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Microscopy techniques for the characterization of modified electrodes in the development of glucose biosensors

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Abstract

Microscopy techniques were used to study films of a hydrotalcite-like compound electrosynthesised on a Pt electrode, entrapping glucose oxidase. SECM study has been carried out to probe local enzymatic activity and to obtain topographic images of the modified electrode, which can be used as glucose amperometric biosensor. Due to the complexity of the investigated system, different configurations were considered, starting from the Pt surface only modified with the inorganic clay, up to the optimized biofilm, with glucose oxidase stabilized by cross-linking with glutaraldehyde. SECM was used to detect the activity of the entrapped enzyme by observing the feedback current in a solution of $K_4[Fe(CN)_6]$ and glucose in phosphate buffer, when $K_3[Fe(CN)_6]$ was generated at the tip.

It was demonstrated that the enzyme distribution was not homogeneous all over the electrode surface, but it reflected the distribution of the underlying electrosynthesised inorganic film. SECM gave only poor topographic information about the complex samples studied, but the images were in good agreement with those provided by other microscopy techniques.

Keywords: Scanning electrochemical microscopy (SECM); Atomic force microscopy (AFM); Scanning electron microscopy (SEM); Glucose biosensors; Local enzymatic activity; Hydrotalcite-like compounds

1. Introduction

Amperometric biosensors employ immobilized enzymes for the conversion of target analytes into electrochemically detectable products, i.e. combine the advantages of electrochemical detection with the high substrate specificity of the enzymes. Various methods have been reported, in the literature, concerning enzyme immobilization on electrode surfaces: they include cross-linked polymers [1], composite ceramic carbon [2,3], sol–gel silica matrix [4–6]. Anyway, there is not a large diffusion in literature about methods for characterizing the biosensor reactive surface and detecting enzymatic activity within the system. Recently, the use of anionic clays in the fabrication of biosensors has been proposed [7,8] as an alternative way of immobilizing enzymes.

The determination of glucose is one of the most popular and well-known biosensor applications. Glucose is of special importance since it is involved in human metabolic processes. Glucose determination in blood deserves a particular attention as it is a fundamental step in the treatment of diabetic patients [9,10]. Furthermore its detection is also important in food and fermentation industry and many papers deal with this subject [1,11,12]. Glucose electrochemical biosensors, based on its enzymatic oxidation mediated by glucose oxidase (GOx), have generated much interest. The enzyme catalyzes the oxidation of glucose to gluconolactone, in the presence of oxygen. In the natural enzymatic reaction, molecular oxygen acts as an electron acceptor for $FADH_2$, being reduced to H_2O_2. It is well known that biosensors activity depends on three main factors: diffusion of the substrate through the biological membrane, enzyme activity within the immobilization matrix and efficiency of the electrochemical transduction step [9,13].

As matrices for the immobilization of biomolecules, we chose anionic clays belonging to the class of layered double hydroxides, also named hydrotalcite-like compounds (Htlc). These materials consist of brucite $Mg(OH)_2$ type layers in which trivalent metal cations partially substitute the bivalent ones and interlayer anions compensate the excess of positive charge. Their

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abbreviated formula can be written as M(II)/M(III)-X, where X represents the interlayer anion. Htlc display some attractive features: high water content providing biocompatible environment for the entrapped molecules, high mobility of analyte and reaction products, non-toxicity and high chemical and hydrolytic stability [7]. Generally, adsorption of enzymes on a matrix causes a drastic decrease in activity due to a modification of the conformation of the protein. On the contrary, the electrostatic interaction between the enzyme and the Htlc matrix is favourable to enzyme adsorption on clay surface [14]. Recently we have proposed a glucose amperometric biosensor [8] based on a simple, rapid and reproducible technique for the immobilization of glucose oxidase within a Htlc material. The clay, directly electrosynthesised on Pt surfaces, behaves as an entrapping matrix and as an electrode modifier, which allows a stable current signal for the oxidation of hydrogen peroxide to be recorded. Furthermore, we demonstrated that no significant changes in the structure of the enzyme occur after the immobilization within the Htlc film.

In the last few years, scanning electrochemical microscopy (SECM) has been successfully applied to the study of enzyme-containing systems, particularly for the investigation of modified surfaces and enzyme patterns [15–18]. In this work, the amperometric biosensor obtained by entrapment of the enzyme GOx within a Htlc layer has been characterized by SECM, in order to probe local enzymatic activity and to study its surface topographic features. Information about surface topography has been compared to that provided by other microscopy techniques, such as atomic force microscopy (AFM) and scanning electron microscopy (SEM).

2. Materials and methods

2.1. Chemicals

$K_4[Fe(CN)_6]\cdot 3H_2O$ and $Al(NO_3)_3\cdot 6H_2O$ were purchased from Aldrich (Milwaukee, WI, U.S.A.), KNO_3 from Carlo Erba (Milan, Italy), $Ni(NO_3)_2\cdot 9H_2O$ and anhydrous D(+)-glucose were obtained from Fluka (Buchs, Switzerland). Glucose oxidase (GOx, E.C. 1.1.3.4, 197,000 units g^{-1}, solid, from Aspergillus Niger), bovine serum albumin (BSA) and glutaraldehyde (GA, 25% w/w, aqueous solution) were purchased from Sigma (St. Louis, MO, U.S.A.).

Phosphate buffer solution (PBS) was prepared with KH_2PO_4 (Merck, Darmstadt, Germany), setting pH value with NaOH "FIXANAL," obtained from Riedel-de Haën (Hannover, Germany).

All reagents were of analytical grade and were used without further purification. All solutions were prepared with water obtained from a Milli-Q system (Millipore, Milford, MA, U.S.A.), further purified by distillation in a glass apparatus.

2.2. Instrumentation

CHI 900B SECM equipment (CH Instruments, Inc., Austin, TX, U.S.A.) was used for the acquisition of SECM images and approach curves.

All the electrochemical measurements were performed in a 1.5 mL Teflon cell (CH Instruments, Inc., Austin, TX, U.S.A.), using a three electrode set up: the working electrode was a Pt disk SECM tip ($a = 5$ μm, RG = 5), the reference electrode was an Ag/AgCl filled with 3 M KCl and the counter electrode was a Pt wire. As substrate, a chemically modified 2 mm diameter Pt disk electrode was used (all electrodes were purchased from CH Instruments, Inc., Austin, TX, U.S.A.).

Before performing chemical modifications and measurements, SECM tip and Pt substrate were carefully polished with 0.05 μm alumina slurry and diamond paste on microcloth pads; moreover, tip electrochemical behaviour was checked by registration of cyclic voltammetry (CV) experiments. CHI 660A Electrochemical Workstation (CH Instruments, Inc., Austin, TX, U.S.A.) was employed to carry out electrosynthesis of Htlc; a three electrode cell configuration was used, with a 2 mm diameter Pt electrode (CH Instruments, Inc., Austin, TX, U.S.A.) to be modified as working, a SCE (AMEL Instruments, Milan, Italy) as reference and a Pt wire (Aldrich, Milwaukee, WI, U.S.A.) as counter electrode. VISTA-100 Scanning Probe Microscope (Burleigh Instruments, Inc., Fishers, NY, U.S.A.) was used to study surface morphology; AFM images were recorded in contact mode, using a Si_3N_4 cantilever with an elastic constant of 32 nN m^{-1}.

EVO 50 EP (ZEISS) scanning electron microscope with an OXFORD INCA 350 EDS microanalysis system was employed to acquire magnified topographic images of the sample and to study its local elemental composition.

2.3. Software tools for SECM data treatment

Data treatment for the normalization of probe approach curves was accomplished with Origin 7 software, while for the acquisition and presentation of SECM bidimensional images Excel, Matlab 7, Surfer 8 and Image J softwares were used.

2.4. Sample preparation

Different kinds of electrode configuration were assembled. Among each series of measurements, electrode surface was rinsed with a 2 M HNO_3 solution, in order to remove deposited material. For each electrode configuration, the procedure of sample preparation is described in the following.

2.4.1. Colloidal Htlc

10 or 20 μL of a colloidal suspension (about 4 g L^{-1}) of Ni/Al–Cl, prepared according to the "coprecipitation method" proposed by Miyata [19], were put on Pt, using a micropipette. Then the electrode was allowed to dry at room temperature until complete solvent evaporation.

2.4.2. Electrosynthesised Htlc

A thin film of Ni/Al–NO_3 was directly electrosynthesised on the electrode surface by reduction of a solution containing 0.225 M $Ni(NO_3)_2$, 0.075 M $Al(NO_3)_3$ and 0.3 M KNO_3, following the procedure described by Scavetta et al. [20]. Electrosynthesis was carried out potentiostatically, applying a