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I am submitting herewith a thesis written by Elizabeth F. Mitchell entitled "Sol-gel Encapsulation of a Bioluminescent Bioreporter." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Microbiology.

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Sol-gel Encapsulation of a Bioluminescent Bioreporter

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Elizabeth F. Mitchell
August 2004

Dedication

I dedicate this work to my family.

Thank you for the love, encouragement, laughter, and prayers you have given me over the years. This would not have been possible without your support.

Acknowledgements

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Abstract

Genetic engineering has allowed for the development of a number of whole-cell bacterial bioreporters. In order for these cells to be used in certain devices and field applications, they must be maintained, and protected, but exposed to environmental conditions. One approach used to accomplish this goal is cellular encapsulation. The recent development of techniques to form inorganic matrices, in particular through the sol-gel process, displays promise as a system of encapsulation. The silica sol-gel process was originally designed for the fabrication of glass, and given that reactions are performed in standard lab conditions and room temperature, the process can be adapted for encapsulation of organic compounds, enzymes, and even whole cells. When mixed with sol, cells become enmeshed within the matrix of silica, forming a gel. The qualities that make silica glass strong, stable, and optically transparent are what also make this method useful for encapsulation of bacterial bioreporters. The nature of the silica matrix allows it to remain stable yet permeable to target analytes, nutrients, and oxygen. In addition, because they are arranged in a matrix, the encapsulated cells are sustained as a system allowing for direct placement of the reporter strain onto a signal processor. The increased understanding of sol-gel chemistry has allowed for the development of techniques that maintain viability of cells while encapsulating them in an inorganic matrix that should be able to withstand conditions in the field or in a sensor device.

In this project, cells of a bioluminescent bioreporter *Pseudomonas fluorescens* 5RL (5RL) were encapsulated in a silica sol-gel matrix. Electron microscopy, bioluminescence,

oxygen uptake, protein concentration, and carbon utilization assays were designed and implemented to investigate the physiological state of encapsulated cells as compared to cells in suspension. Overall, results suggest that cells are not significantly affected by encapsulation. Furthermore, there do not appear to be additional significant mass transfer issues faced by cells inside the gel. Results are promising for the potential field applications of this process towards environmental monitoring, medical diagnostics, agricultural biotechnology, and biocomputing.

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Chapter One

INTRODUCTION AND REVIEW OF LITERATURE

Introduction

Genetic engineering has allowed for the development of a number of whole-cell bacterial biosensors. A biosensor is a genetically engineered reporter cell (bioreporter) that is coupled to a microelectronic device. In order for these cells to be used in certain devices and field applications, they must be maintained and protected, but exposed to environmental conditions. One approach used to accomplish this goal is to encapsulate the cells in a material like alginate or agar. This is not always a feasible option, in part because the organisms can often use the encapsulating material as a nutrient source. In addition, the organic matrix itself may not be able to withstand conditions in the field or in a sensor device. There may also be mass transfer issues that negatively affect physiology of the encapsulated organisms. The recent development of techniques to form inorganic matrices, in particular through the sol-gel process, has the potential to address these problems. The silica sol-gel process was originally designed for the fabrication of glass, and given that reactions are performed in standard lab conditions and room temperature, the process can be adapted for encapsulation of organic compounds, biological ligands, and even whole cells. Silica sol has been described as a “nanoglue” capable of creating a three-dimensional network containing a dispersed solid, in this case cells of a bioluminescent bioreporter *Pseudomonas fluorescens* 5RL (5RL). When mixed with sol, cells become enmeshed within the matrix of silica, forming a gel. The qualities that make silica glass strong, stable, and optically transparent are what also make this

method useful for encapsulation of bacterial bioreporters. Furthermore, because silica gels are inorganic, the encapsulated cells do not use the matrix as a nutrient source. The nature of the silica matrix allows it to remain stable yet permeable to target analytes, nutrients, and oxygen. In addition, because they are arranged in a matrix, the encapsulated cells are sustained as a system allowing for direct placement of the reporter strain onto a signal processor. The increased understanding of sol-gel chemistry has allowed for the development of techniques that will maintain viability of cells while encapsulating them in an inorganic matrix that has potential field applications towards environmental monitoring, medical diagnostics, agricultural biotechnology, and biocomputing.

Hypothesis

Encapsulation in silica sol-gel does not affect bioluminescent bioreporters used for field applications.

Questions to address Hypothesis

Question One: Are cells encapsulated?

If the purpose for the sol-gel technique is to encapsulate cells, it is necessary to determine if cells are in fact encapsulated within the matrix. It is also important to characterize the structure of the gel for future research. To this effect, a protein assay technique will be designed to predict the number of cells encapsulated with the gel. Other experiments will enumerate any cells that are able to escape from the gel. Finally, the gel interior will be visualized under thin section transmission electron microscopy while the surface will be

visualized with scanning electron microscopy. Overall these experiments will begin to characterize the nature of the sol-gel matrix.

Question Two: Is the physiology of encapsulated cells affected?

Once cells are encapsulated it is important to determine if the matrix affects them, especially in respect to their physiological state. This will allow determination of success in the future application of this system for field operations. *5RL* will be used as a model organism in this study because it is an inducible bioluminescent bioreporter. This offers the advantage of utilizing bioluminescence as a measure of the physiological state of cells. In addition, oxygen assays will provide further information on the physiological state of encapsulated cells. These assays will provide a good idea of how cells are functioning within the sol-gel matrix. They will also test whether target analytes are able to diffuse through the matrix to the encapsulated cells. Finally, a long-term study will address the storage capacity of gels for maintaining biosensor cells. This is an important aspect in the feasibility of using this system for real-time monitoring.

Question Three: Is mass transfer an issue for encapsulated cells?

A theoretical problem for cells encapsulated in any material is whether there are mass transfer issues. This would limit the exposure of cells to oxygen and other important nutrients, and for biosensing applications may limit exposure of cells to target analytes. The sol-gel process results in a porous matrix, so it is of intent to test the hypothesis that encapsulated *mass transfer does not limit 5RL*. *5RL* cells are unique in that they cannot degrade the inducing chemical so after induction they should continue to luminesce until

they deplete carbon or oxygen, or if cells die. This can be used to determine whether gels are limiting mass transfer depending on whether added oxygen or carbon will restore bioluminescence. A second test to examine mass transfer issues will be an anaerobic induction experiment. Encapsulated cells will be placed in a sealed chamber with media, and then bubbled with nitrogen to remove oxygen from the test chamber. After bubbling, cells will be monitored for bioluminescence. After sufficient induction time, chambers will be opened and monitored for an increase in bioluminescence. If there are mass transfer barriers the time interval will be significant for oxygen to reach the cells and they may be unable to recover.

Overall these experiments should help elucidate the effect of sol-gel encapsulation on the bioluminescent reporter *5RL*. They should allow a determination on whether this system has potential for use in field applications. It will also be possible to make recommendations on the strengths and weaknesses of this technology.

Review of Literature

Microbial Catabolic Enzymes

Many chemicals utilized by present day industry are synthetic. These chemicals have no equivalent counterparts in nature and along with their complex structure, are resistant to natural degradation (39, 50). As a consequence, they are not readily removed and tend to accumulate in natural systems leading to pollution. However, as a consequence of their persistence, catabolic enzymes have evolved or been engineered to possess the capability

of breaking the chemicals down into less toxic and even biocompatible forms (39, 50). Most of these catabolic enzymes are associated with microbial populations.

Microbial catabolic enzymes typically function in a process where chemicals are detoxified through a series of steps into simple metabolites (39, 50). The genes coding for these enzymes can be maintained on the bacterial chromosome or a plasmid, as well as in the natural bacteriophage genome. Maintenance of the genes is metabolically costly to the cell, so they are only maintained if they are beneficial. For many cells, survival in certain environments is dependent on the gene products to detoxify chemicals and use them as a carbon source. This is why most catabolic plasmids are easily transferable and function in a broad host range. They are often transferred to other species sharing the toxic environment.

The discovery of microbial catabolic enzymes is significant to humans as well. We are affected by the same pollutants and also need ways of detecting and degrading the toxins. Once identified and characterized, bacteria that degrade chemicals may be placed into a new site for bioremediation (25, 39, 50). The genes encoding catabolic enzymes are often duplicated and reinserted back into the original organism for a higher level of catabolic activity. These genes may also be inserted into new species for use in a different environment that contains a similar pollutant but cannot support the growth of the original organism. In most cases it is best to have a native species used in degradation because they are already adapted to the environment. However, genes can often be transferred into laboratory strains. In environmental settings horizontal transfer of genes

is a common feature. Genes are often transferred naturally between species through conjugation and transformation. Genetic engineering can artificially increase the speed and rate of success for this process.

Development of a Bioreporter

The positive regulation of these catabolic operons by the pollutants or intermediate metabolites leads to another application in the biochemical sensing of pollutants. Utilizing promoters for specific analytes, a detection system can be added to the cell, resulting in a bioreporter (2, 12). The genes that code for these detection systems are most often placed behind the promoter of interest. When the cell comes into contact with the target analyte, the promoter is turned on, and a detectable signal produced (2, 12). Common detection systems include Chloramphenicol acetyltransferase, β -galactosidase, firefly luciferase, bacterial luciferase, aequorin, green fluorescence protein, and urogen (12). The advantages and disadvantages of each are discussed in Table 1 (2, 12, 31, 38, 53).

When selecting a system it is important to take into account the cell type being used as well as the potential applications (2). When dealing with environmental pollutants there is a desire for continuous, online, real-time monitoring. The system that best fits this application is bacterial luciferase (12, 31, 47). The most important characteristic of this system is that apart from oxygen, no added substrate is required for the reaction when the full *lux* operon is expressed (31). Bacteria expressing the *lux* gene cassette make all components necessary for the reaction. In addition, there is no need for excitation in the

Table 1. ADVANTAGES AND DISADVANTAGES OF COMMON REPORTER SYSTEMS.

Reporter System	Advantages	Disadvantages
Chloramphenicol acetyltransferase (CAT)	No endogenous activity Automated detection with ELISAs	Requires addition of substrate Requires separation of product from substrate Often uses radioisotopes
β -galactosidase (β -gal)	Sensitive and stable Simple assays (colorimetric and chemiluminescent) Applicable in anaerobic environments	Requires addition of substrate Endogenous activity
Firefly luciferase (LUC)	Sensitive No endogenous activity in mammalian cells	Requires addition of substrate (luciferin), O ₂ , and ATP
Bacterial luciferase (<i>lux</i>)	Sensitive No endogenous activity in mammalian cells Does not require addition of substrate	Requires O ₂ Heat labile above 45°C
Aqueorin	Sensitive No endogenous activity in mammalian cells	Requires addition of substrate and Ca ⁺⁺
Green fluorescence protein (<i>gfp</i>)	Autofluorescent Mutants with different colors available Stable at biological pH	Requires exogenous excitation Moderate sensitivity Long induction period Background fluorescence may interfere
Uroporphyrinogen III methyltransferase (UMT)	Autofluorescent May have a better signal to noise ratio than <i>gfp</i>	Endogenous activity

viewing and measurement of response (47). The need for oxygen limits the system to use in aerobic environments. Another consideration is the heat stability of the luciferase enzyme which has an upper limit 45°C (53). This presents an issue for high temperature environments.

Bioluminescence

The *lux* system in bacteria has been identified in members of *Vibrio*, *Photobacterium*, and *Photorhabdus* genera that occupy marine, freshwater, and terrestrial environments (21, 31). The reaction entails the oxidation of reduced riboflavin phosphate (FMNH₂) and a long chain fatty aldehyde with the emission of blue-green light at 490nm. This reaction is under the control of the *lux* cassette – *luxCDABE*. The luciferase enzyme is coded by the *luxA* and *luxB* genes, which form a heterodimeric protein of 77 kDa (31).

The second requirement of the bioluminescence reaction is the synthesis of aldehydes catalyzed by a multienzyme fatty acid reductase (31). This system is composed of a transferase (LuxD), a reductase (LuxC), and a synthetase (LuxE). These genes are not required for light emission, but are involved in synthesis and recycling of fatty aldehydes. In this reaction, the transferase catalyzes the transfer of myristol-ACP to water, oxygen, and thiol accepters, resulting in myristic acid. Myristic acid is activated by the synthetase forming a myristol acyl-AMP intermediate. In the presence of the reductase, the acyl group is transferred between LuxC and LuxE, and reduced by NADPH to myristyl aldehyde. Myristyl aldehyde and FMNH₂ are reduced by luciferase (LuxAB), resulting in the generation of blue-green light – bioluminescence.

The *luxCDABE* genes have been found in all bioluminescent organisms to date (31). These genes have been successfully transferred to various bacterial strains resulting in the creation of numerous prokaryotic bioreporters. Recently the development of yeast and mammalian bioluminescent cell lines has been accomplished in our lab (20, 38). Again, one reason for the prolific creation of bioluminescent bioreporters is the stand-alone nature of the system. No substrate must be added for measurement to take place, the only requirements are the transcription and translation of *lux* genes along with oxygen (47).

Development of a Bioreporter for Naphthalene

One chemical of interest is naphthalene (CAS# 91-20-3). Naphthalene is an environmental pollutant ranked thirty-sixth in the 2003 Completed Exposure Pathway Site Count Report by the Agency for Toxic Substances and Disease Registry (ATSDR). In this report, naphthalene was found in seventy sites overall and thirty-seven sites recorded on the National Priorities List. It is categorized by the Environmental Protection Agency in Group C as a possible human carcinogen. Naphthalene is known to cause hemolytic anemia in humans. As a result of these and other potential effects of naphthalene exposure, it is desirable to detect and if possible detoxify environmental naphthalene.

The biodegradation of naphthalene occurs naturally in certain polluted environments. The Nah7 plasmid of *Pseudomonas putida* is a model system for naphthalene catabolism. The Nah7 plasmid consists of two operons, an upper and lower pathway, that each function in

a stepwise process (9, 58). The upper pathway, *nahABCDEF*, codes for enzymes that convert naphthalene into salicylate (58). The lower pathway, *nahGHIJK*, encodes enzymes that convert salicylate into pyruvate and acetaldehyde. Both pathways are controlled by the regulator *nahR*, which is activated by salicylate (9, 59). The NahR protein binds to the *nahR* gene and activates transcription of both operons when salicylate is present. When salicylate is absent, but there is presence of naphthalene in the environment, low constitutive levels of the upper pathway enzymes convert naphthalene to salicylate, which then binds to *nahR* and results in induction of both pathways. A study by Yun and Gunsalus used transposon mutagenesis to identify this mechanism of control (59). These authors observed that mutations in the *nahA* gene led to a Nah⁻ Sal⁺ phenotype (salicylate was degraded but not naphthalene). Mutations in *nahG* led to Nah⁺ Sal⁻ phenotype (naphthalene degraded to salicylate, which was not degraded). Finally, mutations in *nahR* gene resulted in a double negative phenotype suggesting that *nahR* was the regulator of both pathways (59). It was previously known that the regulatory gene was located in the 7kb region separating the two operons and the product of this gene was required for the expression of both operons (58).

In order to “sense” the presence of naphthalene and salicylate in the environment, a bioluminescent bioreporter was designed (25). *Pseudomonas fluorescens* 5R (5R) is an environmental strain isolated from contaminated Manufactured Gas Plant soil. Strain 5R carries a catabolic plasmid (pKA1) that shows homology to the model plasmid pNah7. The *luxCDABE* genes were transferred to 5R via the *lux* transposon Tn4431 carried on a suicide vector pUCD623. A bioluminescent construct 5RL was selected and

characterized. The *lux* cassette inserted into *nahG*. Given that the *lux* cassette disrupts the lower operon, *5RL* is not able to convert salicylate into pyruvate and acetaldehyde (25, 59). As a result, salicylate accumulates and there strong constitutive induction of the upper pathway, resulting in a highly luminescent reporter system. As confirmed by naphthalene mineralization assays, [¹⁴C]salicylate was identified as the sole metabolite in *5RL* (25). *Pseudomonas fluorescens 5RL* is a useful bioreporter system because it is easily grown at room temperature, contains a tetracycline resistance gene, and is quickly induced by salicylate (23, 25). Once induced, *5RL* has a high level of bioluminescence, which allows for ease of detection and monitoring. In addition, the mechanism of induction for *5RL* is well characterized.

Development of a Biosensor

Once a bioreporter strain has been designed it may be used in many sensing applications. However, in order to be used as a biosensor the bioreporter must be coupled to a microelectric system/device (2, 22, 47). With a functioning biosensor it is possible to achieve on-line real-time monitoring of target chemicals. In our lab this has been accomplished with the first generation Bioluminescent Bioreporter Integrated Circuit (BBIC) (52). According to Simpson et al., the successful development of a BBIC requires four parts. The first is a reporter cell line that is sensitive to a target substance (52). As mentioned previously, *5RL* is a strain capable of detecting naphthalene and emitting a strong bioluminescent response (3).

The second requirement is an integrated circuit (IC) “designed to perform the desired function” (52). In this case, the IC should be designed to detect bioluminescence. The circuit should be sensitive enough to distinguish the signal from background noise and process the signal into an analog or digital output (12, 52). It may also be beneficial to be able to integrate other types of measurement such as physical properties like pH, temperature, salinity, oxygen concentration, as well as position of the biosensor (2, 12, 52). Finally the IC should be able to communicate the measurements it takes (2, 12, 52).

The BBIC device conceived by Simpson et al. is designed through the complementary-metal-oxide-semiconductor (CMOS) IC process (52). The device measures light emitted by the bioreporter cell, converts light emission to a numerical value, and communicates the results (52). Other measurements may be added to the IC depending on the desired application. A prototype was created with the toluene bioreporter *Pseudomonas putida TVA8* (*TVA8*) (52). *TVA8* was placed on an optical application specific integrated circuit (OASIC) (52). Bioluminescence was detected when the *TVA8* cells were induced with toluene vapor suggesting that the BBIC design was successful (52).

The next requirement for a biosensor is a bioresistant and biocompatible IC coating (52). This will protect both the bioreporter and IC from field conditions. A coating that shows promise for BBIC devices is silicon nitride (SiN) film deposited by molecular-jet chemical-vapor deposition (MJ-CVD) (52). SiN films are resistant to attack by chemical and biological materials (52). They are optically favorable because they act as an antireflective coating to block out background noise (52). The MJ-CVD technique allows

deposition of thin films in a layer-by-layer process (52). Because this technique involves a small orifice, which the source gas passes through, the surface is only exposed for short periods of time and allows a reduced gas load (52).

The final BBIC requirement is a method to adhere bioreporter cells to the IC (2, 52). It is important to keep cells on the IC to allow biosensing measurements to be taken. It is also desirable to prevent the escape of genetically engineered organisms into the natural environment. These goals can be accomplished through encapsulation/immobilization. As with the IC coating, the encapsulation procedure must be able to withstand biological/chemical attack and must be stable in the field (2, 52). In addition, the encapsulation procedure must be gentle enough to preserve cell viability, but allow long-term storage (47). One technique that has been used is encapsulation in soft gels of agar or alginate. These materials are gentle on the cells but have some significant drawbacks. For example, agar may be used as a nutrient source by bioreporter cells and microbial cells from the natural community. Alginate has some improvements as an encapsulation matrix but may dissolve when exposed to phosphate buffer or other chelates (22, 26). Other techniques such as antibody attachment of cells to surfaces and the use of latex for immobilization may be too complicated or costly for routine use (6). One material that shows promise is a silica matrix fabricated through the sol-gel technique.

The Sol-gel Technique

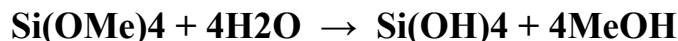
Silica sol-gels are quick and easy to make. They are more structurally stable than soft gels such as alginate or agar (26, 44). They are inorganic and cannot be utilized as a food

source for microorganisms (29). They show resistance to chemical and biological attack. Furthermore, silica gels are porous allowing diffusion of water, oxygen, nutrients, and target analytes, while displaying negligible swelling and remaining nontoxic and biologically inert (11, 29). All of these benefits indicate that silica gels may be a good choice for the encapsulation of bioreporter cells.

The sol-gel process proceeds through several steps as depicted in figure 1. The first step, hydrolysis, involves the catalysis of silicon alkoxides which is close to the natural biomineralization of silica with silicic acid (29, 56). Hydrolysis results in a liquid “sol” that is composed of reactive Si-OH groups (3). The second step, condensation, results in gel formation (56). Condensation occurs spontaneously when organics are added to the inorganic sol (3, 29, 56). Condensation is caused by a pH change and results in the formation of oxo bridges (3, 29). Either acids or bases may catalyze hydrolysis and condensation. Acid catalysis results in a microporous gel (pore size less than two nanometers) while base catalysis results in a mesoporous gel (pores greater than five nanometers) (3). One typical process involves the hydrolysis of tetramethylorthosilicate (TMOS) with hydrochloric acid and water (44). Condensation occurs when cells are

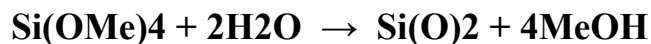
Step 1: Hydrolysis of Silicon Alkoxides

- ❖ Tetramethylorthosilicate [Si(OMe)₄,] water, and acid are mixed to form a sol.
- ❖ The sol is a slightly opaque or clear, viscous liquid.
- ❖ The reaction continues after the sol is formed but the rate of the reaction can be increased by a change in pH.



Step 2: Condensation

- ❖ As mentioned above, condensation occurs spontaneously following sol formation.
- ❖ The consequence of condensation is gel formation.
- ❖ When cells are encapsulated, the addition of cells in a slightly basic medium changes the pH enough to result in condensation and gelation in the space of a few minutes.



Step 3: Ageing of the Gel

- ❖ Silica crosslinking continues after gelation.
- ❖ Crosslinking results in increased elasticity of the gel as well as stiffening and pore shrinkage.
- ❖ Gels must be kept wet to prevent drying. Dried gels are known as xerogels.

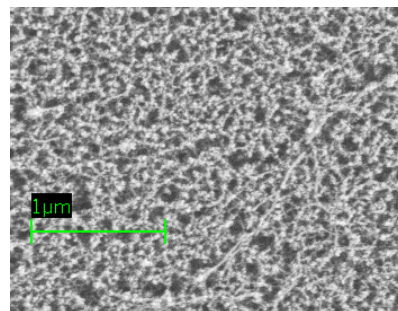
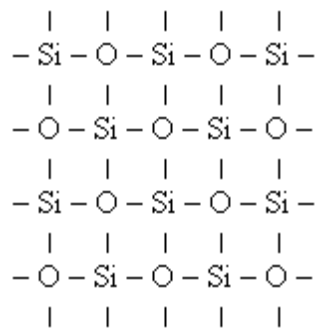


FIGURE 1. THE SOL-GEL PROCESS.

Sol-gel chemistry proceeds through the steps of hydrolysis, condensation, ageing, and densification. Densification is not depicted in this figure because it is not used with encapsulation of cells.

added in slightly basic media. The resulting gel has a pore size of one to ten nanometers (nm) (3).

The next step in the sol-gel process is ageing of the gel. This occurs naturally over time and involves the continued crosslinking of silica molecules in the matrix (29, 56). Ageing also results in stiffening and shrinkage of the gel as well as an increase in gel elasticity (56). After gelation, gels may be air dried to give xerogels or treated with high heat and pressure to make dense ceramics (56). When biological materials are to be encapsulated it is important to prevent drying and keeps gels wet. As gels dry water is expelled and the pores shrink and may collapse. The small pores prevent diffusion and along with the loss of water results in decreased viability (14, 16, 19, 22, 34, 44). The report by Dunn et al. suggests that decreased viability results from the loss of solution instead of pore shrinkage (14). This was confirmed by Bergogne et al. who suggested that 70% water was needed for optimal activity (3). If an application requires the use of xerogels, gels should be made and dried before biological materials are added as in the report of Power et al. (42).

Encapsulation of Enzymes

There are many reports of using the sol-gel process to encapsulate enzymes. Narang et al. encapsulated glucose oxidase in a “sol-gel sandwich” and observed a good activity even after a week of storage (34). Dunn et al. compared encapsulated cytochrome C oxidase to that in solution (14). They observed an increased stability of encapsulated enzyme when exposed to methanol. Sol-gel encapsulation prevented the irreversible aggregation of

enzyme (14). Bhatia and Brinker made a similar observation with horseradish peroxidase and glucose-6-phosphate dehydrogenase (4). The study by Martinez-Pérez et al. combined the indicator amplex red with a uricase-peroxidase system in a sol-gel matrix to form a bioreporter for uric acid in biological fluids (30). They saw no difference in reaction times when compared with enzymes in solution as well as a lower detection limit (30).

Park and Clark described the creation of “solzymes,” arrays of sol-gel encapsulated enzymes (37). This array will allow for high-throughput screening of biocatalytic activity. This is a practical application of sol-gel technology that is low cost, versatile, and involves simple detection and quantification (37). In addition, the solzymes showed better thermostability than enzymes in solution (37). A similar array for the “reagentless fluorimetric detection of glucose” was developed by Rupcich and Brennan (46). Sakai-Kato et al. reported the development of an on-chip enzyme reactor involving encapsulated trypsin (48). The microchip can be utilized for over a week when stored at 4°C while a solution of trypsin is viable for a day (48). The microchip also allows a smaller sample size and shorter reaction time (48). Overall, encapsulated enzyme studies describe increased enzyme stability and detection, with decreased reaction time and detection limits. The matrix stabilizes the enzyme without prohibiting movement and activity.

Encapsulation of Whole Cells

Sol-gel may also be used to encapsulate whole cells. Since cells are metabolically active they require different considerations than enzymes. One debate with the encapsulation of cells is whether they can withstand the condensation reaction with silicon alkoxides. The main concern is the alcohol produced as a result of condensation (29, 56). This has led to a variety of alternate encapsulation methods. The main alternative to the alkoxide route (through the use of silicon alkoxide) is called the aqueous route. This method described by Bhatia and Brinker, utilizes the acidification of aqueous sodium silicate followed by removal of excess sodium (4). The resulting gel is mesoporous and there are no alcohol byproducts formed (10). Other reports suggested the use of additives such as glycerol or polyethylene glycol and glycidoxypopyltrimethoxysilane to improve viability and protect cells from the stress of encapsulation (17, 35, 36). Another technique by Ferrer et al. used a Rotavapor to remove methanol after it had been formed through the alkoxide method (17).

The theory is that release of alcohol will damage the membranes of encapsulated cells (4, 10, 17). Premkumar et al. looked at the consequence of methanol on encapsulated cells in two separate studies utilizing the alkoxide route for sol-gel preparation (43, 44). In these assays they observed general toxicity and genotoxicity bioreporters for induction of bioluminescence from methanol released by the gel. Only a low level of bioluminescence was detected from the organisms suggesting that cells were not stressed by methanol formation. A third study by the lab reported that methanol release was too slow for cells to be affected (45).

An additional concern over the use of sol-gel encapsulation with whole cells is the matrix and whether there will be additional mass transfer issues for the cells (7, 8). Several reports did not find any significant mass transfer issues (24, 44, 45). Premkumar et al. suggested that because the response pattern of cells in solution to those encapsulated was similar, gels did not limit mass transfer for encapsulated cells (45). In another study they compared thick to thin films and saw a similar response in both (44). A delayed response in the thicker films would imply mass transfer issues, which did not occur. Inama et al. observed similar Lineweaver-Burke plots and K_m values from encapsulated cells and cells in solution suggesting there are no mass transfer limitations (24).

The next basic question of encapsulated cells is whether they are able to divide within the silica matrix. While the report by Pope saw the budding of yeast cells from the surface of gels (40) and Armon et al. used gels to grow a surface biofilm (1) it does not appear that cells are able to divide within silica gels. A study by Premkumar et al. followed individual cells through confocal microscopy and did not observe any cellular division (45). In addition, Inama et al. measured the tensile strength of silica gels and saw that it was enough to prevent cellular division (49). In contrast, Branyik et al. observed the formation of a biofilm on gels, which they attributed to division of cells within the matrix (13).

Another question involves how well cells are encapsulated and if they are able to escape from the matrix. As mentioned in the previous paragraph Pope observed the budding of

yeast cells from the surface of gels (40). Inama et al. also observed the escape of yeast cells from the matrix. They were able to increase cellular containment by adding a layer of silica gel without cells to the surface of their gels (24). Premkumar et al. used rinsing to remove cells that were not encapsulated. After rinsing there was less than a five percent decrease in fluorescence and no residual fluorescence observed after gels were removed (45). This suggests that most, if not all cells are well encapsulated, and escaped cells are in all likelihood from the gel surface.

There are also differing observations on the activity of encapsulated cells. Branyik et al. reported a “less effective” oxidation of phenol by encapsulated cells and suggested this could be due to mass transfer limitations or by surface cells utilizing the phenol before it could reach cells on the gel interior (8). Other studies suggested only a slight decrease in activity for encapsulated cells (40, 45). And yet others observed increased activity of cells in sol-gel (10, 15, 35, 44). Fennouh et al. attributed the activity of cellular enzymes to the random dispersion of cells in the gel, which prevents agglomeration (15). Nassif et al. attributed the increased activity to the protective effect of encapsulation while Coiffer et al. suggested that the disruption of the cellular membrane might explain the increase (10, 35). As with encapsulated enzymes, many reports suggested that cells in a sol-gel matrix are protected and remain active after storage (10, 17, 28, 33, 35, 43, 44, 45, 47).

Novel Applications of Sol-gel Encapsulation

A novel feature of the encapsulation of cells into sol-gel is that multiple species may be encapsulated together (45, 47). Because cells are randomly dispersed throughout the gel

one species does not dominate as in natural settings. This would allow the use of a microbial consortium in sensing and degradation applications (45). Gelman et al. utilized this idea by encapsulating acid and base catalysts in the same gel (18, 45). Because the catalysts can only react with chemicals that diffuse into the gel they can be stably maintained without reacting with each other (18). Another idea is to use the advantages of *lux* and *gfp* in the same sensing application by encapsulating two biosensors together (47). The bioluminescent reporter cells would give rapid and sensitive detection while the *gfp* expressing strain could provide information over a long exposure and offer cumulative detection (47). Or as described by Premkumar et al. a *gfp* expressing strain was encapsulated with a strain expressing red fluorescent protein (*rfp*) in the same gel (45). Both markers could be visualized under the confocal microscope and cells from both strains were located adjacent to one another in the gel (45).

The sol-gel technique may also be extended to medical applications. One main area of research is utilizing the sol-gel technique to encapsulate tissue for transplants (5, 41). The sol-gel matrix is porous enough to allow diffusion of nutrients into gels and products out, but small enough to prevent attack of transplanted tissues by antibodies and microorganisms. In diabetic patients, not enough insulin is produced. The transplantation of pancreatic islets of Langerhans would remedy this condition. By encapsulating the islets in silica gel, Pope et al. demonstrated that islets were protected from bacterial infection and antibody attack (41). One mouse that received the encapsulated islets had outstanding performance with blood glucose levels maintained for eleven weeks (41).

The same conclusion was reached in Boninsegna et al. with a gaseous application of silica oxide (SiO_2) onto islets (5).

Another area of application is with dental and orthopedic surgery. Yang et al. developed an epoxy- SiO_2 hybrid cement (57). The hybrid displayed superior mechanical properties as well as a low polymerization temperature and very low cytotoxicity when compared to the currently used Simplex®P cement (57). Meseguer-Olmo et al. developed a sol-gel glass combined with the antibiotic gentamycin ($\text{SiO}_2\text{-CaO-P}_2\text{O}_5$) to be used in orthopedic surgery (32). The glass is applied over implants and bone during surgery and helps prevent infections. Gentamycin concentrations were higher in bone than in other organs indicating that the antibiotic was contained at the surgical site by the sol-gel (32). In addition, sol-gel glass allowed newly formed bone to penetrate the implant while the sol-gel was reabsorbed and the whole process exhibits a low inflammatory response (32). All reports indicated encouragement with results observed and researchers are currently carrying out further studies.

Chapter Two

MATERIALS AND METHODS

Cell Culture

All experiments were performed with *Pseudomonas fluorescens* 5RL. The media used for growth of 5RL cells was Luria Broth (LB) (10g tryptone, 5g yeast extract, 10g NaCl, 1L deionized water) supplemented with 14 µl/ml tetracycline (Fisherbrand). A stock solution of tetracycline was prepared with 14 mg tetracycline in 5ml of sterile water and 5ml of ethanol and stored at -20°C in a light safe container. During experiments Minimal Salts Media (MSM) was utilized (2g NaNO₃, 0.75g KH₂PO₄, 0.003g FeCl₃, 0.1g MgSO₄, 0.005g CaCl₂, 0.25g Na₂HPO₄, 1L-deionized water). For plate count experiments cells were grown on LB plates (10g tryptone, 5g yeast extract, 10g NaCl, 17g agar, 1L deionized water) supplemented with 14 µl/ml Tetracycline and 30ppm sodium salicylate. All media was sterilized in a Consolidated autoclave at 250°C and 20 PSI for 20 min before use. Tetracycline was added after sterilization of the culture medium.

Sol Precursor Preparation

The sol-gel encapsulation technique was adapted from Premkumar et al. (44). To prepare the sol precursor, 8ml of tetramethylorthosilicate (TMOS, ACROS Organics) was added to a clean glass 50ml tube along with 4ml of HPLC-grade water (Fisher Scientific) and 1ml of 0.1M HCl. All reagents were chilled to 4°C before use. The precursor mix was kept on ice and sonicated on setting 4 (20% power) with a Virsonic 300 (Virtis Company) for 10 min until completely mixed. After sonication, the sol was left to age

overnight at 4°C. After ageing sol was either used to make gels or left in storage at 4°C. Each sol mixture was used within ten days of preparation.

Encapsulation of cells

After aging overnight, the sol precursor was removed from cold storage. From a log phase *5RL* culture 3ml was removed and added to 1 ml sol in a 10 ml disposable test tube. The test tube was briefly vortexed to ensure a homogenous mixture and then dispensed in 400µl aliquots into individual microplate wells. The mixing and dispensing step was performed quickly before gelation of the sol-gels. Either Greiner 24-well, solid bottom, black microplates or sterile, clear Costar® 24-well cell culture microplates were utilized. Greiner plates were treated with ethylene oxide for sterilization.

After gelation, “Bio-gels” (*5RL* cells encapsulated in sol-gel) were allowed to dry for five minutes to ensure a thorough reaction and to facilitate crosslinking of silica. After drying, 1 ml of MSM was added to the top of each gel to rinse. Next, MSM was poured off and another 1ml aliquot of MSM was added to gel surface. Plates were sealed with Topseal™ A plate sealers (Perkin Elmer) and stored at 4°C until use. All plates were stored for at least 24 hours before use.

Protein Assay

In order to arrive at conclusions about cells within the sol-gel matrix it is necessary to quantify the number of cells encapsulated. To this effect, the Pierce Coomassie Plus™ protein assay was modified to provide an estimate of encapsulated cell number. Gels

were made as usual and aliquoted into a Costar® microplate. After gelation and 5 minutes of drying, gels were rinsed with MSM media and then 1ml of MSM was added to the top of gels until ready for use.

Coomassie Plus reagent was removed from storage at 4°C and allowed to come to room temperature. When ready for use, media was poured off gels and gels were crushed by tapping with a wooden applicator. 1.5ml Coomassie reagent was added to crushed gels, mixed, and allowed to bind for 15 minutes at room temperature. After 15 minutes of incubation, plates were read in a Packard SpectraCount® Microplate reader at 610 nm.

With the purpose of estimating cell number, a calibration curve was created using known cell numbers from a plate count assay versus protein absorption data. Aliquots of a 5RL log phase culture were diluted and plated as with plate count technique. From the same culture, gels were made, and simultaneously used in a protein assay as described previously. Protein absorption was plotted versus plate counts and a regression equation for cell number was calculated. After the generation of this equation, subsequent protein assay data was inserted to give an estimate of encapsulated cell number.

Escaped Cells Assay

Bio-gels were prepared and placed into a 24-well Costar® brand clear plates. After gelation of Bio-gels, MSM media was placed on top of gels. At time points of 2, 4, 24, 48, and 72 hours 100µl of media was removed from the media on top of random gels in triplicate and plated. An additional aliquot of media was used for serial dilution, which

was also plated in triplicate. Plates were placed in a 25°C incubator and observed for colonies. After 48 hrs plate counts were taken. In addition, at each time point a protein assay was performed on three gels. Gels that were crushed for protein assay were not reused. Plate counts and a protein assay were also performed on the original culture to provide an estimate of initial cell numbers.

Transmission Electron Microscopy

For primary fixation, Bio-gels were cut into 1mm³ pieces and fixed for one hour in a 3% glutaraldehyde buffer (1ml 25% glutaraldehyde, 3.2 ml dH₂O, and 4.2 ml 0.2M Cacodylate buffer). The pieces were then rinsed with 0.1M Cacodylate buffer to remove glutaraldehyde. For secondary fixation, 5ml 4% OsO₄ and 5ml buffer was used. Gels were fixed for one hour. After fixation, gels were dehydrated with ethanol and embedded in Spurr's Embedding Media. After curing the epoxy, the blocks were trimmed and thin sections cut on a Reiker OMU3 microtone with a diamond knife. Sections were placed on 400nm thin bar mesh and stained with uranyl acetate in 50% methanol and lead citrate. A Hitachi H800 Transmission Electron Microscope was used to visualize sections.

Scanning Electron Microscopy

Bio-gels were left intact during fixation for SEM. Gels were fixed for one hour with 3% glutaraldehyde buffer (1ml 25% glutaraldehyde, 3.2 ml dH₂O, and 4.2 ml 0.2M Cacodylate buffer). They were washed with 0.1 M Cacodylate buffer to remove glutaraldehyde and then dehydrated with acetone. Gels were transferred to a mesh holder and critical point drying was performed in a Ladd Critical Point Dryer. Dried gels were

mounted on a stud with copper tape. Next, gels were sputter coated with gold for 20 seconds at 20 milliamps in a SPI Sputter Coater. In an attempt to lessen charging artifacts, gels were painted around the edge with carbon paint. Gels were visualized with a Leo 1525 Scanning Electron Microscope.

Bioluminescence Experiments

Bio-gels were removed from storage and the media was poured off. Gels were rinsed once with MSM by rapid pipetting. Next, 1ml of sterile MSM containing 30ppm sodium salicylate was added to wells containing gels. After its addition, salicylate diffuses through the sol-gel matrix and induced cells as shown by an increase in bioluminescence. Finally plates were sealed with Topseal™ A plate sealers to prevent evaporation of media and drying out of gels.

To mimic the concentration of cells in Bio-gels, 300µl of “Free cells” (non-encapsulated cells in suspension) were added to wells of a Greiner microplate along with 100µl of sterile MSM. Free cells must be stored in LB in order to maintain viability during storage. After addition of cells into the microplate, 1ml of sterile MSM containing 30ppm salicylate was added to each well containing cells. Finally plates were sealed with Topseal™ A plate sealers to prevent evaporation of media.

Negative control wells were prepared with either sol-gels with MSM and salicylate or sterile LB with MSM and salicylate. This provides a background reading of the scintillation counter and insurance against contamination. In addition, non-induced

controls were prepared with Bio-gels and Free cells that were given sterile MSM without salicylate. This provided a background level of bioluminescence from *5RL* cells and allowed determination of true induction.

After all plates were prepared and sealed they were placed in a 1450 Microbeta Liquid Scintillation Counter and monitored for bioluminescence. The Microbeta was kept at room temperature and measurements were taken each hour unless otherwise specified. Readings were given in the arbitrary measurement of the Microbeta, Luminescent Counts Per Second (LCPS).

Oxygen Monitoring

Cellular respiration rate was monitored with a YSI 5300A Biological Oxygen Monitor. The monitor utilizes Clark-type polarographic oxygen probes that were calibrated to 100% air by immersion in air-saturated water. For oxygen experiments Bio-gels were prepared in Costar® plates as usual. A Bio-gel was carefully removed from the microplate and placed into a glass 5356 Micro Oxygen Chamber inside a 5301B Bath. The bath was connected to a MGW Lauda RM6 circulating water bath maintained at 25°C. MSM (3ml) was added to each chamber and allowed to come to temperature. For monitoring of Free cells, 300µl of cells in LB were added to each chamber along with 3ml of MSM, and allowed to come to temperature.

After chambers reached the correct temperature and probes were calibrated to 100%, 30µl glucose (0.55M) was added to the MSM in each chamber. Oxygen probes were immersed

in MSM and the percent air was recorded each minute until 0%. Data was converted from % air to $\mu\text{gO}_2/\text{ml}$ and a respiration rate was calculated. Probes were removed from chambers, rinsed, and stored in sterile water.

Long-Term Experiment

Greiner plates were set up and placed in storage at 4°C as depicted by figure 2. Plate A contained 16 Bio-gels in rows one to four and 4 sol-gels in row six. Bio-gels and sol-gels were prepared as normal. Plate B contained 12 Bio-gels in rows one to three while rows four to six were left empty for Free cells. Plate C was left empty for Free cells in rows one to four, and LB in row six. Aliquots of culture in LB were placed in 15ml disposable tubes for Free cells. A Costar® plate was also prepared with Bio-gels in each well, and designated Plate D.

Six sets of the above plates were prepared and randomly labeled as 0, 2, 4, 6, 8, and 10 weeks. All plates were sealed with Topseal™ plate seals. Plates and tubes were placed in 4°C for storage. Empty plates were kept sealed until use. After overnight ageing the “0 week” plates and tube were removed from storage. MSM was poured off plates A and B. Free cells were aliquoted into plates B and C. MSM with salicylate was added to plates A and C to induce cells. MSM without salicylate was added to plate B for a control. Plates A through C were placed in the Microbeta to monitor bioluminescence. Four Bio-gels were removed from plate D and used in the oxygen monitor as previously described. Four aliquots of Free cells were also tested in the oxygen monitor. In addition, oxygen monitoring was extended with the addition of carbon quantification to estimate the

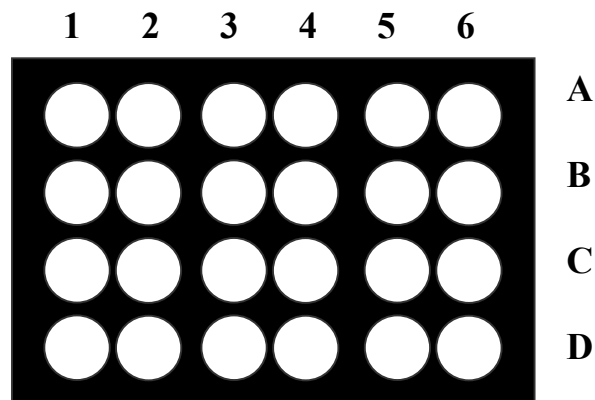


Plate A: Induced Bio-gels (IN Gels) and NC

- Bio-gels in wells A1 to D4
- Sol-gels in wells A6 to D6
- MSM with 30ppm salicylate added to each well

Plate B: Non-induced Bio-gels (UN Gels) and Free cells (UN Cells)

- Bio-gels in wells A1 to D3
- Sol-gels in wells A4 to D6
- MSM added to each well, NO salicylate

Plate C: Induced Free cells (IN Cells) and NC

- Free cells in wells A1 to D4
- MSM in wells A6 to D6
- MSM with 30ppm salicylate added to each well

Plate D: Protein assay and Oxygen uptake

- Bio-gels in all wells
- Clear Costar plates used instead of black Greiner plates
- Bio-gels removed and used for protein and oxygen uptake experiments

FIGURE 2. EXPERIMENTAL DESIGN OF LONG-TERM BIOLUMINESCENCE ASSAY.

Six sets of plates were set up as displayed above. Plates were sealed and stored at 4°C until removed for use at 0, 2, 4, 6, 8, or 10 weeks.

amount of carbon respired (see below). Finally, Bio-gels from plate D were crushed and used in protein assays as described above.

After 2, 4, 6, 8, and 10 weeks additional plate sets were removed and tested for bioluminescence, oxygen, and protein. Results were collected and analyzed. Results were compared statistically with each other to estimate the long-term storage potential of *5RL* Bio-gels and Free cells.

Carbon Quantification

To determine the amount of carbon respired by cells during oxygen monitoring, samples from oxygen chambers were analyzed in a Shimadzu Total Organic Carbon Analyzer (TOC-V_{CSH}). After oxygen monitoring, aliquots were removed from each chamber, placed in 2 ml Eppendorf tubes, and frozen at -70°C for carbon quantification. When ready for testing, aliquots were thawed and 1ml was added to glass wool in a ceramic sample boat and inserted into the Solid Sample Module (SSM-5000A). Samples taken after oxygen monitoring were spun down in a centrifuge to settle any cellular debris. A calibration curve was formulated from TOC peak area values versus known glucose concentrations. A regression equation was generated and used to predict glucose before and after oxygen monitoring to calculate the amount of glucose respired for each sample. This was then compared to the theoretical yield of *Escherichia coli* cells to predict the amount of carbon that would be utilized by *5RL* cells in normal respiration versus the amount given off as a by product (55). These predictions will not be exact because *5RL*

and *E.coli* have different types of metabolic processes, they will only give an approximation.

Anaerobic Induction of Bioluminescence

Bio-gels were made as usual except that 200µl pre-gelled solution was pipetted into 50x15mm shell vials instead of aliquoting into a 24-well plate. After gelation, Bio-gels were dried as usual for five minutes. MSM was placed on top of gels, vials were sealed with parafilm, and left to age at room temperature overnight. Sol-gels were prepared in the same manner except that LB used to make gels was sterile to give a negative control of gels without cells. After cells were removed for encapsulation, the remaining culture was placed in a 15 ml tube for storage (Free cells). Free cells were left in LB to support cells during overnight storage (cells left in MSM alone did not remain viable for the experiment).

To begin the experiment, MSM was poured off vials with gels and 2ml fresh MSM with 1µl resazurin (5mg/ml, Sigma) was added to allow colorimetric monitoring of oxygen. Free cells (200µl) were aliquoted into separate vials with 2ml MSM and 1µl resazurin. All vials were then sealed with Fisherbrand Turnover Septa Rubber Stoppers (No. 13) and sealed around the edge with parafilm. Sealed vials were bubbled with nitrogen via 2.5-inch 25-gauge needles for 10 min. After bubbling, cells were induced with 30ppm salicylate added via syringe, and then monitored for bioluminescence with the Femtomaster FB14 (Zylux Corporation).

Bioluminescence was measured every 15 minutes until the light began to level off. Next, caps were removed and bioluminescence was measured immediately, and again after 5, 10, 15, 30, 60, 90, and 120 minutes. Results for all three groups were plotted and statistics calculated for each.

Why does the Light go Out?

Bio-gels were prepared as usual and dispensed in 400µl aliquots into rows 1-4 of three separate 24-well Greiner plates. Row 5 was used for sol-gels made with sterile LB for no cell, negative controls. After overnight ageing, MSM was poured off Bio-gels and 1ml of fresh MSM containing 30ppm salicylate was added to top of gels and plates were sealed. Plates were then read every five hours in a 1450 Microbeta Liquid Scintillation Counter.

After 150 hours when bioluminescence had peaked and then declined, plates were removed from the Microbeta and subjected to one of three separate treatments. Plate one was opened, the media removed, and gels exposed to air for ten minutes. After air exposure, media was placed back into the plate, and the plate was re-sealed. Plate two was opened, 10µl glucose (0.555M) was quickly added and then the plate was resealed. Plate three was opened, the media removed, and gels exposed to air for ten minutes. After air exposure, media was placed back into the plate along with 10µl glucose (0.555M), and the plate resealed. This gave the treatments of Add Oxy, Add Glu, and Add Both. Sol-gels were unsealed but nothing was added.

After treatment, plates were placed back into the Microbeta reader and observed every five hours. Treatment was repeated after 420 hours with plates placed back into Microbeta reader. At 715 hours the treatment was revised and 10 μ l glucose (0.555M) was added to the Add Oxy plate in an attempt to restore function. Other plates were not given treatment. Plates were read again in the Microbeta reader. Throughout all treatments plates were read for a continuous 865 hours (36 days). Data was graphed and statistically analyzed.

Statistical Analysis

Statistical analysis was performed with SPSS version 12.0 for Windows. The tests utilized in this study included the Independent Samples t-Test, the One Sample t-Test, Regression analysis, univariate Analysis of Variance (ANOVA), and Multivariate Analysis of Variance (MANOVA). ANOVA and MANOVA tests were performed with the univariate and multivariate General Linear Model in SPSS. All tests were performed with an alpha (α) of 0.05. The Dunnet T3 Post Hoc test was utilized for ANOVA and MANOVA. If repeated measures were utilized in an assay, the statistical tests were compensated for by the software or by dividing α by the number of measures. Graphs were plotted in either SPSS or Microsoft Excel. Microsoft Excel was also utilized for calculating the averages and standard error of assays.

Chapter Three

RESEARCH QUESTION ONE: ARE CELLS ENCAPSULATED?

Protein Assay

In order to make quantitative observations on experiments with encapsulated cells it would be beneficial to be able to quantify the number of cells that are encapsulated. Previous studies have attempted to culture cells after encapsulation by crushing the resulting gels and spreading them on a plate (8, 10). However, this method presents some caveats: because cells are enmeshed within the sol-gel matrix it seems improbable that all cells would be released during the crushing step. Therefore, this technique would not give an appropriate estimate of cell numbers. In the present study an alternate technique for estimation of encapsulated cell number is used based on a colorimetric protein assay. The Pierce Coomassie Plus™ protein assay was chosen. This assay utilizes Coomassie Blue G-250 dye that changes from red to blue after binding to the NH_3^+ groups of proteins (6, 51, 54). This type of assay was selected because it is simple, rapid, and has low interference with amino acids and other chemicals (51). In addition the reagent has a long shelf life and high sensitivity when compared to the reagents used in other techniques (49, 51). Sol-gel makes this type of assay possible because it is optically transparent. This allows the resulting assay to be read in a spectrophotometer without interference from the gel itself. The resulting absorbance data is a measurement of encapsulated protein alone.

For quantification of cells in subsequent experiments using the protein assay, a preliminary “known” data set was examined. The assay was performed with dilutions of a

stock culture. Protein measurements were made with the Spectra Count at 610nm, and cell numbers for the stock culture were estimated from plate counts. Next, a regression equation was derived from the comparison of protein data and viable plate counts using a quadratic curve. This type of curve was suggested by the Pierce Protein assay protocol. The resulting equation is: $y = -3.9026 \times 10^{10} + 6.626 \times 10^{10}(x) - 2.095 \times 10^{10}(x^2)$ where y represents the theoretical CFU/ml and x represents the protein absorbance at 610 nm.

When a protein assay is performed, the absorbance values from the plate reader at 610nm can be entered into this equation as X to predict Y , which is theoretical CFU/ml. This can then be used to determine the effect of a specific number of encapsulated cells or can be further modified to represent overall biomass. The regression equation has an adjusted R-squared (R^2) value of 0.9680 that suggests the equation is a good fit with the data (figure 3).

There are a few issues with this method. One such issue is that the equation was derived from cell numbers of a viable plate count method. This method is variable in the number of cells that grow. This gives a high standard error, which makes the assay less accurate. In addition, this technique only shows the viable cells that are able to be cultured. It is possible that there could be cells in a viable/non culture-able state (VBNC). These cells will not grow on plates but will be counted in the protein assay. The Coomassie reagent binds to any type of protein including protein from the LB medium, dead cells, and live cells (including those that cannot be cultured). Protein from LB is discounted by the negative control, which has media but no cells. The dead cells and VBNC cells will

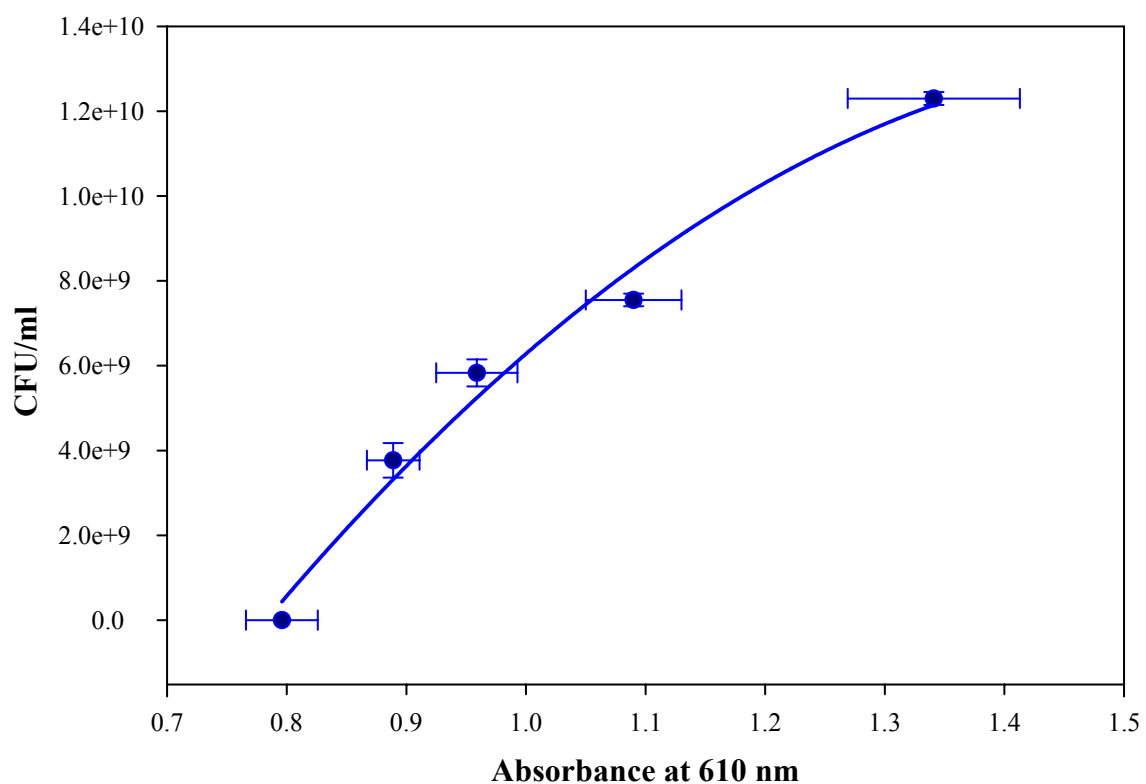


FIGURE 3. STANDARD CURVE FOR PROTEIN CONCENTRATION.

The best fit line has an adjusted $R^2 = 0.9680$ indicating that the curve fits the data well. Each point is the mean of six replicates. X error bars indicate the standard error for protein absorbance, while Y bars indicate the standard error of plate counts.

contribute to the protein assay value but not the plate counts. Overall, it would seem that the plate count method might under-represent the number of cells in an assay. Alternate methods such as staining with acridine orange may be a potential remedy for these issues however, because gels are irreversibly bound in the matrix it may prove difficult to enumerate cells.

Another, more specific issue with the technique is that the protein assay must be followed exactly to give accurate results. In preliminary experiments only 1 ml of Coomassie Plus reagent was used and the results from independent assays were dissimilar. The assay could also be improved by using a plate reader that read at 595nm, which is the optimum absorbance for Coomassie Plus reagent. The curve described above was generated from readings taken at 610nm. The reagent can be read from 575 to 615nm, but if not read at 595nm the assay sensitivity will be reduced.

It is also important to make a negative control gel for each protein assay performed. This will ensure that all conditions are correct. The negative control should be a gel made exactly like Bio-gels being utilized except without cells. For the encapsulation method utilized in these studies the protein reading at 610nm should be greater than or equal to 0.783. If assay readings are lower the regression equation will predict a negative number of cells. The use of a negative control will ensure that there is enough protein in the medium to fit the background of the curve. If protein readings are below 0.783 with the negative control it is necessary to redo the initial experiments and derive a new regression equation for encapsulated cells.

Escaped cells assay

One potential application for sol-gel encapsulated bioreporter cells is to place them onto a signal processor and use the resulting biosensor in the field. A concern with using biosensors in the field is the release of genetically engineered bacteria into the environment. The engineered bacterium may have genes that would give it a metabolic advantage over natural species. The presence of an “escaped” species may change the population dynamics of the environment that it is placed, which may be undesirable for both scientific investigations and the natural environment. To address this concern, an “Escaped” cells assay was performed to quantify the number of cells that escape encapsulation in comparison to those encapsulated. This assay will also provide an idea of the success of the sol-gel technique for encapsulating bacteria.

Escaped cells were cultured from each time point (figure 4). The fewest number of cells were detected at the two-hour time point. After two hours the number of escaped cells increased until twenty-four hours and then began decreasing. At four hours there were five times as many escaped cells than at two hours, at twenty-four hours 1,000 times as many. At forty-eight and seventy-two hours there were two times less cells cultured than at twenty-four hours. Because the media was replaced after the twenty-four and forty-eight hour points the cultured cell number is not quantitative. Cells cultured at the twenty-four hour point escaped in the first twenty-four hour block while cells cultured at the forty-eight hour point escaped in the second twenty-four hour block, and so on. ANOVA analysis was performed on the results. These tests indicate that there are no significant differences between the number cells that escape over time ($p > 0.05$). This

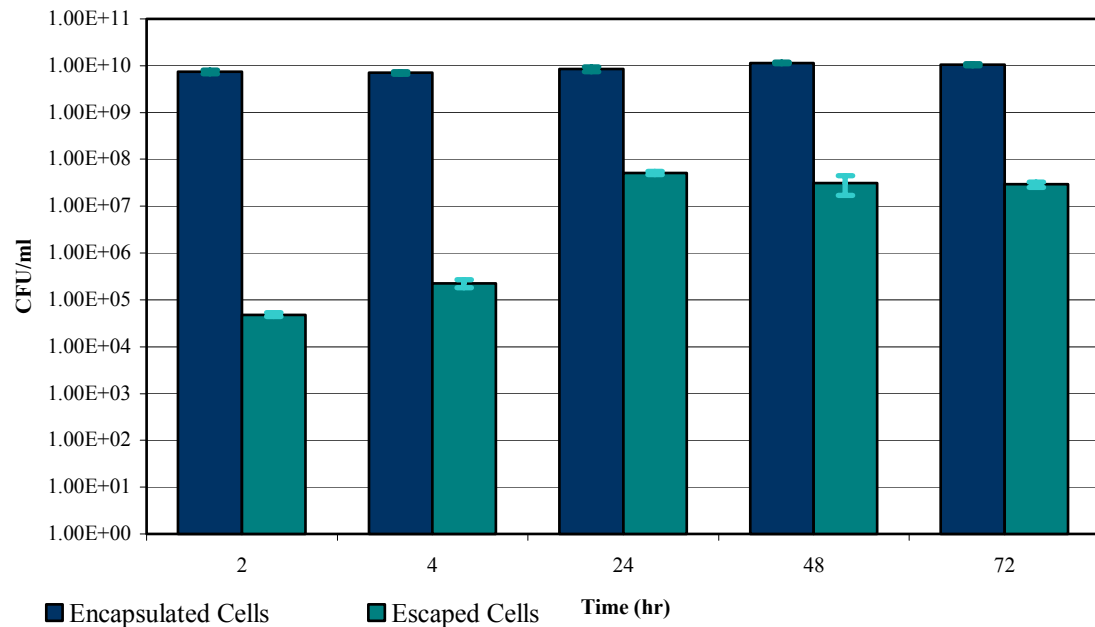


FIGURE 4. ENCAPSULATED VERSUS ESCAPED CELLS.

This graph depicts the number of cells that are predicted by the protein assay to be encapsulated as well as those that were cultured in plate counts as “escaped.” Bars represent the mean of six replicates, with error bars depicting the standard error.

implies that while the number of cells that escape changes over time, the difference is not significant.

The highest estimate of encapsulated cells from a protein assay occurred at forty-eight hours. It is evident that cells remain in the gel (from the high estimate of encapsulated cells) yet there is a high estimate of escaped cells in the previous measure. This suggests that escaped cells may be a few cells that escape and begin dividing in the media on top of gels. This would provide a high plate count for escaped cells while still allowing a high estimate of encapsulated cell number. In addition, some of the encapsulated cells estimated in the protein assay may be cells growing on the gel surface. Gels were rinsed before protein assays were performed but it is not certain if all were removed. ANOVA analysis was performed with SPSS. These tests indicate that there is a significant difference in the number of cells encapsulated over time ($p = 0.010$). This provides support for the idea that cells are forming a surface biofilm on gels or may indicate cellular division inside.

Encapsulated cells were compared with escaped cells in figure 4. (Notice the log scale that is utilized in order to observe escaped cells). At 24 hours, the greatest concentration of escaped cells, there are 160 times more cells encapsulated than cells that have escaped. At all other time points this value is greater (with 150,000 times more cells encapsulated at two hours). MANOVA analysis was performed on the assay results. These tests indicate a significant difference over time ($p = 0.001$) as well as a significant time*type interaction ($p = 0.001$). Type indicates the different groups of encapsulated and escaped.

One interesting observation was that at the two-hour time point encapsulated and escaped cell numbers are following a similar trend ($p= 0.929$). The other time points only show marginal interactions ($p= 0.069, 0.080, 0.069$). These tests could not be extended due to a small sample size.

Overall the tests suggest that while a large number of cells have escaped, this is not close to the larger quantity of cells that remain encapsulated. Premkumar et al. made similar observations from a fluorescence assay with encapsulated bioreporter cells (45). In their study, less than 5% of bioluminescence was attributed to non-encapsulated cells after Bio-gels were rinsed. In addition, there was no lingering fluorescence from non-encapsulated cells after Bio-gels were removed from the fluorescence detector. They concluded that the encapsulated bacteria, solidly embedded in the sol-gel matrix, generated fluorescence. In the current Escaped cells assay, the quantified escaped cells are all less than 0.61% of those that remain encapsulated.

From the above data, it is reasonable to propose that the sol-gel technique provides a matrix for the encapsulation of cells. It is also possible to recommend that gels either be used immediately or left for twenty-four hours before use to allow any cells that are not well encapsulated to be released. After twenty-four hours gels should be thoroughly rinsed and then may be used in experiments. For gels that will be stored long-term it is recommended that media be replaced periodically, especially in the first twenty-four hours to remove escaped cells and inhibit biofilm formation on the gel surface. Gels that are observed with a surface biofilm should be discarded.

Electron Microscopy

Cells encapsulated in a sol-gel matrix are complicated to test owing to the difficulty of removing cells from the matrix. This makes observations on the nature of encapsulated cell physiology challenging, and any cells removed from a gel may be altered. One method that will provide a visual picture of the state of cells within the matrix is to utilize electron microscopy. Electron microscopy will allow a qualitative determination of cell state. One of the critiques of the sol-gel technique is that cellular membranes are disrupted during encapsulation. This is the type of issue that is possible to critique from an electron micrograph. To this effect Bio-gels were used in scanning electron microscopy (SEM) to get an idea of the surface of the gel. Additional Bio-gels were prepared for thin section transmission electron microscopy (TEM) to get an idea of the state of cells within the matrix.

Intact cells were visualized in both TEM and SEM preparations. The cells appear to be randomly dispersed. Their cell membranes are intact. In the SEM images, the porous nature of the sol-gel matrix is evident as shown in figure 5. This is apparent on the gel surface as well as the interior, as seen within the cracks. The pores are small, but they are on the scale of a bacterial cell and should allow adequate nutrients and oxygen to reach the cells. The presence of cells on the gel surface is minimal but it is interesting that there are numerous holes in the shape of a *5RL* bacterial cell as shown (figure 6). Because the encapsulation process is random, it is reasonable to expect that some cells were on the surface as gelation occurred. These cells were removed during rinsing or some other step

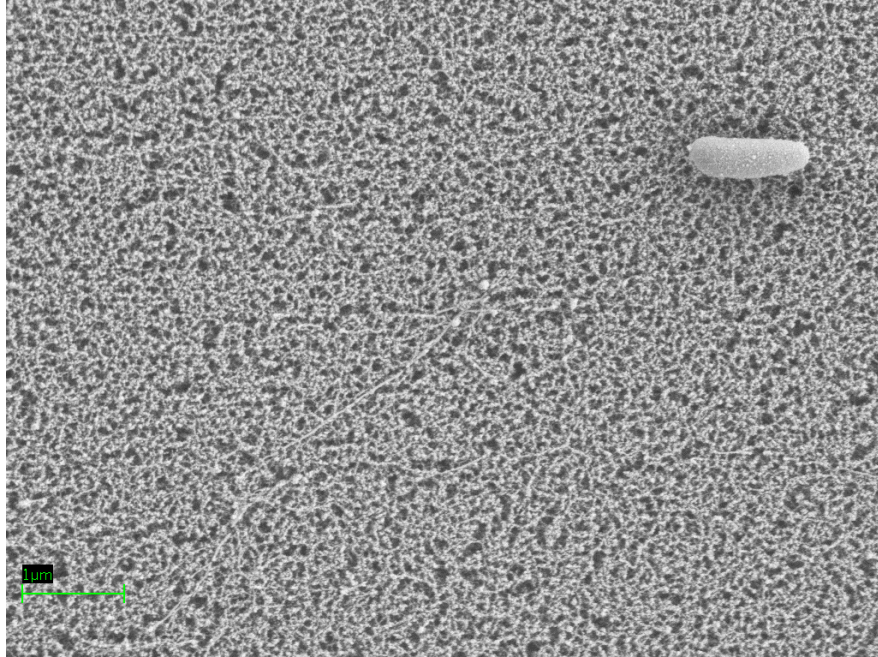


FIGURE 5. THE POROUS NATURE OF THE SOL-GEL MATRIX.
A *5RL* cell in the upper right corner illustrates relative cell/pore sizes (SEM image).

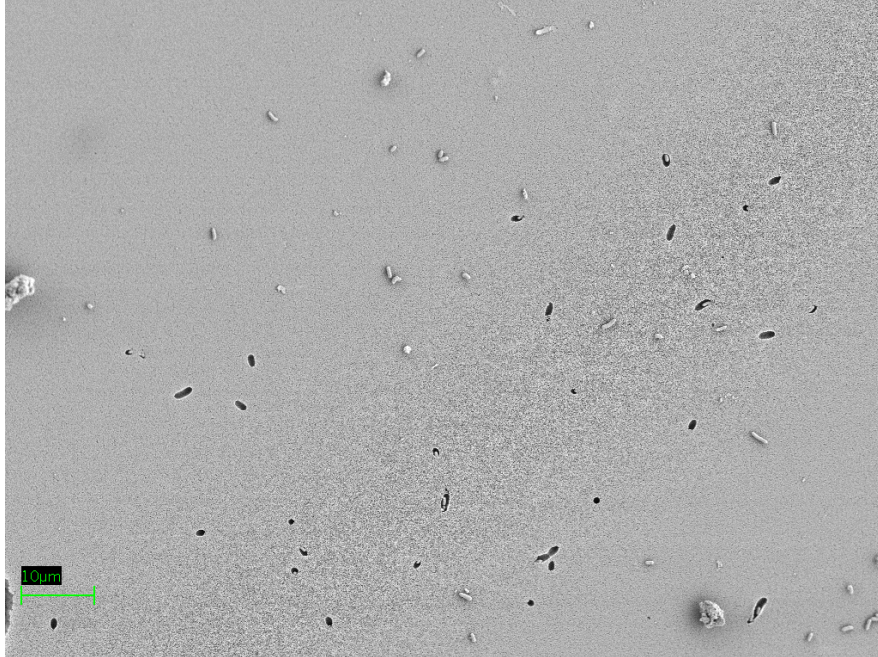


FIGURE 6. THE BIO-GEL SURFACE WITH CELLS AND CELL-SHAPED INDENTATIONS.
There are minimal numbers of cells present on the gel surface but many *5RL* shaped holes in the gel as depicted in this SEM image.

leaving behind an indentation in the gel surface as in figure 7. Also interesting about these indentations are that in some cases cells appear to be using them as an “escape route” (figure 8). It is unclear how important this is in cellular encapsulation. This may be an indication that gels are not as solid as previously thought. Cells may be able to move in and out of the gel depending on the conditions. It is possible that the gel is weaker near the indentation and only adjacent cells may escape. This may only be an artifact of critical point drying during SEM preparation that resulted in drying and pore shrinkage of the gel.

Another interesting feature of these images are the horseshoe and circular shaped cracks as in figure 9. It is unknown why the gel would crack in this particular shape but it was a consistent occurrence in multiple preparations. These cracks allow visualization of the interior of the gel where the same spongy, porous matrix is visible (figure 10). It is also possible to distinguish cells that are inside the matrix, encapsulated by the silica gel.

More information on cells within the gel can be gained from the TEMs. These images were viewed after a thin section preparation. They each show a section from the interior of the gel. Many cells are visualized even though sections are taken from the interior of the gel. This suggests that cells are found throughout the gel instead of solely on the surface. It also suggests that not all cells are able to escape or move in the matrix. When examining individual cells it is possible to see that the cell membrane remains intact as in figure 11. The matrix completely surrounds the cell but does not disrupt the cell membrane, which is important in maintaining cell viability.

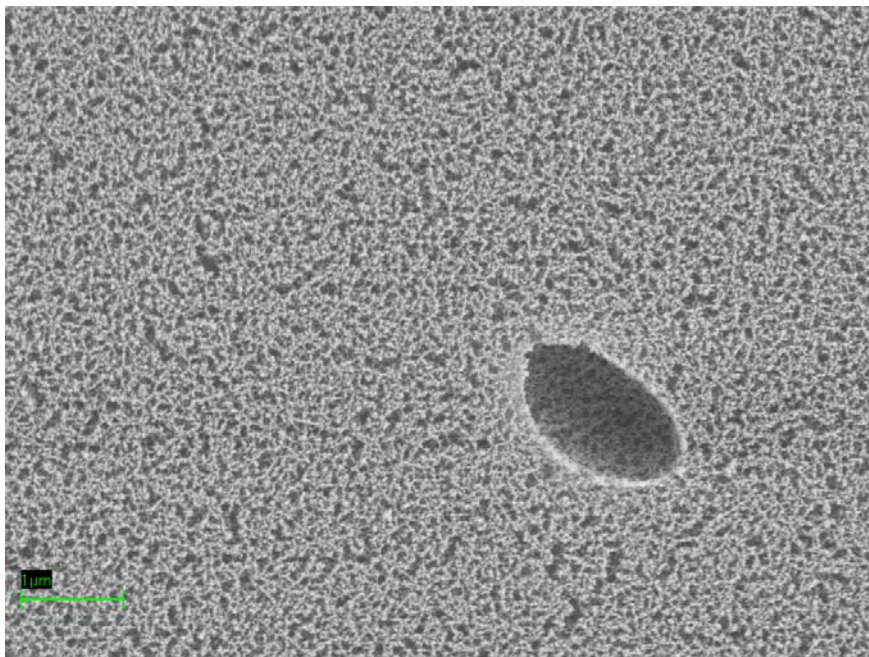


FIGURE 7. 5RL INDENTATION IN GEL SURFACE.

This micrograph depicts an indentation left in the gel surface by a 5RL cell.

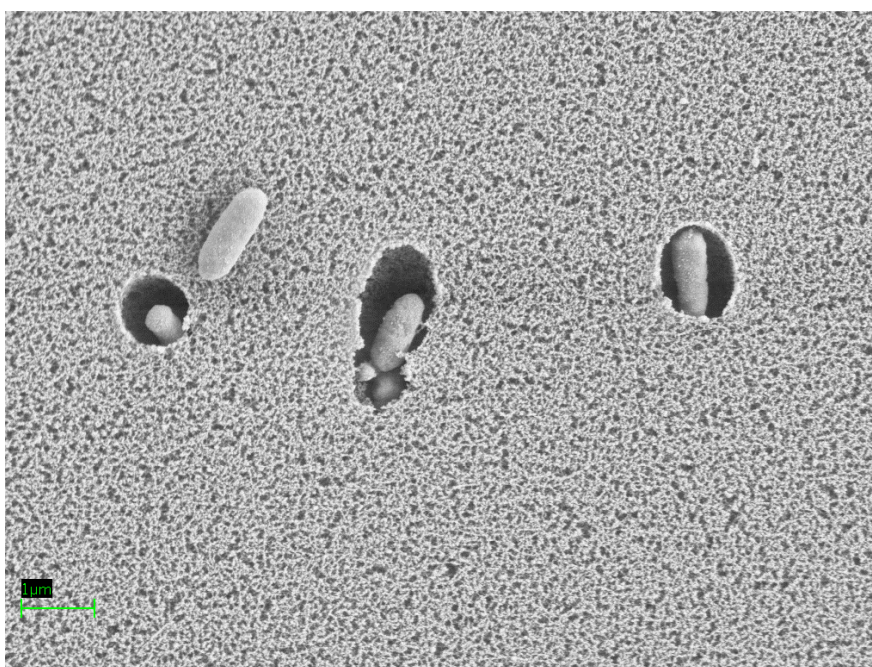


FIGURE 8. ESCAPE OF CELLS FROM SOL-GEL.

There are many holes in the sol-gel matrix and it is unknown whether cells can use these holes as an “escape route.” This image suggests that cells may be able to move through the holes at the gel surface.

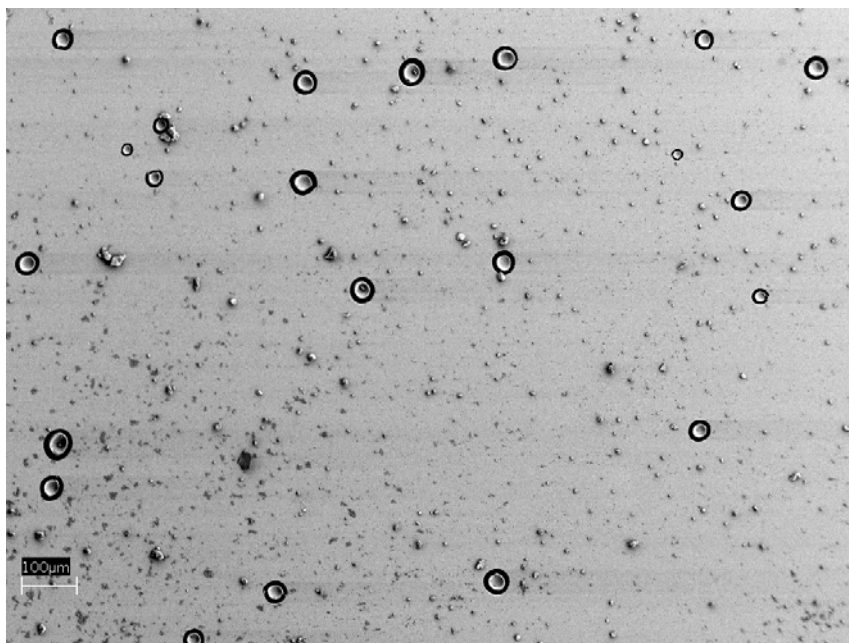


FIGURE 9. CIRCULAR CRACKS VISUALIZED IN NUMEROUS PREPARATIONS.
Also observed were horseshoe-shaped holes. A close up of a horseshoe crack is shown in Figure 10.

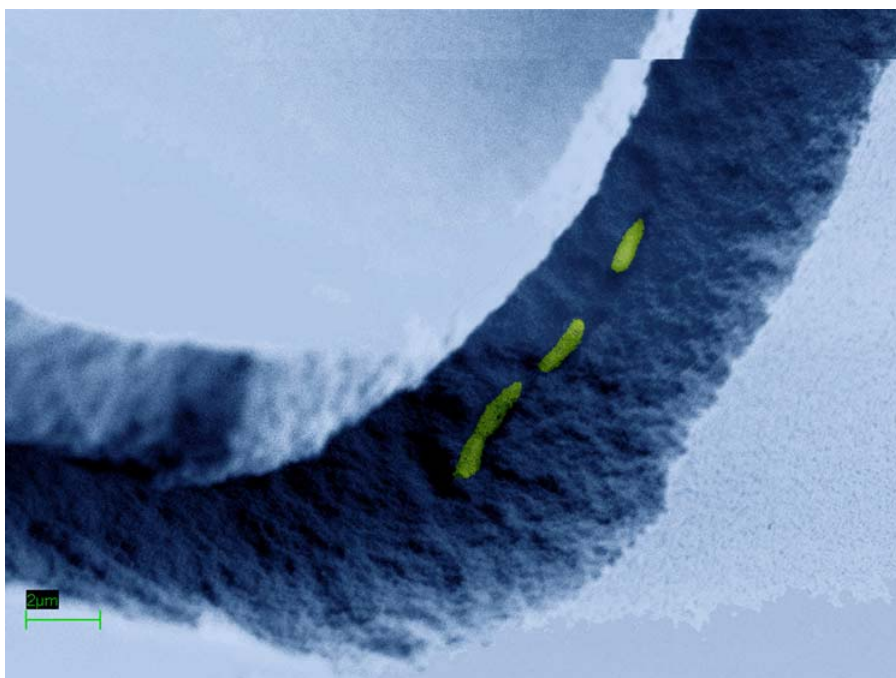


FIGURE 10. FALSE COLOR IMAGE OF HORSESHOE CRACK IN GEL SURFACE.
5RL cells (yellow) may be visualized partially enmeshed in sol-gel matrix.

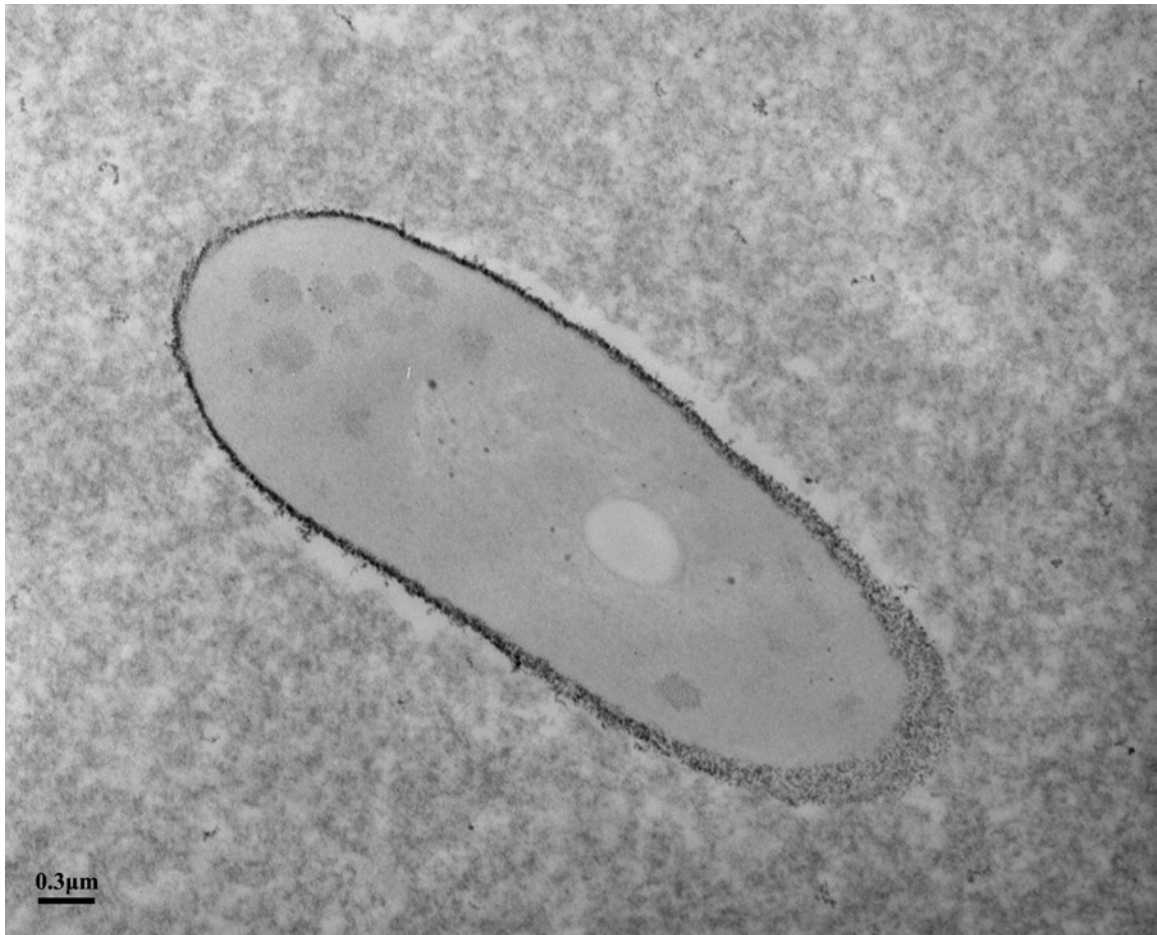


FIGURE 11. TEM OF A 5RL CELL WITHIN THE SOL-GEL MATRIX.

It is evident that cells remain intact during the process of encapsulation. In addition, there is no evidence of damage to the cell membrane.

Another interesting finding was cells surrounded by a putative mucoidal layer (figure 12A.) We do not believe this to be a feature of the sol-gel encapsulation technique, but instead a *5RL* mucoidal phase variant. It is possible to locate both cell types adjacent to each other in the gel as seen in figure 12B. In addition, *5RL* colonies on agar exhibit the same phase variation as in the photo of induced colonies on agar shown in figure 13. What is particularly worthy of note is that the sol-gel matrix does not disrupt the mucoidal layer, which is most likely composed of polysaccharides. This provides evidence for the benign nature of the encapsulation process, both the cell membrane of the *5RL* cell as well as the mucoidal layer in phase variants are preserved. If the gelation process was particularly harsh, damage would be expected, however there is no evidence from the TEM images to suggest that this is true.

Another question about the encapsulation process is whether it is possible for cells to divide within the matrix. When viewing TEMs it is possible to see cells that appear to be in the process of division as seen in figure 14. However, because these cells are seen in freshly made gels as well as gels after seven days it is hard to tell whether these cells were in the process of dividing at the time of encapsulation or if division is occurring post-encapsulation. If division was in progress during encapsulation then it may be that the nature of the matrix prevents the completion of division and separation of cells. Conversely, if division was initiated after encapsulation it may occur more slowly than normal, or the cells may be incapable of complete separation. Overall, it is difficult to draw a conclusion on whether cells are able to divide within the sol-gel matrix.

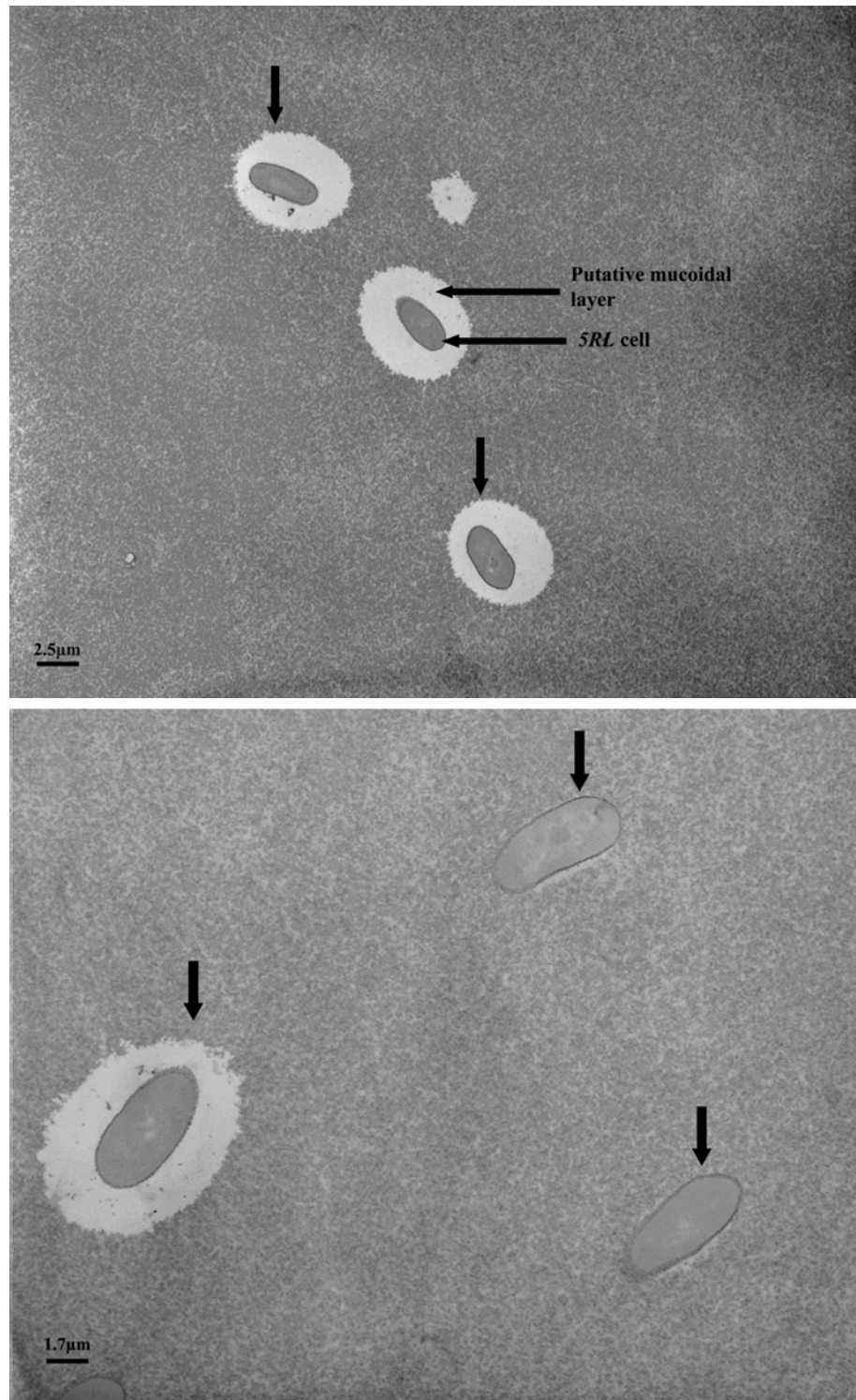


FIGURE 12. 5RL PHASE VARIANTS ENCAPSULATED IN THE SOL-GEL MATRIX.
The white layer surrounding some cells is a putative mucoidal layer. Both variants may be located adjacent to each other in the matrix (TEM images).

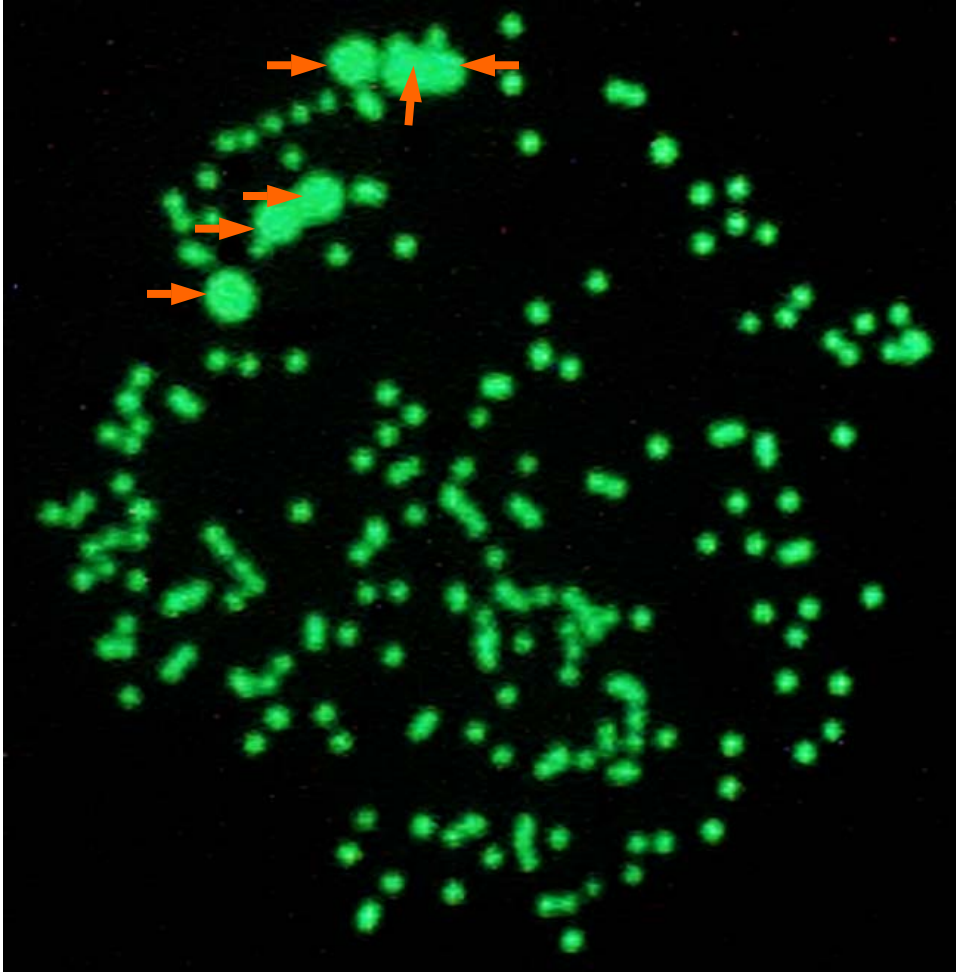


FIGURE 13. INDUCED *5RL* COLONIES GROWING ON AN AGAR PLATE.

This figure depicts a photograph of a plate with induced *5RL* colonies. Phase variants are common when *5RL* is grown on agar. Mucoidal colonies are designated with an orange arrow. The other colonies have a smooth phenotype. This is an LB plate with salicylate added to induce cells.

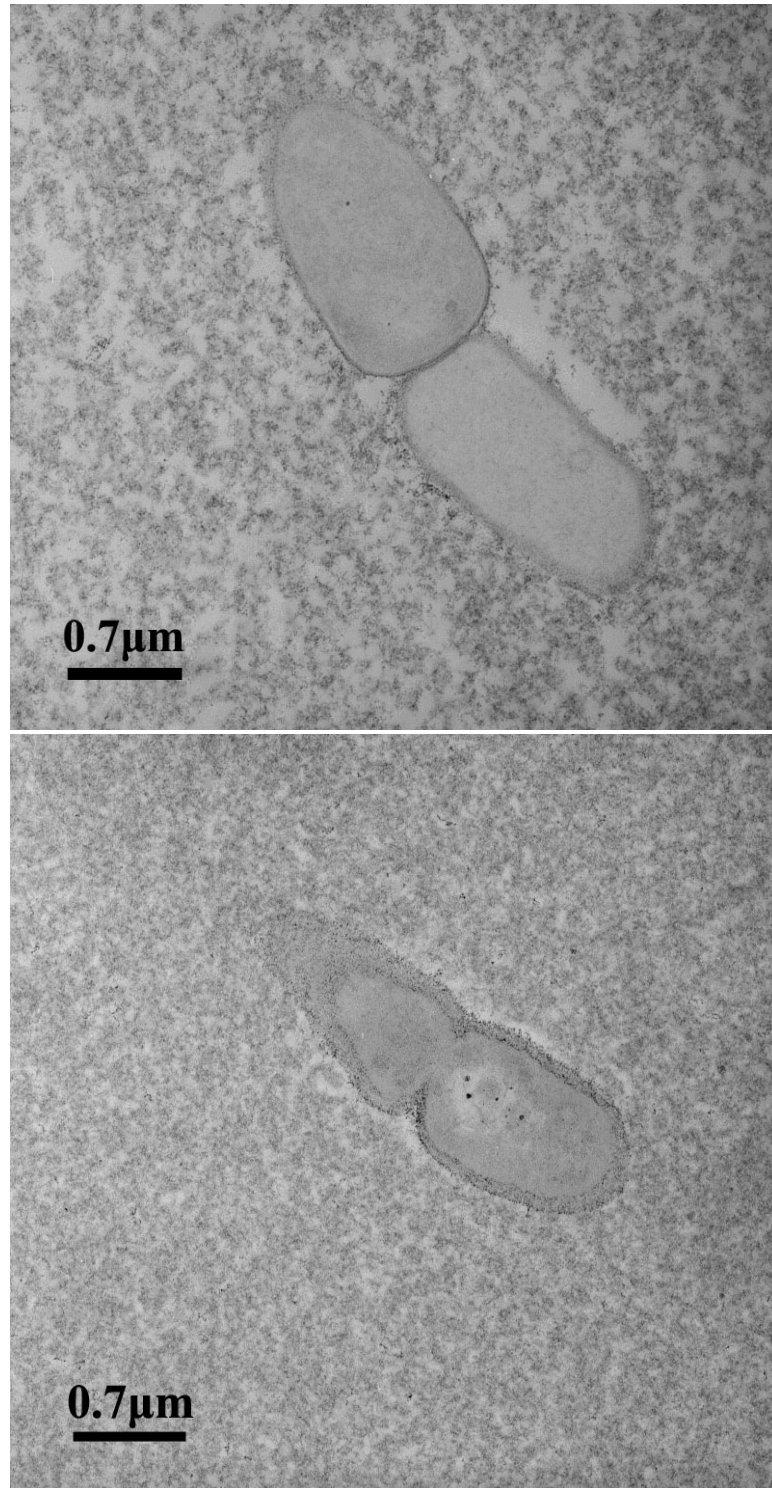


FIGURE 14. TEM IMAGES DEPICTING THE POSSIBILITY OF CELLULAR DIVISION WITHIN THE SOL-GEL MATRIX.

It is difficult to determine whether cells were in the process of division when encapsulated or if division was initiated after the encapsulation event.

Chapter Four

RESEARCH QUESTION TWO – IS CELL PHYSIOLOGY AFFECTED BY ENCAPSULATION?

Bioluminescence

When cells are utilized in field applications it is desirable for them to react in the same manner as in the laboratory. This is often difficult because it is possible to exert more control over laboratory conditions and keep cells in an optimum environment. In this same line of thought it is desirable for encapsulated cells to react in the same manner as free, non-encapsulated cells. There is currently great debate over the effect of encapsulation and whether it is detrimental to cells. In many reports mentioned previously, it was found that sol-gel encapsulation actually provides better stability for enzymes (4, 14, 30). Ideally the same effect is seen with the encapsulation of cells. The physiological state of cells will demonstrate any influence encapsulation has on cells.

One indicator of the physiological state of cells is bioluminescence, which provides a measure of enzyme activity (2). For cells to emit bioluminescence they must have the ability to form a functional luciferase. They must also be able to synthesize the aldehydes necessary for light to be produced. If cells are functional they should have the ability to accomplish these tasks and bioluminescence will be produced. On the other hand, if cell physiology is affected then theoretically bioluminescence will also be affected. *5RL* cells can be measured for induced bioluminescence allowing physiological monitoring of cells.

5RL cells were encapsulated in sol-gel (Bio-gels) and compared to cells in solution (Free cells), as well as appropriate negative controls (Sol-gel, MSM). Sol-gels are gels made with sterile media and MSM is sterile as well. This provides a background measurement of experimental set-up cells. Because *5RL* typically has a high background bioluminescence, non-induced controls were also used (UN Bio-gels and UN Cells). Maximum non-induced background bioluminescence was used as the threshold for induced cells to ensure true induction of *5RL* cells by salicylate. Cells were either encapsulated, or in the case of Free cells, re-suspended before any inducer was added. In addition, cells were encapsulated a minimum of twenty-four hours before experiments to allow reduction in background bioluminescence and ageing of gels. Free cells were stored in LB to ensure their viability. This may impart an advantage to Free cells, however they did not remain viable for experiments when stored in minimal media.

Throughout the course of this project numerous bioluminescence experiments were performed. A typical example is seen in figure 15. Overall, Bio-gels displayed a slightly higher fold induction above the threshold (4.15x) when compared to Free cells (3.89x). However, the actual peak bioluminescence above the threshold was higher in Free cells (5.12×10^6) than Bio-gels (4.71×10^6). An independent samples t-test was used to compare the mean peak bioluminescence above the threshold of Bio-gels and Free cells. This test indicates that there is no significant difference ($p = 0.879$) between the peak bioluminescence of Bio-gels and Free cells. This implies that encapsulated cells are able to respond to induction and emit light, as well as non-encapsulated cells.

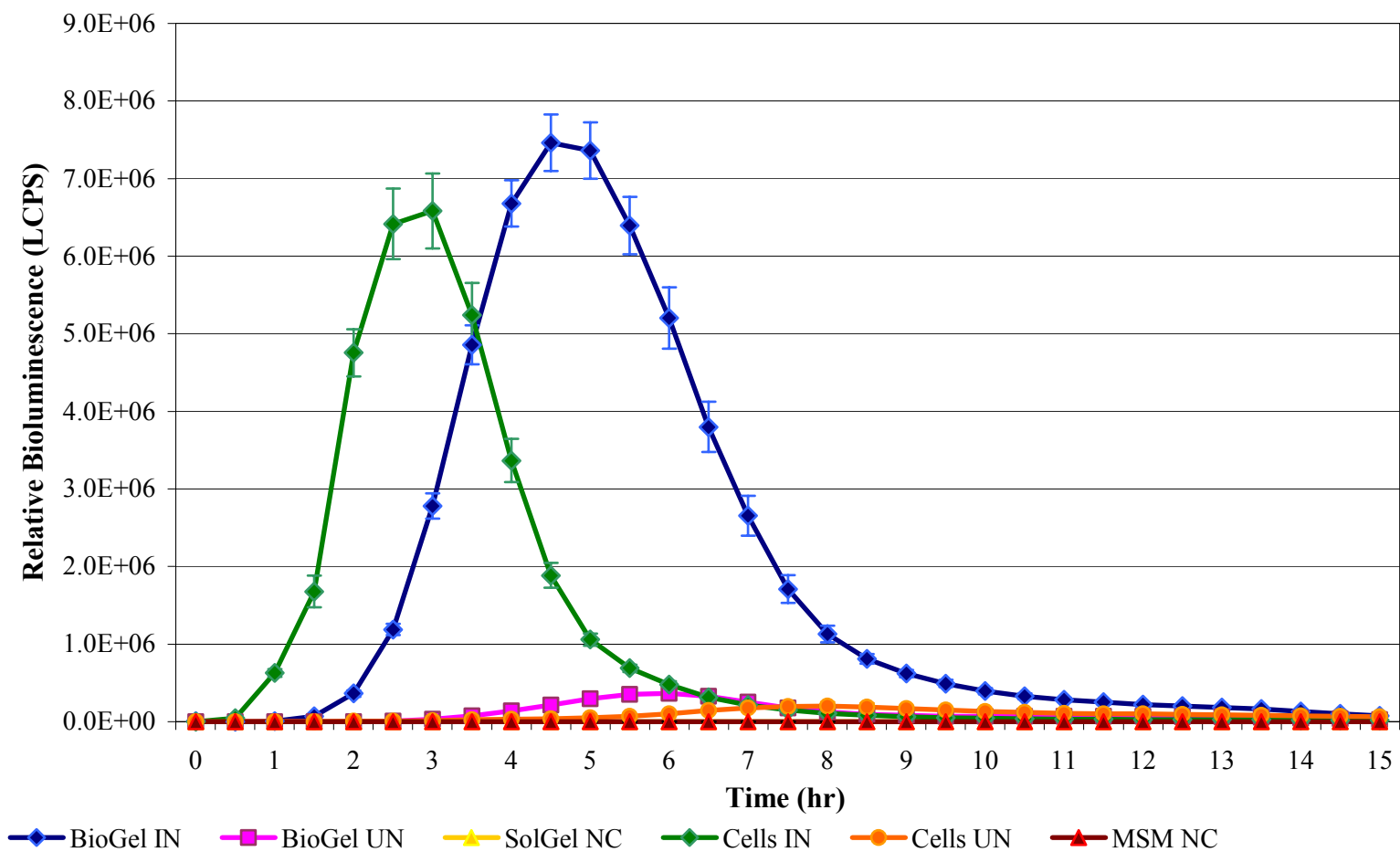


FIGURE 15. TYPICAL BIOLUMINESCENCE COMPARISON BETWEEN BIO-GELS AND FREE CELLS.

IN indicates that cells have been induced by the addition of salicylate. UN indicates non-induced cells. Data points represent the mean of eight replicates. Error bars show the standard error of the assay.

Another question with bioluminescence is how fast cells are able to respond. One theory is that the sol-gel matrix would interfere with response time by slowing diffusion of target analytes through the gel to the encapsulated cells. When comparing Bio-gels and Free cells for the initial induction above the background, the mean time for both groups was four hours. An independent samples t-Test was utilized to examine the mean induction times. The test suggests that there is no significant difference ($p = 0.964$) between the induction time of Bio-gels and Free cells. This test was skewed slightly by one outlying case that had a high background bioluminescence and therefore an induction time of sixteen hours for Bio-gels and seventeen hours for Free cells. However removal of this case did not impact the outcome of the t-Test, there was no significant difference ($p = 0.556$) between the induction time of Bio-gels and Free cells.

The time for induction to reach peak bioluminescence was also compared. As previously, the mean peak induction was equal for both Bio-gels and Free cells, at seven hours. The mean peak induction times were compared in an independent samples t-Test. This test suggests that there is no significant difference ($p > 0.05$) between the peak induction time of Free cells and Bio-gels. These results imply that the sol-gel matrix does not inhibit the induction of encapsulated cells. In addition, when cells are induced they are able to respond in the same capacity whether or not they are encapsulated.

Cellular Respiration

Another measure of physiology is cellular respiration. As cells convert nutrients to energy for use in anabolic, catabolic, and maintenance functions they use oxygen. By

measuring the amount of oxygen utilized to convert a known amount of carbon it is possible to get an indication of the physiological state of cells. Cells that are physiologically inhibited will have a slower rate than healthy cells. This is another determination of physiological effect of sol-gel encapsulation on cells. The experiment was also useful in determining whether transfer of oxygen through the matrix is inhibited.

Cellular encapsulation was carried out as normal and Costar® plates were utilized. Costar® plates allowed easier removal of aged Bio-gels from the plate. Bio-gels were transferred from plates into chambers of a YSI bath along with MSM and glucose. Free cells were added to separate chambers also with MSM and glucose. MSM was utilized as a negative control to ensure proper calibration of oxygen probes. Oxygen probes connected to a 5300A Biological Oxygen Monitor were placed in chambers. Oxygen was measured until 0% air was reached. Results were converted from % Air to $\mu\text{gO}_2/\text{ml}$, respiration rates were calculated, and analyzed.

As with bioluminescence, numerous oxygen experiments were performed during this project. A typical experiment is depicted in figure 16. From this graph as well as by examining the respiration rates, it is evident that Free cells have a faster respiration rate ($0.141 \mu\text{g O}_2/\text{ml} * \text{min}^{-1}$) than Bio-gels ($0.065 \mu\text{g O}_2/\text{ml} * \text{min}^{-1}$) or MSM alone ($0.002 \mu\text{g O}_2/\text{ml} * \text{min}^{-1}$). An independent samples t-test confirms that there is a significant difference ($p < 0.001$) between the mean rate of respiration of Free cells and Bio-gels. On the other hand, Bio-gels have a faster rate than MSM without cells. An independent samples t-test confirms that there is a significant difference ($p = 0.016$) between the mean

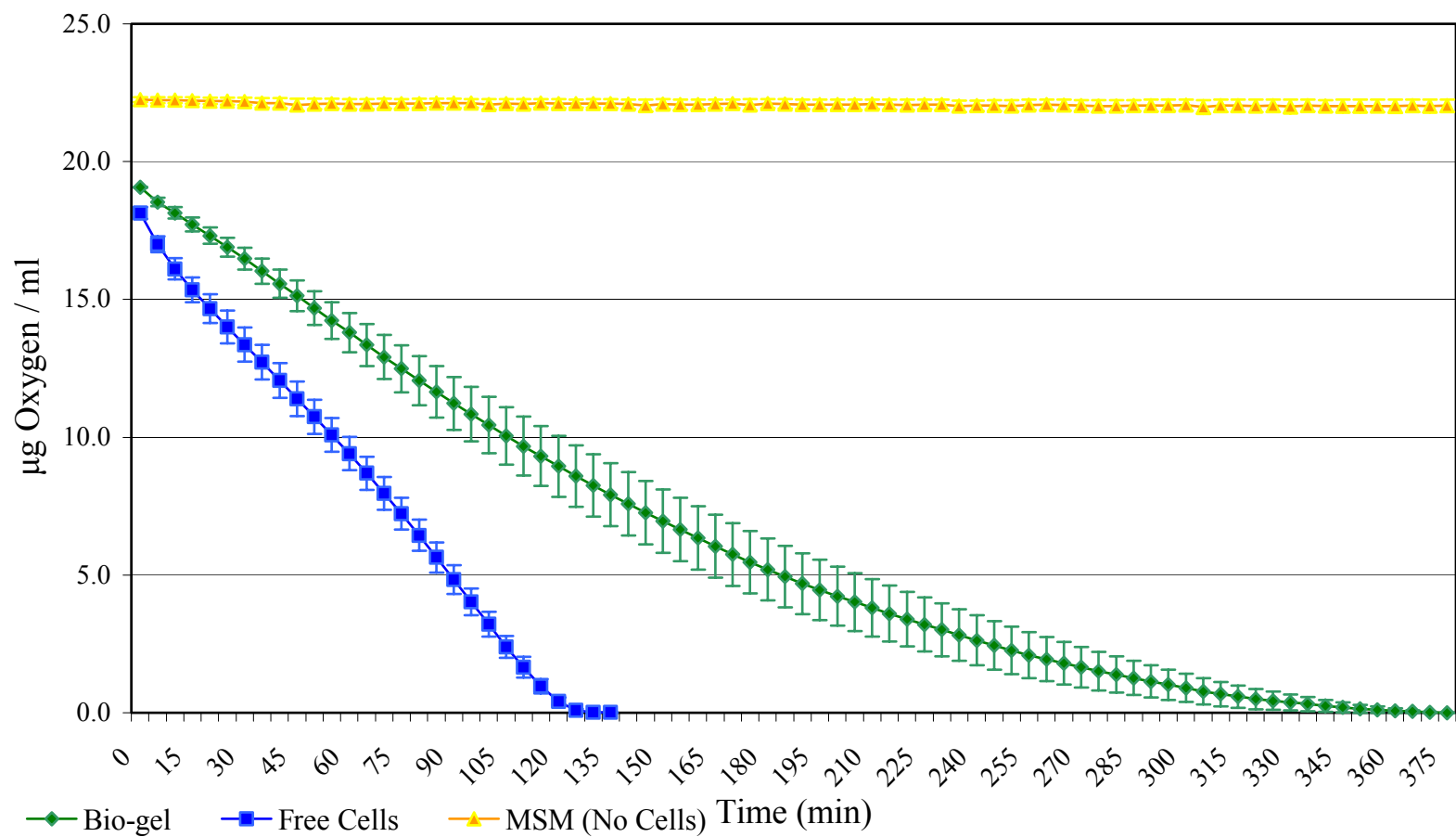


FIGURE 16. TYPICAL OXYGEN EXPERIMENT DEPICTING DEPLETION OF OXYGEN FROM THE TEST CHAMBER.

Bio-gels and Free cells utilize oxygen faster than the MSM without cells. Data was collected in one minute intervals but is show in five minute intervals for ease of viewing. Each data point is the mean of six replicates. Error bars represent the standard error of the assay..

rate of respiration of Bio-gels and MSM. To compare all groups together, an ANOVA analysis was performed. The ANOVA results suggest that there is a significant difference ($p < 0.001$) between the mean respiration rates of the three groups. The Dunnett T3 post hoc test also shows that all groups have significantly different ($p < 0.001$) mean respiration rates.

These results may contradict what was observed with the bioluminescence studies, which indicated that there were no significant differences between Bio-gels and Free cells. One explanation, however is that encapsulated cells are less active inside the matrix. Because it is unlikely that cells are able to divide within sol-gel, the encapsulated cells could require less carbon while still remaining viable. The Free cells will continue to divide while in the oxygen chamber and this could explain their higher rate of respiration. The theory can be backed up by the comparison of Bio-gels to MSM. The Bio-gels have significantly higher respiration rate than media without cells, which indicates that they are metabolically active.

Long-term Experiment

In order to get an overall picture of the physiological state of cells, the previous experiments were combined. Protein concentration, Bioluminescence, and cellular respiration and were all examined for Bio-gels and Free cells over a ten week period. Investigation of a diverse set of parameters on the same cells gave a better idea of impacts on the physiological state of cells. In addition, by performing the experiment

over a ten-week period it will also be possible to investigate the storage potential of encapsulated cells.

Bio-gels were made as usual but for this experiment a separate set of plates was prepared for each interval. Because *5RL* cells cannot completely remove salicylate they do not respond to re-induction (25). Therefore, a new set of samples was required for each interval. However, all plates were prepared at the same time from the same culture to insure uniformity. Greiner plates were prepared for bioluminescence and sealed, while Costar® plates were prepared and sealed for oxygen and protein determinations. Culture for Free cells was aliquoted into separate disposable tubes for each interval. In a preliminary experiment Free cells were added directly to plates but there was a swift decline in viability over the storage period. Consequently, for the actual experiment culture was stored in tubes and aliquoted at the appropriate interval. All plates and tubes were kept at 4°C until use. Every two weeks plates and tubes were removed from storage and bioluminescence was measured on the Microbeta, oxygen was monitored with YSI probes, and protein was determined with the Coomassie method. The results were then analyzed and observations made.

In addition, an extra step was added to the monitoring of cellular respiration. Before and after an oxygen experiment, aliquots were removed and frozen for carbon quantification with the TOC-V_{CSH}. This will allow further investigation into how much carbon is utilized during oxygen monitoring. This test will provide another depiction of the physiological state of cells.

Long-term Protein

First, protein measurements were taken at each two-week interval. Figure 17 depicts the mean concentration from each interval. It appears that protein differs at week four. An ANOVA analysis was performed on the protein data. ANOVA results suggest that there is at least one week that has a significantly different ($p = 0.014$) protein concentration. A Dunnett T3 post hoc test suggests that all groups are different. However, when week four was compared to other groups the significance level was $p = 0.1$ instead of the $p = 0.8$ level for other groups compared against each other. To check this possibility, the ANOVA test was performed without week 4 data. These results suggest that the protein concentration is not significantly different ($p > 0.05$) from week to week. It is unclear why week four would have a change in protein concentration while the other weeks remain similar.

The examination of results from week four indicates that there were two outliers that caused the normal distribution to be skewed to the right. It is possible that these readings have an undue influence on the study. An ANOVA test performed with week four data but without the outliers. Results indicate that the protein concentration is not significantly different ($p > 0.05$) from week to week. The Dunnett T3 Post Hoc test indicates that week 4 is similar to weeks 6, 8, and 10 but significantly different from week 0 ($p = 0.018$) and week 2 ($p = 0.021$).

Long-term Bioluminescence

The second set of experiments tested bioluminescence production. Figure 18 A-F show the bioluminescence for each week. Two comparisons between Bio-gels and Free cells

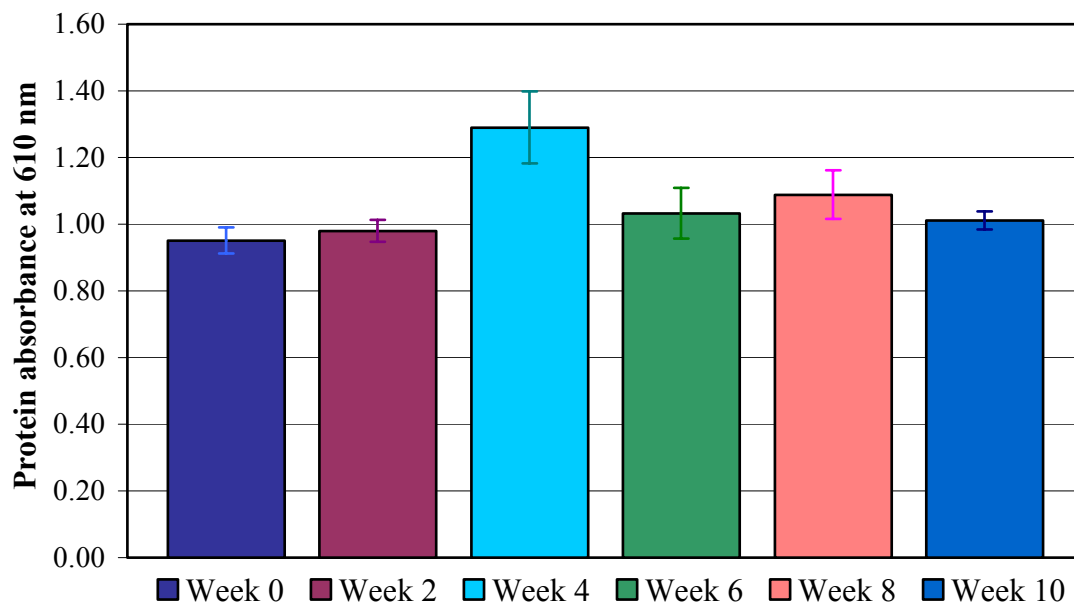


FIGURE 17. PROTEIN ABSORBANCE FROM LONG-TERM EXPERIMENT.

This plot demonstrates that protein concentration of gels differs over the study. The highest concentration is at week four. Bars represent the mean of eight replicates. Error bars indicate the standard error of the assay.

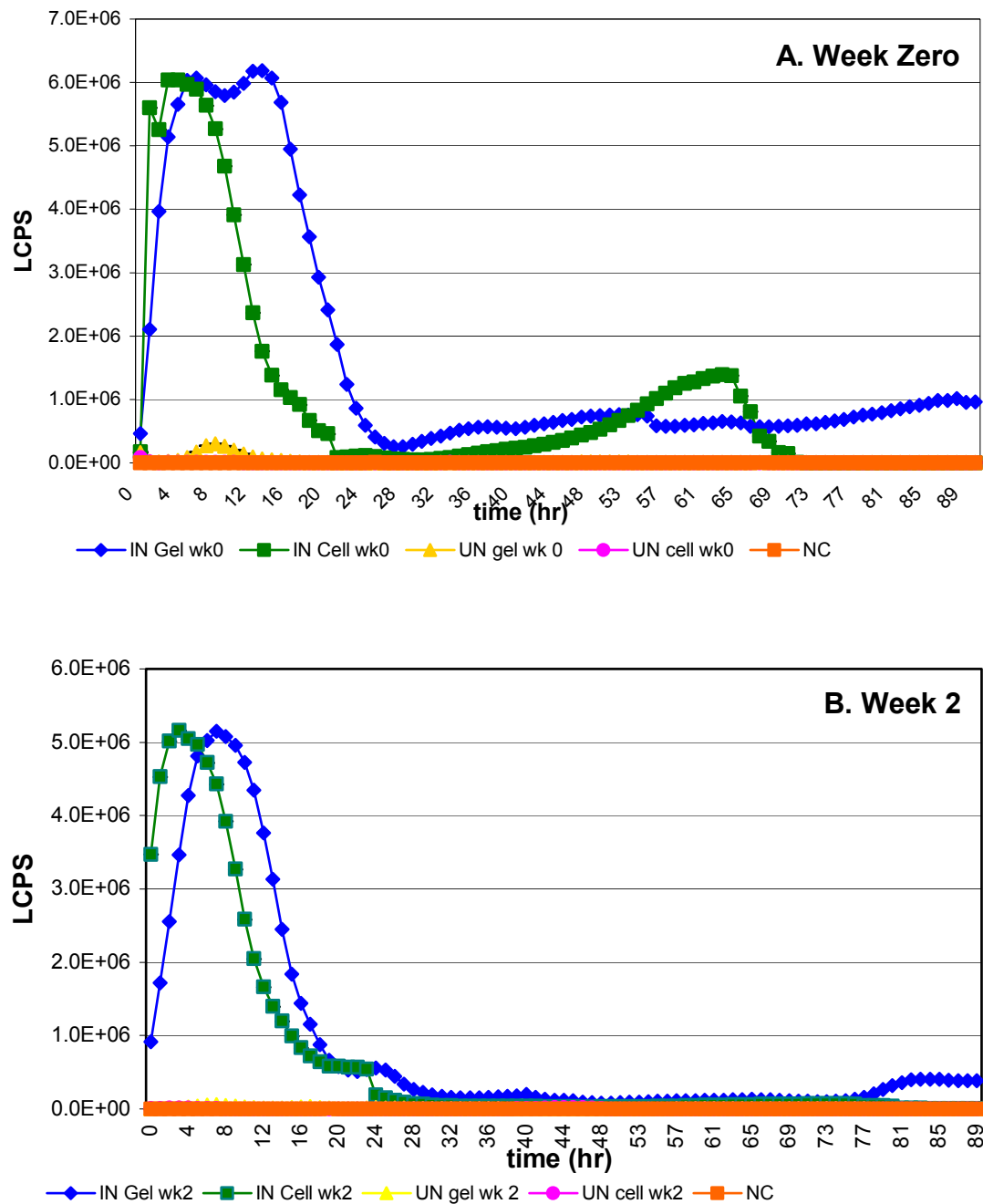


FIGURE 18. LONG-TERM BIOLUMINESCENCE.

These plots indicate that light emission changes over time. Weeks 0 to 6 demonstrate a similar induction cycle. Week 10 is completely different. Data points represent the mean of sixteen replicates. Error bars demonstrate the standard error of the assay. The majority of error bars are too small to visualize.

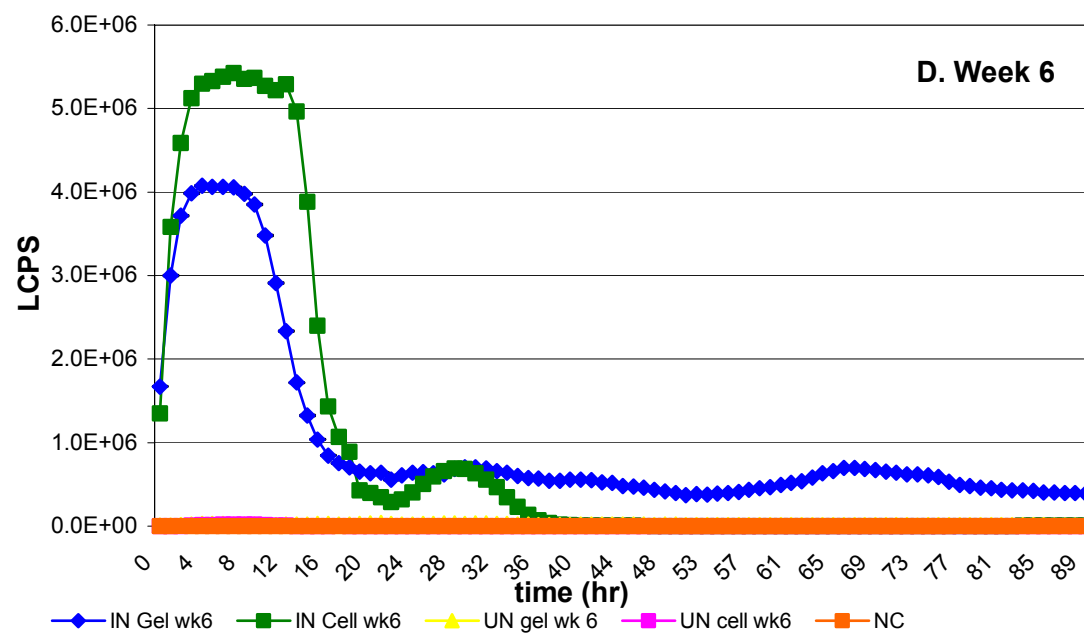
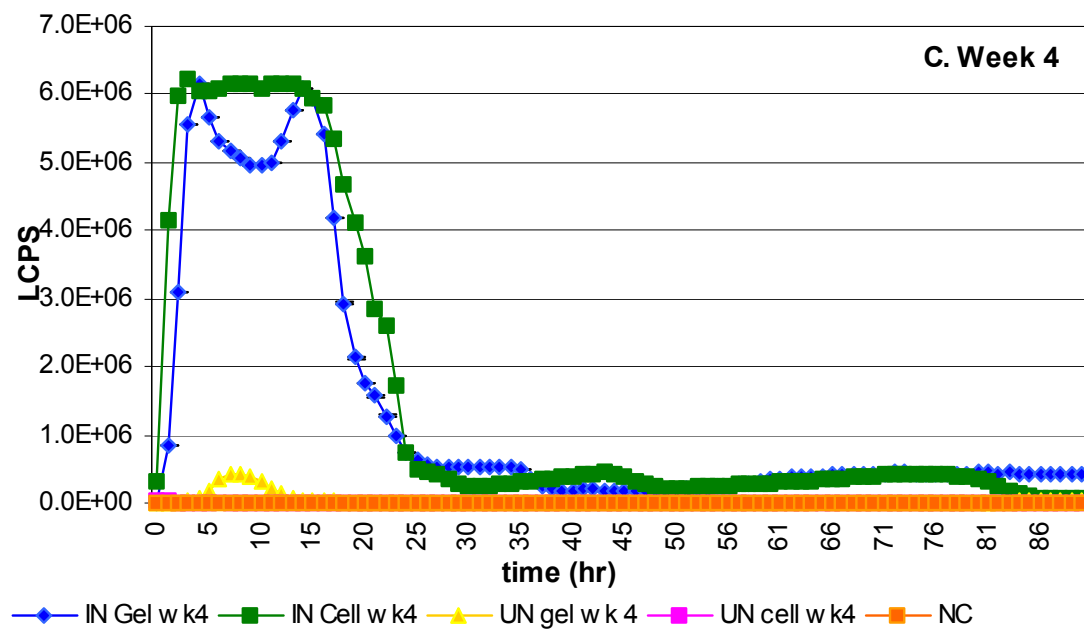


FIGURE 18. CONTINUED.

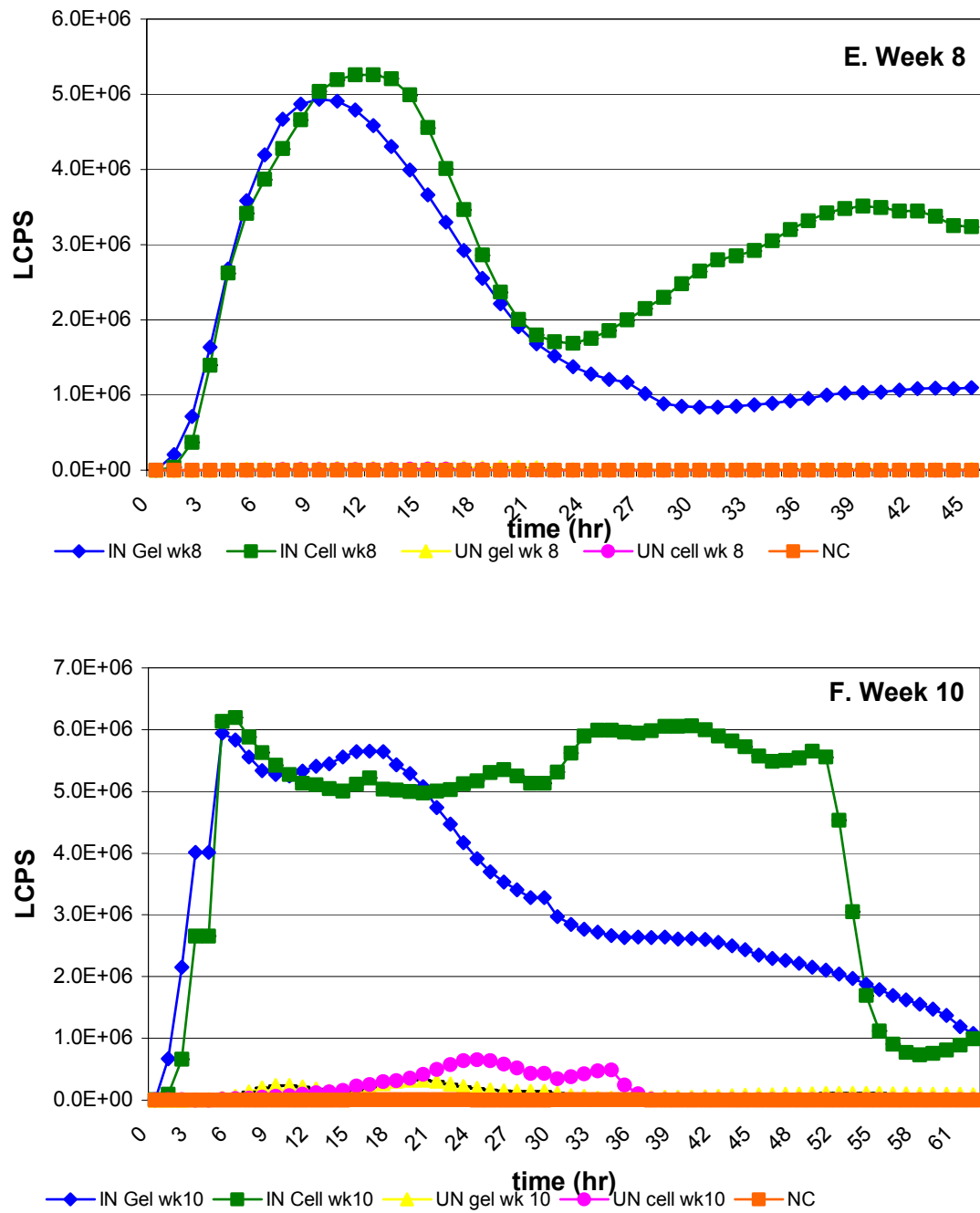


FIGURE 18. CONTINUED.

were made each week: induction above background and peak bioluminescence. Independent samples t-Tests were performed in each case and the results can be seen in Table 2. At weeks zero, two, four, and six, t-Tests indicate significant differences between the comparisons of induction and peak bioluminescence of Bio-gels and Free cells. Free cells are consistently higher except at the beginning of the experiment (week two.)

At weeks eight and ten, independent samples t-Test suggest that induction and peak bioluminescence is not significantly different between Bio-gels and Free cells. At these weeks it appears that Bio-gels are beginning to out-perform the Free cells. At both weeks, while the difference is not significant, Bio-gels have higher induction above background than Free cells. This may predict a future trend implying increased long-term stability of encapsulated cells during storage.

All weeks were compared to one another with MANOVA analysis. When examining bioluminescence the model suggests that there was a significant difference ($p < 0.001$) in induction of bioluminescence over time. The model also suggests that there was no significant difference ($p > 0.05$) between the induction of bioluminescence between Bio-gels and Free cells. In addition, this model suggests that the difference ($p > 0.05$) between the inductions of bioluminescence over time was probably the same for Bio-gels and Free cells. Dunnet T3 post-hoc tests indicate that week ten is similar to all but week eight and weeks two, four, and six are also similar.

TABLE 2. BIOLUMINESCENCE DATA FOR EACH WEEK FROM THE LONG-TERM EXPERIMENT.

Part A lists the induction and peak bioluminescence for Bio-gels and Free cells at each week of the study. The results of an Independent Samples t-Test comparing data from Bio-gels and Free cells at each week are listed in Part B. The two groups are significantly different until weeks eight and ten, which are similar. Data represents the mean of sixteen replicates.

Part A.

Type	Week	Peak Bioluminescence	Induction
Bio-gels	0	5.73×10^6	17 x
	2	4.28×10^6	17 x
	4	4.71×10^6	4 x
	6	2.55×10^6	4 x
	8	4.72×10^6	263 x
	10	5.27×10^6	19 x
Free cells	0	5.08×10^6	37 x
	2	1.73×10^6	2 x
	4	5.68×10^6	23 x
	6	4.07×10^6	6 x
	8	5.22×10^6	201 x
	10	5.53×10^6	10 x

Part B.

Week	Significance of Induction	Significance of Peak Bioluminescence	Is the Difference Significant?
0	< 0.001	0.017	Yes
2	0.002	0.007	Yes
4	< 0.001	0.001	Yes
6	0.047	0.021	Yes
8	0.553	0.402	NO
10	0.246	0.263	NO

When examining peak bioluminescence, MANOVA suggests that overall there was a significant difference ($p < 0.001$) between peak bioluminescence over time. The model also suggests that there was no significant difference ($p > 0.05$) of peak bioluminescence between Bio-gels and Free cells. Finally, the model predicts that the difference ($p < 0.001$) between peak bioluminescence over time was probably not the same for gels and cells. Dunnett T3 post hoc tests indicate that two groups share similarity. Weeks zero, four, eight, and ten are similar. Weeks two and six are similar to one another.

The statistical analysis of bioluminescence suggests differences in the means at each week but similarities in the variance over time. This indicates that Bio-gels and Free cells are responding in the same manner over time. Bio-gels do not appear to be negatively impacted by encapsulation. In addition, the light levels observed at eight and ten weeks may predict a future trend indicating an improved response to induction after long-term storage for encapsulated cells.

Long-term Respiration

The third set of tests involved monitoring of oxygen. Oxygen values as % air were converted into μg of O_2 per ml. A respiration rate was calculated from these values. Independent samples t-Tests were performed at each week between Bio-gels and Free cells. These test indicate a significant difference in respiration rates at week zero ($p < 0.001$), week two ($p = 0.001$), week four ($p < 0.001$), and week six ($p < 0.001$). There was no significant difference at week eight ($p > 0.05$) and week ten ($p > 0.05$) between Bio-gels and Free cells. These results are similar to those observed in the bioluminescence

study, which suggests that encapsulation may improve the stability of cells in log-term storage. Data was also compared over time with an ANOVA. This test suggests that there are significant differences ($p < 0.001$) in respiration rates over time between Bio-gels and Free cells. While there were trends in the differences of respiration rates, there were no significant differences or similarities indicated in the Dunnet T3 post hoc test.

To compliment oxygen monitoring, carbon was quantified. This assisted in the determination of cellular respiration by allowing measurement of initial and final carbon. The carbon recovery rate for *5RL* are not readily available in the literature so values for *Escherichia coli* (*E. coli*) were used. This should give an approximation of carbon respiration that can be applied to *5RL*. It is known that *E. coli* has an 89% carbon recovery rate (55). This suggests that 89% of glucose given to *5RL* cells will be used for cellular processes such as anabolism, catabolism, and cellular maintenance. Cells are composed of 52.4% protein, 16.6% polysaccharide, 15.7% RNA, 9.4% lipid, and 3.2% DNA so carbon utilized can be partitioned into the cellular components (55). This also implies that 11% should be either given off as carbon dioxide or another byproduct.

A glucose standard was created using known glucose concentrations plotted against TOC peak area data (figure 19). Points were analyzed by linear regression and the following equation was generated: $y = 0.026x + 1.336$ with y indicating the concentration of glucose and x indicating TOC peak area. Regression analysis indicates that the line fits the data well ($R^2 = 0.998$). This standard was utilized to predict glucose concentration of each oxygen experiment after oxygen was depleted from the chamber. These values are shown

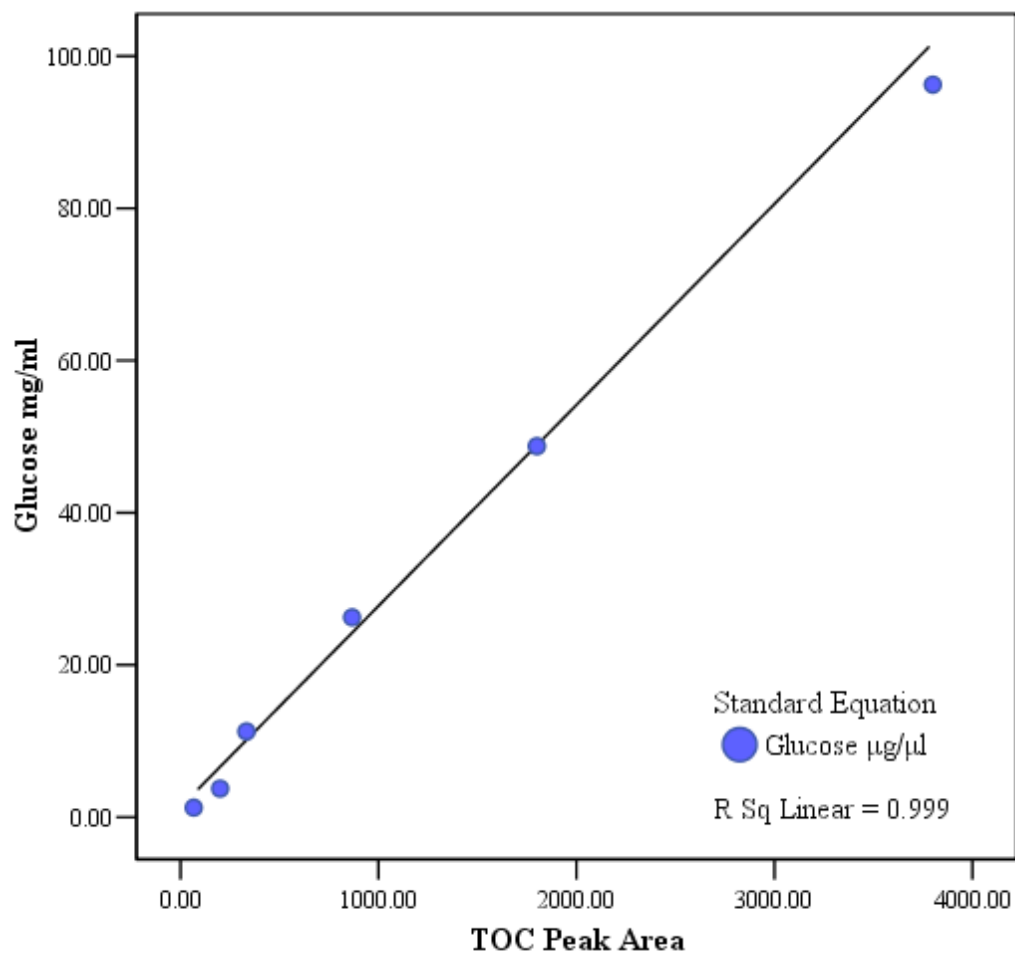


FIGURE 19. GLUCOSE STANDARD.

The glucose standard was created by testing known glucose samples for carbon content. Peak area was plotted vs. glucose concentration. A regression line was generated with an $R^2 = 0.999$, which indicates that the line is a good fit for the data. Data points are the mean of five replicates.

in table 3. It was expected that glucose concentration should decrease after an oxygen experiment because cells use glucose during respiration. This was not the case. In all but four samples the predicted final value was greater than the initial value. Aliquots were spun down prior to analysis to prevent contamination by cellular material but this could be a potential source of carbon. Also, the majority occurs with Bio-gels that still contain some LB medium bound inside the sol-gel matrix. If LB was released from Bio-gels it could add carbon to the TOC analysis.

Of the remaining samples, it is expected that 2.369mg of carbon would be utilized by cells (89%) and 0.293 mg would remain (11%). This was not observed in any sample. Free cells have more carbon remaining than the Bio-gel sample. A one-sample t-Test was utilized to compare predicted values from TOC quantification against the theoretical *E.coli* value of carbon that would not be respired (0.293mg). The test indicates that the predicted values are similar to the theoretical value for week 0 cells ($p > 0.05$), week two cells ($p > 0.05$), week six cells ($p > 0.05$), and week 8 gels ($p > 0.05$). These groups have lower carbon recovery rates than the theoretical *E. coli* rate, which may indicate a difference in *5RL* energetics. The predicted rates for samples tested are: week 0 cells 82.4%, week two cells 85.0%, week six cells 86.2%, and week 8 gels 79.7%. This provides evidence for the theory that encapsulated cells are less metabolically active than cells in solution. In addition, this method may require optimization to get a better response from future data sets but it appears to be useful in predicting the carbon recovery rate for oxygen experiments.

TABLE 3. PREDICTED GLUCOSE IN MILLIGRAMS.

The initial glucose concentration is 2.622mg glucose. The final concentration should be less than the initial but this is not the case in some samples. The theoretical recovery rate for cells is 2.369mg (89%) while 0.293mg will remain (11%). Predicted values are the mean of three replicates.

Type	Week	Predicted glucose (mg)	Difference from Initial Concentration
Bio-gels	0	6.641	- 4.019
	2	7.880	- 5.258
	4	8.507	- 5.885
	6	9.478	- 6.856
	8	2.451	0.171
	10	4.112	- 1.490
Free cells	0	2.161	0.461
	2	2.230	0.392
	4	4.397	- 1.775
	6	2.259	0.363
	8	3.307	- 0.685
	10	3.104	- 0.482

Chapter Five

RESEARCH QUESTION THREE: IS MASS TRANSFER AN ISSUE FOR ENCAPSULATED CELLS?

Anaerobic Induction of Bioluminescence

One problem faced by all matter is the property of mass transfer resistance. Mass transfer involves the transfer of materials through different phases (water, oxygen, and target analytes). In order for these materials to reach the cell they must pass through several phases: from air to media, from media to the cell membrane, and from the cell membrane into the cell. When not addressed, these mass transfer limitations have the potential to negatively impact the viability of bioreporter cells. When utilizing a culture of cells in suspension mixing the culture will negate mass transfer limitations. However, this is not always possible for bioreporter cells inside a microelectronic device or when utilized in applications outside the laboratory. In these situations it is known that cells face some mass transfer resistance.

When utilizing a sol-gel matrix to encapsulate bioreporter cells it is important to determine whether the matrix adds an additional significant mass transfer barrier over that of cells that are in suspension but not mixed. The sol-gel matrix is porous and depending on the pore size (in relation to cells that are encapsulated) the matrix may or may not affect mass transfer. Additionally, it is not possible to negate mass transfer barriers in gels through mixing because the matrix would be destroyed. Mass transfer becomes even more important when utilizing a bioluminescent bioreporter cell because

the bioluminescence reaction requires oxygen for the production of light. The encapsulation of a bioreporter provides an opportunity to test mass transfer in the gel to see if bioluminescence is inhibited, which would indicate problems with the matrix.

Bio-gels and Free cells were induced with salicylate in sealed tubes that had previously been bubbled with nitrogen to remove oxygen. Free cells were not mixed to give an approximation of the normal mass transfer resistance faced by a non-mixed culture. The cells in both cases, as observed by a slight induction of bioluminescence, then used all remaining oxygen. When light levels begin to decline, tubes were opened to allow oxygen to the cells. As seen in figure 20, bioluminescence quickly recovered when tubes were opened and exposed to air. Independent samples t-tests were used to compare initial bioluminescence to that after oxygen became available (table 4.) The alpha value was adjusted at each time point to compensate for repeated measures.

Both Free cells and Bio-gels had a significant increase in bioluminescence immediately after opening the tubes. The experiment was continued to ensure true significance instead of possible type one errors. After five minutes of exposure to air, Independent samples t-test indicates there was no significant increase in bioluminescence of Free cells or Bio-gels. At ten minutes after air exposure Free cells again showed no significant difference while the Bio-gels displayed a significant increase in bioluminescence from the initial reading. At fifteen minutes, and all time points thereafter, both showed a significant change in bioluminescence. The sol-gel negative control group did not show a significant

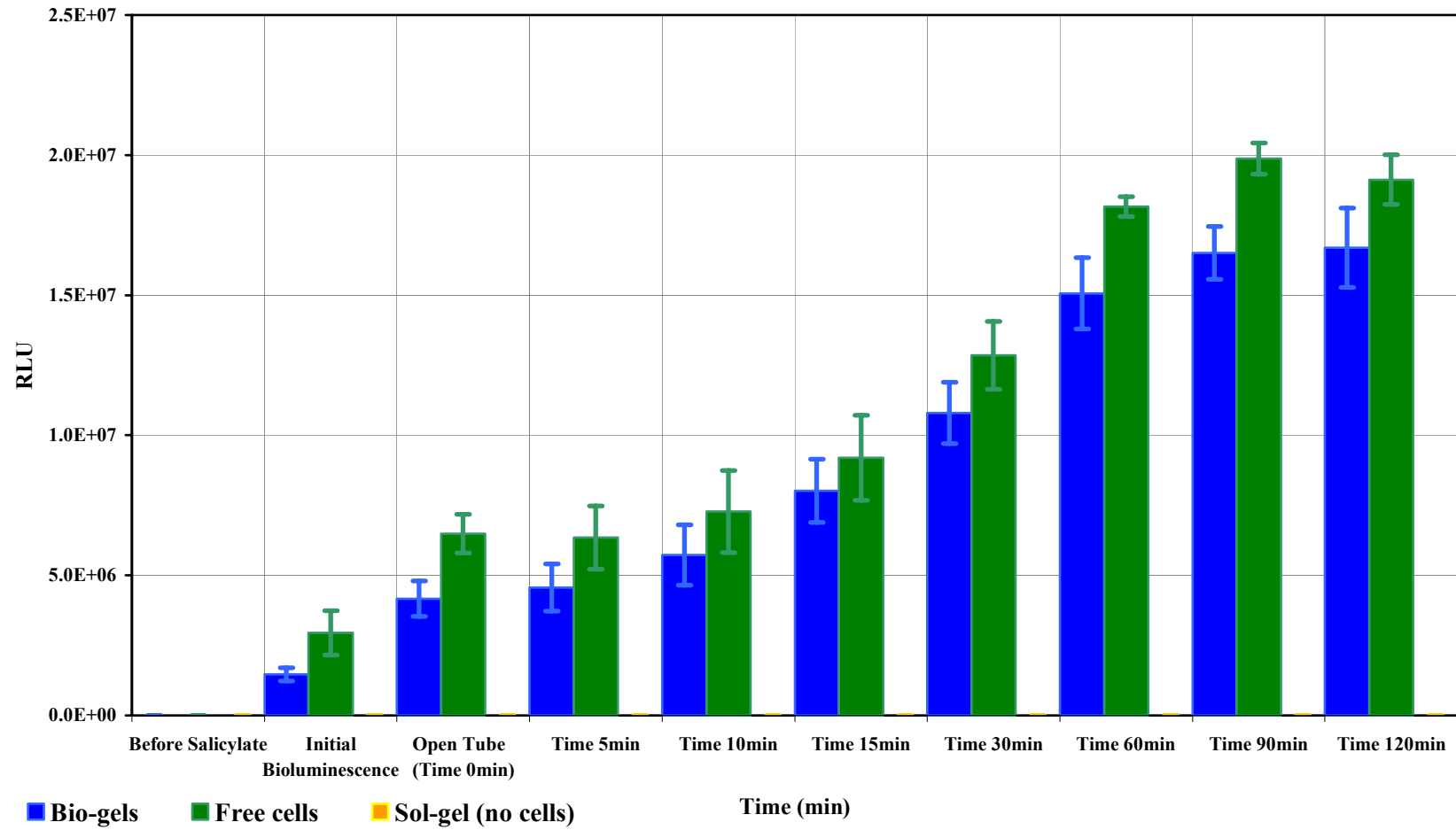


FIGURE 20. ANAEROBIC INDUCTION OF BIOLUMINESCENCE.

Groups were induced with 25ppm salicylate in MSM. Bars show the average of three replicates. Error bars indicate the standard error for each.

TABLE 4. INDEPENDENT SAMPLES T-TEST RESULTS FOR ANAEROBIC INDUCTION EXPERIMENT.

Values were judged to have a significant difference in bioluminescence if their calculated p-value was less than the adjusted alpha (α). Alpha was adjusted to compensate statistically for the repeated measures taken during the experiment.

Type	Time point	Adjusted α	Calculated P-value	Significant Difference?
Free cell	0 min	0.0500	0.028	Yes
	5 min	0.0250	0.064	<i>NO</i>
	10 min	0.0167	0.041	<i>NO</i>
	15 min	0.0125	0.011	Yes
	30 min	0.0100	< 0.001	Yes
	60 min	0.0083	< 0.001	Yes
	90 min	0.0071	< 0.001	Yes
	120 min	0.0063	< 0.001	Yes
Bio-gel	0 min	0.0500	0.009	Yes
	5 min	0.0250	0.035	<i>NO</i>
	10 min	0.0167	0.015	Yes
	15 min	0.0125	< 0.001	Yes
	30 min	0.0100	< 0.001	Yes
	60 min	0.0083	< 0.001	Yes
	90 min	0.0071	< 0.001	Yes
	120 min	0.0063	< 0.001	Yes
Sol-gel (Negative control)	0 min	0.0500	0.253	<i>NO</i>
	5 min	0.0250	0.253	<i>NO</i>
	10 min	0.0167	0.303	<i>NO</i>
	15 min	0.0125	0.927	<i>NO</i>
	30 min	0.0100	0.904	<i>NO</i>
	60 min	0.0083	0.409	<i>NO</i>
	90 min	0.0071	0.626	<i>NO</i>
	120 min	0.0063	0.799	<i>NO</i>

induction of bioluminescence at any point during the experiment. This is to be expected since no cells were encapsulated in this group.

From the t-test it is interesting that Bio-gels showed a recovery in bioluminescence faster than Free cells. With the visual observation of the resazurin indicator, which turns pink in anoxic conditions, it was possible to see the gels were pink while the media above gels was purple. The vials containing Free cells were a purple color. Over time all vials of both Bio-gels and Free cells turned pink in color. This is an irreversible change in color. It is possible to theorize from this observation that since cells are located together in the gel at the bottom of the Bio-gel vials they were able to create a higher demand for oxygen and therefore a gradient in the media. This allowed oxygen from the atmosphere to diffuse through the media faster than with Free cells that were dispersed throughout the vial. Nevertheless, from the t-test data it does not appear that mass transfer in the sol-gel matrix is limiting bioluminescence through the availability of oxygen any more than in non-mixed aqueous media.

Another possible theory to explain the response is that cells are healthier in one condition over the other. This may be true in the case of Free cells because they are stored in rich LB media while Bio-gels are stored in MSM. A preliminary experiment was performed with Free cells stored in MSM and there was not a significant induction in bioluminescence, indicating poor health of the cells. Therefore, Free cells were stored in rich media before subsequent experiments. Overall, the Bio-gels were exposed to harsher

conditions, yet still had a better luminescence response overall. This again implies that the sol-gel matrix does not limit mass transfer.

Why Does the Light Go Out?

The bacterial strain utilized in this project is *5RL*. As discussed in the Review of Literature in Chapter One, *5RL* was created with an insertion of a *lux* cassette into a natural naphthalene catabolic plasmid pKA1 (25). The *lux* cassette inserted into *nahG*, disrupting the lower operon. Because of this, *5RL* cannot convert salicylate into pyruvate and acetaldehyde, so there is accumulation of salicylate. Since salicylate is the inducer of both upper and lower operons, there is continued induction of the upper pathway and in the case of *5RL*, continued bioluminescence.

When using *5RL* in a bioluminescence experiment there is an induction peak and then light declines until it reaches a background level. However, because *5RL* cannot completely break down salicylate the bioluminescence should continue instead of exhibiting a decline. This brings up the question of “what makes the light go out?” The possibilities include the following: inadequate oxygen, inadequate nutrients to convert into reaction precursors, or cell death. Of these possibilities, the lack of oxygen is a concern for encapsulated cells. If the sol-gel matrix is limiting mass transfer then there will not be sufficient oxygen for the bioluminescent reaction.

To get an idea of “why the light goes out” gels were set up as usual in three Greiner plates. Bio-gels were induced with salicylate and then plates were sealed. A peak in

bioluminescence consistent with previous experiments, was observed. After the light had decreased to a background level each plate was subjected to a different treatment. Plate one (Add Oxy) was given oxygen by exposure to air ten minutes. Plate two (Add Glu) was given glucose. Plate three (Add Both) was given both oxygen and glucose. A sol-gel negative control (NC) also followed each treatment but no cells were utilized in this group. Bioluminescence was observed, and after light declined to a background level the treatment was repeated.

As seen in figure 21, all groups had nearly identical initial induction peaks (peak I). ANOVA analysis was carried out with SPSS to compare the induction. Statistics indicate that at least one group is significantly different from the others ($p < 0.001$). The Dunnet T3 post hoc test was to determine where the differences lie. The tests indicated that the bioluminescence of Add Oxy was similar to Add Glu ($p > 0.05$) and Add Both ($p > 0.05$). Also, Add Glu was similar to Add Both ($p > 0.05$) while NC was significantly different from all treatments ($p < 0.001$). It is expected for the NC group to show different bioluminescence because no cells were present. These results are summarized in table 5.

After the first treatment (peak A), Add Oxy had only a small peak measuring 23% of the bioluminescence of the initial induction. In contrast, Add Glu and Add Both had large peaks with a 143% and 155% induction respectively. ANOVA analysis was carried out with SPSS to compare the induction. Statistics indicate that at least one group is significantly different from the others ($p < 0.001$). The Dunnet T3 post hoc test was performed indicating that the bioluminescence of Add Glu was similar to Add Both ($p >$

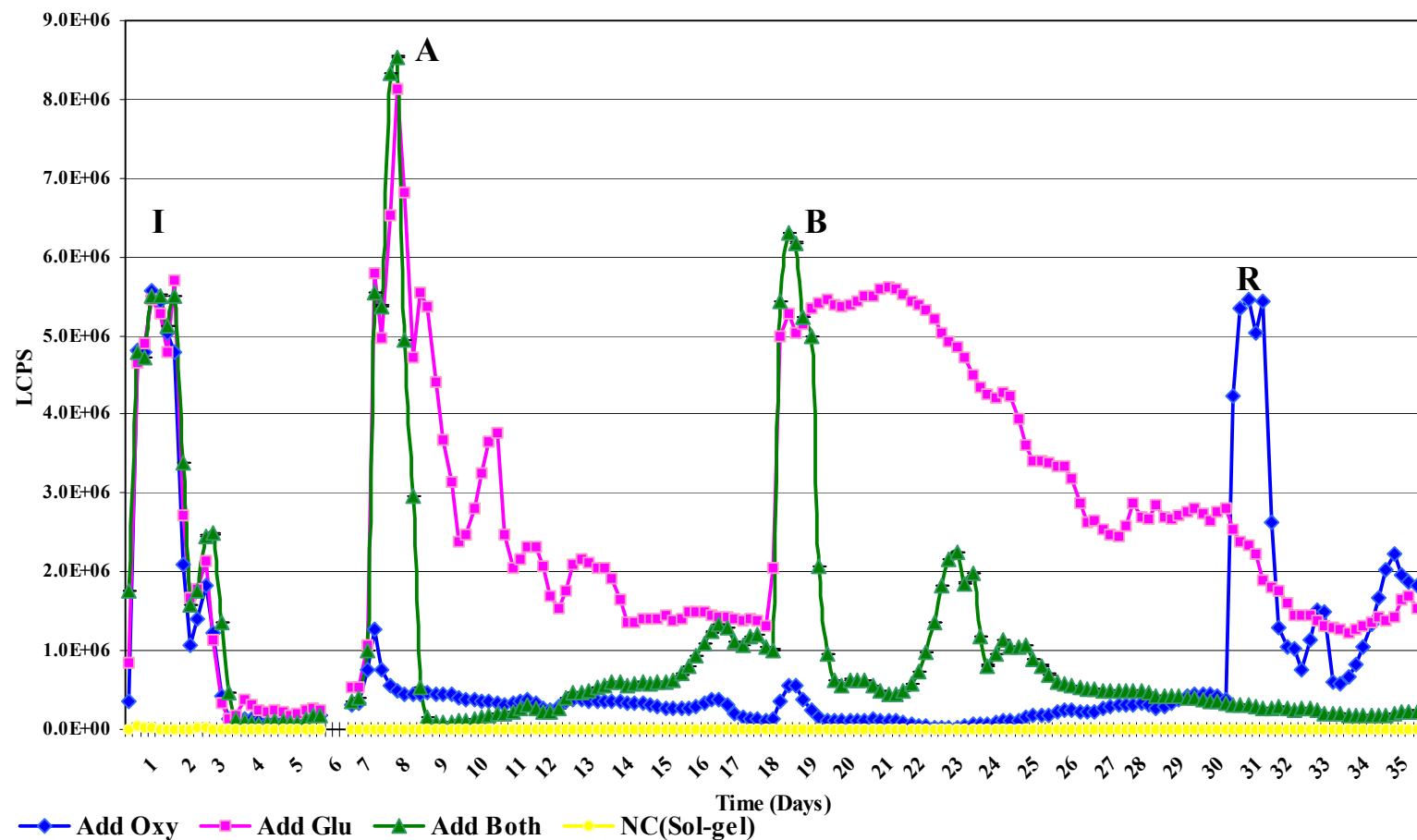


FIGURE 21. OVERALL BIOLUMINESCENCE OF “WHY NO LIGHT?” EXPERIMENT.

Bold black letters identify the peaks. Data points are the average of sixteen replicates. Error bars represent the standard error of the assay. The majority of error bars are too small to visualize.

TABLE 5. SUMMARY OF ANOVA RESULTS AND BIOLUMINESCENCE FROM “WHY DOES THE LIGHT GO OUT?” EXPERIMENT

Part A. ANOVA Results

Treatment Cycle	ANOVA Results	Dunnet T3 Results
Induction	At least one group is significantly different (p<0.001)	NC is significantly different from Add Oxy, Add Glu, and Add Both (p<0.001)
Treatment A	At least one group is significantly different (p<0.001)	Add Oxy and NC are significantly different from Add Glu and Add Both (p<0.001)
Treatment B	At least one group is significantly different (p<0.001)	Add Oxy and NC are significantly different from Add Glu and Add Both (p<0.001)
Restore Bioluminescence	At least one group is significantly different (p<0.001)	All groups are significantly different from one another (p<0.001)

Part B. Bioluminescence of each group.

Treatment Cycle	Type	Bioluminescence	Percent of Initial Induction
Induction	Add Oxy	5.20×10^6	NA
	Add Glu	4.87×10^6	NA
	Add Both	3.75×10^6	NA
Treatment A	Add Oxy	1.11×10^6	23 %
	Add Glu	7.89×10^6	143 %
	Add Both	8.37×10^6	155 %
Treatment B	Add Oxy	4.52×10^5	10 %
	Add Glu	4.30×10^6	98 %
	Add Both	5.25×10^6	114 %
Restore Bioluminescence	Add Oxy	5.04×10^6	98 %

0.05) while Add Oxy and NC were significantly different from all treatments ($p < 0.001$ for each). These results are summarized in table 5.

After the second treatment (peak B), which was identical to the first, a similar pattern was observed. Add Oxy displayed a small peak of 10% bioluminescence of the initial peak while Add Glu and Add Both had large peaks of 98% and 114% respectively. ANOVA analysis was carried out with SPSS to compare the induction. The statistics indicate that at least one group is significantly different from the others ($p < 0.001$). The Dunnet T3 post hoc test was performed indicating that the bioluminescence of Add Glu was similar to Add Both ($p > 0.05$) while Add Oxy and NC were significantly different from all treatments ($p < 0.001$ for each). These results are summarized in table 5.

No additional salicylate was given during any treatment, so bioluminescence is from cells induced in the initial induction period. Encapsulated cells were able to recover bioluminescence after utilizing glucose to make necessary precursor materials better than by receiving oxygen alone. This supports observations in the previous experiment that the transfer of oxygen through the gel is not limiting bioluminescence of cells.

To further test this hypothesis, a treatment of glucose was given to the Add Oxy group in an attempt to restore bioluminescence (peak R). The treatment was successful and bioluminescence was observed at 98% of the initial peak. ANOVA analysis was carried out with SPSS to compare the induction. The statistics indicate that at least one group is significantly different from the others ($p < 0.001$). The Dunnet T3 post hoc test was

performed indicating that all groups displayed different levels of bioluminescence from each other ($p < 0.001$ for all). These results are summarized in table 5.

It is likely that some of the cells were no longer viable at this time point, which explains the lower bioluminescence of Add Oxy than the initial peak. The final treatment was given thirty days after the initial induction. This is an encouragement in itself because cells remained viable for over thirty days when encapsulated in the sol-gel matrix without addition of a carbon source. No special storage was used, and plates were continuously measured at room temperature throughout the experiment. In addition, cells responded to treatment with 98% of the original bioluminescence signal after thirty days, indicating potential for use in long-term studies.

All of the data was compared with a MANOVA analysis using the difference between initial and peak induction at each treatment (figure 22.) The difference of initial to peak bioluminescence was used in the comparison because this experiment utilizes repeated measures and to make a comparison over time, all data must be considered. In addition, statistics were corrected for repeated measures with the SPSS program. The tests show a significant difference between bioluminescence levels of the groups over time ($p < 0.001$) as well as a significant time*type interaction ($p < 0.001$). The Dunnet T3 Post-Hoc test indicates that Add Glu and Add Both did not show significant differences in bioluminescence over time ($p > 0.05$). All other comparisons indicate a significant difference in bioluminescence over time ($p < 0.001$).

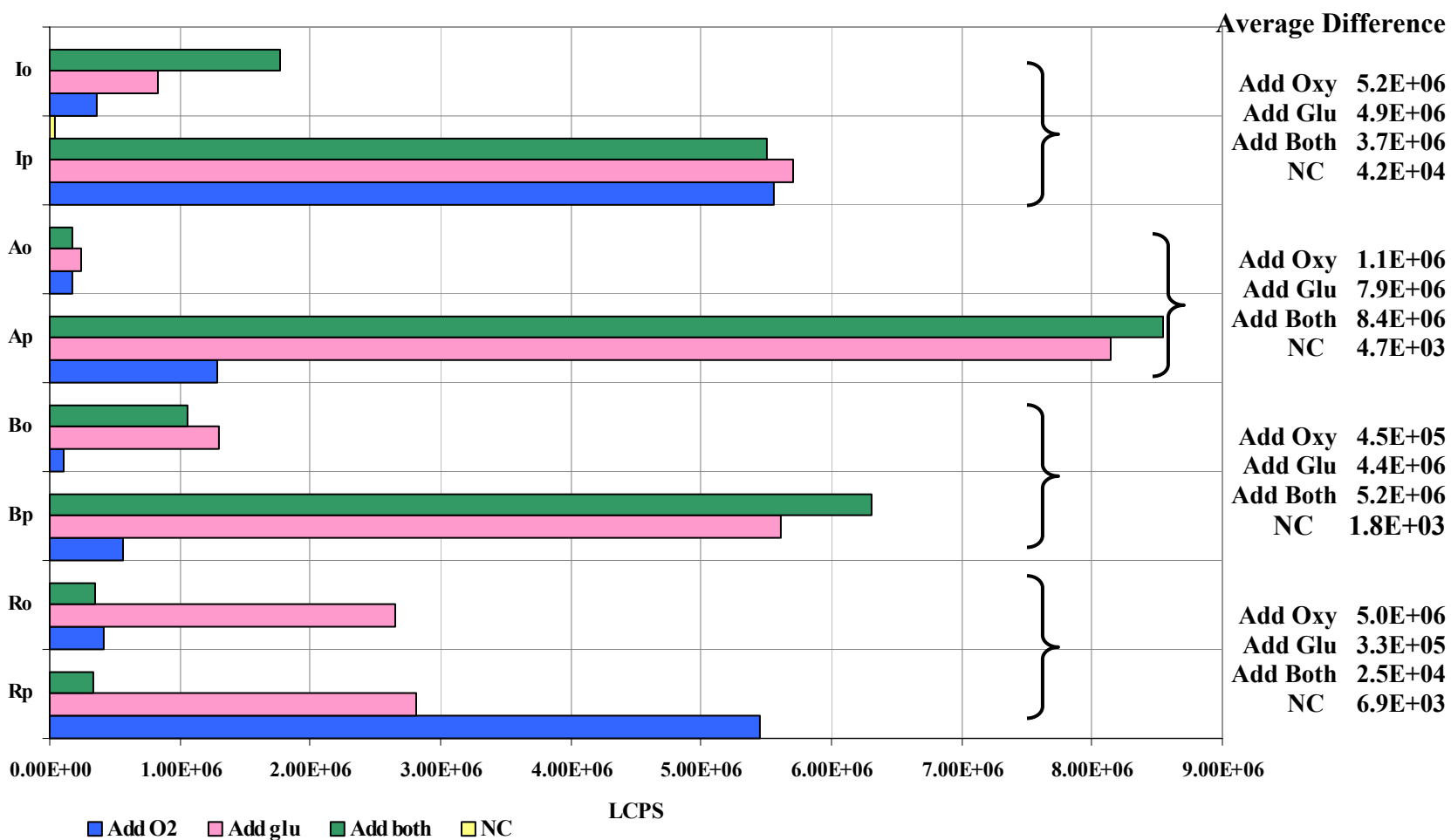


FIGURE 22. DIFFERENCE BETWEEN INITIAL AND PEAK BIOLUMINESCENCE.

Difference values were utilized in MANOVA to test the variance of time and type. Bars represent the mean of sixteen replicates.

Each type was also examined separately over time using their difference values. Add Oxy, Add Glu, and Add Both all showed significant differences in bioluminescence over time ($p < 0.001$) and each treatment had a significant difference from the next ($p < 0.001$). These tests support the results from previous tests that show the change in bioluminescence over time for each group. With Sol-gel (NC) there are not as many differences. There is no significant time interaction of bioluminescence between treatments ($p > 0.05$). There are similarities between each treatment: from Initial to Peak A ($p > 0.05$), from Peak A to Peak B ($p > 0.05$), and from Peak B to Restore ($p > 0.05$). These tests indicate that bioluminescence did not change in the NC group, which is expected because no cells were encapsulated.

These experiments suggest that mass transfer is not an issue for sol-gel encapsulated cells. Bio-gels were not limited by oxygen in either case, which is similar to reports from other labs (24, 44, 45). In the anaerobic induction experiment Bio-gels were able to recover faster than Free-cells. This experiment suggests what would occur when cells are placed on a device to form a biosensor. The encapsulated cells would act as a unit, which may mean a quicker response in the field. Because they are together in the matrix, cells may create a higher oxygen demand, which would allow oxygen to diffuse faster into the device. This would improve the viability of cells. In addition, Bio-gels also were induced more quickly in the anaerobic induction experiment, which was not shown on the graph, but also indicates their superior fit in a biosensor device used in the field. In the second experiment, it is likely that nutrient limitation is the explanation for decrease in bioluminescence. Adding oxygen had a far lesser effect than the addition of glucose.

Encapsulated cells remained viable and were active for over thirty days with continuous measurement at room temperature and only a 2% decrease in bioluminescence from the initial induction. This experiment also indicates that mass transfer does not limit Bio-gels.

Chapter Six

CONCLUSIONS

The study of the encapsulation of cells in a sol-gel matrix has involved many experiments. A brief listing of the results of each is below, to be followed by overall conclusions of the study.

Protein Assay

It is possible to use the Coomassie Blue protein assay to derive an estimate of the number of cells encapsulated in sol-gel. The equation that was derived to predict encapsulated cell number is: $y = -3.9026 \times 10^{10} + 6.626 \times 10^{10}(x) - 2.095 \times 10^{10}(x^2)$. This equation has an adjusted $R^2 = 0.9680$ which indicates that it is a good fit for the model. It was also discovered that the assay has a minimum absorbance value of 0.783 that can be used in the equation. Readings below this value will result in a negative prediction.

Escaped Cells Assay

Escaped cells were detected at each time point over a 72-hour period. The number of escaped cells was significantly less than the number encapsulated indicating that the majority of cells remain enmeshed in the matrix.

Electron Microscopy

SEMs show the porous nature of the matrix. Cells are visualized on the surface as well as cellular impressions in the sol-gel. A number of horseshoe and circular cracks are also

observed in the gel surface. This is also an unknown phenomenon and may be the result of normal gel ageing or could be an artifact of critical point drying used for SEM preparation.

TEMs show cells surrounded by the sol-gel matrix. Their cell membrane does not appear to be disrupted suggesting that cells are not harmed during the encapsulation procedure. Two unique phase variants were visualized in the TEMs: normal and mucoidal. A white layer surrounds the mucoidal variant. This is an unknown phenomenon that may be similar to the mucoidal and smooth colonies that grow when *5RL* is grown on agar.

Bioluminescence

In short-term studies there is no significant difference in the induction of Bio-gels and Free cells. This indicates that the sol-gel matrix does not inhibit the diffusion of salicylate to encapsulated cells. It also indicates that encapsulated cells are not inhibited in their ability to respond to induction, form a functional luciferase, and emit bioluminescence. There is also no significant difference in the response time of Bio-gels and Free cells. This again implies that cells are not affected by encapsulation.

Oxygen Monitoring

Bio-gels and Free cells were able to deplete oxygen from the chamber much faster than media without cells. This suggests that cells are metabolically active whether or not they are encapsulated. Free cells have a faster respiration rate than Bio-gels. This may suggest that diffusion of oxygen or glucose to the encapsulated cells is inhibited. A more likely

possibility is that Bio-gels are less active than Free cells because they are not able to divide within the matrix.

Long-term Study

The long-term study confirms observations made in the previous protein and oxygen monitoring. The protein concentration changes significantly over the ten-week study. This appears to be the result of two outliers in the four-week measurement. However there is a slight change even when these values are removed. This may be due to improper rinsing of gels, which allows “escaped” cells at the surface to be measured, or to another unknown factor.

As with short-term studies, Free cells used oxygen faster than Bio-gels in the long-term experiment. In addition, Bio-gels utilized oxygen faster than the negative control. Oxygen studies show a significant difference in the respiration rate for the first six weeks. Week eight and ten are not significantly different which may indicate that Free cells are losing viability. Data from carbon quantification was not able to confirm the oxygen results. The results that were usable, displayed similarity to the theoretical value of carbon that would remain after respiration, although the predicted recovery rates were lower for *5RL* than *E. coli*. Recovery rates indicate a difference between Free cells and Bio-gels but there were not enough samples for further analysis. The carbon quantification protocol needs optimization.

Bioluminescence data seems to contradict the conclusions reached in protein and oxygen experiments. According to MANOVA analysis, although the difference in bioluminescence over time was significant, the difference between Bio-gels and Free cells was not significant. This is similar to results observed in short term bioluminescence studies. The t-Tests suggested a similar trend between Bio-gels and Free cells in results at weeks eight and ten for bioluminescence and cellular respiration. The main finding of the bioluminescence study was that cells retain the ability to respond to induction by salicylate even after storage for ten weeks.

One detail that affected all of the long-term experiments was the preparation conditions. Encapsulated cells endured more harsh conditions through the encapsulation procedure and during storage and were still able to respond better than Free cells by weeks eight and ten. If Free cells were stored in plates or even tubes of minimal media they did not remain viable for the study. This gives new insight into the long-term stability of sol-gels for use in maintenance of bioreporters.

Why Does the Light Go Out?

After several treatment cycles, it was evident that depletion of nutrients was more important to bioluminescence than depletion of oxygen. This was confirmed by a treatment where glucose was given to the oxygen only group to restore bioluminescence. Bioluminescence was restored to levels similar to the initial induction even after thirty days. This suggests that encapsulated cells do not face mass transfer issues.

Anaerobic Induction of Bioluminescence

Bioluminescence was restored faster to Bio-gels than Free cells after anaerobic tubes were exposed to air. This indicates that encapsulated cells do not face mass transfer issues. In addition, because cells are concentrated in the gel at the bottom of the tube it may have been possible for them to create an oxygen gradient that allowed for their oxygen needs to be met in a more efficient manner than Free cells that were randomly dispersed in the media.

Overall Conclusions

Although there are indications that significant differences exist between Free cells and Bio-gels, it does not appear that encapsulation dramatically affects cells. Encapsulated cells were able to respond above any background or non-induced controls in each experiment indicating that cells are metabolically active. In some instances encapsulated cells respond faster and to a better extent than cells in suspension. One example is in long-term storage. Results suggest that encapsulation may allow for better long-term maintenance of bioreporters. Another advantage is that after gels are prepared they may be stored “as is” until use. This was not possible with Free cells. Bio-gels were maintained at 4°C in microplates with minimal media and glycerol. In the “Why No Light” study they were kept at room temperature for thirty-five days and still displayed bioluminescence levels similar to the initial induction. The results of this project indicate that there is potential for the use of sol-gel encapsulation of bioreporters used in certain devices and field applications.

Future applications of this project include testing Bio-gels in biosensors and field situations. It is important to determine if encapsulated cells can respond as well outside the laboratory. It would also be interesting to attempt encapsulation of various reporter strains in the same gel as suggested in the report by Premkumar et al. (45). This would extend the possibilities of both biosensors and sol-gel encapsulation even further by allowing multiple sensors to be used at the same time.

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