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Graphite−Poly(tetrafluoroethylene) Composite Enzyme Electrodes as Suitable Biosensors in Predominantly Nonaqueous Media

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The performance of a graphite−poly(tetrafluoroethylene) Teflon composite amperometric ferrocyanide-mediated peroxidase electrode in a predominantly nonaqueous medium such as reversed micelles is discussed and compared with the behavior in a medium formed by acetonitrile/water. The composite electrode was constructed by purely physical entrapment of both the enzyme and the mediator into the bulk of the graphite−Teflon matrix with no need of covalent attachments. This biosensor responded rapidly to the changes in the concentration of both hydrogen peroxide and 2-butanone peroxide in reversed micelles formed with ethyl acetate, 0.1 mol L^{-1} dioctyl sulfosuccinate as the surfactant, and a 4% phosphate buffer (pH 7.4) as the dispersed phase. The electrode showed a long-term operation due to the renewability of its surface. Moreover, reproducible responses were obtained with different electrodes fabricated from different composite matrixes. No significant loss of the enzyme activity was observed after four months of dry storage at 4 °C of the composite electrode. Limits of detection of 2.1×10^{-7} and 3.5×10^{-7} mol L^{-1} were obtained for H_2O_2 and 2-butanone peroxide, respectively. The possibility of using this biocomposite electrode in flowing systems, using the reversed micelles as the carrier, has been demonstrated. The kinetic of the enzymatic reaction was faster in a 90:10 acetonitrile/phosphate buffer medium than in reversed micelles, which can be attributed to the higher water content present in the former medium. A similar stability of the biosensor and a slightly better sensitivity for peroxides was observed in the acetonitrile/water mixture when compared with reversed micelles. Finally, the electrode also performed well in the flow injection mode.

Incorporation of enzymes to carbon composite matrixes can be considered as an efficient strategy for the design of electrochemical biosensors. Undoubtedly, carbon paste electrodes modified with enzymes, tissues, and cells constitute until now the most widely used group of composite biomaterials,[1−3] although it is claimed that, in general, they suffer from practical difficulties that limit commercial implementation.[4] Some approaches to improve important properties such as mechanical rigidity, renewability of the surface, fast response, mass production ability, and possibility of coimmobilizing other components have been developed by using rigid graphite−epoxy[5,6] or graphite−poly(tetrafluoroethylene) (Teflon)[7] biocomposite electrodes. Furthermore, unlike modified carbon paste electrodes, the incorporation of biological species into these rigid matrixes is a useful immobilization method to work in nonaqueous media. This compatibility with organic or predominantly organic solvents is specially interesting in order to use electrochemical biosensors as detectors in flowing systems. In this context, trapping of enzymes into Eastman AQ polymeric coatings has been shown to be suitable because of the high stability of these coatings in various organic solvents.[8]

In this paper, the fabrication of a bulk-modified graphite−Teflon amperometric peroxidase-mediated electrode is reported, as well as its behavior in a predominantly nonaqueous medium such as reversed micelles (also called a water-in-oil emulsion). These organized media, consisting of an organic solvent as the continuous phase, an aqueous solution as the dispersed phase, and a surfactant as emulsifying agent, have shown to be suitable working media for developing amperometric enzyme biosensors[9−11] and can be considered as universal solubilization media for both hydrophilic and hydrophobic analytes, thus allowing the enzymatic determination of compounds scarcely soluble in water. Furthermore, in reversed micelles, the amount of water needed for the enzyme operation is very easy to be controlled and optimized. For comparison purposes, the behavior of the enzyme composite electrode in a medium formed by acetonitrile/water at different percentage ratios is also discussed. Hydrogen peroxide and 2-butanone peroxide were chosen as analytical substrates.

EXPERIMENTAL SECTION

Apparatus, Electrodes, and Electrochemical Cell. All amperometric measurements were performed on a Metrohm 641

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VA potentiostat (Herisau, Switzerland) in connection with a Linseis L6512B X-Y recorder (Selb, Germany). Batch measurements were carried out by amperometry in stirred solution. Flow injection experiments were performed by using an arrangement that consisted of an Isco Model Wiz peristaltic pump (Lincoln, NB) and an Omnifit Model 1106 injection valve with variable-injection volumes (Cambridge, England). Other apparatus used were a P-Selecta Ultrasons ultrasonic bath (Barcelona, Spain) and a Carver pellet press (supplied by Perkin Elmer, Norwalk, CT).

The electrochemical cell was a BAS Model VC-2 cell (W. Lafayette, IN) with a BAS RE-1 Ag/AgCl reference electrode and a platinum wire counter electrode. A large-volume (50-mL) glass wall-jet[12] cell was used for flow injection measurements, the electrode potentials being reported vs the Ag/AgCl reference electrode (Model RE-1, BAS).

Graphite−Teflon Composite Enzyme Electrode. Graphite−Teflon−peroxidase−ferrocyanide pellets were prepared in the following manner. 0.170-g sample of graphite (ultra F purity, Carbone of America, Bay City, MI) and 0.014 g of horseradish peroxidase (EC 1.11.1.7, type II, activity 190 units/mg of solid, Sigma, St. Louis, MO) were accurately weighed and thoroughly mixed with magnetic stirring for 2 h in a 0.4-mL suspension of phosphate buffer (pH 7.4) at 4 °C. Then, 0.016 g of potasium ferrocyanide (Sigma) was added, and the resulting mixture was stirred for ∼10 min. Next, water was evaporated by using an airstream until the paste was dried (∼3 h). Then, the paste was blended by hand and an airstream was passed through again for 5 min. The resulting paste, which has a powder consistency, was mixed by hand with 0.7 g of Teflon powder (Aldrich, Milwaukee, WI) and 0.1 g of graphite (in order to obtain a Teflon percentage of 70%). The mixture was pressed into pellets with a 1.3-cm-diameter. Carver pellet press at 10 000 kg cm^{-2} for 10 min. The pellets were ∼0.4 cm thick. Several 3.0-mm-diameter disk portions of the pellet were bored, and each disk was press-fitted into a Teflon tube. Electrical contact was made through a stainless steel screw. Before use, the composite enzyme electrode was immersed in ethyl acetate for at least 8 h. We have observed that this immersion period allows the attainment of much higher amperometric signals, as was observed for enzyme electrodes prepared by direct adsorption of the enzyme onto the surface of a graphite electrode.[9] After use, the electrode was stored at 4 °C immersed in ethyl acetate.

Reagents and Solutions. Other reagents, apart from those mentioned above, were hydrogen peroxide (Sigma), 2-butanone peroxide (Fluka, Buchs, Switzerland), ethyl acetate and acetonitrile (Aldrich), and dioctyl sulfosuccinate (AOT, Sigma). All chemicals were of analytical-reagent grade, and the water used was obtained from a Millipore Milli-Q purification system.

Stock solutions of the peroxides (0.10 mol L^{-1}) were water-in-oil microemulsions prepared by dissolving the appropriate amount in the reversed micelles solution formed with ethyl acetate as organic solvent, 0.1 mol L^{-1} AOT as emulsifying agent, and 0.05 mol L^{-1} phosphate buffer (pH 7.4) as aqueous phase (4%). More dilute standards were prepared by suitable dilution with the same components of the emulsions. Stock solutions of hydrogen peroxide and 2-butanone peroxide in acetonitrile (0.10 mol L^{-1}) were also prepared.

Procedures. Activation of the composite enzyme electrode was accomplished daily by immersion in a stirred 0.5 mM hydrogen peroxide reversed micellar solution and holding at 0.1 V for 6 min. Then, the activated electrode was immersed in the electrochemical cell, and amperometric measurements in stirred solutions were performed at room temperature by applying the desired potential and allowing the transient currents to decay. The electrode was immersed in ethyl acetate between the different experiments. After the composite electrode was used repeatedly, the response obtained decreased appreciably with respect to the original response, so regeneration of the electrode surface was performed by polishing for 5 s on a 150-grit SiC paper.

Amperometric flow injection measurements were carried out by using the above mentioned reversed micelle system as the carrier.

Determination of Hydrogen Peroxide in Solutions for Disinfection, Neutralization, and Storing of Contact Lenses. Samples used (Aosept), obtained from Civa Vision SA, contained 3% hydrogen peroxide. The preparation of the sample consisted of a simple dilution by adding 22.5 μL of the contact lens cleaning solution to 10 mL of reversed micelles. Aliquots of 25.0 μL of this solution were transferred into the electrochemical cell containing 5.0 mL of reversed micelles, and the determination of hydrogen peroxide was accomplished by amperometry at +0.10 V with constant stirring. Both the standard additions method and the standard calibration method were used.

RESULTS AND DISCUSSION

The use of a horseradish peroxidase (HRP) amperometric biosensor in different reversed micellar systems for the determination of 2-butanone peroxide was previously demonstrated by our group.[10] In that work, the immobilization of the enzyme was carried out by direct adsorption on the surface of a graphite disk electrode. Furthermore, graphite−Teflon biocomposites, where the enzyme was covalently attached to graphite, were also used to determine hydrogen peroxide and 2-butanone peroxide.[7]

In this context, it should be remarked that in this work we have fabricated the graphite−Teflon composite enzyme electrode simply by inclusion of the enzyme into the bulk of the graphite−Teflon pellet which constitutes the electrode. This purely physical entrapment of the biological species into the composite matrix involves some important practical advantages with respect to the former biosensor designs. Besides all the advantageous properties of the use of rigid biocomposite electrodes mentioned above, no covalent immobilization of the enzyme is needed, and consequently, the electrode fabrication procedure was easier, faster, and less costly. Some loss of sensitivity due to the covalent linkages is in this way avoided. Futhermore, coimmobilization of mediators can be carried out easily in the same procedure.

The enzymatic reaction employed involved catalytic reduction of peroxides in the presence of ferrocyanide used as a mediator. Thus, the amperometric signal used for monitoring this reaction corresponded to the electrochemical reduction of ferricyanide.

Performance of the Biocomposite Electrode in Reversed Micelles. Both the graphite-to-Teflon ratio used to fabricate the composite electrode and the composition of the reversed micellar system employed were the same as those optimized before.[7,9] Thus, pellets containing 70% (w/w) Teflon and reversed micelles formed with 5 mL of ethyl acetate, 0.1 M AOT as the surfactant, and a 4% phospate buffer (pH 7.4) as the dispersed phase were used in all experiments. Furthermore, the amount of mediator

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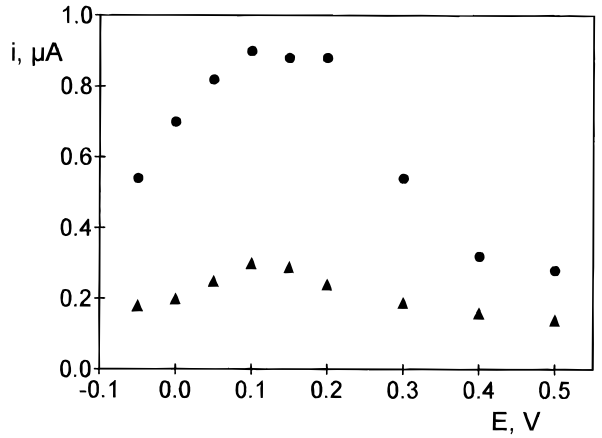

Figure 1. Influence of the applied electrode potential on the steady-state current for 2.0×10^{-5} mol L^{-1} (●) hydrogen peroxide and (▲) 2 -butanone peroxide in reversed micelles.

ferrocyanide (1.6%) employed in the preparation of the composite electrodes gave assure that it was in great excess with respect to the substrate concentration reaching the electrode.

The composite peroxidase electrode exhibited a rapid response to the changes in the concentration of both hydrogen peroxide and 2-butanone peroxide in the reversed micelles, when current−time recordings were obtained at +0.10 V for successive additions of 10 μL of 5.0×10^{-3} mol L^{-1} peroxides stock reversed micelles. Nevertheless, the time to reach the steady-state current for 2-butanone peroxide was longer, as well as the steady-state signal much smaller, than for hydrogen peroxide. This can be explained because the enzymatic reaction takes place in the aqueous microdomains at the electrode surface, a partitioning equilibrium of the substrates between the aqueous and organic microdomains being established.[9,13] As 2-butanone peroxide is scarcely soluble in water, it will take longer to attain this equilibrium, and consequently, the kinetics of the enzymatic reaction will be slower and the steady-state current smaller. Nevertheless, in both cases, the electroanalytical response is fast enough to be useful, which is favored by the absence of a membrane barrier on the electrode surface.

On the other hand, Gorton et al. have demonstrated that the enzymatic reaction involving peroxidase can also be performed without mediator, i.e., by direct enzyme−electrode charge transfer.[14] However, in this case, lower amperometric steady-state signals were obtained because the HRP_{ox}/HRP_{red} system is slower than the mediator systems usually used. A similar behavior was observed for graphite−Teflon peroxidase electrodes in reversed micelles when ferrocyanide was employed as the mediator incorporated to the electrode matrix.

The dependence of the composite enzyme electrode response on the applied potential was evaluated over the potential range from −0.05 to +0.50 V for a hydrogen peroxide or 2-butanone peroxide concentration of 2.0×10^{-5} mol L^{-1} (Figure 1). As expected, a similar shape was observed for both substrates with a maximum response between approximately 0.10 and 0.20 V. A potential of 0.10 V was selected for subsequent studies.

Regarding the stability of the composite bioelectrode, four aspects have been considered: (a) repeatability of the amperometric signal when the electrode surface is regenerated by polishing; (b) repeatability for successive measurements with no surface regeneration; (c) reproducibility for different electrodes fabricated from the same pellet and from different pellets (see preparation of composite electrodes); and (d) effect of the storage time of the pellet from which electrodes are constructed.

One of the most interesting properties of the use of rigid biocomposite electrodes is the possibility of an easy renewability of the electrode surface by simple polishing. Thus, the reproducibility of the signal obtained for a H_2O_2 constant concentration of 50 μM in the reversed micellar system was examined after regeneration of the electrode surface by polishing for ~5 s on a 150-grit SiC paper. In this case, a sharp decrease of the steady-state current was observed from the tenth polishing, the RSD value for the first 10 measurements being 6.6%. Repetitive experiments allowed us to deduce that the number of polishings that could be performed without observing a significant decrease in the amperometric response depended on the thickness of the pellet. Thus, when regeneration of the electrode surface was accomplished so many times that the thickness of the pellet became too low, the electrode was useless and should be changed.

These results indicated that the enzyme composite electrode yields reproducible measurements with the regeneration procedure of the electrode surface and that the enzyme was uniformly distributed into the bulk of the electrode matrix. This represents an important advantage with respect to other enzyme immobilization schemes where a new electrode should be fabricated whenever the enzyme activity is lost.

On the other hand, a set of 10 successive measurements for 50 μM hydrogen peroxide in the reversed micelle with no electrode surface regeneration yielded a RSD value of 6.2% for the steady-state current. Comparing this repeatability with that achieved when the electrode surface was renewed by polishing, it can be deduced that no noticeable differences were observed in both cases. Nevertheless, it has been verified that continuous work with a nonrenewed surface gave rise to a decrease of the amperometric signal from a certain time, probably as a consequence of the surface loss by solubilization of the enzyme or the mediator. In these cases, the initial amperometric response can be recovered by polishing of the electrode surface as commented above.

In conclusion, it can be said that it is not necessary to polish the electrode surface after performing each experiment, which implies that this type of composite biosensors has, in combination with the renewability of the surface, a long-term operation. Under the experimental conditions used in this work, one composite peroxidase electrode could be regularly used during approximately one month. In this period of time, the electrode must be renewed by polishing five or six times.

On the other hand, the reproducibility of the amperometric signal obtained with different composite enzyme electrodes fabricated from the same pellet, and with different electrodes constructed from different pellets, has been also checked. Table 1 summarizes the results obtained for five electrodes, three of them fabricated from the same main pellet, and the other two from different pellets. The steady-state current RSD value for all the five electrodes was 6.5%, whereas for the three electrodes constructed from the same pellet, it was 5.5%. These results allow us to conclude that the fabrication procedure of the composite electrodes is reliable and that reproducible electroanalytical signals

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Table 1. Reproducibility of Different Graphite−Teflon−HRP−Ferrocyanide Electrodes for Measurements of 50 μM H_2O_2 in Reversed Micellar Systems Formed from Ethyl Acetate, 0.1 mol L^{-1} AOT, and 4% Phosphate buffer

pellet	electrode	i, μA	RSD, %	RSD, %
1	1	2.67	5.5	6.5
	2	2.41		
	3	2.43		
2	4	2.37		
3	5	2.49		

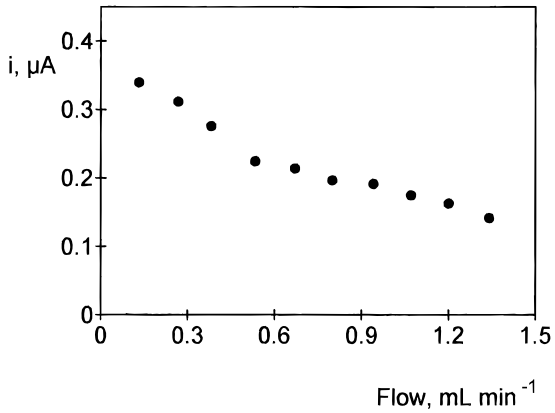

Figure 2. Effect of the reversed micelle flow rate on the peak height for 6.0×10^{-5} mol L^{-1} H_2O_2; applied potential, +0.1 V.

can be achieved with different electrodes constructed in the same manner.

Finally, the influence of the storage period of the main pellet when it was dry-stored at 4 °C in a refrigerator was also evaluated. After four months of storage, no significant loss of the enzyme activity, immobilized into the electrode matrix, was observed, and reproducible amperometric signals were obtained with electrodes fabricated from that stored pellet when compared with those obtained with other electrodes fabricated from the same pellet four months before.

Lineweaver−Burk plots, in the concentration range 1.0×10^{-5}−20.0×10^{-5} mol L^{-1} for both hydrogen and 2-butanone peroxide, were used to estimate the apparent Michaelis−Menten constant ($K_{m,app}$), this being 44 and 104 μM for hydrogen peroxide and 2-butanone peroxide, respectively. These values permit to predict a higher sensitivity for the determination of hydrogen peroxide, although a somewhat more limited linear range at the upper part of the corresponding calibration graph can be expected.

The plots of the steady-state current vs peroxide concentration were linear over the ranges 5.0×10^{-7}−6.0×10^{-5} ($r = 0.998$) and 1.0×10^{-6}−1.0×10^{-4} mol L^{-1} ($r = 0.999$) for hydrogen peroxide and 2-butanone peroxide, respectively. The slopes and intercepts of these calibration plots were $(3.9 \pm 0.1) \times 10^4$ μA mol^{-1} L and 0.01 ± 0.04 μA and $(1.5 \pm 0.1) \times 10^4$ μA mol^{-1} L^{-1} and -0.01 ± 0.01 μA, respectively. Relative standard deviations, calculated from 10 different solutions of each substrate at a concentration level of 1.0×10^{-6} mol L^{-1}, were 7.1 and 7.5% for hydrogen peroxide and 2-butanone peroxide, respectively. The limits of determination and detection were estimated according to the 10× standard deviation and 3 s_b/m criteria, where m is the slope of the calibration graph and s_b is the standard deviation ($n = 10$) of the amperometric signal from 1.0×10^{-6} mol L^{-1} peroxide. These values were 7.1×10^{-7} and 2.1×10^{-7} mol L^{-1}, respectively, for H_2O_2, and 1.1×10^{-6} and 3.5×10^{-7} mol L^{-1} for 2-butanone peroxide. These analytical characteristics are comparable and sometimes better, mainly with regard to the linear ranges of the calibrations graphs and the limit of detection for 2-butanone peroxide, than those reported in the literature for other composite peroxidase electrodes working in aqueous solutions.[14] Furthermore, when comparison is made with organic-phase peroxidase electrodes,[14] the limits of detection obtained with the graphite−Teflon composite enzyme electrode for both hydrogen peroxide and 2-butanone peroxide are considerably better than those reported previously.

As a simple application of this method, hydrogen peroxide was determined in cleaning solutions of contact lenses. As explained in the Experimental Section, only dilution of this solution in the reversed micelles was needed to perform the amperometric measurements.

Slope values of the calibrations obtained by applying the standard additions method (4.1 ± 0.2 μA mol^{-1} L) and the standard calibration method (3.9 ± 0.1 μA mol^{-1} L) showed that no apparent matrix effect existed. The results obtained from five samples yielded a hydrogen peroxide content in the cleaning solution of contact lenses of 3.3 ± 0.1% when the standard additions method was used and 3.3 ± 0.2% when H_2O_2 was determined by interpolation in the calibration graph. The confidence intervals were always calculated for a significance level of 0.05. Both values agree fairly well with the peroxide percentage labeled. The very simple sample preparation and the absence of matrix effect allow the easy automation of the method which, therefore, can be used for quality control of this type of products.

Flow Injection with Amperometric Detection in Reversed Micelles. The use of enzyme-based flow injection analysis has increased considerably in the last years. Furthermore, we have demonstrated recently that reversed micelles can be employed as the carrier in FIA and, consequently, can exploit all the advantages of these media for on-line monitoring applications.[11] Thus, the rapid response exhibited by the composite peroxidase electrode in the batch mode suggested the possibility of using this biocomposite electrode in flowing systems in connection with amperometric biosensing detection. The composition of the reversed micelles used as the carrier solution is the same as that for batch experiments, i.e., ethyl acetate as organic solvent, 0.1 mol L^{-1} AOT as emulsifying agent, and 4% 0.05 mol L^{-1} phosphate buffer of pH 7.4 as dispersed aqueous phase. A potential of +0.10 V was selected to be applied to the biosensor, as well as an injection volume of 100 μL. At this potential, the graphite−Teflon composite peroxidase electrode responds rapidly to injections of hydrogen peroxide microemulsions. The short response time and rapid return to the baseline indicated facile transport of the substrate in the reversed micellar working medium.

As we had seen for enzyme-based FIA of phenols in reversed micelles,[11] the flow injection peak height decreased as the carrier flow rate increased (Figure 2). This is because flow amperometric measurements with enzyme biosensors depend on the rate of the enzymatic reaction. As the measured current does not correspond with the steady state, an increase in the flow rate gives rise to a lower extent in the enzymatic reaction, and therefore, the monitorized substance, which is usually a product of this reaction, produces a smaller amperometric signal. As expected, the peak

width decreased with increasing the flow rate. Taking into account these results, and as a compromise between sensitivity and the smallest peak width, all subsequent experiments were made with the reversed micelle flowing at 0.94 mL min $^{-1}$.

Under the above conditions, the reproducibility of the amperometric response was evaluated from a series of 30 repetitive injections of 100 μL of 1.0×10^{-4} mol L^{-1} hydrogen peroxide. Despite the hydrodynamic conditions, such a response remained practically constant with a relative standard deviation for i_p of 1.9%, indicating a good enzyme immobilization into the electrode matrix. Under these experimental conditions, ~30 samples h^{-1} can be analyzed.

A linear calibration graph for hydrogen peroxide was obtained over the 5.0×10^{-7}–1.0×10^{-4} mol L $^{-1}$ range ($r = 0.999$), the slope and intercept being $(3.4 \pm 0.1) \times 10^3$ μA L mol^{-1} and 0.006 $\pm$ 0.003 μA, respectively. The limits of determination and detection, calculated according to the same criteria mentioned above, were 1.0×10^{-6} and 3.1×10^{-7} mol L^{-1}, respectively. The relative standard deviation, calculated from the signals of 10 differents injections of 2.0 μM H_2O_2, was 4.5%.

Performance of the Biocomposite Electrode in Acetonitrile/Water Media. As mentioned in the introduction, a comparison of the performance of the graphite–Teflon composite peroxidase electrode in predominantly nonaqueous media such as reversed micelles and acetonitrile/water mixtures could be useful in order to ascertain the suitability of these biocomposites as biosensors in these media. An acetonitrile/water medium was chosen because of being widely used as mobile phase in liquid chromatography.

The amperometric response of the biocomposite electrode to successive additions of hydrogen peroxide and 2-butanone peroxide stock solutions in a 90:10 acetonitrile/phosphate buffer (pH 7.4) medium was compared with responses obtained in reversed micelles. The steady-state current was reached earlier in acetonitrile/water for both peroxides; that is, the kinetic of the enzymatic reaction is faster in this medium. This can be attributed to the higher water content present in this case with respect to reversed micelles (4%). Moreover, a single-phase medium is now used instead of the organized water-in-oil emulsions. Taking into account that, as mentioned above, in reversed micelles the enzymatic reaction takes place in the aqueous microdomains at the electrode surface, and that a partitioning equilibrium of the substrates between the aqueous and oil microdomains is established,[9,10] the differences observed in the reaction kinetic between the both media seem reasonable.

The influence of the phosphate buffer percentage on the steady-state current was evaluated over the 1–20% range (Figure 3), using 50 and 100 μM H_2O_2 concentrations. As can be seen, the current increased rapidly with the water percentage for the lower water contents, following which a practically constant response was observed. A 10% phosphate buffer was then selected for further experiments.

Furthemore, the dependence of the composite peroxidase electrode response on the applied potential was also checked between +0.20 and −0.20 V. The steady-state current exhibited a good plateau from +0.10 up to −0.10 V, and consequently, a potential of 0.00 V was chosen for subsequent studies.

The stability of the composite bioelectrode in this medium was examined using a hydrogen peroxide concentration of 50 μM. A

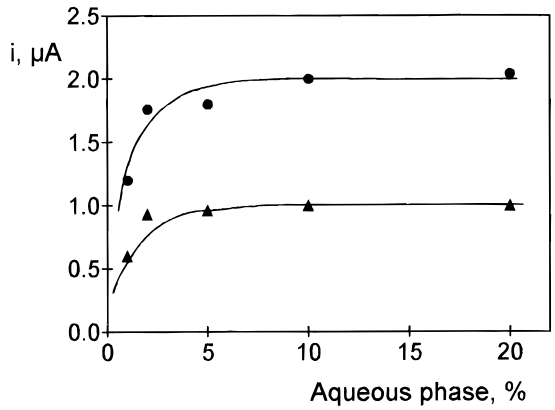

Figure 3. Effect of the phosphate buffer percentage on the steady-state current obtained at a graphite–Teflon composite peroxidase–ferrocyanide electrode for (●) 100 and (▲) 50 μM H_2O_2 in acetonitrile/phosphate buffer media; E_{app} = 0.0V.

RSD value for the steady-state current of 6.7% was obtained (n = 10) with no regeneration of the electrode surface by polishing, whereas RSD was 6.3% (n = 10) when the elecrode surface was renewed by polishing for 5 s on a 150-grit SiC paper before each current–time recording. These values show no differences in the stability of the composite biosensor in the 90:10 acetonitrile/water medium with respect to that occurred in reversed micelles, and, hence, the same conclusions mentioned in that case can be deduced now.

An estimation of the apparent Michaelis–Menten constant was made from Lineweaver–Burk plots. Values obtained were 187 μM for hydrogen peroxide and 608 μM for 2-butanone peroxide. The ranges of linearity achieved from the steady-state current vs concentration plots were 5.0×10^{-7}–1.0×10^{-4} mol L^{-1} (r = 0.999) for H_2O_2 and 1.0×10^{-6}–2.0×10^{-4} mol L^{-1} (r = 0.999) for 2-butanone peroxide. The slopes and intercepts of these linear plots were $(4.4 \pm 0.1) \times 10^4$ μA mol^{-1}L and -0.020 ± 0.001 μA, respectively for H_2O_2, and $(1.90 \pm 0.02) \times 10^4$ μA mol^{-1} L and -0.009 ± 0.003 μA for 2-butanone peroxide. As can be deduced, when comparing with the corresponding calibration plots obtained in reversed micelles, a slightly larger range of linearity and better sensitivity was obtained for both peroxides in the 90:10 acetonitrile/phosphate buffer medium. These results agree with the differences observed in the rate of the enzyme reaction in both media used. The limits of determination and detection, calculated from the standard deviation (n = 10) of the amperometric signal of 1.0×10^{-6} mol L^{-1} peroxide solutions, were 6.6×10^{-7} and 1.9×10^{-7} mol L^{-1}, respectively, for H_2O_2 and 8.0×10^{-7} and 2.5×10^{-7} mol L^{-1} for 2-butanone peroxide.

Finally, the behavior of the graphite–Teflon peroxidase electrode was tested in the flow injection mode using a 90:10 acetonitrile/phosphate buffer mixture as the carrier solution. A sample loop of 150 μL, a flow rate of 0.94 mL min^{-1}, and an applied potential of 0.00 V were used for these experiments. Under these conditions, 20 repetitive injections of 50 μM H_2O_2 in the acetonitrile/phosphate medium yielded a RSD for i_p of 4.5%, indicating again a good stability of the biosensor in the predominantly nonaqueous flowing stream. A linear calibration graph was obtained between 5.0 and 50 μM (r = 0.999), with a slope of 0.200 $\pm$ 0.004 nA μM^{-1} and an intercept of 3.2 $\pm$ 0.1 nA. The limit of detection obtained was 0.9 μM.

CONCLUSIONS

All the above results show that graphite−Teflon composite enzyme electrodes constitute a suitable approach to fabricate robust biosensors able to work in predominantly nonaqueous media in both batch and flowing modes. These devices, besides their compatibility with these media, combine other advantageous practical properties such as the possibility of a bulk immobilization of the enzyme(s), and the mediator if necessary, with no need of covalent attachment of the enzyme to graphite, and an easy and reproducible renewability of the electrode surface.

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