An Organic-phase Enzyme Electrode based on an Apparent Direct Electron Transfer between a Graphite Electrode and Immobilized Horseradish Peroxidase

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A mediatorless horseradish peroxidase (HRP) enzyme electrode operated in nonaqueous media is constructed by cryohydrogel immobilization.

Biosensing with enzyme electrodes is generally based on the measurement of analytes which are soluble in aqueous solution. Organic-phase enzyme electrodes (OPEEs) used in nonaqueous media have only recently attracted attention,[1] due to some distinct advantages, including monitoring of hydrophobic substrates, elimination of microbial contamination, reduction of side reactions, enhanced thermostability, and relatively ease of enzyme immobilization. More interestingly, OPEEs show promising applications in organic synthesis, biocatalytic processes, environmental monitoring, and drug analysis *etc.* However, future development is challenging since enzymes immobilized on OPEEs are confronted with more hostile situations in nonaqueous media. Strictly, it is impossible for the enzyme to retain its catalytic activity under a water-free environment. Enzymes immobilized on OPEEs must retain a thin aqueous film the presence of which is essential for their catalytic activity. However, in organic solvents, especially water-miscible solvents, this layer is easily disturbed or even lost, and the enzyme is deactivated. At present the only way of providing this essential hydration layer for enzymes immobilized (generally adsorbed) on OPEEs is to deliberately add water to the organic solvent before use. Therefore, no OPEEs reported previously[2–8] are strictly operating in pure nonaqueous media, which can cause many problems, *e.g.* enzyme and/or mediator readily detaching from the electrode, and inconvenience in actual operations, especially for monitoring *in situ* or on-line in flow systems.

This communication reports, for the first time, an organic-phase enzyme electrode based on apparent direct electron transfer between a spectrographic graphite electrode and immobilized HRP. We have prepared a polyol cellulose (PC), an aqueous solution of which can be refrigerated to a hydrogel. This cryohydrogel has a semiinterpenetrating network, possesses a high mechanical strength and thermal stability, and can retain its water molecules to some extent in organic solvents. The PC and cryoimmobilization method were initially used to prepare enzyme electrodes, which can be operated in water-free organic media since the immobilization material itself, the cryohydrogel, can provide a suitable aqueous microenvironment for the enzyme to retain its catalytic activity.

The graphite electrode was prepared by inserting a spectrographic graphite rod (5 mm diameter) into a Teflon shrink tubing. The graphite electrode was polished on wet, fine emergy paper, ultrasonicated in deionized water and acetone, successively, and then dried at ambient temperature. For preparation of the enzyme electrode, 5 mg of horseradish peroxidase (HRP, EC 1.11.1. 90 Unit/mg) was dissolved in 200 μl of an 8% aqueous solution of polyvinyl alcohol (PVA-217) plus 2–5% (by mass) of sulfuric acid-treated cotton cellulose. An aliquot (20 μl) of the mixture was spread over the grapite electrode surface and the electrode refrigerated at −4 °C for 24 h, and kept in the refrigerator at 4 °C when not in use. The enzyme electrode was rehydrated with 10 μl of water and dried under ambient conditions for 30 min prior to use. Electrochemical measurements were performed at room temp. and under air (unless otherwise indicated). A platium foil was used as the counter electrode and an Ag/AgCl (sat. KCl) electrode as the reference electrode. The electrolyte solutions contained 0.1 mol dm^{-3} NBu_4ClO_4. Water and aqueous buffers were deliberately excluded from the background solutions. The stock H_2O_2 solution (5×10^{-2} mol dm^{-3}) was prepared in chloroform.

Fig. 1 shows the variation of the reduction current of H_2O_2 with the applied potential at the HRP-modified graphite electrode in chloroform (0.1 mol dm^{-3} NBu_4ClO_4). As can be

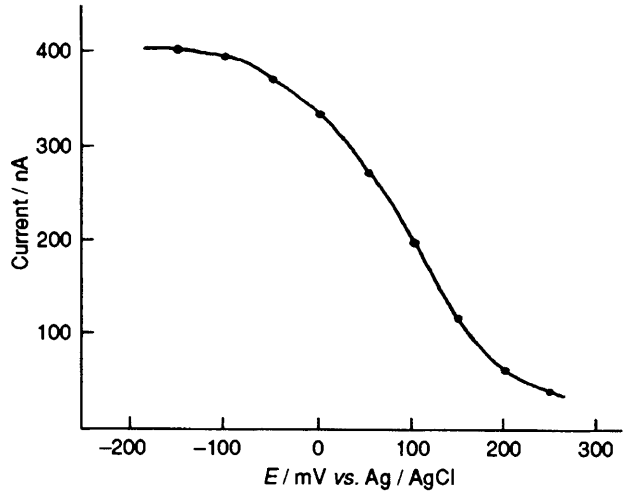

Fig. 1 The dependence of the response to H_2O_2 of the HRP-hydrogel modified graphite electrode upon applied potential for 0.5 mmol dm^{-3} H_2O_2 in chloroform. Stirring rate: 250 rpm.

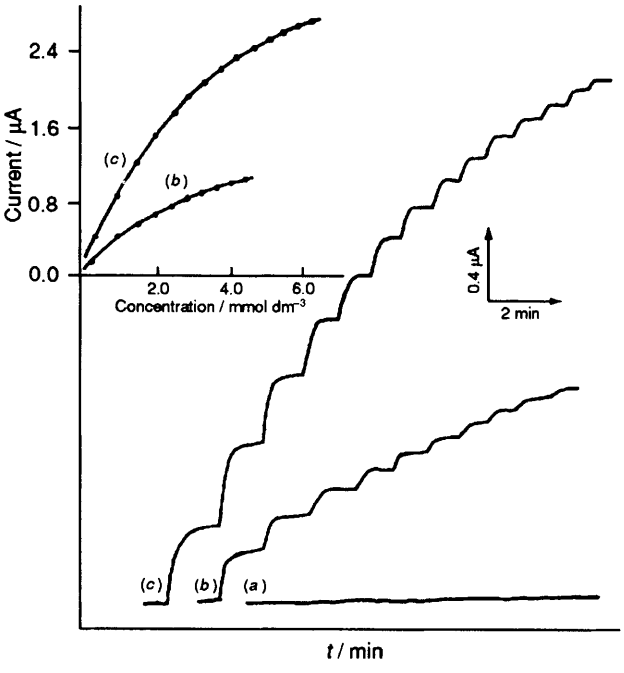

Fig. 2 Amperometric response of the hydrogel modified graphite electrode in the absence (*a*) and presence of HRP [(*b*) and (*c*)] upon successive addition of 50 μl of 5×10^{-2} mol dm^{-3} H_2O_2 solution in 5 ml of chloroform [(*a*) and (*b*)] or chlorobenzene [(*a*) and (*c*)]. Applied potential: 0.0 mV; stirring rate: 250 rpm.

seen, when the applied potential is > +100 mV, the current is small but increases upon reducing the applied potential; it increases more rapidly at *ca.* 0.0 V and finally reaches a plateau at −100 mV. This behaviour differs from that in aqueous solution,[9] where the reduction of H_2O_2 starts at +600 mV, and the current increases upon reducing the applied potential until it levels off at −100 mV. Experiments under nitrogen show a 2–4 fold increase current. This indicates that functionalities[9] on the electrode surface do not act as mediators, and that direct electron transfer from the active sites of the HRP to the graphite electrode occurs.

A variety of organic solvents were investigated and it was found that no response to H_2O_2 was observed in water-miscible solvents, such as acetonitrile or acetone while sensitive responses were obtained in water-immiscible solvents, such as chloroform and chlorobenzene as shown in Fig. 2. However, an HRP–$K_4Fe(CN)_6$ (as mediator) modified graphite electrode prepared as above showed excellent responses both in water-miscible and immiscible solvents. In the former reactive organic molecules can penetrate into the hydrogel, distort the intimate contact of the enzyme to the graphite electrode, and prevent the electrochemical action of HRP.

Platinum and glassy carbon electrodes were also investigated. Fig. 3 shows the amperometric responses of these electrodes modified by PC-hydrogel in the absence [(*a*) and (*b*)] and presence [(*c*) and (*d*)] of HRP. As can be seen, the electrodes modified with HRP show a much larger response to H_2O_2. This implies that unmediated catalytic reactions occur at both glassy carbon and platinum electrodes. The presence of oxygen-containing functionalities on the graphite electrode has been proposed to mediate the electron transfer.[10] However, on the surface of glassy carbon or platinum electrodes, especially the latter, without special treatment, such functionalities (*e.g.* phenolic or quinone groups, *etc.*) would be much less abundant than on graphite; it thus seems reasonable to propose direct electron transfer between the electrodes and HRP.

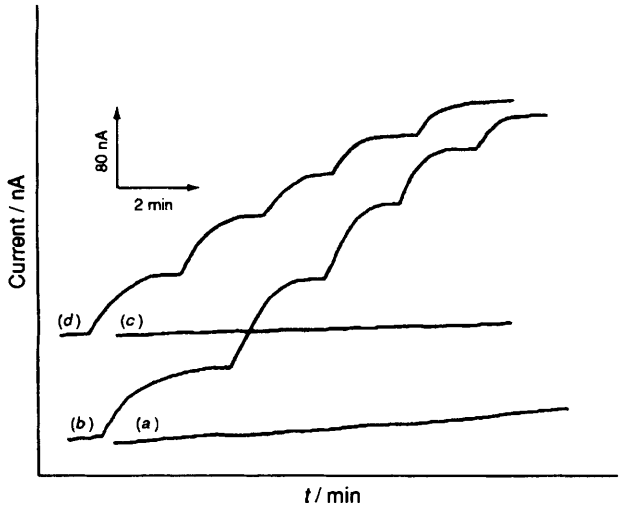

Fig. 3 Amperometric response of the hydrogel modified glassy carbon [(*a*) and (*b*)] or platinum electrode [(*c*) and (*d*)] in chloroform in the absence or presence of HRP. Other conditions are as in Fig. 2.

The HRP-modified graphite electrode prepared by this cryohydrogel method has an excellent performance and can be operated in *pure* organic solvents. By using this enzyme electrode, a stable base current can be obtained within 10 min and the response time is 0.5–2 min; The useful measuring range is up to 5.0 mmol dm^{-3} with a detection limit of 1.3×10^{-6} mol dm^{-3} in chloroform with corresponding values of 7.0 mmol dm^{-3} and 2.5×10^{-7} mol dm^{-3} in chlorobenzene. The enzyme electrode can be conveniently stored in the dried state or immersed in buffer solution, and has a prolonged storage lifetime. The enzyme electrode has been used intermittently for a month with no obvious deterioration in the sensing characteristics being observed. This enhanced stability may be attributed to two aspects. Firstly, because HRP is physically and chemically (generally by hydrogen bondings) entrapped in the hydrogel it will not readily detach from the electrode and secondly the cryohydrogel itself provides an appropriate microenvironment for the immobilized enzyme to retain its activity. It has been reported that lyophilization[11] and polyhydroxyl compounds[12] stabilize the enzymes activity. In the refrigerating process the catalytically active conformation of the enzyme is retained and may explain the enhanced thermal stability for the enzyme thus immobilized.

Such types of OPEEs have prospects for mediatorless measurement of other analytes by coimmobilizing H_2O_2-producing oxidases. Cryoimmobilization on materials such as polyol cellulose could be envisaged for preparing other biosensors.

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