Alcohol sensing membrane based on immobilized ruthenium(II) complex in carboxylated PVC and surface covalently bonded alcohol oxidase

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Abstract

Alcohol sensing membranes coated on overhead transparency films for the continuous monitoring of ethanol, propanol and butanol are presented. Alcohol oxidation catalyzed by alcohol oxidase in conjunction with the fluorescence quenching reaction of oxygen-sensitive dye ion-pair, tris(4,7-diphenyl-1,10-phenanthroline) ruthenium(II) didodecylsulphate was chosen for the determination. Alcohol oxidase was immobilized covalently on a plasticized carboxylated poly(vinyl chloride) membrane and the oxygen-sensitive dye ion-pair was entrapped in the same membrane. The sensing membrane relates oxygen consumption, as a result of enzymatic oxidation, to alcohol concentration. Measurements have been performed in air-saturated alcohol standard solutions of pH 7.0. Storage stability, reproducibility and the effect of pH on sensing membrane performance have been studied in detail. The alcohol sensing membrane proposed here is simple to prepare and has a fairly rapid response time of < 1 min. It has been successfully applied to the determination of the ethanol contents in various spirits. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Ruthenium(II) didodecylsulphate; Alcohol oxidation; Carboxylated PVC

1. Introduction

The rapid and accurate determination of alcohol is receiving attention in fermentation, quality control of beverages and blood analysis. Traditionally, alcohol is determined by gas chromatography and distillation methods [1,2], combined with subsequent measurements of density and refractometry. However, these methods either require distillation of the sample or extensive reagent preparation. In recent years, intensive works have been performed on the development of optical sensors. They have been accepted as advantageous because they can be miniaturized, are cheap to be manufactured and quite safe. Two kinds of optical alcohol sensor device have been developed over the past decade. Only a few sen-
Table 1
Compositions of M₁ to M₄ oxygen-sensitive optode membrane

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Weight of polymer (mg)</th>
<th>Weight of TBP (mg)</th>
<th>Weight of Ru(dpp)₃(DS)₂ (mg)</th>
<th>Emission peak maximum (nm)</th>
<th>Relative fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>M₁</td>
<td>83</td>
<td>83</td>
<td>1.3</td>
<td>593.7</td>
<td>8.2</td>
</tr>
<tr>
<td>M₂</td>
<td>83</td>
<td>166</td>
<td>1.3</td>
<td>593.2</td>
<td>5.7</td>
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<tr>
<td>M₃</td>
<td>83</td>
<td>249</td>
<td>1.3</td>
<td>592.6</td>
<td>3.8</td>
</tr>
<tr>
<td>M₄</td>
<td>83</td>
<td>332</td>
<td>1.3</td>
<td>592.0</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Table 2
Compositions of M₅ to M₁₀ oxygen-sensitive optode membrane

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Weight of polymer (mg)</th>
<th>Weight of TBP (mg)</th>
<th>Weight of Ru(dpp)₃(DS)₂ (mg)</th>
<th>Relative fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>M₅</td>
<td>83</td>
<td>332</td>
<td>2.6</td>
<td>1.8</td>
</tr>
<tr>
<td>M₆</td>
<td>83</td>
<td>332</td>
<td>3.9</td>
<td>2.7</td>
</tr>
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<td>M₇</td>
<td>83</td>
<td>332</td>
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</tr>
<tr>
<td>M₈</td>
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<td>1.6</td>
</tr>
<tr>
<td>M₁₀</td>
<td>83</td>
<td>332</td>
<td>9.1</td>
<td>0.7</td>
</tr>
</tbody>
</table>

sors, upon exposure to alcohol, show fluorescence enhancement of fluorescein derivatives [3] and malachite green [4] or fluorescence quenching of polyaromatic-substitute 1,3-oxazoles, thiazoles [5] and p-N,N-dioctylamino-4'-trifluoroacetylstilbene [6]. In most cases the recognition process is based on the enzymatic recognition of alcohol. The transduction is performed by optically detecting changes in the reduced form of nicotinamide adenine dinucleotide (NADH) concentration, the production of hydrogen peroxide or the consumption of oxygen (O₂) [7–11]. Other enzyme-based amperometric alcohol sensors based on alcohol oxidase have already been described in detail [12,13]. The sensitivity and limit of detection of the electrochemical sensors are far better than the newly developed optical ones. However, optical sensors can offer some advantages over electrochemical sensors such as immunity to electromagnetic interference, possibility of remote and in situ monitoring and ease of fabricating a multi-component detecting system [14]. Considerable research effort will continue to expend in developing biosensors and chemosensors based on optical methods. Wolfbeis and Posch [15] have mentioned the entrapment of alcohol oxidase and tris(2,2'-bipyridyl) ruthenium(II) dichloride into silica gel for fibre optic sensing of ethanol. Although these existing sensors for the determination of alcohol have been successfully realized, there is still a demand for a fast-responsive, mass-produced and light-emitting diode (LED) compatible alcohol sensor.

Recently, Koncki et al. [16] have successfully immobilized an enzyme and a pH-sensitive dye in a polymeric membrane for detection of urea. Borrowing this novel idea, we fabricated sensing membranes based on the immobilization of alcohol oxidase and tris(4,7-diphenyl-1,10-phenanthroline) ruthenium(II) didodecylsulphate (Ru(dpp)₃(DS)₂) onto carboxylated poly(vinyl chloride) (PVC-COOH). Dissolved O₂ optodes were first fabricated by immobilizing the dye ion-pair in plasticized PVC-COOH optode membranes coated on overhead transparency films. Alcohol sensing membrane was subsequently constructed by using a simple and well-known 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide condensation reaction and immobilizing alcohol oxidase on the same PVC–COOH matrix.
Fig. 1. Observed relative fluorescence intensity versus time curves for M₁–M₄ optode membranes while immersed in air-saturated 50 mM phosphate buffer solution at pH 7.0 and 20°C. The plasticizer content is M₁ = 83; M₂ = 166; M₃ = 249; M₄ = 332 mg.
The sensing membrane presented here has three advantages. First, in the past these optode membranes were usually coated on glass or quartz solid supports which are easily broken if they are handle carelessly. The cutting of the glass material into different shapes and sizes is also inconvenient. Presently, we make use of overhead transparencies as our solid support for the mass production of the optode membranes. The use of overhead foils has been recently adapted in the industry for mass production of optode membranes [17]. The transparency is inexpensive, light, strong, water-proof and also has no visible light absorption. It can easily be cut into different shapes and sizes using scissors or a scalpel and be fixed to any sensor heads. Second, using a high-brightness blue LED, combined with a miniature photodiode detection system, the present PVC-based sensing membrane will have the potential for a low-cost, high-performance, simple and
portable alcohol sensor for use in many fields from quality control of beverages to on-line fermentation monitoring. Third, this sensing membrane shows a fast forward and reverse response time by using a highly active and extremely thin enzyme layer deposited on an oxygen-sensitive optode membrane.

2. Experimental

2.1. Chemicals and reagents

Alcohol oxidase (from Hansenula species) with a specific activity of 11 U mg$^{-1}$ solid from Sigma (St. Louis, MO), was covalently immobilized on the oxygen-sensitive membrane via the coupling reagent 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) from Sigma. High relative molecular mass carboxylated poly(vinyl chloride) (PVC-COOH), plasticizer tri-n-butyl phosphate (TBP), tetrahydrofuran (THF) and various kinds of alcohols were purchased from Aldrich Chemical (Milwaukee). Dithiothreitol, sodium dihydrogen phosphate, disodium hydrogen phosphate and trisodium phosphate were obtained from Fluka Chemical (Buchs, Switzerland).

Tris(4,7-diphenyl-1,10-phenanthroline) ruthenium(II) didodecylsulphate dye ion-pair [Ru-(dpp)$_3$(DS)$_2$] was synthesized according to a modified procedure reported in the literature [18]. Standard solutions (0.1 M) of various alcohols were prepared by adding appropriate volumes of alcohols in phosphate buffers (50 mM). A series of standard solutions were then prepared by successive dilutions with buffer solutions.
2.2. Instrumentation

Fluorescence spectra were recorded using a spectrofluorometer, consisting of a lamp power supply (model LPS-220), a xenon lamp (model A1010), and a photomultiplier detection system (model 710), from Photon Technology International (London, Ontario, Canada). A Hewlett-Packard model 5890 Series II gas chromatograph equipped with a flame ionization detector and a HP-20M (Carbowax 20M) column was used to determine the ethanol contents of various spirits.

2.3. Preparation of dissolved oxygen-sensitive optode membrane

A cocktail of polymer solution was prepared by dissolving 5.2 mg of dye ion-pair, 332 mg of TBP and 83 mg of PVC-COOH powder in 2 ml of THF. An aliquot of 0.2 ml of this solution was applied to a dust-free transparency which was fixed in a spin-on device. A membrane of approximately 4 μm thickness was then coated onto the transparency and dried in ambient air before use. The size of the membrane was 36 mm in diameter.

2.4. Assembly of the alcohol sensing membrane

A freshly prepared solution containing 1 mg of EDC and 1 mg of alcohol oxidase per 0.2 ml of water was deposited on the surface of a O₂-sensitive membrane and left overnight at room temperature. Before use, the membrane was put in a phosphate buffer (pH 7.0, buffer concentration 50 mM) for at least 2 h in order to condition it and to remove unbound enzyme.

2.5. Procedures

A membrane coated on a transparency film was fitted into a quartz cuvette well covered with a polytetrafluoroethylene lid and then placed in the cuvette holder of the spectrofluorometer. About 3.5 ml of various test solutions were injected into the cuvette using a syringe. A color filter with 530 nm cut-on wavelength (L.O.T.-Oriel, Leatherhead, Surrey, UK) was used to remove the scattered light from the excitation source. In order to perform the continuous measurements, the old solution was pumped out and the fresh solution was injected instantly. Fluorescence measurements were taken under batch conditions.

3. Results and discussion

3.1. Fabrication of dissolved oxygen-sensitive membrane

Highly lipophilic Ru(dpp)₃(DS)₂ was prepared in 88.4% yield, starting from ruthenium (III) trichloride hydrate, according to the reported procedure [18]. The compound in its solid form is extremely stable and can be stored in the dark for a long period of time without any deterioration.

The oxygen-sensitive membrane is a thin layer of Ru(dpp)₃(DS)₂ dye ion-pair immobilized in the plasticized PVC-COOH. Compared with that in acetonitrile, the emission spectra of Ru(dpp)₃(DS)₂ immobilized on the PVC-COOH are blue-shifted from 614 to 592 nm. The shift of emission peak maximum is caused by the change of the micro-environment surrounding the Ru(dpp)₃(DS)₂ dye ion-pair. The fluorescence excitation and emission spectra characteristic of Ru(dpp)₃(DS)₂ immobilized on PVC-COOH matrix coated on a transparency film or a quartz solid support is very similar. However, the transparency film is cheaper and is easy to cut into different shapes and sizes. Furthermore, prolonged used optode membranes coated on transparency films can be simply disposed and replaced with new ones once their sensing performances deteriorate. The storage of packs of optode membranes is very convenient and they are light and portable. As a result, we decided to mass-produce all our sensing membranes coated on overhead transparency films as the solid support material.

The fluorescence of the dye ion-pair strongly and fully reversibly quenched by molecular O₂. The effects of the ingredients on the optode membrane were investigated in more detail. It can be found that, with increasing plasticizer content, a reduction in the fluorescence intensity of Ru(dpp)₃(DS)₂ fluorophore can be noted due to the decrease in concentration of fluorophore in

Fig. 4. Typical time-response curve of the alcohol sensing membrane for the determination of ethanol solutions. (1) 7.2 mM ethanol; (2) 26.5 mM ethanol; (3) 50 mM phosphate buffer solution at pH 7.0 and 20°C.

the membrane. For membranes M₁ to M₄ (Table 1), with increasing contact time of the membranes with the buffer solutions, it can be observed that the fluorescence intensities gradually increase but the increments gradually slow down (Fig. 1). The duration for membranes M₁ to M₄ reaching 95% of final fluorescence intensity were 4, 3.5, 2.5 and 1.5 h, respectively. It is possible that plasticized PVC membranes undergo substantial water uptake when immersed in aqueous solutions. Plasticizers and water can form a micro-emulsion on the surface of the bulk membrane and this provides a large interfacial area for the uptake of the ion-pair indicator from the bulk to the surface of the membrane. With increasing plasticizer content, the rate of surface adsorption of the indicator increases which constitutes a faster speed in reaching the plateau of the fluorescence intensity shown in Fig. 1. This observation has been fully explained by Mohr and Wolfbeis [19]. We further studied the effect of the contact time of the optode membrane on the fluorescence intensity of Ru(dpp)₃(DS)₂. After the membranes were soaked in the buffer solution for 4 h, the fluorescence intensities of four kinds of membranes all tended to be constant, and kept unchanged overnight. A compromise must be made between the contact time and the fluorescence intensity. For convenience, membrane M₄ was used in all further studies. The fluorescence intensity of membrane M₄ was smaller than that of other membranes; however, it is strong enough for the present work. In addition, higher level of plasticizer content in the film increases markedly the sensitivity of the film towards oxygen as explained by Mills and Thomas [20]. The effect of the plasticizer content
on the fluorescence spectrum has also been studied. It was found that no obvious spectral change occurred except that emission peak maxima were slightly blue-shifted with increasing plasticizer content.

The Ru(dpp)$_3$(DS)$_2$ concentration has a greater effect on the fluorescence intensity (Table 2). The fluorescence intensity reached a maximum when the amount of Ru(dpp)$_3$(DS)$_2$ was at 5.2 mg. At higher concentrations of O$_2$ indicator, the fluorescence intensities significantly decreased which strongly suggests that high content of Ru(dpp)$_3$(DS)$_2$ can cause self-quenching. Thus, Ru(dpp)$_3$(DS)$_2$ content was controlled at 5.2 mg throughout these experiments.

The 90% forward and reverse response can be reached within 20 s. Immobilized Ru(dpp)$_3$(DS)$_2$ is photostable. A linear graph was obtained when $I_o/I$ was plotted against [O$_2$] using Stern–Volmer equation; $I_o$ is the fluorescence intensity recorded in a 100% N$_2$-saturated buffer solution as a function of [O$_2$]. The regression equation is $I_o/I = 0.0218$ [O$_2$] + 0.9912 ($r^2 = 0.994$). The linear range was up to 80% O$_2$-saturated buffer solution. The limit of detection, which is based on three times the standard deviation at zero dissolved O$_2$ concentration, was determined to be 17.6 μM. Although the sensitivity and limit of detection of the O$_2$-sensitive optode membrane is not as good as those described in the literature [21], it is a good substrate for the subsequent alcohol oxidase immobilization and it will be described in the following section.

3.2. Analytical features of the alcohol sensing layer

In order to make an O$_2$ transducer in conjunction with an alcohol sensing layer, alcohol oxidase
is immobilized on PVC-COOH using a simple EDC condensation reaction. The enzyme immobilization procedures are very simple and proceed smoothly under mild conditions. The resulting enzyme membranes show good activity and possess high stability, mechanical resistance and flexibility.

The enzyme is specific for lower primary alcohols, according to the equation

\[ \text{RCH}_2\text{OH} + \frac{\text{alcohol oxidase}}{\text{O}_2} \rightarrow \text{RCHO} + \text{H}_2\text{O} \]

In this study we attempted to show the substrate selectivity of this enzyme from an organism, Hansenula species. As shown in Fig. 2, ethanol is indeed the best substrate for the Hansenula enzyme, the order of reactivity being ethanol > propanol > butanol. The limits of detection were calculated to be 4.0, 7.5 and 6.0 mM for ethanol, propanol and butanol, respectively. But the response to iso-propanol, iso-butanol and tert-butanol was low and the response to methanol was inconsistent.

The dependence of enzymatic activity on the pH of the phosphate buffer (50 mM) was investigated over the pH range 5.0–9.0 (Fig. 3). In this experiment, 42.6 mM ethanol were dissolved in the phosphate buffer solutions. It can be found that the optimum pH is 7.0–8.0. When the working temperatures were raised to 30°C or above, the response to ethanol decreased markedly and it

Fig. 6. Reproducibility of two sets of the alcohol sensing membranes for the determination of butanol in 50 mM phosphate buffer solution at pH 7.0 and 20°C.

<table>
<thead>
<tr>
<th>Set no.</th>
<th>Slope*</th>
<th>y-intercept*</th>
<th>r²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.0023 ± 0.00014</td>
<td>0.00009 ± 0.0026</td>
<td>0.9995</td>
</tr>
<tr>
<td>2</td>
<td>0.0022 ± 0.00014</td>
<td>0.0008 ± 0.0026</td>
<td>0.9961</td>
</tr>
</tbody>
</table>

* 95% confidence limit.
Table 3
Results of the determination of ethanol contents in various commercial spirits

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ethanol concentration (% v/v)</th>
<th>Found (^b)</th>
<th>Reported</th>
<th>GC (^b)</th>
<th>Dilution factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glenfiddich</td>
<td>44.3 ± 1.8</td>
<td>43</td>
<td></td>
<td>43.29 ± 1.20</td>
<td>300</td>
</tr>
<tr>
<td>Herbs liquor</td>
<td>46.5 ± 0.6</td>
<td>48</td>
<td>47.0 ± 0.52</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>Kiu Kiang Chiew</td>
<td>30.6 ± 2.3</td>
<td>NR</td>
<td></td>
<td>31.52 ± 0.32</td>
<td>200</td>
</tr>
</tbody>
</table>

Using the null hypothesis and t-test, there are no significant difference at 95% confidence level between the methods [22].

\(^a\) Manufacturers: Glenfiddich, Scotland; Herbs Liquor, Thailand, Kiu Kiang Chiew, People’s Republic of China.

\(^b\) Average of three measurements.

NR, Not reported.

is probable that higher temperature caused the enzyme inactive.

Response time and reversibility of an alcohol sensing membrane was demonstrated by applying the membrane to two different ethanol concentration solutions alternatively (Fig. 4). It can be noted that the full-steady state is achieved within 1 min, which represents a fairly rapid response alcohol sensing system. The alcohol sensing membrane can be renewed easily by washing it with phosphate buffer. The standard deviation for ten replicate measurements of a 10 mM ethanol solution was 5%.

The storage stability of the immobilized alcohol oxidase is quite good as shown in Fig. 5. When the membrane with the immobilized alcohol oxidase was stored in a 10 mM dithiothreitol solution, i.e. an antioxidant which can prevent the sulphydryl group (-SH) of the enzymes from being oxidized, the enzyme membrane was stable for 7 days.

The reproducibility of the enzymatic membrane fabrication was examined by performing calibrations of butanol with two sets of the enzyme membrane. The relative fluorescence intensities are found to be reproducible (Fig. 6).

3.3. Sample analysis

Three commercial spirits were analyzed for their ethanol contents. The procedure involved dilution of spirits with the working buffer (dilution factor is 200–300) and three repetitive determinations. The concentrations of the diluted sample solutions were calculated from the calibration graph. The results compare favorably with those obtained by gas chromatography (GC) as shown in Table 3. The statistical study for the two methods also demonstrated that there were no significant differences between them at 95% confidence level [22].

4. Conclusion

The alcohol sensing membrane presented here is based on the use of inexpensive overhead transparencies as the solid support, alcohol oxidase as the recognition of alcohols and Ru(dpp)\(_3\):(DS)_2 dye ion-pair as the O\(_2\) indicator, and compatible with LED devices. Therefore, it is ideally suited for mass production of the alcohol sensing membranes. Aside from its specific utility in this alcohol sensing schemes, the method for immobilizing enzyme on PVC–COOH is a very simple procedure for the construction of enzyme bulk optode membranes. A thin enzyme layer in conjunction with a sensitive indicating system results in a transparent film which has a fast response time. The results indicate the potential for a mass-produced and high performance alcohol sensing membrane for use in a wide range of continuous alcohol monitoring.

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References