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A sensitive and disposable electrochemical immunosensor for detection of SOX2, a biomarker of cancer

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ABSTRACT

A novel, sensitive, disposable indium tin oxide (ITO)-based electrochemical immunosensor was developed firstly for simple, rapid determination of Sex-determining region Y-box 2 (SOX2). SOX2 is a cancer biomarker and used for detecting small cell lung cancer, lung adenocarcinoma, squamous cell carcinoma, skin cancer, prostate cancer, and breast cancer. In this study, a disposable ITO thin film based electrode was used as working electrode for biosensing the interaction between SOX2 antigen and anti-SOX2 antibody. In this study, carboxyethylsilanetriol (CTES) was also utilized for electrode modifying so as to obtain self-assembled monolayers. The formed self-assembled monolayers were activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)/N-hydroxysuccinimide (NHS) chemistry and they were used as a heterobifunctional crosslinker and activator, respectively. Anti-SOX2 antibody was used as a biorecognition molecule and was covalently immobilized onto the ITO thin film modified with CTES. Immobilization steps were characterized by electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV), fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM) and atomic force microscopy (AFM). The optimum immobilization conditions such as antibody concentration, antibody and antigen incubation times were examined for the best sensitivity of the immunosensor. Under optimal conditions, this immunosensor had a wide linear detection range (25 fg/mL–2 pg/mL) with a detection limit as low as 7 fg/mL SOX2. Furthermore, the developed SOX2 immunosensor had good storage stability (79.36% of initial activity after 9 weeks), repeatability (3.88% of RSD) and reproducibility (4.25% of RSD). Our developed immunosensor has an acceptable performance for detection of SOX2 antigen, exhibits low detection limit, and has selective and reproducible results in immunoreaction analysis.

1. Introduction

Searching for serological markers, SOX (Sex-determining region Y-box) proteins are defined as immunogenic antigens in different cancer types. SOX proteins are parts of the Sry-like high mobility group super family of developmental transcription factors [1]. Over the last two decades, ~20 genes of the SOX gene family have been defined and classified into groups depending on their protein specificity. They are SOX-B1 (SOX1, SOX2 and SOX3), SOX-B2 (SOX14 and SOX21), and SOX-C (SOX4, SOX11 and SOX12) families. In 1994, the SOX2 protein was explored and characterized in humans. The SOX-B1 family is located on chromosome 3q26.3-q27 and encodes a protein contains 317 aminoacids. SOX2 is comprised of three main domains: N-terminal, HMG (high mobility group) and transactivation domain [2]. SOX2 helps the regulation of cell pluripotency and is closely related to early embryonic development, neural and sexual differentiation. The occurrence and development of malignant tumors indicate that there is a relationship between SOX2 amplification, overexpression and tumor formation. Different studies proved that SOX2 is amplified and overexpressed in various malignant tumors, including lung [1–3], breast [4,5], esophageal cell [3], squamous cell [6,7], colon [8], ovarian [7,9], glioblastoma [2,3], cervical [9], prostate [10], pancreatic cancer [9], gastric cancer [3,9], ameloblastic carcinoma [11], head and neck squamous cell carcinoma [7], cutaneous squamous cell carcinoma [12], testicular germ cell tumor [13]. SOX2 antigens were determined by using immunohistochemical assay [4,9] and real-time fluorescent quantitative polymerase chain reaction [14]. The level of the serum in human embryonic stem cells was found as 8–25 μg/mL. According to our knowledge, this is the first study to quantify serum SOX2 by using a biosensor.

Biosensors are alternative tools for fast and low-cost detection of an analyte with desired sensitivity, selectivity and they have been used for simultaneous monitoring of numerous cancer biomarkers. Several

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biomolecules such as enzymes, antibodies, nucleic acids and other biological/synthetic molecules have been widely utilized as biorecognition element to fabricate biosensors for specific analyte detection with desired specificity [15]. Electrochemical immunosensors have received considerable attention owing to their sensitivity, simplicity, cost-effective production [16]. Electrochemical examination of antigen-antibody interactions can allow for a fast, sensitive, selective and cheap analysis for the detection of cancer biomarker. The electrochemical immunosensor are assayed more easily and rapidly compared to the conventional method such as ELISA. Electrochemical impedance spectroscopy (EIS) is a valuable and sensitive technique to investigate the interfacial features of the modification steps of electrode [17,18]. Beside these, effective identification of membranes can be monitored by using EIS technique. Enzyme-substrate reactions, antibody-antigen interactions, receptor-ligand interactions and probe DNA-target DNA hybridizations can follow successfully by using EIS technique [19]. Impedimetric immunosensors have attracted increasing attention during the recent years, because they are able to direct and label-free analyses and can be an option to analysis for multi-analytes. Additionally, they provide rapid analysis and do not require skilled person [20].

Single frequency impedance (SFI) technique is important for monitoring biorecognition events and interactions such as between antibody-antigen, and also between probe DNA-target DNA interactions. It measures the impedance at a fixed frequency against time. In this study, SFI response was observed during SOX2 incubation period [21,22].

Square wave voltammetry (SWV) is a sensitive electrochemical technique which has been usually utilized as detection method [23]. Among all the voltammetric techniques, SWV has many benefits, such as the sensitive and fast analysis. This technique has been applied for monitoring chemical reaction and determination of some analytes at trace levels [24].

The usage of indium tin oxide (ITO) electrodes as transducer has attracted considerable interest owing to its outstanding properties, such as high electrical conductivity, wide electrochemical working range, good substrate adhesion, stable electrochemical working window and low cost [25–27]. These unique properties make possible the usage of ITO electrode as a transducer for biosensing applications. Transducers have an important role in the fabrication of a successful immunosensor. It ensures an effective layer for the immobilization of biorecognition elements and is used to monitor of an electrical signal resulting from an electrochemical reaction on the electrode surface, on which potential, current and frequency are exposed. The incomparable features of a transducer help to improve the characteristics of an immunosensor [28]. Various studies direct the researcher to utilize ITO based electrodes to use transducer for development of optic and electrochemical biosensors, microfluidic lab on-chip biosensors and electrochemiluminescence analysis [27].

Self-assembly is a method that has attracted significant interest in physics, chemistry and biology. It provides a convenient and simple way of creating a highly ordered thin molecular film with tailored chemical and electrochemical properties. In a self-assembly process, an ultra-thin molecular layer is formed by adsorption of molecules from solution onto a solid surface. Then the adsorbates spontaneously arrange themselves until finally a completely ordered molecular monolayer is formed, a so-called self-assembled monolayer (SAM) [29]. SAM have been used for developing biosensors, microassays, biochips and molecular switches. The SAM formation provide suitable platform to biomolecule immobilization. SAM with different functional ends bind to biomolecule rapidly. In this study, biorecognition elements, anti-SOX2 antibodies, were immobilized on the ITO electrode via a self-assembled monolayer of epoxysilane [27]. Different researchers performed similar methods including cleaning and pretreatment to form a surface of hydroxyl groups to prepare ITO electrode. 3-Isocyanatopropyltriethoxysilane (IPTES) [30], 3-aminopropyltriethoxysilane (APTES) [31–34], N-(2-aminoethyl)-3-aminopropyl-trimethoxysilane [35], 3-mercaptopropyl trimethoxysilane (3-MPTMS) [36], 3-glycidoxypropyldimethoxysilane [20] were used for SAM formation on the ITO electrode in literature. Carboxyethylsilanetriol (CTES) is another silane agent containing carboxyl groups [24,36,37]. It has been used for construction of biosensor material such as boron-doped diamond electrode [38], silicon-based biosensor [37]. Additionally, CTES was used as an immobilization matrix firstly.

In this study, the enhanced electrochemical detection of SOX2, a cancer marker, was performed for the first time in literature. A new platform to immobilize the anti-SOX2 antibody was constructed by assembling CTES on ITO electrode to produce a high effective electrode surface. The SOX2 antigen was analysed by using three different methods; EIS, SFI and SWV. Apart from these electrochemical techniques, SEM was used to identify the layer after immobilization steps. During the study the fabrication parameters of the new immunosensor such as antibody - antigen concentration and antibody-antigen binding period were also optimized.

2. Material and methods

2.1. Reagents and materials

All reagents, which are utilized in the study, were of analytical grade and were obtained from Sigma-Aldrich (St. Louis, M.O., USA). ITO-coated Polyethylene Terephthalate (PET) films, which have a surface resistivity of 60 Ω/square and the transmittance 550 nm (> 79%), and biorecognition elements (anti-SOX2 antibody and SOX2) were obtained from Sigma-Aldrich. Anti-SOX2 antibody, SOX2 and bovine serum albumin (BSA, 0.5%) were prepared by using phosphate buffer (50 mM, pH 7.0) and were kept at −20 °C. A redox probe solution, which contained 1 M KCl, 5 mM $[Fe(CN)_6]^{4-}$, 5 mM $[Fe(CN)_6]^{3-}$ in phosphate buffer (50 mM, pH 7.0) was prepared. Commercial ELISA kit was purchased from LSBio LifeSpan BioSciences, Inc.

2.2. Instrumentation

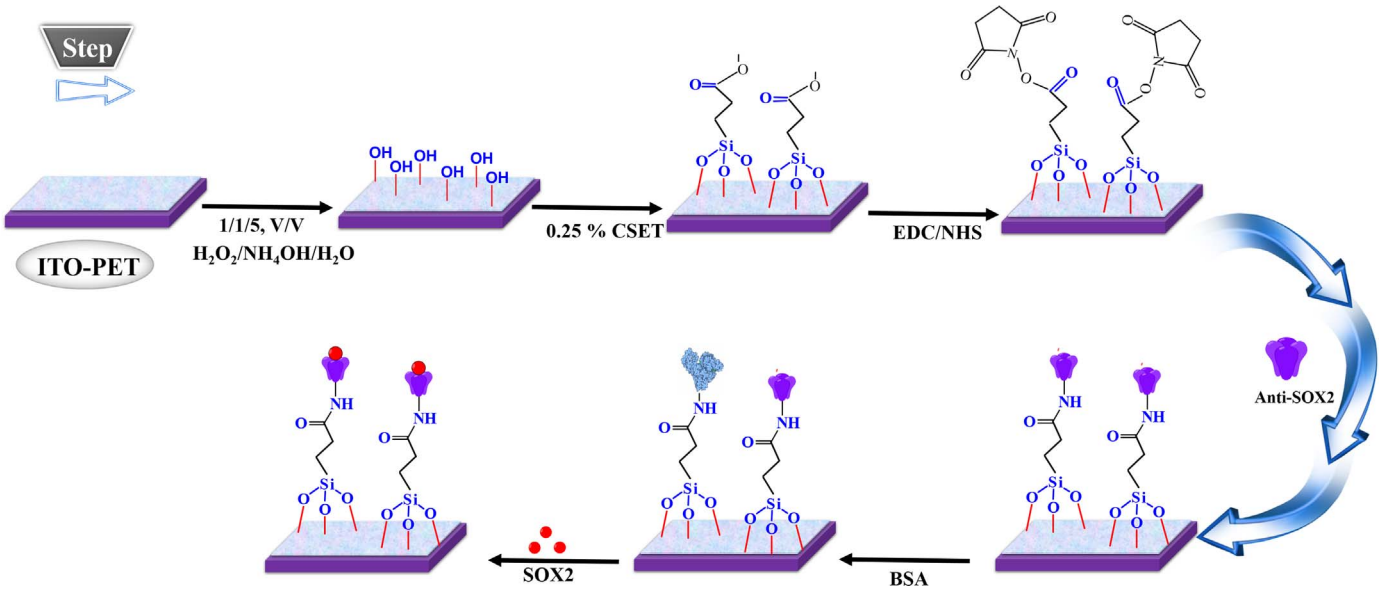
Electrochemical measurements were performed via a Gamry Potentiostat/Galvanostat (Reference 600, Gamry Instruments, Warminster, PA, USA) connected with a computer with an EChem Analyst software. Three-electrode system, consisting of a Ag/AgCl (saturated with KCl) as reference electrode, a platinum wire as a counter electrode and a modified ITO electrode (2×20 mm) as a working electrode, was used in this work. Reference electrode and counter electrode were purchased from BASi (West Lafayette, IN, USA).

2.3. Preparation of electrodes before modification

The ITO electrodes were prepared by ultrasonic washing in an acetone, soap solution and ultra-pure water for 10 min each. Then they were dried at room temperature under argon gas for future use. Afterwards electrodes were dipped into a mixed solution containing ultra-pure water/hydrogen peroxide/ammonium hydroxide (5:1:1, v/v) for 90 min to obtain hydroxylated active ITO surfaces in dark conditions. Then, they were rinsed carefully with ultra-pure water and dried under argon gas. The hydroxylated ITO electrode was dipped in ultra-pure water containing 0.25% CTES (v/v) at room temperature overnight. After carefully washing with ultra-pure water, the CTES modified ITO electrodes were dried under argon gas. Prior to immobilization of an antibody, electrode surfaces should be activated by use of EDC/NHS chemistry. The CTES modified ITO electrodes were activated using EDC (0.04 M) as coupling agent and NHS (0.01 M) as an activator.

2.4. Design strategy of the impedimetric SOX2 biosensor

After washing the unbound EDC/NHS with ultra-pure water, electrodes were immersed into the PBS buffer solution (pH 7.0)



Scheme 1. Fabrication protocol of the biosensor.

containing SOX2 antibody (5 ng/mL). The amino groups (NH_2) of anti-SOX2 antibodies were bound with carboxyl groups of modified ITO electrode via strong amide bond (CO–NH) formation. To remove loosely bound anti-SOX2 on the electrodes, they were rinsed with ultra-pure water. Subsequently, 0.5% BSA solution was employed as blocking agent to block remaining active sites to prevent nonspecific adsorption. The electrode surface was washed with ultra-pure water after each step to move away adsorbed molecules, which was bound nonspecifically. The fabrication process of the immunosensor is presented in Scheme 1.

2.5. Experimental characterization

The detection process was characterized by cyclic voltammetry (CV), EIS, SWV and SFI techniques. These electrochemical measurements (CV, EIS, and SWV) were carried out in 5 mM $K_3[Fe(CN)_6]$/$K_4[Fe(CN)_6]$ (1:1) solution that contains 0.1 M KCl. CV (Scan rate:100 mv/s, step size:10 mV), EIS (Initial frequency: 50,000 Hz, Final frequency:0.05 Hz, points:5), and SWV (Potential scan range: 0–1.2 V, pulse size: 25 mV) measurements were employed during the experiment. FTIR measurements were performed by using Bruker FTIR (Bruker, Vertex 70 ATR, Germany) equipped with diamond. FTIR spectra in the range 4000–400 cm^{-1} were recorded in order to investigate the nature of the chemical bonds formed.

2.6. SEM and AFM studies

Scanning electron microscope images were monitored by using a FEI-Quanta FEG 250. The utilized acceleration voltage was 5 kV. AFM analysis was performed using a AFM PLUS+, NanoMagnetic Instruments. The images were obtained using the tapping mode of AFM. The SEM and AFM measurements were performed at the Scientific and Technological Research Center of NKU (NABİLTEM).

3. Results and discussion

3.1. Immobilization of anti-SOX2 antibody onto CTES modified ITO electrode

The fabrication procedure of the proposed label-free ITO immunosensor is illustrated in Scheme 1. The first stage of the process was cleaning of the ITO thin film surface. The aim of the cleaning is to remove the organic contaminants from the ITO surface, which could affect the response of the biosensor. Then, the hydroxyl groups were formed on the ITO electrode. The silane etriol molecule (CTES), which includes an acetate organo group on a silane etriol inorganic reactive end, is reactive with inorganic –OH substrates. The biosensing platform was constructed by assembling CTES on ITO electrode to create a sensitive surface. CTES is also silane-coupling agent containing carboxyl groups. These carboxyl groups were activated by EDC/NHS incubation. The CTES modified active ITO electrode allows an active surface for immobilization of anti-SOX2 antibody. Anti-SOX2 antibodies were immobilized on the ITO electrode through amide bonding with the reaction between the carboxylic group of self-assembled monolayer and the amino group of an antibody. Lastly, the modified electrode was immersed in BSA (0.5%) solution at room temperature to block possible remaining active sites of bioelectrodes. The ITO/CTES/NHS/EDC/anti-SOX2/BSA bioelectrodes were then washed with ultra-pure water and dried under argon gas, then stored at 4 °C until use. In conclusion, we successfully prepared a high sensitive impedimetric immunosensor for SOX2 detection, for the first time.

FTIR spectra obtained after CTES layer formation (black spectrum) and after anti-SOX2 immobilization (red spectrum) are shown in Fig. 1. The black spectrum shows a peak about 3300 cm^{-1}, and this peak belongs to carboxyl groups of CTES. The red spectrum shows a peak about 1651 cm^{-1} and this peak confirms the amide bond indicating immobilization of anti-SOX2 at the ITO electrode surface [35].

In this work, the manufacturing process was characterized by EIS and CV techniques. $[Fe(CN)_6]^{3-/4-}$ was utilized as a redox couple which ensures electronic amplification and facilitate electron transfer between electrolyte solution and ITO thin film surface. Throughout the study, the stepwise fabrication process was observed by using this redox couple.

Fig. 2A exhibits impedance spectra of ITO/CTES/NHS/EDC/anti-SOX2/BSA immunoelectrode at each modification step. The impedance spectra consist of two parts; a semicircle portion at higher frequencies and a linear portion at lower frequencies. The electron transfer resistance (R_{ct}), which equals the semicircle portion, shows the electron transfer kinetics of the redox couple at the electrode interface. Each reaction step of the modified electrode was analysed by EIS and each electrochemical reaction is presented by an electric circuit. A Randles equivalent circuit for these EIS spectra is shown in Fig. 2 (inset). It consists of the solution resistance (R_s), the charge-transfer resistance (R_{ct}), the Warburg resistance (Z_w) and the constant phase element (CPE) [35,38,39]. In this study, impedance values were fitted to

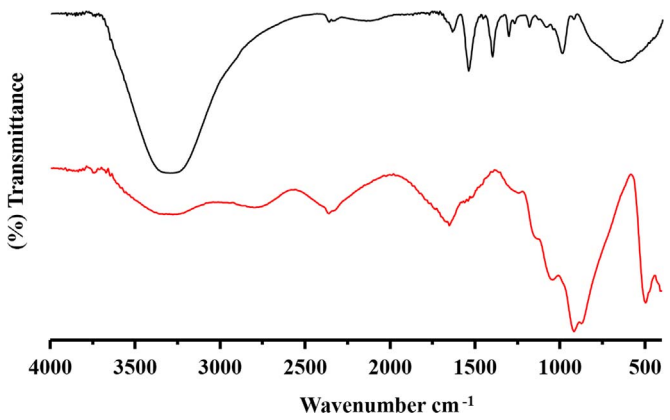


Fig. 1. FTIR spectra after CTES layer formation (black spectrum) and after anti-SOX2 immobilization (red spectrum).

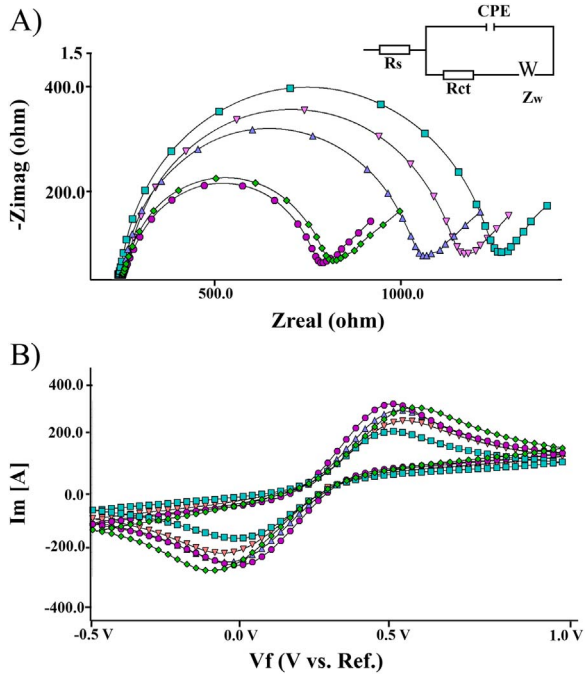


Fig. 2. EIS and CV measurements for step by step fabrication of immunosensor. CVs and EISs were recorded in PBS containing 5 mM $Fe(CN)_6^{3-/4-}$ and KCl 0.1 M. [The Nyquist impedance spectra (A) and cyclic voltammograms (B) obtained for the fabrication procedure are illustrated by the different symbols as; ♦ ITO/OH; • ITO/OH/CTES; ▲ ITO/OH/CTES/NHS/EDC; ▼ ITO/OH/CTES/ NHS/EDC/Anti-SOX2; ■ ITO/OH/CTES/Anti-SOX2/BSA].

Randles equivalent circuit simulation via Gamry Software (Reference 600, Gamry Instruments) and results were corresponded to Randles model. The diameter of the semicircle demonstrated the blocking behaviour of the proposed electrode after each modification step.

During the CTES SAM formation step onto hydroxylated ITO electrode, the impedance spectra also illustrated a decrease in semicircle diameter of Nyquist plot. This decrease indicated that an easy electronic transport at the electrode surface interface. The diameter of the semicircle is further increased due to the insulation of EDC/NHS. After covalent bonding of anti-SOX2 antibodies, a remarkable increase in R_{ct} value was observed owing to their non-conductive properties. The blocking of the remaining active ends by BSA caused an increase in R_{ct}, owing to the insulating influence of BSA on the modified ITO electrode. The EIS results indicated that the proposed immunosensor was developed successfully.

CV is a commonly used method to investigate the electrochemical behaviour of modified electrodes. The differences in oxidation and reduction peak currents in CVs associated with the modification steps in the fabrication process. In this study, CV was used to monitor peak currents of redox couple. Fig. 2B illustrates the CVs of the modified electrode. After self-assembling of CTES on the hydroxylated ITO, the oxidation and reduction peak current values increased. When CTES modified surface was activated with EDC/NHS, the peak current values of redox probe decreased slightly. Covalent bonding of anti-SOX2 on the electrode surface increased the insulating feature of the ITO electrode. Thus, a decrease in peak currents values was seen. Blocking remaining active ends with BSA caused a decrease in the redox peak values due to insulating features of BSA. The CV measurements confirmed that the construction of the bioelectrode was successful.

3.2. Morphological studies by using SEM

The surface morphologies of the ITO based immunosensor fabrication processes were analysed by use of SEM and AFM. These techniques were employed for characterization of the morphology of the immunosensor surface during the layer-by-layer modification. As seen from Fig. 3A and B, hydroxylated ITO electrode had a homogenous surface with a narrow distribution. A root mean square (rms) roughness value of this step was 3.5 nm on a 5×5 μm scale. It can be seen in Figs. 3C and D that the CTES film on the electrode surface is uniform, dense and also rms roughness was found as 5 nm on a 5×5 μm scale. After immobilization of anti-SOX2, the electrode surface showed a densely networked fibril structure as seen in Fig. 2E and F. A rms roughness value of 82.2 nm on a 5×5 μm scale was achieved. This increase was originated from the morphology of antibody molecules onto ITO surface via covalent interactions. Furthermore, BSA immobilized on the ITO electrode illustrated aggregates of BSA protein with regular distribution in SEM (Fig. 3G) and AFM (Fig. 3H). The rms roughness value of 85.4 nm on 5×5 μm scale was found. A different electrode surface was observed after interaction between anti-SOX2 antibody and SOX2 antigen as shown in Fig. 3I and J. Consequently, the rms value increased to 89.1 nm due to high loading of antigen onto electrode. Results of SEM studies indicated the accuracy of EIS and CV.

3.3. Optimization studies of SOX2 biosensor

The experimental parameters such as CTES and antibody concentrations, antibody and antigen incubation time, which have an impact on the immunosensor response were investigated to develop a successful biosensor.

Self-assembled monolayer technique based on silanization process was used in this study. To obtain a well ordered layer of carboxyl groups, hydroxylated ITO electrodes were immersed in CTES solution overnight. To investigate the impact of CTES concentration on the response of the proposed immunosensor, CTES solutions with varied concentrations were tried. The low concentration of CTES was not enough to bind sufficient amount of anti-SOX2 antibody. The response at 0.25% and 0.5% was quite similar. Therefore, 0.5% concentration was chosen.

For examination of the effect of anti-SOX2 antibody concentration for the fabrication of immunosensor, varied concentrations (10 ng/mL, 5 ng/mL, 0.4 ng/mL) were studied. The best response was obtained in 5 ng/mL anti-SOX2 concentration. At the low antibody concentration (0.4 ng/mL), adequate amount of antibodies did not bind to the electrode surface and desired signal could not be obtained. Using of high (10 ng/mL) anti-SOX2 concentration caused low response. When the response was taken into account, 5 ng/mL was chosen as the optimal one. SOX2 calibration curves obtained by this optimization step were shown in Supplementary material Fig. 1.

Another optimization parameter that should be optimized is the incubation duration of anti-SOX2 antibody. The optimal incubation duration was chosen by incubating the immunosensor in anti-SOX2

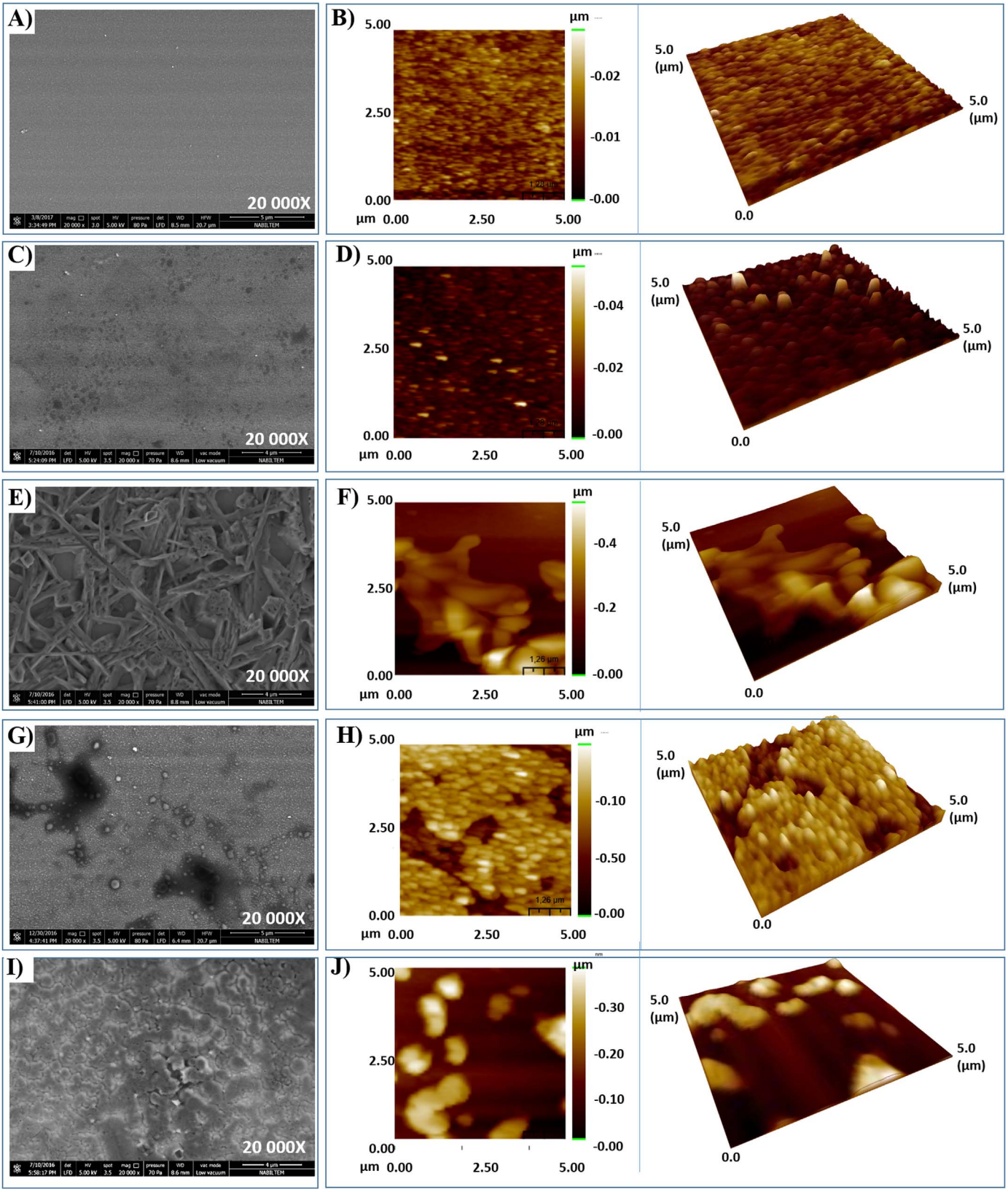


Fig. 3. SEM and AFM images of different electrode surfaces obtained during the biofunctionalization [(A and B) ITO/OH; (C and D) ITO/OH/CTES; (E and F) ITO/OH/CTES/Anti-SOX2; (G and H) ITO/OH/CTES/Anti-SOX2/BSA (I and J) ITO/OH/CTES/Anti-SOX2/BSA/SOX2].

antibody solution for varied periods (30, 45, 60 min). The highest response was achieved by incubating the immunosensor in anti-SOX2 standard solution for 45 min. Calibration curves belonging to these times are given in Supplementary material Fig. 2.

Other important parameter, which is effective on the interaction between antibody and antigen, is the incubation period of SOX2 antigen. To find optimum interaction period, different incubation periods (30, 45, 60 min) were studied. The highest response was

obtained at 30 min. SOX2 calibration curves obtained by the biosensor by using different SOX2 incubation periods were shown in Supplementary material Fig. 3.

3.4. Analytical characterization of the developed biosensor

As it is well known, EIS can be utilized to characterize the stepwise modification of the immunosensor because it is a sensitive and reliable technique to monitor the interface features of modified electrode surface. Under optimal conditions, a series of immunosensors were constructed for the detection of different concentrations of SOX2. A linear relationship was found between the SOX2 concentrations and response, which were calculated by using equivalent circuit model (Fig. 2 inset). This equivalent circuit includes the electrolyte resistance between the working and the reference electrode, R_s; the Warburg impedance, Z_w, associated with the diffusion process of redox probe ions from the bulk solution to the electrode-solution interface; the charge transfer resistance, R_{ct}, double layer capacitance regarding with the capacitance of the complex bioactive layer. R_{ct} introduces the largest difference during the modification process of the biosensor. For this reason, R_{ct}, the proper parameter to monitor the interfacial features of the proposed biosensor during the manufacturing steps was monitored.[40–44].

The quantitative behaviour of the biosensor was assessed by measuring the dependence of ΔR_{ct} on the concentration of SOX2, which was calculated as given;

$$\Delta R_{ct} = R_{ct}(SOX2) - R_{ct}(BSA)$$

R_{ct}(SOX2) is the value of semicircle diameter after SOX2 incubated with immobilized antibody. R_{ct}(BSA) is the value of semicircle diameter after blocking by BSA.

Fig. 4 presents the impedance spectra, cyclic voltammograms and calibration plot of proposed immunosensor, which was incubated in increasing concentration of SOX2 antigen. As shown in Fig. 4A, the resistance (the semicircle diameter of Nyquist plot) increased with the increasing SOX2 concentration. The immunosensor exhibited a wide dynamic range between 25 fg/mL and 2 pg/mL with the coefficient of 0.9995 (Fig. 4B). The lowest concentration detected and quantified are known as limit of detection (LOD) and the limit of quantification (LOQ), respectively. LOD and LOQ were calculated by using the equations 3 S_{blank}/m and 10 S_{blank}/m respectively. S_{blank} and m show the signal of the blank sample and the slope, respectively. By using this equation, LOD and LOQ were calculated as 7 fg/mL and 22 fg/mL, respectively. According the results, the biosensor was found to be suitable for determining the SOX2 concentration. The peak currents for detection of SOX2 on the immunosensor decreased with the increasing concentrations of analytes, just as shown in Fig. 4C. The reason of this decrease was that the increasing nonconductive features of the proteins hindering electron transfer and mass transport of the electrochemical probe.

Surface coverage was calculated by using the equilibrium Q=nFAΓ, where Q is the total charge (C), n the number of electron transferred, F the Faraday constant (96,485 C mol^{-1}), A the electroactive surface area (cm^2) and Γ is the surface coverage (mol cm^{-2}). The surface coverage of hydroxylated ITO electrode was found as 6.45×10^{-8} mol cm^{-2}. The silanization agent, CTES, formed a self-assembled monolayer on ITO surface and Γ was found as 5.92×10^{-8} mol cm^{-2}. After activation CTES with NHS/EDC, the surface coverage was calculated as 6.27×10^{-8} mol cm^{-2}. Thus, much more active densely packed CTES layer was formed on ITO electrode. After immobilization of anti-SOX2 antibody (5 ng/mL), the activated coupled with these antibodies and the surface coverage was calculated as 5.86×10^{-8} mol cm^{-2}. The free active ends bound with BSA (0.5%) that is a globular protein. So that a large surface area (Γ was found as 6.005×10^{-8} mol cm^{-2}) was obtained. In the last step, where SOX2 (0,25 ng/mL) biomarker is detected, Γ was found as 5.73×10^{-8} mol cm^{-2}. The result showed that there is an antibody-antigen interaction between anti-SOX2 antibody and SOX2 antigen.

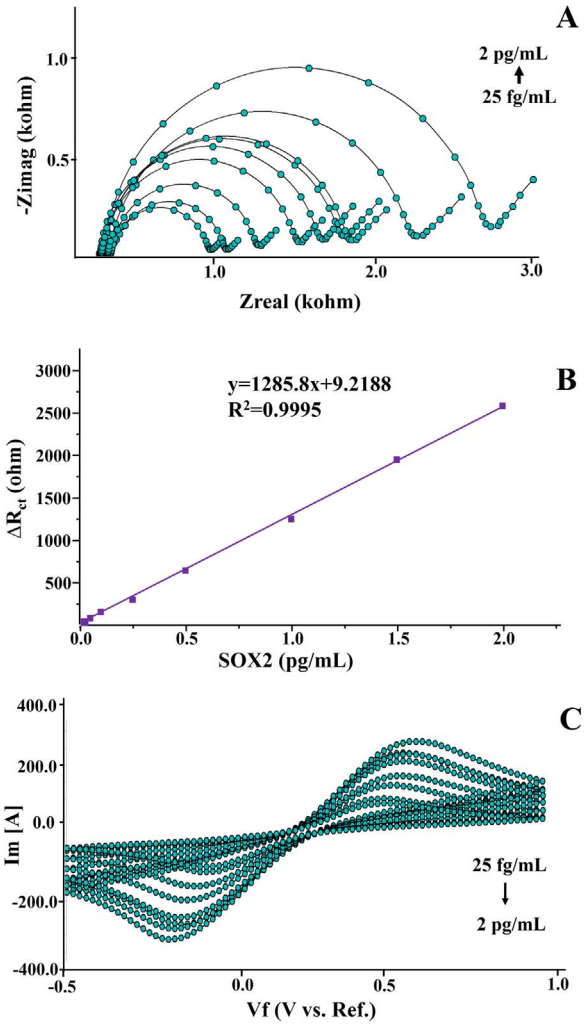


Fig. 4. SOX2 calibration curve of proposed biosensor based on ITO modified CTES [(A) Impedance spectra on ITO surface of the different SOX2 concentrations in 0.1 M KCl solution containing 5.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$ (B) SOX2 calibration plot of the immunosensor (C) CVs of the different SOX2 concentrations in 0.1 M KCl solution containing 5.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$.

Repeatability of a biosensor is to get the same results after measurement under the same conditions. The repeatability of the responses was investigated by analysis of the same concentration of SOX2 (250 fg/mL) using 20 equally prepared electrodes. All electrodes illustrated similar electrochemical response and correlation coefficient, average value and standard deviation were 3.88%, 256.7 fg/mL and 9.97 fg/mL, respectively. The results suggest that the repeatability of the immunosensor were fairly satisfactory.

Since reproducibility was a significant parameter for the immunosensors, it was checked by using 10 different calibration plots of biosensors, which were prepared at the same conditions. All of them had the same linearity in the range from 25 fg/mL to 2 pg/mL for SOX2. The relative standard deviations were also calculated for the slopes and intercepts of the linear curves obtained for these 10 biosensors as 4.25% and 5.14%, respectively. This demonstrated that the reproducibility of the proposed immunosensor for SOX2 detection was acceptable.

The regeneration ability of the immunosensor is critically important for biosensor applications. The biosensor was regenerated after incubating the used electrode in the acidic solution (ultra-pure water: HCl, 0.1%) for 5 min. After regeneration, it was incubated in SOX2 antigen solution (250 fg/mL). As result of EIS measurement, our biosensor showed reusability till 8th regeneration cycle retaining 22%

of its initial response.

Stability of the biosensor is important in the daily life application. When the biosensors were not in use, they stored at 4 °C (in dry form) and they were evaluated every 7 days in a period of 10 weeks. In this way, 79.36% of initial activity was observed after a storage duration of 9 weeks. The results pointed that the developed immunosensor had acceptable stability.

In order to evaluate the analytical reliability and application possibility of the proposed biosensor, five human serum samples were analysed by using proposed immunosensor. The biosensor responses of SOX2 in five human serum samples (with 1,000,000 dilution) were compared with the ELISA method results. The measured values and the values after addition standard SOX2 solution are shown in Table 1. The results showed that acceptable results were obtained by using the biosensor for SOX2 detection and recoveries were between 99.52% and 100.77% for the SOX2. Comparison for the cancer biomarkers detection with other electrochemical biosensors based on ITO electrode is given in Table 1. It can be seen that the biosensor fabricated in this study obtained a satisfying detection limit. What is emphasized in this study is the cheapness, easy modification of the fabricated biosensor.

3.5. Single frequency measurements (SFI)

SFI was carried out to monitor binding characteristics between anti-SOX antibodies and SOX2 antigens. For the SFI experiments, the potentiostat was fixed at a frequency of 80 Hz. The amount of frequency was chosen by evaluating the Bode plots, because it includes important frequency data of the system. Fig. 4 (Supp. Mat.) displays the Bode plot, which was used for chosen the optimum frequency utilized in SFI. Nyquist diagram does not include frequency information, because of this Bode plot have been used mostly. The impedance was monitored at this fixed frequency (80 Hz) as function of time and phase angle for 30 min. The response is illustrated in Fig. 5.

SFI technique is informative to monitor anti-SOX2 antibody and SOX2 antigen interaction. The remarkable difference in the phase angle proved the interaction of antibody-antigen. As seen Fig. 5, time dependent change of impedance was illustrated with green curve and the change in the phase angle was presented with pink curve. Important differences in the charge transfer resistance and phase angle were owing to interaction between SOX2 antigen and anti-SOX2 antibody. Consequently, it can be concluded that, SFI can be utilized for biorecognition event monitoring, or to monitor the differences on the electrode surface which is formed in short time.

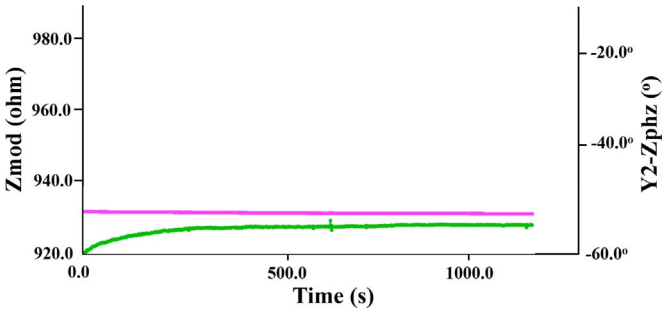


Fig. 5. Single frequency impedance experiment (■ variation is phase angle; pink • variation is impedance; green).

3.6. Square wave voltammetry measurements (SWV)

In this study, a label-free immunosensor for SOX2 cancer biomarker was evaluated by using an alternative method, SWV. The interaction between antigen and antibody forms an insulating layer which prevents the interfacial electron transfer kinetics between the redox probe and the electrode and causes an increase at the electron-transfer resistance and a decrease in SWV current (Supp. Material Fig. 5). Fig. 6 shows the obtained calibration plot of SWV detection. The obtained signals were a function of SOX2 concentration. Its response is linear with the SOX2 concentration over a range from 25 fg/mL–2 pg/mL. The regression coefficient was 0.9539.

4. Conclusion

In this work, silane modified ITO electrode was utilized as a biosensing layer for the development of impedimetric immunosensor. Anti-SOX2 antibodies were effectively immobilized on the ITO electrode surface owing to the unique properties of CTES, which made the label-free detection of SOX2 feasible. The proposed immunosensor has good repeatability, reproducibility and storage stability as well as acceptable precision. It can detect SOX2 antigen in a linear range of 25 fg/mL–2 pg/mL. The LOD and LOQ values were found as 7 fg/mL and 22 fg/mL, respectively. Furthermore, the developed immunosensor can detect the biomarker in serum and its performance is the same as ELISA. Consequently, the proposed immunosensor illustrated

Table 1
SOX-2 determination in human serum samples and comparison of the performance of electrochemical cancer biosensor based on ITO electrode modified with silane agents.

SOX-2 determination in human serum samples

Sample	ELISA Results (fg/mL)	Found by biosensor (fg/mL)	Added SOX2 (fg/mL)	Total found by biosensor	% Recovery	% Relative difference
1	230.78	227.70	250.0	478.91	100.25	+0.25
2	202.92	202.04	250.0	453.63	100.35	+0.35
3	203.62	207.86	250.0	461.41	100.77	+0.77
4	235.53	233.54	250.0	481.24	99.52	−0.48
5	246.71	245.98	250.0	496.40	100.08	+0.08

Comparison of the performance of electrochemical cancer biosensor based on ITO electrode modified with silane agents

Biomarker	Biosensor construction	Linear range	LOD	Reference
Vascular endothelial growth factor	APTES and gold nanoparticle modified ITO	100–600 pg/mL	100 pg/mL	[32]
CYFRA-21-1	APTES and hafnium oxide modified ITO	2–18 ng/L	0.21 ng/mL	[45]
CYFRA-21-1	APTES and zirconia nanoparticle modified ITO	2–22 ng/L	0.122 ng/mL	[28]
Plasmodium falciparum histine rich protein 2	APTES modified ITO	1 pg/mL–100 ng/mL	2.2 pg/mL	[46]
Cry1Ab	APTES modified ITO	1–10 ng/mL	0.37 ng/mL	[47]
Receptor for activated C Kinase 1	TESU modified ITO	14.25–712.5 fg/mL	30 fg/mL	[39]
SOX2	CTES modified ITO	25–2000 fg/mL	7 fg/mL	This work

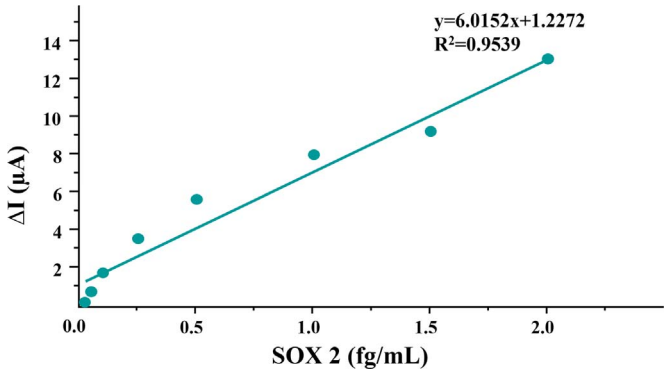


Fig. 6. SOX2 calibration curve obtained by the SWV in 0.1 M KCl solution containing 5.0 mM $K_3[Fe(CN)_6]/K_4[Fe(CN)_6]$.

simple manufacturing, rapid response, a wide linear range and excellent selectivity, and thus it gives an opportunity to use in clinical diagnosis.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.talanta.2017.05.048.

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