Development of an oxygen-rich biosensor using enzymatic reaction

Yue Cui*, John P. Barford and Reinhard Renneberg

Department of Chemical Engineering and Department of Chemistry , Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong

*Author for correspondence (Fax: +852-23580054; Email: kecuiyue@ust.hk)

Key words: Biosensor, oxygen, catalase; hydrogen peroxide

Abstract

This work reports a novel strategy for the development of an oxygen-rich biosensor. The principle is based on an enzymatic reaction between catalase and hydrogen peroxide to release oxygen, thus to increase the oxygen amount in the enzyme matrix. This method improves the determination reliability by alleviating the oxygen dependence.

Introduction

The importance of amperometric biosensors has increased considerably in recent years thanks to the advantages of being highly sensitive, rapid, accurate, economical, and easy to handle (Frew & Hill 1987, Scheller *et al.* 1991, Turner 2005). The biochemical reactions involving oxygen as a reactant are widely used for the constructions of amperometric biosensors. However, these biosensors suffer from problems in some applications, such as bioprocess monitoring and in-vivo measurement, due to the restriction of oxygen solubility or the fluctuation of oxygen concentration, which result in a narrow linear detection range or an unsteady result (Zhang & Wilson 1993, Wang 2001).

Various strategies have been developed to solve these problems which resulted from the oxygen dependence, by using a proper membrane coverage to improve the relative surface availability to oxygen while presenting a diffusion barrier to the substrate (Clark *et al.* 1986, Zhang & Wilson 1993), by replacing oxygen with an artificial electron-acceptor (Cass *et al.* 1984,

Frew & Hill 1987), or by designing oxygen-rich biocomposite electrode materials (Wang and Lu 1998, Wang *et al.* 2000, Wang *et al.* 2001, Wang *et al.* 2002).

This work reports a novel strategy for designing an oxygen-rich biosensor using an enzymatic reaction between catalase and H_2O_2 , which can be employed for some in-vitro applications, such as bioprocess monitoring, where the atmosphere O_2 fluctuation affects the process performance significantly (Namdev *et al.* 1993). The Clark-type oxygen electrode is one of the most typical oxygen-detecting electrodes, having been used for the developments of various biosensors, and the oxygen-permeable membrane in the front of the electrode, such as the Teflon membrane, makes the determinations of analytes free from electroactive interferences (Jobst *et al.* 1993, Chan *et al.* 1999, Mak *et al.* 2003). The measurements of glucose with glucose oxidase (GOD) based biosensors have been widely performed in clinical diagnosis, food analysis and bioprocess monitoring (Bradley and Schmid 1991, White *et al.* 1996, Wang 2001). Therefore, in this work, glucose is used as a representative parameter to construct an oxygen-rich biosensor based on a Clark-type oxygen electrode.

Materials and methods

Apparatus

A potentiostat (Biometria EP30, Germany) and a computer installed with software FIABOLO were used. The Clark-type oxygen electrode (model SEM1/4, Elbau, Germany) was composed of

a Pt working electrode (diameter: 0.5 mm) and an Ag/AgCl reference/counter electrode. A stirred measuring cell (volume: 1ml) was used for the experiments.

Materials

Glucose, glucose oxidase (GOD, EC 1.1.3.4, from *Aspergillus Niger*), catalase (EC 1.11.1.6, from Bovine Liver), disodium hydrogen phosphate, sodium dihydrogen phosphate, nafion® perfluorinated resin solution (5 wt. % in lower aliphatic alcohols and water), and hydrogen peroxide (H_2O_2) were all reagent grade purchased from Sigma-Aldrich (USA). The nitrogen (N_2) and oxygen (O_2) gases were supplied from HKO (Hong Kong). Saturated potassium chloride gel was from Bio Sensor Technologies (Germany). Teflon membrane was from Biological Systems Inc. (USA). Dialysis membrane was from SensLab (Germany).

Preparation of biosensors

Enzyme solution containing $5 \text{ U } \mu\text{l}^{-1}$ GOD and a loading of catalase ranging from $7.5 \text{ U } \mu\text{l}^{-1}$ to $750 \text{ U } \mu\text{l}^{-1}$ was mixed with the diluted nafion (2%) (v/v 1:1). And $1 \mu\text{l}$ of this mixture was spread over a Teflon membrane, followed by drying and storing at 4°C overnight. The enzyme layer sandwiched between a Teflon membrane and a dialysis membrane was fixed on the top of the Clark-type oxygen electrode with a membrane holder and an O-ring. The Teflon membrane facing toward the oxygen electrode received $15 \mu\text{l}$ of saturated potassium chloride gel as

electrolyte before assembling the sensor. The sensor was then screwed into a measuring cell, which was filled with buffer solution, and rehydrated for around 30 min before usage.

Procedure for measurements

The O₂ removal was attained by purging the buffer solution with N₂ gas (for 1 min), the N₂ atmosphere was subsequently maintained over the solution, and the complete O₂ removal was observed when the background current decayed to a constant value which was around zero nA. The O₂ saturation was attained by purging the buffer solution with O₂ gas (for 1 min), an O₂ atmosphere was subsequently maintained over the solution, and the O₂ saturation was observed when the background current reached a constant high value.

Experiments were performed by applying the desired potential to the Clark electrode at -0.6 V versus Ag/AgCl for the reduction of O₂ and magnetically stirring the buffer solution at 300 rpm to obtain a uniform distribution of substrate. When a constant background current was obtained, substrate e was added into the buffer solution so as to give a desired concentration, and until a stationary current, the current difference was recorded in order to plot the current-concentration curve. All measurements were carried out in phosphate buffer (PBS, 50 mM, pH 6.8) at room temperature (20 °C).

Results and Discussion

Construction of the oxygen-rich glucose biosensor

As shown in Figure 1, the principle is as follows: when a constant glucose flux enters the enzyme matrix, the glucose and O_2 in the enzyme matrix are consumed by GOD to generate gluconolactone and H_2O_2 , thus the O_2 amount in the enzyme matrix affects the GOD-catalyzed reaction significantly due to being a reactant. In order to provide an enough amount of O_2 for the enzymatic reaction by GOD under conditions with O_2 fluctuation or restricted O_2 solubility, we employ catalase in the enzyme matrix and H_2O_2 in the buffer solution to release oxygen, thus to increase the oxygen amount in the enzyme matrix. The amount of H_2O_2 produced from glucose is proportional to the O_2 concentration in the enzyme matrix, and it can also serve as a supply for enzymatic reaction by catalase, while in the absence of H_2O_2 in buffer, its amount would be quite small under atmosphere O_2 -deficit conditions, thus there is a need for the presence of H_2O_2 in buffer solution. By using this method, the O_2 concentration in the enzyme matrix can be increased significantly due to the O_2 release from H_2O_2 by catalase.

Investigation of the O_2 release from H_2O_2 by catalase

The Clark-type electrode is based on the detection of O_2 , thus, the electrode current is a reflection of the O_2 amount in the enzyme matrix. In order to examine the ability of the catalase-based enzymatic reaction for the release of O_2 from H_2O_2 , the biosensors were constructed with a constant GOD loading and different catalase, H_2O_2 loadings.

Figure 2 compares the current responses of the biosensors with different catalase loadings in the enzyme matrices ranging from 3.75 U to 375 U, to various injections of H_2O_2 with concentrations ranging from 0 to 2 mM in buffer. As shown in the figure, with the increasing of H_2O_2 concentration in buffer, the current increased in the investigated range due to the increasing release of O_2 from H_2O_2 in the enzyme matrix. However, when the H_2O_2 concentration was too high, the current became unsteady as the time went on. Therefore, a H_2O_2 concentration at 1 mM in buffer was used for other investigations which can result in a steady background current for the measurement of glucose. Also, the sensors with different catalase loadings exhibited different current responses. When the catalase loading was too low, the rate of the enzymatic reaction was also too low to release a high amount of O_2 ; when the catalase loading was too high, it presented a diffusion barrier and the H_2O_2 in buffer was consumed too fast, consequently, the H_2O_2 flux entering the enzyme matrix was not constant and the current was followed inconstant. The optimized catalase loadings obtained in the investigated range were 7.5 U and 75 U in the enzyme matrix. Therefore, a catalase loading at 7.5 U in the enzyme matrix was used for further experiments due to the high O_2 production with a low loading of catalase.

Detection of glucose under different atmosphere conditions

Figure 3 compares the current responses to various concentrations of glucose under three different atmosphere conditions in the measuring cell – O_2 saturated condition, ambient air, and

O₂ deficit condition. In all three cases, the sensor performances were investigated in the presence of H₂O₂ and in the absence of H₂O₂.

As the glucose concentration was proportional to the O₂ consumption in the enzyme matrix, the amount of O₂ in the enzyme matrix was a reflection of its detection range. The O₂ supplied by the ambient air provided an enough O₂ loading for the enzymatic reaction by GOD, therefore, when the O₂ amount in the enzyme matrix was similar to or higher than that obtained under ambient air, the sensitivity to glucose, which was proportional to the current slope, did not change significantly.

In the first case, under O₂ saturated condition, O₂ gas was used to saturate the O₂ concentration in buffer solution, which provided a large amount of O₂ for the enzymatic reaction by GOD, compared to this, the release of O₂ from H₂O₂ by catalase was in a small amount. Due to the high concentrations of O₂ in the enzyme matrix both in the presence of H₂O₂ and in the absence of H₂O₂ in buffer (a. and b.), the detection ranges were quite similar and both were broadened significantly compared to that under ambient air. The sensitivities in the linear detection ranges were also similar to that under ambient air.

In the second case, under ambient air, the sensor in the presence of H₂O₂ showed a broader detection range for glucose (c. ambient air + H₂O₂) than that in the absence of H₂O₂ (d. ambient air), which was resulted from the higher O₂ concentration in the enzyme matrix due to the release

of O_2 from H_2O_2 by catalase. Both situations (c. and d.) showed similar sensitivities in the linear detection ranges.

In the third case, under O_2 deficit condition, N_2 gas was used to drive out the O_2 in buffer solution contained in the measuring cell to ensure the absence of O_2 supplied by the ambient air in the enzyme matrix. As shown in the figure, in the absence of H_2O_2 (f. N_2), the current response was nearly zero for the detection of glucose, making the determination impossible. While in the presence H_2O_2 in the buffer (e. $N_2 + H_2O_2$), there was sufficient O_2 in the enzyme matrix for the GOD-catalyzed reaction due to the release of O_2 from H_2O_2 by catalase, though the O_2 in the measuring cell supplied by the ambient air had been driven out by N_2 gas, and the sensitivity (e.) in the linear detection range was similar to that under ambient air (d.), as shown in the figure.

Hence, the biosensor using the enzymatic reaction between catalase and H_2O_2 resulted in accurate measurements for glucose under different atmosphere conditions (O_2 saturated condition, ambient air, and O_2 deficit condition) by alleviating the oxygen dependence, which ensures the reliable determination under conditions with O_2 fluctuation or restricted O_2 solubility.

Stabilities of GOD and catalase in measurements

The stabilities of the immobilized enzymes of the oxygen-rich glucose biosensor, including GOD and catalase, were investigated by performing different measurements. During this study, the enzyme matrix on electrode was subjected to a continuous exposure to buffer solution and stressed with an 8-hours continuous stirring.

Figure 4 shows the stabilities of GOD in the enzyme matrices for 250 continuous measurements of glucose in the presence and absence of H_2O_2 . As shown in Figure 4 (A), GOD did not lose its activity when being exposed continuously to H_2O_2 over these measurements, which resulted in a similar stable detection performance as that in the absence of H_2O_2 (Figure 4 (B)) thanks to the inborn high stability of GOD. The slight increases of the signal responses during measurements in both cases were probably due to the slight increases of the GOD activities. Therefore, the oxygen-rich glucose biosensor maintained a high working stability for the detection of glucose.

Figure 5 shows the stability of catalase in the enzyme matrix for 250 continuous measurements of H_2O_2 . Catalase maintained a high stability ($>90\%$) for these measurements, and the slight loss of its activity was probably due to the continuous exposure to H_2O_2 in measurements. Therefore, the enzymatic reaction by catalase and H_2O_2 provides a stable release of O_2 for the oxygen-rich biosensor.

Conclusion

In this work, we have demonstrated a new strategy for the development of an oxygen-rich biosensor using enzymatic reaction between catalase and H_2O_2 . This method improves the determination reliability by alleviating the oxygen dependence. Such biosensor could be used in some in-vitro applications where there are fluctuations of O_2 concentration or restrictions of O_2 solubility. Also, the successful construction of the oxygen-rich glucose biosensor provides new possibilities for the developments of other kinds of oxygen-rich biosensors which involve O_2 as a reactant in reactions by several classes of enzymes (such as oxidase, hydroxylase), or enzyme combinations, etc.

Acknowledgement

The authors greatly acknowledge Hong Kong Research Grant Council for the financial support (HKUST6253-02P and 510-0091-02-44000-0).

References

- Bradley J, Schmid RD (1991) Optimization of a biosensor for insitu fermentation monitoring of glucose concentration. *Biosens. Bioelectron.* **6**: 669-674.
- Cass AEG, Davis G, Francis GD, Hill HAO, Aston WJ, Higgins IJ, Plotkin EV, Scott LDL, Turner APF (1984) Ferrocene-mediated enzyme electrode for amperometric determination of glucose. *Anal. Chem.* **56**: 667-671.

Chan CY, Lehmann M, Tag K, Lung M, Kunze G, Riedel K, Gruendig B, Renneberg R (1999) Measurement of biodegradable substances using the salt-tolerant yeast *Arxula adeninivorans* for a microbial sensor immobilized with poly(carbamoyl) sulfonate (PCS) part I: Construction and characterization of the microbial sensor. *Biosens. Bioelectron.* **14**: 131-138.

Clark LC, Noyles LK, Spokane RB, Sudan R, Miller ML (1986) Design and long-term performance of surgically implantable electroenzymatic glucose sensors. *Ann. N. Y. Acad. Sci.*

Frew JE, Hill HA (1987) Electrochemical biosensors. *Anal. Chem.* **59**: 933A-944A.

Jobst G, Urban G, Jachimowicz A, Kohl F, Tilado O, Lettenbichler I, Nauer G (1993) Thin-film Clark-type oxygen sensor-based on novel polymer membrane systems for in-vivo and biosensor applications. *Biosens. Bioelectron.* **8**:123-128.

Mak KKW, Wollenberger U, Scheller FW, Renneberg R (2003) An amperometric bi-enzyme sensor for determination of formate using cofactor regeneration. *Biosens. Bioelectron.* **18**:1095-1100.

Namdev PK, Irwin N, Thompson BG, Gray MR (1993) Effect of oxygen fluctuations on recombinant *Escherichia-coli* fermentation. *Biotechnol. Bioeng.* **41**: 666-670.

Scheller FW, Hintsche R, Pfeiffer D, Schubert F, Riedel K, Kindervater R (1991) Biosensors-Fundamentals, applications and trends. *Sensor. Actuat. B-Chem.* **4**: 197-206.

Turner APF (2005) Biosensors and bioelectronics 20 years on. *Biosens. Bioelectron.* **20**: 2387-2387.

Wang J (2001) Glucose biosensors: 40 years of advances and challenges. *Electroanal.* **13**: 983-988.

Wang J, Chen L, Chatrathi MP (2000) Evaluation of different fluorocarbon oils for their internal oxygen supply in glucose microsensors operated under oxygen-deficit conditions. *Anal. Chim. Acta* **411**: 187-192.

Wang J, Li SF, Mo JW, Porter J, Musameh MM, Dasgupta PK (2002) Oxygen-independent poly(dimethylsiloxane)-based carbon-paste glucose biosensors. *Biosens. Bioelectron.* **17**: 999-1003.

Wang J, Lu F (1998) Oxygen-rich oxidase enzyme electrodes for operation in oxygen-free solutions. *J. Am. Chem. Soc.* **120**: 1048-1050.

Wang J, Mo JW, Li SF, Porter J (2001) Comparison of oxygen-rich and mediator-based glucose-oxidase carbon-paste electrodes. *Anal. Chim. Acta* **441**: 183-189.

White SF, Tothill IF, Newman JD, Turner APF (1996) Development of a mass-producible glucose biosensor and flow-injection analysis system suitable for on-line monitoring during fermentations. *Anal. Chim. Acta* **321**: 165-172.

Zhang YN, Wilson GS (1993) In-vitro and in-vivo evaluation of oxygen effects on a glucose-oxidase based implantable glucose sensor. *Anal. Chim. Acta* **281**: 513-520.

Figure legends

Fig. 1. Schematic representation for the construction of oxygen-rich glucose biosensor.

Fig. 2. Investigation of the O₂ release from H₂O₂ by catalase (n=3) (Sensor: 2.5 U GOD, a loading of catalase, and 1% nafion in 1 µl of matrix per membrane. Buffer: 50 mM PBS at pH 6.8).

Fig. 3. Detection of glucose under different atmosphere conditions (n=3) (Sensor: 2.5 U GOD, 7.5 U catalase, and 1% nafion in 1 µl of matrix per membrane. Buffer: 50 mM PBS containing 1 mM H₂O₂ at pH 6.8).

Fig. 4. Stabilities of GOD in the enzyme matrices for 250 continuous measurements of glucose performed in (A) 50 mM PBS containing 1 mM H₂O₂ at pH 6.8 and (B) 50 mM PBS at pH 6.8 (n=3) (Sensor: 2.5 U GOD, 7.5 U catalase, and 1% nafion in 1 µl of matrix per membrane. Substrate: 2 mM glucose). The relative response (%) was calculated by normalizing the signal to the initial signal of these measurements.

Fig. 5. Stability of catalase in the enzyme matrix for 250 continuous measurements of H₂O₂ (n=3) (Sensor: 2.5 U GOD, 7.5 U catalase, and 1% nafion in 1 µl of matrix per membrane. Buffer: 50 mM PBS at pH 6.8. Substrate: 1 mM H₂O₂). The relative response (%) was calculated by normalizing the signal to the initial signal of these measurements.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5