UNIVERSITY OF CALIFORNIA, SAN DIEGO

Immobilized Lactate Oxidase for Development of a Long-term Implantable Lactate Sensor

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

In

Bioengineering

By

Adam Louis Strobl

Committee in charge:

Professor David Gough, Chair
Professor Pau Chau
Professor Michael Heller
Professor Michael Sailor
Professor Shyni Varghese

2010

The Dissertation of Adam Louis Strobl is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2010

DEDICATION

To my family and friends,

whose support made this work possible.

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Chapter 2 is reproduced, in part, from the following, which are currently in preparation for publication:

- Strobl AL and Gough DA. Lactate oxidase (LOx) immobilized in a natural polymer carrier: parameter effects on stability in a physiologically relevant range. *In Preparation*.
- Strobl AL and Gough DA. Stability of Lactate oxidase (LOx) immobilized in a polyionic matrix. *In preparation*.

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VITA

2002 Rose-Hulman Institute of Technology, BS in Chemical Engineering

2006 University of California, San Diego, MS in Bioengineering

2008 University of California, San Diego, Candidate in Philosophy

2010 University of California, San Diego, PhD in Bioengineering

ABSTRACT OF THE DISSERTATION

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by

Adam Louis Strobl

Doctor of Philosophy in Bioengineering

University of California, San Diego, 2010

Professor David A. Gough, Chair

Development of a continuous, long-term implantable lactate sensor has long been a goal in the field of biosensors. The current standard of care in treatment of diabetes mellitus involves measurement of only one metabolite, glucose, on an infrequent, discrete basis. Continuous monitoring would capture of all metabolic excursions so that treatment decisions could be made on complete time-series information. In addition, the measurement of the metabolite lactic acid would lead to an even better understanding of the patient's metabolic state. Lactate has also been shown to be of importance in many diseases involving compromised circulatory or pulmonary function, and athletes would benefit from continuous measurement of this variable.

Lactate oxidase (LOx), the enzyme used in the construction of our lactate sensor, is known to be quite unstable with respect to enzymatic activity over time. This leads to reduced sensor lifetimes, which can be problematic for an implantable sensor due to the necessity for frequent replacement. This dissertation focuses on the characterization of LOx as it pertains to sensor design.

LOx was immobilized in different constructs and the activity as a function of *in vitro* incubation time was measured. Two unique systems of immobilization were used here: one based on ionic forces and the other based on chemical cross-linking. It was found that good immobilization yield (the amount of active enzyme remaining after immobilization divided by the amount formulated) could be achieved in the ionic immobilization scheme; however, stability was lower than free enzyme in PBS in all cases. Protection of LOx via complexing with high concentrations of the oppositely charged polymer (a polycation) was essential for maximizing stability. Immobilization via chemical cross-linking in a bovine serum albumin matrix also had good process yields when minimum amounts of cross-linking agent were used. In addition, it was found that stability could be enhanced when compared to free enzyme in PBS via immobilizing the enzyme at a pH of approximately 5.5. The parameters found by experimentation were used to determine the linear range as a function of time for a sensor design model.

I Introduction

1.1 Benefits of continuous lactate monitoring for diseased states, critical care and sports

An amperometric, implantable biosensor for the continuous measurement of lactate has been a large goal of the biomedical community due to its usefulness across a wide range of diseased and healthy states. Lactate is not only the end product of glycolysis under anaerobic conditions, but it is also known to be used as an energy source itself, and is an important metabolic shuttle (Gladden LB, 2004). In diabetic populations, it is known that fasting levels of both lactate and pyruvate levels are elevated (Konrad T, et al, 1999). In addition, insulin sensitivity affects lactate kinetics in addition to glucose kinetics (Watanabe RM et al, 1995). It has been shown that dynamic glucose information contains predictive information (Bremer T and Gough DA, 1999; Rahaghi F and Gough DA, 2006); therefore, dynamic lactate information should give new insights into the metabolic state of the diabetic patient. New dynamic metabolic information could be used in order to better predict the onset of diabetic episodes and pave the way for better control of the disease. Also, a better understanding of the dynamic metabolic state could lead to the ultimate end-goal of diabetes management technology: a closed-loop control system (Steil GM et al, 2004).

Beyond diabetes, continuous lactate monitoring would provide important information for the treatment of diseased states involving cardiac or pulmonary impairment, which could impact local or systemic lactate levels. Elevated lactate levels have been observed in patients with sepsis who are undergoing pulmonary failure (Brown, SD et. al., 1996). Lactate levels are also a determinate for the prognosis of patients in shock (Broder, G and Weil, MH, 1964). Additionally, sports medicine would benefit from dynamic lactate measurement. Athletes are currently restricted to lactate level testing in a controlled environment where blood can be

drawn and tested at certain intervals (Beaver, WL et. al., 1985). Continuous monitoring of lactate levels produces a more robust determination of fitness in athletes (Palleschi, G et al, 1990). A monitoring system that could be used in normal training and competition environments could be highly useful for adjusting strategies “on the fly”.

1.2 Results of glucose sensor performance

The first enzyme based sensor was a glucose sensor using glucose oxidase (GOx) paired with an oxygen sensing electrode and was published in 1962 by Clark and Lyons (Clark LC and Lyons C, 1962). The reaction that the enzyme catalyzes is (Tse PHS and Gough DA, 1987):

$$glu\cos e + O_2 + H_2O \xrightarrow{GOx} gluconic \quad acid + H_2O_2 \tag{1.1}$$

The signal in this type of sensor is based on quantification of the reduction of amount of the available co-substrate, O_2. The signal generated in an amperometric sensor is proportional to the flux of O_2 at the surface of the electrode (Gough DA and Leypoldt JK, 1979):

$$i_{O_2} = nFAD_{O_2}\frac{\partial c_{O_2}}{\partial x} \tag{1.2}$$

where:

n = number of electrons liberated (4 in the case of O_2)

F = Faraday’s constant

A = the electrode cross-sectional surface area

D_{O2} = the diffusion constant of O_2

c_{O2} = the concentration of O_2

x = the spatial variable perpendicular to the electrode surface

Glucose oxidase-based sensors remain popular, both because there is a requirement in medical and industrial communities that glucose be measured and because GOx from *Aspergillus Niger* has been shown to be very stable under operating conditions (Tse PHS and Gough DA, 1987). *In vivo* sensors were quickly adopted, and with the advent of the 2-dimensioinal enzyme-electrode system, measurements could be made directly in whole blood using a catheter-type sensor (Gough DA et al, 1985). These sensors were shown not to be operationally limited by enzyme lifetime (Armour JC et al, 1990); however, there are drawbacks to this method, including concerns of thrombogenicity (Schoenfisch MH et al, 2000) and relative invasiveness.

In recent years, commercial continuous glucose monitoring systems have become available (MiniMed Guardian®, DexCom SEVEN®, Abbott Diabetes Care FreeStyle Navigator®). These systems rely on a sensor mounted in needle-type system where the sensor element is inserted subcutaneously and the associated hardware (power source and signal transmitters) remains external. Drawbacks of these systems include the relatively short lifespan of the device (3 day replacement with the MiniMed device (Wood JR and Laffel LMB, 2007), 7 day replacement with the Dexcom device, and 5 day replacement with the Abbott device (Garg SK et al, 2009) and the requirement of frequent calibration (2 to 4 times daily), which may introduce error (Kamath A et al, 2009). This involves blood glucose monitoring by fingerstick, similar to readings a diabetic without a CGM device must make.

A third device-type is the hermetically sealed subcutaneous glucose sensor. This sensor type is entirely implanted in the subcutaneous tissue and can function for longer periods without recalibration (Gilligan BJ et al, 2004). Currently, a device of this type produced by Glysens Inc has been found to function for greater than one year in the porcine subcutaneous environment (Gough DA et al, 2010). These sensors have the disadvantages that interstitial fluid

glucose and lactate may lag behind their respective values in blood, and that physiological differences in tissue can complicate the signal (Makale MT et al, 2005). However, as a minimally invasive implantable sensor, this type of device is ultimately preferable. Therefore, the parameters measured in the laboratory for lactate oxidase are evaluated in a model of a sensor designed for use in the subcutaneous environment.

1.3 Results of previous lactate sensor performance

Lactate sensors have been created using the same sensing-modalities as their glucose-sensing counterparts. The enzyme most easily used here to select for the substrate is lactate oxidase (LOx). The reaction catalyzed by LOx is the following (Baker DA and Gough DA, 1995):

$$lactate + O_2 \xrightarrow{LOx} pyruvate + H_2O_2 \tag{1.3}$$

Although GOx and LOx have similar stabilities in solution (Gough DA and Bremer T, 2000; data presented in this dissertation), LOx does not seem to easily gain large amounts of stability upon immobilization. This leads to LOx being labeled in the literature as an "unstable enzyme" (Gibson TD et al, 1992; Minagawa H et al, 1998). A catheter-type biosensor has been developed for use in order to measure lactate in physiological studies. This type of lactate sensor was created by Baker and Gough and was responsive to high lactate levels (up to 25mM) for more than 1 week during continuous operation under *in vivo* conditions (Baker DA and Gough DA, 1995). This sensor had a relatively large active area, at 3mm in length. However, the functional lifetime of only slightly greater than one week makes it unsuitable for use in human subjects with chronic disease such as diabetes.

Surprisingly, there is little data on the use of lactate sensors in *in vivo* systems; therefore not much is known about the lifetime of other sensor types in this application. However, LOx

has been studied in various immobilized enzyme systems, often under highly accelerated test conditions (Chen Q et al, 1998; Minagawa H et al, 1998). Many other systems are evaluated for use as an *in vitro* testing system, where only the shelf-life of the sensor is tested under refrigerated conditions (Hall CE et al, 1996; Gavalas VG and Chaniotakis NA, 1999). Some of these modalities are discussed further in section 1.4.2.

1.4 Sensing modalities

1.4.1 O_2 versus H_2O_2-based amperometric sensors

There are two basic modalities of sensing (Jablecki M, 2002) in what is generally termed a "first generation" continuous sensor (Liu J and Wang J, 2000). In both systems, an oxidoreductase type enzyme is immobilized in a construct that is placed in contact with an electrode surface. This immobilization reaction is not only important for keeping the enzyme in close proximity to the electrode surface, but also because it has implications for the parameters determining sensor lifetime (see section 1.5). In the first modality, the electrode is potentiostatically biased at about +600mV versus an Ag/AgCl reference electrode; this results in consumption of the reaction product H_2O_2 at the electrode surface. The main disadvantages of this modality are that there are many other components in blood or interstitial tissue that may also be reduced at this electrode bias voltage, and that H_2O_2 causes enzyme inactivation before it diffuses either out of the membrane or to the electrode surface (Tse PHS and Gough DA, 1987). In addition, it is possible for certain designs based on H_2O_2 detection to produce the same sensor value for multiple substrate concentrations (Jablecki M and Gough DA, 2000). Because of these drawbacks, it is generally a better strategy to determine amount of substrate oxidation based upon reduction of the co-substrate O_2 at the electrode surface (the second

modality). In this case, the electrode is biased at –500mV versus an Ag/AgCl reference electrode, where both H_2O_2 and O_2 are electrochemically active (Tse PHS, 1984). A thin silicone rubber membrane is employed to keep polar molecules, including H_2O_2, from being consumed at the electrode (Jablecki M, 2002). There is very little interference from other substrates at the electrode surface in this sensing mode. In addition, the enzyme catalase can be included in the active enzyme layer (Gough DA and Bremer T, 2000); this catalyzes the reduction of H_2O_2, thereby quickly eliminating a source of enzyme degradation and also allowing for regeneration of half of the O_2 utilized in the oxidoreductase reaction. This in turn allows for higher levels of substrate to be measured with the same sensor geometry (Jablecki M, 2002). The overall coupled reaction scheme for LOx then becomes:

$$lactate + \frac{1}{2}O_2 \xrightarrow{LOx,catalase} pyruvate + H_2O \tag{1.4}$$

Sensor simulations shown in chapter 5 are based on this reaction scheme. A signal that is proportional to the amount of substrate being oxidized can be determined by subtracting the signal in the presence of the active enzyme layer from a sensor that is lacking the enzyme but is in the immediate vicinity. For this dissertation, it is assumed that the sensor would operate via O_2 measurement.

1.4.2 Other sensing modalities

In addition to the "1st generation" sensors such as the Clarke-type electrode system, there are other possibilities for sensing the consumption of a substrate via enzyme catalysis. "2nd generation" sensors generally utilize a redox-mediator agent that shuttles electrons back and forth to an electrode. The mediator can be regenerated and can be useful for lowering the operating potential of the electrode to a point where interference from secondary molecules is

acceptably low (Liu J and Wang J, 2000). So-called "3rd-generation" sensors go one step further and attempt to wire the redox reaction at the electrode directly to the enzyme active site (Heller A, 2004).

These 2nd and 3rd-generation biosensors can be highly suited to making very fast measurements of extremely low-concentration analytes. In the case of lactate measurement, we have an analyte that is present in relatively high concentrations (approximately 1mM for a normal adult at rest). In this case, being able to quantify larger concentrations via a linear calibration over a longer period of time becomes the main goal. Because of these requirements, and because glucose-oxidase based 1st-generation sensors have been used successfully as *in vivo* sensors for an analyte that has roughly the same concentration, we have chosen to base our work on the assumption that the 1st-generation system will be used.

1.5 Sensor design and trade-offs

There are various design elements that must be taken into account when creating a sensor for a specific purpose. For example, a sensor that can rapidly determine whether or not a given analyte is present in micro-molar concentrations will have a different design than one that must respond linearly to variations in concentration between 0.5 – 4 mM and have a time-lag no greater than 20 minutes. Generally, a sensor will be functioning in its linear response range as long as it is under "diffusion control". This condition describes a reaction system whereby the rate of substrate consumption is determined by the rate of diffusion of substrate into the active region. The opposite case is that of "reaction control", whereby the rate of substrate consumption is limited by the maximal turnover rate of the enzyme contained within

the active region. The ratio of diffusion effects to reaction effects can be expressed by the following term (Tran-Minh C and Broun G, 1975):

$$\sigma = \left(\frac{k_{cat} c_e \delta^2}{D K_m} \right)^{\frac{1}{2}} \tag{1.5}$$

where:

k_{cat} = maximum intrinsic catalytic rate

c_e = concentration of active immobilized enzyme

δ = thickness of the diffusion layer

D = diffusion coefficient of the limiting substrate

K_m = Michaelis constant for the limiting substrate (at saturating conditions of the other substrate)

As σ increases, the system becomes more diffusion limited. If we construct a 2-D sensor (in which different substrates diffuse in from different directions), such as that shown in figure 1.1, this equation becomes:

$$\sigma = \left(\frac{k_{cat} c_e \delta^2 \xi^2}{D K_m} \right)^{\frac{1}{2}} \tag{1.6}$$

where ξ is a geometry factor taking into account the aperture effect in the design shown below (Jablecki M, 2002):

$$\xi = \sqrt{1 + \frac{3}{2} \kappa^2 \left(1 - \left(\frac{\mu}{\kappa} \right)^4 \right)} \tag{1.7}$$

where:

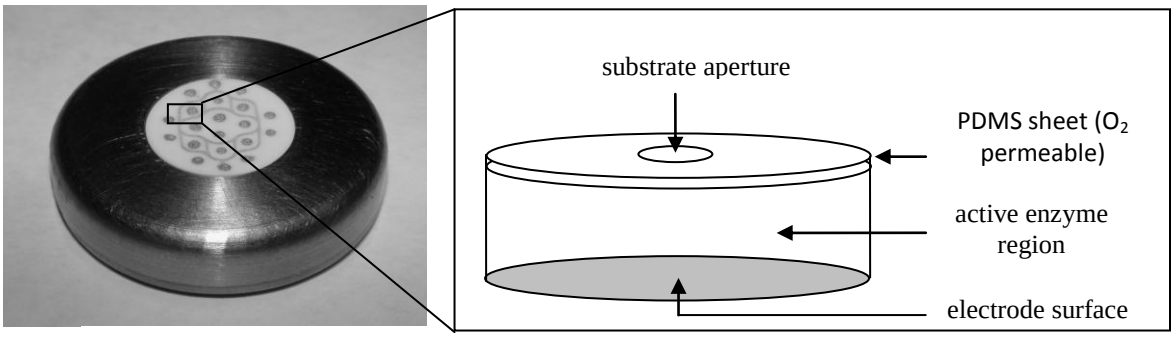
$$\mu = \frac{r_a}{\delta} \tag{1.8}$$

$$\kappa = \frac{r_e}{\delta} \tag{1.9}$$

r_a = aperture radius

r_e = electrode radius

It is quickly seen that the design of the substrate aperture is highly effective at increasing σ at high κ-values (when δ is relatively small); however, it is less effective when using a thicker active enzyme region.



Courtesy Glysens Inc.

Figure 1.1: Picture and expanded schematic of the sensor device.
On the left, an entire sensor device, built by Glysens Inc, is shown. It consists of several electrodes, which (in a glucose sensing configuration) contain the enzyme GOx immobilized near the surface. The proposed design for the substrate-sensing element is shown on the right (Jablecki M, 2002). Access of the substrate is limited by a PDMS membrane. This helps to overcome limitation by the co-substrate in the O_2 depleted subcutaneous environment.

By investigating these equations, we see that there are several design variables in creating a sensor. We may, for instance, increase the thickness of the diffusion layer in which the enzyme is immobilized. The advantage would be increased linear range of the sensor and increased lifetime. This would have the trade-offs of increasing the response time of the sensor

(Gough DA and Leypoldt JK, 1981) as well as decreasing the signal strength at a given concentration. The variable that we are most interested in with regards to this dissertation is that of the active enzyme concentration, c_e. This concentration decreases with time as the enzyme goes through a spontaneous inactivation process. This leads to a first-order decrease of the form (Tse PHS and Gough DA, 1987):

$$c_e(t) = c_{e,0} e^{-\frac{t}{\tau_0}} \tag{1.10}$$

where:

$c_{e,0}$ = initial concentration of active immobilized enzyme

t = time

τ_0= time constant of spontaneous inactivation (1/time)

Here we see that there are two parameters that we can attempt to control in order to increase the lifetime of an enzyme-based biosensor: $c_{e,0}$ and τ_0. This assumes that there are no inactivating chemical agents present. H_2O_2, the reaction product, is a strong inactivating agent (see section 2.4.3.1 for analysis); however, the problem of H_2O_2 generation has been dealt with successfully in glucose sensors by the introduction of catalase as a coimmobilized enzyme (Conway PJ and Gough DA, 1987). Therefore, we focus our efforts here on increasing the active enzyme concentration after the immobilization processes employed, and on increasing the stability of the resulting immobilized enzyme.

1.6 Background on increasing enzymatic stability

It is often noted that enzymatic stability changes with immobilization. This immobilization may take many different forms; adsorption in carbon paste electrodes, physical entrapment in sol-gel matrices, chemical cross-linking in a polymer matrix, etc. In addition,

there are methods by which a bulk immobilization scheme may take place, or ones in which a layer-by-layer buildup of material can occur. In a layer-by-layer (LbL) system, one drives the immobilization by alternating enzymatic and binding layers. The binding can be electrostatic (Wu BY et al, 2007; Ferreira M et al, 2004), chemical (Yingying S et al, 2006), or immuno (such as biotin-streptavidin complex) (Anzai J et al, 1998). Unfortunately, for an *in vivo* continuous sensor such as we are proposing, it would take many such layering steps before a sufficient enzymatic layer could be built up. Like the adsorption-type biosensor constructs, biosensors based on LbL constructs may be better suited to performing fast analysis on diluted samples *in vitro* (Ram MK et al, 2001). Therefore, we have concentrated our efforts on immobilized constructed that can be completed in a bulk reaction.

One such bulk construct is a glutaraldehyde cross-linked natural polymer matrix. It has been known for some time now that the enzyme glucose oxidase from *Aspergillus Niger* gains a large degree of stability when it is immobilized by the glutaraldehyde cross-linking method (Tse PHS and Gough DA, 1987). Other enzymes also exhibit this same basic effect; the hypothesized explanation is that the glutaraldehyde cross-bridges are responsible for bracing the active structure of the enzyme. These cross-bridges may be intra- or inter-molecular, and between either the same molecule (enzyme-enzyme) or a carrier molecule (ie, enzyme-BSA) (Wong SS and Wong LJC, 1992). Glutaraldehyde reacts predominately by a Schiff-Base reaction scheme; however, there are many other side reactions that occur, and the molecule also self-polymerizes (Hermanson GT, 2008). Therefore, the reaction can be difficult to control; besides “bracing” the active structure, glutaraldehyde may also react in a manner that inactivates the enzyme entirely (Tse PHS et al, 1987). For this reason, it is important to consider the amount of enzyme that is still active after immobilization. If activity is too low in the initial preparation, the sensor will

have a very limited range of detection, and a dilution mechanism would have to be utilized (see section 1.5). A simplified diagram of the cross-linking process' effect on the enzyme is shown in figure 1.2b.

Another method favored by researchers for stabilizing enzymes is that of complexation with an oppositely charged polymer (Gibson TD et al, 1995). This method is often used for enzymes that have a charged substrate (like LOx) (Heller J and Heller A, 1998), and that are immobilized via adsorption or physical restraint methods, such as adsorption on a carbon paste electrode (Gavalas VG and Chaniotakis NA, 2000) or trapping within a sol-gel matrix (Chen Q et al, 1998). It is thought that complexation with the flexible, oppositely charged polymer protects the enzyme from potentially inactivating interactions with the host material (Heller J and Heller A, 1998). One method of completing an immobilization (discussed at length in chapter 4), is the complexation of a linear polycation with a linear polyanion (Mizutani F et al, 1995). A simplified diagram of this process' effect on the enzyme is shown in figure 1.2c.

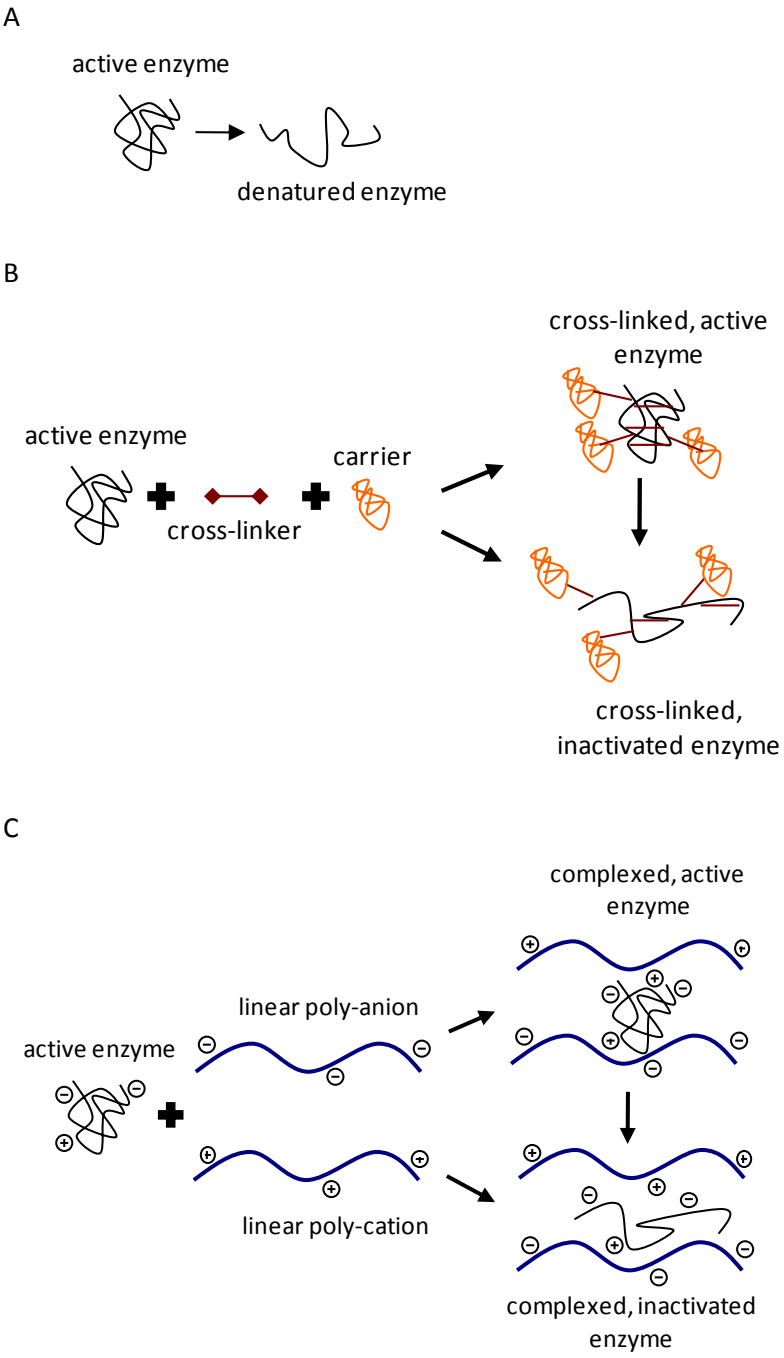


Figure 1.2: Simplified schematic of enzyme immobilization effects
(a) Active enzyme eventually denatures, losing activity. (b) Enzyme can be immobilized via chemical cross-linking; results may inactivate some enzyme molecules but stabilize others. (c) Enzyme can be immobilized via interaction with charged polymers; this also may inactivate the enzyme.

Researchers have also investigated changing the primary structure of enzymes in order to make them more stable. Rational design processes have been proposed, where a mutated version of the protein would be expressed that has certain amino acids deliberately replaced (Kaneko H et al, 2005). This approach is limited by our understanding of the catalytic mechanism of each enzyme under consideration and the resulting effects of amino acid substitution. A pure trial and error approach is hemmed in here by our knowledge of the enzyme and its interactions. A different approach is that of random mutagenesis, followed by screening for a highly thermostable variety of the resultant enzymes (Minagawa H et al, 1995). This process has produced varieties of LOx that are highly stable at very high temperatures. These thermostable varieties have yet to be evaluated in an *in vivo* type situation. One potential drawback to this methodology is that, in order to decrease the time to screen for thermostability, the screening process is performed at very high temperatures (70°C). Because of the possibility that the inactivating mechanism may change at the lower temperatures seen *in vivo* (see section 3.3.2.2.2), the high stability may or may not be present the *in vivo* situation.

1.7 The approach of this dissertation

1.7.1 Immobilization of lactate oxidase via glutaraldehyde cross-linking

The enzyme lactate oxidase from *A. Viridans* was immobilized in a natural polymer matrix via the glutaraldehyde method. This source was chosen as it is a widely available commercial source and has been used by several other groups working with LOx (Chen Q et al, 1998; Moser I et al, 2002). The cross-linking process was refined such that glutaraldehyde was added in a careful and consistent manner. This is important due to the high sensitivity of immobilization yield to the amount of glutaraldehyde used.

Stability testing was conducted on the resultant membranes, with both positive (free LOx in solution) and negative (non-LOx-loaded membranes) controls. Incubation was mainly done at 37°C in pH 7.4 PBS, although higher incubation temperatures were also evaluated for their effect on stability. Process variables for the immobilization were changed to affect the outcome of the immobilization process and hence the final enzymatic stability. The activity assay was carefully validated with regard to the reaction/diffusion problem and with regard to the specific chemical components. Results of this assay validation are given in chapter 2. Results for inactivation parameters of the immobilized enzyme are given in chapter 3.

1.7.2 Immobilization of lactate oxidase via polyionic bonding

LOx from *A. Viridans* was immobilized in a 2-component polyionic matrix. A strong polycation, diethylaminoethyldextran (DEAE-D), was stirred together with LOx and then precipitated out of solution with a strong polyanion, poly(sodium-p-styrenesulfonate) (PSS). The resulting material contains active enzyme bound within the matrix. Enzyme stability in each of the individual matrix components was studied, and the immobilization scheme was formulated such that activity was maintained while the precipitated material did not resolubilize.

Stability testing was conducted, again with positive and negative controls. Process variables were changed and the effect on stability measured under the same incubation conditions as 1.7.1. Results are given in chapter 4.

II A spectrophotometric assay for soluble and immobilized lactate oxidase

2.1 Introduction

In order to measure the stability of the immobilized enzyme systems we create, there must be an activity assay that is reproducible and robust. The assay, repeated over several timepoints for the same immobilized enzyme membrane, should give an accurate measure of the enzymatic activity and not be altered by the assay itself.

Assaying activity of the native, soluble enzyme is straightforward. An aliquot of enzyme solution is added to the test solution and stirred vigorously. As long as substrate concentrations are kept high (easily obtainable in practice), the activity reported by the measurement is very close to the V_{max} value defined by Michaelis-Menten kinetics. However, when confining the enzyme to an immobilized construct, limitation of the reaction rate by diffusion of the substrates into the construct can affect the apparent kinetics of the enzyme (Seong GH et al, 2003). This problem must be dealt with by keeping the substrate concentrations relatively high, the activity of the immobilized enzyme relatively low, and the diffusional effects of the membrane comparatively low (ie, a low value for σ from equation 1.5). Gough and Leypoldt dealt with this issue by using a rotated disk electrode, which gives a well defined boundary layer; the entire system can be solved analytically (Gough DA and Leypoldt JK, 1979). However, we wish to use a spectrophotometric assay to measure LOx activity due to ease of use for multiple samples. In this chapter we attempt to quantify the effect of diffusion on the assay system in order to ensure that measurements of activity and stability parameters are accurate.

Additionally, the membranes are subjected to assay conditions repeatedly throughout a stability experiment. Since we are attempting to measure the spontaneous inactivation of the enzyme at the conditions defined in the experiment, effects of the assay itself must be

minimized. Therefore, we must show that the conditions to which we expose the membranes during testing do not appreciably alter the outcome of the experiments.

2.2 Materials and Methods

2.2.1 Membrane Manufacture

2.2.1.1 Glutaraldheyde cross-linked matrices

For bovine serum albumin carrier matrices, BSA (from OmniPur) was dissolved in the appropriate buffer solution for the pH region considered (sodium acetate, pH 4.5; piperazine, pH 5.5; bis-tris, pH 6.5; PBS, pH 7.4; PBS, pH 8.5: all materials from Sigma) to the amount 20% w/w. The resulting solution was titrated to the desired pH at room temperature using 1 N HCl or 1 N NaOH (Sigma). Concentrated LOx solution in 50 mM PBS, pH 7.4, was made from lyophilized powder (15-20% protein) (from Genzyme or AG Scientific, source: *A. Viridans*) at a ratio of 1 mg powder/ml (approximately 40 U/ml). For collagen carrier matrices, type 1 collagen from bovine Achilles tendon (from Sigma), was denatured and solubilized via autoclaving to a solution strength of 11% w/w in PBS. For one standard batch of BSA-carrier membranes, 200 μl of LOx solution was mixed with 2 g of 20% w/w BSA solution (note that loading was varied during certain experiments; however, solution solids concentrations of the mixture remained constant). The same relative theoretical loading per dry weight of carrier was targeted in the collagen-carrier membranes. The solution was again titrated after mixing to the desired pH at room temperature. For all BSA-carrier batches (and some collagen-carrier batches), 365 μl of 2.5% glutaraldehyde (from Sigma) solution (just enough to affect a solid gel after a 10 to 20 minute reaction period) in distilled water was added to this mixture while stirring, which was allowed to continue for 2 minutes. The solution (still liquid at this time) was then drawn up and

injected into 18 mm diameter wells at an amount of 0.15 ml per well. The wells were made by a thin sheet of PDMS (Sylard 184 silicone elsastomer base and curing agent) over a Petri dish covered in Parafilm "M" (American Can Company), which later aided in membrane removal. The albumin/LOx/glutaraldehyde mixture was allowed to dry to a thin film. This film was then covered by a thick acrylic sheet with holes aligned with the circular enzyme layer films. These holes are backed by a PTFE microporous membrane, 0.45 μm (Millipore), which contacted the dried film, creating a well for glutaraldehyde solution on top of the membrane. Glutaraldehyde solution is poured in the wells, and additional cross-linking is allowed to proceed. The process is shown schematically in figure 2.1. Membranes are removed from the device, dried and weighed, and placed in 10 ml of PBS (pH 7.4) for storage.

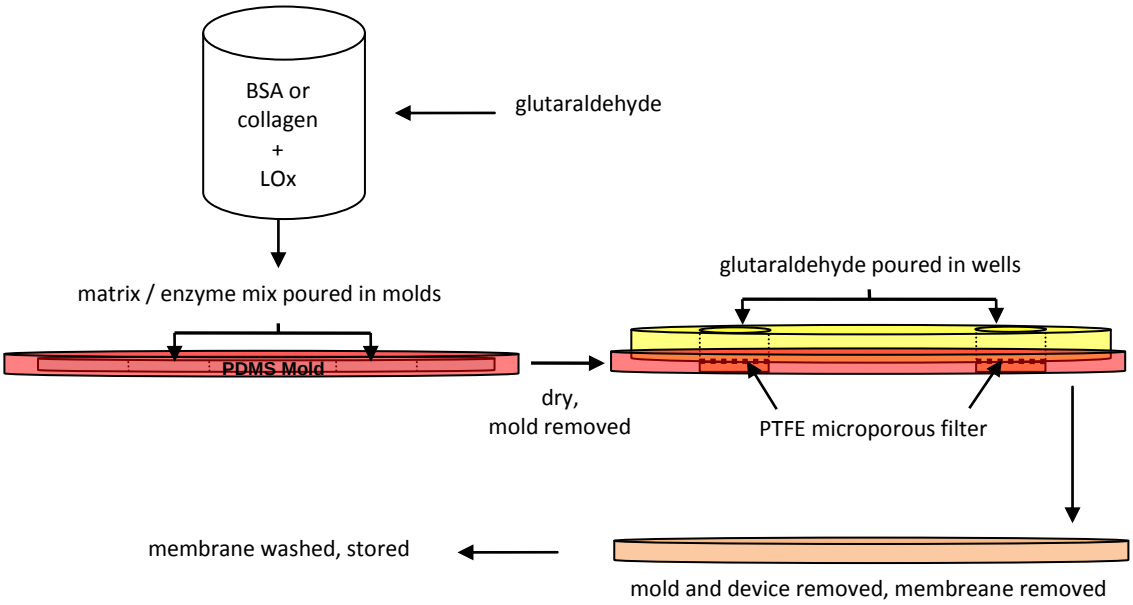


Figure 2.1: Schematic showing the membrane manufacturing process.
LOx is mixed with BSA or denatured collagen solution and glutaraldehyde is added (for all BSA and some collagen batches) at the appropriate pH. The mixture is stirred and thinly spread in wells where it is allowed to solidify and dry. An additional amount of glutaraldehyde is then added to wells atop the membranes, separated by a microporous PTFE filter. After a specified time, the cross-linking agent is removed.

2.2.1.2 Polyionic matrices

LOx used in these experiments was from AG Scientific (15-20% protein, approximately 40 U/mg lyophilized powder). Diethylaminoethyldextran HCl (DEAE-D) (Sigma, average MW = 500,000) and poly(sodium-p-styrenesulfonate) (PSS) (Acros Organics, average MW = 70,000) are dissolved in a buffer solution appropriate to the desired pH of the immobilization reaction and titrated to the required pH value. Proper solution strength for DEAE-D is determined by the experimental conditions; solution strength of PSS was held to 15% w/w throughout experiments. LOx is dissolved in pH 7.4 PBS at a solution strength appropriate for the determined loading of the immobilized construct. For immobilized experiments, LOx is added to DEAE-D solution to achieve the desired concentration and loading, stirred for approximately 10

min, and then allowed to incubate for a specified period of time. In order to effect immobilization, PSS solution is added to an amount of the DEAE-D/LOx solution in one of two ways; either the required amounts are pipetted into a shallow well and mixed directly on the well, or the amounts are mixed in a larger bulk batch in a vial and then transferred to the wells. These wells are formed by a PDMS mold on a parafilm backing in a petri dish container (as with the process outlined in 2.2.1.1). Upon addition of PSS, a viscous precipitate forms which is then dried to a film. Also, additional amounts of PSS were added after drying to a film for some experiments.

In contrast with the cross-linked natural polymer matrices, the film that is created here, when hydrated, does not adhere to itself sufficiently to create a fully-formed membrane alone in solution. Therefore, the material is left on the parafilm backing which allows material to retain a consistent shape. The membrane can then be rehydrated and tested repeatedly. The membranes undergo a wash step whereby some material as well as active enzymatic activity can be lost. The immobilization and membrane formation process is shown schematically in figure 2.2.

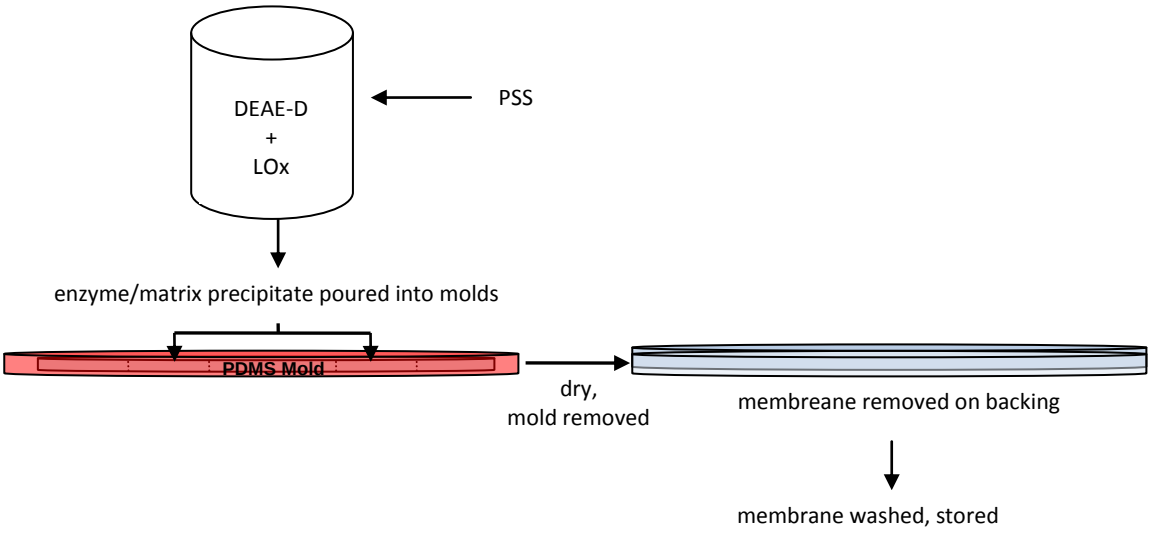


Figure 2.2: Diagram of the process by which the polyionic membranes are created.
PSS is added to a vial containing a DEAE-D/LOx mixture, creating a precipitate. This precipitate is put into a mold, where it is dried to a thin film and removed on a parafilm backing. Membranes contain strongly bound LOx and are suitable for multiple assays among timepoints in stability testing protocols.

2.2.2 Soluble Enzyme Assay

The LOx activity assay is based on the dye o-dianisidine, which absorbs strongly at 500 nm when oxidized. A 1 cm cuvette is filled with 2.4ml 0.21mM o-dianisidine solution, 0.1ml horseradish peroxidase solution at approximately 60 pupurogallin U/ml, and 0.5ml of approximately 0.3M lactate/sodium phosphate solution titrated to pH 7.0, and is equilibrated to 37°C (all materials from Sigma). A known amount of approximately 0.4 U/ml LOx solution (original strength at the beginning of incubation) is stirred in and the absorbance at 500nm is monitored by a spectrophotometer.

2.2.3 Immobilized Enzyme Assay

2.2.3.1 Procedure in glutaraldehyde cross-linked matrices

For the immobilized enzyme assay, the hydrated membrane is added to a cuvette with the same reaction solution as in section 2.2.2. It is stirred continuously until there is noticeable color change (A_{500} is approximately 0.05-0.3), removed to stop the reaction, and the final A_{500} value is measured. The membrane is then washed in PBS and returned to the incubation vial and incubator if completing stability testing with additional timepoints. Results are normalized to the dry weight of the membrane after cross-linking and before testing. This dry weight does not change appreciably for the BSA-carrier membranes.

2.2.3.2 Procedure in polyionic matrices

As with the glutaraldheyde cross-linked matrices, the hydrated membrane is added to the reaction cuvette and is stirred until the required color change occurs. The membrane is again removed to stop the reaction and a final A_{500} value measured. The additional steps below are completed because of material loss during incubation for stability testing.

Since the material is only loosely adhered to the parafilm backing, there is some material lost while incubating during stability testing. After the initial wash step, material was not found to resolubilize; however, some material may slide off of the parafilm backing. This material loss requires that we obtain a dry material weight after each assay. The material weight is obtained by subtracting a tare weight (parafilm backing) obtained at the end of the experiment. Here the material is removed from the backing with acetone. The drying time between reading and re-hydration and re-immersion in the incubation bath is approximately four hours. This dehydration is completed at room temperature in a fume hood. Error in the

calculations arise from this process both in the amount of residual water that remains in the membrane during this drying process (note that driving off all remaining water would denature the enzyme and disallow future testing of the same membrane) and in the amount of enzyme inactivation occurring during this time (expected to be low because the drying is taking place at room temperature). Calculations of the specific activity of the immobilized enzyme (in units / mg of dry material) are then based on the post-assay dry weight.

2.2.3.3 Determination of assay validity

Unlike the soluble enzyme assay, the apparent kinetics measured may not represent the intrinsic enzyme kinetics due to diffusional resistances. See the *Theoretical Aspects* section (2.3) for an explanation. Computational methods are used to determine the relative impact of the reaction rate and diffusion rate on the apparent activity that the assay shows. This is done via the FEM software package COMSOL Multiphysics.

In addition, we investigated the effects of individual components of the assay on the immobilized enzyme, due to the advantages of reusing membranes in testing. For evaluating the effect of the product H_2O_2, incubation conditions chosen were 2mM lactate and 0.1mM H_2O_2 in PBS at 37°C. This was appropriate considering that the H_2O_2 concentration seen by LOx in the membrane is close to conditions expected during the assay process (see *Theoretical Aspects* section). Low activity values and a large incubation volume (which was changed for fresh solution when appropriate) were used to ensure that substrate/product concentrations remained relatively unchanged. The lactate levels used during testing were evaluated for inhibition/inactivation of LOx over an appropriate timespan. To determine the effect of the dye o-dianisidine, comparisons were made among membranes that were naive to the testing

process (ie, a piece of a membrane that has not been previously tested is tested at each timepoint), a membrane that was re-exposed to assay conditions upon each testing (normal testing procedure), and a membrane that was re-exposed to assay conditions and stored in o-dianisidine solution that was oxidized until A_{500} = 0.2 in a 1 cm cuvette.

2.2.4 Analysis Methods

Experimental methods by which various parameters are found are described in each section. All regressions to experimental data are non-linear and are performed by either MATLAB or directly in a spreadsheet by MS Excel. Comparisons of parameters for best-fits are performed by utilizing the confidence intervals generated by MATLAB to perform ANOVA (for more than two experimental conditions) and the student's t-test (two experimental conditions).

2.3 Theoretical Aspects

2.3.1 Soluble Enzyme Assay

The reaction scheme for the spectrophotometric assay is:

$$lactate + O_2 \xrightarrow{LOx} pyruvate + H_2O_2 \qquad (2.1)$$

$$H_2O_2 + (o)dianisidine_{red} \xrightarrow{peroxidase} (o)dianisidine_{ox}$$

Oxidized o-dianisidine absorbs strongly at 500nm. Activity in the soluble enzyme assay is calculated by the following:

$$V_{\max} = \frac{\Delta A_{500/\min} \cdot v_t}{7.5 \cdot v_e} \qquad (2.2)$$

where:

V_{max} = units of activity present in the test solution (U/ml)

$\Delta A_{500/min}$ = change in the absorbance reading at 500 nm per minute

7.5 = extinction coefficient of oxidized o-dianisidine over a 1-cm path-length at 500 nm

v_t = the total volume of the assay solution

v_e = the volume of enzyme solution added

In order to measure the intrinsic kinetics, substrate levels are kept high and reaction times are kept short (2 to 4 minutes).

2.3.2 Immobilized Activity Assay

For the immobilized enzyme assay, the apparent activity of the membrane per mg of dry membrane weight is:

$$V_{\max[app]} = \frac{\Delta A_{500/\min} \cdot v_t}{7.5 \cdot m_m} \tag{2.3}$$

where:

$V_{max[app]}$ = apparent units of activity present in the membrane (U/mg dry weight)

m_m = dry weight of membrane

The activity assay utilized in the testing of enzyme-loaded membranes must be validated with regard to the effect of diffusion of substrates on apparent enzyme kinetics. Reaction controlled conditions, where the apparent maximal reaction velocity ($V_{max[app]}$) of the enzyme in the assay is close to the intrinsic maximal reaction velocity ($V_{max[int]}$), can be achieved by the use of membranes that are relatively thin and that have relatively low amounts of active enzyme (keeping the value of σ (equation 1.5) low). Also, the substrate levels are kept relatively high throughout the procedure, and the reaction medium is vigorously stirred to prevent bulk solution boundary layers.

2.3.2.1 Modeling of the assay system

A one-dimensional model of the system was set up in the FEM program COMSOL to assess the degree of diffusion limitation in the system. This is not substantially different than the full 3-dimensional model because nearly all diffusional limitation is in the axial direction due to the thinness of the membrane; however, the 1-dimensional model is computationally much less intensive. Results of the COMSOL model were compared to the results generated by Bassom (Bassom AP et al, 1997) for the analogous 1-substrate Michaelis-Menten problem and were found to be identical. Boundary conditions are set as the substrate conditions in the bulk solution, and the reaction in the membrane is governed by 2-substrate Michaelis-Menten kinetics:

$$\frac{dc_h}{dt} = \frac{V_{\max} c_l c_{O_2}}{c_l c_{O_2} + K_l c_{O_2} + K_{O_2} c_l} \tag{2.4}$$

where:

c_l , c_{O2}, and c_h = concentrations of lactate, oxygen and H_2O_2, respectively

K_{O2} and K_l = the Michaelis-constants for oxygen and lactate, respectively

V_{max} = the maximum reaction velocity

This reaction rate is in effect only limited by O_2 in the assay system. We assume a homogeneous distribution of enzyme throughout the membrane, which is reasonable given the manufacturing method whereby the enzyme/carrier mixture is vigorously stirred during an initial cross-linking procedure. The system was analyzed at different membrane thicknesses and enzymatic activities, and the apparent kinetic results of the simulation were compared with the intrinsic kinetic parameters input to the model. This is done through the use of a regression of the results of the FEM model to a polynomial form. An excellent fit is found for a calibration

curve, where the apparent activity is taken as the independent variable and the intrinsic activity input into the model is the dependent variable. This fit is a deviation from linearity such that:

$$V_{\max,I}(V_{\max,A},\delta) = a(\delta)\cdot V_{\max,A}{}^{3} + V_{\max,A} \tag{2.5}$$

where:

$V_{max,I}$ = intrinsic activity (U/m^3)

$V_{max,A}$ = apparent activity (U/m^3)

δ = membrane thickness (m)

A cubic function can be used to describe the value of the parameter "a" with respect to the membrane thickness:

$$a(\delta) = b\cdot\delta^3 + c\cdot\delta^2 + d\cdot\delta \tag{2.6}$$

Results are shown with and without this correction applied, and corrected and uncorrected data are fit to equation 1.10 in order to obtain parameters for the spontaneous inactivation of the enzyme. The parameters used in the simulation are shown in table 2.1 and are gathered or estimated from the literature (diffusion constants (Gough DA et al, 1985)) or have been determined in the lab (kinetic constants K_{O2} and K_L for LOx).

The model results are applied to the experimental data (apparent activity) in the case of glutaraldehyde cross-linked BSA membranes to "back-calculate" the estimated intrinsic activity in the immobilized region. The apparent and corrected data are compared in the results and discussion sections of chapter 3.

Table 2.1: The values used in FEM modeling of the enzyme assay system.
D_l is estimated to be slightly higher than that of glucose, and D_h is estimated to be between the values for oxygen and lactate (Gough DA el al, 1985). The maximum membrane concentrations equal the bulk concentrations in solution multiplied by an assumed partition coefficient of 0.8.

Parameter	Value	Units
K_l	0.39	mol/m^3
K_{O2}	0.03	mol/m^3
c_{O2} membrane, max	0.1716	mol/m^3
c_l membrane, max	38.704	mol/m^3
D_{O2}	1.00E-09	m^2/s
D_l	4.00E-10	m^2/s
D_h	6.00E-10	m^2/s

2.3.2.2 Experimental determination of unknown reaction/diffusion modeling parameters

The principles of sensor design in the case of LOx immobilized in the polyionic matrix remain identical to those used in the designs based on natural polymer cross-linked matrices; however, the diffusion constants in this matrix have not been previously measured in the literature. The characteristics that apply to the assay can be analyzed without determining the diffusion coefficient directly. In order to perform this analysis, we turn to the form of the fit to the solution for the FEM model in equation 2.5 and 2.6. There is an added complication here in that the surface of the immobilized matrix is very easily displaced (similar to a very viscous liquid rather than the hydrogel-like constructs measured in chapter 3). Therefore, a thickness measurement of the enzyme layer is not easily performed. We can instead fit experimental data to the form:

$$V_{\max,I}(V_{\max,A}, m) = a(m) \cdot V_{\max,A}{}^3 + V_{\max,A} \tag{2.7}$$

with

$$a = b \cdot m^3 + c \cdot m^2 + d \cdot m \tag{2.8}$$

where m is the mass of the immobilized material.

Here, we make the assumption that the density of the immobilized material is roughly constant, and therefore thickness is proportional to the mass for the same diameter disk. The units of the parameters b, c and d change correspondingly. In sections on immobilization in a BSA matrix, we determined the values of the parameters by the best fit to the COMSOL generated solution using the diffusion constants gathered and estimated from the literature. In the present case, the parameters can be determined experimentally by varying mass and loading and then measuring the resultant apparent activity. Yield is assumed to be constant for the experiment and is included as an additional parameter. The final equation to which the experimental data is then regressed is:

$$V_{loading}(V_{\max,A},\delta) = \frac{(b\cdot m^3 + c\cdot m^2 + d\cdot m)V_{\max,A} + V_{\max,A}}{\phi} \tag{2.9}$$

where:

$V_{loading}$ = the loaded V_{max} formulated

ϕ = yield of the immobilization process (as a fraction)

2.3.2.3 Chemical effects of the assay

One major goal of our testing is to measure spontaneous inactivation, which can be expressed as the first-order process as shown in equation 1.10. However, during the course of the assay, immobilized LOx can be affected by the chemical components of the assay. The product H_2O_2 is known to cause rapid enzymatic inactivation. A simplified expression for this inactivation is as follows (Tse PHS, 1984):

$$\tau_{i_h} = \left(\frac{1/\tau_i - 1/\tau_0}{c_h}\right)^{-1} \tag{2.10}$$

where:

τ_{ih} = time constant of inactivation due to H_2O_2

τ_i= time constant of inactivation measured experimentally

A more accurate value can be ascertained taking into account the concentrations of the other substrates, K_m values of the enzyme, and the changing amount of H_2O_2 experienced by the immobilized LOx as it deactivates (Tse PHS, 1984):

$$c_e(t) = c_{e,0} \cdot \exp - \left(\begin{array}{l} 1/\tau_0 + \left(c_l/\tau_{i_l} + c_h/\tau_{i_r}\right) \dfrac{K_{O_2} c_l}{c_l c_{O_2} + K_{O_2} c_l + K_l c_{O_2}} + \\ \left(c_h/\tau_{i_h}\right) \dfrac{c_l c_{O_2}}{c_l c_{O_2} + K_{O_2} c_l + K_l c_{O_2}} \end{array} \right) t \quad (2.11)$$

where:

τ_{il} = inactivation constant due to lactate

τ_{ir} = inactivation constant of the reduced form of the enzyme by H_2O_2

τ_{ih} = H_2O_2-mediated inactivation constant of the oxidized form of the enzyme complexed with H_2O_2

Note that in the case that τ_{il} and τ_{ir} are much larger than τ_{ih}, the middle term of the exponential can be neglected in determining τ_{ih}. During the course of an inactivation experiment where the bulk c_h is held constant, the value of c_h across the membrane varies with position in the membrane and time. The average value in space is found via the COMSOL model; this value varies in time according to:

$$c_{h,avg} = (c_{h,bulk} + c_{rxn} e^{t/\tau_i}) \cdot \alpha_h \quad (2.12)$$

where:

$c_{h,avg}$ = the average concentration of H_2O_2 across the membrane

$c_{h,bulk}$ = the concentration of H_2O_2 in the bulk solution

c_{rxn} = the initial average H_2O_2 caused by the reaction

α_h = the partition coefficient of H_2O_2 in the membrane

The substrates lactate and O_2 also vary, but the variation is small and does not cause a large deviation in the calculation of the inactivation constants.

In the case of reversible inhibition by assay components, whereby the apparent activity of the enzyme is simply reduced and not eliminated, an apparent K_i value can be calculated based on an assumption of non-competitive inhibition:

$$K_i = \frac{V_{\max[app]} c_i}{V_{\max[\text{int}]} - V_{\max[app]}} \tag{2.13}$$

where c_i is the concentration of the inhibiting agent. Note that this value is only valid for the concentration analyzed and may change if different concentrations are used and non-competitive inhibition is invalid. By measuring these various influences, we can determine if there is any significant effect on the measurement of spontaneous inactivation via repeated measurement of immobilized membrane samples.

2.4 Results and Discussion

2.4.1 Modeling of Assay System and Low-loading Analysis in Glutaraldehyde cross-linked membranes

The result of FEM modeling of the reaction assay system is a curve showing the apparent activity given during testing based on a modeled intrinsic activity (figure 2.3). This curve can be used to back-calculate actual intrinsic values from the apparent values found during assay. However, there is error associated with this curve because of error in the parameters used. Maeda-Yorita et al, 1995, found the value of K_{O2} for LOx from *A. Viridans* to be 0.16 mM at 25°C; however, the value found in our studies was approximately 0.03 mM at 37°C.

We assume that intrinsic K_m values are not changed drastically by the cross-linking, which has been shown to be true for GOx (Tse PHS et al, 1987). Since the membrane is well hydrated, substrate should have about the same access to the active site as before immobilization. Changing the K_{O2} value in the simulations results in a small change in the effectiveness of the apparent measurement. A typical membrane in the stability experiments with a loading of 21 U/g dry weight and 10% immobilization yield would have a $V_{max[app]}/V_{max[int]}$ = 0.91 at the lower K_{O2} value and 0.88 at the higher K_{O2}. The value used for D_{O2} has a larger effect on $V_{max[app]}/V_{max[int]}$, due to the K_{O2} value being accounted for in the rate equation; note that both parameters affect the actual rate of substrate consumption similarly. Since D_{O2} is difficult to measure in the membrane experimentally, this is the largest source of error in the model. Small variations in K_l and D_l have very little effect since lactate is in large excess during the assay.

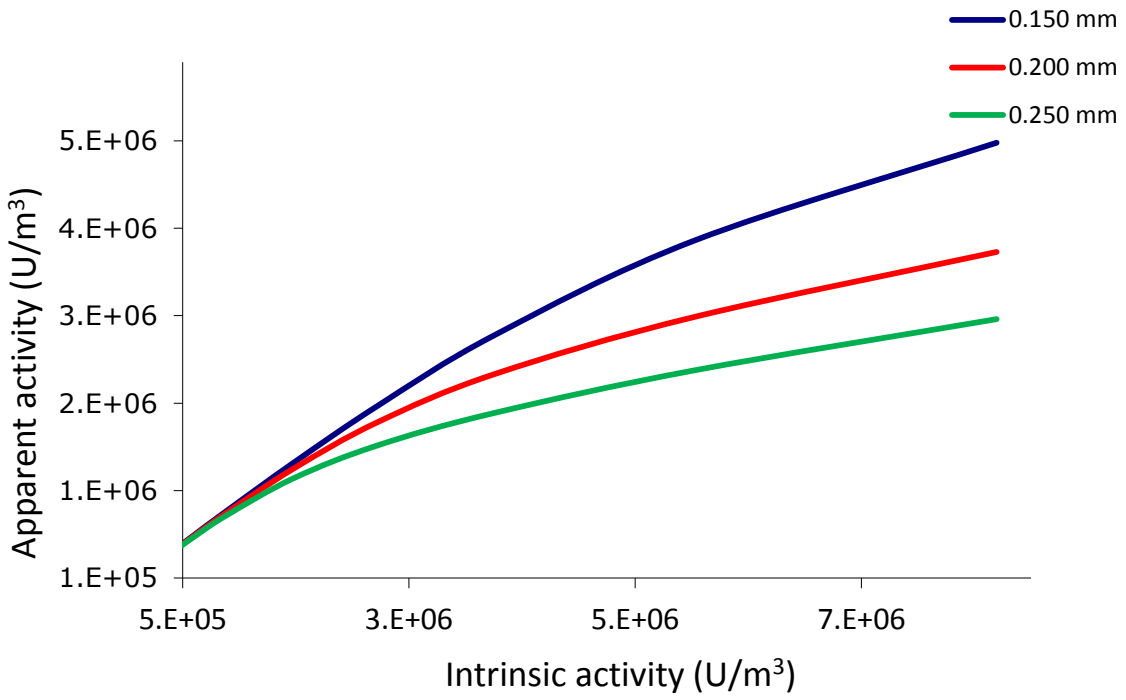


Figure 2.3: Modeling results for the reaction/diffusion system representing the assay. Note that apparent kinetics display attenuation at higher loading levels and at larger thicknesses. The model solution is affected especially strongly by the diffusion constant for O_2, since it is in effect the limiting substrate, and is the largest source of error in the model.

With the constants used in the simulation, $V_{max[app]}/V_{max[int]}$ at time 0 of the incubation is 0.75 to 0.95. $V_{max[app]}/V_{max[int]}$ increases with enzyme inactivation, reaching 0.95 to 0.99 at the end of stability testing. The results from the FEM model can be used to calculate an intrinsic activity from the apparent results of the assay.

The results of measuring activity and process yield in the low-loading membrane group are shown in figure 2.4. Values for approximate corresponding reaction rates of native assays are shown as well. Note that at very low reaction rates, the measured reaction rate of the native enzyme is substantially lower than that predicted by the dilution. When this assay error is accounted for in the results from the immobilized enzyme assays, the yield of the process appears to be constant (ie, there is no statistically significant trend of yield with respect to

formulated activity). This error is due to the inhibition of LOx by the oxidized o-dianisidine over the longer time required by the assay at low activity levels (see section 2.4.3).

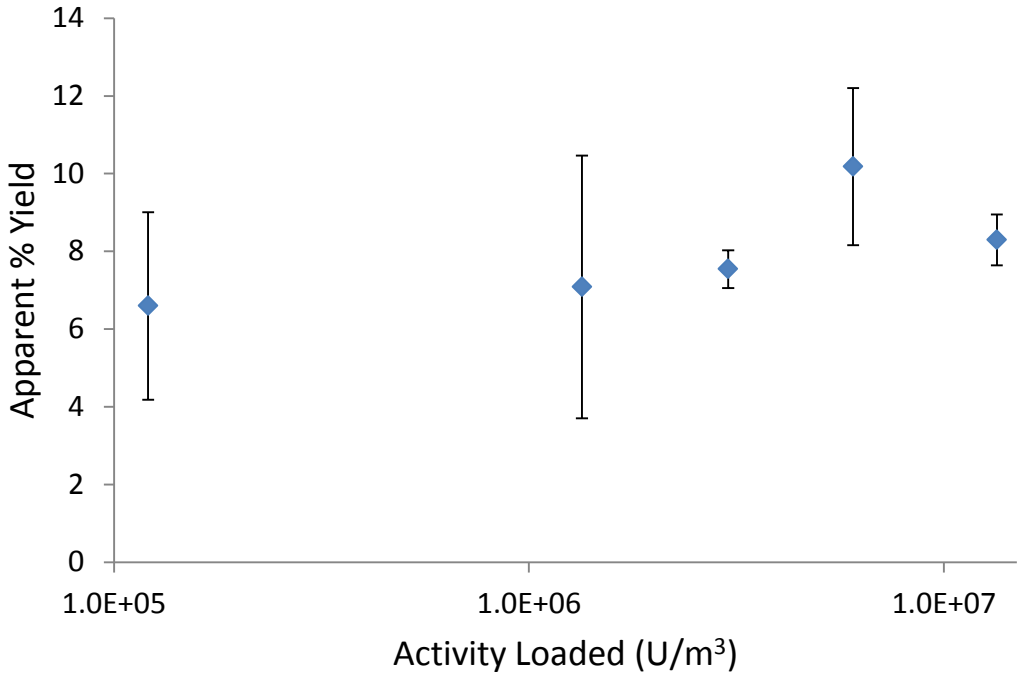


Figure 2.4: Apparent activity of immobilized LOx around the-loading levels used for experiments except those reported in section 3.3.2.2.3 (highly loaded membranes). When corrected for o-dianisidine inhibition (corrected data shown), there is no trend seen in yield, indicating little diffusional resistance at these loading levels. Error bars represent 1 standard deviation (n=5).

Results of the experiment shown in figure 2.4 and the analysis of the rest of the stability results for the manufacturing parameter analysis (see chapter 3, table 3.1: note the high R^2 values for the apparent kinetic fits to equation 1.10) show that diffusion limitation in the membranes is not a large problem when confined to the levels of activity used in these tests. This indicates that in our specific construct, the effective diffusion constant of O_2 may be slightly higher than the values reported in literature; however, no direct measurement was made. Note that the regression analysis of the "corrected" data always yields a lower value for τ than

the regression performed directly on the apparent activity data. This is due to the relatively larger change in the higher activity membranes when back-calculating "intrinsic" activity values.

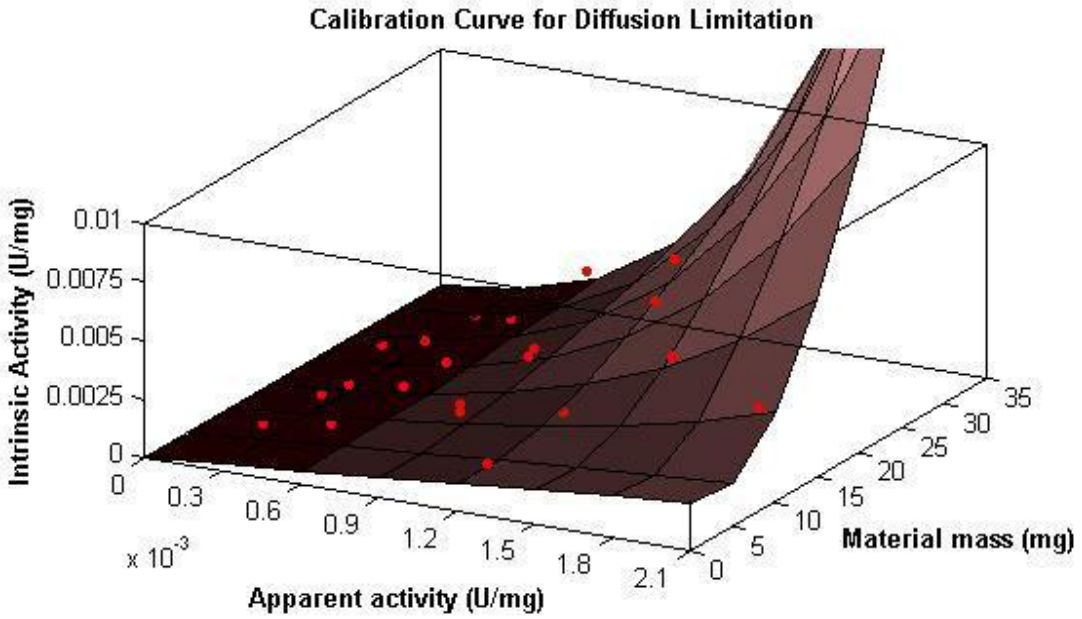
2.4.2 Experimental Data Fit to the Modeled System in Polyionic Membranes

As described in the theoretical section (2.3.2.2), the immobilization was performed and the masses and loadings of the resultant membranes were varied while keeping the diameter constant. The data was regressed to equation 2.7 using a non-linear regression. The regression is plotted in MATLAB and is shown in figure 2.5a. The parameter "a" from equation 2.8 is also plotted as a function of mass. Note that the value of "a" initially has a negative value before the mass of the membrane material reaches approximately 6.5mg. This was allowed in the regression in order to obtain the best fit at higher mass levels (where we require the greatest accuracy in order to ensure the closest "correction" to actual intrinsic values). Therefore, the diffusion correction calculation is neglected when the mass falls below the 6.5 mg cutoff. This experiment was conducted with the second immobilization procedure described in section 2.2.1.2 (whereby the DEAE-D/LOx solution and the PSS solution are mixed in a vial, and the resulting viscous material transferred to wells). However, a loading variation experiment was conducted with the other method of immobilization directly in the well, and the parameters were not found to be significantly different.

It is assumed that the diffusional characteristics of the immobilized polymeric material remain relatively constant for each subsequent experiment. However, the yield parameter does vary considerably. Therefore, in order to perform a correction for the diffusional resistance during assay readings, only the parameters b, c and d are used. The yield is then calculated based on the best-fit of the first-order decay model (equation 1.10) to the corrected data. All

subsequent results of stability experiments are shown as “apparent results” (no correction is applied before regression to equation 1.10) and “diffusion-corrected results” (the correction is applied before regression).

A



B

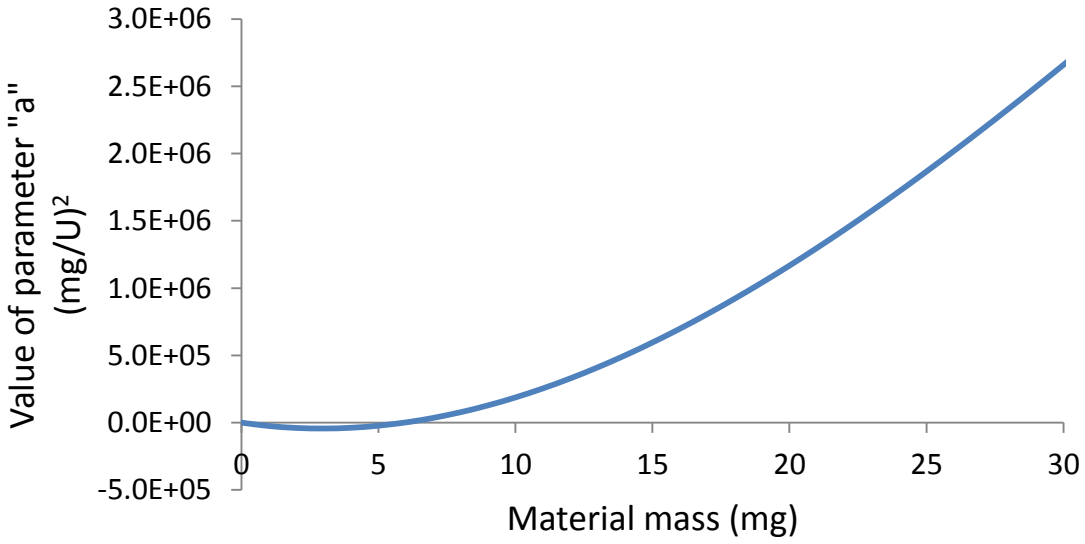


Figure 2.5: Fit of experimental results to the diffusion correction model for polyionic matrices (a) The calibration curve (surface) for diffusional resistance in the assay is determined by fitting a series of experimental data (red) at different masses and LOx loading levels to equation 2.7 derived by the FEM modeling of the assay. (b) The parameter "a" from equation 2.8 is plotted against membrane mass for the best-fit parameters. Note that it is initially negative; no correction is made if mass is less than 6.5 mg.

2.4.3 Chemical Effects of Assay System

The following chemical effects were measured in the BSA-carrier glutaraldhyde cross-linked system. The effects are expected to be quite similar in the collagen-carrier and polyionic systems.

2.4.3.1 Reaction substrate and product effects

The effect of H_2O_2 inactivation on the immobilized enzyme is shown in figure 2.6. At the substrate/product levels tested (2 mM lactate, 0.24 mM O_2, 0.1 mM H_2O_2), inactivation is significantly faster than in PBS alone. Incubation of the membranes in high-level lactate solution (48 mM, the same concentration used in the assay) showed no decrease in activity over a 2-hr incubation period, indicating that substrate-mediated inactivation is insignificant over the total time-period of assay exposure. Using equation 2.11, if τ_{ir} is large in comparison to τ_{ih} for LOx, then we may solve for τ_{ih} directly using the data from the H_2O_2 inactivation experiment. This is reasonable since in another oxidoreductase, GOx, the value for τ_{ir} was found to be 38 times higher than τ_{ih} (Tse PHS and Gough DA, 1987). However, for purposes of determining the rate of inactivation in the assay conditions, the quantity ($K_{O2}/\tau_{ir} + c_{O2}/\tau_{ih}$) is the same for all experiments. This value is found to be 0.31 hrs^{-1}, and the τ_{ih} value is found to be 0.62 mM*hrs.

In order to determine the effect on the spontaneous inactivation measurements made, we must determine the total amount of inactivation caused by the H_2O_2 to which the membrane is exposed. In the case of the pH 5.5 manufacturing condition results shown in section 3.3.2.2.1, this is calculated to be about 1% of the total starting activity of the membrane. Because of this low impact, we report only the spontaneous inactivation parameters calculated from the regression of the experimental data to equation 1.10.

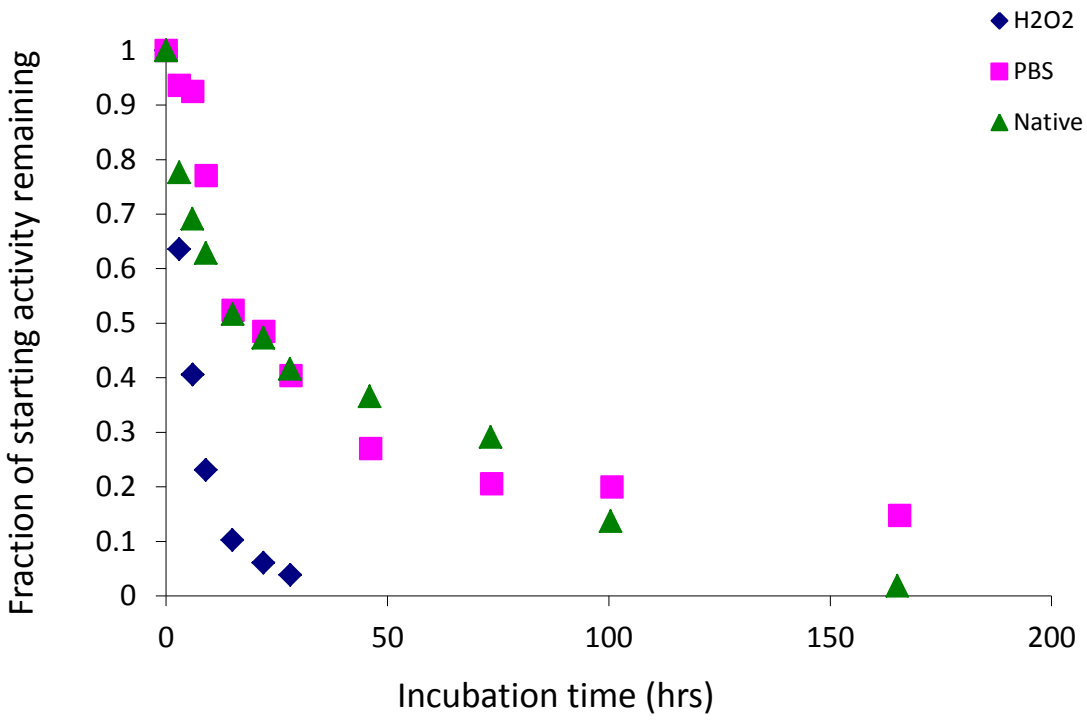


Figure 2.6: The time-dependent inactivation of LOx in the presence of H_2O_2 and the substrates. Conditions: c_I = 2 mM, c_{O2} = 0.21 mM, c_{H2O2} = 0.1 mM, all in 0.1 mM PBS buffer; temperature = 37°C. Membranes are retested at each timepoint. n=1 per timepoint. Control tests are the membrane in PBS and the native enzyme in solution.

2.4.3.2 Effects of the dye o-dianisidine

The reaction product o-dianisidine (oxidized) from the assay was found to induce a decrease in the activity of LOx. This effect was not immediate; there is apparently a certain time required for the dye to complex with the enzyme and inhibit activity. Results are shown for both the soluble enzyme assay and the immobilized enzyme system incubated at 37°C (figure 2.7). After an equilibration period of 40 to 70 min, the apparent activity of the inhibited enzyme in the soluble system is a nearly constant percentage of the enzyme free in PBS. K_i is calculated to be about 0.012 mM^{-1} per equation 2.13. In the immobilized case, exposure to the inhibitor changes due to the wash steps to which the membrane is exposed. Over short-term testing (0 to 3 hours), there is no statistically significant decrease in activity. The average value of the

apparent activity of the naïve-to-assay membranes and the membrane exposed to oxidized o-dianisidine are not different over this time period. In addition, since the repeated testing membrane shows no decrease in activity, we may assume that repeated exposure of the immobilized enzyme to the inhibitory agent over this testing interval (5 minutes total assay time over 5 discrete assays with wash steps) has no effect on apparent activity. Over long-term testing to over 300 hrs of incubation time with 6 additional assays, we see no statistically significant difference in the reported parameter values between the three tests.

A

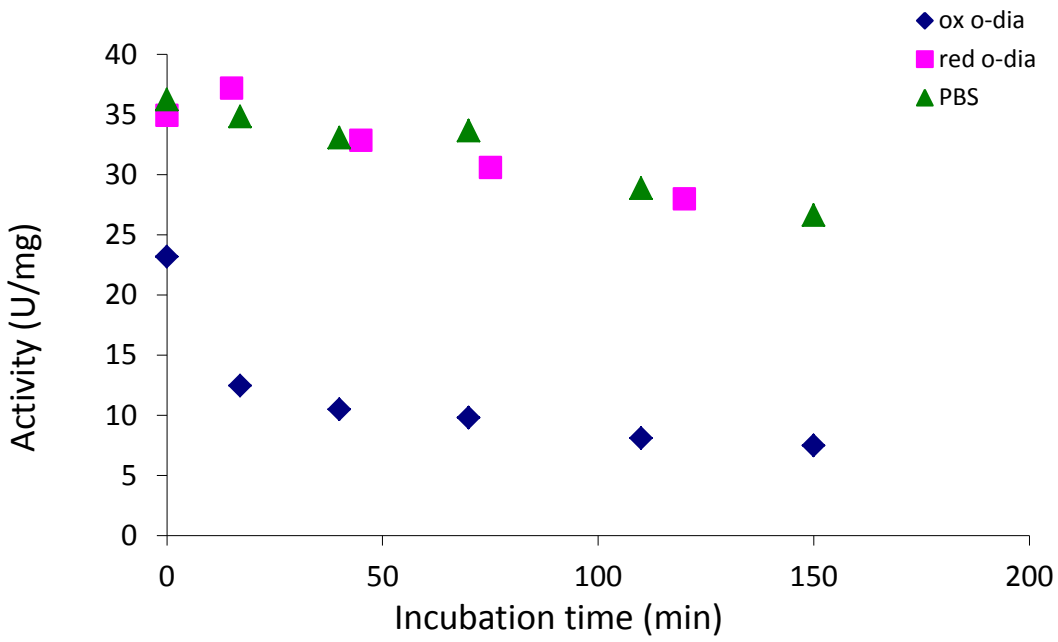
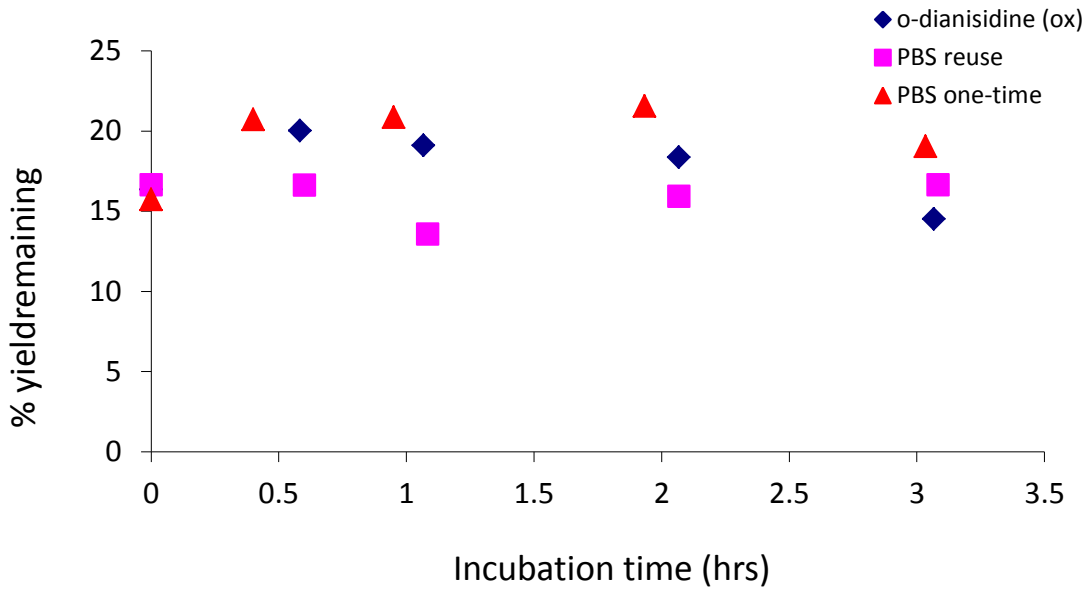


Figure 2.7: Results of the inactivation experiment in the presence of oxidized o-dianisidine. The soluble enzyme assay (a) shows a clear inhibition of activity which becomes constant around the 40 min to 70 min timepoint. The immobilized enzyme assay shows no clear inhibition when subjected to a wash before assay was completed. Results are shown over the short-term (b) and the long-term (c). Experimental groups in (b) and (c) include the membrane stored in oxidized o-dianisidine, the membrane stored in PBS but retested at each timepoint, and a naïve-to-testing membrane group. n=1 per timepoint.

B



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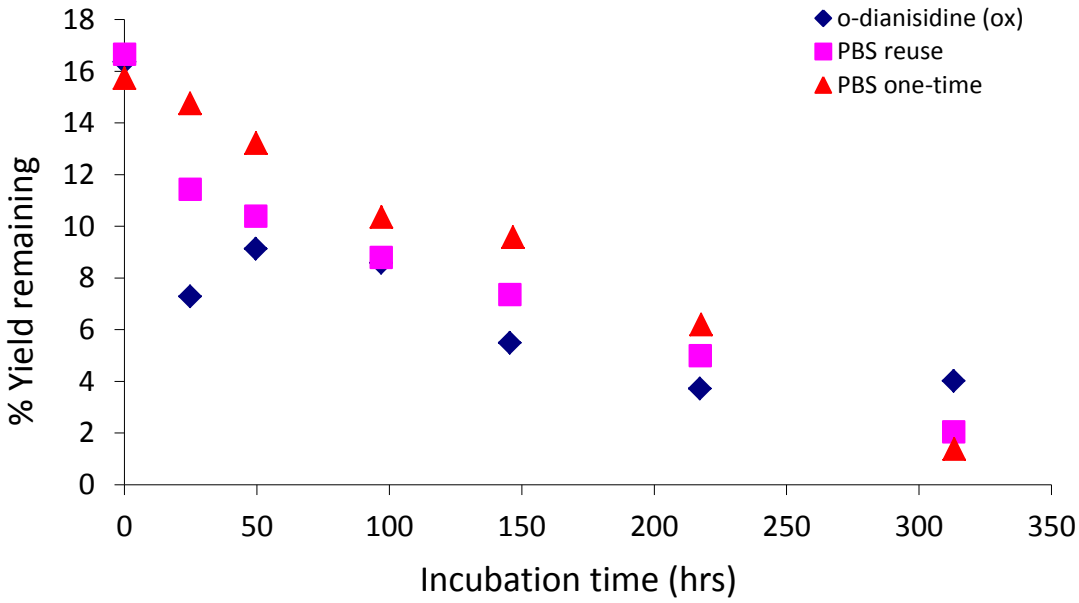


Figure 2.7: Results of the inactivation experiment in the presence of oxidized o-dianisidine, continued.

2.5 Conclusions

From the studies conducted in this chapter, we conclude that the spectrophotometric assay proposed can provide a robust, accurate measure of enzyme activity for repeated measurement stability analysis. There is significant diffusion resistance to the substrates in the system; however, this is mitigated by the use of thin membranes that are loaded with lower levels of the enzyme LOx. Additionally, we can make a quantitative estimate of the diffusion limitation with known or experimentally found parameters allowing the back calculation of an estimated intrinsic activity. The chemical effects of the assay system on repeated measurements of membranes were found to be insignificant.

This chapter, in part, is a reprint of the material as it appears in the following:

- Strobl AL and Gough DA. Lactate oxidase (LOx) immobilized in a natural polymer carrier: parameter effects on stability in a physiologically relevant range. *In Preparation*.
- Strobl AL and Gough DA. Stability of Lactate oxidase (LOx) immobilized in a polyionic matrix. *In preparation*.

The dissertation author was the primary investigator and co-author of these papers.

III Lactate oxidase in a chemically cross-linked matrix

3.1 Introduction

Since the goal of this project is an implantable sensor , the *in vitro* stability testing conducted here mimics physiological conditions. The stability testing of lactate oxidase stability, as well as many other enzymes with biosensor applications, is often completed at relatively high temperatures (often as high as 63°C (Chen Q et al, 1998) or 65°C (Minagawa H et al, 1998)). These accelerated test procedures are used for their expediency; however, the data presented here show that the degree of stability conferred by a process such as cross-linking may change with temperature.

The work reported here uses the manipulation of processing parameters to affect the cross-linking of LOx in such a way that stability is conferred. This begins with a method of cross-linking that delivers glutaraldehyde in a way that is highly controllable such that mechanical stability is conferred to the membrane, yet a relatively high enzymatic activity (yield) is preserved. In order to facilitate stability of LOx activity, the pH is controlled to various values during the cross-linking process. This results in changing the preferential sites for cross-linking to the LOx molecules. Also, by bringing the pH of the solution closer to the isoelectric point (IP) of the enzyme and carrier (4.6 for LOx (Chen, Q et. al, 1998) and 4.7 for albumin (Forciniti, D et. al, 1991)), the molecules can be brought together more effectively; this may allow for more intermolecular bracing at a lower amount of total glutaraldehyde reactions.

3.2 Materials and Methods

3.2.1 Material covered earlier

The immobilized enzyme membranes are created as described in section 2.2.1.1. The soluble enzyme assay method is discussed in section 2.2.2 and the immobilized enzyme assay in 2.2.3.1.

3.2.2 Incubation

Incubation of collagen membranes took place in an oven set to approximately 37°C or in jacketed 1 L capacity reactors with temperature controlled to ±0.2°C by a circulating water bath (Thermo Scientific, model NESLAB RTE7). Incubation of BSA-carrier membranes took place exclusively in the jacketed reactors. All incubation solutions were approximately 20 mL of PBS at pH 7.4. Temperature of the incubation for some BSA-carrier membranes was varied as explained for individual experiments.

3.2.3 Evaluation of Cross-linking Parameter Effects on Yield

The amount of glutaraldehyde added before membrane dehydration (some collagen-carrier experiments and all BSA-carrier experiments) was determined as the amount being just enough to cause the mixture to solidify (the material is free-flowing for several minutes but eventually gels). Optimal times and appropriate glutaraldehyde strength for the 2^{nd} cross-linking step were determined by the active enzyme yield as well as appropriate mechanical rigidity of the resulting membrane.

3.2.4 Evaluation of Cross-linking Parameter Effects on Stability

All stability tests were run with two types of controls: the enzyme solubilized in PBS (positive control) and membranes with no enzyme added (negative controls). Buffer solution in which the membranes were stored (PBS) was also tested for activity to determine if enzyme was being leeched from the membrane. Time points were chosen to attempt to capture the inactivation curve. pH of the process during cross-linking was evaluated for its effect on stability; it was controlled at the values 8.5, 7.4, 6.5, 5.5 and 4.5, and the stability evaluated at 37°C. The most successful (pH 5.5) was then evaluated for stability at 39°C, 41°C, and 50°C. Other values closer to pH 5.5 were evaluated at 41°C, where a similar degree of stabilization (DoS) (see next section, 3.2.5) to the 37°C trial was observed for the pH 5.5 test group.

3.2.5 Analysis/Statistical Methods

In the collagen-carrier yield experiments, multiple membranes were created and tested for activity. The yield was calculated by assaying the native solution that was used to load the collagen material. Results were compared using ANOVA followed by Student's t-tests corrected for multiple-comparisons. For stability testing, one membrane was assayed for activity at each timepoint and then discarded. Parameters are calculated based on an initial value whose activity was quickly washed out, followed by a first-order model of the type in equation 1.10. It was also corrected by allowing for a background value (determined by a negative control in BSA-carrier experiments, see below).

In all cross-linking parameter variation experiments (BSA-carrier membranes), five membranes were assayed for each timepoint during stability testing along with one negative control membrane and three repeated measurements of the native enzyme activity. To

compare amongst experimental groups, the parameters of equation 1.10 were found for each membrane using non-linear regression in MS Excel. A population of parameters could then be compared using ANOVA followed by Student's t-tests corrected for multiple comparisons (where applicable). For comparisons to the native enzyme trials, the three repeated measurements of the native were treated as three separate tests, and the parameters were compared within the respective incubation batch only. Significance is defined as $P<0.05$. A "degree of stabilization" (DoS) was calculated for each trial as the parameter τ for the immobilized enzyme divided by the same parameter for the native enzyme. The values of the negative controls were plotted against time and a student's t-test was used to detect any significant trend. If there was no significant trend for a particular test, then a corrected activity in units U/(mg dry membrane albumin) was calculated by subtracting the global average native control value for each corresponding test. If there was a significant trend, then a best-fit trend value for each time-point was used.

3.3 Results and Discussion

3.3.1 Effects of Cross-linking Conditions on Yield

3.3.1.1 Yield in collagen-carrier membranes

In the collagen-based system, the cross-linking was entirely completed during the step by which glutaraldehyde was added through the PTFE membrane for a period lasting several hours. This process was optimized both for the strength of the glutaraldehyde solution and also for the amount of time for which cross-linking was allowed to occur. Initially, the cross-linking step was allowed to occur with a 2.5% w/w solution of glutaraldehyde for 14 hours. This led to a process yield of only 0.5%. It was determined that process yield could be significantly increased

by changing the glutaraldehyde concentration to 0.25%. The effect of the total time of cross-linking with this concentration was explored. Good process yield was observed and results are plotted in figure 3.1. A reasonably mechanically tough membrane could be made with a cross-linking time of 19 hours or longer, and yield of the resulting membranes significantly drops after the 19 hour cross-linking time. Therefore, we chose 19 hours of cross-linking with 0.25% glutaraldehyde for use in the stability testing portion of the experiments.

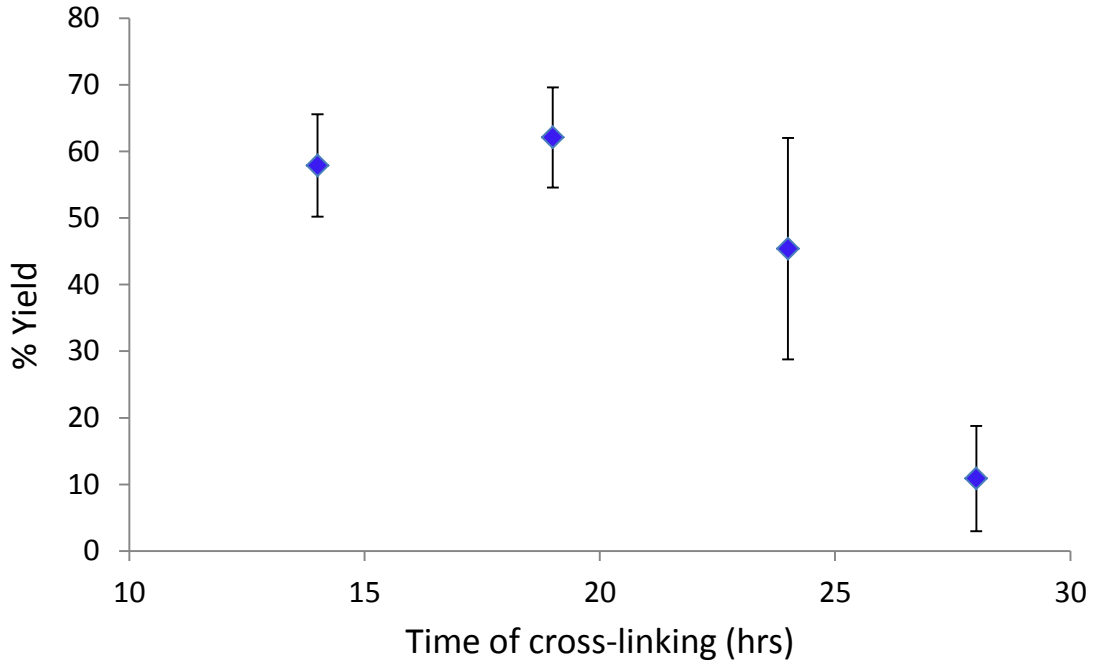


Figure 3.1: The effect of the time of the final cross-linking step with 0.25% glutaraldehyde on the resultant yield of the immobilization process in collagen-carrier membranes.
Results show a statistically significant decrease in yield past 19hrs. Error bars represent 1 standard deviation (n=7).

3.3.1.2 Yield in BSA-carrier membranes

The results of changing the final cross-linking addition strength from 0.25% glutaraldehyde to 1% glutaraldehyde are statistically significant, showing a drop from 11.0% of formulated activity (n=5) to 8.5% of formulated activity (n=5), respectively (a 20 hour secondary cross-linking time was used). The result of varying the cross-linking time on the apparent

activity yield is shown in figure 3.2. These results are for membranes cross-linked at pH 5.5 (shown in testing to promote stability), although this pattern roughly holds for all pH values tested. Both the studies on glutaraldehyde concentration and time of cross-linking show that increased exposure to the cross-linker results in a decreased process yield of active enzyme. This is expected due to the increased chance of an inactivating reaction occurring.

For stability experiments, 20 hrs of cross-linking at 0.25% glutaraldehyde was chosen for subsequent stability studies. The yield is not less than that of lower cross-linking times, and the BSA-carrier membranes were observed to have greater mechanical strength and could withstand the incubation temperatures used in PBS for many weeks without dissolving or changing significantly. This is in contrast to collagen membranes, which were unable to form a stable construct while maintaining high levels of activity. Lower levels of cross-linking produced membranes that would eventually dissolve over the course of the stability protocols. These membranes all used the same amount of cross-linking agent before being shaped in wells and dried to a film. As expected, BSA/LOx mixtures that had no cross-linking treatment did not form a film; these mixtures instead began to crystallize once a solubility limit had been reached.

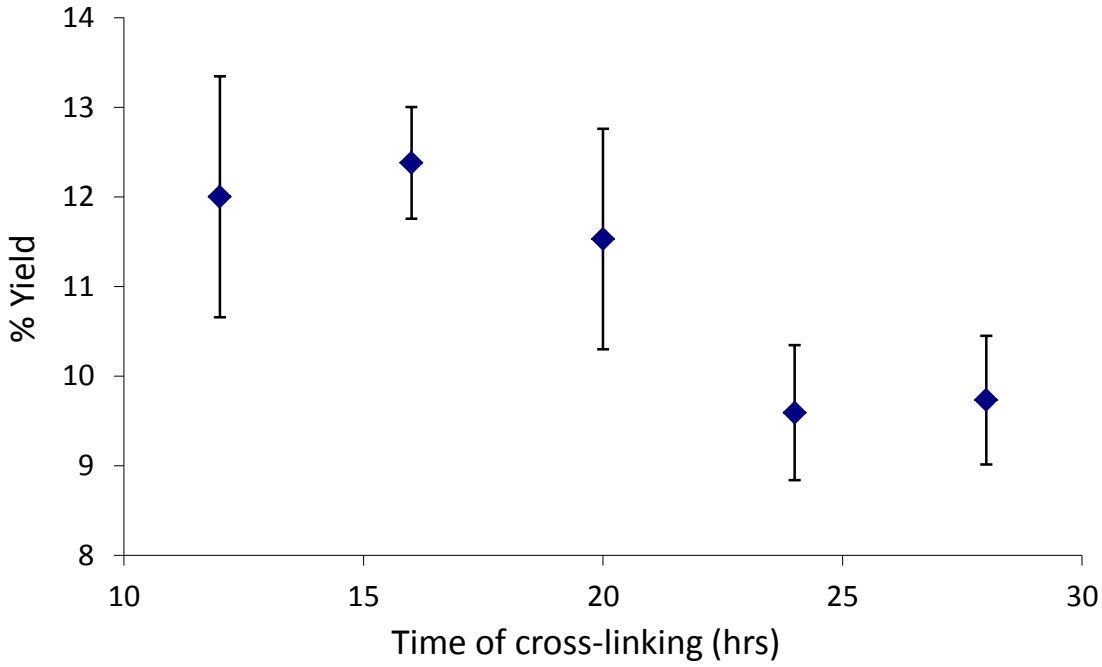


Figure 3.2: The effect of the time of the final cross-linking step with 0.25% glutaraldehyde on the resultant yield of the immobilization process in BSA-carrier membranes.
Results show a statistically significant decrease in yield past 20hrs. Error bars represent 1 standard deviation (n=5).

3.3.2 Effects of cross-linking conditions on stability

3.3.2.1 LOx cross-linked in a collagen matrix

Apparent activity results for incubation of collagen-carrier membranes at 37°C are shown for stability testing in figure 3.3. pH of the solutions during the membrane production process was controlled to either 7.4 or 4.5. Curves shown are non-linear best-fits performed by MATLAB to equation 1.10, corrected by an initial, short-lived value and a long-term stationary value (representing clouding of the assay). In the case of the pH 7.4 groups, we see a clear first-order decay as predicted. However, the pH 4.5 trial produced only an initial activity followed by the long-term stable value. This indicates that no active enzyme was effectively immobilized within the membrane at this pH. Unfortunately, we noticed a tendency of the collagen-carrier membranes to dissolve over time. Also note that the incubation was completed in an oven,

which was determined to have inaccurate and imprecise temperature control. This explains the lower stability of the native enzyme in comparison to other tests, which utilized jacketed reactors with a circulated water bath temperature control system. It was also attempted to create collagen-carrier membranes using the 2-step cross-linking method developed for the BSA-carrier system. These membranes were determined to have a similar process yield to the BSA-carrier membranes (approximately 24%); however, the membranes were determined to still be mechanically unstable under the incubation conditions. Because of this mechanical instability in comparison to the BSA-carrier membranes, BSA was used as the carrier for all remaining testing in this chapter.

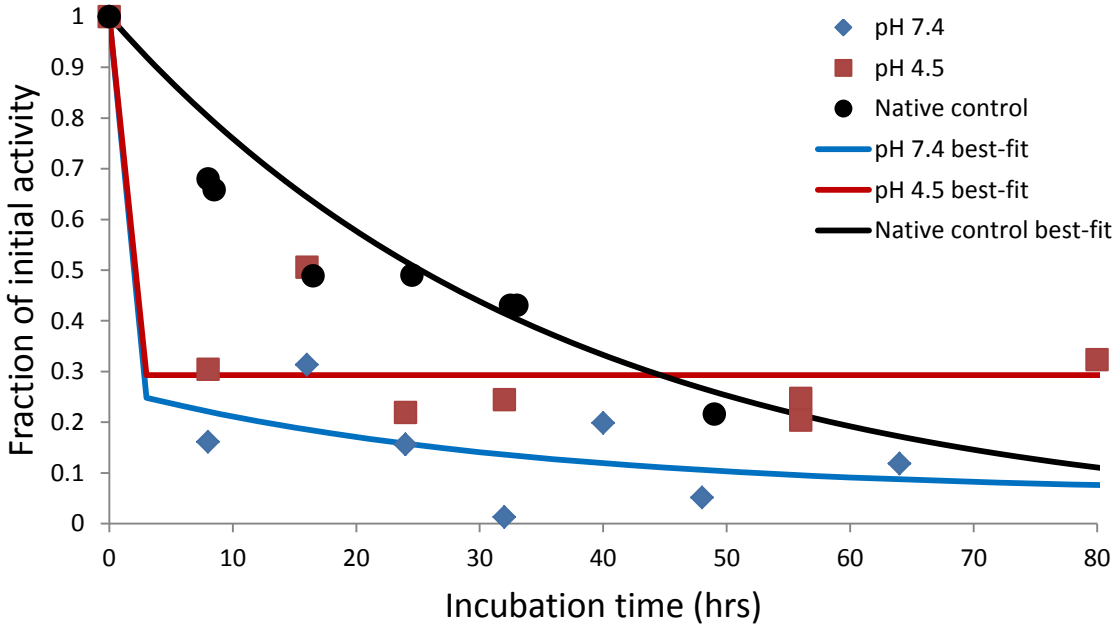


Figure 3.3: Spontaneous inactivation of LOx immobilized in a collagen-carrier matrix incubated at 37°C in PBS.
Membranes were tested once and discarded; each point represents one experimental measurement. Best-fit regressions include an initial, transient activity term and unchanging term representing assay clouding by the collagen material.

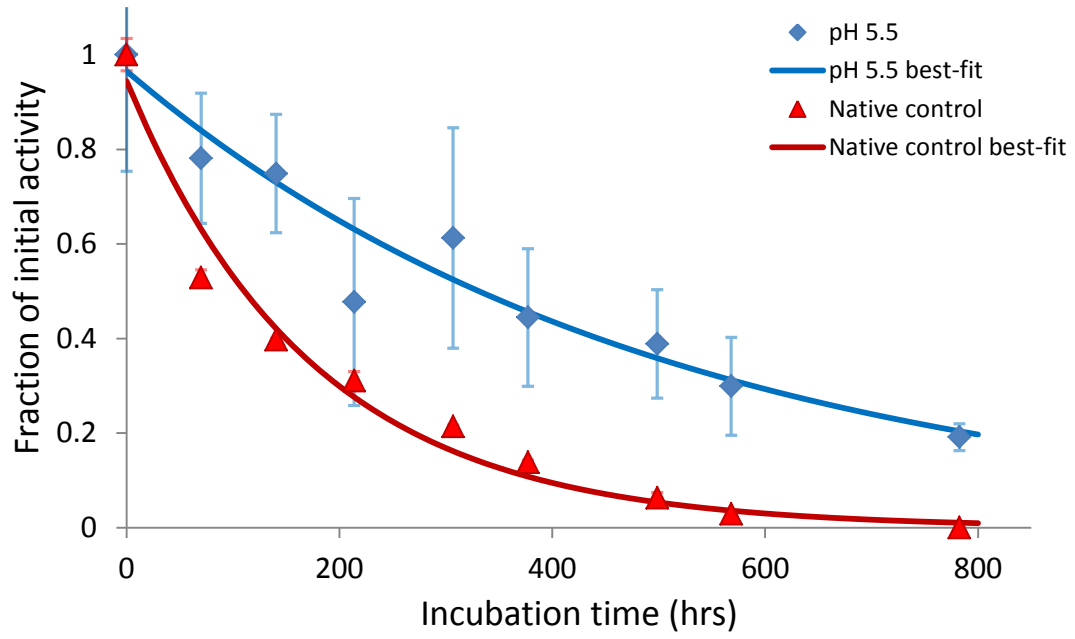
3.3.2.2 LOx cross-linked in a BSA matrix

All parameters for the stability tests are reported in table 3.1 at the end of the Results section. This table is grouped by the temperature of incubation. Statistical significance is shown here in comparison to the native control by highlighting the degree of stabilization values that show statistical significance.

3.3.2.2.1 Effects of Cross-linking pH on Stability

Apparent activity results for incubation of BSA-carrier membranes at 37°C are shown for stability testing in figure 3.4. Results are separated by batch and are shown against the native control. Results for membranes created at pH 4.5 are not shown; these test conditions produced a membrane with no measurable activity. The curves are non-linear best-fits performed by MATLAB using a least-squares regression technique. These are fit to the first-order spontaneous inactivation model from equation 1.10. Membranes produced under test conditions at pH 7.4 and 6.5 produced no significant gain in stability over the native soluble enzyme. LOx immobilized at a pH of 8.5 showed a statistically significant gain in stability over the course of the immobilization experiment; however, this gain only showed a DoS of about 1.3. The gain in stability of the pH 5.5 cross-linking condition membranes was also significant and had a much larger DoS of about 2.9.

A



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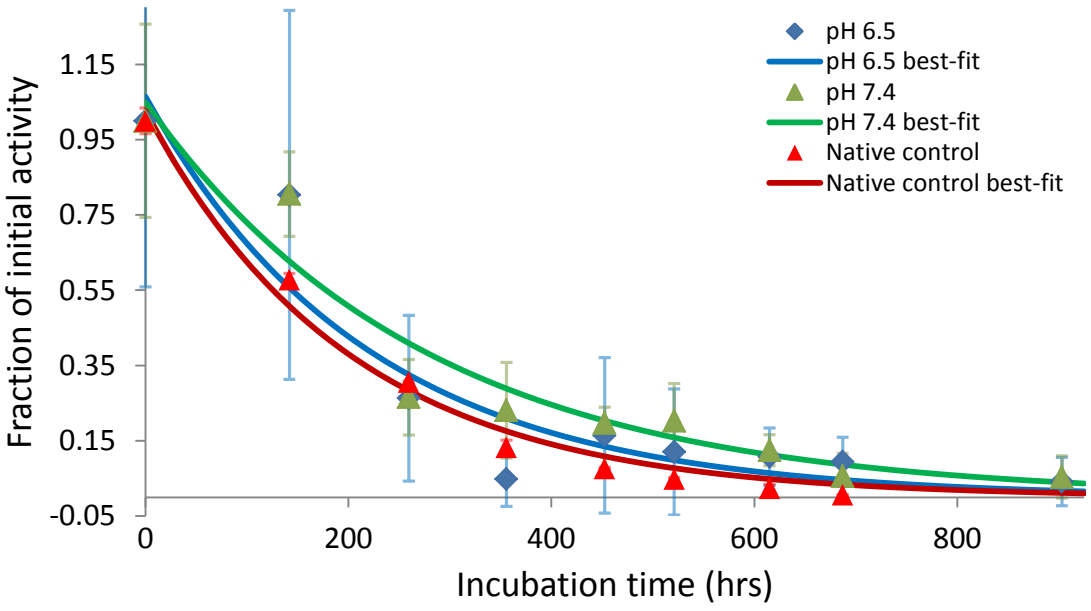

Figure 3.4: Spontaneous inactivation at 37°C of LOx immobilized at different pH values in a BSA-carrier membrane.
Results shown are separated by manufacturing/incubation batch and are compared against native control experiments run concurrently. pH 5.5 (a); pH 6.5 and 7.4 (b); pH 8.5 (c). Error bars represent 1 standard deviation (n=5).

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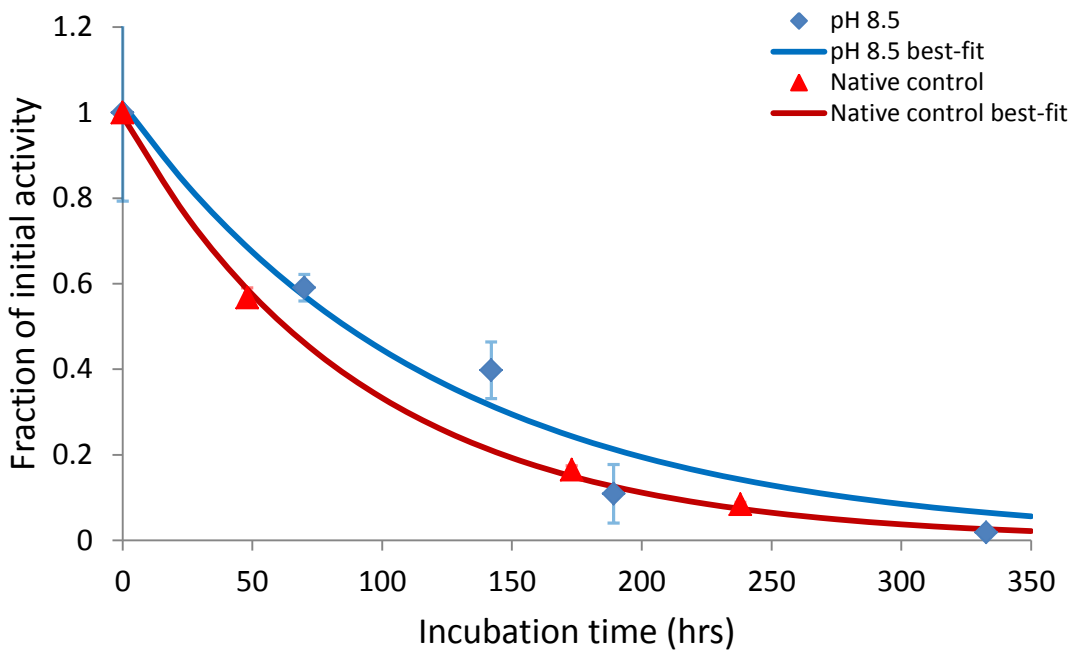

Figure 3.4: Spontaneous inactivation at 37°C of LOx immobilized at different pH values in a BSA-carrier membrane, continued.

The use of glutaraldehyde as a cross-linking agent, although damaging to enzymatic activity, has been shown to aid in stabilization of the remaining activity when used with other enzymes such as GOx (Tse, PHS and Gough DA, 1987). Immobilizing the enzyme at a pH of 7.4 or 6.5 in our experiments does not fix the enzyme in a position that helps to significantly decrease the degradation rate, as the observed rate of decay for these trials was about the same as for the control. When the process is attempted at pH 4.5, no active enzyme is recovered; this is most likely due to the enzyme inactivating in the native state before immobilization occurs (LOx is extremely unstable at this pH). At an immobilization pH of 5.5, it is likely that the amino acid residues are charged and presented such that glutaraldehyde attacks and immobilizes the enzyme in a manner that is stabilizing. In addition, the molecules albumin and LOx are closer to their isoelectric points, meaning that they can become closer associated without as much charge

repulsion. The exact mechanism of this stabilization is not known, as glutaraldehyde cross-linking is not a highly specific cross-linker (Hermanson GT, 2008); different LOx molecules may have differing linkages to the albumin carrier or to each other.

3.3.2.2.2 Accelerated Testing

Accelerated testing was conducted at increased temperatures that were considered physiologically relevant (39°C and 41°C), and outside this range at 50°C. The former temperatures could potentially be reached in an *in vivo* system for varying periods of time, depending on the implant location. pH 5.5 manufacturing conditions were utilized as the basis for these tests, while additional tests were carried out at a manufacturing pH of 5.0 and 6.0, to attempt to narrow in on the pH range giving the greatest degree of stability. Best fit parameter values for all tests are tabulated along with the average R^2 values in table 3.1. Values for the τ parameter of equation 1.10 are plotted versus the temperature of incubation for the pH 5.5 manufacturing tests alongside the soluble enzyme control in figure 3.5.

It is noted that the DoS is not constant with temperature; there could be transitions to different inactivating mechanisms at different temperatures when LOx is immobilized. This hypothesis is supported by the observation that there is relatively more stability offered by immobilization at 37°C and 41°C (where τ is roughly 2 ½ to 3 times the control value) than at 39°C (where τ is roughly the same as the control value). The high DoS also was not seen when the membranes were incubated at 50°C, indicating that relevant information about the stability of the proposed sensor in *in vivo* conditions would have been missed had only accelerated testing been performed. Also, note that, at 41°C, both pH 5.0 and 6.0 immobilization offered some stability gain versus the control. However, neither test showed as high of an inactivation

constant as the pH 5.5 condition, and the pH 5.0 condition suffered from exceedingly low yield. This low yield is most likely due to the same destabilizing effect of low pH that causes membranes cross-linked at pH 4.5 to have a yield of zero.

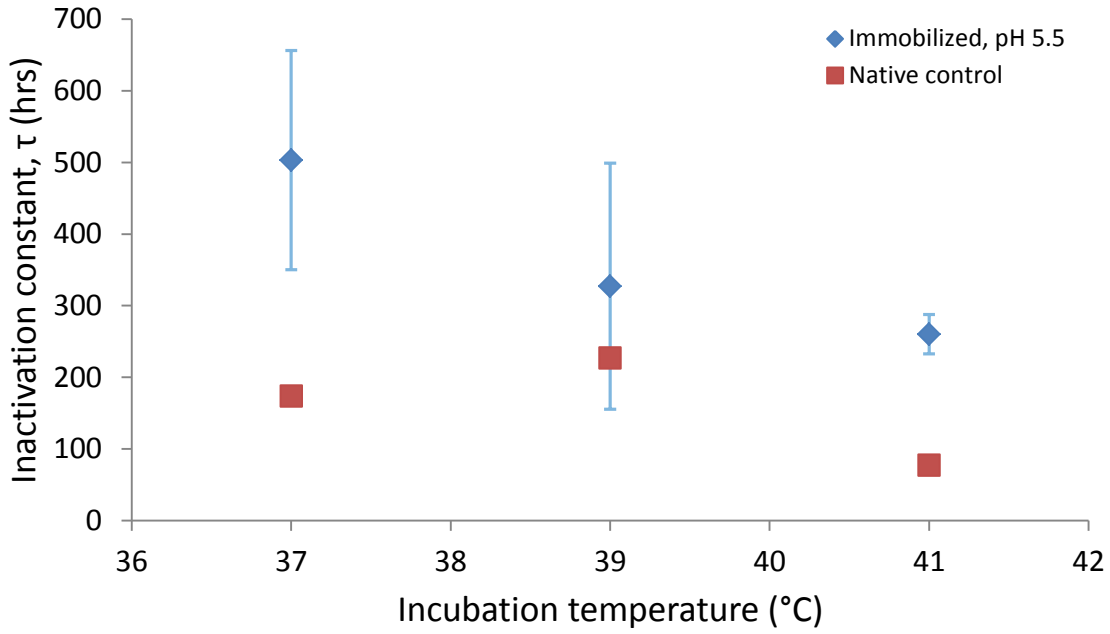

Figure 3.5: The time-constant of inactivation for immobilized LOx and the soluble control plotted as a function of temperature in a physiologically relevant range (37°C to 39°C). Values for 50°C are not shown here but are shown in Table 2.

In addition to the testing done at different pH values, different levels of cross-linker were used to determine if an increase would provide an increase in final stability at 41°C, even if there might be a corresponding decrease in the immobilization yield. Figure 3.6 shows the normalized stability profiles of LOx immobilized using the standard 0.25% glutaraldehyde concentration (during the final cross-linking step) and a 1% concentration. Stability is shown to be greater than native for both cases; however, both the yield and stability parameters of the 1% glutaraldehyde test group are significantly lower than that of the 0.25% glutaraldehyde test group. This indicates that there is no advantage in using higher cross-linking levels under the *in*

vitro testing conditions. These tests were completed at the accelerated condition of 41°C since the DoS offered for the pH 5.5, 0.25% glutaraldehyde test case is roughly equal here to that at the standard of 37°C.

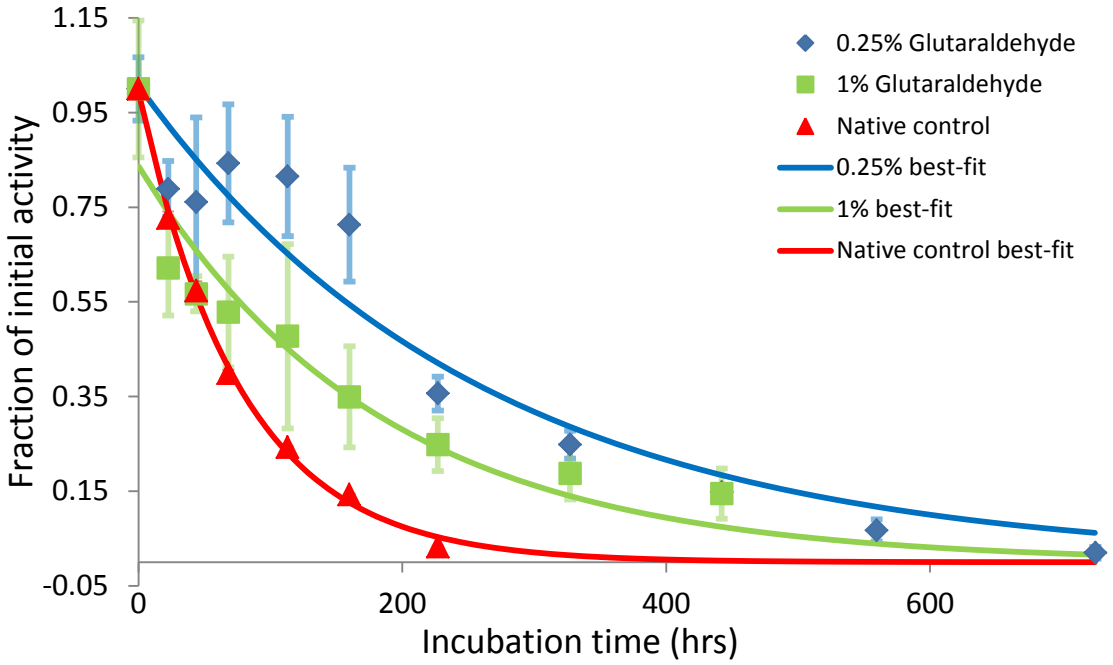

Figure 3.6: Spontaneous inactivation at 37°C of LOx immobilized using different concentrations of glutaraldehyde during the second cross-linking step.
The data is normalized in the figure; however, both yield and stability are significantly lower in the higher glutaraldehyde concentration case.

3.3.2.2.3 Highly Loaded Membranes

In a sensor system, the enzyme loading is desired to be as high as possible. It is therefore advantageous to know if the parameters of yield and stability are roughly invariant as loading levels are increased; however, assay values for V_{max} will be inaccurate due to the diffusion problem described earlier. Results for measuring yield in highly-loaded membranes are shown in figure 3.7a. These are shown alongside solid lines on the figure representing the theoretical predictions of apparent activity based on the FEM model of the assay system. These predictions are scaled to the measured yield of the low-loaded membranes. Modeling results

for different membrane thicknesses are shown parametrically. It can be seen that the measured apparent activities fall roughly in line with the theoretical model predictions, with the exception of the trial formulated at 395 U/g albumin, which appears to have a somewhat lower yield. A very high loading (1184 U/g albumin) was created in a separate batch at higher thicknesses (used due to increased mechanical strength with repeated testing) for use in the accelerated stability study in figure 3.7b. The best-fit solution is found and corrected for diffusional resistance according to the model proposed. The corrected yield value is 47% and the corrected time constant of inactivation is roughly 16 hrs (at about 49°C).

Comparison of the apparent activity of highly-loaded membranes with the FEM model predictions show that the yield of the cross-linking process does most likely not decrease with increasing loading levels (as would be used in *in vivo* sensor construction). Measured results do seem to be slightly lower than predicted at very high levels when thinner membranes are used. It is possible that at these loading levels, the boundary layer in the bulk solution phase (neglected in the modeling analysis due to rapid stirring) becomes significant. However, at the higher membrane thickness used in the accelerated stability trial of the high loading case (figure 3.7b), apparent yield is somewhat greater than model prediction. These results for both the very thin and slightly thicker membranes would fit with the hypothesis of a thin bulk-solution boundary layer caused by inadequate stirring, if the diffusivity of O_2 has been slightly underestimated. These results indicate that stability and process yield are relatively inelastic with the increased loading conditions that may be used in construction of an actual *in vivo* sensor system. However, because of large error propagation in a back-calculation of this type, the evidence is only indirect.

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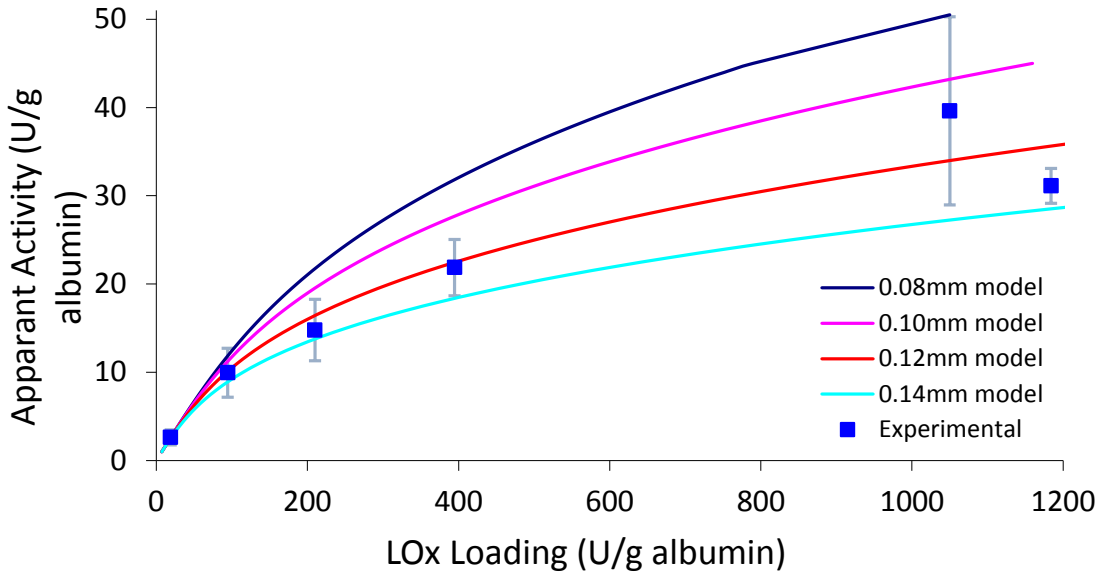

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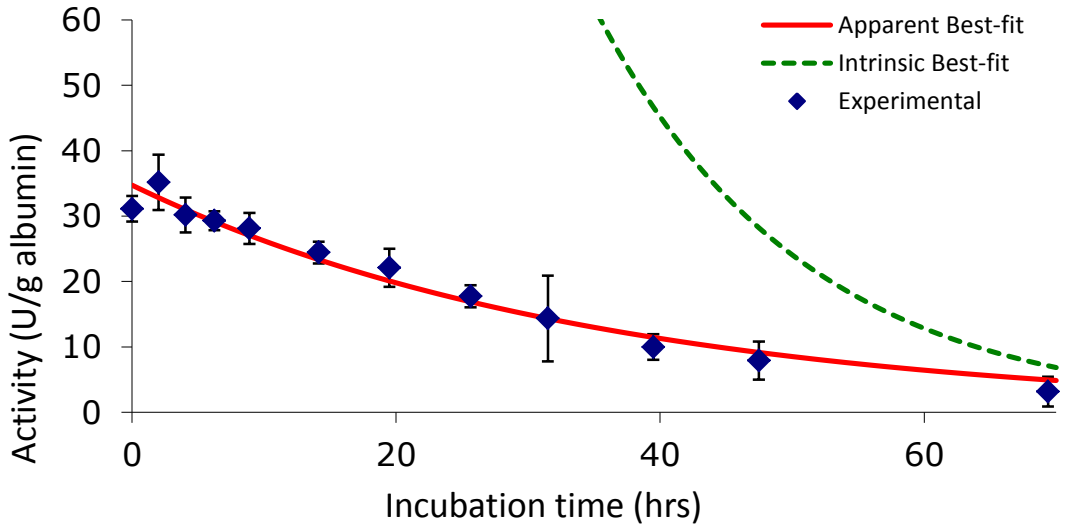


Figure 3.7: Highly-loaded membranes assayed to indirectly determine if yield and stability parameters have been affected.
(a) Results for apparent yield of the membranes are plotted with solid lines showing the predicted values of apparent yield according to the model proposed and scaled to low-loading yield (average thicknesses for experimental data are 0.09mm, 0.11mm, 0.14mm, 0.07mm, and 0.09mm with respect to increase loading levels). (b) A highly loaded level of 1184 U/g albumin (average thickness = 0.17mm) is subjected to stability measurements at 50°C. The apparent and "corrected" best-fit are shown. The corrected time constant of inactivation is roughly 16 hrs.

Table 3.1: The results of the best-fit parameter models to the experimental data for BSA-carrier membranes.
Experiments are grouped by the incubation temperature and the pH of manufacturing is shown in the left-hand column. The parameters are given with and without (in that order, separated by a slash) the diffusion correction based on the FEM model of the assay applied. Red highlighting of the DoS represents statistical significance at in comparison to the native control at $P < 0.05$.

	c_0 (% yield)	τ (hrs)	R^2	DoS
37°C				
pH 5.5	6.73 / 7.02	503 / 485	0.81 / 0.81	2.89 / 2.79
pH 6.5	9.14 / 9.81	219 / 212	0.86 / 0.86	1.09 / 1.05
pH 7.4	14.96 / 17.24	276 / 256	0.91 / 0.91	1.37 / 1.27
pH 8.5	25.14 / 34.96	121 / 92	0.95 / 0.97	1.32 / 1.09
39°C				
pH 5.5	8.92 / 8.93	327 / 321	0.71 / 0.66	1.44 / 1.41
pH 6.0	17.71 / 19.76	206 / 199	0.73 / 0.71	0.91 / 0.88
41°C				
pH 5.5	11.13 / 12.73	260 / 240	0.82 / 0.81	3.36 / 3.10
pH 5.0	2.04 / 2.05	164 / 163	0.93 / 0.93	2.22 / 2.21
pH 6.0	18.40 / 23.86	240 / 198	0.98 / 0.98	3.26 / 2.69
50°C				
pH 5.5	13.10 / 14.37	6.9 / 6.3	0.99 / 0.99	1.09 / 1.00
pH 6.5	19.87 / 26.79	6.2 / 4.7	0.99 / 0.99	0.98 / 0.74

3.4 Conclusions

Utilizing a covalent enzyme immobilization processes developed in the lab, we have studied parameters of manufacturing in order to maximize the active yield and stability of the resultant construct. Collagen and BSA were evaluated as protein carriers for the immobilized LOx; BSA was found to be superior because of its ability to maintain mechanical strength over time at the required cross-linking levels. Ultimate loading potential of this method can only be indirectly determined; however, we present evidence that suggests that yield is roughly invariant with loading. The natural polymer carrier membranes have been investigated for

enzymatic stability at physiologically relevant conditions. We have shown a roughly three-fold increase in the stability of the enzyme when the immobilization procedure is conducted at pH 5.5 in a BSA carrier protein matrix. An increase in the stability of the enzyme leads to a corresponding increase in the expected sensor lifetime regardless of sensor design (ie, the 3-fold increase in stability observed here will increase expected sensor lifetime by the same fold-number). Additionally, for every doubling of loading, we expect to see an increase in the lifetime of approximately 2 weeks (one half-life).

Stability was also tested at 39°C and 41°C, as well as 50°C for comparison to accelerated testing protocols reported elsewhere. The DoS was not constant throughout the incubation temperature range tested, indicating that there are various mechanisms of inactivation that occur at different temperatures, and these mechanisms are effective at slightly different temperatures in the immobilized and solubilized states. Therefore, we recommend evaluating the degree of stability at the temperature at which the immobilized enzyme construct is intended to operate.

This chapter, in part, is a reprint of the material as it appears in the following:

- Strobl AL and Gough DA. Lactate oxidase (LOx) immobilized in a natural polymer carrier: parameter effects on stability in a physiologically relevant range. *In Preparation*.

The dissertation author was the primary investigator and co-author of this paper.

IV Lactate Oxidase in a Polyionic Matrix

4.1 Introduction

In addition to using chemical immobilization methods, it is also possible to immobilize enzymes via electrostatic interaction. In this chapter, the properties of lactate oxidase immobilized within such a matrix are explored. As stated in the introduction chapter, functioning sensor elements have been constructed through the use of this type of immobilization (Mizutani F et al, 1995). These sensors were tested in very low lactate concentrations (linear range extends to about 0.3 mM) and were only evaluated for shelf-life stability. An implantable sensor design must have a linear response to higher concentrations found *in vivo* due to the lack of a system for sample dilution and preparation. As stated earlier, this generally precludes the use of mono-layer or layer-by-layer construction of the sensor surface and immobilized enzyme layer. Therefore, we have chosen to study LOx immobilized in a bulk polyionic matrix. This bulk matrix has similar mechanical properties to the glutaraldehyde cross-linked natural polymer matrices studied in the previous chapter. The matrix could be created in any geometrical shape required by the sensor design and in any required thickness, and it can be held in place by a PDMS surround and possibly a dialysis-type membrane to provide a mechanical seal around the substrate aperture.

The bulk polyionic matrix studied here is created by the interaction of a strong polycation, diethylaminoethyldextran (DEAE-D), and a strong polyanion, poly(sodium-p-styrenesulfonate) (PSS). Materials were chosen due to ready availability and the ability to successfully interact to form a mechanically stable matrix. These materials have slightly differing molecular weights per charge; this led to the study of differing ratios of the components being used. The effect of the individual components in solution on stability of the

enzyme LOx was investigated. The results of this experiment, as well as the suggestions of literature regarding the role of a polycation in enzyme stability (Heller J and Heller A, 1998), led to experiments in which the concentration and amounts of DEAE-D were varied. The time for complexation between DEAE-D and LOx was investigated. Since the pH of the solution in which the immobilization is performed affects the charges on the surface and interior of the enzyme, pH effects were also considered.

4.2 Materials and Methods

4.2.1 Material covered earlier

The process by which LOx is immobilized in a polyionic matrix is covered in section 2.2.1.2. The soluble enzyme assay for the control is described in section 2.2.2. The immobilized enzyme assay is described in section 2.2.3.

4.2.2 Evaluation of LOx stability in individual matrix components

LOx was dissolved in a solution of the individual matrix components at varying concentrations. PSS concentrations were 0.14%, 1.4% and 14% after LOx addition. DEAE-D concentrations were 0.14%, 1.4%, 14% and 24% after LOx addition. Solutions were evaluated for activity after overnight incubation. If significant activity remained (DEAE-D solutions only, see results), stability testing was completed at 37°C.

4.2.3 Evaluation of immobilization parameter effects on yield and stability

All stability tests were run with two types of controls: the enzyme solubilized in PBS (positive control) and membranes with no enzyme added (negative control). Buffer solution in

which the membranes were stored (PBS) was also tested for activity to determine if enzyme was being leeched from the membrane. Time points were chosen to attempt to capture the inactivation curve. The amount of PSS added as a "binder" was evaluated for its effect. This was done in the range of 1:1 to 3:2 PSS:DEAE-D charge ratio. pH of the process during immobilization was also evaluated for its effect on stability; it was controlled at the values 9.5, 8.5, 7.4, 6.5, and 5.5, and the stability evaluated at 37°C. Complexation time of LOx with the polymer DEAE-D before immobilization was evaluated, as was the concentration of DEAE-D that this complexation took place in. Higher concentrations (above 24% wt/wt DEAE-D) were achieved via evaporation during the complexing step.

4.2.4 Analysis methods

All experimental conditions for stability experiments were conducted with 5 immobilized membranes, a positive control (LOx in solution) with 3 repeated measurements per timepoint, and a negative control (a membrane with no LOx loading). Due to the nature of the material studied, some assays resulted in assay solution that was very clouded by immobilized material suspended in the solution. This clearly affected the absorbance reading and these assay values were not used in the calculations. When more than one timepoint for a particular membrane was not included, the results for the particular membrane as a whole were not used in the final calculations. As with the membranes studied in chapter 2, there is a normal, low level clouding that occurs in the assay that does not obfuscate the results. This is controlled for by inclusion of the negative control. Since different immobilization conditions were found to greatly influence the mechanical stability of the membrane and therefore the control values for the assays, each set of control values were analyzed for trends. When no trend was found, the

average control value was used for each timepoint. If trends were apparent, a best-fit trend value was used.

Results were compared statistically in the same manner as those in chapter 2. Here we pay special attention to differences among test groups analyzed at the same time due to larger differences among positive control values for different incubations. It is believed that varying manufacturing conditions of the LOx enzyme from the vendor AG Scientific may have contributed to the higher variability in stability observed as opposed to that from the vendor Genzyme. Positive control tests are treated as three separate measurements of each parameter of equation 1.10, as before. Each test membrane is also treated as a separate measurement of the stability and yield parameters. All test groups are first compared using ANOVA for differences in the stability and yield parameters of the regression, followed by pair-wise t-tests using a Bonferroni correction factor if differences are observed. Statistical significance is defined at $P<0.05$.

4.3 Results and Discussion

Parameter results for all stability experiments are shown in table 4.1 at the end of this section. This table is grouped by experiment (meaning the same manufacturing and incubation group) and further by the sub-section in which it is discussed. Group names are described in the text of the individual sub-sections. Parameters that are significantly different from one another are highlighted in red; when an experiment has one group that is significantly different from the other two groups (and those two groups are not significantly different from one another), then the group that is different is highlighted in red and the groups that are the same are highlighted in green. Comparisons against the native control time constant of inactivation are not shown;

however, there is a statistically significant difference between the native control and immobilized LOx in all cases except the experiment shown in figure 4.2a, where the group labeled 1:1 (see section 4.3.2 for a description) was not different.

4.3.1 Stability of LOx in solution with DEAE-D and PSS polymers

In order to gain insight into the effects of charged polymers on the stability of the LOx enzyme, the stability was first evaluated in solution with only one of the polymers at a time. The enzyme was found to be highly unstable in PSS (polyanionic) solution; after overnight incubation under refrigerated conditions, there was minimal activity left: at 0.14% PSS wt/wt solution, 8.5% of the initial activity remained; at 1.4% PSS, 5.3% remained; at 14% no residual activity could be measured. This is generally what one would expect given a destabilizing agent; the higher the concentration of the polyanion, the lower the amount of activity remaining. We therefore hypothesize that higher levels of PSS in the immobilized construct would lead to greater instability.

The response of LOx stability to the presence of the polycation DEAE-D in solution is quite different. Here the mixture was incubated at pH 7.4 and 37°C. The results are shown in figure 4.1. Here we see a surprising result: the stability of LOx is greater at higher concentrations of the polycation. At low concentrations, the polycation seems to be highly destabilizing. However, the stability is close to that of the native control alone in PBS when the concentration of DEAE-D is increased to 24% wt/wt. Literature states that many enzymes with a charged substrate (such as LOx) require complexation with an oppositely charged polyion in order to retain stability when immobilized on or within a charged environment (Heller J and Heller A, 1998; Cox JA et al, 2003). This effect is often explained as a result of “wrapping” or

"shielding" the enzyme molecule from deleterious charge effects. If we interpret the results of the experiment in this manner, we may then explain the result by hypothesizing that the DEAE-D polycation complexes with LOx more efficiently at higher concentrations. At lower concentrations, the enzyme perceives only the destabilizing effect of charges moving past the molecule and attracting/repulsing residues during random motion. This suggests it may be necessary to allow DEAE-D and LOx to complex at high DEAE-D concentrations in order to retain stability in immobilized form.

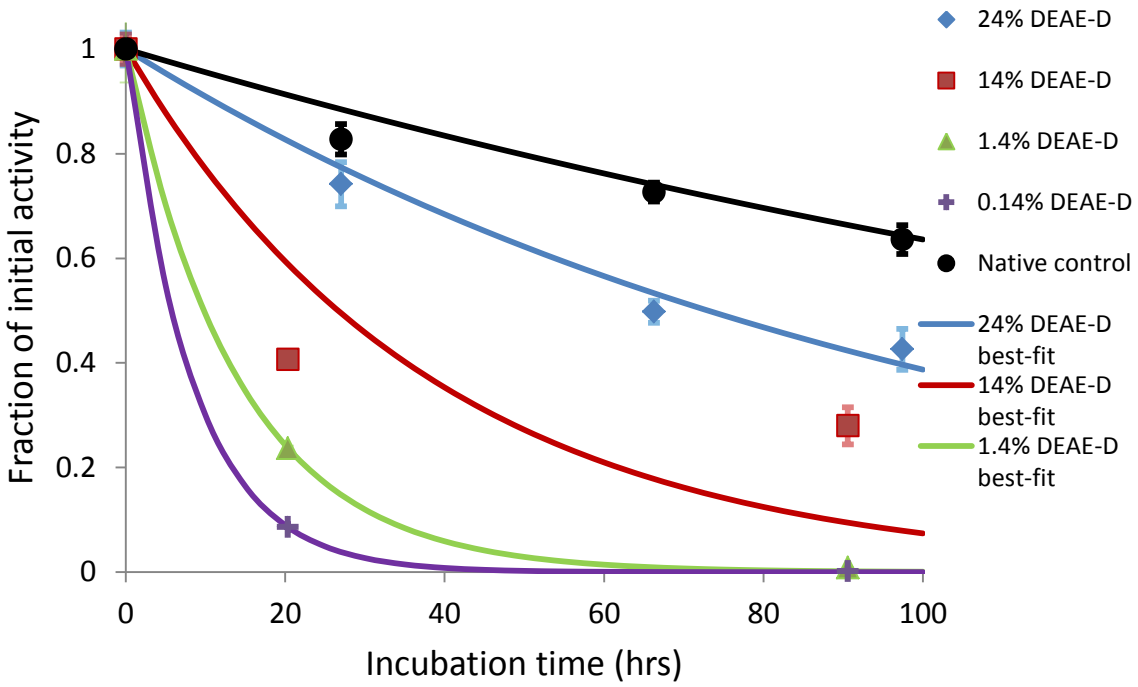


Figure 4.1: The stability of LOx in solution with specific concentrations of DEAE-Dextran. All solutions were titrated to pH 7.4 and incubations were completed at 37°C. Note the increasing stability with increasing concentrations of DEAE-D. Error bars represent one standard deviation in the readings (sometimes too small to see on the above figure). Results are all significantly different from one-another.

4.3.2 Immobilization yield and stability with varying amounts of PSS

Initial development of the immobilization procedure involved determining the range of ratios of the two matrix components that would yield a mechanically stable membrane while

preserving enzymatic activity. Here we evaluated a one-step and a two-step PSS addition process. In the one-step process, the matrix components were mixed in the well and allowed to dry to a film, at which time the membranes were weighed and washed. All immobilizations in this set were completed while buffered at pH 7.4, and the DEAE-D concentration used was 24% (after LOx addition). The immobilization reaction here was done on the well. Results of the one-step addition process are shown against the 2-step addition process in figure 4.2a. Here, the addition of PSS was in a 1:1 charge ratio with the amount of DEAE-D. The 2-step addition utilized an additional aliquot of PSS (in the same amount – referred to on the figure as 1:1 + 1:1) after the immobilized film had initially dried. This was allowed to redry before the weighing and washing steps. Results appear to show a decrease in stability of the immobilized LOx with the additional PSS, although this difference is only statistically significant if the diffusion correction is applied. As seen in the figure, the diffusion resistance in this experiment is quite large, making comparisons somewhat difficult; however, there appeared to be no increase in mechanical stability of the membranes with the secondary addition of PSS, giving this process no perceived advantage.

Since it was determined that there was no advantage to using the 2-step addition system, an experiment was run to determine if yield and stability were affected by the ratio of PSS addition in the one-step process. The charge ratios of PSS to DEAE-D were set to 1:1, 5:4 and 3:2 in the test groups. Results of the stability test are shown in figure 4.2b. Statistical tests show no difference among the groups here for both the yield and stability parameters. After the diffusion-correction is applied, the parameters change by a substantial amount; however, the results still remain statistically the same to one another. The advantage of operating at the higher PSS:DEAE-D charge ratio is a mechanically more stable membrane, allowing greater

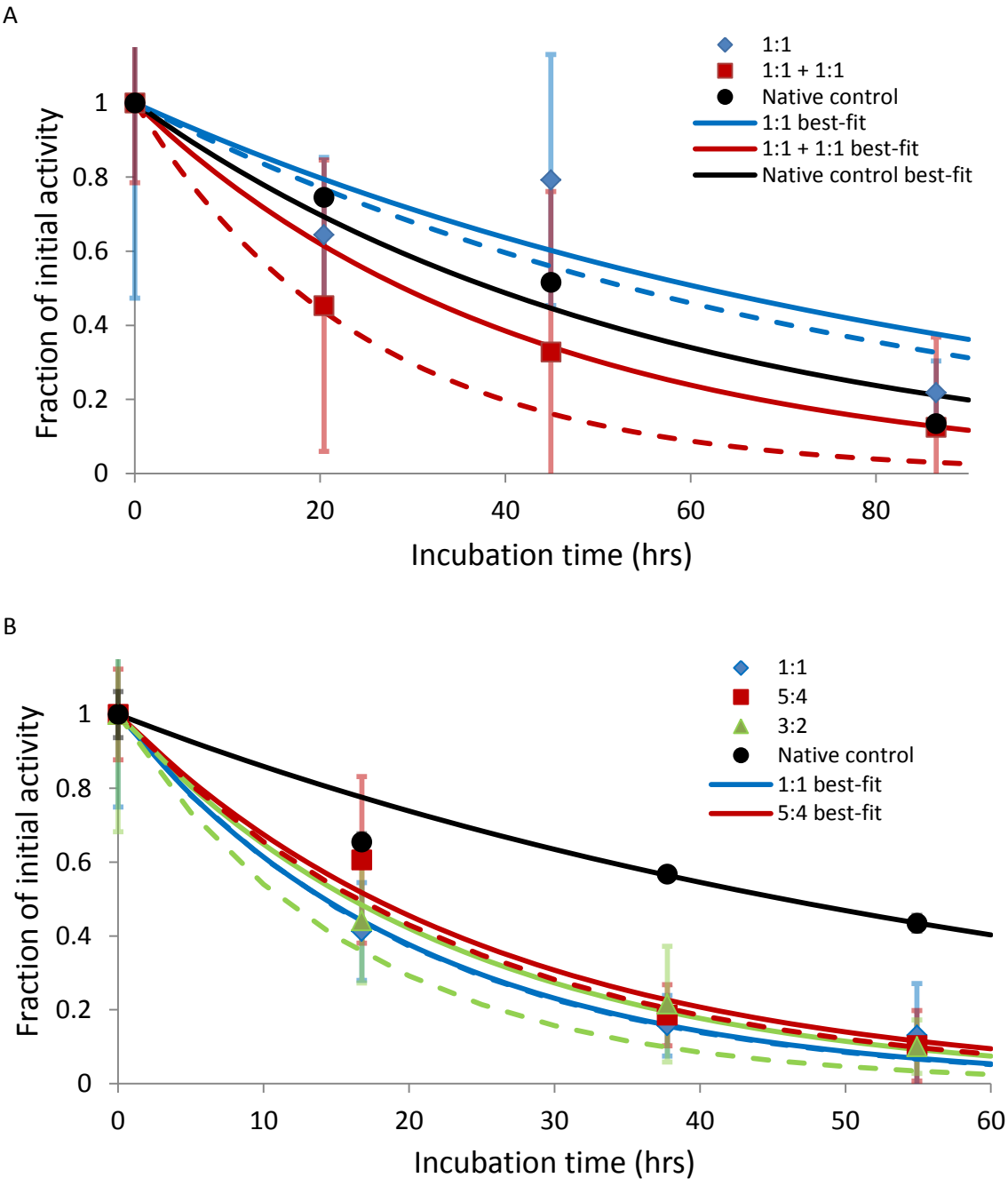


Figure 4.2: Stability testing at 37°C, pH 7.4 of polyionic immobilized constructs with 1-step and 2-step PSS addition processes, and with different PSS:DEAE-D ratios.
(a) A 1-step and 2-step PSS addition are compared. The trials are significantly difference only when the diffusion correction is applied. (b) Variations in the amount of polyanion were used, with charge ratios of 1:1, 5:4 and 3:2 (PSS:DEAE-D). There is no difference in the parameters found, both apparent and corrected for diffusional resistance (dotted). Experimental data shown are the apparent values. Error bars represent one standard deviation.

accuracy in later timepoints. Therefore, we chose to utilize a charge ratio of 3:2 going forward in the experimentation.

4.3.3 pH variation in membrane manufacture

Two separate experiments were conducted to determine the effect that the pH at time of the immobilization reaction would have on the yield and stability parameters of the immobilized enzyme. Conditions of one experiment were pH's 5.5, 7.4 and 9.5, while the other was narrowed in further to pH's 6.5, 7.4 and 8.5. Other conditions were the same as the previous experiment, with a PSS:DEAE-D charge ratio of 3:2. Results of the stability testing are shown in figure 4.3. In the first experiment (figure 4.3a), ANOVA shows a difference amongst the groups for the stability parameter (with all groups having lower stability than the native control). The pH 7.4 test shows the highest stability here. Pairwise t-tests show that the pH 7.4 test has greater stability than the pH 5.5 or pH 9.5 cases. Yield appears lower for the pH 9.5 group; however, this difference is not statistically significant. In the pH 6.5/7.4/8.5 experiment, the stability is not significantly different among the test groups. Only the yield of the pH 6.5 test is shown to be different than the pH 7.4 test group. If the diffusion correction is made based on the parameters identified in section 3.4.1, the yield of the pH 6.5 test appears different than both the pH 7.4 and pH 8.5 test groups.

These test groups indicate that the pH during complexation of LOx with DEAE-D and immobilization via PSS addition does indeed have an effect on the stability of the resultant immobilized enzyme construct. However, the effect is small, and it only shows a negative effect at extreme pH values. It seems that performing these steps at a neutral or slightly alkaline pH allows for the best interaction of LOx with the matrix components.

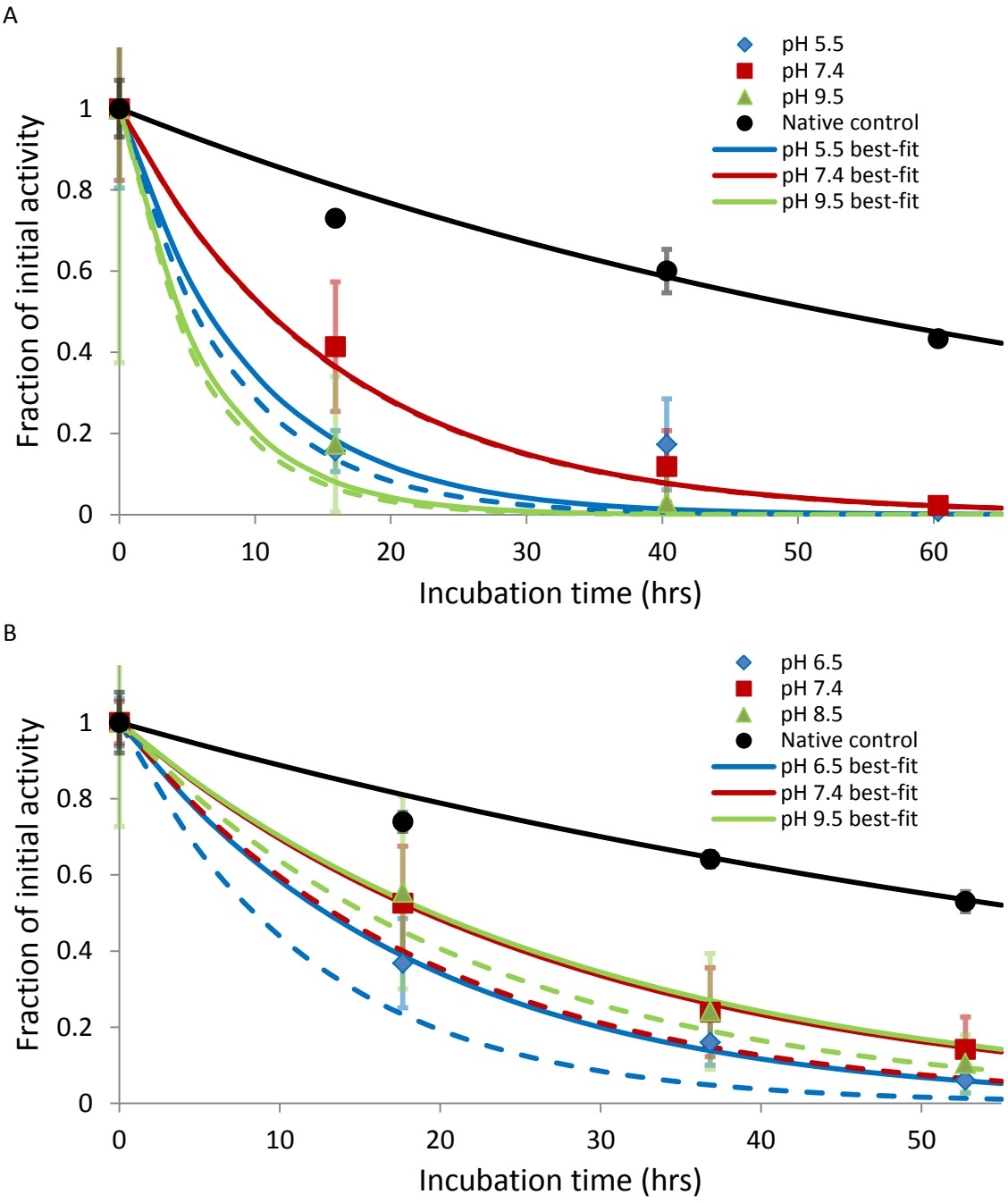


Figure 4.3: Stability testing at 37°C, pH 7.4 of polyionic immobilized constructs created at different pH values.
The pH during the complexation/immobilization reaction was varied between pH 5.5 and pH 9.5 in two separate experiments. In the larger range experiment, there is a significant difference in the stability of the membranes. In the smaller range experiment, there is not a significant difference amongst the groups. The best-fit exponential decays for both the apparent and diffusion-corrected (dotted) data are shown. Experimental data shown are the apparent values. Error bars represent one standard deviation.

4.3.4 Complexation time effect on LOx stability

To partially test the hypothesis that complexation of LOx with the polycation DEAE-D leads to higher stability, we investigated the amount of time needed for the complexation to take place. Here, the time that DEAE-D solution was allowed to incubate with LOx before precipitation with PSS was set at either 10 minutes (during which the solution was stirred together for adequate mixing) or 21 hours (after a 10 minute stirring). The precipitate/immobilized enzyme was created by adding the amount of PSS directly to the vial and stirring. Solutions were kept at pH 7.4, the final charge ratio was 3:2 anion:cation, and the concentration of the DEAE-D/LOx mixture was 24%. Stability results are shown in figure 4.4.

Results show that complexation time is in fact required for the DEAE-D to affect a degree of stability to LOx in the final immobilized construct. Stability and yield parameters were both significantly higher in the case where the polycation and enzyme were allowed to complex for 21 hours prior to immobilization. This indicates that association between the components is not instantaneous and some time is required. The specific point at which optimal association has taken place was not determined in this analysis.

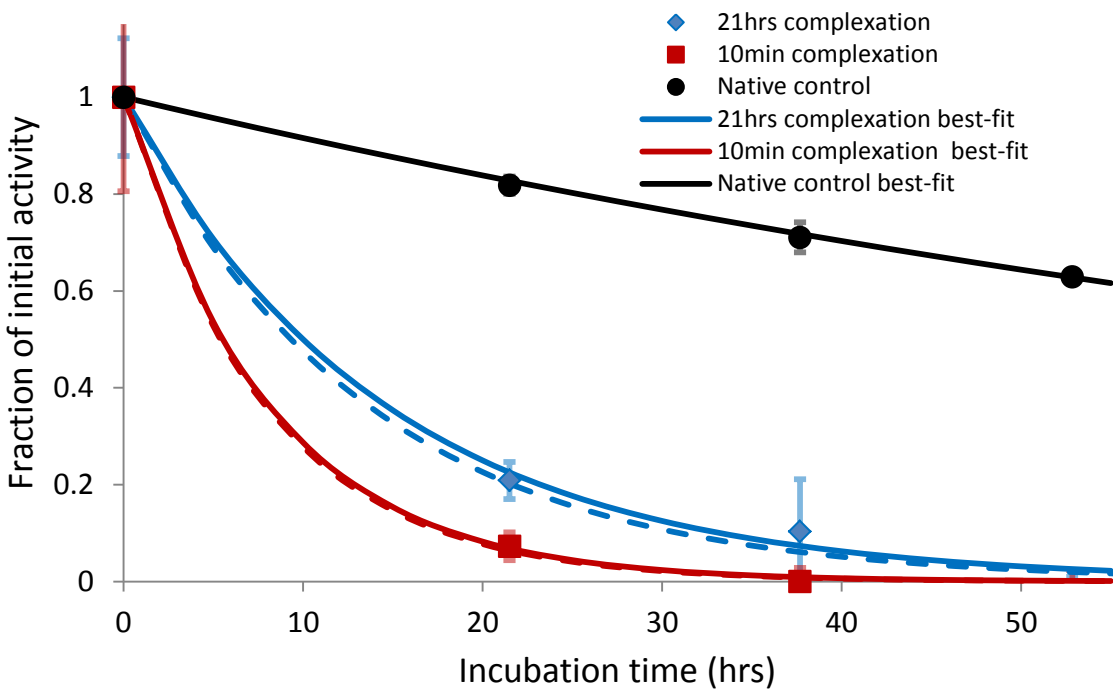


Figure 4.4: Stability testing at 37°C, pH 7.4 of polyionic immobilized constructs for different LOx/DEAE-D complexation times.
LOx was allowed to complex with the DEAE-D solution for either 21 hours or 10 minutes. Results show a statistically significant difference between the stability of the two groups. The best-fit exponential decays for both the apparent and diffusion-corrected (dotted) data are shown. Experimental data shown are the apparent values. Error bars represent one standard deviation.

4.3.5 DEAE-D concentration during complexation effect on LOx stability

The effect of the concentration of DEAE-D during the complexation step was evaluated in two different experiments. In the first, the concentration was varied within a range where it was easily weighed and handled. Concentrations of 9%, 14% and 24% w/w DEAE-D were used during complexation with LOx. At concentrations much higher than 24%, the solution becomes difficult to mix with LOx and to weigh accurately, due to extremely high viscosity. For the second experiment, good mixing was insured by keeping the initial concentration of DEAE-D/LOx to 24%. However, the vials in which the complexation was occurring where exposed to different air/flow conditions to allow for different, higher concentrations via evaporation. Evaporation

was conducted at room temperature (to avoid denaturation) either open to room air, or in a fume hood. Final concentration values were determined via mass balance and were 31% (fume hood evaporation), 29% (room air evaporation) and 24% (closed cap vial). Concentrations were therefore higher in the test groups; however, the concentration was changing and average concentration during the complexing step is less than the final concentration. The complexation/evaporation step was held to 21 hours. Other parameters were the same as those in section 4.3.4.

Results for the first experiment (9%, 14% and 24% DEAE-D) show clear differences among the groups (figure 4.5a). Stability and yield are significantly different as shown by ANOVA, and pairwise t-tests indicate that there are differences between the 9% group and the 24% group, as well as the 14% group and the 24% group for both parameters. This provides further evidence supporting the hypothesis that complexing DEAE-D with LOx provides for enhanced stability, as the components are forced closer together at higher concentrations. The results appear very similar to those results for the stability of LOx in solution with DEAE-D (figure 4.1)

Results for the second experiment, where higher concentrations of DEAE-D were achieved by evaporation of the DEAE-D/LOx mixture, did not show differences among the groups (figure 4.5b). This indicates that either the increase in concentration was not enough to force a closer association of DEAE-D and LOx, or that there is an upper limit on the protective nature of this wrapping action.

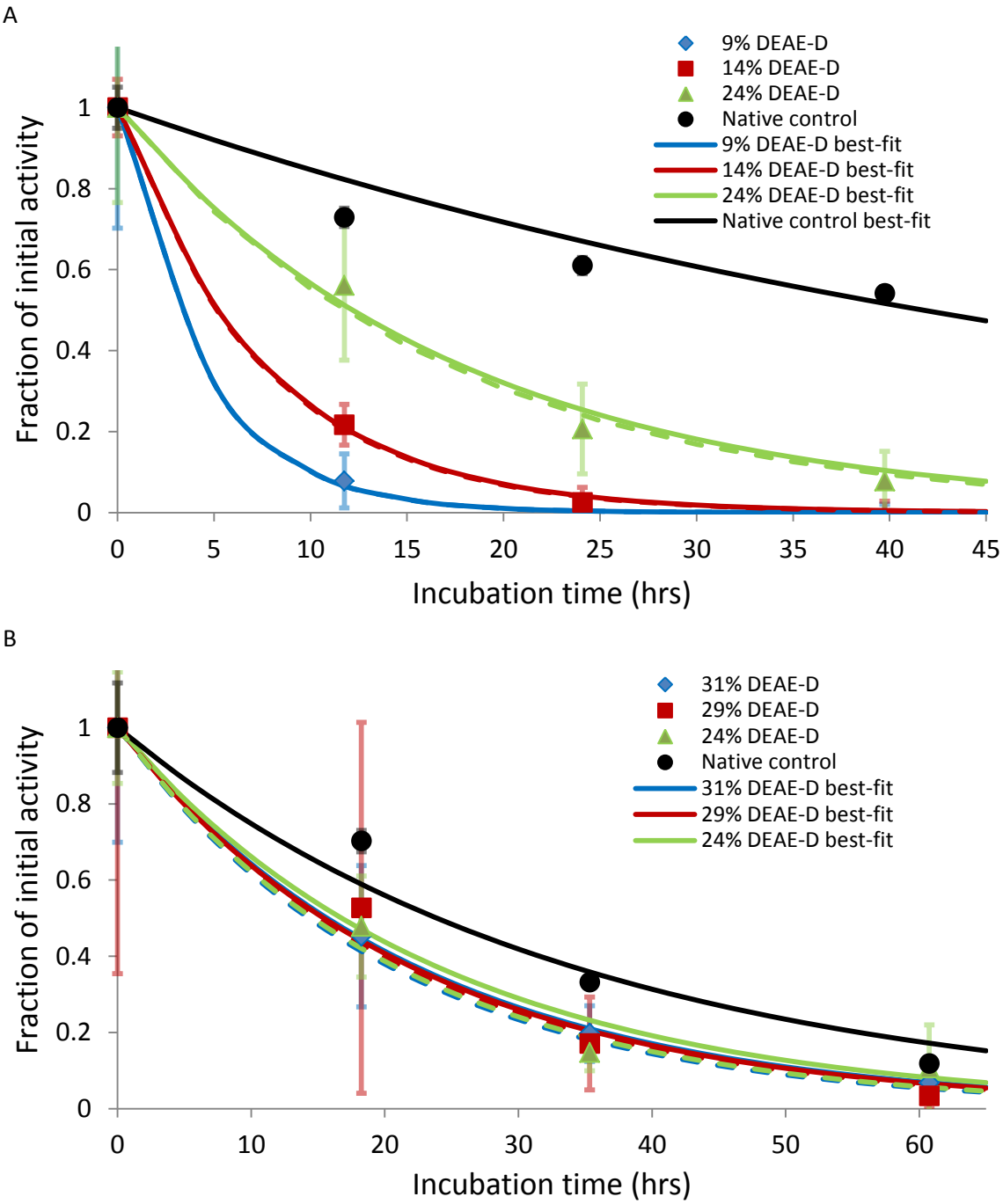


Figure 4.5: Stability testing at 37°C, pH 7.4 of polyionic immobilized constructs created with differing concentrations of DEAE-D.

(a) Concentration of DEAE-D during the complexing/immobilization step is varied within the range 9% to 24%. Results show a statistically significant difference between stability of the groups. (b) Concentrations of DEAE-D are varied by evaporation to achieve higher concentrations (29 and 31%). No differences in stability and yield were seen. The best-fit exponential decays for both the apparent and diffusion-corrected (dotted) data are shown. Experimental data shown are the apparent values. Error bars represent one standard deviation.

4.3.6 Accelerated testing at 39°C

Membranes resulting from the same conditions used in section 4.3.4 were also exposed to incubation at 39°C, and results are shown in figure 4.6. The results from this experiment showed that the stability of the membranes was significantly less than that at 37°C. However, the "21 hour complexation" group experienced a much larger drop in stability and no longer had a greater stability than the "10 minute complexation" group. Therefore, we may conclude that the protection afforded by complexing with the polycation is lost at 39°C. Depending on the various modes of inactivation that are present (see the discussion in chapter 2), this relative stability enhancement may appear again at yet higher temperatures. Because stability was already quite low at 39°C, we did not complete further testing at higher temperatures.

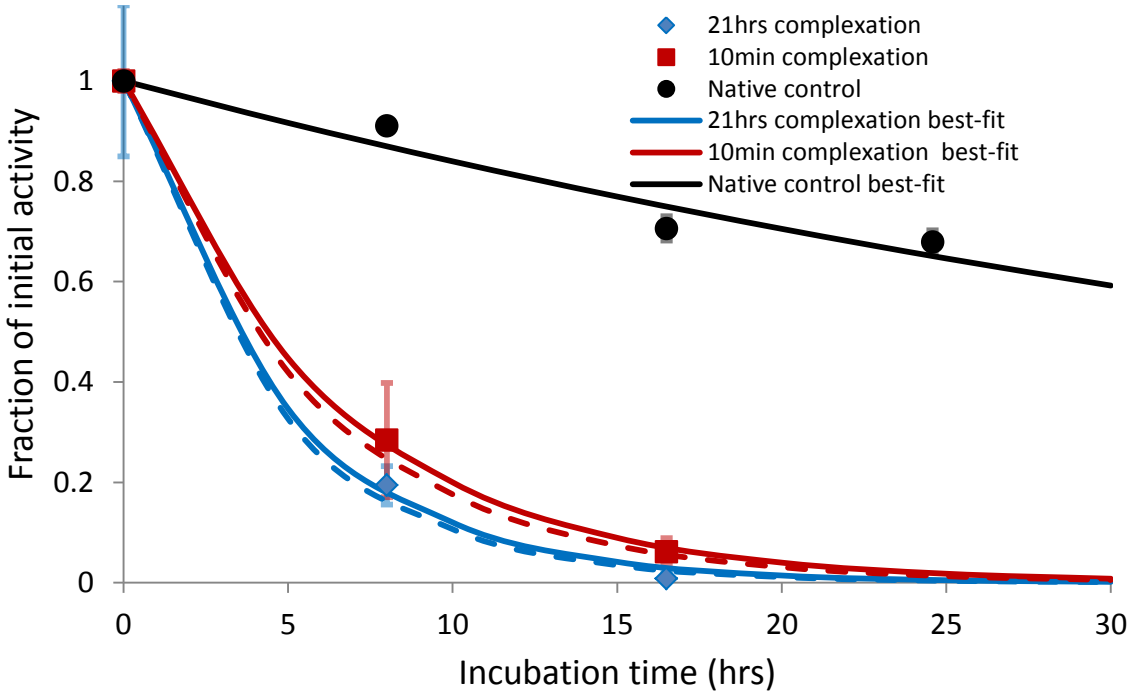


Figure 4.6: Accelerated stability testing at 39°C, pH 7.4 of polyionic immobilized constructs. LOx was allowed to complex with the DEAE-D solution for either 21 hours or 10 minutes. Results do not show a statistically significant difference between the stability of the two groups. The best-fit exponential decays for both the apparent and diffusion-corrected (dotted) data are shown. Experimental data shown are the apparent values. Error bars represent one standard deviation.

Table 4.1: Inactivation parameters for all polyionic experiments.
Descriptions of experimental groups are given in the associated section (left column). Parameters are shown with and without (in that order, separated by a slash) the diffusion correction from section 2.4.2. Each experiment is grouped together, and groups are statistically compared. Black indicates no difference for that parameter in that group; red indicates that the parameters are different; green lettered parameters are not significantly different from one another when another group in that experiment is different.

Section	Group	c_0 (% Yield)	τ (hours)	R^2
4.3.2	1:1	6.00 / 8.08	88.6 / 77.3	0.64 / 0.68
	1:1 + 1:1	6.00 / 10.31	41.9 / 24.6	0.98 / 0.98
	Native	N/A	55.6	0.97
	1:1	9.12 / 9.26	20.5 / 20.3	0.98 / 0.98
	5:4	11.39 / 13.3	25.4 / 23.7	0.94 / 0.94
	3:2	10.70 / 20.8	23.1 / 16.2	0.98 / 1.00
	Native	N/A	66.0	0.94
4.3.3	pH 5.5	8.96 / 11.75	9.42 / 8.02	0.93 / 0.94
	pH 7.4	8.79 / 10.45	17.97 / 15.71	0.97 / 0.97
	pH 9.5	5.09 / 6.16	6.35 / 5.85	0.99 / 0.99
	Native	N/A	75.4	0.96
	pH 6.5	10.03 / 20.48	18.62 / 12.15	0.99 / 1.00
	pH 7.4	7.47 / 11.75	27.5 / 19.3	0.99 / 0.99
	pH 8.5	8.25 / 10.84	28.1 / 22.2	0.96 / 0.99
	Native	N/A	84.2	0.97
4.3.4	21hrs	15.89 / 17.97	14.43 / 13.45	0.98
	10min	11.55 / 12.47	8.01 / 7.82	1.00
	Native	N/A	113.6	0.99
4.3.5	9%	3.27 / 3.32	4.40 / 4.38	0.99 / 0.99
	14%	4.09 / 4.20	7.54 / 7.44	0.99 / 0.99
	24%	11.84 / 12.54	17.58 / 16.87	0.96 / 0.97
	Native	N/A	60.2	0.93
	31%	24.05 / 27.52	22.6 / 20.6	0.99 / 0.99
	29%	22.20 / 23.39	22.3 / 21.7	0.94 / 0.94
	24%	19.36 / 21.78	24.2 / 21.1	0.98 / 0.98
	Native	N/A	34.5	0.96
4.3.6 (39°C)	21hrs	16.62 / 18.55	4.72 / 4.47	1.00 / 1.00
	10min	18.75 / 21.19	6.21 / 5.76	0.99 / 1.00
	Native	N/A	57.3	0.93

4.4 Conclusions

Here we have shown the stability properties of lactate oxidase in a bulk polyionic matrix. This matrix was composed of a polycationic polymer, diethylaminoethyldextran (DEAE-D), that was precipitated out of solution with a polyanionic polymer, poly(sodium-p-styrenesulfonate) (PSS). DEAE-D has been used in other similar enzyme-immobilization processes in order to enhance enzyme stability (Gavalas VG and Chaniotakis NA, 1999). PSS has also been used in order to precipitate linear polycations from solution for use in a thin-film biosensor (Mizutani F et al, 1995). We chose these materials for their ease of use and ability to form a robust precipitate that could be dried to a film and rehydrated for testing.

Testing in the solubilized form revealed that the polyanion was highly degradative to LOx activity; however, the polycation, while always affecting stability negatively, was associated with better enzymatic stability at higher concentrations. Along with literature stating that complexing LOx and a polycation could have a net positive effect on stability, this suggested that in situations where the two components were forced close together, a protective element could be observed. Therefore, we studied parameters that could have some effect on how the components complexed before being precipitated out of solution. These included pH of the solutions, concentration of the polycation, and the time allotted for complexation of DEAE-D and LOx. In addition, because it was known that the polyanion had a negative effect on stability, we determined how much of this “ionic-glue” was necessary for mechanically stability of the construct while still not degrading the enzymatic stability.

It was determined that a charge ratio of 3:2 polyanion:polycation was sufficient to induce a mechanically stable precipitate while preserving the same enzymatic stability. The pH at which the immobilization and complexation steps were completed also had an effect on

immobilized stability, although the effect was not great. Neutral or slightly alkaline pH during these steps was indicated as providing the best stability during incubation. It was determined that a certain time must be allotted for complexation of the enzyme and the polycation before precipitation if maximum stability is to be attained. Here, we used a complexation time of 21 hours. The parameter with the clearest effect of the resulting LOx stability and yield of the immobilization process was the concentration of the DEAE-D during complexation. Higher concentrations were associated with higher stability and yields; however, this affect disappeared at the concentrations higher than 24% w/w, which were obtained by evaporation during complexing.

This chapter, in part, is a reprint of the material as it appears in the following:

- Strobl AL and Gough DA. Stability of Lactate oxidase (LOx) immobilized in a polyionic matrix. *In preparation*.

The dissertation author was the primary investigator and co-author of this paper.

V Simulations of sensor response using the parameters measured

5.1 Introduction

We have attempted to maximize the stability of the enzyme lactate oxidase in immobilized constructs suitable for use in implantable biosensors. In order to predict the response and lifetime on an implantable sensor, the parameters that have been found in the lab can be used in a model of the functioning sensor system. This model is constructed in much the same way as the assay model developed in chapter 2. We employ the FEM program COMSOL Multiphysics to define a sensor geometry, create a mesh, and solve the system of reaction and diffusion equations numerically. Because we do not know the ultimate design constraints of the final sensor (ie, maximum acceptable time-lag, required linear response range), this modeling exercise is only intended to show general trends for the sensor designs, given the stability parameters found.

5.2 Methods and Assumptions

The model is geometrically more complicated than the simple 1-D reaction diffusion model that was sufficient to determine the assay response. The basic design of the sensor is that proposed by Jablecki for a tissue-based glucose sensor (Jablecki M, 2002). This design is built in COMSOL as an axisymmetric 2-D model (ie, the geometry is created in 2-D with a central axis around which the model is rotated). The basic sensor design is shown in figure 5.1. This design has a cylindrical active enzyme region sitting atop of a circular platinum electrode. Surrounding the active region is a PDMS surround (highly O_2-permeable, but impermeable to lactate), with only a small aperture for the substrate lactate to enter. This design both increases the effective σ-value of the sensor (causing it to operate under diffusion-control for a longer

period of time), and allows for a reduced, controlled influx of lactate. This design is successful in helping to counter the effect of the small amount of GOx inactivation due to the substrate glucose in glucose sensors and so would have the same effect for a lactate sensor, in the event that lactate does become inactivating over the long-term (it does not appear to be inactivating over the short-term, as discussed in section 2.4.3.1).

To define the model of the implanted sensor system, we have made several assumptions regarding the nature of the tissue boundary layer and the entire sensor array that would be employed. The center-to-center distance between active electrodes is assumed to be 2mm; therefore, we the model "box" is constructed as having a total radius of 1mm (note that this geometry is not completely accurate to sensor array design, but is sufficient for our purposes). O_2 and lactate then diffuse into the system from a boundary at the top, with the far edge of the sensor (away from the active enzyme region) having a no-flux boundary condition. The tissue boundary layer is set to 400μm in our model; this is sufficient to drive O_2 flux levels at the Pt electrode surface to values seen *in vivo* in a porcine animal model, after a 2-week tissue acclimatization time (Gough et al, 2010). We then assume that lactate and O_2 originate at this same boundary as a constant concentration source. The electrode acts as a sink for O_2, with a boundary value concentration of 0.

In addition to the constants listed in Table 2.1, the diffusion constants of lactate and O_2 must be defined for the tissue boundary layer, and the diffusion constant of O_2 must be defined for the PDMS surround. Tissue values are assumed to be close to values found in PBS. These values are the following: $D_{O2,\ Tissue} = 2.3*10^{-9}\ m^2/s$ (Gough DA and Leypoldt JK, 1980), $D_{l,\ Tissue} = 1*10^{-9}\ m^2/s$ (estimated), and $D_{O2,\ PDMS} = 3.2*10^{-9}\ m^2/s$ (Makale MT et al, 2004)

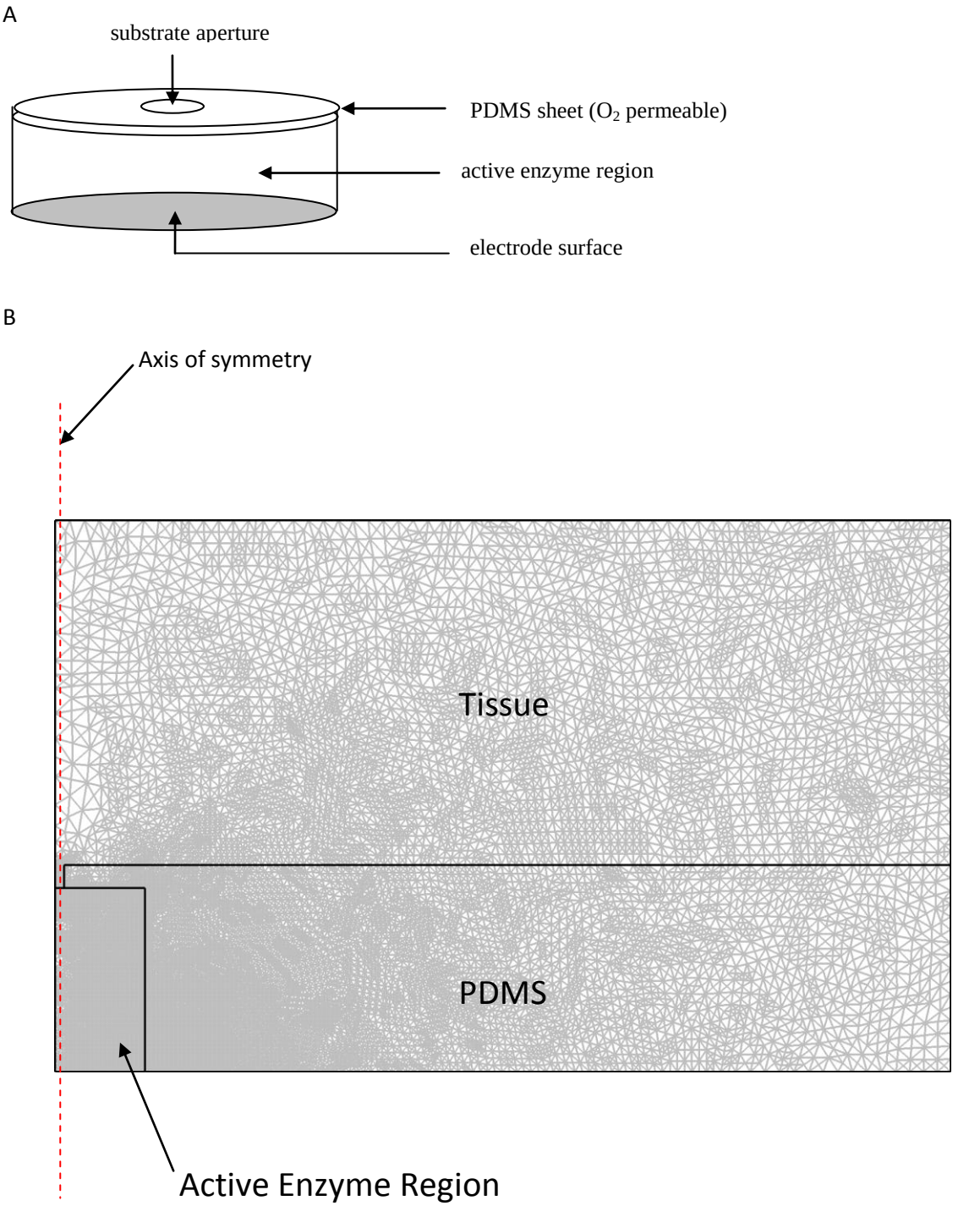


Figure 5.1: Design of the sensor model as built in COMSOL Multiphysics.
(a) A simplified representation of the 3-D design. Note that the PDMS extends on each side of the active enzyme region as well. (b) A labeled version of the axisysmmetric model as built in COMSOL, with meshing shown. Note that mesh is very dense in the active region, especially around the edge of the electrode and the substrate aperture

This constant concentration source is employed in order to study the steady-state sensor response over time only. We do not attempt to quantify time-lag under conditions of varying lactate levels, nor do we attempt to account for short-term variations in O_2 levels. A large short-term reduction in the amount of O_2 available to the sensor would cause a reduction in the linear range of the sensor. However, since there is always an O_2 sensor functioning without the active enzyme layer (in order to provide the base O_2 current which is used to calculate the difference current), this would be a known quantity. Sensor response is recorded with time as a function of the decreasing active enzyme concentration (equation 1.10). The actual value recorded and displayed in the figures is the O_2 different current normalized by the O_2 current in the absence of enzyme:

$$\frac{i_{O_2} - i}{i_{O_2}} \tag{5.1}$$

where "i" is the current generated in the presence of the active enzyme layer. The current is calculated by allowing COMSOL to integrate the O_2 flux over the surface of the electrode and multiplying by Faraday's constant and n = 4 (the number of electrons liberated when O_2 is reduced), as per equation 1.2. The mesh for the FEM solver was generated using an adaptive mesh system built into COMSOL. The mesh result was evaluated by comparing the mass balance according to the integrated flux of O_2 entering and leaving the entire system (ie, the mass balance).

Parameters for equation 1.10 are taken from the results section of chapter 3. The inactivation parameter τ is that from the pH 5.5 manufacturing condition trial at 37°C, rounded to 500 hours. The loading used is the highest loading that was indirectly measured in section 3.3.2.2.3, adjusted to be in terms of units of activity per unit volume by using an averaged

density (found my measuring the thickness of the hydrated membranes) and is rounded to $4.5*10^7$ U/m^3.

5.3 Results and Discussion

As stated in section 1.5, the effect of the substrate aperture is muted at thicker active enzyme layers. The value of the expression $\delta^2\xi^2$ in equation 1.6 is shown in figure 5.2 as δ is varied. We can see that if a very fast sensor is required (ie, a very thin active layer), then the substrate aperture is a highly effective method for extending the lifetime of the sensor. However, at larger thicknesses (some of which are analyzed here for sensor response with time), this effect becomes much less important, although an approximately 9% increase in σ is given even at a thickness of 400 µm.

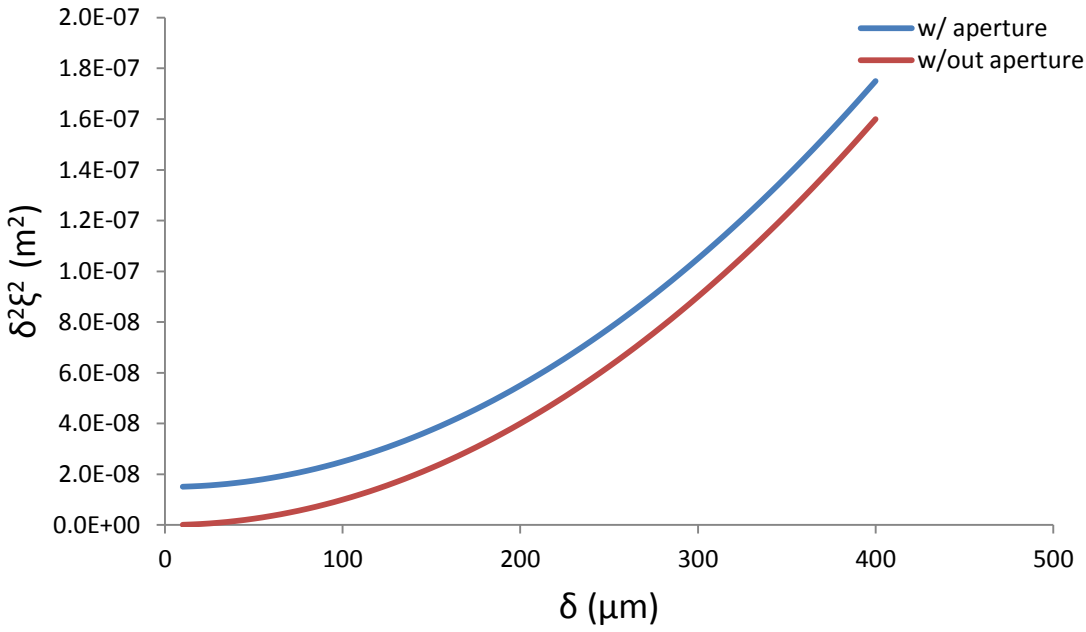


Figure 5.2: The value of the quantity $\delta^2\xi^2$ plotted against δ for a small substrate aperture sensor design on a 100 µm radius electrode.
It is easily seen that the substrate aperture increases the amount of diffusion control in the sensor very effectively at low active area thicknesses, but it is less important at higher thicknesses.

Model sensors with the dimensions described in section 5.2 and with varying active enzyme layer thicknesses were evaluated. The calculated linear range of the 200 μm-thick active layer model sensor is shown in figure 5.3; this range changes with time as shown. It is seen that the linear response range of the sensor is never quite linear. This is due to more of the reaction taking place closer to the electrode at higher substrate concentrations. Reactions taking place closer to the electrode surface will have a larger effect on the flux of O_2 at the surface per unit of substrate oxidized.

In addition, the response of the sensors to specific substrate concentrations with time is shown (figure 5.4). It can be seen that the sensor signal gradually increases with time; this is due to the same effect posited above. After reaching its maximum value, at which point the substrate concentration is the upper limit of the linear range, the signal begins to decrease (the linear range is now below the substrate concentration being traced). Note the large variation in the perceived lifespan of the sensor with different substrate concentrations. If a linear response to only 2.5 mM is desired, the lifespan of the sensor becomes approximately 33 days in a 100 μm thick sensor, 65 days in a 200 μm thick sensor, and 90 days in a 400 μm thick sensor.

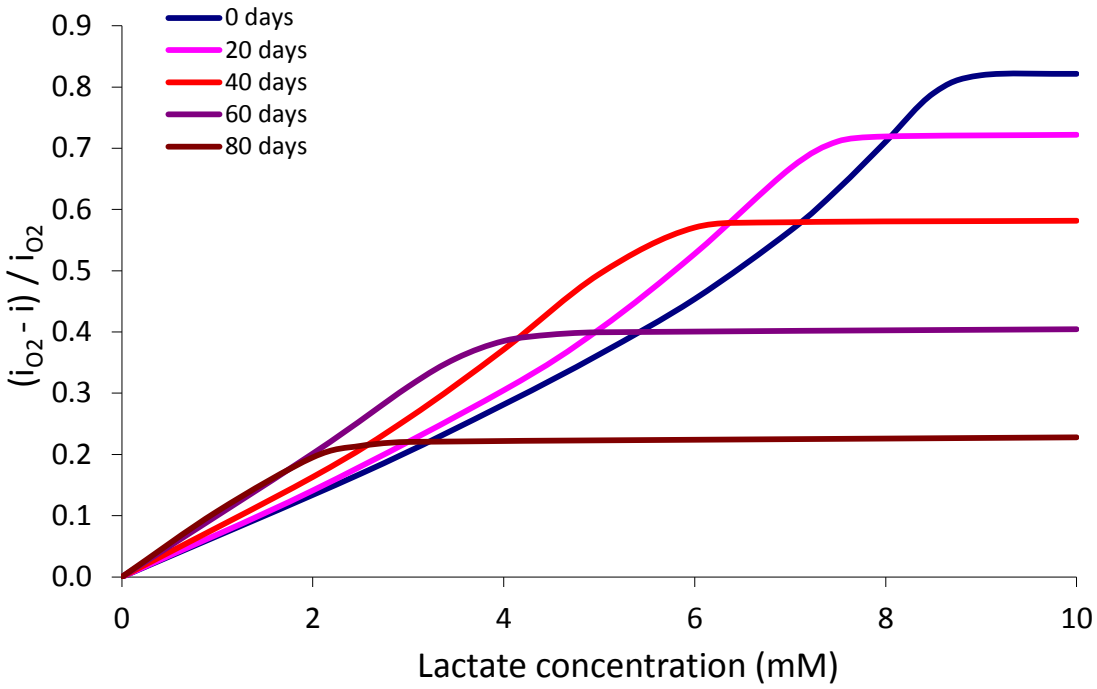


Figure 5.3: The linear range of a sensor with δ_m = 200 µm varied with time in use
As the active enzyme is reduced over time, the linear range decreases in a predictable manner.

A

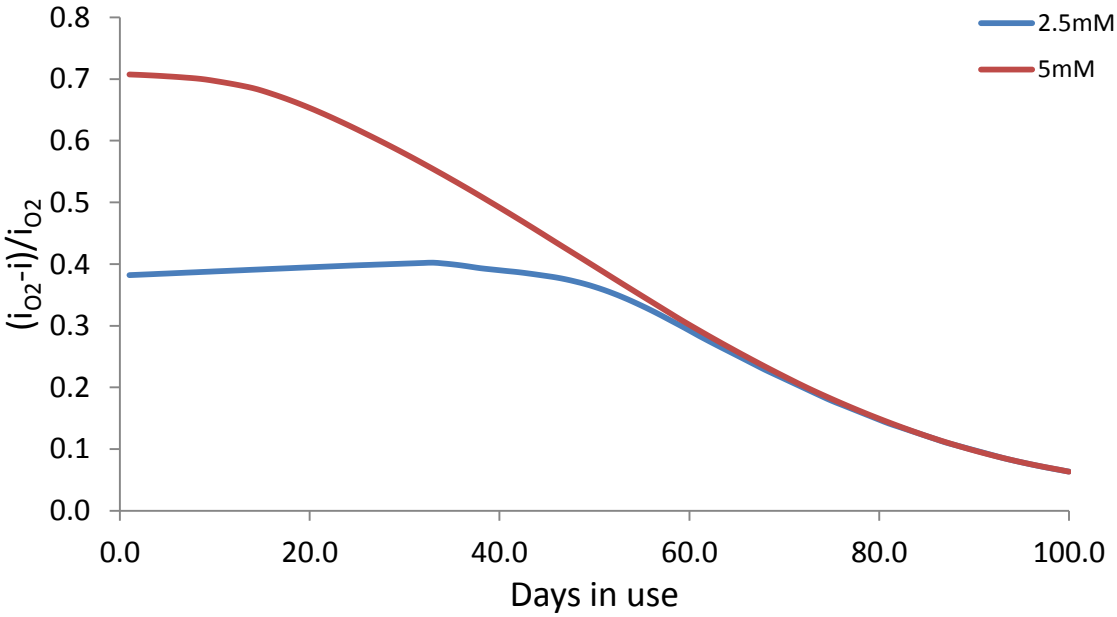
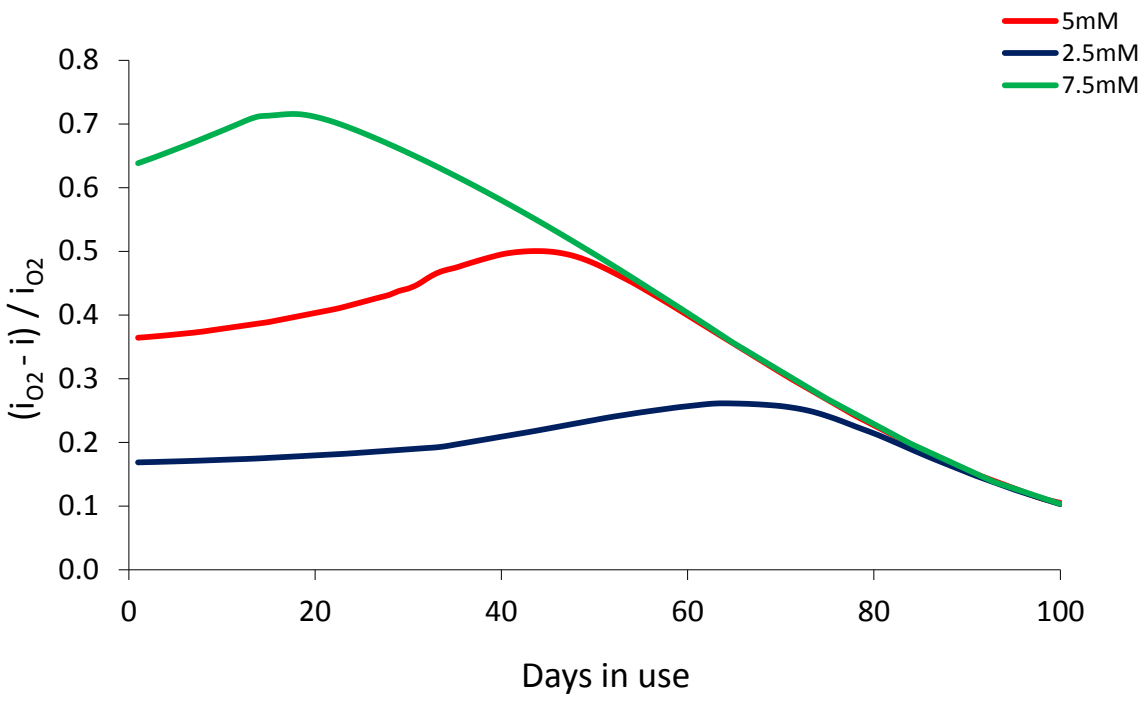


Figure 5.4: The response of modeled sensors over time to given substrate concentrations. Sensors are different in the thickness of the active enzyme layer. (a) 100µm thickness; (b) 200µm thickness; (c) 400µm thickness.

B



C

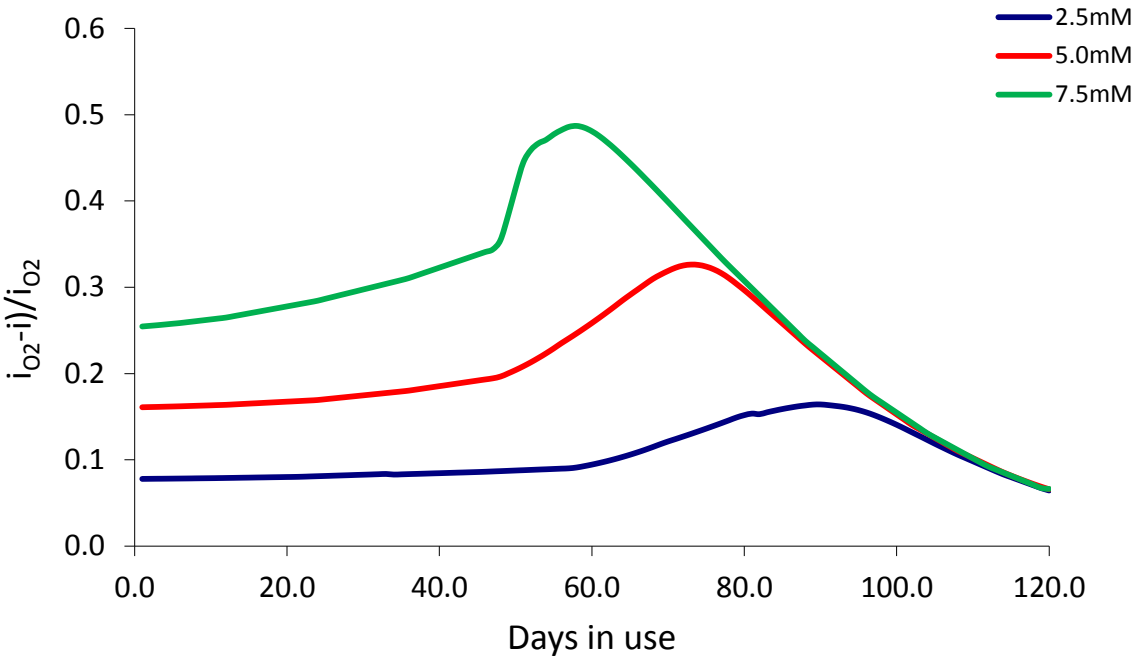


Figure 5.4: The response of modeled sensors over time to given substrate concentrations, continued.

5.4 Conclusions

FEM modeling of the proposed sensor systems gives some insight into the expected lifespan of the sensor in various applications. Depending on requirements for the speed of sensor response and the clinical range of interest, different designs may be employed. The results given here are for the inactivation rate constant found in the lab, along with the initial loading value that we were able to support in the experiments. However, it is quite possible that much higher loading levels may be achieved. Note that for every doubling of the LOx active loading, we would expect an increase in sensor lifetime of approximately 2 weeks (one half-life). Since the maximal loading we attempted to quantify was only approximately 6 mg of LOx protein per g BSA, we could potentially have four doublings and still have the matrix at only approximately 10% enzyme. This would lengthen the lifespan of the 200 µm sensor from 65 days to 121 days.

A larger delay may be tolerable, as in the 400 µm sensor, especially since the thickness of the tissue boundary layer assumed is large in comparison to the thickness of the thinner sensor models (ie, in the thinner models, most response delay would be attributable to tissue effects). Sensor design clearly must be optimized for the individual task for which it will be used.

This chapter, in part, is a reprint of the material as it appears in the following:

- Strobl AL and Gough DA. Lactate oxidase (LOx) immobilized in a natural polymer carrier: parameter effects on stability in a physiologically relevant range. *In Preparation*.

The dissertation author was the primary investigator and co-author of this paper.

VI Conclusions and Future Directions

6.1 Conclusions

A long-term amperometric lactate sensor would be very useful in the treatment of many patient populations, as well as in the field of sports medicine. Currently, the lifetime of these sensors is limited by the stability of the enzyme, lactate oxidase, used to select for the substrate. Previous sensors built on an oxygen-electrode system have been shown to have an *in vivo* lifetime in excess of one week (Baker DA and Gough DA, 1995), which would be sufficient for acute care patients, but not for chronic care patients such as those with diabetes mellitus. Therefore, it is necessary to study LOx in immobilized constructs that would be suitable for use in such a sensor. This work has presented an analysis of LOx in two very different immobilized systems. The analysis was completed by use of an assay system that was controlled for the diffusional resistance presented by the immobilized systems. The assay system was also validated in regards to chemical interactions that could affect final stability measurements.

6.1.1 Chemically cross-linked matrices

One immobilized system that was studied utilized a covalent immobilization scheme. Here we used glutaraldehyde to cross-link LOx within a natural polymer matrix. Chemical immobilization via glutaraldehyde has been used in many immobilized enzyme systems, both for biosensor (Lillis B et al, 2000; Baker DA and Gough DA, 1995) and bioreactor (Mehaia MA and Cheryan M, 1990) systems. It has been used quite successfully to stabilize the enzyme glucose oxidase (GOx) for use in continuous glucose monitoring applications (Tse PHS and Gough DA, 1987; Gough DA et al, 2010). Systems investigated here were based on either collagen or bovine serum albumin as a carrier protein. It was found that using collagen as a carrier had

several downsides, including the inability to create a mechanically stable membrane for the duration of stability testing without inactivating LOx during the cross-linking process. We recommend using BSA as the carrier moiety, since a relatively high yield upon immobilization can be obtained while providing a mechanically robust membrane. A 2-step immobilization procedure was developed in order to precisely control the amount of glutaraldehyde delivered to the reaction system.

Experiments on the system focused on the evaluation of processing parameter effects on the yield and stability parameters of the system. Amount of glutaraldehyde addition was first optimized for active enzyme yield (while still providing for the aforementioned mechanical rigidity) by varying the amounts of glutaraldehyde added to the system. Constructs were tested in a stability protocol to ensure that a greater level of cross-linking did not produce increased stability even if yield were sacrificed. It did not (in fact, stability was slightly lower), which indicated that the parameters influencing amount of cross-linking had been optimized. We attempted to influence the manner of glutaraldehyde attack and cross-linking through manipulation of parameters during the immobilization. It was found that immobilizing LOx with BSA at a pH of approximately 5.5 led to a nearly 3-fold increase of the stability parameter at 37°C. We hypothesize that this relative stabilization may be an effect of bringing the pH closer to the isoelectric point of both LOx and BSA, thereby allowing the proteins to become closer associated. Additionally, different amino acid residues will be exposed to attack at different pH values; this affects how the enzyme is cross-linked in the matrix.

LOx stability was studied both in a physiologically relevant temperature range (37°C, 39°C and 41°C) and at highly accelerated conditions of 50°C. Here we found that the stability conferred on the cross-linked enzyme was not constant across all temperatures studied.

However, the ratio of the inactivation constants of the immobilized enzyme to the native enzyme free in solution (DoS) remained at least 1 for all temperatures studied. Also, the enzyme did not become highly unstable within the 37 - 41°C range, indicating that brief temperature shocks due to illness or environmental conditions would not inactivate an *in vivo* sensor prematurely. The inconsistency of the DoS conferred across temperatures, however, indicates that enzymatic activity stability studies should be conducted at the temperature of the intended application. For example, the stabilizing effect of immobilizing LOx with BSA at pH 5.5 would have been entirely overlooked had the system only been studied at the more convenient temperature of 50°C. This inconsistency can be explained by the presence of multiple mechanisms of LOx inactivation. Certain mechanisms may become the primary cause of inactivation at different temperatures in native and immobilized forms.

6.1.2 Polyionic matrices

The second of the systems studied utilized an ionic immobilization method. The components of this immobilized matrix are a linear polycation complexed with a linear polyanion. Once these 2 components are mixed in the proper ratio, a precipitate is formed that can be used to immobilize LOx. The polycation chosen was diethylaminoethyldextran (DEAE-D), and the polyanion was poly(4-styrenesulfonate) (PSS). The literature indicates that complexation of enzymes (especially those with a charged substrate) with an oppositely charged polymer can affect stability in an immobilized system (Gibson TD et al, 1995; Heller J and Heller A, 1998). This seems to be a necessary step when the enzyme is in direct contact with a charged system such as a carbon paste electrode (Gavalas VG and Chaniotakis NA, 2000) or a sol-gel matrix (Chen Q et al, 1998). This type of immobilized system was investigated in order to

determine its effectiveness as an immobilized enzyme layer that could be used in an implanted biosensor measuring physiological levels of lactate.

LOx was first studied in the individual components of the matrix. We determined that while the polyanion was highly detrimental to LOx activity (the effect increasing with increasing concentration), the polycation was much less detrimental, and in fact greater stability was observed at higher concentrations. This led us to believe that LOx could complex with DEAE-D at high concentrations allowing for the protective effect reported in the literature above. Knowing that high concentrations of PSS were detrimental to enzymatic activity, the amount of PSS used to effect immobilization was kept to a minimum while allowing for good mechanical stability of the immobilized construct. Processing parameters were varied in the construction of the membranes and the resulting stability parameters were measured.

pH was again evaluated as a possibility for changing the way the enzyme was immobilized and hence the final stability of LOx. Here we determined that it was appropriate to produce the complexed enzyme matrix at a neutral or slightly alkaline pH. However, the effect of manufacturing pH on stability was not large. The most important determinate of LOx stability in the immobilized construct was the potential for complexation with DEAE-D via time and concentration. Some minimum time for incubation of DEAE-D and LOx together before PSS addition was necessary to increase stability. Use of higher concentrations of DEAE-D during this complexation was associated with higher stability as well.

6.1.3 Recommendations and applicability of the research

For purposes of creating the enzyme layer in a long-term *in vivo* lactate sensor, we recommend using a BSA-carrier glutaraldehyde cross-linked matrix. This matrix, when cross-

linked at pH 5.5, leads to an immobilized enzyme with a nearly 3-fold gain in stability. This is done while preserving an acceptable active enzyme yield upon immobilization. The matrix can be formed in any geometrical shape based on the required design of a lactate sensor. In modeling simulations, a design with a 200 µm thick enzyme layer and a small substrate aperture is shown to last approximately 65 days when a linear response to 2.5 mM is required. However, this simulation uses only the highest loading we attempted to measure in the lab; for each doubling of this loading, we expect an increase in lifetime of approximately 2 weeks (irrespective of the linear range required). An enzyme loading of 10% wt/wt would give a sensor lifetime of approximately 121 days with this model. Also, the enzyme layer can be thickened if a time-lag induced by the sensor comparable to the tissue time-lag can be tolerated, extending lifetime to 146 days in a 400 µm thick design with an enzyme loading of approximately 10%. Whatever the final design chosen for a functioning *in vivo* lactate sensor, the lifetime is increased by 3-fold due to the gains in LOx stability achieved by this work.

6.2 Future directions

If this 3-fold gain in stability found in our studies prove to be less than is required for use of a lactate sensor in the intended application, there are several lines of research that could potentially help to extend the lifetime of LOx, as well as other enzymes. We have focused here on a processing approach to lengthening the lifetime of LOx, while still maximizing the available activity after the immobilization reaction. There are other materials that could be considered as an immobilization carrier for LOx, as well as other homo- and heterobifunctional reagents that could be used as cross-linkers. Some of these cross-linkers are much more specific than

glutaraldehyde; this would allow targeted experiments to determine which functional groups could be utilized to possibly extend the lifetime of LOx.

A relatively complete treatment of possible conjugation techniques and cross-linkers is given in *Bioconjugate Techniques* (Hermanson GT, 2008). A protocol could be developed whereby a carrier molecule is conjugated to specific functional groups on the enzyme. That carrier could then be further cross-linked to other carrier molecules in order to form an immobilized membrane that could be hydrated, much like the BSA-carrier membranes described in chapters 2 and 3. The protocol would proceed thusly: (1) A heterobifunctional agent is conjugated to the enzyme. The other functional group of the agent is chosen not to react with the enzyme. (2) A carrier molecule is functionalized such that it will react with the free end of the heterobifunctional agent at many sites. (3) The functionalized carrier and the enzyme are allowed to react. (4) A homobifunctional agent reacting with left-over functionalized groups on the carrier molecules is added to cross-link the mixture to a solid structure that can be hydrated. Several appropriate functional groups on LOx could be targeted, including hydroxyl and amine groups. Parameters that could be optimized include the molar ratios of the conjugation agents and the length of the cross-bridges.

In addition, there are a number of hydrogel materials that could be conjugated to LOx via the same cross-linking agents. These materials would have good substrate transport and could be set as hydrogels by a number of methods, including small changes in temperature, pH or ionic strength (Galaev IY and Mattiasson B, 1999). The number of possibilities for using different materials and cross-linkers is astounding; however, these all may run into the same limits of stability imposed by the basic structure of the active LOx tetramer. In order to extract a more stable conformation, it may be necessary to change the primary structure of the molecule.

There are several ways in which analysis of possible changes to the primary structure of an enzyme can be accomplished. The crystal structure of LOx has been recently published (Leiros, I et al, 2006); therefore, it is possible to analyze the structure for sites which may be weak points for stability. An analysis of these potential weak points may lead to a variety of amino acid substitutions. In addition, powerful modeling tools can be used to look quantitatively at forces among the different amino acid interactions, allowing for a more robust analysis (Korkegian A et al, 2005). Rational design methods employed by Kaneko et al led to an LOx molecule mutated at a residue intended to improve interactions among the subunits with higher stability at 60°C to 70°C. The same group also has employed a random mutagenesis procedure, whereby mutations are created and then screened for stability at accelerated temperatures (Minagawa H et al, 1998). A procedure whereby the mutations were completed multiple times produced a yet more thermostable version of LOx (Minagawa H and Kaneko H, 2000). Because of the high temperatures used, it is currently unknown if these mutants would be able to produce a high DoS over the wild-type enzyme at physiological conditions.

These types of thermostable mutant enzymes could be immobilized and tested at *in vivo* conditions in order to determine if high temperature thermostability here translates into high stability at physiological conditions. In addition to immobilization at the best-case conditions determined in this dissertation, the enzyme could be tested for pH stability, and immobilization could potentially be conducted closer to the isoelectric points of LOx and BSA without extreme loss in yield. In short, there are still many unexplored avenues both for various immobilization procedures and the use of mutated enzymes. Long-term stability testing of LOx is relatively unexplored, even in the variety of sensor constructs that have been created. Use of thermostable mutant LOx enzymes with the chemical immobilization methods discussed in this

dissertation may provide for the best research avenue going forward, if a more stable LOx construct is required for the given sensing application.

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