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Methods of cellular integration and techniques for improving encapsulated cell viability for the purpose of developing living materials and cell based bio-sensing materials

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Methods of Cellular Integration and Techniques for Improving Encapsulated Cell Viability For the Purpose of Developing Living Materials and Cell Based Bio-Sensing Materials

BY

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A.S., Chemistry, San Juan College, 2006
B.S, Chemical Engineering, University Of New Mexico, 2010

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ABSTRACT

Living materials offer the potential for detection-reporter systems based on living cells that are genetically tailored to sense target analytes with high levels of specificity and accuracy. A biotic or living material would allow the development of simple, hand held devices that could be utilized for many applications including industrial process monitoring, environmental remediation, military and defense applications and drug delivery. Many living materials have been developed and characterized but they still lack heightened and prolonged encapsulated cell viability necessary to make the material a candidate for widespread use.

One particular material offers several advantages to increase yeast cell viability by integrating the cells into a lipid-silica templated mesoporous silica matrix using an evaporation induced assembly that selects specifically for living cells. This type of integration yielded increased cell viability versus other conventional methods by introducing an artificial fluid lipid bi-layer between the cell plasma membrane and the

surrounding silica which aided in protecting the integrated cells. Despite viability improvements, integration by this method still lacks the desired and necessary prolonged cell viability. During the integration process cells are threatened by stresses such as desiccation or oxidation and incompatibility with the bio-nano interface. To reduce the negative impacts of such stresses and further promote integrated cell viability, strategies inspired by extremophillic organisms were investigated by incorporating trehalose, manganese-phosphate buffer and fluid/non-fluid liposomes into the integration process.

It was discovered that incuding manganese phosphate buffer with the integration process offered average viability increases of 3-7% versus cells integrated without the buffer. Findings based on a superoxide indicating fluorescent assay suggest that inclusion of manganese phosphate buffer reduces oxidative stress immediately upon integration and that oxidative stress is likely a primary stress in the first few hours of integration. Addition of fluid (DOPC) or non-fluid (DPPC) lipid liposomes also had an impact on integrated cell viability. Both types of lipids offered initial increases in integrated cell viability – non-fluid lipids offered the largest gains of nearly 4-5 fold over a standard control, and 3 times higher than fluid lipid liposomes. Inclusion of non-fluid liposomes (DPPC) also had the best effect of maintaining cell viability during prolonged encapsulation, surpassing viabilities numbers seen for standard silica matrices or matrices containing fluid lipids (DOPC) up to 15 days.

Incorporating trehalose had the biggest positive effect on integrated cell viability, surpassing the viability of yeast cells integrated without trehalose by 5 fold, offering initial viabilities near 25-30%. Optimum relative humidity ranges were also established for producing films with and without trehalose. Films containing trehalose providing

integrated cell's with increased viability at higher relative humidities where as cells integrated into matrices without trehalose preferred mild to moderate humidity levels near 18-20%.

As an alternative to the lipid templated mesoporous-silica encapsulation method shown above, three different aqueous silica gels were also tested for their ability to encapsulate an engineered bio-sensing strain of *E. coli*. This particular strain of bacteria is capable of detecting theophylline, a small organic compound with a chemical structure similar to caffeine, and responding by producing green fluorescent protein (GFP). Cells encapsulated in each gel were subjected to tests that would determine long term viability trends and the ability of the cells to detect theophylline during prolonged encapsulation. The limits of theophylline detection and GFP response curves of the encapsulated cells were also analyzed.

Findings indicate that gels made with poly-glycerated silicate (PGS) offered improved viability over gels formed with sodium-silicate based methods – by a margin of 10%-20 after encapsulation for 2-5 weeks. All three aqueous silica matrices offered profound improvements in cell viability (initially and during prolonged encapsulation) versus cells integrated using standard or modified lipid template mespoporous silica matrices. Furthermore, cells encapsulated in PGS gels were slower at producing equivalent GFP fluorescent intensities, under conditions of identical theophylline exposure, versus gels made from sodium-silicate solutions. Theophylline detection thresholds of encapsulated cells had theophylline limits near 1 uM for all three gels which surpassed the reported theophylline detection threshold of the cells tested in solution by a factor of 10. Although each gel shared the same dynamic limit of detection, each matrix

offered a distinct fluorescent response curve and also had a concentration range where integrated cells were observed to respond to theophylline exposure best. To that end, it is concluded that material properties/differences of each silica matrix had profound impacts on cell viability, cell ability to grow colonies post encapsulation, theophylline detection response curves and the overall fluorescent intensities of integrated bacterial cells.

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Introduction

In recent years, advances in cell biology and materials engineering have spawned a new revolution to develop materials that incorporate bio-molecules and living cells into 3D, organic and inorganic matrices.[1-5] These materials are of particular interest because they offer potential solutions to problems involving military and defense applications, industrial process monitoring, environmental remediation, drinking water contamination, tissue engineering and drug delivery.[4-8] An enormous volume of work exists on the topics of protein, enzyme and bio-molecule encapsulation which has been successfully performed and documented.[2,3,6-9] Unfortunately, work that is focused on integrating living cells and organisms is less abundant, owing to the increased difficulty of performing such tasks.[4,6,10-11]

To date, techniques for integration/encapsulation of biomolecules and living cells have relied on sol-gel processes that utilize modified organo-silanes, alkoxide or sodium silicate based chemistries although, other methods for encapsulation do exist.[1, 3, 10-13] Innovative as these methods may be, the majority of integration methods do not meet the requirements for maintaining living cell viability and activity when encapsulated for extended time periods. Common challenges associated with living cell encapsulation include exposure of biological components to alcohol solvents, cyto-toxic reactants and bi-products, highly acidic conditions, sensitivity to matrix topography, desiccation, oxidation and osmotic stress. These challenges hinder integrated cell viability and adversely affect cell metabolic activity and gene up/down regulation necessary for cell based bio-sensing. This ultimately limits the functionality of a living material and therefore the ability to produce a bio-sensing analytical device. One should not be

discouraged however, because suitable materials and methods continue to emerge that are worthy of a second look.

One such material was developed exclusively by members of our research group, specifically to address the challenges mentioned above.[10,11,13] The material is formed using a cell directed integration process (CDI) by which cells are incorporated into a 3D mesoporous bio/nano interface, characterized by localized lipid bi-layers enveloped by a thin layer of porous, condensed silica.[5,10,13] Several subsequent studies and publications have resulted from this method/material, all of which confirm its innovative biocompatibility; however, this method of integration can be further improved by applying known extremophile techniques that would alleviate major stresses and enhance cell viability.[14-15] Extremophiles are organisms that have evolved to survive in environments that would be otherwise unsuitable for any normal yeast, bacteria or mammalian cell.[14-15] Continued research involving extremophillic organisms has inspired techniques that can be applied to encapsulating matrices and cell integration to counteract the stresses that cells experience.

The first technique for mitigating known CDI stresses is based on the extremophile *D. radiodurans*, a type of bacteria with a very high resistance to oxidative stress and ionizing radiation.[16] This organism is known to have high ratios of Mn^{2+} to $Fe^{2+/3+}$ which are proposed as the direct cause for the organisms oxidative resistance.[16,17] By incorporating manganese (specifically manganese phosphate buffer) into the CDI process, it is theorized that cell viability will increase by reducing oxidative stress.

The second extremophile technique for increasing viability is the addition of liposomes in lipid templated mesoporous silica films. Studies involving the thermophile

Methanolocacus jannaschii (*M. jannaschii*) present findings where the bacterium undergoes a change from the more fluid diether membrane lipids to the more thermostable tetraether lipids upon exposure to high temperature.[22-23] The tetraether lipids are formed by the fusion of two diether lipids and form a monolayer which spans the entire cell membrane. Previously, the addition of liposomes using cell directed assembly (CDA), an integration process similar to CDI, resulted in multilayered fusion of lipids around integrated cells which can span nearly 1 μm in thickness.[11] It is believed that similar behavior will occur when incorporating lipids into the CDI process which may result in higher cell viability.

The final extremophile technique involves the addition of trehalose to the CDI method. Trehalose is a non-reducing, disaccharide sugar molecule believed to provide many micro-organisms, plants and insects with a high tolerance to desiccation and drought; other studies also reported that trehalose is effective at combating oxidative stress.[18, 19, 20] Also investigated in this body of work is the effect of humidity on cell viability for cells integrated into lipid-templated mesoporous silica matrices in the presence or absence of trehalose. This is important because the presence of water (in the film or ambient) during or after the CDI process can directly affect the rate of silica condensation and the amount of water available to the cells; impacting integrated cell behavior and viability as a result.

As an alternative to lipid templated mesoporous-silica encapsulation method, three novel aqueous silica gels were also investigated as a method for living cell encapsulation. Aqueous silica gels demonstrate exceptional bio-compatibility because they are formed with sodium phosphate buffers at near neutral pH levels, offering

suitable ion concentrations and high levels of water content.[1-3,6] They also reduce the encapsulated subjects exposure to harmful chemicals and ethanol co-products, eliminate the need for higher acid concentrations and reduce problems related to desiccation. To further promote a biocompatible environment, aqueous gels can have an ameliorant (in this case glycerol) incorporated into the gel. Glycerol is known to be effective at protecting cells during cryogenic storage and offering protective properties during some methods of cell encapsulation.[2, 6, 21]

To test and characterize all three aqueous silica gels, an engineered bio-sensing strain of *Escherichia coli* (*E. coli*) was chosen as the organism for encapsulation. It was engineered with an artificial riboswitch mechanism which enables cells to detect theophylline and respond by producing green fluorescent protein (GFP). The behavior of these particular *E. coli* has been well documented in solution however, their ability to function when encapsulated is less understood.[22, 23] Riboswitches are mechanisms for creating cell based sensor-reporter systems for detecting analytes.[22,24] Characteristic riboswitches are an RNA molecule(typically a cloned aptamer, naturally or synthetically derived) placed on the 5' untranslated region of mRNA or DNA directly upstream of genes that are to be regulated.[22,24] When the aptamer encounters its specific target, the riboswitch is switched on (typically via conformational change) and ribosomes can then bind the mRNA and begin expression. The goal was to encapsulate riboswitch *E.coli* in aqueous silica matrices that would allow cells to remain viable during prolonged encapsulation as well as maintain ability to detect and report theophylline exposure.

The work presented in this thesis is focused on understanding, exploiting and optimizing methods and materials related to sol-gel chemistry for the purpose of

encapsulating living micro-organisms. This work will aid in the development of robust, functional living biotic materials that will further the development of bioelectronics, bio-analytical devices, platforms for drug delivery and tissue engineering as well as environments to conduct in depth biological studies that could extend from yeast and bacteria to mammalian and cancerous cells. Presented herein are the experimental results of incorporating extremophile based strategies for the purpose of enhancing and prolonging integrated cell viability as well as experimental studies to characterize and understand the behavior of a bio-sensing strain of *E. coli* encapsulated in novel aqueous silica matrices.

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Chapter 1

Extremophile-Inspired strategies for Mediating Stresses upon Cell-Directed Integration into Lipid-Templated Mesoporous Silica

1.1 Introduction

Living materials are a revolutionary class of organic and inorganic hybrid materials that are characterized by the direct incorporation of living cells and bio-molecules into a materials 3D architecture. These materials are the subject of increased research efforts because they offer the potential to solve many issues that affect millions of people around the world every day.[1-3] Some of the many areas that could benefit from such materials include military and defense applications, industrial process monitoring, environmental remediation, tissue engineering and drug delivery.[3-5,] Despite many advances in the fields of materials engineering and biology several biocompatibility issues arise that prevent implementation of living materials into the platforms of cell based sensing devices.[4]

To date, many methods exist for integrating/encapsulating bio-molecules and cells into 3D architectures, each with its own set of advantages and disadvantages.[5] Many methods of integration rely on sol-gel chemistry which is based on aqueous organo-silicon, alkoxide, or sodium-silicate compounds. These compound first undergo acid or base catalyzed hydrolysis; and then condensation reactions that form a silica matrix to encapsulate a bio-molecule or cell.[5,6] While sol-gel techniques are generally effective, integrating living cells becomes problematic due to the use of acidic and basic catalysts, cyto-toxic solvents, reactants and generation of toxic co-products.[3,5] It is also suspected

that integrated cells are subjected to high levels of oxidative stress, desiccation and osmotic stress.[7,8]

Several years ago, members of our research group developed a novel method for encapsulating cells in silica thin films that offered simplicity and bio-compatability.[9, 10] This method, cell directed integration (CDI), is based on sol-gel chemistry that starts by preparing a sol-gel solution that contains a short chain lipid with a phosphocoline head group (*di*C_6PC).[3, 5, 11] The sol-gel is deposited onto a substrate forming a weakly condensed lipid/silica thin film mesophase. Cells suspended in droplets are applied to the mesophase, dissolving it, and then actively reconstruct the evaporating surface to create a fully 3D bio/nano interface, composed of localized lipid bilayers enveloped by a condensed silica.[11] A key advantage of CDI is the use of lipid to form the mesophase (versus more toxic surfactants) and the multilayered lipid bi-layer that results from the integration process. This bi-layer provides cells with a biocompatible interface which prevents them from contacting the silica framework which possesses polar silanol groups suspected to cause integrated cell lysis, presumably through electrostatic and hydrogen bonding interactions.[1]

Integrated cell viability is a top priority for creating a biotic material although increasing long term viability remains a challenge because stresses such as desiccation, oxidation and interactions with silica interface prevent prolonged encapsulation. To address these extreme stresses, experiments were conducted which employed extremophile inspired tactics to allow cells to tolerate highly stressful environmental conditions. Extremophiles are organisms that survive in environments that are otherwise un-inhabitable by evolving unique natural defenses to combat intense local stress. Of

particular interest are extremeophiles such as *D. radiodurans* which is known to resist ionizing radiation and oxidative stress by accumulating high levels of manganese(II) ions or the thermophile *Methanolocacus jannaschii* (*M. jannaschii*) which undergoes changes in lipid fluidity in response to high temperature exposure. Also being considered are different insects, bacteria and plants that are all believed to resist severe desiccation by utilizing a disaccharide sugar molecule called trehalose.[12, 22, 23]

Presented herein are the results of incorporating extremophile inspired strategies of manganese-phosphate, fluid and non fluid liposomes and trehalose with the CDI process in an effort to further enhance integrated cell viability.

1.2 Results and Discussion

Cell Directed Integration

Cell directed integration (CDI) is an active process based on sol-gel chemistry that starts by preparing a sol-gel precursor solution (see experimental section) that contains a short chain lipid with a phosphocoline head group (*di*C_6PC).[3, 5, 11] The sol-gel is deposited onto a substrate forming a weakly condensed lipid/silica thin film mesophase. Cells suspended in droplets are applied to the mesophase, dissolving it, and then actively reconstruct the evaporating surface to create a fully 3D bio/nano interface, composed of localized lipid bilayers enveloped by a lipid and condensed silica.[11] For the bulk of this chapter the sol-gel precursor solution and the lipid templated mesoporous silica matrices that are formed using this solution will be indicated to as the standard or control. The cell directed integration (CDI) process has been well established however, continued research is aimed at developing and quantifying methods for improving

integrated cell viability over prolonged periods of encapsulation. The reason: increased and prolonged viability is paramount to every aspect of research involving encapsulated or integrated cells.

In an attempt to optimize this standard system it was necessary to consider the stresses that could be directly affecting integrated cell viability and the measures that could be implemented to alleviate or eliminate the stress entirely. It was determined that strategies evolved by naturally occurring extremophillic organisms to survive extreme environments could be artificially applied to, or incorporated with the integration process. The extremophile inspired strategies chosen include manganese(II)phosphate to address oxidative stress, and trehalose to counteract the effects of desiccation. Fluid and non-fluid lipid liposomes were also incorporated into the CDI process to determine if they offer additional protection to the cells in the presence of the *di*C_6PC lipid bi-layer. Fluid lipids may offer the advantage of increased fluidity of the artificial lipid bi-layer and self-healing properties as well. Non-fluid lipids could have decreased self-healing properties however they could offer the advantage of forming a robust lipid shell between the cell membrane and the silica host. *Saccharomyces cerevisiae (S. cerevisiae*, S288C yeast) were chosen as the experimental organism of choice because they are a model eukaryote and are fairly robust compared to more delicate mammalian and plant cells.

Method for analyzing viability according to the live dead viability assay.

In order to analyze the effects of including an extremophile inspired strategy, a method for assaying integrated cell viablity was necessary. The assay chosen is the *Fungalite* yeast vitality kit, which uses a membrane integrity indicator (propidium iodide)

and a cell permeable component (AM/CFDA) to assay yeast cell viability. In the presence of compromised membrane the propidium iodide (PI) component will stain cells red – indicating dead. The acetoxymethyl ester (AM) of the esterase substrate 5-carboxyfluoresceindiacetate (AM/CFDA) will stain cells green (indicating viable) in the presence of functional non-specific esterases. Cells that indicated esterase activity and compromised membrane integrity would stain orange or yellow and were not counted as viable. One limit to this investigation is that the assay tests for non-specific esterase activity and membrane permeability which are designed to test cells in solution however, when cells are encapsulated this is only a measure of cell viability and not an overall indication. It is important to note that when assaying cells within a matrix, membrane permeability does not equate to dead from the standpoint of bioactivity which may still be preserved despite the results of viability assays.

All viability measurements were taken and evaluated by the following protocol: On each substrate was prepared a single mesophase thin film in which cells were integrated by applying 0.5 μL droplets to the surface and allowing CDI to proceed. Each substrate was given 6 or 7 droplets which would dry to form a community/colony of integrated cells. When assaying for viability at a given data point, the entire substrate was assayed as a whole and each itegrated colony of cells created by a single droplet was considered a part of the population to be analyzed for the substrate. Each colony was measured for its percent viability from which an average (M_1) and standard deviation (SD_1) was derived. In some cases, a single colony with a viability percentage well outside the range of $M_1 \pm SD_1$ was removed from the population and a new mean (M_2) and standard deviation (SD_2) were determined. No substrate exmained had less than 5

droplets to use in statistical analysis. The final standard deviation for a substrate *(SD_1* or *SD_2*) was used for determining the standard error using the formula $SE = SD_x/\sqrt{n}$. The standard error was used to apply inferential error bars to the data.

When using standard error bars, wide inferential bars indicate large error; short inferential bars indicate high precision and the amount of error and the distance associated between points can be correlated with P test values to indicate statistical significance. This is especially so when analysis involves representative studies with small population sizes where more robust tests such as Chi squared analysis cannot be performed. Error bars given by 95% confidence interval are approximately $M \pm 2xSE$ indicating that such error bars capture the true mean on 95% of occasions. Therefore $M \pm 2xSE$ intervals are quite good approximations to 95% CIs when *n* is 10 or more, but not for small *n*.; if $n = 3$, you need to multiply the SE bars by 4.[23] If a figure shows SE bars you can multiply them in width to approximate the statistical significance. For this chapter, if double the SE bars don't overlap, $P < 0.05$, and if double the SE bars just touch, P is close to 0.05, if double the error bars overlap then $P > 0.05$ and there is less statistical significance between those data points.[23] When $P < 0.05$ the data indicates statistical significance where as $P \approx 0.05$ is considered to have mild significance and values $P = 0.1$ through 0.95 indicate there is very little or no statistically significance difference between data points.

Incorporating Manganese(II)phosphate with the CDI integration process

Manganese(II)phosphate solutions were prepared by balancing molar ratios of Mn^{2+} and PO_4^{3-} utilizing manganese(II)sulfate and a sodium phosphate buffer. The

maximum amount of this solution that could be incorporated with the sol-gel precursor recipe, while maintaining a stable solution that produced quality films, was 4 mM (see experimental methods). Having developed a protocol to include manganese and phosphate, several cell integrated samples of the films as well as Sol-E control films were prepared and viability was monitored. Results of the study can be seen on Figure 1-1.

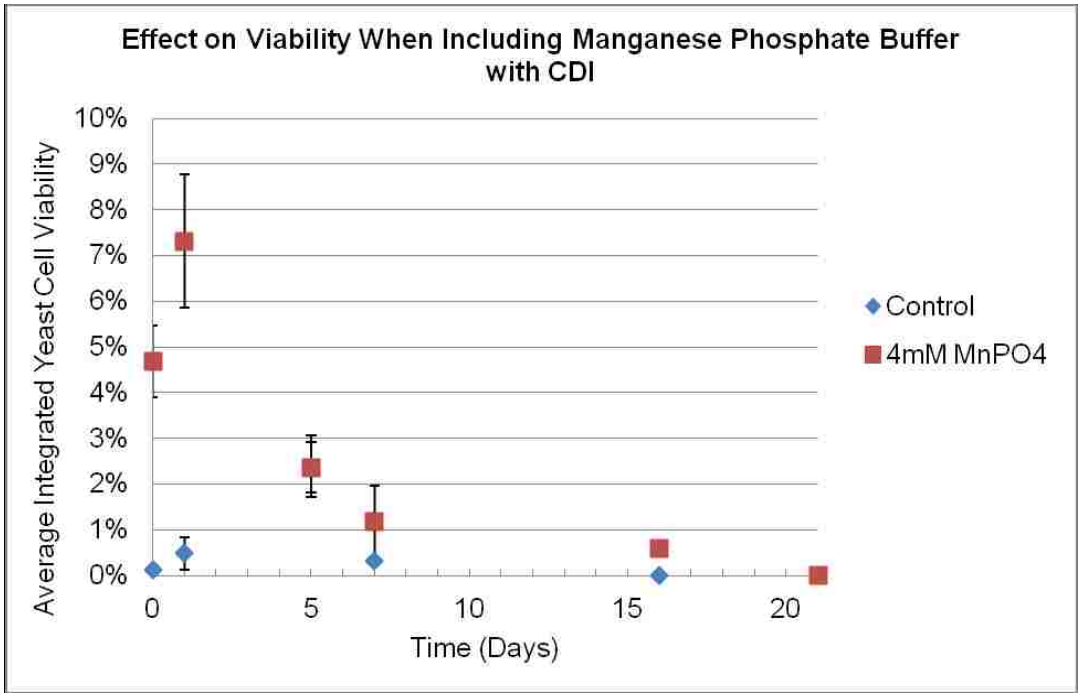

Figure 1-1: Comparing the percent viability of CDI integrated S288C yeast cells when films are modified to include 4.0 mM manganese(II)phosphate buffer versus a standard mesoporous silica film. Error bars represent the standard error of n=5 measurements.

Figure 1-1 illustrates that incorporating manganese-phosphate buffer into the silica matrix does offer an increase in initial viability and allows prolonged encapsulation with extended viabilities of less than 1% for nearly 3 weeks before both types of silica matrices converge at zero viability. Cells integrated into control mesoporous silica matrices without manganese phosphate buffer exhibited low initial signs of cell viability

however, the viability of the control peaked near day 5, only to drop to less than 1% by day 7. The films containing manganese phosphate had higher initial viabilities and little or no effect during prolonged cell encapsulation. The effects of including manganese phosphate were mild as neither type of silica matrix had viabilities above 10%.

As a compliment to the viability study based on the live/dead assay, a study to investigate the presence of superoxide species using the MitoSOX™ Red superoxide indicator was also conducted. This would provide insight to whether or not the manganese phosphate buffer was reducing or eliminating reactive oxygen species or superoxides. The MitoSOX™ Red assay utilizes an indicator which permeates the cell plasma membrane and specifically targets the mitochondria of living cells and, upon oxidation, produces 2-hydroxyethidium which is highly fluorescent when bound to nucleic acids. The MitoSOX™ Red mitochondrial superoxide indicator has excitation/emission maxima of approximately 510/580 nm. The reagent is rapidly oxidized by superoxide but not as quickly by other reactive oxygen (ROS) or reactive nitrogen (RNS) species. Yeast cells in solution were tested using 5 a μM MitoSOX™ solution prepared using 0.1 M DPBS which could also be used to assay integrated cells by applying 200-300 μL to the substrate. As a control, cells were assayed in solution before and after exposure to .10% hydrogen peroxide as well as after allowing cells to dry on a clean glass substrate. Figure 1-2 and Figure 1-3 illustrate the findings based on the superoxide indicator.

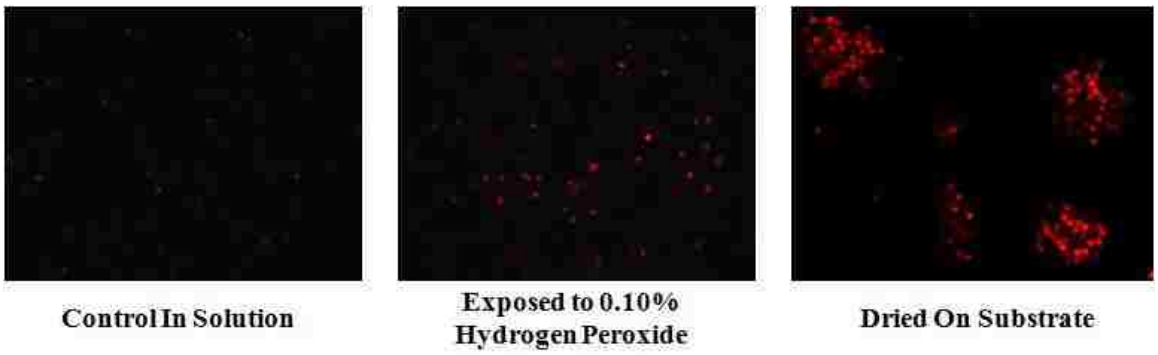

Figure 1-2: Images of yeast cells before and after exposure to 0.01% hydrogen peroxide and cells dried on a clean glass substrate.

When tested in solution, the level of superoxides observed in yeast is low, which can likely be associated with normal metabolic activity. Each of the methods used to test cells as a control (as demonstrated in Figure 1-2) yielded two distinct peaks in fluorescent intensity which is summarized in Figure 1-3. Exposing cells to a 0.10% solution of hydrogen peroxide had a noticeable impact on both the fluorescent intensity of cells that assayed positive for superoxides as well as the percentage of cells testing positive among the population. Data suggests that the fluorescent intensity of cells is 50% higher for cells exposed to 0.10% hydrogen peroxide versus a control. The percentage of cells percieved as testing positive for ROS/superoxides increased when yeast cells were allowed to dry on clean glass substrates and were assayed for superoxide species. Fluorescent intensities measured after assaying suggest that desiccation alone causes a 170% increase in fluorescent intensity over cells tested in solution and a 20% increase over cells tested after exposure to 0.10% hydrogen peroxide.

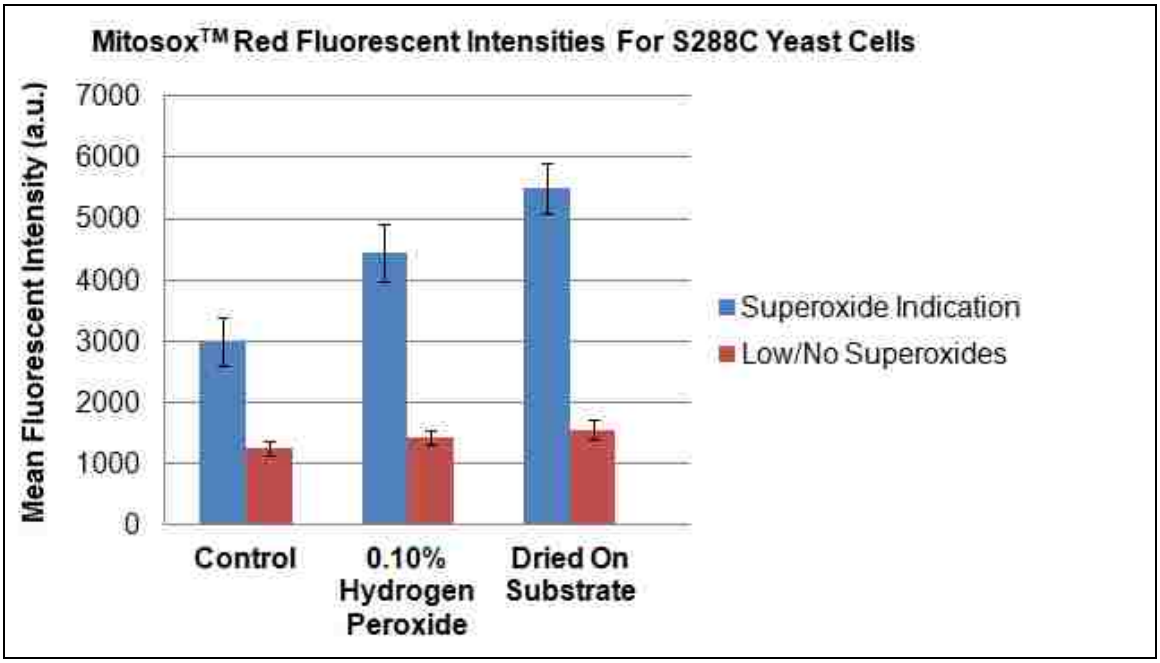

Figure 1-3: Mean fluorescent intensities of S288C yeast cells assayed using the MitoSOX™Red superoxide indicator. Fluorescent intensity is indicated in arbitrary units (a.u) and error bars represent the 95% confidence of samples measured.

This distinct difference in fluorescent, proposed as a discerning characteristic of cells experiencing oxidative stress, was also observed for cells integrated into lipid templated mesoporous silica matrices formed with and without the addition of manganese phosphate buffer (illustrated in Figure 1-4). Utilizing this distinction, a method to approximate the amount of cells experiencing increased superoxide concentrations was developed by using the lower fluorescent intensities as baselines to represent cells with insignificant/typical concentrations of superoxides and associating cells with higher intensities directly to high levels of oxidative stress. This allowed an average to be taken for each droplet dried onto a single substrate from which an average and standard error for the substrate as a whole could be calculated and presented graphically; this process

allowed statistical analysis that is analogous to the method used to determine integrated cell viability by considering cells with arbitrary fluorescent intensities ≥ 3000 a.u. as cells experiencing increased oxidative stress. The results of the Mitosox^{TM} Red superoxide analysis are presented in Figure 1-5.

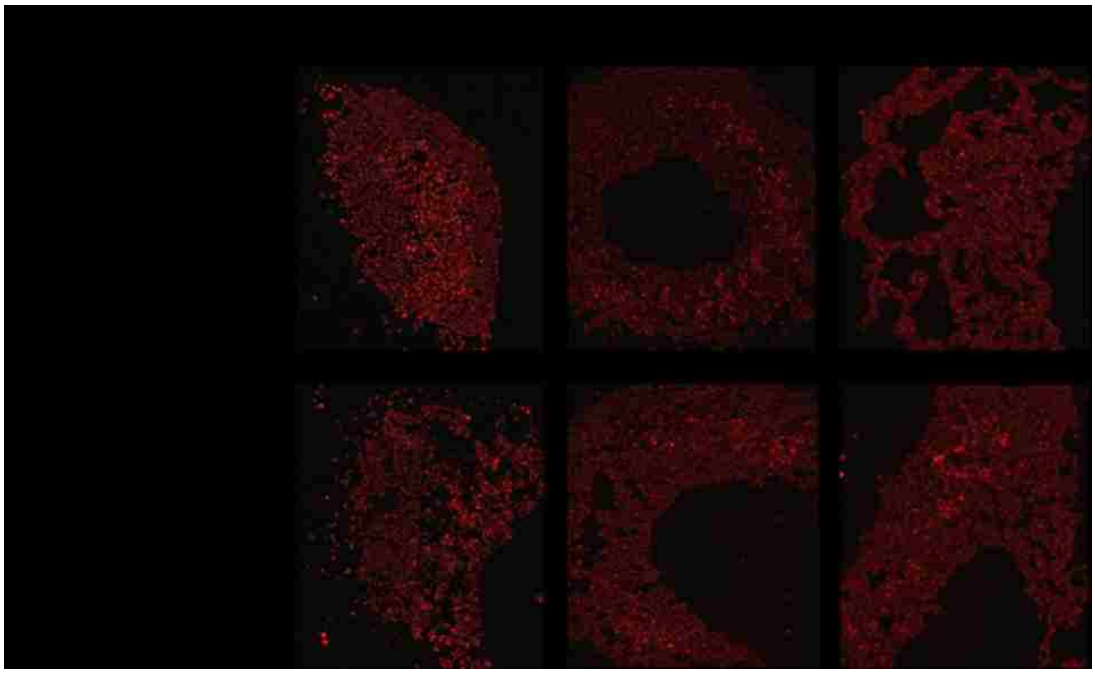

Figure 1-4: Fluorescent images of integrated yeast cells assayed for super-oxides using the Mitosox^{TM} Red Superoxide indicator.

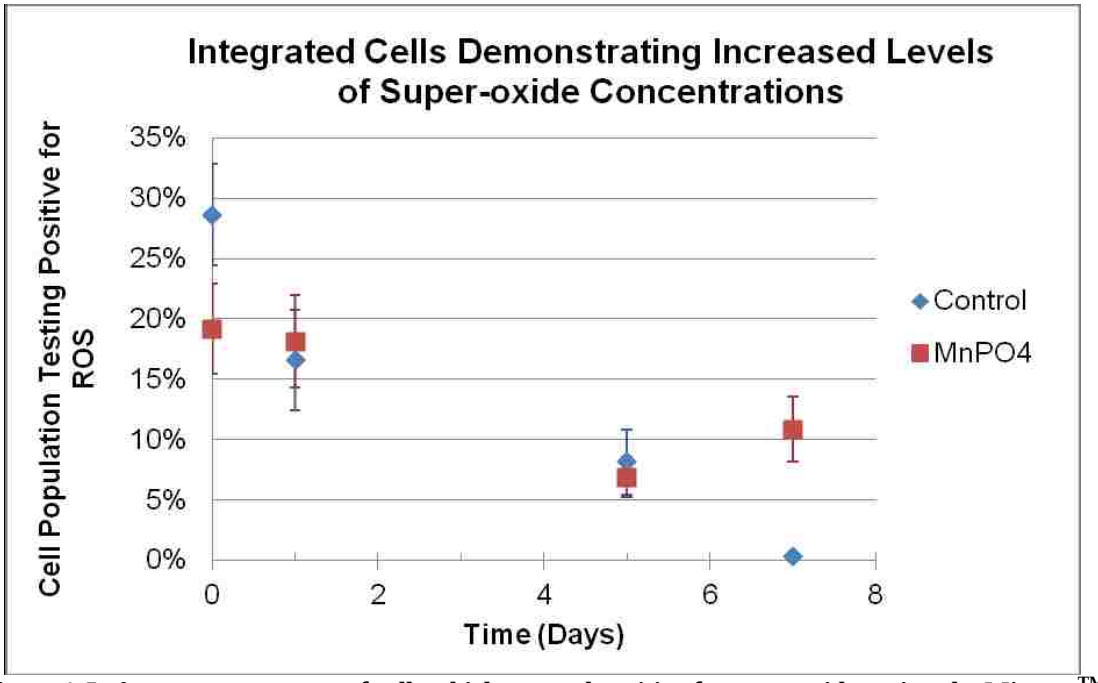

Figure 1-5: Average percentage of cells which assayed positive for superoxides using the Mitosox™ Red assay. Error bars represent the standard error of n=5 measurements.

Findings based on the Mitosox™ Red assay correlate well with initial viabilities presented in Figure 1-1. Cells integrated in control matrices were had higher levels of superoxides and lower cell viabilities relative to cells integrated in silica matrices containing manganese phosphate, which had lower concentrations of superoxides and higher viabilities. Conversely, when tested on day 1, cells integrated in the presence of manganese-phosphate had higher viabilities however cells integrated into either silica matrix were demonstrating similar levels of oxidative stress; a trend which continued through day 5. Data obtained via the Mitosox™ Red assay combined with viability data suggests that oxidative stress has a larger effect on cell viability in the first few hours of integration than any other time in the study. Findings also suggest that desiccation and the drying process may have a larger effect on superoxide concentrations than the condensing silica matrix. Supporting data indicates that 41.7 +/- 3.8% of cells dried on a substrate indicated oxidative stress whereas only 20-30% of cells tested indicated

oxidative stress when integrated into manganese containing or standard silica matrices respectively.

The upward trend in oxidative stress which was observed on day 7 (Figure 1-5) for cells integrated into matrices containing manganese phosphate is not well understood. This trend could indicate that despite having lower viability numbers, these cells continued experiencing limited metabolic activity which allowed the generation of superoxides. This is more likely than the alternative: that the condensing silica continues producing superoxides and ROS; especially because superoxides and oxidative radicals are highly reactive and therefore a fixed concentration of ROS would be expected to decrease rapidly. Furthermore, it is not well understood why some integrated samples demonstrate peak viabilities on days after initial integration instead of at time zero. It is believed that in the first hours or days after CDI, cells may be actively adjusting to their environment and/or could be in a state of dormancy where esterase enzymes could be down regulated as the cell copes with integration stresses. Currently our group is performing RNA based Gene Chip experiments to probe the state of encapsulated cell metabolic activity, which will provide more insight into the trends being observed.

The manganese dependent oxidative resistance is supported by research involving the oxidative irradiation of bacteria with varying ranges of manganese:iron ratios. Studies indicate that in the presence of higher manganese ratios, the more radiation the bacteria could resist.[12] Other research efforts have shown that Mn^{2+} affiliates itself with peptides, proteins and some enzymes, protecting them from oxidative stress although the exact mechanism is unclear.[14] Alternative studies suggest that Mn^{2+} may catalytically scavenge reactive oxygen species (ROS) and super oxide species from the cytoplasm inside the

cell.[13] Works such as these propose that manganese allows favorable ROS quenching via cyclic behavior between oxidative states; whereas iron participates in oxidation/reduction cycles based on Fenton chemistry which can be harmful to the cell.[12-14] From this data it is concluded that manganese phosphate may offer protection against oxidative stress during CDI integration of yeast, particularly in the first hours of cell integration.

Incorporating fluid and non-fluid liposomes into the CDI integration process

The next technique for preserving cell viability was the incorporation of ~120 nm fluid (DOPC) or non-fluid lipid liposomes (DPPC) with the CDI process. Previously, the addition of liposomes using cell directed assembly (CDA), an integration process similar to CDI, resulted in multilayered fusion of lipids around integrated cells which can span nearly 1 um in thickness.[3] What had not been previously studied were the direct effects liposomes have on integrated cell viability, especially in the long term. It is believed that incorporating lipids into the CDI process will result in a similar multilayered layered fusion of lipids which may result in higher cell viability. Fluid or non-fluid liposomes were incorporated into the sol-gel precursor solution which was used to prepare weakly condensed, mesophase lipid/silica thin films which contained the DOPC or DPPC liposomes. These films then had S288C yeast encapsulated via CDI. These samples were assayed for viability over the course of several days and the results can be seen in Figure 1-6.

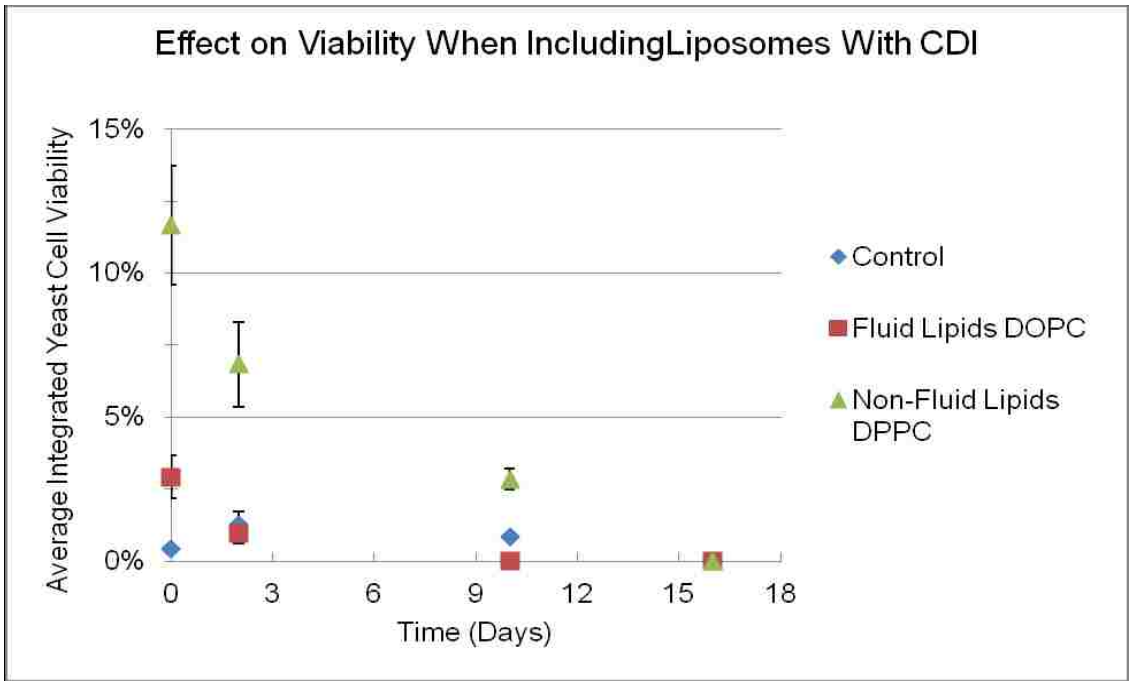

Figure 1-6: Effect on yeast cell viability when cells are CDI integrated with 120 nano-meter fluid (DOPC) or non-fluid (DPPC) lipid liposomes. Error bars represent the standard error of n=5 measurements.

Data presented in Figure1-6 indicates that non-fluid lipid liposomes offer increased protection that is not offered by the fluid lipid liposomes or the *di*C_6PC lipid bi-layer of the standard control alone. Yeast cells that were CDI integrated into lipid templated mesoporous silica films in the presence of non-fluid (DPPC) lipid liposomes had initial viabilities higher than that of fluid (DOPC) or standard control counterparts; they also offered higher viabilities than films prepared with manganese phosphate in the matrix. Yeast cells integrated into silica matrices containing fluid liposomes (DOPC) had initial viabilities which were higher than those of the standard control but trends did not demonstrate ability for DOPC lipids to improve or prolong encapsulated cell viability versus the control. By day 16 all of the cells in each of the three samples were assayed as dead.

Findings indicate that the inclusion of fluid (DOPC) lipid-liposomes produced an opposite and undesired effect of lowering the viability to levels below what was seen for standard lipid templated mesoporous silica films made without liposomes. It is believed that the DOPC based liposomes likely increased the fluidity between the cell and the bio-nano interface however this did not have a positive overall effect on yeast cell viability. The reason for decreased viability caused by fluid (DOPC) lipid liposomes is not conclusive but it is proposed that the non-fluid liposomes (DPPC) may have enhanced viability by effectively thickening and stabilizing the lipid layer at the bio-nano interface, providing cells with additional protection from the condensing silica and the topography of the silica shell. This is based on published research that investigated the thermophyle *Methanococcus jannaschii* (*M. jannaschii*) which utilizes changes in lipid fluidity to survive high temperature environments.

Studies of the bacteria *M. jannaschii* indicate that cells undergo a change from relatively fluid diether membrane lipids to the more thermostable tetraether lipids upon exposure to high temperature.[22-23] These tetraether lipids are formed by the fusion of two diether lipids and form a monolayer which spans the entire cell membrane. It is believed that DOPC or DPPC lipids may self assemble along with the *di*C_6PC lipids to produce a composite membrane that is more or less fluid than the original *di*C_6PC lipid bi-layer. Similar to the *M. jannaschii* bacteria which survive by modifying membrane fluidity, encapsulated cells may be benefiting from a more stable, less fluid lipid layer. Additional fluorescent microscopy studies by our group, such as FRAP and FRET, are being implemented which utilize fluorescently tagged lipids for further understand the lipid-lipid interactions during CDI.

Incorporating trehalose with the CDI integration process

The final strategy examined was the incorporation of trehalose into the CDI process as a counter measure to protect cells from desiccation. Early experiments with trehalose were performed by preparing a trehalose solution within which to suspend yeast, and then apply droplets of the suspension on the prepared mesoporous silica thin film for integration. This method proved ineffective because the droplet would form a viscous gel that often took several hours to dry. Prolonged drying times were undesirable because the integration process relies on evaporation in order to concentrate and condense the silica matrix around the cells. Moreover, the same method also proved itself very inefficient for integrating cells as nearly every cell would wash away when a light rinse was applied to the sample/substrate. An alternative approach was taken by dissolving trehalose directly in the sol-gel precursor solution (see experimental section). Incorporating the trehalose directly into the matrix, proved more effective for integrating cells and immediate increases in cell viability were observed. Incorporation of trehalose at various concentrations revealed a positive correlation between trehalose concentrations and integrated cell viability. This positive correlation is illustrated in Figure 1-7.

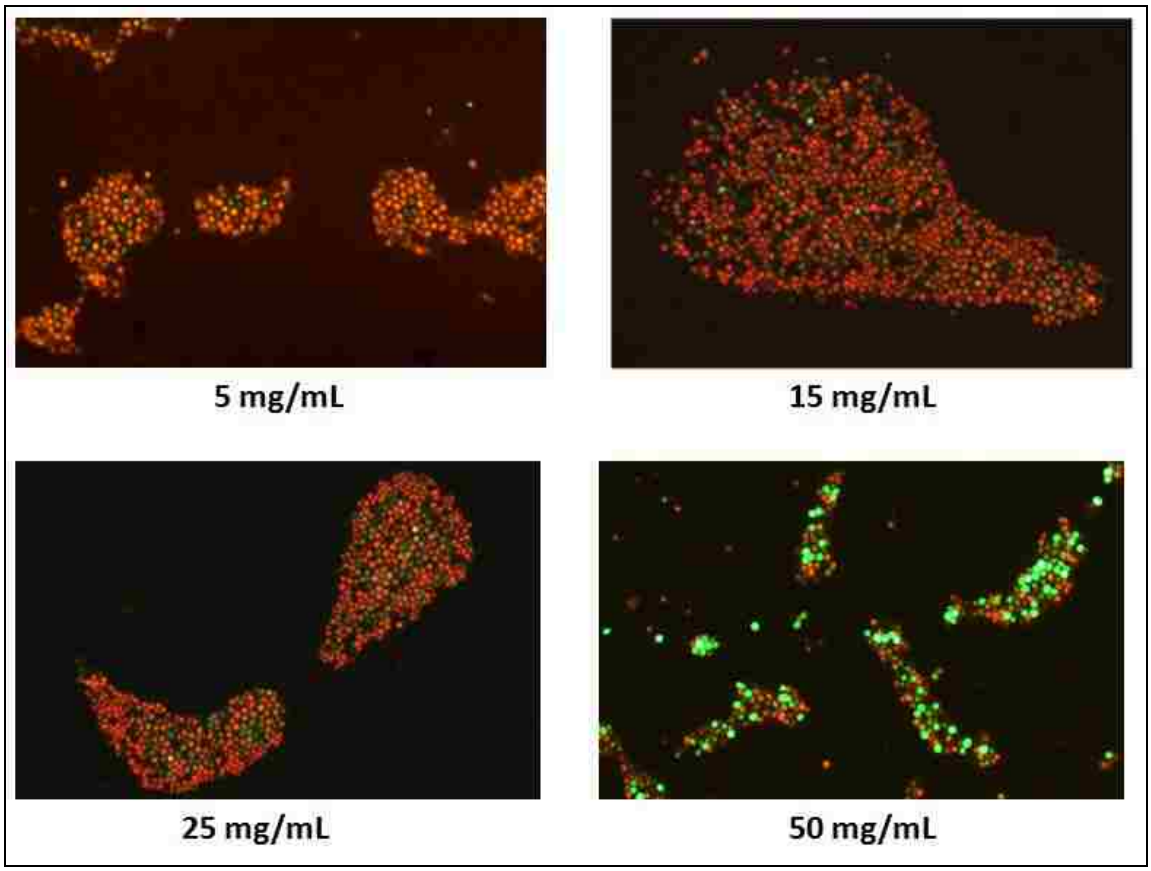

Figure 1-7: Fluorescent microscopy images illustrating the positive correlation between trehalose concentration and CDI integrated S288C yeast cell viability. Viability was measured using CFD,AM/PI viability 1 hour after the droplets evaporated.

Several trials were conducted to determine the effective range of trehalose concentration which was deemed to be 15-50 mg dissolved trehalose per mL of sol-gel precursor solution. Concentrations above 50mg/mL did produce an effect of slightly increased viability at the expense of partial and incomplete cellular integration. Cells integrated into films with trehalose levels above 50mg/mL would wash away when even the slightest rinse was applied to the sample (much like cells that were applied via suspension in a solution of dissolved trehalose). Once the trehalose CDI process was characterized and reproducible, substrates containing trehalose were prepared and monitored for viability. Two concentrations of trehalose were chosen for investigation,

50 mg/mL and 22 mg/mL, which were compared to standard mesoporous silica thin films prepared without trehalose. The results of the study are presented in Figure 1-8.

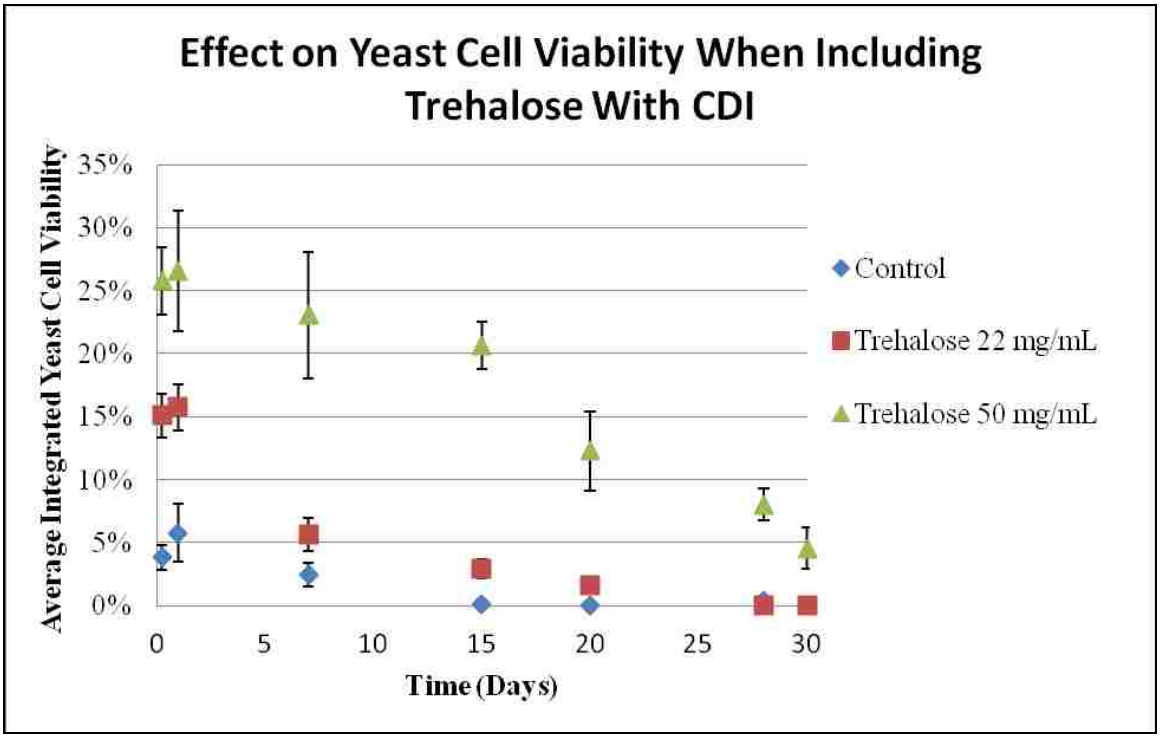

Figure 1-8: Effect of including trehalose within the mesoporous silica thin film on S288C yeast cell viability. Error Bars represent the standard error of n = 5 measurements

As shown by Figure 1-8, cells that were integrated into matrices formed with trehalose exhibited viabilities 3 to 5 times higher than those observed for samples prepared without trehalose. Higher levels of trehalose provide integrated cells with increased and extended cell viability over cells integrated into standard mesoporous silica films. Yeast that were integrated into matrices formed with sol-gel precursors containing 50 mg/mL trehalose also exhibited higher and prolonged viabilities relative to the samples prepared with 22 mg/mL trehalose. Supporting data from an alternative study also saw results where trehalose had a favorable increase on cell viability and behavior when subjected to desiccative stress. In this study, arsenic(III) bio-reporting bacteria were

subjected to preservation via vacuum drying. During the study cells were tested for viability using positive analyte detection as well as performing culturability studies. The study concluded that cells exposed to extracellular trehalose using 34% solutions exhibited cell viability via positive analyte detection, but not culturability, after being stored dry for over 12 weeks.[20]

It is common to see integrated cells have viabilities which are lower initially, then raise and peak near days 1-4; the viabilities of all three samples were observed to mildly increase from time zero to day 1. This could be an indication that in the first 2-48 hours cells are continuing to adjust favorably to their encapsulated environment. Two theories are proposed which could explain how trehalose provides higher prolonged viabilities when incorporated into the mesoporous silica films.

The water replacement theory proposes that trehalose replaces structural water that is lost during drying and participates in hydrogen bonding with other molecules.[17] The vitrification theory postulates that trehalose forms its own type of encapsulating matrix which traps bio-molecules, reduces molecular motion, prevents membrane diffusion and free radical diffusion; due mostly to the characteristically high glass transition temperature which is not particularly sensitive to small amounts of water.[18] In times of high desiccation or drought, many organisms will utilize these properties of trehalose to adapt and survive, once the organism is rehydrated it will quickly resume metabolic activity. In addition trehalose, much like glycerol, is often used a cryo-protectant to disrupt the crystal structure of ice and protect cells during freezing. In a similar fashion, during CDI, trehalose could be acting as a poragen that would cause the silica to condense with an amorphous structure rather than being well ordered or

crystalline.[19] Some strains of *S. cerevisiae* do not readily internalize or produce high levels of trehalose, even during mild or moderate stress, while other strains do.[21] Typically, trehalose uptake or production in yeast is initiated by high levels of heat shock.[20] The S288C strain of *S. cerevisiae* is not known to readily uptake or produce trehalose when in solution.

To see what effect trehalose has on the ordered mesophase structure of the condensed mesoporous silica, films were made with increasing levels of trehalose and tested using low angle X-Ray diffraction (XRD). XRD data for standard lipid templated mesoporous silica films that contained no trehalose exhibit an intense peak near $2\Theta = 3^{\circ}$. Films prepared with sol-gel precursors that contained up to 5 mg/mL trehalose had minimal resemblance to standard control mesoporous silica films as tested using low angle XRD. By including only small amounts of trehalose the peak at $2\Theta = 3^{\circ}$ loses its intensity and becomes an unspecified amorphous region. When including trehalose in concentrations of 10-50 mg/mL, the expected peak at $2\Theta = 3^{\circ}$ is not observed, and cannot be discerned from neighboring background noise. Indicating that no well ordered meso-structure is present. This data is summarized in Figure 1-9.

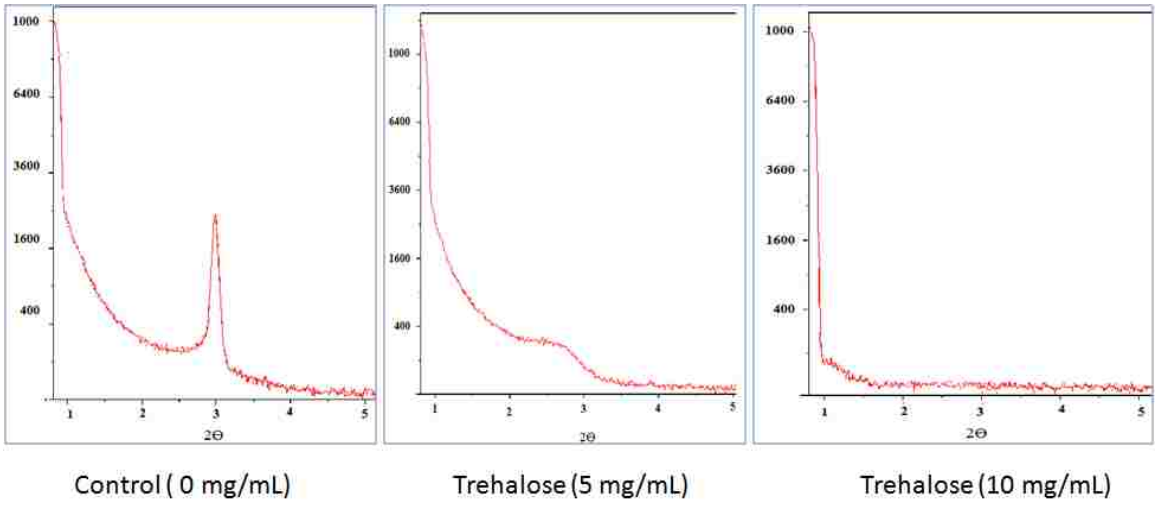

Figure 1-9: Comparison of low angle X-Ray diffraction profiles for lipid templated mesoporous silica films made with or without trehalose.

Contact angle was also measured for samples prepared with trehalose to see what effect the trehalose had on state of water in the film and the hydrophilic or hydrophobic nature of the films. The results are given in Figure 1-10.

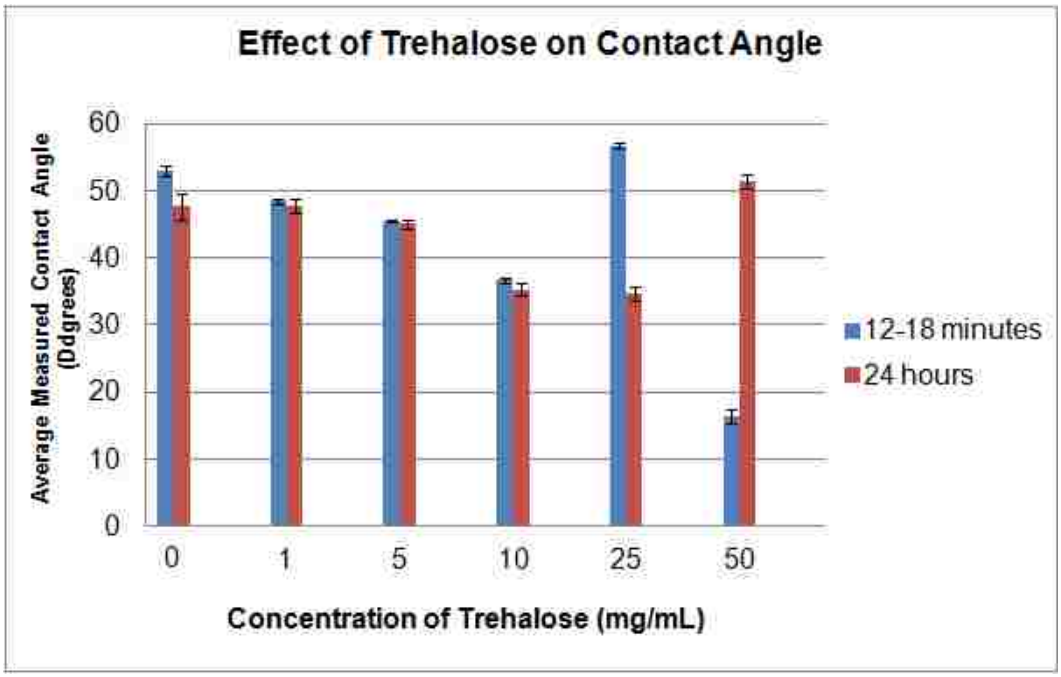

Figure 1-10: Comparing contact angle measurements of lipid templated mesoporous silica matrices formed with or without trehalose. The control is denoted by the 0 mg/mL concentration. Contact angle measurements taken at 12-18 minutes are representative of the time when cells/droplets would be applied to the matrix for integration. The time point at 24 hours was taken to allow films to continue condensing before measuring. Error bars represent the standard error of n=5 measurements.

Findings presented in Figure 1-10 suggest that when films are tested 12-18 minutes after spinning, there is a decrease in contact angle that is positively correlated with increases in trehalose concentration. This occurs for all concentrations except 25 mg/mL where a high contact angle, even higher than that observed for the control, is seen. When allowed to condense for 24 hours, films measured with trehalose continued to exhibit decreased contact angle as a function of increased trehalose concentration with a minimum occurring near 25 mg/mL trehalose concentrations. However, the samples containing 50mg/mL trehalose tested at 24 hours had substantially higher contact angles than the other samples tested. Trehalose has a strong affinity for water and according to XRD data it also disrupts the ordered structure of the condensed mesophase and as such,

the decreased contact angles observed for higher trehalose concentrations are not surprising. It is however, counter intuitive to see a higher contact angle for the 25 mg/mL concentration at 12-18 minutes, or the higher contact angle observed for the 50 mg/mL concentration the next day. The reason for this is not well understood but it is an indication that trehalose causes noticeable changes in the way the thin films form, interact with water and likely the rate that silica condensation occurs – which is also water dependent.

To further understand the mechanism by which trehalose promotes and extends integrated cell viability, a study investigating the effects of humidity on cell viability was conducted. This study would address the impact that including trehalose (at 50 mg/mL concentrations) or excluding trehalose has on integrating cells into mesoporous silica matrices. For each humidity value tested, the humidity was kept constant while the droplets dried, and remained constant for another 5 hours before assaying for viability. What was observed for the mesoporous silica control was lower viability at either humidity extreme (desiccated or humidity up to 35%) with a viability maximum occurring near 18% relative humidity. Figure 1-11 illustrates the results of the humidity study. It is important to note that identical stocks of yeast were not used at each humidity value tested however, all stocks of cells used for integration had average viabilities of 89.1%-92.5% +/- 1.3% based on the AM/CFDA, PI assay.

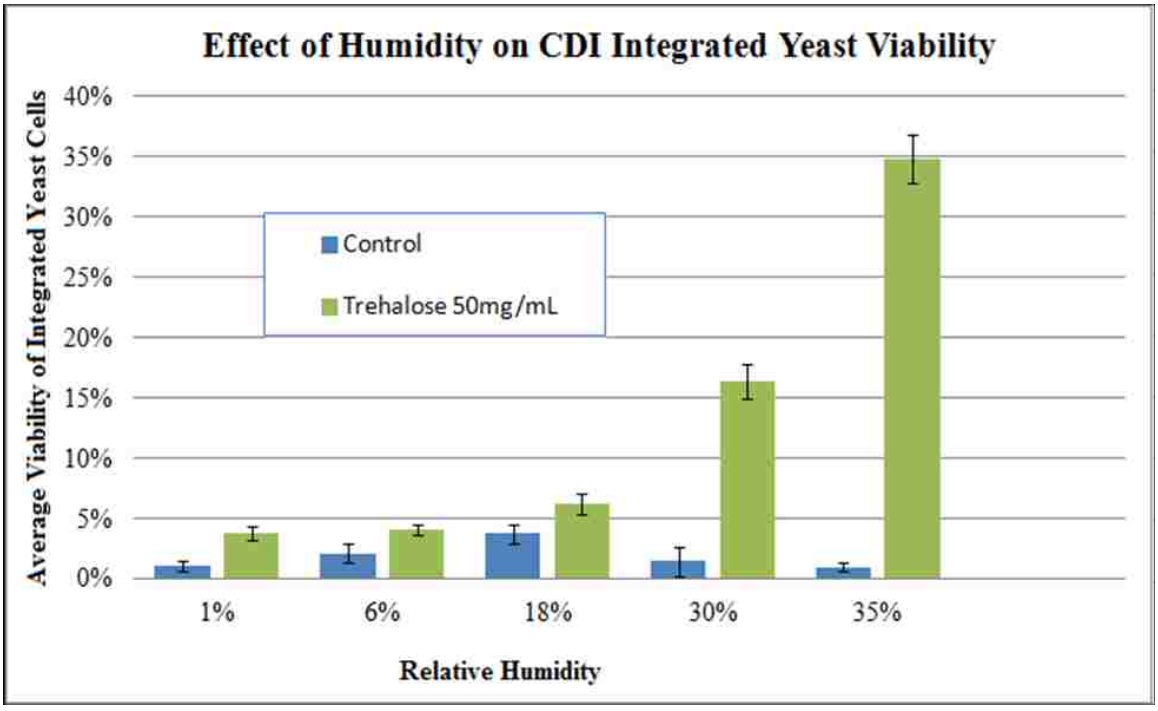

Figure 1-11: Effect of humidity on viability of S288C yeast cells 5 hours after being encapsulated in mesoporous silica thin films prepared with or without addition of trehalose. Error bars represent standard error of n=5 measurements.

Yeast cells integrated into mesoporous silica thin films had a maximum viability of 4.1% at 18% relative humidity, indicating that moderate humidity levels optimize cell viability. This trend suggests that an optimal amount of water may have been present that created a balance between the rate of silica condensation and amount of water that could be delivered to cells via the capillary effects of the porous silica matrix.[10] Meanwhile the strong correlation between relative humidity and increased viability for the trehalose containing matrices indicates that trehalose may enable the cells to utilize and sequester atmospheric moisture, greatly impacting cell viability. Relative humidity values above 35% did not promote cell integration or accurate measures of viability because the majority of cells integrated would wash off the film immediately upon rinsing. This behavior is supported by the fact that increased humidity would provide more water

which is necessary to allow the silica to condense. If the partially condensed thin film were to approach fully condensed levels before the cells/droplets were applied to the substrate, the cells would not undergo evaporative induced self assembly to become integrated.[10] Furthermore, for humidity values up to 35%, the silica matrix may have already formed several small chain silica polymers, yet remained only partially condensed which would release ethanol bi-products which could then evaporate. In the event of increased condensation and ethanol evaporation, the cells would be exposed to less toxic ethanol during integration, which would result in higher viabilities.

Findings indicate that including trehalose with CDI does promote increased and prolonged cell viability, with a strong dependence on the level of atmospheric moisture- at least for initial viability studies. Data suggests that trehalose offers significant advantages against desiccation however, it is not known if this effect is due to water replacement or vitrification theories. The vitrification theory postulates that trehalose forms its own type of encapsulating matrix which traps bio-molecules, reduces molecular motion, prevents membrane diffusion and free radical diffusion. The water replacement theory proposes that trehalose replaces structural water that is lost during drying and participates in hydrogen bonding with molecules and cellular components, protecting or preserving them in a stable state that may be returned to a native state when water is reintroduced to the system.

Findings from XRD and contact angle studies also indicate that trehalose reduces the ordered structure of the lipid/silica mesophase but does not prevent silica condensation or the ability for cells to self-assemble the silica nano-structure. Currently our lab is evaluating the effect of various sol-gel encapsulation techniques on

gene expression which may provide additional insights that are not immediately available by these experiments alone.

1.3 Conclusions

In summary, multiple extremophile strategies have been applied to the CDI process in an effort to improve integrated yeast cell viability. Results indicate that including such strategies does indeed have a net positive influence on cell viability. Cells that were integrated into films containing manganese-phosphate buffer exhibited mild increases (up to 5-7% increases in initial viability, and 1-2% for almost 3 weeks) over mesoporous silica control films that did not contain manganese-phosphate buffer. Furthermore, when including data obtained using the Mitosox^{TM} assay, findings suggest that manganese phosphate does have a mediating effect against oxidative stress. It is not clear however, if the oxidative stress is caused by the evaporation process and desiccation or if it is due to the presence of the matrix, particularly during matrix condensation. Of the three extremophile inspired strategies tested, incorporating manganese-phosphate had the smallest effect on increased and prolonged cell viability.

Incorporating fluid and non-fluid lipid liposomes directly into the matrix during the CDI process also had noticeable effects on integrated cells viability. It is believed that non-fluid lipid liposomes work constructively with the *di*C_6PC lipid bi-layer to offer cells a more biocompatible environment at the bio-nano interface and may be protecting cells from polar silanol functional groups on the backbone of the silica matrix. Yeast cells integrated which included non-fluid lipids (DPPC) had assay based initial viabilities near 13% and consistently demonstrated 3-5 times higher average viabilities than cells integrated with fluid lipids (DOPC) or the standard control which contained no lipids.

Fluid liposomes(DOPC) offered initial increases of up 5% average cell viability versus the standard control mesoporous silica matrices formed without liposomes. Fluid liposomes however, have the opposite effect during prolonged encapsulation, causing a decrease in integrated cell viability.

Trehalose had the most profound impact on increasing and prolonging cell viability. When integrating cells by the CDI method, trehalose demonstrated positive correlations between cell viability and the amount of trehalose incorporated into the silica matrix. Matrices formed via CDI that contained 50mg/mL trehalose increased integrated cell viability by nearly 6 fold over the mesoporous silica control, offering initial cell viabilities near 30%. In addition, trehalose has demonstrated to be highly efficient at utilizing moisture from the atmosphere and providing integrated cells with initial viability numbers upwards of 30% when the relative humidity is near 35%. Not only did trehalose containing films outperform standard mesoporous silica films at each humidity level examined, they also enhanced and prolonged long term viability. Humidity and desiccative stresses are certain to be strong factors in the formation of standard mesoporous silica matrices, which was evident by the peak seen at 18% relative humidity and the drop in viability as the humidity increases or decreases from that value upon integrating cells and during sample storage. The methods by which trehalose increases cell viability have yet to be verified exactly; however, based on the results of the humidity study it is certain that desiccation is a primary concern when integrating cells. Other studies with trehalose also suggest that trehalose may help to reduce oxidative stress, which is also believed to be a factor during CDI. Currently our research group is investigating the ability of trehalose to protect cells from oxidative stress during CDI as

well as evaluating the effect of various sol-gel encapsulation techniques on gene expression which may provide additional insights that are not immediately available.

1.4 Materials

Sodium chloride, sodium hydroxide, manganese(II)sulfate, hydrochloric acid (12.1N HCl), Dulbecco's phosphate buffered saline (DBPS, 1X), D+glucose (dextrose), ampicillin-sodium salt, tetracycline hydrochloride, triethylsilyl propionitrile and tetraethoxyorthosylicate (TEOS) were purchased from Sigma Aldrich (St. Louis, MO). Absolute ethyl alcohol (99.9% ACS reagent, absolute EtOH) was purchased from Fischer Scientific (Pittsburgh, PA). 1,2-Dihexanoyl-*sn*-glycero-3-phosphocholine(*di*C_6PC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids (Alabaster, AL). The LIVE/DEAD® *Funga*Light™ Yeast Viability Kit (L34952) and the MitoSOX™ Red superoxide indicator was purchased from Invitrogen (Carlsbad, CA). 13mm Acrodisc® Syringe Filters with GHP Membrane were purchased from the Pall Life Sciences **(**Ann Arbor, MI). Bacto™ Peptone, Bacto™ Tryptone and Bacto™ Yeast Extract were purchased from BD & LD (Sparks, MD).

1.5 Experimental Methods and Procedures

Culture Media and Propagating Cell Lines

Saccharomyces cerevisiae (*S. cerevisiae,* S288C) yeast cells were inoculated from a YPD agar plate and incubated at 31.7°C in 50mL of YPD growth media for 7 days or until stationary (G0) phase. YPD growth media was prepared by dissolving 10 g Bacto™

Yeast Extract, 20 g Bacto^{TM} Peptone, 20 g dextrose in 1000 mL DI water which was then sterilized by autoclaving. Growth media was dosed with 1.0 mL of ampicillin sodium stock (50mg/mL) and 1.0 mL of (0.01 mg/mL) tetracycline HCl directly prior to inoculating the cells.

Preparing Silica Sol-Gel Precursor Solution

Preparing a standard lipid templated mesophase silica thin film was done by first preparing the A2** stock. A2** stock is a pre-hydrolyzed TEOS solution that is prepared by combining 61 mL TEOS, 61 mL of 200 proof EtOH, 0.2 mL of 0.07 N HCl and 4.9 mL of DI water and refluxing the mixture for 90 minutes at 60°C. The final molar ratio of ingredients being 1: 4: 5×10^{-5}: 1. This solution was stored in Nalgene containers in the freezer at -20°C with careful attention to minimizing thermal cycling and exposure to humidity. This solution was allowed to age for 3-5 days before first use.

To prepare the mesoporous silica precursor solution 0.20 mL of 200 proof EtoH was combined with 0.25 mL of A2** stock. To this was added 0.160 mL of 0.05 N HCl and 0.4 mL of DI water. The final solution was used to dissolve 30 mg *di*C_6 PC lipid. This solution was aged at room temperature for 20 minutes then syringe filtered using a 0.2 μm GHP membrane filter.

Preparing Silica Sol-Gel Precursor Solutions Containing Manganese Phosphate (4 mM)

To prepare lipid templated mesoporous silica thin films containing manganese-phosphate, the same precursors as a standard Sol-E solution were used: 0.20 mL of 200 proof EtOH, 0.25mL A2** stock and 0.160 mL of 0.05 N HCl. The manganese-phosphate addition is performed by substituting the DI water with 0.4 mL of 10mM manganese phosphate

solution. The final solution was used to dissolve 30mg *di*C_6 PC lipid, then aged 30 minutes and syringe filtered using a 0.2 µm GHP membrane filter. The manganese phosphate buffer solution was prepared by dissolving 12.68 mg of manganese sulfate into 15 mL of 10 mM sodium phosphate buffer, pH 6.8. The final concentration of manganese and phosphate in the sol-gel precursor solution was 4 mM.

Preparing Silica Sol-Gel Precursor Solutions Containing ~120 nm Lipid-Liposomes (DOPC/Fluid or DPPC/Non-Fluid)

Fluid lipid liposomes were formed using 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and non-fluid lipid liposomes were formed using 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC). To from the liposomes, 2.5 mg of DOPC or DPPC lipid was lyphillized under vacuum for approximately 4 hours. The lyophilized lipids were rehydrated to a concentration of 2.5 mg/ml using 0.5x PBS. The rehydrated lipds were then subjected to 11 extrusion cycles in an *Avanti* Mini-Extruder equipped with a polycarbonate membrane with 100 nm pores. The resulting liposomal solutions were characterized via Dynamic Light Scattering (DLS) using a Zetasizer Nano-Series DLS Instrument which confirmed the mean diameter of the liposomes. This procedure was used for both DPPC and DOPC lipids.

Preparing mesoporous silica precursor solutions with fuid or non-fluid lipid liposomes was very similar to making a standard mesoporous silica precursor solution. First, 0.20 mL of 200 proof EtoH was combined with 0.25 mL of A2** stock. To this was added 0.160 mL of 0.05 N HCl and 0.4 mL of DI water. This solution was aged 15 minutes and then syringe filtered using a 0.2 µm GHP membrane filter. For the last step, 100 µL of fluid or non-fluid liposome stock solution was added to the precursor solution.

Liposomal addition was performed last because when adding the liposomes before filtering an undesirable portion of them was lost to the filter.

Preparing Silica Sol-Gel Precursor Solutions Containing Trehalose

0.20 mL of 200 proof EtOH was combined with 0.25 mL of A2** stock. To this was added 0.160 mL of 0.05 N HCl and 0.4 mL of a given concentration of trehalose dissolved in DI water. The DI water was combined with trehalose immediately before using, effective concentration ranges were 10-100 mg trehalose per mL DI water. The final solution was used to dissolve 30mg *di*C_6 PC lipid, then aged 20 minutes and syringe filtered using a 0.2 µm GHP membrane filter.

Preparing Mesoporous Silica Thin Film Preparation and Cell Integration

Round glass substrates (#1.5, 25mm) were prepared by first etching them for minimum 6 hours in 0.01M sodium hydroxide bath. Next, the substrates were washed using 2% Alconox solution followed by rinsing with warm tap water and a rinse with DI water. The substrates were then rinsed off using 190 proof ethyl alcohol (190 EtOH), dried with nitrogen and UVO treated for 5 minutes.

Sol-gel solutions were spin-coated onto the substrates by spinning for 35 seconds at 2000 RPM using a Laurel© spin coater (Model WS-400B-6NPP/LITE) under nitrogen environment where the relative humidity was < 15%. The volume of sol-gel used to prepare each film was 150 µL which was deposited using a 200 µL pipette dispenser. Once the films were coated to the substrates, the sample was allowed to set covered, at ambient conditions for approximately 15 minutes before cells were applied for integration.

The *S. cerevisia* (S288C) yeast cells to be integrated were first collected by centrifugation at 10,000 rpm for 30 seconds then washed 3 times in DI water and resuspended in DI water at a final concentration of 10^5-10^6 cells/mL. Films/substrates ready for cell integration had 0.5 µL volumes of the washed, diluted cells applied to their surface via a micro-pippette tool. The droplets were allowed to dry and samples could then be stored for various periods of time before being analyzed. Directly prior to testing, a light rinse was applied to the films for approximately 10 seconds, followed by nitrogen drying to remove un-integrated cells/material.

Assaying for Viability: FungaLight™ CFDA, AM/Propidium Iodide Yeast Vitality Kit

The *Fungalite* Yeast Vitality Kit uses a membrane integrity indicator (propidium iodide) and a cell permeable component (AM/CFDA) to assay yeast cell viability. The propidium iodide (PI) component will stain cells red (indicating dead) and the acetoxymethyl ester (AM) of the esterase substrate 5-carboxyfluoresceindiacetate (AM/CFDA) will stain cells green (indicating viable). Upon entering the cell the AM/CDFA component will be cleaved by nonspecific esterases, resulting in the emission of green fluorescent signal. The characteristic excitation and emission maxima are 492/517 nm for CFDA/AM and 490/635 nm for propidium iodide. Cells that were in solution or integrated into a silica matrix could be assayed by following a standard protocol. 1.5 µL of CFDA solution (1 mg in 100 µL DMSO) and 1.5 µL of PI solution (20 mM in DMSO) were added to 1.5 mL of 0.1X PBS, pH 6.8. Cells that indicated esterase activity and compromised membrane integrity would stain orange or yellow and were not counted as viable.

Assaying for Superoxides: MitoSOX™ Red superoxide indicator

The MitoSOX™ assay utilizes an indicator which permeates the cell plasma membrane and specifically targets the mitochondria of living cells and, upon oxidation, produces 2-hydroxyethidium which is highly fluorescent when bound to nucleic acids. The MitoSOX™ Red mitochondrial superoxide indicator has excitation/emission maxima of approximately 510/580 nm. The reagent is rapidly oxidized by superoxide but not as quickly by other reactive oxygen (ROS) or reactive nitrogen (RNS) species. Yeast cells in solution were tested using 5 a µM MitoSOX™ solution prepared using 0.1 M DPBS. Samples to be assayed using MitoSOX™ Red were incubated at 37˚C for 10 minutes.

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Chapter 2

Characterization of Bio-Sensing Riboswitch *Escherichia coli* Encapsulated in Novel Aqueous Silica Gels

2.1 Introduction

Sol-gel materials capable of incorporating bio-molecules and living organisms into 3D organic and inorganic matrices are gaining popularity as their potential to meet growing demands in industrial, medicinal, military, food process and environmental applications becomes more viable.[1-4] In particular, functional biotic materials could be used for bio-catalysis, drug delivery, tissue engineering, and a variety of platforms to detect analytes, toxins and even viral/bacterial genetic material.[5-8] The encapsulation of biological molecules, including enzymes, has been well established by several scientists and global research efforts.[8-10] Meanwhile, encapsulation of living cells remains more challenging.[11,12]

Conventional bio-analytical detection platforms seek to take advantage of simple detection and reporter processes.[13,14] Examples of well studied detector bio-molecules include horseradish peroxidase, glucose-6-phosphate dehydrogenase and glucose-oxidase.[4,9,10] Preferred reporter bio-molecules include bacterial luciferase (*Lux*), aequorin and green fluorescent protein (GFP, although other colors are available) that offer a bioluminescent/fluorescent response making them easily detected[13,14] A general reporter organism is a genetically engineered cell that contains detect and report combinations that exploit the natural advantages of the host.[13] Sensor-reporter mechanisms have been incorporated into nematode, plant, bacterial, yeast, mammalian and cancerous hosts with

specificity for detecting hundreds of organic, inorganic and biologically produced compounds.[7, 13, 14]

Riboswitch mechanisms are a prime example of a cell based sensor-reporter approach for detecting analytes and controlling the downstream gene expression of reporter molecules.[14,15] Characteristic riboswitches are an RNA molecule (typically a cloned aptamer, naturally or synthetically derived), placed on the 5' untranslated region of mRNA or DNA directly upstream of genes that are to be regulated.[14,15] When the aptamer encounters its specific target ligand, the riboswitch is switched on (typically via conformational change) and ribosomes can then bind the mRNA and begin expression – this is illustrated in Figure 2-1.

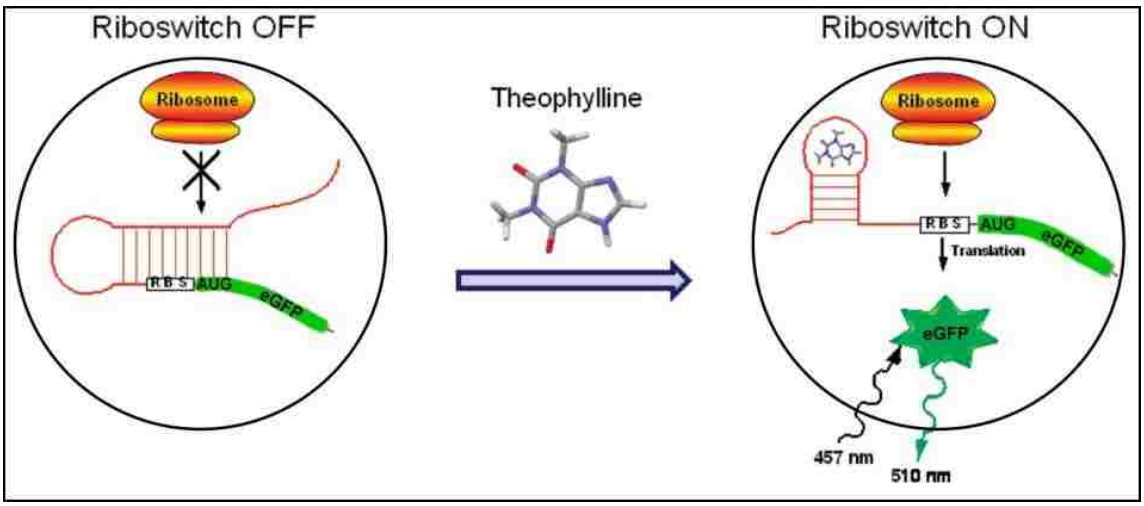

Figure 2-1: Illustration depicting the mechanism of riboswitch activation upon induction with theophylline.

Encapsulating and utilizing organisms that contain riboswitch mechanisms has been gaining increased research interests due to their tailorability in detector-reporter combinations.[14,16] The bio-sensing organism chosen for this study is a genetically modified strain of *Escherichia coli* (*E.coli*) that was engineered with a riboswitch plasmid to detect theophylline and respond by producing green fluorescent protein

(GFP).[14] The behavior of these particular *E. coli* has been well documented in solution however, their ability to function when encapsulated is less understood.[14,19]

To date, many methods for encapsulating bio-molecules or living cells rely on sol-gel techniques that utilize hydrolysis and condensation reactions of organo-silanes to create a porous silica framework, though other methods exist.[9-12, 14, 17] A particularly novel method for living cell encapsulation/integration developed by our research group is cell directed integration (CDI). Cell directed integration (CDI) is an active process based on sol-gel chemistry that starts by preparing a sol-gel precursor solution (containing a short chain lipid *di*C_6PC) that is deposited onto a substrate forming a weakly condensed lipid/silica thin film mesophase.[22-24] Cells suspended in droplets are applied to the mesophase, dissolving it, then actively reconstruct the evaporating surface to create a fully 3D bio/nano interface where cells are enveloped by localized lipid bi-layers and condensed silica.[23] The evaporation of the sol-gel/droplets is a distinct and necessary component of the CDI process but this and other similar methods of integration expose the cells to many stresses including alcohol solvents, cyto-toxic reactants and bi-products, highly acidic conditions, sensitivity to matrix topography, desiccation, oxidation and osmotic stress. Upon integrating cells, these samples are stored under ambient or artificial environmental conditions but are not kept in aqueous solutions therefore cells are vulnerable to further desiccative stress.

These challenges limit prolonged storage and activity of the encapsulated biological subject and as a consequence, the materials applicability suffers. An effective biomaterial would be cost efficient, reliable, device compatible and, above all, offer simplicity, especially where access to equipment and resources is limited. To fulfill these

requirements, the biomaterial would have several qualities: (i) the matrix requires and consists of minimal cytotoxic chemicals/catalysts, (ii) the encapsulating matrix provides high diffusivity and fluidity enabling controllable, easy access to oxygen, nutrients and target molecules which more closely resembles the natural environment where the biological target performs best, and (iii) the bio-nano interface remains compatible with cell topography by reducing the exposure to polar silanol groups found on the silica backbone which are believed to disrupt cell membranes; presumably through electrostatic and hydrogen bonding interactions.

To develop a material which meets these strict requirements, an alternative to the CDI method is proposed where riboswitch *E. coli* are instead encapsulated in different aqueous silica gels that maintain a highly diffusive, 3D water micro-environment. The first two types of aqueous silica gels, AQ or AQ+Gly (the latter of which contains glycerol) are prepared using sodium silicate as a silicon source.[10]. The third gel is a poly-glycerated silicate with a 10:1 ratio glycerol:silicon being used recently for the first time to stabilize living cells for cell based sensing applications.[18,19] All three of these aqueous gels demonstrate exceptional bio-compatibility because rather than being integrated in a thin silica film via a process such as CDI, cells are instead encapsulated in aqueous monolith composed of a silica framework which gels when a silicon precursor is combined with phosphate buffers at near neutral pH levels, catalyzing the silica formation. This method for cell encapsulation offers the advantages of suitable ion concentrations, high levels of water content and reduced acid and ethanol concentrations; which effectively reduce the chemical cyto-toxicity, osmotic and desiccative stresses of the encapsulation process. Furthermore glycerol, known to protect cells during cryogenic

storage by preventing water from crystallizing into ice, has been incorporated into sol-gel based encapsulating matrices in the hopes that the protective properties can be passed down and utilized.[4,9,20] It is hypothesized that the addition of glycerol (either in solution or covalently bound to the silica backbone) will further increase biocompatibility by changing the porosity and structure of the condensed silica framework and/or by reducing the concentration of polar silanol groups that are presumed to lyse cell membranes due to electrostatic and hydrogen bonding interactions.

The primary goal is to explore and refine silica based encapsulation strategies which will enable the riboswitch *E.coli* to remain viable and accessible during prolonged periods of encapsulation, enabling sensitive detection upon theophylline exposure. The goal in investigating these bacteria was to prove that one or all of the aqueous gels will allow full functionality of the riboswitch mechanism by meeting or exceeding the 0.01 mM theophylline detection limit of cells as tested in solution.[14]

2.2 Results and Discussion

Experimental overview

Encapsulating organisms in aqueous silica gels is a process characterized by the ability of a stable, acidic sol-gel precursor to form a gel when mixed with salt solutions and buffers which raise the solution's pH and catalyze the formation of the silica framework. This procedure begins with a single-batch of cells, stable silica-precursors (sol-gel precursors) and sodium-phosphate buffers that have been prepared to a desired pH and molar concentration. These gels offer the cells a hospitable environment with compatible ion concentrations and levels of acidity. Research began by confirming and validating the behaviors of each aqueous silica gel as previously characterized.[10, 18, 19] The experimental methods section offers more detail about the protocols to prepare the

sodium-silicate based gel (AQ) and the glycerol modified counterpart (AQ+Gly), as well as the poly-glycerated silicate gel (PGS).

First, are presented the experimental findings of a five week study testing cells for viability via live/dead fluorescent dye viability assay as well as a complimentary plating study to monitor the ability of cells to be freed from the matrix and then reproduce. The next goal in this work is validating and characterizing the fluorescent minima/maxima of both of the positive control (pSAL:GFPa1His) and riboswitch (pSAL:RS12.1GFPa1His) BL21 *E. coli* cell lines encapsulated in the matrix over time. Finally, theophylline detection thresholds observed for each of the gels was also investigated and the results are presented herein.

Assessing cell viability and riboswitch activity during prolonged encapsulation

It is highly important that any cell-based materials, and resulting analytical device(s), extend the usefulness of encapsulated or integrated subjects by maintaining viability and metabolic activity for as long as possible. A study was conducted which would determine the affects each particular matrix have on cell viability during prolonged encapsulation. Aqueous monoliths from each type of sol-gel precursor were created, allowing 5 weeks of encapsulated storage while conducting regular inductions of riboswitch activity as well as assaying for viability and colony forming units by extracting cells from the monoliths and plating them on growth plates.

Assaying for viability was done with fluorescent microscopy using the *Bac*Light LIVE/DEAD bacterial viability assay (Kit L7012, *Invitrogen*), which utilizes two components for staining cells with fluorescent markers. SYTO-9^{TM} is a membrane permeant dye that binds with nucleic acids of living bacteria, staining them green.

Propidium iodide (PI) is a membrane impermeable compound that enters damaged or membrane compromised bacteria (associated with dead/dying cells) staining them red.

In order to assay the encapsulated cells first, 500 µL of assay solution was applied to the tops of the monoliths which were allowed to incubate for 30-40 minutes at 37˚C degrees. Next, a sample was prepared by removing a small portion of the center of the monolith (approx 20-25 mg in mass) and then breaking the sample with a scapula and pressing it firmly between two 25 mm round microscope cover discs. This sample was imaged randomly 10-15 times to obtain a statistical population of cells, in each image the number of fluorescently stained cells was counted to obtain a percent viability. Cells that stained orange or yellow were not considered viable. The percent viability of all images from a sample was used to obtain average (M_1) and standard deviation (SD_1) at each time point. From this data was derived the standard error using the formula $SE = SD_1/\sqrt{n}$ where n is no less than 10.

Stationary phase (G0) BL21-RS cells were first assayed for viability using the *Bac*Light™ LIVE/DEAD assay and were found to be 92.4 +/- 1.7% viable. Upon mixing, gels were allowed to sit for 8 hours before taking the first time point, followed by acquisition of several data points as the study progressed. Theophylline induced riboswitch activity was also tested initially and weekly to determine the impacts of prolonged encapsulation on responsiveness and cell metabolic activity. Figure 2-2 depicts the resulting viability trends observed from the 5 week encapsulation study.

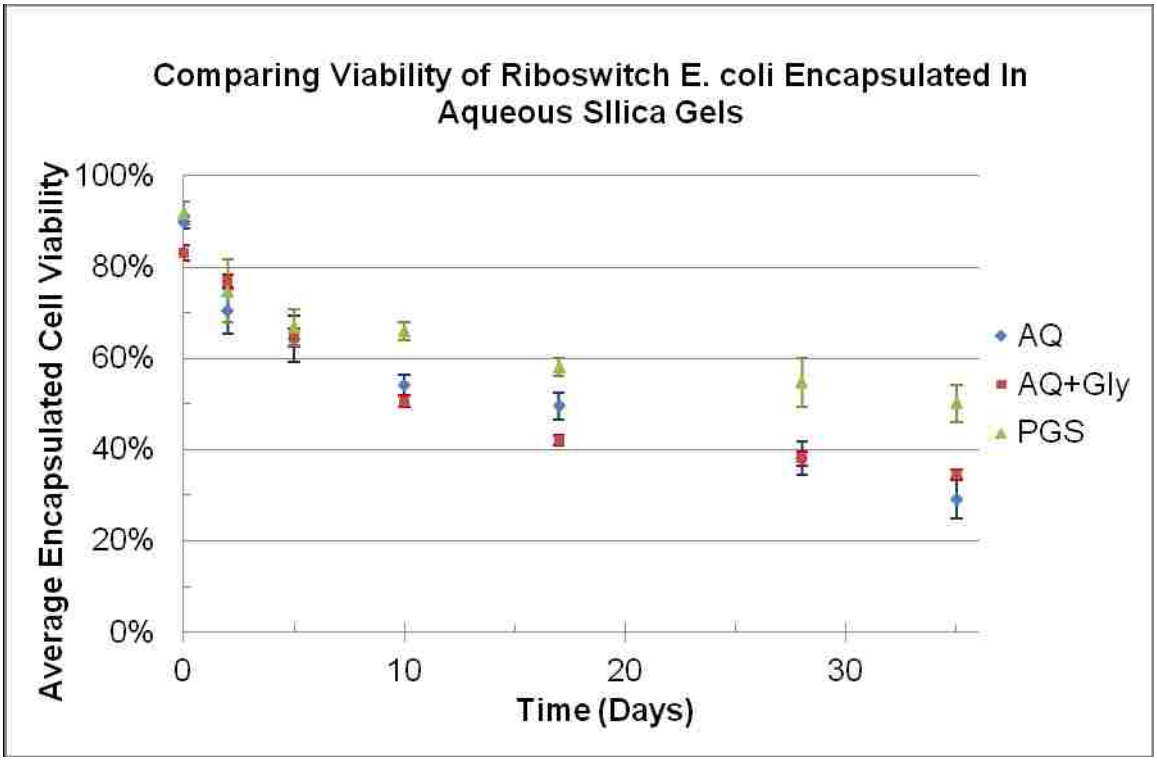

Figure 2-2: Average percent viability of BL21-RS cells encapsulated in AQ, AQ+Gly or PGS based aqueous silica matrices. Error bars represent the standard error of n = 10.

As shown by Figure 2-2, encapsulating cells using any of the three aqueous silica gels resulted in greater than 30% retained viability after 30 days. The gels all had similar initial viabilities, a trend which continued for 5 days. By day 10, the AQ and AQ+Gly based matrices diverge from viability trends observed from PGS based matrices. This divergence continues to grow through day 35 where the PGS based matrices were observed to provide 15-20% higher viabilities than the AQ or AQ+Gly silica gels. These findings indicate that the simple addition of glycerol to the AQ+Gly based gels does not provide increased biocompatibility in the same manner that PGS aqueous gels do.

One explanation for this behavior could be inherent to the differences in the ways that each gel forms. Varied levels of synteresis were observed for the aqueous gels and the rate and extent that each gel condenses differs as well. AQ and AQ+Gly based gels

condense quickly and are known to undergo high levels of synteresis which squeeze encapsulated cells, resulting in compromised membranes and cell lysis. PGS based silica gels condense much slower and undergo less synteresis over time which could explain the observed trends when using the *Bac*Light™ LIVE/DEAD bacterial viability assay.

As a compliment to the viability study based on the live/dead assay a plating study was also conducted. Each week a 16.5 +/- 1.3 mg portion of each gel was removed, then pressed into a 1.7 mL micro-centrifuge tube and resuspended in 0.1 M phosphate buffered saline. The solution was mixed rapidly for 30-40 seconds via vortexing. After re-suspending the cells and streaking on an LB agar+ampicillin growth plate, the plate was incubated for 24 hours, after which the number of colonies forming units was recorded.

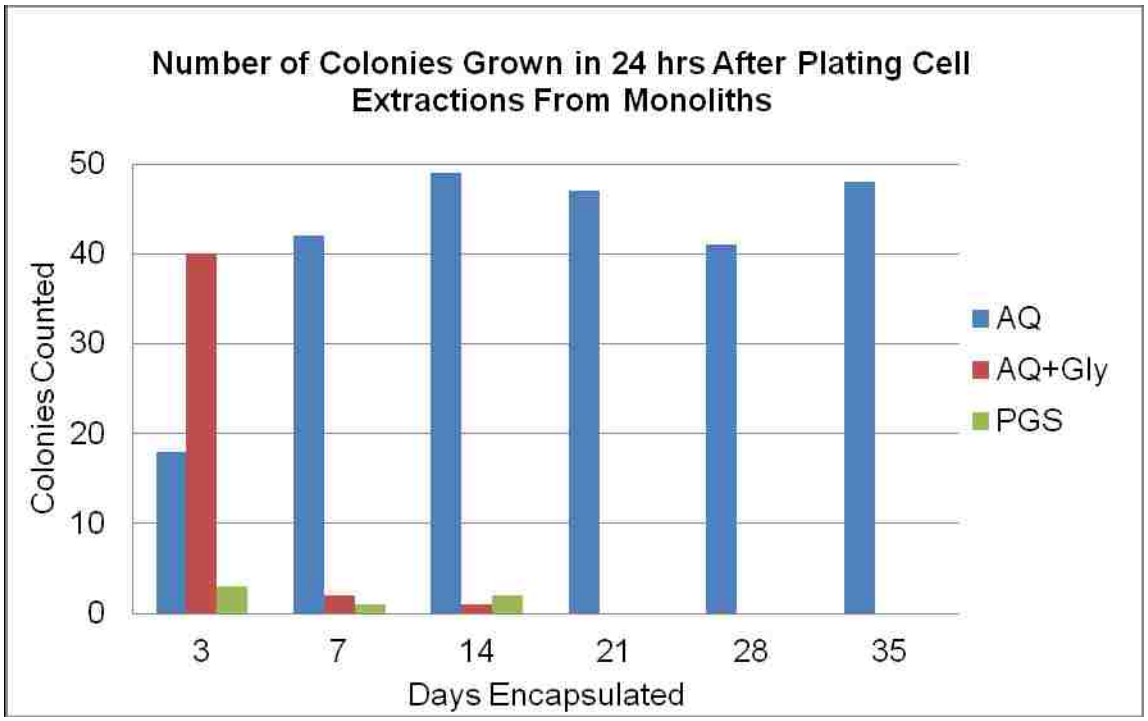

Figure 2-3: The number of colonies grown by extracting cells a 16-18 mg sample from a monolith and using the sample to streak cells onto an agar growth plate which was incubated for 24 hours. The time in days indicates how long cells had been encapsulated.

Findings based on plating studies indicate that despite having consistently lower average viability based on the *Bac*Light LIVE/DEAD bacterial viability assay, the cells encapsulated in AQ based silica gels were viable and able to reproduce on solid substrates for the duration of the study. Conversely, cells encapsulated using PGS aqueous gels exhibited higher average viabilities but did not offer high levels of reproduction on solid substrates, especially after the second week. Initially, silica gels formed with AQ+Gly offered relatively higher levels of encapsulated cell reproduction but this did not continue for the duration of the study. Additionally, the two matrices prepared with glycerol had higher prolonged viabilities (especially PGS matrices) according to the assay but they also had the least ability to form colonies. Glycerol is well known for being an osmolyte and offering cryo-protective properties but it is also argued that at higher concentrations glycerol disrupts cell membranes and prevents bacterial growth.[4, 9, 20] Therefore the presence of glycerol may be linked to the limited ability of cells to reproduce when streaked on growth plates after long periods of encapsulation in PGS or AQ+Gly matrices.

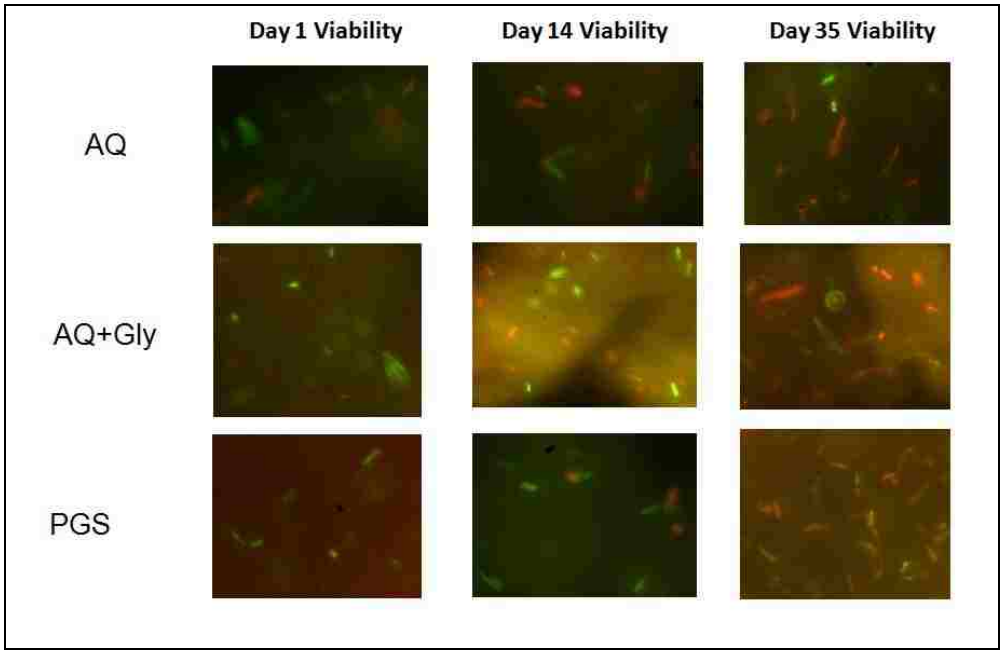

Figure 2-4: Comparing time lapse fluorescent microscopy images of encapsulated BL21-RS cells assayed for viability.

Over the course of the study, encapsulated cells were exposed to 2 mM theophylline concentrations at regular intervals to test their ability to detect and respond to external stimuli via GFP expression. Cells suspended in solution and/or encapsulated cells were theophylline induced using a nutrient deprived solution that would limit potential cell growth during induction (see experimental methods). Cells were prepared for microscopy using the same technique as when assaying for viability except that cells were incubated for 6 hours before imaging. Findings confirm that cells encapsulated in each aqueous silica gel were able to recognize and respond to the theophylline analyte when encapsulated for up to 5 weeks, as illustrated by Figure 2-5.

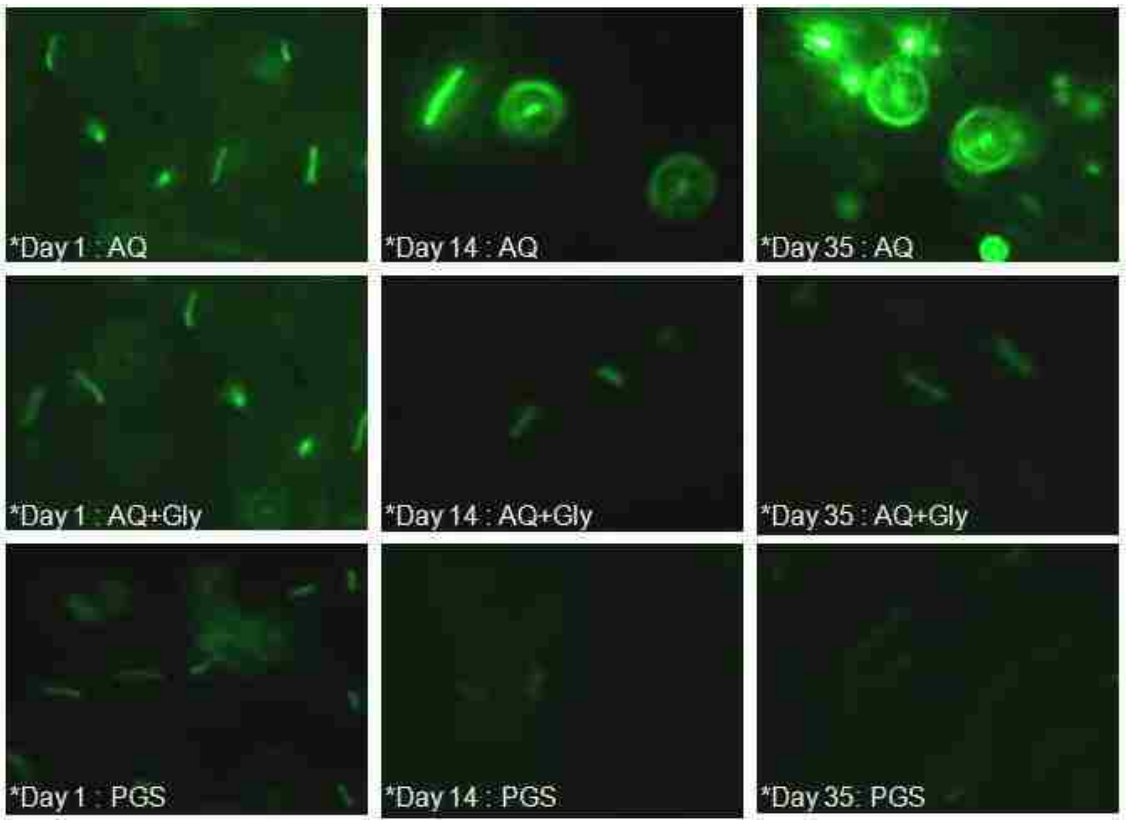

Figure 2-5: Comparing time lapse images of 2mM theophylline activation of encapsulated BL21-RS cells. All images were taken using the same microscope and imaging software at identical settings

Of particular interest are the behavioral differences in GFP expression by cells encapsulated in different matrices as a function of time. Cells encapsulated in AQ and AQ+Gly matrices are observed to have consistently higher initial GFP fluorescent intensity than cells in PGS silica gels. Cells encapsulated in AQ silica gels were observed to maintain or even increase the intensity level of theophylline induced GFP expression. Meanwhile, cells encapsulated in AQ+Gly or PGS silica gels exhibit decreased levels of GFP emission intensity. This might be counterintuitive to expectations based on the *Bac*Light LIVE/DEAD bacterial viability assay, where higher viability cell populations might be expected to show higher GFP expression levels.

However based on the plating study, a positive correlation between encapsulated cell colony-forming ability and theophylline induced GFP expression is evident.

Time course fluorescent profiles and riboswitch behavior of cells encapsulated or in solution

The fluorescent intensity of GFP expression was imaged using fluorescent microscopy and samples were prepared for imaging in the same manner as described above. When measuring fluorescent intensity a single sample was imaged randomly 10-15 times to obtain a statistical population of cells where the fluorescent intensity of each cell was measured using imaging software. From these measurements the mean intensity (M_1) and standard deviation (SD_1) at a given time point was determined. From this was derived the standard error using the formula $SE = SD_1/\sqrt{n}$ where n was no less than 25. All fluorescent measurements are presented in non-normalized arbitrary units.

Determining the behavior of fluorescent behavior of riboswitch ON/OFF and positive control cells was accomplished by growing cultures of each cell line to stationary (G0) phase then measuring their resulting fluorescent intensities. This established a baseline that all subsequent measurements could be compared to. One study documented the effects of incubating both cell lines at 37˚C for 6 hours in standard inducing media (see experimental methods) with and without theophylline. This step confirmed that theophylline did not affect the positive control cell line, and also provided baseline measurements which outlined the effects of the inducing process and media in the absence of theophylline. Shown in Figure 2-6, are images that depict the typical behavior of each cell line and the GFP expression of 2mM theophylline induced riboswitch "ON" cells in solution.

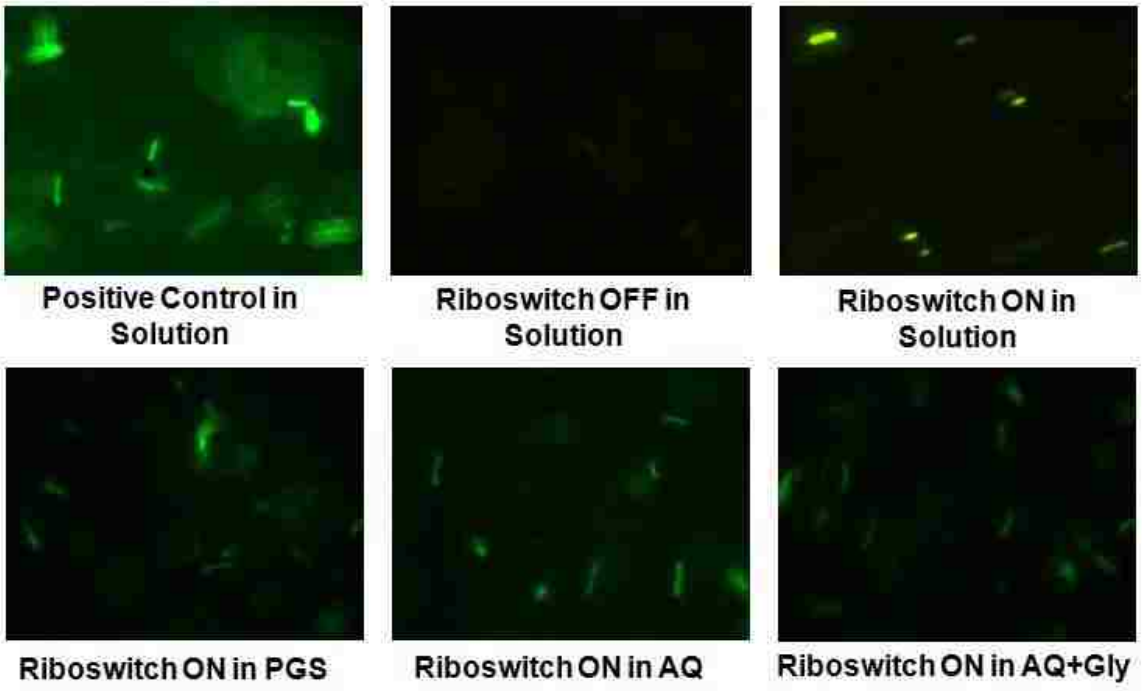

Figure 2-6: (Top) Images demonstrating the fluorescent behavior of positive control and riboswitch ON/OFF in solution after 6 hours. (Bottom) Images of 2mM theophylline induced riboswitch activity of BL21-RS cells encapsulated in each aqueous silica matrix.

The characteristic time course profiles for riboswitch ON/OFF behavior, beginning at time zero (theophylline addition), and extending several hours until the fluorescent intensity of the resulting GFP expression reached a peak or plateau was determined. These results are compiled in Figure 2-7 which compares positive control and riboswitch ON/OFF profiles for cells in solution and encapsulated in all three matrices.

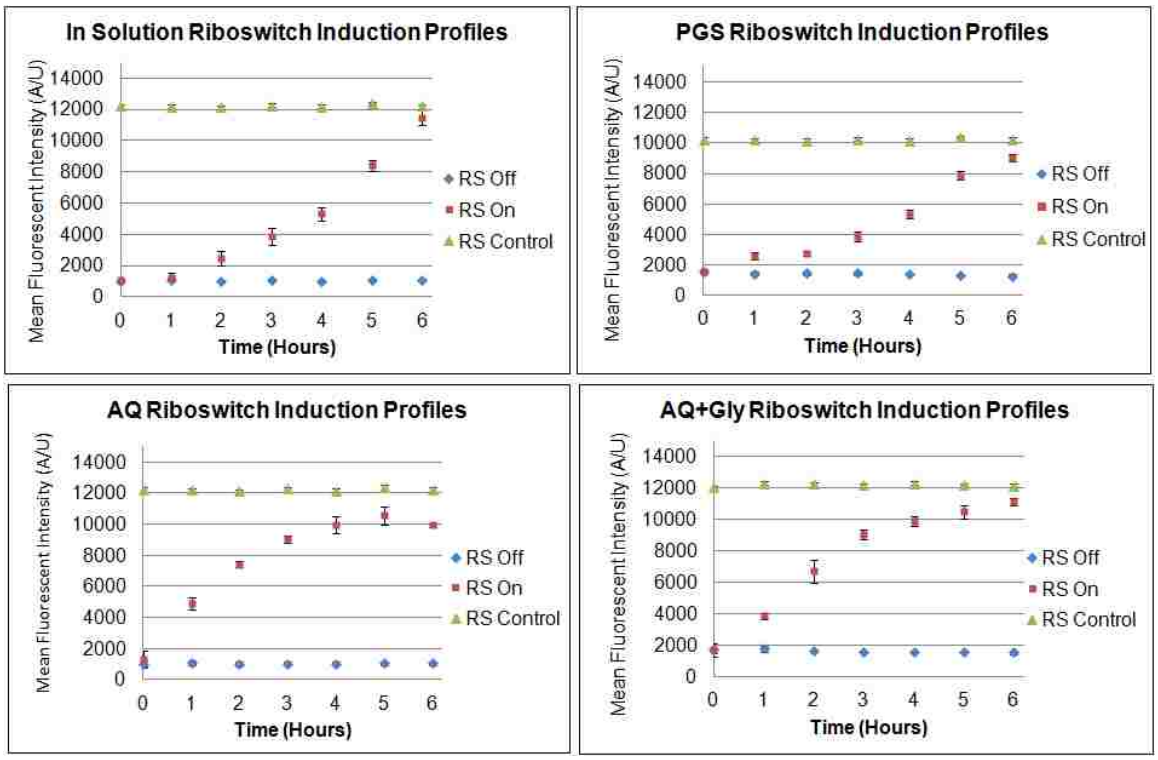

Figure 2-7: Time course profiles for riboswitch behavior when in solution or encapsulated in each of the three aqueous silica matrices. Shown for each graph is induction in the absence of theophylline, "RS Off" while cells activated with 2mM theophylline are denoted by "RS On". Positive control cells are denoted by "RS Control". Cells tested were encapsulated for 2-3 days prior to analysis. Error bars represent standard error of n ≥ 25 data points. Fluorescent intensity is given in arbitrary units (a.u.)

Findings indicate that cells tested in solution had the highest GFP fluorescent intensities for positive control and riboswitch OFF cells as well as lowest fluorescent intensity baselines (riboswitch OFF) versus cells that were encapsulated in aqueous silica gels. Positive control cells also had higher intensities than were observed for riboswitch ON cells when tested in solution or when encapsulated. Cells left in the riboswitch OFF state were observed to decrease in fluorescent intensity with respect to time although it is not well understood why this behavior occurs.

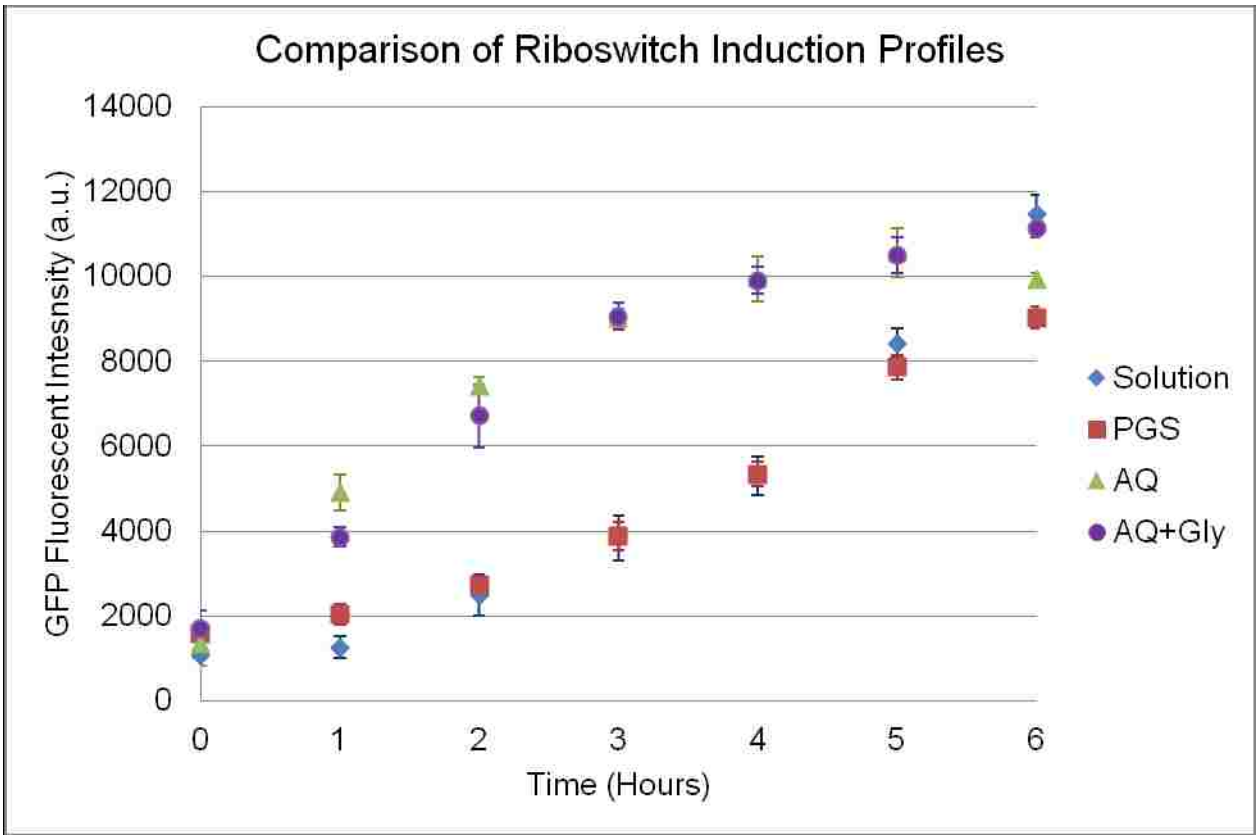

Figure 2-8: Comparing time course profiles for riboswitch induction using 2mM theophylline. Intensity is given in arbitrary units (a.u.). Cells tested were encapsulated for 2-3 days prior to analysis. Error bars represent the standard error with n≥25 data points.

Figure 2-8 provides a comparison of riboswitch ON induction profiles using a 2mM theophylline inducing media. The most notable features of this comparison are expedited rates of GFP expression seen by cells encapsulated in AQ and AQ+Gly matrices until 6 hours post induction when the mean fluorescent intensity of cells in solution reach a maximum at 6 hours that is similar to that observed for encapsulated cells. Up to four hours post induction, PGS encapsulated cells have a profile that is nearly identical to cells induced in solution however, 5 and 6 hours post induction the encapsulated cells have lower fluorescent intensities. When mesured at 6 hours, the lower fluorescent intensity of PGS based matrices could also be an optical property inherent to

the silica matrices where photons become scattered and there is loss of photons entering and exiting the matrix. Findings presented in Figure 2-7 and Figure 2-8 suggest that cells encapsulated by AQ and AQ+Gly offer a quicker response to theophylline detection versus PGS gels; not only in the rate they produce reporter protiens but also in the over all final fluorescent intensity. This does not however, rule out the possibility of final intensity measurements being influenced by optical properties of the gels but it seems unlikely. This behavior is likely due to metabolic energy/resources being diverted from normal processes and cell growth/division to producing metabolites, leaving the cells poised to respond to theophylline induction. Similar types of behavior have been observed by researchers who encapsulated plant cells in aqueous sol-gels and discovered that encapsulated cells were capable of producing 10-100 times more metabolites than non-encapsulated control cells.[25,26]

Theophylline response profiles for determining detection thresholds

Finally, the limiting threshold for theophylline detection by encapsulated BL21-RS cells encapsulated in each matrix was measured. Previous work involving riboswitch *E. coli* tested in solution showed the cells to have detection thresholds as low as 0.01mM theophylline.[14] In this study, nine solutions with theophylline concentrations ranging from 0.005-0.5 mM were allowed to incubate over each silica matrix. Fluorescent measurements were recorded at 3, 6 and 24 hours to determine the theophylline response profiles, which are given in Figures 2-9 through Figure 2-11.

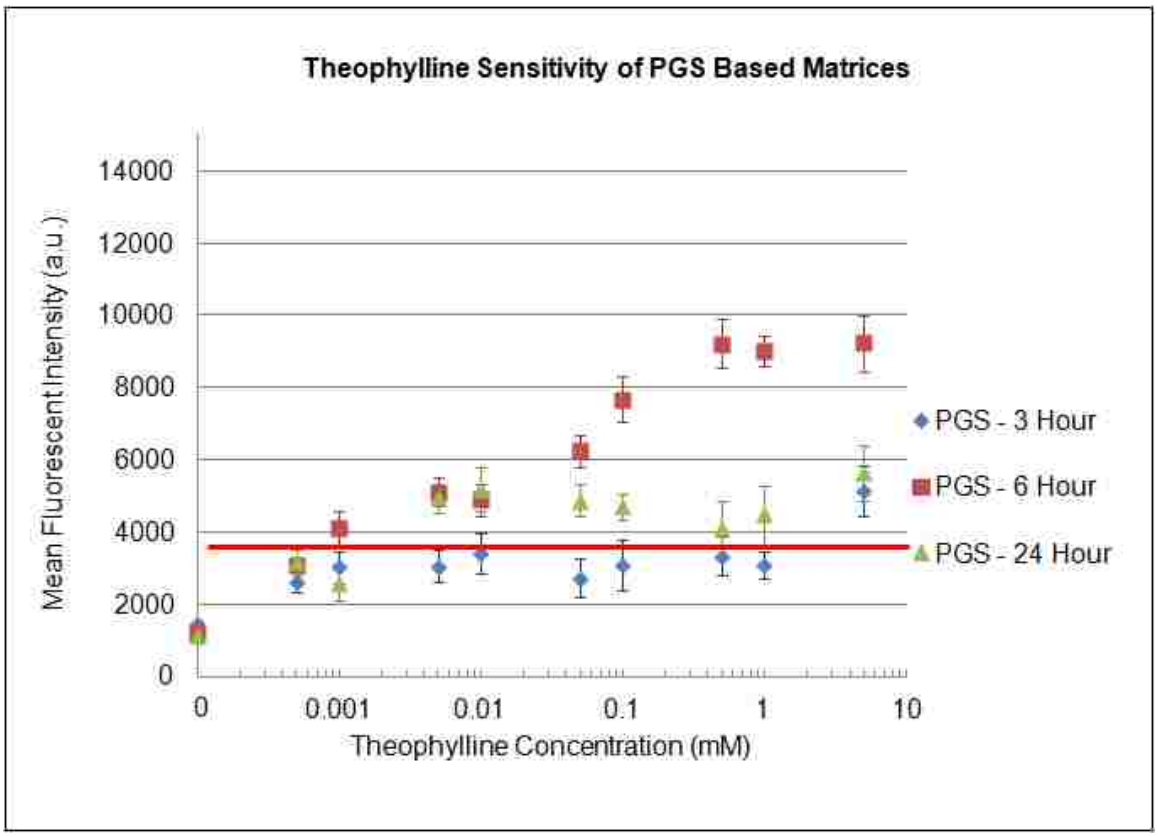

Figure 2-9: Theophylline response curves taken for varying concentrations of theophylline induction at 3, 6 and 24 hours to determine threshold of detection for PGS based aqueous silica gels. Cells tested were encapsulated for 2-3 days prior to analysis. Fluorescent intensity is represented in arbitrary units (a.u.) Error bars represent standard error of n≥25 data points.

Figure 2-9 depicts the theophylline response curves observed for cells encapsulated in PGS aqueous gels. Fluorescent intensity peaks at 6 hours, with the 3 hour and 24 hour time points having lower average intensities. To determine the threshold of detection, the mean fluorescent intensity of riboswitch off cells was measured (M_1) which would provide a value that allows safe estimate of the threshold of detection. The value M_1 is used to approximate the standard minimal fluorescent intensity (M_{fm}) where $M_{fm} = 3*M_1$ represented by the red bar. The lowest theophylline concentration tested with a fluorescent intensity value that appears above M_{fm} after 6 hours is chosen as the theophylline limit of detection (LOD). Findings indicate that PGS based aqueous gels offered encapsulated cells a theophylline limit of detection (LOD)

threshold is 1 µM theophylline. The LOD of cells encapsulated in AQ and AQ+Gly matrices is given in Figure 2-10 and Figure 2-11 respectively.

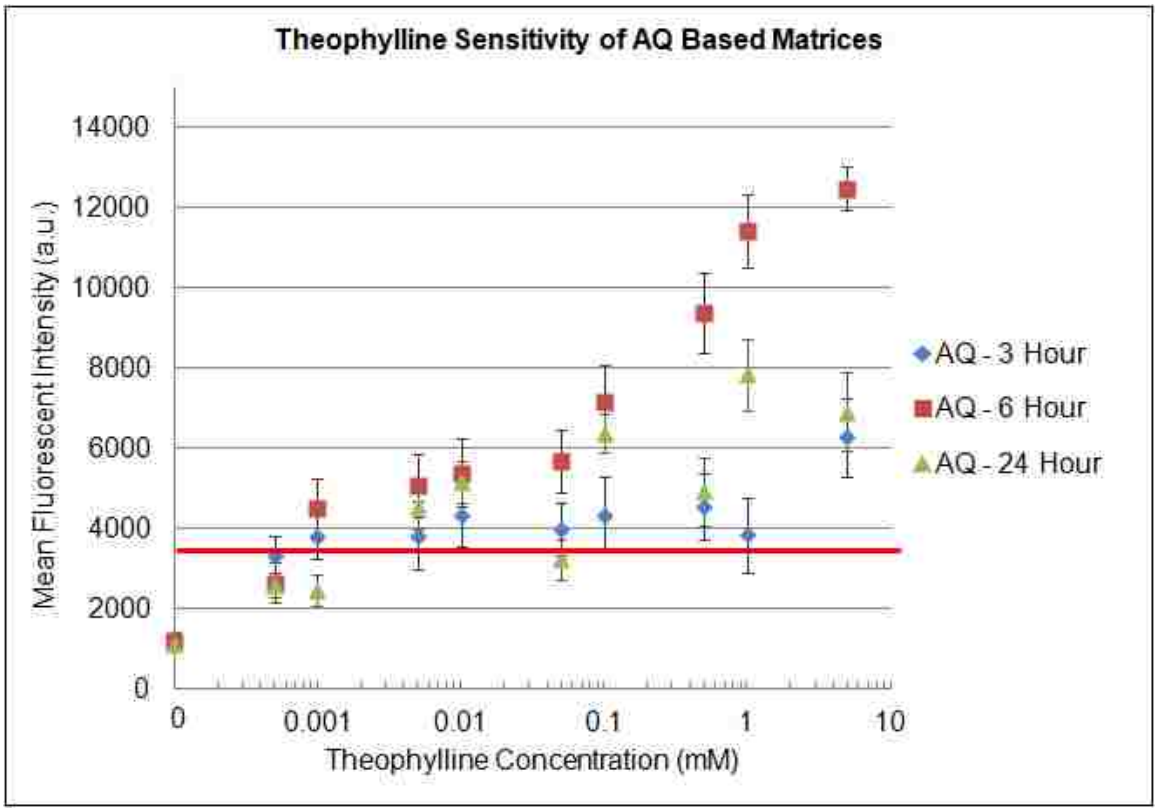

Figure 2-10: Theophylline response curves taken for varying concentrations of theophylline induction at 3, 6 and 24 hours to determine threshold of detection for AQ based aqueous silica gels. Cells tested were encapsulated for 2-3 days prior to analysis. Error bars represent standard error of n≥25 data points.

Shown in Figure 2-10, cells encapsulated in AQ based silica gels have a theophylline LOD of 1 µM. Findings presented in Figure 2-11 demonstrate that AQ+Gly based silica gels also allow cells to detect 1 µM levels of theophylline. When encapsulated in silica gels formed with AQ+Gly, fluorescent intensities associated lower theophylline concentrations are seen to plateau sharply from 0.05 to 0.01 mM while cells encapsulated in AQ matrices had 18-20% higher fluorescent intensities for the same concentration range. The AQ+Gly based matrices may offer less sensitive theophylline

detection at near micro molar concentrations however these matrices do offer noticeable increases in fluorescent intensity at the same dynamic range but exhibit lower limits of detection with saturation occurring at lower concentrations.

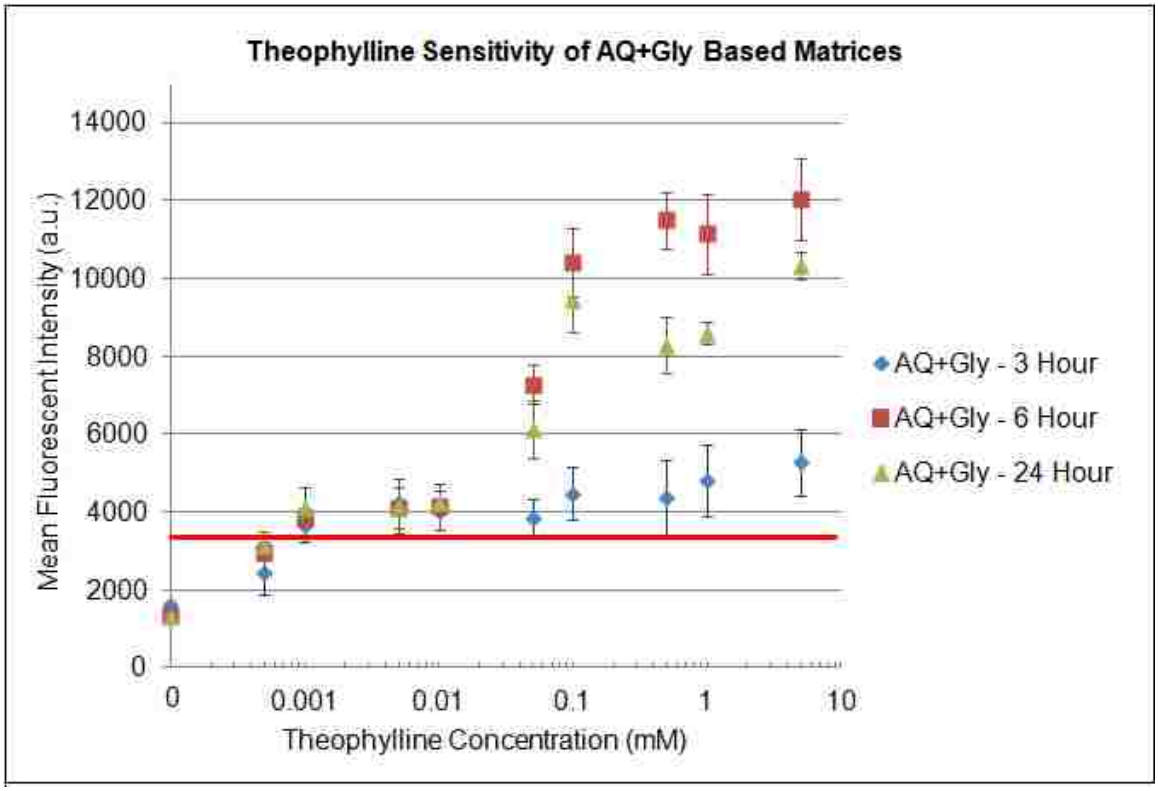

Figure 2-11: Theophylline response curves taken for varying concentrations of theophylline induction at 3, 6 and 24 hours to determine threshold of detection for AQ+Gly based aqueous silica gels. Cells tested were encapsulated for 2-3 days prior to analysis. Error bars represent standard error of n≥25 data points.

Examination of Figures 2-9 through 2-11 reveals trends that are common among each aqueous silica matrix. First, each gel typically had its highest fluorescent intensity observed at the 6 hour time point, generally the next highest intensity observed at 24 hours and the lowest intensities observed for the 3 hours time point. This data also indicated that the response curves observed at 6 hours are the best choice for determining the LOD for cells encapsulated in each matrix.

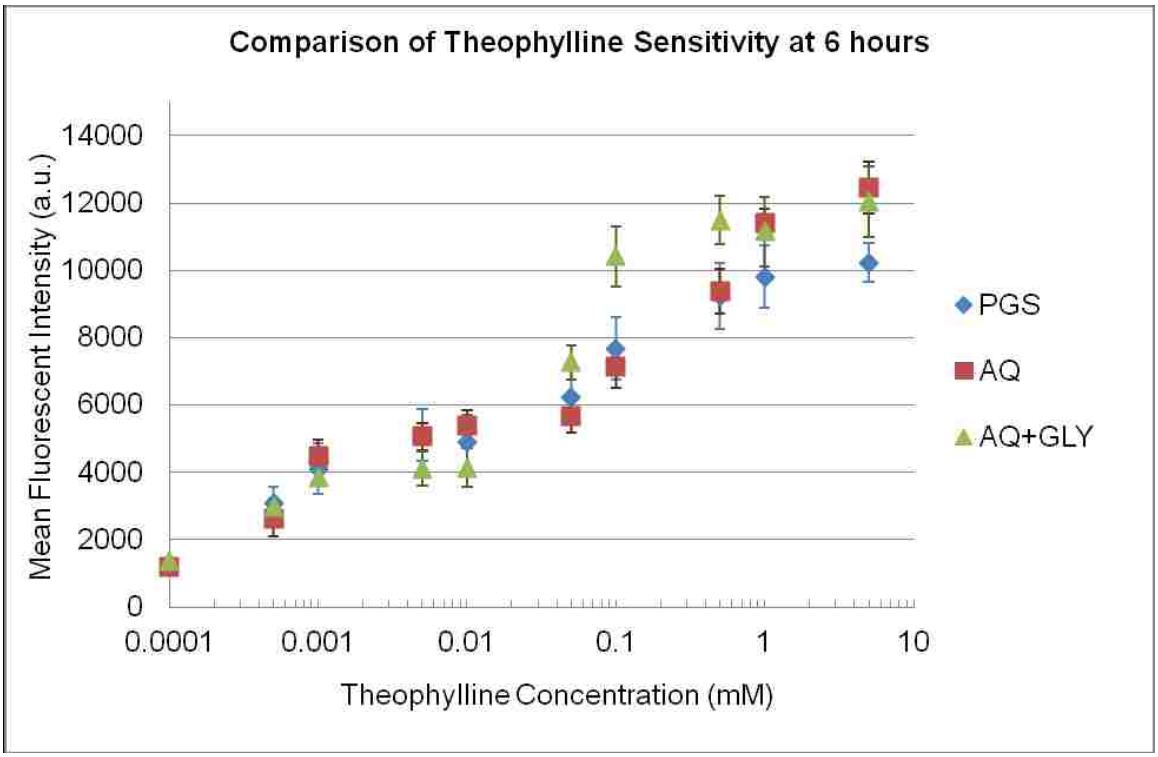

Figure 2-12: Comparison of theophylline sensitivity response curves taken at 6 hours. Cells tested were encapsulated for 2-3 days prior to analysis.

Figure 2-12 offers a combined comparison of theophylline detection thresholds observed for each gel taken at 6 hours. This figure demonstrates the small statistical significance between each aqueous silica gel and its respective detection response curve but also highlights the places where one or two gels offer strengths or weaknesses. At saturation, AQ and AQ+Gly matrices had cells with higher fluorescent intensities than cells within PGS derived matrices. For the concentration range between 0.01-0.5 mM, AQ+Gly based gels offered the most robust response to theophylline detection.

To understand the effects each matrix has on cell encapsulation it is important to relate the trends observed by the viability assay, plating study and induction behavior. It is observed that AQ based gels offer limited viability (as assayed by *Bac*Light LIVE/DEAD bacterial viability assay) but do offer consistent colony-forming capacity as

well as theophylline induction, and interestingly, increased rates of theophylline induced GFP expression (see Figure 2-7 and Figure 2-8). In most cases, AQ+Gly based gels had similar characteristics, except for the ability to form colonies during prolonged encapsulation.

One proposed explanation is that the AQ and AQ+Gly aqueous silica gels are not as adequate at protecting cells from membrane lysis or disruption relative to PGS derived matrices. Compromised membrane itegrity combined with high analyte diffusivity and un-restricted enzyme/ribosomal access could allow quickened theophylline induced GFP expression. Moreover, the cell and its contents are encased by the hydrated silica in a cage shaped to the cell's 3D morphology which would also keep the necessary cytoplasmic components preserved, localized and accessible. One particular study proposed this type of behavior by using similar sodium-silicate based solgel chemistry to encapsulate *E. coli* and test for Beta-Galactosidase enzymatic activity.[21] The same study went on to conclude that a lysed/compromised membrane offered quicker and more profound enzymatic response in aged, encapsulated cells than was seen for cells tested as a control in solution; this was despite the compromised membrane that should render the cell not viable. This theory is further strengthened by the fact that PGS gels have an induction profile very similar to that of cells induced in solution, implying that PGS gels offer increased preservation of membrane integrity when encapsulated cells are stored deprived of nutrients.

The second explanation is that in an effort to self-mitigate the stresses of aqueous sol-gel encapsulation (particularly mechanical stress or starvation) the cells may have enabled their selves more access to outside chemicals in order to search for sustenance,

ultimately making it easier for the theophylline to permeate the cell membrane. The limited availability of nutrients could also influence the way that cells use their resources in that cell metabolism becomes focused on metabolite or enzyme production and less on cell division or growth. This would be similar in behavior to plant cells which were encapsulated in aqueous silica gels which demonstrated a 10-100 fold increase in secondary metabolite production versus free cells.[25-26] This type of stress response behavior is not well understood, though some have explored the topic.

A third explanation is that cells encapsulated in AQ based matrices are the only cells that are truly viable, especially after increased amounts of time encapsulated, and that cells encapsulated by AQ+Gly and PGS matrices are in a state of declining, preserved metabolic activity. This idea is supported by trends of consistent culturability and theophylline induction, as well as increased rates of response to theophylline induced GFP expression over 6 hours; a combination of characteristics not seen by the AQ+Gly or PGS based matrices. How viability versus culturability translates into riboswitch performance as judged from GFP fluorescent intensities is at present unclear. Further inquiry into the state of integrated cell metabolism is being performed using a Gene Chip assay which will provide further insight into the topic.

2.3 CONCLUSIONS

In summary, three methods for encapsulating bio-sensing *E. coli* cells into aqueous, sol-gel based silica matrices have been characterized. Each matrix displays its own unique characteristics and offers different qualities (desired/undesired) that make them candidates for applications in bio-sensing analytical devices. The processes for

encapsulating cells in these matrices are biocompatible with the added bonus of minimizing toxic solvents, chemical precursors and co-products as well as minimizing or eliminating negative impacts caused by desiccation or osmotic stresses. As a result, all three aqueous silica gels offer prolonged viability (up to 30%) of encapsulated cells and maintain the ability to detect and respond to theophylline induction for periods up to 5 weeks.

AQ based silica gels exhibit high initial cell viabilities but lower relative extended cell viabilities based on a *Bac*Light LIVE/DEAD Bacterial Viability Assay. However, AQ-based encapsulation allows cells the ability to form colonies when grown on solid substrate. Cells encapsulated in AQ based matrices were capable of theophylline detection and GFP response up to 5 weeks, and interestingly, these cells had theophylline induced fluorescent intensity which increased the longer the cells were encapsulated. The AQ derived silica matrices also allowed encapsulated Riboswitch *E. coli* a theophylline limit of detection near 1 μM. When measured six hours after theophylline induction, encapsulated cells exhibited increasing fluorescent intensities that were positively correlated with increasing theophylline concentrations. When examining the time course fluorescent profiles for cells induced with theophylline, cells encapsulated in AQ matrices responded with increased GFP expression which resulted in higher fluorescent intensities being observed versus cells induced in solution for the same amount of time.

Encapsulation of Riboswitch *E. coli* using AQ+Gly derived matrices affected cell behavior in a manner similar to AQ based matrices. AQ+Gly derived matrices allowed 1 μM theophylline detection, had similar cell viabilities to AQ derived gels through the majority of the study and cells responded to theophylline induction with similar

timecourse profiles. However, unlike AQ based matrices, cells encapsulated using AQ+Gly derived matrices reduced the ability of cells to reproduce when streaked onto solid substrates. Cells induced with theophylline over the course of 5 weeks did not demonstrate increases in fluorescent intensity with respect to time. Instead, AQ+Gly encapsulated cells behaved similarly to cells encapsulated in PGS matrices, where theophylline induced intensity decreased as a function of time. Furthermore, cells encapsulated within AQ+Gly matrices did not offer GFP fluorescent expression that would allow one to distinguish between theophylline concentrations ranging from 1-10 µM; AQ and PGS derived matrices did offer this ability. However, using AQ+Gly matrices did offer increased GFP response to theophylline induction near 100 µM concentrations.

PGS based matrices were most distinct from AQ and AQ+Gly matrices. PGS derived matrices allowed encapsulated cells to detect and respond to theophylline induction with a 1 µM limit of detection. Cells encapsulated using PGS matrices had lower overall intensities as compared to cells within AQ, AQ+Gly matrices or cells in solution. These cells also had higher assayed viabilities and nearly no ability to reproduce on solid substrates – even in the first 3 weeks. When examining time course profiles and limits of theophylline detection, PGS encapsulated cells were more liken to the riboswitch ON behavior of cells tested in solution. Similar to cells encapsulated in AQ+Gly matrices, PGS encapsulated cells had theophylline induced fluorescent intensities that decreased as a function of time.

Bio-sensing *E. coli* encapsulated in all three matrices offer the ability to detect the theophylline target analyte at concentrations near 1 µM. However, the behavior of cells

responding to theophylline induction varies based on the aqueous silica matrix used. From this data and the differences observed in cell behavior, many conclusions arise. Findings indicate that the encapsulating matrix has profound influence of cell reproduction, assayed viability and GFP expression profiles. It is also concluded that assayed cell viability does not couple well with cell reproduction and it is believed that glycerol may negatively impact the reproduction of post encapsulated cells. Also, neither cell viability nor reproduction post encapsulation is coupled with theophylline induced cell activity; which is most obvious when comparing viability and induction behavior between cells encapsulated in PGS and AQ derived matrices. Data suggests that AQ and AQ+Gly based matrices cause increased cell plasma membrane permeability (based on Baclite viability assay) however, this is not an indication of decreased activity or even cell death (as demonstrated by the plating study or the increases/decreases in theophylline induced fluorescent intensity).

It is believed that these encapsulation methods would be broadly applicable for encapsulating other bio-sensing organisms, enzymes and other bio-molecules of interest with similar levels of success; depending on the desired sensitivity and concentration range to be studied. The glycerated sols presumably mediate cellular interactions with silanol groups, which are believed to disrupt membranes of cells integrated into lipid templated mesoporous silica films, presumably through hydrogen bonding and electrostatic interactions. Explaining perhaps the PI staining associated with compromised membranes and the increased incidence of un-viable cells observed for AQ based gels. However whether, when confined within a 3D matrix, to what degree membrane permeability translates into loss of viability / biofunctionality is an important

question that we will continue to address. In particular it appears that for AQ matrices the GFP expression becomes brighter and less localized in the cells over time. It is believed that this behavior is largely due to cells focusing their resources towards producing enzymes/metabolites instead of cell growth or division. Further work will investigate if this is evidence of loss of membrane confinement of cellular components yet retention of activity within a silica compartment.

2.4 Materials

Stable and functioning positive control (pSAL:GFPa1His) and riboswitch (pSAL:RS12.1GFPa1His) BL21 *Escherichia coli* (*E. coli*) cell lines were obtained from Svetlana Harbaugh at the Air Force Research Laboratory, Wright-Patterson Air Force Base, Dayton, Ohio. Aqueous solutions were prepared using de-ionized (DI, nano-pure) water that was produced using an on-site ion-exchange purification system and was measured to be no less than 18 Mega-Ohms (MΩ). Sodium chloride, sodium hydroxide, theophylline, Dulbecco's phosphate buffered saline (DBPS, 1X), ampicillin-sodium salt, sodium-silicate solution, tetraethoxyorthosylicate (TEOS), tetraethylorthosilicate (TEOS), anhydrous glycerol, titanium(IV) isopropoxide (97%) and theophylline were purchased from Sigma Aldrich (St. Louis, MO). Sodium phosphate (dibasic, heptahydrate) and sodium phosphate (monobasic, monohydrate) were purchased from EM Science (Darmstadt, Germany). Bacto™ Yeast Exctract, Bacto™ tryptone and agar were purchased from Becton Dickinson and Company (Sparks, MD). The LIVE/DEAD *Bac*Light Bacterial Viability Assay (Kit L7012) was purchased from Invitrogen (Carlsbad, CA).

2.5 Experimental Methods and Procedures

Propagation of Cell Lines

Positive control (pSAL:GFPa1His) and riboswitch (pSAL:RS12.1GFPa1His) BL21 *Escherichia coli* (*E. coli*) cell lines be tested or encapsulated were taken from nitrogen cryogenic storage and inoculated into 50 mL of LB broth, dosed with 1 mL of ampicillin stock solution (50mg/mL), then incubated overnight at 37C degrees. The LB broth recipe called for dissolving 10g bacto-tryptone, 5g yeast extract and 10g NaCl (sodium chloride non-idiozed) in 1L of DI water; pH adjusted to 7.5 using NaOH. Broth was sterilized by autoclaving. Directly prior to inoculation, 1mL ampicillin stock (50mg/mL) was added to each flask. Cultured cells were streaked onto LB+Ampicillin agar plates and incubated for 24 hours. LB-Agar + ampicillin (50ug/mL) plates were produced by following the LB broth recipe above and adding 15 grams agar per 1L of broth. This mixture was autoclaved to sterilize and melt the agar. To the warm (~50˚C) and sterile agar-broth solution was added 1mL of ampicillin sodium stock (50mg/mL), then the mixture was divided into sterile plates. Cultured agar plates allowed cells lines to be propagated by these methods for 30 days before a new plate is required; otherwise cells risked dropping their respective plasmids.

Synthesis of 10:1 Poly-glycerated Silicate (PGS)

Poly-glycerated Silicate (10:1) was synthesized by following these procedures using glass equipment, paying careful attention to inert atmospheric control and anhydrous conditions/materials in all steps. A 100 mL round bottom flask was equipped with a stir bar and nitrogen-purge then attached to a reflux condenser and charged with

63.4 g anhydrous glycerol (688mmol). The glycerol was heated to 60˚C at which point a mixture of 10.21g tetraethoxyorthosylicate (TEOS, 49.0 mmol) and 1.02g titanium(IV)isopropoxide (3.0 mmol) was added in a drop wise manner with rapid stirring over a period of 15 minutes. After addition of the TEOS and catalyst the mixture was refluxed at 130˚C for 3 hours. The ethanol co-product was removed using vacuum distillation at 130˚C and ~10 mTorr for 2-3 minutes. The product was a viscous fluid with a clear to opalescent white appearance. Typical yields were 92-96% of theoretical which corresponded with the theoretical formula $Si(C_3H_7O_3)_4 \cdot 10C_3H_8O_3$; however, it is certain that inadvertent side products and isomers are present in the final product.

Preparation of PGS Derived Aqueous Gels

Stationary phase cells were pelleted using Eppendorf 5424 centrifuge by spinning at 10,000 RPM. Cells were washed 3x and resuspended in 0.1M sodium-phosphate buffer, pH 6.0, diluted from stock concentration to ~10^7cells/mL. Equal volumes of PGS and buffer suspended cells were combined in 1.7 mL polypropylene micro-centrifuge contains to yield a mixed volume of 500 µL. Gelation occurred in 60-90 minutes with a final cell density near 10^6-10^7cells/mL; gels were formed and stored at room temperature.

Synthesis of Sodium-Silicate based Aqueous Sol-gel (AQ)

All steps were performed using polypropylene centrifuge tubes/contains. 1.654 mL of Sodium silicate solution (26.5% SiO_2; 10.6% Na_2O) was added to 6.8 mL of DI water. This mixture was immediately added to 3.08 g of highly acidic H^+ cation-exchange resin (Dowex 50WX8-100) with mixing to bring the pH near 4. Next the resin was removed via multiple steps of high speed centrifugation and collecting the supernatant (vacuum filtration is also an option). Hydrochloric acid (2.0M) was added to

the cleaned solution at a rate of 7.1 µL per mL-collected, to bring the pH down near 2.0. This solution can be stored in the refrigerator for up to 48 hours before using.

Preparation of Sodium-Silicate based Aqueous Gel + 20% Glycerol (AQ+Gly)

To the above listed solution is added 20% glycerol by volume.

Preparation of AQ and AQ+Gly Derived Aqueous Gels

Stationary phase cells were pelleted using an Eppendorf 5424 centrifuge by spinning at 10,000 RPM. Cells were washed 3x and resuspended in 1.0 M sodium-phosphate buffer, pH 7.0, with a 2.5 fold dilution from their stock concentration. AQ or AQ+Gly were combined with the buffer suspended cells in a 5:1 ratio. Monoliths prepared by this method had 480 µL initial volumes using 400uL of AQ or AQ+Gly and 0.8 µL of suspended cell. Gelation of AQ derived gels formed in 10-15 seconds and AQ+Gly gelation occurred in 25-30 seconds. Final cell density was near 10^6-10^7cells/mL; all gels were formed and stored at room temperature.

Assaying for Viability

The *Bac*Light LIVE/DEAD Bacterial Viability Assay (Kit L7012, *Invitrogen*) uses two components for staining cells with fluorescent markers. The first component, SYTO-9™, is a membrane permeant dye that binds with nucleic acids of living bacteria, staining them green. The second component, propidium iodide (PI), is a membrane impermeable compound that enters damaged or membrane compromised bacteria, staining them red. Characteristic excitation/emission maxima for these dyes are about 480/500 nm for SYTO-9™ and 490/635 nm for propidium iodide.

Encapsulated cells and cells in solution could be assayed by adding 2.5uL of SYTO-9™ component and 1.5uL of propidium iodide component to a 1.7mL

contains/cuvette and diluting to 150 µL using DPBS (0.1X, pH 6.9. Each of the three aqueous silica monoliths (~500uL) was given 500uL of the above prepared viability assay and incubated for 25-30 minutes (in solution) or 30-40 mintues (if encapsulated) at 37˚C.

Inducing Riboswitch Mechanism (Riboswitch ON)

The standard solution for inducing the riboswitch mechanism of encapsulated cells, or cells in solution, was prepared at theophylline concentration of 2.0 mM in a 1.7mL micro-centrifuge cuvette/contains. This also contained 45uL of LB broth, 150 µL Dulbecco's PBS (1X, pH 6.9) and 15uL of ampicillin stock solution (50mg/mL) then diluting with DI water to 1.5 mL. After applying the solution the cell suspension or aqueous gels were put into an incubator at 37˚C for 6 hours.

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SUMMARY AND FUTURE OUTLOOK

In summary, described herein are novel improvements on existing integration strategies and new methods and materials for encapsulating living micro-organisms into 3D silica matrices. This work demonstrates the impact that nano-materials have on integrated cells and provides insight for modifying and optimizing encapsulating environments for the purpose of producing living biotic materials. Extremophile inspired strategies were artificially incorporating with a living cell integration process by incorporating manganese-phosphate buffer, non-fluid lipid (DPPC) liposomes or trehalose with the integration process. These additives mitigate the stresses experienced by cells being integrated into lipid templated mesoporous silica matrices by producing a localized affect that mimics the conditions created by extremophiles to survive. These additives were successfully included in the CDI process and all demonstrated some ability to increase integrated cell viability.

As an alternative to lipid templated mesoporous silica matrices, aqueous silica gels were also investigated as potential material for living cell encapsulation. It was discovered that changes in the silica gel and the microstructured framework had noticeable effects on integrated cell viability and behavior. It is postulated that viability according to an assay, viability according to cell reproduction with plating studies, and cell activity as indicated by theophylline induced GFP expression are not all coupled the same way among different silica gels. Furthermore, glycerol and glycerated precursors also impacted cell viability and activity but also had noticeable effects on the rate of gelation as well as the level of gel synteresis over time.

The effects of such modified integration methods were carefully studied and analyzed to truly understand the mechanisms by which the effects were produced so that they can be further refined for future work. Several questions regarding cell activity, cell viability and its relationship to the encapsulating silica matrix remain un-answered. To further the impact of this work and answer such questions, a handful of experiments are proposed.

One proposed study would further investigate the mechanism(s) by which trehalose creates such considerable increases in cell viability. It is clear that trehalose and the availability of water have clear impacts on cell viability and data from manganese-phosphate studies also reveals that dessication by itself may be causing increased levels of oxidative stress on integrated cells. A stronger correlation could be made if trehalose were studied for its ability to reduce oxidative stress and even resist direct exposure to known reactive oxides and superoxides. This idea stems from studies where trehalose has shown to offer protective properties against cell desiccation, and more importantly, oxidation.

One study demonstrates protection against oxidation by subjecting two different strains of yeast to known ROS to see how trehalose protected against oxidative stress. The yeast were exposed to 10% solutions of external trehalose, then washed, and exposed to either menadione (a source of intracellular superoxide) or tert-butylhydroperoxide (TBOOH), an organic superoxide that preferentially attacks the plasma membrane. The study concluded that extracellular trehalose addition to a strain of yeast that does not produce trehalose still provided defense against the menadione. Meanwhile, extracellular exposure to trehalose offered no protection against TBOOH, the reason being that the

exterior of the cells had to be washed, removing the trehalose from the surface of the cells. This is of primary interest because in this system trehalose is likely to accumulate at the cell surface and if oxidative stresses were occurring outside of the cell during matrix condensation, trehalose could offer significant protective advantages. A similar approach could be taken with integrated cells, to see if extracellular trehalose is in fact protecting against oxidation, dessication or both.

The results of the riboswitch and aqueous silica gel experiments could be further understood and benefit highly from conventional materials analyses, the results of which could be coupled with the trends observed for Riboswitch *E. coli* or by cells that will be integrated using these matrices in the future. This could be accomplished by converting the aqueous gels into aero gels using solvent exchange and critical point CO_2 drying. Doing so would allow many tests such as powder XRD, nitrogen adsorption/desorption, SEM or TEM to be used to analyze the porous structure and silica framework of the encapsulating matrix. The aerogel process has been attempted with these gels but the results were unsuccessful.

There is another experiment that riboswitch *E. coli* and all three aqueous matrices would benefit from. This experiment would consist of a plating and/or culture study that would seek to replicate previously encapsulated cells much like the plating study conducted as a compliment to the viability assay. However in this study, the generations that arise from previously encapsulated cells should be analyzed to compare theophylline induced behavior and response curves to normal control cells in solution and their encapsulated ancestors. This would provide insight to how the matrix effects the cells

and if the cells return to a regular metabolic state more representative of the integrated cells or normal control cells that have not been integrated.

The results of all of these studies and the works presented in this paper may be broadly applied to other similar systems and classes of living materials. Future work should consist of encapsulating/integrating multiple types of organisms with varying levels of complexity which could include bacteria, yeast, bio-sensing organisms, mammalian cells or cancerous cells that can be investigated in stable, well-defined environments.