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Amperometric Biosensors Based on Bulk-Modified Epoxy Graphite Biocomposites

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Different amperometric glucose biosensors based on chemically and biologically modified epoxy-graphite composites have been fabricated. The bulk of the resulting biocomposites behaves as reservoirs of enzyme and metallic catalysts for a first-generation biosensor, and also of redox mediators when the biosensor is a second-generation one. The response characteristics have been compared with those of a conventional membrane-type glucose biosensor.

1. Introduction

Great efforts are being made recently in developing new sensors to improve their selectivity, sensitivity, robustness, cost, manufacturing complexity and applicability to the monitoring and control of clinical, environmental and industrial processes. This field has taken advantage of the availability of new materials and has yielded a generation of composite-based transducers. One material used in the construction of voltammetric biosensors has been the conductive graphite-epoxy composite. Conventional amperometric biosensors consist of a conductive electrode with the immobilization of the biological material close to the active electrochemical surface, for example, by attaching modified membranes.[1] Recently, the conductive material forming the electrode has been modified by merging biological material with the conductive resin, resulting in bulk modification of this composite, as shown schematically in Fig. 1. The procedure is reduced to a simple mixing of the graphite, epoxy components, enzyme and auxiliary materials with curing at low temperatures to assure enzymatic activity.

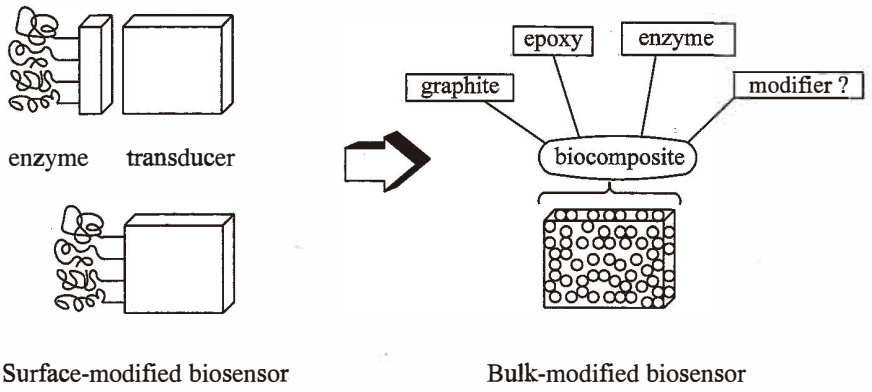

Fig. 1. The transition from surface-modified biosensors to bulk-modified biosensors.

2. Materials and Methods

2.1 *Reagents*

To fabricate the electrodes, graphite powder with a particle size of 50 μm (Merck), epoxy resin Epo-Tek H77 (Epoxy Technology, Billerica, MA, USA), gold power (32,659-3 [7440-57-5], Aldrich), palladium powder (36,666-6 [7440-05-3], Aldrich), tetrathiafulvalene (TTF) (Fluka) and glucose oxidase (GOD) (G-2133 type VII from *Aspergillus niger* obtained from Sigma) were used.

2.2 *Auxiliary electrodes*

To characterize and evaluate the working electrodes, a platinum auxiliary electrode and a double-junction Ag/AgCl reference electrode (Orion 92-02-00) were used.

2.3 *Fabrication of the biosensors*

2.3.1 *Amperometric glucose biosensor based on epoxy-graphite/GOD*

The glucose biosensor was fabricated by applying a nylon-6,6 membrane to the surface of the electrode.[1] Previously, GOD had been immobilized covalently on the nylon surface. The immobilization process[2,3] consisted of activating the nylon with dimethyl sulfate in order to form an imido ester group. The nylon membrane containing the enzyme was fixed to the electrode by a dialysis membrane and an O-ring, using Parafilm as a sealant.

2.3.2 *Amperometric glucose biosensor based on the biocomposite epoxy-graphite-catalyst-GOD*

In this case, the sensitive composite material was prepared by mixing the catalyst, graphite and epoxy resin in a ratio 1:4:16 by weight. The catalyst is a mixture of gold and

palladium in a ratio of 3:2 by weight. For each gram of the resulting composite, 15 mg of the GOD enzyme was added (Fig. 2(a)).

2.3.3 *Amperometric glucose biosensor based on the biocomposite epoxy-graphite-TTF-GOD*

The biosensor composite material was prepared by graphite powder dispersion in an epoxy resin. The mixture was a 1:4 weight ratio of TTF and conductive resin. The resulting mixture was thoroughly homogenized; 15 mg of GOD was added for each gram of the graphite-epoxy-TTF mixture (Fig. 2(b)).

3. Results

The amperometric enzymatic glucose electrode was used for comparison of the performance characteristics of the biocomposite electrodes. The amperometric transducer is fabricated with a conductive epoxy graphite composite and the enzyme is chemically immobilized on a nylon layer. This electrode is a first-generation electrode, which

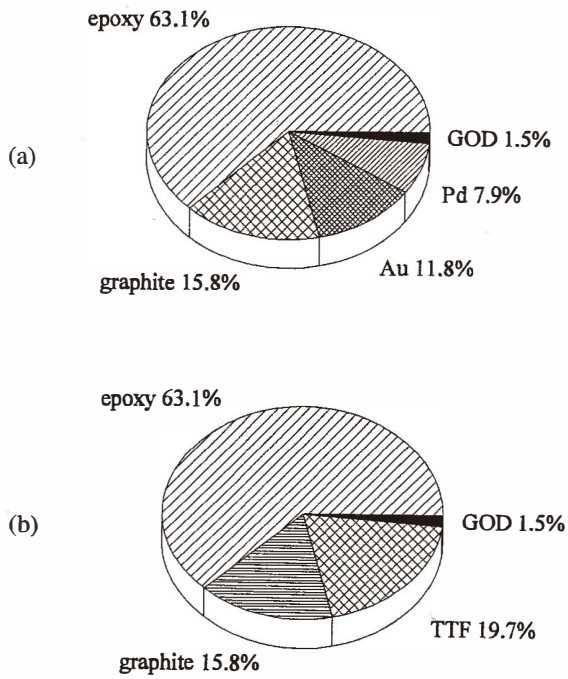

Fig. 2. Composition of the two biocomposites tested for the fabrication of glucose biosensors with (a) Au-Pd catalyst and (b) TTF as redox mediator.

amperometrically measures a product of the enzymatic reaction.

$$\text{D} - (+) - \text{glucose} + O_2 \xrightarrow{GOD} \text{gluconic acid} + H_2O_2$$

$$H_2O_2 \xrightarrow{E} O_2 + 2H^+ + 2e^-$$

The chemical reaction involved is the well-known oxidation of glucose by means of GOD, and the hydrogen peroxide product is measured by its oxidation at the graphite-epoxy electrode at a working potential of 1150 mV. Due to the high working potential employed, foreign species can be oxidized as well, giving rise to interference problems. This is the main disadvantage of this sensor; however, it can be overcome by using electrochemical modifiers.

The performance characteristics of this conventional biosensor, as outlined in Table 1, can be summarized as good sensitivity and ample linear working range, of 10^{-2} to 10 mM of glucose. When the sensitivity is calculated from the log I (nA) versus log of glucose concentration (M) plot, the mean value for 5 consecutive calibrations is 6.56, showing an excellent reproducibility of 1.10% (RSD). The lifetime of this biosensor is longer than two months, and the response time is relatively high, due to the physical barrier of the

Table 1
Performance characteristics of the different amperometric glucose biosensors.

Biosensor [references]	Epoxy/graphite/ GOD [1]	Epoxy/graphite/ Au/Pd/GOD [4]	Epoxy/graphite/ TTF/GOD [5]
Configuration	Enzymatic membrane	Biocomposite	Biocomposite
Detection	H_2O_2	H_2O_2	TTF
Working potential (mV)	1150	900	150
Linear working range (mM glucose)	0.01–10	0.01–2	0.1–2
Response time	32 s	6 s	11 s
Lifetime	> 2 months	> 3 months	3 days
Regeneration	no	possible	possible
Interference levels	high	high	low
Ascorbic acid (0.1 g/l)	> 100 %	> 100 %	13 %
Citrate (5.0 g/l)	–*	–*	–*
Urea (0.1 g/l)	–*	–*	–*
Uric acid (0.1 g/l)	> 50 %	> 100 %	–*

* undetectable
(Measurements in a pH 7.0 buffered solution, 0.1 M phosphate and 0.1 M KCl.)

enzymatic membrane. The interference levels of some species that are found in the samples with glucose are also shown in Table 1.

In the bulk-modified biosensors, the biological material is trapped within the conductive matrix instead of being immobilized on the attached membrane. When the composite is modified by incorporating GOD for amperometric measurement, further materials must be incorporated. This is necessary to monitor the products of the enzymatic reaction and to regenerate the enzyme. Two methods[4,5] are shown here: the use of tetrathiafulvalene as redox mediator and the use of a hydrogen peroxide catalyst (Au-Pd).

The first conductive matrix tested incorporated gold-palladium as a catalyst for the oxidation of hydrogen peroxide. The use of this catalyst allows the detection of hydrogen peroxide at a lower potential, 900 mV, thereby reducing the interference problems. The performance characteristics of this biocomposite, also shown in Table 1, are good sensitivity and an ample linear working range, which are somewhat less than those of the conventional biosensor. The sensitivity and reproducibility values, calculated as above, can be separated in those calibrations performed successively and calibrations carried out after the regeneration of the biosensor. For 13 calibrations performed over three months with a single unregenerated biosensor, the sensitivity has a value of 6.12 with a variation of 2.18% (RSD). This variation corresponds to an initial rise of its value, and the reach of a steady value after *ca.* one week, inferring a progressive humectation of the bioactive material on the electrode surface. These figures show that the immobilization procedure is satisfactory, and the biocomposite remains active for periods longer than three months. When values from the first calibration after regeneration of a single electrode unit are compared, the mean sensitivity result is 5.63 and its reproducibility is 2.14% (RSD) (randomly dispersed values corresponding to five cycles). This reproducibility figure is directly related to the bulk distribution of bioactive material, which proves to be homogeneous. It is also shown how the enzymatic activity is maintained in the bulk of the biocomposite. Besides, the fabricated bulk-modified biosensor shows a high response rate, with a typical response time of 6 s, highly favored by the absence of diffusion barriers always present in conventional biosensors. Concerning selectivity, the interference levels remained high since the working potential continued to be relatively high, 900 mV.

The second biocomposite tested incorporated TTF as a redox mediator. This kind of electrode is designed particularly to overcome interference problems. The sequence of redox reactions involved is:

$$\text{D} - (+) - \text{glucose} + \text{GOD}_{ox} \longrightarrow \text{gluconolactone} + \text{GOD}_{red}$$

$$\text{GOD}_{red} + 2\text{TTF}^{+} \longrightarrow \text{GOD}_{ox} + 2\text{TTF}^{0} + 2\text{H}^{+}$$

$$2\text{TTF}^{0} \xrightarrow{E} 2\text{TTF}^{+} + 2\text{e}^{-}$$

Measurement is based on the oxidation of the TTF^0 mediator, which has previously been reduced by GOD_{red}, which is involved in the biocatalytic oxidation of glucose. The working potential of this system is controlled by the mediator and is a relatively low 150 mV.

The performance characteristics are also summarized in Table 1. The linear working range results are somewhat smaller than those of the previous biosensors mentioned, 0.1 to 2 mM of glucose. The response rate, with a typical response time of 11 s, is much higher than that of the enzymatic membrane biosensor. The main advantage of this third biosensor is the reduced interference level of foreign species, which is very low. The disadvantage of the design is that it has a short lifetime, 3 days, due to the loss of oxidated TTF, which is water-soluble. This drawback is compensated with the main advantage of these bulk-modified biosensors, their easy regeneration by simple polishing of the surface to expose a fresh surface interface of bioactive material. Other solutions to this problem would be to use charged membranes (i.e. the use of Nafion[6]) or covalent linking of the mediator to avoid its loss. With this biocomposite, the response characteristics are degraded from day to day due to the loss of mediator. Nevertheless, sensitivity values obtained in the first calibration after polishing of the same device are highly reproducible, with a mean value of 3.58 (log I(nA) versus log of glucose concentration (M)) and the estimated variability 1.4% (RSD), corresponding to five regeneration and calibration sequences.

4. Discussion

The two biocomposite designs presented here realize biosensors that integrate a voltammetric transducer and both enzymatic and redox systems in a single rigid unit. Moreover, the working potential is reduced, which lowers the response time and interference level for foreign species, thereby qualitatively increasing the stability of the signal. The resulting biosensors have ample working ranges (0.1–2 mM glucose) and fast response times (typical value: 10 s).

The main advantages of the bulk-modified biosensors are that they can be easily fabricated and exhibit long term preservation of the enzyme activity, longer than 3 months for the graphite-epoxy-Au-Pd-GOD biosensors. A fresh bioactive surface can be produced by a simple polishing procedure, as the bulk of the biocomposite acts as a reservoir of biocatalytic materials, mediators and catalysts. Furthermore, these biocomposites allow for easy machining of the biosensing material and simplified manufacturing by screen-printing technologies.

The feasibility of using other enzymatic systems, although not presented here, has also been verified. Preliminary results of an acetylcholinesterase inhibition biosensor for pesticide determination have been obtained.[7]

In summary, these bulk-modified biosensors are rigid, machinable and polishable single amperometric devices. They have low cost, allowing for disposable use and for mass production. The development time for new sensors is very short as there is no need to study a particular chemical immobilization process. These fabricated units are polishable, and the calibration parameters after polishing are reproducible. This is favored since the enzymes retain their bioactivity in the rigid graphite-epoxy matrix.

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