A novel label free immunosensor based on single-use ITO-PET electrodes for detection MAGE1 protein

Burcu Özcan[a]*, Mustafa Kemal Sezgintürk[b]

[a]Namık Kemal University, Faculty of Science, Chemistry Department, Biochemistry Division, Tekirdağ, Turkey

[b] Çanakkale Onsekiz Mart University, Faculty of Engineering, Bioengineering Department, Çanakkale, Turkey

***Corresponding author:**

e-mail: burcuozcannku@gmail.com

Phone: 0 (286) 218 00 00

Abstract

This study is aimed to design a label free biosensor based on transparent ITO-PET (Indium tin oxide- polyethylene terephthalate) electrode to determine MAGE1 protein. Firstly, indium tin oxide (ITO) electrodes were modified with $NH_4OH/H_2O_2/H_2O$ to obtain the OH groups on the surface. Later, the surface of ITO electrodes were treated with 3-glycidoxypropyltriethoxysilane (3-GOPE). After SAM formation, anti- MAGE1 protein was covalently immobilized on modified ITO electrodes. The parameters such as SAMs concentration, the concentration and incubation time of anti-MAGE1 solution were optimized. For determining the immobilization steps and optimization of the biosensor, electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV) were utilized. To determine analytical characterization of designed biosensor; linear range, repeatability, reproducibility, regeneration processes were studied. In optimum conditions, the linear range of the biosensor was determined as 0.01 pg-1.28 pg/mL. Square wave voltammetry technique was applied to the biosensor. These steps were realized in K_3 [Fe $(CN)_6$]/K_4 [Fe$(CN)_6$] redox probe. Moreover, Single frequency technique was used to understand the interaction between antiMAGE-1 and MAGE-1 protein. This step was realized in pH 7 phosphate buffer. And also, shelf life of designed biosensor was investigated. SEM (Scanning Electron Microscope) technique was used to determine the morphology of the surface in every step. The other important parameter for this study was Kramer's Kronig transform. It is used to determine whether the impedance spectra of the designed biosensor are affected from the deviation obtained because of external factors. Lastly, the designed biosensor was applied to real human serum and the results were compared with ELISA (Enzyme-Linked ImmunoSorbent Assay) test.

Keywords: ITO-PET electrodes, MAGE1, cyclic voltammetry (CV), electrochemical impedance spectroscopy (EIS), label free immunosensor

1. Introduction

Tumor markers are produced by cancer or by other cells of the body in response to cancer or certain benign (noncancerous) conditions. Most tumor markers are made by normal cells as well as by cancer cells. Nevertheless, they are produced at much higher levels in cancerous conditions [1]. Tumor markers are used to help detection and diagnosing some types of cancer. Human melanoma cells state antigens recognized by cytolytic T lymphocytes derived from the blood of the tumor-bearing patient or from tumor infiltrating lymphocytes [2]. A number of these antigens are encoded by genes of the MAGE family [3, 4]. The MAGE genes are stated not only in melanomas but also in tumors of other histological origins [5, 6]. These genes are silent in normal tissues apart from testis and placenta [7, 8]. Recent data remark that the expression of the MAGE genes in testis is restricted to the germ-line cells [9, 10]. Because male germ-line cells lack expression of the major histocompatibility complex molecules, they are not expected to present MAGE-encoded antigens. With regard to cancer immunotherapy, the potential usefulness of the antigens encoded by the MAGE genes largely rests on the tumor specific expression of these genes [11].

A silane compound is a monomeric silicon- based molecule containing four constituents. Silanes that contain at least one bonded carbon atom are called organosilanes. Functional silane compounds containing an organo- functional or organo- reactive arm can be used to conjugate biomolecules to inorganic substrates [12]. The appropriate selection of the functional or reactive group for a particular application can allow the attachment of proteins, oligonucleotides, whole cells, organelles, or even tissue sections to substrates. The organosilanes used for these applications include functional or reactive groups such as hydroxyl, amino, aldehyde, epoxy, carboxylate, thiol, and even alkyl groups to bind molecules through hydrophobic interactions [13].

ITO layers are transparent conducting oxide films and use in many fields such as optoelectronic devices, touch panels. And also, using ITO film electrodes for biosensors have been efficiently increased due to their many features such as high electrical conductivity, low cost and wide working area [14].

In this work, a label-free immunosensor for sensitive detection of MAGE1 protein was designed by using transparent ITO-PET electrodes. For this purpose, 3-GOPE was used as SAM solution. Anti- MAGE1 solution was covalently immobilized onto the ITO electrodes

by the help of 3-GOPE. All immobilization steps such as SAMs concentration, incubation time and concentration of anti-MAGE1 solution were optimized. Two main techniques EIS and CV were used for immobilization steps in designed immunosensor. Moreover, an efficient technique -single frequency impedance- was used to characterize the binding of MAGE1 and anti-MAGE1 protein. The impedance measurements were taken versus time at a fixed frequency. Reproducibility, repeatability and regeneration studies were also done for determination of analytical characterization of fabricated biosensor. It provided a wide detection range. However ITO coated PET electrodes were disposable, they could be regenerated. The surface morphology of the electrode in each immobilization step was observed by using SEM. Finally, the applicability of this biosensor to the real human serum was very important and the results of this process were compared with ELISA test. The proposed biosensor assay demonstrated good agreement with ELISA test.

2. Experimental

2.1. Materials and methods

All reagents and ITO-coated PET films were bought from the company named as Sigma Aldrich (USA). The transmittance and resistivity of the surface were 550 nm (>79%) and 60 ohm/square, respectively. MAGE-1 and antiMAGE-1 solution prepared with pH 7.0 phosphate buffer (50 mM) were also bought from Sigma Aldrich. 0.5% (BSA) was also prepared with phosphate buffer. Commercial ELISA kits were bought from LSBio LifeSpan BioSciences, Inc. All electrochemical experiments were generated 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) was utilized as a working electrode in three electrode system for the electrochemical measurements. Moreover, an Ag/ AgCl and a platin wire were utilized as a reference electrode and a counter electrode, respectively.

2.2.Construction of ITO-based immunosensor

Firstly ITO-PET electrodes were ultrasonically cleaned with acetone, soap solution, and ultra-pure water. After this process, the electrodes were treated with ultra-pure water including hydrogen peroxide (1:7, v/v) and ammonium hydroxide (1:7, v/v) to create hydroxyl groups on the surface. After this procedure the electrodes were washed with ultra-pure water and dried with argon gas. Later, the electrodes were immersed in 3-glycidoxytriethoxysilane (in toluene) for SAM modification and incubated with this solution

overnight. Silane groups were interacted with OH groups on the surface, anti-MAGE1 protein could be immobilized onto the surface for 1 hour. After the attachment of anti-MAGE1 protein on the surface BSA was used as a blocking agent.

2.3. Characterization of the anti-MAGE1 based immunosensor

All electrode modification and immobilization processes were characterized by using cyclic voltammetry and electrochemical impedance spectroscopy. All electrochemical measurements were realized in potassium ferricyanide/ potassium ferrocyanide (1:1) solution including 0.1 M KCl. For CV measurements, the applied potential was chosen between -0.5 V and 5 V. (step size: 20 mV; scan speed: 50 mV/s). Impedance spectra were collected in the frequency range between 10,000 and 0.05 Hz. Morphological changes obtained in each step of fabricated biosensor were observed with Scanning electron microscopy (FEI-Quanta FEG 250 model) at the Scientific and Technological Research Center of Namık Kemal University (NABILTEM).

3. Results and Discussion

3.1.Immobilization steps of the proposed biosensor

The first step for the immobilization of anti-MAGE1 was SAM formation. After the OH groups were generated on the surface, the electrodes were incubated with 3-GOPE solution. This silane-coupling agent can interact with OH groups on the electrode surface. The advantage of this type of functional silane derivative is to promote the bonding of an organic molecule to an inorganic particle, surface or substrate [15].

Two methods, EIS and CV, were used to characterize the fabrication processes for the biosensor. All measurements were realized with K_3 [Fe $(CN)_6$]/K_4[Fe$(CN)_6$] redox probe. This solution enables easy electron transport over the electroactive species on the surface [16]. The EIS spectra and the CV voltammograms for the fabricated biosensor are shown in Figure 1.

Fig 1 A B C

As can be seen in Fig. 1A, the significant anodic or cathodic peaks belonging to bare electrodes could not be monitored because of high electrical resistance. Nevertheless, anodic

and cathodic peak currents efficiently increased with formation of OH groups on the electrode surfaces. The reason for this change was possibly an increase in electron transfer rate of the redox probe. After the formation of hydroxyl groups on the surfaces, the electrodes were incubated with 3-GOPE solution overnight. One of the most useful silane modification agents is glycidoxy compound containing reactive epoxy groups [17]. The electrode surfaces covalently coated with this silane-coupling agent can be used to conjugate thiol-, amine- or hydroxyl- containing ligands. Thus, 3-GOPE solution can be used to link inorganic silica or other metallic surfaces containing –OH groups with biological molecules containing any of these three functional groups [13]. When Figure 1A is examined, it could be seen that the anodic and cathodic peak currents decreased. Negatively-charged epoxy groups of the SAM solution hindered diffusion of the negatively-charged redox probe onto the surface by generating impulsion. After SAM formation, anti-MAGE1 solution was covalently immobilized onto the silane-modified surface with the help of epoxy groups. The diffusion of the redox probe could be easily generated compared to 3-GOPE solution. It may be a result of anti-MAGE1 inhibiting the negative epoxy ends of SAMs. Hence, the anodic and cathodic peak currents increased. In the last step, BSA protein was used to block the free active ends. BSA treatment caused a decrease in anodic and cathodic peaks because of its insulating form.

EIS is largely used as a standard characterization technique for determining the construction and characterization steps of biosensors [18]. This technique can identify diffusion-limited reactions [19] and provide information on the capacitive behavior of the system [20]. Moreover, this method can provide information about the electron transfer rate of reaction. EIS data may be presented as a Bode plot or a Nyquist plot which have two sections: a semicircle part and a linear portion. The semicircle part and the linear part can be attributed to electron transfer resistance (Rct) and diffusion limited process, respectively [21]. Having looked at Figure 1B, the EIS spectra belonging to each immobilization step can be seen. The Rct value incidental to the OH formation step efficiently increased after the electrode surface was modified with 3-GOPE solution because of its epoxy groups. These negatively-charged functional groups pushed the negatively-charged redox probe and as a consequence inhibited the diffusion of the redox probe onto the electrode surface. When the modified electrodes were immobilized with anti-MAGE1 solution, the electron transfer resistance decreased in comparison with the previous step. The amine groups of anti-MAGE1 protein interacted with epoxy groups of 3-GOPE solution. Nevertheless, the unbounded epoxy ends were blocked with BSA which caused an increase in the electron transfer resistance. The reason for this

could be that the surface became more insulated. EIS measurements are widely examined by fitting to an equivalent circuit model shown in Figure 1C.

The scheme for the immobilization process is shown in Scheme 1.

Scheme 1

3.2.Optimization procedures for the biosensor

Optimization studies, which include SAMs concentration, incubation time and concentration of anti-MAGE1 solution, allow determination of the best fit conditions for a sensitive, repeatable and reproducible biosensor.

Firstly, the electrode surface was cleaned and in the following step, hydroxyl groups were generated on the ITO surface. Afterwards, the hydroxylated surface was modified with 3-GOPE solution which generated a strong monolayer for immobilization of anti-MAGE1 protein. The first optimized parameter was SAM concentration. So as to detect the influence of the concentration of 3-GOPE solution on the response of the biosensor, 0.1%, 0.5%, 1% and 1.5% 3-GOPE concentrations were studied for the fabricated biosensor. The calibration curves belonging to each different concentration of 3-GOPE are shown in Fig 1S. The charge transfer resistance values related to these concentrations were 3650, 2160, 3040 and 3540 Ω, respectively. The maximum Rct values were obtained when 0.1% 3-GOPE solution was used. When the Rct values obtained from EIS spectra were plotted versus the concentrations of MAGE-1 solution, it was proven that 0.1% 3-GOPE concentration provided better and linear results. The MAGE-1 protein calibration curves showed that higher concentrations could not provide good results compared with 0.1% 3-GOPE solution and the signals belonging to these concentrations were decreased. Besides, the highest concentration (1.5% 3-GOPE) would probably not allow a stable monolayer to be generated because high concentration of 3-GOPE solution caused denaturation of anti-MAGE1 protein. Namely, even if anti-MAGE1 was immobilized onto the modified surface, it could not attach to the MAGE1 protein. Due to these reasons, the optimal concentration of 3-GOPE was chosen as 0.1% for the constructed biosensor.

The second optimization parameter was concentration of anti-MAGE1 solution which is one of the most important steps for a good and linear biosensor. Different concentrations of anti-MAGE1 were tested to determine how the response of the biosensor was affected by the varied concentrations of anti-MAGE1 solution. The Rct values belonging to the used

concentrations of anti-MAGE1 solution (1.1 ng/mL, 5.5 ng/mL, 13.75 ng/mL, 27.5 ng/mL, 55 ng/mL) were 1340, 2120, 1380, 694 and 1360 Ω, respectively. The calibration curves belonging to each different concentration of anti-MAGE1 are shown in Fig. 2S. When the calibration curves are examined, the concentrations of anti-MAGE1 higher than 5.5 ng/mL could not provide good results. The intense surface possibly caused a loss of activity in productivity of the biosensor. The concentration 1.1 ng/mL could not provide a good result compared to 5.5 ng/mL. So the optimal concentration of anti-MAGE1 solution was chosen as 5.5 ng/mL.

Finally, the last optimization parameter was the incubation time for anti-MAGE1 protein. For that purpose, anti-MAGE1 solution with known concentration was immobilized onto the modified surfaces of ITO electrodes during different incubation periods (30 min, 60 min, and 75 min). The longer incubation period (75 min) caused a decrease in the charge transfer resistance. It probably resulted from surface damage. The damage was possibly caused by the high antibody amount on the surface because this created an insulating effect, which reduced the charge transfer resistance on the surface. However, the charge transfer resistance for 30 min and 60 min were close to each other. The calibration curves belonging to each period can be seen in Fig 3S. Consequently, 30 min was chosen as the incubation period for anti-MAGE1 protein.

3.3.Analytic characteristics of the anti-MAGE1 based immunosensor

The analytical productivity of the anti-MAGE1-based immunosensor was investigated by evaluating varied concentrations of MAGE-1 solution. In this study, the charge transfer resistances belonging to MAGE-1 concentrations were determined with the help of the model given in Fig. 1C. To acquire a calibration graph for the proposed biosensor, the following equation was used.

$$\Delta R_{ct} = R_{ct\ (anti\text{-}MAGE1/MAGE1)} - R_{ct\ (anti\text{-}MAGE1)}$$

$R_{ct\ (anti\text{-}MAGE1/MAGE1)}$ is the charge transfer resistance value after MAGE-1 binds to anti-MAGE1 and $R_{ct\ (antiMAGE1)}$ is the charge transfer resistance value when anti-MAGE is immobilized onto the surface [22].

Fig 2 A B C

Fig. 2 displays the cyclic voltammograms and Nyquist plots belonging to the optimized biosensor with increasing concentrations of MAGE-1 solution. The semicircle diameter of the EIS spectrum (Fig. 2A) increased with increasing MAGE-1 concentrations. Moreover, the linear detection range was determined between 0.01 pg mL^{-1} and 1.28 pg mL^{-1} with a regression coefficient (R squared) of 0.9958%.

Limit of detection (LOD; $3*S_{blank}/m$) and limit of quantification (LOQ; $10*S_{blank}/m$) were calculated as 0.0078 pg mL^{-1} and 0.026 pg mL^{-1}, respectively. S_{blank} and m are the signal of blank sample and slope, respectively [23]. This wide linear range could allow the determination of MAGE-1 protein sensitively. The calibration curve of the proposed biosensor is shown in Fig. 2B.

The CV voltammograms (in Fig. 2C) decreased with increasing MAGE-1 concentration because the increasing MAGE-1 concentrations generated an insulator effect on the modified electrode surface and inhibited the diffusion of the redox probe to the surface.

Apart from these two techniques, EIS and CV, the square wave voltammetry technique which is very fast and sensitive [24] was utilized to prove the EIS and CV results. In SWV, it was easily seen that the peak currents decreased with binding of MAGE-1 protein to the modified electrodes. The differences between the peak currents obtained after BSA treatment and binding of MAGE-1 protein to the surface increased with increasing concentration of MAGE-1. With each incubation of MAGE-1 protein, the surface became more insulating because of higher MAGE-1 amounts on the surface.

Fig 3

For investigation of the repeatability of anti-MAGE1-based biosensor, the same concentration of MAGE-1 solution (0.32 pg mL^{-1}) was utilized under the same conditions. It could be demonstrated that a perfect repeatable biosensor was obtained when the average value (0.3079 pg mL^{-1}), standard deviation (± 0.012596 pg mL^{-1}) and coefficient of variation (4.09 %) were calculated.

Reproducibility is one of the most important parameters in the fabrication of a sensitive, linear and good biosensor. To investigate the reproducibility of the biosensor, ten different biosensors were prepared under the same circumstances. The results obtained from these immunosensors indicated that the proposed biosensor showed excellent reproducible

characteristics. Relative standard deviation (RSD) values belonging to slope values for MAGE-1 calibration graphs were 7.3%.

Kramers-Kronig (K-K) relations can be utilized to appraise the impedance data quality [25]. The real part of a spectrum can be procured by an integration of the imaginary part or the imaginary part can be obtained by an integration of the real part. Goodness of fit values can be obtained by using the K-K transform. In practice, if the calculated model overlaps with the experimental impedance spectrum, it demonstrates that the biosensor has good linearity and accuracy and the biosensor is not affected by extrinsic parameters [26]. Consequently, the K-K transforms can be seen in Fig. 4S and goodness of fit values are given in Table 1.

3.4.Regeneration and storage life of the biosensor

To utilize the biosensor in practical applications, reusability of the 3-GOPE-modified sensor was a considerable necessity. In the regeneration step, we first prepared the electrode with known method. Later, the electrode was incubated in MAGE-1 solution. EIS measurement was taken at this step. After the first EIS measurement, the same electrode was treated with 10 mM HCl (0.1%) acid solution to remove the weak interactions such as hydrogen bonds or van der Waals between MAGE-1 and anti-MAGE1 protein for 5 min. Then, EIS measurement was taken again. Unsurprisingly, the Rct value obtained after HCl application to the electrode surface was decreased because the MAGE-1 protein was removed from the biosensor's surface. After the first HCl treatment, the electrode was incubated in MAGE-1 protein solution and the EIS measurement was taken again. It could be seen that the Rct value increased and was similar to the Rct value obtained from the first EIS measurement.

As mentioned above, the same electrode was secondarily incubated in the regeneration solution (HCl, 0.1 %) for 5 minutes. After that, Rct value decreased again as expected. When looking at the EIS measurements, it could be seen that the Rct values belonging to BSA and the obtained Rct values after each HCl treatment were similar to each other. It could be explained if the weak interactions between MAGE-1 and anti-MAGE1 were only removed from the electrode surface. If 3-GOPE or anti-MAGE-1 were removed the electrode surface, the Rct values after HCl treatment would decrease more than the values obtained from BSA treatment. This process could be repeated four times for the present biosensor. After the fifth HCl solution pretreatment, the biosensor began to become damaged and lost its activity. The

biosensor preserved 95% of its initial activity after the first regeneration step and after the second regeneration 90% of its initial activity remained. After the fourth regeneration 80% of its initial activity was present. Finally, at the end of the fifth incubation with acid solution, the biosensor was destroyed and so MAGE-1 could not bind to the biosensor's surface. Even if the ITO-PET electrode was a disposable electrode, the ITO-based biosensor could be regenerated four times. This is one of the most important advantages of the biosensor especially when it could be applied in clinical use.

To determine storage life of the fabricated biosensor, the electrodes were prepared in accordance with the procedure at the optimum conditions. The electrodes were kept at 4 °C for long time periods (8 weeks). The biosensor had the same activity for two weeks. After 3 weeks, it insignificantly lost 0.33% of its activity. After 7 weeks, it preserved 93.18% of its activity. Finally in the eighth week, the biosensor did not produce a response (Fig. 4). It could be concluded that the biosensor has stability for long-term storage.

3.5. Single frequency technique

As is known, EIS is utilized for investigation of immobilization and characterization steps. In this study, unlike EIS, single frequency technique could be used to indicate the binding of MAGE-1 to anti-MAGE1 with the help of impedance measurements at a fixed frequency, which was chosen by using Bode plots versus time. 30 Hz and 300 seconds were chosen as the fixed frequency and time, respectively. The measurements were taken in phosphate buffer (pH 7.0). The result is displayed in Fig. 5.

Fig 5

3.6.Morphological monitoring of the electrode surface

In morphological monitoring of different biosensor surfaces, the SEM technique was utilized and related pictures can be seen in Fig 6. In A, the bare electrode surface and hydroxylated electrode surface are shown in A and B, respectively. After hydroxylation, the surface was modified with 3-GOPE and is seen in C. The electrode surface after anti-MAGE1 immobilization is shown in D and the surface after BSA treatment is shown in E. Finally, the MAGE-1 bound surface is seen in F.

3.7.MAGE-1 detection in real human serum

ELISA is an efficient and certain method to measure antigen or antibody concentration [27]. However, this technique is expensive and time-consuming in contrast to label- free immunosensors [28].

In this process, the aim was to determine whether the proposed biosensor could detect MAGE-1 protein in real human serum supplied by Namık Kemal University Faculty of Medicine (with ethics committee permission). The ELISA technique was also used for the same purpose and the results obtained from these two techniques were compared with each other. The detection range of MAGE-1 for this ELISA kit is in the range of 0.625 - 40 ng/mL. The comparison of these techniques is shown in Table 1. When the results are examined, the biosensor results of MAGE-1 in clinical serum samples were in a good agreement with the standard ELISA results. As is seen, the current biosensor could sensitively detect MAGE-1 in human serum.

4. Conclusion

In this study, we reported a design of a sensitive, low-cost and high performance biosensor for detection of MAGE-1. The single-use and flexible ITO-PET electrode was used as a working electrode. 3-GOPE which is a silanization agent was firstly used for biosensors and it was proven that it successfully generated an active layer. Optimization parameters such as SAMs concentration, antibody concentration were analyzed and the optimum conditions were determined for a high sensitive and accurate biosensor. We also proved the accuracy of the biosensor by using SEM and SWV. The designed biosensor has a long lifetime and it is very advantageous and necessary parameter for clinical use. Moreover, our biosensor could be regenerated. Finally, the proposed biosensor was applied to real human serum samples. The results were compared with ELISA test. The results of these two techniques are close to each other. It suggests that the designed biosensor in this study can be used as a diagnostic tool in clinical field.

Acknowledgment

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

References

[1] W. Bigbee, R.B. Herberman, Tumor markers and immunodiagnosis. In: R.C. Bast, D.W. Kufe, R.E. Pollock (Eds), Cancer Medicine, 6th ed., BC Decker Inc., Hamilton, Ontario, Canada, 2003.

[2] T. Boon, J.C. Cerottini, B. Van den Eynde, P. van der Bruggen, A.Van Pel, Tumor antigens recognized by T lymphocytes, Annu. Rev. Immunol.12 (1994) 337-365.

[3] P. van der Bruggen, C. Traversari, P. Chomez, C. Lurquin, E. De Plaen, B. Van den Eynde, A. Knuth, T. Boon, A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma, Science 254 (1991) 1643-1647.

[4] B. Gaugler, B. Van den Eynde, P. van der Bruggen, P. Romero, J.J. Gaforio, E. De Plaen, B. Lethe, F. Brasseur, T. Boon, Human gene MAGE-3 codes for an antigen recognized on a melanoma by autologous cytolytic T lymphocytes, J. Exp. Med. 179 (1994) 921-930.

[5] F. Brasseur, M. Marchand, R. Vanwijck, M. Herin, B. Lethe, P. Chomez, T. Boon, Human gene MAGE-1, which codes for a tumor-rejection antigen, is expressed by some breast tumors, Int. J. Cancer 52 (1992) 839-841.

[6] P. Weynants, B. Lethe, F. Brasseur, M. Marchand, T. Boon, Expression of MAGE genes by non-small-cell lung carcinomas, Int. J. Cancer 56 (1994) 826-829.

[7] E. De Plaen, C. Traversari, J. J Gaforio, J.P. Szikora, C. De Smet, F. Brasseur, P. van der Bruggen, B. Lethé, C. Lurquin, P. Chomez, O. De Backer, T. Boon, K. Arden, W. Cavenee, R. Brasseur, Structure, chromosomal localization, and expression of 12 genes of the MAGE family, Immunogenetics 40 (1994) 360-369.

[8] C. De Smet, C Lurquin, P. van der Bruggen, E. De Plaen, F. Brasseur, T.Boon, Sequence and expression pattern of the human MAGE2 gene, Immunogenetics 39 (1994) 121-129.

[9] P. Chomez, R. Williams, O. De Backer, T. Boon, B. Vennstrom, The Smage gene family is expressed in post-meiotic spermatids during mouse germ cell differentiation, Immunogenetics 43 (1995) 97-100.

[10] K.Takahashi, S. Shichijo, M. Noguchi, M. Hirohata, K. Itoh, Identification of MAGE-1 and MAGE-4 proteins in spermatogonia and primary spermatocytes of testis, Cancer Res. 55 (1995) 3478-3482.

[11] A. Jassim, W. Ollier, A. Payne, A. Biro, R.T.D. Oliver, H. Festenstein, Analysis of HLA antigens on germ cells in human semen, Eur. J. Immunol. 19 (1989) 1215-1220.

[12] M. Longhaei, M. Sadeghi, B. Ghalei, M. Shahrooz, The role of compatibility between polymeric matrix and silane coupling agents on the performance of mixed matrix membranes: Polyethersulfone/MCM-41, Journal of Membrane Science 513 (2016) 20-32.

[13] G. T. Hermanson, Silane coupling agents, third ed., Bioconjugate Techniques, 2013.

[14] M. Z. H. Khan, Effect of ITO surface properties on SAM modification: A review toward biosensor application, Cogent Engineering 3 (2016) 1170097.

[15] J. J. Chruściel, E. Leśniak, Modification of Thermoplastics with Reactive Silanes and Siloxanes, in: A.Z. El-Sonbati (ed.), Thermoplastic Elastomers, InTech, Rijeka, 2012.

[16] B. Özcan, M. K. Sezgintürk, Graphene oxide based electrochemical label free immunosensor for rapid and highly sensitive determination of tumor marker HSP70, Talanta 160 (2016) 367-374.

[17] Y. Xie, C. A. S. Hill, Z. Xiao, H. Militz, C. Mai, Silane coupling agents used for natural fiber/polymer composites: A review, Composites: Part A 41 (2010) 806-819.

[18] D. Grieshaber, R. MacKenzie, J. Vörös, E. Reimhult, Electrochemical Biosensors - Sensor Principles and Architectures, Sensors 8 (2008) 1400-1458.

[19] R. Sharma, S.E. Deacon, D. Nowak, S.E. George, M.P. Szymonik, A.A.S. Tang, D.C. Tomlinson, A.G. Davies, M.J. McPherson , C. Wälti, Label-free electrochemical impedance biosensor to detect human interleukin-8 in serum with sub-pg/ml sensitivity, Biosensors and Bioelectronics 80 (2016) 607-613.

[20] J. K. Bhattarai, Y. H. Tan, B. Pandey, K. Fujikawa, A. V. Demchenko, K. J. Stine, Electrochemical impedance spectroscopy study of Concanavalin A binding to self-assembled monolayers of mannosides on gold wire electrodes, Journal of Electroanalytical Chemistry 780 (2016) 311-320.

[21] Ç. Sayıklı Şimşek, M. N. Sonuç Karaboğa, M. K. Sezgintürk, A new immobilization procedure for development of an electrochemical immunosensor for parathyroid hormone detection based on gold electrodes modified with 6-mercaptohexanol and silane, Talanta 144 (2015) 210-218.

[22] M. Ç. Canbaz, M. K. Sezgintürk, Fabrication of a highly sensitive disposable immunosensor based on indium tin oxide substrates for cancer biomarker detection, Analytical Biochemistry 446 (2014) 9-18.

[23] M. N. Sonuç Karaboğa, Ç. Sayıklı Şimşek, M. K. Sezgintürk, AuNPs modified, disposable, ITO based biosensor: Early diagnosis of heat shock protein 70, Biosensors and Bioelectronics 84 (2016) 22-29.

[24] L. Li , H. Zhao, Z. Chen, X. Mu, L. Guo, Aptamer biosensor for label-free square-wave voltammetry detection of angiogenin, Biosensors and Bioelectronics 30 (2011). 261-266.

[25] B. Demirbakan , M. K. Sezgintürk, A novel immunosensor based on fullerene C60 for electrochemical analysis of heat shock protein 70, Journal of Electroanalytical Chemistry, 2016.

[26] M. Urquidi-Macdonald, S. Real, D. D. Macdonald, Application of Kramers-Kronig Transforms in the Analysis of Electrochemical Impedance Data II. Transformations in the Complex Plane, J. Electrochem. Soc. 133 (1986) 2018-2024.

[27] A. T. Perestam, K. K. Fujisaki, O. Nava, R. S. Hellberg, Comparison of real-time PCR and ELISA-based methods for the detection of beef and pork in processed meat products, Food Control 71 (2017) 346-352.

[28]F. Malvano, D. Albanese, A. Crescitelli, R. Pilloton, E. Esposito, Impedimetric Label-Free Immunosensor on Disposable Modified Screen-Printed Electrodes for Ochratoxin A, Biosensors 6 (2016) 33.

Table 1.

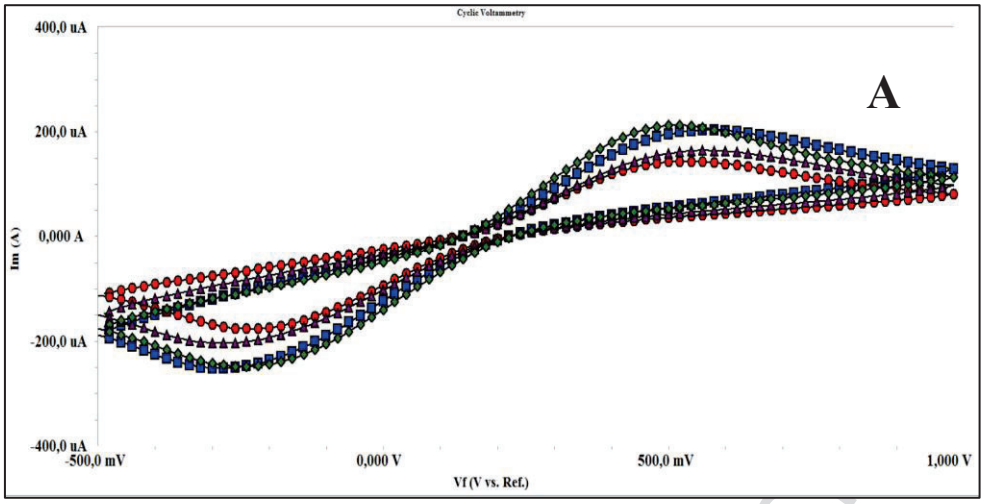
The results of Kramer's Kronig transforms, sample analysis and comparison of used methods to determine HSP70.

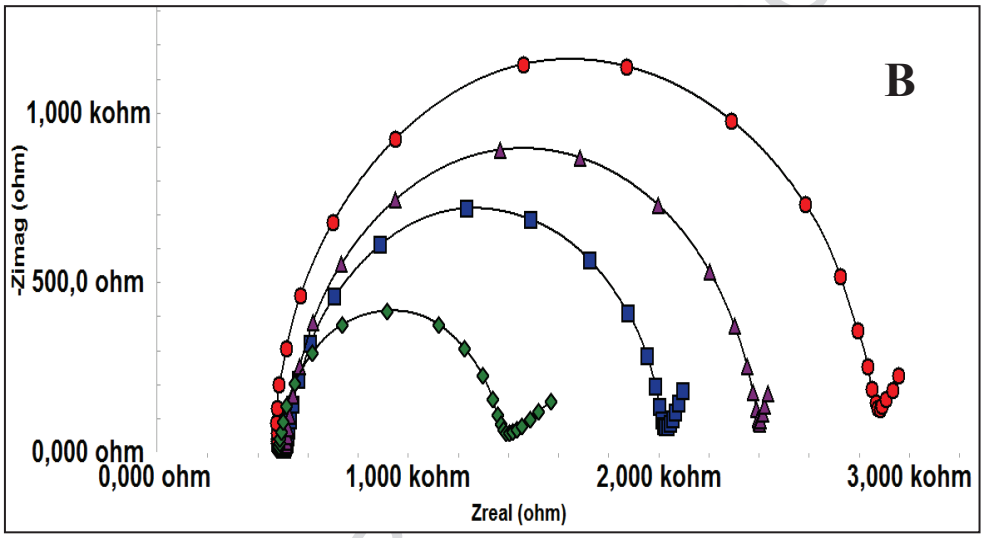
The surface of biosensor	Goodness of fit values
ITO/OH	5.109
ITO/OH/3-GOPE	13.65
ITO/OH/3-GOPE/anti-MAGE1	7.014
ITO/OH/3-GOPE/anti-MAGE1/BSA	595.2
ITO/OH/3-GOPE/antiMAGE1/BSA/MAGE1	10.59

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

	ELISA test	The proposed biosensor	Difference (%)
Sample 1	10.414 ng/mL	10 ng/mL	3.97
Sample 2	20.69 ng/mL	20.5 ng/mL	0.92
Sample 3	21.03 ng/mL	20 ng/mL	4.89

FIGURES





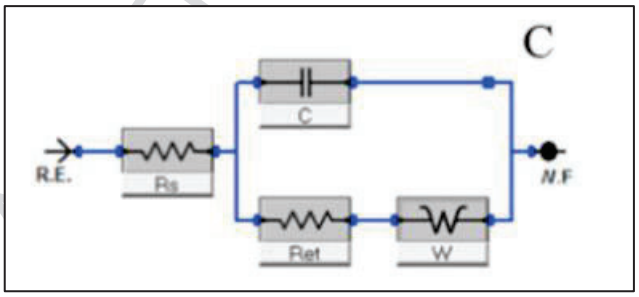
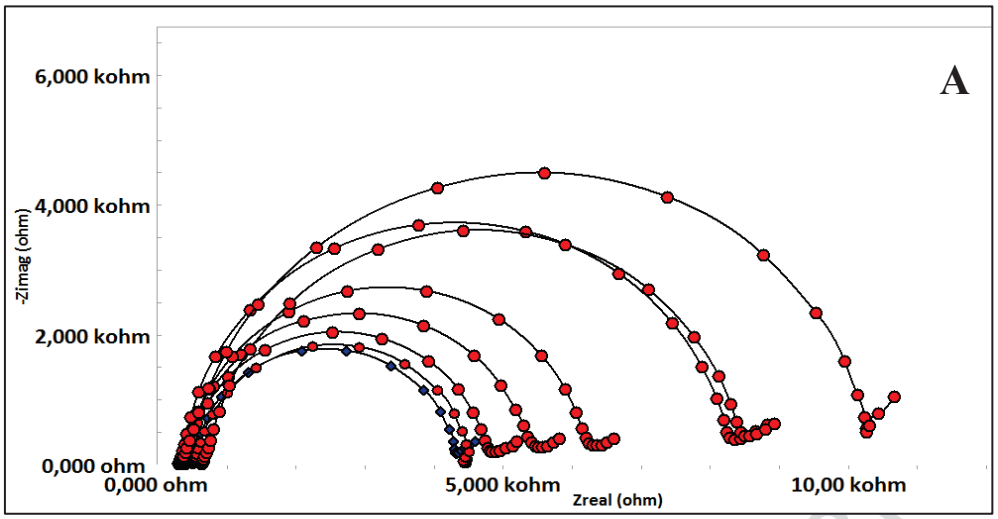
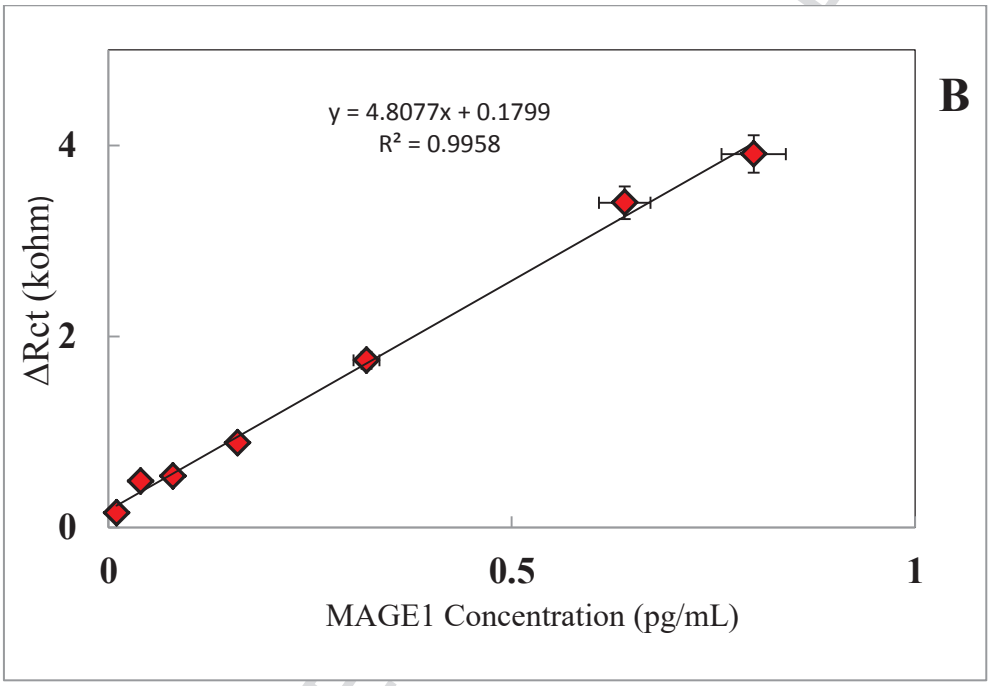


Fig 1. A.B.C





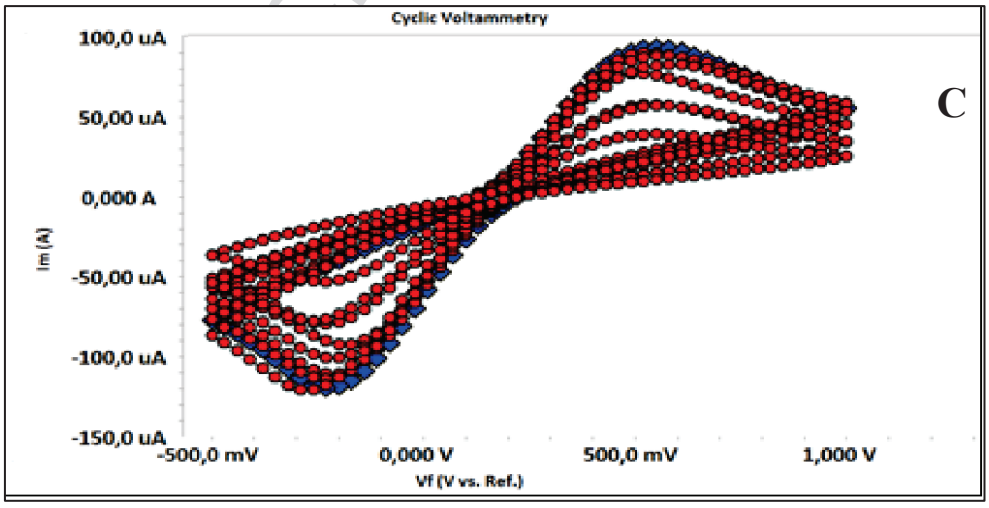


Fig 2 A.B.C

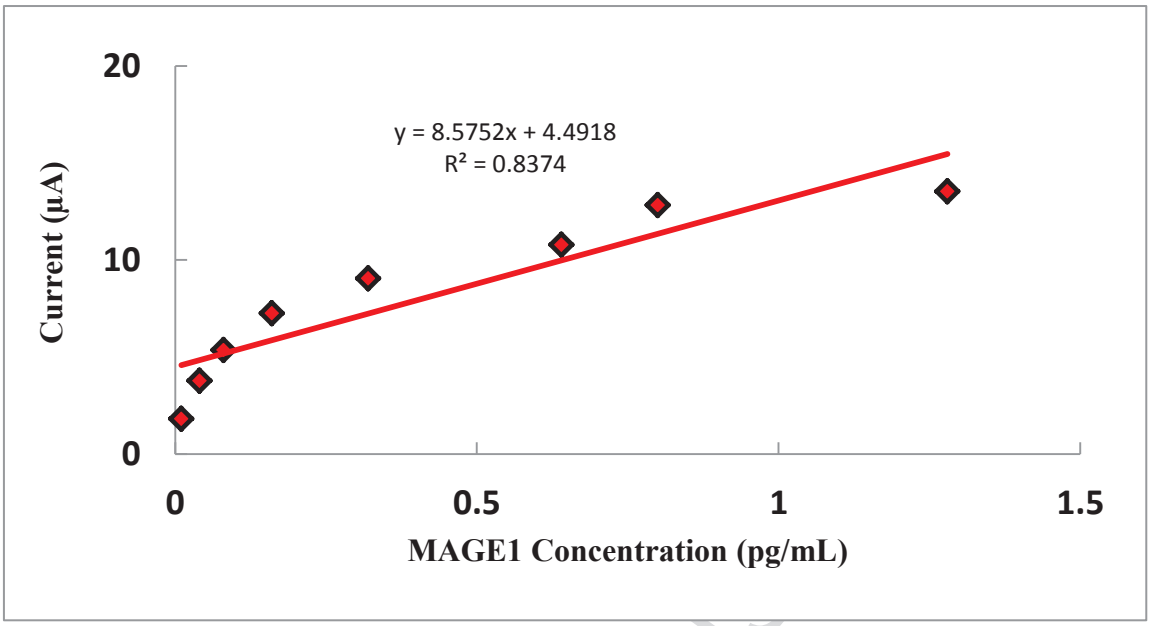


Fig 3.

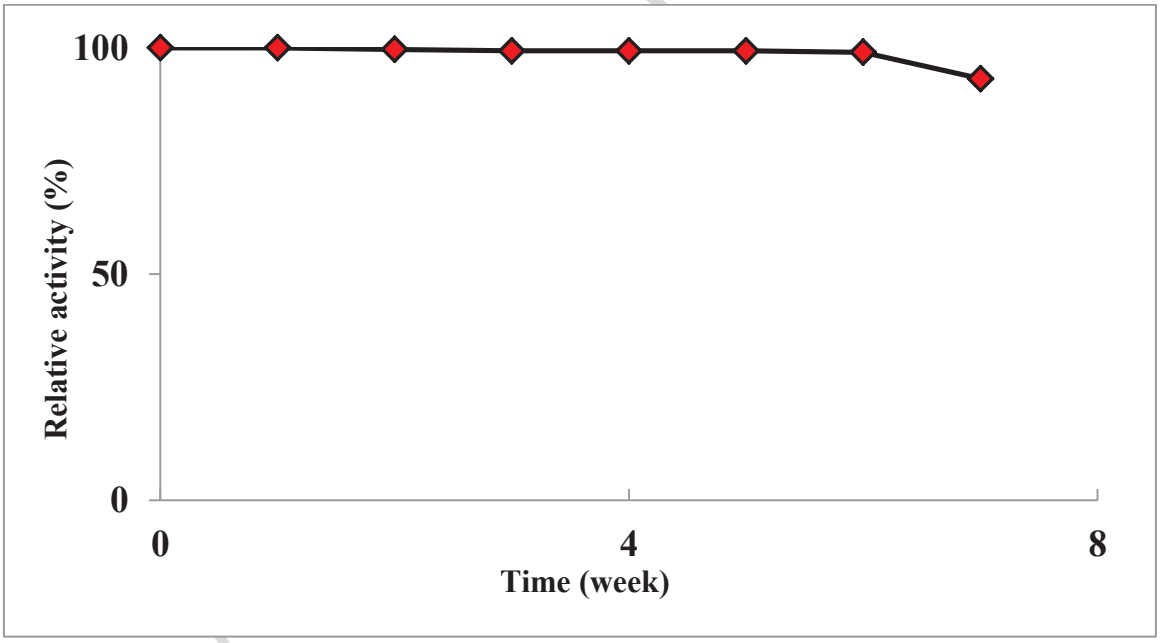


Fig 4.

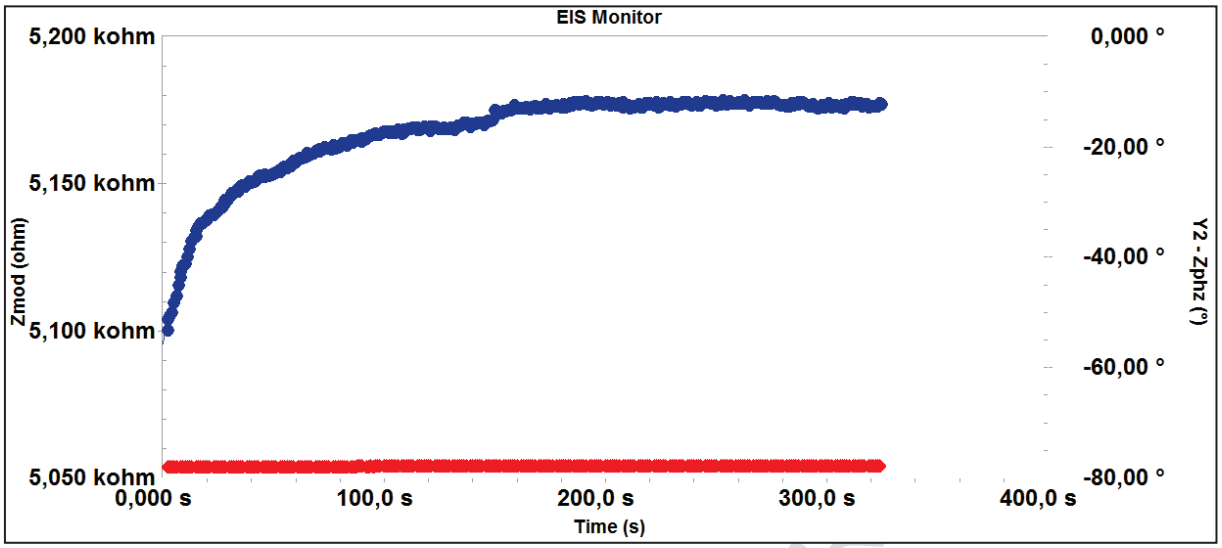


Fig 5.

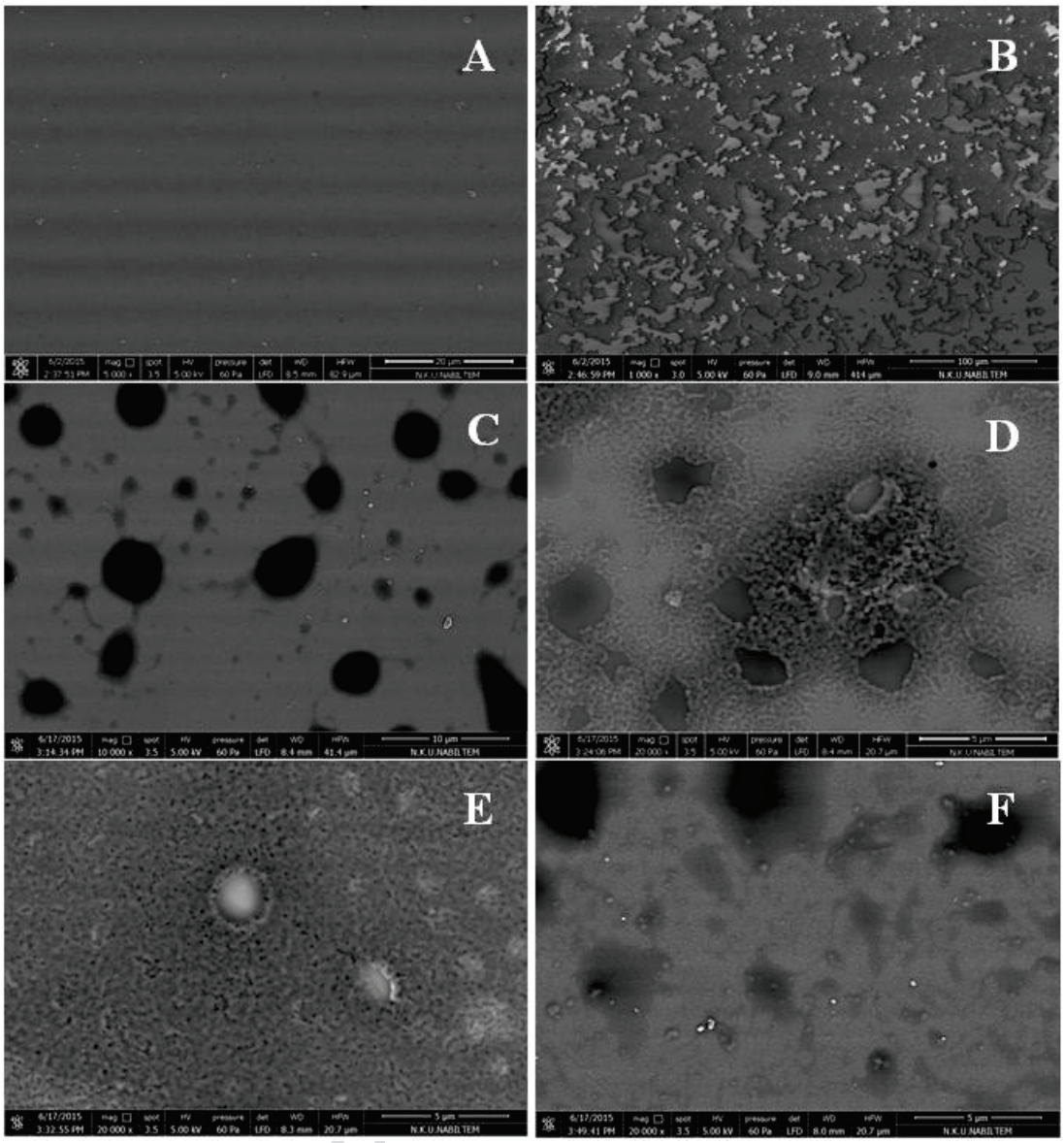


Fig 6.

Figure and Scheme Legends

Figure 1 CV voltammograms (A) and EIS spectra (B) of immobilization steps. Green (--♦-♦-): ITO/OH, purple (▲-▲): ITO/OH/3-GOPE, blue (-■-■-): ITO/OH/3-GOPE/anti-MAGE1, red (-●-●-): ITO/OH/3-GOPE/anti-MAGE1/BSA. C) Equivalent circuit model applied to fit the impedance measurements

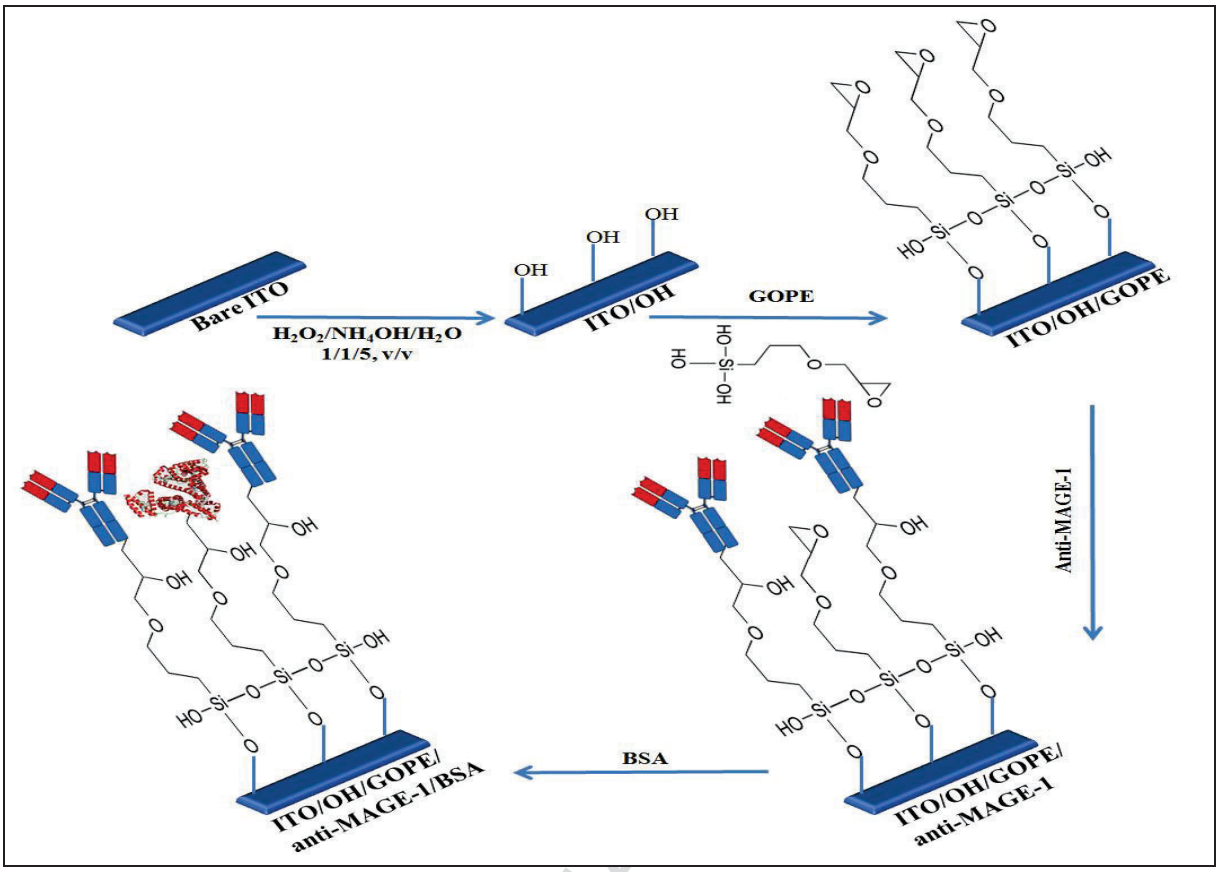
Figure 2 MAGE-1 calibration curve study for the ITO based biosensor. (A) Impedance spectra obtained for the different MAGE-1 concentrations (B) MAGE-1 linear calibration plot drawn by the impedance differences extracted from (A). (C) CV voltammograms obtained for the different MAGE-1 concentrations.

Figure 3 MAGE-1 calibration curve obtained by the SWV.

Figure 4 The storage stability of the designed biosensor.

Figure 5 Single frequency impedance measurement.

Figure 6 SEM characterization of designed biosensor surfaces. A) bare ITO-PET surface B)ITO-PET/OH C) ITO-PET/OH/3-GOPE D)ITO-PET/OH/3-GOPE/antiMAGE1 E) ITO-PET/OH/3-GOPE/anti-MAGE1/BSA F) ITO-PET/OH/3-GOPE/anti-MAGE1/BSA/MAGE1



Scheme 1. Representation of immobilization steps of anti-MAGE1 onto ITO-PET electrodes.

Highlights

- Single Frequency Impedance was used for identifying the antibody- antigen interaction.
- The new biosensor showed high analytical performance with a linear range 0.01 pg/mL- 1.28 pg/mL.
- CV, EIS and SEM methods were used for characterization of the electrode surfaces.
- Kramers Kronig transform was performed on the experimental impedance data.
- The proposed biosensor was applied to real human serum samples. The results were compared with ELISA test.