ENHANCING THE LONGEVITY OF ION-SELECTIVE ELECTRODE ARRAYS IN BIOREACTORS

By

HAJIME FUCHIDA

A thesis in partial fulfillment of
the requirements for the degree of

Master of Science in Chemical Engineering

WASHINGTON STATE UNIVERSITY
Department of Chemical Engineering

DECEMBER 2007

To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of HAJIME FUCHIDA find it satisfactory and recommend that it be accepted.

_____
Chair

_____

_____

ACKNOWLEDGMENT

I started this program with a deficiency in knowledge of Chemical Engineering as I was admitted into a conversion program. My completion of a degree would never be achieved without the excellent instruction of the Chemical Engineering faculty. I also wish to acknowledge all the assistance of numerous people who have devoted their time and energy to the completion of my degree.

First of all, I would like to thank my advisor, Dr. Bernard Van Wie, for his guidance, encouragement, and high expectations. I also appreciate his family and their generosity in hosting cultural events during Thanksgiving and Christmas breaks. I would not have accomplished my project without their thoughtful support. Secondly, I would like to thank Dr. David Kidwell and Dr. Kazuhiko Ishihara for their contribution of technical support and donation of a polymer sample. Next, I appreciate all of my collaborators in our laboratory: Dan Rieck, Chris Detzel, Sarah Haarsma, Natasha Godwin, Bingwen Liu, Harvey Doty, and Tai Le. I am grateful for their technical assistance and effective discussions about this project, and I really enjoyed spending time with them in our laboratory. Also, I would like to acknowledge the School of Chemical Engineering and Bioengineering, and the National Science Foundation for the great research opportunity and their financial support.

I met many friends in Pullman. I enjoyed my life with them and will carry away priceless memories. I would like to thank all of my friends for the memorable days we spent together.

Finally, I would like to thank my committee members, Dr. Bernard Van Wie, Dr. Cornelius Ivory, and Dr. Su Ha, for their valuable comments on this project.

ENHANCING THE LONGEVITY OF ION-SELECTIVE ELECTRODE ARRAYS IN BIOREACTORS

Abstract

by Hajime Fuchida, MS.
Washington State University
December 2007

Chair: Bernard Van Wie,

It has been observed that Ion-Selective Electrodes (ISEs) suffer performance loss after long time use in bioreactors. It is speculated that protein adsorption inactivates ISEs by inhibiting ion permeation. The amphiphilic polymer, poly(2-methacryloyloxyethylphosphorylcholine-co-butyl methacrylate) or poly(MPC-co-BMA), was coated on ISEs to improve their longevity when exposed to bioreactor effluent for long time periods. It was hypothesized that the polymer coating would prevent protein adsorption by forming a hydrogel on the ISE surface, rendering the protein adsorption process reversible.

In this project, K^+ ISEs made with valinomycin as the ionophore were used to determinate the effects of the polymer coating on response slopes, limits of detection (LODs), interference with NH_4^+, and longevity in batch bioreactor effluent, by coating the ISEs with five different polymer solutions: 0%, 0.01%, 0.10%, 1.0%, and 10% by weight.

The results showed that the polymer coated ISEs maintained a Nernstian response through long time exposure to bioreactor effluent as expected. However, the polymer coating tended to simultaneously reduce the ISE LOD, with a negative

correlation between the weight percent of polymer and the LOD. The polymer coating tended to increase ISE interference from NH_4^+. This implies that the reduction of the ISE LOD is not severe enough to negatively affect ISE performance in most bioreactor applications because of the relatively high K^+ content in the growth media. Therefore, the results validate the use of poly(MPC-co-BMA) to coat ISEs and render them suitable for applications involving biological fluids.

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS iii

ABSTRACT v

LISTOF FIGURES ix

LIST OF TABLES x

DEDICATION xi

CHAPTER

1. INTRODUCTION 1
2. THEORIES 4
    - ION SELECTIVE ELECTRODES 4
    - PREVENTION OF PROTEIN ADSORPTION 8
    - IONS IN BIOREACTORS 11
3. MATERIALS AND METHODS 13
    - REAGENTS AND MATERIALS 13
    - ELECTRODE FABRICATION 14
    - EXPERIMENTS 16
4. RESULTS 19
    - UNIFORMITY OF UNCOATED ISES 19
    - POLYMER EFFECTS ON SLOPES AND LODS 22
    - POLYMER EFFECT ON LONGEVITY 29
5. CONCLUSIONS 35

6. REFERENCES 36

LIST OF FIGURES

Figure 2.1. Mechanism of ISE ion transport 4

Figure 2.2. The geographical determination of LODs 6

Figure 2.3. K^+ ISE responses with changes of K^+ and NH_4^+ concentration..... 6

Figure 2.4. The mechanism of protein adsorption on polymer surface 9

Figure 2.5 Chemical Structure of Poly(MPC-co-BMA) 10

Figure 3.1. The standard addition method..... 17

Figure 3.2. Experimental setup for measuring ISE longevity 18

Figure 4.1. The initial calibration of K^+ ISEs with $K^+$and $NH4^+$ 21

Figure 4.2a. The calibration of coated K^+ ISEs with K^+ 23

Figure 4.2b. The calibration of coated K^+ ISEs with NH_4^+ 24

Figure 4.3. Interference of valinomycin with NH_4^+ 27

Figure 4.4. ISE response in a bioreactor over 4.5 days..... 30

Figure 4.5. The responses of the non-coated electrodes. 32

Figure 4.6. The responses of the coated electrodes 32

Figure 4.7. Response slope changes over 4.5 days..... 33

LIST OF TABLES

Table 2.1: Intracellular and extracellar inorganic ion..... 11

Table 3.1: Standard addition methods scheme..... 17

Table 4.1: The response slopes and LODs determined from the K^+ calibration and corrected response slope and LOD shifts with polymer coating. 24

Table 4.2: The response slopes and LODs determined from the NH_4^+ calibration and corrected response slope and LOD shifts with polymer coating. 24

Table 4.3: Estimated interference coefficient of K^+ ISE with NH_4^+ in logarithmic scale. The interference coefficient is getting larger at higher K^+ concentrations.... 26

Dedication

This thesis is dedicated to my mother and father

who provided both emotional and financial support

Chapter 1

Introduction

Mammalian cell culture is widely used for manufacturing a variety of excreted products such as monoclonal antibodies[1], hormones[2], and antibiotics[3]. However, these cultures are highly susceptible to changes of their environmental conditions caused by the accumulation of toxic metabolites such as carbon dioxide, ammonium and lactate ions. A narrow pH range of 6.8 – 7.2 is required to keep the cultures alive[4]. However, carbon dioxide decreases the pH which provides negative effects on cell growth. Also, ammonium inhibits cell growth at 5 mM[5] level while lactate inhibits at 40 mM[6] level. Currently, there is an emphasis on improvement of the yield in smaller bioreactors by using dense cell population cultures to improve yields in smaller bioreactors[7,8]. A Continuous Centrifugal Bioreactors (CCBR) was recently developed for monoclonal antibody production from hybridoma cell lines and can maintain population densities of 10^8 or more cells/mL[9,10]. Yet the smaller reactor volume and dense suspension allow the environment surrounding the cells to change rapidly. Therefore, it is critical that means for on-line monitoring be devised to detect and allow correction for shifts in pH or buildup of lactate and ammonium ions.

There are numerous techniques to monitor species in bioreactors including ion chromatography[11], wet chemistry assays[10], and ion-selective electrodes (ISEs) [12]. Ion chromatography and wet chemistries, however, are problematic when continuous on-line

monitoring is important as there is a need to remove samples and process them off line. This is time consuming and may not allow for speedy adjustments to the process to maintain operation within an optimal range. Cost is also an issue with the requirement of expensive analytical equipment and process reagents. The deficits of the ISEs have been discussed in terms of their drift[13] and protein adsorption on the membranes[14, 15]. Drifting can be solved with frequent calibration[13], and it is thought that protein adsorption can be inhibited by introducing 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer[15] which can dramatically reduce adsorption or make it at least reversible.

There are various types of MPC polymers depending on the companion monomer used to build the polymer with MPC. Poly(2-methacryloyloxyethyl phosphorycholine-co-n-butyl methacrylate) or poly(MPC-co-BMA) is one of such MPC polymers consisting of MPC and n-butyl methacrylate(BMA) units. In terms of applications for use of the poly(MPC-co-BMA), success has been demonstrated in decreasing protein adsorption on glucose sensors[16] and artificial catheters[17,18]. Though poly(MPC-co-BMA) has been used to coat ISEs and no significant effects in response slopes or limit of detection (LOD) were observed[19], no long-term studies there have been conducted for their use in continuous monitoring of cell culture bioreactors.

In this paper we will demonstrate the use of poly(MPC-co-BMA) in long-term continuous monitoring of effluent from a batch bioreactor. We coated poly(MPC-co-BMA)

onto ISEs specific for measuring the activity of K^+. LODs are compared for coated and non-coated ISEs and slopes are compared to theoretical values on the basis of the Nikolskii-Eisenman equation[12]. We discuss calibration and interference issues in culture medium and show studies to illustrate the optimal concentration of coating copolymer.

Chapter 2

Theory

2.1 Ion Selective Electrodes

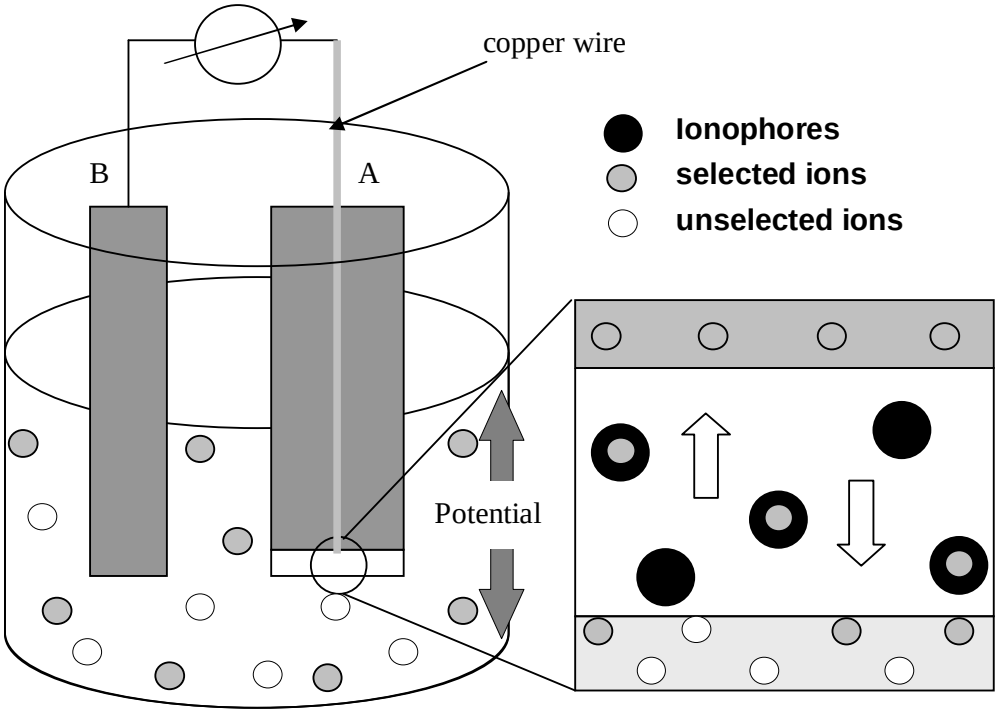

Figure 2.1: Mechanism of ISE ion transport.

ISEs are specific for certain ions and measure ion activity in a solution. The selectivity for and transport of ion species is accomplished by lipid-soluble molecules called ionophores. Ionophores are thermodynamically stable in the organic liquid phase that makes up 50 – 70% of the composition of typical ISE membranes. This is because ionophore molecular structures are such that the molecules have a hydrophobic exterior. Ionophores are also able to diffuse in the membrane which aids in molecular transport. The mechanism of ISE transport and means for electrical measurement is described in Figure 2.1. ISE arrays

consist of an ISE electrodes (A) and a reference electrode (B). When ionophores encounter ions at membrane boundaries, they bind to the ions according to ion fit within coordination sites. The bound ions are carried by ionophores across the membrane, and released on the opposite side of the membrane. This ion transport generates a potential difference between the reference and ISE electrodes. The potential difference is detected as an electromotive force (emf) with a multimeter or specially designed electronic board. The detected potential difference for a homogeneous solution is expressed by the Nernst equation[12].

$$E = E^{o} + \frac{2.303 \cdot R \cdot T}{z_A \cdot F} \cdot Log[a_A] \qquad (1)$$

where a_A is the activity of the primary ion "A" , R, T, F and z represent the ideal gas constant, temperature in Kelvin, Faraday's constant, and the charge on the ion of interest, respectively.

The coefficient in front of the logarithm of the activity for the primary ion "A" is the response slope, and it has a value of approximately 59 mV at room temperature for a monovalent ion "A". It is an essential parameter for sensors since higher response slopes provide better precision for data analysis. The LOD is another important parameter used to evaluate ISE performance. The LOD is defined as the concentration at which the ISE response just reaches the lower concentration limit of Nernstian behavior[12]. Figure 2.2 shows graphically the LOD on a calibration curve.

Interference effects with ISE are observed, however, when they are used in heterogeneous solutions. Since ionophore molecular affinity is based on of physical and chemical properties such as their size and electrical charge interference occurs when ions with similar properties coexist in the same solution. For instance, K^+ electrodes respond quite well to concentration changes of NH_4^+ though the NH_4^+ concentration typically must be at least an order of magnitude higher than that of K^+ for the same relative change in ISE voltage. Figure 2.3 shows actual data indicating interference of K^+ with NH_4^+ ions. These effects can be modeled with the Nikolskii-Eisenman equation (2) which the ISE emf in such

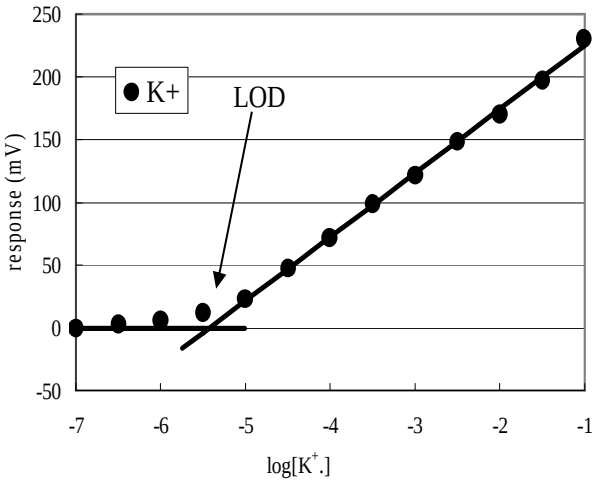

Figure 2.2: The graphical determination of LOD.

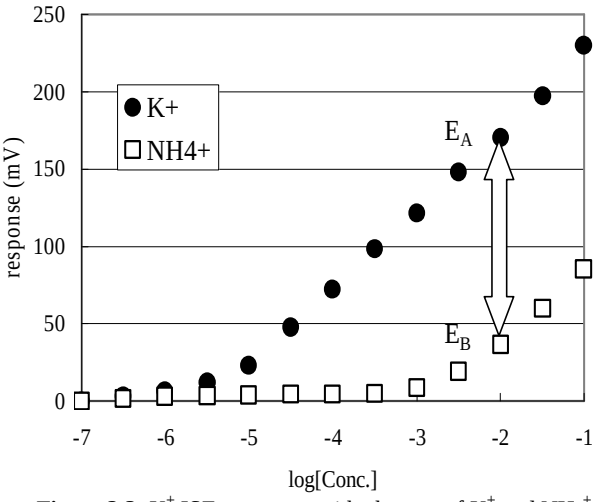

Figure 2.3: K^+ ISE responses with changes of K^+ and NH_4^+ concentration.

heterogeneous solutions[21].

$$E = E^{o} + \frac{2.303 \cdot R \cdot T}{z_A \cdot F} \cdot Log\left[a_A + K_{AB}^{Pot}\left(a_B\right)^{z_A/z_B} + K_{AC}^{Pot}\left(a_C\right)^{z_A/z_C} + ...\right] \qquad (2)$$

where a_A, a_B, a_C are the activities of primary ion "A", and secondary ions "B", "C", etc. Also, R, T, F and z represent the ideal gas constant, temperature in Kelvin, Faraday's constant, and the charge on the primary ion, respectively. K_{AB}^{Pot} is the selectivity coefficient indicating the ratio of ISE response of the secondary ion over the primary ion, and the x_A/x_i represents the ratio of the valance state of the primary ion to that of secondary ion i.

Selectivity coefficients may be determined by the separate solution method by comparing the responses of species "A" and "B" each contained in a separate solution to which the ISE is exposed. The interference coefficients are calculated with equation (3)[21].

$$\log K_{AB}^{Pot} = \frac{(E_B - E_A)z_A F}{2.303RT} + (1 - \frac{z_A}{z_B})\log a_A \qquad (3)$$

where E_B, E_A are the respective experimental emf values of ion "A" and ion "B" taken at the same activity level for each ion (see Figure 2.3).

Once the activities of interfering ions and their interference coefficients are known, the Nikolskii-Eisenman equation can be used to obtain the actual activity of the primary ion.

2.2 Prevention of Protein Adsorption

For ISEs placed in protein solutions water molecules surround both the proteins and the polymer surfaces due to van der Waals forces. Approach of protein towards the polymer surface eliminates water molecules bound to protein and the polymer. This drives protein conformation changes that allow the hydrophobic parts of the protein to face the polymer surface directly. Since this dehydration increases the entropy, the protein adsorption process is irreversible. This process is illustrated in Fig. 2.4 and is known as dehydration. This protein adsorption must be inhibited and Ishihara *et al* report that high surface water fraction levels cause any protein adhesion to take place reversibly[15,18]. The group has shown that an effective solution results when MPC polymer is applied to the polymer surface which serves to retain water molecules at the surface.

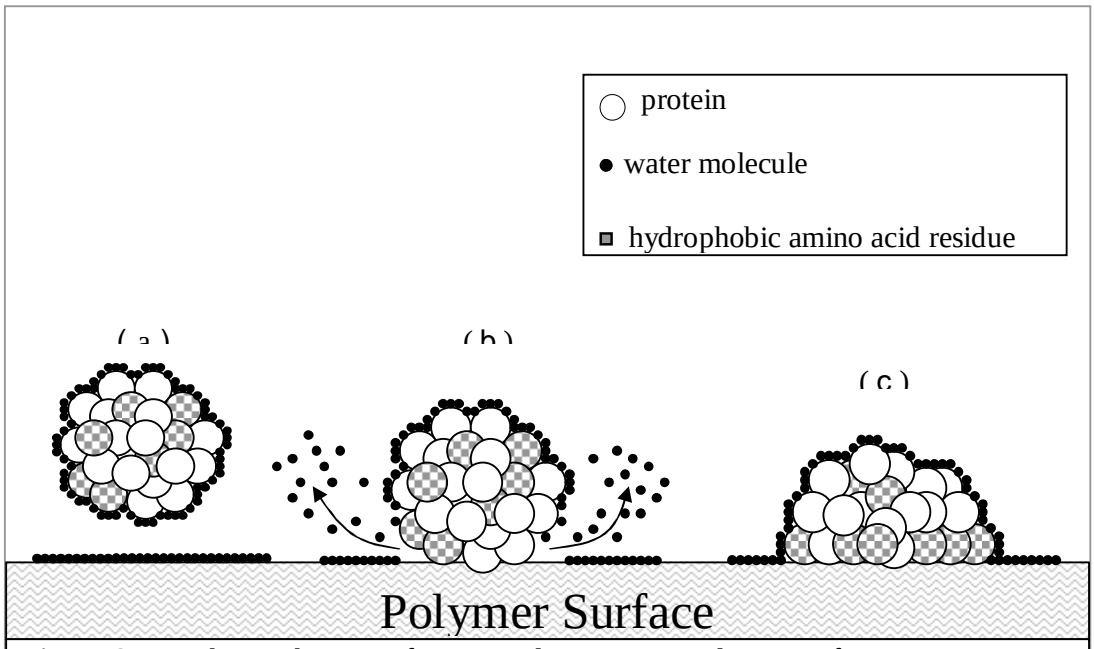

Figure 2.4: The mechanism of protein adsorption on polymer surface. Protein surrounding water molecules approach the polymer surface (a). The water molecules located between the protein and the surface are removed as the proteins reach the polymer surface (b). Once protein attaches to the polymer surface, it changes its conformation and binds to the surface irreversibly with hydrophobic interactions (c).

The protein adsorption phenomenon is believed to occur on ISE membranes because of membrane hydrophobicity and this phenomenon is believed to be the cause of significant reduction in ISE performance in the presence of serum and/or cell culture proteins.

In this work we postulate that MPC polymer coating can reduce or eliminate the decline in ISE performance by preventing membrane adsorption of protein. The chemical structure of poly(MPC-co-BMA) is indicated in Figure 3.5 and shows two distinct units involved in the structure, a hydrophilic unit to capture water molecules and a hydrophobic unit for binding to a polymer surface.[9] The polymer carries no net charge and forms a hydrogel which functions to keep a high water fraction on the polymer surface. The MPC

polymers are produced by polymerizing MPC units with various alkyl methacrylates or styrene and the polymer characteristics are dependent on the composition. PMB30 consists of 30 mole percent MPC and 70 mole percent BMA, and is a commonly used MPC polymer because of its extraordinary biocompatibility compared with other polymers[9], and it has been tested for use on ISE membranes for reduction in adhesion of blood platelets.[19]

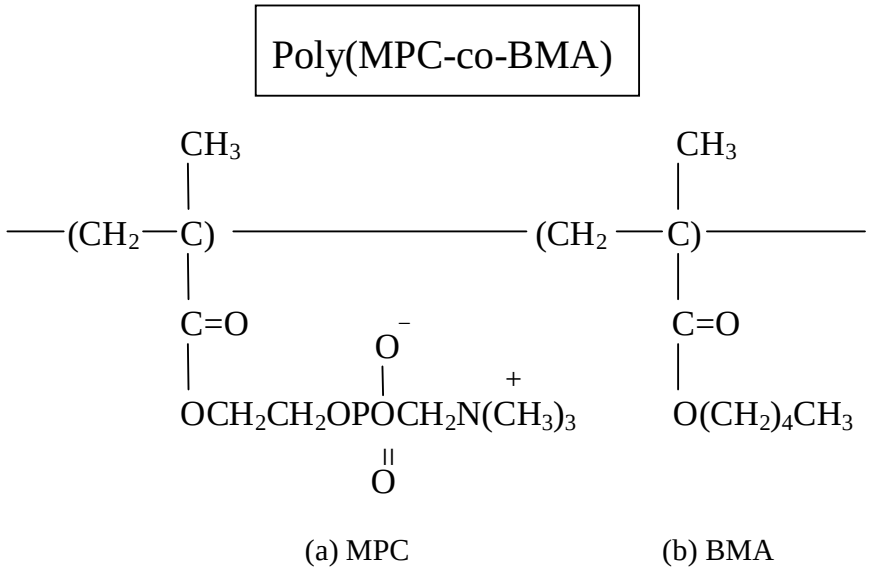

Figure 2.5 Chemical Structure of Poly(MPC-co-BMA). In this work PMB30 is used, which consists of a 3 to 7 ratio of hydrophilic MPC units (a) to hydrophobic BMA units (b).

2.3 Ions in Bioreactors

It is important to monitor ion concentrations in bioreactors to optimize the cell growth. Some ions inhibit cell growth such as NH_4^+ and excess H^+, e.g. below pH 6.8, others are essential for cell growth such as the electrolytes, K^+, Na^+, Ca^{++}, Mg^{++} and Cl^-, while others may be used to indicate cell health and cell lysis.

Figure 2.1: Intracellular and extracellar inorganic ion

	extracellular	intracellular
Na^+	155 mM	10 mM
K+	5.4 mM	141 mM
Ca^{2+}	1.8 mM	<0.5 mM
Mg^{2+}	0.8 mM	29 mM
Cl^-	118 mM	4 mM
HCO_3^-	44 mM	10 mM
SO_4^{2-}	0.8 mM	1 mM

Regarding detection of cell lysis with ISEs we can consider there is there is a significant difference in extracellular and intracellular inorganic ion concentration as summarized in Table 2.1[18]. Intracellular K^+, Mg^{2+} concentrations are remarkably higher than extracellular values and similarly, extracellular Na^+ and Cl^- concentrations are much higher than intracellular levels. When cells are lysed, cytoplasm mixes with the medium and will result in major changes in the ion concentrations in the medium causing concentrations of K^+, Mg^{2+} to increase, and Na^+ and Cl^- concentrations to drop simultaneously. In contrast, cell growth gives opposite effects and will cause bring K^+, Mg^{2+} concentrations to go down and take Na^+ and Cl^- to go up. A simple mass balance on each ionic species, based on reactor cell numbers and feed and effluent flow rates, will allow one to establish the maximum and minimum concentrations that can be expected and assist in estimating the percent viability and rate of cell growth or cell death.

Cell metabolism will also release NH_4^+, CO_2 and lactate into the culture medium. NH_4^+ inhibits cell growth at 5mM and leads to cell death at 12 mM[5] while lactate prevents cell growth at 40 mM[6]. In addition the lactic acid, CO_2 and NH_4^+ affect medium pH. An optimal pH range of 6.8-7.2 is required to keep cells alive[4]. Therefore, it is necessary to monitor the metabolite concentrations for optimization of cell production. Of course it is straightforward to measure acidity with a pH probe and can be measured with an ISE containing a ionophore. What will complicate matters is that K^+ electrodes have some selectivity for NH_4^+, NH_4^+ ISEs have some selectivity for K^+ and the NH_4^+ to NH_3 equilibrium is affected by pH. Hence, it is important to measure K^+, NH_4^+, and pH in concert to improve accuracy. Yet, this paper is focused on determining performance of only one of the ISE types, that for K^+ and how poly(MPC-co-BMA) will enhance ISE longevity. Once progress is made here advances can be made for measuring different kinds of ions, by developing a suite of ISEs each with a different ionophore in the ISE membrane.

Chapter 3

Experimental Methods

3.1 Reagents and Materials

Our ISE arrays consist of seven ISE electrodes surrounding a reference electrode. We use 18 AWG gauge copper wires (Radio Shack) coated with a polymer membrane consisting of Valinomycin, polyvinyl chloride (PVC) powder, and 97 % pure Bis(2-ethylhexyl) sebacate (all from Fluka). The reference electrode is a liquid filled type with a 99.99 % pure silver wire inserted (Prince & Izant Company) into sodium nitrate (Aldrich) electrolyte as the reference solution, and covered with a reference membrane made from PVC (Fluka), and polyethylene glycol (PEG) (Sigma-Aldrich). The membrane components for both electrodes are dissolved into 99% tetrahydrofran (THF) (Sigma-Aldrich). The array body is built from a PVC rod (McMaster-Carr), and PD190 Epoxy Adhesive (McMaster-Carr) is used to bond wires to the body and to seal the reference solution. Poly(MPC-co-BMA), which is coated onto ISE membranes, was donated by Dr. Ishihara.

Dulbecco's Modified Eagle's Medium (DMEM) (Gibco) is used as the cell growth medium and the basic solvent for calibration solutions comprised of different concentrations of potassium chloride (Sigma-Aldrich). The mouse hybridoma MM1A cell line is used in the long term ISE longevity experiments. Cells were obtained from Professor. W.C. Davis

of the Department of Veterinary Microbiology and Pathology at Washington State University.

3.2 Electrode Fabrication

A PVC rod is cut to a 1 inch length, and the cut end faces are made flat with 120 grit sandpaper. Seven holes are drilled in the face parallel with the central axis with a #58 (0.0420") drill bit with holes in a circular pattern near the circumference at 45^{o} angles from each other, and a lager hole made into the central axis with a #38 (0.1015") drill bit for the reference electrode. Seven bare 18 AWG gauge copper wires are cut to 1.5 inch lengths. These wires are coated with DP 190 Epoxy® adhesive and inserted into the circumferential holes till wire just comes out from the front of the rod surface. After the epoxy is completely dry, the array is sanded with 120 grit sandpaper (J type cloth backing; 3-M). The procedure is repeated with 220, 320, 400, 600, 800, and 1200 grit silicon carbide sandpapers (3M, Buehler) to polish the copper wires. Drops of water are used on sandpapers starting at 400 grit on up to carry away sandings. In a final step, the array is polished with a 0.3 micron Type-N alumina powder slurry (Wendt Dunnington) on a cotton polishing cloth. After the polishing step, 30 μL of reference membrane solution, made by mixing100 mg PVC powder and 200 mg polyethylene glycol in 2 mL THF and heating in a

water bath to aid dissolution[20], is pipetted into the center hole from the polished side. Then the array is hung facing down. When the membrane is dry, it is examined with a dissecting microscope to assure there are no holes or large air bubbles. A saturated sodium nitrate solution filling is pipetted into the opposing end of the center reference hole. An Ag/AgCl reference electrode, made by etching a silver wire in $FeCl_3$/HCl PC-Board etching solution, is inserted into the hole and the back side of the array is fully covered with epoxy to seal in the sodium nitrate solution. For the next step, 3 µL of K^+ ISE membrane, comprised of 1% Valinomycin, 65.8% sebacate and 33.2 % PVC dissolved at a net 100 mg/mL concentration in THF [2], is pipetted onto the surface of each polished copper wire.

So that membrane forms on the surface of the polished side of array with being pulled by gravity. The membrane should be dried to move onto the next step. Reference membrane solution is made by mixing100 mg PVC powder, and 200 mg ethylene glycol in 2 mL THF. The mixture may need to be heated by water bath to make PVC and ethylene glycol dissolved completely. Membrane forms by evaporating THF from the solution.

Once membranes are completely dry, 0.25 µL of poly(MPC-co-BMA) solution is pipetted onto the membrane and allowed to dry overnight in a vacuum chamber. Poly(MPC-co-BMA) solution is made by dissolving poly(MPC-co-BMA) into ethanol to

obtain cocktails of 0.01%, 0.10%, 1.00%, 10.0% polymer concentration by weight. Then each electrode is coated with a different polymer solution including a 100% ethanol control.

3.3 Experiments

There were two types of experiments performed, the first to understand the effects of the poly(MPC-co-BMA) on ISE performance and the second to assess ISE longevity when continuously exposed to bioreactor effluent.

Calibration curves for both K^+ and the interfering NH_4^+ responses are obtained for both non-coated and polymer coated ISE arrays placed in a 50 mL of distilled water in a crucible and monitored with an Environmental Monitoring System (EMS) board, developed at the Naval Research Laboratoryl[20] as shown in Figure 3.1. The arrays are assessed with the standard addition method by adding to the crucible KCl (or NH_4Cl) solutions of 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4} M as indicated in Table 3.1. Then the response slopes and the LODs are determined for each electrode along with the interference coefficients of NH_4^+ on K^+ ISEs as determined with Equation (3).

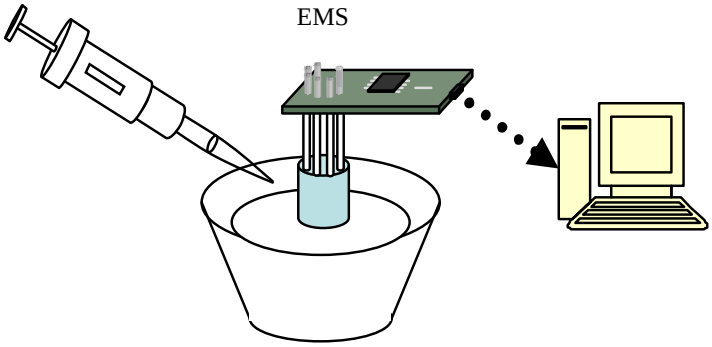

Figure 3.1: The standard addition method
The standard addition method is used by placing electrode arrays in a 50 mL crucible and determining calibration curves between concentrations of 10^{-7} and 10^{-1} M.

.

Table 3.1: Standard addition methods scheme

order	standard concentration (M)	addition volume (μM)	total concentration log[(M)]
1	10^{-4}	50	-7.0
2	10^{-4}	109	-6.5
3	10^{-3}	34	-6.0
4	10^{-3}	110	-5.5
5	10^{-2}	34	-5.0
6	10^{-2}	110	-4.5
7	10^{-1}	34	-4.0
8	10^{-1}	110	-3.5
9	1	35	-3.0
10	1	110	-2.5
11	3.5	100	-2.0
12	3.5	320	-1.5
13	3.5	1000	-1.0

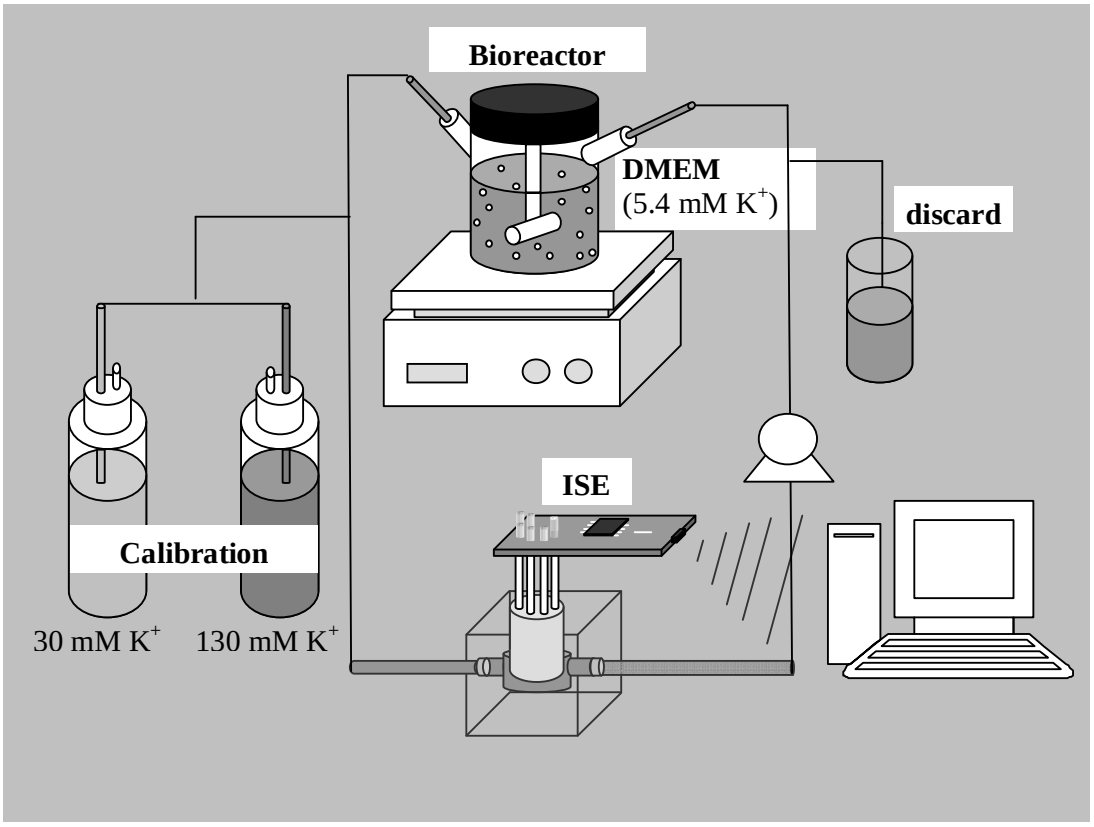

Figure 3.2: Experimental setup for measuring ISE longevity.
The electrical potential is measured with the NRL board and data sent to a computer via an IR link. Medium containing cells is circulated through the ISE array and calibrations performed twice daily using medium containing 30 mM and 130 mM K^+.

Long term experiments are run with the ISE arrays and the batch setup shown in Figure 3.2

Approximately 6 x 10^6 hybridoma cells and 400 mL of DMEM are place into the bioreactor.

The bioreactor contents are circulated past the ISE array at a rate of two mL per minute.

The calibration solutions consist of 30 mM K^+ and 130 mM K^+ are made with DMEM and

KCl and calibrations are made twice daily.

Chapter 4

Results

4.1 Uniformity of Uncoated ISEs

At first, K^+ ISE electrodes were calibrated with the standard addition method by adding ever increasing amounts of K^+ or NH_4^+ prior to polymer coating; this is important since each ISE electrode has its own individual variation in performance. This initial calibration is required so each electrode can be analyzed individually after polymer addition.

Figure 4.1 summarizes the results from the initial calibration of K^+ ISEs showing averaged mV readings and standard deviation error bars for electrode responses (N=6) to standard additions of K^+ and the interferent NH_4^+, respectively. From these data one can calculate the average response slopes and LODs. For K^+ additions shown in Figure 4.1 the average slope and LOD were determined to be 51.6 ± 0.8 mV/log [C] and -4.79 ± 0.02 which corresponds to very small relative standard deviations (RSDs) of 0.6 % and 0.2 %, respectively. It is noted that the 0.2 % RSD for the LOD is on a logarithm scale which translates to an actual variance of 2.3 % in actual LOD concentration between electrodes. One can then assess the uncertainty that might result when using an average LOD and slope from a collective set of electrodes to translate voltage data into concentrations. We can take an extreme case that begins with the lowest K^+ concentration of 5.4 mM in the base culture medium and assess the impact of uncertainties from voltage values in a dense 2 x 10^8 cell/mL

culture that undergoes complete cell lysis that results in a 53 mM K^+ medium concentration. Taking the voltage and its corresponding standard deviation at the base 5.4 mM concentration we can use standard deviations around an average slope to see what concentrations could yield similar mV readings at the 53 mM K^+ concentration. We begin with a voltage one standard deviation above the average mV reading at 5.4 mM K^+ and a slope one standard deviation above the average slope. We then project this line up to the lower standard deviation limit of the average voltage one would expect at 53 mM. The concentration corresponding to this voltage is 43 mM K^+. Doing just the opposite by taking the lower standard deviation at 5.4 mM and the slope one standard deviation below the average we look for the intersection with the higher standard deviation voltage at 53 mM K^+; this results in a concentration that corresponds to 65 mM. Though this is a wide range a rise in voltage of this magnitude would certainly be an indication of cell lysis. A much better way to operate is to calibrate an individual electrode immediately before each monitoring event. Then we are concerned about standard deviations around the points corresponding to 5.4 mM and 53 mM K^+ due to random noise around a given voltage; there is little or no uncertainty in the slope just determined from the calibration. In this case we find a concentration range of 50 mM (-5.4%) to 56 mM (+4.8%). In reality we can do much better than this because any reported voltage is the average of at least 100 points randomly and tightly clustered about a mean value with standard deviations on the order of 0.1 mV. Therefore, we have very high

certainty that the averages are trustworthy representations of the voltage assigned to a certain concentration.

When considering the NH_4^+ interferent, however, Figure 4.1 reveals a more significant variation in the response among the various electrodes. The figure shows an average response slope of 50.2 ± 0.5 mV / log [C] which is 2.7% below that for the K^+ calibration and LOD of -3.85 ± 0.06 which is an order of magnitude higher in concentration than that for the K^+ calibration. The respective relative standard deviations are 1.0% and 1.6%. Again, the response slopes are virtually identical although the LODs show slightly greater variation with uncertainties in the actual concentration values to be as high as 17%. These uncertainties are most important when determining the selectivity coefficients for NH_4^+ interference on the K^+ ISE and will be discussed later.

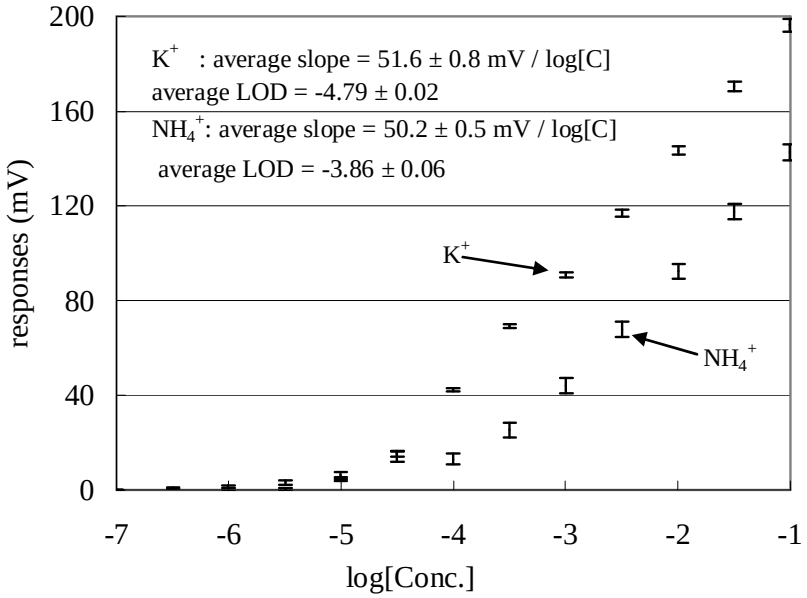

Figure 4.1: The initial calibration of K^+ ISEs with $K^+$and

4.2 Polymer effects on slopes and LODs

The calibration curves for K^+ and the interferent NH_4^+ for K^+ ISEs coated with poly(MPC-co-BMA) solutions of 0%, 0.01%, 0.1%, 1.0% and 10% are presented in Figure 4.2 (a) and (b) with slopes and LODs summarized in Table 4.1. Compared with the results of the initial calibrations for K^+ calibrations in Figure 4.1, there is an average increase in slope of 6.14 ± 0.61 mV for the K^+ curves. LODs show an improvement from the -4.79 log of concentration to a value of -5.07 for the non-coated electrodes and to -4.99 for those coated with 0.01% polymer. Increasing polymer percentages show increasing values, i.e. decreasing sensitivity, down to values of -4.35 and -4.36 for the 1% and 10% polymer solutions as indicated by the shifting of curves to the right.

It is noted that the increase in slope for all electrodes and improved LODs for the uncoated and low polymer percentage are attributed to the fact that all electrodes, including uncoated controls, are kept in a vacuum to dry the polymer solution completely. These improvements are best explained by more complete evaporation of leftover THF solvent from the membranes that takes place in the drying step. Since water is soluble in THF it is possible that besides K^+, other ions can be transported through the membranes thereby lowering the selectivity and sensitivity to the primary K^+ ion. Despite the changes observed in the basic character of the ISE responses the relative effects of polymer coating can be calculated by taking the baseline shifts in slope and LOD observed for the control ISEs and

adding them to the polymer coated ISEs – any deviation of the actual measured values and this correction can then be attributed to the polymer coating itself. The lower parts of Table 4.1 summarize the results of these calculations showing an average reduction in slope on the order of 1 mV / log [C] with no specific trend that correlates to percent polymer in the coating solution. On the other hand, there is a clear trend that the more concentrated polymer coatings lead to higher LODs (lower sensitivity) that level off for the 1.0% and 10% coating solutions. However, the LOD for the 10% polymer coating solution is still -4.36 which can allow one to detect to as low a concentration as 43.7 μM K^+ which is well below the 5.4 mM level of the standard DMEM culture medium.

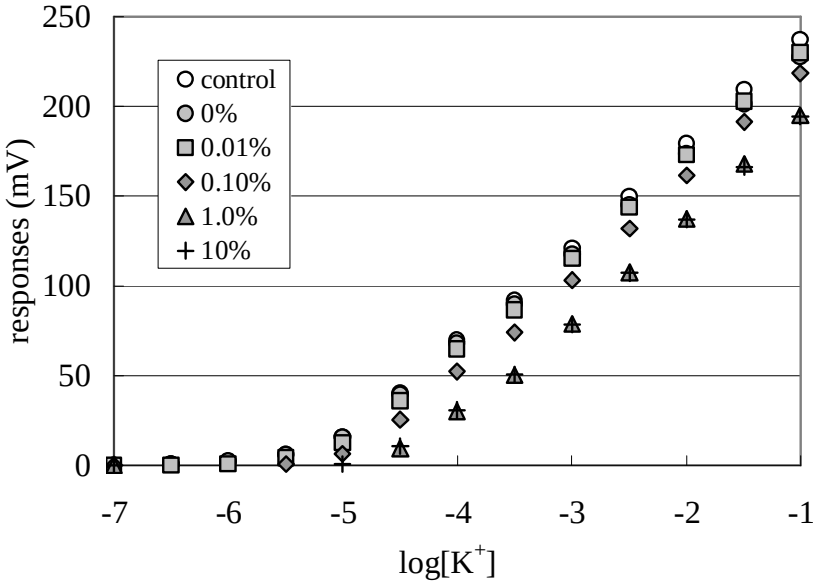

Figure 4.2a: The calibration of coated K^+ ISEs with

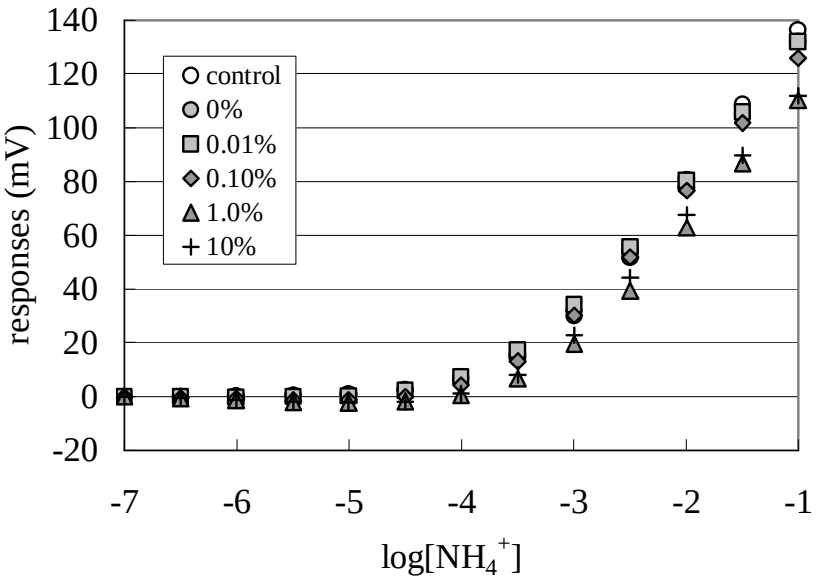

Figure 4.2b: The calibration of coated K^+ ISEs with

Table 4.1: The response slopes and LODs determined from the K^+ calibration and corrected response slope and LOD shifts with polymer coating.

% polymer	control	0%	0.01%	0.10%	1.0%	10%
K^+ slope	58.5	55.4	57.8	58.2	58.6	58.0
K^+ LODs	-5.07	-5.12	-4.99	-4.77	-4.35	-4.36
K^+ slope shift	0	-1.50	-1.21	-0.83	-0.14	-1.12
K^+ LOD shift	0	-0.01	0.09	0.31	0.71	0.73

Table 4.2: The response slopes and LODs determined from the NH_4^+ calibration and corrected response slope and LOD shifts with polymer coating.

% polymer	control	0%	0.01%	0.10%	1.0%	10%
NH_4^+ slope	55.2	53.9	51.2	49.7	47.7	45.2
NH_4^+ LODs	-3.47	-3.45	-3.57	-3.54	-3.32	-3.48
NH_4^+ slope shift	0	-0.46	-4.11	-5.88	-7.86	-10.42
NH_4^+ LODs shift	0	-0.11	-0.21	-0.20	-0.04	-0.15

Figure 4.2 (b) and Table 4.2 show the slope and LOD results for NH_4^+ calibrations with the K^+ ISEs. Again, after polymer addition and further drying, an improvement in response slope is observed for all electrodes with an increase to 55.2 mV/log [C] for the non coated electrode – as in the original calibrations the NH_4^+ slope is below that for the K^+, but this time is 6% below the improved value registered for the K^+ curve. The individual variance for each polymer coated electrode can again be compared one to another by adding the improvement for the non-coated ISE to each electrode and observing the difference between the observed and calculated values. For NH_4^+ curves the response slopes tend to decrease with increasing polymer solution concentration to a maximum of -19 % for the 10% polymer coating solution. Meanwhile LODs show an average decrease of 0.12 which corresponds to a decrease in selectivity of 32 % for a given concentration, however, since there is no specific trend over the range of polymer concentrations, this may be due to random variation in performance between electrodes.

Also of interest is the change in selectivity of the K^+ electrode for NH_4^+. Because the response slopes for NH_4^+ are dependent on the polymer concentration and the baseline slope is less than that for K^+, the interference coefficients are changing with concentration changes of K^+. Interference coefficients calculated with Equation (3) are shown in Table 4.3 and are plotted in Figure 4.3 as a function of K^+ concentration with polymer

concentration as a parameter. One concludes that higher polymer coating concentration leads to more interference and that this increase is at a maximum value at 10^{-3} M K^+ (1 mM) with a 270% increase in the selectivity coefficient on a non-logarithmic basis. There's a diminishing impact on the coefficient with log increases of percent polymer from 0.01% to 10% polymer coating solution with average increases of 170% in the coefficient between 0.01 and 0.1%, of 35% between 0.1 and 1% and of 15% between 1 and 10% at 10^{-3} M K^+ (1 mM).

Table 4.3: Estimated interference coefficient of K^+ ISE with NH_4^+ in logarithmic scale. The interference coefficient is getting larger at higher K^+ concentrations.

[K^+]	**control**	**0 %**	**0.01%**	**0.10%**	**1.0%**	**10%**
-3.00	-1.51	-1.48	-1.37	-1.24	-1.00	-0.94
-2.50	-1.62	-1.58	-1.49	-1.35	-1.16	-1.07
-2.00	-1.67	-1.63	-1.57	-1.43	-1.26	-1.18
-1.50	-1.70	-1.64	-1.64	-1.51	-1.37	-1.30
-1.01	-1.71	-1.62	-1.66	-1.57	-1.44	-1.40

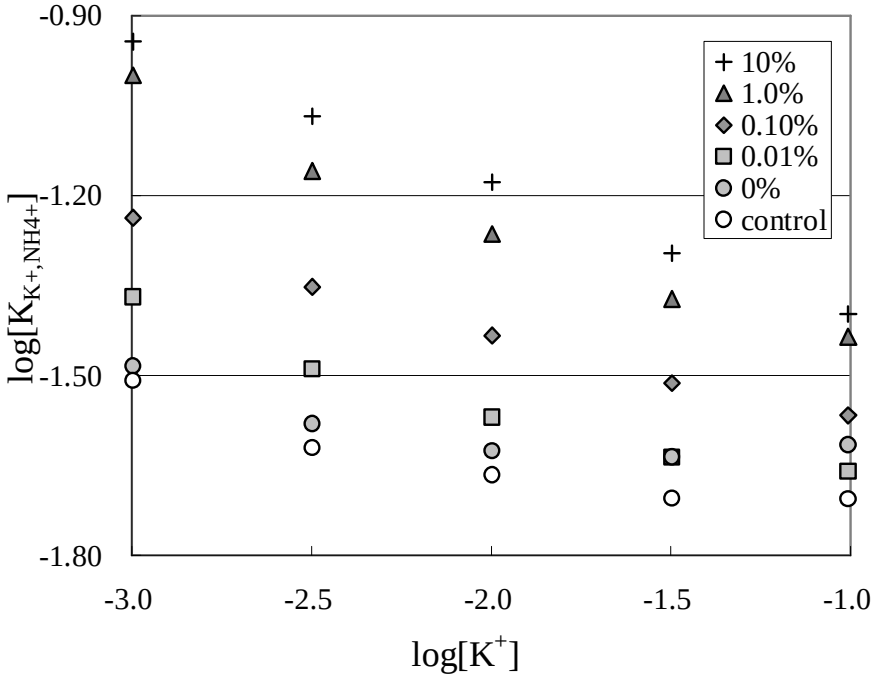

Figure 4.3: Interference of valinomycin with NH_4^+

At this point we can discuss the consequence of using the various coating procedures. First, all of the electrodes presented may be used for determining K^+ levels starting at the 5.4 mM ($10^{-2.3}$ M) base medium value to a 53 mM ($10^{-1.3}$ M) value that would be seen if all the cells in a dense 2 x 10^8 cells/mL culture were lysed and released their contents simultaneously into the medium with an assumption of a 15 μm diameter cell. The maximum concentration of NH_4^+ ion will be at a level where cell death occurs somewhere between 10 (10^{-2} M) to 25 mM ($10^{-1.6}$ M) according to unpublished data from our lab. As long as a corresponding NH_4^+ electrode is available one can use two equations like that shown in Equation (2) to estimate activities for K^+ and NH_4^+ from which corresponding

selectivity values could be determined from Table 4.3 and one like it for K^+ interference on an NH_4^+ electrode. An iterative procedure could then be used to determine the actual activities. This approach has greater limitations as the log of the selectivity coefficients approach 0.0 in which case the ISE would not be selective for one species over the other. Nevertheless, faster convergence of our proposed procedure will occur for the lower more negative selectivity coefficients and for the ISEs represented in Figure 4.3 and Table 4.3 these ISEs will have the advantage of requiring less polymer. Also, of importance are the uncertainties in the selectivity coefficients if one were to use an average slope and LOD for the NH_4^+ response. The impact of these uncertainties on the selectivity coefficient can be found from Equation 3 when using standard deviations in E_B and E_A to calculate the maximum and minimum value of K_{AB}. Then one can do a sensitivity analysis using Equation 2 to determine the uncertainties in calculated activity based on measured voltages. For this one finds an uncertainty of 17% in an activity for the 0.10% polymer concentration determined in the 53 mM range where cell lysis would cause a maximum value in K^+ concentration in the presence of 25 mM NH_4^+ with the assumption that we can use the same standard deviation in response voltage determined for the initial calibration of the collective set of six ISEs.

We also note the recent work which shows improvements in ISE LODs to the picomolar range by controlling the magnitude and direction of flux of the interested ion through the ISE membrane[23]. Future work should include an assessment of polymer affects on these ISEs i.e. whether the enhanced sensitivity is impeded by coating and whether this will be important for a given ISE application. It is also important to address concerns about any economic impact of coating as this could increase ISE cost. We note however, that production of the poly(MPC-co-BMA) used in this paper has increased to the point where more than 12 tons per year are available at low cost Hence, the most important issue is how well the polymer protects ISEs from response degradation as will be discussed in the next section.

4.2 Polymer effects on longevity

For the experiment on longevity in a bioreactor a coating solution of 0.1% polymer was selected after personal communication with Prof. Ishihara who pioneered use of the polymer for preventing protein adhesion in various types of medical implants[15,17]. Selection of this concentration is supported by the previous sets of experiments as K^+ slopes are unaffected and the LODs for K^+ and NH_4^+ are minimally affected.

Figure 4.6 shows results from the long term experiment highlighting the calibrations performed at the various time intervals while truncating the vast amount of data between those intervals when only reactor medium is flowing by the sensor array. The darker lines show responses for polymer coated electrodes while the lighter ones indicate the response for non-coated ISEs. A rapid decline in response slope for the non-coated electrodes is evident within two days as compared to the polymer coated electrodes which maintain response slopes over the 6 days. By the end of this experiment there is no response to 30 mM K^+ solution for the non-coated electrodes and only a minimal change in response of 24 mV out of an expected 38 mV to the 130 mM K^+ solution.

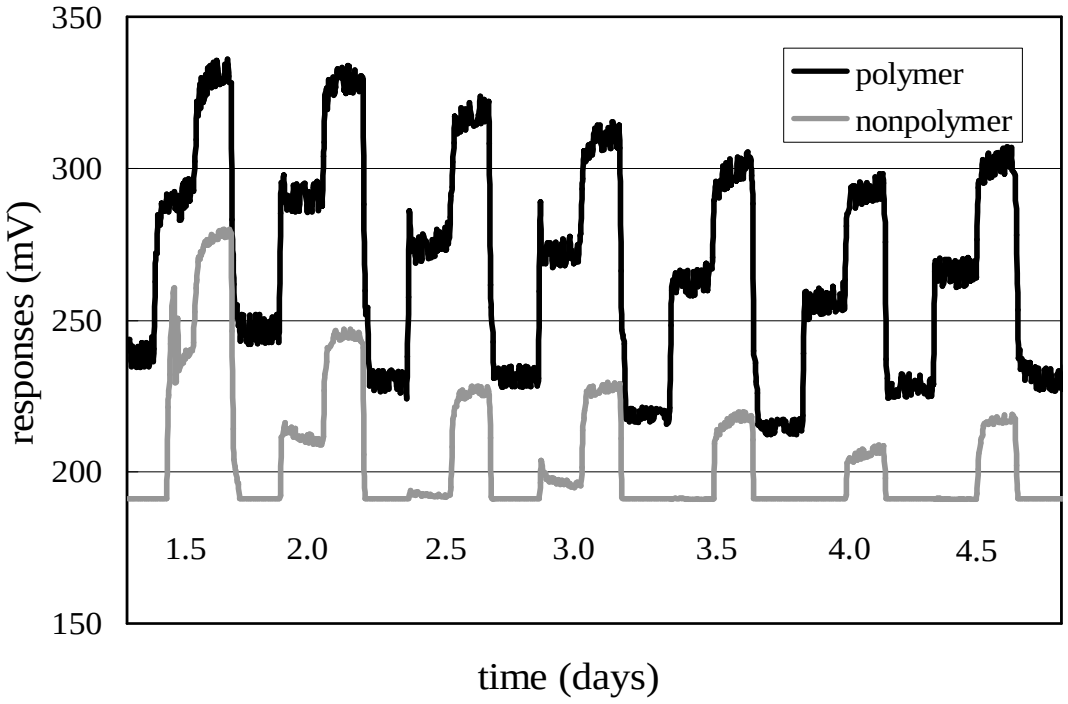

Figure 4.4: ISE response in a bioreactor over 4.5 days.

The responses for the coated and non-coated ISEs to the corresponding 30 (-1.52) and 130 mM (-0.89) scale K^+ concentrations are plotted in Figures 4.5 and 4.6, respectively along with the theoretical response based on the Nernst equation. The most distinctive features of these plots are the contrast in linearity and response slope between the non-coated and coated electrodes. Non-coated electrodes show non-linear trends after the second day with diminished responses both to the 30 mM and 130 mM calibrants and no response at all to the 30 mM calibrant after 3.5 days. By Day 2 the response slope has dropped from 61 mV/log [C] to 48 mV/log [C] and to 38 mV/log [C] after 4.5 days. In contrast, the response slopes of coated electrodes show minimal decline and with strong responses to both calibrants. The coated electrodes perform with an approximate 55 mV/log [C] response slope after 4.5 days which is 9.8 % below the theoretical slope of 61 mV/log [C] at 37˚C – this is still adequate for detecting concentration levels in the medium especially if calibrations are done before each measurement. Figure 4.7 summarizes the changes in response slope between the 30 mM concentrationand 130 mM concentration levels over the 5 day experiment. The figure shows the rapid decline in performance of the non-coated electrodes even for the higher concentration calibration and the steady performance for the coated electrodes. Based on these data we conclude that poly(MPC-co-BMA) definitely improves the longevity of the K^+ ISEs and allows ISEs to retain their sensitivity to the lower concentrations of K^+.

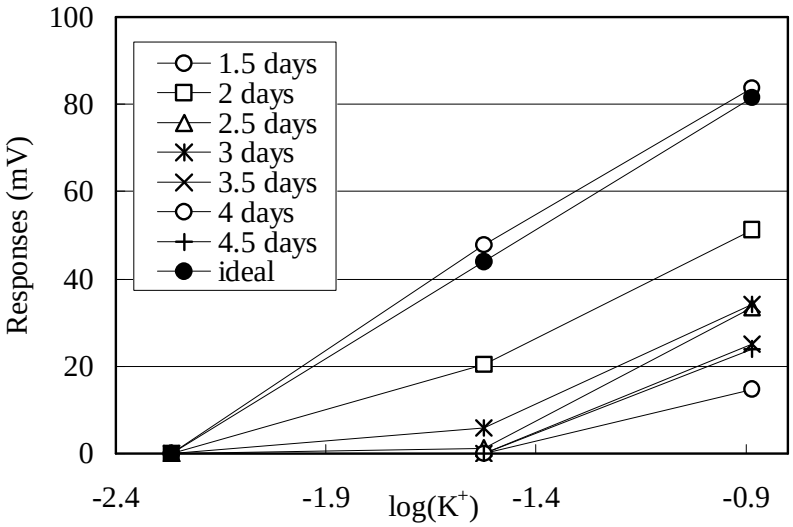

Figure 4.5: Responses of non-coated electrodes. Compared with the ideal response line, the responses tend to lose their linearity and slopes with time of exposure to

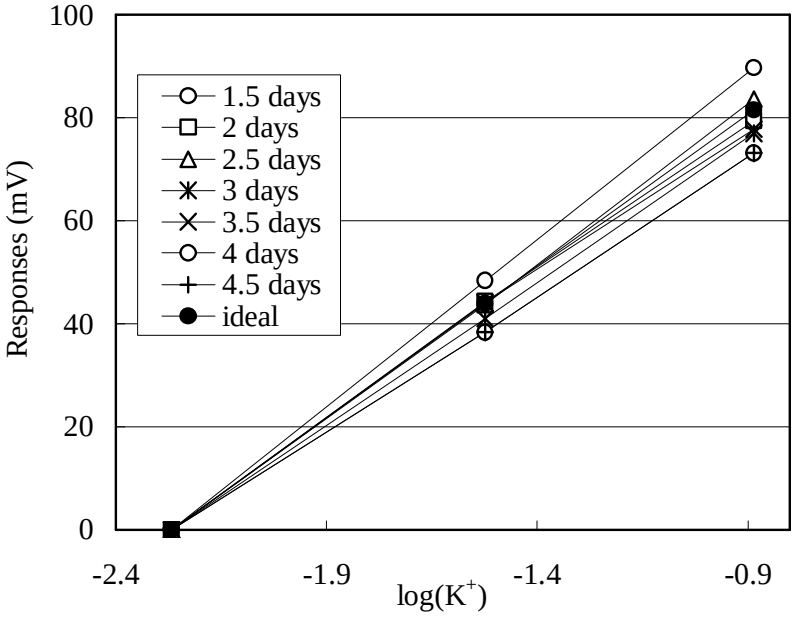

Figure 4.6: Responses of electrodes coated with 0.1 % polymer. Response slopes hold their linearity though there is a small decrease in slope with time of exposure to bioreactor effluent.

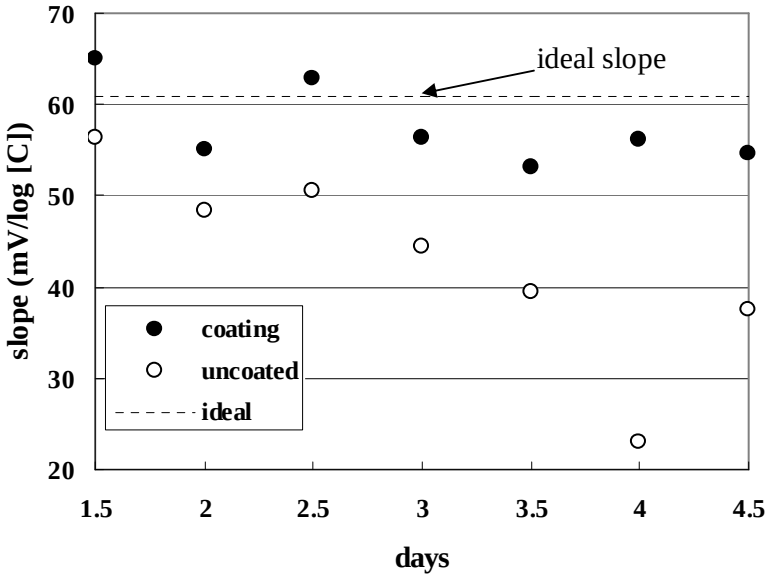

Figure 4.7: Response slope changes over 4.5 days.

It should be noted that cell death occurred after 5 days and was attributed to culture transition to a high pH of 9.4. The most probably reason for the pH increase is production of the metabolite NH_4^+ which will be in equilibrium with NH_3 and known to be very toxic to cells[6]. Responses were monitored for a 6^{th} day as ISEs continued to be exposed to not only proteins, but also significant cell debris in the culture medium. Though calibrations were not peformed during this period, there was no apparent change in the coated electrode performance.

Chapter 6

Conclusions

ISEs were developed to monitor ions in bioreactor effluent with the goal of producing a monitoring system suitable for maintaining optimal cell growth. To reduce problems associated with protein adsorption related deterioration of ISE sensitivity, poly(MPC-co-BMA) was applied to coat and protect ISE membranes . The most important result is that the polymer coating does truly enhance the longevity of valinomycin-based K^+ ISEs. ISEs coated with 0.1 % polymer solution showed near theoretical Nernstian responses and good LODs even after 6 days of exposure to bioreactor effluent. This is in contrast to non-coated ISEs which show greatly diminished response in slope and LOD after only 2 days.

It is noted that the employment of the polymer causes a measurable decline in the K^+ LOD and NH_4^+ interference coefficient. Nevertheless, K^+ concentrations in bioreactor effluent can still be monitored readily with the modified ISEs since the decline in LOD still results in detection limits that are two orders of magnitude below the 5.4 mM or higher K^+ levels found in culture medium . Because of the polymer concentration effect on NH_4^+ selectivity, it is important to determine such effects a priori and to include a NH_4^+ ISE to account for interference affects especially where rather large NH_4^+ concentrations are anticipated.

These results lay the foundation for polymer coating applications to other kinds of ISEs where it is anticipated that the coating procedure will function equally well since the present results Finally, we note recent work showing improvements in ISE LODs to the picomolar range[23]. It will be particularly important to note the impact of polymer coating on LODs and response slopes for such applications.

References

[1] S. Farid, “Established Bioprocesses for Producing Antibodies as a Basis for Future Planning” Adv Biochem Engin/ Bioechnol 101(2006) 1-42.

[2] T. Nottorf, W. Hoera, H. Buentemeyer, S. Siwiora-Brenke, A. Loa, J.Lehmann, “Production of Human Growth Hormone in a Mammalian Cell High Density Perfusion Process” Cell Technology for Cell Products (2007) 789-793.

[3] G. Seth, P. Hossler, J. Chong Yee, W. Hu, “Engineering Cells for Cell Culture Bioprocessing-Physiological Fundamentals” Adv Biochem Engin/ Bioechnol 101(2006) 119-164.

[4] C. Detzel, B. Van Wie, W. Davis, “BioProcess Monitoring and Control of Media Conditions in a Continuous Centrifugal Bioreactor” Abstracts of Papers, 229th ACS Meeting, San Diego, CA, USA, March 13-17, 2005.

[5] D Mason, “Hybridoma Culture and Modeling in a Centrifugal Bioreactor”, Washington State University, Department of Chemical Engineering Master’s Thesis, 2004.

[6] S. Ozturk, M.Riley, B. Palsson, “Effect of Ammonia and lactate on Hybridoma Growth, Metabolism, and Antibody production” Biotechnology and Bioengineering 39(1992) 418-431.

[7] D. Anderson, D. Reilly, “Production technologies for monoclonal antibodies and their fragments.” Current Opinion in Biotechnology 15 (1990) 456-62.

[8] K. Chen, Q. Liu, L. Xie, P. Sharp, D. Wang , “Engineering of a mammalian cell line for reduction of lactate formation and high monoclonal antibody production,” Biotechnology and Bioengineering 72 (2001) 55-61.

[9] B. Van Wie, M. Elliott, J. Lee, “Development and Characterization of a Continuous Centrifugal Bio-Reactor,” Biotechnology Bioengineering Symp., 17 (1986), 336-344.

[10] B. Van Wie, T. Brouns, M. Elliott, W. Davis, “A Novel Continuous Centrifugal Bioreactor for High-Density Mammalian and Microbial Cells” Biotechnology and Bioengineering, 38 (1991) 1190-1202.

[11] R. Smith, “Ion Chromatography Applications,” CRC Press Inc., 1988.

[12] E. Bakker, P. Buhlmann, E. Pretsch, “Carrier-Based Ion-Selective Electrodes and Bulk Optodes. 1. General Characteristics,”Chem. Rev. 97 (1997) 3083-3132.

[13] Y. Miyahata, K.Yamashita, S. Ozawa, Y. Watanabe, “Shift and drift of electromotive forces of solid-state electrodes with ion-selective liquied membranes” Analytica Chimica Acta 331(1996) 85-95.

[14] R. Lu, J. Lee, K. Park “Calculation of solvation interaction energies for protein adsorption on polymer surface,” Journal of Biomaterials Sciecne Polymer Edition 3 (1991) 127-147.

[15] K. Ishihara, H. Nomura, T. Mihara, K. Kurita, Y. Iwasaki, N. Nakabayashi “Why do phospholipid polymers reduced protein adsorption?” Journal of biomedical material research, 39(1998) 323-330

[16] M. Sakakida, *et al*, “Development of ferrocene-mediated needle type glucose sensor covered with newly designed biocompatible membrane, 2-methacryloyloxyethyl phosphorycholine-co-n-butyl methacrylate,” Medical Progress though Technology 21 (1995) 91-103

[17] S. Ho, N. Nakabayashi, Y. Iwasaki, T. Boland, M. LaBerge, “Frictional properties of poly(MPC-coBMA) phospholipid polymer for catheter applications,” Biomaterials 24(2003), 5121-5129.

[18] K. Ishihara, “Bioinspired phospholipid polymer biomaterials for making high performance artificial organs,” Science and Technology of Advanced Materials, 1 (2000), 131-138

[19] M. Berrocal, R. Johnson, I. Badr, M. Liu, D. Gao, L. Bachas, “Improving the Blood Compatibility of Ion-Selective Electrodes by Employing Poly(MPC-co-BMA) a Copolymer Containing Phosphorylcholine as a Membrane Coating,” Analytical Chemistry, 74(2002) 3644-8.

[20] D.Kidwell, “Ion Selective Electrode for Direct Organic Analysis in Saliva, Sweat, and Suface Wipes,” U.S. Patent Application Publication, US 2003/0121779 A1 (2003)

[21] R.Buck, E.Lindner, “Recommendations for Nomenclature of Ion-selective Electrodes Pure & Appl. Chem., 66 (1994) 2527-2536.

[22] N. Tietz, “Fundamental of Clinical Chemistry Third Edition” W.B.Saunders Company, 646-647, 1987.

[23] E. Bakker, M. Meyerhoff, “Ionophore-based membrane electrodes: new analytical concepts and non-classical response mechanisms,” Analytica Chimica Acta 416 (2000), 121-137