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Electrochemical immunosensor based on chitosan/conductive carbon black composite modified disposable ITO electrode: An analytical platform for p53 detection

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ABSTRACT

In this study, we fabricated a label-free electrochemical immunosensor for sensitive and selective detection of tumor marker p53. This immunosensor was based on chitosan/carbon black composite (Chitosan-CB) layer coated ITO electrode. This composite was utilized for enhancement of the conductivity of the immunosensor. Anti-p53 antibodies were captured on the modified ITO electrode through the cross-linking of chitosan and glutaraldehyde. Electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) techniques were utilized for electrochemical characterization of the proposed immunosensor. Moreover, the biosensor construction steps were monitored by using scanning electron microscopy (SEM) and atomic force microscopy (AFM). The immobilization of anti-p53 antibodies on the electrode surface was investigated by using Fourier-transform infrared spectroscopy (FTIR) and Raman spectroscopy. The change in impedance which formed during the specific interaction between anti-p53 antibody and p53 antigen was used to detect p53. Under optimized experimental conditions, the fabricated immunosensor had a wide linear range of 0.01–2 pg/mL and low detection limit of 3 fg/mL. The fabricated immunosensor had good sensitivity, stability and repeatability. Furthermore, it was successfully applied to analyze p53 in human serum.

1. Introduction

Cancer is a dangerous disease and has the second highest death rate in the world. The early detection of cancer is possible by measuring cancer biomarkers in biological fluids (Srivastava and Gopal-Srivastava, 2002). The accurate and sensitive detection of cancer biomarkers facilitates the treatment of cancer. Up to the present, a lot of techniques such as enzyme-linked immunosorbent assay (ELISA), radioimmunoassay and fluorescence immunoassay methods are employed for cancer diagnosis. These methods have some drawbacks such as high cost, long pretreatment steps, and poor selectivity. To overcome these disadvantages biosensors are convenient tools for cancer biomarkers detection (Amani et al., 2018; Aydın et al., 2018a; Verbarg et al., 2017). Biosensors contain a biorecognition layer on the transducer surface. This layer is specific to the desired analyte. Transducer can be optical, piezoelectric or electrochemical. Furthermore, this layer is composed of enzymes, antibodies or aptamers which have high specificity to target analytes. The most preferred transducer is electrochemical due to its low-cost, easy usability, and rapid response (Bahadır and Sezgintürk, 2015). Among different electrochemical techniques, electrochemical impedance spectroscopy (EIS) is the most attractive technique due to its sensitivity and label-free feature. The main principle of the EIS method is based on monitoring of change in impedance during the biorecognition events formed on the electrode surface. Additionally, EIS is a non-destructive technique and it utilizes very small potentials for examinations. Label-free impedimetric immunosensors provide many benefits such as easy fabrication, fast response, low cost and high selectivity. Furthermore, they do not require any labels. Because of these features of impedimetric immunosensors, they have gained interest (Arkan et al., 2015; Bahadır and Sezgintürk, 2015; Törer et al., 2018).

Protein p53 has an important role in cell growth control and DNA repair process. Loss of p53 function induces tumor formation and gene mutation due to conformational variation in the structure of the p53 protein (Chen et al., 2012). The level of p53 antigen in human serum from cancer patients are ranged from 0.52 ± 0.23 ng/mL to 1.03 ± 0.59 ng/mL (Attallah et al., 2003). Also, p53 is a sign in different cancer types such as colorectal (Attallah et al., 2003), small cell lung cancer (Segawa et al., 1997). Therefore, the accurate detection of

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Table 1
Comparing analytical parameters of different immunosensors for the detection of p53 (A), results of real human serum experiments (B).

A) Working electrode	Transduction technique	Concentration range	LOD	Reference
Graphene oxide (GO)/streptavidin-gold nanoparticles modified GCE electrode	Electrochemical	0.2–2 pM 2–200 pM	30 fM	(Afsharan et al., 2016a, 2016b)
Streptavidin modified-gold nanoparticles-thiolated GO modified GCE	Electrochemiluminescence	0.2–200 pM	22.8 fM	(Afsharan et al., 2016a, b)
Multiwalled carbon nanotubes doped nylon 6 composite fibers modified GCE	Electrochemical	2 pg/mL–2 ng/mL	1 pg/mL	(Wang et al., 2015)
Poly(cysteine) and AuNPs modified GCE electrode	Electrochemical	0.0369–50 pM 0.018–2.5 pM	48 fM	(Hasanzadeh et al., 2017)
Polymer GMA modified ITO electrode	Electrochemical	0.02–4 pg/mL	7 fg/mL	(Aydın et al., 2018)
Chitosan/Super P modified ITO electrode	Electrochemical	0.01–2 pg/mL	3 fg/mL	This study

B) Sample	Found by the biosensor	Added p53 amount	Total found	% Recovery	% Relative difference
Human serum 1	0.46 pg/mL	0.2 pg/mL	0.65 pg/mL	98.87	− 1.13
Human serum 2	0.35 pg/mL	0.2 pg/mL	0.55 pg/mL	99.66	− 0.34
Human serum 3	0.44 pg/mL	0.2 pg/mL	0.66 pg/mL	101.97	1.97
Human serum 4	0.43 pg/mL	0.2 pg/mL	0.61 pg/mL	97.59	− 2.41
Human serum 5	0.51 pg/mL	0.2 pg/mL	0.71 pg/mL	100.03	0.03

p53 antigen level in human serum is very important. Enzyme-linked immunosorbent assay (ELISA) is mostly utilized method for p53 biomarker detection. This technique is successful, but it has some disadvantages such as long analysis steps and high cost commercial kit requirements. Therefore, biosensors gained attention for biomarker detection. A variety of electrochemical biosensors have been utilized for p53 detection and some of these biosensors are summarized in Table 1A. A magnetic particle based immunosensor was developed for p53 detection. To increase the success of biosensor, carbon nanospheres and protein cage nanoparticles was employed. Detection limit of this biosensor was 10 fg/mL (Chen et al., 2012). In another study, gold nanoparticles/graphene oxide (Au NPs/GO) modified glassy carbon electrode (GCE) was utilized biosensor platform. A signal was monitored after biotinylated capture anti-p53 antibody immobilized GCE electrode interacted with p53 and secondary anti-p53 labeled with horseradish peroxidase (HRP). Detection limit of this biosensor was 30 fM (Afsharan et al., 2016a, 2016b).

Single frequency impedance (SFI) analysis is part of the EIS method. In this method, a single frequency is utilized as an excited signal instead of wide frequency range. This technique has been used for investigation of immunoreaction formed between antibody and antigen (Aydın and Sezgintürk, 2017b; Singal and Kotnala, 2017).

The most important factors that affect the performance of a biosensor are biorecognition layer and transducer. Designing of electrochemical biosensors based on biorecognition element immobilization enhances the sensitivity of unmodified electrode. Therefore, a lot of electrode modification strategies have been utilized in literature (Bhalla et al., 2016). Indium tin oxide (ITO) is considered to be a promising platform in biosensor applications because of its excellent properties such as excellent optical transparency, good electrical conductivity, wide electrochemical working window and stable electrochemical and physical properties. All these properties make ITO substrate a useful platform to develop a biosensor. To fabricate a biosensor by using ITO substrate as working electrode, firstly, ITO substrate should be modified for biorecognition element immobilization. Different techniques such as silanization (Aydın and Sezgintürk, 2017b; Bahadır and Sezgintürk, 2016; Gündoğdu et al., 2017), self-assembled monolayer formation (Aydın et al., 2017, 2018b), electropolymerization (Kim et al., 2016), electrophoretic deposition (Kumar et al., 2017) and spin coating (Aydın et al. 2018c) have been utilized to modify ITO substrate (Aydın and Sezgintürk, 2017a). Spin coating is one of the most common technique to form thin films on the substrates. This technique is best for fastening without attaching to the surface. For example, polymers with a lot of active ends groups could not bind to the electrode surface chemically; therefore, this technique is the most preferable one. It is utilized in a wide range of applications in industry and science (Yuan et al., 2014). The advantage of this technique is its ability to quickly and easily produce very uniform films. Spin coating technique includes the application of a thin film on the substrate surface by coating a solution of the desired material in a solvent while it is rotating. Firstly, a drop of fluid including desired material was dropped onto the center of a platform and then the platform was spun at high speed. Centripetal acceleration will provide a film formation on the substrate. This technique has been used for biosensor development in different applications (Aydın et al., 2018b).

Chitosan is a proper matrix for the immobilization of biosensing elements. It is a natural polymer which has amino groups on its surface. It has good biocompatibility and forms film on the transducer. Because of these properties, it is preferred as an interface material. However, its utilization in the fabrication of an electrochemical immunosensor is limited due to its non-conductive feature and low solubility in different solution. To increase the conductivity of interface material, a wide range of nanomaterials have incorporated with chitosan (Aydın et al. 2018c). Conductive transducing material or conductive agent modified transducer material have high conductivity and enhance the electron transfer between electrode surface and electrolyte. Because of this, electrodes modified with conductive materials have gained attention in the literature (Curulli et al., 2012; Wang et al., 2014; Zhang et al., 2015). Among these, carbon black is a good candidate owing to its low cost. Moreover, the nanomaterial/chitosan composite provides a robust platform for biosensing applications. Carbon black (CB) is a useful carbon material and is utilized in different applications (da Silva et al., 2018; Mondal et al., 2018). It has conductive property and a large surface area. Different conductive composites made by CB are usually utilized in sensor development (Lounasvuori et al., 2018). The most attractive feature of CB is the uniform dispersion of CB in matrices. The incorporation of chitosan into CB produces a new type of biocomposite materials. Considering the above-mentioned benefits of chitosan and super P, we have integrated them in a biosensor fabrication to improve biosensor characteristics. To the best of our knowledge, this new biocomposite has not been utilized for p53 detection thus far.

In this study, we developed a new impedimetric immunosensor by using chitosan/carbon black composite modified disposable ITO electrode for ultrasensitive detection of p53, a cancer biomarker. The reason for the usage of this composite was the non-conductive property and low solubility of the chitosan. To overcome these drawbacks, we integrated chitosan with CB and CB was used for improvement of the conductivity of immunosensor and for amplifying the electrochemical signal. Chitosan, which is a natural polymer, had a lot of amino groups on its surface, therefore it was the best candidate for biorecognition element immobilization platform. This composite was suitable for antibody attachment via glutaraldehyde crosslinking. We prepared a homogeneous composite slurry composed of chitosan and CB and the homogeneous dispersion of chitosan in slurry provided an increase in

the ease of use of chitosan. The homogeneous dispersion of chitosan was characterized by using SEM and AFM measurements. To construct the immunosensor, spin-coating technique was utilized. Spin-coating method provided a homogeneous layer on the electrode surface. Also, this electrode construction technique included simple and non-complex modification steps and the fabricated biosensor could detect the p53 antigen in an ultra-low sample volume. Different variables affecting the biosensor performance were studied and optimized. This developed biosensor was employed to determine p53 antigen in the urine and serum samples.

2. Experimental

2.1. Reagents and materials

All chemical reagents were of analytical grade and utilized as received, and aqueous solutions were prepared with ultrapure water. Chitosan (Sigma-Aldrich, low molecular weight), Glutaraldehyde (Sigma-Aldrich, 25% Aqueous Solution), Acetone (Sigma-Aldrich, 99.9%), and N-Methyl-2-pyrrolidone (NMP, Sigma-Aldrich, 99%) were used without further purification. Monoclonal anti-p53 antibody and p53 antigen were purchased from Sigma–Aldrich. Anti-p53 antibody, p53 antigen and BSA were prepared by using phosphate buffer (PBS, pH 7.4, 5 mM) and stored at − 20 °C. Indium tin oxide (ITO) electrodes (5 mm x 20 mm, 60 Ω/cm^2), were obtained from Sigma-Aldrich. These protein solutions were prepared in 5 mM phosphate buffer (pH 7.4). Clinical real serum and saliva samples were obtained from the NKU Hospital. TIMCAL & Carbon Super P® conductive carbon black (99%) as conductive agent and polyvinylidene fluoride (PVDF, 99.5%) as binder material in composite slurry were purchased from MTI Corporation.

2.2. Apparatus

All electrochemical analyses were carried out using a potentiostat-galvanostat (Gamry 1000) with a conventional three electrode system, including a working electrode (chitosan-super p modified ITO electrode), a reference electrode (Ag/AgCl) and a counter electrode (platinum wire). All electrochemical measurements were performed in phosphate-buffered saline (PBS, pH 7.4) containing 5 mM $[Fe(CN)_6]^{3-}$/ $[Fe(CN)_6]^{4-}$ as a redox probe. The reference and counter electrodes were obtained from BASi Company. This device operated in a potential range from 0 to 500 mV at a scan rate of 100 mV/s for CV measurements. The impedimetric measurements were performed at 50,000 Hz and 0.05 Hz for initial and final frequency, respectively. The spin-coating procedure was carried out using a spin-coater (MTI VTC-50). The FTIR analyses were performed using a Perkin Elmer Company Spectrum 100 FTIR infrared spectrometer in the range of 4000–400 cm^{-1} at room temperature and the results were used without any corrections. Dispersive Raman analyses were carried out with a Thermo Scientific Company DXR Raman spectrometer equipped with a 780-nm excitation laser and a confocal microscope. The morphological investigations of composite modified ITO-PET electrodes were carried out using SEM (FEI-Quanta FEG 250 Model). Furthermore, Atomic Force Microscopy (AFM) images of the composite modified ITO-PET electrodes were recorded by using a NanoMagnetics company AFM PLUS model. The AFM images were obtained using the tapping mode of AFM with a commercial tapping silicon (Si) cantilever.

2.3. Preparation of the Chitosan-CB composite

The conductive (Chitosan-CB) composite interface material coated ITO-PET electrodes were prepared by the spin coating method as depicted in Scheme 1. The appropriate weight ratios of the chitosan and binder (polyvinyldenefluoride) were dissolved in mix solution (acetone 1.6 mL/ NMP 0.4 mL). The solution was mixed, stirred and sonicated continuously until the solution mixture took on a homogeneous viscous liquid appearance at 40 °C for 100–120 min. The conductive agent (carbon black) was added to chitosan solution and the mixture was homogenized by blending in a magnetic bar with magnetic stirrer at a maximum rotation speed for one hour.

2.4. Preparation of the ITO based immunosensor electrode

The immunosensor electrode fabrication procedure is schematically demonstrated in Scheme 1. Chitosan-CB composite coated ITO-PET electrodes were fabricated by using spin-coating technique. The rectangle patterned (5 mm x 20 mm, 60 Ω/cm^2) ITO-coated PET electrodes were cleaned by sonication in acetone, soap solution and ultrapure water for 10 min each and dried by using argon. The prepared homogenized black conductive slurry was dropped on the ITO electrode surface. Then, the spin-coater was accelerated to 1000 rpm rotational speed for sixty seconds and then dried under reduced pressure at room temperature.

2.5. Fabrication process of the electrochemical immunosensor

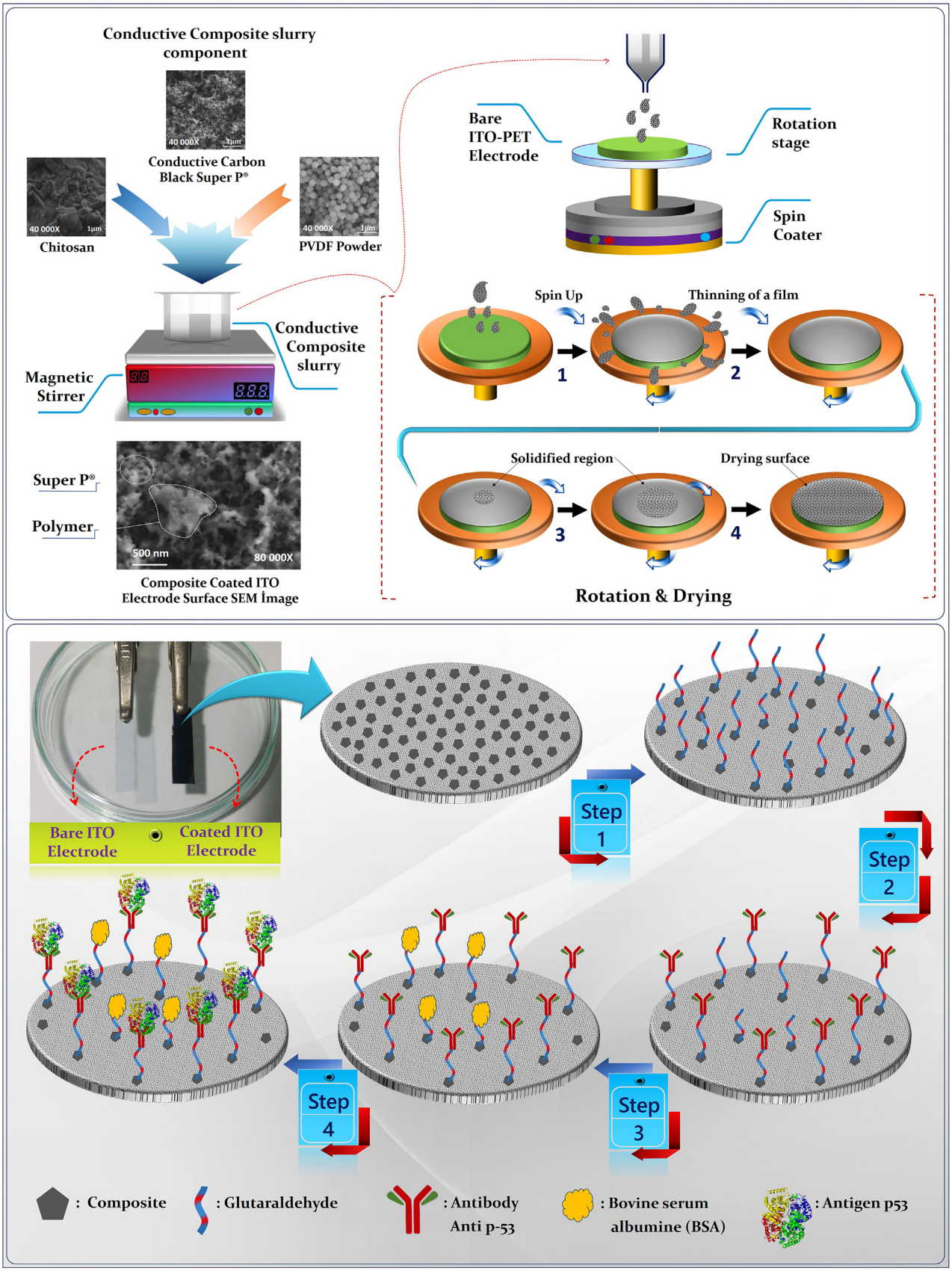
The fabrication steps of the immunosensor are shown in Scheme 1 and this process includes four steps. In the first step, the prepared Chitosan-CB composite modified ITO electrodes were dipped into pH 7.4 PBS solution containing glutaraldehyde (1%) (GLU) at room temperature for 15 min to activate amino groups on the chitosan for coupling to antibody. Anti-p53 antibodies were immobilized onto chitosan composite via glutaraldehyde crosslinking. The bifunctional—CHO groups of glutaraldehyde react with—NH_2 sites on the chitosan to facilitate bonding of the antibody to chitosan. Then, ITO electrodes were rinsed with ultra-pure water to eliminate free glutaraldehyde molecule. In the second step, the prepared ITO electrode was dipped in PBS solution containing anti-p53 antibodies. After immobilization of anti-p53 antibodies on the ITO electrodes, electrodes were rinsed with ultra-pure water to remove unbound antibody molecules. In the third step, free aldehyde groups of chitosan were blocked by using BSA solution for 1 h. The aim of this step was to prevent nonspecific interaction between free groups on the ITO electrode and p53 antigen. In the final step, fabricated biosensor was ready to detect p53 antigen.

3. Result and discussion

3.1. Chemical characterization of Chitosan-CB composite modified electrode

The chemical structure of conductive composite interface material coated ITO-PET electrodes was characterized by FTIR and RAMAN spectral techniques to show the success of the prepared homogenous composite coated ITO-PET electrode and immobilization of anti-p53 antibody. Infrared (FTIR) spectroscopy is an important and widely used spectral technique for identifying chitosan in composite. It is based on the vibrations of the atoms of a molecule. Furthermore, FTIR and Raman spectra of carbon black and chitosan and EDX spectra of carbon black and chitosan-carbon black composite were added in Supp. Inf. File Fig. SI-1 and Fig. SI-2, respectively.

The FTIR spectra of prepared chitosan-CB composite coated ITO electrode before anti-p53 antibody immobilization (blue line) and after anti-p53 antibody immobilization (red line) are shown in Fig. 1A and B, respectively. The FT-IR spectra of prepared chitosan-CB composite coated ITO electrode surface showed important absorption bands to identify the characteristic functional groups. As seen in Fig. 1A, the stretching vibrations of -OH bond of chitosan in composite were observed at 3350 cm^{-1} which was overlapped by -NH_2 stretch peaks (Kumar et al., 2012). The signals observed around 2921 cm^{-1} and 2867 cm^{-1} were originated from C-H stretching vibration (Fei Liu et al., 2001; Shanmugasundaram et al., 2001). The bands at 1648 cm^{-1} and 1585 cm^{-1} were attributed to NH_2 group and γ-NH_2 bending,



Scheme 1. The schematic representation of electrochemical immunosensor formation for p53 detection based on chitosan-super P composite modified ITO electrode.

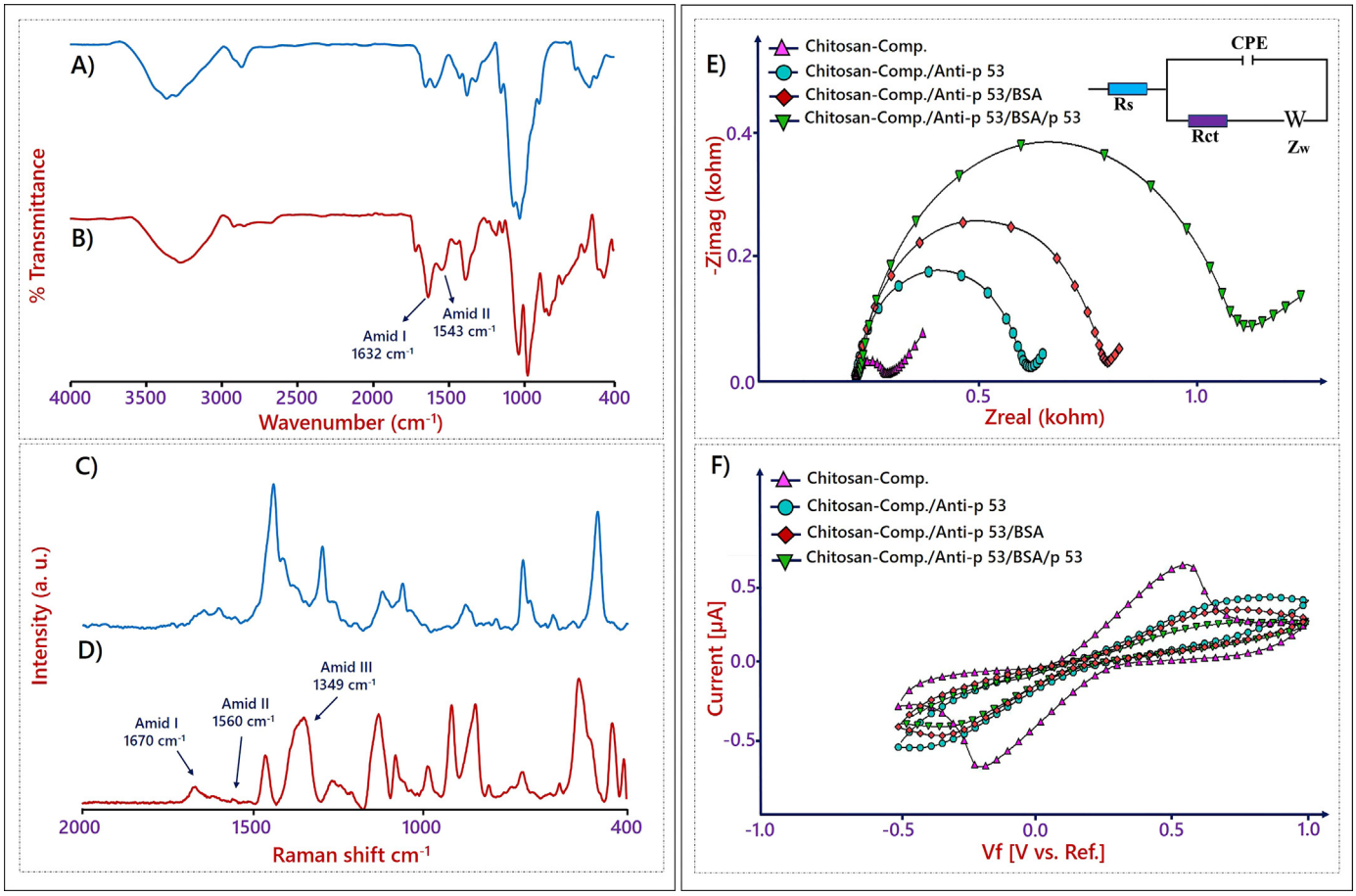


Fig. 1. FTIR (A and B) and Raman spectra (C and D) before antibody and after antibody immobilization; the stepwise fabrication procedure of the biosensor was monitored by EIS (E) and CV (F).

respectively (Qi et al., 2004). The band at 1150 cm^{-1} was attributed to the stretching vibration of C-C-O bonds in chitosan backbone. At red line in Fig. 1B, there were broad and intense bands at 1632 cm^{-1} and 1543 cm^{-1} which were originated from amide I and amide II, respectively (Aydın et al., 2017).

The chemical structures of composite modified ITO electrode and anti-p53 antibody immobilized ITO electrodes were also investigated via Raman spectroscopy (Figs. 1C and 1D). The Raman fingerprint region can be a good show of functional groups of chitosan in the composite. The bending and stretching vibration of C-C-O bonds of chitosan backbone were observed at 478 cm^{-1}, 877 cm^{-1} and 1120 cm^{-1}, respectively (Fig. 1C) (Dollish et al., 1974; Socrates, 2001). Raman spectroscopy is an available and a very reliable spectral technique to show functional groups of protein in the characteristic peaks in the analysis of compound (Ren et al., 2014; Rygula et al., 2013). The Raman spectrum in the regions of 1640–1680 cm^{-1}, 1480–1570 cm^{-1} and 1235–1300 cm^{-1} originated from amide I, amide II and amide III, respectively (Aydın et al. 2018c; Kurouski et al., 2015). The Raman spectrum in Fig. 1D has a broad amide I band at 1646 cm^{-1} which illustrate C=O stretching and out of plane C-N stretching (Srinivasan et al., 2013). The small peak at 1553 cm^{-1} was originated from the amide II bond which consisted of C-N stretching and N-H bending (Uversky and Permiãkov, 2007). The amide III bond was observed at 1297 cm^{-1} which originated from undefined secondary structures in protein (Maiti et al., 2004).

3.2. Electrochemical detection of P53 antigen

The stepwise fabrication and electrochemical behavior of the immunosensor were investigated using EIS in 50 mM PBS solution (pH 7.4) containing 0.1 M KCl 5 mM $Fe(CN)_6^{3-/4-}$.

EIS is a successful technique to investigate interface characteristics of modified electrodes. The impedance spectrum contains a semicircle portion at higher frequencies regarding the electron transfer limited process and a linear part at lower frequencies corresponding to the diffusion limited process. The semicircle diameter in the impedance spectrum is associated with the electron transfer resistance (R_{ct}). This parameter controls electron-transfer kinetics of the redox probe at the electrode surface and can be utilized as a proper signal for monitoring of the modification at each step. To control diffusional impedance, the warburg impedance (w) is utilized and it indicates the diffusion layer of infinite thickness. These parameters are located in Randles equivalent circuit and this circuit includes electrolyte resistance R_s, CPE apart from these parameters (Aydın and Sezgintürk, 2017b; Liu et al., 2013). The Randles equivalent circuit was used to fit all data obtained during EIS measurements (Fig. 1E inset).

Fig. 1E shows the impedance spectra of the proposed biosensor obtained during the stepwise modification process. The Chitosan-CB composite modified ITO electrode has a small semicircle due to the low transfer resistance of the modified electrode. In addition, EIS and CV data of bare ITO electrode and CB/ITO electrode are shown in Fig. SI-3 and Fig. SI-4, respectively. After antibody immobilization on the ITO electrode surface, the R_{ct} greatly increased. This increase was originated from an insulation layer composed of p53 antibodies formation. Thus, the electron transfer between the electrode and electrolyte solution was decreased. The remaining aldehyde groups were blocked by BSA and an insulating protein layer was formed on the ITO electrode. As seen in Fig. 1E, after BSA blockage an enlarged diameter of the

semicircle was obtained. Finally, the interaction of p53 antigen with its antibody immobilized on the ITO electrode increased the R_{ct} because of the antibody-antigen adduct formation. This interaction formed a blocking layer on the electrode surface and hindered the diffusion of the redox couple on the electrode surface.

For the CV measurements ferri-ferro redox couple was employed as a marker to investigate the changes obtained during fabrication procedure. As seen in Fig. 1F, chitosan-CB composite modified ITO electrode had higher peak currents than other steps due to high conductivity of CB. The antibody immobilization on the electrode surface showed a decrease in peak currents, indicating that the electron transfer of redox couple was obstructed. After blockage of aldehyde groups with BSA, the currents were decreased. This decrease demonstrated that the remaining sites were successfully blocked. Finally, as expected, anti-p53 antibody and p53 antigen coupling resulted in a decrease in peak currents. The results of CV measurements were consistent with EIS measurement results and showed successful interaction between antibody and antigen.

3.3. Optimization of experimental variations

The effect of chitosan amount in composite was significant because chitosan indicated the number of amino groups on the ITO electrode surface. Therefore, 6 different chitosan concentrations (2.5 mg, 5 mg, 10 mg, 15 mg, 20 mg and 30 mg) were tried. The signal at 2.5 mg chitosan concentration was too lower than other levels. When 5 mg chitosan was utilized, the maximum signal was obtained. When the chitosan concentration was increased, a gradual decline in the signal was observed. Therefore, 5 mg was chosen as optimum value (Fig. 2A). The relative standard deviation (RSD) of this step was calculated as 4.91%. When electrodes were prepared higher than this concentration, the hydrophobicity was increased on the electrode surface. Influence of antibody level on the biosensor signal was also examined by using six different levels (0.0985 ng/mL, 0.4925 ng/mL, 1.97 ng/mL, 3.94 ng/mL, 7.88 ng/mL, 23.64 ng/mL). At the smallest antibody concentration (0.0985 ng/mL), a low signal was obtained. In addition, the results illustrated a decrease was obtained in biosensor signal with increasing antibody level. The possible reason was that the higher amounts of antibodies increased the disorder in alignment of the attached antibody and resulted in steric hinderance of the immunoreaction (Fig. 2B). Because of this, 0.4925 ng/mL was selected as optimal concentration for the biosensor preparation. The relative standard deviation (RSD) of this step was calculated as 4.86%. The effect of the incubation duration was examined to increase the sensitivity of the immunosensor response. Because of this, antibody and antigen incubation time experiments were performed. To optimize antibody incubation time, chitosan/super P composite modified electrode was dipped in PBS solution containing anti-p53 antibodies and incubated for different periods from 15 to 90 min. Compared to calibration curves of these periods (Fig. 2C) and obtained signals, 30 min was adequate for incubation. Additionally, 15 min was not adequate for incubation. During increasing antibody incubation times, decreases in biosensor signals were observed. This may be due to denaturation during the incubation process. The relative standard deviation (RSD) of this step was calculated as 3.65%. To optimize antigen incubation time, antibody immobilized electrodes were incubated for different durations from 15 to 90 min in PBS solution

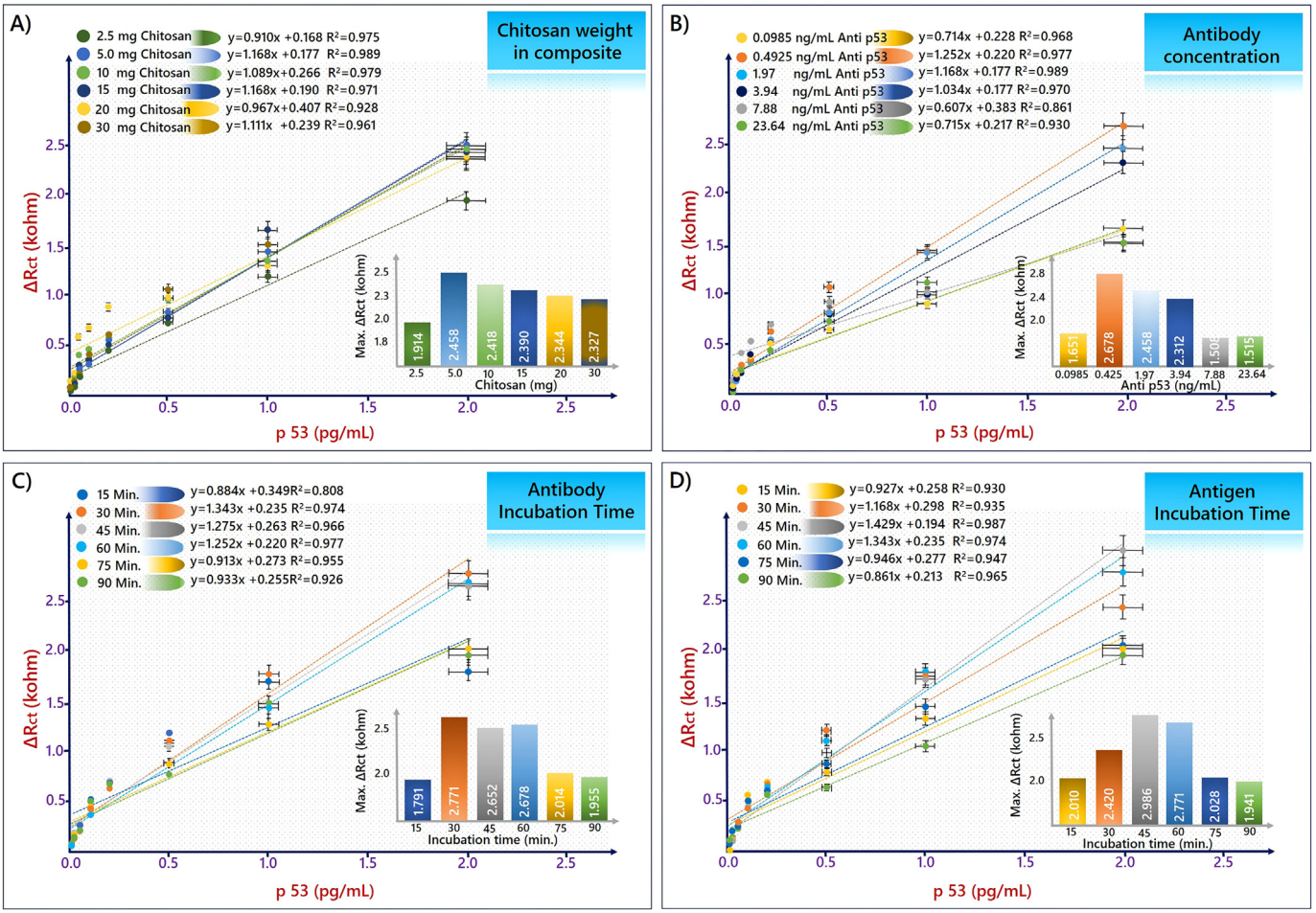


Fig. 2. Optimization of experimental variations; chitosan concentration (A), anti-p53 antibody concentration (B), anti-p53 antibody incubation time (C), p53 incubation time (D).

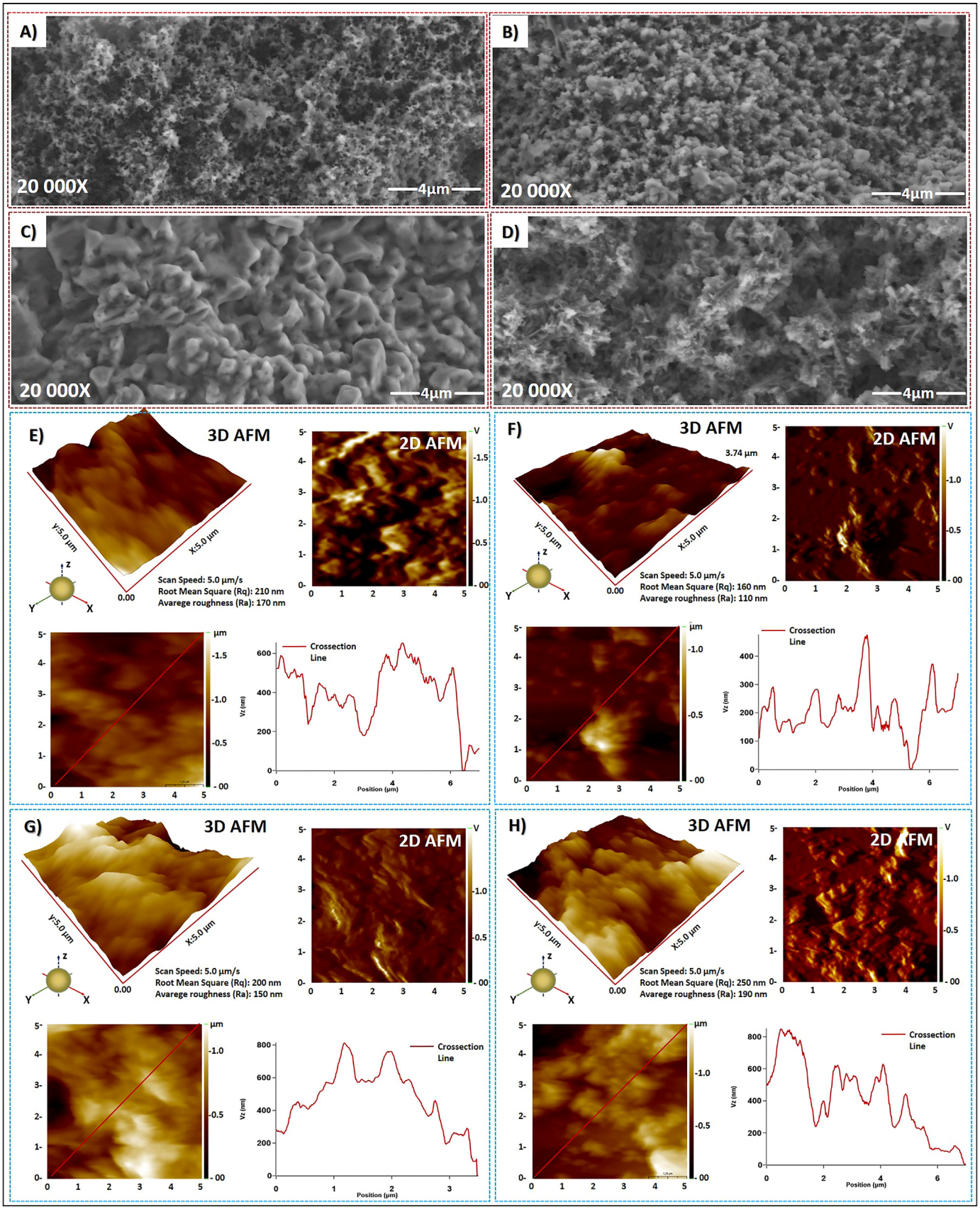


Fig. 3. SEM and AFM images of composite modified ITO electrode (A and E), anti-p53 antibodies immobilized electrode surface (B and F), after BSA blockage of free aldehyde groups (C and G), after interaction between anti-p53 antibodies and antigens (D and H).

containing p53 antigens. Based on the results obtained after incubations, maximum signal was obtained after 45 min incubation. 15 min and 30 min incubation in antigen solution was not enough for binding of p53 antigen to p53 antibody. Because of this, 45 min was selected as the optimum antigen incubation duration for antigen immobilization (Fig. 2D). The relative standard deviation (RSD) of this step was calculated as 4.1%. During increasing antigen incubation times, decreases in biosensor signals were seen. This may be due to denaturation during the incubation process.

3.4. Morphological characterization of the immunosensor

SEM and AFM were employed to analyze the surface morphology of the proposed biosensor. Field emission scanning electron microscopy was used to evaluate the structure of the modified ITO electrode. In addition, the AFM images with the height profiles of the ITO modified with chitosan-CB, chitosan-CB/anti-p53 antibody, chitosan-CB/anti-p53 antibody/BSA and chitosan-CB/anti-p53 antibody/BSA/p53 were evaluated. SEM and AFM images of the ITO/chitosan-CB composite in Fig. 3A and E revealed that the dense composite was uniformly dispersed on the surface of the ITO electrode. As seen in SEM image, the chitosan-CB composite had a porous matrix. This porous matrix provided a large surface area for immobilization of the antibody. Furthermore, this structure provides a high surface/volume ratio, facilitating amplification of electrochemical signals. The root mean square (RMS) value of this surface was found as 210 nm by using AFM. After antibody immobilization, the surface was changed. As seen in Fig. 3B and F, the porous property of the electrode surface was decreased and also a smooth surface was observed. The RMS value of this step was found as 160 nm. This decrease in RMS value supported the smooth surface formation. The blockage of free aldehyde ends was performed by BSA coupling. It is clearly seen that the BSA coverage present on the modified ITO electrode and this image was consistent with the literature. The RMS value of this step was found as 200 nm. After interaction between anti-p53 antibody and p53 antigen, the surface morphology was changed. As seen in Fig. 3D and H, protein aggregates were seen. The RMS value of this step was measured as 250 nm. The height of the chitosan-CB/anti-p53 antibody/BSA/p53 was not increased. These observations suggest that proteins with flexible conformations may change their conformation during the specific interaction.

3.5. Analytical characterization of the immunosensor

A Nyquist plot of the electrochemical impedance spectrum is used for measurement of the charge transfer resistance (R_{ct}) and Fig. 4A displays the Nyquist plots of the developed biosensor after incubation in a PBS solution containing different concentrations of p53 antigen for 45 min. As is seen from the plots, the R_{ct} increased along with the increasing p53 concentrations. The calibration curve (Fig. 4B) clearly showed that there was a linear relationship between the R_{ct} and p53 concentration over a range of 0.01–2 pg/mL. The limit of detection and limit of determination for p53 antigen were found as 3 fg/mL and 10 fg/mL, as it was obtained from the signal-to-noise characteristics of the data at S/N ratio of 3 and 10, respectively. The relative standard deviation (RSD) of the calibration curve was calculated as 1.41%.

SFI measurements were performed to monitor the immune-interaction specificity of the designed immunosensor by monitoring impedance during the measurement at fixed frequency. As seen in Fig. 4D, an increase was monitored in impedance value at fixed frequency. This increase proved the interaction between anti-p53 antibody and p53 antigen.

The repeatability of the biosensor was evaluated by successive determination of 0.30 pg/mL p53 antigen for 20 times using equally prepared immunosensor under optimal experimental conditions. The relative standard deviation was calculated to be 4.47%. The reproducibility of the immunosensor was evaluated by preparing 10 different biosensors independently. The relative standard deviation was found to be 2.74% showing an excellent reproducibility (Fig. 4E).

The regeneration of the proposed biosensor was examined by repeatedly monitoring of 0.1 pg/mL p53 antigen binding. Under optimized experimental conditions, acidic solution (ultra-pure water HCl 0.1%) was used for regeneration of the biosensor. Following each binding event, the electrode was regenerated for 5 min in the acidic solutions. After regeneration the proposed immunosensor was tested without and with p53 antigen. After three cycles, the surface of the proposed immunosensor got damaged. This result showed the reusability of the immunosensor (Fig. 4F).

Selectivity is an important parameter to obtain accurate results. Because of this, the selectivity was tested by using a mixture solution containing different biomarkers (Interleukin-1β, Interleukin-8, Tumor Necrosis Factor-α) and p53. The biosensor showed higher signal when it was incubated in mixture solution including p53 antigen. As seen in Figure SI-5, this immunosensor had excellent selectivity for p53 antigen.

Long storage stability shows the robustness of the biosensor. For the storage stability, the electrodes were kept at 4 °C for 10 weeks. After three weeks storage, this immunosensor had 75% initial activity (Fig. 4F).

The fabricated biosensor was applied to the determination of p53 in serum samples and the results are summarized in Table 1B. For the determination of p53 antigen by fabricated immunosensor in human serums, they were diluted 1000-fold. To test reability of the proposed immunosensor, p53 antigen standards were added. This biosensor had acceptable recovery (97.59–101.97). Considering the acceptable recovery in real samples, the obtained results showed the applicability of immunosensor.

4. Conclusion

In this article, we described a new impedimetric immunosensor based on the Chitosan-CB composite modified ITO electrode for p53 detection. Carbon black is highly conductive material and chitosan is natural and biocompatible polymer including a lot of amino groups. These features make them the best couple for immunosensor fabrication. Moreover, the difficult-to-use (due to low solubility) chitosan was used in this study in a simple and fast way to manufacture biosensor. In addition, carbon black promoted electron transfer due to good conductivity. The obtained results confirmed our visions. Moreover, chitosan-CB composite efficiently immobilized anti-p53 antibody and could be used in the preparation of other impedimetric immunosensors for detection of clinically or environmental interested biospecies. The combination of carbon black and chitosan provided a composite bioelectrode matrix and a robust immunosensor in which the antibody is directly attached to the electrode surface. Furthermore, the construction of the platform for the immunosensor was very simple and cost-effective and the nanocomposite was coated in one step on the electrode surface. In this study, anti-p53 antibodies were used as biorecognition elements and bound to amino groups via glutaraldehyde crosslinking. The immunosensor construction steps were monitored by electrochemical techniques (EIS, CV) and by morphological techniques (SEM and AFM). The interaction between anti-p53 antibodies and p53 antigens were monitored by using SFI technique and the quantification of P53 antigen was performed by EIS technique. Furthermore, the detection approach of the affinity reaction occurred on the chitosan-carbon black composite modified ITO electrode implies a significant improvement of the analytical characteristics achieved for the determination of p53 antigen with respect to previous immunosensor designs. This label free impedimetric immunosensor had good selectivity, low detection limit (3 fg/mL), wide linear range (0.01–2 pg/mL), good reproducibility and repeatability. This label-free immunosensor exhibited a fast response for p53 antigen with long-term stability and high selectivity. Also, Interfering species such as Interleukin-1β, Interleukin-8,

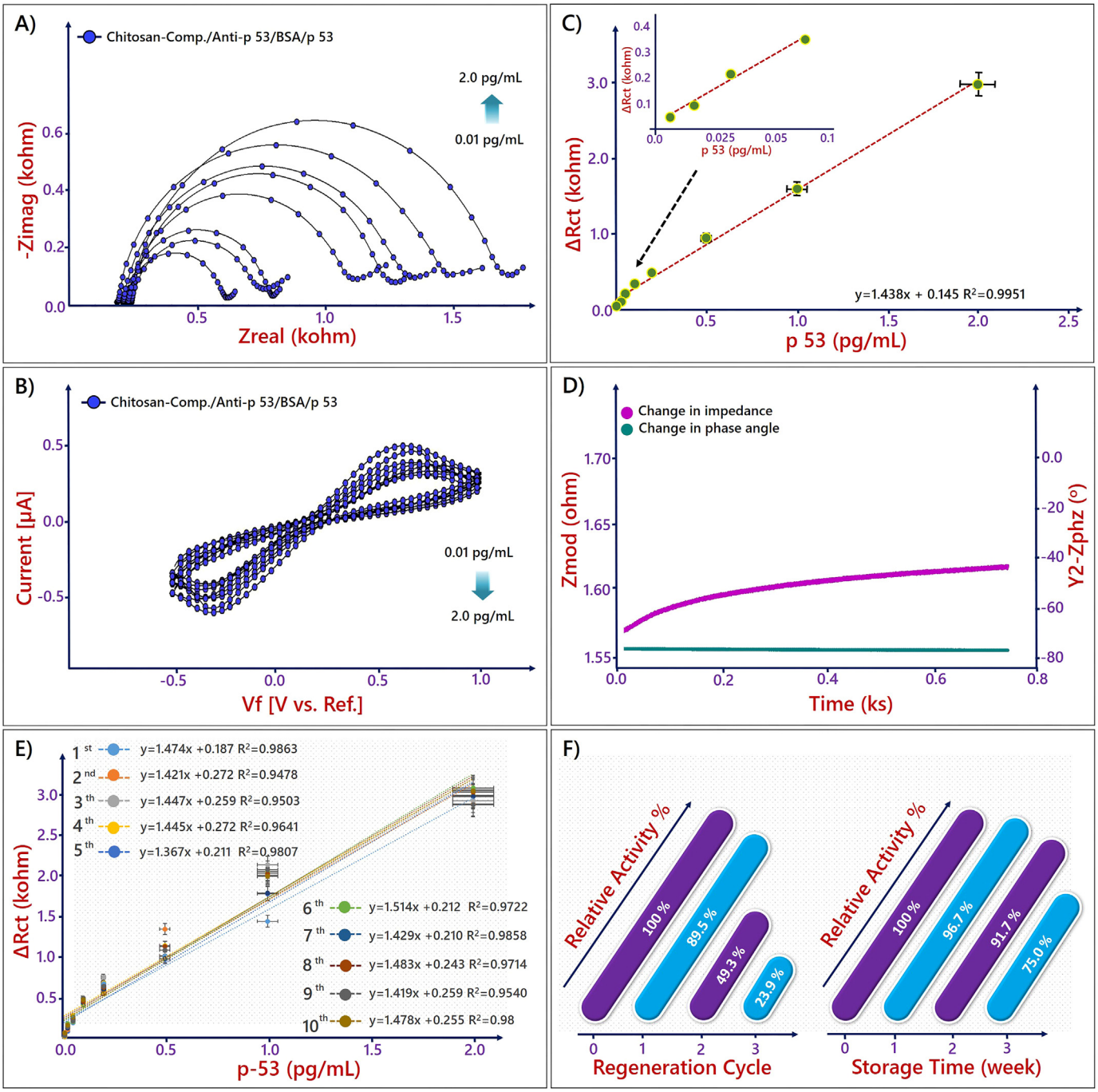


Fig. 4. EIS (A) and CV (B) results of increasing concentration of p53, calibration curve of proposed immunosensor (C), SFI result during antibody and antigen interactions (D), reproducibility of the immunosensor (E), regeneration and storage stability of the immunosensor (F).

Tumor Necrosis Factor-α did not significantly change the value of impedance of immunoelectrode. Moreover, the designed biosensor was applied for p53 antigen detection in human serum and this immunosensor can determine p53 antigen in human serum without sample treatment except a simple dilution with PBS buffer. The recovery results displayed that the method was simple and sensitive enough for the determination of p53 in serum samples. This new immunosensor provided a convenient, low cost, specific and sensitive way for p53 detection and this method could be used in the clinical analysis. In addition, this technique will a very attractive alternative to the existing methods due to simplicity, sensitivity and inexpensive property. Also, the possibility of miniaturization makes our immunosensor as suitable screening biotool for practical applications.

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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.bios.2018.09.008.

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