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A novel sensitive doxorubicin impedimetric immunosensor based on a specific monoclonal antibody–gold nanoaprticle–sol–gel modified electrode

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ABSTRACT

In this work, ultrasensitive electrochemical doxorubicin impedance immunosensor was investigated based on immobilization of a specific monoclonal antibody on gold nanoparticles (GNPs) associated with a thiol base sol–gel (TBSol–Gel) modified gold electrode. Scanning electron microscopy (SEM) and atomic force microscopy (AFM) were employed for characterization of the various layers that were formed at the electrode surface. The redox couples of 1.0 mmol L^{-1}, $Fe(CN)_6^{4-/3-}$ species on the electrode surface was followed to study the layers formation and determination process, using electrochemical impedance spectroscopy (EIS) and cyclic voltammetry (CV). After optimization of analytical parameters, the relative charge transfer resistance (R_{ct}) increases linearly with doxorubicin concentration in the ranges of 0.1–1.0 and 2.5–50.0 pg mL^{-1}, with a detection limit of 0.09 pg mL^{-1}. A high association constant of 1.4×10^{11} L mol^{-1} was obtained for the affinity of doxorubicin toward the immobilized antibody on the electrode surface. The capability of the proposed immunosensor for the determination of doxorubicin in spiked human serum and urine samples was examined by standard addition method, and the results show that the immunosensor is a useful tool for the determination of doxorubicin in the biological samples.

1. Introduction

Doxorubicin is an anthracycline antibiotic that has been extensively used for chemotherapeutic treatment of a variety of cancers, such as lymphoblastic leukemia, soft tissue and osteogenic sarcomas, breast and thyroid. Doxorubicin is also known by the trade name of “adriamycin” that intercalated by DNA. Most serious drawback effect of doxorubicin is life-threatening heart damage. The detection of doxorubicin in biological and clinical samples is very important because of its high cardiotoxicity effects [1–3].

To date various methods such as liquid chromatography [4–6], electrophoresis [7,8] and spectrometry [9,10] have been developed and introduced to measure doxorubicin which are time-consuming and expensive and have low sensitivity. Several general electrochemical methods have been reported as simple and inexpensive methods for doxorubicin determination [11–13]. These electrochemical methods have not had sufficient selectivity and sensitivity. As well, biological samples have a complex matrix and generally contain of low doxorubicin concentration. Therefore, electrochemical impedance-based immunosensors that are efficient methods proposed to overcome these problems. Impedimetric immunosensors are label-free electrochemical immunosensors based on the detection of the binding between immobilized antibody and antigen that are known as a sensitive and non-destructive technique [14,15].

The immobilization of biomolecules such as antibody on the surface of electrode is the most important step in the impedimetric immunosensors to generate stable, reproducible and selective biosensors. Antibodies are physically adsorbed on the gold surface; however, the adsorption of antibodies directly onto the bulk metal surface leads to the denaturation and the reduction of their bioaffinity. Antibodies that are adsorbed on the surface of noble metals nanoparticles can retain their activity because of the biocompatibility of these nanoparticles mainly GNPs [16–18].

Modification of the electrode surface with various functional groups such as SH and NH_2 provide a suitable substrates to bind the GNPs to the electrode surface. Self-assembly process is commonly used with dithiol groups. In 2011, Rezaei et al. reported an impedimetric immunosensor based on the immobilized antibodies on the surface of the gold electrode modified with 1,6-hexanedithiol and GNPs [19]. Although this method is sensitive, but due to stability limitation, foul odor and toxicity of dithiols, their application is difficult. Furthermore, in this method, the gold

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electrode was modified by immersion into a 1,6-hexanedithiol solution for 7 h and a solution of colloidal gold nanoparticles for 44 h to obtain the GNP-modified gold electrode (GNPs/HDT/gold electrode) which is very time consuming.

Sol–gel method is one of the more practical methods to modify the electrode surface. This method provides a simple, rapid and stable way to design modified surfaces with various functional groups that can be further used as functionalized sites to immobilize gold nanoparticles and antibodies [20,21].

The aim of this research is to develop a rapid and sensitive doxorubicin immunosensor using sol–gel method for modification of the electrode surface to facile immobilizing of antibody. The gold nanoparticles deposited electrochemically on the surface of 3-(trimethoxysilyl)-1-propanethiol sol–gel matrix to retain the bioactivity of the immobilized antibodies on the electrode surface. The electrochemical behavior of the modified electrode was investigated employing cyclic voltammetry (CV). Electrochemical impedance spectroscopy (EIS) was used to study the interaction of the doxorubicin and antibody immobilized on the electrode surface.

2. Experimental

2.1. Reagents

The mouse monoclonal doxorubicin antibody and doxorubicin hydrochloride were purchased from Enzo Company. Hydrogen tetrachloroauratetrihydrate ($HAuCl_4 \cdot 3H_2O$) and 3-(trimethoxysilyl)-1-propanethiol (MPTS) were obtained from Merck. Bovine serum albumin (BSA), potassium ferrocyanide, potassium ferricyanide and all chemicals were of analytical grades and purchased from Merck and Sigma, and doubly distilled water was used in preparation of all solutions.

2.2. Apparatus

Electrochemical measurements were performed with a potentiostat-galvanostat Autolab with a three-electrode cell containing a saturated Ag/AgCl (3 mol L^{-1} KCl) reference electrode and a platinum electrode was used as an auxiliary electrode. Unmodified or modified gold electrode with different layers was applied as working electrode. The system was run on a PC by GPES and FRA 4.9 software. Cyclic voltammetry (CV) and electrochemical impedance spectroscopy (EIS) were carried out in a probe solution containing 1.0 mmol L^{-1} $K_3[Fe(CN)_6]$ and $K_4[Fe(CN)_6]$ and 0.1 mol L^{-1} KCl in 0.1 mol L^{-1} phosphate buffer with pH=5.0. In CVs, potential was cycled from −200 to 600 mV, with a scan rate of 50 mV s^{-1}. The EIS measurements were recorded within a frequency range of 10 kHz to 10 mHz at the potential of redox couple $Fe(CN)_6^{4-/3-}$ (200 mV).

The surface of modified gold electrode was characterized by scanning electron microscopy (SEM) (Zeiss, Germany), and atomic force microscope (AFM) (Bruker, Germany).

2.3. Preparation of TBSol–Gel

The TBSol–Gel for the self-assembly was prepared by mixing absolute ethanol, MPTS and aqueous hydrochloric acid (0.1 mol L^{-1}) at the volume ratio of 1.5:1:1, and stirring the mixture for 10 min at 50 °C until a homogeneous solution resulted [22].

2.4. Fabrication of the immunosensor

Before modification, gold electrode was carefully polished with 0.05 μm alumina slurry on a polishing cloth pad and rinsed with water. Then the electrode was washed ultrasonically in ethanol-water (1:1, v/v) for 5 min. The electrode was then subjected to electrochemical pretreatment using potential cycling from –200 to 1500 mV with a scan rate of 100 mV s^{-1} in a 0.5 mol L^{-1} H_2SO_4 solution until a stable cyclic voltammogram was obtained. Then the electrode allowed drying at room temperature.

The cleaned gold electrode was left in the thiol base sol–gel for 10 s and then was stored at room temperature for 15 min. After that, it rinsed with water to remove the physically adsorbed materials and to complete the sol–gel formation process.

For the immobilization of gold nanoparticles, the pretreated electrodes were immersed into a 0.1% of $HAuCl_4 \cdot 3H_2O$ solution (about180 s), at the potential of −200 mV. After electrochemical deposition of gold nanoparticles, the modified electrode rinsed with water and dried at room temperature.

The immobilization of the antibody on the electrode surface was carried out with the following procedure: 15 μL of 20.0 μg mL^{-1} doxorubicin monoclonal antibody solution in phosphate buffer (0.1 M, pH 5.0) was dropped on the surface of the GNP/TBSol–Gel modified electrode for 2 h at room temperature in a high-humidity condition. Then, a 1.0% BSA solution was applied to the electrode surface for 30 min to block the non-specific sites. Lastly, the electrode was washed with PBS solution to remove any unstable adsorbed molecules from the electrode surface and dried with nitrogen gas to finish electrode modification. This electrode (MAb/GNP/TBSol–Gel/Au electrode) was used to determine doxorubicin throughout the experiment.

2.5. Immunoreaction and doxorubicin detection

To facilitate the formation of antibody and antigen interactions the MAb/GNP/TBSol–Gel/Au electrode was immersed into 5 mL antigen solutions containing 0.1–50.0 pg mL^{-1} doxorubicin, 0.1 mmol L^{-1} phosphate buffer solution with pH 5.0 at 4 °C for 30 min. Subsequently, the electrode was soaked in the same buffer solution to remove nonspecifically absorbed antigen. Then it was transferred into the electrochemical cell containing probe solution for impedimetric measurements.

Electrochemical impedance spectroscopy was used as a suitable technique to study the interaction of the doxorubicin and antibody immobilized on the electrode surface. The charge transfer resistance (R_{ct}) can be used as a signal to monitor the immobilized doxorubicin concentration on the surface of the MAb/GNP/TBSol–Gel/Au electrode. Variation of the relative resistance ($[R_{ct}(i) - R_{ct}(0)]/R_{ct}(0)$) was used as a signal for the immunosensor, where $R_{ct}(0)$ and $R_{ct}(i)$ are the electron transfer resistance of MAb/GNP/TBSol–Gel/Au electrode before and after immersing in the doxorubicin solution, respectively.

2.6. Preparation of real samples

Human serum sample was obtained from the Health Center of Isfahan University of Technology. To precipitate proteins, the human serum was mixed with the trichloroacetic acid solution (10%, w/v) with a 1:1 (v/v) ratio. Then, it was centrifuged for 10 min with 1200 rpm and the supernatant was used without further pretreatment. The urine sample was collected from healthy person and was centrifuged (10 min at 1200 rpm). After filtering, it diluted 10 times with doubly distilled water and was used without any additional pretreatment. 0.5 mL of each samples were used in each experiment and the pH adjusted by 4.5 mL of 0.1 M PBS solution with pH 5.0. The standard addition method was used to determine doxorubicin in the spiked samples. Before EIS analysis, the MAb/GNP/TBSol–Gel/Au electrode was immersed into 5.0 mL of the treated samples at room temperature for 30 min.

3. Results and discussion

3.1. Characteristics of MAb/GNP/TBSol–Gel/Au electrode

The characteristics and morphology of the electrode at each assembly step were studied using SEM, AFM, CV and EIS.

3.1.1. SEM and AFM characterization

The surface morphology, structure and dispersing state of gold nanoparticles were studied using scanning electron microscopy and atomic force microscopy. Fig. 1A shows SEM images of the TBSol–Gel/Au electrode (a) and GNP/TBSol–Gel/Au electrode (b and c). These pictures showed that the sol–gel formed homogeneously on the surface of the gold electrode. In addition, it can be seen that a homogeneous surface and good dispersion of GNPs on the surface of sol–gel. Fig. 1B shows an AFM topology of the surface of TBSol–Gel/Au electrode (a) and GNP/TBSol–Gel/Au electrode (b and c). These images indicate a clear morphological change between GNPs and TBSol–Gel modified electrode. After electrodeposition, the presence of GNPs on the surface of the TBSol–Gel/Au electrode is clearly reflected in the 3D AFM image that show electrodeposited gold particles have medium thickness between 40 and 60 nm. SEM and AFM results demonstrate that thiol base sol–gel with –SH functional groups can be used as a good matrix for gold nanoparticles electrodeposition to give a stable and homogeneous thin film.

3.1.2. Electrochemical characterization

Cyclic voltammetry provides useful information of the electrode surface changes during the fabrication processes. Fig. 2 shows the cyclic voltammograms of the bare Au electrode (curve a), TBSol–Gel/Au electrode (curve b), GNP/TBSol–Gel/Au electrode (curve c) and MAb/GNP/TBSol–Gel/Au electrode (curve d) at the potential range from 100 to −600 mV and scan rate of 50 mV s^{-1}. 1.0 mmol L^{-1} $Fe(CN)_6^{4-/3-}$ in 0.1 mol L^{-1} phosphate buffer solution (pH=5.0) and 0.1 mol L^{-1} KCl were used as a redox probe and supporting electrolyte, respectively. As indicated in Fig. 2b, after treatment of the bare gold electrode with thiol base sol–gel, the redox peak currents was decreased in comparison with bare electrode. This may be explained by the fact that sol–gel forms a passive film on the surface of the electrode, thus, the electron transfer between the redox probe in the solution and the electrode surface was blocked. As can be seen in Fig. 2c, after the immobilization of GNP, the current was significantly increased and the reversible behavior of the redox probe was appeared. Immobilization of antibody on the GNPs on the electrode surface caused the decrease of the peak current of the redox couple (Fig. 2d) due to the passivation of surface with binding of antibody molecules.

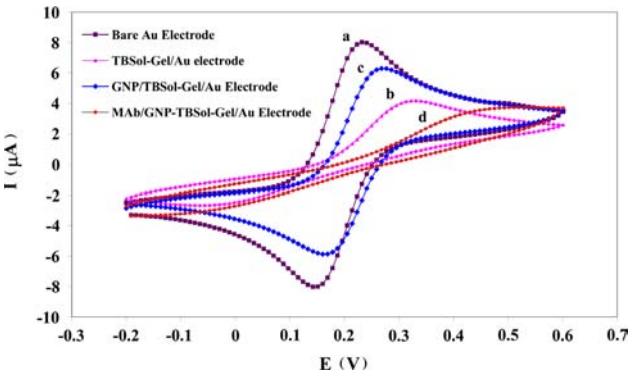

Fig. 2. The cyclic voltammetric curves (a) bare Au electrode, (b) TBSol–Gel/Au electrode, (c) GNP/TBSol–Gel/Au electrode and (d) MAb/GNP/TBSol–Gel/Au electrode in 1.0 mmol L^{-1} $Fe(CN)_6^{4-/3-}$ in 0.1 mol L^{-1} phosphate buffer pH 5.0 containing 0.1 mol L^{-1} KCl and with a scan rate of 50 mV s^{-1}.

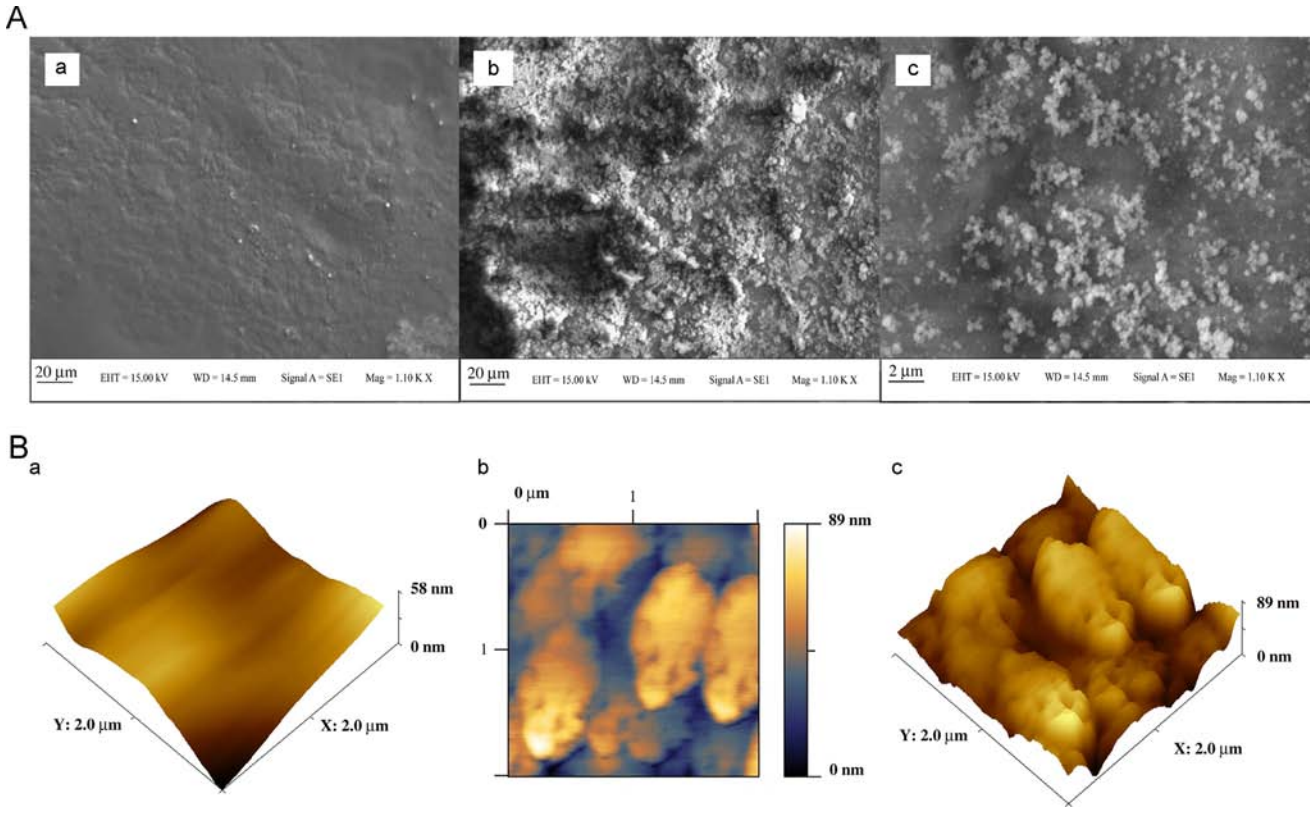

Fig. 1. SEM (A) and AFM (B) images of (a) TBSol–Gel/Au electrode and (b and c) GNP/TBSol–Gel/Au electrode.

The Nyquist diagrams of impedance spectra show a semicircle part at high frequencies, corresponding to the charge transfer limited process and a linear part at lower frequencies, resulting from the diffusion-limiting step of the electrochemical process. In the case of very fast electron transfer processes semicircle has a very small size, so the impedance spectrum that is observed includes only the linear part, while very slow electron transfer processes due to kinetic limitation are characterized by a large semicircular region without diffusion limitation. The semicircle diameter illustrates the charge transfer resistance (R_{ct}), which indicates the behavior of the electrode surface for the redox couple for each step of the modification and therefore it can be used as a signal to characterize the modification steps. According to the obtained results from Nyquist diagram (Fig. 3), it can be seen that at the bare Au electrode (Fig. 3a), a semicircle with R_{ct} about 219.8 Ω was obtained. However, the diameter of the semicircle was obviously increased by modification of the electrode with sol–gel. In the presence of GNPs at a surface of the thiol base sol–gel modified electrode, the resistance to electron transfer was decreased (Fig. 3c, R_{ct} = 16.4 kΩ). However, after the immobilization of doxorubicin-specific antibody onto GNP/TBSol–Gel/Au electrode, the R_{ct} was increased. These results adequate with voltammetric data that suggests the immobilized molecules strongly bind with gold nanoparticles and block charge carriers in the surface of the electrode. Nyquist diameter of MAb/GNP/TBSol–Gel/Au electrode (Fig. 3e) in the presence of doxorubicin as an antigen is much larger than solution without doxorubicin (Fig. 3d), because the antigen–antibody interaction increases the resistance of the electrode surface.

3.2. Influence of the experimental parameters

The influence of experimental parameters including GNPs electrodeposition time, and doxorubicin incubation time were studied to find the optimum conditions.

3.2.1. The effect of the electrodeposition time

The effect of the electrodeposition time of the gold nanoparticles on the electrochemical behavior of the modified TBSol–Gel/Au electrode was studied using cyclic voltammetry. These investigations were performed by electrodepositing at the potential of −200 mV in 0.1% $HAuCl_4$ solution for deposition time of 60, 120, 180 and 240 s. According to the obtained results (Fig. 4), the peak current amplifies at the first, and then reaches to a steady amount

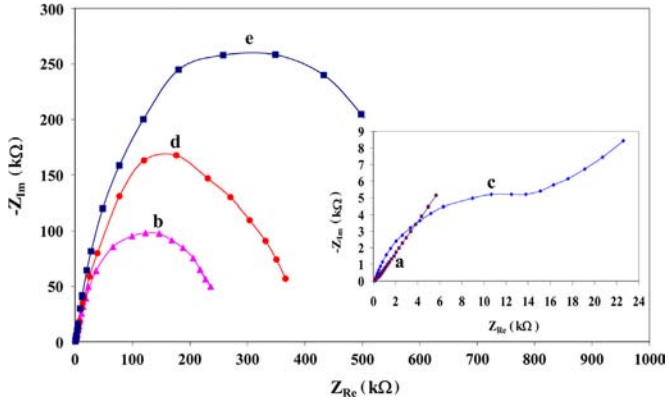

Fig. 3. (A) The Nyquist plots of (b) TBSol–Gel/Au electrode, (d and f) MAb/GNP/TBSol–Gel/Au electrode before and after incubation in doxorubicin solution and inset (a) bare Au electrode and (c) GNP/TBSol–Gel/Au electrode, in 1.0 mmol L^{-1} $Fe(CN)_6^{4-/3-}$ in 0.1 mmol L^{-1} phosphate buffer pH 5.0 containing 0.1 mmol L^{-1} KCl. A voltage of 200 mV and a frequency range of 0.1 Hz to 10 kHz was used.

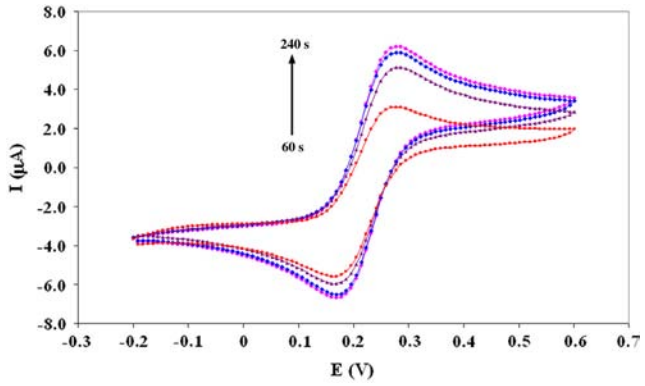

Fig. 4. Effect of the electrodeposition time of GNPs on the surface of TBSol–Gel/Au electrode in 1.0 mmol L^{-1} $Fe(CN)_6^{4-/3-}$ in 0.1 mol L^{-1} phosphate buffer pH 5.0 containing 0.1 mol L^{-1} KCl and with a scan rate of 50 mV s^{-1}.

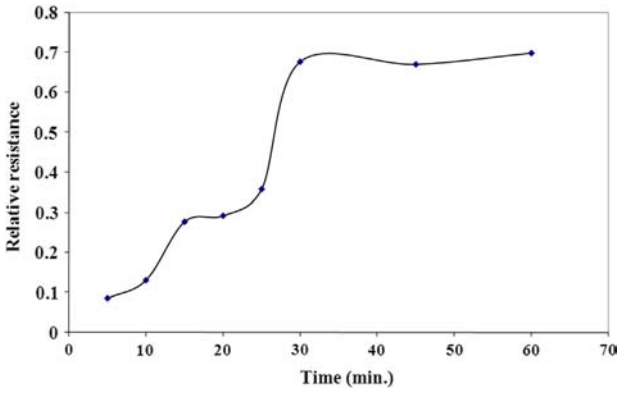

Fig. 5. Effect of the incubation time in a 30.0 pg mL^{-1} doxorubicin solution.

after 180 s. Thus, electrodeposition time of 180 s was selected as the optimum time for the gold nanoparticles deposition.

3.2.2. The effect of the incubation time

Doxorubicin was accumulated on the surface of MAb/GNP/TBSol–Gel/Au electrode due to its interaction with the doxorubicin-specific monoclonal antibody. To find the optimum incubation time of doxorubicin, EIS technique was used using MAb/GNP/TBSol–Gel/Au electrode that was immersed into the PBS solution (pH 5.0) containing 30 pg mL^{-1} at room temperature for different incubation times up to 60 min. Fig. 5 shows the variation of the relative resistance of the redox probe with incubation time. It was observed that the relative resistance increases until approaching to a constant value after about 30 min. After 30 min, due to the saturation of the electrode surface, electrode response was constant. Thus, in all following experiments incubation time of 30 min was selected as the optimum condition.

3.3. Evaluation of the immunosensor

3.3.1. Determination of doxorubicin with the immunosensor

Electrochemical impedance spectroscopy was used to make the calibration curve. Fig. 6 shows electrochemical impedance spectra of the MAb/GNP/TBSol–Gel/Au electrode in different concentrations of doxorubicin. Under the optimized conditions, relative resistance was used to obtain the calibration curve. The calibration curve of the proposed immunosensor is linear in two concentration ranges of 0.1–1.0 and 2.5–50.0 pg mL^{-1} with regression equations of $y = 0.261x + 0.206$, $R^2 = 0.989$,and $y = 0.006x + 0.496$, $R^2 = 0.990$ (where x is concentration of doxorubicin and y is relative

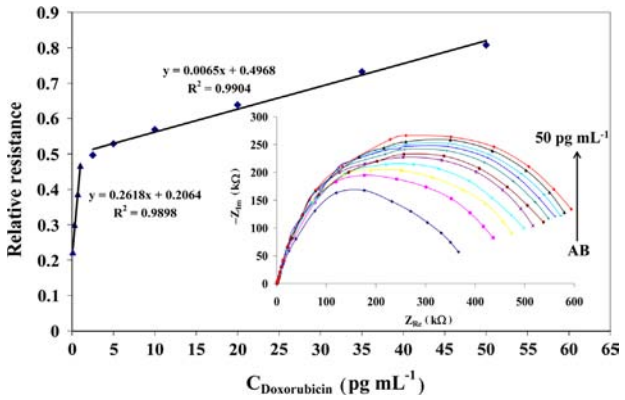

Fig. 6. Calibration curves for the determination of doxorubicin using the proposed immunosensor. Inset shows Nyquist diagrams of the MAb/GNP/TBSol–Gel/Au electrode before and after immobilaization of 0.1–50.0 pg mL^{-1} of doxorubicin.

resistance as signal), respectively. The detection limit of the immunosensor was 0.09 pg mL^{-1} ($3s_b/m$).

3.3.2. Repeatability, reproducibility and stability of the immunosensor

To obtain the repeatability of the electrode, a relative standard deviation (RSD) of 6.9% was obtained for five repetitive measurements of an antigen sample with a concentration of 10.0 pg mL^{-1}. Reproducibility of the electrode construction was obtained 10.5% using five electrodes prepared independently under the same conditions with a concentration of 10.0 pg mL^{-1}.

In order to investigate the stability of the fabricated immunosensor, the MAb/GNP/TBSol–Gel/Au electrode was stored in the refrigerator at 4 °C for three weeks. No significant reduction in its performance was observed when performing EIS measurements compared to fresh samples. The experimental results showed that the impedance remained the same during the first week, and decreased gradually but retaining 90.0% of initial value after three weeks of storage. These results indicate that the repeatability, reproducibility and stability of the proposed immunosensor are acceptable.

3.3.3. The antibody–antigen association constant

The association constant (K_a) of the antibody–antigen interaction shows the binding affinity of the antigen to the antibody and can have a wide range from 10^5 L mol^{-1} to 10^{12} L mol^{-1}. The association constant of the surface-immobilized antibody and its binding to a specific antigen according to Langmurian theory is explained using $K_aC=[R_{ct}(i)-R_{ct}(0)]/R_{ct}(0)$ equation [23]. Thus, the calibration curve's slope can be used to measure the affinity of doxorubicin toward the antibody immobilized on the surface of the modified electrode. The slope of the first linear portion was used to measure the association constant, and an association constant of 1.4×10^{11} L mol^{-1} was obtained. This association constant can indicate that the bioactivity of the immobilized antibodies on the electrode surface is high and in adequate with the results that obtained in the previous works.

3.3.4. Response characteristics

Response characteristics of the proposed immunosensor are compared with previously reported immunosensor for determination of doxorubicin based on gold electrode modified with 1,6-hexanedithiol (HDT) and GNPs [19]. One of the main different between MAb/GNP/TBSol–Gel/Au electrode and antibody/GNP/HDT/gold electrode is that sol–gel formation on the electrode surface is a simple and very rapid method with a stable coating, whereas the self assemble method using dithiol which was used in the mentioned method is a time consuming method. It can be found that in compared with antibody/GNP/HDT/gold electrode, the proposed immunosensor (MAb/GNP/TBSol–Gel/Au electrode) offered lower detection limit for doxorubicin determination.

Table 1
Determination of doxorubicin in spiked human serum and urine samples.

Sample	Added (pg mL^{-1})	Detected (pg mL^{-1})[a]	Recovery (%)
Human serum	5.0	5.07 ± 0.56	101.3
	10.0	9.94 ± 0.75	99.4
	15.0	16.27 ± 0.19	108.5
	20.0	19.35 ± 0.21	96.8
Urine	5.0	4.77 ± 0.44	95.3
	10.0	9.59 ± 0.82	95.9
	15.0	15.56 ± 0.47	103.7
	20.0	19.88 ± 0.43	99.4

[a] Standard deviation ($n=3$).

3.4. Real sample analysis

Finally, the analytical application of the immunosensor for the determination of doxorubicin in complex biological matrix has been studied. The concentration of doxorubicin was determined in the spiked human serum and urine samples under the optimized conditions. The standard addition method was used for the analysis of prepared samples and results are summarized in Table 1. As can be seen, the results measured by the proposed immunosensor are satisfactory and the recoveries are in the range of 96–108%. Thus, the proposed immunosensor can be used for determination of doxorubicin concentration in clinical samples.

4. Conclusion

In this work, a highly sensitive immunosensor to determine doxorubicin using a specific monoclonal antibody immobilized on gold nanoparticles coupled with a thiol base sol–gel modified gold electrode is studied. Electrode surface modification with TBSol–Gel provide a simple and rapid way to form a homogeneous matrix on the electrode surface with –HS groups that can be used as functionalized sites to immobilize gold nanoparticles and antibody. The high surface-area of the gold nanoparticles can enhance the immobilization density of adsorbed antibodies on the electrode surface and can maintain their bioactivity. The proposed immunosensor, in compare to previously reported immunosensor, has a better sensitivity. Furthermore, the time used for the electrode preparation in this method is lower than prior method.

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