Pergamon

Talanta, Vol 41, No 3, pp 455–459, 1994

0039-9140/94 $6 00 + 0 00

DEVELOPMENT OF AN AMPEROMETRIC BIOSENSOR FOR THE DETERMINATION OF PHENOLIC COMPOUNDS IN REVERSED MICELLES

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(*Received* 21 *April* 1993 *Revised* 7 *June* 1993 *Accepted* 12 *July* 1993)

Summary—The possibilities of amperometric enzyme electrodes in reversed micellar systems for the determination of phenol, 4-chloro-3-methylphenol and 2,4-dimethylphenol are illustrated The used enzymatic reaction consisted of the oxidation of the phenolic compounds by oxygen, catalysed by tyrosinase The reduction of the liberated quinones was amperometrically detected The concentration of the components of the reversed micelles, as well as the potential applied to the tyrosinase electrode have been optimized The stability of the enzyme electrode with time was also evaluated The effect of the analyte solubility in water upon the analytical performance of the electrode was explored Advantages of amperometric biosensors in reversed micelles are shown with respect to aqueous media and organic phase enzyme electrodes

The development of amperometric biosensors is an area of growing interest in many branches of science due to their potential for detecting chemicals at analytically useful levels. Recent advances in this area can be found in literature.[1–4] Regarding amperometric enzyme electrodes, although most of their applications have been carried out in aqueous medium, the use of organic-phase enzyme electrodes has been recently reported [5] Such electrodes exhibit some advantages, such as an extended scope of analytes (*i.e* hydrophobic compounds) a decrease in the interference of hydrophilic ionic species or side reactions and an easier fabrication.

On the other hand, catalysis by enzymes entrapped in hydrated reversed micelles is becoming a new trend in molecular biology because of its utility in the understanding of the enzyme functioning in natural lipid systems [6] Reversed micelles are three-component systems composed of an organic solvent (the continuous phase), water (the disperse phase) and a surfactant. Recently, it has also been demonstrated that oil-in-water microemulsions can be used as a suitable medium for performing enzymatic assays.[7]

The aim of this work is to investigate the possibilities of amperometric biosensors in reversed micelles or water-in-oil emulsions. These emulsions can provide universal solubilization media for both hydrophilic and hydrophobic analytes Moreover, the amount of water necessary for the hydration of the enzyme is very easy to control in such emulsions, and as in organic solvents,[8] a relative facility for the enzyme immobilization on the electrode surface can be expected if working in reversed micellar systems where enzymes are almost insoluble.

In this work, the possibilities of amperometric enzyme electrodes in reversed micelles for the determination of phenolic compounds included in the US Environmental Protection Agency List of Priority Pollutants[9] are illustrated.

EXPERIMENTAL

Apparatus, electrodes and electrochemical cell

The measurements were performed on a EG&G PAR mod. 362 Scanning Potentiostat, in connection with a Linseis LY 16100 A recorder. The electrochemical cell was a BAS mod. VC-2 cell with a BAS RE-1 Ag/AgCl

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reference electrode and a platinum wire counter electrode. Measurements were carried out by amperometry in stirred solutions

The enzyme electrode was prepared daily as follows: a 3-mm diameter graphite (U50-2, Ultra Carbon Co.) disk electrode was polished for 5 sec on a 150 grit SiC paper (such a pretreatment is necessary to generate a rough electrode surface which allows the strong adhesion of the enzyme); a 10 μL aliquot of the tyrosinase stock solution was placed on the electrode covering the whole electrode area, and allowed to dry using a heat gun during 15 min; the electrode was immersed in ethyl acetate for 12 hr at 4°C (we have observed that this waiting period allows the attainment of much higher amperometric signals) Prior to the preparation of a new electrode, the old enzyme electrode was cleaned with water.

Reagents and solutions

Tyrosinase (EC 1.14.18.1, activity 2100 units/mg solid, Sigma); dioctyl sulfosuccinate (AOT, Sigma); phenol, 4-chloro-3-methylphenol, 2,4-dimethylphenol (Aldrich); ethyl acetate (Aldrich).

Tyrosinase stock solutions were prepared by dissolving 1 mg of enzyme in 100 μl of 0.05*M* phosphate buffer (pH 7.4). A 0.10*M* AOT stock solution ethyl acetate was also prepared. Stock solutions of the phenolic compounds (0.10*M*) were water-in-oil microemulsions prepared daily, formed with ethyl acetate as organic solvent, 0.10*M* AOT as emulsifying agent and 0.05*M* phosphate buffer (pH 7.4) as aqueous phase. More dilute standards were prepared by suitable dilution with the same components of the emulsion.

Procedure

In order to activate the enzyme electrode, the tyrosinase electrode was immersed daily in a 0.4 m*M* phenol reversed micellar solution and kept at −0.2 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. Between the different experiments the electrode was immersed in ethyl acetate.

Calibration plots for phenolic compounds were obtained by adding fixed volumes of the phenolic compound stock microemulsions every 90 sec. Amperometric measurements were carried out at −0.2 V.

RESULTS AND DISCUSSION

The enzymatic reaction employed involves the oxidation of the phenolic compounds by oxygen, catalysed by tyrosinase. The reduction of the liberated quinones products was monitored amperometrically.

Figure 1 shows current–time recordings obtained at the enzyme electrode (a) and the plain graphite electrode (b) at −0.20 V immersed in reversed micelles formed with 5 ml of ethyl acetate, 0.1*M* AOT and 4% of water, for successive additions of 20 μl of a 5 m*M* phenol stock microemulsion. As it can be observed the tyrosinase electrode exhibits a rapid response to the changes in the substrate concentration, the steady-state current being reached in 20 sec, no signal was observed after phenol additions when the graphite electrode was used. The fast response observed is favoured by the absence of a membrane barrier on the electrode surface.

Optimization of experimental conditions

Firstly, the concentration of the components of the reversed micelles was optimized. Thus, Fig. 2 shows the effect of AOT concentration and water percentage of the amperometric response for a phenol concentration of 1.6 m*M*. The current vs % H_2O plot exhibits a maximum for percentages between 4.6 and 5.0%, indicating that, in this range, the enzyme hydration on the electrode surface is optimum for its enzymatic activity. For water percentages higher than 5% a decrease in the current, as well as a considerable increase in the noise of the amperograms registered were observed. A slight turbidity also appeared at these higher water percentages which can be due to changes in the

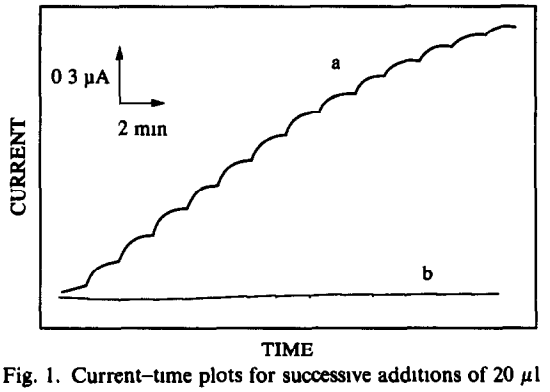

Fig. 1. Current–time plots for successive additions of 20 μl 5 m*M* phenol at (a) tyrosinase, (b) graphite electrodes in reversed micelles, 5 ml ethyl acetate, 0 10*M* AOT, 4% of water, applied potential, −0.2 V

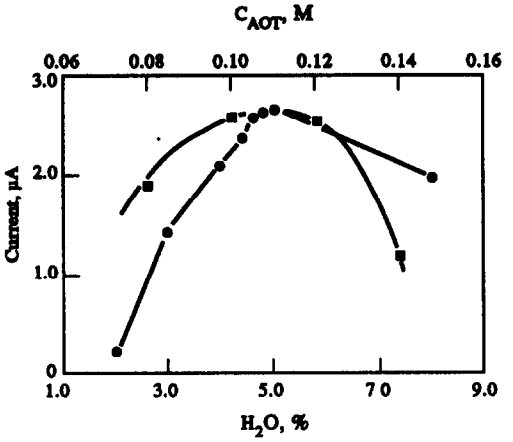

Fig 2 Influence of (■) AOT concentration and (●) water percentage on the steady-state current for 1 6 m*M* phenol in reversed micellar systems Applied potential, −0 2 V

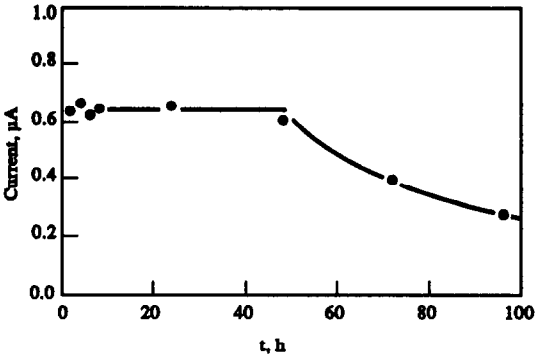

Fig 4 Steady-state current dependence on time for a tyrosinase electrode in reversed micelles, 5 ml ethyl acetate, 0 10*M* AOT, 4% of water, applied potential, −0 2 V

emulsion structure. From the current vs surfactant concentration plot one can deduce that the highest signals were obtained for AOT concentrations between 0.10 and 0.12*M*.

These results suggest that the enzyme reaction depends strongly on the reversed micelles composition. This behaviour is similar to that observed for enzyme reaction rates in water-in-oil microemulsions; they are found to vary with the water content[6] (often an optimum is found where the inner diameter of the micelle equals the diameter of the protein). The ratio $[H_2O]$/[surfactant] can be correlated with micellar dimensions; the higher this ratio the larger is the radius of the micellar particle.[6] As deduced from Fig. 2, the amperometric response showed a maximum for a $[H_2O]$/[AOT] ratio of 25 which can be related to the size of the hemi-micelle formed at the electrode surface.

The dependence of the tyrosinase electrode response on the applied potential was evaluated over the 0.1 to −0 35 V range (Fig. 3), using a 1.6 m*M* phenol concentration in a water-in-oil emulsion formed with 0 10*M* AOT and 4.6% of water. The current increased rapidly between +0.1 and −0.25 V, following which a gradual decrease in the response was observed. Moreover, at potentials more negative than −0.25 V, the appearance of a white precipitate adhered to the electrode surface was detected, which may be due to polymerization of the *o*-quinone produced in the enzymatic reaction at these potentials. Consequently, a potential of −0.20 V was chosen for subsequent studies.

The stability of the enzyme electrode with time was examined using a phenol concentration of 0.08 m*M*. Although a water percentage of 4.6% should provide the highest currents, the tyrosinase electrode was stable for approximately 1 hr. However, its stability is remarkably improved by working with a lower water percentage. Thus, Fig. 4 shows the current dependence on time for an electrode when a 4 0% of water was used in the reversed micelle system. The behaviour observed is that expected

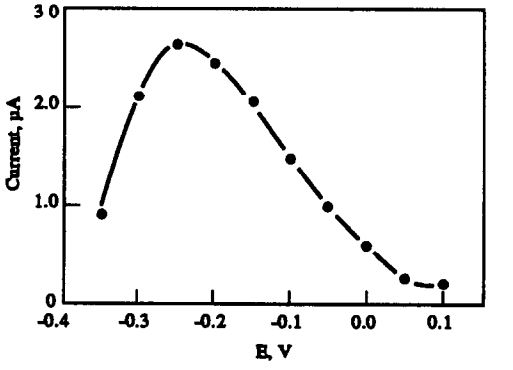

Fig. 3 Influence of the applied potential on the steady-state current for 1 6 m*M* phenol in reversed micelles, 5 ml ethyl acetate, 0 10*M* AOT, 4 6% of water

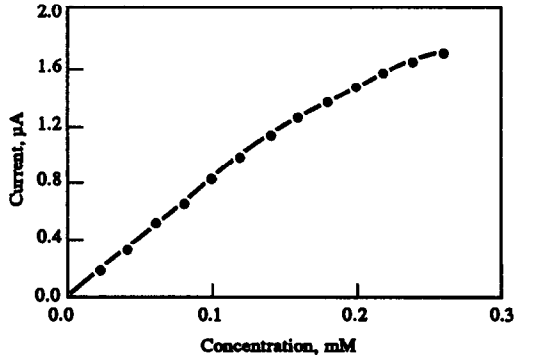

Fig 5. Calibration plot for phenol at a tyrosinase electrode in reversed micelles, 5 ml ethyl acetate, 0 10*M* AOT, 4% of water, applied potential, −0 2 V

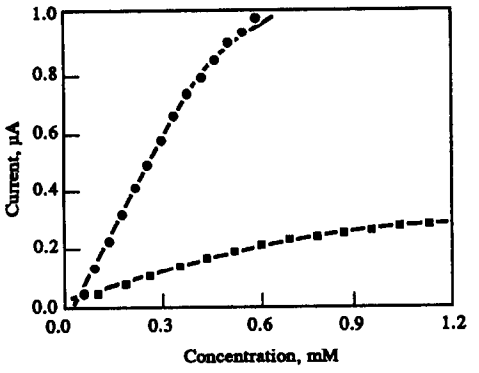

Fig 6 Calibration plots for (●) 4-chloro-3-methylphenol and (■) 2,4-dimethylphenol at a tyrosinase electrode in reversed micelles, 5 ml ethyl acetate, 0 10*M* AOT, 4% of water, applied potential, −0 2 V

for this kind of electrodes [10] Measurements made on the first day were performed at intervals of 1 hr, whereas later on, they were made daily Good stability was found over the first 48 hr (the relative standard deviation over this period being 4 7%) A similar stability was found when a water percentage of 3.0% was used, but, as stated in Fig. 2, sensitivity was lower than that obtained with 4% of water

On the other hand, the measured currents for two phenol concentration levels, 1.6 and 0.08 m*M*, remained practically constant when measurements were carried out with 8 different tyrosinase electrodes The relative standard deviations for these series were 7.1% and 7 2%, respectively

Taking into account the above data, the optimal experimental conditions used for analytical purposes with the enzyme electrode were: water percentage, 4%, AOT concentration, 0.10*M*, applied potential, −0 2 V

Calibration plots

Figure 5 illustrates the dependence of the steady-state current on the phenol concentration. The linear range obtained was 0 02–0.14 m*M* ($r = 0\ 999$), the slope and intercept being 7 92 μA/m*M* and 0.012 μA, respectively. A levelling is observed for phenol concentrations higher than 0.14 m*M*, as expected for enzymatic reactions. The limits of determination and detection were calculated according to the 10 × standard deviation and $3s_b/m$[12] criteria, where m is the slope of the calibration graph and s_b is the standard deviation ($n = 10$) of the signal from 0.02 m*M* phenol. These values were 0.018 m*M* and 6 μ*M*, respectively.

In order to verify the suitability of reversed micelle systems as working media for both hydrophilic and hydrophobic substrates, the behaviour of two phenolic compounds scarcely soluble in water, 2,4-dimethylphenol and 4-chloro-3-methylphenol (or *p*-chloro-*m*-cresol), was tested in the same experimental conditions mentioned above for phenol. Both phenolic compounds are included in the list of priority pollutants of the US Environmental Protection Agency (EPA).

Figure 6 shows the steady-state current dependence on the concentration for both phenolic compounds. Linearity was observed within the ranges 0.04–0.36 m*M* ($r = 0.999$) and 0.08–0.64 m*M* ($r = 0.999$) for 4-chloro-3-methylphenol and 2,4-dimethylphenol, respectively. The slopes and intercepts of these linear ranges were 2.05 μA/m*M* and −0.02 μA, and 0 23 μA/m*M* and 0 048 μA, respectively. The determination and detection limits, calculated from the standard deviation ($n = 10$) of the signal from 0.04 m*M* 4-chloro-3-methylphenol and from 0.08 m*M* 2,4-dimethylphenol, were 0.04 m*M* and 0.013 m*M* for 4-chloro-3-methylphenol, and 0 096 m*M* and 0.029 m*M* for 2,4-dimethylphenol. As it can be observed, sensitivity followed the trend: phenol > 4-chloro-3-methylphenol > 2 4-dimethylphenol. This trend agrees with the water solubility order for these compounds. This can be due to the enzymatic reaction taking place in the aqueous microdomains at the electrode surface, a partitioning equilibrium of the substrates between the aqueous and oil microdomains being established.[13] On the contrary, linearity is lost at higher concentrations in the sequence 2,4-dimethylphenol > 4-chloro-3-methylphenol > phenol.

The concentration dependence data were also used to estimate the apparent Michaelis–Menten constant ($K_{m,app}$), this being 114, 254 and 454 μ*M* for phenol, 4-chloro-3-methylphenol and 2,4-dimethylphenol, respectively

CONCLUSIONS

The above results demonstrate the possibility of using reversed micelle systems as suitable media for developing enzyme amperometric biosensors These water-in-oil emulsions can be considered as universal solubilization media for both hydrophilic and hydrophobic analytes, thus allowing the enzymatic determination of

compounds scarcely soluble in water. Moreover, the enzyme can be immobilized on the electrode surface in a very simple fashion. The easy control of the water content needed for the enzyme operation is an additional advantage with respect to organic phase enzyme electrodes.

Acknowledgements—Financial support from the Spanish CICYT (project ALI 92-0049) and the Comunidad de Madrid (project COO 9/91) is gratefully acknowledged

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