

# **Evaluating bacterial cell immobilization matrices for use in a biosensor**

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### **Abstract**

A biosensor is proposed that contains bacteria that naturally effluxes potassium ions when threatened by electrophilic species. *Pseudomonas aeruginosa* is an activated sludge isolate and possesses the characteristic potassium efflux response. It has been immobilized in calcium alginate beads, photopolymer disks, and a thermally reversible gel in order to ultimately incorporate the immobilized system into a functional biosensor. The potassium efflux and cell viability were measured in the immobilized matrices.

Wastewater treatment is of utmost importance; however, processes are easily upset. Upsets can be caused by various electrophiles found in the environment, and can cause serious health effects to people or the environment downstream from an upset. Electrophiles can cause the activated sludge in wastewater treatment facilities to deflocculate, and untreated water can be lost downstream. Devising a detection system for proactively sensing electrophiles prior to an upset is an important complementary goal.

Immobilization systems have been evaluated including photopolymer coated alginate beads and sol gel coated alginate beads. The thermally reversible gel, NIPA-co-AAc (N-isopropylacrylamide-co-acrylic acid), shows promise as an immobilization matrix for the bacteria; however its high lower critical solution temperature (LCST) of ~33°C is problematic for typical, ambient applications. Another thermally reversible copolymer, N-isopropylacrylamide-co-N-acryloyl-6-amino caproic acid (NIPA-co-AcACA) was synthesized; however, it did not form a continuous matrix; making it

useless as an immobilization scheme for biosensors. Alginate beads fall apart easily in bacteria media, but are structurally stable in potassium solutions. Cells immobilized in alginate beads seemed to efflux four times less potassium than did planktonic controls, while cells in thermally reversible gels effluxed a comparable amount of potassium as planktonic controls. This result may indicate a tighter matrix around the alginate immobilized cells, not allowing proper diffusion of potassium out of the matrix.

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## Table of Contents

Chapter 1: Literature Review	
Introduction.....	1
Biological element.....	5
Immobilization matrices.....	9
Alginates.....	10
Photopolymers.....	12
Sol gels.....	13
Thermally reversible gels.....	15
Experimental objectives.....	16
References.....	17
Chapter 2: Evaluating immobilization matrices for capturing <i>P. aeruginosa</i>	
Abstract.....	23
Introduction.....	23
Experimental.....	25
Results and Discussion.....	31
Conclusions.....	35
References.....	37
Chapter 3: The compositional dependence of lower critical solution temperature (LCST) and gel structure of N-isopropylacrylamide (NIPA) based thermally reversible copolymer gels	
Abstract.....	47
Introduction.....	48
Experimental.....	50
Results.....	52
Discussion.....	52
Conclusions.....	53
Acknowledgements.....	53
References.....	54
Additional information.....	56
Chapter 4: Future Work.....	57
References.....	67

## **List of Tables**

### **Chapter: 1**

Table 1 Some biosensors used in pollution determination.....	2
--	---

### **Chapter: 2**

Table 1 Efflux potential from immobilized cells.....	46
--	----

### **Chapter: 3**

Table 1 Results table of the NIPA-co-AAc copolymers in the DSC.....	55
---	----

## List of Figures

### Chapter 1

Figure 1	General schematic of a biosensor.....	4
Figure 2	Schematic of a stressed cell and the potassium efflux response.....	8
Figure 3	Schematic of deflocculation.....	8
Figure 4	General schematic of a desirable immobilization matrix.....	10
Figure 5	G and M blocks of the alginate structure (Top), and the illustration of alginate chains forming ionically crosslinked structures with the addition of calcium ions (Bottom).....	11
Figure 6	Schematic of a thermal gel transition.....	15

### Chapter 2

Figure 1	Conceptual picture of alginate bead stability using the texture analyzer. The bead was placed on a table under a force probe at time 0 (a), the bead was under force until rupture (b), and the force probe hit the table (c).....	39
Figure 2	The photopolymerization of poly(ethylene glycol) diacrylate with Irgacure 2959 as the initiator (Top). The size of the resulting photopolymer disks next to a quarter (Bottom).....	40
Figure 3	Output spectrum of a blacklight used for photopolymer curing.....	41
Figure 4	Synthesis of the thermally reversible gel NIPA-co-AAc.....	41
Figure 5	Alginate beads tested under compression after incubation in solutions containing 0.6, 0.3, and no potassium in DI water. Each test consisted of at least three samples and showed no degradation of alginate beads over the two week time period.....	42
Figure 6	HPLC chromatograph of the cured photopolymer disks supernatant after suspension in water. The peak is indicative of unpolymerized monomer in solution with the cured photopolymer disk. Curing times longer than 8 minutes show full cure of the sample. HPLC data was collected for 25 minutes with no other peaks showing in that time.....	43
Figure 7	NMR spectra of the 98 mole% NIPA-co-AAc polymer. The spectrum was integrated over the entire range. The peak at 6.2 ppm shows the OH and NH groups from the AAc and NIPA, respectively. The peak at 4 ppm is due to the single CH resonance in the NIPA and was designated a value of 1. All other peaks were normalized to it. The group of peaks on the right was from all of the rest of the hydrogen in the sample and its integrated intensity is slightly larger than 9. Because there are 9 hydrogen atoms unaccounted for in the NIPA, the 0.05 is considered to be from acrylic acid. There are three hydrogen atoms in the acrylic acid making the AAc composition considered 2 mole%.....	44
Figure 8	Water retention in NIPA copolymers (95 mole% top, 98 mole% bottom) as detected by DSC.....	45
Figure 9	LIVE/DEAD <sup>®</sup> stain of alginate (top left) right after immobilization, thermal gel (top right) after ten days of immobilization, and photopolymer (bottom) right after immobilization.....	46

### **Chapter 3**

Figure 1 DSC of 98 mole% NIPA copolymer in water.....	55
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## Chapter 1: Literature review

### Introduction

Biosensors are self-sufficient devices utilizing biological elements in conjunction with a transducer that converts biological expressions into a useful signal. Biosensor classification can be grouped by either biological element such as whole cell, DNA, enzymatic, or antibiotic, or by transducer such as optical, electrochemical, or thermal to name a few (Rodriguez-Mozaz *et al.*, 2005). In recent years, biosensors used for environmental monitoring have become increasingly prevalent as summarized in Table 1. Of particular interest in the environment are pollutants contaminating waterways, drinking, and wastewater. The last of which is very important, since a process upset occurring at a wastewater treatment facility could cause serious problems down stream, including impact on our waterways and drinking water. Some pollutants disrupting our natural environments include pesticides containing chlorinated compounds, heavy metals, and other electrophiles.

Electrophiles can be found in landfills from discarded paints and resins, in industrial waste from disinfectants, preservatives, or paper manufacturing, or in pesticides, to name a few (Solod 1998). These types of electrophiles are toxic to fish, wildlife, and are potentially hazardous to humans. Wastewater treatment facilities are particularly susceptible to these toxins as the electrophiles have the potential to deflocculate activated sludge used in processing wastewater (Bott and Love 2002).

**Table 1 Some biosensors used in pollution determination**

<b>analyte</b>	<b>environment</b>	<b>biological element</b>	<b>transducer</b>	<b>reference</b>
pesticides and estrone	river water	antibodies	optical	Rodriguez-Mozaz <i>et al.</i> , (2004), Mallat <i>et al.</i> , (2001), Mallat <i>et al.</i> , (1999)
phenols	wastewater	enzymes	electrochemical	Nistor <i>et al.</i> , (2002)
linear alkyl benzene sulphonate (LAS)	river water	bacteria	electrochemical	Nomura <i>et al.</i> , (1998)
toxicity	wastewater	bacteria	electrochemical	Farre <i>et al.</i> , (2001), Philp <i>et al.</i> , (2003)
alkanes	groundwater	bacteria	optical	Sticher <i>et al.</i> , (1997)
estrogens and xenoestrogens	lake water and wastewater	estrogen receptor (EC)	optical	Seifert <i>et al.</i> , (1999)
biological oxygen demand (BOD)	river water	bacteria	optical	Chee <i>et al.</i> , (2000)
zinc dichromate chromate	soil	bacteria	optical	Ivaska <i>et al.</i> , (2002)
mercury and arsenite	soil	bacteria	optical	Petanen and Romantschuk (2002)
<i>chlamydia trachomatis</i> (DNA)	river water	DNA	electrochemical	Marrazza <i>et al.</i> , (1999)

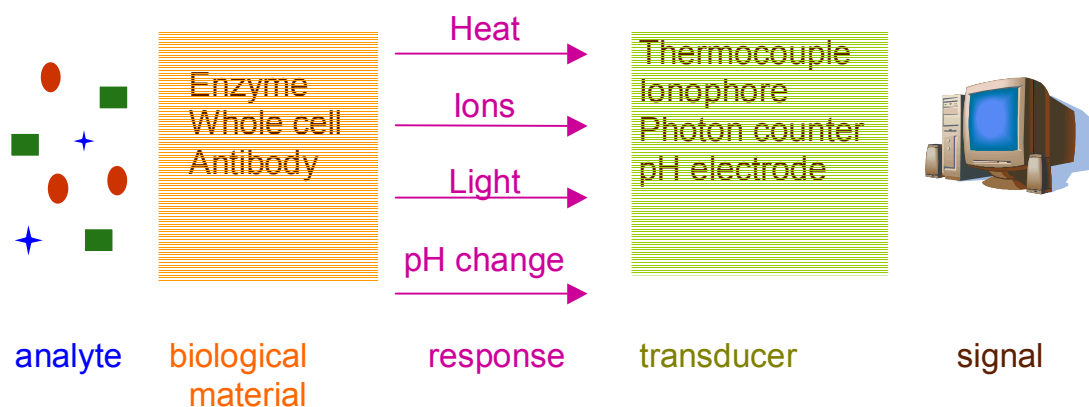
One commercially available biosensor is sold under the trade name MICREDOX and is manufactured by Lincoln Technology. Its main function is to determine

biochemical (or biological) oxygen demand (BOD) in samples; however, it has been recently used to test for biotoxicity as well (Tizzard *et al.*, 2004). BOD is a test whose results are indicative of the relative amounts of oxygen required by aerobic microorganisms to decompose organic matter in a sample, such as in wastewater (Liu *et al.*, 2004).

Activated sludge used in wastewater treatment facilities consist of various types of bacteria prevalent in the environment that ingest and metabolize sewage. The sludge breaks down the waste, and in turn, grows and multiplies. The bacteria are cultured and form larger colonies known as flocs. The flocs are easily separated from the residual treated sewage when the bacteria and sewage (or mixed liquor) are conveyed to the clarifier. Because the flocs occupy a larger area than individual cells, they quickly fall to the bottom of the clarifier. If the bacteria have deflocculated, they will not be easily settled out of the mixed liquor, and could be sent along in the process with the purified aqueous stream. The deflocculation of the activated sludge could result in a complete shut down of the wastewater treatment facility and loss of untreated water downstream (Love 2003).

Deflocculation events, called upsets, can cause treatment capabilities to be compromised for months, which also translate into large monetary fines. The wastewater industry not only affects people's water and environment, but also their pockets. Wastewater facility operations, capital, and general maintenance expenditures are an astounding \$30 billion in the U.S.A. (EPA 2002). This figure continues to increase as most of these electrophiles are not biodegradable, making their threat to the environment long term.

Potential episodes are difficult to predict and traditional sampling techniques are time consuming and can get quite expensive (Belkin 2003). An online, continuous monitoring sensor would allow treatment facilities to take early action against harmful electrophiles before an upset event occurs. Biosensors are emerging as very useful in this capacity as they are easy to use, low cost, and respond quickly to pollutants (Horsburg *et al.*, 2002). A general schematic of a biosensor is shown in Figure 1.



**Figure 1 General schematic of a biosensor.**

In order for biosensors to be considered as real time monitoring semi-permanent devices, they need to be rugged, incorporate a large number of cells to ensure a large signal, and operate remotely with little maintenance required (Baeumner 2003). Cellular immobilization is the key to these complex design and integration problems.

Immobilization schemes need to be tailored for geometry, structural stability in the field, biological stability of the cells, chemical stability in the presence of harsh wastewater contaminants, and diffuse rapidly enough to allow for short analysis times. Immobilization schemes must also co-localize the biological element with the transducer to ensure proper signal throughput. Common immobilization methods are physical adsorption to a surface, covalent binding to a surface, and entrapment (Collings *et al.*, 1997). Natural immobilization hydrogels typically have poor structural stability and

synthetic gels often require toxic chemicals or processing conditions for immobilization (Philip *et al.*, 2003). The goal of this work is to compare the performance of hydrogels, structurally reinforced natural hydrogels, and other synthetic gels for use as bacterial immobilization matrices for biosensors.

### **Biological Element**

The biological elements used in biosensors are the most important part of the biosensor. The choice of biological element used in each biosensor depends on the analytes being monitored, the storage capacity of the element, and the environmental and operational stability of the element. The sensors need to be sensitive to small quantities of analyte and reliable. Portability is desirable as well. Biological elements include enzymes, DNA, antibodies, whole cells, dead cells, and receptors, to name a few (D'Souza 2001). Whole cell biosensing is also very useful in detecting general upsets (Belkin 2003, Paitan *et al.*, 2003).

Viable whole cells are particularly valuable as they utilize respiratory and metabolic functions to sense analytes. They also have the capability of demonstrating the potential effects of certain analytes to living organisms (Bousse 1996). Cell expression can be positive as in the case of cell growth or mitosis. Adverse expressions can also be observed as in the case of protective responses or even death. Microorganisms are also able to easily adapt to their environment and metabolize a wide range of chemicals (D'Souza 2001). Microbial cells are also low cost, have large population sizes, grow rapidly, and require little maintenance (Dennison 1995).

When dealing with electrophiles, there are several classes that need to be tested for when using conventional methods to monitor wastewater. A different analytical method for each electrophilic chemical must be used on each sample of water taken in order to determine whether each electrophile was indeed present. With a microbial biosensor, the microbes sense all electrophiles, making them a more general, proactive sensing device, capable of early warning against process upset downstream.

Often, the whole cells used for environmental pollution monitoring have been genetically modified bacterial strains. The genetic modification entails making certain bacteria express a luminescence gene that allows the cells to possess a non-native fluorescence when exposed to certain analytes (Keane *et al.*, 2002). This scheme may be useful, but the genetic modification of these cells is tedious, expensive, and risky. There is a threat that these genetically modified organisms could be released inadvertently into the environment, causing regulatory concerns. These cells are often immobilized using the natural bacterial ability to form biofilms. While this is an effective immobilization in certain instances, the bacteria used in wastewater treatment facilities are susceptible to turbulent waters, causing the biofilms to slough off and be lost downstream. The analytes being screened are capable of deflocculating biofilms, causing mobilization of previously immobilized cells.

*P. aeruginosa* is a Gram negative bacterial strain that is present in activated sludge in most wastewater treatment facilities in America (Snaider *et al.*, 1997). Because of this, it is an ideal candidate for use in a sensor as it has already shown its ability to thrive in the wastewater environment. Most Gram negative bacterial cells have a glutathione (cysteine tripeptide) buffer system that allows them to modulate the

reduction/oxidation (red ox) changes triggered by internal or external stimuli within their cells (Filomeni *et al.*, 2002). Oxidative stressors, such as electrophiles, trigger this response.

Glutathione (N-(N-L- $\gamma$ -Glutamyl-L-cysteinyl)glycine) is present within the cell as a reduced sulfhydryl (GSH) and as two different oxidized translations. GSH is used as a first responder to the attack. Once the electrophilic analytes enter the cell, the GSH is first to react with the stressors by conjugating with the electrophiles, protecting other intracellular molecules from attack. This reaction causes a  $K^+$  to efflux out of the cell. In order to retain charge balance within the cell,  $H^+$  is transported into the cell through an antiport, causing the intracellular matrix to acidify. The added acidity further protects other sensitive molecules within the cell by initiating conformational structure changes, making them less susceptible to prolonged attack by electrophiles, as shown in Figure 2 (Love and Bott 2002). The  $K^+$  release is thought to be the cause of deflocculation in activated sludge present in wastewater treatment facilities (Bott and Love 2004). Activated sludge bacteria typically have a negative charge surrounding them, and bind together to form flocs with the presence of divalent cations. Monovalent cations do not have the same effect. The  $K^+$  efflux produces a monovalent positive charge environment surrounding the cell. If all cells in the floc have this same positive charge surrounding them, the cells will repel from each other. This results in deflocculation and ultimately process upset, as shown in Figure 3. Glutathione is produced intracellularly, and only prolonged oxidative stresses will deplete the supply, eventually causing cell degeneration and death (Filomeni *et al.*, 2002).  $K^+$  is effluxed quantitatively, making attack by

electrophiles easily spotted in a biosensor by monitoring  $K^+$  content directly downstream of immobilized bacteria.

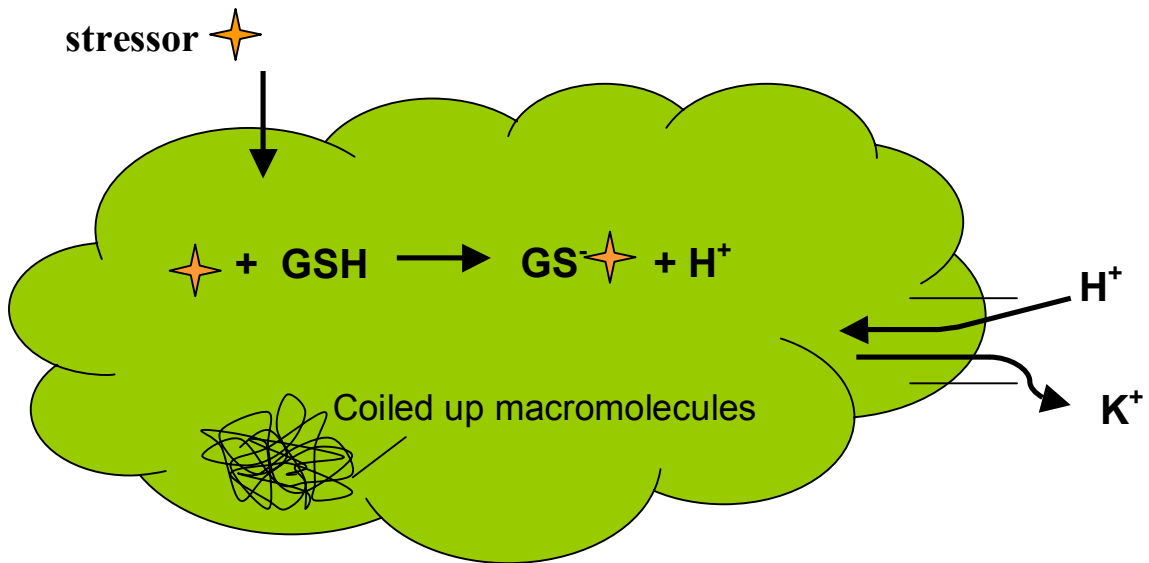


Figure 2 Schematic of a stressed cell and the potassium efflux response.

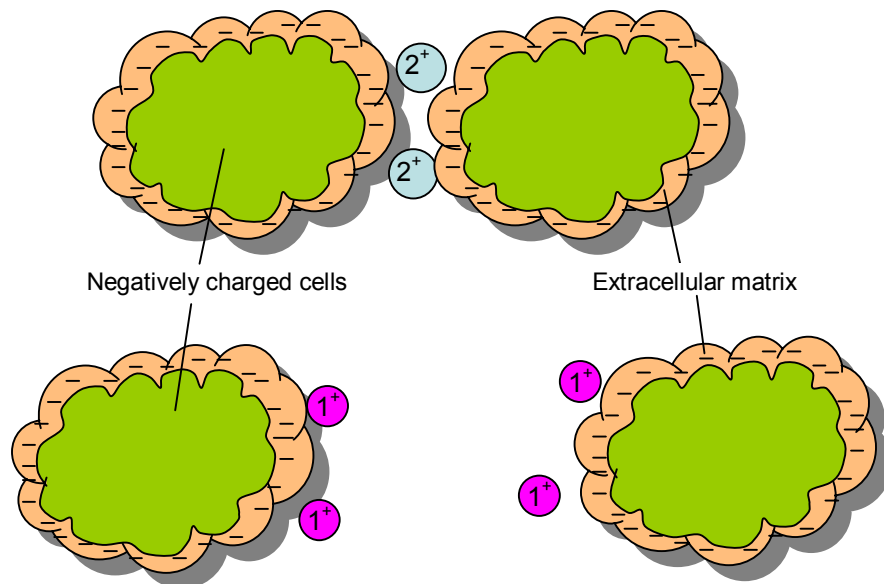


Figure 3 Schematic of deflocculation



## **Immobilization Matrices**

With the biological element chosen, developing a benign immobilization medium is the next step. Immobilization matrices must prevent the bacteria from dislodging from the matrix and flowing downstream, yet still enable trafficking with the environment and signal transduction (Premkumar *et al.*, 2002). An ideal immobilization matrix would be functional at ambient temperatures, survive harsh wastewater conditions including contaminated water and turbidity, and allow the flow of nutrients and oxygen and analytes through the matrix along with wastes and signal out. It would also prevent cell flow within the matrix. A simple schematic of a desirable immobilization matrix is shown in Figure 4.

There are several types of immobilization matrices used for whole cells studied today. These include systems that produce hydrogels as a response to different triggers. These triggers include ions, heat, light, or other chemicals that would also act as electrophiles. When designing the immobilization matrices, the activation of the immobilization scheme must be carefully considered. For instance, excessive heat during immobilization processing could kill bacteria, as could exposure to certain chemicals. Ultraviolet light can cause intracellular mutations or death. Any of these could cause premature bacterial stress without any electrophiles being present in the environment, giving false positive signals in the biosensor. Hydrogels are particularly useful due to their high water content, pliability, biocompatibility, and easily controlled diffusion characteristics (Elisseeff *et al.*, 2000, Li 1998, Uludag *et al.*, 2000). Some common hydrogels are those found in contact lenses and include poly(ethylene glycol, poly(vinyl

alcohol), poly(methacrylic acid), and poly 2-hydroxyethylmethacrylate (Smeds and Grinstaff 2001).

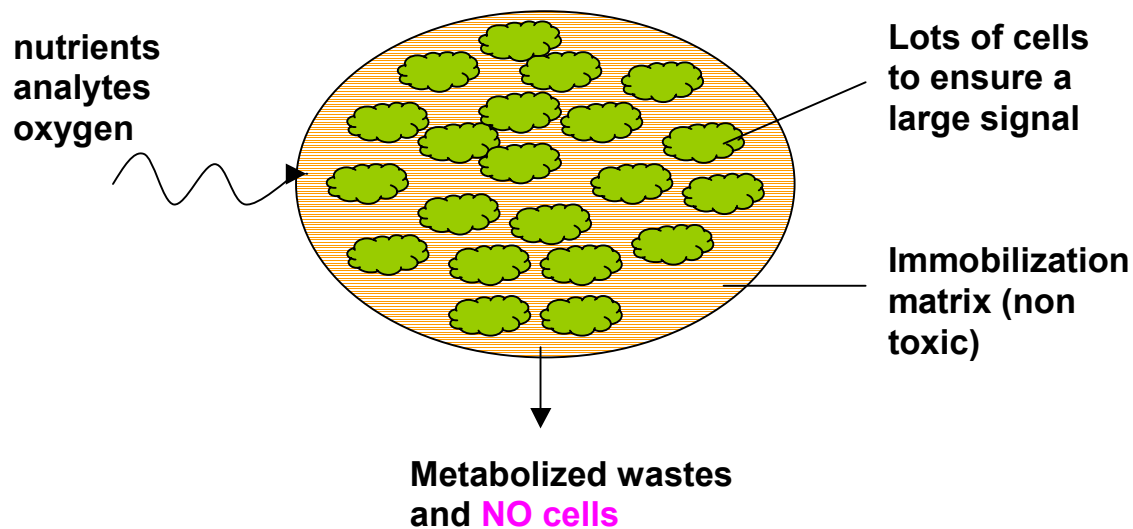


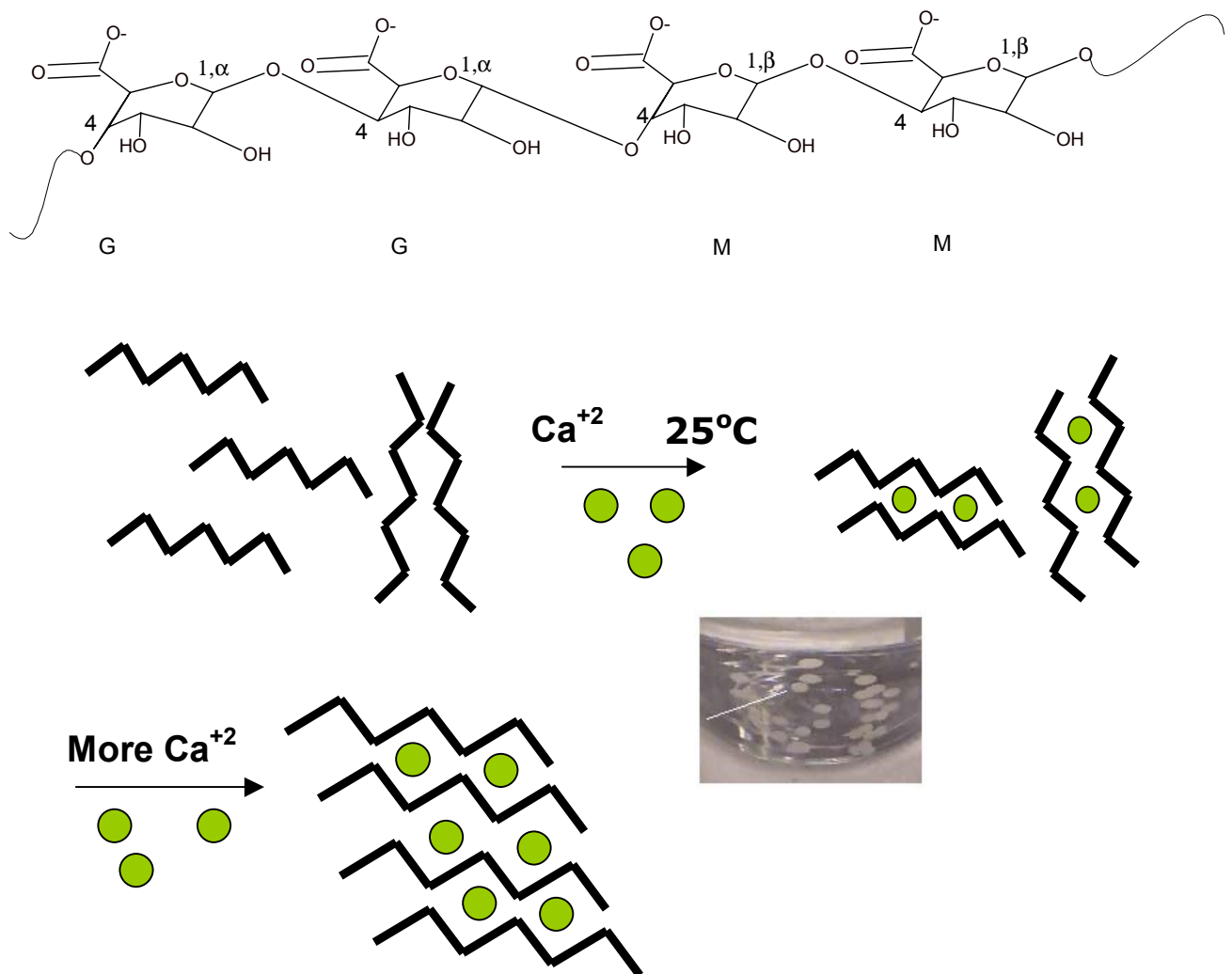
Figure 4 General schematic of a desirable immobilization matrix.

### Alginates

Common immobilization matrices include naturally occurring alginates. Alginates are produced by some strains of seaweed and are a variety of polysaccharide. Alginates are formed by converting mannuronic and guluronic acid into their salt forms of mannuronate (M) and guluronate (G). They are copolymers consisting of (1-4) linked  $\beta$ -D-mannuronic acid and  $\alpha$ -L-guluronic acid (Smidsrod *et al.*, 1990). Alginates are linear polymers comprised of blocks of M and G, or alternating GM blocks. The alginate used in this project is from Norway and is from the leaf of the seaweed *Laminaria hyperborea*. It is comprised of 60-75% of the G constituent.

Alginates are often used in the food industry as gelling compounds, such as in the production of the meat-like chunks found in pet food. Alginates are ionically crosslinked between the carboxylic acid elements through divalent ions like  $\text{Ca}^{+2}$ , as illustrated in Figure 5. Because their crosslinks are ionic as opposed to covalent, they are easily

broken apart by cationic scavengers such as sodium citrate and chelators such as ethylene diamine tetra acetic acid (EDTA) (Smeds and Grinstaff 2001, Lu *et al.*, 2000). In addition to the weak bonding structure, natural hydrogels are also susceptible to biodegradation, making their use somewhat limited depending upon the cell type being immobilized.



**Figure 5** G and M blocks of the alginate structure (Top), and the illustration of alginate chains forming ionically crosslinked structures with the addition of calcium ions (Bottom).

## Photopolymers

Another commonly used class of immobilization matrices is photopolymers. Most photopolymers utilize visible or ultraviolet light to crosslink the monomers used in the formation of the matrices. Some photopolymers utilize harsh chemical initiators to facilitate the polymerization. A photon from the light source breaks the photoinitiator into groups of highly energized radicals. The radicals then react with the resident monomer in solution and initiate the usually unstable thermoset polymerization (Bryant *et al.*, 2000).

Photopolymers used as immobilization matrices are often in the form of micropatches formed by photolithographic techniques that are typically 50-500  $\mu\text{m}$  in critical dimension. The smaller microstructures result in a capture of 1-3 cells per structure (Koh *et al.*, 2002). The result is a structure that can support cells for long periods; however, the small size does not allow the capture of many cells, lowering signal response (Zhan *et al.*, 2002 and Heo *et al.*, 2003). Another drawback to the photolithographic technique is a loss of cells. When the cells and photopolymer solution are placed on a treated substrate, a mask is placed over the substrate and a light shone through the mask. Only the areas of the solution that are exposed to the light source become gelled. This implies that any solution on the substrate that has not been gelled will be washed away.

A photoinitiator known to be non-toxic to mammalian cells is 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone. It is sold by Ciba Specialty Chemical Company under the alias Irgacure 2959 (Williams *et al.*, 2004). Due to its low toxicity levels, Irgacure 2959 was used in the experiments found in later chapters as the

photoinitiator in the polymerization of poly(ethylene glycol) diacrylate. Irgacure 2959 is an ultraviolet light activated photoinitiator, but it is sensitive to visible light. This sensitivity makes it a good candidate for curing in the violet spectral region of the electromagnetic spectrum. Curing times are typically longer when using the lower frequencies, but they are less detrimental to the overall well being of the cells being encapsulated.

A photopolymer was also used in the following chapters as a structural reinforcement of alginate beads by coating the beads with the photopolymer prior to polymerization. A similar method has been demonstrated to increase strength of alginate immobilization matrices by more than twice the alginate innate strength (Lu *et al.*, 2000).

### **Sol gels**

Sol gels are hybrid organic-inorganic compounds that are a bridge between glasses and polymers (Livage 1997). Sol gels are rigid, thermally and structurally stable, and transparent, making them particularly useful for the immobilization of cells that are luminescent or show other visible changes when sensing the environment. These techniques are called ‘chimie douce’ and are based on solution chemistry (Fennouh *et al.*, 1999). The reactivity of organic and inorganic species is typically quite different and can result in phase separation between the two constituents in the hybrid matrices. Because of this, bonds must be made between the inorganic and organic components of the hybrid. There are two classes of hybrids, one exhibiting weak, van der Waals and hydrogen bonds, and the other exhibiting strong covalent bonds. Using the chimie douce

technique, metastable, yet kinetically stable phases can be formed, and covalently bonded, strong matrices can be achieved (Livage 1997).

Silica gels are typically made by the hydrolysis and condensation of silicon alkoxides that produces alcohol, a cell killer (Nassif *et al.*, 2002). Recently, aqueous silica gels formed at room temperature have been found to effectively immobilize viable bacterial cells (Brinker and Scherrer 1990, Yu *et al.*, 2004). The aqueous silica provides a non-toxic and biologically inert environment that shows an increase in overall cell viability when formed in the presence of glycerol (Nassif 2003). The benign process occurs in steps as follows:

$\equiv\text{Si-OR} + \text{H}_2\text{O} \Rightarrow \equiv\text{Si-OH} + \text{ROH}$  (hydrolysis producing a soluble, hydroxylated monomer)

$\equiv\text{Si-OH} + \text{RO-Si}\equiv \Rightarrow \equiv\text{Si-O-Si}\equiv + \text{ROH}$  (polymerization and alcohol condensation)

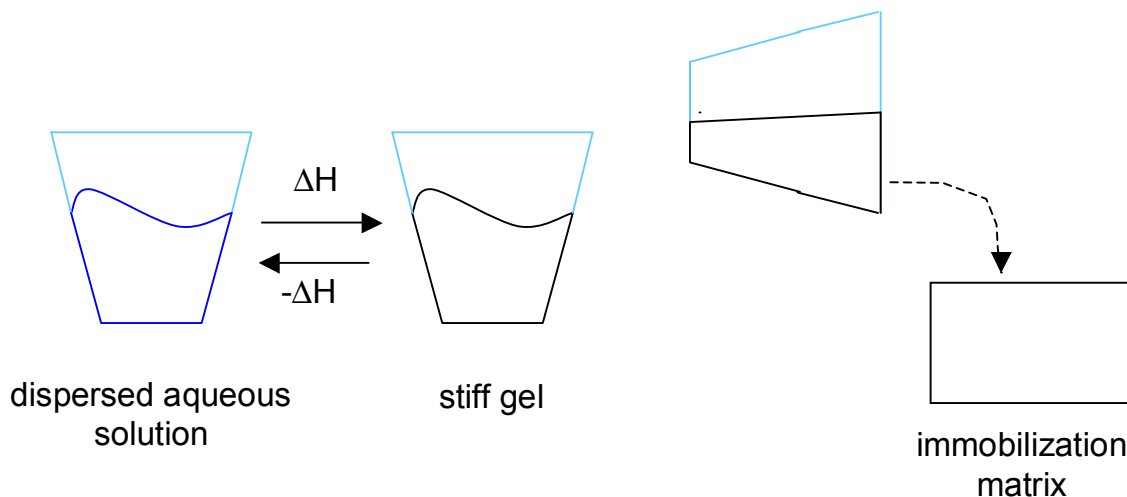
$\equiv\text{Si-OH} + \text{HO-Si}\equiv \Rightarrow \equiv\text{Si-O-Si}\equiv + \text{H}_2\text{O}$  (water condensation)

where R is an alkyl group such as methyl or ethyl. Water can then be removed to by drying to produce the stiff gel (Chen and Lin 2003). In this study, sol gels were used as coatings over alginate beads as reviewed by Coradin *et al.*, 2003 and performed by Kuncova *et al.*, 2002.

Cell immobilization is simple when using alginate beads and reinforcing them in the sol gel. The immobilization step occurs during alginate gelation, and the sol gel is formed over the bead by mixing the beads in a prepolymerized sol gel solution, and drying the prepolymer in order to form the sol gel.

## Thermally reversible gels

Thermally reversible gels, while most commonly used in drug delivery systems, are quickly being viewed as a potential matrix for immobilization. In aqueous solutions, thermally reversible gels undergo volume-phase transitions about a certain temperature as shown in Figure 6 (Sun *et al.*, 2003). They form collapsed, dehydrated, hydrophobic gels above a lower critical solution temperature (LCST) and swollen, hydrated, hydrophilic dispersed solutions below the LCST. Cells can be easily immobilized by mixing cells with the aqueous thermally reversible gel solution at low temperatures. When the solution temperature is raised above its LCST, the cells will be immobilized within the hydrogel matrix.



**Figure 6 Schematic of thermal gel transition.**

The most common thermally reversible gel is the poly(N-isopropylacrylamide) (PNIPA) gels. PNIPA is comprised of hydrophilic amide and hydrophobic isopropyl groups in its side chains (Xue and Hamley 2002). The change in phase of the hydrogel is due to a disruption of the hydrophilic and hydrophobic balance within the structure causing a macroscopic phase transition (Sanchez *et al.*, 2004, Xue *et al.*, 2004).

The gel can also transform during other environmental changes such as pH, electric field, or ionic strength (Gehrke and Cussler 1989, Ohmine and Tanaka 1982, and Tanaka *et al.*, 1982). PNIPA is often copolymerized with other hydrophilic or hydrophobic monomers in order to modulate the LCST. The most commonly studied comonomer is acrylic acid (AAc) (Xue *et al.*, 2001, Xue *et al.*, 2001, Huglin *et al.*, 1997, Velada *et al.*, 1998). Another reason to copolymerize PNIPA with another monomer is to increase water absorbency in the hydrogel. This option raises the LCST and causes the overall thermal sensitivity to decrease, making the compositional design a trade off between maintaining a lower LCST and raising water absorbency (Ni and Zhu 2004).

### **Experimental Objectives**

The overall objectives of this research were:

- Make immobilization matrices with commercially available products as well as through polymer synthesis
- Quantify, compare, and observe the immobilization matrices response to different environmental conditions (i.e. heat, light, turbulence)
- Immobilize *Pseudomonas aeruginosa* in the matrices and test dynamic cell viability.

The specific results and methods used to meet these objectives will be discussed within the following chapters of the thesis.



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## Chapter 2: Evaluating immobilization matrices for capturing *P. aeruginosa*

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### Abstract

*P. aeruginosa* has been immobilized in calcium alginate beads, photopolymer disks, and a thermally reversible gel in order to ultimately incorporate the immobilized system into a functional biosensor. The bacteria responds to electrophiles with a glutathione-gated K<sup>+</sup> efflux and this expression, along with the bacterial viability, were measured in the immobilized state after dosing. Other immobilization systems have also been evaluated including photopolymer coated alginate beads and sol gel coated alginate beads. The thermally reversible gel, NIPA-co-AAc (N-isopropylacrylamide-co-acrylic acid), shows much promise as an immobilization matrix for the bacteria; however its high lower critical solution temperature (LCST) of ~33°C is more problematic for typical, ambient measurements due to increased hardware requirements.

**Keywords:** alginate, photopolymer, immobilization matrix, thermally reversible gel

### Introduction

The proposed biosensors are designed to detect electrophiles in water supplies and are crucial to the well being of both the environment and the general public. Electrophiles cause oxidative stress in cells, initiating a stress response mechanism and potentially cell death. Electrophile threats include heavy metals and organic compounds containing chloro- and nitro- constituents. These types of threats can come from water

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run-off near mining sites, hazardous waste treatment facilities, other industrial facilities, and agricultural procedures.

In addition to directly affecting surface waters, toxic loading of electrophiles is one factor causing process upset in wastewater treatment plants. The most prevalent modes of process upset are ineffective biological oxygen demand (BOD) removal, deflocculation, ineffective nitrification, and sludge bulking, while the most common causes of these upsets are high flow, toxic organics, oil and grease, and heavy metals (Love and Bott 2000). Unfortunately, the cause of the upset is commonly not discovered until well after the event, if at all. Several of the causative factors can be grouped as electrophilic chemicals, such as the heavy metals and toxic organics listed earlier (Love and Bott 2000). Applying a biosensor to detect electrophilic threats to wastewater treatment plants and surface waters would allow better monitoring procedures, leading to improved water quality while reducing the potential of these threats affecting water supplies down stream through proactive sensing.

Several groups have developed biosensors for specific compounds by genetically modifying bacterial strains with reporter genes. For example, engineered strains have been developed and immobilized to create sensors that respond to phenol (Philp 2003; Gu 2002; Reshetilov 2002), polynuclear aromatic hydrocarbons (PAHs) (Semple 1998), and heavy metals (Virta 1998). The difficulty in commercializing a genetically modified organism limits its implementation, and the need for a general sensor for many types of toxins still exists.

The biosensor under development here employs the innate protective response of the bacterial cells *P. aeruginosa* as they efflux potassium when stressed by electrophiles.



When an electrophile permeates a bacterial cell, the reaction between the electrophile and the glutathione (N-(N-L- $\gamma$ -glutamy-L-cysteinyl)glycine) found in *P. aeruginosa* spontaneously activates the glutathione-gated potassium efflux (GGKE) mechanism (Love and Bott 2002). The cell then effluxes potassium out of the cell (Apontoweil and Berends 1975a and b). The GGKE results in an increase in hydrogen ions within the cells and expels potassium ions from inside the cells in order to maintain charge balance within the cell. The hydrogen ions acidify the cytoplasm, causing the DNA to supercoil, protecting it from oxidative damage (Ferguson *et al.*, 2000). This process is thought to be responsible for the deflocculation of activated sludge at wastewater treatment facilities (Bott and Love 2004). By harnessing the intrinsic cellular response and monitoring the potassium dynamics, the cells in this biosensor remain in the wild state, not engineered, and thus fewer risk-based controls exist for their use, commercialization, and eventual disposal.

The active component of the biosensor, the bacterial cells, will ultimately be incorporated into a microfluidic device. Determining the ideal matrices for bacterial immobilization is the major challenge in the development of the biosensor. The purpose of this research is to create an immobilization strategy for bacterial cells as a functional element in a biosensor for electrophilic compounds.

## **Experimental**

### **Bacteria concentration**

Five liters of *P. aeruginosa* were grown overnight to mid-log growth state. The cells were concentrated by centrifugation in multiple bottles for 20 minutes at 4420 x g and the supernatant was discarded. The cells were re-suspended in 20 mL of fresh media

and divided into aliquots containing an equal number of cells for the immobilization. After immobilization, all forms of immobilized bacteria were immediately placed in media consisting of  $\text{NaH}_2\text{PO}_4$  0.15g;  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  0.3g;  $\text{NH}_4\text{Cl}$  0.05g;  $\text{NaCl}$  0.025g, Bis Tris 1.0g;  $\text{MgSO}_4^{(2-)} \cdot 7\text{H}_2\text{O}$  0.246g;  $\text{CaCl}_2$  0.0147g;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  2.5mg;  $\text{ZnCl}_2$  0.25mg;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  0.185mg;  $\text{CuSO}_4$  0.030mg;  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$  0.006mg;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  0.001mg;  $\text{H}_3\text{BO}_3$  0.03mg; and 0.89mL glacial acetic acid per liter of media. All media supplies were used as received from Fisher Scientific (Pittsburgh, PA).

### **Alginate**

Alginate beads were formed by dissolving 2 wt% Protanal LF 10/60 alginate (FMC Biopolymer, Philadelphia, PA) in distilled (DI) water and dropping it from a 21.5 gauge syringe tip into a 10 wt% aqueous calcium chloride (Fisher Scientific, Pittsburgh, PA) solution. When immobilizing bacteria, the bacteria was added as 10 vol% to the alginate solution and stirred before adding drop wise into the  $\text{CaCl}_2$  solution. Approximately 20 ml total alginate solution was used for each batch of immobilized bacteria testing, producing ~900 beads with an average diameter between 2-3 mm.

### **Alginate Stability**

Alginate beads without cells were isolated for two weeks in DI water and solutions containing 0.3 and 0.6 mg potassium per 1.1 mL of DI water. These solutions represented the possible potassium efflux ranges based on the typical number of cells immobilized per bead. The beads in DI water containing no potassium were used as a control. This was done in order to determine the effect of potassium on the bead stability. Ten beads were used for each study. The beads were tested under compression on days 0, 1, 7, and 14 using a texture analyzer (TA-XT2i Texture Analyzer, Texture

Technologies Corporation, Scarsdale, NY). The test speed was 0.1 mm/s using a 5 kg load cell. Rupture was identified as the peak of the force curve before reaching the stage as shown in Figure 1.

### **Photopolymer**

Photopolymer disks were made by mixing polyethylene glycol (400) diacrylate SR 344 (Sartomer, Exton, PA) with 1 wt% 2-hydroxy-1-[4-(2-hydroxyethoxy)phenyl]-2-methyl-1-propanone Irgacure 2959 as initiator (Ciba, Tarrytown, NY) and adding ~22 wt% DI water. The solution was dropped from a pipet onto a polyethylene sheet and cured for 10 minutes using a standard black light found at any party store. Larger amounts of DI water in prepolymer solution resulted in an incurable solution. Resulting disks were approximately 0.5 cm in diameter and 0.1 cm in thickness, as shown in Figure 2. When immobilizing bacteria, the DI water was omitted from the solution, and concentrated bacteria were added in 22 wt% to the solution before dropping onto the sheet.

Curing times were determined using a high performance liquid chromatograph (HPLC). Disks were made and cured using the same light source for 8, 9, 10, and 11 minutes and placed in 1 ml water for approximately 10 minutes. The water surrounding the photopolymer was injected into an Agilent HPLC with water as the mobile phase and octadecyl (C18) as the stationary phase. The HPLC was equipped with a diode array detector (DAD) and variable wavelength detector (VWD) set at 254 and 450nm respectively. The stationary phase had 100 Å pore size with 400 m<sup>2</sup>/g surface area. The water surrounding the disks was injected in 100 µl volumes and pushed through the system at 1 ml/min.

When coating alginate with the photopolymer, the alginate beads formed using the process described in the previous section were added to the prepolymer not containing water or bacteria and gently shaken for 30 seconds. The solution was poured out onto the sheet so that all beads were in a single layer. The entire solution was cured using a commercial black light (Spencer Gift, 345-400 nm) with the spectrum similar to that shown in Figure 3. The beads were then isolated from the cured mass.

### **Sol gel**

The sol gel formation was a modification of the process done by Kuncova *et al.*, 2002. One mole methyltrimethoxysilane (Aldrich Chemical Co., Milwaukee, WI) was stirred with 3 moles of DI water and a drop of hydrochloric acid from a plastic, disposable pipet. The solution was left at room temperature over night to prepolymerize. The alginate beads formed above were washed in TRIZMA buffer (Aldrich Chemical Co., Milwaukee, WI), spread in a net, and dipped in the prepolymerized solution. The beads were dried in an oven at 50°C for one minute and the process repeated three times, using fresh solutions each time.

### **Thermally reversible gels (NIPA-co-AAc)**

All chemicals were purchased from Aldrich Chemical Co. in Milwaukee, WI and used as received, unless indicated otherwise. Chemicals included acrylic acid (AAc), N-isopropylacrylamide (NIPA), 2,2'-azobisisobutyronitrile (AIBN), acetone, benzene, ether, and 1,4-dioxane. Distilled water was also used.

Free radical solution polymerization was used to synthesize the AAc copolymers in a similar fashion to other published procedures (Au *et al.*, 2003) (Figure 4). NIPA was dissolved in 1,4-dioxanes and placed in a three neck, round bottom flask. AAc was

added in 2 mole% and the three neck flask was purged with nitrogen. AIBN (~0.2 mole%) was dissolved in benzene and placed into an addition funnel and added drop wise into the round bottom flask, purged with dry nitrogen. After AIBN was added, the addition funnel was replaced with a reflux condenser, and the entire apparatus lowered into a mineral oil bath at 70°C. The mixture was stirred with a stir bar and left for 18 hrs under purge. The mixture was then cooled to room temperature and dissolved in acetone. The dissolved polymer was precipitated into ether. The polymers were then dried in an oven at 100°C, prior to characterization. The copolymer was dissolved in 90 wt% DI water. Bacteria were added to the solution and the temperature was raised above its lower critical solution temperature (LCST) of 34°C.

All polymers were dissolved in deuterated chloroform and characterized using proton nuclear magnetic resonance (<sup>1</sup>HNMR) on a Varian Inova 400 instrument. A differential scanning calorimeter (DSC) (DSC7, Perkin Elmer) was also used to determine relative water adsorption content. Aqueous solutions of 10 wt% gel were used as samples. They were placed in the DSC and ramped up from 25°C to 115°C at 5°C per minute, and left at 115°C for 10 minutes in order to determine how much water was lost during heating.

### **LIVE/DEAD<sup>®</sup> staining**

LIVE/DEAD<sup>®</sup> staining was done as described in Linares *et al.*, 2004. A LIVE/DEAD<sup>®</sup> BacLight<sup>™</sup> Bacterial Viability Kit (Molecular Probes, Eugene, Oregon, USA) was used with a fluorescent microscope (Zeiss Axioscope 2 Plus, Thornwood, NY, USA) and camera system (Zeiss AxioCam MRm, Thornwood, NY, USA) to visually compare the numbers of live and dead cells at 20x and 40x magnification. The

LIVE/DEAD<sup>®</sup> stain uses propidium iodide, red coloring, to identify dead cells, and SYTO 9 dye, green stain, to identify live cells. The kit contents were mixed one to one and three  $\mu\text{L}$  of the mixture were added to one mL of cells. To stain all gels, an aliquot of the gel was placed in a micro centrifuge tube with one mL of water and stained with the same amount of dye. Alginate beads were dissolved in PA M9 media consisting of  $\text{NaH}_2\text{PO}_4$  3.0 g;  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  6.0 g;  $\text{NH}_4\text{Cl}$  1.0 g;  $\text{NaCl}$ , 0.50 g;  $\text{MgSO}_4^{2-} \cdot 7\text{H}_2\text{O}$  0.246 g;  $\text{CaCl}_2$  0.0147g;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  2.5 mg;  $\text{ZnCl}_2$  0.25 mg;  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  0.185 mg;  $\text{CuSO}_4$  0.030 mg;  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$  0.006 mg;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  0.001mg;  $\text{H}_3\text{BO}_3$  0.03 mg; glacial acetic acid 0.89 mL per liter solution before staining, and thermal gels were cooled below their LCST, making them liquid, before staining.

### **Potassium Efflux**

Potassium efflux experiments were done as described in Linares *et al.*, 2004b. After *P. aeruginosa* cells were concentrated as described above, the cells were either immobilized or re-suspended in fresh media (planktonic) and divided evenly in 100 mL amounts. The bacteria was placed in flasks, situated on stir plates and aerated using an aquarium pump to ensure oxygenation during the experiment. One milliliter pipet tips were placed on the end of the air tubing in each flask to reduce bubble size and to prevent contamination of the tubing.

Typical experiments included six flasks, three were shocked with 50 mg/L of N-ethyl maleimide (NEM), a known electrophilic toxin, and three control flasks remained un-shocked. Samples for potassium measurement were taken over a one-hour time period for planktonic experiments, and a two-hour time period for immobilized experiments by filtering the media through 0.2  $\mu\text{m}$  nitrocellulose MCE filters (25 mm

diameter, Fisher Scientific, Pittsburgh, PA). Samples were acidified with concentrated nitric acid and diluted one part to ten parts with nanopure water. One milliliter of diluted sample was removed and replaced with one mL of 12.7 g/L cesium chloride solution (Alfa Aesar, Ward Hill, MA) to lower sodium interference.

Potassium standards were prepared using a potassium reference solution (Fisher Scientific, Fairlawn, NJ) and the same concentration of cesium chloride was added to standards. The samples were analyzed on an Atomic Absorption Spectrometer (AA) (5100 PC Atomic Absorption Spectrometer, Perkin Elmer, Norwalk, CT). All glassware used in the potassium measurements was prepared by acid washing in 10% nitric acid, followed by triple rinsing with nanopure water. Five days after initial efflux experiments, cells were shocked with NEM again, and potassium efflux monitored in the same manner.

## **Results and Discussion**

Alginate beads containing cells proved to have little stability during experiments. The beads disintegrate during aeration and lose their integrity. By the end of experiments lasting more than five days, the alginate beads were completely degraded. Because the alginate matrix degraded after only five days of polymerization, experiments were conducted to determine if the potassium efflux from the cells caused the breakdown of the polymer structure. The alginate polymerizes by substitution of calcium for sodium within the matrix, and matrix stability was hypothesized to be affected by re-equilibration in sodium. Because of the alginate's fast degradation, it was thought that the potassium effluxed from the cells aided in the matrix degradation; however, the compression tests

performed on the beads in potassium solution over two weeks time showed no effect of the potassium on bead stability, as shown in Figure 5.

Photopolymers were considered cured when the water surrounding the photopolymer no longer contained any unpolymerized constituents. Figure 6 shows the chromatographs of the water solutions after curing for 8, 9, 10, and 11 minutes. The eight-minute cure shows that there is still monomer left in solution as indicated by the large peak present at 3 minutes. The peak is substantially smaller when the polymer was cured for 9 minutes, and does not seem to get significantly smaller when the cure time was extended to 10 or 11 minutes. Because of this, it was decided that the 10 minute cure was sufficient to complete polymerization.

Cured photopolymer disks were very rigid and stable over long periods, unless vigorously shaken. The disks were quite brittle, making them more susceptible to fracture. Combining the less stable alginate beads with the more rigid matrices of the photopolymer or sol gel was considered. Alginate beads coated with sol gel or photopolymer survive weeks without dissolution in concentrated ethylenediamine tetra acetate (EDTA) solution, which dissolves alginate quickly.

Thermal gels also remain structurally stable after weeks as long as the temperature remains above 34°C. As shown in Figure 7, the NMR spectrum showed that the structures desired were indeed synthesized. The spectrum was integrated over the entire range, and then peaks were picked. The peak at 6.2 ppm shows the OH and NH groups from the acrylic acid and N-isopropylacrylamide, respectively. The peak at 4 ppm was determined to be due to the single CH resonance in the NIPA. This integrated peak area was then designated a value of 1, and all other peaks were normalized to it. Since



NIPA is at least 98 mole% of the sample, the assumption is that an integrated relative value of 1 for that peak would be appropriate. If there was 50% NIPA in the sample, a value of 0.5 would have made more sense. The group of peaks on the right was from all of the rest of the hydrogen in the sample and its integrated intensity is slightly larger than 9. Because there are 9 hydrogen atoms unaccounted for in the NIPA, the 0.05 is considered to be from acrylic acid. Since there are three hydrogen atoms in the acrylic acid (not including the hydrogen attached in the OH group), the 0.05 is divided by 3, and this number (0.016666) is the relative amount of acrylic acid in the sample. The relative amount is then divided by the total sample ( $1+0.016666$ ) and the mole fraction of AAc is approximately 0.016. Due to experimental and analytical errors, this can be considered 2 mole%, which is what we expected based on the initial composition of the mixture.

Data obtained from the DSC regarding water retention was inconsistent. Three samples were run for each of the NIPA-co-AAc gels. The PNIPA and 90 mole% NIPA-co-AAc exhibited no transition or water loss up to 115°C. One of the three samples of the 95 and 98 mole% NIPA copolymers showed transitions, as shown in Figure 8. The 95 mole% NIPA copolymer showed transitions at 85°C and 107°C while 98 mole% NIPA had one transition appear at 98°C. The endotherms for the 95 mole% NIPA sample added together are approximately 8 J/mg, and the endotherm for the 98 mole% NIPA is much smaller at 2.6 J/g. This along with the fact that residual water in the 95 mole% NIPA sample came out over 100°C shows that the water content captured and effectiveness of capture is greater for the 95 mole% NIPA sample. This is expected to be due to the fact that the copolymer has a higher hydrophilic content, making it more likely to absorb more water than a sample with less hydrophilic content.

### **LIVE/DEAD<sup>®</sup> stain**

Sol gel and photopolymer coated beads have not yet been tested as immobilization matrices, so no data exist on *P. aeruginosa* LIVE/DEAD<sup>®</sup> stain due to lack of time. *P. aeruginosa* survived well in alginate matrices as well as in thermal gel matrices, as shown in Figure 9. The photopolymer does not dissolve and auto fluoresces in the green under the fluorescence microscope. Using the LIVE/DEAD<sup>®</sup> stain, it is impossible to differentiate live cells from the background. There is a large red area in the picture, thus it is apparent that the cells do not survive immobilization well in the matrix, as shown in the top right of Figure 9.

### **Potassium efflux**

Sol gel- and photopolymer- coated beads have not yet been tested as immobilization matrices, so no data exist on *P. aeruginosa* potassium efflux in these matrices. Given the results from the LIVE/DEAD<sup>®</sup> stain, further tests were conducted using only the alginate and thermal gel systems. The potassium efflux of alginate immobilized cells was at a maximum one hour after initial dosing. The maximum efflux potential of the immobilized cells was  $(1.3 \pm 0.1) \times 10^{-10}$  mg K<sup>+</sup> per cell, as shown in Table 1 (Linares *et al.*, 2004). This value is at least 4 times lower than the efflux from planktonic cultures, which may be a result of poor permeation through the matrix walls.

The efflux potential from the thermal polymer immobilized cells was higher than that from the alginate and comparable to the results for planktonic culture (Table 1). The efflux observed one day after immobilization was  $(4.6 \pm 0.8) \times 10^{-10}$  mg K<sup>+</sup>/cell, which is four times greater than that of the alginate immobilized cells. The efflux experiments performed on bacteria that were immobilized for five days showed a drastic decrease in

observed potassium efflux from alginate immobilized cells and efflux from thermal gel immobilized cells was not detected. This reduction in efflux could be attributed to the low potassium concentrations present in the media, which did not allow the cells to uptake the proper  $K^+$  concentrations needed to replenish their internal reservoirs.

## **Conclusions**

Three different immobilization matrices were made and evaluated for general stability, cell viability, and general functionality. The matrices were alginate beads, photopolymer disks, and thermal gels. The alginate beads were good immobilization matrices for short periods of time; however, during long periods, they disintegrated, releasing the cells. The potassium concentration in the aqueous solution surrounding the alginates following electrophilic shock exhibited four times less efflux than did planktonic controls, suggesting lower matrix permeation.

The photopolymer disks, while structurally stable under mild conditions, were brittle and easily fractured if shaken vigorously. Photopolymers also seemed rather lethal to the cells as observed by LIVE/DEAD<sup>®</sup> staining. This may have been due to the inherent toxicity of the prepolymerized solution, or an inability for oxygen and nutrients to permeate the matrix.

The thermal gels were stable over long periods (>10 days), as long as they remained above their LCST. Cells immobilized in the thermal gels showed potassium efflux potentials comparable to those of the planktonic controls, and LIVE/DEAD<sup>®</sup> staining indicated that the gels were non-toxic to the *P. aeruginosa*. An improvement to this immobilization matrix would be to have a thermally reversible gel matrix that had a lower LCST, allowing room temperature analysis and operation of experiments.

Two other hybrid matrices were also suggested, sol gel- coated alginate beads and photopolymer- coated alginate beads; however, no data have been gathered regarding their performance with cells. It should be noted that because the alginate immobilized cells produced a smaller potassium efflux potential, the coated alginates are already less sensitive than corresponding thermal gel immobilization matrices.

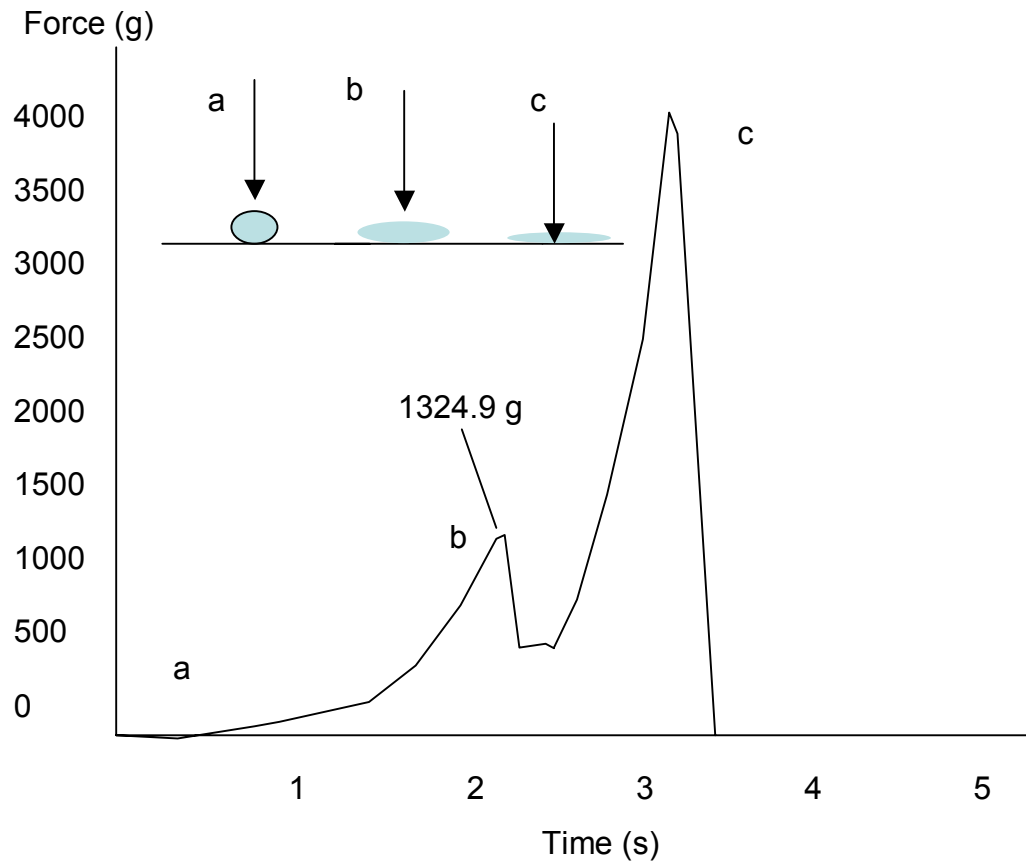
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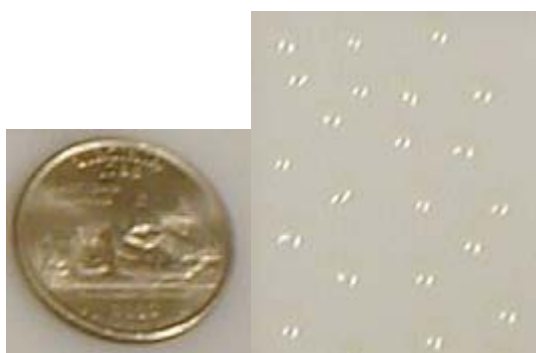
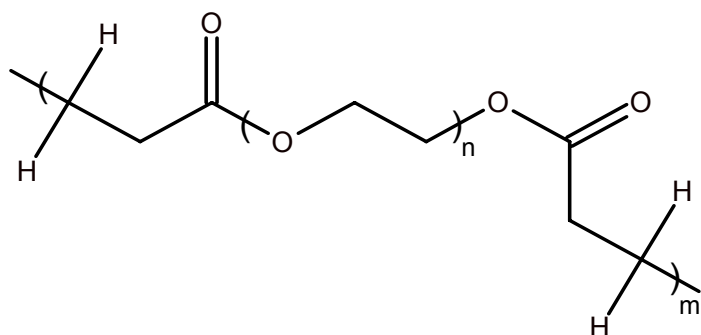
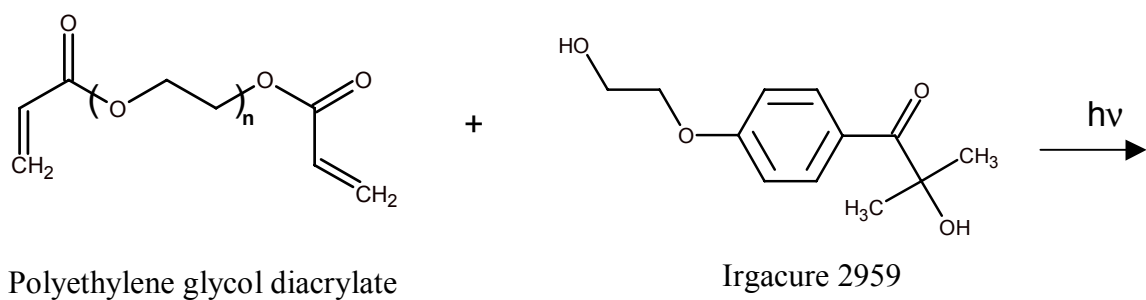
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**Figure 1** Conceptual picture of alginate bead stability using the texture analyzer. The bead was placed on a table under a force probe at time 0 (a), the bead was under force until rupture (b), and the force probe hit the table (c).



**Figure 2 The photopolymerization of poly(ethylene glycol) diacrylate with Irgacure 2959 as the initiator (Top). The size of the resulting photopolymer disks next to a quarter (Bottom).**



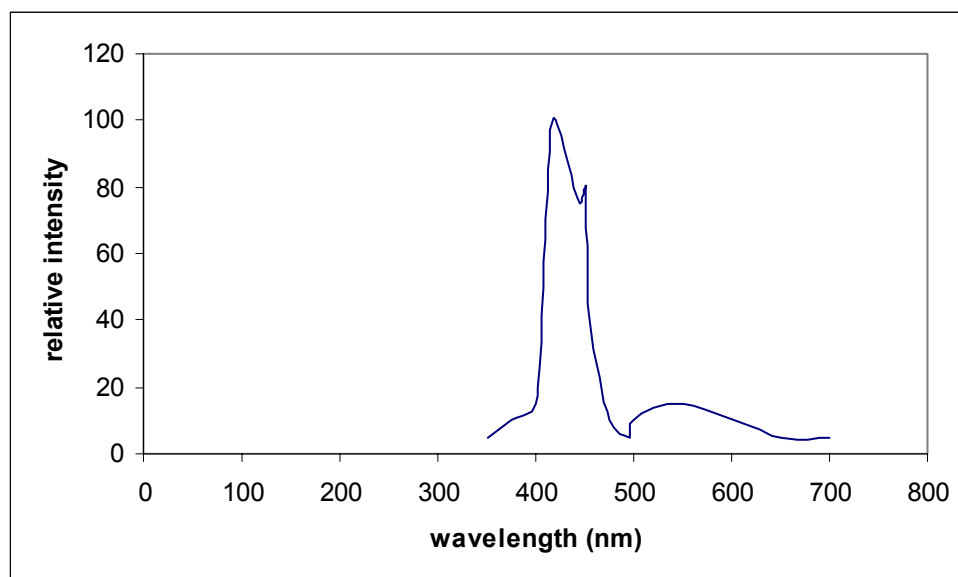


Figure 3 Output spectrum of a blacklight used for photopolymer curing

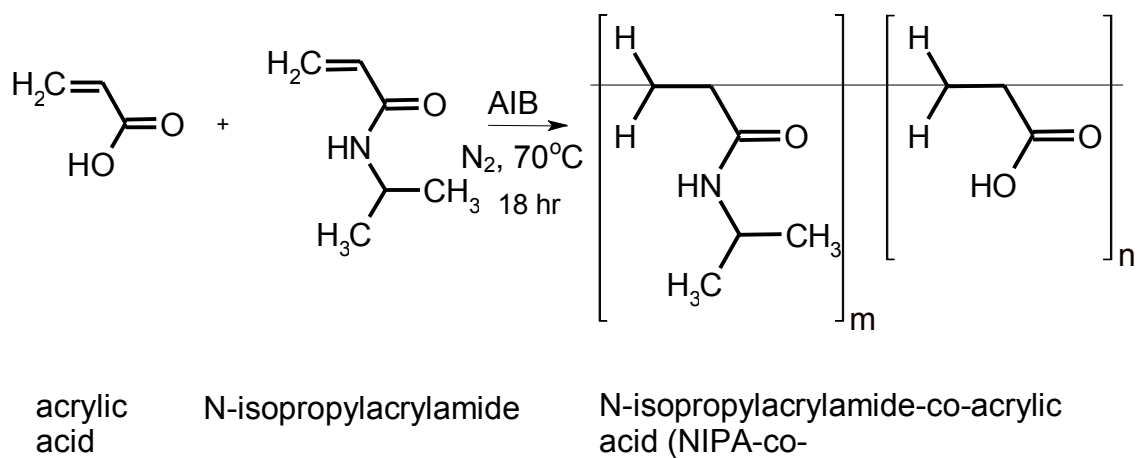
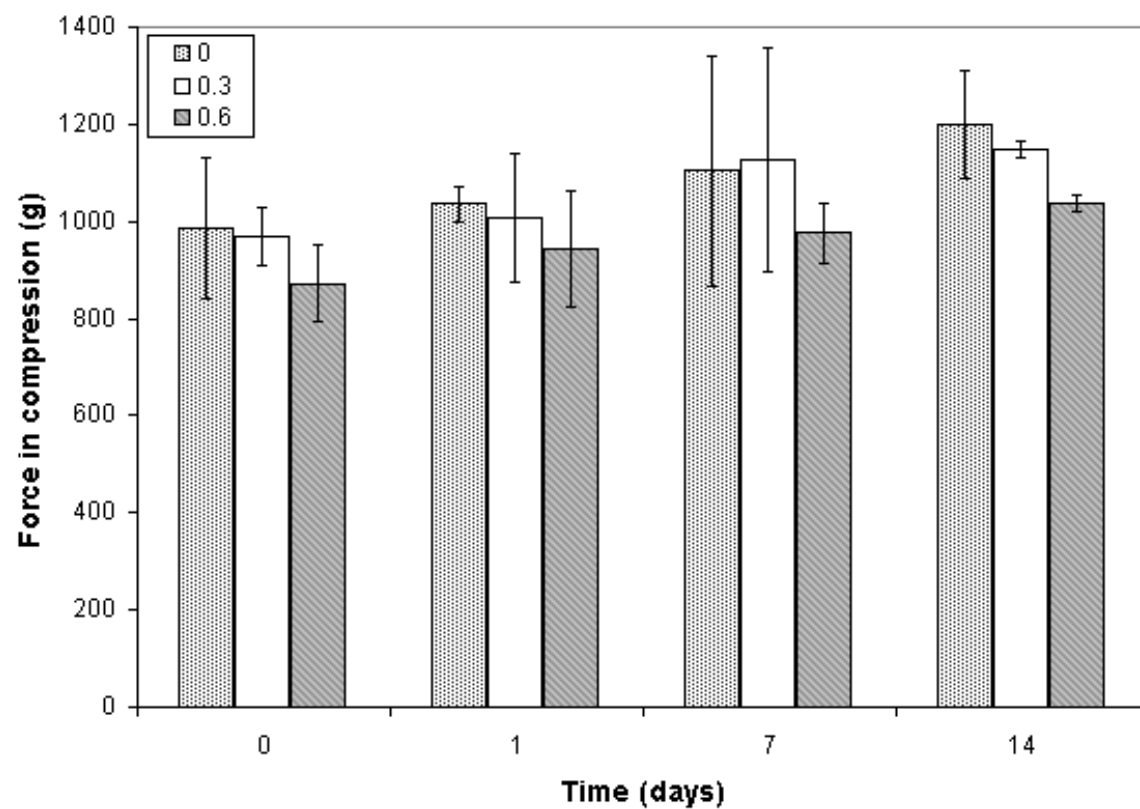
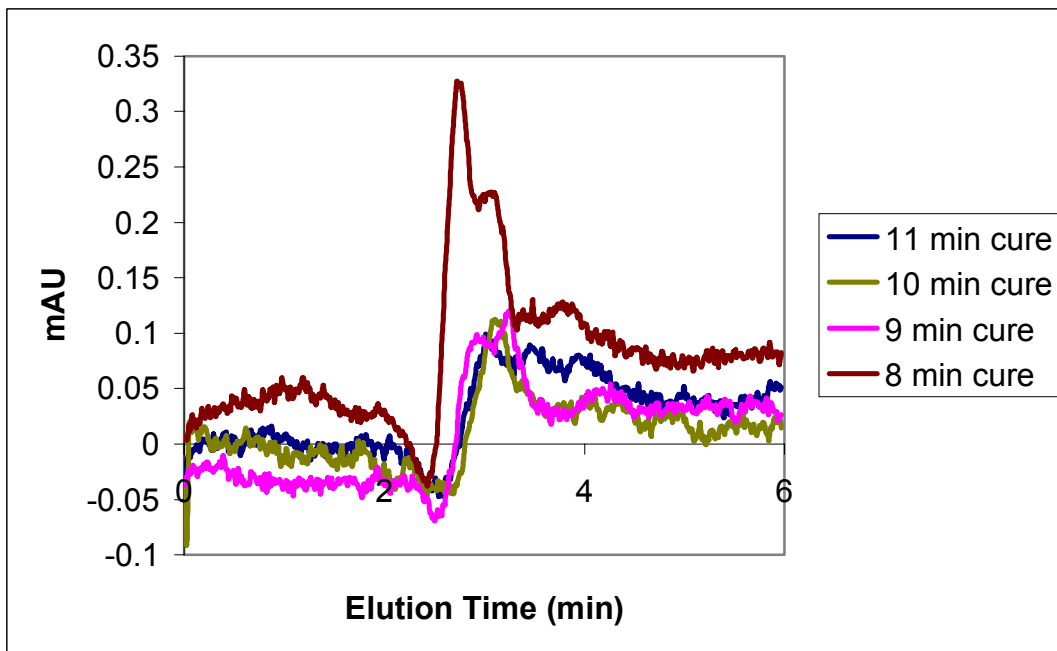


Figure 4 Synthesis of the thermally reversible gel NIPA-co-AAc



**Figure 5** Alginate beads tested under compression using a texture analyzer after incubation in solutions containing 0.6, 0.3, and no potassium in DI water. Each test consisted of at least three samples and showed no degradation of alginate beads over the two week time period.



**Figure 6 HPLC chromatograph of the cured photopolymer disks supernatant after suspension in water. The peak is indicative of unpolymerized monomer in solution with the cured photopolymer disk. Curing times longer than 8 minutes show full cure of the sample. HPLC data was collected for 25 minutes with no other peaks showing in that time.**

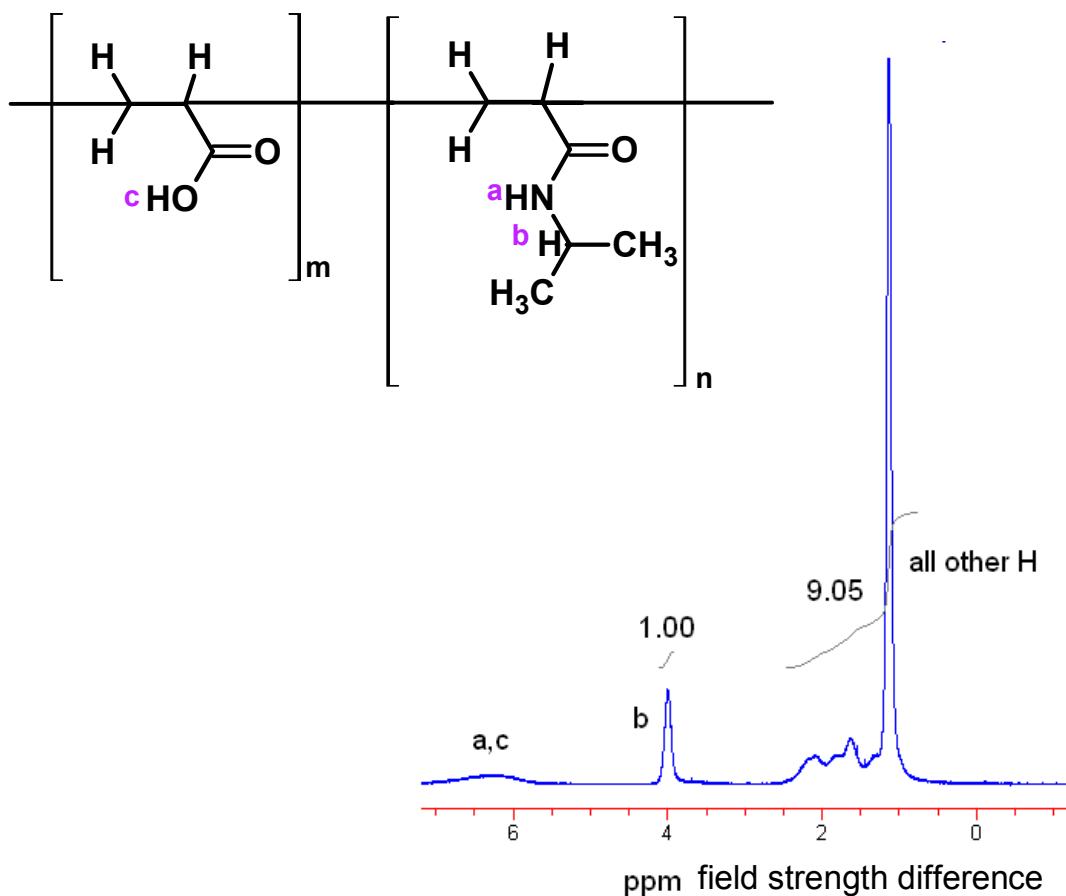
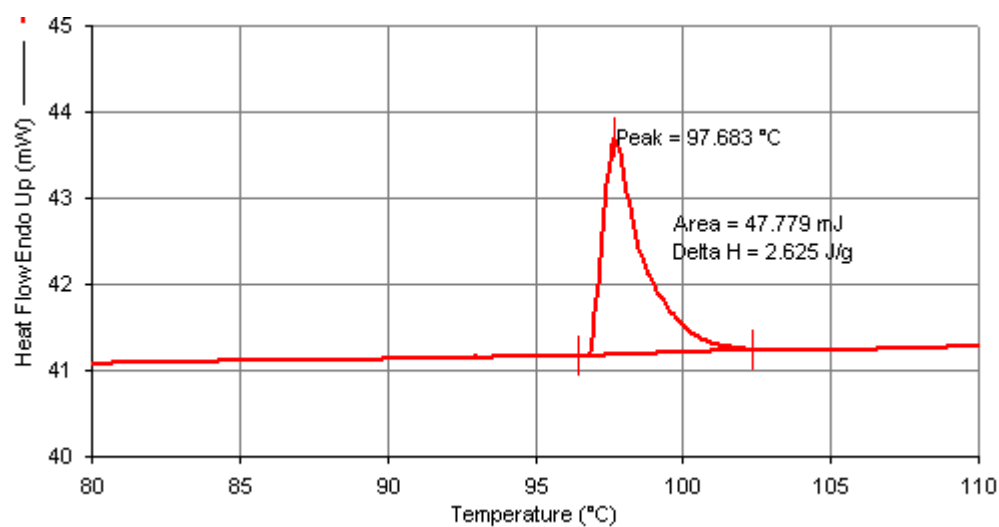
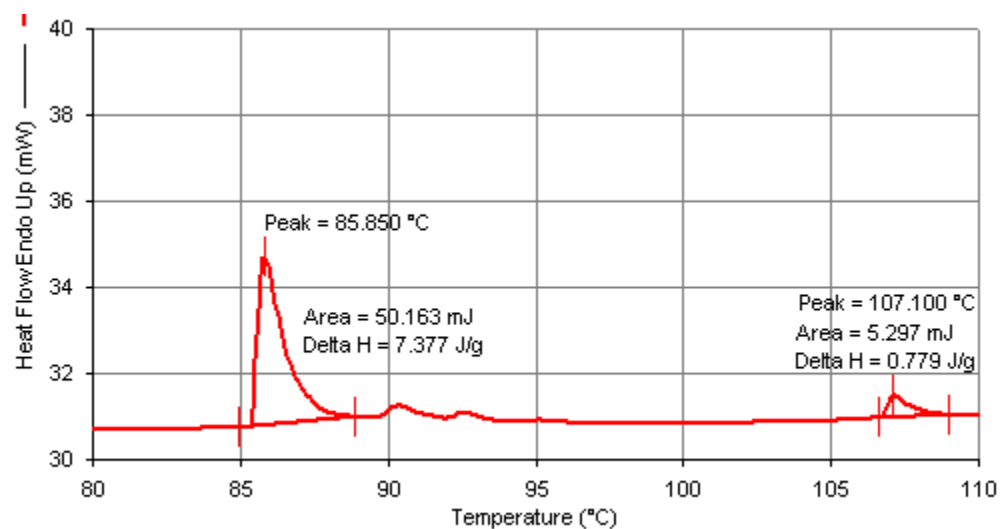
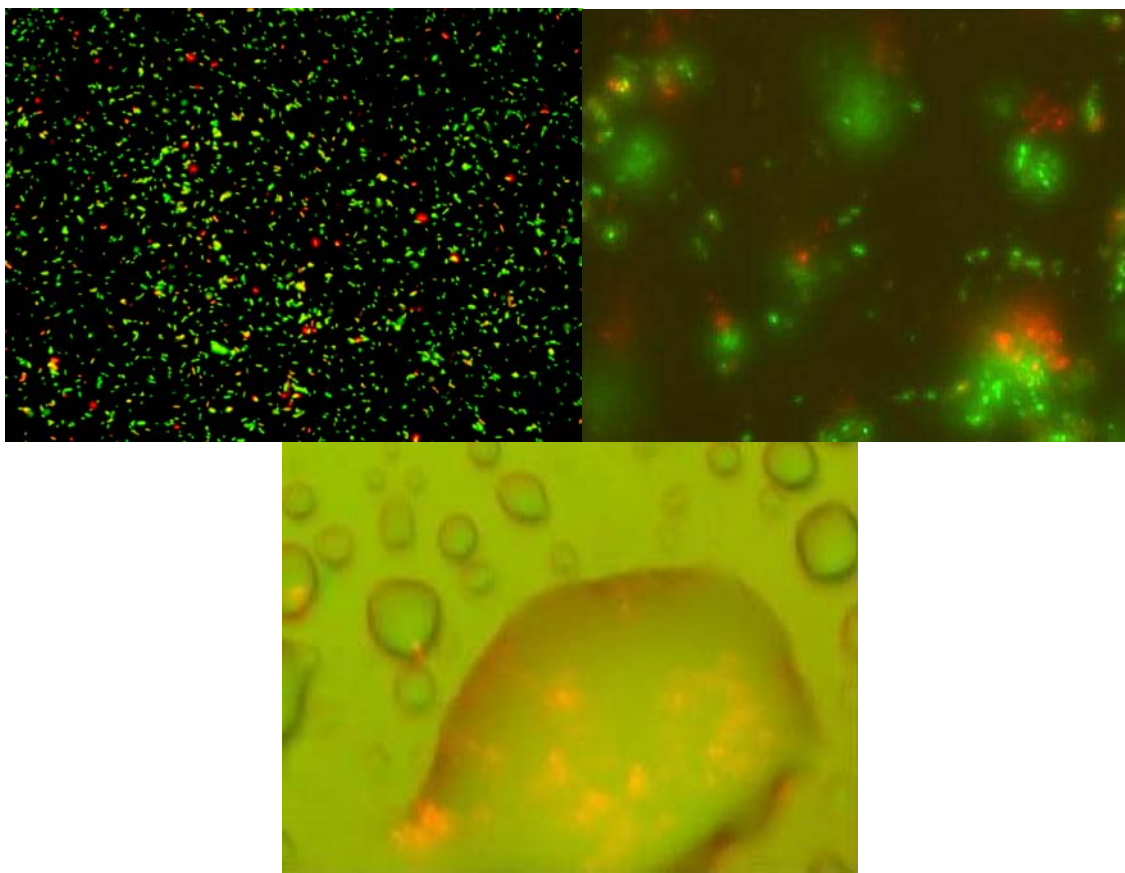


Figure 7 NMR spectra of the 98 mole% NIPA-co-AAc polymer. The spectrum was integrated over the entire range. The peak at 6.2 ppm shows the OH and NH groups from the AAc and NIPA, respectively. The peak at 4 ppm is due to the single CH resonance in the NIPA and was designated a value of 1. All other peaks were normalized to it. The group of peaks on the right was from all of the rest of the hydrogen in the sample and its integrated intensity is slightly larger than 9. Because there are 9 hydrogen atoms unaccounted for in the NIPA, the 0.05 is considered to be from acrylic acid. There are three hydrogen atoms in the acrylic acid making the AAc composition considered 2 mole%.



**Figure 8** Water retention in NIPA copolymers (95 mole% top, 98 mole% bottom) as detected by DSC.



**Figure 9** LIVE/DEAD<sup>®</sup> stain of alginate (top left) immediately after immobilization, thermal gel (top right) after ten days of immobilization, and photopolymer (bottom) immediately after immobilization. Red cells indicate dead cells while green cells are alive.

**Table 1** The efflux potential from immobilized *P. aeruginosa* in two polymer matrices on day one as compared to the average of four planktonic experiments.

<b>Matrix</b>	<b>Efflux Potential (mg K<sup>+</sup> / cell)</b>
None (Planktonic)	$(4.50 \pm 0.4) \times 10^{-10}$
Alginate	$(1.3 \pm 0.1) \times 10^{-10}$
Thermal	$(4.6 \pm 0.8) \times 10^{-10}$

### **Chapter 3: The compositional dependence of lower critical solution temperature (LCST) and gel structure of N-isopropylacrylamide (NIPA) based thermally reversible copolymer gels**

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#### **Abstract**

N-isopropylacrylamide (NIPA) has been known to copolymerize with varying amounts of acrylic acid (AAc) (0-10 mole%) to form thermally reversible polymer gels. As a structural comparison, N, N-*tert*-butylacrylamide (NTBA) was substituted for NIPA to determine whether differences arose in both structure and the transition temperatures of the thermal reversion. The hydrophilicity of the NIPA gels was also modulated by copolymerizing NIPA with the hydrophilic component N-acryloyl-6-aminocaproic acid (NIPA-co-AcACA) as a substitute for acrylic acid. It was observed that the NTBA derived copolymers were not soluble in water and had no lower critical solution temperature (LCST) in methanol solutions. Differential scanning calorimetry (DSC) indicated that water soluble NIPA copolymers exhibited a varying LCST between 32 and 36°C, and no correlation was shown between endothermic magnitude of transition, or LCST with mole fraction of AAc. The NIPA-co-AcACA copolymers exhibit a LCST at ~19°C; however, they do not form continuous matrix gels at that temperature.

*Keywords:* NIPA; hydrogel; LCST

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## Introduction

Aqueous based, thermally reversible hydrogels are soluble in water and remain as liquid dispersions at temperatures below their lower critical solution temperature (LCST). At temperatures above their LCST, these polymers usually form a hydrophobic solid precipitate or gel. This can be compared to the coil to globule phenomena found in dilute solution viscosity experiments [1]. Thermally reversible polymer hydrogels based on poly(N-isopropylacrylamide) (PNIPA) are emerging as useful matrices for drug delivery and for cellular and bacterial immobilization [2,3,4]. PNIPA possesses both hydrophilic amide and hydrophobic isopropyl groups that allow it to form different mesostable structures at various temperatures in aqueous solutions [5]. Several factors affect the resulting gel structure including the composition, the presence of co-polymerizing species, the cross linker content, and the synthesis history [6]. The copolymerization of N-isopropylacrylamide (NIPA) with acrylic acid as a hydrophilic component is common practice [7], and is studied here as well.

The use of thermally responsive gels is limited due to their specific gelation temperature, relating to a sort of spinodal decomposition [6,8]. There is a large potential for these gels to be used as matrices for immobilizing biological materials in biosensors, bioreactors, and cellular scaffolds [6, 9]. The gels can fixture the biomass while allowing the flow of nutrients, oxygen, toxins, and metabolized wastes through the structure. If the cells are not immobilized properly, the biomass can be washed away from the sensor or reactor function, rendering it useless. Hydrogels are also widely used as drug delivery scaffolds. The hydrophilic characteristics of the gels allow drugs to become more bio-available. The LCST is important when targeted delivery of drugs is needed. For



instance, anti-cancer drugs can be selectively accumulated in tumors using the innate hyperthermia of the tumor [10].

Many biosensors using immobilized biological elements are used in the environment to test for toxins [11]. The current technology typically uses assays with biological elements tethered to the substrates, alginates, or sol gels. This tethering method is not always stable or simple to process. Modulation of the LCST of thermally reversible polymer gels would be very useful for creating either a cellular scaffolds or viable, in-situ biosensors [12]. In an ambient environment, the temperature is rarely high enough to keep the gels swollen for very long. If the gels are cooled, the biological element will disperse from the thermally reversible immobilized medium and be lost. If the LCST of the gel can be lowered, there is potential for the matrix to remain functional in normal, ambient conditions, eliminating the need for external heaters and other extraneous materials. With the goal of lowering the LCST, a group of NIPA copolymers were synthesized, characterized, and some exploratory immobilization experiments conducted with known bacterial cultures, using the LCST as the trigger for immobilization.

Hydrogels were synthesized using free radical polymerization. NIPA monomers were copolymerized with N-acryloyl-6-aminocaproic acid (AcACA) hydrophilic monomers, and both NIPA and N-tert-butylacrylamide (NTBA) were copolymerized with acrylic acid (AAc) in varying amounts in each formulation. NTBA was chosen because its structure is very similar to NIPA, with the only difference being an additional methyl group.

## Experimental

### Materials

All chemicals were purchased from Aldrich Chemical Co. in Milwaukee, WI and used as received, unless indicated otherwise. Chemicals included 6-aminocaproic acid (6ACA), tetramethylethylenediamine (TEMED), sodium hydroxide, hydrochloric acid, ammonium persulfate, ethyl acetate, sodium sulfate, petroleum ether, acrylic acid (AAc), N-isopropylacrylamide (NIPA), N,N-*tert*-butylacrylamide (NTBA), poly(N-isopropylacrylamide) (PNIPA), 2,2'-azobisisobutyronitrile (AIBN), acetone, benzene, ether, and 1,4-dioxane. Distilled water was also used.

### Synthesis of hydrogels

Free radical solution polymerization was used to synthesize the AAc copolymers in a similar fashion to other published procedures [13]. NIPA or NTBA was dissolved in 1,4-dioxanes and placed in a three neck, round bottom flask. The hydrogels were synthesized with and without the addition of AAc. Several concentrations of the AAc were evaluated in the hydrogels, including 10 mole%, 5 mole%, and 2 mole%. The AAc was added in the proper concentration, and the three neck flask was purged with nitrogen. AIBN (~0.2 mole%) was dissolved in benzene and placed into an addition funnel and added drop wise into the round bottom flask, purged with dry nitrogen. After AIBN was added, the addition funnel was replaced with a reflux condenser, and the entire apparatus lowered into a mineral oil bath at 70°C. The mixture was stirred with a stir bar and left for 18 hrs under purge. The mixture was then cooled to room temperature and dissolved in acetone. The dissolved polymer was precipitated into ether for the NIPA copolymers

or water for the NTBA copolymers. All of the formed polymers were then dried in an oven at 100°C, prior to characterization.

NIPA-co-AcACA was synthesized in a two step procedure found elsewhere [14]. The N-acryloyl-6-aminocaproic acid (AcACA) was made first by adding 10 ml acryloyl chloride drop wise to a beaker containing 13.16 g 6-aminocaproic acid, 4 g NaOH, and 80 ml water. The entire beaker was cooled in an ice bath and stirred over a stirring plate. The pH was kept at approximately 7.6 by the addition of the appropriate quantity of 10M NaOH. The product was extracted using ethyl acetate and acidified to pH 5 using HCl. The solution was then extracted 3 more times in ethyl acetate. The remaining product was dried over anhydrous sodium sulfate overnight, and then poured into 500 ml petroleum ether. The new solution was kept refrigerated for about one week, or until crystals appeared to stop growing. The crystals were removed from the solution and dried in air.

After the AcACA was made, 0.185 g of the AcACA and 0.45 g of the NIPA (20:80 mole%) were dissolved in 50 ml water. 5 g of ammonium persulfate was added and the solution purged with nitrogen. 0.5 ml TEMED was then added to the solution, and the reaction proceeded overnight at 37°C while stirring and being purged. Product was removed and washed with cold water. The remaining product was then dried overnight on a 50°C hotplate. Structures were confirmed using proton nuclear magnetic resonance ( $^1\text{H}$  NMR).

### **Differential Scanning Calorimetry (DSC)**

Perkin Elmer cryogenic DSC (Pyris-1, DSC-7) measured the temperature transitions of the hydrogel solutions. The AAc copolymers were dissolved in 90 wt% DI

water, and scanned from -10 to 55°C, down to 3°C, and back to 55°C at a rate of 10°C per minute. The same thermal scan was used for NTBA copolymers that were dissolved in 90 wt% methanol. NIPA-co-AcACA copolymers were dissolved in 90 wt% DI water and scanned from 10 to 30°C at a rate of 5°C per minute.

## Results

The NIPA copolymers precipitated into a brittle, white solid, while the NTBA polymers were yellowish and brittle. The NTBA copolymers were not soluble in water, but were soluble in alcohol. The NTBA copolymers in methanol solutions exhibited no LCST when characterized by DSC at the temperatures tested. The LCST for NIPA copolymers was easily identified using DSC. Figure 1 shows the DSC curve of the 98 mole% NIPA copolymer. The top peak on heating shows the LCST while the lower peak upon cooling is identified as the recrystallization peak. All of the AAc copolymers exhibited a similar heat of melting, as shown in Table 1.

The LCST did change slightly with the addition of the AAc; however, there was no correlation between AAc mole fraction and LCST change in relation to the NIPA, also shown in Table 1. The NIPA-co-AcACA copolymer exhibited a LCST onset at 19°C; however, it does not form a continuous matrix.

## Discussion

The 98 mole% NIPA copolymer dissolved in water at room temperature with some shaking, and precipitated into a solid matrix quickly at 40°C. The 98 mole% NIPA also remained swollen longest after the removal of heat.

The DSC curves proved that the onset of the LCST is indeed a sharp transition for the NIPA-co-AAc system and can be modified slightly. The size of the endotherm

caused by the transition was relatively insensitive to the addition of AAc, with no distinguishable trends. It is obvious that changing the acrylic acid concentration in the copolymers has a small but notable effect on the LCST. Kara *et al.*, commented that the original gel synthesis conditions lead to nuances and heterogeneities that may override the specific gelation temperature associated with the LCST [4]. This would suggest that more attention to the actual variations in the gel polymerization conditions may lead to better overall control of the LCST. Nevertheless, this knowledge can help determine other hydrophilic monomers that may be able to lower the LCST even further.

The NIPA-co-AcACA copolymer precipitated into a dispersed second phase upon heating; however, it did not form a continuous matrix. The solution only changed from clear and colorless to white and cloudy, making it a poor candidate for immobilization of cells in biosensors or scaffolds for drug delivery.

## **Conclusions**

After synthesizing and characterizing NIPA and NTBA copolymers with 0-10 mole% AAc, it was determined that there is some minor change in LCST (33-36°C) with the addition of AAc in NIPA. The mole fraction of AAc addition does not affect the LCST significantly. A NIPA copolymer incorporating a different hydrophilic monomer, such as AcACA, achieved a lower LCST, but it did not allow a stiff, agglomerated matrix to form. NTBA did not dissolve in water, and showed no gelation characteristics when dissolved in methanol.

## **Acknowledgements**

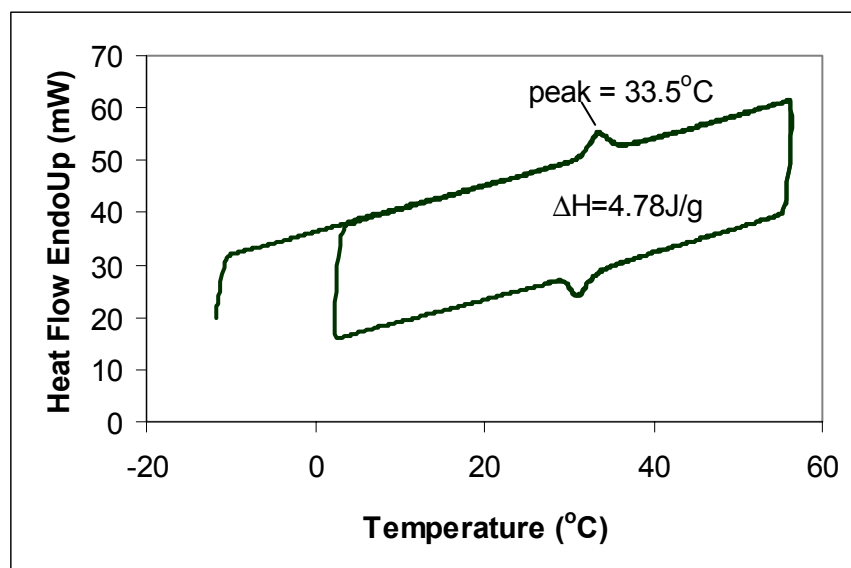
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**Table 1 Results table of the NIPA-co-AAc copolymers in the DSC. A minimum of three samples were run at each condition.**

<b>copolymer</b>	<b>LCST (°C)</b>	<b>Heat of melting (J/g)</b>
PNIPA	35.8+/-0.4	4.6+/-0.1
98 mole% NIPA with AAc	33.4+/-0.2	4.0+/-0.2
95 mole% NIPA with AAc	33.7+/-0.1	4.8+/-0.3
90 mole% NIPA with AAc	33.0+/- 0.40	4.1+/-0.2
NIPA-co- AcACA	19.1+/-1.5	NA



**Figure 1 DSC of 98 mole% NIPA copolymer in water**

### **Additional Information**

A 15 wt% aqueous solution of the NIPA-co-AcACA hydrogel was also put into an alginate solution and a psyllium husk solution in order to observe the effects of polysaccharides or fibrous materials had on the gelation of the hydrogel. The NIPA-co-AcACA hydrogel formed a solid gel upon entering the 3 wt% alginate solution; however, not all of the water was consumed within the matrix. The psyllium husk solution showed no effect on the overall dissolution of the hydrogel. Both solutions were placed in a refrigerator overnight with no additional change noticed. Both solutions were also placed on a 70°C hot plate for an hour, with no noticeable change.



## **Chapter 4: Future Work**

The matrices should be tested for cell viability and matrix stability for at least one month. Shock dosing experiments also need to be performed on the aging cells in order to ensure proper matrix permeability and cell performance. These measurements can be done simultaneously; however, each matrix should be tested in the same manner at least three times using fresh cells, media, and matrix material every time. Once a matrix has been identified as stable and non toxic to the cells, the system needs to be tested in conjunction with the optical detection system. Matrices and cells also need to undergo a freeze-thaw regimen once a good system has been identified. This freeze-thaw mechanism would simulate storage and shipping conditions of the biological element necessary for the commercial biosensor. The cells would need to be frozen in their matrix for various time periods, thawed, and the same experiments performed as above (viability, matrix stability, and potassium efflux). Once all of the tests have been performed and a working system has been identified, a working prototype can be developed and tested in real wastewater conditions.

Diffusion of potassium out of the matrices must also be determined in order to examine the value of an alginate bead matrix with reinforcements. Some approaches to determine the diffusion are to make beads, place them in a potassium solution containing known quantities of potassium and testing the solution over time for potassium content. This would allow one to observe how much potassium is flowing into the alginate matrix and one can assume that the same amount flowing into the matrix would also be allowed to flow out. An alternative is to gel the alginate in the presence of potassium, i.e. make the calcium chloride solution contain a known amount of potassium. Then place the

beads into a vial of potassium free water, and test the potassium present in the solution over time. There are several other more complex methods available in the literature (Benyahai and Polomarkaki 2005, Guilherme *et al.*, 2002, and Lewinska *et al.*, 2002)

Another item of interest is the calcium chloride solution. It has been noted in several papers that the calcium chloride solution used to gel the beads has been of varying concentration, typically in the 0.1-4 wt% range, (Favre *et al.*, 2001, Zhang and Furusaki 2001, Garbayo *et al.*, 2002, Lewinska *et al.*, 2002, Bajpai and Sharma 2004, and Benyahai and Polomarkaki 2005) as opposed to the 10 wt% solutions used in our study. The gelling times have also varied. These two parameters could cause the alginate beads to have a tighter alginate matrix, making diffusion through the matrix more difficult. Garbayo *et al.* also noted that electrostatic interactions due to the calcium clusters surrounding the bead could play a part in the diffusion of certain charged species.

The photopolymers are also very interesting as their stability is not contingent on temperature or surrounding media. Their major stability limitation is brittle fracture due to shaking or other vigorous treatment. In practice, the immobilized cells will be harnessed in place in the biosensor, making fracture no longer a concern.

Tests should be done in order to identify the ultimate cause of cell death in the photopolymer matrix. A similar diffusion test as described above in the alginate section should be performed on various size photopolymer disks. Preliminary tests show that cells are not viable after immobilization in the photopolymer matrix. This may be due to poor diffusion, toxicity of the photopolymer, exposure to the curing lamp for extended periods, or some other problem yet to be determined. Others have concluded that photopolymers in the form of small micro patches used for cell immobilization have been

successful (Heo *et al.*, 2003, Zhan *et al.*, 2002, and Koh *et al.*, 2002). These micro patches require microfluidic channels as described by Duffy *et al.*, 1998 along with photomasks allowing micron resolution of micro patches in the photopolymer. The micro patches only allow a few cells to be encapsulated per patch, making the required surface area of a chip with enough cells to produce a readable signal quite large. The micro patch construction is also problematic as it causes several cells to be washed off of the substrate after curing sections using the photomask. The incorporation of the patches in the microfluidic channels is good for getting nutrients and oxygen to the cells; however, some cell adhesion to the patches must be present in order to keep the cells from flowing through the system. Their curing times were also much shorter, on the order of seconds, making exposure to harmful wavelengths limited.

While the thermally reversible gels are impractical for certain applications because of their high LCSTs, some thought has been put into lowering their LCST. It is realized that even an LCST of 20°C would still be difficult to maintain in the cooler climates, it is still an interesting problem. Some work has been done on the effect of a hydrophobic monomer on the LCST of NIPA hydrogels (Ni and Zhu 2004). They demonstrated that the addition of a hydrophobic monomer significantly decreased the LCST of the resulting hydrogel. Their work suggests that the addition of a cross linker in small amounts will increase the water uptake capacity of the resulting hydrogel. They demonstrated that the addition of hydrophilic monomers increases the LCST, but also increases the overall absorbance capacity of water. With this in mind, it would be a good idea to copolymerize NIPA hydrogels with NTBA with and without the addition of 2

mole% acrylic acid, and with and without a cross linker. A good place to start in this synthesis would be to do the following:

1. Procure the materials including NIPA, NTBA, AAc, a cross linker (such as tera(ethylene glycol) diacrylate), an initiator (such as benzoyl peroxide), distilled water, and dioxane
2. Mix water with twice the volume dioxane. Mix in NIPA to a concentration of 2M. Place this solution in a round bottom flask and stir with a magnetic stir bar. Add 2 mole% AAc (with respect to the NIPA) and continue stirring. Add 40 mole% NTBA (with respect to the NIPA) to the mixture. After stirring for ~10 minutes, add 0.5 mole% (with respect to the total monomer content) dissolved in dioxane.
3. Stir at room temperature under nitrogen until all constituents have dissolved. Add drop wise, under nitrogen, 0.5 mole% initiator. Stir for ~20 minutes. Lower the round bottom flask into a preheated (55°C) oil bath and allow stirring and purging for 24 hrs.
4. The resulting gel should be washed with DI water thoroughly and dried.

The hydrogel should have a low (~20°C) transition temperature, and should gel with retention of >90% water. The NIPA-co-NTBA hydrogels should be tested in the same manner as the NIPA-co-AAC hydrogels using *P. aeruginosa* cells. All matrices should be tested over longer periods of time as well.

With respect to the optical detection system, optical ion sensors (optodes) combine the advantages of high selectivity and reversible recognition of ionic species with a simple to recognize optical transition (Morf et al.1990). Poly(vinyl chloride) (PVC) is chemically inert, exhibits a high tensile strength, and is compatible with most plasticizers, making it the most commonly used polymer host for optodes (Eugster et al. 1994, Morf et al. 1989). Plasticized PVC has a tendency to allow the plasticizer to leach its matrix, making optode lifetime, and ion permeability decrease causing sensitivity and selectivity to also decrease rapidly (Moody et al. 1992, Oesch et al. 1980, Reinhoudt et al.

1994). There is a need to develop unplasticized optodes. Poly(vinyl butyral) (PVB) is an amorphous polymer with a low glass transition point and known for its use in laminated materials (Gopal et al. 1996). It is especially attractive as it has high ionic mobility through its matrix due to its amorphous nature (Papke et al. 1982). The heavy pendant group present in the polymer allows it to remain amorphous. The low glass transition temperature also contributes to the high ionic mobility at ambient conditions. PVB exhibits good adhesion to a variety of substrates making it an excellent candidate for laminated materials, and the optode discussed here.

The polymer host in conjunction with a neutral, cationic sensitive ionophore, lipophilic anionic sites (negative charges), and a protonated chromoionophore (positive charges) comprises the optode. The overall optode charge is neutral. The desired cation permeates the polymer host and binds to the ionophore. This causes the film to become positively charged, so a proton from the chromoionophore is sacrificed. This deprotonation causes a shift in spectrum of the optode.

There are several types of optodes, including flow through, waveguide, films or sensing plates, and fiber optic devices (Hismoto and Suzuki 1999). The optode discussed here is in the form of a flow through film. The configuration was chosen to take allow for continuous, rapid measurements of a flow through device along with the easy determination and disposability of the plates. The chromoionophore chosen for these experiments is ETH 5294 and it shifts its fluorescent peak with deprotonation. Fluorescent sensitive chromoionophores allow sensors to be smaller than their absorbance sensitive counterparts (Shortreed et al. 1997). BME 44 is the ionophore selected for its selectivity to potassium ions.

It is imperative for the operation of the biosensor that both the PVC and PVB systems are tested thoroughly. The optimal wavelength for excitation must be determined for each sensor, along with the corresponding emission. Both systems must incorporate the same amount of each constituent and be made in the same manner to ensure proper comparison of results. After excitation and emission wavelengths are determined, the optodes must be tested in the presence of potassium in both acidified and neutral conditions. The tests should be run over time to determine relative diffusion of the ions in the optode, and comparisons of the two systems performance must be noted. A simple recipe for the construction of testable samples follows:

#### Optode Film Preparation

##### Key

Chemical Name	Nickname
poly(vinyl butyral)	PVB
poly(vinyl chloride)	PVC
tetrahydrofuran	THF
Bis(2-ethylhexyl) sebacate	plasticizer/DOS
chromoionophore I/N-Octadecanoyl-Nile blue	chromoionophore/ ETH 5294
potassium ionophore III/2-Dodecyl-2-methyl-1,3-propanediyl bis[N-[5'-nitro(benzo-15-crown-5)-4'-yl]carbamate]	ionophore/BME 44
potassium tetrakis [3,5-bis(trifluoromethyl)phenyl]borate	lipophilic anion/KTFPB

1. Dissolve polymer in solvent (at least 2 days prior to the rest of the preparation)

- For PVB, dissolve 15 wt% in cyclohexanone (i.e. 1.5g PVB in 8.5g cyclohexanone)
- For PVC, dissolve 15 wt% in THF (i.e. 1.5g PVB in 8.5g THF)

Be sure all polymer is dissolved in a homogeneous solution before continuing (no chunks, gobs, or phase separation)

2. Place the ionophore, chromoionophore, and lipophilic anion in a vessel in the proper concentrations as shown below:

- The KTFPB is used as 10mmole/kg concentration, with the polymer (not the entire solution). KTFPB's molecular weight is 902.31g/mole.

$$\frac{10mmole}{kg} * \frac{1mole}{1000mmole} * \frac{1kg}{1000g} = \frac{0.0000010mole}{g}$$

$$\frac{902.31g}{mole} * \frac{0.000010mole}{g} * xg = 0.0090xg$$

- Where x = the amount of polymer in the optode solution. If you are using ten grams of polymer solution in your optode film, then that corresponds to 1.5 grams of polymer, making x = 1.5g, and the amount of KTFPB needed is 1.5g\*0.0090 = 0.0135g

The other constituents are added in a similar manner, but the concentrations are shown below:

Name	Molecular Weight	Concentration
ionophore	967.06g/mole	20.2mmole/kg
chromoionophore	583.85g/mole	9.9mmole/g

3. After the three components are added into a clean, dry vessel, the THF needs to be added. In the case of PVC optode solutions, the PVC polymer solution can be added. In the case of the PVB optode, an amount of THF equal to 10 wt% of the total solution must be added first. For instance, if the entire solution before adding the THF is equal to 11.05g, the THF needed follows the following formula:

$$\frac{yg}{11.05g + yg} = 0.10$$

Where y = the number of grams of THF needed in solution

4. Once the THF has been added, all the components have been dissolved (no chunks, etc.), the PVB solution can be added to the PVB optode solutions. After everything has been mixed well, the plasticizer can be added to the proper solutions (the PVC and the plasticized PVB (PVBP)).
5. The plasticizer is used in 2/3 concentration with the polymer; therefore, multiplying the amount of polymer (x) in solution by 66% gives you the amount of plasticizer needed.

Below is a table of the solutions, their components and amounts needed for a 10-12g solution. Each solution must then be filtered through a 2.75  $\mu\text{m}$  syringe filter and cast (three drops from the syringe filter) onto 9x22 mm glass cover slips. The films should then be covered and allowed to dry overnight on a level surface before testing. This process results in films approximately 0.1 mm thick.

additive	fraction	g	total sample
PVB	15 wt%	1.5000	1.5000
plasticizer	66 wt% (polymer)	0.9999	2.4999
ionophore	20.2 mmole/kg	0.0293	2.5292
chromoionophore	9.9 mmole/kg	0.0079	2.5371
lipophilic anion	10 mmole/kg	0.0135	2.5506
cyclohexanone	85 wt% (polymer)	8.5000	11.0506
THF	10 wt% (total)	1.2279	12.2785
additive	fraction	g	total sample
PVB	15 wt%	1.5000	1.5000
plasticizer	0 wt%	0.0000	1.5000
ionophore	20.2 mmole/kg	0.0293	1.5293
chromoionophore	9.9 mmole/kg	0.0079	1.5372
lipophilic anion	10 mmole/kg	0.0135	1.5507
cyclohexanone	85 wt% (polymer)	8.5000	10.0507
THF	10 wt% (total)	1.1168	11.1675
additive	fraction	g	total sample
PVC	15 wt%	1.5000	1.5000



plasticizer	15 wt%	0.2650	1.7650
ionophore	20.2 mmole/kg	0.0293	1.7943
chromoionophore	9.9 mmole/kg	0.0079	1.8022
lipophilic anion	10 mmole/kg	0.0135	1.8157
THF	85 wt% (polymer)	8.5000	10.3157
cyclohexanone	0 wt%	0.0000	10.3157

Other casting techniques could be used as in the case of spin casting or doctor blades. Spin casting will result in thinner solutions; however, the film will not be uniform on the edges of the substrate used. Spin casting techniques would also need to have the polymer concentration of the films adjusted. Thicker solutions may cause premature evaporation of the solvent on the top of the film, making the resulting film uneven and possibly lumpy. Very thin solutions spun at high speeds will cause the resulting film to be very thin, making signal output weak. Doctor blades are often difficult to control and may cause the edges of the resulting film to be thinner than the rest of the film. Various substrates can be used when using a doctor blade, as opposed to the spinner. Spinners require ridged substrates while doctor blades function well on any surface.

The PVB films that do not contain plasticizer will be green until exposure to acidic solutions. Upon exposure to acidic solutions, films may turn blue-green over time. The plasticized PVB films will be a darker green or brown-green prior to exposure to acidic solution, and will turn green to blue-green after exposure to the acidic solution. Neutral solutions should have no effect on the films. PVC films will be a dark red or purple to begin with, and may turn blue when in acidic solution over time. Any spots that show up on the film are due to inhomogeneities in the starting solution. All solutions must be well mixed prior to film casting. The proper excitation and emission

wavelengths, along with homogeneous solutions and good casting techniques will make testing of the optode films simple and relevant.

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