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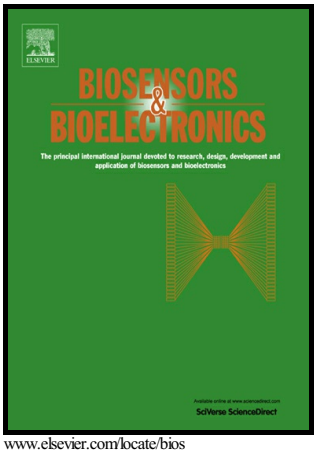

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A highly selective electrochemical immunosensor based on Conductive Carbon Black and Star PGMA polymer composite material for IL-8 biomarker detection in human serum and saliva

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Abstract

A new approach to enhance the electrochemical performance of biosensor was attempted by using Super P© carbon black/Star polymer composite material. In this study, we developed an electrochemical IL 8 biosensor by modification with a conductive composite including Super P, polyvinylidene fluoride (PVDF) and star polymer (SPGMA) of disposable ITO electrode surface. The Super P carbon black as carbonaceous material had a high conductivity and was used for the enhancement of electron transfer between electrode surface and electrolyte. Anti-IL 8 antibodies were utilized as biorecognition molecules and bound to epoxy groups of star polymer covalently. The chemical characterization of antibody immobilization on this composite was performed by using Fourier-transform infrared spectroscopy (FTIR) and Raman spectroscopy. The characterizations of stepwise modification of this immunosensor were performed by electrochemical techniques such as Electrochemical Impedance Spectroscopy (EIS), Cyclic Voltammetry (CV) and Single Frequency Impedance (SFI); and morphological techniques such as Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM). Several variables that affect the immunosensor performance were optimized. Under optimum conditions, a wide linear range 0.01-3 pg/mL and low detection limit 3.3 fg/mL were obtained. Super P-star polymer composite modified immunosensor was easy, sensitive, cheap and reliable analytical method for IL 8 detection. The applicability of the proposed immunosensor to determine IL 8 in saliva and serum samples were examined. The results of biosensor and Enzyme-linked Immunosorbent Assay (ELISA) kit were in compatible. Consequently, it was concluded that the electrochemical immunosensor offers a potential approach for IL 8 detection in clinical applications.

Keywords: Protein IL 8, Electrochemical impedance spectroscopy, Star-polymer, Poly(glycidyl methacrylate), Conductive Super P® Carbon Black.

1. Introduction

Cancer is a disease of epoch and it threats human health significantly. The identification of biomarkers has a significant role in the early stage of cancer (Sheikhpour et al. 2017; Wang et al. 2017; Wu and Qu 2015). Biomarkers are present in saliva, serum, plasma and tissue. The biomarker measurement is usually performed in serum samples. However, recent research has displayed that a variety of protein ingredients in blood are also present in saliva (Abdul Rehman et al. 2017). Saliva, has attracted attention due to being easily accessible biological fluid(Khan et al. 2018). The collection, storage and transfer of saliva samples is easy. The analysis of saliva provides a desirable and promising platform for the diagnosis of different types of cancers (Chianeh and Prabhu 2014; Dong and Pires 2017; Liu and Duan 2012; Radhika et al. 2016; Zhang et al. 2015). For the determination of biomarkers, a variety of methods such as immunoassays, chromatographic techniques have been utilized. Biosensors are an innovative alternative for detection of biomarkers due to their accuracy and rapidity. Biosensors are composed of an active sensing element responsible for the analyte specificity and a transducer to convert the recognition event into a detectable electric signal (Junior et al. 2018). Moreover, electrochemical immunosensors have attracted interest in the quantitative determination of biomarkers due to low cost, fast analysis and ease of use. To improve the sensitivity of electrochemical immunosensors, a lot of materials such as carbon nanotube (CNT), graphene oxide, metal nanoparticles, conducting polymers have been used to amplify signal (Campuzano et al. 2017; Chikkaveeraiah et al. 2012; Dolatabadi and de la Guardia 2014).

Interleukin 8 (IL 8) belongs to CXC chemokine family and known as CXCL-8 (Rajkumar et al. 2014). It is a potential neutrophil chemoattractant and regulates biological processes through interactions with relative receptors (Sun et al. 2014). Furthermore, IL 8 is a multifunctional pro-inflammatory cytokine, which attracts and activates neutrophilic granulocytes and is produced in response to inflammatory stimulation. It induces neo-angiogenesis by activating the vascular endothelial growth factor pathway and promotes the activity of matrix metalloproteinases 2 and 9 and also metastatic potential of malignancy (Kim et al. 2015). A lot of studies reported that an increase in IL 8 concentrations is associated with melanoma, breast, renal, gastric, ovarian, pancreatic and colorectal cancer (Rajkumar et al. 2014; Sun et al. 2014; Xie 2001). Moreover, overexpression of IL 8 is related to the disease progression of urogenital cancers, including transitional cell carcinoma of the bladder and prostate cancer (Lehrer et al. 2004). Also, IL 8 is an important and potential diagnostic biomarker in oral cancer. Oral cancer is foremost cancer type in the neck and head class. At the early stage detection of oral cancer, the survival rate is 60-80% and the survival rate in advanced stages of oral cancer is 30-40% (Tang et al. 2015). Determination of biomarkers of early disease is important in disease prognosis. IL 8 is a salivary biomarker for early stage oral squamous cell carcinoma. Different analytical techniques such as capillary electrophoresis (Rovin et al. 2002), High Performance Liquid Chromatography (HPLC) (Coyne 2008) have been utilized in determination of IL 8 levels with high sensitivity and selectivity. However, these techniques require a lot of time, expensive devices and maintenance. Enzyme-linked Immunosorbent Assay (ELISA) is a most popular technique for IL 8 biomarker determination (Liao et al. 2016). However, this technique has some limitations such as large and expensive device requirements, time-consuming pre-treatment steps (Sharma et al. 2016). Because of this, rapid, easy, highly sensitive and selective analytical techniques for IL 8 determination are required. Different techniques based on electrochemical (Sharma et al. 2016; Torrente-Rodríguez et al. 2016; Wan et al. 2011) and optical (Domnanich et al. 2011; Dong and Pires 2017) biosensing techniques have been developed. However, the electrochemical techniques are popular and a lot of studies have been performed to develop selective and sensitive biosensors (Heydari et al. 2016; Sandbhor Gaikwad and Banerjee 2018). To obtain ultrasensitive biosensors different amplification strategies using nanomaterials such as CNTs (Wan et al. 2011), magnetic beads (Torrente-Rodríguez

et al. 2016), Fe_3O_4 nanoparticles (Tang et al. 2015) have been fabricated. A carbon nanotube based ultrasensitive multiplexing electrochemical immunosensor was developed by Wan et al. (2011). A disposable screen-printed carbon electrode array (SPCE) and multi walled carbon nanotube (MWCNT) were utilized as an electrode material and electroactive label, respectively. These labels were loaded with horseradish peroxidase (HRP) and anti-rabbit immunoglobulin G (IgG). The detection limit of this study was 8 pg/mL (Wan et al. 2011). In another study performed by Torrente-Rodriguez et al., magnetic nanoparticles were used as signal enhancer and dual screen-printed carbon electrodes were used as antibody immobilization matrix. The detection limit of this study was 72.4 pg/mL (Torrente-Rodríguez et al. 2016). Tang et al. (2017) developed an electrochemical sensor based on Fe_3O_4@graphene oxide@molecularly imprinted polymer nanoparticles. These nanoparticles were assembled on gold thin film electrode (Tang et al. 2015). Another biosensor based on carboxylic acid terminated monothiol alkane poly(ethylene glycol) self-assembled gold electrode was constructed for IL 8 detection. These carboxylic groups were activated with NHS/EDC chemistry and used for the binding of anti-IL 8 antibodies. A low limit of detection (80 fg/mL) was obtained (Dong and Pires 2017).

Electrochemical Impedance Spectroscopy (EIS) is a selective and label-free method to determine a variety of analytes, especially biomarkers. This method enables to follow the changes in capacitance or charge-transfer resistance associated with the specific interaction between desired molecule and the biorecognition element on the modified electrode surface (Arora et al. 2017; Bahadır and Sezgintürk 2016; Bandarenka 2013; Bogomolova et al. 2009; Moya 2013; Xu et al. 2013). Biosensors based on EIS detection principle have some superiorities such as low-cost, simple application procedure, ease of miniaturization. In recent years, numerous EIS immunosensors have been developed and fabricated based on monitoring interfacial feature variations after interaction between antibodies and antigens(Wang et al. 2016). The interaction between antibody and antigen can be monitored by Single Frequency Impedance (SFI) technique. The detection principle of this technique is based on impedance measurement at one frequency periodically. The changes at impedance are followed versus time (Aydın et al. 2017; Aydın and Sezgintürk 2017; Canbaz and Sezgintürk 2014; Karaboğa et al. 2016; Özcan et al. 2014).

Immobilization of biorecognition element on the electrode surface is the most important stage of biosensor fabrication. The immobilization success of biorecognition element on the electrode surface effects the biosensor stability and sensitivity. Polymers are the best candidate for production of convenient matrix for biorecognition molecule immobilization. The use of polymers in biosensor applications increased with the work of Shirakawa et al. on electrical conductivity of polyacetylene. Several types of polymers such as polyaniline, polythiophene, polypyrole, polycarbazole have been used in biosensing applications (Ruecha et al. 2014). To use polymer as interface material in biosensor applications, solubility and feasibility are important factors (Teles and Fonseca 2008). Star polymers are convenient interface materials for immobilization of biorecognition element due to branched shape and a lot of branching arms. They have several same or different linear polymer chains that attached to only one branching point. Compared to linear polymers and dendrimers, star polymer has remarkable features such as, high solubility, globular shape, low cost and easy feasibility (Gao and Matyjaszewski 2009). However, studies based on star polymer modified immunosensors are very rare in literature. Therefore, this study will open the door in combination of star polymer and biosensor technology.

Carbonaceous materials such as graphite, graphene, fullerene, carbon onions, carbon nanotube (single walled-SWCNT and multi walled-MWCNT) have significant role in nanotechnology and industrial applications. They attract more attention of researchers owing to their excellent chemical and physical features such as large surface area, strong adsorption capability, high chemical stability, extremely high mechanical strength and good electrical conductivity. These materials are widely used for fabricating electrochemical systems such as batteries and sensors. Furthermore, carbonaceous

materials have been frequently used for electrode modification because carbon is a good conductor, electrochemically inactive, cheap and robust. Several shapes and sizes of carbon nanomaterials are utilized for electrode modification strategy due to their robust property (Gaddam et al. 2017; Singh et al. 2018). Among these carbonaceous materials, Carbon black Super P® attracts attention owing to its excellent electrochemical properties such as high electrical conductivity, chemically stability, extraordinary electrochemical and physical properties, large surface area, and low cost (Bandodkar et al. 2016). CB material is easy to obtain and the conductive properties of it are lasting. Furthermore, it can greatly adjust the resistivity of composite material. Because of this, the conductive composite made by CB is usually used in sensor construction (Liu et al. 2018). An amperometric biosensor was designed for the detection of NADH and NADPH by using an iron oxide/carbon black composite (Fe_2O_3/CB) modified glassy carbon electrode. This composite provided NAD(P)H oxidation under a very low overpotential without the employment of the mediators or chemical treatment (Kim et al. 2010). Ania et al. (2018) developed a glucose biosensor by using CB modified electrode. In this study, carbon black facilitated the electron exchange between the electroactive center of glucose oxidase and the support (Ania et al. 2018).

Spin coating is a practical method for coating of flat surfaces. To produce uniform coated electrode surface, firstly, a small fluid drop is immersed on the center of surface and then this surface is spinned at high speed. Thus, a uniform surface is formed by the spreading of fluid. This method is used usually for coated surface formation on different substrates such as silicon wafers, sensors and membranes. In sensor application, this technique focuses on spin-coating of polymer films on the sensor surfaces (Norrman et al. 2005). El-Said and Choi (2014) formed a polystyrene (PS) film (El-Said and Choi 2014) and Singh et al. (2016) formed a thin film of graphene oxide(Singh et al. 2016) on the ITO electrode.

Herein, a simple and sensitive label-free impedimetric immunosensor was fabricated and used for IL 8 antigen detection. In our work, disposable ITO electrode decorated by conductive composite material was utilized firstly as a support for anti-IL 8 antibodies immobilization. For this purpose, firstly, a four-armed star shaped poly(glycidyl methacrylate) with high solubility and good feasibility was synthesized. Secondly, homogenized conductive composite slurry was prepared by mixing star polymer (SPGMA), Super P carbon black conductivity agent and polyvinyldenefluoride (PVDF) binder in optimized ratio.

Finally, homogenized conductive slurry was coated with spin coating method on the disposable ITO electrode surface. The usage of super P composite in immobilization matrix material could improve the sensitive detection of IL 8 effectively. Furthermore, the biosensor preparation procedure is simple and requires less steps than other fabrication procedure like self-assembly. In the present study, conductive composite material coated electrode usage decreased the detection limit of IL 8 compared with the other IL 8 biosensors found in literature. Electrochemical techniques (EIS, CV and SFI) and morphological techniques (SEM, AFM) were utilized to characterize the immunosensor construction steps. The applicability of the proposed immunosensor was examined in the determination of IL 8 antigen in serum and saliva samples.

2. Experimental

2.1. Materials

The glycidyl methacrylate (GMA) (97%), Pentaerytritol (99%), N,N,N′,N″,N″ Penta-methyl-diethylenetriamine (99%), Copper(I) bromide (98%), α-Bromoisobutyryl bromide (98%), anisole (99.7%), acetone (99.9%), and N-Methyl-2-pyrrolidone (NMP) (99%), Triethylamine (TEA) (≥99%) were all of analytical purity (obtained from Sigma-Aldrich) and used as-received, without further purification. Indium tin oxide (ITO) electrodes (5mm x 20mm, 60 Ω/cm^2), monoclonal anti-IL 8

antibody (produced in chicken; binding constant 1.74 x 10^7 M; No reactivity detected to recombinant IL-1β, IL-6 or TNFα), IL 8 human recombinant antigen (expressed in *E. coli*) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich. These protein solutions were prepared in 5 mM phosphate buffer (PBS, pH 7.4). All chemical reagents were of analytical grade and utilized as received, and aqueous solutions were prepared with ultrapure water. Clinical real serum and saliva samples were obtained from Namık Kemal University Hospital. TIMCAL Carbon Super P® conductive carbon black (+99 %) as conductive agent and polyvinylidene fluoride (PVdF, 600000 g/mol, 99.5%) as binder material in composite slurry were purchased from MTI-XTL Corporation.

2.2. Instrumentations

EIS, CV and SFI techniques were carried out by using Gamry 1000 potentiostat/galvanostat connected to a three-electrode cell. A conventional three- electrode cell composed of platinum wire as an auxiliary electrode, an Ag/AgCl electrode as a reference electrode and a modified ITO electrode as a working electrode was utilized. All electrochemical measurements carried out in phosphate-buffered saline (PBS, pH 7.4) containing 5 mM $[Fe(CN)_6]^{3-}$/$[Fe(CN)_6]^{4-}$ as a redox probe. This device operated in a potential range from 0 to 500 mV at a scan rate 100 mV/s for CV measurements. The impedimetric measurements were performed at 50000 Hz and 0.05 Hz for initial and final frequency, respectively. The spin-coating process was performed using a traditional spin-coater (MTI VTC-50). Attenuated Total Reflection Fourier Transform Infrared (ATR-FTIR) measurements were performed using a Bruker Company Vertex 70 FTIR infrared spectrometer in the range of 4000–400 cm^{-1} at room temperature and the results were used without any corrections. Dispersive Raman measurements were performed with a Thermo Company DXR Raman spectrometer equipped with a 780-nm excitation laser. The morphological investigations of polymer and modified ITO were carried out using SEM (FEI-Quanta FEG 250 Model). AFM images were recorded by using a AFM PLUS+, Nanomagnetic Instruments. The images were obtained using the tapping mode of AFM with a commercial tapping silicon (Si) tip and the estimated tip radius is less than 10 nm. Images were acquired at solution 256 x 256 points within the range of 5μm/s scan rate and were subjected to first-order flattening.

2.3. Preparation of the super P- star polymer modified electrode

The preparation process of conductive composite interface material coated ITO-PET electrodes includes four stages, as depicted in Scheme 1. First, composite interface materials were prepared by mixing the synthetized star polymer (10 mg, SPGMA), carbon black conductivity agent (40mg, Super P) and polyvinyldenefluoride binder (10 mg, PVDF) in the presence of 1.8 ml Acetone and 0.2 ml NMP. Second, the mixture was homogenized by blending in a magnetic bar with magnetic stirrer maximum rotation speed for 60 minutes. Third, the obtained homogenized slurry was spread over the entire surface of the ITO electrode with spin coater. Finally, the spin-coater was accelerated to 1000 rpm rotational speed and maintained there for sixty seconds and then dried under reduced pressure at room temperature.

2.4. Fabrication of the electrochemical impedimetric immunosensor

The immobilization procedure schematized in Scheme 1. Prior to the modification of ITO electrode, it was washed by sonication in acetone, soap solution and ultra-pure water for 10 min. Next, the composite modified ITO electrode was prepared as described above. After washing with ultrapure water, the modified electrode was immersed in PBS solution containing 2.5 ng/mL anti-IL 8 antibody for 45 min. After that, the electrodes were rinsed with ultra-pure water to remove loosely attached antibodies. Then, the electrodes were immersed in PBS solution containing BSA. Finally, the electrodes got ready to measure IL 8 antigens.

Scheme 1. Schematic illustration of the composite preparation procedure and the fabrication process of the immunosensor.

3. Results and Discussion

3.1. Chemical Characterization of Super P®/Star Polymer Composite Modified Electrode

The star polymer with glycidyl side-groups was synthesized from two-step route sequence (Scheme 1). Esterification and ATRP chemistry techniques were used in the first and final steps to obtain star-shaped polymer (SPGMA). Tetra-armed SPGMA polymer were synthesized and characterized according to our previous report (Aydın et al. 2018a). The chemical interaction between composite material coated ITO surface and anti-IL 8 antibody were characterized by FTIR and RAMAN spectral technics. The FTIR spectra of conductive composite interface modified ITO-PET electrode surface before anti-IL 8 immobilization (blue line) and after anti-IL 8 immobilization (red line) are shown in Fig. 1A and 1B, respectively.

Fig. 1. FTIR and Raman spectra before and after anti-IL 8 antibodies immobilization.

The result from FTIR in Fig. 1A revealed that the characteristic band of epoxy group in polymer appears at 904 cm^{-1} and 842 cm^{-1} (Lei et al. 2016). The strong peak at 1724 cm^{-1} can be assigned to the typical C=O stretching vibration of carbonyl groups (Aydın et al. 2018b). When two FTIR spectra were analysed, reduction of the distinct characteristic peaks of epoxy group at 904 cm^{-1} and 842 cm^{-1} provided further evidence of bond formation of polymer and antibody having taken place (Çakmak et al. 2009). For the antibody immobilization (at red line in Fig. 1B), the characteristic peaks corresponding to C=O stretching at 1640 cm^{-1} (amide I) and N−H bending at 1543 cm^{-1} (amide II) were observed, demonstrating that the amide linkages were formed between the epoxy groups of polymers and the terminal amino group of anti-IL 8 antibody. Besides, the chemical interaction of polymer and antibody were also examined with Raman spectroscopy. Contrary to FTIR spectra, the Raman spectral technique has been proved to be a convenient tool to identify proteins, nucleic acids, large molecules and assemblies, because large molecules which have multiply bonded or electron-rich groups produce more intense Raman bands (Kurouski et al. 2015). Moreover, IR absorption spectra of proteins and nucleic acids are complicated and many bands are overlapped (Thomas Jr 1999). The Raman spectra of modified ITO-PET electrode before anti-IL 8 immobilization (blue line) and after anti-IL 8 immobilization (red line) are also shown in Fig. 1C and 1D, respectively. The Raman spectra showed that epoxy ring stretching vibrations of polymer were observed at 1260 cm^{-1} and 910 cm^{-1}. Amide I, II and III regions are monitored mostly between 1650–1680 cm^{-1}, 1480-1570 cm^{-1} and 1235-1300 cm^{-1}, respectively (Kitagawa and Hirota 2002). As shown in Fig. 1D, amide I, amide II and amide III bonds were observed at 1670, 1560 and 1349 cm^{-1}, respectively.

3.2. Electrochemical characterization of immunosensor fabrication

For the electrochemical characterization of the proposed immunosensor fabrication, electrochemical impedance spectroscopy and cyclic voltammetry were utilized. EIS is an effective method for analysing the interface features of surface modified electrodes. In the EIS spectra, the

semicircle part is related to the electron transfer limiting process at high frequency and linear portion is related to the diffusion limiting process at low frequency. The electron transfer resistance (R_{ct}) can be directly measured by the semicircle diameter and it is associated with the electron transfer kinetics of the redox probe in the electrode interface. To fit the impedance spectra, an equivalent circuit was utilized. This circuit contains the electrolyte solution resistance (R_s), the electron transfer resistance (R_{ct}), the Warburg impedance (Z_w) associated with redox probe diffusion from the bulk of the electrolyte to the electrode solution interface, constant phase element (CPE), associated with the double layer capacitance(Dutta et al. 2017; Huang et al. 2008; Junior et al. 2018; Liu et al. 2009; Wang et al. 2016).

Fig. 2. Impedance (A) and voltammetric (B) characterization of the build-up process of proposed immunosensor. (Inset: Equivalent electrical circuit).

Figure 2 illustrates EIS spectra and cyclic voltammograms of electrode fabrication process. As illustrated in Figure 2A, the R_{ct} of the polymer modified electrode was small. This small Nyquist plot illustrated the super P-GMA composite successfully fixed on the ITO electrode. When the anti-IL 8 antibodies were modified on ITO/super P-GMA, the R_{ct} increased evidently. Since the nonconductive property of the antibody decreased the conductivity of the electrode surface and prevented the transfer of redox probe. After blockage of polymer epoxy groups with BSA, an increase was observed in R_{ct} value. BSA is a protein like antibodies and has nonconductivity property. Therefore, they markedly affect the electrochemical impedance, which was consistent in the previous studies (Aydın et al. 2018a). After the recognition reaction between antibody and antigen, the impedance increased remarkably. It could be attributed to the fact that nonconductive property increased on the ITO surface and further impeded the charge transfer between the redox probe and the electrode surface.

The redox probe illustrates a reversible cyclic voltammogram with cathodic and anodic peaks. As seen in figure 2B, after super P-GMA composite modification, reversible peaks with high current were seen. A decrease in peak current was monitored after anti-IL 8 antibodies immobilization. Amino groups of anti-IL 8 antibodies bonds to epoxy groups of SPGMA polymer covalently. After blockage epoxy groups with BSA, nonconductive layer was increased on the ITO electrode. Cyclic voltammogram shows a decrease in the peak current due to decrease in the diffusion capacity of redox probe. The interaction between anti-IL 8 antibody and IL 8 antigen caused a decrease in peaks current due to more blocking effect of proteins on the electrode surface. All results obtained from EIS and CV results confirmed that the impedimetric immunosensor was successfully constructed, and the increase in the impedance originated from increasing IL 8 antigen concentrations.

When compared these results to the EIS responses obtained in our previous work (Aydın et al. 2018a), super P-star polymer composite modified electrode indicated that this composite film was more sensitive surface for IL 8 antigen detection. All result confirmed that super P-star polymer would enhance the sensitive detection of IL 8 antigen, which may be attributed to the excellent electrical features.

3.3. Morphological characterization of immunosensor fabrication

SEM and AFM images of the immunosensor construction steps are shown in Figure 3. As seen Figure 3A and 3G, a well distributed super P/SPGMA polymer composite was observed. The SEM images of PVDF and Super P were illustrated 3E and 3F, respectively. Comparing figure 3A, 3E and 3F, it was concluded that polymer PGMA was distributed homogenously. The root mean square

(RMS) value of this step was 400 nm (Fig. 3G). After anti-IL 8 antibody immobilization, the modified ITO surface was changed completely as seen Figure 3B and 3H. The surface appeared to be smoother than the previous one. Furthermore, the surface was coated with anti-IL 8 antibodies. This result was consistent with AFM results. The RMS value was measured as 660 nm after anti-IL 8 immobilization (Fig 3H). After BSA blockage (Figure 3I), a noticeable increase was observed in roughness value (3081 nm). And the electrode surface looked like typical BSA SEM figures in our previous work (Fig. 3C) (Aydın et al. 2018a). The surface topography changed after interaction between IL 8 antibody and IL 8 antigen, suggesting a selective recognition (3D and 3J). The RMS value was measured as 340 nm.

Fig. 3. SEM and AFM images of composite modified ITO electrode (A and G), anti-IL 8 antibodies immobilized electrode surface (B and H), after BSA blockage of free epoxy groups (C and I), after interaction between anti-IL antibodies and antigens (D and J).

3.4. Optimization of immunosensor fabrication conditions

To obtain a sensitive immunosensor, fabrication conditions containing polymer concentration, anti-IL 8 antibody concentration and incubation time, and IL 8 antigen incubation duration were optimized. Firstly, the effect of polymer weight existing in the composite on EIS performances was studied. Three composites composed of with different star polymer weight were prepared and applied for immunosensor construction. The calibration curves after studying with these concentrations are illustrated in Figure 4A. As shown in Figure 4A, 10 mg polymer was used in the following experiments due to high response. At the weight of 20 mg polymer, disturbances were seen on the electrode surface. At the weight of 5 mg polymer, a low signal was obtained. The sensitivity of electrochemical immunosensor closely depended on the anti-IL 8 antibody concentration. Therefore, three different anti-IL 8 antibody concentrations were used for optimization of antibody concentration. As seen Figure 4B, the highest signal was obtained at 2.5 ng/mL antibody concentration. Low antibody concentration (0.4 ng/mL) caused low biosensor signal and high antibody concentration (10 ng/mL) had similar signal at 2.5 ng/mL antibody concentration. Therefore, 2.5 ng/mL antibody was chosen as optimum value. Another important parameter was antibody incubation period. In this optimization step, three different durations were tried and the maximum signal was achieved at 45 min (Fig. 4C). The last optimization parameter was IL 8 incubation period. To optimize this parameter three different durations were utilized. This optimization parameter was important to monitor interaction between antibody and antigen (Figure 4D). In the period of 30 min and 60 min, the signals were similar. At 45 min, a highest signal was obtained and therefore, 45 min was chosen as optimum incubation duration.

Fig. 4. Optimization results of experimental conditions; (A) polymer concentration, (B) antibody concentration, (C) antibody incubation time, (D) antigen incubation time.

3.5. Ultrasensitive impedimetric detection of IL 8 antigen

Under the optimal experimental conditions, the proposed immunosensor was applied to quantitatively detect different concentrations of IL 8 antigen. Obtained EIS responses are shown in

Figure 5A. As shown in figure 5A, more antigens interacted with anti-IL 8 antibodies on the electrode surface, and the variations at ΔR_{ct} was linear with the IL 8 antigen concentration in the range of 0.01 to 3 pg/mL with an extremely low detection limit (LOD) 3.3 fg/mL (S/N=3) and low determination limit (LOQ) 11 fg/mL (Figure 5 C). The regression equation was ΔR_{ct}= 0.827 [IL 8] + 0.01. As shown in figure 5B, peak currents of the immunosensor were decreased in the presence of gradually increased concentrations of IL 8. The changes in current values clearly suggested an ultrasensitive signal between anti-IL 8 antibody and different concentrations of IL 8 antigens on the modified electrode surface. Because of amounts of specific biorecognitions, the redox probe transfer to the electrode surface was gradually hindered and caused a decrease in peak currents.

The interaction between anti-IL 8 antibody and IL 8 antigen was followed by SFI technique. In this technique impedance measurement was performed at constant frequency value. The constant frequency value was determined by using Bode Plot. As seen in Figure 5D, an increase was observed in impedance value (purple). This increased proved the interaction between antibody and antigen.

Fig. 5. (A and B) EIS and CV responses of the immunosensor at different IL-8 concentrations, (C) calibration curve obtained after EIS measurements, (D) Single frequency impedance result.

3.6. Repeatability, reproducibility, regeneration, stability and specificity of the proposed immunosensor

The repeatability and reproducibility of the immunosensor were assessed. To examine the repeatability of the immunosensor, twenty independent electrodes were prepared for the detection of 0.250 pg/mL IL 8 antigen. The relative standard deviation (RSD) of twenty independent electrodes was 5.72%, confirming the immunosensor repeatability. To evaluate the reproducibility of the immunosensor, ten immunosensors (composed of 80 independent electrodes) were prepared under the same condition. They have similar responses and the linear curves of these immunosensors are illustrated in Figure SI-1A. All of these immunosensors showed the same linearity in the range between 0.01 pg/mL and 3 pg/mL for IL 8. The relative standard deviation (RSD) of ten immunosensors was 3.37%, confirming reproducibility of the immunosensor. To test the reusability of the immunosensor, the fabricated electrode (after IL 8 antigen immobilization) was immersed in acidic solution (ultra-pure water including HCl, 0.1%) for 5 min. Then, this electrode was washed by using ultra-pure water. To perform interaction between antibody and antigen, this electrode was immersed in PBS solution containing IL8 antigen. The excessive amount of IL 8 antigens was removed by washing in ultra-pure water. Then, the impedimetric signal was measured. This process continued until signal disappeared. As shown in figure SI-1B, the signal was decreased gradually during the process. The total loss in the signal was about 89.72% after 5 cycles (Figure SI-1B). This signal proved the reusability of the biosensor. The stability of the impedimetric immunosensor was further assessed by measuring the EIS responses of 0.250 pg/mL IL 8 antigen. The immunosensors were stored at 4 ^{0}C in dry form. After 6 weeks, the EIS results of the immunosensor decreased to about 66.7% of its initial value, indicating the long-term stability of the immunosensor (Figure SI-1C).

Specificity and accuracy are important parameters in the fabrication of a biosensor. To test specificity of the proposed immunosensor, two PBS buffer solutions were used, one included interference materials (different biomarkers such as Tumor necrosis factor alpha, interleukin 1β and interleukin 1α and drugs such as ampicillin, amoxycillin, erythromycin) at a relatively high concentration and IL 8 antigen and the other included only interference materials at high concentration. Biosensor did not give response to these inference materials. Also, the interference studies proved the specificity of the immunosensor. Furthermore, the practical usability of the

immunosensor was tested by using real serum and saliva samples. The measurements of IL 8 concentrations in serum and saliva samples were performed after dilution of these samples 200 and 50-fold, respectively. To verify the accuracy of the immunosensor, the sample results were compared to the ELISA results. The results showed that the sample results measured by biosensor were compatible with the ELISA results (Table SI-1A). The analytical properties of the biosensors for IL 8 quantification are summarized in Table SI-1B. After the investigation of the biosensors as summarized in Table SI-1B, it was concluded that our developed biosensor had faster and easier preparation procedure than the other biosensors that require nanoparticles or expensive chemicals. Moreover, our immunosensor had the lowest detection limit among those previously reported.

4. Conclusion

In this study, we successfully fabricated a facile immunosensor for rapid determination of IL 8, a cancer biomarker. By construction of this immunosensor, ITO electrode was firstly modified by composite composed of Super P, PVDF and star polymer. This composite provided a conductive and convenient matrix for IL 8 antibody immobilization. Furthermore, with the help of this composite, a much lower detection limit (3.3 fg/mL) and wide detection range (0.01-3 pg/mL) were obtained. The antibody immobilization on the modified ITO electrode surface was monitored FTIR and Raman Spectroscopy. Moreover, the modification steps were followed by using SEM and AFM morphological techniques. The interaction between anti-IL 8 antibody and IL 8 antigens was monitored by SFI technique. Our immunosensor illustrated good reproducibility and repeatability and high stability. The analytical applicability of the proposed immunosensor was examined by its application for IL 8 detection in serum and saliva samples. Furthermore, these results are in compatible with ELISA results. Consequently, this modification electrode method provided a new strategy for the design of IL 8 antigen detection sensor.

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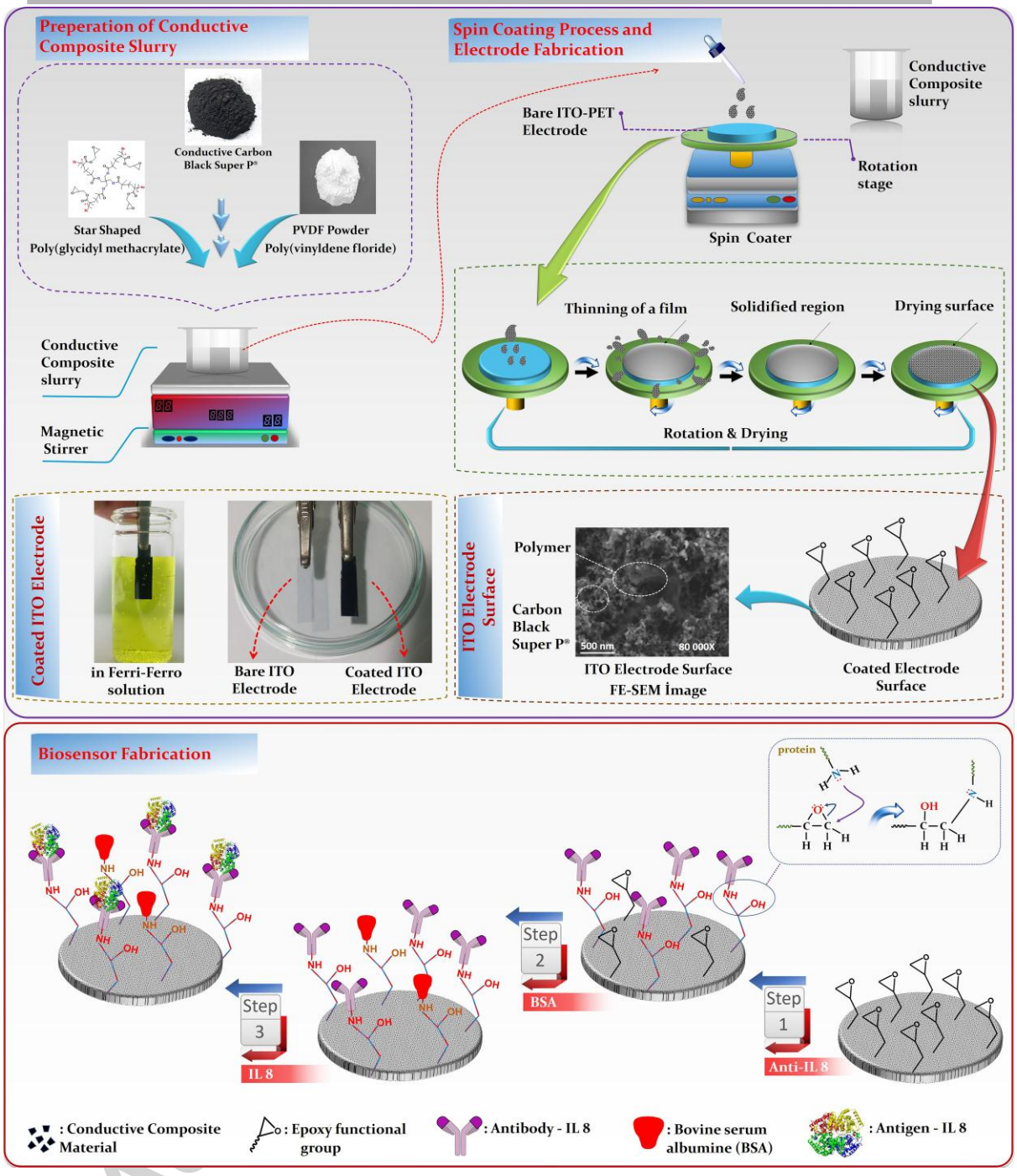

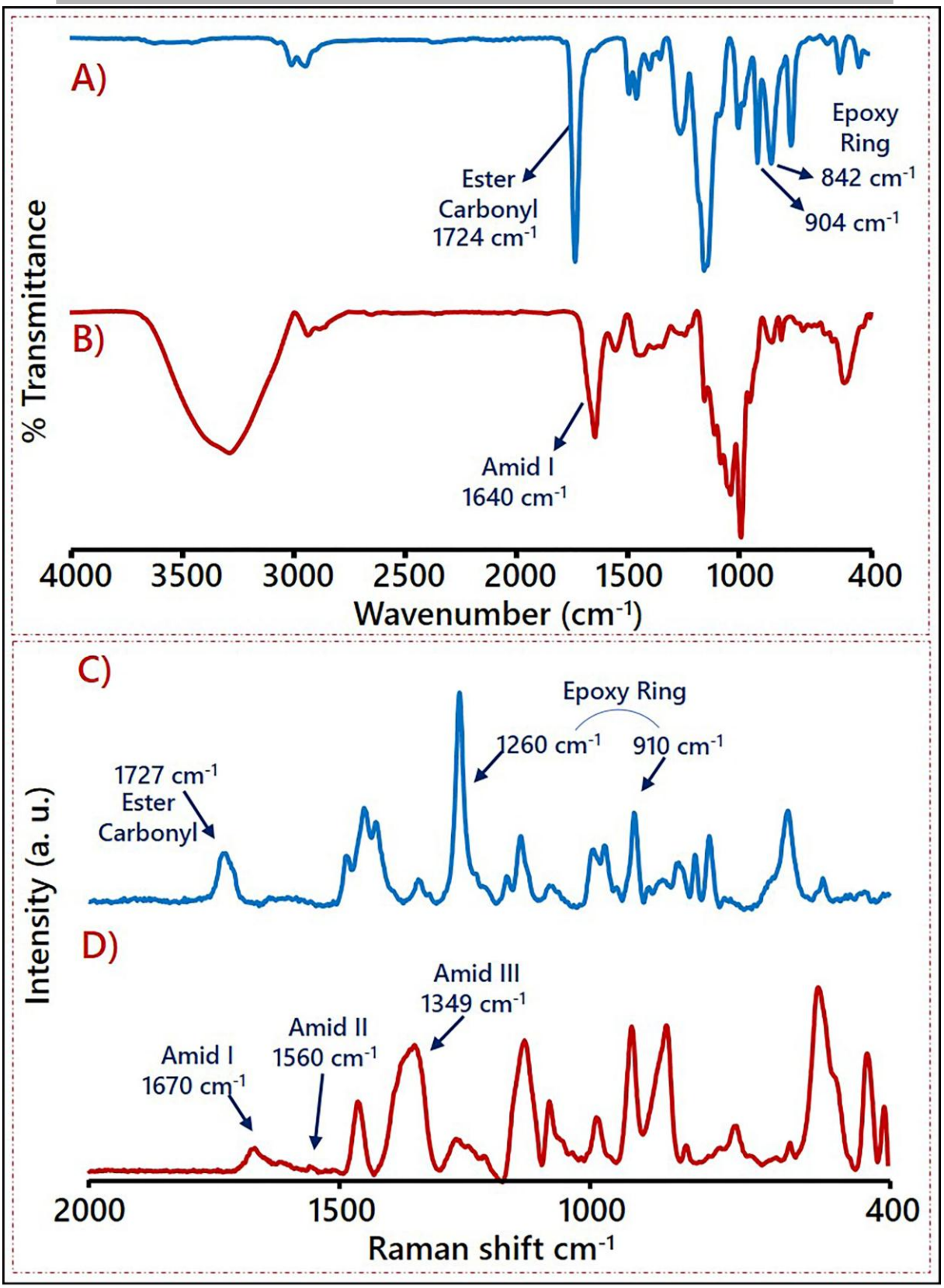

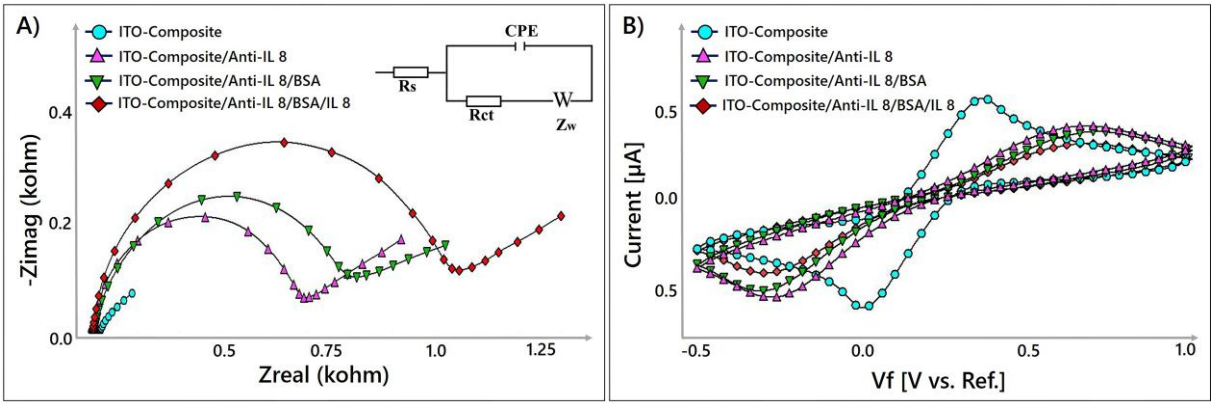

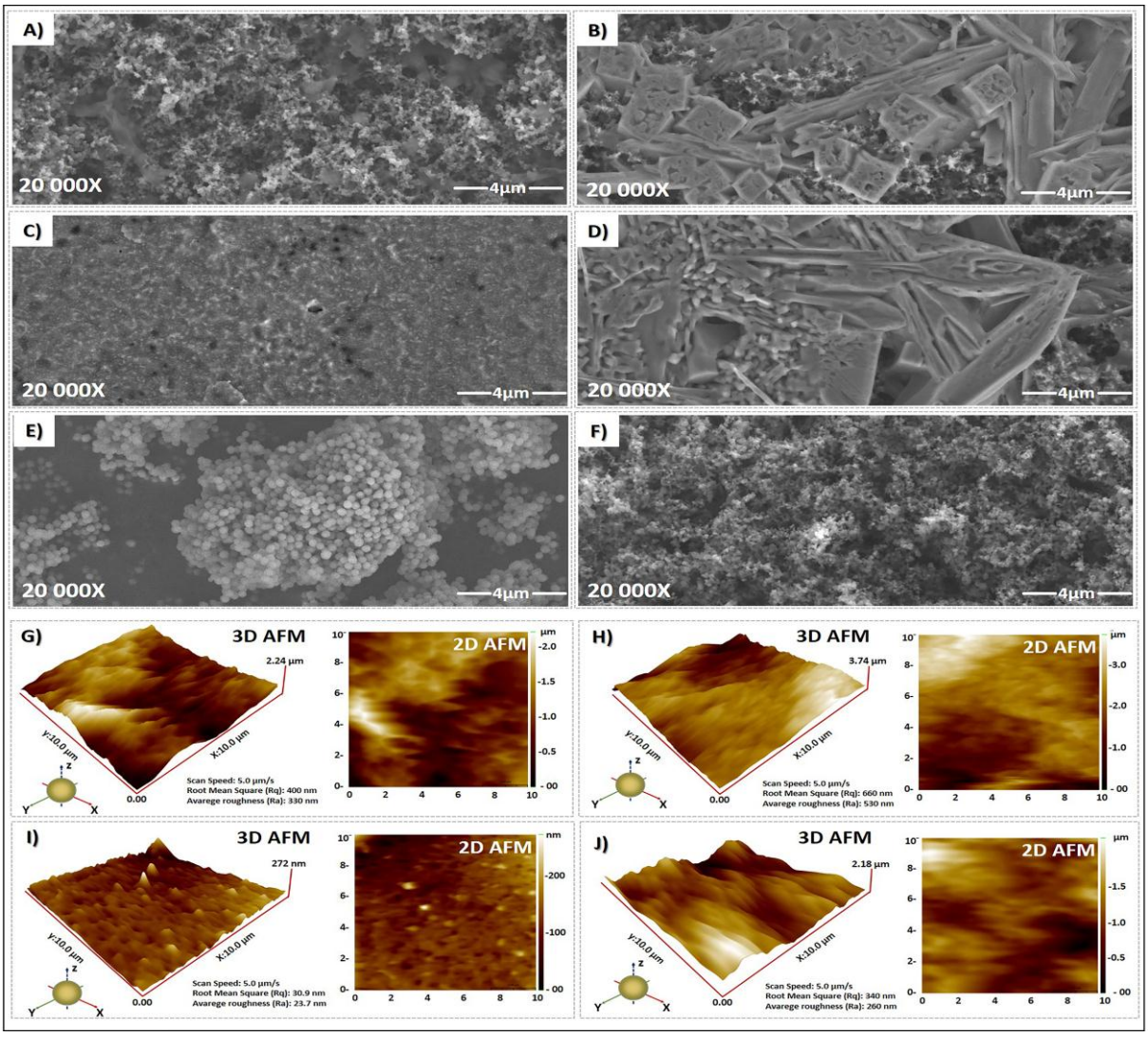

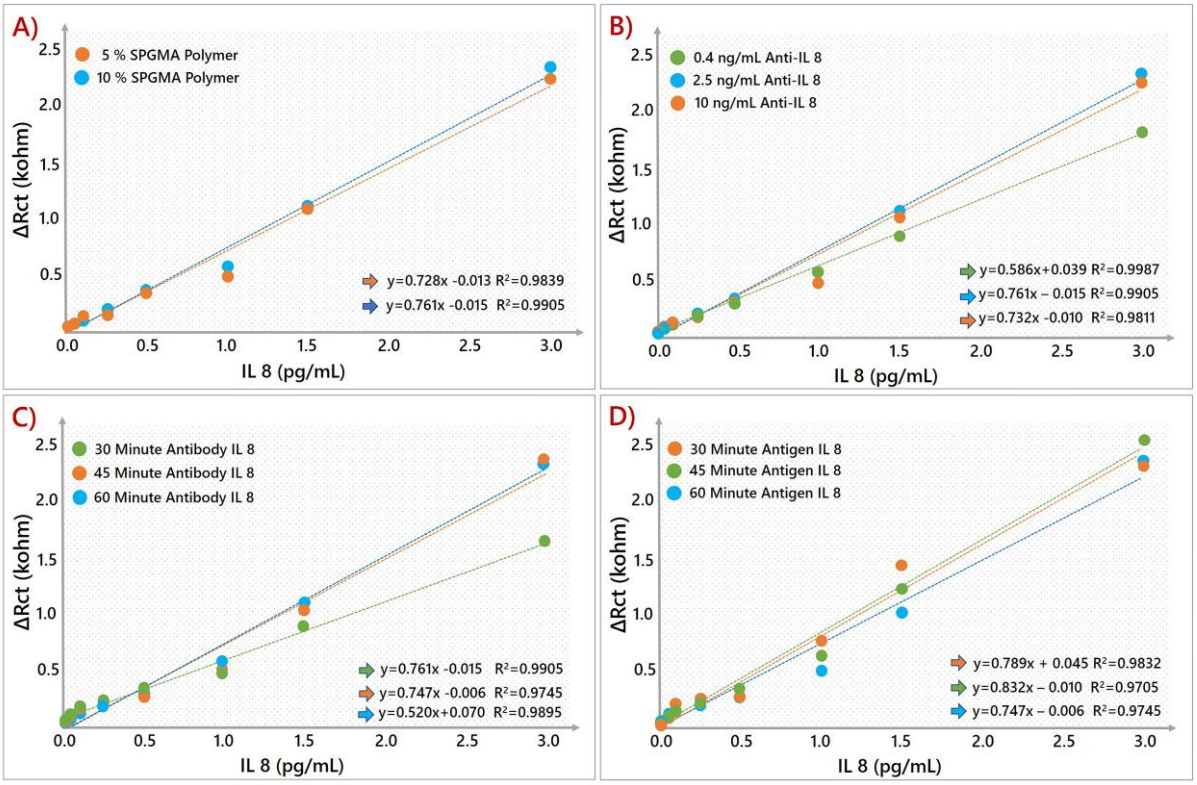

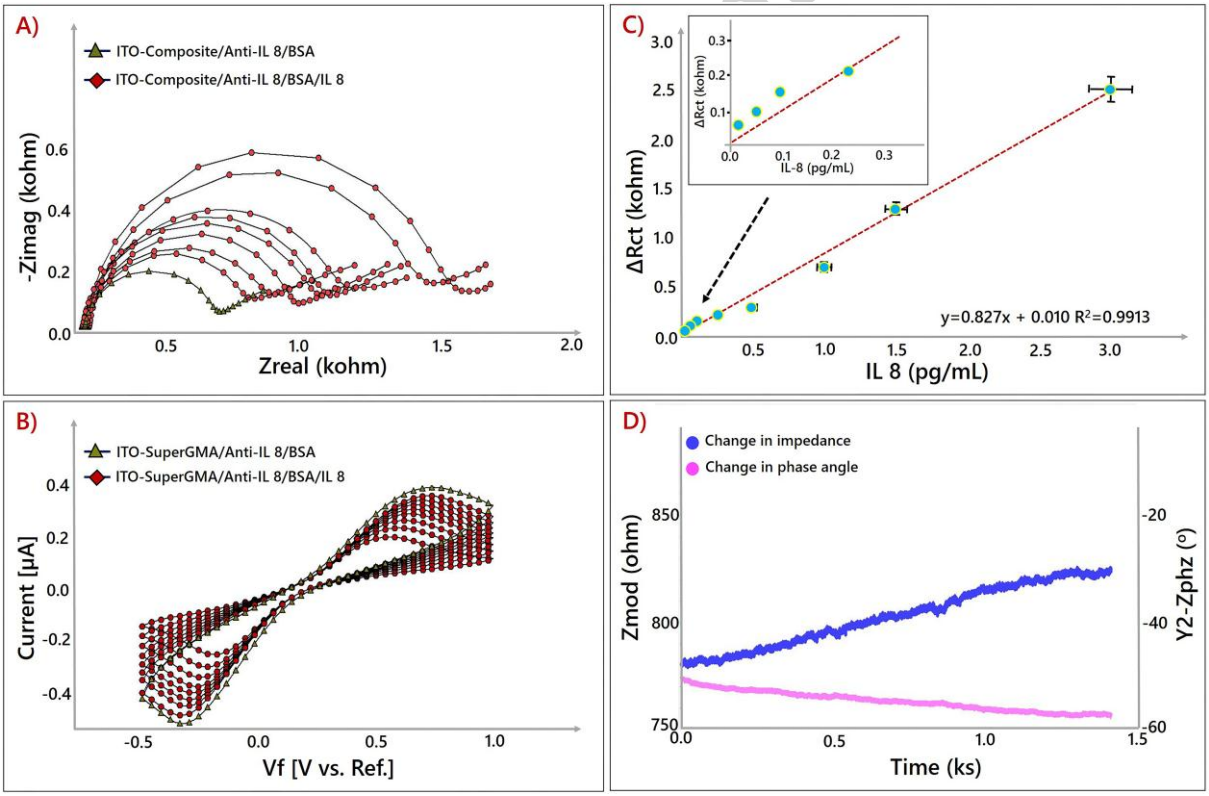

Highlights

- An electrochemical label-free immunosensor was introduced for IL 8 detection.
- A conductive composite material was used to prepare the proposed immunosensor.
- The immunosensor could detect interleukin 8 as low as 3.3 fg/mL.
- The applicability of the biosensor was determined in real serum and saliva samples.