ELECTROPHORETIC PROTEIN DEPOSITION: A NEW ENZYME IMMOBILIZATION METHOD FOR THE DEVELOPMENT OF AMPEROMETRIC BIOSENSORS

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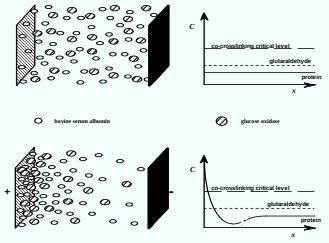
AIM OF THE WORK

Electrodeposition [1] is an enzyme immobilization method based on the well-known electrophoretic phenomena of proteins under the influence of an electrical field. In the original method [1], the enzyme is mixed to a collagen dispersion at a pH value different from their isoelectric points to form macromolecular complexes which migrate to, and deposit on, an electrode surface held at an appropriate electric potential. In spite of the interest of such a method, quite similar to the enzyme entrapment in electrosynthesized polymers, the so-called "*electrochemical immobilization*" [2], up to now few papers have been devoted on this subject [3-4]. These studies do not deal with the understanding of both chemical and electrochemical processes involved in protein electrodeposition, which are particularly significant for the proper development of biosensors. For example, a study of a suitable electrochemical technique, able to control the protein deposition while minimizing the undesirable but collateral faradaic processes (i.e. O_2 evolution), cannot be found in the relevant literature. More important, the realization of an useful biosensor, free of interference and fouling problems (which arise in real matrices analysis) has not yet been achieved with this approach.

The electrodeposition method, herewith called "*electrophoretic protein deposition*" (EPD), has been investigated in our laboratory with the aim to develop a novel approach in amperometric biosensor realization. The influence of some chemical and electrochemical parameters on the protein deposition has been studied with several electrochemical methodologies. Galvanodynamic and potentiodynamic techniques have been compared in terms of membrane quality, thickness and spatial control of protein deposition. An electrochemical quartz crystal microbalance study permitted further insights about the growth of proteic deposit on the electrode surface. The enzyme electrodes so obtained have been further characterized to realize the feasibility of EPD procedure for the development of an useful biosensor. In this respect, the realization of amperometric biosensors using the hybrid approach [5] has been drawn out to this novel enzyme immobilization procedure. In particular, EPD of co-crosslinked bovine serum albumin/glucose oxidase membranes coupled with electrosynthesized non-conducting films of poly-2-naphthol[6] or poly-o-aminophenol[7] permitted the realization of glucose biosensors with anti-interference and anti-fouling performances so interesting to assure a future employment for real sample analysis.

ELECTROPHORETIC PROTEIN DEPOSITION

MECHANISM



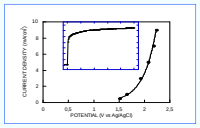
The electrode is dipped on a supporting electrolyte containing the enzyme (e. g. glucose oxidase), an inert protein (e. g. bovine serum albumin) and the crosslinker (e. g. glutaraldehyde). Both the protein and the crosslinker concentrations need to be optimized so to avoid any appreciable co-crosslinking in solution.

By using a proper electrical field and the correct chemical conditions (e. g. pH, ionic strength), electrophoretic migration of both proteins occurs so to induce their preferential accumulation and subsequent immobilization by chemical linking onto the desired electrode

Enzyme co-crosslinking, a classical enzyme immobilization method, can be carried out only onto the electrode surface under electrochemical control achieving all the advantages allowed by electrochemical immobilization.

ELECTROCHEMICAL TECHNIQUES FOR ELECTROPHORETIC PROTEIN DEPOSITION

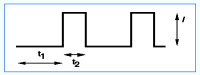
Galvanostatic technique



Galvanostatic (as well as potentiostatic) deposition produced a foamy deposit near the electrode surface as well as an evident oxygen evolution. Whenever the current density applied for protein deposition, the corresponding electrode potentials reached values so high to promote water oxidation (see figure). As in the case of metal electroplating, gas evolution heavily interfers or hampers the deposition process.

Galvanodynamic technique

Galvanostatic experiments show that electrophoretic protein deposition requires high current (or potential) values while minimizing oxygen evolution at the electrode surface.



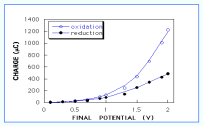
Accordingly, a proper current pulse sequence revealed successfull. Anyway, the deposition of a satisfying proteic layer required a careful optimization of some electrochemical parameters (i.e. t_1, t_2, I).

Analysis of the relevant E-t transients showed that the electrode potential at the rest time (i.e. t_1) increased during the application of the current pulse sequence, whatever t_1, t_2 and I values. These experimental findings suggest a notable platinum oxide formation, which is known to enhance the undesired oxygen evolution.

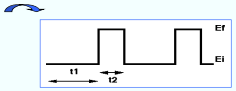
In spite of its effectiveness, current pulse technique does not allow any proper control of the electrode potential.

As a consequence, a potentiodynamic technique would assure a significant improvement of the electrophoretic protein deposition process.

Potentiodynamic technique

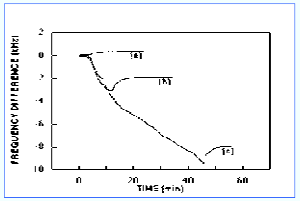


If a double potential step experiment is carried out by fixing the initial potential E_i while increasing the final potential E_f of potential pulses, oxidation charge resulted higher than reduction charge suggesting also in these cases an irreversible oxygen evolution enhanced by platinum oxide formation.



Protein deposition was optimized by applying at the electrode the proper potential pulse sequence: in particular E_i must be enough negative to promote entirely the stripping of platinum oxide which inevitably forms during the application of E_f.

ELECTROCHEMICAL QUARTZ CRYSTAL MICROBALANCE (EQCM) STUDY



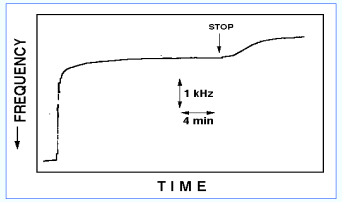
EQCM profiles for EPD acquired during the application of current pulses for (b) short and (c) long deposition time. (a) Electrogravimetric profile acquired in the presence of the only supporting electrolyte (phosphate buffer, I 0.1 M, pH 7).

Apparently, the application of the current pulse sequence at the electrode produces a roughly constant growth of the proteic deposit (≈ 0.1 μg/min, i.e. ≈ 1 micron/h). It is interesting to note that stopping the current pulse application hampered the EPD process.

EQCM profiles seem to suggest the possibility of a thickness control by proper using of the deposition time. Anyway, co-crosslinking kinetics seriously obstructed this approach.

EQCM profiles acquired during the application of potential pulse sequence were significantly different from the previous ones. In fact, a steep mass change was followed by a steady state mass value which was roughly dependent on the final potential E_f applied.

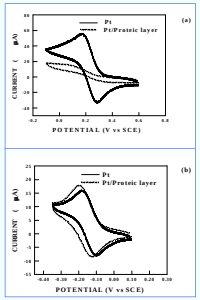
Hence, a suitable pulse sequence application could permit an electrophoretic protein deposition of the desired thickness for all the time required for proper co-crosslinking reaction.



EQCM profile for EPD acquired during the application of potential pulses

An AC impedance EQCM study is currently in progress in our laboratory to corroborate these experimental findings.

ELECTROCHEMICAL BEHAVIOUR OF MODIFIED ELECTRODE



Voltammetric profiles of potassium ferricyanide (a) and hexaammineruthenium (III) chloride (b) at both bare and modified platinum electrode with proteic layer. Supporting electrolyte: phosphate buffer I 0.1 M, pH 7. Scan rate: 50 mV/s.

Permeability of the proteic layer can be explained in terms of an electrostatic interaction towards the analytical probes so that just species with an opposite charge can efficiently permeate through the deposit to reach the electrode.

Codeposition of a permselective electrosynthesized film is **necessary** to prevent interferences and fouling problems.

Pt/proteic layer/film electrodes

Electrosynthesis of permselective films onto the proteic layer. The presence of a proteic clothing onto the electrode does not hamper the electrosynthesis of polymeric films, apart from a certain slowing down of the growth because of the diffusion of monomer through the deposit.

How to codeposit the proteic layer with a permselective film ?

Pt/film/proteic layer electrodes

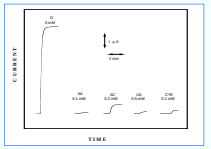
Electrophoretic deposition on electrodes previously modified with permselective films.

Pt/film/proteic layer: the electrodic perturbation necessary for the electrophoretic deposition causes a certain modification in the permselectivity characteristics of the films previously polymerized.

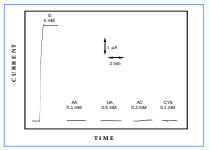
Pt/proteic layer/film: permselectivity of films is better preserved when any EPD process is carried out after polymer electrosynthesis.

Between poly-2-naphthol (P2NAF) and poly-o-aminophenol (PoAP), the first was the film which showed the best permselectivity behaviour.

Biosensor Pt/GOD/PoAP



Biosensor Pt/GOD/P2NAF



Current-time responses for successive additions of glucose (G), ascorbic acid (AA), uric acid (UA), acetaminophen (AC) and cysteine (CYS) to phosphate buffer supporting electrolyte (pH 7, I 0.1M). Rotation rate: 1000 rpm.

ELECTRODE MODIFICATION BY ELECTROSYNTHESIZED NON CONDUCTING FILMS

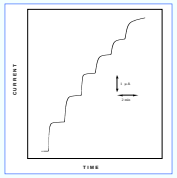
BIOSENSORS PERFORMANCES

Biosensor Pt/PoAP/GOD

Current-time responses of a typical rotating disk Pt/PoAP/GOD electrode for successive additions of a glucose standard solution to a phosphate buffer supporting electrolyte (I 0.1 M, pH 7). Glucose additions refer to a total glucose concentration of 1.25, 2.49, 3.72, 4.95, 7.39 mM; rotation rate: 1000 rpm.

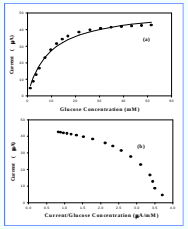
- **Response Time**: 4÷5 seconds
- **Sensitivity**: 0.3÷0.6 μA/(mM mm²)
- **Stability**: after about 40 days of using sensitivity was the 70% of the initial value

Biosensor Pt/GOD/P2NAF



Current-time responses of a typical rotating disk Pt/GOD/P2NAF electrode for successive additions of a glucose standard solution to a phosphate buffer supporting electrolyte (I 0.1 M, pH 7). Glucose additions refer to a total glucose concentration of 1.25, 2.49, 3.72, 4.55, 6.17, 8.60 mM; rotation rate: 1000 rpm

Biosensor Pt/PoAP/GOD



Glucose calibration curve (a) and relevant Eadie-Hofstee plot (b) obtained at a typical rotating disk Pt/PoAP/GOD electrode. The solid line in (a) is the Michaelis-Menten fitting of the experimental data.

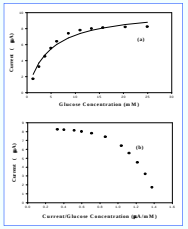
The experimental current-concentration values show a significant deviation from the Michaelis-Menten model of enzymatic catalysis

Possible explanation:

Due to the thickness of the enzymatic layer, diffusion of substrates and/or enzymatic reaction products through the electrodic membrane may become a step kinetically determinant

MIXED KYNETIC CONTROL

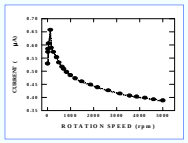
Biosensor Pt/GOD/P2NAF



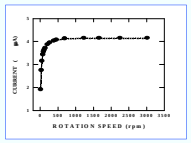
Glucose calibration curve (a) and relevant Eadie-Hofstee plot (b) obtained at a typical rotating disk Pt/GOD/P2NAF electrode. The solid line in (a) is the Michaelis-Menten fitting of the experimental data.

HYDRODYNAMIC BEHAVIOUR

1. Thin enzyme layer



2. Thick enzyme layer



Responses to glucose 1.5 mM for different rotation rates at a (1) thin and a (2) thick layer glucose biosensor, obtained by potentiodynamic EPD. Supporting electrolyte: phosphate buffer solution, I 0.1 M, pH 7.

1. Enzymatic catalysis seems to be the step kinetically limiting on the increasing of rotation rate

2. Current increases with rotation rate until reaching a steady state value. This behaviour indicates a process under diffusive control through the membrane.

Possibility of passing from enzymatic to diffusive control by varying opportunely the proteic layer thickness

CONCLUSIONS

The aim of this work was to develop a novel method for amperometric biosensor realization based on **electrophoretic protein deposition**.

Using a suitable electrochemical technique (galvanodynamic or potentiodynamic) it is possible to control the protein deposition directly onto the electrode surface, while minimizing collateral faradic processes, such as O_2 evolution. Particularly, potentiodynamic thechnique revealed more advantageous as it allows a better control on the faradic processes occurring at the electrode surface.

The hybrid approch of biosensors realization was adopted by coupling co-crosslinked bovine serum albumin/glucose oxidase membranes with electrosynthesized non conducting films of poly-2-naphthol or poly-o-aminophenol.

The electrophoretic protein deposition revealed compatible with the approach employing electrosynthesized permselective films.

Glucose biosensors with anti-interference and anti-fouling performances were obtained. It would be quite interesting a future employment of these devices for real sample analysis.

References

1. W.R. Vieth and K. Venkatasubramanian, in *Methods in Enzymology* (Edited by K. Mosbach),, Vol. XLIV, Ch. 19, Academic Press Inc., Orlando (1976)
2. P.N. Bartlett and J.M. Cooper, *J. Electroanal. Chem.* **362**, 1 (1993)
3. K.W. Johnson, *Sensors and Actuators B* **5**, 85 (1991)
4. D.J. Strike, N.F. deRooij and M. Koudelka-Hep, *Sensors and Actuators B* **13-14**, 61 (1993)
5. A. Guerrieri, G.E. De Benedetto, F. Palmisano and P.G. Zambonin, *Biosensor & Bioelectronics* **13(1)**, 103 (1998)
6. R. Ciriello, T. R. I. Cataldi, D. Centonze and A. Guerrieri, Electroanalysis (1999) submitted for publication
7. R. Ciriello, A. Guerrieri, paper in progress.