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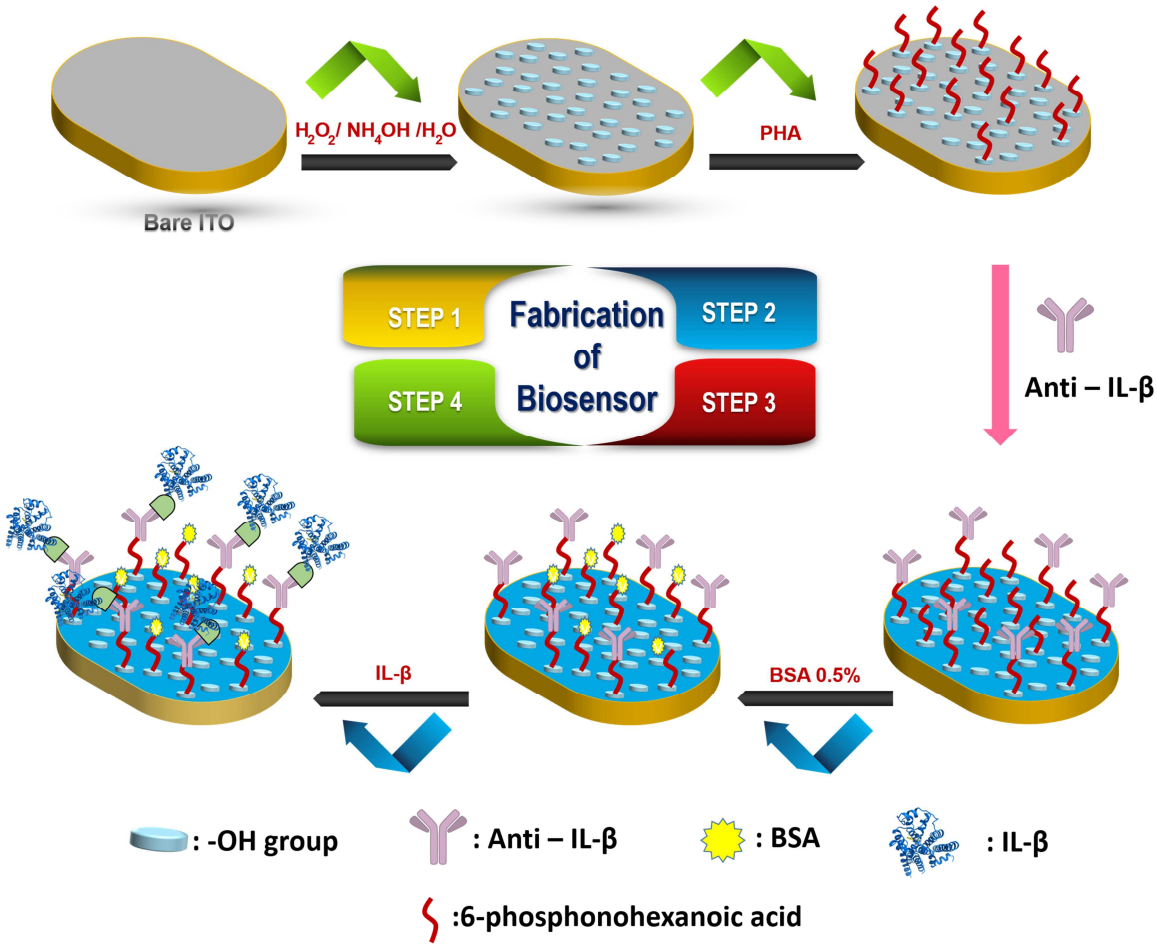
Bare ITO
H_2O_2/ NH_4OH /H_2O
PHA
STEP 1
STEP 2
STEP 3
STEP 4
Fabrication of Biosensor
Anti – IL-β
IL-β
BSA 0.5%
: -OH group
: Anti – IL-β
: BSA
: IL-β
:6-phosphonohexanoic acid

A disposable and ultrasensitive ITO based biosensor modified by 6-phosphonohexanoic acid for electrochemical sensing of IL-1β in human serum and saliva

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Abstract

In this study, we constructed a new and sensitive ITO based electrochemical immunosensor for detection of interleukin 1β (IL-1β), a cancer biomarker found in serum and saliva. 6-phosphonohexanoic acid (PHA) was used as a biomolecule immobilization matrix for the first time. Anti-IL-1β antibody was utilized as a biorecognition molecule that immobilized onto carboxyl groups of 6-phosphohexanoic acid (PHA) via amide bond. Selective interaction between anti- IL-1β antibodies and IL-1β antigens was investigated by Electrochemical Impedance Spectroscopy (EIS), Cyclic Voltammetry (CV) and Single Frequency Impedance (SFI) methods. The surface characterization of the immunosensor was performed by fourier transform infrared spectroscopy (FTIR), raman spectroscopy, scanning electron microscopy (SEM), SEM-energy dispersive X-ray spectroscopy (EDX) and atomic force microscopy (AFM) in order to illustrate individual steps of biosensor construction. Under the optimized experimental conditions, the change in impedance was proportional to IL-1β concentrations in the range of 0.025-3 pg/mL (R^2=0.99) with detection limit of 7.5 fg/mL. The reproducibility, repeatability, stability, and specificity of the developed immunosensor were analyzed. In addition, the developed immunosensor was successfully utilized for the determination of IL-1β in serum and saliva samples by using the standard addition method with recoveries of 96.7-105.4%. This immunosensor was applicable for the requirements of routine analysis with respect to performance, functionality and cost.

Keywords: 6-phosphonohexanoic acid, interlekin-1β, indium tin oxide, electrochemical impedance spectroscopy, single frequency impedance

1. Introduction

Biomarkers are unique molecular signatures of certain diseases such as different types of cancer and skin melanoma. They are secreted in body fluids and also present in human blood and saliva [1, 2]. Saliva is an aqueous biofluid and comprised of a wide range of analytes from organic species (protein, DNA, mRNA) to inorganic species (Na^+, Cl^-, Ca^{2+}, HCO^{3-}, Mg^{2+}, NH_3). The accurate determination of these biomarkers is important in order to diagnose the diseases [3, 4]. Therefore, ultrasensitive methods should develop for determination of biomarkers. The utilization of saliva for disease diagnostic has gained attention due to being safe to handle, easy to collect and store, being a non-invasive method [5]. Also, saliva is one of the most important tool for diagnosis of different types of cancer such as oral [4, 6], breast [7], lung [7] and head [3, 4].

Interleukin-1β (IL-1β) is a key inflammatory cytokine secreted upon infection, cellular injury, or antigenic challenge. This cytokine directly acts on several cell types and induces on inflammatory state [1, 8]. The largest producers of IL-1β are macrophages; and apart from macrophages IL-1β is produced by epidermal tissue, mucosa epithelial cells, acinar and ductal cells of the salivary glands [9, 10]. IL-1β is a biomarker in the human lung, colon, breast, oral carcinoma and skin melanomas [9, 11]. The IL-1β concentration in biological fluids is very low. In healthy population, IL-1β concentration is ~212.8 pg/mL and <10 pg/mL in serum and saliva, respectively. Clinically relevant ranges are ~753.7 pg/mL and >10 pg/mL in serum and saliva, respectively [12]. For the determination of the low IL-1β concentration, sensitive analytical methods are required; therefore, ELISA kits are usually found as commercial. Apart from ELISA kits, biosensors have been developed for IL-1β detection. However, there are a limited number of biosensors for IL-1β detection in literature. The reported biosensors in literature and commercial ELISA kits are summarized in Table SI-1A. The cost of commercial ELISA kit (320 $) is higher than biosensor, and the measurement protocol is very long [13]. Therefore, biosensors are attractive tools for IL-1β determination. For the sensitive detection of IL-1β, different biosensors with different modification strategies and several detection techniques have been utilized. Krause et al. (2015) developed a sensitive microfluidic array amplified with magnetic beads and gold nanoparticles (AuNPs) for IL-1β and other cancer biomarkers detection to investigate mucositis risk in cancer patients [14]. A fiber-optic particle plasmon resonance sensor was fabricated by Chiang et al., and the detection principle was based on molecular binding of IL-1β to anti-IL-1β conjugated AuNPs. The linear range and detection limit of this biosensor were 0.05-10 ng/mL and 21 pg/mL, respectively [15]. Baraket et al. (2017) developed a biosensor based on eight gold microelectrodes as working electrode placed onto silicon substrates. The linear range and detection limit of this biosensor were 1-15 pg/mL and 0.3 pg/mL, respectively [16]. In sum, the early

determination of cancer biomarkers is significant in identification and treatment of diseases; therefore, we aimed to develop a more ultrasensitive biosensor than the reported biosensors in literature.

Immunosensors based on high specific biorecognition of antigens by antibodies is the basic analytical technique for selective and sensitive determination of cancer biomarkers. In the determination of cancer biomarkers, various types of immunosensors such as electrochemical, optic and piezoelectric have been developed [17]. In recent years, electrochemical immunosensors have been popular for determination of different analytes such as metal ions, cancer biomarkers, pesticides, and aflatoxins due to their excellent features such as convenience, low cost, fast response, ease-of-use and ultra-high sensitivity [18]. Electrochemical immunosensors modified by using different strategies with different types of electrodes such as metal electrodes, carbon/graphite electrodes, metal oxide electrodes provide more advantages than traditional techniques, including high selectivity, sensitivity, and reliability, and usability in on-line applications [19].

Electrochemical impedance spectroscopy (EIS) is a successful and an informative technique for monitoring of electrochemical features of biological interfaces [20-22]. Impedimetric biosensors measure impedance that includes resistance and capacitance. As a result of biochemical interactions, a small amplitude voltage signal is obtained as a function of frequency. The basic construction procedure of an impedimetric immunosensors is based on the direct immobilization of antibodies onto the electrode surface. This procedure prevents the labelling process that is normally required by other electrochemical biosensors and shortens the detection time [23, 24].

Single Frequency Impedance (SFI) is an alternative technique for monitoring of molecular interactions formed on the electrode surface. The basic principle of this technique is based on impedance measurements at a fixed frequency, and the changes in kinetic parameters are followed during the experiment. Furthermore, this technique is able to monitor total impedance in a single frequency versus time. An increase in impedance value is observed when antibodies interact with their antigens [20, 25-27].

ITO material has attracted attention in recent years due to their excellent properties such as low cost, good optical transparency, good electrical conductivity, wide electrochemical working window, good substrate adhesion, stable electrochemical and physical properties [28]. Several studies have been performed for the fabrication electrochemical [29-31] and optical sensors [32-34] based on ITO materials as working electrode. In a typical arrangement, a biorecognition element is immobilized on the modified ITO electrode surface. Interface matrix is important in detection of analyte because the biorecognition events occur at interfaces [35]. Different methods such as electrophoretic deposition [36, 37], adsorption [38], silanization [39, 40] and electropolymerization [41-43] have been used for modification of ITO electrodes. Self-assembled monolayer (SAM) formation on the ITO electrode is a remarkable technique due to simple preparation, good reproducibility, high stability, and it has been

used in a lot of studies [44, 45]. Until now, silanization technique has been used for ITO based immunosensor development. The basic principle of silanization technique is based on self-assembling of silane groups on hydroxyl terminated surface. The silane based chemicals are densely packed on ITO surface, and they act as a crosslinker when their Si-O bonds react with surface hydroxyl groups of ITO electrode [44, 46]. Apart from silane groups of chemicals, phosphonic acid groups of chemicals can be linked to hydroxyl group of activated ITO electrode. However, studies based on phosphonic acid modified biosensors are not found in literature. Phosphonic acids have gained interest because they produce SAMs on a range of metal oxide surfaces than silanes and thiols [47]. 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 [45]. They undergo chemisorption through hetero-condensation by reacting with hydroxyl groups on metal oxide surface. The SAM of phosphonates supplies a platform for immobilization of biorecognition elements [48]. Therefore, in this study, we utilized 6-phosphohexanoic acid (PHA) to construct the immobilization matrix for biorecognition element, and we investigated the immobilization of IL-1β antibodies on the PHA modified ITO electrode surface for the first time. Therefore, this study will prove the usability of PHA as immobilization matrix in biosensor technology.

In this work, we developed a novel sensitive IL-1β immunosensor based on the specific interaction between anti-IL-1β antibody and IL-1β antigen on the PHA modified ITO electrode. For this purpose, firstly, ITO electrode modified with PHA and PHA monolayer was formed on the ITO electrode surface. This formed monolayer was utilized as an immobilization matrix. Then, carboxyl groups on 6-phosphohexanoic acid modified electrode were activated by using EDC/NHS chemistry. The activated carboxyl groups were reacted with the amino groups of antibodies, and antibodies were covalently immobilized to the sensing surface. The residual active carboxyl groups were blocked by using bovine serum albumin (BSA). Finally, the proposed ITO electrode was ready to use as an analytical tool for IL-1β antigen detection. The electrochemical characterization of the proposed immunosensor was performed by using EIS and cyclic voltammetry (CV) methods. The immobilization of biorecognition elements (anti-IL-1β antibodies) on the PHA modified surface was confirmed scanning electron microscopy (SEM), atomic force microscopy (AFM), fourier transform infrared spectroscopy (FTIR) and Raman Spectroscopy. The applicability of the proposed IL-1β immunosensor in real samples such as spiked human serum and saliva was also studied. As a result, the new biosensor was suitable for the impedimetric determination of IL-1β in saliva and serum samples.

2. Experimental

2.1. Reagents and materials

Anti-IL-1β antibody and IL-1β proteins and bovine serum albumin (BSA) were obtained from Sigma-Aldrich. 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 x 20 mm, 60 Ω/cm^2) were obtained from Sigma-Aldrich. Other utilized chemicals were of analytical grade. $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$, phosphate buffer solution (PBS, pH 7.4) were prepared by using ultrapure water (specific resistance of 18.2 MΩ cm). Antibody, antigen, and BSA solutions were prepared by using PBS and stored -20 ^oC until use. The real serum samples were obtained by research ethics committee approval with the number of 2013/86/07/05 from Namık Kemal University, Faculty of Medicine. Saliva samples were collected from the volunteers in NKU Research Lab.

2.2. **Apparatus**

Electrochemical measurements of modified electrodes were carried out on Gamry Potentiostat, Galvanostat (Reference 1000, Gamry Instruments, USA) by using a conventional three electrode system with a modified ITO sheet as a working electrode, a platinium wire as a counter electrode, and Ag/AgCl electrode as a reference electrode. The reference and counter electrode were acquired from BASi (West Lafayette, IN, USA). The EIS measurements were carried out in ferri-ferro solution that prepared by using PBS, 5 mM ferricyanide and 5 mM ferrocyanide.

Scanning electron microscope (SEM, FEI, Quanta FEG 250 Model) and atomic force microscope (AFM, AFM PLUS+, Nanomagnetic Instruments) were used to evaluate the surface modifications. To monitor surface end groups, FTIR (Bruker Vertex 70 ATR Model) and Raman (Thermo DXR Raman spectrometer equipped with a 780 nm excitation layer and a confocal microscope) spectroscopy were utilized.

2.3. Procedures

2.3.1 Impedimetric immunosensor manufacturing

Before modification, ITO sheets were cleaned by sonication in acetone, soap solution and ultra-pure water for 10 min. Following that, the cleaned ITO sheets were incubated into a solution of $H_2O_2/NH_4OH/H_2O$ (1:1:5) for 90 min at room temperature. Then, the electrodes were rinsed with ultra-pure water and dried under argon gas. Afterwards, the hydroxylated ITO sheets were immersed into PHA solution (1 mM) prepared by a mixed solution of dimethyl sulfoxide (DMSO) and H_2O (1:1) overnight at room temperature, allowing the self-assembly of PHA onto the hydroxylated ITO via P-O bond. The ITO sheets were thoroughly washed with ultra-pure water to remove the loosely bound phosphonic acid molecules; then, the carboxyl groups of PHA formed on ITO electrode were activated by incubating in a solution of 4 mM EDC and 4 mM NHS in PBS buffer for 1 hour. After being rinsed with ultra-pure water, the activated ITO electrodes were immersed in PBS solution containing IL-1β

antibodies for 45 min. Finally, to minimize the non-specific interaction, the electrodes were immersed in BSA solution for 1 h at room temperature to block free carboxyl ends. After being washed with ultrapure water, the proposed immunosensor (designated as ITO/PHA/NHS/EDC/anti-IL-1β/BSA) was ready for detection of IL-1β antigen.

2.3.2 Electrochemical Measurement

EIS was used to investigate the immobilization process and to monitor the interaction between anti-IL-1β antibody and IL-1β antigen. The impedimetric feature of the resulting biosensor was investigated in 50 mM pH 7.4 PBS containing 5 mM $Fe(CN)_6^{4-/3-}$ and 1 M KCl, and monitored within a frequency range of 0.05-50000 Hz. After immobilization of each step, Nyquist plot was recorded to monitor biosensor behavior. The Nyquist plot was used to evaluate the impedance results. CV measurements were also utilized to characterize each electrode modification steps. The CV measurements were carried out from -0.5 to 1 Vs. reference electrode with a scan rate of 100 mV/s.

3. Results and Discussion

3.1 Characterization by Electrochemical Impedance Spectroscopy and Cyclic Voltammetry

The fabrication procedure of the proposed immunosensor is composed of four steps: (i) cleaning and hydroxylation of ITO substrate, (ii) SAM formation by using PHA including carboxyl groups, (iii) activation of carboxyl groups by using NHS/EDC chemistry, (iv) anti-IL-1β antibodies immobilization on the PHA modified electrode via amide bond formed between the carboxyl group of PHA and amino group of antibody, (v) blocking of remaining free carboxyl groups on ITO electrode to prevent nonspecific interaction. The schematic diagram of the immunosensor fabrication is shown in Scheme 1.

Scheme 1. The schematic representation of the IL-1β electrochemical immunosensor fabrication

In this study, EIS was used as characterization technique to analyze interfacial features of the ITO electrode during the modification steps. The EIS measurements were carried out in ferri-ferro solution. 5 mM ferricyanide and 5 mM ferrocyanide were utilized as redox probe. EIS spectra include real and imaginary parts and known as the Nyquist plot composed of a semicircle part and a linear part. The semicircle at high frequency part shows the electron transfer limited process, while the linear at low frequency part shows the diffusion limited process. The resistance of electron transfer limited process can be calculated by analyzing the semicircle diameter. The inset in Figure 1A includes Randles equivalent circuit. The Warburg impedance (Z_w) is the diffusional impedance based on diffusion of electrons from the electrolyte to the electrode surface. The solution resistance (R_s) is the resistance between the reference electrode and working electrode. The charge transfer (R_{ct}) is responsible for the electron transfer kinetics of the redox probe at the interface of electrode. The constant phase element (CPE) indicates the capacitance because of dielectric layer [49].

Figure 1. EIS measurements (A) and cyclic voltammograms (B) of the proposed immunosensor

Fig. 1A shows the Nyquist plots of different surface condition of ITO electrode. After PHA layer was successively immobilized on the ITO electrode surface, a remarkable increase in R_{ct} value was observed, which implies that a dense, insulative layer was constructed. Furthermore, after PHA layer formation on the ITO electrode, the resistance increased due to the strong electrostatic repulsion between the negative charge on the electrode surface and the redox probe present in the solution. As seen in Figure 1A, a loop was observed. This loop may be originated from the PHA insulating layer formation and increasing resistance [50]. After activation of carboxyl groups with EDC/NHS chemistry, the R_{ct} decreased notably. The cause of this decrease was the partial compensation of the negative charges of the electrode. The R_{ct} value further increased after immobilization of anti-IL-1β antibodies on the surface, which proved the successful immobilization of antibodies and protein structure hindered the diffusion of electrons toward the electrode surface. After being incubated in the BSA solution, the R_{ct} increased obviously, which means the blockage of free remaining carboxyl groups. After IL-1β antigen immobilization on the ITO electrode surface, an antibody-antigen complex was formed and this formed complex generated a protein layer that formed a barrier for electron transfer.

Furthermore, the fabrication process of the immunosensor was investigated by using CV measurements. As can be seen from 1B, the peak current decreased after SAM formation due to its insulator property. After activation of carboxyl groups by using EDC/NHS chemistry, peak currents increased. Anti-IL-1β antibody immobilization on the electrode surface caused a decline in peak current, which suggested that the antibodies formed a barrier to the electron transfer between electrode and electrolyte interface. After the addition of BSA, the peak currents decreased remarkably which indicated that remaining binding sites were blocked by BSA. After introducing IL-1β antigens onto electrode surface, a slight decrease was observed in peak currents that indicated the specific interaction was formed successfully.

3.2 FTIR and Raman Studies

The FTIR spectra of the PHA modified ITO electrode and anti-IL-1β antibody immobilized ITO electrode are shown in figure 2A and 2B, respectively. The broad peak of P-O-H between PHA and hydroxyl groups on ITO surface was observed between 2550-2700 cm^{-1} (figure 2A) that indicated the characteristic peak of phosphonate bond [51, 52]. PHA modified ITO had the absorption peak at 1635 cm^{-1} (figure 2A) that indicated the presence of carboxyl groups (C=O bonds) [46, 53]. The similar peaks are seen in figure 2C (Raman spectra). After immobilization of antibodies on PHA modified ITO electrode, the peaks observed at 1648 cm^{-1} and 1546 cm^{-1} (figure 2B) originating from amide bonds in protein [54]. 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, amide II and amide III regions are monitored mostly between 160-1680 cm^{-1}, 1480-1570 cm^{-1} and 1235-1300 cm^{-1}, respectively [55]. As shown in figure 2D, amide I, amide II and amide III bonds were observed at 1669 cm^{-1}, 1549 cm^{-1} and 1281 cm^{-1}, respectively.

The Energy Dispersive X-ray spectroscopy (EDX) study was carried out to determine phosphonate groups on ITO electrode after PHA modifications. EDX spectrum showed that corresponding peak of P confirmed the presence of SAM layer on the ITO electrode. In sum, as seen in figure 2E, hydroxylated ITO electrode included oxygen (O), indium (I) and tin (Sn) elements and after SAM formation on the ITO electrode, carbon (C), oxygen (O) and phosphorous (P) elements were seen (Figure 2F).

Figure 2. FTIR spectrum (A) and Raman spectra (C) of ITO/PHA and FTIR spectrum (B) and Raman spectra (D) of ITO/PHA/anti-IL-1β, EDX results before PHA modification (E), and after PHA modification (F)

3.3 Morphological Characterization of the Modified Electrodes

Figure 3 illustrates the SEM and AFM images of the immunosensor obtained after each immobilization step. As seen in figure 3A and 3B, a smooth surface was attained after activation of ITO electrode. Figure 3C and 3D show SEM and AFM images obtained after PHA modification, from which we can see that PHA was uniform distributed on the surface. The RMS values of hydroxylated and PHA modified ITO electrode were 2.85 nm and 6.08 nm, respectively. It is clear that PHA formed a dense and well-ordered SAMs on the ITO electrode surface. After antibody immobilization on the modified surface, the surface morphology was changed (Figure 3E and 3F). After antibody immobilization, the RMS value was 118.11 nm on 5 x 5 µm scale. The increase in RMS value confirmed the amide bond formation on ITO electrode. The blocking of active carboxyl ends resulted in a coverage of BSA on the ITO electrode (Figure 3G and 3H), and the RMS value was 88.45 nm on 5 x 5 µm scale. As a result of specific interaction between anti-IL-1β and IL-1β, globular structures were observed (figure 3I and 3J). After antibody-antigen interaction, the RMS value was found as 124.21 nm on a 5 x 5 µm scale. The increment in RMS value supported the specific interaction.

Figure 3. SEM and AFM images of the immunosensor obtained after each immobilization step; ITO/OH (A and B), ITO/OH/PHA (C and D), ITO/OH/PHA/anti-IL-1β (E and F), ITO/OH/PHA/anti-IL-1β/BSA (G and H), ITO/OH/PHA/anti-IL-1β/BSA/IL-1β (I and J)

3.4 Optimization of Experimental Condition

Optimization of the experimental parameters is important and necessary to fabricate a sensitive, stable, repeatable and reproducible immunosensor. The PHA concentration, antibody concentration and incubation time, antigen incubation time were optimized.

The SAM formation on the electrode surface is important for biorecognition element coupling. Because of this, three different PHA concentrations (1 mM, 2.5 mM and 5 mM) were examined. The signals of 1 mM and 2.5 mM PHA concentrations were similar and higher than the signal of 5 mM PHA concentration. Therefore, 1 mM PHA concentration was chosen as optimal level due to lower fabrication cost (See Figure SI-1). The amount of biorecognition element used in the fabrication of the biosensor is an important parameter for obtaining a successful biosensor with high performance because it affects the detection capability of the biosensor. To obtain maximum signal, the amount of anti-IL-1β antibody was optimized. Three different amounts (0.4 ng/mL, 2 ng/mL, 5 ng/mL) of anti-IL-1β antibody were tried. When 2 ng/mL anti-IL-1β antibody concentration was utilized, the maximum signal was obtained (See Figure SI-2). Therefore, 2 ng/mL 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 incubation time was not enough to bind of antibodies on ITO electrode surface. However, the signal of 45 min and 60 min were nearly similar (Figure SI-3). 45 min incubation time was chosen as optimum incubation time due to being time-saving. The last optimization parameter is antigen incubation time. The prepared immunoelectrodes were incubated in PBS solution containing IL-1β antibodies in different times (30, 45 and 60 min). The short incubation time (30 min) was inadequate for IL-1β antigens binding to their antibodies. The signals of 45 min and 60 min were similar, so 45 min incubation time was chosen as optimum value (Figure SI-4). The optimum results of experimental variables are summarized in Table SI-1B.

3.5 Impedimetric detection of IL-1β

To investigate the analytical performance of the immunosensor, we measured IL-1β standard solutions with different concentration under the optimum experimental conditions. The linear detection range of IL-1β biosensor was calculated by using this equation.

$\Delta R_{ct}=R_{ct}(\text{IL-1}\beta)- R_{ct}(\text{BSA})$

Where $R_{ct}(\text{IL-1}\beta)$ is the value of R_{ct} when IL-1β antigen incubated with immobilized antibody, $R_{ct}(\text{BSA})$ is the R_{ct} of immunosensor after BSA blockage.

Figure 4. (A) Impedance measurement of different concentrations (0.025-3 pg/mL) of IL-1β, (B) Cyclic voltammograms of different concentrations (0.025-3 pg/mL) of IL-1β, (C) Calibration curve drawn by fitting the results to Randles equivalent circuit, (D) Single Frequency Impedance result of anti-IL-1β antibody-IL-1β antigen interaction.

Figure 4A and figure 4B show the impedance spectra and cyclic voltammograms of increased IL-1β concentrations. Nyquist plots and cyclic voltammograms illustrated an increase of impedance and a

decrease in peak currents, respectively. The cause of this increase and this decrease was the blocking effects of proteins and the electrode surface became thicker with the increasing concentration of IL-1β.

To determine the analytical calibration range, various concentrations of standard IL-1β solution were used. As can be seen in figure 4A, when the IL-1β concentration increased, the R_{ct} increased in accordingly because the formation of increasing number of anti-IL-1β antibody – IL-1β antigen complexes resulted an increase in hindering of the electron transfer reaction of the redox probe on the electrode interface. An excellent linear correlation was found between the immunosensor response and the concentration of IL-1β over the range of 0.025 pg/mL to 3 pg/mL with equation $\Delta R_{ct}(k\Omega) = 0.8504$ [IL-1β] (pg/mL) + 0.0358 (R^2=0.99). Furthermore, slope error and intercept error were calculated as 0.062 and 0.017, respectively. The limit of detection (LOD) and limit of quantification (LOQ) were calculated as 7.5 fg/mL (three times the standard deviation of the blank) and 25 fg/mL (ten times the standard deviation of the blank), respectively.

The process related to anti-IL-1β antibody and IL-1β antigen coupling was monitored by SFI technique. In this technique, total impedance was measured at a single frequency versus time and the kinetic parameters were monitored during the analysis. Firstly, the frequency value was determined from Bode Plot. This measurement was performed in Ferri-Ferro solution. The point corresponding to the maximum value in the Nyquist plot was selected from the Bode plot. In this measurement, 20 Hz was chosen as the frequency value (Figure SI-5). After that, this electrode immersed in PBS solution including IL-1β antigen and the impedance was measured at 20 Hz. As shown in figure 4D, a significant increase was observed in impedance value (pink) during SFI measurements. This result proved the specific interaction between anti-IL-1β antibody and IL-1β antigen.

The repeatability of proposed immunosensor was carried out by recording impedance signal of independent prepared 20 electrodes at same concentration of IL-1β (0.5 pg/mL). The relative standard deviation of the measurement results was found as 4.56%. This result indicated the acceptable repeatability and feasibility. The reproducibility of the proposed immunosensor was calculated on ten different immunosensors (prepared with 80 electrodes) and a good relative standard deviation 5.42% was found. As seen figure 5A, these immunosensors had same linearity. The attained results illustrated the high precision and acceptable reproducibility of the immunosensor.

Storage stability has a significant role in clinical applicability; therefore, we investigated the shelf-life of the proposed immunosensor. For storage stability test, firstly we modified ITO electrode and immobilized anti-IL-1β antibody on this modified electrode. After BSA blockage step, we stored it in fridge. After storage of the immunosensor at 4 ^{o}C (in dry form) for 10 weeks, the impedance signal remained 26.45% of the initial value after 7 weeks for 0.5 pg/mL, which suggests good stability (Figure 5B).

Reusability is another important parameter in declining the cost of immunosensor. The basic of this test is composed of testing the robustness of the biosensor. In this test, firstly ITO electrodes (ITO/PHA/anti-IL-1β/BSA) were exposed to drastic conditions (immersed in acidic solution; ultra-pure water; HCl, 0.1%) for 5 min and then they were immersed in PBS buffer including IL-1β antigen. The signals obtained before and after exposure were recorded. As seen figure 5C, we attained good responses during 6 cycles, and after 6 cycles, the surface got damaged.

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

To appraise the specificity of the proposed biosensor, the impedance signal of the biosensor was recorded in the presence of some drugs (amoxicillin, ampicillin, aspirin) and proteins (receptor activated kinase C1, tumor necrosis factor α, interleukin 8). As seen figure SI-6, the proposed immunosensor gave response only to the solution containing IL-1β antigen. This result illustrated high selectivity of the immunosensor.

In order to testify the practical application, the developed immunosensor was used to detect IL-1β in human serum and saliva samples. Serum samples were supplied by Namık Kemal University hospital. Saliva samples were collected from the volunteers in NKU Research Lab. Prior to saliva collection volunteers rinsed their mouth with ultra-pure water in order to remove debris and moisturise. Saliva collection started after 10 minutes of rest. Samples were visually controlled before being immediately frozen and stored at 80 ^{o}C until analysis. One ml of clear whole saliva centrifugated at 2600 g for 15 min to remove cell pellets and debris. All the real serum and saliva samples were diluted 400 and 20 fold, respectively. Additionally, the recovery test of the developed biosensor was examined by spiking the samples with certain amounts of IL-1β antigens. The recovery studies were carried out to evaluate the effect of other proteins towards the developed method. The recovery values were found on the range of 99.70%-105.39%, which indicated that the recovery of the immunosensor was appropriate for real samples application (Table SI-1C). The developed electrochemical biosensor showed good reliability and applicability for determination of IL-1β. Therefore, it can be concluded that PHA sensing platform was a promising tool for determination of IL-1β in human serum and saliva.

The analytical performance of the proposed immunosensor is compared to the previous reported sensing methods for IL-1β (Table SI-1A). Electrochemical immunosensors based on antibody-antigen interactions are known as the best candidate for the detection of IL-1β due to their high sensitivity, selectivity and easy usability. Several biosensors with different type modification methods and with different transduction processes have been fabricated for IL-1β determination. As seen in Table SI-1A, the LOD and linear detection range of our developed immunosensor is better than that of other IL-1β sensor reported in the literature. The high sensitivity and wide linear detection range of the proposed immunosensor can be attributed to the well-ordered SAM layer formation.

The immunosensor is a convenient, low-cost, sensitive and specific technique whereas the ELISA technique is high-cost and requires a lot of preparation steps. Another advantage of this immunosensor is reusability. However, ELISA kit is disposable, and reusability of this assay is impossible. Consequently, our immunosensor provides a cheap, accurate, reliable and sensitive analysis for IL-1β detection.

4. Conclusion

A novel immunosensor based on PHA modified ITO electrode was fabricated and used for electrochemical detection of IL-1β in human serum and saliva samples. The SAM of phosphonates firstly was constructed on ITO electrode, and they provided a good platform for further immobilization of anti-IL-1β antibodies. Electrochemical characterizations were performed by using EIS, CV and SFI techniques, but for quantification of IL-1β antigens, EIS technique was utilized. A linear relationship between R_{ct} and IL-1β concentration ranging from 0.025-3 pg/mL was obtained with a detection limit of 7.5 fg/mL. The developed immunosensor was simple, easy to fabricate, high specific, highly reusable and sensitive for the detection of IL-1β. It exhibited high selectivity for IL-1β in presence of some drugs and some biomarkers. Moreover, this biosensor was successfully applied to determination of IL-1β in human serum and saliva samples. The experimental results proved that the proposed method was accurate, convenient and rapid. Consequently, this phosphonate SAM based ITO electrode can serve as a proper electrode material in biosensor development, and other phosphonate based chemicals can be used in the modification of electrodes. Thus, this immunosensor can be commercialized due to simple preparation steps and low cost.

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Highlights

- An impedimetric immunosensor was fabricated by using 6-phosphonohexanoic acid (PHA) as an immobilization matrix.
- The analytical immunosensor is a rapid and sensitive method for determination of interleukin-1β (IL-1β).
- The immunosensor is able to detect IL-1β at low concentration in real human serum and saliva samples.
- The proposed immunosensor can detect IL-1β with wide linear range (0.025-3 pg/mL) and low detection limit (7.5 fg/mL).