Analytical Biochemistry 554 (2018) 44–52

Contents lists available at ScienceDirect

Analytical Biochemistry

journal homepage: www.elsevier.com/locate/yabio

# An impedimetric immunosensor for highly sensitive detection of IL-8 in human serum and saliva samples: A new surface modification method by 6-phosphonohexanoic acid for biosensing applications

Elif Burcu Aydın[a,*], Mustafa Kemal Sezgintürk[b]

[a] Namık Kemal University, Scientific and Technological Research Center, Tekirdağ, Turkey
[b] Çanakkale Onsekiz Mart University, Faculty of Engineering, Bioengineering Department, Çanakkale, Turkey

ARTICLE INFO

*Keywords:*
Interleukin-8
6-Phosphonohexanoic acid
Indium tin oxide
Electrochemical impedance spectroscopy
Single frequency impedance spectroscopy

ABSTRACT

In this study, we fabricated a sensitive and label-free impedimetric immunosensor based on 6-phosphonohexanoic acid (PHA) modified ITO electrode for detection of interleukin-8 (IL-8) in human serum and saliva. PHA was first employed to cancer biomarker sensing platform. Anti-IL-8 antibody was used as a biorecognition element and the detection principle of this immunosensor was based on monitoring specific interaction between anti-IL-8 antibody and IL-8 antigen. The morphological characterization of each electrode modification step was analyzed by scanning electron microscopy (SEM), SEM-energy dispersive X-ray spectroscopy (EDX) and atomic force microscopy (AFM) while electrochemical characterization was performed by electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV) and single frequency impedance (SFI) techniques. Moreover, the antibody immobilization on the electrode surface was proved Fourier-transform infrared spectroscopy (FTIR) and Raman Spectroscopy. This proposed impedimetric immunosensor exhibited good performances with a wide linear in the range from 0.02 pg/mL to 3 pg/mL as well as a relative low detection limit of 6 fg/mL. The impedimetric immunosensor had a good specificity, stability and reproducibility. This study proved that PHA was a suitable interface material to fabricate an electrochemical biosensor.

## Introduction

Interleukin (IL)-8 is an inflammatory chemokine which is present in the C-X-C chemokine subfamily [1,2]. IL-8 is expressed by several cell types such as activated monocytes and macrophages, T cells, neutrophils, NK cells and also endothelial cells, a wide variety of epithelial cells and fibroblasts [3,4]. An increase of IL-8 expression is observed in a lot of cancer types such as brain, breast, cervical, colorectal [2], gastric, lung, melanoma, ovarian, prostate, renal, oral [5], and thyroid [6]. In various cancers, high IL-8 levels have been identified as a prognostic factor [7]. Furthermore, Velásquez et al. (2014) reported that high level of IL-8 is a sign of myocardial infarction [8]. Wong (2006) reported that IL-8 protein level is high in oral cancer patients (750 ± 236 pg/mL) than control groups (250 ± 130 pg/mL) [9]. Furthermore, the IL8 protein threshold value is 600 pg/mL to discriminate oral cancer patients from healthy individuals [10,11]. In healthy individuals, the IL-8 basal clinical level is 5–10 pg/mL [12].

The early diagnosis of cancer and thereby the early diagnosis of certain protein biomarkers is important for human health but it is difficult because the level of protein biomarker in serum is very low [13]. Biomarkers are usually detected by traditional techniques such as enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, fluorescence immunoassay, and electrophoretic immunoassay [14]. However, they have some disadvantages such as difficult operation processes, requiring a great deal of time. ELISAs are the basic analysis method of IL-8 protein detection in clinic applications at the moment [12].

Despite the widely utilization of ELISA tests, they are limited in point-of-care (POC) analysis because they require large and expensive instruments, additional chemicals, and a lot of time for application of the protocol [12]. Therefore, there are increasing efforts to fabricate rapid, sensitive and selective immunosensors. Electrochemical immunosensors have different advantages such as high sensitivity, low cost and rapid response [15]. IL-8 biosensors found in literature are summarized in Tables SI–1. Wan et al. (2011) developed a multiplex, amperometric immunosensor based on screen printed carbon electrode (SPCE) array as working electrode for IL-8 detection. Anti-IL-8 antibody was the primary antibody and immobilized on SPCE array while horseradish peroxidase (HRP)-multiwalled carbon nanotubes (MWCNTs)—anti-rabbit IgG composite was used as multi-labeled

* Corresponding author.
*E-mail addresses:* b_e_bahadir@hotmail.com, ebbahadir@nku.edu.tr (E.B. Aydın).

https://doi.org/10.1016/j.ab.2018.05.030
Received 28 February 2018; Received in revised form 27 May 2018; Accepted 30 May 2018
Available online 11 June 2018
0003-2697/ 

nanoprobe. The detection limit was 8 pg/mL [13]. An electrochemical magnetosensor was fabricated by Torrente-Rodriguez et al. (2016) in order to detect protein IL-8 and IL-8 mRNA in human saliva. The detection limits were found as 0.21 nM for IL-8 mRNA, 72.4 pg/mL for IL-8 protein, respectively [10]. Another study for IL8 detection, conducted on the screen printed dual carbon electrode, which was used as working electrode and the amperometric detection was performed by using HRP enzyme labeled biosensing. An optical microfluidic biosensor based on absorbance detection principle was developed for salivary protein biomarkers (IL-1β, IL-8, MMB-8). This biosensor includes an organic photodetector made of polythiophene-C70 bulk. The detection limit was 80 pg/mL. The unspiked human saliva samples were tested and the results of saliva samples were in accordance with commercial ELISA test [11]. In recent years, the detection of a few fg/mL of IL-8 in saliva was performed by Sharma et al. by using an impedimetric biosensor. They used carboxylic terminated monothiol alkane PEG acid film modified gold electrode and the detection of human IL-8 protein was monitored by EIS technique [12].

Biosensing platform of a biosensor is important for high sensitivity, specificity detection of target analytes. Antibodies, aptamers, enzymes, nucleic acids are used as sensing molecules and the signal is occurred after biorecognition events and this signal is measured by chemical, electrical and optical techniques [15]. The quantifiable signal is associated to the concentration of the analyte. Therefore, the biorecognition molecule immobilization has an important role in achieving high sensitivity and selectivity with long shelf life [16]. ITO is an excellent electrode material and has been utilized in optic and electrochemical sensors and biosensors. Furthermore, ITO has many interesting advantages such as a wide potential window and stable electrochemical and physical features [17,18]. In typical biosensor system, biorecognition elements are immobilized on the electrode surface and these elements selectively bind the desired analyte molecules and they result in detectable interfacial changes [19]. Interface has an excellent role in detection of analyte because most of the biological reactions occur at interfaces [20]. To prepare effective interface for binding of biorecognition elements, different techniques such as electropolymerization [21,22], silanization [23–26], electrophoretic deposition [27,28], and electrochemical deposition [29,30] have been used in ITO based biosensors. Silanization methods have been usually used in literature due to its several advantages; easy to prepare, forming stable monolayer and providing uniform immobilization matrix [16,17]. In silanization technique, siloxane bonds are formed between hydroxylated electrode surface and silane agents [17]. However, studies based on phosphonate based biosensors are not found in literature. Apart from silane self-assembled monolayer (SAM), phosphonate SAM can be used for SAM formation on ITO surface. Phosphonic acids have gained interest due to their ability to produce SAMs on the metal oxide surface than silanes and thiols. Phosphonates have three oxygens with a carbon attached directly to phosphorus. The lack of hydroyzable P-O-C linkage increases the stability in aqueous solution and forms SAMs easily [31]. Phosphonic acids have a spontaneous affinity to form SAM on different surfaces; for example, phosphonic acids are bound more strongly to metal oxide surfaces than carboxylic acid [32]. The SAM of phosphonates provides an immobilization matrix for biomolecules after several electrode surface modifications [33]. Therefore, in this study we utilized 6-phosphonohexanoic acid to construct the immobilization matrix for biorecognition element and we investigated the immobilization of IL-8 antibodies on the PHA modified ITO electrode surface including carboxyl active ends, for the first time.

Electrochemical impedance spectroscopy (EIS) is a sensitive and one of the oldest electroanalytical technique and has attracted attention in recent years. This method is usually used for monitoring of changes in electrode-electrolyte interfacial features induced by antibody-antigen interaction, DNA hybridization or enzyme-substrate catalysis. The electrode-electrolyte interface is important for several areas of modern science and technology. In addition, impedance measurements are used in different fields of electrochemistry, such as electrode kinetics, double layer studies, batteries, corrosion, solid-state chemistry and biosensing [34–36]. It is successful and informative method for the characterization of bio-functionalized electrodes and the determination of some analyte from small ions to large proteins [37,38]. It has been mostly used in the design of electrochemical biosensors. The principle of this method is based on the analysis of the response of the system under investigation to alternating current probing signals. During the EIS experiments, three electrodes were usually utilized; a working electrode (the object under investigation), a counter electrode (to apply the electric current) and a reference electrode (to provide a known reference point) [39]. Compared with other redox labelled electrochemical techniques such as cyclic voltammetry (CV), differential pulse voltammetry (DPV) and square wave voltammetry (SWV); EIS provides a lot of advantages such as high sensitivity, easy signal quantification, less destructive effect on the measured biological interactions [37,40]. Electrochemically inert species is measured by EIS, when the measurements are carried out in the presence of redox probe, such as ferricyanide, which undergoes oxidation and reduction at the electrode surface [41].

Single Frequency Impedance (SFI) is an electrochemical impedance spectroscopy technique where a single frequency is utilized as an excited signal instead of wide frequency range. By using this technique, the interaction between antibody and antigen can be monitored easily. The most common use is in impedance-based sensor development and in on-line sensor systems. Unlike standard EIS, it does not provide a spectrum. Instead of EIS, SFI impedance technique measures electrochemical impedance at one frequency periodically. The measurement is made with a constant DC potential applied to the cell. This technique is suitable, simple and inexpensive for the analysis of clinical and on field assays [24,42,43].

In this work, a novel phosphonate based immunosensor was developed for the first time. PHA modified ITO electrode was prepared in simple steps at room temperature. Further, this immunosensor was fabricated by immobilizing anti-IL8 antibodies on PHA modified ITO electrode. The biosensing principle of the immunosensor was based on specific interaction between anti-IL8 antibody and IL8 antigen. The electrochemical characterization of PHA modified electrode was performed by recording CV and EIS responses. Apart from EIS and CV, the performance of the bio-electrode was thoroughly evaluated SFI measurements. Moreover, the antibody immobilization on the electrode surface was investigated with FTIR and Raman spectroscopy. The prepared working electrode showed excellent IL-8 sensing properties such as good repeatability and high sensitivity. In addition, the developed immunosensor was reusable and thus this property decreases the cost of biosensor. Therefore, a simple, low-cost and label-free immunosensor was constructed for determination of IL8. This immunosensor has remarkable advantages of simple fabrication process, low detection limit and less interference from other substances present in human serum and saliva samples.

## Experimental

### *Materials and chemicals*

6-phosphonohexanoic acid (PHA), hydrogen peroxide ($H_2O_2$), ammonium hydroxide ($NH_4OH$), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC), N-hydroxysuccinimide (98%) (NHS), indium tin oxide (ITO) electrodes (2 mm × 20 mm, 60 Ω/cm$^2$) and biomolecules (anti-IL-8 antibodies, IL-8 antigens and bovine serum albumin) were purchased from Sigma Aldrich. Anti-IL-8 antibodies (produced in goat), IL-8 antigen (expressed in *E. coli*), and bovine serum samples (BSA) solutions were prepared by using phosphate (PBS) buffer (pH 7.4). Ferricyanide solution ($[Fe(CN)_6]^{3-/4-}$, 5.0 mM) was prepared by dissolving potassium ferricyanide and potassium ferrocyanide with PBS (1 M, pH 7.4) and utilized as the supporting electrolyte medium.

All solutions were prepared using ultra-pure water. MyBioSource Human Interleukin 8 ELISA Kit (Detection Range 6.25 pg/mL - 200 pg/mL and sensitivity 1.0 pg/mL) was utilized for comparison of immunosensor results. Serum samples were supplied by Namık Kemal University hospital. Saliva samples were collected from the volunteers in NKU Research Lab.

*Apparatus*

The detection process was performed on the electrochemical workstation (Potentiostat/Galvanostat Reference 1000, Gamry Instruments, Warminster, PA, USA) and was characterized by cyclic voltammetry (CV), EIS and SFI techniques. These electrochemical measurements (CV and EIS) were carried out in 5 mM $K_3[Fe(CN)_6]$/$K_4[Fe(CN)_6]$ (1:1) solution that contains 0.1 M KCl. CV (Scan rate:100 mv/s, step size:10 mV), EIS (Initial frequency:50000 Hz, Final frequency: 0.05 Hz, points:5) measurements were employed during the experiment. Modified ITO electrode, platinium wire and Ag/AgCl (KCl) were utilized as the working, counter and reference electrodes, respectively. FTIR measurements were carried out by using Bruker FTIR (Bruker, Vertex 70 ATR, Germany) equipped with diamond. FTIR spectra in the range 4000–400 $cm^{-1}$ were recorded to analyze the nature of the chemical bonds. Dispersive Raman measurements were carried out with a Thermo company DXR Raman spectrometer equipped with a 780 nm excitation laser and a confocal microscope with 3 objectives. After each modification step of biosensor preparation, the modified ITO electrodes were characterized using AFM (AFM Plus+, Nanomagnetic Instruments) and SEM (SEM, FEI, Quanta, FEG 250 model). Thermo Scientific™ Multiskan™ FC Microplate Photometer was used as ELISA kit reader.

*Fabrication of the biosensor*

Before the utilization of ITO sheet as an electrode material, a sheet of ITO film was cleaned ultrasonically in acetone, soap solution and ultrapure water, respectively, for 10 min. To form hydroxyl ends on bare ITO sheets, they immersed a solution containing $NH_4OH$:$H_2O_2$:$H_2O$ (1:1:5) for 90 min. The activated electrodes were thoroughly cleaned with ultrapure water. After it was dried with a stream of argon gas, the ITO sheet was immersed in a tube containing 1 mM PHA overnight. A layer of self-assembled monolayer composed of phosphonic acid and carboxyl end groups were first immobilized on the surface of ITO electrode. In this step, phosphonic acid end groups of PHA monolayer were bound to hydroxyl groups of hydroxylated ITO electrode covalently and P-O bonds were formed. Thus, a monolayer containing carboxyl end groups were formed on the ITO electrode surface. After that, anti-IL8 antibodies were covalently attached to the activated -COOH groups of PHA by using EDC (4 mM)/NHS (4 mM). Then, the activated -COOH ends were blocked by BSA. Thus, an immunosensor was prepared successfully for IL8 detection.

## Results and discussion

*Design procedure of PHA based electrochemical impedance immunosensor*

The design procedure of the immunosensor fabrication is shown in Scheme 1. Firstly, the ITO sheets were cleaned by using a reported protocol mentioned above for elimination of organic materials because these contaminants may affect the results. As shown in Scheme 1, the cleaned ITO electrode was activated by dipping in basic solution containing $H_2O_2$. After being rinsed with ultrapure water, covalent attachment of phosphonic acid ends group of PHA and hydroxyl ends groups of hydroxylated ITO were performed through P-O bond formation. Thus, a SAM coverage was formed on the ITO electrode. To immobilize biorecognition elements, anti-IL8 antibodies, these carboxyl groups were activated EDC/NHS chemistry. EDC and NHS acted as a coupling agent and an activator, respectively. To prevent nonspecific interaction that could be formed between carboxylic acid groups of PHA and other proteins, BSA was utilized. Consequently, the fabricated immunosensor based on phosphonic acid interface matrix got ready for IL8 detection.

The stepwise biosensor fabrication procedure was monitored by EIS and CV, and the resulting Nyquist plots and cyclic voltammograms are illustrated in Fig. 1. The EIS data were fitted to Randles equivalent circuit (inset in Fig. 1A). This circuit composed of 4 parts: the solution resistance ($R_s$), the charge transfer resistance ($R_{ct}$), the constant phase element (CPE) and Warburg impedance ($Z_w$). $[Fe(CN)_6]^{3-/4-}$ was utilized as a redox probe for the electrode kinetics at the interface. In Nyquist diagram, the diameter of the semicircle reflects the $R_{ct}$ of redox probe conversion on the electrode surface. The $R_{ct}$ value varied in each modification step. Furthermore, the most important parameter is $R_{ct}$ and is used as the main indicator in the Faradic EIS detection. The concentration of analyte in the sample is calculated by using $R_{ct}$ values based on before and after interaction between desired analyte and functional molecule modified the electrode [19,44].

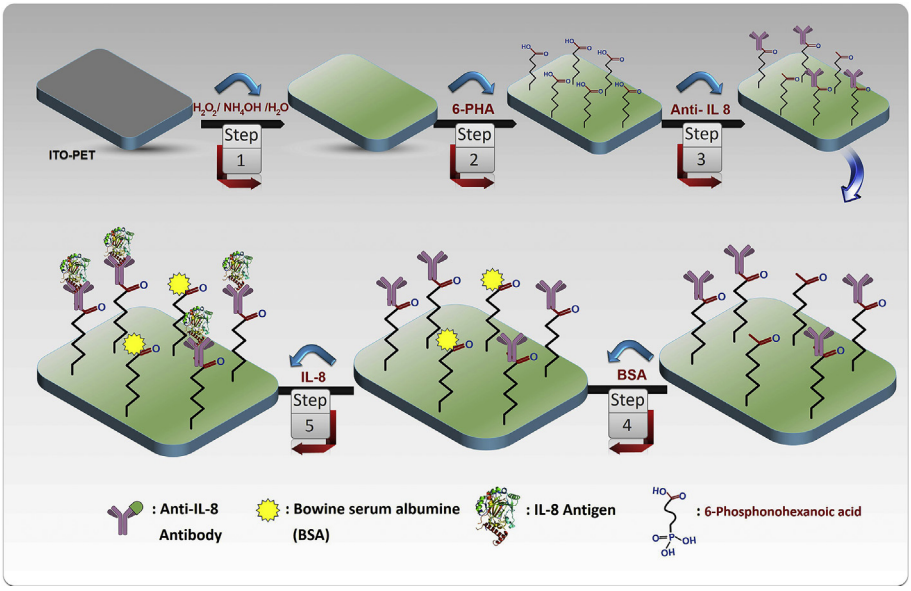
As seen in Fig. 1A, hydroxylated ITO electrode had a small $R_{ct}$ and a huge increase was seen in $R_{ct}$ after self-assembly of PHA on the hydroxylated ITO surface. This increment illustrated that dense, well-ordered SAMs were formed on ITO electrode. Moreover, PHA monolayer formation on the hydroxylated ITO sheet increased the resistance because of the strong electrostatic repulsion between the negative charge on the electrode surface and the redox probe present in the solution. After activation of carboxyl end groups of PHA modified on ITO electrode, a decrease was seen in $R_{ct}$. The cause of this decrease was the partial compensation of the negative charges of the electrode. Anti-IL8 antibodies bound the carboxyl groups of PHA via amide bond, therefore an insulative layer was formed on the ITO electrode, thus the semicircle diameter increased. Then, the free active carboxyl groups present on ITO electrode were blocked by BSA to prevent nonspecific adsorption on the electrode surface. Thus, a dramatic increase in $R_{ct}$ was monitored. After interaction between anti-IL8 antibodies with IL8 antigens, an enlarged $R_{ct}$ was found.

The modified electrode was further characterized with CV measurements. CVs of the biosensor at different steps are illustrated in Fig. 1B. After PHA SAMs formation on the hydroxylated ITO electrode, a significant decrease was seen in peak current. The activation of carboxyl of PHA increased the peak current. However, after immobilization of anti-IL8 antibody, BSA, IL8 in sequence, the peak currents decrease gradually. These results were in accordance with those of impedance results.

*FTIR and Raman studies*

The FTIR spectra of the PHA modified ITO electrode and anti-IL-8 antibody immobilized ITO electrode are illustrated in Fig. 2A and B. The peak of P-O-H between PHA and hydroxyl groups on ITO surface was observed between 2550 and 2700 $cm^{-1}$ that indicated the characteristic peak of phosphonate bond [45,46]. PHA modified ITO had the absorption peaks at 1695 $cm^{-1}$ and 1418 $cm^{-1}$ that indicated the presence of carboxyl groups (C═O bonds) [47,48]. The similar peaks are seen in Fig. 2C (Raman spectra). After immobilization of antibodies on PHA modified ITO electrode, there were the peaks observed at 1648 $cm^{-1}$ and 1546 $cm^{-1}$ originated from amide bonds in protein (Fig. 2B) [49]. The molecular structure of proteins is usually investigated by using Raman spectroscopy. Raman spectral technique has been proved to be a convenient tool to identify amide bonds. Amide I, II and III regions are monitored mostly between 1650 and 1680 $cm^{-1}$, 1440-1570 $cm^{-1}$ and 1235-1300 $cm^{-1}$, respectively [50]. As shown in Fig. 2D, amide I, amide II and amide III bonds were observed at 1662, 1449 and 1259 $cm^{-1}$, respectively.

In order to verify the PHA layer formation, energy-dispersive X-ray spectroscopy (EDX) was carried out. The experimental composition of




**Scheme 1.** Fabrication process of impedimetric immunosensor for IL-8 detection.

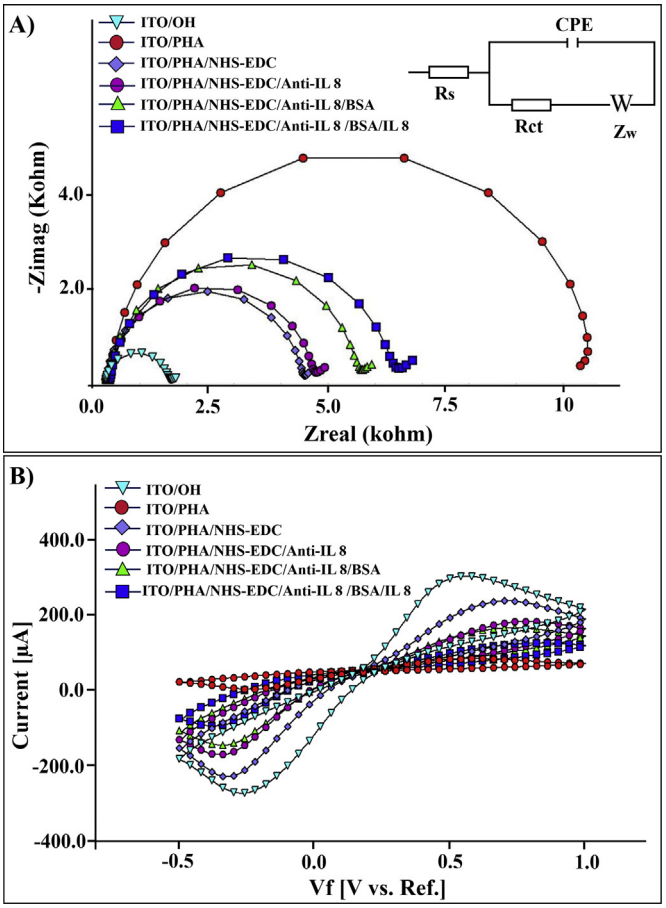



**Fig. 1.** (A) Nyquist plots and (B) cyclic voltammograms of the proposed immunosensor after each biosensor construction step. The inset in (A) is Randles equivalent circuit for EIS.

PHA modified ITO electrode was determined by analyzing the characteristic of EDX images. The SEM-EDX images of hydroxylated ITO electrode and PHA modified ITO electrode were illustrated in Fig. 2E and F, respectively. As seen in Fig. 2E, before PHA modification, indium (I), tin (Sn), oxygen (O) peaks were observed on the hydroxylated ITO electrode. While the characteristic peak of phosphorous (P) on the EDX spectrum proved the introducing of PHA layer on the ITO electrode (Fig. 2F). Additionally, the camera images of ITO electrode were compared. It is obvious that a layer of PHA film was assembled onto the ITO surface. All these results clearly confirmed the successful formation of PHA layer on ITO electrode surface.

*Morphological characterization of the proposed biosensor*

AFM and SEM were employed for the characterization of the morphology of the biosensor surface during the layer-by-layer modification. As seen from Fig. 3A and B, the PHA modified ITO electrode surface exhibited a uniform distributed layer. The root mean square (RMS) value of PHA modified ITO electrode was 1.70 nm. The anti-IL8 antibody immobilization on the PHA modified ITO electrode changed the surface morphology. Protein aggregates were clearly distributed on the PHA modified ITO electrode surface, indicating the loading of anti-IL8 antibodies (Fig. 3C and D). After anti-IL 8 antibodies immobilization the RMS value was found as 69.30 nm. The blockage of free remaining ends of PHA, caused a layer on the electrode surface (Fig. 3E and F). The interaction between anti-IL8 antibody and IL8 antigen is illustrated in Fig. 3G and H. The RMS values of these steps were 8.3 nm and 34.7 nm, respectively. Consequently, the immobilization process was performed successfully as seen in SEM and AFM images.

*Optimization studies of experimental conditions*

Optimization of the experimental parameters is important and necessary to construct a sensitive, stable, repeatable and reproducible biosensor. Different amount of PHA concentration, antibody concentration and incubation time, antigen incubation time were analyzed to obtain optimal experimental parameters.

The SAM formation on the electrode surface for immobilization of biorecognition element is important. Therefore, three different PHA concentrations (1 mM, 2.5 mM and 5 mM) were examined. The signal of 1 mM and 2.5 mM PHA concentrations were similar and higher than the signal of 5 mM PHA concentration. Because of this, 1 mM PHA concentration was chosen as optimum level. The calibration plots belonging to these PHA concentrations are illustrated in Figure SI-1A. The sensitivity of the proposed biosensor was largerly depended on the level of anti-IL-8 antibody. Therefore, three different amounts of antibody were utilized. The signal was low when 0.5 ng/mL antibody was

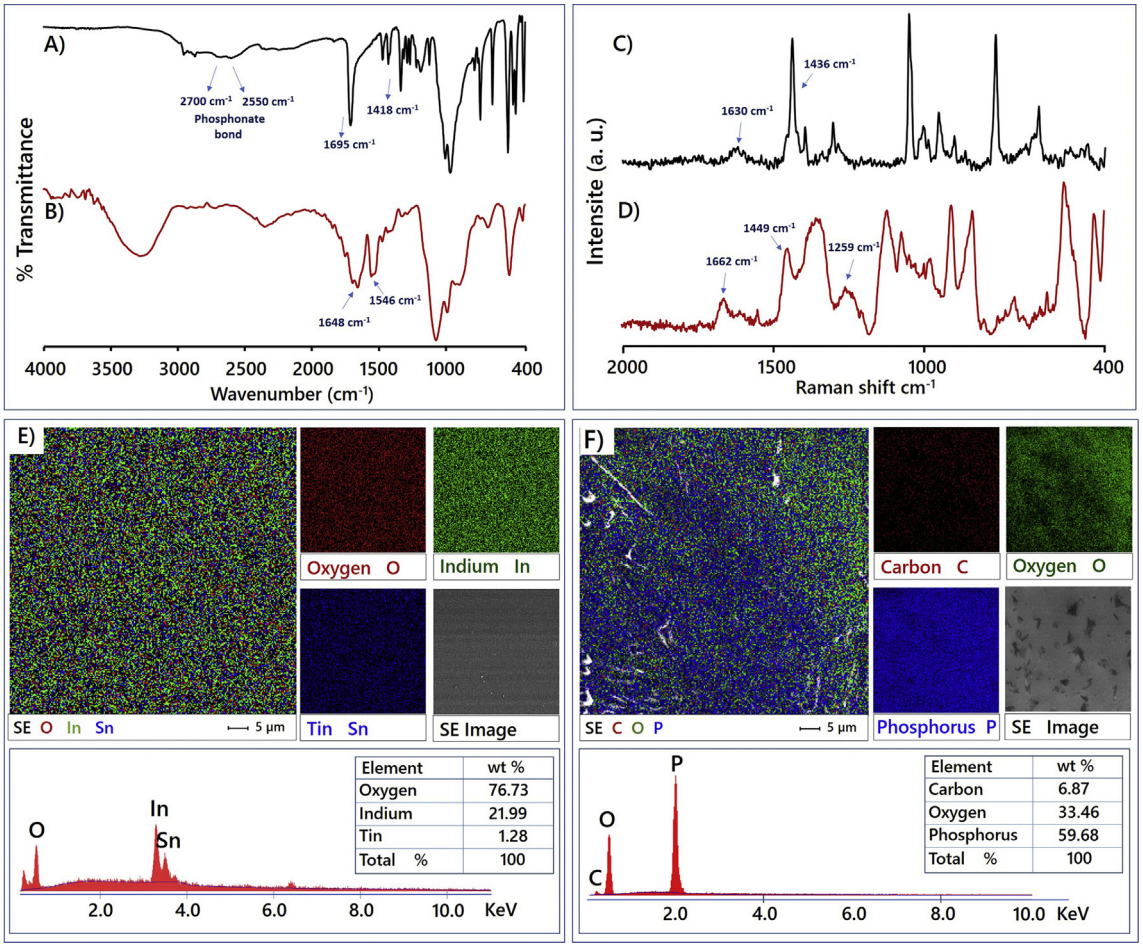



**Fig. 2.** FTIR spectra and Raman spectra of the proposed immunosensor before (A and C) and after (B and D) anti-IL-8 antibody immobilization, respectively. SEM-EDX images of before (E) and after (F) PHA modification.

utilized. In addition, the signals were the same after incubating the prepared electrodes in PBS solution containing 2.5 ng/mL and 5 ng/mL anti-IL-8 antibody concentration (Figure SI-1B). Therefore, 2.5 ng/mL antibody concentration was chosen as optimum amount. Another important optimization parameter is biorecognition element incubation time. This parameter is significant because it affects the binding efficiency of antibody. PHA modified ITO electrode was incubated in PBS solution containing antibodies for three different incubation times (30, 45, 60 min). 30 min period was not enough to bind of antibodies on ITO surface. However, the signal obtained after 45 min incubation time was the highest signal (Figure SI-1C) and 45 min incubation time was chosen as optimum incubation time. The last optimization parameter is antigen incubation time. The fabricated immunoelectrodes were incubated in PBS solution containing IL-8 antibodies in different times (30, 45 and 60 min). The short incubation time (30 min) was insufficient to bind IL-8 antigens to their antibodies. The signals of 45 min and 60 min were similar therefore of this 45 min was chosen as optimum value (Figure SI-1D).

### *Analytical features of the proposed IL8 biosensor*

Under optimum conditions, EIS measurements were performed to determine the detection limit and detection range of the proposed immunosensor by using ten fabricated immunoelectrodes incubated in various concentration of IL-8 antigen solutions. The linear detection range of IL-8 biosensor was calculated by using this equation.

$\Delta R_{ct} = R_{ct}(IL8) - R_{ct}(BSA)$

Where $R_{ct}$(IL-8) is the value of $R_{ct}$ when IL-8 antigen incubated with immobilized antibody, $R_{ct}$(BSA) is the $R_{ct}$ of immunosensor after BSA blockage.

Typical Nyquist plots and CVs of the immunosensor after incubating in different concentrations of IL8 antigen are shown in Fig. 4A and B, respectively. It is apparent that the $R_{ct}$ value illustrated a dependence upon the concentration of IL-8 antigen. As the concentration of IL8 antigens increased, the $R_{ct}$ values increased; however, the peak currents decreased. An increase in the amounts of specific recognitions hindered the redox probe transfer from the solution to the electrode surface. The calibration curve showed a wide detection range from 0.02 pg/mL to 3 pg/mL with an $R^2$ of 0.999. The regression equation is $\Delta R_{ct}$ (kΩ) = 1.0215 [IL-8] + 0.0493. Limit of detection (LOD) and limit of quantification (LOQ) were determined to be 6 fg/mL (signal to noise of three) and 19 fg/mL (signal to noise of ten), respectively. The linear detection range and LOD are proper for routine analysis in comparison with those reported in previous researches (Tables SI–1). As seen in Tables SI–1, the detection limit of our developed immunosensor is better than that of other IL-8 biosensor reported in the literature. The high sensitivity of the developed immunosensor can be attributed to the well-ordered SAM layer formation.

In this study, the interaction between anti-IL 8 antibody and IL-8 antigen was monitored by using SFI technique. This technique proved the coupling of antigens to their antibodies. Firstly, immunoelectrodes were prepared until immunoreaction step and this immunoreaction was monitored by using SFI technique. SFI measurement performed in PBS solution including IL 8 antigen. In SFI measurements 31 Hz was chosen as the frequency value from Bode Plot (Figure SI-2). As seen in Fig. 4D,

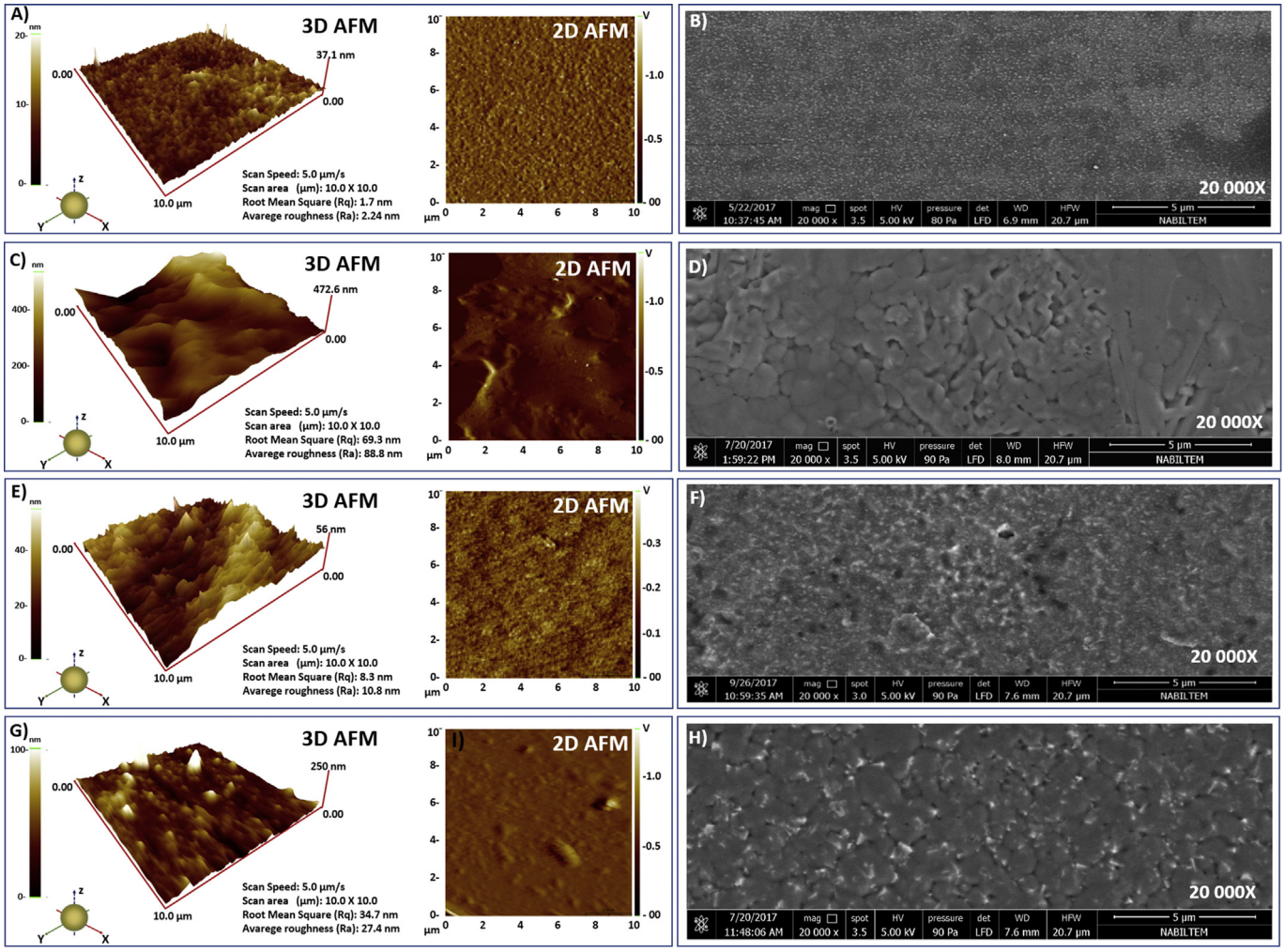



**Fig. 3.** SEM and AFM images of the proposed immunosensor after each biosensor construction step (A and B) ITO/PHA, (C and D) ITO/PHA/anti-IL-8 antibody, (E and F) ITO/PHA/anti-IL-8 antibody/BSA, (G and H) ITO/PHA/anti-IL-8 antibody/BSA/IL-8.

an increase was seen in impedance value (pink). This increase illustrated that the antibody-antigen specific interactions.

The repeatability of proposed immunosensor was performed by recording impedance signal of independent modified electrodes at same concentration of IL-8 (0.5 pg/mL). Relative standard deviation (RSD%) for twenty immunosensor, which were fabricated at the same conditions was 4.49%. The low RSD value showed that the repeatability of proposed immunosensor for IL-8 detection was suitable. The reproducibility of the proposed immunosensor was examined by fabricating 10 biosensors prepared under same conditions. The RSD (%) for ten immunosensor was 5.20% and this low RSD (%) illustrated the reproducibility of immunosensor (Fig. 5A).

The storage stability of the immunosensor was investigated by storing the immunoelectrodes in dry form at 4 °C for 10 weeks. The IL-8 biosensor remained almost 71% of its impedance signal suggesting an acceptable stability that required for practical applications after 7 weeks. This high stability can be originated from the prepared uniform platform and covalent attachment of the anti-IL8 antibodies on the modified electrode surface (Fig. 5B).

Reusability is a significant criteria to decrease the cost of immunosensor. The basic of this test is based on the testing the robustness of the biosensor. In this test, firstly ITO electrodes were exposed to drastic conditions (alkaline or acidic) and then the signal measured before and after exposure. As seen in Fig. 5C, we attained good responses during 3 cycles and after 5 cycles, the surface got damaged.

The feasibility of the immunosensor for analytical applications was examined by measuring IL8 in human serum and saliva. For the measurement of IL-8 concentration by proposed immunosensor in saliva and serum samples, these samples were diluted 200 and 50 fold, respectively. Furthermore, the same serum and saliva samples were tested by using ELISA kit. As seen in Tables SI–1B, the results obtained from both methods were compared and both methods were in good agreement for the determination of IL-8 in complex samples. When the analysis cost of biosensor and ELISA kit were compared, ELISA had higher cost (500 $) than biosensor construction (1 $) costs. Furthermore, the regeneration ability of biosensor decreases the analysis cost; therefore, biosensor will replace the ELISA kit analysis. Furthermore, the preparation procedure of ELISA kit was very long and the procedure included tiring operation steps.

Apart from real samples analysis, selectivity test was performed by using interference materials such as different biomarkers (vascular endothelial factor, receptor activated kinase C1, interleukin 1β, interleukin 1α at a relatively high concentration (100 ng/mL)) and drugs (aspirin, amoxicillin, ampicillin, erythromycin at a relatively high concentration (100 ng/mL)). They were added PBS solution containing IL-8 antigen (0.5 pg/mL) and without IL-8 antigen. As seen in figure SI-3 and figure SI-4, the interference materials did not affect the immunosensor response. These results demonstrated the high selectivity of the developed immunosensing strategy.

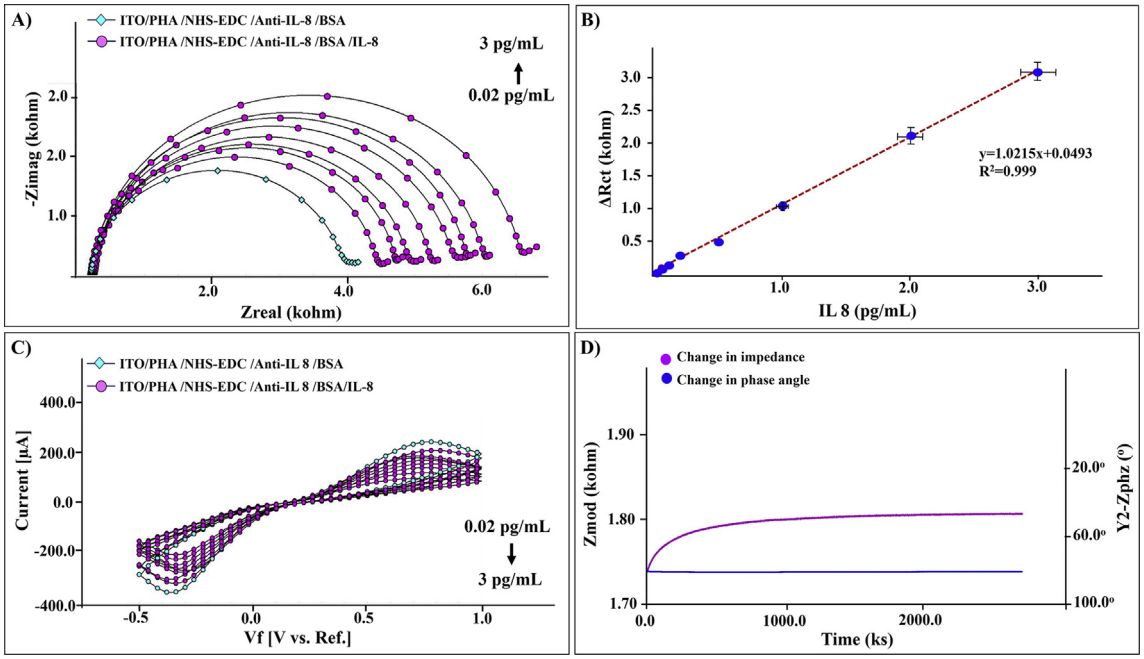



**Fig. 4.** (A) Impedance measurement of different concentrations (0.02–3 pg/mL) of IL-8, (B) Cyclic voltammograms of different concentrations (0.02–3 pg/mL) of IL-8, (C) Calibration curve obtained by fitting the results to Randles equivalent circuit, (D) Single Frequency Impedance result of anti-IL-8 antibody and IL-8 antigen interaction.

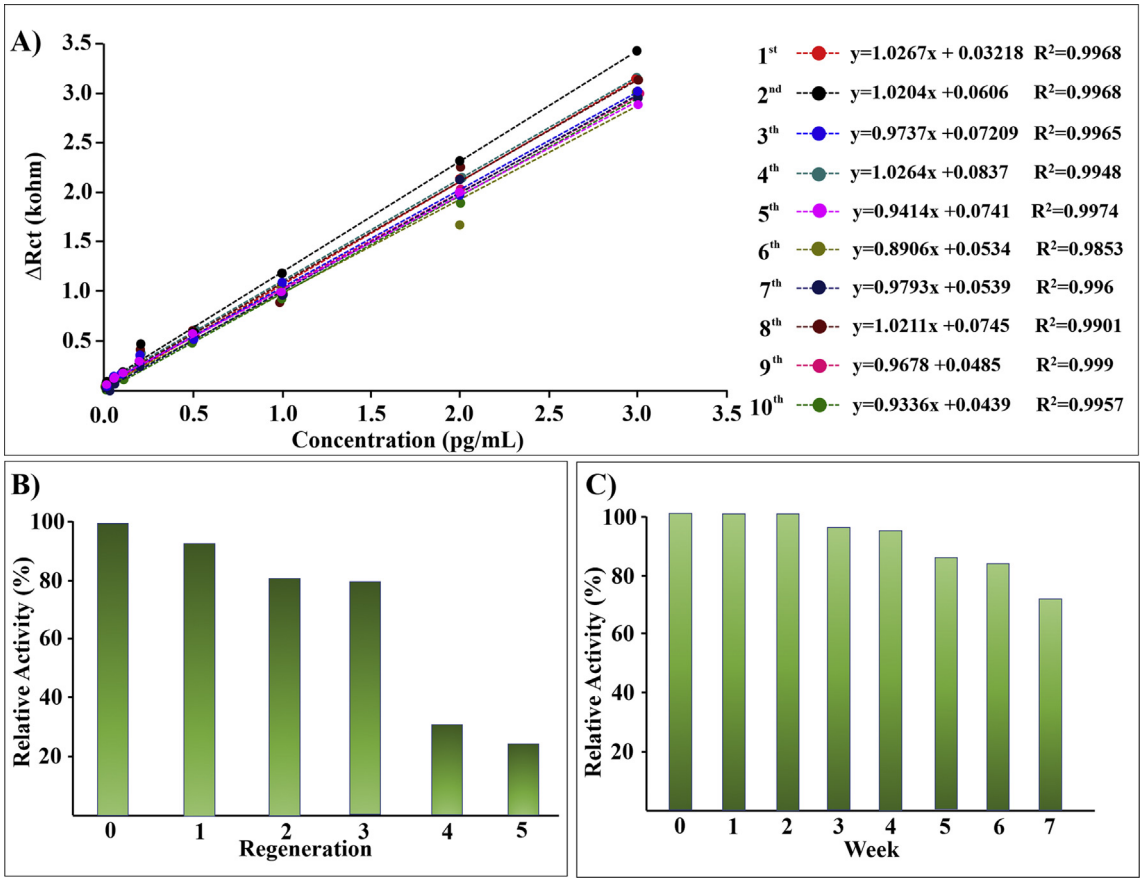



**Fig. 5.** Results of reproducibility (A), reusability (B) and storage stability (C).

## Conclusion

A new and practical immunosensor for IL-8 was developed with the utilization of PHA as interface material for the first time. The usage of PHA possess the advantages of forming a dense monolayer on metal oxide surface and has carboxyl groups on its end groups to bind the biorecognition elements. Electrochemical characterization was performed by using EIS and CV techniques, but the quantification of IL8 antigen was performed EIS technique. The interaction between anti-IL8 antibody and IL8 antigen was monitored by using SFI technique. Furthermore, the step by step electrode modification was observed by using SEM and AFM imaging. The binding of phosphonate groups on the hydroxylated ITO electrode was monitored by using SEM-EDX technique. The immobilization of anti-IL 8 antibodies was proved by FTIR and Raman measurements. The fabricated IL-8 biosensor exhibited wide linear detection range (0.02–3 pg/mL), low detection limit (6 fg/mL) and good stability (7 weeks). Moreover, satisfactory results were obtained after analyzing the real human serum and saliva samples. In a word, the developed IL8 immunosensor had proven to be a promising alternative method for determination cancer biomarkers in clinical analysis.

## Acknowledgment

The financial support from TÜBİTAK (The Scientific and Technological Research Council of Turkey, Project number: 113 Z 678) is gratefully acknowledged.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ab.2018.05.030.

## References

[1] K. Matsushima, J.J. Oppenheim, Interleukin 8 and MCAF: novel inflammatory cytokines inducible by IL 1 and TNF, Cytokine 1 (1989) 2–13.
[2] Q. Sun, F. Sun, B. Wang, S. Liu, W. Niu, E. Liu, C. Peng, J. Wang, H. Gao, B. Liang, Interleukin-8 promotes cell migration through integrin αvβ6 upregulation in colorectal cancer, Canc. Lett. 354 (2014) 245–253.
[3] N. Mukaida, A. Harada, K. Yasumoto, K. Matsushima, Properties of pro-inflammatory cell type-specific leukocyte chemotactic cytokines, interleukin 8 (IL-8) and monocyte chemotactic and activating factor (MCAF), Microbiol. Immunol. 36 (1992) 773–789.
[4] M. Braiek, Y. Yang, C. Farre, C. Chaix, F. Bessueille, A. Baraket, A. Errachid, A. Zhang, N. Jaffrezic-Renault, Boron-doped diamond electrodes modified with Fe3O4@ Au magnetic nanocomposites as sensitive platform for detection of a cancer biomarker, Interleukin-8, Electroanalysis 28 (2016) 1810–1816.
[5] K. Rajkumar, G. Nandhini, R. Ramya, P. Rajashree, A.R. Kumar, S.N. Anandan, Validation of the diagnostic utility of salivary interleukin 8 in the differentiation of potentially malignant oral lesions and oral squamous cell carcinoma in a region with high endemicity, Oral Med. Oral Surg. Oral Pathol. Oral Radiol. 118 (2014) 309–319.
[6] K. Xie, Interleukin-8 and human cancer biology, Cytokine Growth Factor Rev. 12 (2001) 375–391.
[7] P. Zarogoulidis, F. Katsikogianni, T. Tsiouda, A. Sakkas, N. Katsikogiannis, K. Zarogoulidis, Interleukin-8 and interleukin-17 for cancer, Canc. Invest. 32 (2014) 197–205.
[8] I.M. Velásquez, P. Frumento, K. Johansson, A. Berglund, U. de Faire, K. Leander, B. Gigante, Association of interleukin 8 with myocardial infarction: results from the stockholm heart epidemiology program, Int. J. Cardiol. 172 (2014) 173–178.
[9] D.T. Wong, Towards a simple, saliva-based test for the detection of oral cancer. 'Oral fluid (saliva), which is the mirror of the body, is a perfect medium to be explored for health and disease surveillance.', Expert Review of Molecular Diagnostics 6 (2006) 267–272.
[10] R. Torrente-Rodríguez, S. Campuzano, V.R.-V. Montiel, M. Gamella, J. Pingarrón, Electrochemical bioplatforms for the simultaneous determination of interleukin (IL)-8 mRNA and IL-8 protein oral cancer biomarkers in raw saliva, Biosens. Bioelectron. 77 (2016) 543–548.
[11] T. Dong, N.M.M. Pires, Immunodetection of salivary biomarkers by an optical microfluidic biosensor with polyethylenimine-modified polythiophene-C 70 organic photodetectors, Biosens. Bioelectron. 94 (2017) 321–327.
[12] R. Sharma, S.E. Deacon, D. Nowak, S. George, M. Szymonik, A. Tang, D. Tomlinson, A. Davies, M. McPherson, C. Wälti, Label-free electrochemical impedance biosensor to detect human interleukin-8 in serum with sub-pg/ml sensitivity, Biosens. Bioelectron. 80 (2016) 607–613.
[13] Y. Wan, W. Deng, Y. Su, X. Zhu, C. Peng, H. Hu, H. Peng, S. Song, C. Fan, Carbon nanotube-based ultrasensitive multiplexing electrochemical immunosensor for cancer biomarkers, Biosens. Bioelectron. 30 (2011) 93–99.
[14] S. Campuzano, P. Yánez-Sedeño, J.M. Pingarrón, Electrochemical bioaffinity sensors for salivary biomarkers detection, Trac. Trends Anal. Chem. 86 (2017) 14–24.
[15] E.B. Bahadır, M.K. Sezgintürk, Applications of electrochemical immunosensors for early clinical diagnostics, Talanta 132 (2015) 162–174.
[16] S.K. Vashist, E. Lam, S. Hrapovic, K.B. Male, J.H. Luong, Immobilization of antibodies and enzymes on 3-aminopropyltriethoxysilane-functionalized bioanalytical platforms for biosensors and diagnostics, Chem. Rev. 114 (2014) 11083–11130.
[17] E.B. Aydın, M.K. Sezgintürk, Indium Tin Oxide (ITO): a promising material in biosensing technology, Trac. Trends Anal. Chem. (2017).
[18] X. Li, Z. Zheng, X. Liu, S. Zhao, S. Liu, Nanostructured photoelectrochemical biosensor for highly sensitive detection of organophosphorous pesticides, Biosens. Bioelectron. 64 (2015) 1–5.
[19] E. Bahadır, M. Sezgintürk, Label-free, ITO-based immunosensor for the detection of a cancer biomarker: receptor for Activated C Kinase 1, Analyst 141 (2016) 5618–5626.
[20] J. Narang, C. Singhal, N. Malhotra, S. Narang, A.K.P.N.R. Gupta, R. Kansal, C. Pundir, Impedimetric genosensor for ultratrace detection of hepatitis B virus DNA in patient samples assisted by zeolites and MWCNT nano-composites, Biosens. Bioelectron. 86 (2016) 566–574.
[21] N. Prabhakar, Z. Matharu, B. Malhotra, Polyaniline Langmuir–Blodgett film based aptasensor for ochratoxin A detection, Biosens. Bioelectron. 26 (2011) 4006–4011.
[22] W.A. El-Said, J.-W. Choi, Electrochemical Biosensor consisted of conducting polymer layer on gold nanodots patterned Indium Tin Oxide electrode for rapid and simultaneous determination of purine bases, Electrochim. Acta 123 (2014) 51–57.
[23] A. Gündoğdu, E.B. Aydın, M.K. Sezgintürk, A novel electrochemical immunosensor based on ITO modified by carboxyl-ended silane agent for ultrasensitive detection of MAGE-1 in human serum, Anal. Biochem. (2017).
[24] E.B. Aydın, M.K. Sezgintürk, A sensitive and disposable electrochemical immunosensor for detection of SOX2, a biomarker of cancer, Talanta 172 (2017) 162–170.
[25] M.B. dos Santos, S. Azevedo, J. Agusil, B. Prieto-Simon, C. Sporer, E. Torrents, A. Juárez, V. Teixeira, J. Samitier, Label-free ITO-based immunosensor for the detection of very low concentrations of pathogenic bacteria, Bioelectrochemistry 101 (2015) 146–152.
[26] H. Zeng, D.A.Y. Agyapong, C. Li, R. Zhao, H. Yang, C. Wu, Y. Jiang, Y. Liu, A carcinoembryonic antigen optoelectronic immunosensor based on thiol-derivative-nanogold labeled anti-CEA antibody nanomaterial and gold modified ITO, Sensor. Actuator. B Chem. 221 (2015) 22–27.
[27] S. Kumar, S. Kumar, S. Tiwari, S. Augustine, S. Srivastava, B.K. Yadav, B.D. Malhotra, Highly sensitive protein functionalized nanostructured hafnium oxide based biosensing platform for non-invasive oral cancer detection, Sensor. Actuator. B Chem. 235 (2016) 1–10.
[28] I. Tiwari, M. Singh, C.M. Pandey, G. Sumana, Electrochemical genosensor based on graphene oxide modified iron oxide–chitosan hybrid nanocomposite for pathogen detection, Sensor. Actuator. B Chem. 206 (2015) 276–283.
[29] A. Singh, M. Choudhary, M. Singh, H. Verma, S.P. Singh, K. Arora, DNA functionalized direct electro-deposited gold nanoaggregates for efficient detection of Salmonella typhi, Bioelectrochemistry 105 (2015) 7–15.
[30] A.K. Yagati, J.-C. Pyun, J. Min, S. Cho, Label-free and direct detection of C-reactive protein using reduced graphene oxide-nanoparticle hybrid impedimetric sensor, Bioelectrochemistry 107 (2016) 37–44.
[31] F. Mastrangelo, G. Fioravanti, R. Quaresima, R. Vinci, E. Gherlone, Self-assembled monolayers (SAMs): which perspectives in implant dentistry? J. Biomaterials Nanobiotechnol. 2 (2011) 533.
[32] R. Quiñones, A. Raman, E.S. Gawalt, Functionalization of nickel oxide using alkylphosphonic acid self-assembled monolayers, Thin Solid Films 516 (2008) 8774–8781.
[33] R.V. Jagadeesh, V. Lakshminarayanan, Adsorption kinetics of phosphonic acids and proteins on functionalized Indium tin oxide surfaces using electrochemical impedance spectroscopy, Electrochim. Acta 197 (2016) 1–9.
[34] J. Muñoz, R. Montes, M. Baeza, Trends in Electrochemical Impedance Spectroscopy involving nanocomposite transducers: characterization, architecture surface and bio-sensing, Trac. Trends Anal. Chem. (2017).
[35] E.B. Aydın, M. Aydın, M.K. Sezgintürk, Highly sensitive electrochemical immunosensor based on polythiophene polymer with densely populated carboxyl groups as immobilization matrix for detection of interleukin 1β in human serum and saliva, Sensor. Actuator. B Chem. 270 (2018) 18–27.
[36] M. Aydın, E.B. Aydın, M.K. Sezgintürk, A disposable immunosensor using ITO based electrode modified by a star-shaped polymer for analysis of tumor suppressor protein p53 in human serum, Biosens. Bioelectron. 107 (2018) 1–9.
[37] X.Y. Zhang, L.Y. Zhou, H.Q. Luo, N.B. Li, A sensitive and label-free impedimetric biosensor based on an adjunct probe, Anal. Chim. Acta 776 (2013) 11–16.
[38] M. Labib, S. Martić, P.O. Shipman, H.-B. Kraatz, Electrochemical analysis of HIV-1 reverse transcriptase serum level: exploiting protein binding to a functionalized nanostructured surface, Talanta 85 (2011) 770–778.
[39] A.S. Bandarenka, Exploring the interfaces between metal electrodes and aqueous electrolytes with electrochemical impedance spectroscopy, Analyst 138 (2013) 5540–5554.
[40] M. Labib, P.O. Shipman, S. Martić, H.-B. Kraatz, Towards an early diagnosis of HIV infection: an electrochemical approach for detection ofHIV-1 reverse transcriptase enzyme, Analyst 136 (2011) 708–715.
[41] M. Behpour, S.M. Ghoreishi, E. Honarmand, M. Salavati-Niasari, Comparative

electrochemical study of new self-assembled monolayers of 2-{[(Z)-1-(3-furyl) methylidene] amino}-1-benzenethiol and 2-{[(2-sulfanylphenyl) imino] methyl} phenol for determination of dopamine in the presence of high concentration of ascorbic acid and uric acid, Analyst 136 (2011) 1979–1986.
[42] S. Singal, R.K. Kotnala, Single frequency impedance analysis on reduced graphene oxide screen-printed electrode for biomolecular detection, Appl. Biochem. Biotechnol. (2017) 1–12.
[43] E.B. Aydın, M. Aydın, M.K. Sezgintürk, A highly sensitive immunosensor based on ITO thin films covered by a new semi-conductive conjugated polymer for the determination of TNFα in human saliva and serum samples, Biosens. Bioelectron. 97 (2017) 169–176.
[44] L. Zhang, C. Yu, R. Gao, Y. Niu, Y. Li, J. Chen, J. He, An impedimetric biosensor for the diagnosis of renal cell carcinoma based on the interaction between 3-aminophenyl boronic acid and sialic acid, Biosens. Bioelectron. 92 (2017) 434–441.
[45] L. Daasch, D. Smith, Infrared spectra of phosphorus compounds, Anal. Chem. 23 (1951) 853–868.
[46] R. Luschtinetz, G. Seifert, E. Jaehne, H.J.P. Adler, Infrared Spectra of Alkylphosphonic Acid Bound to Aluminium Surfaces, Macromolecular Symposia, Wiley Online Library, (2007), pp. 248–253.
[47] S.K. Arya, A.K. Prusty, S. Singh, P.R. Solanki, M.K. Pandey, M. Datta, B.D. Malhotra, Cholesterol biosensor based on N-(2-aminoethyl)-3-aminopropyl-trimethoxysilane self-assembled monolayer, Anal. Biochem. 363 (2007) 210–218.
[48] K. Khaldi, S. Sam, A. Gouget-Laemmel, C. Henry de Villeneuve, A. Moraillon, F. Ozanam, J. Yang, A. Kermad, N. Ghellai, N. Gabouze, Active acetylcholinesterase immobilization on a functionalized silicon surface, Langmuir 31 (2015) 8421–8428.
[49] J. Kong, S. Yu, Fourier transform infrared spectroscopic analysis of protein secondary structures, Acta Biochim. Biophys. Sin. 39 (2007) 549–559.
[50] T. Kitagawa, S. Hirota, Raman Spectroscopy of Proteins, Handbook of Vibrational Spectroscopy, (2002).