Hydrogen peroxide biosensor based on microperoxidase-11 entrapped in lipid membrane

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Abstract

A highly catalytic activity microperoxidase-11 (MP-11) biosensor for H₂O₂ was developed to immobilizing the heme peptide in didodecyldimethylammonium bromide (DDAB) lipid membrane. The enzyme electrode thus obtained responded to H₂O₂ without electron mediator or promoter, at a potential of +0.10 V versus Ag | AgCl. A linear calibration curve is obtained over the range from 2.0 × 10⁻⁵ to 2.4 × 10⁻³ M. The biosensor responds to hydrogen peroxide in 15 s and has a detection limit of 8 × 10⁻⁷ M (S/N = 3) Providing a natural environment with lipid membrane for protein immobilization and maintenance of protein functions is a suitable option for the design of biosensors.

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Keywords: Microperoxidase-11; Lipid membrane; Didodecyldimethylammonium bromide; Biosensor

1. Introduction

The properties of enzyme-based amperometric biosensors are predominately governed by the ability of the immobilized biomolecule (enzyme) to exchange redox equivalents (electrons) with the electrode at an appreciable electron transfer (ET) rate. On the first sight, the simplest ET pathway would be the direct electron tunneling between the enzyme’s active site and the electrodes surface. However, since the prosthetic group of many enzymes is deeply buried within the protein shell, the rate of a direct ET is governed by the ET distance, according to the Marcus’ theory (Marcus and Sutin, 1985; Marcus 1993).

Hydrogen peroxide is the product of the reactions catalyzed by a large number of oxidases, and it is essential in chemical, biological, clinical and many other fields, so the determination of hydrogen peroxide is practically important. Peroxidase based H₂O₂ biosensor is promising because of its simplicity and high sensitivity (Wang et al., 1999; Wang and Dong, 2000; Wang et al., 2000; Jia et al., 2002).

The peroxidases are ferric heme enzymes, which catalyze the oxidation of a variety of substrates by hydrogen peroxide. One of the heme model species for the peroxidases can be obtained from enzymatic cleavage of cytochrome C (cyt.c) (Aron et al., 1986; Wang and Vanwart, 1989), microperoxidase-11 (MP-11), which still exhibits peroxidase activity (Munro and Marques, 1996). Its primary structure where numbers refer to positions in the native cyt.c is shown in Fig. 1. The heme group is covalently bound to the undecapeptide via two thioether bonds between two cysteines and the vinyl groups of the heme; His-18 is coordinated to the heme iron on the “proximal” site, as in native cytochrome. Though the peroxidase activity of a heme peptide is lower than that of intact peroxidase (Baldwin et al., 1987; Gooding et al., 2001), MP-11 is much smaller in size with a molecular weight of 1881, so they could carry out direct and rapid ET to or from an electrode with ferrous/ferric transition (Razumas et al., 1989; Mabrouk, 1996).

The interest in forming phospholipid film on solid substrates is to identify a model system that exhibits
membrane mimetic behavior like Langmuir–Blodgett (LB) films and black (bilayer) lipid membranes (BLMs), but with the additional advantages of ease and reproducibility of preparation, long-term stability, and formation on a support that is conducive to surface analysis and is electrically conductive. Furthermore, the presence of the lipid film could greatly reduce the interference and effectively exclude hydrophilic electroactive material from reaching the detecting surface (Tien et al., 1998), which might cause undesired reactions. In addition, the cast lipid film could be utilized as the model of a biological material, which may supply a biological environment on the surface of the detecting surface. Thus, the cast lipid film was an ideal choice to develop a new class of biosensor.

A serious problem in the application of biosensors in real systems, especially in biocolloids consists in possible interferences of the electrode surface with various electrode poisons mainly proteins. It has been shown by Kissinger and Hart (1973) that modification of carbon electrode by lipids can substantially improve the sensitivity and stability of a biosensor. For example, the implanted lipid modified carbon electrode has been successfully used for in vivo monitoring of ascorbate oxidation in rat striatum for several months (Ormonde and Neill, 1990). The modification of carbon electrode by phospholipids can provide charged functionalities of the electrode surface, which leads to a better selectivity of the sensing thanks to the electrostatic attraction or repulsion effects (Kaufmann et al., 1997). In contrast to ‘hard’ immobilization of enzymes onto the solid substrates, the surrounding lipids provide also a necessary conformation mobility of macromolecules, which resulted in a better amplifying effect. However, the lipids are soluble in the organic phase, so the biosensor cannot be used in non-aqueous media. Comparing with the sol-gel composite, the lipid has poor mechanical strength.

In this paper, we cast the mixture containing didodecyldimethylammonium bromide (DDAB) lipid vesicles and MP-11 onto a glassy carbon (GC) electrode to form enzyme entrapped lipid film. MP-11 in lipid film exhibited high catalytic activity for H2O2 and the lipid membrane supplied MP-11 a biological environment.

2. Experimental

2.1. Reagents

DDAB was obtained from Acros (Belgium). Microperoxidase-11 (MP11) from horse heart (Sigma Chemical Co.) was used without further purification. Hydrogen peroxide (30%) was purchased from Beijing Chemical Reagent Co. (Beijing, China). The solution of H2O2 was prepared daily. The phosphate buffer contained 10 mM phosphate buffer with 0.1 M NaCl. Pure water (18.2 MΩ) was used throughout, obtained by means of Millipore Q water purification set. All other chemicals were of reagent grade and used as received.

2.2. Sample preparation

Aqueous solution of MP11 was prepared in 10 mM phosphate buffer at pH 6.9. MP11 concentrations were measured spectrophotometrically using a molar absorptivity of 1.76 × 10^3 M⁻¹ cm⁻¹ at 395 nm (Wilson et al., 1977). For the preparation of DDAB vesicles, a dry film of 10 mg of lipid was produced under rotary evaporation from a stock solution in chloroform and then organic solvent was removed by purified nitrogen. The lipid film was hydrated to the desired concentration with 10 mM phosphate buffer, pH 6.9. The resulting unilamellar liposome suspension was sonicated for several hours (in average about 2 h) in a bath sonicator, until a clear suspension of small unilamellar vesicles was obtained (Pinheiro et al., 1997). The vesicle sizes of a few representative samples were determined by dynamic light scattering measurement on a DynaPro-MS/X dynamic light scattering instrument. The DDAB lipid vesicles had diameters ranging from 40 to 70 nm.

2.3. Preparation of enzyme modified electrode

A GC electrode (diameter 3 mm) was first polished with sand paper followed by 1.0, 0.3, and 0.05 μm alumina slurry, respectively, then sonicated in deionized water bath for 1 min for three times and then dried at room temperature. MP11 (0.1 mg) and the vesicle of DDAB (10 mg ml⁻¹) were mixed. 5 μl of this mixture was spread evenly with a microsyringe on to the surface of the GC electrode. Films were dried gradually over
night with a small bottle covered over the electrode to serve as a closed evaporation chamber, followed by a period of standing in air. The resulting electrode was rinsed with water and stored at 4 °C when not in use.

2.4. Apparatus

FT-IR absorbance spectra of the dried films on CaF₂ were recorded on a 520 Fourier transform infrared spectrometer (Nicolet, USA) equipped with a DTGS detector and OMNIC E. S. P. software. The films were recorded on a 520 Fourier transform infrared spectrometer (Nicolet, USA) equipped with a DTGS detector and OMNIC E. S. P. software. The films were dried in a dry-air atmosphere at room temperature for 1 day unless otherwise stated. Twenty scans were collected and averaged for each spectrum. The method for the films on CaF₂ was the same with that used in enzyme electrode preparation.

Absorbance spectra in the Soret (350–450 nm) regions were obtained for samples recorded at room temperature on a Cary 500 Scan UV–vis–NIR spectrophotometer (VARIAN, USA).

Cyclic voltammetry was carried out with a CHI 630 A electrochemistry workstation (CHI, USA). All electrochemical experiments employed a three-electrode cell (5 ml, single electrolyte compartment) with a GC working electrode, a platinum wire auxiliary electrode and an Ag/AgCl (sat.KCl solution) reference electrode. The potential of Ag/AgCl (sat.KCl solution) was taken as 0.198 V versus SHE. The buffers were purged with purified nitrogen (N₂) for 20 min prior to a series of experiments. A nitrogen environment was kept over solutions in the cell for exclusion of oxygen.

3. Results and discussion

3.1. FT-IR spectra of MP11-DDAB films

The Fourier transform infrared (FTIR) spectroscopy of a protein provides a wealth of information on structure and environment of the protein backbone and of the amino acid side chains. The amide I and II infrared bands have been used widely for monitoring conformational changes in proteins (Kumosinski and Unruh, 1993). Absorbance from DDAB does not occur in the amide region (Fig. 1, curve a). The MP11 film (Fig. 1, curve b) showed the large asymmetric peak at 1653 cm⁻¹ assigned to the amide I stretch and the two peaks at 1539 and 1559 cm⁻¹ attributed to the antisymmetric stretch of the carboxylates (Wright et al., 1997). However, for DDAB-MP11 film (Fig. 1, curve c) comparing to MP11 film, the main difference was that the peak at 1559 cm⁻¹ which was assigned to the α-carboxyl of glutamate disappeared and replaced by a more broad peak centered at 1534 cm⁻¹. From the results we obtained that the carboxylates of MP11 molecule could interact with head group of DDAB lipid. Our group also found that MP11 could adopt α helix in the full hydrated lipids and insert into the lipid membrane (Huang et al., 2001).

3.2. The current response of the electrode to hydrogen peroxide

The voltammetric behavior of the GC electrode coated by MP-11-DDAB film could be repeatedly scanned without significant decrease of peak currents and was stable in buffer for more than 2 weeks. In the absence of H₂O₂, the background current of the enzyme electrode is observed in Fig. 2(a). After 0.2 mM H₂O₂ was added into the buffer solution and the mixture was stirred for about 3 min, the reduction of H₂O₂ was performed on the enzyme electrode. An enhancement in reduction current is shown in Fig. 2(b). The electrocatalytic current rises from ca. 0.2 V, being reported also for other electrocatalytic systems consisting of MP-11 (Razumas et al., 1992; Lötzbeyer et al., 1994; Willner et al., 1998; Patolsky et al., 1999). This indicates that the current does not result from simple ET mediation via ferric/ferric transition of the heme peptide but does result from the reduction of a high-valent heme-oxygen complex such as compound I or compound II of peroxidase, because the applied potential is much more positive than the redox potential for ferric/ferric transition. This phenomenon results from the positive potential shift of microperoxidase-11 upon its complexation with H₂O₂. The positive shift in the reduction of H₂O₂ by MP-11 as compared with the redox potential of MP-11 itself, is attributed to the formation of an intermediate Fe(III)-peroxide species, which is reduced at the positive potential (Dams, 1991). From Fig. 2, we also could obtain that the electrode exhibited the highest activity at the potential of 0 V. When the potential
reaches to the low potential, it causes the reduction of O2; when the potential reaches to the high potential, interference from coexisting electroactive species could be significant. We select 0.1 V as the optimal working potential.

The effect of pH on the response of the MP-11 electrode was investigated over the range 5–10. As is known, a number of factors might cause different pH dependence of the sensor sensitivity. Tatsuma had pointed out if the immobilized microperoxidase reacted with H2O2 in the same manner as peroxidase, the reaction could be formulated as follows (Tatsuma and Watanabe, 1991):

\[
ferric \text{ heme peptide} + \text{H}_2\text{O}_2 \rightarrow \text{compound I} + \text{H}_2\text{O} \quad (1)
\]

\[
\text{compound I} + e^- + \text{H}^+ \rightarrow \text{compound II} \quad (2)
\]

\[
\text{compound II} + e^- + \text{H}^+ \rightarrow \text{Ferric heme peptide} + \text{H}_2\text{O} \quad (3)
\]

\[
[(\text{HN})\text{Fe}^{III}-\text{L}] + \text{HO}_{\text{OH}} \rightarrow [(\text{HN})\text{Fe}^{III}-\text{OOH}] + \text{HL} \quad (4)
\]

\[
[(\text{HN})\text{Fe}^{III}-\text{OOH}] \rightarrow [(\text{HN})\text{Fe}^{IV}=\text{O}]^+ + \text{OH}^- \quad (5)
\]

\[
[(\text{HN})\text{Fe}^{III}-\text{OOH}] + \text{H}^+ \rightarrow [(\text{HN})\text{Fe}^{IV}-\text{OH}] + \text{HO}^* \quad (6)
\]

Ferric heme peptide + H2O2

\[
\rightarrow \text{compound II} + \text{HO}^* \quad (7)
\]

\[
\text{compound II} + e^- + \text{H}^+ \rightarrow \text{Ferric heme peptide} + \text{H}_2\text{O} \quad (8)
\]

Up to date, there has been no conclusive evidence for the liberation of HO* even in the case where the hemolytic cleavage is envisaged. If one assumes that the hemolytic cleavage is accompanied by the proton and ET to the leaving group, the reaction could be represented by Eq. (1). So reaction 1 or 7 is dependent on pH. Further, a lower pH prevents heme molecules from dimerization, though it detaches imidazole of His from the heme via protonation. Several factors can be the causes for the pH dependence. The elucidation of this point is, however, beyond the scope of the present work. The pH effect on the sensor response for 0.2 mM H2O2 is illustrated in Fig. 3. The enzyme electrode shows a large response to H2O2 between pH 6.0 and 8.0, and the current response reaches the maximum value at pH 7.3.

A current–time plot of the enzyme electrode on successive step changes of H2O2 concentration is demonstrated in Fig. 4. When an aliquot of H2O2 is added into the buffer solution, the reduction current rises steeply to reach a stable value, the response time (reaching 90% of the maximum response) is less than 15 s, which indicates a fast diffusion process and a high activity of the MP-11 in this enzyme-biomembrane system (Fig. 4a). The linear range of H2O2 concentration spans between 2.4 mM and 20 uM with a correlation coefficient of 0.994 (Fig. 4b), the sensor has a detection limit of \(8 \times 10^{-7}\) M at a signal-to-noise ratio of 3.

The dependencies of biosensor response on ionic strength and buffer capacitance were investigated. The effect of ionic strength on the response of the enzyme electrode was studied by varying the solution with different [NaCl] ranged from 100 to 500 mM. Actually, due to the insulating characteristic of biomembrane, ionic strength did not affect the response of the enzyme electrode (data not shown). In the same time, different capacitance of phosphate buffer ranged from 10 to 100 mM was also investigated, no observable change was found for the response of the enzyme electrode (data not shown).

The effect of substances that might interfere with the response of the enzyme electrode was studied (Table 1).
The inhibition current obtained for each interfering substance present at a concentration of 0.2 mM was compared with the current obtained in the presence of 0.2 mM \( \text{H}_2\text{O}_2 \), and this ratio was used as a criterion for the selectivity of the biosensor. Similar to a previous report, glucose, sucrose, lactic acid did not cause any observable interference (Wang et al., 1999), only ascorbic acid produced a negative bias; as for imidazole, it reduced the response of the electrode to \( \text{H}_2\text{O}_2 \) greatly which supported the formation of a heme-oxygen compound, because the strong ligation of imidazole to the sixth coordination site of heme Fe(III) may interfere with the oxygen complex formation (see Table 1). In the same time the effect of \( \text{O}_2 \) was also investigated. After purging dissolved oxygen by nitrogen bubbling for 20 min, an \( \text{H}_2\text{O}_2 \) solution was added (0.2 mM) to the system. Subsequent reintroduction of oxygen did not reduce the response; oxygen did not interfere with the peroxidative activity of MP-11 under the present conditions. Further, the background current was independent of the dissolved oxygen. This meant that the influence of oxygen to the activity of MP-11 electrode was negligible in this system. It is obviously that the lipid film on the surface of the electrode can greatly reduce the interference and effectively excludes hydrophilic electroactive material from reaching the detecting surface (Tien et al., 1998).

The reproducibility of the sensor was examined at a \( \text{H}_2\text{O}_2 \) concentration of 0.2 mM with the same sensor, and the relative standard deviation is 5.6% (\( n = 8 \)). The storage stability of the enzyme electrode was tested over 2 weeks period. When the electrode was stored in the refrigerator at 4 °C (dry), no apparent decrease in the response to 0.2 mM \( \text{H}_2\text{O}_2 \) was found over 2 weeks.

The electrode-to-electrode reproducibility was estimated from the response to 0.2 mmol \( \text{L}^{-1} \) \( \text{H}_2\text{O}_2 \) at six different biosensors. This series yielded a mean current response of 354 nA and a relative standard deviation (R.S.D.) of 4.2%.

The good electrochemical response, stability and reproducibility of the enzyme electrode demonstrates the high activity of the MP-11, this can be attributed to the model membrane system, which supplies a biological environment on the surface of the GC electrode to maintain the active configuration of enzyme. So high activity of MP-11 is retained. This also demonstrates that the multiplayer lipid membrane is an ideal choice to immobilize enzyme for constructing the third-generation biosensor based on GC electrode.

4. Conclusion

A high bioelectrocatalytic activity for the reduction of \( \text{H}_2\text{O}_2 \) could be obtained by entrapping MP-11 in lipid film. Further, the presence of the lipid bilayer greatly reduces the interference (such as glucose and sucrose) and effectively excludes hydrophilic electroactive compounds (such as lactic acid and ascorbic acid) from reaching the electrode surface, which may cause undesired reactions. In the meanwhile, the lipid membrane provides the peptide a biocompatible environment performing its function. Thus, from the selectivity and biocompatibility points of view, the microperoxidase-11 entrapped and lipid membrane based biosensor is an ideal choice to develop a new class of electrochemical biosensor for \( \text{H}_2\text{O}_2 \).

Acknowledgements

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References


Table 1
Inhibition of the interferences to the enzyme electrode

<table>
<thead>
<tr>
<th>Potential interfering substance</th>
<th>Current ratio a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>0</td>
</tr>
<tr>
<td>Sucrose</td>
<td>0</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>0</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>−0.06</td>
</tr>
<tr>
<td>Imidazole (0.053 mM)</td>
<td>−0.48</td>
</tr>
</tbody>
</table>

a Current ratio is the current of interfering substance compared with that of 0.2 mM hydrogen peroxide.