University of Alberta

Development of a Sulfate Microelectrode for Profiling Environmental Biofilms

by

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Abstract

Microelectrodes can be used for profiling environmental biofilms. Currently there is no sulfate microelectrode available for studying sulfate reduction in biofilms. In this study, a sulfate microelectrode was fabricated and tested in measurement of environmental biofilms for the first time. The composition of the sulfate-sensing membrane in the microelectrode was optimized. The sulfate microelectrode, with a tip diameter of 20 μm, displayed satisfying selectivity to SO_4^{2-}. It exhibited log-linear response to sulfate concentration change with a Nernstian slope of -25.8 mV per concentration decade. The linear detection range was 10^{-5} M to 10^{-2} M SO_4^{2-} with a detection limit of 10^{-5} M SO_4^{2-} in water. The response time was 90 seconds. The sulfate microelectrode was applied on environmental biofilm samples. The sulfate profile obtained indicated the sulfate concentration change inside the biofilm. In conclusion, a sulfate microelectrode with satisfying characteristics was fabricated successfully and was useful for characterizing environmental biofilms.

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1.0 Introduction

1.1 Overview

A sensor is a device that converts a nonelectrical input quantity into an electrical output signal (Gardner *et al*., 2001). A microsensor, usually with a small tip-diameter of 1-20 μm (Santegoeds *et al*., 1998), is a miniature sensor. Microsensors include electrochemical microsensors and fiberoptic microsensors. The electrochemical microsensors can be further divided into three groups: potentiometric microelectrodes, amperometric microelectrodes, and micro-biosensors. The microsensor technique has been extensively applied in environmental research because it has considerable advantages: it can measure chemical and physical variables with high spatial resolution; it allows for determination of microenvironmental conditions in environmental samples with little interference from other factors; and is suitable for *in situ* measurement with little disturbance to the samples. Examples of environmental samples that have been studied using microsensors are sediments (Reimers, 1987), soils (Meyer *et al*., 2002), and biofilms (Bungay *et al*., 1969).

A biofilm is a complex aggregate of microorganisms attached to inert surfaces (Lewandowski and Beyenal, 2007). Biofilms are present everywhere, in surface and ground waters, in drinking water pipes and wastewater treatment

plants, and on other technical equipment (e.g., in medical offices and laboratories), and even on human teeth (Wanner and Bauchrowitz, 2006). These natural and engineered systems are influenced by environmental biofilms with undesirable effects: microbiologically influenced corrosion of metals and biofilm caused problems in drinking water or food processing; however, biofilms have positive functions such as decontamination of polluted water. Therefore, a better understanding of biofilms will help people manage their negative effects and take advantage of their positive effects.

One of the positive effects of the biofilm is its application in environmental engineering to effectively treat wastewater (e.g. sulfate-rich wastewater). In various kinds of biofilm reactors where sulfate-rich wastewater was treated, sulfate reduction is an important microbial process in biofilms. Sulfate can be reduced biologically to sulfide under anaerobic conditions, and sulfide can combine with hydrogen to form hydrogen sulfide (H_2S) or combine with metal to form metal sulfide usually in the form of precipitation. It is important to understand this sulfur circulation process inside biofilms because it has significant effect on performance and maintenance of biofilm reactors and it is the theoretical foundation for the design of biofilm reactors as well.

The dynamics of sulfate reduction inside biofilms have been investigated by microelectrodes for H_2S (Kuhl *et al*., 1998) and S^{2-} (Kuhl and Jorgensen, 1992), O_2 (Revsbech, 1989) and pH (Ito *et al*., 2002) separately or in

combination. To our knowledge, however, the internal sulfur cycle in biofilms is not yet well described in terms of the mass balance between biofilm and bulk solution. The deficiency is largely due to the lack of microelectrode for determination of sulfate in micro-environment. Therefore, this study aims to develop a sulfate microelectrode, which is needed to directly study the sulfate concentration within biofilms so that the overall efficiency of sulfate reduction can be determined.

1.2 Objectives

The overall objectives of this study were to develop a sulfate microelectrode for the determination of sulfate in biofilms and improve the characteristics of the sulfate microelectrode.

2.0 Literature Review

This section summarizes analytical methods for biofilms characterization, introduces theory of ion-selective electrodes (ISEs), and reports research progress on sulfate electrodes.

2.1 Analytical methods for biofilms characterization

Biofilms in water and wastewater treatment have been studied by conventional methods such as the characterization of biofilm mass, biofilm density, total protein and adenosine-triphosphate (ATP) (Lazarova and Manem, 1995). However, these parameters are not sufficient to describe biofilm activity. With the development of advanced technologies, there have been more analytical methods available for characterization of biofilms (Denkhaus *et al*., 2007): molecular techniques, separation techniques, detectors, surface and interface characterizing techniques, spectrometry, and microsensors. These interdisciplinary methods would allow for better investigation of different aspects of biofilms. Among them, microscopic, molecular, and microsensor techniques are three popular techniques.

Microscopy, belonging to surface and interface characterizing techniques, is a basic tool used for understanding any biological process such as assessment

of microbial populations. Confocal Laser Scanning Microscopy (CLSM) is an important microscopic technique and provides optical images of biological samples. It is a powerful tool to study biofilms because it can obtain biofilm images of the fine structure of the interior of the biofilms by an optical sectioning process. Compared to conventional microscopy, it allows for three-dimensional (3D) reconstruction of a complex biofilm sample which is alive and fully hydrated without fixation and dehydration. However, the CLSM also has some drawbacks such as low resolution and being unsuitable for thick and opaque biofilms. Nevertheless, CLSM is an excellent image analysis method for biofilm structure observation and characterization.

Recently various kinds of molecular techniques have increasingly become popular for studying the species composition of microbial communities and the spatial distribution of their inhabitants. The molecular techniques include nucleic acid probes (oligonucleotide probe, DNA microarray) of specific bacterial DNA sequences from target microorganisms, pulsed field gel electrophoresis (PFGE), denaturing-gradient gel electrophoresis (DGGE), fluorescent *in situ* hybridization (FISH), and polymerase chain reaction (PCR) for amplification of DNA (Denkhaus *et al*., 2007). The molecular techniques can identify different bacterial cells in biofilms, thereby contributing to a better understanding of spatial organization and genetic activities of microbial communities in biofilms. Aoi (2002) reviewed advances and limitations in

molecular techniques, particularly focusing on FISH and related techniques. FISH is a cytogenetic technique used to detect specific DNA sequences on chromosomes. Fluorescent probes are used to bind to parts of chromosomes. The hybridized cells can be observed by fluorescent microscopy. This technique is a powerful tool to identify specific bacteria and microbial diversity in biofilms. It is easy to perform and is fast to obtain accurate results. The major limitation is the requirement of specific DNA fragments that recognize specific bacterial DNA sequences.

Microsensors are powerful tools to determine microenvironment conditions and *in situ* bacterial activity by measuring chemical activities in biofilms with high spatial resolution (Santegoeds *et al*., 1998). Most microsensors used in biofilm research are electrochemical microsensors. A considerable number of electrochemical microsensors have been developed for measuring different kinds of chemical species. Among them, the amperometric microelectrodes can determine oxygen (Revsbech, 1989), hydrogen sulfide (Jeroschewski *et al*., 1996), hydrogen (Ebert and Brune, 1997), and nitrous oxide (Revsbech *et al*., 1988) concentrations. Potentiometric microelectrodes that have been successfully developed include pH (Kohls *et al*., 1997), ammonium (de Beer and Van Den Heuvel, 1988), nitrate (de Beer and Sweerts, 1989), nitrite (de Beer *et al*., 1997), and sulfide (Revsbech *et al*., 1983) microelectrodes.

Amperometric microelectrodes measure the current generated by

electrochemical reaction of the analyte occurred at the tip of the microelectrode (Santegoeds *et al*., 1998). The current, measured by a picoammeter, is proportional to the concentration of the analyte. The amperometric microelectrodes are relatively mature and frequently used technologies due to their better selectivity and long lifetime. The oxygen microelectrode is a well-known and widely used amperometric microelectrode for its great stability and excellent performance. As the type of oxygen microelectrodes evolved from the separate (the reference electrode is external) to the combined (the working, reference, and guard electrode are in one body), the performance of oxygen microelectrodes has improved dramatically. Revsbech and Ward (1983) constructed an oxygen microelectrode with stable signal, pH independent, and insensitivity for interference ions. A combined oxygen microelectrode was fabricated, evaluated, and applied in a field study to measure dissolved oxygen (DO) concentration in wastewater biofilms (Lu and Yu, 2002). The study showed that the oxygen microelectrode had fast response time and stable reading with good calibration linearity. The DO concentration profile indicated that oxygen penetration in the biofilm was less than 0.5 mm. The application of oxygen microelectrodes was reviewed by Lu and Yu (2002) as well.

Potentiometric microelectrodes are based on charge separation of ions across a membrane (Santegoeds *et al*., 1998). An electrical potential difference is generated between the working electrode and the reference electrode, and

related to the concentration of the analyte. The potentiometric microelectrodes are easy to fabricate but have short lifetime. Ammonium, nitrate, and pH microelectrodes were used to evaluate substrate and pH effects in a nitrifying biofilm (Zhang and Bishop, 1996). The ammonium, nitrate, and pH profiles were obtained to demonstrate that either a pH decrease of 1.4-1.6 units or an increase in glucose or ammonium loading caused inhibition of the biofilm nitrification process. De Beer *et al*. (1997) employed nitrate and nitrite microelectrodes for nitrification and denitrification studies on wastewater biofilms. High nitrite concentrations were found in narrow zones of less than 1 mm in the biofilms.

Microelectrode measurements have been used for the study of sulfate reduction in various environmental biofilm samples. A sulfide microelectrode combined with oxygen and pH microelectrodes was used to study sulfide dynamics in aerobic biofilms by Kuhl and Jorgensen (1992). It was demonstrated that oxygen respiration occurred in the upper layer of the biofilm, while sulfate reduction happened in anoxic layer of the biofilm. A microelectrode for detection of hydrogen sulfide (H_2S) in sediments and biofilms was developed by Kuhl *et al*. (1998). The first profile of H_2S was obtained by using the well-performed microeletrode. Their data showed that sulfate reduction occurred in the deeper anoxic part of the sediment.

A combination of techniques has been effectively applied in studies of

sulfate reduction in biofilms. For example, both microelectrodes and molecular techniques were first used to study sulfate reduction in a trickling-filter biofilm by Ramsing *et al*. (1993). The distribution of sulfate reducing bacteria (SRB), O_2, and H_2S was determined in photosynthetic biofilms by using oligonucleotide probes and microelectrodes. The SRB existed heterogeneously in biofilms. The study indicated a negative correlation between spatial distribution of SRB and O_2 profiles. Okabe *et al*. (1998) studied microzonation of O_2 respiration, H_2S oxidation, and sulfate reduction by microelectrodes and determined vertical distribution of SRB in aerobic biofilms grown on rotating disk reactors by oligonucleotide probes. Both results corresponded well showing that sulfate reducing activity was restricted to a narrow anaerobic zone in the middle of the biofilm.

In conclusion, a combination of advanced techniques is available to provide more direct information on relationships between *in situ* spatial organization of microorganisms and their *in situ* activity in biofilms.

2.2 Theory of ISEs

Defined by the International Union of Pure and Applied Chemistry (1976), ISEs are potentiometric sensors whose potentials linearly depend on the logarithm of the activity of a given ion in the solution being measured. The ISEs

contain a sensing membrane that responds to a given ion selectively. The electromotive force (EMF) is generated between the working electrode and the reference electrode so that it can be used to calculate the activity of the specific ion. The cell assembly is shown in Fig. 1.

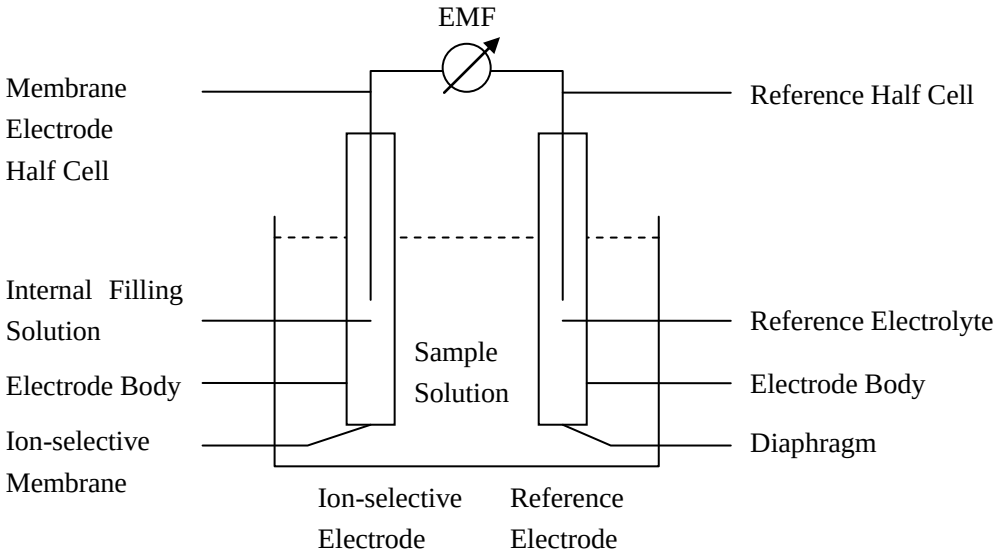

Figure 1 Schematic cell assembly of an ISE (Adapted from Ammann, 1986)

There are three main types of ISEs: solid state, glass, and liquid membrane electrodes. In this study, a sulfate microelectrode is a liquid membrane ISmE which is a miniaturization of ISE. A liquid membrane ISmE with its tip configuration is shown in Fig. 2.

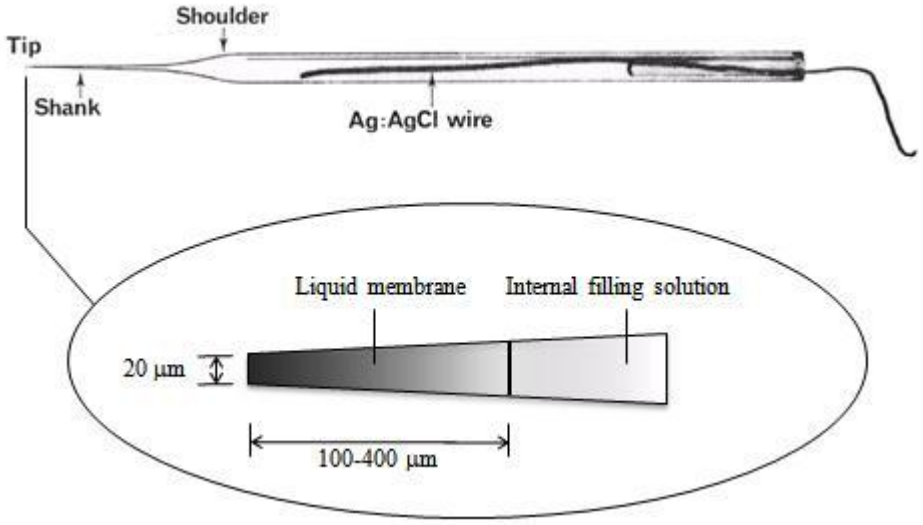

Figure 2 A liquid membrane ISmE with its tip configuration (Adapted from Tomas, 1978)

The liquid membrane ISmE has the following advantages: (1) it is a non-destructive method of measuring ions; (2) it does not change the activity of ions in the sample; and (3) the fabrication procedure is straightforward.

2.2.1 Characteristics of ISmE

The performance of an ISmE can be evaluated by several characteristics (Miller and Wells, 2006): slope, selectivity, detection limit, and response time. A good ISmE should have a near ideal slope, a small selectivity coefficient for interference ions, a low detection limit, and a fast response time.

A potential difference is generated between internal filling solution and

sample solution. The ideal relationship between the potential and activity of the ion of interest, i, is log-linear, following the Nernst equation. In most cases, more than one ion is present in sample solutions and the ISmE does not have ideal selectivity for the ion of interest. Thus, the interference ion, j, present in sample solutions should be taken into account as it contributes to the measured EMF. An extended Nicolsky-Eisenman equation was proposed to deal with this situation:

$$\mathrm{EMF} = \mathrm{E}_0 + \mathrm{s}\log\left[a_i + \sum_j K_{ij}^{pot} (a_j)^{Z_i/Z_j} \right] \quad (1)$$

where

EMF: electromotive force [mV];

E_0: reference potential [mV];

s: Nernstian slope, $\mathrm{s} = \frac{2.303RT}{Z_i F} = 59.2/Z_i$ ([mV/decade], 25℃);

a: ion activity;

R: gas constant (8.314 J $K^{-1}mol^{-1}$);

T: absolute temperature [K];

F: Faraday equivalent (9.6487×10^4 C mol^{-1});

Z_i: charge number of the ion, i;

K_{ij}^{pot}: potentiometric selectivity coefficient.

The selectivity coefficient is a measure of the preference of the electrode for the detected ion, i, with respect to the interfering ion, j. For an ideal ISmE,

all of the selectivity coefficients should be zero. In practice, a selectivity coefficient less than 1 indicates a preference for the detected ion, i, relative to the interfering ion, j. The selectivity coefficient can be determined by three methods: the separate solution method, the fixed interference method, or the fixed primary ion method. The selectivity coefficients are not constant parameters under all conditions. They are dependent on both the method used and on the conditions of the measurement.

A schematic representation of an ideal ISmE calibration curve is shown in Fig. 3. At 25 ℃, the ideal slope, s, is 59.2 mV/decade for a monovalent cation, i; -59.2 mV/decade for a monovalent anion, i; 29.6 mV/decade for a bivalent cation, i; and -29.6 mV/decade for a bivalent anion, i.

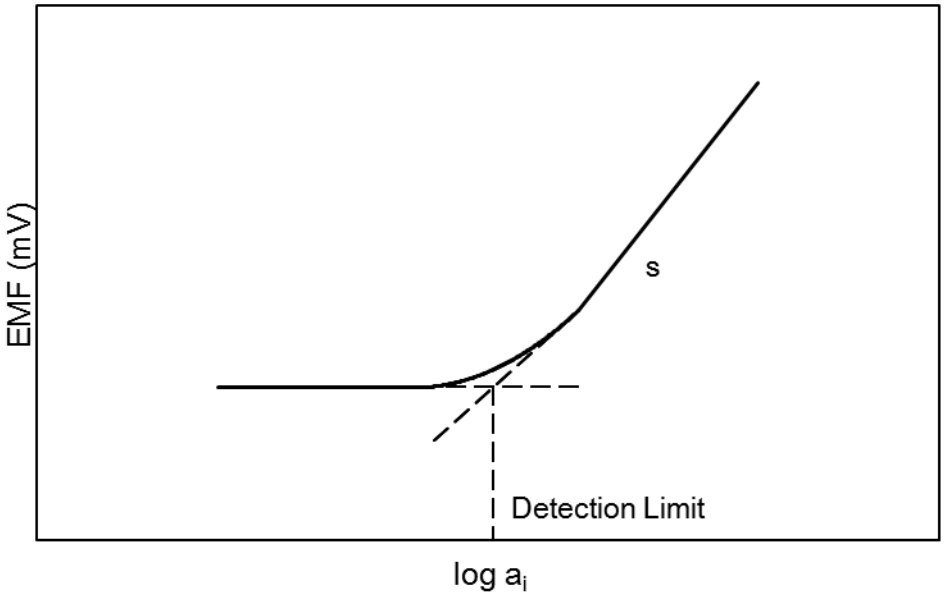

Figure 3 An ideal calibration curve of an ISmE (Adapted from Ammann, 1986)

The detection limit is the lowest ion activity that can be detected, which is the intercept of the two asymptotes of the linear response curve and the non-linear response curve. The sensor exhibits linear response at high activities but deviates from linearity at low activities. The detection limit may depend on the tip geometry and composition of the ion-selective membrane. It can also be affected by the presence of interfering ions. No useful information about ion activity can be provided below the detection limit and it is better to use the linear portion of the calibration curve.

The response time is another important parameter of an ISmE. It is the length of time that elapses between the point when an ion-selective electrode and a reference electrode are brought into contact with a sample solution and the point when the potential is within l mV of its steady-state value (IUPAC, 1976).

The experimental conditions used should be stated, i.e., the stirring rate, the composition of the solution, the composition of the solution to which the sensor was exposed prior to this measurement, the history and preconditioning of the sensor, and the temperature at the time of measurement.

2.2.2 Ion selective membrane

The ion selective membrane, also called cocktail or liquid membrane, is the most important substance in an ISmE because it determines microelectrode

properties. It generally includes four main components: ionophore, plasticizer, additive, and matrix. The ionophore is the critical component that is sensing the ion of interest. The plasticizer forms the bulk of the cocktail. It serves the purpose of solubilizing ionophore and lipophilic additives. The properties of lipophilicity, viscosity, and the ability to plasticize the matrix are important. The main function of an additive is to improve the performance of the membrane. It can reduce interference in the sample, reduce response time, lower the electricity membrane resistance, and improve ion selectivity. The matrix provides mechanical stability to a liquid membrane but possibly increases the electrical resistance of the liquid membrane. The most common matrix is polyvinyl chloride (PVC). The addition of the matrix can enhance the stability of the liquid membrane. If a matrix is present, all the components are usually dissolved in a solvent such as tetrahydrofuran (THF).

2.3 Research progress on sulfate electrodes

Over the past few decades, extensive efforts have been made to develop ISEs. One of the important aspects has been the miniaturization of the ISE. There have been many sulfate-selective electrodes made in laboratory and applied in various aqueous samples. However, the existing sulfate electrodes are not suitable for measurement in biofilms because the tip size is as large as 7 mm

(Nishizawa *et al*., 1998) and the use of ISEs with such large tips may alter the microenvironment in biofilms. Thus, a sulfate microelectrode with high spatial resolution and accuracy is needed.

The components of cocktail for conventional sulfate electrode were optimized by Nishizawa *et al*. (1998). The use of a bis-thiourea ionophore resulted in ion-selective electrodes with a remarkable higher selectivity for sulfate than any other previous reported ionophore-based ion-selective electrodes (Nishizawa *et al*., 1998; Nishizawa *et al*., 2004). The ionophore used in the study became commercially available at Sigma Aldrich in 2008. In addition to this ionophore, there have been other ionophores such as hydrotalcites (Morigi *et al*., 2001), a bis-pyrylium derivative (Ganjali *et al*., 2004), and a complex of copper (Ardakani *et al*., 2006), but none of them is commercially available. The bis-thiourea ionophore in combination with other components as the cocktail was used for fabricating the sulfate microelectrode in this study.

Summary

Intensive research has been conducted on environmental biofilms. Modern advanced analytical methods (microsensors, CLSM, and FISH, etc.) enable characterization of biofilm structure and activity at microscale and help us to

better understand biofilm communities. Oxygen, hydrogen sulfide, and pH microelectrodes have been used to study sulfate-reducing process in biofilms. However, use of a sulfate microelectrode to study sulfate-reducing and sulfide-oxidizing phenomenon in biofilms has not been reported. Therefore, this thesis research will design and fabricate a sulfate microelectrode in order to better study sulfur cycle inside biofilms.

3.0 Materials and Methods

3.1 Fabrication of sulfate microelectrodes

The procedure for sulfate microelectrode fabrication includes two main steps: the preparation of the cocktail and the preparation of the micropipette. The cocktail determines the performance and critical characteristics of the microelectrode and should be prepared carefully.

3.1.1 Cocktail preparation

The membrane components were as follows: 1) sulfate ionophore (Fluka 17892): $C_{22}H_{22}N_4S_2$ (1,3-[Bis(3-phenylthioureidomethyl)] benzene), 2) plasticizer (Fluka 73732): o-NPOE (o-nitrophenyl-n-octylether), 3) additive (Fluka 91661): TDDMACl (tridodecylmethylammonium chloride), 4) matrix: PVC (Fluka 81392), and 5) solvent (Sigma-Aldrich 83360): THF. All chemicals were Selectophore® grade. All solutions were prepared by ultra pure water (ELGA Ltd., England, M-B121148G) and salts of the highest purity available. The structure of ionophore is shown in Fig. 4.

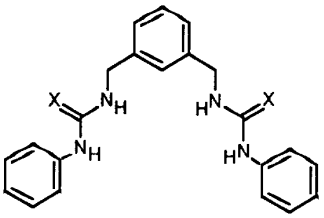

Figure 4 Structure of 1,3-[Bis(3-phenylthioureidomethyl)] benzene, $C_{22}H_{22}N_4S_2$ (MW 406.57) (Nishizawa *et al*., 1998)

In the first experiment, I tested the cocktail for a sulfate selective electrode proposed by Nishizawa *et al*. (1998). The composition of that cocktail is listed in Table 1.

Table 1 The composition of the cocktail for the fabrication of sulfate selective electrodes

Item	Composition
Sulfate Ionophore	1 wt%
TDDMACl	50 mol% [a]
o-NPOE	89 wt%
PVC	10 wt%

[a] Molar ratio relative to the ionophore.

However, sulfate microelectrodes fabricated based on this recipe had very slow response. Therefore, several modified recipes as detailed in Table 2 were

tested.

Table 2 The composition of modified cocktail recipes

Item	Modified Recipe 1	Modified Recipe 2	Modified Recipe 3
Sulfate Ionophore (wt%)	1	2	3
TDDMACl (wt%) [a]	0.7	1.4	2.1
o-NPOE (wt%)	93.3	91.6	89.9
PVC (wt%)	5	5	5
THF [b]	Ca. 2 volumes	Ca. 2 volumes	Ca. 2 volumes

[a] 50 mol% relative to the ionophore

[b] volume relative to the o-NPOE

The amount of ionophore used in the cocktail was critical to produce good microelectrodes. In practice, all the components used were of very small quantities (e.g., 5 mg), which would make weighing procedure more difficult. All the components were weighed directly into one 2 mL vial to avoid unnecessary waste or residue if using several weighing tools. This weighing method should be practiced to make sure accurate amounts of chemicals were weighed into the small vial.

De Beer *et al*. (1997) documented a procedure for cocktail preparation for a

nitrite microelectrode: first a solution of 7% (wt/wt) nitrite ionophore and 1% (wt/wt) additive in plasticizer was prepared; then 10% (wt/wt) PVC was added into the mixture and dissolved within 24 hours. And finally approximately 3 volumes of THF was added and mixed carefully. This is the only detailed procedure of cocktail preparation the author can find in published literature for microelectrodes. When this procedure was followed for fabricating the sulfate microelectrode, a solution of ionophore and additive in plasticizer could not be obtained because this sulfate sensing ionophore did not dissolve in the plasticizer to form a uniform solution. Therefore, the procedure was modified based on my own experience (see 4.1).

3.1.2 Sulfate microelectrode fabrication procedure

The sulfate microelectrode fabrication procedure includes (1) preparation of standard solutions, (2) preparation of electrolyte, (3) preparation of Ag/AgCl wire, (4) silanization of micropipettes, (5) back-filling and front-filling, and (6) cell assembly.

3.1.2.1 Standard solutions

Standard solutions for calibration used were a series of 4 solutions with K_2SO_4 concentrations in water: 10^{-5}, 10^{-4}, 10^{-3}, and 10^{-2} M SO_4^{2-} , i.e., 0.96, 9.6,

96, and 960 mg L^{-1} SO_4^{2-}.

3.1.2.2. Internal filling solution (electrolyte)

The electrolyte used was 10 mM K_2SO_4 + 10 mM KCl solution without any buffer ions in order to reduce possible interference from buffer ions. The electrolyte was degassed by vacuuming.

3.1.2.3. Ag/AgCl wire

A silver wire was submerged in 1 M HCl solution for 1 min until it was coated with a smooth grey layer of AgCl. The Ag/AgCl wire served as an internal reference electrode.

3.1.2.4. Silanization of micropipettes

Each glass micropipette was pulled from 1.2 mm (outer diameter) borosilicate glass capillary with internal filament (World Precision Instruments, USA, 1B120F-6) by using a microsensor-controlled vertical pipette puller (World Precision Instruments, USA, PUL-100). The sharp tip of the micropipette was broken by using a pair of tweezers under microscope to approximately 20 μm in diameter. The micropipette was then silanized by dipping the tip into a silanizing agent, N,N-Dimethyltrimethylsilylamine (Selectophore® grade, Sigma-Aldrich, 41716) for 10-15 s. A number of silanized

micropipettes were placed with tips up on a rack in a covered glass desiccator. They were subsequently heated at 180℃ in an oven (Fisher Scientific, Canada, 3511FS) over night. A hydrophobic surface was obtained for optimal adhesion of the liquid membrane. After silanization, the micropipettes were cooled down to room temperature for use.

3.1.2.5. Back-filling and front-filling

The internal electrolyte was injected by using syringe into a micropipette from the back of the stem (back-filling) until a tiny drop was observed at the tip. The tip of the micropipette was immersed into the cocktail (prepared by modified recipe 1, 2, or 3) for 20-30 s (front-filling). By capillary force a column between 100 and 400 μm was formed at the tip of the micropipette, visible under microscope, and served as the liquid membrane. The filled micropipette was then left to dry for 3-4 hours until the THF in the liquid membrane was evaporated so that the membrane had adequate mechanical strength. The filled micropipette should not be placed in a place with strong ventilation; otherwise the membrane could become segmented arising from the fast evaporation of THF. An Ag/AgCl wire was inserted into the shaft from the back of it. A batch of the microelectrodes was conditioned in 10 mM K_2SO_4 solution at room temperature for 3 hours in order to compare with those that were not conditioned.

The microelectrode was fixed vertically on a rack. The tips of the microelectrode and a reference milli-electrode (Microelectrodes, Inc., USA, MI-401) were immersed in each standard solution, as prepared in 3.1.2.1, and the EMF was recorded by a sensitive high resistance electrometer (Keithley, USA, 6517).

3.1.2.6. Cell assembly

The ISmE has two half-cells. One consists of the liquid membrane, the internal filling solution and the internal reference electrode. The other half-cell contains an external reference millielectrode in contact with a reference electrolyte.

The following cell type was used (as shown in Fig.2):

Ag | AgCl | 3 M KCl | Sample solution ‖ membrane ‖ 0.01 M K_2SO_4, 0.01 M KCl | AgCl | Ag.

3.2 Sulfate microelectrode calibration

The sulfate microelectrode was calibrated in a series of standard sulfate solutions to obtain the relationship between EMF and sulfate concentrations. A calibration curve was then plotted and used to convert EMF values to sulfate concentration for samples measured by the sulfate microelectrode. The setup for

calibration was similar to the measurements on biofilms (see 3.4).

3.3 Environmental biofilm samples

The biofilm samples for testing the sulfate microelectrode were taken from an oxygen-based membrane-aerated biofilm reactor (MABR), which was designed by Shuying Tan and operated by Sabinus Okafor and Shuying Tan in our research group (Tan *et al*., 2010).

3.4 Determination of sulfate concentrations in biofilms

The EMF measurements were performed at room temperature (21±1℃). The setup for biofilms measurement is shown in Fig. 5.

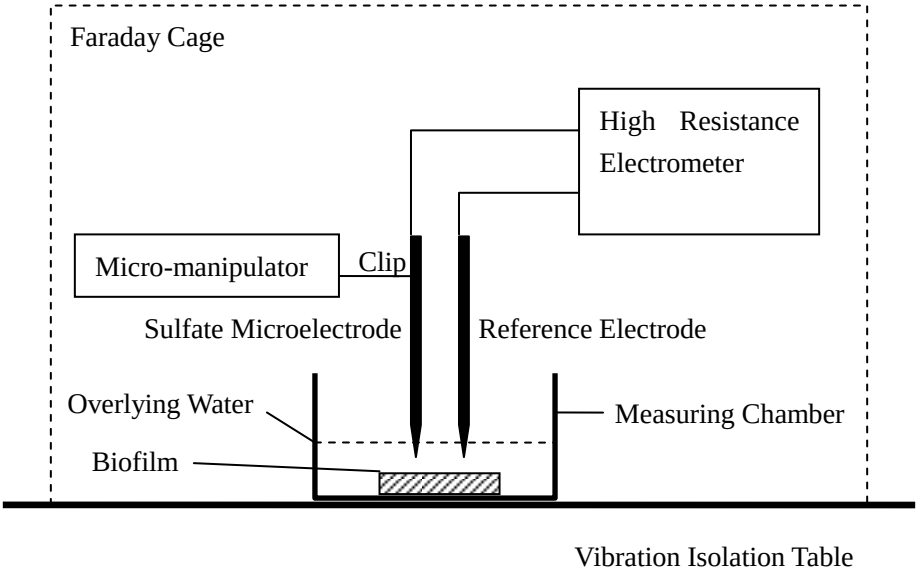

Figure 5 The setup for sulfate determination in biofilms

The setup for biofilm measurement included three main components: the measuring chamber, the interference minimization system, and the microelectrodes and the electrical equipment. The biofilm was submerged in de-ionized water (see 4.4 for further discussion) in a measuring chamber. Circulation was not provided in the chamber to avoid fluid movement induced disturbance to the sulfate microelectrode. All equipment was placed on a high performance vibration isolation table (Technical Manufacturing Corporation, USA) to avoid the effect of mechanical vibration. To minimize electrical interference, the setup was shielded in a Faraday cage (Technical Manufacturing Corporation, USA). Both the cage and the table were connected to a grounding line which was directly in connection with the earth. A sulfate microelectrode was mounted onto a micro-manipulator (World Precision Instruments, USA, M3301L). A milli-electrode used as the reference electrode was also placed in the measuring chamber. The sulfate microelectrode and the reference electrode were connected through cables to the high resistance electrometer which was used to record EMF reading. After pre-calibration in the standard sulfate solutions, the microelectrode was slowly moved toward and then into the biofilm sample in steps of 20 μm. After sample measurement, the same microelectrode was re-calibrated in the standard sulfate solutions (post-calibration).

4.0 Results and Discussion

4.1 Optimization of liquid membrane composition

During the microfabrication procedure of the ISmE, the major challenge was to prepare suitable liquid membrane different than the ISE. When sulfate ISE was prepared, the liquid membrane was completely dried after THF evaporated, and then a disk of membrane was cut out and mounted on an electrode body (Nishizawa *et al*., 1998). However, this method was unfeasible for ISmE due to its delicate tip. Therefore, the status of the liquid membrane needed to be considered. A status of liquid membrane suitable for ISmE could be obtained by adding more THF, but without evaporation of it. As stated in 3.1.1, the liquid membrane composition was optimized for the sulfate microelectrodes. In the modified procedure, the solid components (i.e., ionophore, additive and PVC) of the cocktail were weighed directly into a 2 mL brown vial (Fisher Scientific, Canada, B7999-1A) (to avoid light) according to each aforementioned recipe (Table 2). The liquid components (i.e., plasticizer and THF) were added immediately. At this point, the cocktail was still in suspended appearance. Then the cocktail was heated at 50℃ in a water bath for 3 to 4 hours until the cocktail became a clear and transparent solution. But this cocktail was too thin for making the sulfate microelectrode. A thick gel (semi-solid state) was the best thickness for the cocktail; otherwise it would

cause segmentation in the membrane, which was the main problem during the front-filling procedure. Therefore, the amount of THF added to the cocktail was critical. Too little THF could not dissolve the other four components, while too much THF results in a thin cocktail that would not be mechanically strong.

Determining the optimal amount of THF to add was rather difficult and usually had to be based on personal experience and trial and error. A number of mixing methods were tried to make the cocktail more uniform: shaking solution by vortex, warming the solution up to 50℃, stirring and pumping the solution with a syringe, and sonication of the solution for one hour. As results, warming up to 50℃ was effective in obtaining a uniform cocktail solution. The sonication did not appear to have a positive effect on the uniformity of the cocktail. The other mechanical mixing methods were not effective and were not recommended because they would likely generate bubbles in the cocktail.

Previously it has been shown that the addition of PVC with THF as solvent reduced the electrical resistance of the membrane and thus improved properties of the membrane (Ammann *et al*., 1987). In this study, the THF played an important role in the preparation of the cocktail because the ionophore and additive could not be fully dissolved in the plasticizer alone. However, excessive THF could dissolve all the organic components in the cocktail. The key technical challenge was with the amount of THF used. When too little THF was used, not all the components can be dissolved, whereas too much THF would

cause a very thin cocktail. When the cocktail is too thin, two segments of the membrane would form at the microelectrode tip after front filling. The segments should be avoided.

4.2 The performance of sulfate microelectrodes

A number of properties were tested to evaluate the performance of sulfate microelectrodes. The calibration curves (Fig. 6, Fig. 7, and Fig. 8) of microelectrodes are displayed based on the three recipes tested (see Appendix for all raw data). The average calibration curve slopes for Recipe 1, Recipe 2, and Recipe 3 were -13.9±0.5, -25.8±5.1, -39.8±2.6 mV/decade, respectively. Among the three compositions studied, the membrane incorporating 2% ionophore, 1.4% TDDMACl, 91.6% o-NPOE and 5% PVC exhibits best calibration curve slope (-25.8 mV/decade), which is close to the ideal slope of -29.6 mV/decade. The sulfate microelectrodes showed a good selectivity towards sulfate ions. Therefore, this composition was used to study properties of the sulfate microelectrode, such as linear concentration range, detection limit, and response time.

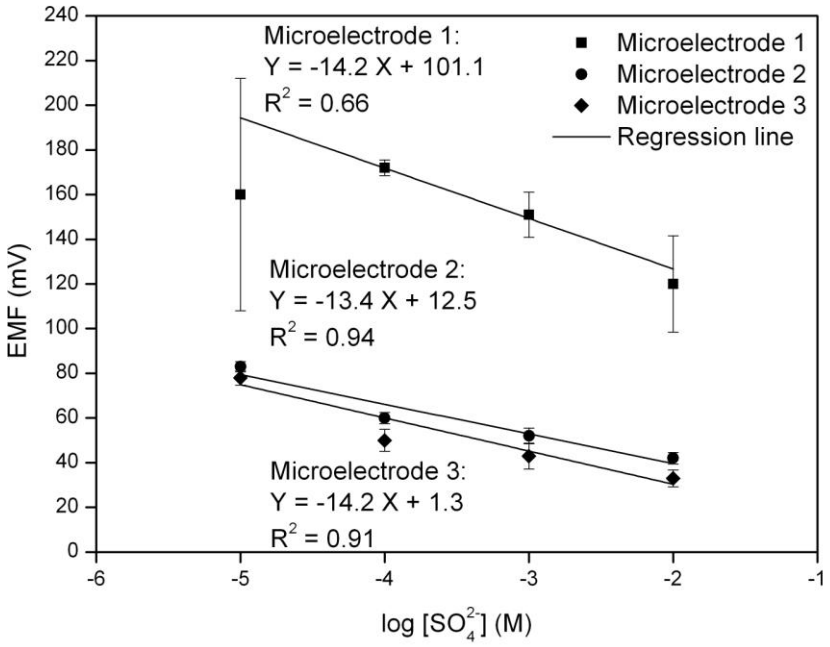

Figure 6 Calibration curves of microelectrodes based on Recipe 1 (Error bars indicate the standard deviation, n=3)

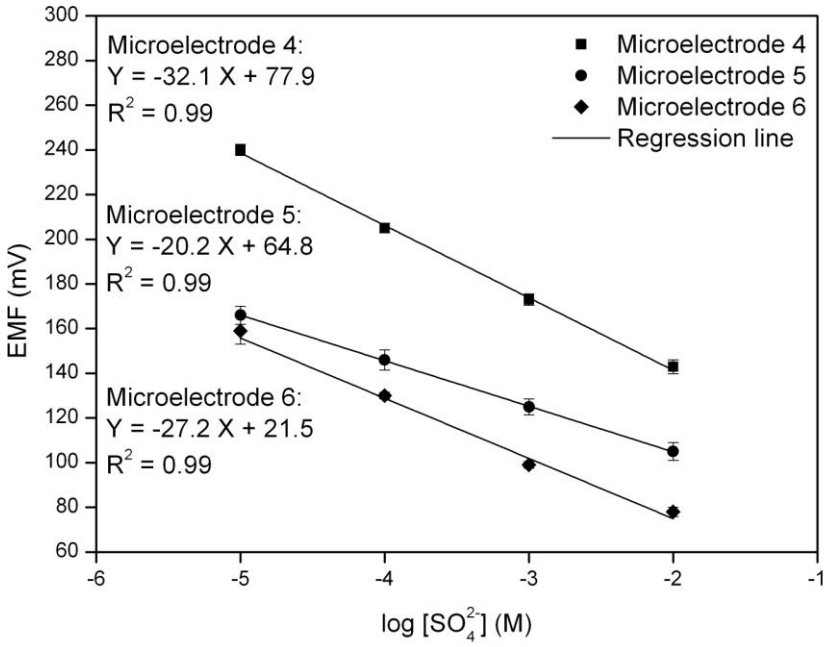

Figure 7 Calibration curves of microelectrodes based on Recipe 2 (Error bars indicate the standard deviation, n=3)

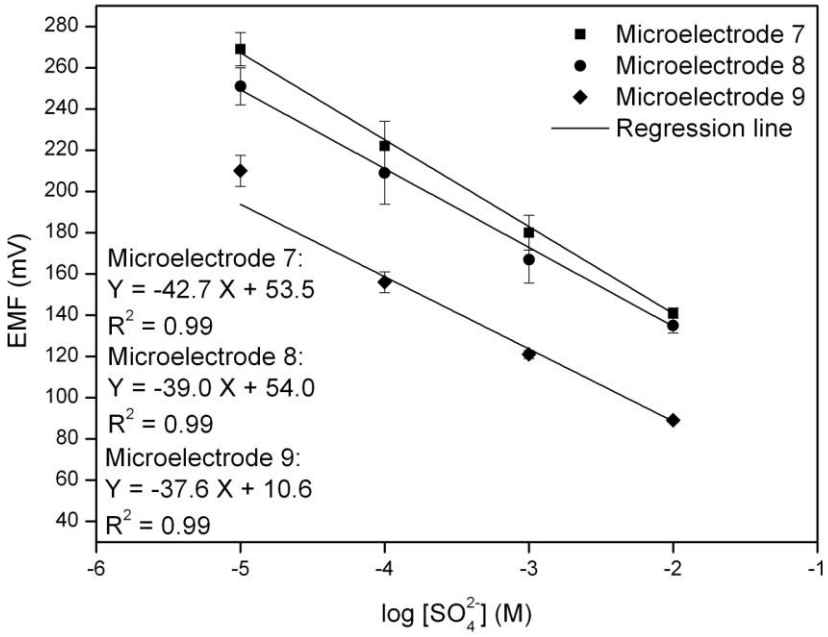

Figure 8 Calibration curves of microelectrodes based on Recipe 3 (Error bars indicate the standard deviation, n=3)

The calibration curves of sulfate microelectrodes based on Recipe 2 displayed good Nernstian slopes close to -29.6 mV/decade with high R^2 values of 0.99.

The sulfate microelectrode exhibited log-linear response between 10^{-5} M (0.96 mg/L) and 10^{-2} M (960 mg/L) with a detection limit of 10^{-5} M sulfate. When 10^{-6} M (0.096 mg/L) sulfate was tested it fell outside the linear response range (data not shown). Domestic wastewater typically contains sulfate concentrations from 20 to 500 mg/L (Lens *et al.*, 1998), which is covered within the detection range. As a result, the sulfate microelectrode can be used for

measuring sulfate concentrations.

The response time of the sulfate microelectrode was evaluated. According to International Union of Pure and Applied Chemistry, the practical response time was the length of time that elapsed for the electrode to reach the 90% of the final value (Lindner *et al*., 1986). In this study, the EMF was recorded at 10 time points and the practical response time was estimated (Fig. 9). As seen in Fig. 9, the response time was decided to be approximately 90 s.

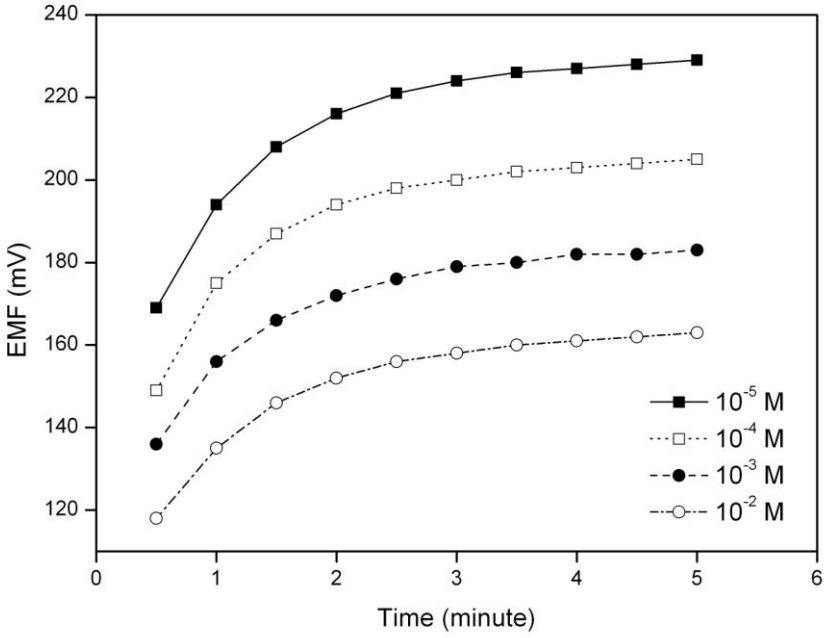

Figure 9 Evaluation of the response time of the sulfate microelectrode

A preliminary exploration was made to improve properties of the liquid membrane by incorporating carbon nanotubes in fabricating the sulfate microelectrode. Carbon nanotubes are allotropes of carbon with a cylindrical

nanostructure. They have many novel properties and can be applied in many fields such as nanotechnology and electronics. Multi-walled carbon nanotubes (MWCNT) have been proven to be efficient transducers of the ion-to-electronic current (Crespo *et al*., 2009). A simple method was reported by Zhu *et al*. (2009) to prepare all solid-state ion-selective sensors with excellent electrical potential stability and sensing properties using MWCNT. They developed ISE with much improved stability and excellent sensing properties. The same approach was applied in the preparation of the cocktail for sulfate microelectrodes. The MWCNT was dispersed in THF under sonication for 3 hours and then mixed with the cocktail. But it appeared to make no improvement on microelectrode performance. The slopes even increased to -45 to -48 mV/decade, which was much higher than the ideal slope (-29.6 mV/decade). Future work is needed to explore the influence of the MWCNT on the ISmE.

Table 3 lists the tip size, slope, linear range, detection limit, and response time of some other ISmEs against the proposed sulfate microelectrode. As can be seen from the table, the detection limit and response time of the tested sulfate microelectrode are comparable to other ISmEs. Therefore, the proposed sulfate microelectrode is suitable for the measurement on biofilms as other ISmEs are.

Table 3 Comparison of properties of the tested SO_4^{2-} ISmE with other ISmEs

ISmE	Tip size (μm)	Nerstian slope (mV/decade)	Linear range (M)	Detection limit (M)	Response time (s)	Reference
The tested SO_4^{2-}	20	-25.8	10^{-5} - 10^{-2}	10^{-5}	90	This study
Ca^{2+}	1	24.7	$10^{-9.2}$ - 10^{-3}	$10^{-9.2}$	N/A	Ammann *et al*., 1987
NH_4^+	1	50-55	10^{-5} - 10^{-1}	10^{-5}	60	de Beer and Van den Heuvel, 1988
NO_3^-	1	55	10^{-5} - 10^{-1}	10^{-5}	30	de Beer and Sweerts, 1989
NO_2^-	10-15	N/A	10^{-7} - 10^{-2}	10^{-7}	10 - 15	de Beer *et al*., 1997
$H_{3-x}PO_4^{x-}$	5-20	-31.5	10^{-5} - 10^{-1}	10^{-5}	60 - 120	Wang and Bishop, 2010

Whether a conditioning step is required before calibration of microelectrodes has been controversial (Lindner and Umezawa, 2008). Based on Recipe 2, the effect of conditioning step during fabrication was examined by calibrating sulfate microelectrodes made with and without conditioning them prior to measurements (Fig. 10 and Fig. 11). The average slopes of the conditioned and unconditioned microelectrodes were 24.9±4.9 and 25.4±4.3 mv/decade, respectively. All tested microelectrodes exhibited good linear response to concentration change. For each calibrated microelectrode, the membrane was checked under microscope after each calibration and found no apparent visual damage. It was concluded that the conditioning step had no obvious effect on the sulfate microelectrode characteristics.

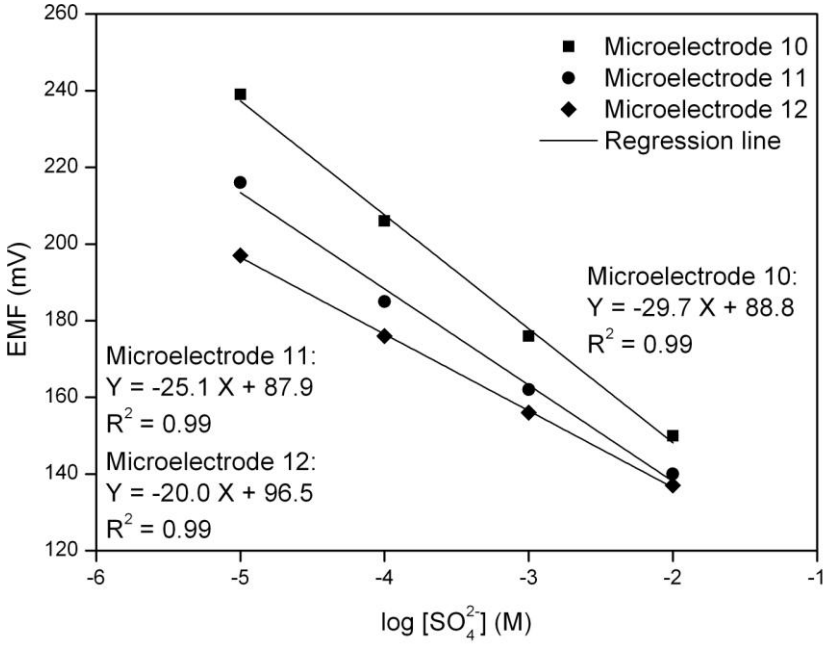

Figure 10 Calibration curves of the conditioned microelectrodes

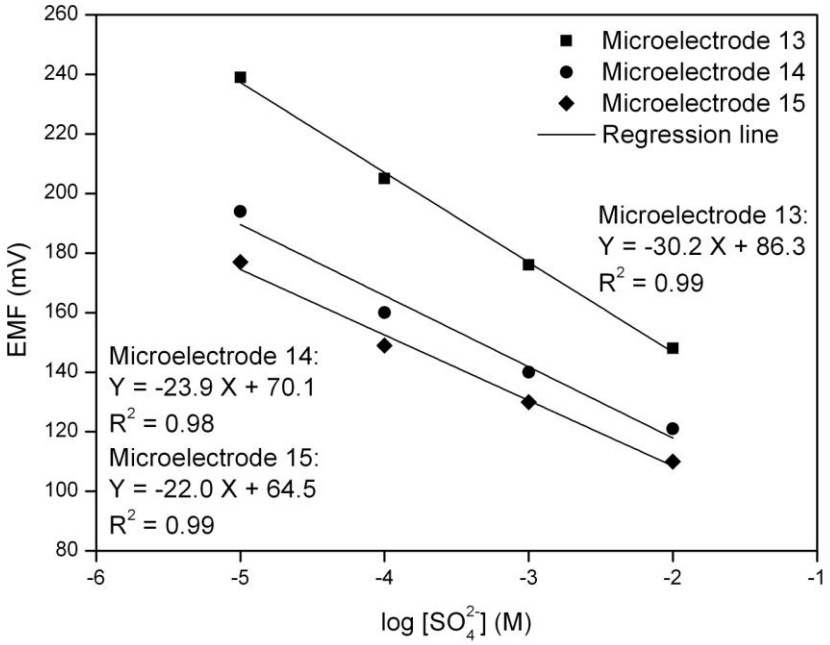

Figure 11 Calibration curves of the unconditioned microelectrodes

De Beer *et al*. (1997) applied a hydrophilic coating on the tip of nitrite microelectrodes before conducting calibrations. The microelectrodes were first dipped in a solution of 10% cellulose acetate in acetone, and then dipped in the bovine serum albumin (BSA) containing glutaraldehyde. After drying, a cross-linked protein layer was formed. The water insoluble and firmly fixed protein layer could prevent liquid membrane from contact with hydrophobic interference substances in samples. The coating also resulted in more sturdy microelectrodes with more stable signals than uncoated ones. This method was tested on the sulfate microelectrodes. However, the result was unsuccessful,

because no matter how fast the microelectrode was dipped in cellulose acetate the membrane at the microelectrode tip was dissolved immediately by cellulose acetate. Thus the protein layer could not be formed due to the different property of this liquid membrane.

High electrical resistance of ISmE is a significant problem when the ISE is miniaturized to the ISmE. In this experiment, this factor also had a huge effect on microelectrode performance. The microelectrodes with tip diameter under 20 μm did not respond linearly to the concentration change due to extremely high resistance (>500 MΩ). However, it is difficult for a microelectrode with tip diameter larger than 30 μm to hold this liquid membrane. Consequently, it was decided to control the tip diameter around 20 μm.

Overall, the sulfate microelectrodes exhibited a log-linear response down to a sulfate concentration of 10^{-5} M in water. According to the calibration data, the sulfate microelectrodes are well-functional.

4.3 Environmental biofilm application

The sulfate microelectrodes were applied on measurement of a piece of biofilm sample from the MABR in our laboratory. A pre-calibration and a post-calibration were performed by the same sulfate microelectrode before and after measurement (Fig. 12).

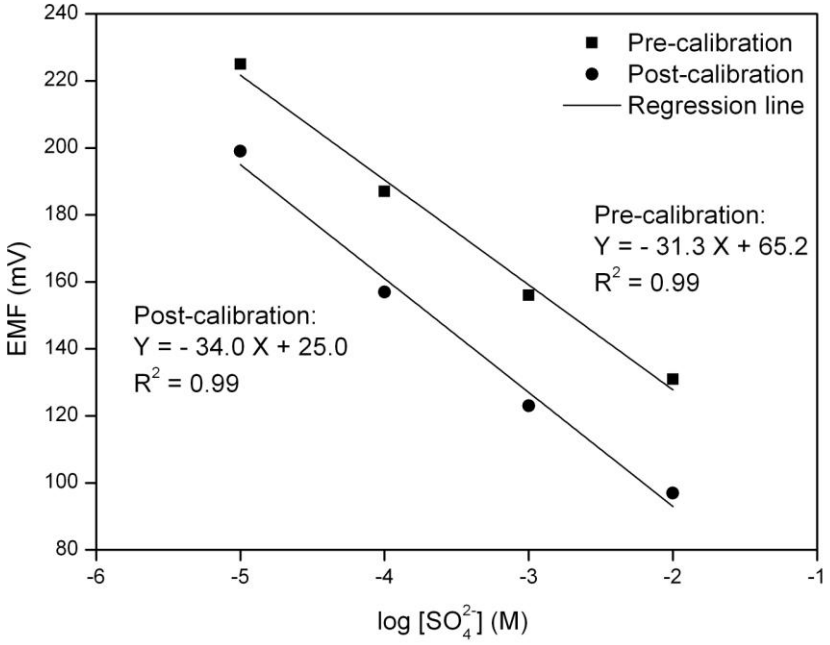

Figure 12 Calibration curves of the sulfate microelectrode used for the measurement of the MABR biofilm

As shown in Fig. 12, this sulfate microelectrode exhibited excellent performance. After measurement, the liquid membrane at the microelectrode tip was unchanged. One profile was obtained, showing the concentration change of sulfate along the biofilm depth (Fig. 13).

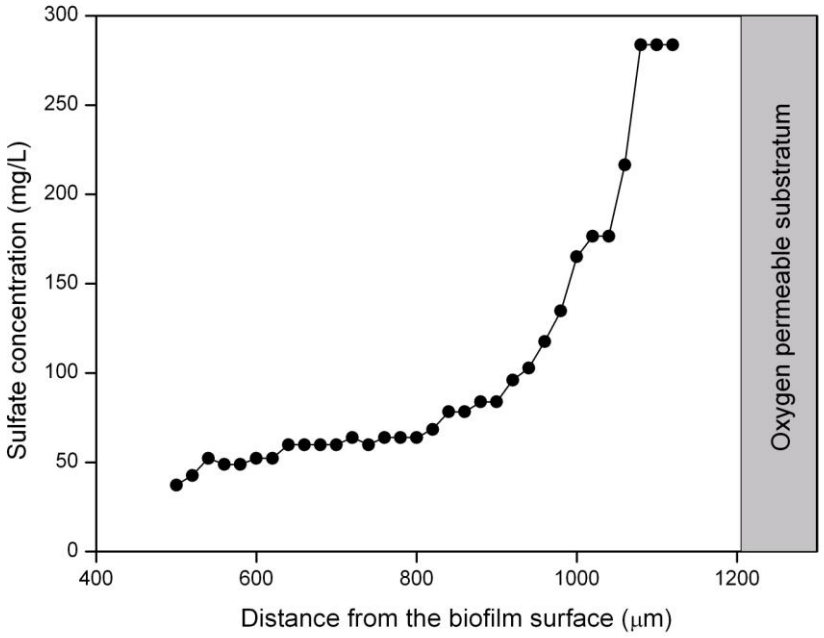

Figure 13 Sulfate profile within the MABR biofilm

This profile shows that the sulfate concentration increases with depth. The sulfate concentration gradually increased from 37.2 mg L^{-1} on the biofilm surface to 283.7 mg L^{-1} at bottom of the biofilm without obvious turning point in the profile. The results were consistent with reactor operating conditions (Tan *et al.*, 2010). Sulfate was reduced in the anoxic zone and accumulated in the aerobic zone of the biofilm.

4.4 Limitations of the sulfate microelectrodes

As mentioned in 3.4, the measurement was taken using pure water as the environment surrounding the biofilm. The synthetic wastewater for the MABR was initially used as the medium surrounding the biofilm, but the microelectrode could not detect the sulfate concentration. Hence, pure water was used instead of synthetic wastewater.

Irregular behaviors of sulfate microelectrodes were observed during a number of measurements, including sudden potential shifts, signal drift, sudden increase in response time. These behaviors would happen unpredictably and affect the performance of microelectrodes. The damage of microelectrodes may be caused by direct contact with water-insoluble biomass. A chemical change of the membrane caused by a hydrophobic substance was suspected as well because little physical change of the membrane was observed after measurement.

5.0 Conclusions and Recommendations

In this thesis, a sulfate microelectrode was successfully developed for the first time by exploring the appropriate composition of liquid membrane which enabled the microelectrode to respond to sulfate concentration change. The liquid membrane of the sulfate microelectrode was optimized to a composition of 2% ionophore, 1.4% additive, 91.6% plasticizer and 5% PVC by weight. The sulfate microelectrode, with a tip diameter of 20 μm, exhibited log-linear response to sulfate concentration change between 10^{-5} M (1.0 mg L^{-1}) and 10^{-2} M (960 mg L^{-1}) SO_4^{2-}. The detection limit was 10^{-5} M (1.0 mg L^{-1}) SO_4^{2-} and the response time was 90 s. However, the microelectrode was sometimes unstable and sensitive to interference substances in biofilm samples. Nevertheless, one sulfate profile was obtained, indicating that the sulfate microelectrode can detect sulfate concentration change in environmental biofilms.

Based on these results, I have the following recommendations for future research: cocktails with different compositions between 1% and 3% ionophore should be explored to produce better liquid membrane; selectivity coefficients need to be determined for further examination of the microelectrode characteristics; and a suitable method is needed to insulate the liquid membrane from the interfering ions in samples

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7.0 Appendix

Raw data

Table A1 EMF response (mV) for three recipes

Recipe	$[SO_4^{2-}]$	10^{-5} M	10^{-4} M	10^{-3} M	10^{-2} M
1	Microelectrode 1	100	172	163	142
		188	176	146	99
		192	169	145	118
	Microelectrode 2	82	60	55	42
		86	63	52	44
		82	58	48	39
	Microelectrode 3	80	56	50	37
		79	48	40	31
		74	47	40	30
2	Microelectrode 4	242	207	174	146
		240	206	175	144
		237	203	170	140
	Microelectrode 5	170	150	129	109
		165	146	124	106
		162	141	122	101
	Microelectrode 6	165	130	98	80
		158	129	99	79
		153	132	101	76
3	Microelectrode 7	277	234	189	143
		269	221	179	141
		261	210	172	138
	Microelectrode 8	260	226	180	139
		251	204	162	134
		242	197	159	132
	Microelectrode 9	210	161	123	89
		203	155	121	88
		195	151	119	90

Table A2 EMF response (mV) for conditioned and unconditioned microelectrodes

	$[SO_4^{2-}]$	10^{-5} M	10^{-4} M	10^{-3} M	10^{-2} M
Conditioned	Microelectrode 10	239	206	176	150
	Microelectrode 11	216	185	162	140
	Microelectrode 12	197	176	156	137
Unconditioned	Microelectrode 13	239	205	176	148
	Microelectrode 14	194	160	140	121
	Microelectrode 15	177	149	130	110

Table A3 Response time of the sulfate microelectrode (mV)

Time (minute)	10^{-5} M SO_4^{2-}	10^{-4} M SO_4^{2-}	10^{-3} M SO_4^{2-}	10^{-2} M SO_4^{2-}
0.5	169	149	136	118
1	194	175	156	135
1.5	208	187	166	146
2	216	194	172	152
2.5	221	198	176	156
3	224	200	179	158
3.5	226	202	180	160
4	227	203	182	161
4.5	228	204	182	162
5	229	205	183	163

Table A4 The sulfate profile within the biofilm from the MABR

Distance from biofilm surface (μm)	EMF (mV)	Sulfate Concentration (mg L^{-1})
0	62	out of range
100	22	out of range
200	33	out of range
300	-69	out of range
400	-96	out of range
500	141	37.2
520	139	42.6
540	136	52.2
560	137	48.8
580	137	48.8
600	136	52.2
620	136	52.2
640	134	59.8
660	134	59.8
680	134	59.8
700	134	59.8
720	133	63.9
740	134	59.8
760	133	63.9
780	133	63.9
800	133	63.9
820	132	68.4
840	130	78.3
860	130	78.3
880	129	83.8
900	129	83.8
920	127	96.0
940	126	102.7
960	124	117.6
980	122	134.7
1000	119	165.0
1020	118	176.6
1040	118	176.6
1060	115	216.4
1080	111	283.7
1100	111	283.7
1120	111	283.7