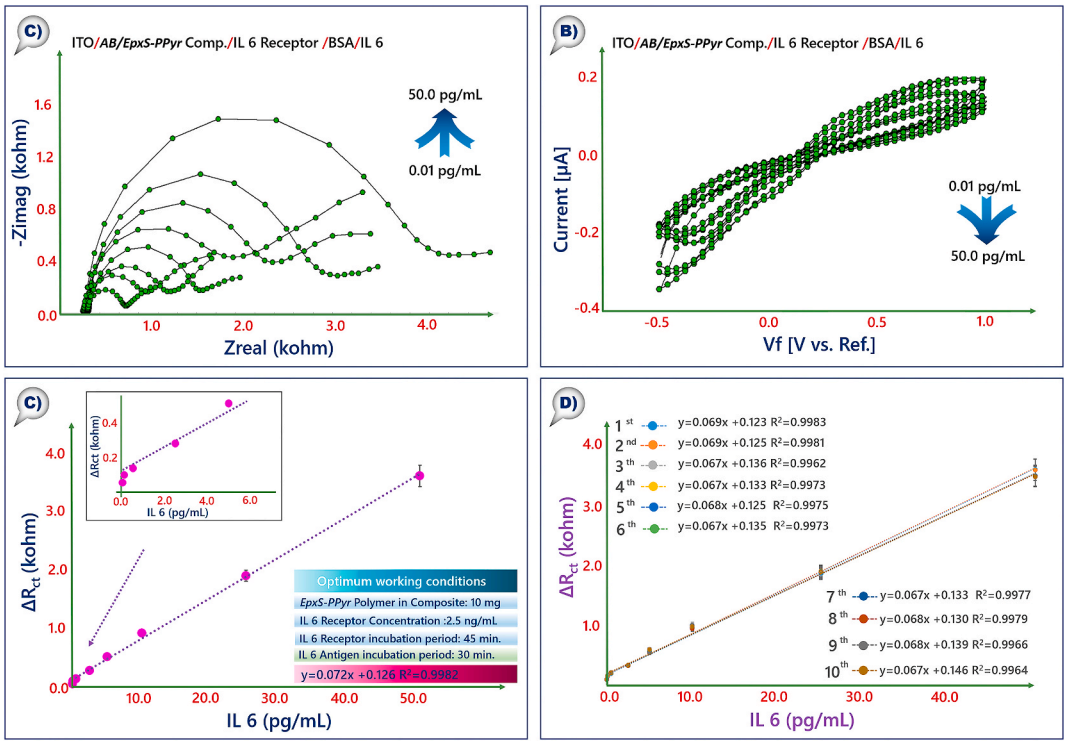


Fig. 4. EIS (A) and CV (B) responses of proposed biosensor in PBS solution containing different concentrations of IL 6 antigen (0.01, 0.1, 0.5, 2.5, 5, 10, 25, 50 pg/mL, respectively), linear relationship between impedimetric responses and the IL 6 antigen concentrations (C), reproducibility of the proposed biosensor.

The fixed frequency is determined by using Bode plot [44]. In this study, this technique was applied to the immunosensor to characterize the binding of IL 6 antigen to IL 6R which was attached onto polymer modified ITO surface. The SFI result of this biosensing system is illustrated in figure SI-1. As seen in this figure, the variation in the impedance (red) was a function of time and the blue curve illustrated the change in the phase angle as a function time. This considerable change was an evident of the interaction between IL6 antigen and IL 6R.

3.6. Study of repeatability, reproducibility, selectivity, stability and regeneration

The repeatability, reproducibility, stability, and selectivity of immunosensors are significant parameters in electrochemical measurement of biomarkers. Repeatability of the proposed biosensor was tested by using 20 immunoelectrodes prepared through the same procedure and these electrodes were used for IL 6 concentration (5 pg/mL) determination. The relative standard deviation (RSD) of the 20 analyses was 3.55%, indicating good repeatability (Figure SI-2). In order to test the reproducibility of the designed biosensor, 10 immunosensors were fabricated through the same procedure for determination of IL6 antigen. The RSD of this analysis was calculated as 1.3% indicating excellent reproducibility (Fig. 4D).

To evaluate the selectivity of the designed immunosensor, different inherent biomarkers (TNF α, p53, IL-1β, IL 1α, and IL 8) were employed during measurement of IL 6 antigen. Although the concentration of interferent biomarkers was 100 times higher than the IL 6 level, the EIS response of interfering substances was much lower than IL 6 antigen. The obtained result proved the selectivity of immunosensor for IL 6 detection (Figure SI-3). The storage-stability of the developed immunosensor was also tested. Ten immunosensors were stored at 4 °C, and the impedimetric signals were measured every week. The impedimetric response retained 80.1% of its initial activity after 5 weeks (Figure SI-4). In addition, the reusability of the immunosensor was investigated by using diluted HCl (10 mM). Firstly, the prepared bioelectrode (ITO/*AB/EpxS-PPyr*/IL 6R/BSA) was used for IL 6 antigen measurement and then, this bioelectrode was immersed in the prepared acidic solution and incubated there 5 min. After incubation, the signal of the bioelectrode was recorded. After regeneration of bioelectrode, it was immersed in IL 6 antigen solution. This process continued until the bioelectrode did not give suitable signal. As observed in fig. SI-5, the immunosensor had 90.9 of its initial activity after 3 cycles.

3.7. Practical applications of the proposed immunosensor in human serums

To investigate practicability of the immunosensor in human serum samples, 5 different serum samples were obtained from Tekirdağ Namık Kemal University Hospital. Before the analyses of IL 6 antigen, these samples were diluted 5 folds. After dilution with PBS, the IL 6 concentration of the samples were measured by using the proposed immunosensor. The recovery experiments were carried out by using standard addition technique and the recoveries were calculated between 99.9% and 105.4%. Table 1B illustrates the human serum samples results and recovery rates. As seen Table 1B, the designed immunosensor could detect IL 6 antigen concentration with high sensitivity and selectivity. Moreover, this immunosensor was suitable for real applications in human serums.

4. Conclusion

In this work, a label-free electrochemical IL 6 immunosensor was developed by using AB/EpxS-PPyr coated ITO electrode. The fabrication process of the immunosensor was investigated by using EIS and CV methods. Under the optimum experimental conditions, sensitive impedimetric response to IL 6 antigen with a wide linear range (0.01–50 pg/mL) and low LOD (3.2 fg/mL) were obtained. This composite increased the electrical conductivity of electrode surface and the EpxS-PPyr polymer offered large active epoxy side groups for IL 6R immobilization. In addition, this composite provided biocompatible and stable

surface. Furthermore, the applicability of the proposed immunosensor for selective and sensitive determination of IL 6 was also investigated. The designed immunosensing tool was successfully applied for IL 6 quantification to human serum samples with high recovery rates. In conclusion, the developed immunosensor could be an alternative tool for rapid sensitive detection of IL 6 in human serum samples.

Credit author statement

Elif Burcu Aydın: biosensor fabrication, electrochemical measurements, Software, Writing - original draft. **Muhammet Aydın:** Monomer synthesis, polymer synthesis, chemical characterization, morphological characterization, morphological measurements, Software. **Mustafa Kemal Sezgintürk:** Biosensor fabrication, electrochemical measurements, Writing - original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2020.121596.

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