Cell Response to Silica Gels with Varying Mechanical Properties

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Dedication

To my family for all of their support and encouragement throughout my education.

Abstract

Sol-gel encapsulation has a variety of applications in biotechnology and medicine: creating biosensors, biocatalysts, and bioartificial organs. However, encapsulated cell viability is a major challenge. Consequently, interactions between cells and their 3D microenvironment were studied through rheological, metabolic activity, and extraction studies to aid in the development of new gel protocols. The cells were encapsulated in variations of three silica sol-gels with varying stiffness.

It was hypothesized that the cell viability and the amount of extracted cells would depend on gel stiffness. For two gels, there was no apparent correlation between the gel stiffness and the cell viability and extracted cell quantity. These gels did strongly depend on the varying gel ingredient, polyethylene glycol. The third gel appeared to follow the hypothesized correlation, but it was not statistically significant. Finally, one gel had a significantly longer period of cell viability and higher quantity of extracted cells than the other gels.

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Chapter 1: Introduction

1.1 Background

There are many different applications in biotechnology and medicine for the encapsulation of biological materials in sol-gel matrices. Sol-gel matrices are particularly appealing because the sol-gel process is relatively mild, so the biological materials are not harmed [1]. In biotechnology, sol-gel encapsulation has been used for biosensors and biocatalysts [2].

The encapsulation of biomolecules in sol-gels has been used to create biosensors that can detect a variety of chemicals, drugs, and toxins [3, 4]. Encapsulated *Moraxella* spp. cells have been used for the detection of organophosphates [5]. Encapsulated *Bacilis subtilis* have been used for the detection of water pollutants [4]. Glucose oxidase encapsulated alone and with horseradish peroxidase has been used for the detection of glucose [6, 7]. Encapsulated myoglobin has been used for the detection of dissolved oxygen, and encapsulated manganese myoglobin has been used for the detection of nitric oxide [6]. Five strains of genetically engineered bioluminescent *E. coli* have been encapsulated together and used to detect heat shock, oxidative stress, fatty acids, peroxides, and genotoxicity [8].

Encapsulated biocatalysts are used to form bioreactors for a variety of applications, such as producing chemicals, food, cosmetics, or drugs [4]. In fact, some current commercial immobilized catalysts include CTIS CACHy, catalysts for asymmetric cyanohydrin synthesis; MetaChip, for rapid testing of drug toxicity against specific cells;

and a number of lipases from Fluka, which are commonly used for esterification reactions [9]. Additionally, immobilized lipases have been successfully used to produce biodiesel, but they need to be made more economical before they can be used commercially [10]. Many more studies have been done in this area. For example, encapsulated yeast spores, which are used for the conversion of carbohydrates to ethyl alcohol and carbon dioxide, have been shown to have similar activity to that of free cells [11]. Immobilized yeast was also used in a bioreactor to convert fumaric acid to L-malic acid [12].

Biocatalysts have also been used for bioremediation purposes. One example of this is the use of entrapped *Bacillus sphaericus* cells for the bioremediation of uranium from mining waste pile waters [4]. Another example is the use encapsulated microbial carriers in a packed bed reactor for phenol degradation during wastewater treatment [13].

In medicine, sol-gel encapsulation is used for controlled drug release platforms, bioartificial organs, bioactive materials, and tissue-derived cell growth [14]. The ability to release drugs in a controlled manner from bioartificial organs, implantable prosthesis, and other biomedical devices would improve patient convenience and the effectiveness and safety of the product [15]. There have been studies on controlling the release rate of heparin, ibuprofen, gentamicin, dexmedetomidine, and *cis*-platin [15-18].

There have been recent studies that have used cell encapsulation to create bioreactors that act as an artificial liver or pancreas. The encapsulation of the cells protects them from the immune system [14]. One example of this is the encapsulation and transplantation of pancreatic islets for the treatment of diabetes [19-22]. Bioactive materials are being used for reinforcement of damaged bones. These materials, usually

calcium phosphates or bioglasses/bioactive glass ceramics, aide in the reconstructive process [14, 23, 24]. These materials ideally prevent rejection from the immune system and degrade naturally in the body over time [24]. Tissue-derived cell growth could be useful for growing and detecting viruses as well as creating antiviral vaccines [25].

In conclusion, the encapsulation of biomolecules has extensive applications in both biotechnology and medicine. There are a number of reasons why it is beneficial to encapsulate biomolecules. Generally encapsulation protects cells from extreme conditions. In biosensors, the gel also prevents cells from proliferating, which is very important for the sensor to produce reliable and repeatable results. In biocatalysts, encapsulation prevents molecules from being released into the solvent, which is important in many different applications. Encapsulation protects cells from being attacked by the hosts' immune system in bioartificial organ and bioactive material applications.

1.2 Gel Chemistry

Silica sol-gels have been successfully used for encapsulation of biomolecules in many of the applications described previously. Silica is widely used for encapsulation because it has many desirable features such as biocompatibility, optical transparency, chemical inertness, mechanical strength, high surface area and porosity, enhanced thermal stability, and resistance to microbial attack [5, 26-29]. It is also available at a relatively low cost, prevents leaching, does not swell significantly in the presence of organic solvents, and protects the biomolecules by forming a cage around them [4, 5, 26, 27]. The porosity,

structure, and the processing and surface conditions of silica gels can also be tuned, which is a desirable characteristic for most applications [30].

In order to form a silica sol-gel, a precursor is required [31]. Common precursors are metal alkoxides, particularly tetramethyl orthosilicate (TMOS) and tetraethyl orthosilicate (TEOS), because of their reactivity with water [29, 31]. A hydrolysis reaction results when a metal alkoxide is combined with water [31]. During hydrolysis, a metal atom attaches to a hydroxyl ion (OH group) and a silanol moiety is formed and usually an alcohol is released [3, 31]. It is common to add a catalyst to promote and accelerate the hydrolysis reactions [31]. Common catalysts include potassium hydroxide, ammonium hydroxide, acetic acid, and hydrofluoric acid [29, 31]. Some sol-gel protocols have other additives such as silica nanoparticles to enhance the mechanical properties of the gel and prevent degradation [32].

The products of two hydrolysis reactions can cause a condensation reaction [31]. The silanol moiety from each reaction combines to produce a siloxane and release either a water or an alcohol molecule [3, 31]. The silanol moieties and the siloxanes attach to another through condensation reactions to create silicon dioxide matrices. Eventually, one large molecule will extend throughout the sol. At this point, the gel point, the sol is considered a gel. The gel reactions continue after the gel point, which is called aging [31]. The sol-gel process can be seen in Figure 1. Sol-gel process

The precursor will mainly determine the kinetics of the sol-gel process [31]. However, there are many other parameters that affect gel structure such as pH, temperature, solvent, catalyst, water/silica molar ratio, and aging conditions.

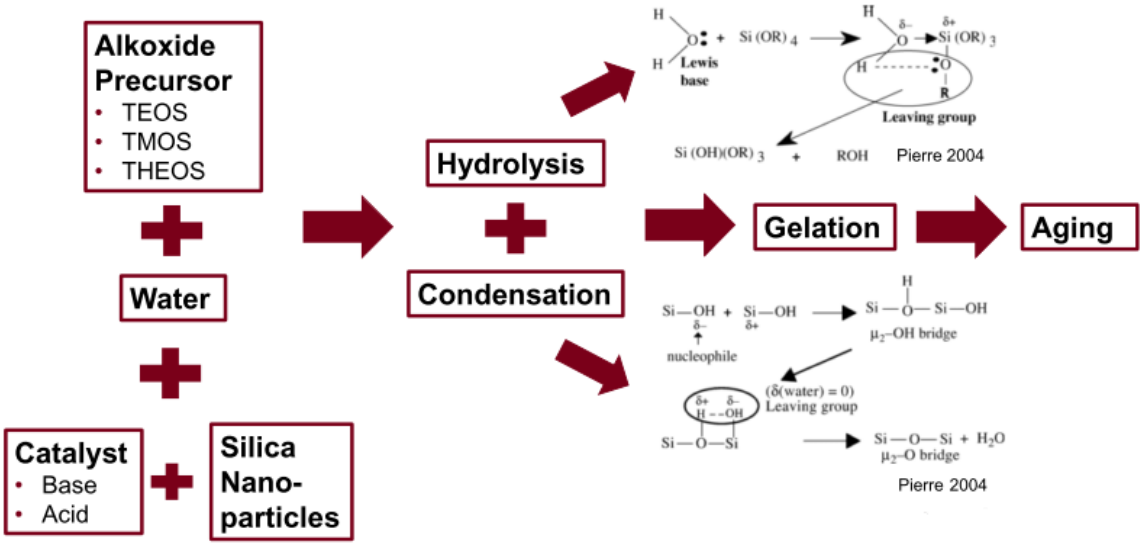

Figure 1. *Sol-gel process*

1.2.1 Effects of pH

Below pH 2 there are slow gelation times and growth occurs through aggregation. Between pH 2 and 7, there is rapid aggregation of molecules and the gelation times generally decrease as the pH increases. Above pH 7, growth occurs through the addition of monomers to large particles instead of particle aggregation and gel times are dependent on the particle size distribution [31].

1.2.2 Effects H_20:Si Molar Ratio

Weakly branched sols are produced when a low H_20:Si molar ratio is used with an acid catalyst. Conversely, highly condensed sols are produced when a high H_20:Si molar ratio is used with an acid or base catalyst [31, 33]. Ratios below 2 favor alcohol condensation reactions, whereas high ratios favor water condensation reactions and more complete hydrolysis before condensation [31].

1.2.3 Effects of Catalyst

As stated previously, the addition of a catalyst promotes and accelerates hydrolysis. If the catalyst is an acid, the strength of the concentration is the sole determiner of how much the hydrolysis is affected. If the catalyst is a base, the nature of the solvent plays a significant role in how much the hydrolysis is affected. Overall, it has been reported that acids are more effective catalysts than bases [31].

Although, catalysts are generally not necessary for condensation reactions, they can be beneficial. The effects of the catalyst is complicated by the hydrolysis reactions, but the pH of base or acid catalyzed sol does have an effect on the condensation rate. Below 2 pH, the condensation rate decreases to a minimum at 1.5 pH. Then, between 2 and 4 pH the condensation rate increases. Between 4 and 8 pH, the rate decreases slightly. Above 8 pH, the sols cease to form a gel [31].

1.2.4 Effects of Solvent

Solvents are commonly added to prevent liquid-liquid phase separation during hydrolysis and to control the silica and water concentrations. More recently, the use of solvents to dry gels without cracking them has been studied [31].

1.2.5 Cell Encapsulation

Although the sol-gel process is relatively mild and performed at room temperature, the extreme pH conditions, alcohol by-products, and the shrinking that occurs during the aging process can be harmful to the cells [14, 34]. Many applications require that the encapsulated cells are viable to work. As a result, a number of ways have been developed to minimize the detrimental effects that the sol-gel process has on the cells [14]. One

procedure breaks gelation into a low pH hydrolysis step and a neutral pH condensation step with storage in between [5, 8, 14]. The storage time allows the alcohol to slowly evaporate over a day or so, after which the pH is neutralized by the addition of a base and cells in their culture media. In some cases, it is not necessary to add a base [14]. Slight modifications to this procedure have been made to remove more alcohol. Conroy, et al., distilled the sol for 1 hour at 85°C or 18 hours at 60°C [27]. Ferrer, et al., used controlled vacuum evaporation which improved the viability of encapsulated *E. coli* cells [34].

This sol-gel process can be modified more to further minimize its harmful effects on cells. A hybrid gel can be created using silicates and organic additives [14]. For example, Conroy, et al., added polyethylene glycol (PEG) to control the pore size of the gel [27]. Ferrer, et al., added 3-Glycidoxypropyl trimethoxysilane (GPTMS) and PEG to their silica gels to attempt to improve cell viability by either improving the surface interactions or reducing gel shrinkage during the aging process. The organic additives did in fact improve the viability of the encapsulated *E. coli* [34].

There have been a number of other additives that have successfully improved the biocompatibility of sol-gels. One common additive is alginate [14]. The encapsulation of pancreatic islets for diabetes treatments performed by Lim and Sun was one of the first studies that used silica-alginate sol-gels [19, 35]. Lim and Sun opened the door for many encapsulation studies that used silica-alginate sol-gels [12, 21, 22, 36].

Another way of reducing the detrimental effects of extreme pH, alcohol, and aging on the encapsulated cells is the use of sodium silicate or colloidal precursors instead of the traditional precursors. This method also requires the neutralization of the pH of the sol

before the addition of cells [14]. However, these precursors do not produce any alcohol and have been used successfully for the encapsulation of enzymes, horseradish peroxidase and glucose-6-phosphate dehydrogenase, and cells, sulfate-reducing bacteria and *Moraxella* spp. cells [5, 26, 37]. The addition of glycerol to these aqueous silica gels was shown to further increase the viability of encapsulated *E. coli* [11]. Although there are no alcohol by-products that result from these precursors, they do release a high Na^+ concentration that can be harmful to the cells if it is not eliminated through an acidic cation-exchange resin [3]. These precursors can also cause osmostic shock from the high production of sodium chloride [38].

Additionally, using the Biosil process for encapsulation can minimize the detrimental effects of sol-gel gel process on the cells. In the Biosil process, the cells are attached to a solid, flat surface and subjected to a nitrogen steam containing silicon alkoxides and trimethoxysilane or methyldiethoxysilane. This eliminates excess liquid and consequently the stress induced by gel shrinkage during the aging process [14].

1.3 Motivation

Encapsulation of all types of biomolecules is currently used in a large number of applications and it has the potential to be used in many more. Therefore, the ability to encapsulate cells in a way that preserves their viability would be beneficial. For instance, it would aid in the development of biosensors for monitoring chemicals and micro environmental changes in many applications, bioreactors for bioremediation and transplantation therapies, and biomimetic studies for better understanding cell behavior [4, 5, 8, 13, 14, 19-22]. Understanding the behavior of a cell type during encapsulation

could be useful for diagnostic purposes because it may allow you to identify the cell type from a biopsy. In any biomimetic study or diagnostic application, it would be useful to have the ability to extract the cells from the gel and culture them so that more tests could be run on the cells in the future.

Despite promising results, cell sol-gel encapsulation was still in its infancy 7-10 years ago. In fact, the use of sol-gels was still in its infancy when compared to widely used polymers such as polysaccharides [39]. Within the realm of sol-gel encapsulation, the encapsulation of enzymes and proteins had been studied much more thoroughly than whole cell encapsulation [3, 34, 37, 40]. Additionally, the amount of animal cell encapsulation reports was small when compared to those of microbe encapsulations [3].

The studies that had been done mostly focused on the encapsulation of pancreatic islets, yeast cells, or *E. coli*. One of the first cell encapsulation studies was the Lim and Sun's encapsulation of Langerhan islets for the design of a bioartificial pancreas [19]. Many others further investigated the encapsulation of pancreatic islets for the treatments of diabetes [21, 22]. On the other hand, Carturan, et al., encapsulated *S. cerevisiae*, which is a species of yeast. Pope, et al., and Branyik, et al., studied the encapsulation of yeast as well [27, 30, 40]. Finally, the encapsulation of *E. coli* was investigated by Livage, et al.; Premkumar, et al.; Finnie, et al.; and Gill and Ballesteros [8, 30, 37, 40, 41]. These are some examples of the studies that have been done.

Throughout these studies a number of challenges remained. One challenge was that the porosity and mechanical properties of the gel needed to be improved, and pore shrinkage during aging, syneresis, needed to be reduced [2]. Common silica sol-gels had

pores that were orders of magnitude smaller than the cells causing diffusion to be extremely slow [27]. The viability of encapsulated cells was also a major challenge [3, 30, 39]. In order to increase the viability of the encapsulated cells, new gelation procedures would need to be developed that are milder and more stable. New procedures for cell encapsulation cannot be efficiently investigated without the careful studies of the changes in the metabolic activity of the cells and their interactions with the gel matrix [3].

Recently there has been a lot of investigations on the encapsulation of cells in sol-gels. Many of these investigations have attempted to understand cell behavior better in order to develop improved sol-gel protocols. In fact, there have been a number of papers that have used sol-gel encapsulation to study the effects of matrix stiffness on tumors and cancer cell lines. For example, Liu, et al., studied the growth of tumorigenic cells in fibrin gels of varying stiffness [42]. Pathak, et al., studied how the matrix stiffness affected tumor cell migration [43]. Ananthanarayanan, et al., studied the relation of gel stiffness to the metastasis of malignant brain tumors [44].

Many investigations have shown that the gel stiffness influences cell behavior in 2D, but little is known about these interactions in 3D [45]. Recently, there have been a few studies on these effects in 3D. Pek, et al., developed a 3-D thixotropic gel in which cells could be cultured without the use of trypsin, and the effect of matrix stiffness on mesenchymal stem cells could be investigated [46, 47]. Peyton, et al., has studied the influence of matrix stiffness on smooth muscle cells in 3D; however, the results were complicated by gel degradation from cell reorganization and further studies were required

[45, 48]. Therefore, there remains a need for further investigation on the effects of matrix stiffness on cell behavior in 3D.

There have also been many studies on new protocols for sol-gel encapsulation throughout its development that are less harmful to cells. These changes in include neutralizing the sol before the cells are added, evaporating the alcohol, using aqueous precursors, and the Biosil method. These were described in the gel chemistry section. Despite these changes, maintaining the viability of the cells is still a major challenge and requires more investigation [4]. There are also no known studies on cell extraction for silica sol-gels. As a result, there is a need for the investigation of the interaction between cells and the gel matrices in 3D so that new gel protocols that maintain cell viability and allow for cell extraction can be developed.

1.4 Experimental Overview

The experiments were meant to address the current needs in sol-gel cell encapsulation: to maximize the time that cells are viable within the gel and the quantity of cells that can be extracted from the gel and continue to proliferate, viable cells. The studies were performed on 3D gels since 2D gels have been thoroughly investigated and 3D gels better mimic the natural environment of the cells. Human Foreskin Fibroblast (HFF) cells were used for all the experiments because they are easy to work with and inexpensive to culture. However, performing all of the experiments with one cell line may be limiting since gel protocols generally are not universal. Also, understanding the behavior of HFF cells and the developing sol-gel protocols that work specifically for HFF cells would probably not have as many applications as some other cell lines.

Long-term cell viability during encapsulation and after extraction has not been a focus of many investigations. However, there have been a few investigations on the growth and cell migration of tumors showing that human glioma cells spread and proliferate more in stiffer gels and non-metastatic murine mammary carcinoma cells undergo apoptosis in high solid stresses [44, 49]. It has been demonstrated that the way in which cell behavior, morphology and migration, is affected by gel stiffness varies based on cell type [50]. This would suggest that cell viability during encapsulation and after extraction would depend on cell type as well.

Intuitively, gel chemistry and structure would affect the way in which the cells react to changes in the mechanical stiffness of the gel. From 2D culture, it can be seen that cells generally spread, proliferate, and are viable on very stiff surfaces (polystyrene). This is expected because the stiffness allows the cells to easily attach to the surface and the scaffold does not apply stress on the cell. In 3D the dynamics are quite different. It is possible that a gel could be very stiff and have large pores that allow cells to attach and proliferate without applying stress to them. However, most silica sol-gels are stiff matrices with pores that are much smaller than the encapsulated cells and do not allow cells to proliferate [51]. Additionally, the silica sol-gels generally shrink during the aging process, syneresis [29]. In such a gel, the cell would not be able to attach to anything, and the gel would start to place stress on the cell during syneresis or proliferation attempts. In such a scenario, a stiffer gel would apply more stress to a cell and possibly induce apoptosis or necrosis. Therefore, it is hypothesized that the length of time the cells are

metabolically active (relates to cell viability), and the quantity of viable cells that can be extracted from the gel decrease as the stiffness of the gel increases.

In order to test this, the concentration of one of the gel ingredients was modified and the following items were measured: the mechanical stiffness of the gel, the metabolic activity of the cells encapsulated in the gel, and the quantity of viable cells extracted from the gel. The measurements were taken through rheological studies, metabolic activity studies, and extraction studies as explained in Chapter 2.

1.5 Gel Formulations

1.5.1 SNP-PEG Gels

Three different gels were studied. The first gel studied was a silica sol-gel that was created using tetrakis(2-hydroxyethoxy)silane (THEOS), culture media, silica nanoparticles, and in some instances PEG. These gels will be referred to as silica nanoparticle PEG gels or SNP-PEG gels. The metabolic activity of the cells encapsulated in gel and the stiffness of the gels was found for a number of PEG concentrations. The amount of each of the gel ingredients for the formulations used in the metabolic activity studies can be seen in Table 1.

Table 1: *SNP-PEG gel formulations given in volume percentages*

Name	THEOS (v/v %)	NexSil 125 (w/w %)	PEG (v/v %)	Media (v/v %)
N0	9.1	0.42	0.0	90.9
N2.3	9.1	0.39	2.3	88.6
N4.5	9.1	0.37	4.5	86.4
N9.1	9.1	0.33	9.1	81.8
N13.6	9.1	0.29	13.6	77.3
N15.9	9.1	0.27	15.9	75.0

As noted previously, the sol-gel process, although relatively mild, can be harmful to encapsulated cells. A number of variations have been made to the traditional silica sol-gel and sol-gel process to reduce these effects. These procedural variations have successfully increased the viability of encapsulated cells, but cell viability remains as a major challenge in cell encapsulation.

The new silica precursor, THEOS, is completely water soluble [52, 53]. As a result, there is no need to add organic solvents like alcohols which are commonly used with TEOS and TMOS [52]. Additionally, THEOS gels produce ethylene glycol as a by-product rather than alcohol [53]. The ethylene glycol is believed to be compatible with the cells. Due to these reasons, THEOS was the precursor of choice for this study. It was combined with silica nanoparticles and culture media, the solvent. The sol did not require any further catalyst to gel, so that the gelation process occurs at a mild pH. In fact, gelation occurred within minutes.

There are a few disadvantages to the SNP-PEG gels as well. First of all, the stress that the gel exerts on the cell as a result of syneresis during the aging process has not been fully addressed. Secondly, silica and silica nanoparticles have been shown to cause cell membrane damage and possible apoptosis or necrosis at high doses. Small doses appear to be harmless to the cells, but the reactions to silica vary based on the cell line [54]. The addition of PEG may reduce these effects because it increases cell viability by either reducing the syneresis of the gel or reducing the effects of the silica on the cells [34].

Another disadvantage is that cells generally cannot proliferate in silica gels due to their rigid structures and small pores [51]. Consequently, the cells may become

mechanically stressed when they attempt to proliferate. It is possible that the disruption in proliferation induces a major change in cell behavior and activity when they prevent because it generally does not occur in their natural environments [29].

Additionally, the SNP-PEG gels have covalent bonds that cannot be broken easily. This would make it difficult to extract the cells from the gel without applying too much stress and killing them. Also, the metabolic activity of the cells was greatly reduced after being encapsulated 24 hours in the SNP-PEG gels leaving a small window of time in which the cells could be extracted after encapsulation. Therefore, the SNP-PEG gels would be a poor choice for cell extraction. Consequently, the extraction studies were not performed on the SNP-PEG gels.

Despite the disadvantages of these gels, they do solve extreme pH condition and alcohol by-product problems without requiring lengthy and complicated protocols. Organic polymers have been used to tune the mechanical properties of gels [34]. In this study, the PEG concentration was changed in order to change the mechanical properties of the gel. Since silica gels are rigid and PEG is an elastic material, it was hypothesized that increasing the PEG concentration would soften the gel.

1.5.2 Particulated SNP-PEG Gels

The second gel studied, consisted of particulating (mixing) two of the gel formulations from the SNP-PEG gels prior to cell encapsulation. The particulation was accomplished through sonication. A sonicator is a device that causes a probe, which is generally placed into a liquid sample, to vibrate at high frequencies and agitate the sample. These gels will be called Particulated SNP-PEG gels. The metabolic activity of

the encapsulated cells, the stiffness of the gel, and the number or viable cells that could be extracted from the gel, and the proliferation rate of the extracted cells were found for the two different particulated gels that were investigated. Particulationof the gels was done to break up the covalent bonds and make the extraction process easier.

Since a lot of the differences in metabolic activity between the different SNP-PEG gel formulations were not statistically significant, only N0 and N4.5 were particulated and studied. These gel formulations were chosen because they represent the total range of metabolic activity found in the SNP-PEG gels, and would probably be representative of the range of metabolic activity that would be found in the Particulated SNP-PEG gels. The rheological studies, metabolic activity studies, and extraction studies were all performed on the Particulated SNP-PEG gels to see how the cells were affected by simply particulating the gel and to find out how many viable cells could be extracted from them.

Since the Particulated SNP-PEG gels have the same ingredients as the SNP-PEG gels, some of the advantages and disadvantages are the same. For example, these gels have a mild pH and they do not produce alcohol by-products. However, they have some additional advantages over the SNP-PEG gels. The cells are not exposed to the potentially harmful hydrolysis and condensation reactions that occur during gelation. The particulation of the gel disrupts the aging process of the gel and the resulting syneresis. The process of breaking the covalent bonds in the gel may also reduce the stress on the cells from proliferation attempts and allow the viable cells to be extracted from the gel.

On the other hand, there are still silica and silica nanoparticle interactions that could be harmful to the cells.

These gels appeared to have a lot of advantages, and it was hypothesized that the cells would be metabolically active for a longer period of time than the SNP-PEG gels. These gels were also expected to follow the same or similar trends to the SNP-PEG gels since they had the same ingredients. Therefore, the addition of PEG was expected to increase the time period that the cells were metabolically active and to weaken the gel.

1.5.3 FS-PEG Gels

The third gel studied, was a thixotropic silica sol-gel. Thixotropic means that light titration turned the gel into a liquid and the removal of the shear stress resulted in the gel quickly solidifying again. This would allow the cells to be easily mixed into the gel when it was in a liquid state and be encapsulated quickly when the shear stress was removed [46]. It was created using TEOS, fumed silica (FS), acetic acid, PEG, ammonium hydroxide, and culture media. These gels will be referred to as the FS-PEG gels. They were modified from the protocol developed by Pek, et al. [46]. The metabolic activity of the encapsulated cells, the stiffness of the gel, and the number of viable cells that could be extracted from the gels were found. The ingredient concentrations of the different FS-PEG gel formulations can be seen in Table 2.

Table 2: *FS-PEG gel formulations*

Name	TEOS: Acetic Acid (v:v)	TEOS-Acetic Acid: PEG-Cabosil (v:v)	Cabosil: PEG (w/w %)
F5	1:9	3:5	5
F7.5	1:9	3:5	7.5
F10	1:9	3:5	10

The Particulated SNP-PEG gels had good metabolic activity, but a lot of cells were lost during the extraction process. Pek, et al., used the thixotropic silica-PEG gel for cell culture in a recent study. They were able to culture mesenchymal stem cells in the gel for over one week and rat hepatic stellate and murine calvarial osteoblastic cells in the gel for over three weeks. These gels allowed the cells to proliferate and be passaged without the use of Trypsin, which can have harmful long term effects on cells [46]. The group also used this gel to study how mesenchymal stem cells were affected by matrix stiffness [47].

These gels do have some disadvantages as well. These gels produce alcohol as a by-product. However, the particulation process includes rinsing of the gel in an attempt to minimize the amount of alcohol in the gel before the cells are incorporated. The process is also lengthy; it takes a few days to prepare them.

Since there are many advantages to using these gels and they have been successful in other studies, it was hypothesized that the encapsulation and extraction of cells from these gels would be more successful than the SNP-PEG and Particulated SNP-PEG gels due to its thixotropic nature. The rheological studies performed by Pek, et al. showed that the storage modulus increased with increasing amounts of Cabosil. This is expected because the Cabosil increases the number of silanol groups and thus advocates van der Waals forces or hydrogen bonding [46]. Therefore, it was expected that the modified gels used in these experiments would also have a storage modulus that increases with increasing Cabosil concentrations. In order to test these hypotheses, the rheological, metabolic activity, and extraction studies were performed on modified versions of the gel.

Chapter 2: Materials and Methods

2.1 General Materials and Methods

2.1.1 Cell Culture

HFF cells were obtained from the American Type Cell Collection (Manasas, VA). The cells were cultured in DMEM (Invitrogen Co.; Carlsbad, CA) supplemented with 15% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Mediatech Inc.; Herndon, VA). They were incubated at 37°C with 5% CO_2. For cell passaging, the cells were washed twice with phosphate buffered saline (PBS), and trypsinized for 6 minutes in the incubator with 0.25% trypsin-EDTA. Once the cells detached, the trypsin was neutralized by adding twice the amount of culture media, the solution was centrifuged for 5 minutes at 800 rpm, the culture media was aspirated, and the cells were resuspended in new culture media. Then, the desired ratio of cells was seeded into a flask with culture media.

2.1.2 Rheological Studies

The rheology tests were performed on an AR-G2 Rheometer from TA instruments (North Castle, DE). A conical plate with a 40 mm diameter, a 2º incline, and a truncation gap of 50 μm was used in conjunction with a Peltier plate. A cone and plate geometry was used to keep the strain constant throughout the sample. The tests were run at 25ºC. In order to prevent the gels from drying during the tests, 1.3 mL of distilled water was placed inside the solvent well, and the solvent trap covers were used throughout. Figure 2 shows the rheology setup.

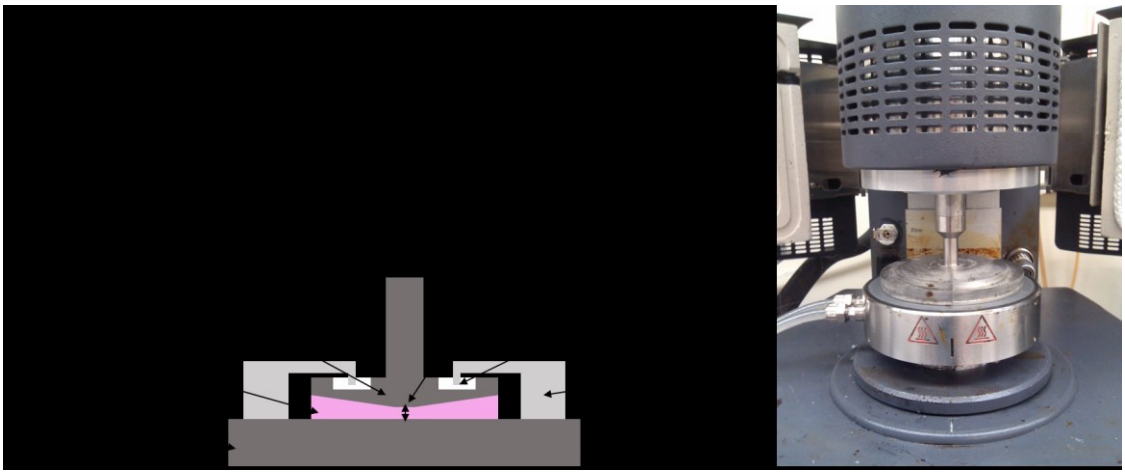

Figure 2. *Rheology setup*

There are three main types of materials: elastic solids, viscous fluids, and viscoelastic materials. Silica sol-gels are viscoelastic materials. Viscoelastic materials exhibit properties of both elastic solids and viscous fluids. When an elastic solid is strained, the stress on it is proportional to the strain. Both the strain rate and the amount of strain that was applied to the material at a previous point in time do not matter. The stress on a viscous fluid depends on the strain rate. Similar to the elastic solid, both the amount of strain and the strain rate applied to the fluid at a previous point in time do not matter. Conversely, the stress depends on the amount of strain applied, the strain rate, and the history of the applied strains in a viscoelastic material.

The complexity of viscoelastic materials makes their characterization more difficult. There are numerous models that combine properties of elastic solids and viscous fluids in different ways to determine parameters of a material and predict its behavior. However, there is a way to characterize such materials using energy and energy storage terms of cyclic loading results. The storage modulus, G', is the amount of stress necessary to shear the elastic portion of the material by 1% strain. The loss modulus, G'', is the amount of

stress necessary to shear the viscous portion of the gel by 1% strain. Since the storage modulus relates to the elastic portion of the gel, it was used as a measure of the gel stiffness and was determined from the rheology tests.

2.1.3 Metabolic Activity Studies

A 96 well plate was used for the metabolic activity studies. There are 8 rows and 12 columns in a 96 well plate. Two columns were used for each experimental condition. Every experiment had live cells in media, dead cells in media, live cells in a gel, and dead cells in a gel. Some experiments had live cells and/or dead cells in more than one gel formulation and some used more than one plate. The cells were killed by incubating them in 70% Ethanol for 30 minutes. A live-dead fluorescence assay containing Hoechst (H) and Propidium Iodide (PI), which were obtained from Sigma-Aldrich Corp. (St. Louis, MO), was used in conjunction with a Nikon T200 microscope (Nikon Instruments Inc.; Melville, NY) to make sure that the cells were dead.

Each row represents a time point, which was either 12 or 24 hours. An appropriate amount of an alamarBlue® Assay (Invitrogen) was added to one row at each time point. The assay turned pink if it was metabolized by the cells, otherwise it remained blue. The fluorescence, which can be correlated to metabolic activity, was determined by a Gemini EM microplate spectrofluorometer (Molecular Devices, LLC; Sunnyvale, CA). An excitation of 540 nm and an emission of 600 nm were used for the experiments. Figure 3 shows how the 96 well plate is organized for the metabolic activity studies, the dye color for live and dead cells, and a scanning electron micrograph of an encapsulated cell.

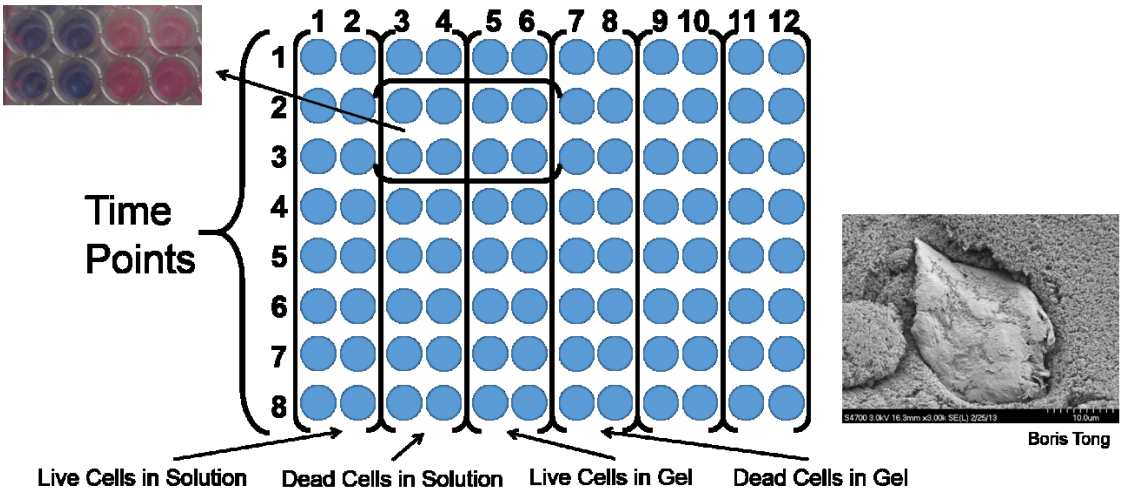

Figure 3. *Metabolic activity setup*

Each plate was read three times and each experiment was performed three times. For each well in a given experiment, the three different read values were averaged. The average and standard deviation was then found at each time point and experimental condition from the averages of the three read values.

Since each experiment was performed at different points in time and with different cells, sometimes the fluorescence of the live cells in solution varied much more significantly between experiments than within the experiments. In order to compare the fluorescence between different experiments, all of the fluorescence values within an experiment were normalized with respect to the average value of the live cells in solution over the entire experiment. This was done before the averages and standard deviations were found.

2.1.4 Extraction Studies

For the extraction studies, all three experimental trials were performed at the same time with the same batch of gel and different flasks of cells. There was one well for a

particular condition and time point for each flask. Five time points of one day were used to determine the proliferation rate of the extracted cells. The first of which was also used to determine the percent recovery of cells.

The cells were encapsulated in the desired gel, extracted 24 hours later, and seeded in a petri dish with 8 mL of culture media. Then, the cell density was found in each petri dish at the given time point. The first time point was one day after the cells were extracted. The cell passage procedure was followed through centrifugation, then all but 1 mL of the solution was pipetted off. The remaining solution and cells were mixed thoroughly and centrifuged a second time. Most of the culture media was pipetted off, the cells were mixed thoroughly with the remaining media, the volume of the solution was measured, and the cell density was found with a hemocytometer. The amount of cells in each petri dish was calculated using the cell density and the dilution volume. An overview of the extraction study setup and methods can be seen in Figure 4.

The average of the three flasks was found each day and plotted against time. A trend line of the plot was used to find the rate at which the cells were proliferating. This rate was compared to the rate at which free cells proliferated when 2,000; 5,000; and 10,000 cells were seeded in a petri dish. There were three flasks of cells and five time points of one day used for all three seed amounts.

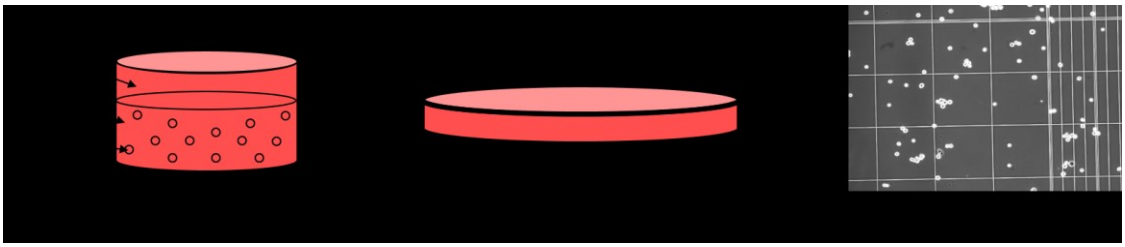

Figure 4. *Extraction setup and method*

In order to determine the percentage of cells recovered, the number of cells from day one was divided by the amount of cells that were encapsulated in each well. The amount of cells encapsulated in each well was determined by the encapsulation density and the volume of cells that went into each well. The cells were encapsulated in the same manner as the metabolic activity studies for that gel, but at a density five times greater so a more accurate cell count could be obtained.

2.2 SNP-PEG Gel Materials and Methods

2.2.1 Rheology

A Time Sweep was used to find the storage modulus of the SNP-PEG gels. Since these gels synthesize very quickly, the gels were prepared mostly on ice and the Peltier plate was kept at 5°C until the test was started. The low temperatures caused the reaction rates to slow down and prevented condensation reactions before the test was started. The strain was kept at a constant 0.1% and the rotational velocity was kept at a constant 1 rad/s. The tests were run for differing lengths of time between 20 minutes and 4 hours. An example of a Time Sweep test for a SNP-PEG gel can be seen in Figure 5.

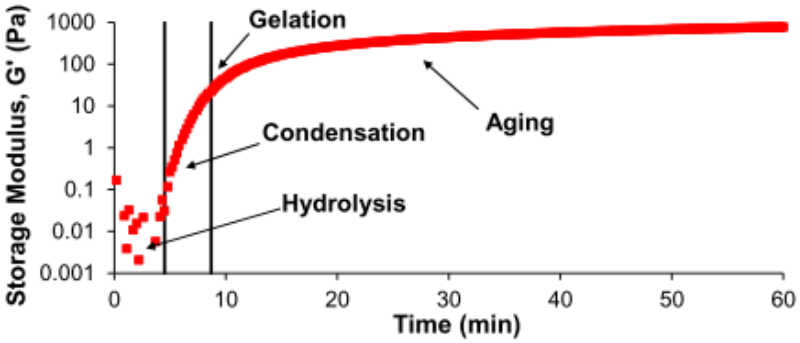

Figure 5. *Time Sweep Test of N13.6 – Storage Modulus vs. Time*

In order to prepare the gel, 20 μL of 85 nm silica nanoparticles was mixed with 1 mL of human prostate carcinoma cells (LNCaP) culture media. The sol was kept in a 4°C refrigerator until it was ready to be used. The silica nanoparticles that were used were Nexsil^{TM} 125-40 Silica Sol washed three times with ultra pure water. They were obtained from NYACOL Nano Technologies, Inc. (Ashland, MA). The LNCaP culture media consisted of DMEM/F12 supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 10^{-9} M of Dihydrotestosterone.

Separately, aliquots of 60 μL of THEOS (Gelest Inc.; Morrisville, PA) and the appropriate amount of 4-Arm 2 kDa PEG-OH (PEG) were made. The PEG was obtained from Creative PEGWorks (Winston Salem, NC). For gel synthesis, the THEOS was put on ice while the silica-media sol was vortexed for 30 seconds. Then, appropriate amounts of silica-media sol and PEG were mixed together. The mixture was vortexed for two minutes and then put on ice for three minutes. After the three minutes, 300 μL of the sol was added to the THEOS and pipetted up and down seven times before 300 μL of LNCaP culture media was added and the entire mixture was vortexed for 10 seconds.

Then, 585 μL of gel was pipetted onto the Peltier plate, the conical plate was lowered from 1,500 to 50 μm, and the test was started immediately. For gels with 9.1% PEG, there was a 30 second equilibration time and a short wait for the plate to reach 25°C which is not believed to have affected the tests in any way. The final PEG concentrations were 0, 2.3, 4.5, 6.8, 9.1, 11.4, 13.6, 15, and 15.9% (v/v).

In order to plot and analyze how the storage modulus changes with PEG concentration, the storage modulus at a few time points were taken from each trial. The

average and standard deviations of the three trials was found for each time point and gel formulation. The chosen time points were 20 minutes, 60 minutes, and 240 minutes. These time points were chosen to see if the way in which the storage modulus varied with PEG concentration changed over time. It should be noted that not all gel formulations were performed for all the time points and at most one trial was performed at the 240 minute time point for all gel formulations.

2.2.2 Metabolic Activity

First, 20 μL of 85 nm silica nanoparticles was mixed with 1 mL of culture media. Then, PEG was mixed with the silica-media sol at volume percentages of 0, 5, 10, 20, 30, and 35%. Aliquots of 8 μL of THEOS were made. The cells were suspended in media at a density of 500,000 cells/mL. The gel was synthesized by adding 40 μL of the silica-media-PEG sol to a THEOS aliquot, quickly pipetting up and down a couple times, and vortexing for 10 seconds. The cells were encapsulated by adding 40 μL of cells suspended in culture media and carefully pipetting up and down a few times. Then, 42 μL of cells encapsulated in gel was pipetted into each well. After at least five minutes, 50 μL of culture media was placed on top of each gel. There should be 10,000 cells in each well. The final PEG concentration in the gel was 0, 2.3, 4.5, 9.1, 13.6, and 15.9% (v/v). The cells generally did not live for more than two days within the SNP-PEG gels, so 12 hour time points were used for the experiments.

2.3 Particulated SNP-PEG Gel Materials and Methods

2.3.1 Gel Synthesis

The gel was prepared by mixing 20 μL of 85 nm silica nanoparticles with 1 mL of culture media. Then, PEG was mixed with the silica-media sol at volume percentages of 0 and 10% producing final PEG concentrations in the gel of 0 and 4.5%. Aliquots of 43.5 μL of THEOS were made. Then, 220 μL of the silica-PEG-media sol was added to each aliquot of THEOS and quickly pipetted up and down a couple times before vortexing it, adding 220 μL of culture media, and vortexing it again. The samples were allowed to gel for 15 minutes after they were mixed. Afterward, culture media was mixed with the samples and they were combined for sonication. The gel was then sonicated at 30% for 5 minutes and centrifuged at 1500 rpm for 5 minutes.

2.3.2 Rheology

The gels used for the rheology tests were synthesized in the manner described in the previous section. Then, aliquots of 464 μL of gel were made and 340 μL of culture media was gently mixed into the gel. After twenty minutes, 400 μL of culture media was gently added on top of the gel. The gel was then put in the 37°C, 5% CO_2 incubator for three days. The excess media was pipetted off the top and the rheology tests were performed after the three days had passed.

A Strain Sweep was used to find the storage of the gels. In order to perform the Strain Sweep test, 650 μL of gel was pipetted onto the Peltier plate, the conical plate was lowered from 1,500 μm to 50 μm, and the test was started after a 10 minute equilibration

time. The strain was varied from 0.05 to 125% during the test and the rotational velocity was kept at a constant 1 rad/s. An example of a Strain Sweep test can be seen in Figure 6.

The average of the first 13 storage modulus values, from 0.05 to 50% strain, was taken from each trial. These values were chosen because the gel was within the linear elastic region. An average and standard deviation was taken of the average storage modulus values found for three different trials. These values were used to analyze the statistical significance of the differences between the gel formulations and create a plot of the storage modulus as a function of the PEG concentration.

2.3.3 Encapsulation

The gel was made in the manner described in the gel synthesis section. Then, aliquots of 55 μL of gel were made. The cells were suspended in culture media at a density of 500,000 cells/mL for metabolic activity studies and 2,500,000 cells/mL for extraction studies. The bottom of the wells were coated with 18-20 μL of the corresponding SNP-PEG gel. Then, 40 μL of suspended cells were carefully mixed with 55 μL of gel and 42 μL of cells encapsulated in gel was put into two wells. After at least 5 minutes, 50 μL of media was gently placed on top of each gel. Each well should contain 10,000 cells in the metabolic activity studies and 50,000 cells in the extraction studies.

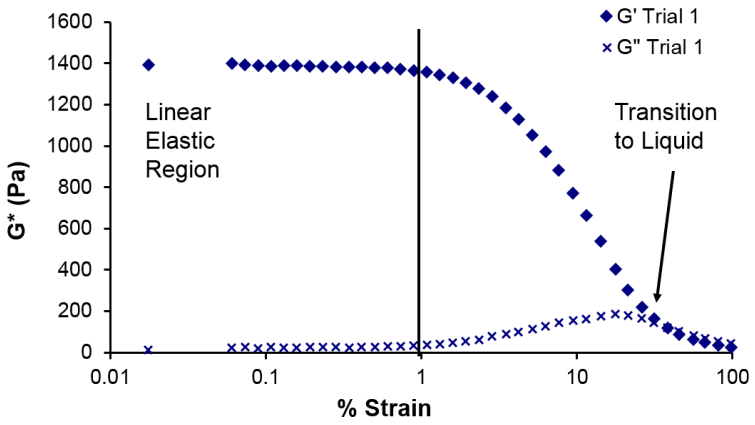

Figure 6. *Strain Sweep Test of F10 – Storage Modulus vs. % Strain*

2.3.4 Extraction

In order to extract the cells, 100 µL of 0.25% Trypsin-EDTA was added to each well and the 96 well plate was put on the orbital shaker for 10 minutes at 150 rpm. Then, 50 µL of media was added to each well to neutralize the Trypsin. The gel was carefully pipetted up and down and stirred two to three times. The well was rinsed out with 100 µL of culture media. The cells were seeded in a petri dish with 8 mL of culture media.

2.4 FS-PEG Gel Materials and Methods

2.4.1 Gel Synthesis

TEOS (Sigma-Aldrich) and 0.5 M acetic acid solution were combined at a volume ratio of 1:9 (TEOS: acetic acid). The solution was sonicated on ice for 30 minutes at 30% and then kept in a 4ºC refrigerator until it was ready to be used. Separately, Cabosil M5 (Cabosil) was added to the PEG based on weight percentages of the amount of PEG. Cabosil is a specific brand of fumed silica that was obtained from Sigma-Aldrich. The TEOS-acetic acid solution was added to the Cabosil-PEG solution at volume ratios of 3:5 (TEOS-acetic acid: PEG-Cabosil) and vortexed for 13-15 minutes. The final pH of the

gel was adjusted to 7 by adding 0.5 M ammonium hydroxide solution. The gel was allowed to age for 70 hours before it was particulated. In order to particulate the gel, culture media was mixed with it, it was sonicated at 30% for 5 minutes, and it was centrifuged for 5 minutes at 1500 rpm. This process was performed two or three times based on the color of the culture media after centrifugation (until it was pink). For large quantities of gel, culture media was mixed with the gel before it was sonicated for a few minutes and divided into two or three batches. This was done to ensure that all of the gel was thoroughly mixed during sonication.

2.4.2 Rheology

The gel used for the rheology tests was synthesized in the manner that explained in the previous section. Then, aliquots of 700 μL of gel were made. The gel was vortexed for one minute and then 105 μL of culture media was gently mixed in with it. After 20 minutes, 400 μL of culture media was gently added on top of the gel. The gel was put into a 37°C, 5% CO_2 incubator for 3 days. Then, the excess media was pipetted off the top of the gel and the rheology tests were performed. The same rheology tests were performed in the same manner on the FS-PEG as the Particulated SNP-PEG gels.

2.4.3 Encapsulation

Aliquots of 500 μL of gel, which was prepared in the manner described in the gel synthesis section, were made. The cells were suspended in culture media at a density of 1,500,000 cells/mL for the metabolic activity studies and 7,700,000 cells/mL for the extraction studies. The gel was vortexed for one minute, which would liquefy the gel, then 75 μL of cells suspended in culture media was quickly and carefully mixed into the

gel and distributed 50 μL into each well. Each aliquot of gel produced enough gel for 6-8 wells. After at least five minutes, 50 μL of culture media was carefully placed on top of each gel. There should have been 10,000 cells/well for the metabolic activity studies and 50,000 cells/well for the extraction studies.

2.4.4 Extraction

In order to extract the cells, 50 μL of culture media was added the each well. The gel was pipetted carefully up and down and stirred two to three times with the pipette. Each well was rinsed out with 50 μL of culture media. The cells were seeded in a petri dish with 8 mL of culture media.

Chapter 3: Results

3.1 SNP-PEG Gel Results

3.1.1 Rheological Studies

It was hypothesized that increasing the PEG concentration in the SNP-PEG gels would decrease the storage modulus of the gel. Figure 7 shows the rheology results as a function of PEG concentration. The rheology results appeared to follow the hypothesized trend when PEG is initially added and for PEG concentrations between 9.1 and 13.6%. However, the gels with PEG concentrations below 9.1% did not follow the hypothesized trend. The low PEG concentrations did show evidence of phase separation. Phase separation occurs when the gel is separated into two domains, a silica (structure) domain and a solvent (macropore) domain, which come together to form one continuous gel. Water soluble additives, such as PEG, can induce phase separation. The silica to PEG ratio is the major parameter that determines the phase separation, and thus the macropore and structure size can be controlled by this ratio [55]. The resulting heterogeneity in the gel could explain the lower storage modulus values. There also seemed to be a transition in the gel between 13.6 and 15% PEG where the storage modulus stopped decreasing. Generally, the rheology results appeared to follow a trend, but not a strictly decreasing trend like was hypothesized.

A number of the gels used for the rheological studies were selected for metabolic activity studies. The gels that were selected can be seen in Table 1. These particular gels were chosen so the entire range of PEG concentrations was represented as well as the

various transitions within the gel. Figure 8 shows the rheology data at 20 and 60 minutes for the gel formulations used in the metabolic activity studies.

Most of the differences between the gel formulations used for the metabolic activity studies were not statistically significant (95% confidence, n=3) at 20 or 60 minutes due to the small sample size. At 20 and 60 minutes, the differences between the N9.1 and all of the other gels used for the metabolic activity studies were statistically significant. At 20 minutes, the difference between the N2.3 and N15.9 gel was statistically significant. Data was not collected at 60 minutes for the N15.9 gel, so it is not known as to whether or not the differences would be statistically significant at that time. Therefore, there was too much experimental uncertainty to determine a trend statistically between the storage modulus and the PEG concentration.

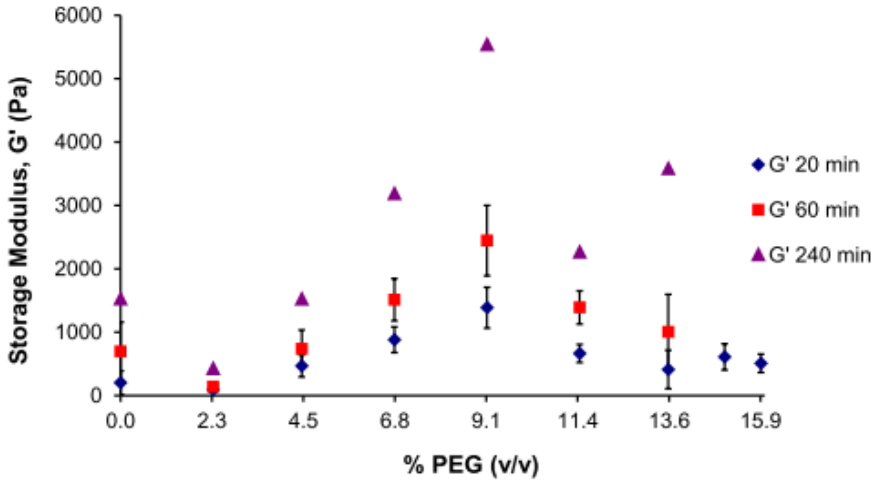

Figure 7*. Storage Modulus vs. PEG concentration (v/v %) for SNP-PEG gels*

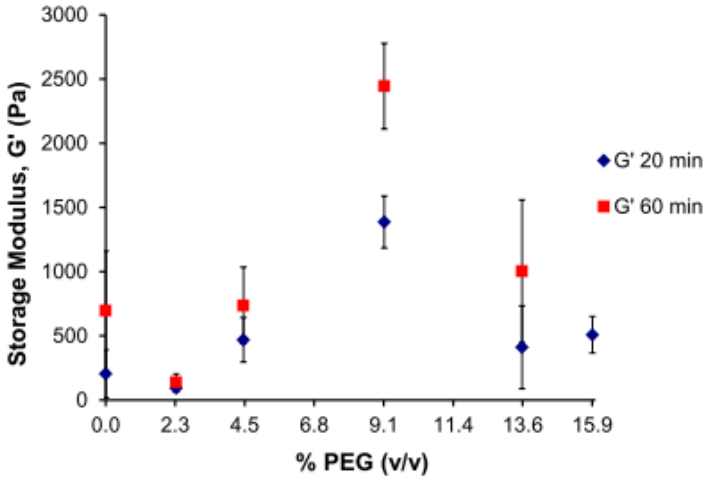

Figure 8. *Storage Modulus vs. PEG concentration (v/v %) at 20 and 60 minutes for the SNP-PEG gels used for the metabolic activity studies*

3.1.2 Metabolic Activity Studies

The hypothesis that the length of time the cells are metabolically active will decreases as the stiffness of the gel increases would suggest that the cells should die the fastest in N9.1 and the slowest in N2.3 and N0. However, this was not the case. Figure 9 shows the normalized metabolic activity (normalization method explained in Chapter 2) results of the SNP-PEG gels as a function of time. The plot appeared to have a similar shape to the rheology results with N4.5 as a peak instead of N9.1. Due to the variation involved in the experiments, only a few of the differences between the gel formulations were statistically significant with a 95% confidence level and n=6 (3 experiments each with 2 wells at every condition and time point, see Chapter 2). N0 was significantly lower than all of the other gels with the exception of N15.9 for the first two time points. At 12 and 24 hours, N4.5 and N9.1 were significantly higher than N13.6 and N15.9. Additionally, N4.5 was significantly higher than N2.3 at 24 hours. Therefore the gels with intermediate PEG concentrations, 2.3, 4.5, and 9.1%, generally were significantly higher (95% confidence)

than gels with extreme PEG concentrations, 0, 13.6, and 15.9%. A more detailed trend could not be determined due to the experimental variation.

3.1.3 Metabolic Activity vs. Rheology

Figure 10 shows the normalized metabolic activity as a function of the storage modulus. There was no apparent correlation between the metabolic activity and the storage modulus. The intermediate PEG concentrations (N2.3, N4.5, and N9.1) appeared to have relatively equal metabolic activity values over a wide range of storage modulus values. The extreme PEG concentrations (N0, N13.6, and N15.9) appeared to have lower metabolic activity values despite having storage modulus values within the range covered by the intermediate PEG concentrations. Therefore, there did not appear to be a correlation between the metabolic activity and the storage modulus. Rather, the PEG concentration appeared to affect both parameters. However, there was too much experimental variation to have statistically significant differences in values between the different PEG concentrations to be certain.

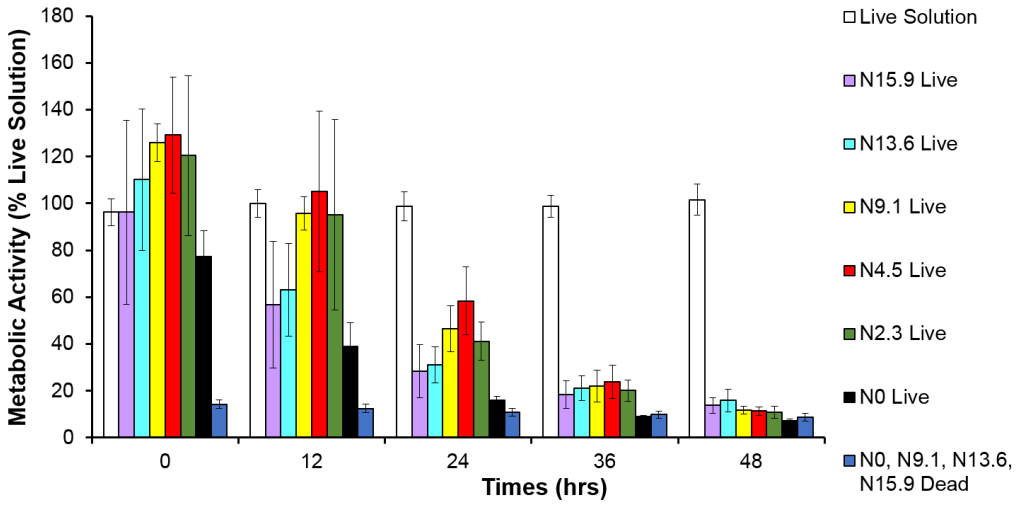

Figure 9. *Normalized Metabolic Activity vs. Time for SNP-PEG gels*

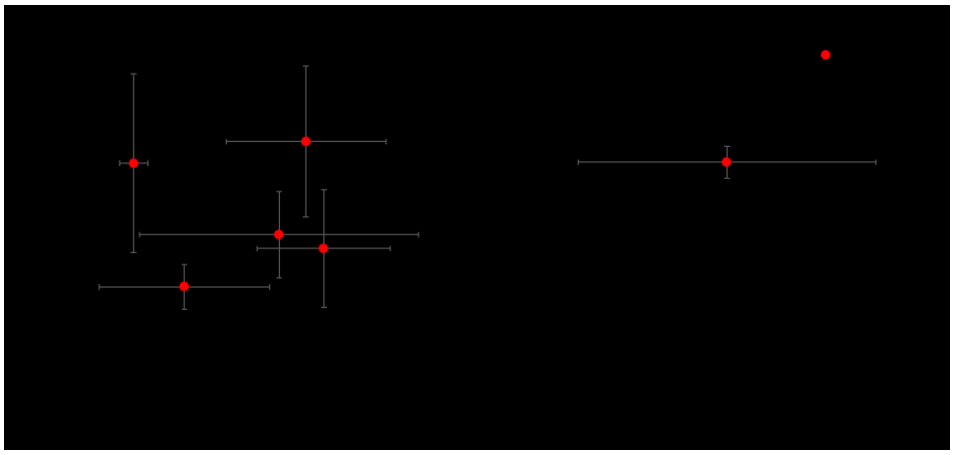

Figure 10. *Normalized Metabolic Activity at 12 hours vs. Storage Modulus at 20 minutes for SNP-PEG gels shown with error bars for both the metabolic activity and storage modulus*

3.2 Particulated SNP-PEG Results

3.2.1 Rheological Studies

The rheology results from the Particulated SNP-PEG gels are shown in Figure 11. The Particulated N0 gel tended to be heterogeneous after particulation. As a result, there was too much variation in the Particulated N0 gel to determine any sort of trend. The differences between the two gels were not statistically significant (95% confidence, n=3).

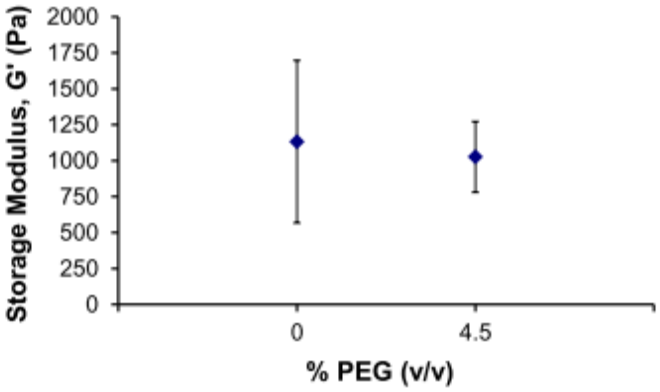

Figure 11. *Storage Modulus vs. PEG concentration (v/v %) for the Particulated SNP-PEG gels*

3.2.2 Metabolic Activity Studies

The normalized metabolic activity is plotted as a function of time in Figure 12. The cells in the Particulated N4.5 gel were metabolically active longer than the cells in the Particulated N0 gel. The cells still had over 50% metabolic activity after 6 days in the Particulated N4.5 gel, whereas they stopped being metabolically active after 4 days in the Particulated N0 gel. The difference between the two gels was statistically significant for all time points except day 2 with a 95% confidence level and n=6, and for all time points after day 2 with a 99% confidence level and n=6. Therefore, there was a significant difference in the metabolic activity of the two gel formulations.

3.2.3 Metabolic Activity vs. Rheology

Figure 13 shows the normalized metabolic activity as a function of storage modulus. The metabolic did seem to increase as the storage modulus decreased. However, the variation in the storage modulus of the Particulated N0 gel was too large to distinguish between the storage modulus of the two gel formulations. This can be seen in Figure 13 which includes the error bars from both the metabolic activity and the storage modulus measurements. There was a significant difference in metabolic activity between the two gels, but not between the storage moduli. Consequently, the storage modulus did not appear to have an effect on the metabolic activity. The PEG concentration appeared to be the only parameter affecting the metabolic activity.

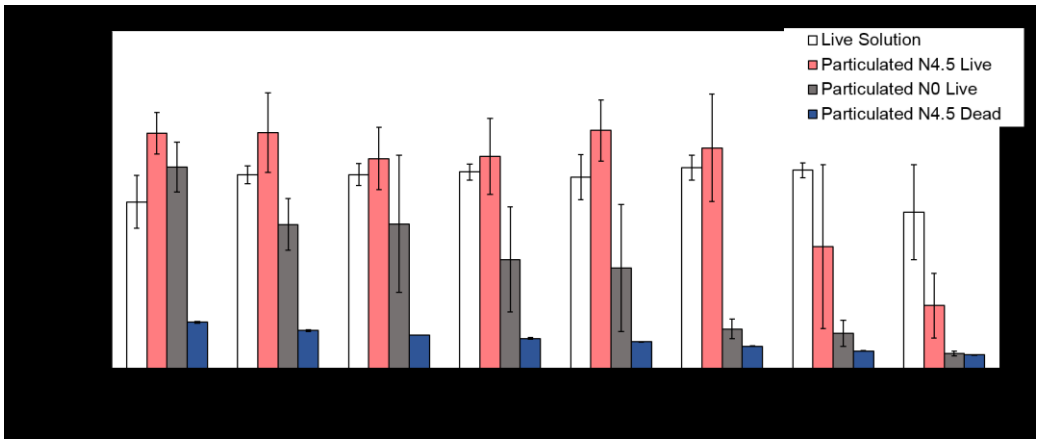

Figure 12. *Normalized Metabolic Activity vs. Time of the Particulated SNP-PEG gels*

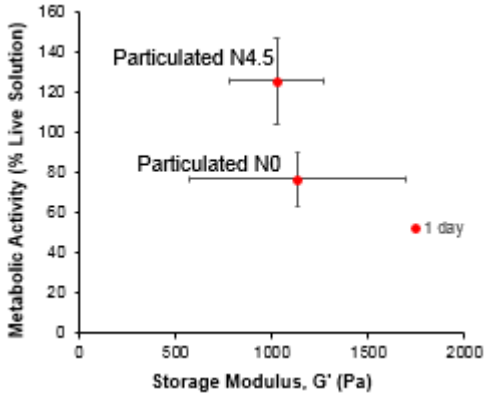

Figure 13. *Normalized Metabolic Activity at 1 day vs. Storage Modulus for the Particulated SNP-PEG gels shown with error bars shown for both the metabolic activity and the Storage Modulus*

3.2.4 Extraction Studies

Figure 14 shows the percentage of cells that were recovered from extraction. This figure shows that 55% of cells were recovered from the Particulated N4.5 gel and 14% of cells were recovered from Particulated N0 gel. The difference between these values was statistically significant (95% confidence, n=3). Figure 14 shows the number of cells in each petri dish as a function of days after extraction. The trend line equations and R^2 values from Figure 14 can be seen in Table 3. It can be seen from the trend line equations

that the proliferation rate of the extracted cells from both gels was about the same as the proliferation rate of the cells that were never encapsulated.

3.3 FS-PEG Gel Results

3.3.1 Rheological Studies

It was hypothesized that the stiffness of the gel, storage modulus, would increase with increasing amount of Cabosil. The experimental results from the rheological studies can be seen in Figure 15. Indeed, the storage modulus appeared to increase with increasing Cabosil. However, the differences were not statistically significant (95% confidence, n=3).

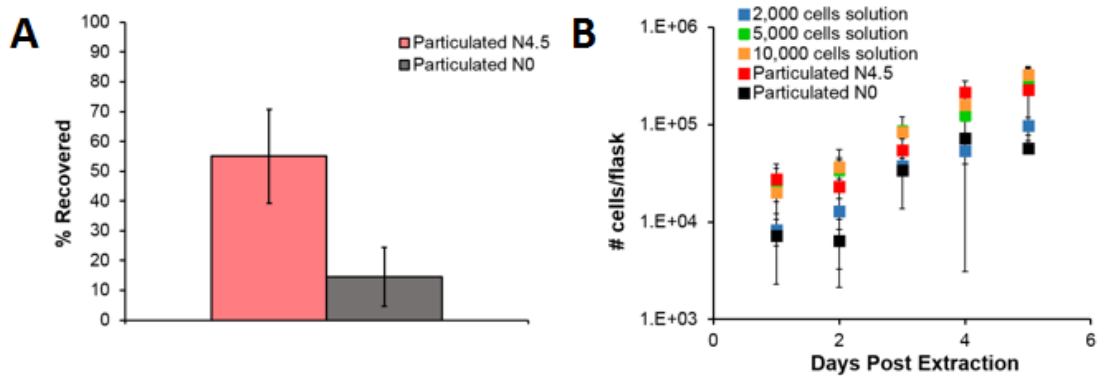

Figure 14. *Extraction Study results for Particulated SNP-PEG gels:*
A. *Percentage of cells recovered from extraction* ***B.*** *Number of cells per flask vs. Days post extraction*

Table 3: *Trend line Information from Figure 14B*

	Trend line Equation	R^2
2,000 cells solution	y = 4,300exp(0.64x)	0.98
5,000 cells solution	y = 11,000exp(0.64x)	0.98
10,000 cells solution	y = 9,600exp(0.70)	1.00
Particulated N4.5	y = 10,000exp(0.64x)	0.87
Particulated N0	y = 3,200exp(0.66x)	0.82

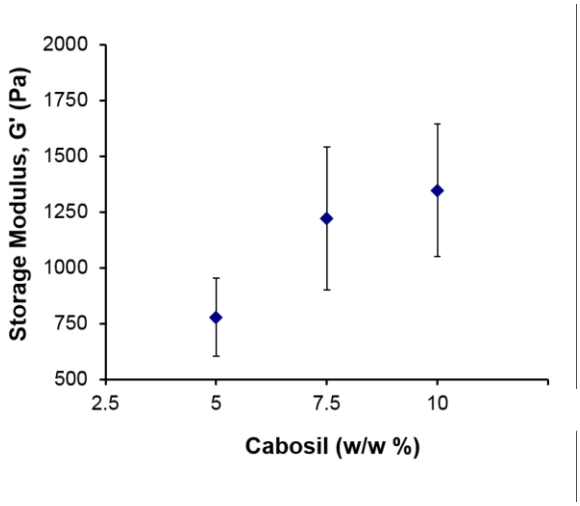

Figure 15. *Storage Modulus vs. Cabosil concentration as a weight percentage for FS-PEG gels*

3.3.2 Metabolic Activity Studies

The hypothesis that the length of time that the cells are metabolically active inside the gel and the quantity of viable cells that can be extracted from the gel increases as the storage modulus decreases suggests that the F5 gel should be metabolically active the longest, then the F7.5 gel, and finally the F10 gel (based on the rheology results). The metabolic activity study results can be seen in Figure 16. After the first day, the cells seemed to follow the hypothesized trend. However, the differences between the metabolic activities of the gel formulations were not statistically significant (95% confidence, n=6).

3.3.3 Metabolic Activity vs. Rheology

Figure 17 shows the normalized metabolic activity of the FS-PEG gels as a function of storage modulus. The metabolic activity decreased slightly as the storage modulus increased, which agreed with the hypothesis. However, the differences between the gel formulations for both the metabolic activity and the storage modulus were not statistically significant (95% confidence).

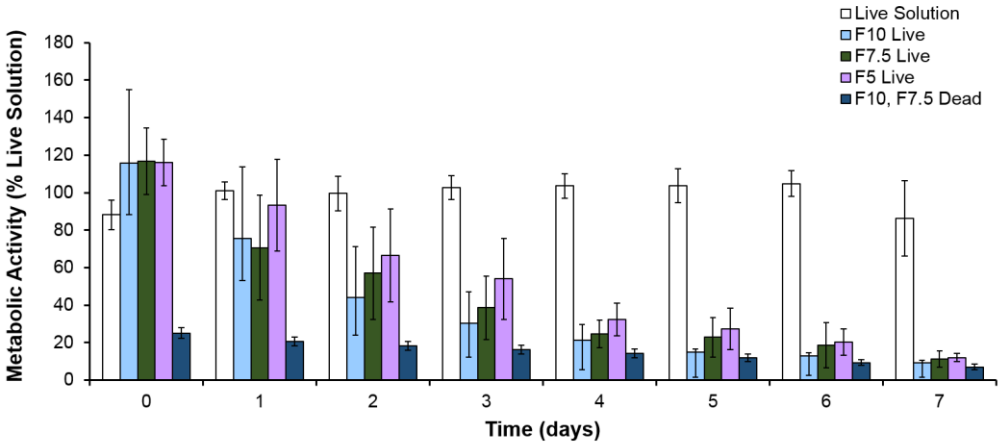

Figure 16. *Normalized Metabolic Activity vs. Time for the FS-PEG gel formulations*

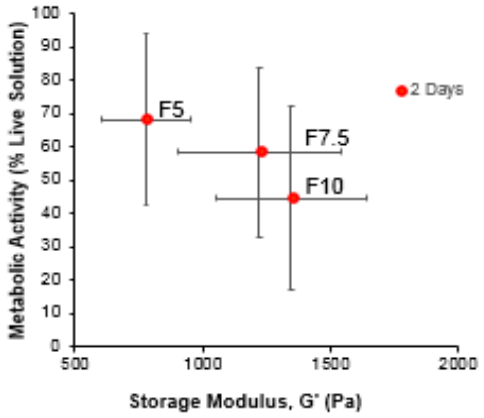

Figure 17. *Normalized Metabolic Activity at 2 days vs. Storage Modulus for FS-PEG gel formulations*

3.3.4 Extraction Studies

There was a very small amount of cells that were recovered from the FS-PEG extraction experiments. This can be seen in Figure 18. This was investigated further by dying the cells with CellTracker™ Green (Invitrogen) before the cells were encapsulated, so that the cells could be seen in the petri dish. The bottom of the well was also coated with a SNP-PEG gel to ensure that the cells were totally encapsulated and did not attach to the well. When the cells were extracted, large chunks of gel were floating around in the

petri dish with cells trapped inside them (Figure 19). An attempt was made to break up the gel more by pipetting the contents of the petri dish up and down with a 10 mL pipette. This did significantly break up the gel chunks (Figure 19). However, the cells seemed to remain trapped inside the smaller chunks of gel and the percent recovery did not significantly improve. Several other variations were made to the extraction procedure but nothing significantly improved the percent recovery.

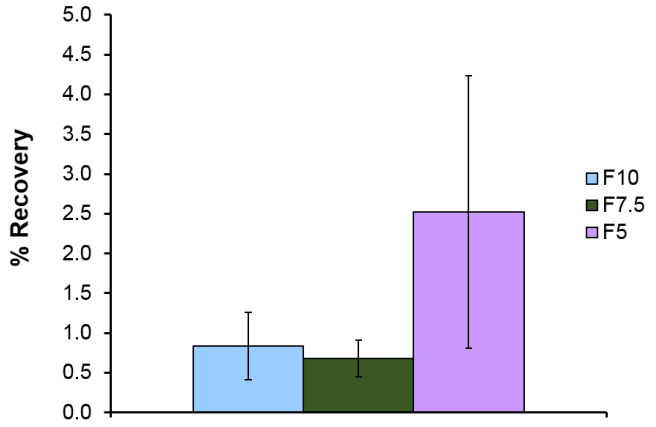

Figure 18. *Percentage of cells recovered from the FS-PEG gel formulations*

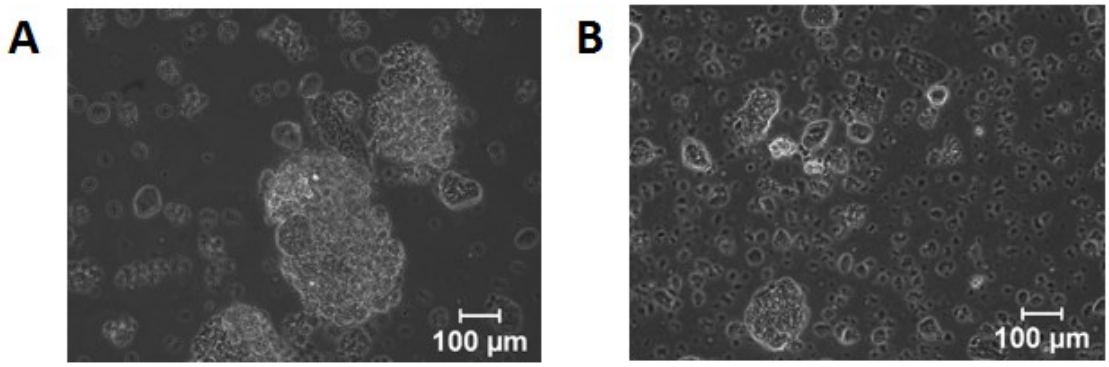

Figure 19. *Pictures of petri dish that cells were seeded in after extraction was performed 2 hours post encapsulation from F10 gel:* ***A.*** *Extraction procedure performed as described in Methods section* ***B.*** *A 10 mL pipette was used on same petri dish to try to break up gel chunks further*

Chapter 4: Discussion

4.1 SNP-PEG Gel Discussion

The hypothesis that the storage modulus will decrease due to increasing PEG concentration was partially incorrect. This is attributed to the fact that phase separation occurred at PEG concentrations below 9.1%. The phase separation could be seen by the opacity of the gels. The hypothesis that the length of time that the cells are metabolically active would increase as the storage modulus decreased was incorrect for the SNP-PEG gels. This could be seen by a few different things. First of all, the cells encapsulated in the gel formulation with the highest storage modulus, N9.1, were not metabolically active for the shortest time period. Secondly, the gel formulations that have statistically significant differences in storage modulus values were not the same gels with statistically significant differences in metabolic activity. Lastly, no correlation could be seen between the metabolic activity and the storage modulus. Therefore, the length of time cells are metabolically active did not appear to depend on the storage modulus at all.

This does not agree with the fairly established notion that the stiffness of the gel affects cell viability in 2D. However, studies have shown that cells can behave quite differently when cultured on a 2D scaffold than a 3D scaffold. This suggests that 2D results do not necessarily apply to 3D studies [48]. There have been a few studies that have shown the stiffness of a 3D gel affecting mesenchymal stem cell differentiation, glioma cell morphology and motility, and tumor growth [42, 44, 47, 49].

There may be a few explanations for the results that were obtained. First, the range of stiffness in the gels that were used may not have been significant enough to produce a

change in cell viability. The rheology results show that something other than the PEG concentration would have to be changed if stiffer gels are desired. HFF cells may be less sensitive to mechanical stimuli than the stem, glioma, and tumor cells. It has been shown that the reaction of the cell to mechanical stiffness is dependent on the cell type [50]. It is also important to note that there have only been a small number of 3D investigations on how mechanical stiffness affects cell behavior.

Additionally, other parameters may be much more important to cell viability than gel stiffness making it difficult to see any effects. For instance, the PEG concentration appeared to play an important role in the stiffness of the gel and the metabolic activity of the cells. There were not a lot of gel formulations that have statistically significant differences at 20 minutes, but there did appear to be a trend. The differences may become statistically significant if later time points were used and/or more trials were performed. There were many statistically significant metabolic activity values between extreme and intermediate PEG concentrations. The extreme PEG concentrations had lower metabolic activity values than the intermediate PEG concentrations.

PEG and GPTMS were shown to improve encapsulated cell viability by reducing gel shrinkage during aging and/or improving the interactions between the cells and the gel [30, 34]. This would explain the large difference between N0 and N2.3, N4.5, and N9.1. The cells encapsulated in the N0 cells had 39% of their metabolic activity at 12 hours and were no longer metabolically active at 24 hours. On the other hand, the cells encapsulated in N2.3, N4.5, and N9.1 still had full metabolic activity at 12 hours, 40-60% metabolic activity at 24 hours, and were inactive after 36 hours. The N13.6 and N15.9 gels appeared

to have a higher metabolic activity than N0 at all of the time points. However, their metabolic activities were quite a bit lower than the other gels that contained PEG. They had 55-65% metabolic activity at 12 hours, 25-35% metabolic activity at 24 hours and were inactive after 36 hours. At 12 and 24 hours N4.5 and N9.1 had a significantly higher metabolic activity than N13.6 and N15.9. A possible explanation for the lower metabolic activity of N13.6 and N15.9 is that the cells were becoming stressed during the hydrolysis reactions of the gels due to the longer gelation times. The effects that gelation times have on cells is unknown [29]. Therefore, the PEG concentration played an important role in the metabolic activity of the encapsulated cells.

Even though the addition of PEG increased the length of time that the encapsulated cells were metabolically active, the cells still lost their metabolic activity after being encapsulated for 36 hours. There could be a number of things contributing to the loss of metabolic activity. First of all, the cells could be harmed by continuous interactions with the silica and silica nanoparticles, which have been shown to be harmful to cells at high doses [54]. Aging often produces syneresis and restructuring into denser gel matrices causing the surface of the gel to have a greater area of contact with the cells and place greater mechanical restrictions on them. This has been have suggested to affect cell viability [29]. Therefore, syneresis may cause the gel matrix to place mechanical stresses on the cells and reduce viability.

The cells could also be stressed as a result of attempting to proliferate since cells are generally not able to proliferate in silica sol-gels due to their stiffness [29, 51]. Consequently, the cells become larger as they prepare to proliferate and become stressed

by the external pressure exerted on them by the constraining gel matrix. The metabolic activity results showed a dramatic loss in metabolic activity at 24 hours and no activity at 36 hours and the proliferation study results showed that the HFF cells generally divided within 24 hours. The metabolic activity results and cellular division rate suggests that stress from proliferation attempts did affect the cell viability in the SNP-PEG gels.

4.2 Particulated SNP-PEG Gel Discussion

There are a few different conclusions that could be drawn from the Particulated SNP-PEG gel results. First of all, there was no apparent correlation between the metabolic activity and the storage modulus. This was due to the fact that there was too much variability in the rheology results of the Particulated N0 gel to see any correlation between the storage modulus and the PEG concentration. Even though the difference between the storage moduli of the two gel formulations could not be seen, there was a significant difference between the metabolic activity of the cells and the quantity of viable cells that were able to be extracted from the two gel formulations. These results suggest that the stiffness of the gel did not affect the cell viability; rather, the PEG concentration had a large effect on the cell viability.

Although the hypothesis that the stiffness of the gel would affect cell viability was incorrect, the addition of PEG was shown to improve cell viability as expected. As stated previously, organic polymers improve cell viability by improving the interactions between the cells and the gel and/or reducing the amount of syneresis [30, 34]. The improvement in interactions between the cells and the gel seems to be more plausible

because the aging process was disrupted and its effects reduced during the particulation process.

Despite the addition of PEG and the particulation, the cells in Particulated N4.5 did eventually die after being encapsulated for 6 days. There a few possible explanations for this phenomenon. Similar to the SNP-PEG gels, there may have been some residual negative interactions between the silica or silica nanoparticles and the cells. Additionally, there may have been stress on the cells from proliferation or attempted proliferation since sol-gels do not usually allow cells to proliferate [29]. It is not known as to whether or not the particulation process made it possible for the cells to proliferate. However, the proliferation cycle of the HFF cells is less than 24 hours, so it does not seem probable that the stress from proliferation caused the loss of metabolic activity. Consequently, it is likely that there was still negative interactions between the silica or silica nanoparticles and the cells. The cells may have experienced a lack of nutrients as well because the culture media was never changed during the test.

The percent of viable cells recovered from the Particulated N4.5 gel was much higher than the Particulated N0 gel. However, 45% of the cells were still lost during extraction in the Particulated N4.5 gel. There could be a couple of different phenomena causing this to occur. The cells could have become stressed and undergone apoptosis or necrosis during the extraction procedure. The cells could have gotten trapped inside of the gel which prevented them from attaching to the petri dish as well. Although almost half of the cells were lost during the extraction procedure, the cells that were recovered proliferated at a normal rate.

4.3 FS-PEG Gel Discussion

In the results of the FS-PEG gels, there appeared to be a correlation between the storage modulus and the amount of Cabosil in the gel. There also appeared to be a correlation between the metabolic activity and the storage modulus. However, the differences between the gel formulations for both the metabolic activity and the storage modulus were not statistically significant. Therefore, it could not be determined if there was a correlation between these parameters. In order to determine if there is a correlation between these parameters, the confidence interval would have to be increased by either performing more trials of the same Cabosil concentrations or by expanding the Cabosil concentrations used.

The cells were viable inside the FS-PEG gels for 3 or 4 days. The eventual death of the cells may have been caused by a variety of things. The cells may have used up the nutrients in the culture media because it was not changed during the experiment. There may have been negative effects from remaining alcohol or the silica and silica nanoparticles within the gel. There may have been stress on the cells from proliferation or attempted proliferation or syneresis. It is not known as to whether or not HFF cells can proliferate in these gels like the cells investigated by Pek, et al. [46, 47]. Nevertheless, stress from cell proliferation or attempted proliferation does not seem probable since the normal proliferation rate was less than 24 hours and the cells lived 3 or 4 days. It also does not seem probable that syneresis would cause the loss in metabolic activity since the aging process was disrupted by particulation.

There was a very small quantity of viable cells that were able to be extracted from the FS-PEG gels. Upon further investigation, the cells appeared to be stuck inside chunks of gel. Although some variations in the extraction procedure broke up the chunks of gel greatly, the quantity of viable cells extracted from the gel did not improve significantly. This could be the result of the gel re-forming too quickly for the cells to be fully extracted or of the gel not fully liquefying under the shear stress applied during the extraction.

4.4 Gel Comparison

Since the rheology tests of the SNP-PEG gels differed so greatly from the Particulated SNP-PEG and FS-PEG gels, the rheology results of all of the gels could not be compared directly. Therefore, only the storage modulus between different formulations of the same gel were compared. Also, the metabolic activity studies and the extraction studies of the different gels could be compared.

It can be seen from the metabolic activity results that the cells were metabolically active the longest in the Particulated N4.5 gel, 6 days. In fact, the cells encapsulated in the Particulated N4.5 gel had a significantly higher metabolic activity than all of the gel formulations from the other gels after the initial encapsulation with a 95% confidence interval and after the first day with a 99% confidence interval. The cells encapsulated inside the Particulated N0, F5, F7.5, and F10 gels were metabolically active the second longest, 3 to 4 days. The cells encapsulated inside the SNP-PEG gels were metabolically active the shortest amount of time, 24 to 36 hours. The Particulated N0, F5, and F7.5 gels had a significantly higher metabolic activity than the SNP-PEG gels after the first day

with a 99% confidence interval. The F10 gel had a significantly higher metabolic activity than the SNP-PEG gels after the first day with a 99% confidence interval. This seems to suggest that particulation significantly improves the viability of encapsulated cells. This could be the result of reduced syneresis, gel stiffness, or stress from proliferation or proliferation attempts. The particulation may have improved cell viability by washing away of harmful by-products as well.

There was a much higher quantity of viable cells that were able to be extracted from the Particulated SNP-PEG gels, 55% from Particulated N4.5 and 14% from Particulated N0, than the FS-PEG gels, less than 3% for all formulations. As stated previously, it is thought that the cells are trapped within the FS-PEG preventing extraction. However, the relative quantity of viable cells that could be extracted seemed to be related to the relative metabolic activity between different gel formulations of the same gel at the time point at which they were extracted. Also, many more cells were able to be extracted from the Particulated N4.5 gel than the Particulated N0 gel. As stated previously, the PEG is thought to improve the cell viability and extraction.

The short length of time that the cells were viable would limit the applications that the SNP-PEG gels could be used for. They could be used for biomimetic studies, so that HFF or other cell behavior could be understood better and compared. Eventually this information may expand into other biotechnology and medicine applications. One example would be the ability to identify a cell through an encapsulation study. This would allow for abnormalities to be identified and a diagnosis to be made if necessary in a patient. This maybe be particularly useful for the diagnosis of cancer. The long term

metabolic activity and extraction rate of the HFF cells from make the Particulated SNP-PEG gels good candidates for many different sol-gel applications. Similar to the SNP-PEG gels, they could be used for biomimetic studies, cell type comparisons, and diagnostic tests. The ability to extract the cells and reuse them would be an added benefit over the SNP-PEG gels. These gels could be investigated for other applications like the development of biosensors, biocatalysts, or bioartificial organs. The FS-PEG gels could be used for similar applications as the Particulated SNP-PEG gels. However, since the Particulated SNP-PEG gels had better viability and extraction results they would be better candidates for the encapsulation applications.

4.5 Conclusions

The results suggest that the PEG concentration was the most important parameter for the length of time that the encapsulated cells were metabolically active for both the SNP-PEG and the Particulated SNP-PEG gels. The PEG concentration also appeared to be the most important parameter for the quantity of viable cells that could be extracted from the Particulated SNP-PEG gels. Consequently, the addition of PEG to the SNP-PEG and Particulated SNP-PEG gels was beneficial. It should also be noted that the cells encapsulated in the FS-PEG and Particulated SNP-PEG gels had a significantly higher metabolic activity than the SNP-PEG gels. This suggests that the particulation process was beneficial.

The Particulated SNP-PEG gels were the best gels for encapsulating HFF cells because the cells were metabolically active inside them for the longest time period and

the largest quantity of viable cells could be extracted from them. This was particularly true of the Particulated N4.5 gel.

4.6 Future Work

There was a lot of information revealed about how HFF cells reacted to different changes in their environment. Any additional information about cell behavior can aid in the development of new gel protocols that increase cell viability and open the door to new applications. New information on cell behavior or gel protocols that allow for biomimetic studies could also have a large impact on medicine. The ability to extract the cells could revolutionize the research and testing of cells and tissues. It would allow cells to be reused so that multiple tests could be performed on them. Therefore, a number of experiments that would expand on the findings of this study are recommended for future tests.

The correlation between the storage modulus and the metabolic activity of the SNP-PEG gels could be explored further by keeping the PEG concentration at a constant value and changing the silica nanoparticle size. This would remove the effects on metabolic activity that the PEG concentration has making it easier to see if there is a correlation between the storage modulus and the metabolic activity of the gel.

The N4.5 gel appeared to have a higher metabolic activity than all of the other SNP-PEG formulations with N9.1 and N2.3 just slightly lower. It may be beneficial to see whether a SNP-PEG gel with 6.8% PEG has a higher or lower metabolic activity than the N4.5 gel. If the gel has a significantly higher metabolic activity, then the 6.8% PEG formulation should also be used in the Particulated SNP-PEG studies.

More studies on the Particulated SNP-PEG gels could be very beneficial. Performing the studies on more PEG concentrations, such as the ones used for the SNP-PEG gels, may reveal a lot of information. It could determine with more certainty if there is a correlation between the storage modulus and the PEG concentration and the metabolic activity and the storage modulus. It would also provide some information as to whether or not the cells are affected by the gelation time. It may also be beneficial to change the media at least one time during the metabolic activity study to see if the cells were dying from a lack of nutrients.

The correlation between the storage modulus and the metabolic activity of the FS-PEG gels could also be explored further. The confidence interval would need to be increased to determine if there is a correlation. This could be done by performing more repeats of the same Cabosil concentrations or by performing experiments with different Cabosil concentrations. Performing the studies on a gel formulation without Cabosil may be particularly interesting because the metabolic activity appeared to increase as the Cabosil concentration was decreased. Therefore, it is hypothesized that the gel formulation without Cabosil would have a higher metabolic activity than the F5 gel. A gel formulation without Cabosil may also have better extraction results because Cabosil stimulates hydrogen bonds and/or Van der waals interactions in the gel [46]. If the gel bonds are weaker, the cells may not get trapped in it.

Additionally, it would be useful to understand how other cell lines react to changes in their environment. Understanding how different cell types react to different gel formulations may lead to the ability to identify cells through metabolic activity studies

and have huge implications in diagnostic applications. In order to understand the cell behavior of other cell types, the metabolic activity and extraction studies could be performed on different cell lines, especially cancer cell lines. The results from the HFF cells would provide a good comparison for such tests and allow one to see if the different gels, gel formulations, and mechanical properties affect the cancer cells differently. Such understanding could be useful for medicine and other scientific applications as well.

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