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11-(Triethoxysilyl) Undecanal Agent-Based Biosensor System Using Disposable ITO-PET Electrode for Tumour Necrosis Factor-Alpha Detection

Tümör Nekroz Faktörü-Alfa Tespiti için Tek Kullanımlık ITO-PET Elektrot Kullanan 11-(Trietoksisilil) Undekanal Ajan Bazlı Biyosensör Sistemi

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ABSTRACT

In this study, a label-free electrochemical biosensor system based on a disposable indium tin oxide polyethylene terephthalate (ITO-PET) electrode modified with the 11-(triethoxysilyl) undecanal (11-TESU) agent was developed for the detection of tumour necrosis factor-alpha (TNF-α) in serum. The developed biosensor was observed with electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV) techniques, square wave voltammetry (SWV) and single frequency impedance (SFI) technique which is utilized for the specific interaction between anti-TNF-α and TNF-α antigen. In addition, scanning electron microscopy was used to look at how the morphology of each ITO-PET surface changed (SEM). All parameters such as 11-TESU concentration, anti-TNF-α concentration and anti-TNF-α incubation time, were optimized. The biosensor system was characterized by measuring its linear determination range, repeatability, reproducibility, reusability, storage stability, and surface coverage. The TNF-α electrochemical biosensor showed high levels of repeatability and reproducibility as well as a large dynamic range of detection (from 0.03 pg mL^{-1} to 3 pg mL^{-1}). The LOD and LOQ for the biosensor were extremely low at $1x10^{-4}$ pg mL^{-1} and $5x10^{-4}$ pg mL^{-1}, respectively. It was applied to real samples to determine whether the proposed biosensor would be useful in clinical settings.

Key Words

Indium tin oxide (ITO), 11-(triethoxysilyl) undecanal (11-TESU), Tumour necrosis factor-alpha (TNF-α).

ÖZ

Bu çalışmada, TNF-α' nın insan serumunda tümör nekroz faktörünün saptanması için 11-(trietoksisilil) undekanal (11-TESU) ajanı ile modifiye edilmiş tek kullanımlık indiyum kalay oksit polietilen tereftalat (ITO-PET) elektroduna dayalı etiketsiz bir elektrokimyasal biyosensör sistemi geliştirilmiştir. Geliştirilen biyosensör, anti-TNF-α ve TNF-α arasındaki spesifik etkileşim için kullanılan elektrokimyasal empedans spektroskopisi (EIS), döngüsel voltammetri (CV) teknikleri, kare dalga voltametri (SWV) ve tek frekans empedans (SFI) tekniği ile gözlemlendi. Ek olarak, her bir ITO-PET yüzeyinin morfolojisinin nasıl değiştiğini (SEM) incelemek için taramalı elektron mikroskobu kullanıldı. 11-TESU konsantrasyonu, anti-TNF-α konsantrasyonu ve anti-TNF-α inkübasyon süresi gibi tüm parametreler optimize edildi. Biyosensör sistemi, lineer tayin aralığı, tekrarlanabilirlik, tekrar üretilebilirlik, rejenerasyon, depolama kararlılığı ve kaplanan yüzey alanı ölçülerek karakterize edildi. TNF-α elektrokimyasal biyosensör, yüksek tekrarlanabilirlik ve tekrar üretilebilirliğin yanı sıra geniş bir tayin aralığı (0,03 pg mL^{-1}'den 3 pg mL^{-1}'e) gösterdi. Biyosensörün LOD ve LOQ değerleri, sırasıyla $1x10^{-4}$ pg mL^{-1} ve $5x10^{-4}$ pg mL^{-1}' dir. Önerilen biyosensörün klinik ortamlarda yararlı olup olmayacağını belirlemek için gerçek örneklere uygulandı.

Anahtar Kelimeler

İndiyum kalay oksit (ITO), 11-(trietoksisilil) undekanal (11-TESU), Tümör nekrozis faktör alfa.

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INTRODUCTION

The 19 ligands and 29 receptors that make up the tumour necrosis factor superfamily regulate many diverse bodily processes. [1]. Involved in systemic inflammation, tumour necrosis factor-alpha (TNF-α) is also a key biomarker that helps regulate the immune system. It causes many physiological and pathological changes, including fever, apoptotic cell death, and cachexia [2-3]. TNF-α is often expressed as a type II integral membrane protein. This is the case in the majority of instances. [4]. Immune regulatory macrophages, T cells, fibroblasts, natural killer cells, mast cells, immune, endothelial, non-osteoblasts, granulocytes, smooth muscle cells, and keratinocytes manufacture this multifunctional cytokine [5]. TNF-α, a key proinflammatory cytokine in the tumour microenvironment (TME), affects all aspects of breast cancer progression, including tumor cell proliferation and survival, metastasis, epithelial-to-mesenchymal transition (EMT), and recurrence.TNF-α may be regarded as a pro-tumorigenic cytokine on the assumption that TNF-α levels in breast cancer ills are correlated with their clinical condition [6]. In addition to its involvement as a pro-inflammatory cytokine in preserving intestinal integrity and the pathophysiology of intestinal inflammation, TNF-α is also primarily described as an anti-apoptotic cytokine. Immune and intestinal epithelial cells have been demonstrated to release TNF-α in mice and during inflammation in individuals with chronic inflammation [7-8]. Furthermore, increased TNF-α secreting cells in the intestinal tissue lead to the raised TNF-α levels seen in patients with chronic intestinal inflammation [9].

Good electrical characteristics and optical clarity make indium tin oxide a potential electrode for various devices [10]. Materials that are both electrically conductive and transparent to light have attracted increased attention from the technological community. ITO thin film is widely employed in optoelectronic and electrochemical applications because of its excellent transmittance and conductivity [11]. Modifying its surface is one effective method for enhancing the electroanalytical activity of an ITO electrode in biosensor development design. Amperometric and potentiometric sensors based on various working electrode types may detect transmitters produced from single cells. The ITO electrode is widely used in the electrochemical detection of biomolecules because of its strong electrical conductivity and excellent photo-penetrability [12]. The aldehyde endings of 11-TESU make it a perfect agent for forming a highly structured self-assembled monolayer. This way, attaching antibodies to the electrode surface without a crosslinker was possible. The 11-TESU agent makes the design of the sensor very practical. The purpose of this research is to develop an electrochemical technique for measuring TNF-α that does not require the use of a labeled electrode. Various techniques were used to optimize and characterize the biosensor, including EIS, CV, SFI, and SWV. A scanning electron microscope was used to monitor the surface morphology changes along the immobilization procedure. Analysis of human serum samples using the standard addition method to investigate the clinical potential of the developed immunosensor. The designed biosensor system was shown to have great sensitivity for examining blood samples and outstanding repeatability, reusability, storage life, and longevity.

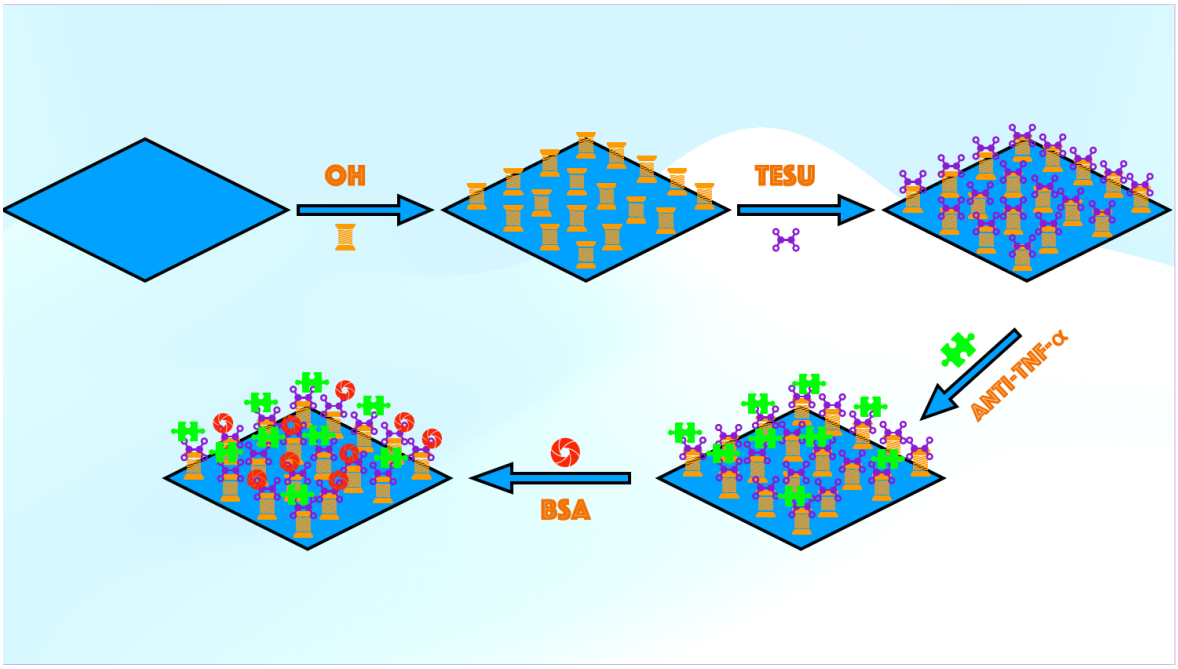
MATERIALS and METHODS

Materials and Instruments

All chemicals (11-TESU, ammonium hydroxide, hydrogen peroxide, ethanol solutions), biorecognition materials (BSA protein (bovine serum albumin) anti-TNF-α and TNF-α antigen, human serums) and ITO-PET electrodes were purchased from Sigma-Aldrich (St. Louis, M.O., USA). Anti-TNF-α antibodies, TNF-α , and BSA were prepared using a phosphate buffer (50 mM, pH 7.0) and kept at –20 °C. A potassium ferro/ferricyanide solution in PBS (phosphate buffer (50 mM, pH 7.0)) was prepared. Counter (a platinum wire) and reference electrodes (Ag/AgCl saturated with 3M KCl) were purchased from BASi (West Lafayette, USA). In this study, a disposable ITO-PET (as a working electrode (2 x 20 mm)) was used. All EIS, CV and SWV analyses were performed with a potentiostat (Gamry Reference 600). The SEM measurements were carried out at the NABİLTEM (Tekirdağ Namık Kemal University, Scientific and Technological Research Center).

The Immunosensor Design and Fabrication

Disposable ITO-PET (indium tin oxide polyethylene terephthalate) electrodes were used to design the suggested biosensor. The disposable electrodes were first sonicated in an ultrasonic bath for 10 minutes in the following solutions: acetone (1 mL), soap solution (1 mL),, and ultrapure water (1 mL), (18.2 M Ω cm deionized). After soaking the electrodes for 90 minutes in a solution of NH_4OH, H_2O_2, and ultra-pure water (1: 1: 5, v/v), the ITO surface



Scheme 1. The immobilisation steps of the TNF-α sensor.

was hydroxylated and became active. The electrodes were modified with 11-TESU (11-(trietoksisilil) undecanal) (in ethanol solution) and dried with pure argon gas overnight. Next, an anti-TNF-α protein was left to incubate with the electrodes for half an hour. Finally, BSA (0.5%, w/v) was used as a blocking agent on the electrode surface for 1 h to prevent non-specific interactions. The disposable ITO-PET electrodes used in the proposed immunosensor were sterilized between uses by rinsing them in ultra-pure water and drying them in an argon stream. Each one of the electrochemical processes was performed at a steady 25 °C. Scheme 1 outlines the procedures that were followed to create the immunosensor.

Measuring principle for the proposed immunosensor

The immobilization steps of the designed immunosensor were carried out using cyclic voltammetry and electrochemical impedance spectroscopy. Electrochemical impedance tests were performed in the 10.000 Hz – 0.05 Hz frequency range. The square wave voltammetry results were in a possible range of (0 V to 1.5 V; frequency: 25 Hz; pulse size: 25 mV; equal. time: 2 s). A potential range (-0.5 V to 1 V; step size: 10 mV; scan rate: 100 mVs-1) was used for the CV technique. All electrochemical tests were performed on a biosensor device (Gamry Reference 600) at 25 °C.

Human serum analysis of the biosensor system

Biosensors were tested using serum samples to evaluate the reliability of the immunosensor system. In this experiment, three ITO-PET electrodes were prepared with varying TNF-α concentrations (0.5 pg/mL and 2 pg/mL). The standard addition method was used for the investigation, and the biosensors were applied to the human serum samples. This study was carried out by diluting the concentrations of purchased human serums according to the detection range of the biosensor for a valid response. The standard addition method was employed to determine TNF-α in serum, diluting the samples with phosphate buffer (0.05 M, pH 7.0). The calibration graph of the TNF-α sensor system was used to derive equations for calculating the RSD and recovery values.

RESULTS and DISCUSSION

Immobilization process of the 11-TESU-modified biosensor system

The EIS and CV methods were utilized throughout the development of this biosensor system. The immobilization procedure of the constructed biosensor was quite successful due to the sensitive measurements of the EIS technique [13]. The first step of immobili-

zation was to activate the hydroxyl groups formed on the surface of the working electrode. Following this process, the semiconductor surface of ITO-PET became electrically conductive. It can be seen the impedance spectra of the hydroxylation (the purple mark) in Fig.1. In the EIS technique, the ΔRct value, which expresses the surface resistance, is observed as a low signal on the conductive surface. Depending on this result, the low signal obtained after hydroxylation is seen in the immobilization spectrum of the designed sensor in Fig.1. The next step is surface immobilizing the 11-TESU agent with highly active aldehyde and silane groups. With this step, the hydroxyl and silane groups interacted to form a stable covalent layer on the surface. This layer increased the saturation of the surface. Accordingly, the ΔRct value (the green mark), which is the surface resistance, also increased in Fig.1. In the next step, after what formed the siloxane bond between the silane ends of the 11-TESU agent and the hydroxyl groups, the interaction with the aldehyde groups at the other end of the agent with anti-TNF-α took place. After this process, the ΔRct value (the blue mark) increased even more due to the diffusion effect of the redox probe. The EIS signal rose after immobilization with anti-TNF-α. The blocking agent (BSA 0.5%, w/v) was utilized for the final step in immobilization to eliminate any chance of non-specific interactions. After incubation with the BSA protein, as seen in Fig.1, the EIS signal (the red mark) increased because electron transport was decreased on the electrode surface. Additional data verification was achieved by measuring with both the CV and EIS methodologies. In Fig.1, the hydroxylated electrode (the purple mark) had the largest CV peak currents. The conductive of the ITO-PET electrode surface is responsible for this effect. After the 11-TESU agent was immobilized, the peak currents (the green mark) decreased. The siloxane link between the substance and the hydroxyl groups is responsible for this decrease. After the anti-TNF-α immobilization, the peak currents (the blue mark) decreased more than before the step. In the CV technique, the peak currents are expected to approach each other as the conductivity on the surface increases. Accordingly, the surface insulation increased with the effect of BSA in the last immobilization stage. The anodic and cathodic peak currents (the red mark) at this stage also became very close in Fig.1.

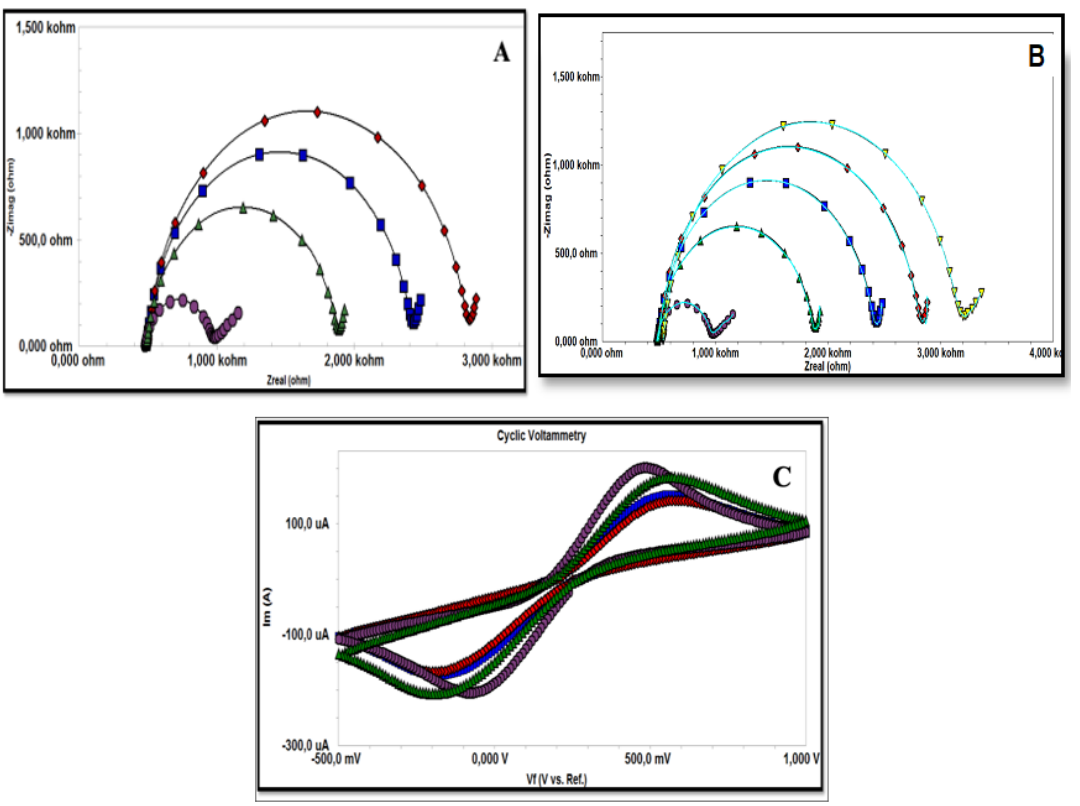


Figure 1. (A) EIS spectra, (B) Kramer's Kronig Transform and (C) CV voltammograms of the TNF-α biosensor system.

Optimization Steps for Improving TNF-α Immunosensor Performance

Optimization studies of the 11-TESU agent

Each parameter of the designed immunosensor was subjected to extensive optimization experiments. The first optimization step belongs to the concentration of the 11-TESU agent. The proposed immunosensor was ready with the different 11-TESU concentration values (0.1 %, 0.25 % and 0.75 %) in ethanol solution. These 11-TESU concentrations were used to calibrate the biosensor systems and obtain standard graphs for the proposed immunosensor. The optimum 11-TESU concentration is shown in Fig.S1. Firstly, the immunosensor system was incubated with the 0.1 % concentration value. A more concentrated 11-TESU concentration of 0.25% was preferred for subsequent optimization after what achieved a good linear graph of this investigation. The developed sensor system was shown to produce a stronger signal when 11-TESU concentrations rose. For this reason, 0.75 % 11-TESU was utilized in the final optimization research. This decrease may be related to the reduction in the stability of the surface at high concentrations. After reviewing all the available evidence, a concentration of 0.25 % 11-TESU was optimal.

Optimization studies for the concentration and incubation period of anti-TNF-α

The ideal concentration and incubation times of anti-TNF-α were also studied as part of the optimization procedure. Preparations of the biosensor system were made at 0.5, 2 and 10 ng/mL anti-TNF-α concentrations to study the impact of anti-TNF-α concentration on the immunosensor answer. Fig.S1 illustrates the standard graphics of this study. The figure shows that 2 ng/mL and 10 ng/mL gave better results at the selected concentration values. The 2 ng /mL was selected as the optimal anti-TNF-α concentration from these two concentrations, which showed very close answers to each other. This optimum value was made considering the consumption of antibodies, which directly affects the cost of the proposed biosensor.

After identifying the optimum anti-TNF-α protein concentration, the research was conducted to detect the optimum incubation duration for optimum anti-TNF-α, another crucial parameter. The biosensor systems were prepared three times (half an h, forty-five min and one h) to determine the best incubation time for the anti-TNF-α. In these three different periods, the sensor is given, which will be experienced in 30 min in the best way. With this study supporting that the designed sensor is fast and practical, the optimum anti-TNF-α was picked as 30 min (Fig.S1.C).

Characterization Studies of the TNF-α Immunosensor

The calibration graph of the proposed immunosensor was generated after extensive immobilization and optimization investigations were carried out. Results from the calibration curve indicated a dynamic range of 0.03 pg/mL to 3 pg/mL. The constructed immunosensor was tested and shown to have LOD and LOQ values of 10^{-4} and $5x10^{-4}$ pg/mL, respectively. These values are derived from the formula [k*Sblank/m], where k is a constant (k = 3 in LOD and k = 10 in LOQ), Sblank is the standard deviation, and m is the mean (slope of the calibration curve). Additionally, the EIS and CV results were obtained in the rising concentrations of TNF-α antigen. Ten different concentrations were used to achieve a great linear range for sensitive detection using the suggested immunosensor. The TNF-α antigen incubation time on the modified ITO-PET electrode surface was 30 min during all procedures. Fig.2 shows the calibration graph of the immunosensor, increasing impedimetric data at several concentrations, and CV voltammograms.

The repeatability of the designed TNF-α biosensor was examined in the analytical characterization of biosensors. The disposable working electrodes (20 pieces) were prepared under the same conditions. The immobilized electrodes were incubated at a constant concentration value (1.25 pg/mL) of the TNF-α antigen. Based on these results, the average value is 0.013, the standard deviation is 0.1171 pg/mL, and the coefficient of variation is 1.23% of the suggested sensor.

The reproducibility of the TNF-α biosensor system was examined by preparing ITO-PET electrodes under optimal circumstances at different periods. The seven different biosensors were built for this work and obtained their EIS results within the determination range of the designed biosensor. The RSD value was found 3.78% (for the slope). Standard plots of the work showed that the immunosensor had excellent reproducibility (Fig. 2).

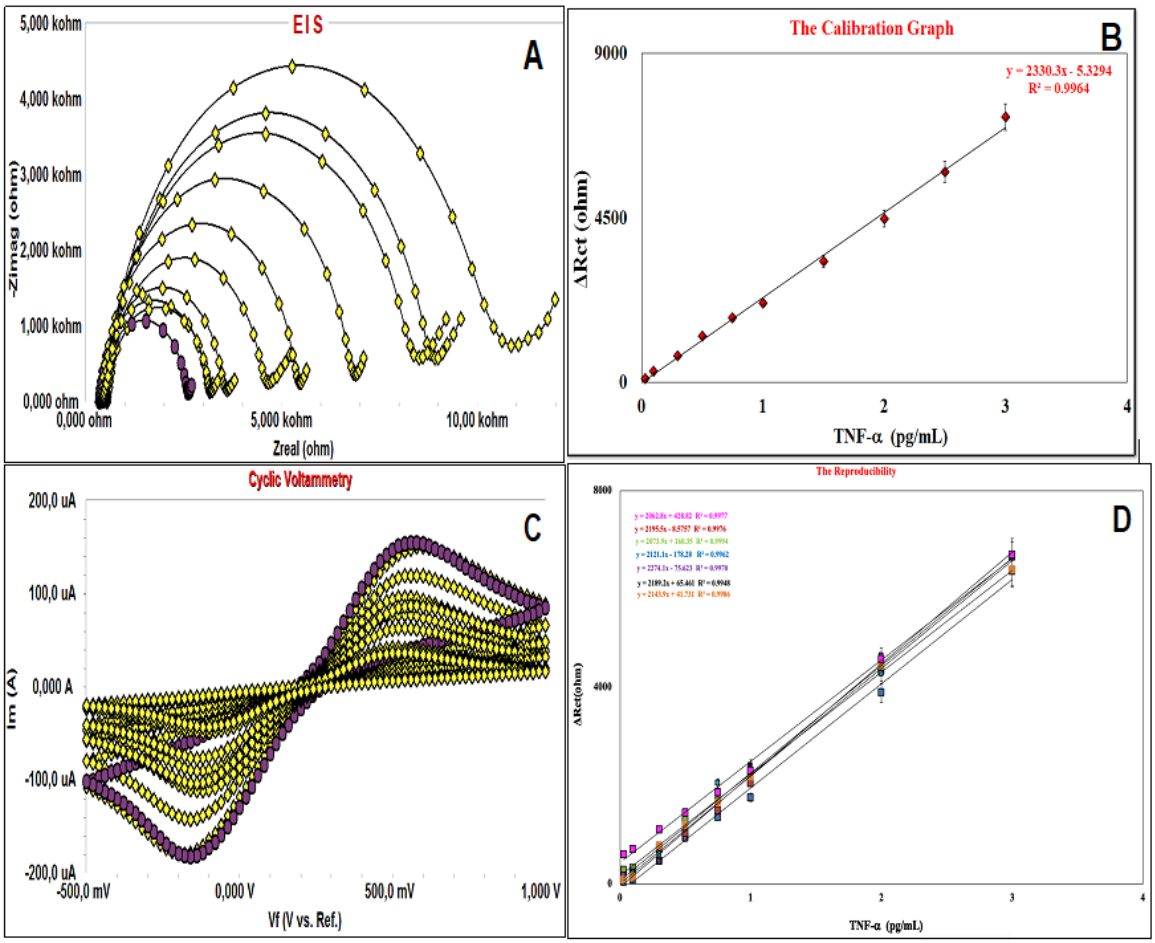


Figure 2. (A) EIS spectra, (B) Calibration curve, (C) CV results of the detection of the TNF-α at increased values, (D) Reproducibility.

The reusability of an immunosensor system is an important feature of surface stability and working electrode expense. A disposable ITO-PET electrode was prepared for this study. After TNF-α (1.25 pg/mL) was immobilized on the ITO-PET electrode, the EIS measurement was performed. After immobilization, the TNF-α antigen was stripped off the ITO-PET surface with an acid solution (10 mM HCl, 2 min). This period continued until the ability to bind proteins was greatly reduced or lost. The ITO-PET surface was regenerated with this procedure 11 times. Half was kept safe up to the 11th bond thanks to the ITO-PET electrode's surface activity. The total activity loss between the first and eleventh binding was 54.61 %. This result is a remarkably good performance for a disposable ITO-PET electrode in terms of reuse. The results obtained are encouraging, considering the potential future applications of the biosensor. The reusability study is presented in Fig.3.

Analytical investigations of the suggested immunosensor included a study of the surface coverage using the Laviron equation (Q=nxFxAxΓ; n: slope, F: Faraday constant (96485 C mol^{-1}), A: Electrode surface area (cm^2), Q: Charge (C) and Γ: Coated surface area (mol/cm^2). The voltammetric method was employed to conduct the analysis, with the ITO-PET electrode surface being scanned at 10 different speeds (10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mV s^{-1}) for each immobilization procedure. Calculated data were put into the Laviron equation for the peak currents obtained from the CV measurements, and the ITO-PET surface area was determined. The data analysis revealed that the hydroxylation ITO-PET surface was initially calculated as $4.86x10^{-8}$ mol/cm^2 and was calculated to be $1.54x10^{-5}$ mol/cm^2 after TNF-α linking. This study response shows that TNF-α was immobilized on the ITO-PET surface.

A crucial factor in immunosensor clinical applications is how long it can store it. It was determined that the constructed biosensor had an eight-week shelf life. There was an investigation of the biosensor's storage lifetime over eight weeks. Eight disposable ITO-PET electrodes were monitored using the EIS method after incubating in 1.25 pg/mL TNF-α for one week. Up to their use, the ITO-PET electrodes were stored in the same circumstances at +4 °C. After the end of the 8th week, the total activity was calculated for the proposed immunosensor. After 8 weeks, the biosensor showed a total activity decrease of 6.9 %, according to this study's findings (Fig.3).

For the analytical investigation, the TNF-α values were detected using SWV. The study was conducted within the determination range (0.03 - 3 pg/mL) of the designed immunosensor. The standard graph of the study is presented in Fig. S2. Research conducted by SWV compared the proposed immunosensor to several electrochemical methods.

The impedance of the designed biosensor was measured using the single-frequency technique for the other characterization study. The assessment of biosensors, the monitoring of processes, and the analysis of slow time-dependent changes in a biosensor surface are all possible applications of the single-frequency impedance technique [14]. This method can study the dynamics of anti-TNF-α and TNF-α antigen interactions. The impedance was measured in real-time as a function of time and phase angle at a constant frequency of 20 hertz. The Bode plot displayed the study's constant frequency using the Gamry Potentiostat/Reference 600. The time-dependent impedance changes in the proposed immunosensor are given in Fig.3. Moreover, the blue curve indicates the impedance measurement, and the red curve indicates the phase angle in Fig.3. This procedure, carried out in phosphate buffer, demonstrates the binding of the TNF-α antigen versus time (pH 7.0).

Biosensors must be able to discriminate between many analytes to detect their targets with any degree of precision. To investigate the selectivity of the immunosensor, several potential interferences were added, including SOX2 protein (1.25 pg/mL), ST2 protein (1 pg/mL), leptin (0.8 pg/mL), and D-glucose (2 pg/mL) solution. Additionally, the analysis was undertaken using a combination of all these possible interferences. The mixed solution contains SOX2, ST2, leptin and D-glucose solution. Also, another solution

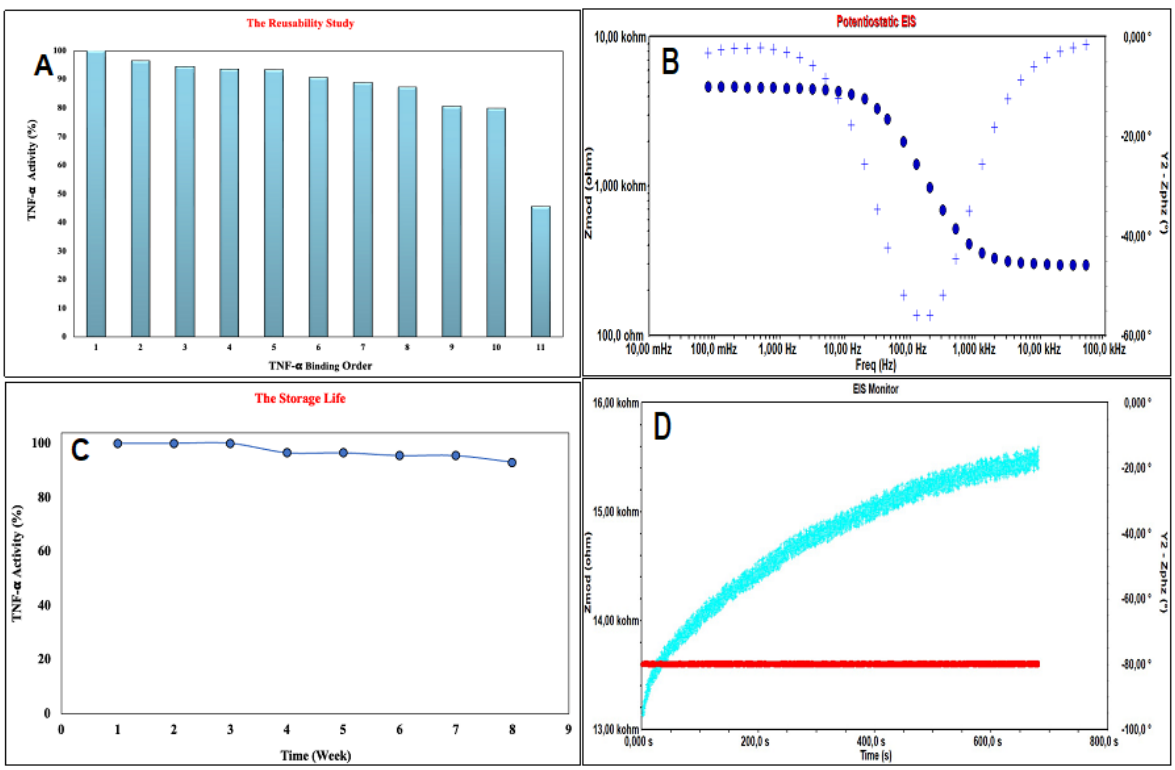


Figure 3. (A) Reusability, (B) Bode plot for SFI analysis, (C) Storage Life, (D) EIS measurement in the SFI.

Table 1. Comparative analysis of analytical characteristics of several TNF-α immunosensors described in the scientific literature.

Immobilization Method	Methods	Detection Range	LOD	Reference
SPEAu/MMPs	Chronoamperometry	1-15 pg/mL	0.3 pg/mL	[15]
Si_3N_4/TESUD	Capacitance	1-30 pg/mL	1.0 pg/mL	[16]
EDAC/NHS	Differential puls voltammetry	76-5000 pg/mL	38 pg/mL	[17]
Nano anti-TNF-α	Potentiometric	0.1-10 mg/mL	0.015 mg/mL	[18]
BioMEMS	EIS	1-15 pg/mL	1.0 pg/mL	[19]
Ab-TNF-α-HRP	Chronoamperometry	1-30 pg/mL	1.0 pg/mL	[20]
ITO-Ab-TNF-α	EIS	10-100 pg/mL	1.0 pg/mL	[21]
Nanowell array assay	EIS	10-500 ng/mL	10 ng/mL	[22]
ITO-PET/11-TESU	EIS	0.03-3 pg/mL	$1x10^{-4}$ pg/mL	This work

includes the given potential interferences and TNF-α antigen (1.25 pg/mL). The biosensor responses were investigated for these solutions to understand whether the biosensor was specific for the TNF-α antigen. As shown in Fig.S3. the immunosensor was largely unaffected by most of the interfering chemicals, whereas the Rct value was significantly elevated by the TNFα solution. It was observed that the response of the constructed biosensor was slightly affected in the analysis of the mixed solution, including TNF-α. This increase may be since while the anti-TNFα antibody will recognize and bind to the TNFα antigen, potential interferences can also bind to the active antibody. When the total results of the method are considered, it is highly selective for TNF-α.

Table 1 compares the developed immunosensor to the numerous TNF-α biosensors published in the scientific literature. The suggested sensor has a more practical immobilization method than the other biosensor systems, according to Table 1. The immobilization method determined for TNF-α was carried out with high-cost SPEAu working electrodes, for example, in the study in reference 13. This work presents a sensor system designed to detect TNF-α protein with ITO-PET electrodes that are less expensive and have a greater detection range. The differential pulse voltammetry technique was preferred for the electrochemical determination of TNF-α' in the study presented in Reference 15. In this proposed sensor study, the electrochemical impedance technique, a more sensitive technique than the voltammetry technique, was used for the electrochemical determination of TNF-α. In addition, this immobilization procedure was executed using a low-cost electrode, ITO-PET, and a more sensitive technique, EIS. The detection range, like the picogram, has a very precise measurement level. All these features highlight the designed sensor in the literature.

The TNF-α immunosensor: A Morphological Analysis

Analytical experiments included scanning electron microscopy to characterize the immunosensor's morphology. After each immobilization stage, the surface morphology was analyzed using scanning electron micrography. Fig.4 displays SEM images of the TNF-α immunosensor. The first morphological analysis shows the bare surface of the ITO-PET electrode. There is no immobilization process on the surface; therefore, the surface seems homogenous. Fig.4. the second image belongs to the hydroxylated surface. The morphological differences between the image's surface features are readily apparent. The SEM image of the 11-TESU immobilization was examined in Fig.4. The next image belongs to the anti-TNF-α antibody immobilization step in Fig.4. The morphological appearance of the BSA protein immobilization is shown in Fig.4. Also, the morphology of this image is very different from the previous one. This distinction is another sign that BSA immobilization has been successful. Finally, Fig.4 presents the electrode surface image of the TNF-α antigen. The results of SEM characterization follow electrochemical characterization techniques.

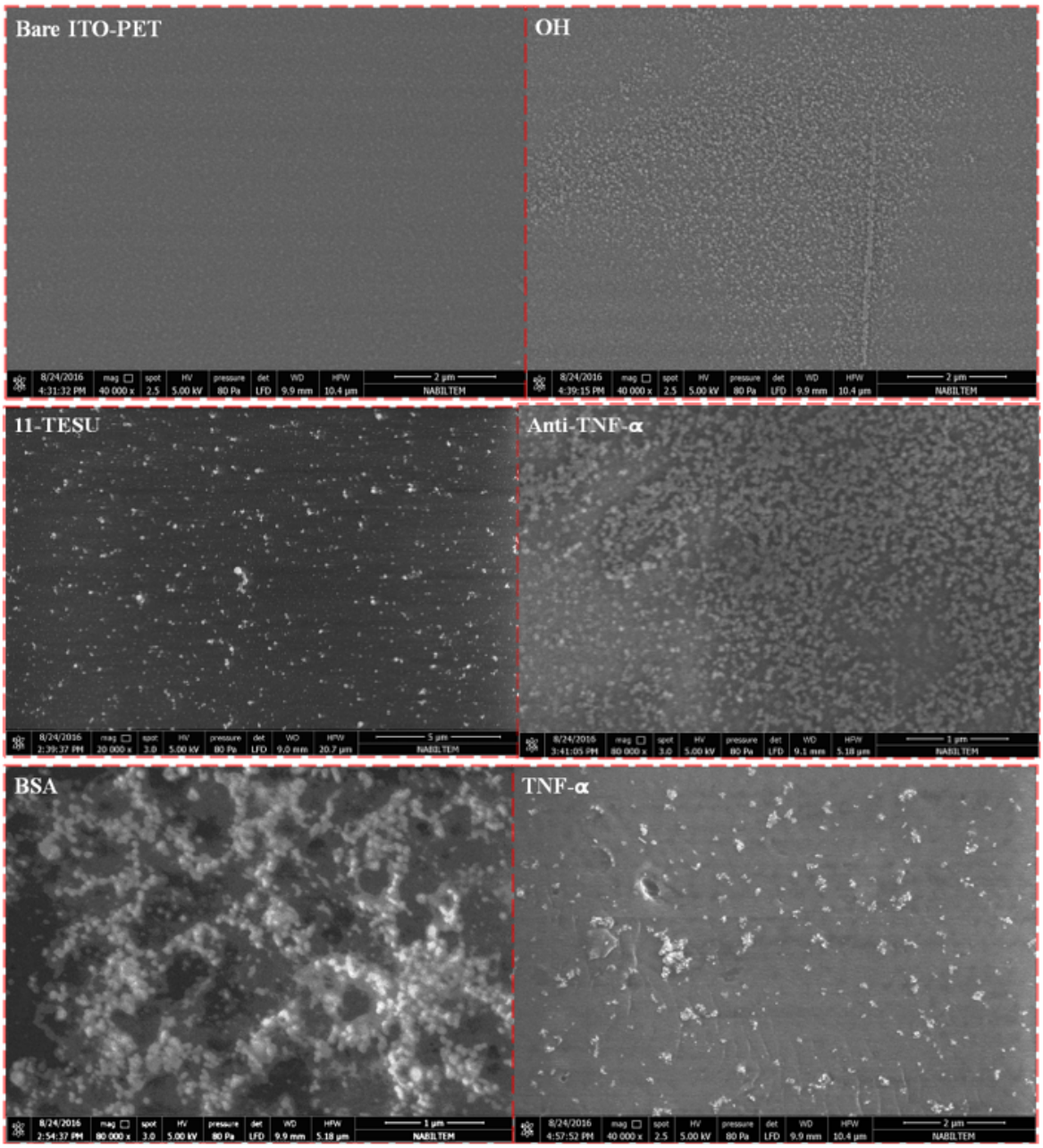


Figure 4. (1) SEM images of bare ITO-PET electrode, (2) OH, (3) 11-TESU, (4) Anti-TNF-α, (5) BSA, (6) TNF-α.

Quantification of Tumor Necrosis Factor-α in Human Serum

The samples from four human serums were used to identify the TNF-α antigen using the conventional addition method and assess the utility of the proposed immunosensor in clinical settings. The EIS data were generated using serum samples containing 0.5 pg/mL and 2 pg/mL TNF- TNF-α protein. All serum samples were diluted via buffer solution (PBS, pH:7.0). Utilizing the formula derived from the calibration graph, determined the RSD % and recovery values for each serum sample (Table 2).

Table 2. Investigation of the TNF- biosensor using serum samples.

Serum Number	TNF-α Concentration Value (pg/mL)	Standard Addition Value (pg/mL)	Measurement Conc. Value (pg/mL)	RSD (%) (n=3)	Recovery (%)
1	9.0	0.5	9.8/9.4/9.6	1.22	101.05
		2	10.92/11.25/11.03	1.05	100.54
2	5.1	0.5	5.7/5.8/5.4	2.07	100.53
		2	7.3/7.0/7.1	1.64	97.67
3	9.8	0.5	10.5/10.2/10.4	1.10	100.58
		2	11.6/11.8/11.9	0.99	99.66
4	6.3	0.5	6.8/6.9/6.8	1.71	100.44
		2	8.3/8.5/8.48	1.39	99.05

Conclusion

This study presents the design of a highly sensitive, outstanding, repeatable and reproducible TNF-α immunosensor with disposable, low-cost, useful ITO-PET electrodes. The early detection of TNF-α protein is crucial because of its many useful functions in metabolism. The proposed immunosensor was constructed by a strong covalent modification process using an 11-TESU silane material that does not need crosslinkers. Moreover, the proposed immunosensor demonstrates effective reusability and long-time storage capability properties. Furthermore, the SEM method, surface coverage research, and single-frequency impedance technique confirmed the validity of all immobilization procedures. EIS and CV techniques were used to characterize all immobilization and optimization experiments, which are powerful electrochemical analysis methods. The TNF-α immunosensor exhibits a very good detection range (0.03 pg/mL – 3 pg/mL). The proposed immunosensor could be tested to detect the TNF-α antigen in human serum samples. The TNF-α immunosensor can analyze at the level of sub-picogram and over a wide detection range.

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