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Redox Competition and Generation-Collection Modes Based Scanning Electrochemical Microscopy for the Evaluation of Immobilized Glucose Oxidase Catalysed Reaction

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Redox competition (RC-SECM) and generation-collection (GC-SECM) modes of scanning electrochemical microscopy were applied for the evaluation of glucose oxidase (GOx) modified not conducting poly(methyl methacrylate) surface. The current vs distance curves in RC-SECM mode were registered at -600 mV vs Ag/AgCl in order to determine local O\textsubscript{2} concentration, taking into account that the O\textsubscript{2} is consumed in GOx catalysed enzymatic reaction. This measurement was performed in phosphate-acetate buffer, pH 6.6, with 0-30 mmol L\textsuperscript{-1} of glucose, using platinum ultramicroelectrode (UME) as moving working electrode in three-electrodes electrochemical cell. The UME current, which is related to oxygen reduction rate, decreased by addition of glucose to the solution. Another part of investigation was performed in GC-SECM mode at +600 mV vs Ag/AgCl in order to measure local H\textsubscript{2}O\textsubscript{2} concentration, which is formed during GOx catalysed enzymatic reaction. The same SECM mode was used for imaging of GOx catalysed reaction without any redox mediator. The distance for imaging was chosen from both RC-SECM and GC-SECM experiments results. The RC-SECM and GC-SECM modes are described and processes, which occurred on the UME and GOx-modified surfaces, are revealed.

Introduction

Biosensors are systems composed of enzyme(s) or other biological origin materials, which are immobilized on surfaces, where biological part is responsible for selective reaction with analyte and generation of electrical signal. However during the development of biosensors certain technical problems were encountered, which should be solved in order to increase the applicability of these analytical devices. Among these problems the increase of efficiency, stability, reliability and analytical applicability of biosensors are the most important tasks in biosensorics. Therefore localized evaluation of biocatalytic activity of redox enzymes immobilized on the surface could be very attractive for biosensor design\textsuperscript{1, 2} or development of biofuel cells\textsuperscript{3}. Scanning electrochemical microscopy (SECM) is an innovative method, which could be applied for the surface-activity analysis of enzymatic biosensors\textsuperscript{4,6}. Initially the SECM was designed as a method suitable for the investigation of electrochemically active surfaces\textsuperscript{7,8}. The most important part of the SECM is an ultramicroelectrode (UME) with a radius ranging from few nm to 25 µm\textsuperscript{9}. The UME usually is moved by positioners in three directions – x, y, z in the solution close to the surface of interest. Mostly the UME is switched as a working electrode in the electrochemical system consisting of two, three or four electrodes\textsuperscript{9}. One of the most informative SECM modes is based on vertical movement of UME vs sample because it allows to register the current changes vs distance over the sample. From these curves measured in feedback (FB) mode the distance of UME from sample surface can be determined, the evaluation of electrochemically active surfaces can be performed, and reaction kinetics can be calculated\textsuperscript{10-14}. Current flow in FB mode is caused by oxidation/reduction reaction, occurred at the UME. The feedback mode can be positive or negative, depending on the changes of current, when the UME is approaching to the sample surface. In negative feedback mode, the current signal is decreasing due to blocked diffusion of redox compounds. In positive feedback, the current signal is increasing, because the redox compounds are formed and/or regenerated at the sample surface\textsuperscript{7}. In present our research the evaluation of glucose oxidase (GOx) catalyzed reaction by SECM without mediator shows that the current vs distance
dependence, which is based on O₂ reduction current, has negative feedback behavior. Feedback can be consumptive, when the O₂ is consumed on the surface, which is evaluated18-20. For this phenomenon, the redox competition mode (RC-SECM) was suggested by Schuhmann’s group19. In this mode, the UME and sample compete for the same analyte in solution. During the experiment oxygen reduction current mostly remains constant unless the UME is approaching to the oxygen consuming area. This effect could be measured at a bipotentiostatic mode, then both UME and surface are held at oxygen reduction potential18,20. W. Schumann’s group has reported several works on application of redox competition modes of SECM (RC-SECM), particularly: (i) the RC-SECM mode was used for the characterization of the performances of a biosensor employing the local electrocatalytic activity of the GOx immobilized within a polymer hydrogel matrix on the top of Prussian Blue-modified glassy carbon electrodes and some particular potential was applied to these electrodes20; (ii) the evaluation of local bio-electrocatalytic activity in RC-SECM mode was described when GOx was immobilized on biofuel cell cathode and again particular potential was applied to the electrode1.

Another important regime of SECM is generation-collection (GC-SECM) mode. In this mode, the UME is only registering currents, which are caused by the reaction products21, 22. Usually the UME passively detects the redox compounds, which are generated at the surface. The problem is that the reaction on the sample occurs continuously, independently on the operation of the UME. But after some adaptations it is possible to measure concentrations of reaction products in real time23-25. In our research GC-SECM based measurements were performed by the registration of H₂O₂ oxidation current, where the H₂O₂ is the product of glucose oxidase (GOx) catalyzed reaction.

Both RC-SECM and GC-SECM experiments can be carried out in constant height and constant distance modes. In constant height mode the UME is moved only laterally in the x and y directions, while in constant distance mode UME can be moved in x-y-z directions26. The constant height mode is appropriate for the evaluation of smooth surface (roughness is smaller than the UME radius) samples27. In this mode the UME current depends on the distance between UME and surface of interest and on the reactivity of compounds immobilized on the surface. Resolution studies of SECM in constant height mode shows quantitative correlation of decrease in resolution and the increase in distance between UME and sample28. To determine the distance, which is the most suitable for appropriate resolution of SECM constant height mode measurement, the current vs distance dependence could be measured in feedback mode by approaching the UME to the surface of interest; and distance between UME and sample could be calculated from the SECM theory, where \( \frac{i_2}{i_0} \) (ratio of UME current and steady-state current far from electrochemically active surface) can be related to \( \frac{d}{a} \) (the ratio of distance between sample and UME and UME radius)26.

The main aim of recent work was to find the appropriate glucose concentration and UME distance from the surface of interest for the SECM-based imaging of GOx-modified surface. The RC-SECM mode was used for the determination of UME distance from surface and for the evaluation of GOx catalyzed reaction. The O₂ reduction current was registered, and current decrease while approaching the GOx-modified surface was observed. This current decrease shows oxygen consumption by GOx. The generation-collection mode was used for the imaging of GOx catalyzed reaction at chosen distance from the surface of interest. The UME distance, which was the most suitable for the imaging, from the surface of interest was chosen after the evaluation of measurement results at both RC-SECM and GC-SECM modes. It should be noted that there are some principle differences between recent our work and previously overviewed researches18-20 published by W. Schumann’s group: (i) we have used redox competition mode for the evaluation of enzymatic reaction while the GOx was immobilized on insulating surface and no external potential was applied; (ii) in researches conducted by W. Schumann’s group an redox mediator was used, while we have performed the SECM measurements without any redox mediator.

**Experimental**

**Materials**

Glucose oxidase (EC 1.1.3.4, type VII, from Aspergillus niger, 215.3 units mg⁻¹ protein) 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 using distilled water. Sodium acetate trihydrate, potassium chloride, monopotassium phosphate, and sodium dibasic phosphate were obtained from Reanal (Budapest, Hungary) and Lachema (Neratovice, Czech Republic).

**Immobilization of glucose oxidase**

A cylindrical poly(methyl methacrylate) (plastic) cell surface was kept in a closed vessel over a 25% solution of glutaraldehyde for 10 min. Then 1.6 µL of 10 mg mL⁻¹ GOx solution was dropped on the surface and it covered 1.13 mm² surface area. Then it was dried at room temperature, in order to get 14 µg mm⁻² GOx layer. After that, modified surface was kept in a closed vessel over a 25% solution of glutaraldehyde for 10 min at room temperature and then it was washed with buffer.

**Measurements by SECM**

SECM and disk-shaped Pt UME from Sensolytics (Bochum, Germany) were used for experiments. The platinum wire (diameter 10 µm, purity 99.99 %) was sealed in borosilicate glass. SECM measurements were performed in both RC-SECM...
and GC-SECM modes in buffer without mediator. Three-electrode electrochemical cell was applied, with UME-based scanning probe, which was switched into three electrode circuit as a working electrode. Pt electrode was used as a counter electrode and Ag/AgCl in 3M KCl was applied as a reference electrode. Current vs distance dependences in RC-SECM mode were registered while applying the potential of -600 mV vs Ag/AgCl. First, the UME was moved with 1 µm s⁻¹ speed in vertical direction until it touched the unmodified plastic surface. From this measurement the distance was calculated using equation (1). In this case, the negative FB was observed due to hindered diffusion. Second, the UME was retracted out to 200 µm distance from the surface of interest and positioned to another place, which is modified by GOx, and the UME was approaching the GOx-modified surface. These measurements were performed in phosphate-acetate buffer, pH 6.6, with glucose concentrations in the range from 0 to 30 mmol L⁻¹. Each measurement was repeated three times, and the mean value was used for further calculations. Current vs distance dependences in GC-SECM mode were registered while applying the potential of +600 mV vs Ag/AgCl by approaching GOx-modified surface from 1 mm to calculated ‘zero’ distance. The imaging of the GOx-modified surface in GC-SECM mode was performed at +600 mV vs Ag/AgCl at the 40 µm distance from surface.

Results and discussion

Registration of ‘approaching’ curves in RC-SECM mode

The SECM based measurements could be divided into two principally different modes based on positive or negative feedbacks. The distance between UME and the surface of interest can be determined by recording approach curves. In this kind of experiments measurement results could be plotted as normalized current i_t/\text{i}_{T,0} dependence on normalized distance d/a; where: i_t – measured current while UME is approaching the sample, d – distance, i_{T,0} – the steady-state current when the UME is placed very far from the surface, a – radius of the UME. Steady state current is expressed as \text{i}_{T,0} = 4nFDCa, with n the number of electrons transferred per molecule; where F is the Faraday constant, D and C are the diffusion coefficient and the initial concentration of the measured substance (e.g. oxygen).

In negative FB mode the current decreases if UME is approaching the surface of interest. When the same analyte is consumed on both UME and on the sample surfaces, the process is called RC-SECM mode. This mode could be applied for the evaluation of local bio-electrocatalytic activity of enzymes, such example with enzyme immobilized on biofuel cell cathode has been reported by Schuhmann’s group. Moreover, the characterization of the performances of a biosensor employing the local electro-catalytic activity of GOx immobilized within a polymer hydrogel matrix on the top of glassy carbon electrodes has been successfully performed in similar way. In both here mentioned cases, the enzyme was immobilized on conducting surface, which during SECM investigations was held at selected potential. Unlike to mentioned researches, in our system GOx was immobilized on the insulating surface and therefore no potential was applied to GOx-modified surface. Despite of this, the competition of two processes (O₂ consumption on the GOx-modified surface with 2-electrons transfer and O₂ consumption on the UME with 4-electrons transfer) during SECM measurements can be described as RC-SECM mode. The processes, which occur on the UME and GOx-modified surfaces, in RC-SECM mode when negative potential is applied to the UME, are revealed in figure 1. In the solution without any redox mediator the reduction of dissolved O₂ occurs on the UME, and additionally the O₂ is consumed by GOx catalyzed reaction. Therefore the O₂ reduction based UME current decreases when the UME is approaching to the surface. However, in this case another factor such as blocked diffusion of O₂ to UME also has significant influence for the measurement of current vs distance.

In order to determine the distance of UME from surface, the O₂ reduction current is usually measured while approaching electrode to the insulating surface. The current vs distance dependence was registered in buffer, while applying -600 mV vs Ag/AgCl potential and approaching unmodified plastic surface (Fig. 2, buffer); during this process the distance was calculated by equation (1). Further measurements by adding glucose to solution were performed in the same fixed x-y position, therefore results of SECM measurements were always mostly affected by two factors: (i) the hindered diffusion when the UME appears close to surface of interest and (ii) the consumption of O₂ by GOx catalyzed reaction.

![Fig. 1. Schematics of processes occurring during SECM measurements on both GOx-modified and UME surfaces in RC-SECM mode without any redox mediator. In this scheme gluconolactone is abbreviated as GLL, and glucose as GLC.](image-url)
The layer of consumed O$_2$ is increasing by consecutive addition of glucose to solution. Hence, the most significant influence to the change of the current has O$_2$ concentration, but not blocked diffusion. Another evidence for this fact is that the current remains at the same level ($i_T/i_T\infty=0.1$) if glucose concentration is in the range from 10 mmol L$^{-1}$ to 30 mmol L$^{-1}$ and UME is at close distance (from 0 to 4 d/a) from surface of interest. In this case the layer, which contains lower concentration of O$_2$, is thicker due to much faster O$_2$ consumption. The dependence of O$_2$ consumption on glucose concentration at different distances from the GOx-modified surface is linear (Fig. 3). At closer distances the current decreases by 25–100% what is clear evidence of O$_2$ consumption.

**GC-SECM mode based measurements**

In the GC mode (Fig. 4), the H$_2$O$_2$ oxidation current on UME was registered. The highest concentration of H$_2$O$_2$, which is formed during GOX catalyzed reaction, is close to the GOX-modified surface. Therefore the current of UME is significantly increasing when approaching to the surface modified by GOX and this increase of UME current is related to the rate of enzymatic reaction.

![Fig. 4. Schematics of SECM processes occurring on GOx-modified and UME surfaces in GC mode without any redox mediator. In this scheme gluconolactone is abbreviated as GLL, and glucose as GLC.](image)

The H$_2$O$_2$ concentration profile (Fig. 5) was determined by registration of current vs distance dependence. However, the estimation of registered current vs distance dependence at GC mode has some disadvantages: (i) the dependence of current vs distance is changing over the time, because the enzyme is continuously consuming both substrates (glucose and O$_2$) and the concentrations of products (H$_2$O$_2$ and gluconolactone) in solution are increasing within course of the reaction; (ii) the current increases by approaching the surface modified by GOX only at within certain distance range, at which the hindered diffusion effect still does not take place; (iii) from current vs distance dependence curves it is not possible to estimate exact distance of UME from the surface, therefore by approaching the surface of interest the UME could be crashed or sample could...
be damaged by UME. To avoid these negative effects, the measurement was performed immediately after the addition of glucose, and distance of measurement was chosen from negative feedback dependence of current vs distance, measured while approaching plastic surface at -600 mV (Fig. 2, in buffer). The measurement of GC current vs distance dependence was started at 1 mm distance between UME and surface. It was determined that the current in GC mode is decreasing more slowly, when the UME is approaching the surface of interest, comparing to that in RC-SECM mode. This phenomena can be explained as follows: H$_2$O$_2$ diffusion from the GOx-modified surface is fast, therefore the increase in current comparing to the measurement without any glucose can be observed even at 1 mm distance. However, the concentration of H$_2$O$_2$ is highest at closest point, and this can be related to continuously proceeding enzymatic reaction, which is producing the H$_2$O$_2$. Here, the effect of hindered diffusion is not seen, because the measurement distance was calculated from negative feedback measurement carefully to avoid sample damage. Thus, both modes are important for the determination of the most suitable distance for the imaging.

**Fig. 5.** Current vs distance curves registered while approaching the GOx-modified surface in the presence and absence of glucose. UME potential was +600 mV vs Ag/AgCl.

The distance of 40 µm was chosen from both approaching curves (Fig. 2, in buffer and Fig. 5), because: i) the current related to O$_2$ reduction at the UME is at 0.85 of normalized steady-state value (applied potential was -600 mV vs Ag/AgCl); ii) the current related to H$_2$O$_2$ concentration is at maximal value (applied potential was +600 mV vs Ag/AgCl). The similar results were obtained inside biosensor, based on GOx$^{29}$. Authors of this research found that the distance is the same for maximal current while measuring H$_2$O$_2$ concentration by GC-SECM mode and for change of maximal current while measuring O$_2$ concentration by RC-SECM mode.

**Fig. 6.** UME current registered at the interphase between the GOx-modified and not modified surfaces in the presence and absence of glucose in buffer; the UME was operating in GC mode at +600 mV vs Ag/AgCl potential at 40 µm (d/a=8) distance.

These conditions allow to scan at the distance at which the hindered diffusion still does not take place – the current related to O$_2$ reduction at the UME (Fig. 2, in buffer) in this distance does not differs by more than 20 % from the steady-state value. On the other hand, the H$_2$O$_2$ concentration is at maximal level (Fig. 5) therefore it allows to perform the measurements at highest resolution.

The horizontal scanning, which was performed at constant 40 µm (d/a=8) from GOx-modified surface, is illustrating that the UME current in the absence of glucose is low (0-3 nA), and the UME current increases up to 24 nA in the presence of 10 mmol L$^{-1}$ of glucose (Fig. 6). The imaging was performed immediately after the addition of glucose, however the diffusion of H$_2$O$_2$ from GOx-modified surface is very fast and the current after addition of glucose increases not only in close proximity to the GOx-modified spot of surface (24 nA, x-coordinate from 100 to 200 µm), but also in surrounding area (9 nA, x-coordinate from 0 to 100 µm). At the same time in current vs distance curve (Fig. 5) the current changes from 8 nA if UME is far (600-1000 µm) from GOx-modified surface to 20 nA close (20 µm) to GOx-modified surface. Tendencies in current changes of approaching curve (Fig. 2) and 3D images of UME current registered at the interphase between the GOx-modified and not modified surfaces (Fig. 5) are very similar, what is an evidence that appropriate distance was chosen for the imaging.

**Conclusions**

The SECM is a powerful tool for the investigation of enzyme-catalyzed reactions. Comparing two different SECM operation modes (RC-SECM and GC-SECM), we have determined that both modes are suitable for the investigation of GOx-modified surfaces in the absence of redox mediators. In RC-SECM mode negative potential was applied for the determination of O$_2$ consumption on the GOx-modified surface, and the most robust O$_2$ consumption was found at the highest glucose...
concentration. Moreover, the current vs distance curves show that the thickness of layer with decreased O\textsubscript{2} concentration ranges approximately 20 µm from GOx-modified surface. From the same experiment, the dependence of current vs glucose concentration was calculated and the highest current change, which indicates the highest O\textsubscript{2} consumption rate, was found close (at 20 µm) to the GOx-modified surface.

Another part of investigations was performed in GC mode at positive UME potential. The current vs distance curve illustrates that the H\textsubscript{2}O\textsubscript{2} concentration significantly differs at 0 to 600 µm distance from GOx-modified surface, while O\textsubscript{2} concentration significantly varies at 0 to 100 µm (20 d/a) distance from surface. This means that H\textsubscript{2}O\textsubscript{2} diffuses very far from surface after the enzymatic reaction, while O\textsubscript{2} is consumed close to surface modified by GOx. Contrary the concentration of H\textsubscript{2}O\textsubscript{2} is highest close to the surface, where the enzymatic reaction takes place. If horizontal scan is performed by SECM, the choose of appopriated distance is very important factor, because at closer distances measurement results can be distorted due to hindered diffusion or fast O\textsubscript{2} consumption. The selection of distance, which is the most suitable for the horizontal scan, should be performed taking into account concentrations of both compounds (consumed O\textsubscript{2} and formed H\textsubscript{2}O\textsubscript{2}), which appears close to the GOx-modified surface. In particular experiment at 40 µm distance of UME form surface of interest was the most optimal for horizontal scanning while taking into account both phenomena. The GC mode based horizontal scan measurements show the most significant increase of UME current (from 0 to 24 nA), when glucose is added to the solution. This can be explained by formation of H\textsubscript{2}O\textsubscript{2} during enzymatic reaction.

Acknowledgments

The work was supported by Research Council of Lithuania, Support to research of scientists and other researchers (Global Grant), Enzymes functionalized by polymers and biorecognition unit for selective treatment of target cells (NanoZim’s), Project Nr. VP1-3.1-ŠMM-07-K-02-042

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