SENSITIVE ELECTROCHEMICAL METHOD DEVELOPMENT
For "in vivo" Measurement of ROS in Ethanol Induced Stress

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Abstract: The role of reactive oxidizing species (ROS) is proved within numerous physiological processes, including aging, signal transduction and some kind of immune functions. Nowadays ROS and oxidative stress gain increasing attention in connection with a wide spectrum of diseases. In case of Asian people, the enzyme taking part in the ethanol metabolism, the aldehyde dehydrogenase is absent or mutated, that can result in liver tissue damage upon extensive alcohol consumption. Most of the ROS species are electrochemically active therefore the applications of electrochemical methods are the most promising for *in situ* or *in vivo* monitoring or quantification of them. In our work development and improvements of selective and sensitive method for electrochemical detection of these molecules and radicals are attempted. We prepared ultra thin size-exclusion layer by electropolymerization of m-phenylenediamine monomer on the surface of the Pt working electrode to ensure its selectivity. We have worked out the optimal circumstances for the selective layer preparation and tested its stability and function. In order to enhance the sensitivity of ROS detection a new amperometric method, the periodically interrupted amperometry (PIA) was developed and applied. With this approach we succeeded selective and sensitive detection of H_2O_2 *in vitro*.

1 INTRODUCTION

The participation of reactive oxidizing species (ROS) in numerous physiological processes, including aging, signal transduction and some kind of immune functions is proved and their role is intensively investigated. The ROS induced oxidative stress is gaining growing attention in health care sciences owing to its involvements in development of a wide spectrum of diseases, such as dermatological, neuronal, immunological disorders.

For example, it is well known that metabolism of ethanol causes oxidative stress in liver tissue. Oxidative stress is generated through the various pathways related to ethanol metabolism (e.g., ALDH, microsomal ethanol oxidizing system), thus leading to hepatic disease. We have previously reported that one of the genetic polymorphisms affects oxidative stress caused by ethanol using model animals (Matsumoto, 2007 and 2008). However, in that studies the experimental animals had to be sacrificed for the analysis. Obtaining closer view and saving life of experimental animals could be resulted by using a proper method for local monitoring of ROS species. Therefore, we decided to introduce into our studies new effective and reliable methods that allow in vivo monitoring.

Following changes of local concentration of ROS in different areas of living subjects has been a challenge for decades in experimental life sciences. Since the life time of these species is quite short, local detection is badly needed for understanding their roles in different processes. They are electroactive. Therefore application of electrometric

methods has been attempted in different times for gaining information about different steps of physiologic happenings.

Fortunately ROS species are small molecules, some of them even are highly volatile. Therefore size exclusion modifying layer or "gas dialysis" membrane employed on the electrode surface can dramatically improve the chance of selective voltammetric or amperometric detection.

Recently dramatic selectivity improvements have been achieved by employing electrochemically prepared polymer layers (Nagy, 2002).

Up till now the performance of ROS measuring microelectrodes were investigated mostly in vitro conditions, however in vivo experiments were also performed.

In order to increase sensitivity of detection with coated amperometric electrodes the method of periodically interrupted amperometry (PIA) has been introduced (Nagy, 2006). It employs short train of measurement electrode potential pulses separated by longer, equal relaxation periods. This measuring program allowing time for reloading of the diffusion layer provides higher current signal and therefore improved sensitivity as well as lower limit of detection.

In this paper we shortly introduce our recent results achieved working out a sensor and a method applicable for ROS measurements. Molecule modeling, in situ atomic force microscopy (AFM) and quartz crystal microbalance (QCM) experiments combined with controlled potential electrolysis (Pribyl, 2010) were employed in developing the selectivity providing polymer layer. That part of the work will be also discussed.

2 EXPERIMENTAL

2.1 Instrumentation

2.1.1 Quartz Crystal Microbalance (QCM)

Standard gain oscillator (10 MHz basis) connected to frequency counter was used for QCM experiments. Data were collected by LabTOOLs software (Petr Skládal). Gold covered QCM sensors (ICMFG, USA) with optically polished surface were used in all experiments.

2.1.2 Atomic Force Microscopy (AFM)

NTgra Vita (NT-MDT, Russia) equipped with a large-scanner head was used for the AFM experiments. HA-NC tips (NT-MDT, Russia) were used in all cases. Typical settings: resonance frequency of the tip in air ~100 kHz (dumped to about 20 kHz in liquid). Scanning speed was 0.8 Hz.

2.1.3 Electrochemistry

AUTOLAB 12 electrochemical workstation controlled with software of GPES version 4.9.009 for Windows (Eco Chem B.V., Netherlands) and CHI type 760C (CH Instruments Inc. Austin, Texas USA), electrochemical workstations were used in voltammetric experiments.

The measuring programs were taken from the standard working menu of the apparatus.

PalmSens (PalmSens, Netherland) instrument driven by PalmLite software was used for electrochemical procedures in case of QCM and AFM studies.

2.1.4 Molecular Modeling

HyperChem Professional 7.52 (academic version) chemical software served for estimation of charge distribution in monomers involved, for guessing their orientation on platinum surface as well as to determine and draw the structure and spatial configuration of the electropolymer.

2.2 Measuring Methods

Controlled potential electrolysis was applied for the deposition of the size exclusion layer, with 0.6 V constant potential. Studying the electropolymer formation with QCM, the gold film on the crystal served for working electrode, while silver-chloride coated silver wire reference and stainless steel auxiliary electrodes were used. The polymer formation was carried out in 0.1 M KCl. Chronoamperometric method was applied for detection of H_2O_2 detection. A three electrode cell was used, where the Pt working electrode was covered with selective layer. 1mm OD Pt and 1 mm OD Ag served as counter and reference electrodes respectively.

2.3 Chemicals and Reagents

All reagents were of analytical grade and used without any purification. All solutions were made with double distilled water. The pH 7.4 physiological phosphate buffer solution (PBS buffer) was produced by the Pharmacy Institute of Medical Faculty, University of Pécs. The hydrogen peroxide

was purchased from Molar Chemicals (Hungary, 03650-203-340). The m-phenylene- diamine (mdpa) used for the preparation of the size exclusion layer (SEL) was obtained from Sigma (USA, A7030). The bovine serum albumin was also Sigma product. The 30% hydrogen peroxide was diluted to 100 mmolL^{-1} by distilled water and the concentration of the diluted solution was determined by iodometric titration. The solution was further diluted for the amperometric electrode calibration.

3 RESULTS AND DISCUSSION

For ROS measurement H_2O_2 was our model compound. To receive the best structured SEL detailed study was made.

3.1 Modeling the Layer

To obtain the thinnest, most effective SEL molecular modeling work was made. First the fine charge distribution of each atom of the monomer was calculated. The high charge values (-0.38) of the nitrogen atoms and the delocalized π bond of the carbon ring determine the possible orientation of the mpda monomer on the platinum electrode surface.

3.2 Deposition of Ultrathin SEL

Relatively high electrode potential is needed for detecting through amperometric oxidation hydrogen peroxide or other ROS species. The presence of other electroactive species would present serious interference, especially at high concentration. Therefore the size exclusion layer essentially needed for selective ROS detection.

The following experimental conditions resulted in optimal SEL according to our studies:

Controlled potential electrolysis at 0.4V vs. Ag/AgCl for 5s in 10 mmolL^{-1} solution of m-phenylenediamine prepared with pH=7.4 phosphate buffer as solvent.

While working out of the procedure, we examined the effectiveness of the SEL making amperometric measurements in stirred buffer solution at 0.65 polarization voltage.

The selectivity check was performed for each freshly prepared SEL in our further studies and only well performing electrode was used. The SEL was accepted as good one if the current change was smaller than 5 pA after ascorbic acid concentration change from 0 to 0.21 mmolL-1. (The electrode diameter was 1mm.)

3.3 Testing the Thickness of the SEL

As it is well known the quartz crystal microbalance (QCM) detects the mass of the surface deposited material through the frequency change of its piezoelectric quartz crystal resonator.

Monitoring of the resonance frequency in the real time allows to follow directly the kinetics of the surface processes without need of other detectors. Electropolymerization of mpda monomer in 10 mmolL-1 concentration solution, at constant potential 400 mV was carried out. The curve in Figure 1 shows, the change of the resonance frequency of QCM gold plated sensor in time. Amperometric polymerization measurement was performed five times for 5 s, and once for 60 s.

The first deposition looks effective, changing the frequency by 452 Hz, while the other deposition steps are much smaller. The deposited mass of mpda is about 375.16 ng which corresponds to a few molecular layer thickness of monomer.
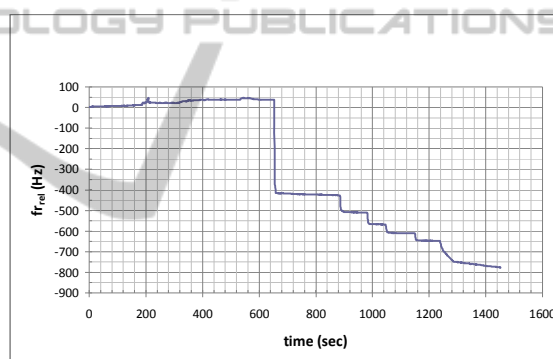

Figure 1: Monitoring the layer formation by quartz-crystal micro balance (QCM). The first stair represents the formation of the 30 nm thick layer in 5 s.

3.4 Topography of Ultrathin SEL

As it is well known, atomic Force Microscopy (AFM) is a high-resolution scanning technique allowing visualization of surface morphology and mechanical properties in a sub nanometer scale. This high-resolution imaging method was applied to check the surface structure of the p-mpda layer deposited. Scanning the surface mechanically with well-defined, precise movements, the changes were detected. The well formed column structured layer could be observed that gives 30 nm average thicknesses as can be seen on Figure 2.
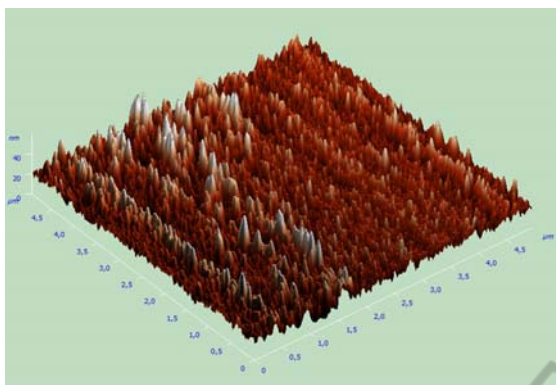
Figure 2: The AFM topology of the ultrathin p-mpda covered working electrode.

3.5 Cell Development for *in vivo*

Alcohol induced ROS measurements *in vivo,* are planed. A new cannula type electrode cell has been developed for experiments in body fluids of anesthetized experimental animals e.g. lack of ALDH2, knockout mice. The electrochemical cell- - incorporated micro sized working electrode has the same sensitivity as an OD 1 mm disk electrode.

3.6 PIA Measurement

The H_2O_2 is a relatively stable ROS. Its stability in two different media was checked with the amperometric H_2O_2 sensor. In one case, the 5 cm^3 PBS buffer, containing 35 g L^{-1} bovine serum albumin, (known free radical scavenger) was pipetted into the measurement cell and the amperometric current was recorded at 0.7V electrode potential. The solution was kept under intensively stirring. After steady reading was obtained, 10 μL doses of 1 mmol L^{-1} H_2O_2 solution were added. After each addition the current increased and achieved steady value. The current was potted against concentration in order to obtain calibration curves. The calibration was performed by chronoamperometric and PIA methods in PBS buffer solution.

4 CONCLUSIONS

Sensitive and selective ultrathin size exclusion layer covered electrode was developed for ROS measurement. In combined application of AFM-QCM-electrochemistry the electrodeposition of poly-phenylenediamine layer was followed and studied.

PIA method developed in our laboratory, has substantial benefits when small concentration of ROS species needed to be followed in presence of other electroactive species.

Further experiments are in progress for development of methods capable of following concentration changes of ROS resulted by alcohol induced oxidative stress *in vivo,* in body fluids of anesthetized experimental animals.

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