UNIVERSITY OF PÉCS

Doctoral School of Chemistry

Developement of a novel amperometric technique applying expediently modified electrodes for designing efficient analytical methods

PhD Thesis

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Supervisor: Dr. Géza Nagy D.Sc

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1. Introduction

Electrochemical methods played an important role in the development and progression of instrumental chemical analysis. Electrogravimetry, developed by Wolcott, Gibbs and Roots more than 150 years ago has to be mentioned among the earliest applied quantitative analytical methods. Similarly, Coulometry – the analytical apply of conductivity measurements – roots in the 18th century. In 1883, Robert Behrend applied potentiometry for indication of titration endpoints. Nernst equation – which gives the theoretical background of direct potentiometry – was introduced in 1889. The potentiometric glass electrode, which is used in our days for pH measurements was brought into practice by Max Cremer in the beginning of the previous century (1908). With the development of the dropping mercury electrode and the mechanics enabling the potential scanning, it was possible to establish the method of polarography. The method was developed by Jaroslav Heyrovsky, the first publication about polarography appeared in 1922.

The evolution of certain electroanalytical methods, the improvement of their performance and their widespreading have been often facilitated by results of other scientific fields or the electroanalytical utilisation of those. Just think about it, how the discovery of the ion-selective electrodes contributed to the popularity of potentiometry. Similarly the appearance of the electronic potentiostats or personal computers caused a rapid progress in the field of voltammetry.

My experimental research, which served as the basis of the dissertation was in the field of electroanalytical methods developement. At the beginning of my work I became aware, that the development of the tools and methods of electrochemistry in the recent past could give scope for the improvement of certain electroanalythical methods. Thus improving their effectiveness and exploring new areas of use.

In the second part of the previous century several biosensors using native plant or animal tissues as catalytic layer were developed by researchers all over the world. The stability of the reaction layers compared to biosensors applying concentrated, purified enzymes was remarkably higher. Furthermore, such sensors are considerably cheaper. In spite of this no such electrodes are used in everyday analytical practice. This can be explained by that the structure, size and enzyme activity of the native layer do not allow reliable analysis in the concentration range of practical samples. It appeared promising to study the possibilities of improving the analytical performance of those biosensors using the combination of applying novel chemically modified electrodes, optimising the layer thickness and utilising innovative electrochemical data collection methods.

It is beneficial if the analysis can be performed without sampling. In this case an error during sampling or inaccuracies due to sample instability are excluded. Theoretically selective electrochemical sensors can be easily lead in solution samples, and after calibration the measured sign is able to indicate sample concentration. In case of voltammetry measurements two problems arise. Firstly the diffusion properties of the sample matrix and the calibration solutions can be different. Secondly, the reagents/chemicals found in the samples can passivate the electrode during the electrolysis. These effects are often appearing in structured samples, tortuous sediments or biological samples as well.

It seemed important to analyse the distortion effects of these phenomena on in-line analysis. I set the aim to study how can the signed measuring technical problems be eliminated by the appropriate alteration of the voltammetric sample collection program, or applying novel results on modified electrodes.

During my experimental research I joined the research group-dealing with designing and improving selective electroanalytical sensors and methods- at Department of General and Physical Chemistry, University of Pécs.

In the course of my work, I tried to broaden the area of analytical application of amperometric sensors. The studied sensors were prepared by the modification of bare voltammetric electrodes in all cases. I fabricated and studied working electrodes modified by biocatalytic layer and diffusion layers. By the employment of the aforementioned modified electrodes in more or less separate applications, studying the methods aiming at different chemical species detection and via the modification of them, I managed to improve the analytical performance characteristics of the methods. In each case, the use of a relatively new amperometric detection method, previously used by few, has fundamentally contributed to the achievement of the results. The further development and application of the novel amperometric detection technique serves as an important connecting link between the sub-areas discussed in the dissertation.

2. Objectives

- Investigation of the possibility of expanding the measuring range of an amperometric biosensor measuring dopamine using a polyphenol oxidase enzyme-containing native banana tissue.
  - As part of this, selection of banana tissue most suitable for sensor fabrication based on electrochemical and spectrophotometric enzyme activity measurements.
  - Selection of detection potential providing the most favorable selectivity and sensitivity via recorded dynamic voltammograms
  - Using Scanning Electrochemical Microscopy (SECM) mapping of dopamine and dopamine quinone concentration profiles resulting from competitive processes in the reaction layer and based on this, determining the optimal reaction layer thickness
  - My goal was to develop the most favorable detection program for the studied biosensor. To this end, I kept in mind that in the case of amperometric detection, the concentration of dopamine quinone on the electrode surface is zero; thus, only material from the environment that reaches the surface can be measured, while the layer close to the electrode is depleted. With the use of periodically interrupted amperometric detection - used in our research group first time - there are rest periods between the measurement periods during which the layer can recharge and the signal increases.
- Development of a modified electrode and voltammetric detection method for direct amperometric determination of L-ascorbic acid in model tortuous media.
- Investigation of electrode passivation, which often occurs during the measurement of real samples, especially yellow peppers, as well as confounding effects due to the difference between the viscosities of standard solutions and the sample matrix and the possibilities of their reduction.
- Direct voltammetric determination of vitamin C in peppers without sample pretreatment; investigation of the effect of ions from steel and metal knives used in food processing on the L-ascorbic acid content of peppers in contact with air.
- Investigation of the applicability of the amperometric Clark-electrode -which is widely used for oxygen measurement and contains a built-in diffusion layer- in tortuous media and sediments. Studying the introduction of a periodically interrupted amperometric detection program for samples where diffusion of the electroactive species to the electrode surface is hindered.

3. Tools and methods used

Preparation of crude enzyme extract from bananas for measurement of polyphenol-oxidase enzyme activity

I placed the peeled, sliced banana in phosphate buffer (pH=7.33) in a beaker. I put the beaker in a water bath for heat treatment. The buffer solution was then filtered from the banana and homogenized using a hand mortar. The resulting sample was centrifuged, resulting in an enzyme-containing liquid phase above the tissue debris. The resulting melanins were removed by filtering the extract through a 20 μm pore diameter syringe filter.

Measurement of polyphenol-oxidase enzyme activity

In the course of my work, I prepared a special amperometric thin-film cell for the comparative study of the enzyme activity of catalytic layers from different plant tissues. The body of the measuring cell is made of EpoFix (Struers GmbH) polymer. A glassy carbon (GC) (d=1 mm) working electrode, a silver quasi-reference electrode (d=2 mm) and a platinum counter electrode (d=3 mm) were embedded in the polymer. The thin-film type reaction space (volume 60 μl) was designed by gluing an annular spacer to the end plate of the cell positioned so that the empty part of the ring was above the electrodes. During the measurement, a filter paper disc was placed on the surface of the cell body containing electrodes to ensure a uniform thickness distribution of the reaction mixture in the thin film cell. During the measurements, the reaction space was covered with a cap to prevent the volume loss of the reaction mixture caused by evaporation. The filter paper disc to be placed at the bottom of the reaction chamber was soaked in phosphate buffer and dried. I then pipetted the enzyme-containing extract into the reaction space of the microcell and connected the electrodes to the potentiostat, observing the amperometric current using a potential of -0.2 V. After the appearance of a constant current value, the dopamine solution was introduced into the thin-layer cell with a microsyringe. As a result of the enzyme catalysed reaction, dopamine quinone appeared on the surface of the electrode and I received a cathodic amperometric current. The slope of the current-time trace indicates the rate of enzyme-catalyzed reaction.

Reference method for the determination of enzyme activity

To determine the polyphenol oxidase activity of the enzyme extract made from banana, I used the standard method of Sigma-Aldrich spectrophotometric enzyme activity measurement as a

reference method. The assay is based on an indirect spectrophotometric method that utilizes the L-ascorbic acid oxidizing ability of polyphenol oxidase.

Redox titrating as a reference method for measuring vitamin C content in solutions

Samples containing L-ascorbic acid were titrated with iodine solution in the presence of starch indicator, the end point was indicated by a slight bluishness of the solution.

Applied instruments

In the voltammetric measurements I used a CHI type 760C (CH Instruments, Austin, TX, USA) electrochemical measuring instrument. For the scanning electrochemical microscopic measurements I used one of the devices developed in the laboratory of the Department of General and Physical Chemistry of the University of Pécs. The chemical microscope consists of three main parts: an electrochemical measuring station - eDAQ Picostat (model EA 162) and e-cable ED410 (eDAQ, Australia) - , a moving unit and a measuring cell. A computer and an optical camera are connected to the device.

Flow Injection Analysis system

The home-made Flow Injection System includes a home-made wall-jet measuring cell. Other units of the system: peristaltic pump (ISMATEC SA Mini-s 620m Ismatec SA, Switzerland), injector (Laboratory MIM, Budapest, Hungary), injector loop volume: 25 µl, dispersion coil.

Model biosensor measuring cell

To determine the optimal thickness of the bananaotrode biosensor enzyme layer, I constructed a model biosensor measuring cell model. The reaction layer was located in the lower part of a glass tube. The signal transducer SECM measuring tip was a carbon paste microelectrode. To form the reaction layer, banana tissue was homogenized with phosphate buffer (pH=7.33), and then the resulting pulp was filled into a glass tube, one end of which was pre-sealed with a dialysis membrane (Technikon Autoanalyzer accessories). The glass tube modeling the biosensor dipped into a measuring cell containing a sample solution. With a magnetic stirrer, I induced intense convection in the measuring cell, with which I wanted to ensure the formation of steady transport conditions on the sample solution side of the membrane. I placed the reference and counter electrodes in the buffer and connected them to the potentiostat. The working electrode attached to the SECM could move in the tube containing the enzyme layer.

Electrodes

For the measurements, I usually used an OP-0830P type saturated calomel reference electrode (Radelkis, Budapest, Hungary) and a 1 cm^2 platinum plate counter electrode.

Preparation of carbon paste microelectrode

Graphite powder (<20 μm particle size, ALDRICH Chemistry) and nujol oil (Pannon Pharmacy) were homogenized in a porcelain mortar. To prepare the electrode body, a borosilicate capillary (Sutter Instruments Co., USA) was pulled out over a flame and then the tapered end was fused. To form the tip of the microelectrode, I carefully sanded the capillary from the sealed end until I reached the appropriate internal diameter (50 μm), and then polished it smoothly with aluminum polishing powder. From the rear end of the capillary made in this way, I inserted a thin copper thread to ensure electrical contact, and from the tapered end of the capillary I filled it with carbon paste, polishing its surface smoothly.

Preparation of a "bananatrode" biosensor

I made contact between a Glassy Carbon (Alfa Aesar, VWR International Ltd., Budapest) rod 0.5 cm long part and a suitable copper wire using silver epoxy (Epo-Tek, ET-H20E-8-0028, ET-PMS-0028, JP Kummer GmbH.) conductive adhesive and then inserted it in a glass tube of appropriate diameter and embedded in EpoFix (Struers GmbH) polymer. After polymerization, the electrode surface was prepared by grinding and wet polishing. In case of the bananatrode biosensor, a 130 μm thick spacer ring made of insulating tape was placed on the 4 mm diameter GC electrode, and the pre-homogenized banana pulp was filled into the resulting cavity with a spatula then covered and attached with a dialysis membrane closely to the electrode.

GC working electrode for measuring L-ascorbic acid in a tortuous medium

The 1 mm diameter, home-made GC working electrode was cleaned and polished before use. If the electrode surface was found to be sufficiently clean and of sufficient activity, I covered it with a moistened dialysis membrane (TechnikonTM Pre-mount type "C", Technikon Instruments, USA), taking care not to trap air between the surface and the membrane, and secured the membrane with thread.

Clark-electrode

The oxygen meter Clark-cell used in the experiments was made at home. I melted a 1 mm diameter platinum wire into the capillary end, and then inserted a small piece of solder and a thin copper wire from the back end of the capillary. I formed the electrical contact over a flame. I gently polished the fused end of the glass tube until the platinum disc appeared in the middle. Then I wet-polished it with alumina polishing powder. Next to the working electrode, a chlorinated silver fiber was used as a counter and reference electrode. The end of the glass tube was closed with a stretched poly-(tetrafluoroethylene) membrane cap (Radelkis, Budapest, Hungary). I filled a bicarbonate buffer internal electrolyte with pH=9.5 containing 0.1 M KCl inside the cell body, and then the glass body containing the electrodes was placed and fixed in the tube so that the platinum disk working electrode fit directly to the Teflon membrane, leaving only a thin film of liquid between them.

4. Results, Thesis points

The results of the experimental and evaluation work can be summarized as follows:

I. I was the first to demonstrate by recording dynamic voltammograms that the selection of the optimal working potential is key for a "bananaotrode" biosensor, thereby improving selectivity and sensitivity to the dopamine quinone to be measured, as unwanted interferences e.g. the presence of oxygen can be minimized, , which is also involved in the enzymatic reaction. This is -0.2 V vs. Hg / Hg_2Cl_2 reference electrode. I first applied periodically interrupted amperometric detection method in case of a natural tissue-based biosensor first, and I developed the program by determining the optimal potentials and measurement timing. The sensitivity of the biosensor operated in this way increased significantly compared to the conventional amperometric detection, while the value of the detection limit decreased from $1.05 * 10^{-5}$ mol / dm^3 to $2 * 10^{-6}$ mol / dm^3.

II. I confirmed the local product enrichment in the natural banana-containing reaction layer of the biosensor as a result of competitive processes by on-site measurements. For mapping the concentration profiles of dopamine and dopamine quinone in the reaction layer, I successfully used a scanning electrochemical microscopic method with a carbon paste microelectrode tip. At a distance of 130-160 μm, a local maximum is formed in the concentration of dopamine quinone. I designed the reaction layer thickness of the "bananaotrode" based on this.

III. I verified the error of the measurement during the amperometric L-ascorbic acid determination in sediment as a model tortuous medium by local measurements. To eliminate the interfering effect, I developed a method: I used a built-in diffusion layer modified glassy carbon working electrode and short-term chronoamperometric detection. I have shown that with increasing diffusion layer thickness, the time for which the diffusion concentration profile is in the modified layer increased proportionally, making it insensitive to changes in the diffusion conditions of the environment.

IV. I developed a method to determine the direct L-ascorbic acid content of fibrous yellow pepper juice without sample preparation. The experimentally proved electrode passivation, viscosity difference and tortuosity caused measurement errors were eliminated using a dialysis membrane as a built-in diffusion layer on the glassy carbon working electrode and a measuring program with 90 s charging/equilibrating time and short-term chronoamperometric detection, and viscosity correction. I performed electrochemical measurements directly on pepper slices and based on these I proved that yellow pepper tissue loses its L-ascorbic acid content significantly faster when I bring into contact with an iron knife comparing to the case, using a stainless steel or ceramic knife.

V. I showed by in-situ measurements that the use of a Clark-cell suitable for oxygen measurement in sediments brings in a significant measurement error due to the problem of tortuosity. I have demonstrated that an appropriately chosen periodically interrupted amperometric detection program provides results close to true values.

5. List of Publications

Publications related to the thesis

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