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A novel method for glucose determination based on electrochemical impedance spectroscopy using glucose oxidase self-assembled biosensor

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Abstract

A method is developed for quantitative determination of glucose using electrochemical impedance spectroscopy (EIS). The method is based on immobilized glucose oxidase (GOx) on the topside of gold mercaptopropionic acid self-assembled monolayers (Au-MPA-GOx SAMs) electrode and mediation of electron transfer by parabenzoquinone (PBQ). The PBQ is reduced to hydroquinone (H_2Q), which in turn is oxidized at Au electrode in diffusion layer. An increase in the glucose concentration results in an increase in the diffusion current density of the H_2Q oxidation, which corresponds to a decrease in the faradaic charge transfer resistance (R_{ct}) obtained from the EIS measurements. Glucose is quantified from linear variation of the sensor response ($1/R_{ct}$) as a function of glucose concentration in solution. The method is straightforward and nondestructive. The dynamic range for determination of glucose is extended to more than two orders of magnitude. A detection limit of 15.6 μM with a sensitivity of 9.66×10^{-7} $\Omega^{-1}\,mM^{-1}$ is obtained.

Keywords: Electrochemical impedance spectroscopy; Glucose oxidase; Impedimetric biosensor; Mercaptopropionic acid; Self-assembled monolayers

1. Introduction

Electrochemical detection of physiological species, such as glucose, has been the subject of several researches. Great efforts have been devoted to the fabrication and characterization of a large variety of amperometric enzyme biosensors [1–4]. The enzyme dissolved in electrolyte solution or immobilized on a solid electrode serves as a redox centre and reacts selectively with biological species. The reaction product may be used directly or by mediation of a reversible redox couple to determine the species of interest. However, mediated electron transfer is the most efficient process and typically used for biosensors construction [5,6]. Immobilization of enzyme on a solid electrode will decrease the distance between conducting substrate and enzyme redox centre; therefore, the reduced or oxidized mediator will be produced near or inside the diffusion layer, which, in turn, increases the sensitivity and selectivity of the sensor [7,8].

The enzyme may be immobilized in a thin layer at the sensor surface in different ways; as using polymers [9], carbon paste [10], monolayers [11] and multilayer self-assemblies [12]. Among them, immobilization via covalent attachment of enzyme to the functionalized self-assembled monolayers (SAMs) [11–13] is especially useful where miniaturization of the sensor in nanometre scales is required [14]. The functionalized SAMs formed on gold surface are ordered molecular assemblies, which are widely used for the immobilization of proteins and enzymes in biosensors fabrication [15–17].

The biosensors usually contain two basic components connected in series: (i) a biochemical recognition system, which translates information from biochemical domain into a chemical or physical output signal, and (ii) a transducer, which serves to transfer the sensor signal from the output domain of the recognition system to mostly an electrical domain. An electrochemical biosensor is a biosensor with an electrochemical transducer [18]. Most of the biosensor electrochemical transducers are based on potentiometric [19,20] or amperometric detections. The amperometric detections are based on measurement of the current resulting from electrochemical oxidation or reduction of an electroactive species, which is (i) a biocatalytic product (e.g. hydrogen peroxide) or (ii) a redox couple mediating enzyme and the electrode. However, amperometric biosensors have their own inherent limitations, such as

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relatively low output current density, noisy response and gradual deterioration of the enzyme activity that mainly originates from high overvoltage applied to the biosensor. Many attempts have been made to overcome these limitations [5]. For example, composite materials [21], functionalized polymers [22], metal oxides [23] and self-assembled monolayers [7,24] have been investigated. These improvements have been focused on modification of the recognition system. However, it is necessary to seek new electrochemical transducers based on other methods such as electrochemical impedance spectroscopy (EIS) [25].

The EIS is a powerful, nondestructive and informative technique, which is usually used for characterization and study of corrosion phenomena [26], fuel cells and batteries [27], coatings and conductive polymers [28], adsorption behaviour of thin films [29], the SAMs [30,31] and electron transfer kinetics [32]. Recently, the EIS has been used in analytical chemistry to trace modification steps of chemically modified electrodes based on SAMs and to quantify the inorganic [33,34] or biological [35–37] species in solution. The basis of the recognition in these systems has been the blocking of electron transfer kinetic of a redox probe at the SAMs–solution interface by complexation or precipitation reaction connected to analyte, and thus the analyte is recognized indirectly [38,39].

Up to our knowledge, there has been no previous report on glucose biosensors based on faradaic impedance transducers and soluble mediators without any complexation or precipitation biocatalytic reaction at the electrode–solution interface. In this work, glucose oxidase (GOx) is used as an ideal enzyme [40] and immobilized covalently on the topside of the gold mercaptopropionic acid self-assembled monolayers to produce Au-MPA-GOx SAMs. Next, the sensor is used to determine glucose in the presence of parabenzoquinone (PBQ) mediator using EIS. The basis of the recognition system in this work is diffusion of glucose to the sensor. The data are presented and discussed from which a new method is proposed for glucose determination based on the EIS measurements.

2. Experimental

2.1. Chemicals

Glucose oxidase (GOx) (from *Aspergillus niger* 20,000 units/g, EC 1.1.3.4), β-D-Glucose, *N*-hydroxysuccinimide (NHS), parabenzoquinone (PBQ), 3-mercaptopropionic acid (MPA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) and other chemicals were of commercial sources (Merck or Sigma) and used as supplied without further purification except parabenzoquinone that was recrystallized from hot solution of *n*-hexane. All solutions were prepared with double-distilled water. Phosphate buffer solutions (PBS) contained 0.05 M KCl, 0.05 M K_2HPO_4/KH_2PO_4 were used and the pH was adjusted with NaOH or H_3PO_4 dilute solutions. The glucose stock solution was prepared in PBS (pH 7.0) and left at 4 °C overnight to allow the equilibration of the anomers.

2.2. Electrode modification

The polycrystalline gold working electrode (0.0314 cm^2, Azar electrode Co. Urmia, IRAN) was polished using aqueous slurries of alumina (0.3 to 0.05 μm), sonicated in water/chloroform/water for 5 min, and then cleaned electrochemically by cycling the electrode potential between 0.000 and +1.500 V vs. SCE in 0.5 M sulfuric acid until reproducible voltammograms were observed [41]. A roughness factor of 1.8 ± 0.1 was obtained from ratio of the real to geometric surface area of the electrode [42] and attempted to maintain it constant in all experiments [43]. The cyclic voltammograms obtained on the electrode in the presence of reversible marker, $Fe(CN)_6^{3-}$, showed a peak separation that confirms the safety of the system ($\Delta E_p \cong 60$ mV). Immediately before modification, the electrode was thoroughly rinsed with distilled water. Cleaned gold electrode was modified by placing into a 25:75 (v/v) water/ethanol solution containing 20 mM MPA for 12 h to form Au-MPA electrode. The modified electrode was washed with the same ethanolic solution, dried in argon stream, and activated in PBS (pH = 5.5) containing 0.002 M EDC and 0.005 M NHS for 2 h. Then, the electrode was rinsed with the same PBS and immediately placed in PBS (pH = 5.5) containing 500 μg/ml of the GOx enzyme for at least 1.5 h to fabricate Au-MPA-GOx SAMs electrode, washed with PBS, and used for electrochemical measurements.

2.3. Electrochemical measurements

A conventional three-electrode cell, consisting of Au-MPA-GOx modified electrode as working, a saturated calomel electrode (SCE) as reference and a platinum foil with large surface area as auxiliary electrode, was used for electrochemical measurements. The measurements were carried out using Potentiostat/Galvanostat EG&G 273A equipped with EG&G FRA 1025 and interfaced through PCII-GPIB IEEE NI-488.2 card. The EIS, cyclic voltammetry (CV) and chronoamperometry data acquisition were performed using EG&G Powersine™ and EG&G M270® softwares. The electrochemical characterization of Au-MPA SAMs electrode was performed in the presence of 0.5 mM $Fe(CN)_6^{3-}$ redox probe using EIS and CV. Quantitative determination of glucose was performed in the presence of 5 mM PBQ as a mediator by chronoamperometry and EIS methods. All impedance measurements were performed in the frequency range 10 kHz to 100 mHz using a 5 mV alternating voltage superimposed on DC potentials. For the characterization of Au-MPA SAMs electrode, the DC potential was formal potential of the redox couple (i.e. $E^{0\prime}$ of $[Fe(CN)_6]^{3-/4-}$). Quantitative determination of glucose was performed in different DC potentials. Other experimental conditions are described in the respective figures.

The EIS data were approximated using Equivcrt 4.55® software and complex nonlinear least square (CNLS) approximation method [44], from which electron transfer kinetics as charge transfer resistance (R_{ct}), double layer capacitance (C_{dl}) and solution resistance (R_s) were extracted for $Fe(CN)_6^{3-}$ or PBQ. The modified Randles' model in which C_{dl} was replaced

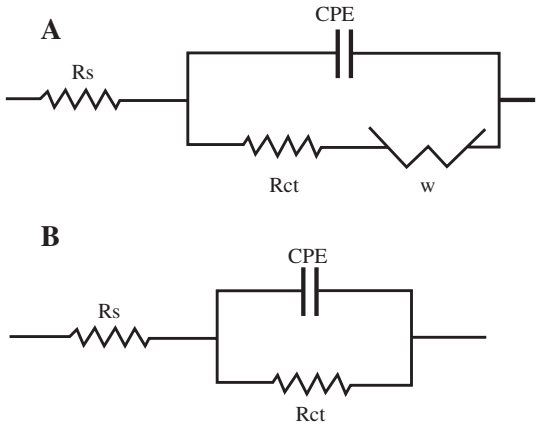

Fig. 1. The modified Randles' equivalent circuit models: (A) includes the Warburg element to consider the diffusion controlled process at low frequency region and (B) without Warburg element for only charge transfer controlled process.

by frequency-dependent constant phase element (CPE), $Z_{CPE}=Y_0\,(j\omega)^{-\alpha}$ and $\alpha=1-\varphi$ (φ is equal to one for complete smooth electrode) was enough to explain experimental data [45]. Two types of modified Randles' model were used (Fig. 1A and B).

2.4. Analytical procedure

The Au-MPA-GOx SAMs modified electrode was allowed to settle to a stable current over a period of at least 1 h in the background solution. Background solution was the PBS (pH 7.0) with 5 mM PBQ as the mediator. Prior to each experiment, the solutions were bubbled with high-purity argon gas for at least 20 min and blanketed with the same gas during the experiments to eliminate oxygen interference. All experiments were carried out at room temperature. Once the electrode background current was stable, additions of β-D-glucose were made from the stock solution using micropipette. For chronoamperometric measurements, the electrode potential was kept constant at +0.600 V vs. SCE, the currents were measured and plotted vs. glucose concentration to obtain calibration curve. For impedimetric measurements, the complex plane plots were recorded after each addition, the data were approximated using CPE model, and the parameters as R_{ct}, C_{dl}, φ and R_s were extracted from which the calibration curves were plotted using $1/R_{ct}$ vs. glucose concentration.

3. Results and discussion

3.1. Fabrication and characterization of Au-MPA SAMs electrode

3.1.1. Formation of SAMs

For enzyme electrode, a short alkyl chain alkanethiol, usually three carbons long, is used so that the grafted redox centre (e.g. an enzyme) being as close as possible to the electrode. An additional advantage of the short alkyl chain is that a relatively disordered SAM is formed which means the underlying metal is still electrochemically accessible [8]. In this study, MPA was selected as a short alkyl chain to bind GOx into the Au electrode surface and form Au-MPA-GOx SAMs biosensor. The schematic diagram of biosensor preparation is shown in Fig. 2. The formation of SAMs was traced by CV and EIS.

The complex plane plots obtained on clean Au and Au-MPA electrodes are shown in Fig. 3. The data were approximated using CPE model from which the R_{ct} and other model parameters were extracted [46,47]. The bare gold electrode shows a very small semicircle (curve a, $R^0_{ct}=1114\,\Omega$ from the high frequency range semicircle) followed by straight line indicating domination of mass diffusion limiting effect on the electron transfer process (curve a, in the low frequency range). The respective semicircle diameter at the high frequencies range, corresponding to the charge transfer resistance at the electrode surface, increases upon the MPA SAMs formation on the gold electrode surface (curve b, $R_{ct}=3730\,\Omega$).

The formation of MPA SAMs on the electrode surface has produced a larger barrier to the interfacial charge transfer, which is revealed by increasing diameter of the semicircle in the spectrum. However, the charge transfer resistance is not infinitive and the diffusion line (Warburg impedance) still

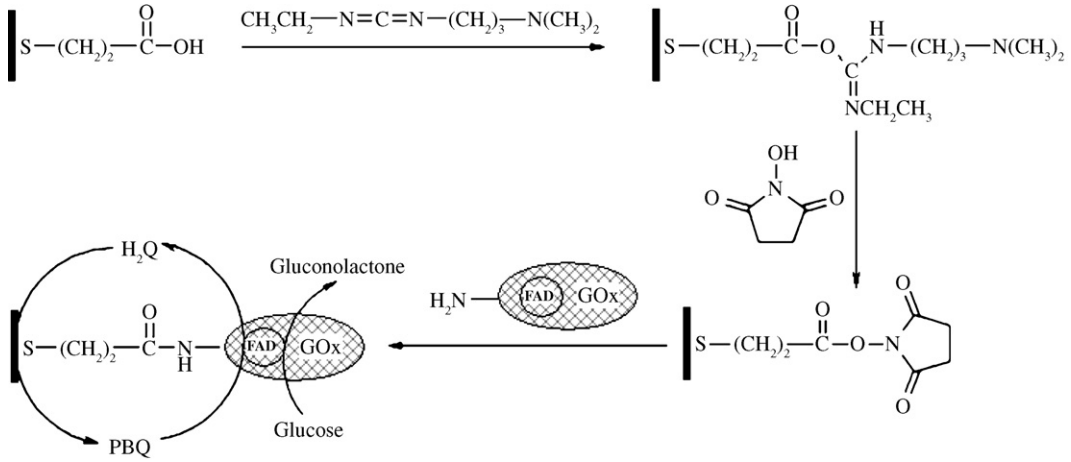

Fig. 2. Schematic illustration of the immobilization of GOx on gold electrode using a SAM of MPA and EDC-NHS as coupling agents.

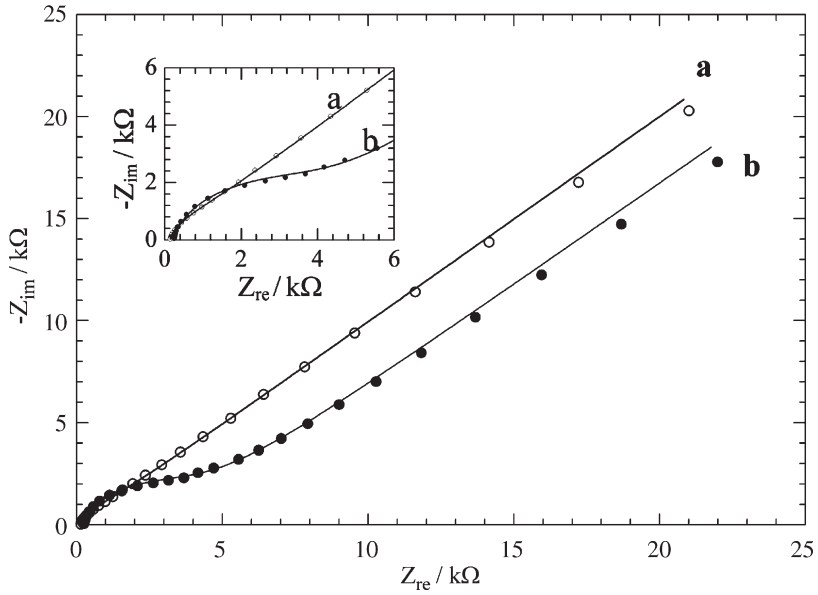

Fig. 3. The complex plane plots obtained on (a) bare gold and (b) Au-MPA SAMs electrodes in 0.1 M PBS in the presence of 0.5 mM $Fe(CN)_6^{3-}$, pH 5.5, coating time in 20 mM MPA ethanolic solution is 12 h, E_{DC} = +0.200 V (vs. SCE), E_{ac} = 5 mV and frequency range: 10 kHz to 100 mHz. The inset shows expansion the high frequency range.

exists. This means that the surface is not completely blocked and the charges can penetrate to the gold surface producing faradaic current. Since the EIS measurements are made at formal potential where no overpotential acting on the electrodes, the EIS results particularly R_{ct} indicate the information of surface coverage [48].

3.1.2. Determination of surface coverage of Au-MPA SAMs

The surface coverage, Γ, may be evaluated for MPA SAMs by integrating the cathodic peak associated with desorption process obtained in NaOH solution by CV [49]. The charges consumed for the MPA desorption, measured from the first cyclic voltammograms (corrected for background) was 53 μC/cm^2. Assuming an one-electron reduction process (Au-SR+ $e^- \rightarrow$ Au+RS^-) [50,51], the charge of 53 μC/cm^2 was further converted to a surface concentration of 5.55×10^{-10} mol/cm^2. Comparing this value with ideal value of 7.68×10^{-10} mol/Cm^2 for the packing arrangement close to ($\sqrt{3} \times \sqrt{3}$), R30 [52] shows that almost 73 ± 7% of the gold electrode surface is covered by MPA. It should be emphasized that Au-MPA SAMs is stable in the applied potential range between +0.800 and −0.700 V vs. SCE, which is a suitable potential window to study most of biological redox species. The partial surface coverage (θ) was also estimated using R_{ct}^0 = 1114 Ω and R_{ct} = 3730 Ω for uncovered and covered gold electrodes (Fig. 3, curves a and b), and the relation $\theta = (1 - R_{ct}^{\circ}/R_{ct})$, as 70 ± 5%. This value is in good agreement with the value obtained by CV. However, it should be mentioned that it is difficult to find a perfect baseline in CV plots.

3.2. Enzyme immobilization

To bind enzyme molecules tightly onto the Au electrode surface, the amine groups of the GOx were coupled to the acidic head groups of Au-MPA SAMs through the formation of imide groups using EDC and NHS, according to the literature [53]. Therefore, the immobilized enzyme on the gold surface and the solubilized glucose in the electrolyte solution could react near the surface of the gold electrode. However, when glucose reduces the FAD of glucose oxidase, $FADH_2$ forms, which cannot directly go to the electrode surface and oxidize, because of fixation of FAD to the enzyme molecule. To solve this problem, PBQ was chosen as a mediating agent between glucose oxidase and electrode [54].

Fig. 4 shows the cyclic voltammograms obtained on the bare Au (curve a), Au-MPA SAMs (curve b) and the enzyme electrode, Au-MPA-GOx SAMs (curve c) in the presence of PBQ. While the redox probe exhibits reversible electrochemical features at the bare Au electrode, immobilization of the MPA

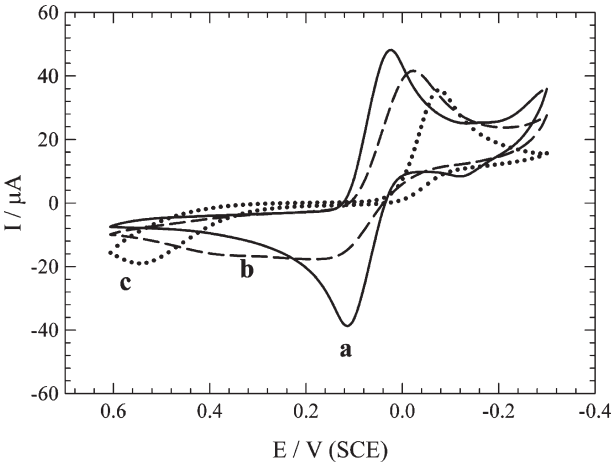

Fig. 4. Cyclic voltammograms obtained on (a) bare gold, (b) Au-MPA SAMs and (c) Au-MPA-GOx SAMs electrodes in PBS, pH 7, in the presence of 5 mM PBQ. Scan rate is 100 mV s^{-1}.

SAMs onto the electrode results in a peak separation. Upon assembly of the enzyme on the Au-MPA SAMs, the interfacial charge transfer between redox probe and the electrode is further blocked and maximum peak separation occurs. These results are consistent with the fact that immobilization of enzyme on the electrode retards interfacial electron transfer kinetics of the redox probe. It is also apparent (Fig. 4, curve c) that the anodic diffusional current is located in the potentials region after +0.550 V vs. SCE. Therefore, the DC potentials in this region (e.g. +0.600 V) were selected for chronoamperometric experiments to ensure that measurements were carried out at the limiting anodic diffusional currents and maximum sensitivity was obtained.

3.3. Glucose measurement

3.3.1. Chronoamperometry

Chronoamperometric measurements were performed to ensure that GOx is immobilized onto the Au-MPA SAMs and the electrode response in the glucose measurements is not an artefact. Thus, the potential of the Au-MPA-GOx SAMs electrode held at +0.600 V vs. SCE where PBQ oxidation current is controlled by mass diffusion, glucose was added stepwise to the solution and the current was monitored. No direct oxidation for glucose was observed in the absence of PBQ, whether Au, Au-MPA or Au-MPA-GOx SAMs used as working electrodes. However, the background currents were decreased upon modification of Au by self-assembled monolayers (Au-MPA SAMs) and bilayers (Au-MPA-GOx SAMs), respectively. A remarkable increase in anodic current was observed only for Au-MPA-GOx SAMs electrode in the background solution (PBS, pH 7 and 5 mM PBQ) and stepwise addition of glucose. A detection limit less than 0.084 mM (for $n=5$) with a sensitivity of 0.38 ± 0.2 μA/mM in the linear range 0–20 mM was found for the prepared sensor. This relatively large standard deviation, besides the high applied DC overpotential and the gradual deterioration of the enzyme activity (which is in turn an inevitable result of high applied DC potentials) limit the application of amperometric method in this case.

3.3.2. Electrochemical impedance spectroscopy

The EIS is frequently used to determine the amount of redox probe that associated with changes in the capacitance and/or electron transfer resistance at the electrode surface. The equation $R_{ct}=RT\times(n^2F^2Ak_{ct}[S])^{-1}$ may explain the relation between bulk concentration of the redox probe and charge transfer resistance [35,39,55], where R is the ideal gas constant, T is the absolute temperature, n is the number of transferred electrons per one molecule of the redox probe, F is faraday constant, A is geometric surface area of the electrode (cm^2), k_{ct} is potential dependent charge transfer rate constant and [S] corresponds to the concentration of the redox probe (mol/cm^3).

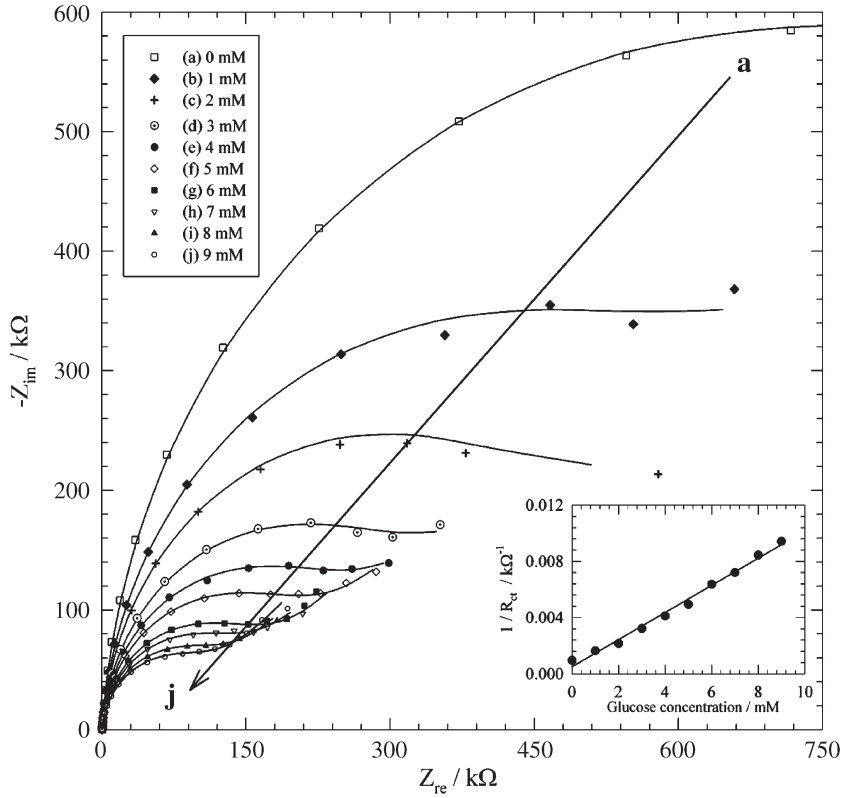

Fig. 5. Complex plane plots obtained on Au-MPA-GOx SAMs electrode for different concentrations of β-D-glucose, in 0.05 PBS pH 7 containing 0.05 M KCl, in the presence of 5 mM PBQ, $E_{DC}=+0.320$ V vs. SCE, $E_{ac}=5$ mV and frequency range: 10 kHz to 100 mHz. Symbols show the experimental data and lines show the approximated results. The inset shows calibration curve obtained using $1/R_{ct}$ as a function of glucose concentration at $E_{DC}=+0.320$ V vs. SCE, linear regression equation: Y (kΩ) $=5.05*10^{-4}+9.66*10^{-4}$C (mM), $r^2=0.991$.

The Au-MPA-GOx SAMs electrode was examined as a biocatalytic interface to produce H_2Q from PBQ using faradaic impedance spectroscopy. It can be considered that both redox reactions, (PBQ/H_2Q) and (GOx-FAD/GOx-$FADH_2$), are fast and reversible; therefore, the extent of H_2Q formation is controlled by the concentration of glucose (the matter is schematically presented in Fig. 2) and one may replace $[S]=k_1$ [glucose], where k_1 is a constant. If all other parameters are also constant, a linear relation as $1/R_{ct}=k$[glucose] is simply found, in which k includes all constants. As a result, the values of the charge transfer resistances gradually decrease upon addition of glucose to the test solution. The extent of the decrease in R_{ct} depends on the magnitude of the applied DC potential that converts H_2Q to PBQ, provided to the AC potential is small and the diffusion layer produced by DC potential is not perturbed by the AC potential [55]. Consequently, the sensitivity of $1/R_{ct}$ as a function of glucose concentration depends on the magnitude of the applied DC potential. To examine the Au-MPA-GOx SAMs electrode response in this manner, the electrochemical cell was assembled, the additions of β-D-glucose were made from the stock solution and the complex plane plots were recorded after each addition at a fixed DC potential. For the clarity, only some of the complex plane plots are presented in the panels. Fig. 5 shows the complex plane plots obtained on Au-MPA-GOx SAMs electrode in deaerated solution (PBS pH 7, 5 mM PBQ) at +0.320 V DC and 5 mV AC potentials, in different

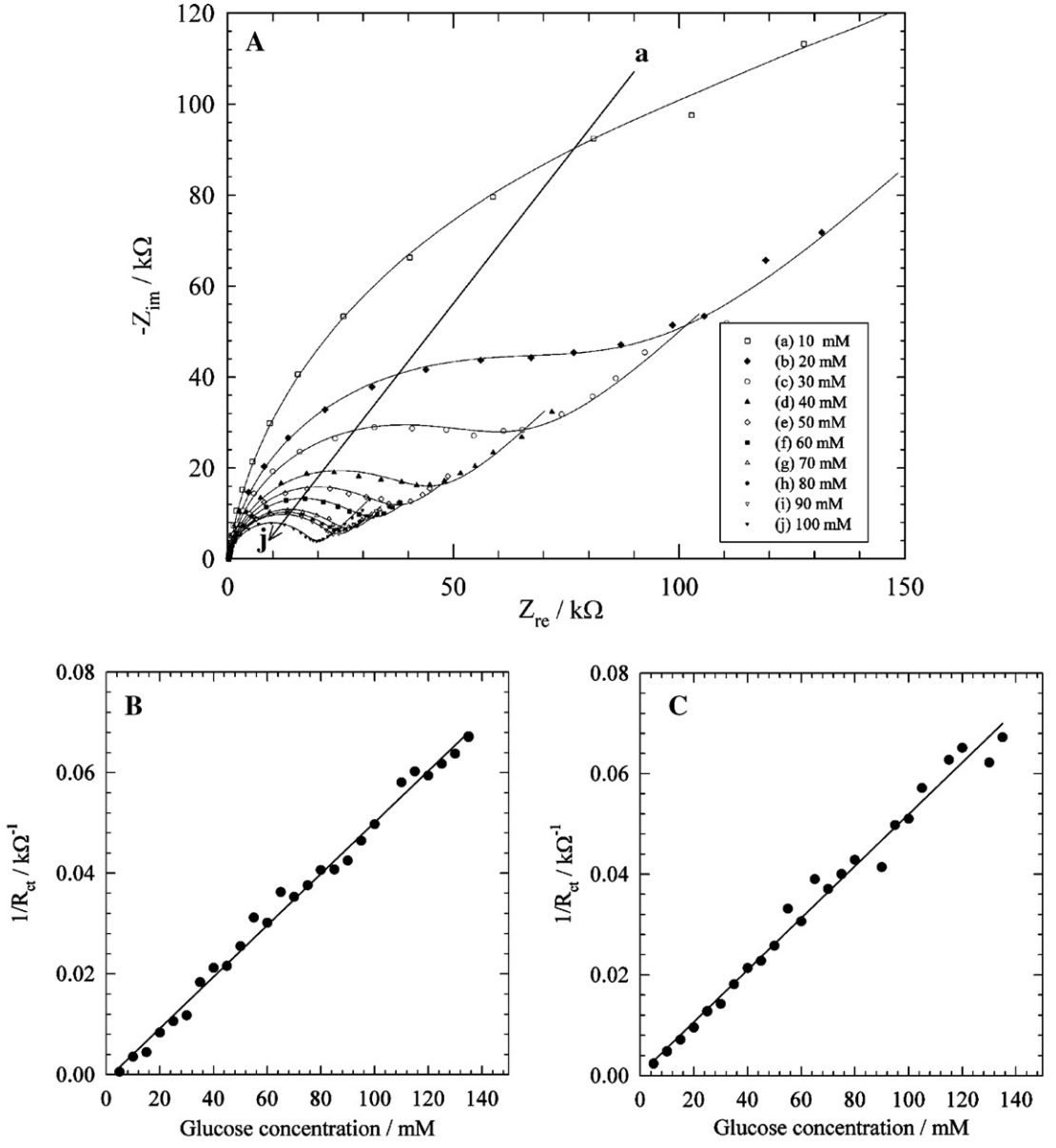

Fig. 6. (A) Complex plane plots obtained on Au-MPA-GOx SAMs electrode for different concentrations of β-D-glucose, in 0.05 M PBS pH 7 containing 0.05 M KCl, in the presence of 5 mM PBQ, E_{DC}=+0.280 V vs. SCE, E_{ac}=5 mV and frequency range: 10 kHz to 100 mHz. Symbols show the experimental data and lines the approximated results. The calibration curves obtained using $1/R_{ct}$ as a function of glucose concentration at E_{DC}=+0.280 V vs. SCE; (B) first curve with linear regression equation: Y (kΩ)=$1.12*10^{-6}+5.13*10^{-7}$C (mM), r^2=0.992 and (C) second curve obtained after 30 h, linear regression equation: Y (kΩ) =$3.72*10^{-7}+5.15*10^{-7}$C (mM), r^2=0.985.

concentrations of glucose. The results were approximated using CPE model and kinetic parameters such as R_{ct}, C_{dl} and R_s were extracted. The variation of $1/R_{ct}$ vs. glucose concentration produced a calibration curve with a short linear behaviour, i.e. 0–10 mM (Fig. 5, inset). The reproducibility of five electrodes was examined at a glucose concentration of 5 mM and a relative standard deviation (RSD) less than 15% was obtained.

The dependency of k_{ct} on DC potential allowed us selecting the potential for more extended calibration curves. Therefore, the impedimetric titration experiments were repeated at another DC potential. The complex plane plots and corresponding calibration curves obtained at +0.280 V are displayed in Fig. 6 (A,B,C). One linear range at +0.280 V (5 mM to 135 mM β-D-glucose) was observed. Such a wide range of linear response has not been reported for glucose calibration curves in literature yet. Also, the DC potentials applied here to construct the calibration curves are less anodic than that applied in amperometric experiments (+0.600 V). This behaviour reveals that partial change in the glucose concentration during data acquisition in the EIS measurements is less than that of amperometric experiments. It means that more accurate data are obtained by the EIS method. Additionally, by applying an appropriate DC potential, one may determine the amount of glucose in different concentration ranges without any necessity to dilute the test solutions.

To study the stability of the biosensor, the impedimetric measurements were repeated at DC potential +0.280 V using the same electrode, but after 30 h of recording the first calibration curve (the related complex plane plots are not presented). Then, the EIS data were approximated and the second calibration curve was extracted (Fig. 6C). Relative variation in slope of the calibration curves was less than accepted experimental error (see Fig. 6B and C). This behaviour reveals the good stability of the biosensor response as a function of time. One has to remember that over 60 complex plane plots (each complex plane plot includes 30 points and needs almost 15–20 min to be recorded) are obtained on the biosensor and still it responses very well. The sensor did not show such a stability in amperometric measurements, where it was forced to work under limiting current densities (high overpotentials, +0.600 V).

4. Conclusion

Our results show that the electrochemical impedance spectroscopy can be used as a basis for detection of glucose. A wide linear relation with more than two orders of magnitude was found between $1/R_{ct}$ and glucose concentration. Since the DC potentials applied in the EIS measurements (e.g. +0.280 V) are less anodic than that applied in amperometric measurements (e.g. +0.600 V), more accurate data can be obtained in the EIS method. Thus, the inherent problems due to high DC overvoltage applied in amperometric measurements can be overcome in impedimetric method. However, the method is still time consuming (at least 15 min is necessary to acquire one spectrum and approximate data). The work is in progress to solve this problem.

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