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Short Communication

Scanning electrochemical impedance microscopy for investigation of glucose oxidase catalyzed reaction

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ABSTRACT

In this research biointerface based on immobilized glucose oxidase (GOx) was evaluated by scanning electrochemical impedance microscopy (SEIM), which consisted of merged scanning electrochemical microscopy (SECM) and electrochemical impedance spectroscopy (EIS). The gluconolactone, which is quickly hydrolyzed to gluconic acid, is produced during the enzyme-catalyzed glucose oxidation reaction. Gluconic acid formed above an enzyme-modified not-conducting plastic surface, was evaluated by EIS technique. A two electrode cell consisting of a scanning probe, which was based on 10 μm diameter ultramicroelectrode and stationary platinum counter/reference electrode was applied for the measurement. Locally measured solution impedance depends on the gluconic acid concentration close to the ultramicroelectrode surface and on the ion diffusion, which is hindered when the electrode is approaching close to the GOx-modified surface. EIS results were evaluated by applying an equivalent circuit consisting of elements representing solution resistance, double-layer capacitance, charge-transfer resistance and Warburg impedance. Solution resistance was calculated and showed to be dependent on the position of ultramicroelectrode. Also it was observed that the thickness of the conducting layer and gluconic acid concentration both are changing in time. The results indicate that here proposed SEIM technique could become a valuable tool for the investigation and characterization of enzyme-modified surfaces of biosensors and biofuel cells.

1. Introduction

Electrochemical impedance spectroscopy (EIS) is a powerful, nondestructive and informative technique, which has been successfully applied for the characterization of glucose oxidase (GOx) based biosensor surfaces [1–5]. Moreover the EIS could be applied for glucose concentration measurements in electrochemical systems based on GOx-modified electrodes. However, conventional EIS based techniques represents only averaged response of the entire electrochemical system. In order to get more advanced mapping of electrochemical system scanning electrochemical microscopy (SECM) merged with EIS (SEIM) eventually could be applied. In SEIM based technique localized impedance measurements could be performed in the range of frequencies when the surface of interest is scanned by ultramicroelectrode (UME). The result of SEIM could be visualized by mapping one of calculated parameters, *e.g.* charge transfer resistance or double layer capacitance as a function of 3D coordinates [6,7]. Conventional EIS produces averaged data, while the combination of SECM and EIS is providing information about local corrosion phenomena, which could help to identify the location of defects induced by corrosion [7]. In addition comparison of conventional EIS with localized EIS was done during the investigation of degradation of polyester coil-coated galvanized steel [8] and in the evaluation of the degradation of some coatings, which were based on deposited organic compounds or polymeric layers [9,10]. Another alternative technique, which was used for the determination of localized EIS data, is an impedance imaging combined with atomic force scanning electrochemical microscopy (AFM-SECM) [11]. In this technique the AFM-based topography and alternating current SECM (AC-SECM) based signals are recorded simultaneously with ring-shaped microelectrode, which is integrated on the AFM probe. Structured glass/gold substrate was evaluated by such advanced AFM probe and significant increase of current was registered when the probe was placed over spots consisting of gold [11].

The evaluation of enzymatic reaction intensity could be estimated by conventional SECM techniques [12,13]. However in conventional SECM the most commonly used electrochemical

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method is based on Faradaic response when fixed potential is applied to the UME in order to register the concentration of electrochemically active enzymatic reaction products [14,15]. Moreover, it should be noted, that the potential applied to the electrode drives the electrochemical system far from the equilibrium, and then the response to this perturbation is observed as disturbing signal. In order to avoid this problem the applicability of technique based on SECM in which the UME is modulated by selected frequency alternating current (AC-SECM) can be applied. The AC-SECM based technique also allows to study local corrosion processes in the entire solution volume without any redox mediator [16]. Using AC-SECM method the approach curves were recorded at several different frequencies and they demonstrated negative feedback behavior while the UME was approaching an insulating surface [17]. This phenomena is similar to that observed in Faradaic SECM based methods, however, different approach curves were observed when different frequencies were applied [18]. In order to advance this technique, the entire electrochemical impedance spectra can be registered at every measurement point and then the electrochemical system can be evaluated using the most suitable and informative equivalent circuits. This technique is called scanning electrochemical impedance microscopy (SEIM). In the case of SEIM conventional approach curves, which represent Ohmic resistance and/or other equivalent circuit parameters *vs* distance are plotted. The concept of SEIM was described recently in the evaluation of localized corrosion processes by Schuhmann's group [7,19]. When SEIM was applied for localized evaluation of corrosion processes the sample was minimally influenced by UME, which was applied as a scanning probe, and it was demonstrated that domains, which have different electrochemical properties, could be easily distinguished by SEIM even if they have similar topography and/or morphology [7]. In another research, impedance dependence on UME distance from insulating sample was revealed at feedback mode SEIM [20]. The spherical diffusion, which has most significant influence far from insulating surface, was evaluated by Cole–Cole impedance evaluation method, and the radial diffusion, which has most significant influence when the UME is approaching insulating surface, was evaluated by Cole-Davidson impedance evaluation method. It was determined that the low-frequency part of the EIS in a thin-layer between UME and surface of interest is controlled by both above mentioned types of diffusion. Moreover it was also demonstrated that the SEIM in feedback mode is suitable for constant-height based imaging. When SEIM was acting in feedback mode the local information on both topography and surface reactivity was obtained from the simultaneous analysis of the current and electrolyte-resistance variations [21]. Fundamental aspects of SEIM were investigated comparing UME responses while it was approaching to different surfaces *e.g.*: (i) insulator surface, (ii) conducting surface not-connected to electric circuit, and (iii) conducting surface, which was connected to electric circuit and was held at constant potential [22]. By this research it has been shown that the admittance of the UME located at relatively small distance from the surface of interest mostly depends on the distance between UME and the surface and on interfacial properties of the surface. Therefore the SEIM based imaging is informative even without any redox mediators. When AC-SECM and SECM methods are compared, it should be noted that the AC-SECM is performed at single frequency, while the SEIM is performed in broad range of frequencies what enables to select the most suitable equivalent circuit and to calculate number of EIS parameters. Therefore parameters, which are derived from SEIM evaluations, better describe an electrochemical system than the parameters that are derived from AC-SECM data. We believe that the SEIM could be suitable for the investigation of biosensor and biofuel cell surfaces in order to evaluate localized activity of immobilized enzymes and/or to perform advanced evaluation of the diffusion of enzymatic reaction products.

The idea of this research is coming out from results of some investigations, which were described in earlier researches: (i) many researches were based on the EIS application for the evaluation of enzymatic reaction when the enzymes were immobilized on surfaces of electrodes [23]; (ii) conventional SECM is very often used for the investigation of enzyme-modified insulating surfaces while applying constant potential to UME [12–15]. Hence by assumption of previous achievements we have expected that it is possible to merge the EIS technique with SECM in order to get efficient SEIM system, which will be suitable for the evaluation of enzyme-modified surfaces. However, according to our best knowledge up to now no research, in the area of application of SEIM for the investigation of reaction product diffusion from enzyme-modified insulating surfaces, was reported.

The main aim of this research was to merge EIS with SECM into efficient SEIM system and, using developed SEIM for the investigation of solution conductivity at different positions, to demonstrate the suitability of SEIM for the evaluation of reaction product diffusion from enzyme-modified surface in real time.

2. Materials and methods

2.1. Materials

Glucose oxidase (EC 1.1.3.4, type VII, from *Aspergillus niger*, 215.3 units mg^{-1}, molar mass 160 kDa, polymer length 583 amino acids [24]) and 25% glutaraldehyde solution were purchased from Fluka Chemie GmbH (Buchs, Switzerland). D-(+)-Glucose was obtained from Carl Roth GmbH&Co (Karlsruhe, Germany). Before investigations glucose solutions were allowed to mutarotate overnight. All solutions were prepared in deionized water purified with Millipore S.A. (Molsheim, France).

2.2. Immobilization of glucose oxidase

A cylindrical poly(methyl methacrylate) (plastic) cell (diameter 55 mm, height 13 mm) was kept in a closed vessel above a 25% glutaraldehyde solution for 10 min to adsorb/graft glutaraldehyde onto the plastic surface. Afterwards 6 μL of 40 mg/ml GOx solution were dropped on the surface and dried in room temperature. Then the sample was again kept over 25% glutaraldehyde solution for 10 min to cross-link the enzyme and afterwards the GOx-modified surface was thoroughly washed with deionized water. Roughness of layer was measured by AFM: the roughness of unmodified plastic was 4 nm, while after enzyme immobilization it was 84 nm. Therefore supposed thickness of enzyme layer was 80 nm.

2.3. Impedance measurements

Scanning electrochemical microscope from Sensolytics (Bochum, Germany) was used for all experiments. The working electrode (WE) was situated as a SECM probe, which was approaching the substrate that was immersed in 0.1 mol L^{-1} glucose solution in deionized water. UME was purchased from Sensolytics GmbH (Bochum, Germany) and it was used as a working electrode. The UME was based on 5 μm radius platinum disk and RG (the ratio between the radius of entire electrode, which is including insulating part, and the radius of conducting part only) value was higher than 10 μm. Platinum wire was used as a counter/reference electrode (CE/RE). The distance between UME and substrate surface was determined by registration of current in feedback mode SECM while UME was approached to the surface of interest. EIS measurements were performed in the range of frequencies from 100 kHz to 20 mHz with sinusoidal

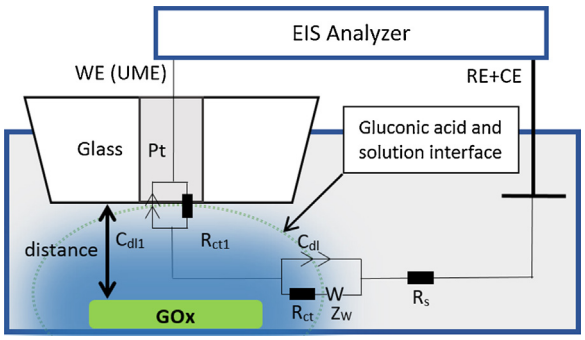

Fig. 1. Experimental scheme used for the measurement of EIS data. Equivalent circuit used for the fitting of EIS data was simplified to: R_s – uncompensated solution resistance, R_{ct} – charge transfer resistance, C_{dl} – pseudo double layer capacitance, W – Warburg impedance. C_{dl1} – double layer capacitance on UME/solution interface, R_{ct1} – charge transfer resistance through UME/solution interface. GOx – glucose oxidase, WE (UME) – ultramicroelectrode, which was applied as working electrode, RE + CE – platinum electrode, which was applied as reference and counter electrode.

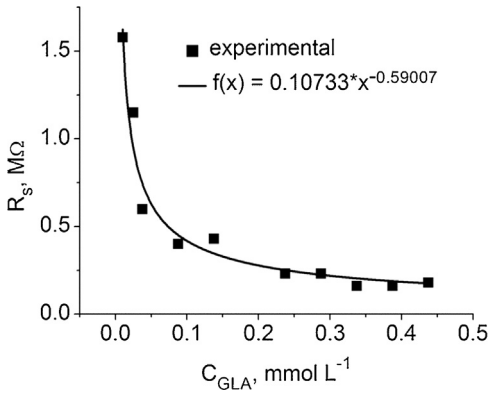

Fig. 2. Calibration curve for solution resistance dependence on gluconic acid (GLA) concentration.

current of 5 mV of root mean square (RMS) amplitude in every step; the UME to the GOx-modified surface from 1.5 mm to 50 μm was approached stepwise, each step was of 250 μm. The speed of UME movement from one step to another was applied as 1 μm s^{-1}. The EIS measurements to acquire dependence of solution resistance on gluconic acid concentration were performed at constant distance. Gluconic acid concentration was changed from 0.01 to 0.45 mmol L^{-1} and EIS measurements were performed in the same range of frequencies.

The equivalent circuit, which describes our system, is showed in Fig. 1. The mathematical impedance dependence, according to a simplified equivalent circuit, is expressed as:

$$Z = \frac{Z_{Cdl} \cdot (R_{ct} + W)}{Z_{Cdl} + (R_{ct} + W)} + R_s \quad (1)$$

where Z_{Cdl} is double-layer impedance (Eq. (2)), R_{ct} –charge-transfer resistance, W – Warburg impedance (Eq. (3)), and R_s is Ohmic resistance of solution.

Double layer impedance is expressed as a constant-phase element:

$$Z_{Cdl} = \frac{1}{Q(j\omega)^{\alpha}} \quad (2)$$

where Q – the capacitance, j – imaginary unit, ω – angular frequency, α – empirical constant.

Infinite diffusion Warburg impedance was calculated as:

$$Z_W = \frac{1}{\sigma\sqrt{j\omega}} \quad (3)$$

where σ – Warburg capacitance, j – imaginary unit, ω – angular frequency [25].

Solution resistance between the UME and counter electrodes was uncompensated. Over the course of time and within distance from the GOx-modified surface only concentrations of formed reaction products are varying in our system. Out of all in GOx-catalyzed reaction formed products only gluconic acid tends to dissociate, therefore the concentration of gluconic acid mostly affects the conductivity of the solution and therefore changes of calculated R_s are mainly related to the variation of gluconic acid concentration.

3. Results and discussion

EIS is based on the measurement of alternating current, which is registered when a sinusoidal excitation signal of low voltage amplitude is applied to electrochemical system. As a result, the AC-current with a shifted phase is registered by working electrode. The measured signal is based on complex impedance, which depends on the solution resistance, double layer capacitances of both working and counter electrodes, and charge-transfer resistance between these two electrodes [26]. Here, the working electrode in applied SEIM equipment is an UME. This electrode is scanning in all three directions and therefore it can be applied for the investigation of selected 3D space of solution. The obtained data were analyzed using equivalent circuit, which is represented in Fig. 1. The SEIM is used more rarely, than the AC-SECM, because scanning at one desired frequency takes less time. But the advantage of SEIM is that the EIS measurements are performed in wide range of frequencies at each point of the UME position. Such measurements allow to evaluate the response of the electrochemical system, to develop 3D model of the system, also to determine the most suitable equivalent circuit and their parameters, which in the most optimal way to describe the system [7]. What is the most important, such measurements allow to find the values of equivalent circuit parameters in selected volume of the electrochemical cell. Reaction products, which affect solution's conductivity, diffuse from the active sites of GOx-modified surface into the solution; therefore the SEIM with moving UME is one of the best choice to study the diffusion of formed reaction products in selected volume of electrochemical cell. Due to the fact that the EIS measurements take a long time, the conductivity changes in time were also evaluated and this issue was taken into account.

The oxidation of glucose in the presence of O_2 is catalyzed by GOx, and in this reaction hydrogen peroxide (H_2O_2) and D-glucono-1,5-lactone, which is rapidly hydrolyzed to β-D-gluconic acid, are produced:

$$\beta\text{-D-glucose} + O_2 \xrightarrow{GOx} \text{D-glucono-1, 5-lactone} + H_2O_2 \quad (4)$$

$$\text{D-glucono-1, 5-lactone} + H_2O \rightleftarrows \beta\text{-D-gluconic acid} \quad (5)$$

Therefore, the changes in impedance spectra are mostly related to the accumulation of gluconic acid because its concentration mainly affects the surface capacitance and Ohmic-resistance of solution. Due to diffusional limits gluconic acid concentration decreases with increasing distance between UME and GOx-modified surface, this fact was approved by Faradaic SECM approach curves [14,15]. To verify the dependence of solution resistivity on gluconic acid concentration, the impedance spectra were measured at different concentrations of gluconic acid. Equivalent circuit values were fitted corresponding to measured impedance spectra and equivalent circuit (Eq. (1)), and then the dependence of solution resistance on the concentration of gluconic acid was found (Fig. 2). The R_s value decreases by addition of gluconic acid to the solution.

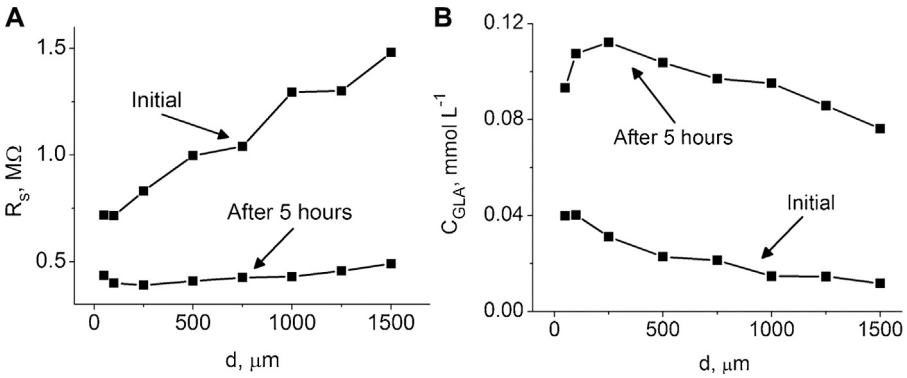

Fig. 3. (A) Calculated solution resistance dependence on the distance of UME from the GOx-modified surface in 0.1 mmol L^{-1} glucose solution; (B) dependences of calculated gluconic acid concentration on the distance of UME obtained from results presented in (A).

Further, the measurements of impedance at different distances from enzyme-modified surface were performed, and the same impedance model (Eq. (1)) was fitted to the results. Solution resistance was decreasing from 1.5 to 0.7 MΩ while the UME was approaching to the GOx-modified surface (Fig. 3A). These changes are related to the gluconic acid diffusion from GOx-modified surface into the solution. The gluconic acid concentration was recalculated according to the resistance of solution (Fig. 3B). When the UME approaches toward the GOx-modified surface it first encounters the gluconic acid solution, which initially was very diluted; however while approaching closer to the GOx-modified surface the UME reached a point where the gluconic acid concentration was the highest, due to superposition of several factors: close diffusion distance from active site of the enzyme and sufficient gluconolactone/water ratio, which is necessary for both hydrolysis of gluconolactone and dissociation of formed gluconic acid. Calculated gluconic acid concentration was changing from 0.011 mmol L^{-1} (at 1.5 mm from GOx-modified surface) to 0.040 mmol L^{-1} (at 50 μm from the GOx-modified surface). After 5 h of reaction course the resistance of the solution remained approximately 0.4 MΩ and it became similar in all distances, suggesting that the gluconic acid has spread in all volume of the solution. Gluconic acid concentration changed from 0.076 mmol L^{-1} (at 1.5 mm from GOx-modified surface) to 0.093 mmol L^{-1} (at 50 μm from the GOx-modified surface). At the distances from 250 to 1500 μm gluconic acid concentration is increasing while approaching to the GOx-modified surface. Below the distance of 100 μm the UME is entering a near-surface zone where the current of UME is blocked by limited diffusion of ions in such thin solution layer.

4. Conclusions

The impedance measurements of the solution at different distances from the GOx-modified surface enabled to evaluate the concentrations of gluconic acid formed in the solution after glucose oxidation during enzymatic reaction. Measured properties of the electrochemical system vary in time and depend on the distance of UME from the GOx-modified surface. If GOx-catalyzed reaction lasted for 5 h the concentration of gluconic acid increased by 6.5 times at 1500 μm distance and by 3.6 times at 250 μm distance from the GOx-modified surface. Measurements show not only differences in gluconic acid concentration in the solution, but also that the diffusion of ions to the UME is blocked when the UME is approaching to the GOx-modified surface by 100 μm or closer. Here demonstrated results show that the SEIM technique could become a valuable tool for the investigation and characterization of biosensors and biofuel cells because the SEIM could be applied without any mediators, without significant perturbation of designed bio-system, and in real-time when the biosensor or biofuel cell is operating.

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