International Journal of Biological Macromolecules 130 (2019) 245–252

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

Highly sensitive and cost-effective ITO-based immunosensor system modified by 11-CUTMS: Analysis of SOX2 protein in real human serum

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ARTICLE INFO

Article history:
Received 18 December 2018
Received in revised form 19 February 2019
Accepted 19 February 2019
Available online 20 February 2019

Keywords:
Biosensor
SOX2
Disposable ITO-PET electrode

ABSTRACT

In this study it is aimed to construct an immunosensor system for detection of Sex-determining region Y-box 2 (SOX2) antigen by using Indium tin oxide- polyethylene terephthalate (ITO-PET) as working electrode. Firstly, ITO-PET electrode surfaces were hydroxylated by using $NH_4OH/H_2O_2/H_2O$ and were incubated with 11-cyanoundecyltrimethoxysilane (11-CUTMS), respectively. After silanization process, anti- SOX2 was immobilized on modified ITO-PET electrodes. All immobilization processes were examined with Electrochemical impedance spectroscopy (EIS) and Cyclic voltammetry (CV). The basic parameters such as 11-CUTMS and anti-SOX2 concentrations, anti-SOX2 and SOX2 incubation period were optimized. The immunosensor prepared under optimal conditions gave a response for a wide concentration range of SOX2 protein (0.02 pg mL^{-1}–2 pg mL^{-1}) and the limit of detection was determined as low as 0.013 pg mL^{-1}. And also, the immunosensor had good repeatability, reproducibility, reusability and long shelf life. Scanning Electron Microscope (SEM) method was utilized to observe the morphologies of the electrode surfaces belonging to all steps. Lastly, seven different real human serum were analyzed with the constructed immunosensor and Enzyme-Linked Immuno-Sorbent Assay (ELISA). The results found with these methods were analogised with each other.

1. Introduction

SOX2 is a transcription factor that is comprised in the maintenance of embryonic stem cell pluripotency [1] and in multiple progressive processes, containing lung branching morphogenesis [2]. In tumor gene expression studies, analyses of genes known to be active in progression and differentiation have shown that SOX2 is overexpressed in poorly differentiated subtypes of cancer [3]. There are several studies which demonstrate that SOX2 levels increase in various types of cancer such as lung [4], prostate [5], breast, colon, glioblastoma [3], ovarian, cervical and pancreatic cancer [6].

Biomarkers are molecules that indicate a normal or abnormal biological conditions in the blood, other body fluids and tissues [7]. Biomarkers are used to monitor changes in disease and health status, risk assessments, clinical diagnosis, disease diagnosis and prognosis, pharmaceutical industry and many other areas [8]. There are a lot of studies and techniques such as Enzyme-linked Immunoassay (ELISA), radio immunoassay and fluorescence immunoassay methods used for detection of biomarkers in human serum [9,10]. ELISA is one of the most widely used spectrophotometric methods for determination of biomarkers in biological fluids [11]. This technique has a lot of advantages such as reproducibility, specificity and quantitative data in clinical analysis and scientific research [12]. However, it has some disadvantages such as long process period, short shelf life, high cost and the narrow dynamic range [13]. Moreover, the detection limit of conventional ELISA is barely less than the nanomolar concentration level. In immunosensors the same receptor surface can be reused for many measurements. The antibody based surface is strong during the process and this provides an economic advantage in contrast to commercial kit assays like ELISA [14].

Electrochemical biosensors have become a focus of attention in recent years due to their high sensitivity, being selective, simple and useful [15]. Since most antigen and antibody molecules are electrochemically inert, the label-free EIS method is improved to detect the immunospecies by analyzing the change of impedance [16,17]. Moreover, the EIS allows to understand the characteristics of the electrical features in the biological interface without causing destruction [18].

Silanization agents can achieve to form a stable bond between inorganic and organic materials [19]. Cyanoundecylsiloxanes are the most useful organosilanes as they have polarizing properties at both low temperatures and high temperatures. Moreover, organo-functional silanes have low surface tensions which provide high adhesion between different materials due to their reactivity. These agents can form the necessary interactions and transition phase between the formed monolayer and the substrate [20].

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https://doi.org/10.1016/j.ijbiomac.2019.02.112
0141-8130/

ITO-PET disposable electrode has attracted considerable attention due to its good electroconductivity [21], cheapness and high transparency [22], which identify it as an perfect material for transparent electrodes in flexible, printed electronics and biosensors [23–25].

In this work, a single-use immunosensor based on ITO-PET electrode was constructed for determination of SOX2 antigen. The electrode surface was first silanized by using 11-CUTMS agent. The modified ITO-PET flexible electrodes were incubated with anti- SOX2 antibody. 11-CUTMS concentration, anti-SOX2 concentration, anti-SOX2 and SOX2 incubation period were optimized for development a sensitive immunosensor. CV and EIS were used for examining the immobilization processes in constructed biosensor. Regeneration, reproducibility and repeatability processes were also study for determining the characterization of the biosensor. The constructed biosensor could detect SOX2 protein in a wide detection range. The electrode surfaces belonging to the immobilization steps were morphologically monitored via the SEM technique. The immunosensor was prepared to apply to the human serum and the analysis results were compared with ELISA test. The results were consistent with the ELISA test results and this harmony promisingly demonstrated the biosensor's usefulness in the clinical field.

2. Experimental

2.1. Solutions, samples and reagents

All chemicals and ITO-PET films (the transmittance of the surface: 550 nm (>79%), the resistivity: 60 Ω/square) were purchased from Sigma Aldrich (USA) Company. SOX2 and anti-SOX2 solutions that were prepared with phosphate buffer (pH 7.0; 50 mM) were also bought from Sigma Aldrich and were kept at −20 °C. Commercial ELISA kits were purchased from LSBio LifeSpan BioSciences, Inc.

2.2. Instrumentation

All electrochemical experiments were realised by using a Gamry potentiostat/galvanostat (Reference 600, Gamry Instruments, Warminster, PA, USA) interfaced with a PC via an EChem Analyst. ITO-PET electrode (2 mm × 20 mm), an Ag/AgCl and a platinum wire were utilized as a working electrode, a reference electrode and a counter electrode in three electrode system, respectively.

2.3. Preparation of ITO-PET electrodes before modification

Firstly, the ITO-PET electrodes were sonicated with pure acetone, soap solution and ultra pure water for cleaning procedure, respectively. To obtain a good and stable silanized surface with 11-CUTMS, the electrode surfaces were first hydroxylated. For this reason, the electrodes were treated with a mixed solution including ammonium hydroxide/hydrogen peroxide/ultrapure water (1:1:5, v/v/v) for 90 min. Afterwards, the electrodes were rinsed with ultra pure water and unwatered with pure argon gase. The hydroxylated electrodes were dipped in 11-CUTMS (0.5%) solution (in toluene and ethanol) overnight. Then the electrodes were gently washed with water and dried again with argon gase to remove the physically adsorbed 11-CUTMS molecules.

2.4. Construction of the impedimetric based sensor

After silanization process, the electrodes were immersed in 100 μL of anti-SOX2 solution (10 ng mL^{-1}) for 45 min. The amino groups of anti-SOX2 protein were covalently interacted with cyano ends of 11-CUTMS silanization agent. Afterwards, the electrodes were carefully washed with water and dried with argon gase as gently as possible. The electrodes were incubated in BSA (0.5%) blocking agent to block the functional active cyano ends after immobilization. After these processes, the electrodes were stored at +4 °C until use. The bare and modified electrode surfaces were stepwise named as ITO, ITO-OH, ITO/11-CUTMS, ITO/11-CUTMS/anti-SOX2, ITO/11-CUTMS/anti-SOX2/BSA. The fabrication process is shown in Scheme 1.

2.5. Electrochemical measurement parameters

EIS and CV techniques were utilized for electrochemical detection and experimental characterization. All measurements were performed in 5 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (1:1) (ferri ferro cyanide) solution that contains 0.1 M KCl. In CV experiments, the potential range was determined as −0.5 V–1 V (step size: 10 mV; scan rate: 100 mV/s). In EIS, the range of frequency was between 50.000 and 0.05 Hz (points: 5). In Square wave voltammetry measurements, potential scan range was 0–1.2 V (pulse size: 25 mV). The morphologies of the electrode surfaces in each step were investigated by using Scanning Electron Microscopy (FEI-Quanta FEG 250 model) at the Scientific and Technological Research Center of Namık Kemal University (NABILTEM).

3. Results and discussions

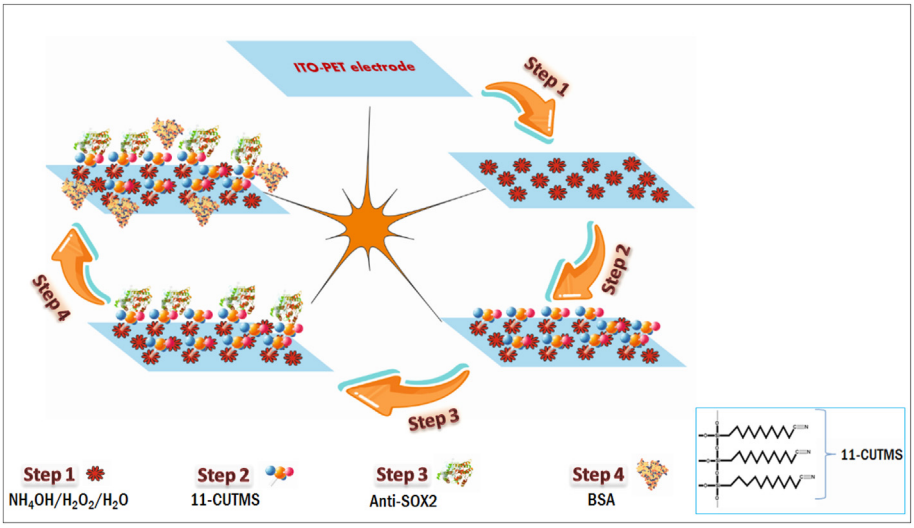
3.1. Electrochemical characteristics of the modified electrodes

In the biosensing system for SOX2 detection, the stable self assembled monolayer was formed by 11-CUTMS on the hydroxylated ITO-PET surface. 11-CUTMS is one of the most useful organo-functional silanes to prepare the cyano functionalized SAM on the electrode surface. 11-CUTMS has –OCH3 group at one end that can interact with the formed hydroxyl groups and this agent can be covalently bound to the amine groups of the antibody via cyano groups at the other end. After the incubation of the electrodes with 11-CUTMS overnight, anti-SOX2 solution was immobilized on the modified electrode surface. Then, BSA was utilized for blocking if there were active cyano groups which did not interact with the amine groups of the anti-SOX2.

Two basic techniques have been used in all immobilization phases: EIS and CV. Ferri ferro cyanide solution which provides electron transport over the electroactive species on the surface easily was utilized as a redox probe [26]. The EIS spectra and CV voltammograms are seen in Fig. 1.

In Fig. 1A, EIS spectra for immobilization steps are shown. An increase in the impedance curve semi-diameter was observed after the hydroxylated electrode surfaces were incubated with the silanization agent. Formation of SAM with 11-CUTMS on the surface resulted in hydrophobic interactions due to the long aliphatic chain portion. And also, the negatively charged -CN ends of 11-CUTMS agent pushed the negatively charged ferri ferro cyanide solution. As a result, the redox probe became difficult to diffuse to the surface, the resistance and therefore the diameter of semicircle increased. Then, anti-SOX2 was immobilized on the electrode surface via the covalent attachment of cyano groups of 11-CUTMS and the amino groups. The formed organic structure was a secondary ketimine and this immobilization step was reflected in the EIS spectrum as a significant increase in the charge transfer resistance. It proved that the layer was successfully modified with anti-SOX2 and insulating protein layer was formed and thus the diffusion of the redox probe became more difficult. After covalent immobilization of the antibody, BSA protein was used to block the unbounded cyano ends. The diffusivity of the redox probe became even more difficult and the Rct value was increased as the surface became more insulated.

In Fig. 1B, CV voltammograms belonging to the immobilization steps are seen. Because of the extremely high electric resistance, the anodic and cathodic peaks of bare electrode could not be observed. By allowing the electrodes to incubate with the 11-CUTMS solution after the hydroxylation process, anodic and cathodic peak currents significantly decreased. The reason for this decrease was the negatively charged -CN groups of 11-CUTMS solution. These groups prevented the diffusivity of the ferri ferro cyanide redox solution to the electrode surface. After anti-SOX2 antibody immobilization, the redox peaks decreased even



Scheme 1. Systematic diagram of immobilization steps.

more due to the decrease of the charge transfer rate. This was because of an increase of the biolayer thickness generated on the electrode surface. And finally, cathodic and anodic peak currents further reduced by the treatment of BSA blocking agent.

A Randles equivalent circuit model which includes charge transfer resistance (Rct), Warburg impedance (Zw) and ohmic resistance (Rs) in association with the resistance of electrolyte solution between reference electrode and ITO-PET working electrode is given in Fig.1A [27]. Zw and Rs are not affected from immobilization procedures of SOX2 immunosensor but Rct is affected from dielectric properties in the electrode/electrolyte interface [28,29].

3.2. Optimization of experimental conditions

To obtain an optimal and stable electrochemical signal, efficient parameters such as the incubation period and concentration of anti-SOX2,

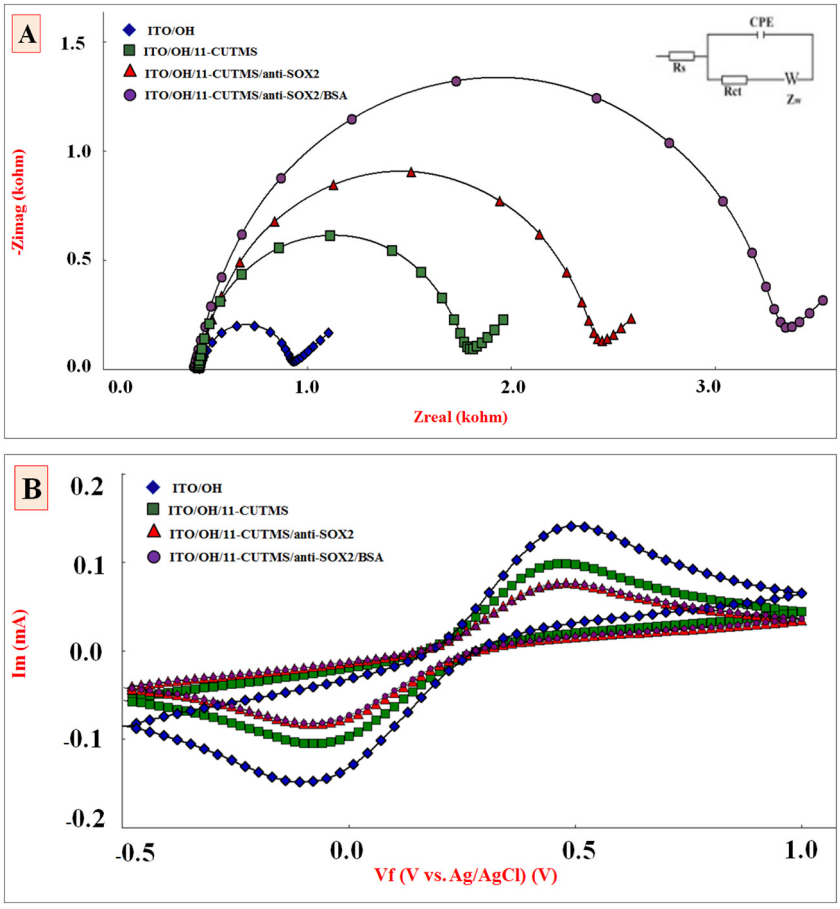


Fig. 1. A) EIS spectra and B) cyclic voltammograms for the immobilization steps of the constructed biosensor blue (-♦-♦-): ITO/OH, green (-■-■-): ITO/OH/11-CUTMS, red (-▲-▲-): ITO/OH/11-CUTMS/anti-SOX2, purple (-•-•-): ITO/OH/11-CUTMS/anti-SOX2/BSA.

the concentration of 11-CUTMS and the incubation time of SOX2 protein were optimized. Firstly, the effect of the 11-CUTMS concentration was discussed. Based on the taken results, with the increasing concentrations of 11-CUTMS solution from 0.1% to 1%, Rct values increased and then decreased and the maximum Rct values were observed at 0.5% 11-CUTMS concentration. Therefore, 0.5% 11-CUTMS concentration was used as optimum concentration. SOX2 calibration curves for this optimization step are illustrated in Fig. 2A.

After the optimization of 11-CUTMS silanization agent concentration, the antibody concentration was optimized. For this purpose, 3 different anti-SOX2 concentrations (2 ng mL^{-1}, 10 ng mL^{-1} and 50 ng mL^{-1}) were discussed and the best response was observed when the modified electrode was incubated in 10 ng mL^{-1} of anti-SOX2 protein. At higher concentrations, electrode surface degeneration probably started. Therefore, 10 ng mL^{-1} of anti-SOX2 solution was selected as optimum one. SOX2 calibration graphs for this optimization step are given in Fig. 2B.

Another critical parameter for a linear and sensitive immunosensor is the incubation time of anti-SOX2 antibody. The electrodes were incubated with anti-SOX2 solution for various times (30, 45, 60 min). The charge transfer resistance decreased at lower incubation period (30 min) compared with the resistance at 45 min. The highest response was taken by incubation of the electrode in anti-SOX2 solution for 45 min. Actually, the similar response was obtained when the electrodes were incubated in antibody for 60 min. But we preferred to chose 45 min for saving on time. SOX2 calibration curves can be seen in Fig. 2C.

After all parameters were optimized, the incubation time of SOX2 antigen which is the other extremely important step was examined. Three periods (30, 45, 60 min) were studied for this purpose and the highest response was obtained when the electrode was incubated in SOX2 protein for 30 min. The calibration curves for this step are shown in Fig. 2D.

3.3. Performance of the biosensor

The analytical characterization of the proposed biosensor was detected by the analysis of different concentrations of SOX2 protein in optimum circumstances by EIS method. A linear calibration graph was obtained with different SOX2 concentrations and biosensor response. The following equation is used to obtain the calibration graph.

$$\Delta Rct = Rct\,(anti{-}SOX2/SOX2) - Rct\,(anti{-}SOX2)$$

Rct (anti-SOX2/SOX2) is the charge transfer resistance belonging the binding of SOX2 and anti-SOX2; Rct (anti-SOX2) is the charge transfer resistance when the electrode was immobilized with anti-SOX2 protein.

In Fig. 3, the impedance spectra, CV voltammograms and calibration graph of designed biosensor are shown. As can be seen in Fig. 3A, the resistance and the semicircle diameter of the impedance curves increased with the increasing SOX2 antigen concentrations. A wide linear detection range (0.2 pg mL^{-1}–20 pg mL^{-1}) was exhibited with the proposed biosensor and the regression coefficient (R squared) was calculated as 0.9957 (Fig. 3C). LOD (Limit of detection) and LOQ (Limit of quantification) values were found by using $3{*}S_{blank}/m$ and $10{*}S_{blank}/m$ equations [30], correspondingly. S_{blank} is the standard deviation and m is the slope.

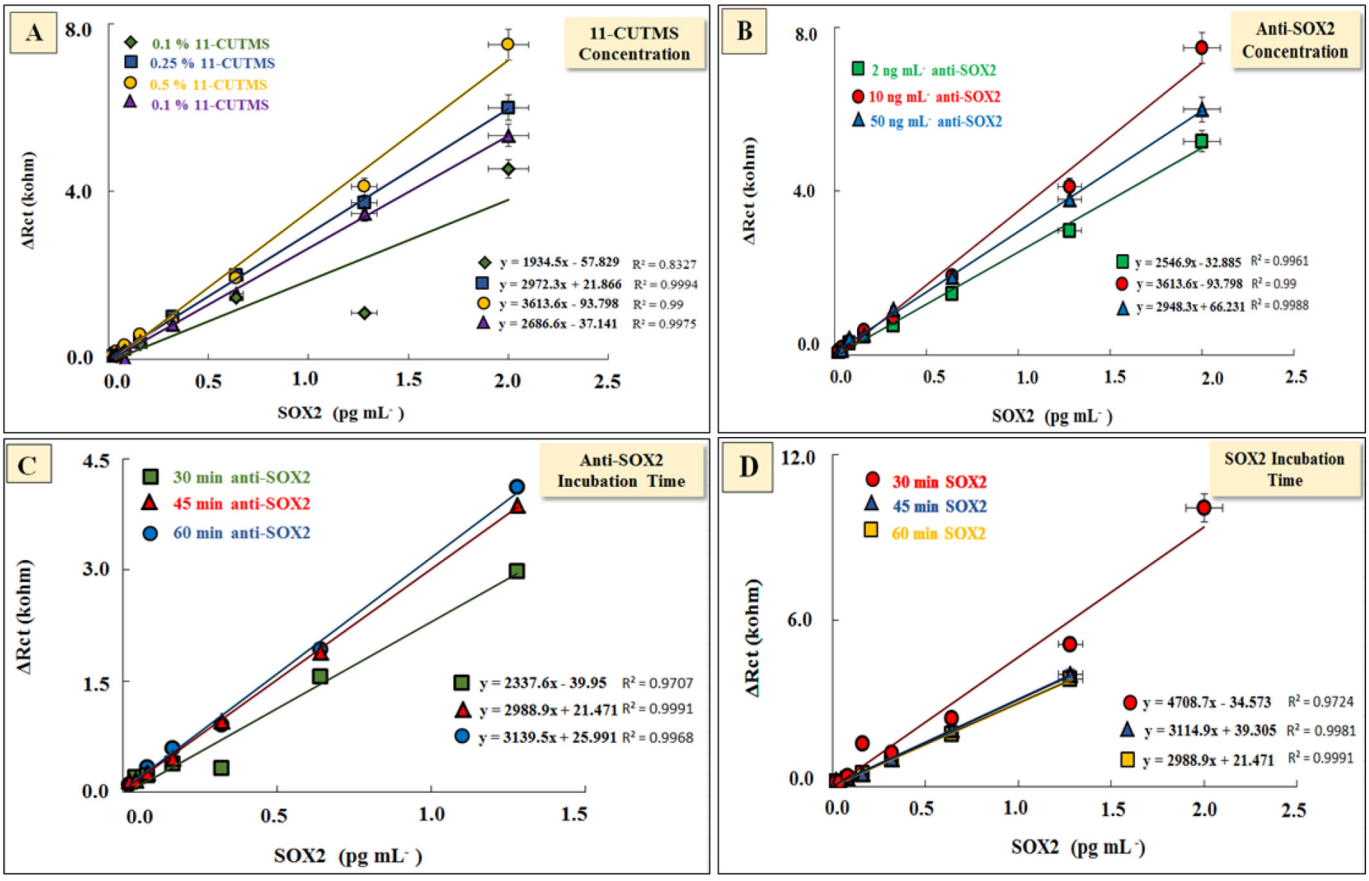


Fig. 2. Optimization steps for the proposed biosensor. A) The effect of the 11-CUTMS concentration to the biosensor response [green (-♦-♦-): 0.1% 11-CUTMS, blue(-■-■-): 0.25% 11-CUTMS, yellow (-•-•-): 0.5% 11-CUTMS, purple (-▲-▲-): 1% 11-CUTMS] B) the effect of the anti-SOX2 concentration to the biosensor response [green (-■-■-): 2 ng mL^{-1} anti-SOX2, red (-•-•): 10 ng mL^{-1} anti-SOX2, blue (-▲-▲-): 50 ng mL^{-1} anti-SOX2] C) the calibration curves for the optimization of the anti-SOX2 incubation time [green (-■-■-): 30 min incubation time of anti SOX2, red (-▲-▲-): 45 min incubation time of anti SOX2, blue (-•-•-): 60 min incubation time of anti SOX2] D) the calibration curves for the optimization of the SOX2 antigen incubation time [red (-•-•-): 30 min incubation time of SOX2, blue (-▲-▲-): 45 min incubation time of SOX2, yellow (-■-■-): 60 min incubation time of SOX2].

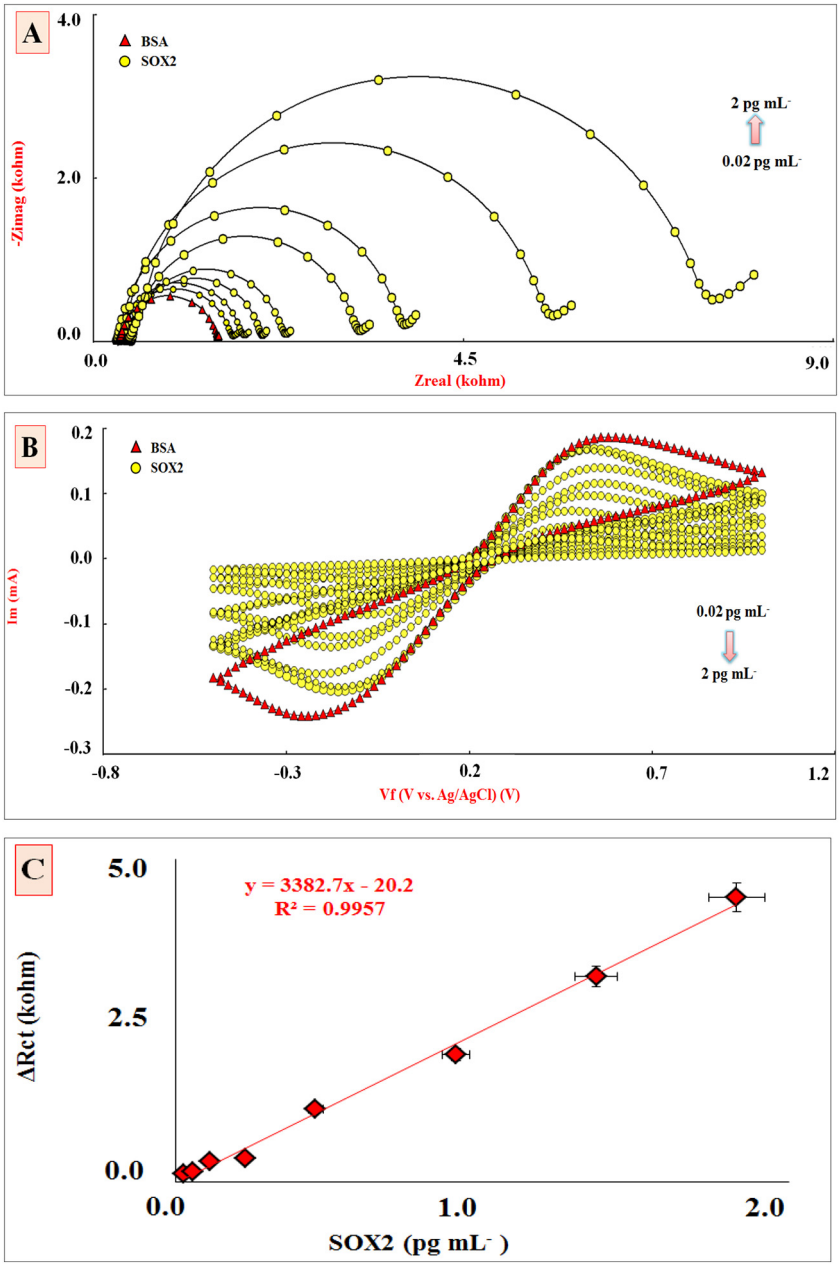


Fig. 3. The study for detection the linear range of the proposed biosensor. (A) Impedance spectra acquired from different SOX2 concentrations (B) CV voltammograms obtained from different SOX2 concentrations [red (-▲-▲-): BSA, yellow (-•-•-): SOX [2] (C) SOX2 linear graph plotted by the impedance differences extracted from (A).

LOD and LOQ values were found as 0.013 pg mL^{-1} and 4.44 pg mL^{-1} by using these equations.

When looking at the CV voltammograms (in Fig. 3B), it was clearly seen that the peak currents decreased with increasing SOX2 concentrations because each increasing SOX2 concentration created a nonconductive influence on the surface and hindered the diffusion of the ferri ferro cyanide solution to the electrode surface.

One of the most important and needed features for a sensitive biosensor is repeatability. To investigate the repeatability of the proposed immunosensor, 20 different electrodes prepared under the same conditions were incubated with the same concentrations of SOX2 protein (0.32 pg mL^{-1}). All modified electrodes showed similar electrochemical response. The mean value, the coefficient of variation and the standard deviation were calculated as 0.33206 pg mL^{-1}, 3.42% and 0.010958 pg mL^{-1}, respectively.

Another important parameter for obtaining a sensitive immunosensor is reproducibility. Therefore, 10 different biosensors were constructed in the same experimental conditions and 10 different calibration graphs (in Fig. 4A) were obtained. All prepared immunosensors showed similar linearity to detect the SOX2 protein (from 0.02 pg mL^{-1} to 2 pg mL^{-1}). The relative standard deviations for the slope and intercept values calculated by using the equations of these 10 different calibration graphs were found as 0.96% and 4.19%, respectively.

SWV was also utilized to evaluate the performance of the proposed biosensor. Similar to the conditions in EIS measurements, the ITO-PET electrodes were prepared and increasing concentrations of SOX2 antigen were carried out to the biosensor. The measurements were taken in ferri ferro cyanide redox probe like EIS and CV. A calibration graph based on the change in peak current in SWV is illustrated in Fig. 1S.

3.3.1. Reusability, storage life and Kramers-Kronig transform for the proposed immunosensor

As mentioned earlier, the proposed ITO-PET based immunosensor is low-cost [31]. However, even so, it is still a great advantage that the

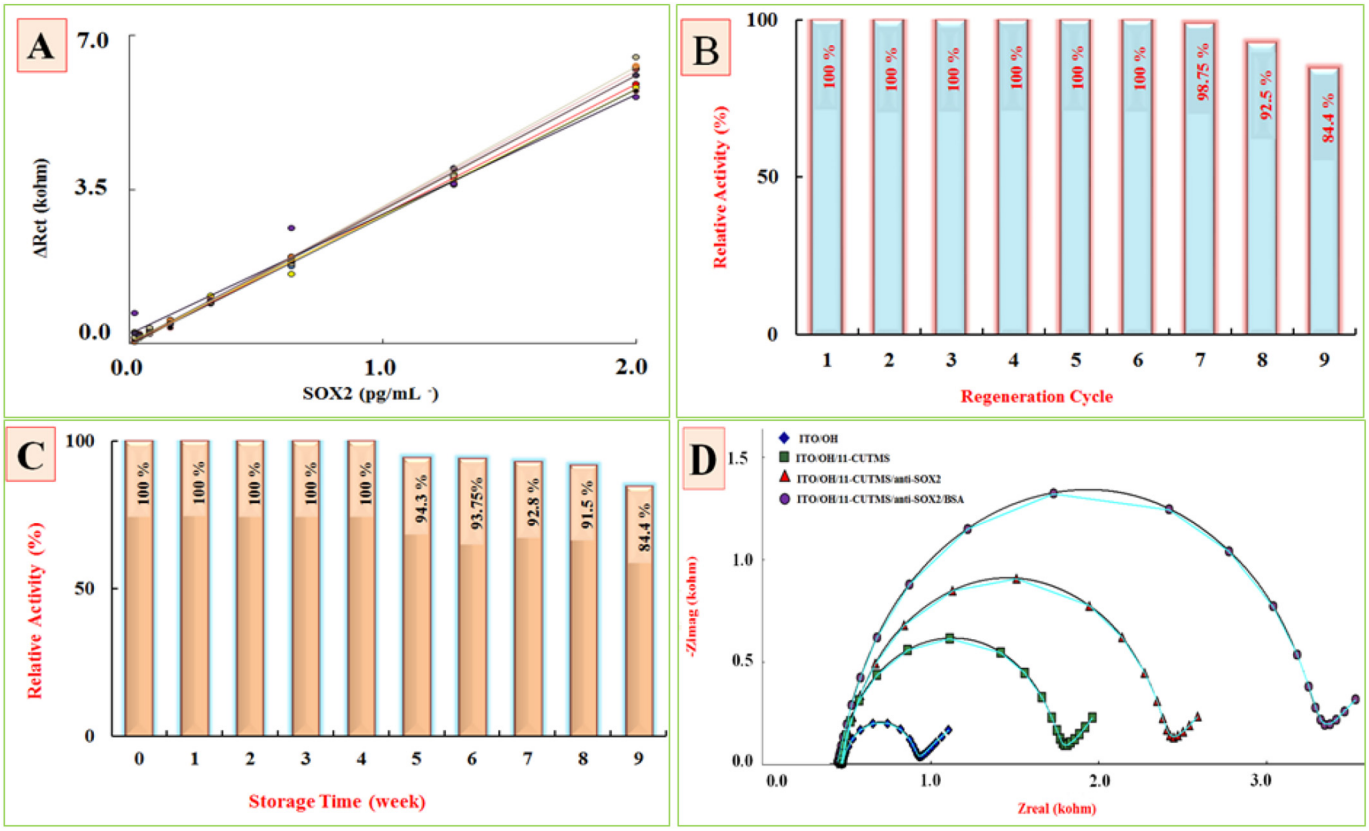


Fig. 4. Analytical studies of the proposed immunosensor. A) The calibration curves obtained from the reproducibility study B) results of reusability C) storage stability of the immunosensor D) Kramers-Kronig Transforms of designed biosensor based on anti-SOX2.

electrode can be reused for clinical use. In order to determine the reusability of the designed biosensor, the electrode was first prepared under optimum conditions and incubated with SOX2 protein. After the first impedance measurement, the electrode was incubated in 0.1% HCl solution (10 mM) for 5 min to eliminate the weak interactions such as hydrogen bonds and Van der waals interactions between anti-SOX2 and SOX2 solution. Impedance measurement was again performed and the Rct value decreased as expected. Then, the electrode was again incubated with the SOX2 antigen at the same concentration with the previous step. Looking at the EIS curve this time, it was detected that the Rct value rose. This process could be repeated 9 times in this way and after ninth acid treatment, the surface became to be damaged. The proposed biosensor was able to save 84.4% of its initial activity (Fig. 4B). However, it completely lost its activity in the later process.

Detecting the storage life of the ITO based immunosensor is an important procedure for clinical use. It is also commercially advantageous to be able to develop a biosensor with long shelf life. 10 different electrodes were prepared under the same conditions in order to determine the storage life of the proposed immunosensor and kept at 4 °C. One of the prepared electrodes was incubated with SOX2 protein (0.32 pg mL^{-1}) every week and EIS measurement was examined. After the tenth week, the constructed biosensor lost 15.6% of its activity to determine the SOX2 antigen (Fig. 4C). However, a response could not be taken from the biosensor in the eleventh week. The biosensor's ability to protect 84.4% of its activity over a 10-week period is a positive result.

To evaluate the impedance data quality, Kramers-Kronig (K_K) transform was used [32]. It is utilized to detect whether the impedance spectra of the developed biosensor are affected from the diversion occured from external factors. In Kramers-Kronig transform, reel part of impedance data is calculated with using imaginative part or imaginative part is calculated with using reel part [33]. This K_K method is excessively considerable to comment and investigate the EIS [34]. Fig. 4D illustrates the plots of Kramers-Kronig transform. The goodness of fit values for step-by step biosensor surfaces are given in Table 1.

3.3.2. SEM images of step-by-step immobilization process

All immobilization steps were observed via the Scanning Electron Microscopy. The morphological properties of the step-by-step modification of the proposed immunosensor can be seen by using SEM

Table 1
The results of Kramers-Kronig transforms and real human serum samples analyses.

The results of Kramers-Kronig transforms	
The surface of biosensor	Goodness o fit values
Kramers-Kronig transforms for different layers of the anti-SOX2 based biosensor	
ITO/OH	4.618u
ITO/OH/11-CUTMS	635.5n
ITO/OH/11-CUTMS/anti-SOX2	290.1n
ITO/OH/11-CUTMS/anti-SOX2/BSA	48.05n
ITO/OH/11-CUTMS/anti-SOX2/BSA/SOX2	1.248u

Comparison of the results obtained by using ELISA and the proposed immunosensor

Sample	ELISA (ng mL^{-1})	The proposed biosensor (ng mL^{-1})	Difference
1	4.12	3.98	3.4
2	3.077	3.23	4.74
3	2.71	2.68	1.2
4	2.71	2.61	3.69
5	2.42	2.36	2.48
6	3.14	3.06	2.55
7	3.29	3.33	1.2

characterization technique in Fig. 5. Fig. 5A shows the bare and smooth electrode surface. When the electrode surface was hydroxylated, it had a homogeneous appearance as seen in Fig. 5B. The morphological change on the surface after modification with 11-CUTMS is observed in Fig. 5C. The surface appearance of the modified electrode surface became pea-like by covalent immobilization of the antibody (Fig. 5D). After BSA blocking agent treatment, the electrode surface became denser as seen in Fig. 5E. After interaction between anti-SOX2 and SOX2, the electrode surface had a view as seen in 5F.

3.3.3. SOX2 detection in real human serum

Lastly, seven different serum samples were taken from Namık Kemal University Research Hospital (by ethics committee decision) in order to test the applicability of the sensor to real serum samples. Serum samples (with 10.000 dilution) were analyzed with 7 different biosensors that were optimally prepared. At the same time, the SOX2 detection was performed in real human serum by using the ELISA technique (the detection range for SOX2: 0.312 ng mL^{-1}–20 ng mL^{-1}). As is known, ELISA is an effective technique for assaying antigen and antibody concentration. However, this method is both time-consuming and expensive when compared to immunosensors. When we looked at the results, it was seen that the results obtained with our biosensor were in accordance with the standard ELISA results (Table 1). Similar results have shown that the proposed biosensor, a cheap and sensitive system, has succeeded the determination of SOX2 protein in real human serum samples. Moreover, Kruskal Wallis test (at a 95% confidence interval) was used to determine whether there were statistically significant differences between the analysis results obtained by two methods for 7 different human serum samples. This non-parametric test technique was utilized because the sample number was <30. As a result of the test, p value was calculated as 0.4232 (chi-squared = 6). Since the p value was >0.05, it was found that the difference between the results was not statistically meaningful. Thus, the results obtained with two methods were coherent with each other. And also, Box plot graphs for analysis results with two methods are given in Fig. 2S. When the graphs were examined, it was seen that the averages and the distributions were very close to each other for both methods.

4. Conclusion

In this study, a single-use and low-cost ITO-PET based immunosensor achieved to detect SOX2 antigen successfully. The sizes

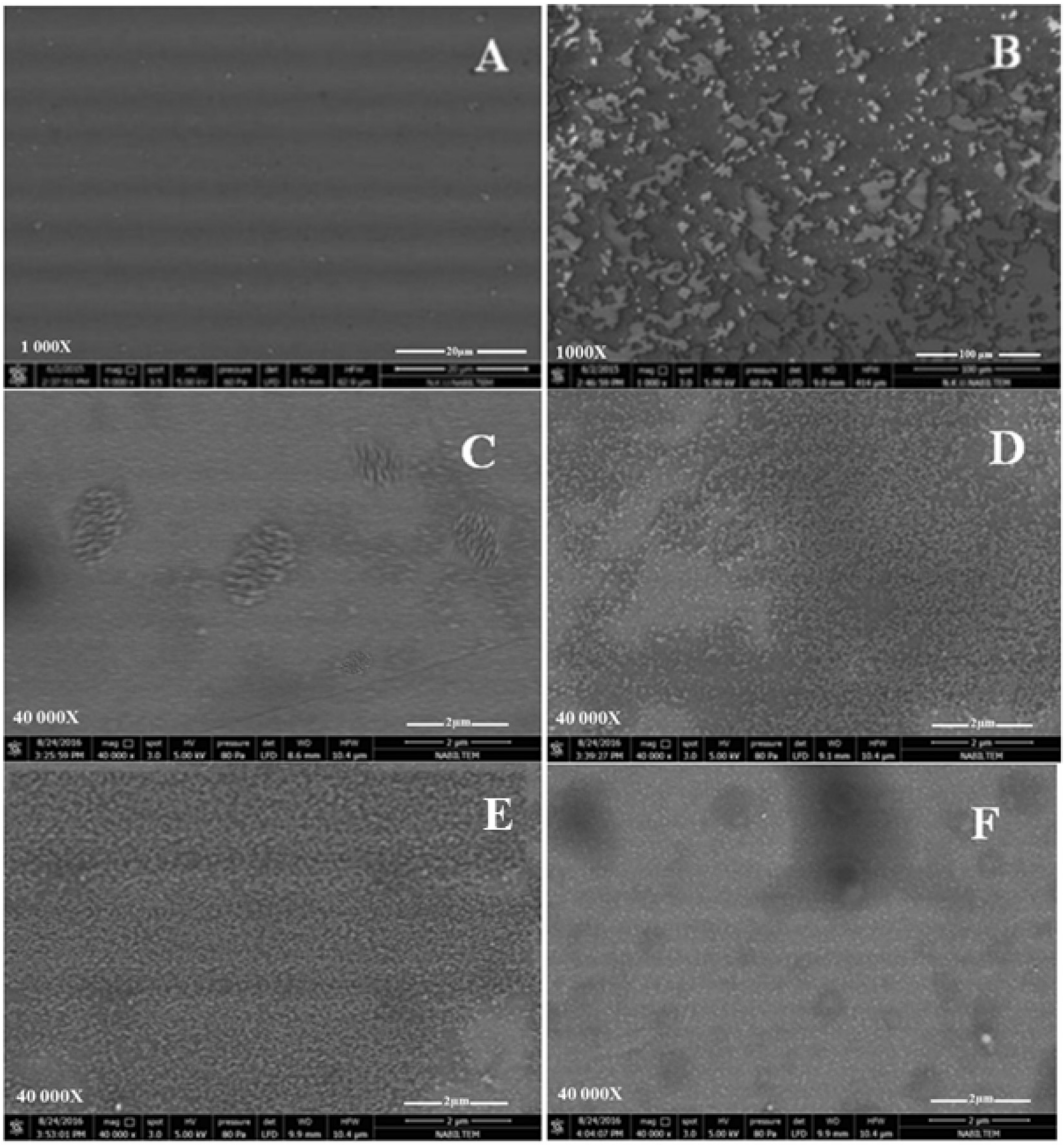


Fig. 5. SEM images of the different electrode surfaces obtained from the fabrication of the biosensor A) bare ITO B) ITO/OH C) ITO/OH/11-CUTMS D) ITO/OH/11-CUTMS/anti-SOX2 E) ITO/OH/11-CUTMS/anti-SOX2/BSA F) ITO/OH/11-CUTMS/anti-SOX2/BSA/SOX2.

of ITO- PET sheets are 1 ft. × 1 ft. × 5 mil. One package contains 5 sheets. The price of a package is 112 euros. The electrodes were cut to size 2 × 0.5 cm. It is clearly shown that an electrode is very cheap (about 0. 021 euros). 11-CUTMS agent generated a great stable monolayer for immobilization of biorecognition element on the electrode surface. The use of 11-CUTMS to generate SAM provided some advantages such as the chemical structure of the agent, the possibility of practical measurement, and no need for cross-linking. And also, the studies in which 11-CUTMS is utilized for SAM formation in biosensing systems are quite rare. The optimization parameters which are necessary to obtain a sensitive and linear immunosensor were analyzed. By using the SEM technique, we observed the electrode surface for each step. Thanks to a good modification of the electrode surface, the biosensor determined SOX2 antigen at low concentrations (0.02 pg mL^{-1}–2 pg mL^{-1}). The constructed biosensor was tried for real human serum and the datas were compared with ELISA test datas. The results were in harmony with each other and this proved that the biosensor could be applied to the human serum and it is undoubtedly very advantageous for clinical use. And also, Kruskal Wallis test was utilized to determine the meaningfulness of the differences between the analysis results obtained with the biosensor and ELISA test for real human serum. It was determined that the difference between the results was not significant and the results were compatible with each other because the calculated p value was >0.05.

Acknowledgment

This study was financially supported by TÜBİTAK (The Scientific and Technological Research Council of Turkey, (Project Number: 113 Z 678), this assistance is greatly acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2019.02.112.

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