ANALYTICA CHIMICA ACTA 612 (2008) 75–82

available at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/aca

A novel approach based on ferricyanide-mediator immobilized in an ion-exchangeable biosensing film for the determination of biochemical oxygen demand

Hailing Chen[a], *Tingxiu Ye*[a], *Bin Qiu*[a], *Guonan Chen*[a], *Xi Chen*[a,b,*]

[a] *Key Laboratory of Analysis and Detection Technology for Food Safety, Ministry of Education, Department of Chemistry, Fuzhou University, Fuzhou 350002, China*

[b] *State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361005, China*

ARTICLE INFO

Article history:
Received 2 December 2007
Received in revised form
31 January 2008
Accepted 1 February 2008
Published on line 9 February 2008

Keywords:
Biochemical oxygen demand
Ferricyanide
Polysiloxane
Ion-association
Seawater

ABSTRACT

A novel biochemical oxygen demand (BOD) sensing method employing a ferricyanide (FC) mediator immobilized in an ion-exchangeable polysiloxane was developed. The ion-exchangeable polysiloxane containing alkylammonium groups (PAPS-Cl) was synthesized by sol–gel reaction of 3-(aminopropyl)trimethoxysilane (APTMOS) catalyzed by hydrochloric acid. FC was combined in PAPS-Cl via ion-association and the product was labeled as PAPS-FC, which was employed for a modified glassy carbon electrode. The apparent diffusion coefficient (D_{app}) of FC on the modified glassy carbon electrode was 9.8×10^{-11} cm^2 s^{-1}. In a three-electrode system, a linear relationship between the anodic current responses and glucose/glutamate (GGA) concentration was obtained up to 40 mg O_2 L^{-1} ($r = 0.994$) when the reaction mixture was incubated for 30 min. Single sensor and piece-to-piece reproducibility were less than 3.8 and 7.7%, respectively. The effects of dissolved oxygen, pH, temperature and co-existing substances on the BOD responses were studied. The sensor responses to nine pure organic substances were compared with the conventional BOD_5 method and other biosensor methods. Detection results of seawater samples were compared with those obtained from the BOD_5 method and showed a good correlation ($r = 0.988$).

1. Introduction

Biochemical oxygen demand (BOD) has been used widely for several decades as an index of organic pollution in industrial wastewater, effluent or natural water. The authorized assay for measuring biodegradable organic levels is the 5-day biochemical oxygen demand (BOD_5) described by the American Public Health Association Standard Methods Committee [1]. BOD_5 is a measure of the quantity of dissolved oxygen consumed by microorganisms during the biological oxidation of organic solutes over a 5-day incubation period. However, this determination requires not only a 5-day period but also complicated procedures and skilled analysts to obtain reproducible results. Therefore, it is time-consuming and not suitable for in situ determinations or on-line measurements [2].

The long duration of the BOD_5 assay is the most significant drawback especially where rapid feedback is essential for environmental monitoring and/or process control. Consequently, considerable effort has been expended on the development of rapid BOD techniques. Since Karube et al. [3] first developed a rapid and reliable biosensor for BOD determination in 1997, several other types of microbial BOD sensor have been

* *Corresponding author at*: State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361005, China. Tel.: +86 592 2184530; fax: +86 592 2184530.
E-mail address: xichen@xmu.edu.cn (X. Chen).

0003-2670/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.aca.2008.02.006

applied [4–9]. Oxygen-based BOD biosensors, however, exhibit low detectivity and narrow response ranges, due to limited dissolved oxygen (8.84 mg L^{-1} O_2 at 1 atm, 20 °C) [10], which is the natural electron acceptor in aerobic microbial catabolism. Fluctuating oxygen level in the sample also causes poor reproducibility and reliability, unless sufficient oxygen supply is incorporated into the biosensor [11].

More recently, bacterial catabolism has been monitored by replacing oxygen with redox mediators that capture electrons from a redox molecule in the electron transport chain [12–15]. An oxidized mediator is reduced during its interaction with a reduced electron transport chain molecule and the reduced mediator is then quantified by an electrochemical method such as amperometry, voltammetry or coulometry. In recent studies [10,16–27], great attention has been paid to ferricyanide (FC), which has been proved to be an efficient mediator for shuttling electrons from the redox center of reduced bacterial enzymes to the electrode surface in the presence of organic compounds. As with conventional aerobic catabolism in the oxidation of organic compounds, the FC-mediated microbial system still involves the oxidation of organic substrate to CO_2 (Eq. (1)). However, the electrons derived from this process, instead of being transferred to O_2 to produce H_2O, are passed to the FC ion, which is reduced to ferrocyanide and then later re-oxidized to FC at a working electrode (anode).

$$\underset{\substack{\text{reduced substrate}\\(\text{i.e. organic material})}}{CH_2O} + H_2O + 4[Fe(CN)_6]^{3-} \xrightarrow{\text{Microorganisms}} \underset{\text{oxidized substrate}}{CO_2} + 4H^+ + \underset{\text{ferrocyanide}}{4[Fe(CN)_6]^{4-}} \quad (1)$$

The most significant advantage of using FC in place of O_2 for BOD assay is its high solubility. This allows for higher microbial populations without the rapid (rate-limiting) depletion of the electron acceptor. Moreover, the need for excessive dilution of samples is greatly reduced. Typical linear working ranges for the FC-mediated BOD assay are from 0 to 200–300 mg O_2 L^{-1} for the standard glucose/glutamate (GGA) solution [10,16,18,24,26]. This is greater than both the authorized method and reported values for BOD biosensors [3,4,6]. However, FC was generally applied in an aqueous solution in the reported FC-mediated biosensing approaches, and its concentration kept at a high level, i.e. 40 mmol L^{-1} [10,27], 55 mmol L^{-1} [16,19,24]. This caused a large waste of FC.

Although some of the FC-mediated BOD sensors previously reported have been designed for environmental application, it seems that the performance of these sensors is still not satisfactory. Their limitations, such as (a) the microorganisms immobilized in current BOD assay are not able to be applied in the samples containing a higher concentration of salt such as seawater and (b) FC was generally applied in an aqueous solution, which made inconvenient for on-line application. To date, to our knowledge, the use of FC immobilized in a thin film as the mediator for determination of BOD has not been reported. In this paper, we reported the synthesis of an ion-exchangeable polysiloxane containing alkyalammonium groups by sol–gel reaction of 3-(aminopropyl)trimethoxysilane (APTMOS) catalyzed by hydrochloric acid. The resulting solution of poly(3-aminopropyl)siloxane hydrochloride (PAPS-Cl) was mixed with FC aqueous solution and then the FC ion was successfully immobilized in the polymer via ion-association. The product, PAPS-FC, was placed on a glassy carbon electrode (GCE), and then TiO_2 sol–gel was placed on the PAPS-FC layer to protect it. We used organically modified silicate (ormosil)–polyvinyl acetate (PVA) as a matrix, noted in Lin et al. [8], in order to immobilize *Exiguobacterium marius, Bacillus horikoshii* and *Halomonas marina* from seawater. Compared to a single microbe, the combination of multi-microorganisms can facilitate the assimilation of a broad range of organic compounds [28]. A GCE with its PAPS-FC layer, TiO_2 and microbial film was used as the working electrode and applied for BOD measurement.

2. Experimental

2.1. Reagents

3-(Aminopropyl)trimethoxysilane (APTMOS) and dimethyldimethoxysilane (DiMe-DMOS) were purchased from Fluka AG (Buchs, Switzerland). Tetramethoxysilane (TMOS) and PVA were purchased from Aldrich (Milwaukee, WI, USA). GGA stock solution was prepared by adding 0.0750 g glucose and 0.0750 g L-glutamic acid to 100 mL phosphate buffer (PB) solution (0.01 mol L^{-1} KH_2PO_4/0.01 mol L^{-1} Na_2HPO_4, pH 7.7). The stock solution corresponds to a BOD of 1000 ± 185 mg O_2 L^{-1} [2]. Lower strength BOD standards were prepared by appropriate dilution of the GGA stock solution in PB solution. All other chemicals used in this study were of analytical reagent grade and all solutions were prepared in deionised distilled water.

2.2. Apparatus

Electrochemical responses were measured with a CHI 660 Electrochemical Analyzer (CHI Co., Shanghai, China). A standard three-electrode cell consisting of an Ag/AgCl (saturated KCl) reference electrode, a platinum wire auxiliary electrode, and a modified GCE working electrode was used. All potentials referred to the Ag/AgCl (saturated KCl) reference electrode. A LEO-1530 scanning electron microscope (SEM) (Oxford Co., UK) with an accelerating voltage at 20 kV was used to record the film images. An EA1110 VarioEL (CARLO ERBA, Italy) was used to analyze the elements. FTIR measurements were used to analyze the chemical bonding, and FTIR spectra were recorded using an FT-IR740SX spectrometer (Thermo Nicolet Co., USA). An FACS Aria flow cytometery (BD Co., USA) was used to control the concentration of the microorganisms. An UV–vis spectrophotometer (DU 7400, Beckman, USA) was used to determine the growth curve of the microorganisms.

2.3. Microorganisms and cell cultures

Three microorganisms, *E. marius*, *B. horikoshii* and *H. marina*, were applied in the BOD sensing film. *E. marius, B. horikoshii* and *H. marina* were obtained from the College of Oceanog-

raphy and Environmental Science, Xiamen University. These microorganisms were cultured in a medium containing 0.1% peptone, 0.1% yeast cream, 0.1% sodium acetate, 0.1% vitamin B and 0.1% microelements in seawater, respectively. All microorganisms were grown under standard aerobic conditions in a rotating shaker at 35 °C for 24 h to achieve their initial stationary phase, and then harvested by centrifugation at 6000 rpm for 10 min at room temperature, washed twice with phosphate buffer of pH 7.7 and resuspended in the same buffer solution. The quantities of microorganisms after a day of cultivation were 2.88×10^9, 2.84×10^9 and 1.97×10^9 cell mL^{-1}, respectively. In addition, the quantities of microorganisms in PB solution cultured asynchronously were adjusted to be 10^9 cell mL^{-1}, which indicated the quantities almost remained constant. The microorganisms were used on the day of cultivation.

2.4. *Immobilization of microbial cells*

Ormosils were prepared by mixing TMOS, DiMe-DMOS and 0.01 mol L^{-1} HCl (1:1.2:1, v/v/v). The precursors were stirred at 60 °C for about 2.5 h. After cooling to room temperature, 500 μL ormosils was mixed with 500 μL 5% (w/w) PVA. Then 200 μL of microorganisms (*E. marius*: *B. horikoshii*: *H. marina*, 1:1:1, v/v/v) in solution were added into 200 μL ormosil–PVA mixture.

2.5. *Fabrication of the biosensor*

Before being used, a GCE (6 mm in diameter) was polished sequentially with 1.0, 0.3 and 0.05 μm α-Al_2O_3, rinsed thoroughly with deionized water after each polishing step, sonicated in deionized water successively and then allowed to dry at room temperature.

2.5.1. *Synthesis of TiO_2 sol–gel*

Tetrabutyl titanate (1 mL) was added in dropwise to a mixture of 16 mL ethanol and 0.7 mL hydrochloric acid in an aqueous solution (1:1, v/v) in an open conical flask, and then polyethylene glycol was added. The reaction solution was magnetically stirred for 6 h. Finally, the product was transferred to a sealed vial and sonicated for 20 min.

2.5.2. *Synthesis of PAPS-Cl and PAPS-FC*

APTMOS was mixed with 0.50 mol L^{-1} HCl (1:10, v/v) in an open vial, and the mixture stirred for 2 h at room temperature. The process for the synthesis of PAPS-Cl is shown in Scheme 1. The ideal chemical formula of PAPS-Cl $[Cl/NH_3\ (CH_2)_3SiO_{1.5}]_n$ [29] was employed. The results of element analysis were: C, 19.5; H, 6.5; N, 8.2; Cl, 30.2; Si, 21.3; O, 14.3%, which were quite similar to the theoretical calculation.

PAPS-Cl solution was mixed with FC (10 mmol L^{-1}) aqueous solution (1:3, v/v), and the solution was placed in the dark until the color of the mixture faded, which indicated that the ferricyanide ion was successfully exchanged to the polymer via ion-association. The solution was poured into a large amount of acetone to precipitate PAPS-FC product. The precipitates were collected by filtration, washed with acetone, and then dried at room temperature. The resulting product PAPS-FC and PAPS-Cl were measured by FTIR. The FTIR spectrum of PAPS-FC exhibited a new peak at 2034 cm^{-1} corresponding to –C≡N stretching, which confirmed that $[Fe(CN)_6]^{3-}$ was combined with PAPS-Cl.

2.5.3. *Fabrication of the BOD_{FC} biosensor*

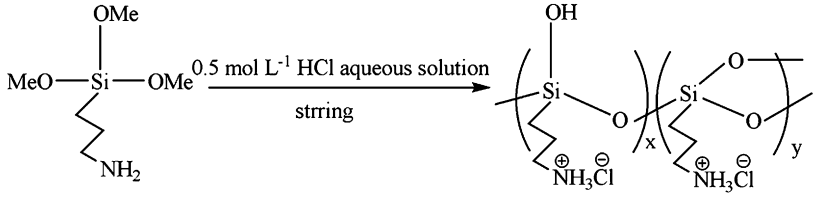
An aliquot (3.0 μL) of the PAPS-FC solution obtained was placed onto the GCE surface and allowed to dry in the dark for 24 h (Fig. 1a). Then the same volume of TiO_2 sol–gel was placed on the PAPS-FC layer to protect it (Fig. 1b) and the modified electrode was successfully fabricated. The thickness of the modified film was estimated to be 30 μm via SEM (Fig. 2). Twenty microliters of the mixture of sol immobilized microbial cells was spread onto the dried modified film in order to construct the BOD_{FC} biosensing film.

3. Results and discussion

3.1. *General properties of $[Fe(CN)_6]^{3-}$ ions in the modified electrode*

Cyclic voltammetry was used to study the redox behavior of $[Fe(CN)_6]^{3-}$ ions in the modified electrode. Fig. 3a shows cyclic voltammograms (CVs) of PAPS-Cl film and PAPS-FC coated by TiO_2 sol–gel on a GCE, respectively. For the electrode coated with PAPS-FC film protected by TiO_2 sol–gel, an obvious reversible CV response, whose anodic and cathodic peak potentials were at 0.267 and 0.212 V. Comparatively, as shown in Fig. 3a-B, no redox current could be found for the PAPS-Cl modified film. The CV profile of PAPS-FC was quite similar to that of ferricyanide in aqueous solution shown in the inset of Fig. 3a. This result indicates that FC ion immobilized in the polymer kept its original electrochemical activity well, which ensured a sufficient sensitivity and reproducibility for the BOD_{FC} application.

Apparent diffusion coefficient (D_{app}) is an important parameter used to study the mechanism of charge transmis-



3-(Aminopropyl)trimethoxysilane PAPS-Cl

Scheme 1 – Synthesis of PAPS-Cl.

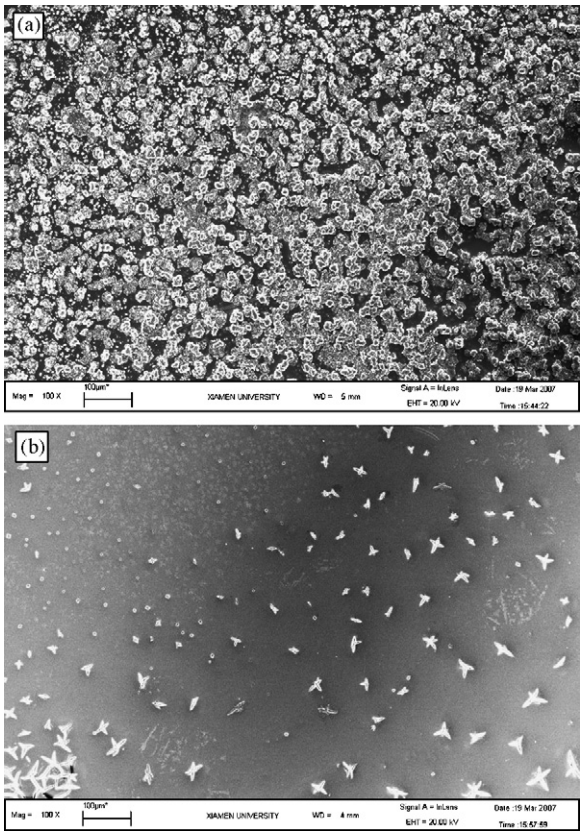


Fig. 1 – SEM images of the GCE coated with (a) PAPS-FC film and (b) the PAPS-FC film protected by TiO_2 sol–gel.

sion in the modified film on an electrode surface. In this study, D_{app} of $[Fe(CN)_6]^{3-}$ ions was estimated using a chronoamperometry [30] method and Cottrell's equation (Eq. (2)),

$$Q(t) = \frac{2nFAD_{app}^{1/2}Ct^{1/2}}{(\pi^{1/2})} \quad (2)$$

where $Q(t)$ is charge at time t, F is Faraday's constant, A is the electrode area in cm^2, n the total number of electrons involved in the electrode reaction, and C the concentration of the redox compound in mol cm^{-3}.

Eq. (2) indicates $Q(t)$ is linear with $t^{1/2}$, and the experiment confirmed this. $Q(t)$ was gained from the integral of anodic

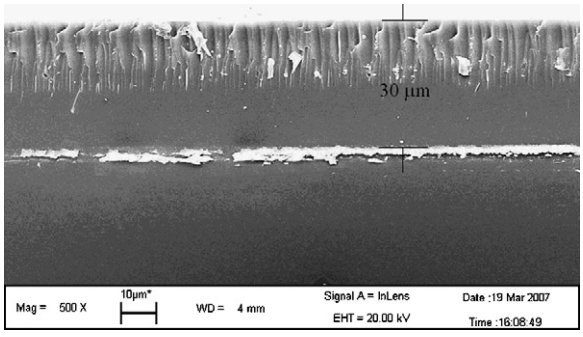


Fig. 2 – SEM image showing the thickness of the PAPS-FC film protected by TiO_2 sol–gel.

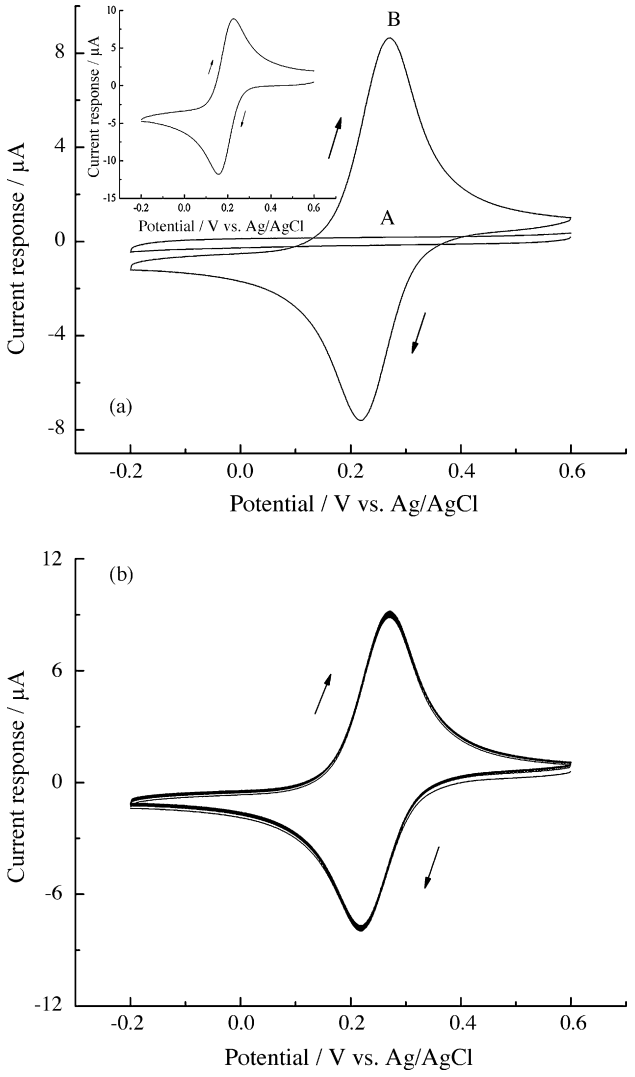


Fig. 3 – (a) Cyclic voltammograms of (A) PAPS-Cl film and (B) PAPS-FC film coated by TiO_2 sol–gel on a GCE separately; and (b) successive potential cycling (100 cycles) of the modified electrode. The inset was CV of ferricyanide in aqueous solution, the electrolyte was 0.01 mol L^{-1} PB solution, the scan rate was 50 mV s^{-1}.

peak swept at low scan rate. Using the plot of $Q(t)$ versus $t^{1/2}$, electrode area and C, the diffusion coefficient was calculated to be 9.8×10^{-11} cm^2 s^{-1}.

3.2. *Effect of the FC ion concentration in the PAPS-FC solution*

The dependence of FC ion concentration in the PAPS-FC solution on the sensor response was examined. When PAPS-Cl was mixed with FC solution (10 mmol L^{-1}) in the ratio of 6:1 (v/v), the resulting FC ion concentration in the PAPS-FC solution was about 1.4 mmol L^{-1} and the PAPS-FC solution was fabricated as the biosensing film. The anodic current response increased proportionally to the GGA solution up to 5.0 mg O_2 L^{-1} (0, 2.5, 5.0 mg O_2 L^{-1}) and then remained constant in the higher concentrations of GGA. The effect of biosensing films with

different concentrations of FC on 100 mg O_2 L^{-1} GGA was studied. The sensing response for 100 mg O_2 L^{-1} GGA solution increased with the increased concentration of FC. Finally, the ratio of PAPS-Cl solution to FC (10 mmol L^{-1}) aqueous solution (1:3, v/v) was adopted.

Compared with the previous reports [10,16,19,24,27], the concentration of FC used in this approach was much lower. In addition, FC was immobilized in the modified film and the dosage of PAPS-FC dropped on the GCE surface was only 3.0 μL. Obviously, less amount of FC was consumed in this method.

3.3. *Ormosils–PVA material for microorganism immobilization*

Although PVA is a kind of nontoxic and hydrophilic material to the immobilization of microbial cells and is capable of maintaining the maximum microbial activity, it readily dilates in water and even flakes away from the microbial film. The mixing PVA with ormosil can effectively overcome these shortages. The presence of DiMe-DMOS in the ormosil increases the film hydrophobicity since two methyl groups in DiMe-DMOS do not undergo hydrolysis through the typical sol–gel process. Si–CH_3 group replaces the Si–OH groups in DiMe-DMOS consequently enhances the surface hydrophobicity. Furthermore, the increase of DiMe-DMOS content in the sensing film improves its response and the flexibility. However, further increase the DiMe-DMOS content will not guarantee the sufficient solubility of PVA. Under our preparation conditions, the optimized volume ratio of DiMe-DMOS to TMOS was selected as 1.2:1. In addition, although a higher PVA content in the BOD sensing film caused higher response intensity and less response time, the film began to dilate and flake away from microbial film when the percentage of PVA reached at 8%. In the experiment, a content of 5% PVA in ormosil was selected.

3.4. *Effect of dissolved oxygen, pH, temperature and co-existing substances*

The influence of dissolved oxygen on the sensor response was studied. Sample solutions with different BOD values were oxygen saturated or deaerated by purging oxygen-free nitrogen gas for 20 min. For a definite sample solution, there was no significant difference comparing the response obtained from oxygen saturated state or that obtained from the deaerated solution, which confirmed that ferricyanide is a preferable electron acceptor to oxygen under the experimental condition.

The physiological state of the microorganisms, in particular their respiratory activity, strongly depends on the solution pH. BOD responses in different pH solution from 5.5 to 8.3 were investigated at a constant BOD concentration of 100 mg O_2 L^{-1}. The response current increased with the increase of solution pH, reached a maximum value at pH 7.3 and then decreased slightly. Generally, according to the measurement, the pH of the seawater around Xiamen area is about 7.7. In subsequent experiments, the solution pH was adjusted to 7.7 using phosphate buffer solution.

Temperature is an important factor affects the activity and metabolism of microorganism. An appropriate temperature promotes the respiration and the activation of microorganisms. For the film of sieved bacteria from seawater, the optimum temperature was studied in the range 15–45 °C, and found that a suitable temperature was 35 °C. The current response reversely decreased when the temperature exceeded the value due to the inactivation and denaturalization of microorganisms in the sensing film. Considering the stability of PAPS-FC modified film turned worse at a higher temperature due to the quick leakage of $[Fe(CN)_6]^{3-}$ from PAPS-FC, 25 °C was chosen in the experiment for longer lifetime of BOD sensor.

Chloride ion is a common component of seawater with an average concentration of 3.2%. In general, chloride concentration affects the activity of microorganisms, but the activity of the selected microorganisms was not obvious change since the microorganisms were all selected and cultivated from the seawater. In addition, although the higher concentration of chloride ion might affect the stability of PAPS-FC film simultaneously due to the electrostatically driven exchange mechanism [31]. Therefore, the electrolyte containing only phosphate buffer (0.01 mol L^{-1}) was selected.

The effects of co-existing compounds including some metal ions at 1 mg L^{-1} on the current response in 10 mg O_2 L^{-1} GGA solution were investigated. As shown in Table 1, the prepared BOD sensing films defended the effects of most metal ions well, but Ag^+, Pb^{2+} or Cu^{2+} led to an obvious decrease for the response by 12.68, 10.75 and 8.12% owing to their toxicity to the microorganisms. Fortunately, the concentrations of toxic cations are generally at a lower level in seawater.

3.5. *Stability of the sensor*

Under the selected optimum conditions, the sensor was immersed in PB solution and the stability of the sensor was evaluated by monitoring its CV response. The concentration of the supporting electrolyte affects the sensor stability since a competitive exchange between the supporting electrolyte and $[Fe(CN)_6]^{3-}$ occurred on the sensing film. Obviously, although a suitable concentration of the supporting electrolyte

Table 1 – Effect of some coexisting metal ions on the response of BOD_{FC} sensor

M^{n+}	$\Delta I/I_0$ (%)	M^{n+}	$\Delta I/I_0$ (%)	M^{n+}	$\Delta I/I_0$ (%)	M^{n+}	$\Delta I/I_0$ (%)[a]
Al^{3+}	−4.58	Fe^{3+}	4.26	Ba^{2+}	−4.12	Sn^{2+}	2.32
Cu^{2+}	−8.12	Zn^{2+}	−3.58	Ni^{2+}	2.52	Pb^{2+}	−10.75
Co^{2+}	1.62	Mg^{2+}	3.54	Ag^+	−12.68	–	–
Cr^{3+}	−1.32	Mn^{2+}	−3.03	Ca^{2+}	2.21	–	–

[a] $\Delta I = I - I_0$, where I was the current response after coexisting substance added, and I_0 was the current response without coexisting substance.

was a necessary factor for electrochemical measurement, higher concentration of supporting electrolyte was not suitable for the $[Fe(CN)_6]^{3-}$ immobilization. In the experiment, 0.01 mol L^{-1} PB solution was selected. According to the experimental results, the sensor current response became stable after five reduplicate measurements. In the following 0.5–4 h application, only a 5.2% current intensity decrease could be found for the prepared sensor.

3.6. Calibration curve for GGA

Various concentrations of the standard GGA solution were applied to obtain a calibration curve. Before the determination, the working electrode was immersed in 0.01 mol L^{-1} PB solution at pH 7.7 for about 0.5 h until the response current was steady (Fig. 3b). Then the corresponding anodic current of each concentration of GGA solution was recorded after the biosensing film was soaked in the GGA solution for 30 min incubation. Cyclic voltammograms of BOD_{FC} biosensor in the absence and the presence of GGA solution were given in Fig. 4a. The current response increased with the addition of GGA solution. As shown in Fig. 4b, the biosensor presented a linear relationship with the anodic current intensity of the BOD_{FC} biosensing film and GGA solution over the range of 1.2–40 mg O_2 L^{-1} ($y = 2.907 + 0.037x$, $r = 0.994$, $n = 5$). The detection limit was calculated as 0.8 mg O_2 L^{-1} according to the specific formula [32]. The repeatability for each single sensor was evaluated at 20 mg O_2 L^{-1} GGA solution (seven replicates). The relative standard deviation was calculated to be 3.8%. The reproducibility of the sensor was performed using six biosensing films prepared in the same batch, and was 7.7% at the same concentration of GGA.

3.7. Sensor responses to organic substances

BOD determination is used to determine the amount of oxygen which is utilized as microorganisms oxidize biodegradable organic substances in various water samples. A BOD sensor should, therefore, have the ability to detect a wide range of organic substances. Hence, the BOD values for various kinds of pure organic substances were measured using the BOD_{FC} sensor to confirm the characteristics of the method. The BOD values obtained by the sensing film were determined using the same procedure described above for GGA measurement. A solution of each pure organic substance was prepared at 50 mg L^{-1} and each sample was repeatedly analyzed five times. Table 2 indicates the substrate specificity of the BOD_{FC} sensor method relative to the reported data from BOD_{DM} [10],

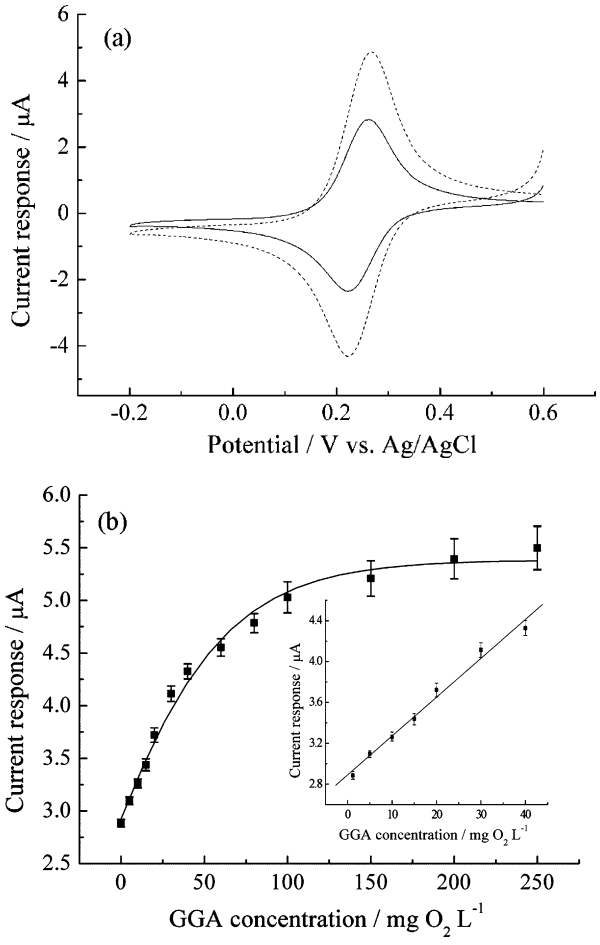


Fig. 4 – (a) Cyclic voltammograms of BOD_{FC} biosensor in the absence (solid line) and the presence of 100 mg O_2 L^{-1} GGA (dash line) and (b) calibration curve for GGA. The inset indicates the linear response (0.01 mol L^{-1} PB solution, and the scan rate was 0.01 V s^{-1}). Each sample was repeatedly analyzed five times.

Table 2 – Comparison of BOD values of various organic samples

Substrate	BOD_{FC} [a]	BOD_{DM} [10]	BOD_{SM} [25]	BOD_{SPV} [33]	BOD_{LUM} [34]	BOD_5 [10]
Glucose	0.65	1.13	1.54	0.66	0.62	0.50–0.78
Fructose	0.38	0.74	0.35	0.73	0.57	0.71
Histidine	0.52	0.63	0.27	0.34	–	0.55
Sucrose	0.42	0.40	0.35	0.45	0.50	0.49–0.76
Glycine	0.36	0.25	–	0.36	0.50	0.52–0.55
Citric acid	0.48	0.18	–	0.18	–	0.63–0.88
Glutamic acid	0.16	0.15	0.59	0.40	0.73	0.64
Soluble starch	0.12	0.03	–	0.07	0.02	0.22–0.71
Lactose	0.02	0.07	0.02	0.04	0.31	0.45–0.72

Values are expressed in mg O_2 mg^{-1} substrate.

[a] Concentration of each pure organic substance was 50 mg L^{-1}. Each result represents the average of five reduplicate measurements.

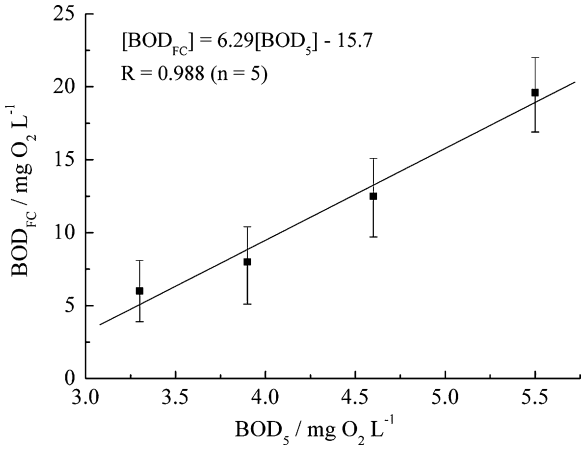


Fig. 5 – Correlation of the BOD values for sea samples calculated using the BOD_{FC} method with those obtained using the BOD_5 method (28 April 2006, sampling places: E118°06′04.0″, N24°26′00.9″; E118°05′31.4″, N24°26′07.8″; E118°05′53.0″, N24°26′05.0″; E118°06′32.1″, N24°25′53.4″; $n = 5$).

BOD_{SM} [25], BOD_{SPV} [33], BOD_{LUM} [34] and BOD_5 [10] for these compounds.

It was obvious that the sensing response to glucose was higher than that to the other substances. This was probably due to the activity of glucose dehydrogenase located on the cytoplasmic membrane and also to the metabolic processes involved in the respiratory chain. The responses of sucrose and histidine obtained by our BOD_{FC} sensing film were quite similar to those obtained by BOD_5. While the most important requisite for the microorganisms used with the BOD sensor is to have a wide substrate spectrum for degradation, it would be extremely unlikely to present the same spectra for different microbial species. Therefore, the results indicated that the BOD_{FC} approach is sufficiently adequate to be applied to BOD determination.

3.8. *Real samples application*

Generally, the BOD value of clean seawater is lower than 5 mg O_2 L^{-1}. The BOD sensor was used to determine the BOD values of seawater from around Xiamen University, China, and the results were compared with the standard method BOD_5 (Fig. 5). Seawater samples from different sampling sites were filtered through a membrane filter (10 μm) before being tested using the BOD_{FC} method. The activity of microorganisms was evidently increased after an effective activation. As can be seen from Fig. 5, BOD_{FC} values were generally larger than those obtained using BOD_5 method. A main reason could be considered that microorganisms in the BOD_{FC} sensing film was adequately activated, which increased the ability of microorganisms to decompose organic substances. Although visible standard deviations were found in Fig. 5, the obtained results by the BOD_{FC} and BOD_5 methods presented a good correlation ($r = 0.988$, $n = 5$). These results demonstrated that the BOD_{FC} method is valid for the determination of BOD in seawater.

4. Conclusions

In this study, we constructed a novel FC-mediated biosensor by immobilizing *E. marius*, *B. horikoshii* and *H. marina* from seawater. The mediator, FC, was combined on the polymer film via ion-association. A calibration curve ranging from 1.2 to 40 mg O_2 L^{-1} was obtained, and the minimum measurable BOD was 0.8 mg O_2 L^{-1}. In addition, the BOD_{FC} sensing responses to pure organic substances were examined, and the results were synchronously compared to the conventional BOD_5 method and other BOD sensor methods. In this investigation, the BOD_{FC} method offers a wide range of assimilability to several categories of organic substances. Finally, comparing the responses of seawater samples obtained by BOD_{FC} with the standard BOD_5 method, the BOD_{FC} method was proved to be valid for BOD determination.

Acknowledgements

This research was financially supported by the National High Technical Development Project (863 project) Foundation (2006AA09Z160), the National Nature Scientific Foundation of China (NSFC, No. 20775064) and the key project of NSFC (20735002), which are gratefully acknowledged. We express our sincere thanks to Professor John Hodgkiss of The University of Hong Kong for his assistance with English and Professor Nianzhi Jiao, State Key Laboratory of Marine Environmental Science, Xiamen University, for his kind provision of the microorganisms.

REFERENCES

[1] Standard Methods for the Examination of Water and Wastewater, 19th ed., American Public Health Association, 1995.
[2] Japanese Industrial Standards Committee, JISK 0102, Japanese Standards Association, Tokyo, 1989.
[3] I. Karube, T. Matsunage, S. Mitsuda, S. Suzuki, Biotechnol. Bioeng. 19 (1997) 1535.
[4] Z.R. Qian, T.C. Tan, Water Res. 32 (1998) 801.
[5] K. Tag, M. Lehmann, C. Chan, R. Renneberg, K. Riedel, G. Kunze, Sens. Actuators B 67 (2000) 142.
[6] I.S. Chang, J.K. Jang, G.C. Gil, M. Kim, H.J. Kim, B.W. Cho, B.H. Kim, Biosens. Bioelectron. 19 (2004) 607.
[7] S. Rastogi, A. Kumar, N.K. Mehra, S.D. Makhijani, A. Manoharan, V. Gangal, R. Kumar, Biosens. Bioelectron. 18 (2003) 23.
[8] L. Lin, L.L. Xiao, S. Huang, L. Zhao, J.S. Cui, X.H. Wang, X. Chen, Biosens. Bioelectron. 21 (2006) 1703.
[9] L.L. Xin, X.D. Wang, G.M. Guo, J.S. Cui, X.H. Wang, X. Chen, Meas. Sci. Technol. 18 (2007) 2878.
[10] H. Nakamura, K. Suzuki, H. Ishiknro, S. Kinoshita, R. Koizumi, S. Okuma, M. Gotoh, I. Karube, Talanta 72 (2007) 210.
[11] K. Riedel, Bioelectrochem. Bioenerg. 25 (1991) 19.
[12] G. Ramsay, A.P.F. Turner, Anal. Chim. Acta 215 (1988) 61.
[13] P. Ertl, E. Robello, F. Battaglini, S.R. Mikkelsen, Anal. Chem. 72 (2000) 4949.
[14] P. Ertl, E. Robello, F. Battaglini, S.R. Mikkelsen, Anal. Chem. 72 (2000) 4957.

[15] P. Ertl, S.R. Mikkelsen, Anal. Chem. 73 (2001) 4241.
[16] K. Morris, K. Catterall, H.J. Zhao, N. Pasco, R. John, Anal. Chim. Acta 442 (2001) 129.
[17] K. Morris, H.J. Zhao, R. John, Aust. J. Chem. 58 (2005) 237.
[18] N. Pasco, K. Baronian, C. Jeffries, J. Hay, Appl. Microbiol. Biotechnol. 53 (2000) 613.
[19] N. Pasco, K. Baronian, C. Jeffries, J. Webber, J. Hay, Biosens. Bioelectron. 20 (2004) 524.
[20] N. Pasco, J. Hay, A. Scott, J. Webber, Aust. J. Chem. 58 (2005) 288.
[21] K.H.R. Baronian, A.J. Downard, R.K. Lowen, N. Pasco, Appl. Microbiol. Biotechnol. 60 (2002) 108.
[22] S.P. Trosok, B.T. Driscoll, J.H.T. Luong, Appl. Microbiol. Biotechnol. 56 (2001) 550.
[23] K. Catterall, K. Morris, C. Gladman, H.J. Zhao, N. Pasco, R. John, Talanta 55 (2001) 1187.
[24] K. Catterall, H.J. Zhao, N. Pasco, R. John, Anal. Chem. 75 (2003) 2584.
[25] N. Yoshida, K. Yano, T. Morita, S.J. McNiven, H. Nakamura, I. Karube, Analyst 125 (2000) 2280.
[26] N. Yoshida, J. Hoashi, T. Morita, S.J. McNiven, H. Nakamura, I. Karube, J. Biotechnol. 88 (2001) 269.
[27] N. Yoshida, J. Hoashi, T. Morita, S.J. McNiven, K. Yano, H. Nakamura, I. Karube, Analyst 126 (2001) 1751.
[28] L. Suriyawattanakul, W. Surareungchai, P. Sritongkam, M. Tanticharoen, K. Kirtikara, Appl. Microbiol. Biotechnol. 59 (2002) 40.
[29] Y. Kaneko, N. Iyi, T. Matsumoto, K. Fujii, K. Kurashima, T. Fujita, J. Mater. Chem. 13 (2003) 2058.
[30] F.C. Anson, Anal. Chem. 36 (1964) 975.
[31] C.C. Hsueh, M.M. Collinson, J. Electroanal. Chem. 420 (1997) 243.
[32] J.W. Si, Complication of Marine Environment Parameters, Monitoring Technologies and Detection Methods, 1st ed., Oceanpress, Beijing, 2005, p. 204.
[33] Y. Murakami, T. Kikuchi, A. Yamaura, T. Sakaguchi, K. Yokoyama, Y. Ito, M. Takiue, H. Uchida, T. Katsube, E. Tamiya, Sens. Actuators B 53 (1998) 163.
[34] C.K. Hyun, N. Inoue, E. Tamiya, T. Takeuchi, I. Karube, Biotechnol. Bioeng. 41 (1993) 104.