UNIVERSITY OF WISCONSIN – MADISON
DEPARTMENT OF BIOMEDICAL ENGINEERING
BME 301 – DESIGN

Cancer Cell Scaffold

Final Report

Vivian Chen – Co-Leader

Sarah Czaplewski – BWIG

Vanessa Grosskopf – Communicator

Josh Kolz – Co-Leader

Sarah Sandock – BSAC

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Abstract

Cell scaffolds are substrates onto which cells can adhere and grow. 3D cell scaffolds promote cell growth to a high density in bioreactors, which are used to maintain the viability of cells. The client desires high cell density in order to obtain clear MRI signals to measure cancer cell metabolic rate. The objective of this project is to develop a scaffold that promotes high density cell growth to $5x10^7$ cells/mL and maintains viability throughout the experiment. Collagen-coated and non-coated polystyrene microcarriers were chosen as the cell scaffold. T47D cells were seeded on the microcarriers and developed a peak density of $3.4x10^7$ cells/mL with the collagen-coated microcarriers after four days. This is about 70% of the desired cell density. The results demonstrate that cells were able to attach and proliferate on the microcarriers, rendering them useful for MRI studies. By optimizing the microcarrier culture protocol, a higher cell density may be achieved.

Background

MRI ^{13}C Hyperpolarization

Hyperpolarization of ^{13}C-labeled compounds,such as pyruvate, provide high contrast in magnetic resonance imaging (MRI) and can be used to track metabolic pathways [1]. Hyperpolarization involves inducing nuclear polarization of a material beyond the thermal equilibrium conditions [1]. This is accomplished by cooling a small amount of ^{13}C-labeled pyruvate to 1.4K. At this low temperature, the spins of the electrons are aligned with the magnetic field, and microwave irradiation will transfer the spin to the nuclei, polarizing the protons [1]. During imaging, it is the decay of the hyperpolarized protons that enables the ^{13}C-labeled compounds to be tracked. ^{13}C-labeled pyruvate is ideal for such studies because ^{13}C has a relatively long decay time (approximately one minute) compared to other compounds, is inexpensive, and degrades via known metabolic pathways [1].

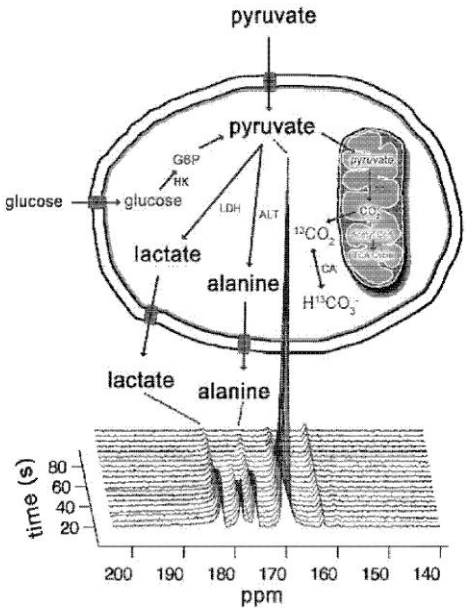

Figure 1: Schematic demonstrating the metabolic breakdown of pyruvate and the resulting signals [1].

This method can be applied to stage cancer and assess progress of treatment by injecting ^{13}C-labeled pyruvate into a cell culture and monitoring its metabolism [2]. Metabolic activity, especially glycolosis, is up-regulated in cancerous tissues. Glycolysis is the conversion of glucose to pyruvate to smaller metabolites such as lactate and alanine. Thus, using MRI, the amount of pyruvate breakdown

products can be measured to evaluate the celluar metabolism and assess the agressiveness of cancer cells [2]. Figure 1 shows a diagram depicting the breakdown of pyruvate and the chemical shifts observed from these compounds.

The client for this project would like to apply this method to study various cancer cell lines. To accomplish this, a bioreactor and cell scaffold are needed to sustain high cell density and viability in order to obtain clear signals in the MRI machine.

Bioreactors

A bioreactor is a system used to grow and sustain cell cultures by delivering nutrients to cells and removing cellular waste. There are many types of bioreactors available commercially. However, there are two basic types of reactors that other bioreactors are based on: the stirred tank reactor and the tubular reactor. The stirred tank bioreactor contains propellers that stir the contents in the bioreactor tank such that the fluids and gases perfused are homogeneous. The tubular reactor uses plug-flow, and nutrient concentration decreases from the inlet to the outlet [4]. A bioreactor system of either type is needed for the MRI hyperpolarization experiment described previously to culture cancer cells at a high density. Figure 2 shows an example of a bioreactor containing a cell scaffold.

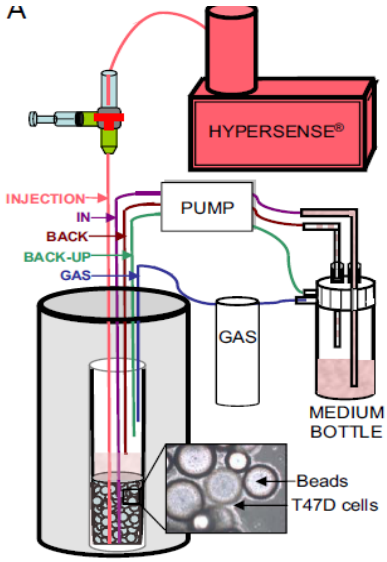

Figure 2: Diagram of bioreactor system including injection and output lines for cell media and gases. A scaffold that consists of many microscopic beads is contained within this bioreactor [3].

Cell Scaffolds

The cell scaffold provides a site for cell attachment and is encased within the bioreactor [4]. There are various methods and materials that can be used to efficiently culture the cells in the bioreactor without being substantially affected by the perfusion system. 2D culture environments, in which growth occurs on a single plane, would not create a high enough density to observe a clear signal during MRI imaging. Additionally, 3D cultures, in which cells grow in multiple planes, better mimic the physiological environment as compared to 2D structures that may change certain properties of the cells, including the phenotype [5]. For this reason, the team will focus on scaffolds with 3D structures. Such scaffolds offer a large surface area for cell attachment which is desirable for growing cell cultures to high densities. Scaffolds can be coated with extracellular matrix (ECM) components that promote the adhesion and proliferation of cells. The 3D structure also allows better diffusion of nutrients for growing cells. Depending on the micro- or macrostructures of the scaffolds, cells may even be protected from hydrodynamic damage in the bioreactor [4].

Cancer Cells

The focus of this project is to promote cancer cell adhesion and proliferation on a cell scaffold. To accomplish this, it is important to understand the biology of cancer cells and the conditions in which they thrive. In general, cancer cells exhibit unregulated growth due to increased or unregulated production of growth factors and ECM components [6]. Included in excessive growth are up-regulated metabolic processes such as lipid synthesis and degradation, bioenergetics, and glycolysis [3]. Cancer cells also demonstrate unregulated cell division leading to higher proliferation rates [6]. This property will be advantageous to this project because cancer cells will naturally proliferate and can thrive in many environments.

Several human cells lines are of particular interest to the client, two of which will be studied in this project. These cell lines are all adherent cancer cell lines and thus need a scaffold to grow on. The human cell lines of interest include:

- Breast cancer: T47D
- Prostate cancer: PC-3, LNCaP, DU145
- Brain glioma: U87, U251
- Brain glioblastoma: 99T

Project Motivation

In order to observe the metabolism of cells, MRI imaging is used to track the decay of ^{13}C-labeled pyruvate. The samples of cells used during the MRI imaging process have to be of high density and viability for images to be clear. The main type of cells that the client is concerned with is cancer cells, which are self-proliferating and tend to overproduce ECM components. This makes cancer cells relatively easy to culture to obtain high cell density and viability. However, different cell lines have different requirements in terms of culture media and attachment surface. Hence, it is necessary to find the optimal scaffold for all cell lines to achieve the maximum cell density and viability.

Design Criteria

There are several key design criteria the cell scaffold needs to fulfill. The first and most important is that the scaffold needs to have a large surface area to volume ratio. This is crucial for the cells to grow to a high density in a limited space. The density that the cells need to reach in order to successfully measure their metabolism is $5x10^7$cells/mL. Another important condition is to maintain cell viability for the duration of seeding, growth, and imaging of the cells, which should take approximately four to five days. Maintaining cell viability means that the cells remain alive and nourished. The scaffold should also allow the perfusion of both oxygen and media to continuously nourish the cells as well as to remove all cell waste. An additional requirement the scaffold needs to satisfy is to ensure proper inoculation of the cells. The scaffold should allow the cells to be easily seeded, and ensure growth and

proliferation. Finally, the scaffold should not contain any ferrous material that would interfere with the MRI machine's magnet and must be within the $3,000 budget for the entire bioreactor and cell scaffold project.

Design Alternatives

Encapsulation

The first design alternative involves the formation of beads that contain the cancer cells. The most common type of scaffold is a calcium alginate bead as shown in Figure 3. These beads can be created with one of two methods, which are similar but have key differences. The first way to create the beads starts by creating a solution of 2% sodium alginate solution mixed with the cells in a one to one ratio. This solution is then drawn into a 1-cc syringe fitted with a 24-gauge angiocatheter that has a 23-gauge needle pierced at its hub to act as the positive electrode in the electrostatic casting process. The syringe is then placed in a syringe pump system set up so droplets fall orthogonal to the calcium chloride solution. This solution has a concentration of 125mM and contains a grounded electrode. This allows an electrostatic potential to be created across the angiocatheter tip and calcium chloride solution using a high voltage DC source. In this method the bead size is controlled by adjusting the applied potential [7]. The other way to create calcium alginate beads does not use an electrostatic potential to control bead size. The protocol is the similar to the first procedure except the beads are made entirely by dropping the sodium alginate-cell solution into the calcium chloride solution with a syringe and syringe pump [8]. The latter method is older and the sizes of the beads vary greatly, meaning the cell densities inside vary as well. Therefore the method of using an electrostatic potential is a better method although it is more costly because a high voltage DC source is needed.

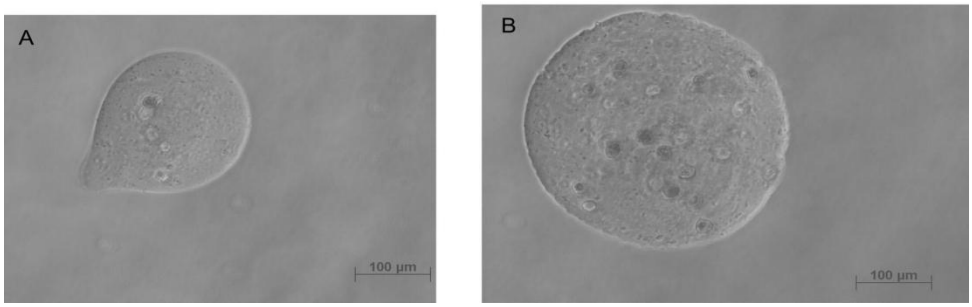

Figure 3: Photos of cells encapsulated in calcium alginate beads, showing the range of sizes that can be obtained using the electrostatic potential method [7].

This method of encapsulation has a strong presence in hyperpolarized ^{13}C spectroscopy research and NMR-compatible bioreactor systems to assess cellular metabolism. The hyperpolarized ^{13}C is able to penetrate the beads and be taken up by the cells. This method has been shown to allow cell growth to a density between $5x10^7$ cells/mL and $1.2x10^8$ cells/mL which is consistent with the density that the client is looking for [3]. This technique has also been used to assess cancer cells in past experiments but not

with the target cell types. This technique is very cost efficient, estimating under $10 per experiment. The main disadvantage of this type of scaffold is the time-consuming construction, as it would take over a day to fabricate enough beads to run a single experiment.

Microcarriers

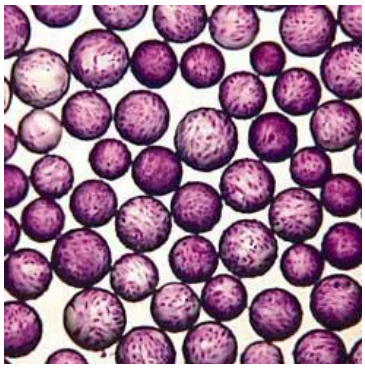

Figure 4: Photograph of microcarriers seeded with cells [2]

Microcarriers are spherical beads that usually range from 60-300 microns in diameter (Figure 4). They can be made from a variety of materials such as dextran (a polysaccharide), glass, polystyrene, acrylamide, and collagen [9]. The microcarriers can have different surface modifications which promote cell adhesion. They may be coated with collagen, FACT which is a modified collagen, or ProNectinF which is a protein polymer containing many copies of RGD, a cell attachment ligand [9]. Positive or negatively charged molecules may also be introduced to the bead's surface. The beads may be nonporous or have micro- or macropores which enhance the surface area of the bead and allow for a greater cell density. Microcarriers are often used with bioreactors because of their versatile composition and because the spacing in between the beads allows for adequate perfusion of fluids. For the design alternatives, three different microcarriers were considered: Biosilon Nunclon Delta Microcarriers, Cytodex 3, and SoloHill Microcarrier Beads.

Biosilon Nunclon Delta Microcarriers

The Biosilon microcarrier is a nonporous polystyrene bead that undergoes a surface treatment that adds charges to the polymer to make the beads more hydrophilic. They are radiation-sterilized and come ready to use, but are not autoclavable. The beads range from 160-300 microns in diameter [10]. The client provided a paper in which these beads were used in studies of metabolism in T47D breast cancer cells [2]. The cell density of 5×10^7 cells/mL which the client desires was achieved using 0.5 g of these beads, so the surface area of these beads is sufficient for the needs of this project. Metabolism of the cells was monitored using NMR perfusion in a bioreactor [2]. These beads were sold by Krackeler Scientific, Inc. and when enquiring about the price, it was discovered that the product was discontinued. This was not known when this design was first considered. However, these beads were expensive due to the specialized surface modification. Other distributors have not been found, but it is possible that with further research one could be found.

Cytodex 3

Cytodex 3 is a microporous microcarrier sold by GE Healthcare. It is made of cross-linked dextran and has a coating of acid-denatured porcine collagen. The beads come in powder form and must be swelled in PBS prior to use. The beads range from 60-87 microns in diameter [11]. This diameter is

smaller than that of the Biosilon beads, thus a greater amount of beads would have to be used to achieve the same cell density as with the Biosilon beads. 10 g costs $141.00, and assuming 0.5 g is needed for an experiment, the estimated cost is $7.05 per experiment [12]. This type of microcarrier was used to investigate the differences between metabolite levels through enzymes regulating phospholipid and mitochondrial metabolism in normal mammary epithelial cells and in breast cancer cell lines 21PT and 21NT. The NMR perfusion experiments for this study were done using bioreactors [12]. Thus, these beads have been used with cancer cells and with a bioreactor, but have not been used with any of the specific cell lines for this project.

SoloHill Microcarriers

The SoloHill microcarrier is a nonporous polystyrene bead with a porcine collagen coating. The beads range from 125-212 microns in diameter and have a surface area of 360 cm^2/g of beads [13]. This diameter is smaller than the Biosilon bead, so greater amount of beads would have to be used to achieve the same cell density. 20 g cost $160.70 from Sigma-Aldrich, thus the estimated cost per experiment is $4.02 assuming 0.5 g of beads is used [13]. Before use, the beads must be suspended in deionized water and then autoclaved [14]. This microcarrier has been used with studies using a mouse mammary tumor cell line EMT6, even though this is not one of the project specific cell lines. Mancuso *et al.* (2004) used this microcarrier in a bioreactor with EMT6 to develop a method "for obtaining high signal-to-noise ^{13}C NMR spectra of intracellular compounds in metabolically active cultured cells" [15]. The specific cell lines for this project should adhere to the collagen coating because Wozniak and Keely (2005) as well as many others have used 3D collagen gels with T47D breast cancer cells [16].

Hollow Fibers

The third category of design alternatives is the hollow fiber scaffold as seen in Figure 5. This type of scaffold is commercially sold pre-incorporated into a perfusion bioreactor system consisting of a plastic outer casing containing a cartridge of several thousand hollow tubule membranes in a parallel array [17].These thousands of fibers provide a large surface area ideal for cell attachment. Cells are most commonly grown in the extra-capillary space (ECS) because of the larger area for growth and proliferation, but cells can also be grown in the lumen of the tubules [17]. When cells are grown in the ECS, they adhere and proliferate on the outside of the tubule membranes. Nutrients (medium and oxygen) and experimental agents (^{13}C-labeled pyruvate or drugs) are perfused

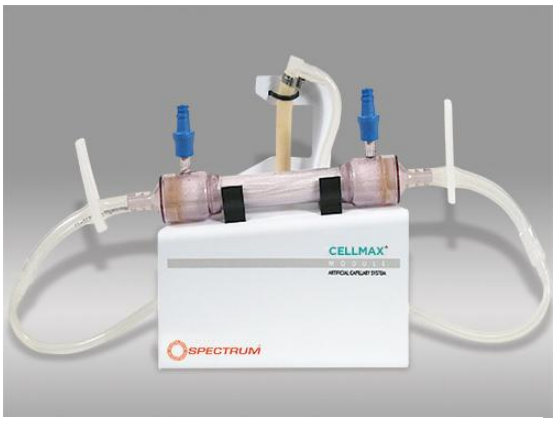

Figure 5: Photo of Spectrum Labs CellMax hollow fiber bioreactor [18].

through the input ports and into the hollow fibers. The nutrients are exchanged for metabolic waste products across the permeable tubule membrane and the waste products are carried out via the output ports [17]. Figure 6 shows a diagram of the setup described.

FiberCell Sytems Inc. and Specturm Labs are two companies that produce these hollow fiber bioreactor systems. Common materials for the hollow membrane include polysulfone, polypropylene, regenerated cellulose, and polyethylene [18, 19]. The bioreactors also are available in a variety of sizes. For this MRI hyperpolarization experiment, a smaller bioreactor is required to fit within the bore of the MRI magnet. The suppliers also recommended the polypropylene membranes because these fibers have established protocols for being coated with extracellular matrix (ECM) proteins to further promote cell adhesion and growth. Furthermore, the molecular weight cutoff of the membranes can also be chosen based upon the size of the molecules needed to diffuse across the membrane. The specific option that was investigated for this project was a polypropylene fiber bioreactor with a surface area of 100 $cm^2$and a cost of $420 [18].

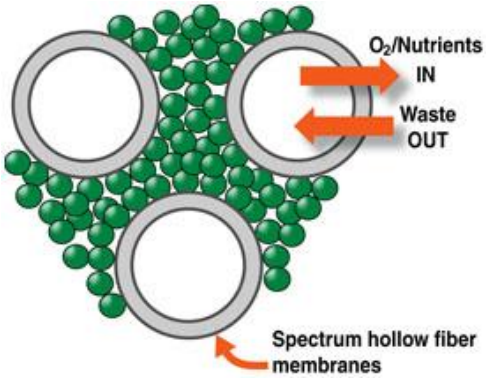

Figure 6: Diagram of cells growing in the extra-capillary space and nutrient/waste diffusion through hollow fiber scaffold [18].

There are several advantages to this system with the most important being larger surface area leading to increased cell density [20]. However, the total surface area of a hollow fiber scaffold is less than that of a gram of the SoloHill microcarriers (100 cm^2 versus 360 cm^2/g microcarriers). The thousands of tubules provide consistent and physiologic perfusion allowing the bioreactor to sustain cell cultures for up to six months [1]. Because the conditions within the bioreactor mimic the physiologic environment, studying the cellular metabolism in the hollow fiber bioreactor may provide results closer to what is seen *in vivo*. Another advantage of the hollow fiber scaffold is that the cells are protected from high shear forces of perfusing medium because the membrane acts as a barrier [20]. This will minimize the amount of cells that detach from the scaffold during use. Additionally, hollow fiber bioreactors have been used with cancer cell lines in the past including brain glioma U87 and several breast cancer cell types [21]. However, there are also several shortcomings with this design. For instance, the membranes may cause noise in the MRI and would require preliminary testing to determine if the bioreactor cartridge would be usable for the MRI hyperpolarization experiment [2]. Also, the ECM proteins used to coat the fibers can be very expensive. Lastly, the bioreactor is difficult to autoclave, requiring a specific protocol with a slow ramp up time to the maximum temperature [2]. Even then, leaks in the membranes can occur which would lead to loss of cells due to perfusion. For the purposes of this project, if the scaffold is expensive it must be reusable and the hollow fiber scaffold may not be able to accomplish this.

3D Scaffold Structures

Porous Structures

Porous structures such as alginated (Figure 7) and fibrin scaffold types have macroporous structures that allow for easy cell seeding and nutrient delivery [22]. Both alginated and fibrin scaffolds provide a defined substrate commonly used for cancer cell lines to study disease states and drug models [23].

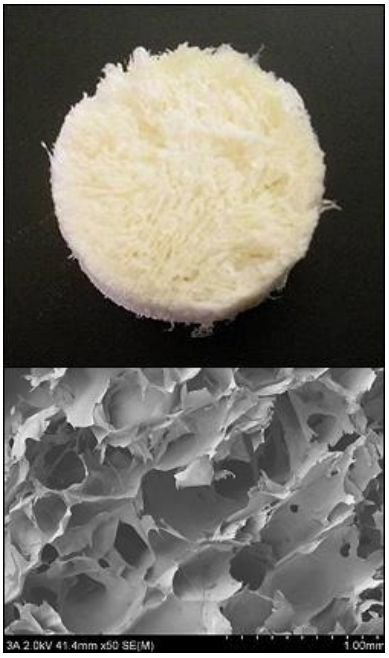

Figure 7: Dry alginated scaffold (top). Microstructure of dry alginated scaffold (bottom) [26].

Generally a solution of proposed substrates, for example 4% chitosan-alginate, 2% acetic acid, 4% alginate for an alginated scaffold, are added to a solution, mixed with a blender, and then cast in welled plate and frozen at -20^{o}C for eight hours [5]. Samples are then lyophilized, sectioned and cross-linked in a 0.2 M $CaCl_2$ solution for 10 minutes in vacuum conditions. Samples are washed for several minutes to remove salts and are sterilized in 70% ethanol for an hour. Scaffolds are stored in PBS in a shaker to remove ethanol. Scaffolds are then ready for use and seeded with cells.

The complex methods of fabrication for alginated and fibrin scaffolds require very specific resources and time that it seems the graduates students working on this project do not find attractive [5, 24]. Additionally, upon looking for commercial products of this type, it seems that they come pre-arranged in various plate formats that would not be compatible with the final bioreactor container. There has been a previous study that utilized alginated hollow fibers [25], but the disadvantages of hollow fiber usage, as discussed in the hollow fiber design alternative section, prevent this from being a viable option. Additionally, the use of this scaffold is dependent on the size and shape of the bioreactor cartridge which is presently unknown.

ECM Protein Gel Structures

ECM proteins provide structure and anchorage to mammalian cells *in vivo*. In labs, ECM proteins, commonly collagen, are used to provide cell cultures with anchorage, provide extracellular cues, and better mimic the *in vivo* environment. ECM proteins are typically used in 2D coating applications. A surface is coated with said protein to encourage attachment of cells to a plate or surface with complex geometry.

ECM proteins are usually expensive. Depending on the type of protein, source, and purity, these proteins can range from $50-500/ml. When performing a literature review, no bioreactor used a scaffold of pure ECM protein. This product is often used as a coating in cases such as hollow fibers or microcarrier beads [2, 16]. An ECM coating would provide the scaffold with an environment that

strongly encourages cell attachment. For the cell lines of interest, any ECM protein would encourage attachment since cancer cells are known to overproduce many ECM proteins to create their own niche.

Design Matrix

Table 1 shows the design matrix used to assess the four design alternatives. The four design options are listed in the left-most column. The microcarriers and 3D scaffold structures categories were broken down into three subgroups each because there is a wide variety available on the market. Each subgroup was scored as a separate design. The criteria used to rate the design alternatives are listed in the top row with their respective weights. These weights total to 100; thus, each design was scored out of 100 possible points.

Of the seven criteria, surface area was weighted the highest with 25 points because it is proportional to the maximum density that cells in culture can attain and high cell density was the most important requirement given by the client. Attaining a high cell density is required to visualize the ^{13}C-labeled pyruvate.

The design alternatives were also weighed based on their presence in bioreactor research and use with the target cell lines. The scores in these categories were based on a literature search conducted by the team. The highest scores were given to designs that have been used in previous studies with the target cell lines. The next highest scores were given to designs having precedence with cancer lines not specific to this project as many cancer cells lines require the same conditions for adhesion and proliferation. These categories of cell specificity and presence in bioreactors were weighted highly (20 and 15, respectively) because it is extremely important to ensure that the chosen design will be compatible with the cells and bioreactor being used before the scaffold can be purchased.

Furthermore, cost and ease of fabrication were given a weight of 10 each because the client did not emphasize the importance of these categories; however, the selected scaffold will need to be fabricated at least 2-3 per week by the client. Thus, experiment preparation should be simple and inexpensive to replicate. A time consuming and expensive scaffold will impede the progress of the client's research. Therefore, a cost effective solution must be chosen for the final design.

The remaining two categories, maintaining cell viability and phenotype, refer to the condition of the cells. In certain cases, materials can cause changes in cell phenotype, which is undesirable for this project. The cells need to remain viable and in their standard proliferative phenotype throughout the duration of the experiment to properly observe the metabolism of pyruvate. Although together these categories hold significant weight, all of the scaffold design alternatives are capable of maintaining cell viability and phenotype based on the literature search conducted by the team. Thus, all design alternatives received the maximal score.

After researching the seven previously described criteria for all design alternatives, the scores seen in Table 1 were given. The 3D gel structures scored lowest of all alternatives. This was primarily due to the lack of use in bioreactors. Research into theses gel structures revealed that use in perfusion

bioreactors can cause damage to the scaffold and can cause cells to fall off the structure. Additionally, the surface area for cell attachment in these 3D gel scaffolds is much smaller than that with the other design alternatives which is a major drawback for this project.

Although higher than the 3D gel structures, encapsulation also scored low on the design matrix. The largest disadvantage of calcium alginate encapsulation is that fabrication is complex and time consuming. This is not ideal for an experiment that is going to be repeated frequently. Furthermore, no journal articles or papers could be found that used encapsulation with the cell lines of interest. Thus, fewer points were given in the cell specificity category.

The hollow fiber scaffold scored very high compared to the 3D gel scaffolds and encapsulation; however, the shortcomings of this design prevented it from being the best alternative. The greatest disadvantage was the possibility of damage due to autoclaving. Considering the cost of the hollow fiber bioreactor cartridge, it must be used for several experiments necessitating that it must be sterilized (ideally via autoclave). Thus, potential damage to the fiber membranes from the autoclave will increase the cost because new scaffolds would need to be purchased frequently. Additionally, the ECM protein coatings are expensive and would further increase the cost and complexity of preparation for each experiment.

Microcarriers as a category are a more cost effective solution than hollow fibers and offer a large enough surface area for the cell densities desired for MRI hyperpolarization. In previous studies, microcarriers have been used successfully in bioreactors and with the cell lines of interest. Based on this precedence, microcarriers will likely be an excellent option to accomplish the objectives of this project. Of the three specific microcarriers considered, the polystyrene beads coated with collagen from SoloHill scored the highest. This high score was received because it has a lower cost than the Biosilon Nunclon alternative and has been used with the specific cells lines of interest unlike the Cytodex 3 microcarriers. Therefore, the team chose to pursue the SoloHill microcarriers as the final design.

Table 1: Design matrix for the cancer cell scaffold showing the collagen coated polystyrene beads attained the highest score.

Type of Matrix	Description/ subcategory	Surface Area (Density) 25	Cell Specificity 20	Presence in Bioreactors 15	Cost 10	Maintains phenotype 5	Viability 15	Ease of Fabricat-ion 10	Total 100
Encapsulation	Calcium Alginate	21	14	12	9	5	15	1	77
Microcarriers	*Cytodex 3*	22	17	15	9	5	15	10	93
	Biosilon Nunclon microcarriers	23	19	15	8	5	15	10	95
	Collagen Coated Polystyrene microcarriers	23	19	15	9	5	15	10	96
Hollow Fibers		24	18	15	6	5	15	8	91
3D Scaffolds	*Alginated Bought/made*	15	14	0	1/8	5	15	8/1	58
	Fibrin Bought/made	15	14	0	1/8	5	15	8/1	58
	ECM Gels	15	14	0	2	5	15	8	59

Final Design

As shown in the design matrix (Table 1), the team chose the collagen-coated polystyrene SoloHill microcarrier (Part Number C102-1521) as the final design [27]. A schematic of the microcarrier is shown in Figure 8. While the diameter of these beads is smaller than that of the Biosilon beads, it is a reasonable compromise to use these beads because they are cheaper and readily available. Since they are cheaper, it will not cost much more to use more beads to achieve the cell density required. This type of microcarrier has been used with the mouse mammary tumor cell line EMT6 [15]. This is not one of the specific cell lines, but most cancer cell lines should behave in a similar manner concerning cell adhesion. Furthermore, this type of microcarrier has a strong presence bioreactor research. The coating on the beads is collagen, and it has been shown that T47D breast cancer cells adhere to collagen gels [16]. Cell viability should not be a problem because T47D cells have been cultured on collagen in previous studies and were viable. Non-coated polystyrene microcarriers from SoloHill (Part Number P102-1521) were also used in testing to provide a comparison to the collagen-coated microcarriers [27].

The team also worked to develop a final protocol design for culture of T47D cells on both coated and non-coated microcarriers can be found in Appendix C.

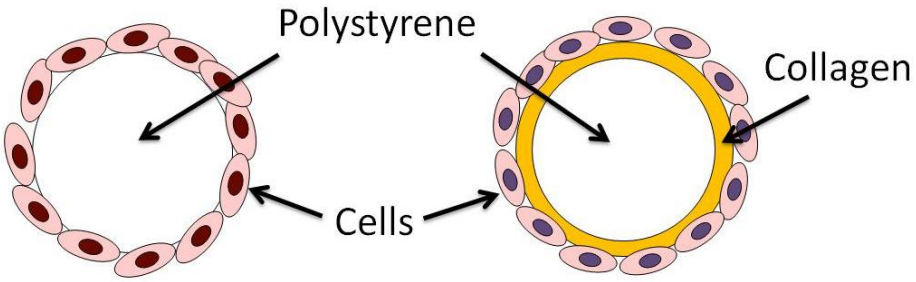

Figure 8: Schematic of both non-coated and collagen-coated microcarrier designs.

Ergonomics

The primary concern is that the user is able to easily insert and remove the scaffold from the bioreactor. This should not be a problem when using microcarriers, as the beads will simply be placed into the cartridge which will be compatible with the bioreactor. They will be easy to remove as well because they will not be tightly packed within the cartridge. Much of the ergonomics for this project cannot be controlled because the team will be purchasing the scaffold and almost no modifications can be made to it.

Ethical Considerations

All experiments will be done *in vitro* meaning there will be no subjects, human or animal, although human cancer cells will be used. Also, there are currently no standards or guidelines for cell culturing techniques set by organizations or the government because the cells are not being used for any type of cell therapy. However, since human cancer cells will be used, experiments should be conducted under biosafety level 2 precautions in order to preserve the integrity of the cells and the experimental lab environment. This will ensure the safety of lab personnel who may come into contact with the same environment as the cells. The scaffold will be designed by the standards and guidelines of the client.

Testing

Two discrete assays were needed for testing. The first test was intended to show that the microcarriers are able to maintain sufficient quantities of attached cells. The second test was to ensure that the microcarriers are unable to pass through a filter, leaving the bioreactor cartridge. Both tests are vital to integration with the MRI compatible bioreactor.

Microcarrier Cell Densities

The goal of the overall project is to sample the MRI signal of pyruvate taken up by cells to access their metabolic activities. The microcarriers serve as a scaffold to fill the 3-dimensional space of the bioreactor cartridge while allowing the cells to grow to high densities with minimal phenotype change. Keshari, et.al. used JM1 rat heptoma cells at a density of $5x10^7$ cells/ml in their probed culture to develop a clear MRI signal. This density of cells or greater should develop a clear signal and this experiment hoped to reach this density.

Materials

Cell lines used in testing included both human breast cancer cell line - T47D from the Beebe lab [Madison, WI] and brain tumor cell line - 99T glioblastoma from the Patankar lab [Madison, WI]. Both non-coated and collagen-coated polystyrene microcarriers were purchased from SoloHill [Ann Arbor, MI]. 10% FBS and 5% penicillin streptomycin were added to both premade RPMI-1640 media for T47D cells and premade DMEM media for 99T cells. Other chemicals, including 0.05% trypsin, trypan blue, and SigmaCote, were used in the protocol. All glassware were coated with SigmaCote to prevent cell attachment. All liquid materials were purchased from Invitrogen [Carlsbad, CA] and Sigma Aldrich [St. Louis, MO].

Culture Conditions

In the initial culture, 99T cells were incubated at 37°C with 5% CO_2 while on top of a magnetic stir plate with a stir bar in the culture spinning at 60 rotations per minute. Styrofoam insulation was placed between the culture bottles and stir plates to prevent overheating of the culture. After learning that the stir bar was breaking the microcarriers and knocking cells off as shown in Figure 9, the team decided to stir the next culture testing the T47D cells with a rotating shaker plate. The rotating shaker plate the team had access to was a self-contained incubator which kept the culture at 37°C but did not manage the CO_2 levels. For the first 48 hours, the microcarriers seeded with cells were kept in suspension by spinning at 80 rotations per minute, which was the lowest speed needed to keep the microcarriers in suspension. After adding more media, the spinning speed was increased to 120 rotations per minute. Because the T47D cells were not kept at a constant CO_2 level, the culture media was changed daily.

Protocol

To assess the density of the cells, a 1-3 mL sample was taken from each culture once a day. The sample was subsequently trypsinized and the cells were counted with a hemocytometer as detailed in Appendices B and C.

Results and Discussion

In the 99T glioblastoma culture, the cells were not quantitatively counted daily because the initial counting protocol used by the team was inadequate (see Appendix B). After consultation with experts, the problems were resolved but by this time the exponential growth phase of the cells had passed. Therefore, a growth curve for 99T cells on the microcarriers was not developed. However, some cell counts were made and can be found in Appendix D. While optimizing the cell counting protocol, qualitative assessments of cell attachment were made using microscopic imaging. Some of these images are show in Figure 9**.** From these images, it was observed that the collagen-coated microcarriers promoted better cell attachment and proliferation as compared to the non-coated microcarriers. Furthermore, it was seen that nearly 40 percent of both non-coated and collagen-coated microcarriers were damaged by the magnetic stir bar by the end of the culture period.

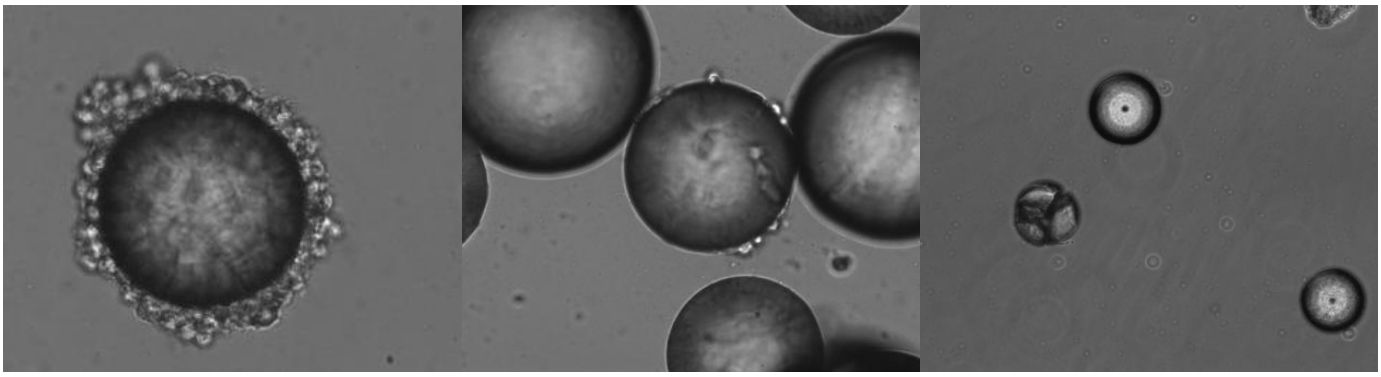

Figure 9: Images of microcarriers seeded with 99T cells. Left image shows collagen-coated microcarrier with significant cell attachment at 20x magnification. Middle image shows non-coated microcarriers with minimal cell attachment at 20x magnification. Right image shows damage caused to microcarriers by the magnetic stir bar at10x magnification.

Based on the inadequacies of the 99T cell culture protocol, the team made several key adjustments as listed in Appendix C. This enabled daily quantitative counting of the T47D cells to be taken and the results are shown in Figure 10**.** The exact cell densities are shown in Appendix D. In this graph, the cell densities were determined by dividing the total number of cells by the volume the microcarriers occupied. As shown in the cell density graph, there was an initial decrease in cell number 24 hours after seeding. This was

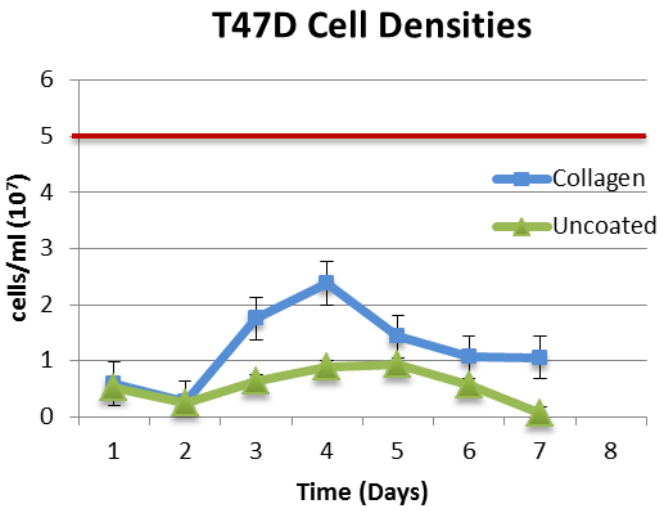

Figure 10: Graph demonstrating daily T47D cell densities, where the red line is the cell density specified by the client.

expected because there will always be some cell loss as not all can attach to the microcarriers. The overall trend of better cell attachment and proliferation on the collagen-coated microcarriers as observed with the 99T cells was again seen in the T47D cell culture. A peak cell density of 2.38×10^7 cells/mL was observed in the collagen-coated microcarriers at day four which represents a 300% increase in cell number after seeding. However, this density is only 48% of the client-specified cell density. For the non-coated microcarriers, a peak cell density of 9.31×10^6 cells/mL was observed at day 5 which represents a 57% increase from the initial seeding density. As indicated by Figure 10, the cell densities of the non-coated and collagen-coated microcarriers decreased after days 4 and 5, respectively. The team believes this can be attributed to the lack of 5% CO_2 presence in the shaker plate incubator and the use of tightly sealed glass bottles as culture vessels which limited gas exchange.

Furthermore, qualitative assessments of the T47D cells were also made using microscopic imaging. The images shown in Figure 11 demonstrate that there was no damage to the microcarriers and that the collagen-coated microcarriers showed better cell attachment and proliferation as compared to the non-coated.

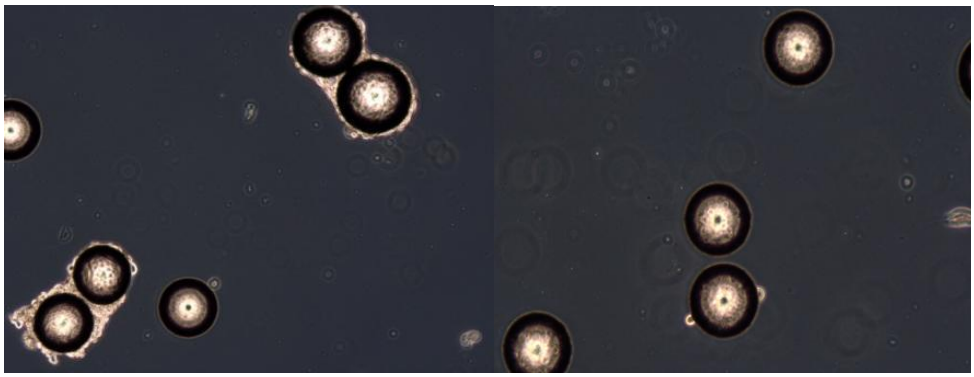

Figure 11: Images of microcarriers seeded with T47D cells at 10x magnification. Left image shows collagen-coated microcarrier with significant cell attachment. Right image shows non-coated microcarriers with minimal cell attachment at 20x magnification.

Filtering Microcarriers

The media perfusion system flow through the cartridge will be strong enough to pick up the microcarriers. To prevent the microcarriers from entering the system, a filter screen will be placed at the top of the cartridge as pictured in Figure 12**.**

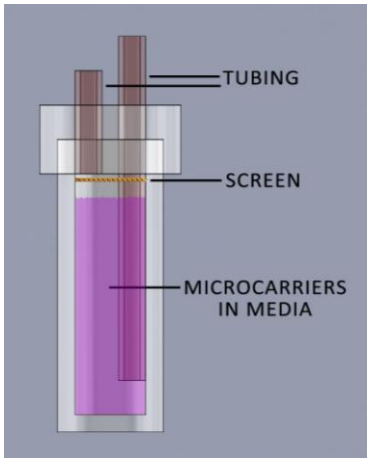

Figure 12: Design of bioreactor cartridge using SolidWorks by bioreactor team, edited to include filter and media.

Materials

The size of the microcarrier beads ranged from 125 – 212 μm in diameter, hence, a MR-compatible brass screen of 106 μm mesh size was manipulated for filter purposes. A solution of non-coated polystyrene microcarriers suspended in deionized water was

used. The ejection of 100-1000 μL pipette was used to simulate the flow in the bioreactor and 0.6 mL Eppendorf tubes was used to capture the flow through.

Protocol

The testing solution had a density of 192.5 microcarriers per 0.1 mL. This solution was placed in a pipette and forced through the filter placed above a 0.6 mL Eppendorf tube. The flow through was then tested for microcarriers under a microscope.

Results and Discussion

Each sample contained no microcarriers proving the filter successful in blocking microcarrier passage. The filter was then observed under a microscope to check for blockage of the filter due to wedging of microcarriers in the mesh. The microarriers, as seen in Figure 13, were too large to wedge into the mesh and moved freely on the surface of the filter. The filter was given to the bioreactor team for integration into their cartridge.

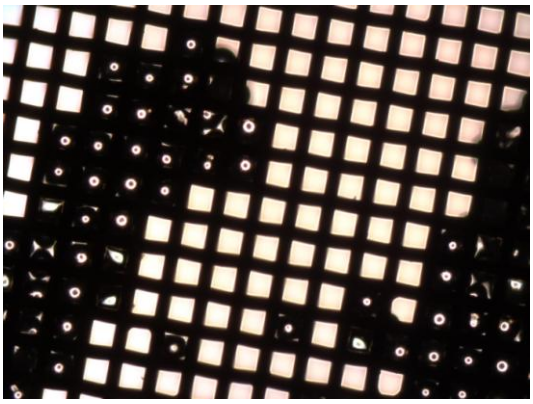

Figure 13: 106 micron mesh size, brass filter under 10x magnification.

Budget

This bioreactor project was separated into two different design projects, a cell scaffold project and a bioreactor project. The client gave both groups a total budget of $3000. For the entire project, the cell scaffold team spent a total of $1,563.88 and the bioreactor team spent $1,100. Therefore the entire project was under budget. Based on the expenses from the experiments, it is assumed that each additional microcarrier experiment will cost approximately $225 for the microcarriers, media, and lab supplies, with the cells adding an extra expense if they need to be purchased.

Future Work

The next step for this project is to test the microcarriers with cells on them in the designed bioreactor cartridge. This will be done to test for leaks, to make sure the filter works properly with the bioreactor cartridge, and insure the cells and microcarriers are infused properly with media throughout the duration of an experiment. This will also help to determine the exact amount of microcarriers needed for proper infusion and a clear MRI signal.

Next, the minimum cell density for clear MRI signals needs to be determined. This is extremely important in saving the client and his graduate students both time and money. The minimum cell density may actually be lower than the client's desired cell density of $5x10^7$ cells/mL because this number is only from a paper in which a clear MRI signal was detected, not a general density used for

MRI studies. Therefore if the client is culturing the cells on the microcarriers for longer than needed, both time and expensive materials are wasted.

In future semester, different cell culturing protocols and techniques along with better equipment will be used to try to promote more efficient cell attachment to all microcarriers in the culture. This semester, many of the microcarriers in culture with the cells were coated with cells, but others had no cell attachment whatsoever. Therefore if improvements can be made to either the protocol or the technique to promote a more efficient attachment, higher cell densities will be achieved which means less culturing time and therefore time and money spent per experiment. Also, the equipment used could have hindered the cell growth from achieving the desired cell densities. Therefore in the future, a shaker plate that can be used in an incubator and spinner vessels will have to be used so the cells can grow in a proper environment.

Additionally, different cell lines will be cultured on the microcarriers in order to determine different cell line's individual attachment and proliferation characteristics. This will be repeated several times to determine the accuracy of the results. In doing this, the general shape and general features of a growth curve for microcarrier culture can be developed. This would help researchers to know what to expect when culturing cells on microcarriers.

Once all these are done, the next step is to contact SoloHill about possibly implementing the newly designed protocol into their microcarrier information. In that way, all researchers looking to use microcarriers for MRI based studies will have a protocol to quickly grow cells on the microcarriers to the needed density for MRI studies.

Conclusion

This semester a protocol for cancer cell cultures on microcarriers to be used in a bioreactor for MRI metabolic pathway studies was developed. Through testing it was found that microcarriers are able to promote cell attachment and proliferation in a 3D space. It was also seen that collagen-coated microcarriers promote quicker attachment and allow for greater proliferation than non-coated polystyrene microcarriers. Furthermore, the protocol for using microcarriers as a scaffold for MRI studies will save researchers both time and money. Although it was observed that microcarriers are able to promote cell attachment and proliferation, because of insufficient equipment only approximately 50% of the client's desired cell density was achieved. In order to optimize the cell densities in the future, better equipment, more experience, and improved techniques are necessary.

Despite the target density not being reached, the density reached using the protocol may be sufficient for the MRI study. The clarity of the signals developed is dependent on the overall amount of pyruvate uptake by the cells. The T47D cell line expresses an elevated number of pyruvate transporters relative to other cell lines tested for these transporters. This characteristic might offset the low cell density, allowing for a clear MRI signal.

References

[1] Rowland, I., Peterson, E., Gordon, J., & Fain, S. (2010). Hyperpolarized13C MR. *Current Pharmaceutical Biotechnology*, *11*, 709-719.

[2] Harris, T., Eliyahu, G., Frydman, L., &Degani, H. Kinetics of hyperpolarized 13C1-pyruvate transport and metabolism in living human breast cancer cells. *PNAS*, *106*(43), 18131-18136.

[3]Keshari, K., Kurhanewicz, J., Jeffries, R., Wilson, D., & Dewar, B. Hyperpolarized 13C spectroscopy and an NMR-compatible bioreactor system for the investigation of real-time cellular metabolism. *Magnetic Resonance in Medicine*, *63*, 322-324.

[4] *Cell Culture Bioreactors.*University of Minnesota. Retrieved February 9, 2011, from http://hugroup.cems.umn.edu/Cell_Technology/Notes/Cell%20Culture%20Bioreactors.pdf

[5] Leung M., Kievit, F. M., Florczyk, S. J., Veiseh O., Wu J., Park J. O., Zhang M., Chitosan-Alginate Scaffold Culture System for Hepatocellular Carcinoma Increases Malignancy and Drug Resistance. Springer Science.**27**: 1939-1948. June 2010.

[6] Weinberg, R, and D Hanahan. "The hallmarks of cancer." *Cell* 100.1 Jan. (2000): 57-70.

[7]Chandrasekaran, P., Seagle, C., Rice, L., MacDonald, J., & Gerber, D. A. (2006). Functional analysis of encapsulated hepatic progenitor cells. *Tissue Engineering, 12*(7), 2001-2008.

[8] Lee, K. H., Lee, P. M., &Siaw, Y. S. (1993). Immobilization of aminoacyclase by encapsulation in poly-L-lysine-stabilized calcium alginate beads. *Journal of Chemical Technology and Biotechnology, 57*(1), 27-32.

[9]Varani J, Dame M, Beals TF, Wass JA. (1983). Growth of three established cell lines on glass microcarriers.BiotechnolBioeng**25**:1359-1372.

[10]*BiosilonNunclon Δ Microcarriers*. Krackeler Scientific, Inc. RetrievedFebruary19, 2011, from http://www.krackeler.com/products/fid/2141

[11]*Cytodex 3.* GE Healthcare. Retrieved February 23, 2011, from http://www.gelifesciences.com/aptrix/upp01077.nsf/Content/Products?OpenDocument&moduleid=167176&cmpid=ppc000029

[12] Samuel, Singer, Souza Kerry, and Thilly G. William. (1995). Pyruvate Utilization, Phosphocholine and Adenosine Triphosphate (ATP) Are Markers of Human Breast Tumor Progression: A 31P- and 13C-Nuclear Magnetic Resonance (NMR) Spectroscopy Study. *Cancer Research* **55**: 5140-145.

[13] *Sigma-SoloHill Microcarrier Beads*. Sigma-Aldrich. Retrieved February 23, 2011, from http://www.sigmaaldrich.com/catalog/ProductDetail.do?N4=Z378674|SIGMA&N5=SEARCH_CONCAT_PNO|BRAND_KEY&F=SPEC&lang=en_US

[14] *Technical Brief: SoloHill Microcarriers*. SoloHill Engineering, Inc. Retrieved March 6, 2011, from http://www.solohill.com/Solohill/Reference/Technical_Brief:_SoloHill_Microcarriers/

[15] Mancuso, A., NJ Beardsley, S. Wehrli, S. Pickup, FM Matschinsky, and JD Glickson. (2004). Real-time Detection of 13C NMR Labeling Kinetics in Perfused EMT6 Mouse Mammary Tumor Cells and BetaHC9 Mouse Insulinomas. *BiotechnolBioeng* **87**(7): 835-48.

[16] Wozniak, Michele A., and Patricia J. Keely. (2005). Use of Three-dimensional Collagen Gels to Study Mechanotransduction in T47D Breast Epithelial Cell. *Biol. Proced.* **7**(1): 144-61.

[17] Whitford, W. &Cadwell, J. (2009). Interest in hollow-fiber perfusion bioreactors growing.*Bioprocess International*. 54-63.

[18] Cell Max Hollow Fiber Bioreactor (2010). *Spectrum Labs*. Retrieved February 26, 2011, from http://www.spectrumlabs.com/cell/MaxCarts.html

[19]Cartridges (2010). *FiberCell Systems*. Retrieved March 5, 2011, from http://www.fibercellsystems.com/products_cartridges.htm

[20] Gillies, R., Ratcliff, R., Galons, J., McGovern, K., Scherer, P., Lien, Y., & Job, C. (1993). Design and application of NMR-compatible bioreactor circuits for extended perfusion of high-density mammalian cell cultures. *NMR in Biomedicine*, *6*, 93-104.

[21] Bartusik, D., Tomanek, B., Siluk, D., Kalizan, R., &Fallone, G. (2009). The application of 19F magnetic resonance ex vivo imaging of 3D cultured breast cells to study the effect of tocopherol. *Analytical Biochemistry, 387,* 315-317.

[22]*AlgiMatrix 3D Culture System.* Invitrogen. Retrieved February 16, 2011, from http://tools.invitrogen.com/content/sfs/brochures/AlgiMatrix%20Flyer.pdf

[23]Dr. Keenan, Tom. Personal Interview.11 February 2011.

[24] Lam, T., Linnes, M., Giachelli, C. & Ratner, B. D. (2007).Mechanical testing and optimizing cell seeding on porous fibrin scaffolds. *Journal of Undergraduate Research in Bioengineering*.

[25]Hoesli, C. A., Luu, M., Piret, James M. (2009). A novel alginate hollow fiber bioreactor process for cellular therapy application.*Biotechnology Progress*. **26**(6): 1740-1751.

[26] *Biomaterials Symposium*. University of Maryland. (2010). Retrieved March 3, 2011, from http://www.bioe.umd.edu/news/news_story.php?id=5312

[27] *SoloHill Microcarrier Beads*. SoloHill Engineering, Inc. Retrieved February 23, 2011, from http://www.solohill.com/files/microcarrier_beads.pdf

[28]*Counting cells with a hemocytometer protocol* (2006, October). Retrieved March 7, 2011, from http://www.mnstate.edu/provost/CountingCellsHemocytometer.pdf

Appendix

A. Product Design Specifications

Project #60: Cell Scaffold

Team Members

Vivian Chen – Co-Leader
Sarah Czaplewski – BWIG
Vanessa Grosskopf – Communicator
Josh Kolz – Co-Leader
Sarah Sandock – BSAC

Problem Statement

Assessing the progression and response to treatment of cancer may be possible by characterizing the metabolic state of cancer cells. Currently, our client uses MRI hyperpolarized carbon-13 labeled pyruvate to evaluate the metabolism of cancer. The objective of our project is to develop a cell scaffold for maintaining the cancer cells within a MR-compatible bioreactor. Cells must adhere and proliferate on this scaffold to sufficiently high densities and sustain viability for visualization of the pyruvate.

Client Requirements

- Grow specified cancer cells to a high density
- Maintain cell viability
- Use materials for scaffold that are compatible with the MRI machine and bioreactor
- Allow sufficient nutrients and experimental agents (i.e. ^{13}C-pyruvate) to reach the cells

Design Requirements

1. **Physical and Operational Characteristics**
    a. *Performance Requirements:* Cells must attach to scaffold and populate it. The scaffold should be coated with a substance that provides evenly distributed cell attachment sites. Also, the chemical interaction between the cells and the scaffold must be minimized. Ideally, a universal scaffold will be used for all cell types tested including Lymphoma K562, Leukemia NKL, Prostate PC3, DU145, LNCaP, U251 and U87 Brain Glioma, and T47D human breast cancer cells.

    b. *Safety:* No ferrous materials may be used in the construction of the scaffolds for the safety of the person conducting the study using the MRI machine.

    c. *Accuracy and Reliability:* Scaffold should be able to culture cells to a density of approximately $5x10^7$ cells/mL within five days and maintain this density for the duration of the experiment which lasts approximately 1 hour. It should sustain cell viability (75%) for five days which includes the culture time and the duration of one experiment. Also,

the scaffold must grow cells to approximately the same density in every use for easy comparison between experiments.

d. *Shelf Life:* The 3D structure of the scaffold should last 5 years and if a separate coating formulation is used, it should last at least one year.

e. *Life in Service:* The scaffold can either be disposable or reusable. If it is disposable, the scaffold will be used for about five days which includes the culture time and the duration of one experiment (1 hour). If the scaffold is reusable, it should last for at least one month.

f. *Operating Environment:* Scaffold will be perfused in a medium to the specific cell type being tested and housed in a MR-compatible bioreactor.

g. *Ergonomics:* User should be able to easily apply and remove scaffold from bioreactor. User should be able to easily assess viability and density of cells on scaffold.

h. *Size:* In previous studies, cell scaffolds were placed within an NMR tube inside a bioreactor. The bioreactor we are using is currently being developed by another team and the inner chamber size has not yet been specified. However, the bioreactor must be able to fit in the 3 inch diameter bore of the MRI machine magnet. Thus, the 3D structure of the scaffold will be smaller than 3 inches in diameter and the coating on the structure should be less than 1 mm thick.

i. *Weight:* The weight of the scaffold should not exceed 10 grams to prevent tipping of the bioreactor.

j. *Materials:* The scaffold materials should minimize chemical interaction with the cells and be biocompatible. Also, the material used should be MR-compatible and contain no ferrous metals. If the scaffold is designed to be reusable, the materials should be autoclave safe.

k. *Aesthetics:* The appearance of the scaffold is not pertinent to this project.

2. **Production Characteristics**
   a. *Quantity:* The quantity depends on if the scaffold is designed to be reusable or disposable. However, the team should focus on making one scaffold which could be reproduced by the client as he sees fit. Also, one generic scaffold for all cell types is ideal but a scaffold tailored to each of the 8 cell types specified would suffice if needed.

   b. *Product Cost:* The budget for the bioreactor and cell scaffold together is between $2000 and $3000. The scaffold should take up a smaller portion of this budget.

3. **Miscellaneous**
   a. *Standards and Specifications*: The scaffold will be designed with the standards and specifications set by our client. Biosafety level 2 constraints must be followed when culturing cells, since we will be working with human cells.

b. *Competition*: Currently, there are a wide variety of scaffold structures and coating used in cell culture. Many types are commercially available such as microcarriers (small beads coated with ECM proteins), hollow fiber scaffolds, and 3D gel structures made of ECM proteins. Still, many other methods have been developed by research labs such as electrostatic calcium alginate encapsulation and the formation of cellular spheroids.

B. 99T Glioblastoma Cell Culture Protocol

(Highlighted text shows changes made to initial protocol)

Preparation for Culture

1. Make medium: DMEM + 10% FBS + 1% Pen Strep
2. Silianize 500 mL bottels to be used in culture to prevent unwanted cell adhesion
3. Place 2.9 of microcarriers in silianized bottle with 30 mL of DI water and autoclave for 30 minutes at 121°C
4. Autoclave micropipette tips, eppendorf tubes, and magnetic stir bars

Lifting the Cells of a Culture Flask

1. Sterilize all materials and hood with ethanol before using
2. Remove medium from T75 flasks containing cells
3. Add 5 mL of sterile PBS without Ca^{2+}or Mg^{2+} to rinse away any extra medium and remove from flask
4. Add 2.5 mL of .05% trypsin per flask, swirl to coat bottom, and put in incubator for 5 min
5. Add 7.5 mL of medium to deactivate trypsin
6. Transfer solution of media and cells (10mL) to a centrifuge tube from each flask
7. Spin in centrifuge at 1000 rpm for 5 minutes
8. Aspirate off liquid media to leave cell pellet
9. Resuspend in 10-13 mL of media and mix well

Counting Cells from Flask

1. In 1.5 mL eppendorf mix 40 uL trypan blue and 40 uL of resuspended cell solution
2. Inject 10 uL of trypan blue/cells into hemocytometer
3. Micropipette 40 uL trypan blue (counting dye) to Eppendorf tube
4. Micropipette 40 ul of well-mixed cell solution to same Eppendorf tube and micropipette up and down to mix
5. Sterilize hemocytometer with ethanol and wipe with kim wipe to dry
6. Place glass slide over hemocytometer grid
7. Micropipette 10 uL of well-mixed dye and cell solution into groove of hemocytometer under the glass slide. The solution should spread out over the grid surface by capillary action
8. Take hemocytometer to microscope at 10x magnification
9. Grid will look like this

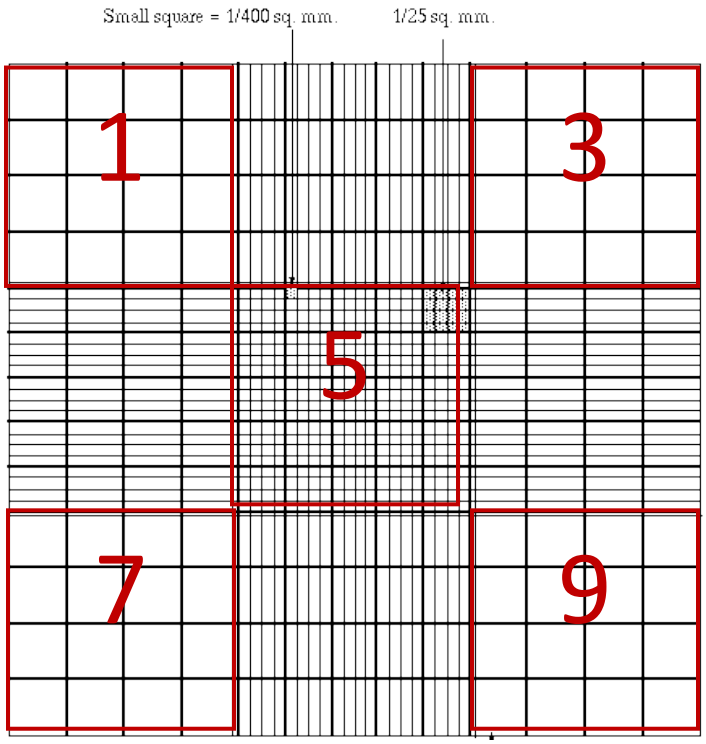

Figure 1: Grid of hemocytometer showing the numbered squares which should be counted.

10. Count cells in areas 1, 3, 5, 7, and 9 only and record. Use following equation to determine number of cells/mL present (2 is a dilution factor and 5 is the number of squares counted)

Equation 1: $\frac{cells}{mL} = \frac{total\ cell\ count * 2 * 10^4}{5}$

11. Multiply number determined in equation by the number of mL used to resuspend cells. This gives you the total number of cells you have
12. Repeat three times to average cell count
13. Clean hemocytometer with ethanol

Seeding the Cells

1. Add remaining resuspended medium/cell mixture to silanized bottles with sterile stir bar
2. Add enough medium to total 200 mL
3. Place bottles in incubator on magnetic stir plate and spin at 60 rpm

Maintaining Culture

1. Continuously spin collagen-coated microcarriers
2. Alternate spinning non-coated microcarriers after first 18 hours. Spin 3 minutes on and 30 minutes of for 8 hours to promote spreading
3. Spin both coated and non-coated continuously for rest of culture

Initial Cell Counting Method from Microcarrier Culture

1. Remove 2 mL sample from culture and put in 15 mL centrifuge tube
2. Add 3 mL of medium
3. Let microcarriers settle
4. Aspirate off medium

5. Add 5 mL of PBS
6. Let microcarriers settle
7. Aspirate off PBS
8. Add 450 uL of .25% trypsin to samples and put incubator for 5 minutes
9. Add 3-5 mL of medium
10. Centrifuge at 1000 rpm for 5 minutes
11. Aspirate media
12. Resuspend microcarriers in 2 mL of medium
13. In 1.5 mL eppendorf mix 40 uL trypan blue and 40 uL of resuspended cell solution
14. Inject 10 uL of trypan blue/cells into hemocytometer
15. Micropipette 40 uL trypan blue (counting dye) to Eppendorf tube
16. Micropipette 40 ul of well-mixed cell solution to same Eppendorf tube and micropipette up and down to mix
17. Sterilize hemocytometer with ethanol and wipe with kim wipe to dry
18. Place glass slide over hemocytometer grid
19. Micropipette 10 uL of well-mixed dye and cell solution into groove of hemocytometer under the glass slide. The solution should spread out over the grid surface by capillary action
20. Take hemocytometer to microscope at 10x magnification
21. Count cells in areas 1, 3, 5, 7, and 9 only show in Figure 1 and record.
22. Use Equation 1 to calculate cells/ml in sample
23. Multiply number determined in equation by the number of 450 uL. This gives you the total number of cells you have in the sample
24. Next multiply the total amount of medium in the culture (200 mL to begin) and divide by the sample size initially taken from the bottle (3 mL) to get total number of cells in culture
25. Repeat three times to average cell count

Revised Cell Counting Method from Microcarrier Culture

1. Remove 3 mL sample from culture and put in 15 mL centrifuge tube
2. Let microcarriers settle
3. Aspirate off medium
4. Add 5 mL of PBS
5. Let microcarriers settle
6. Aspirate off PBS
7. Add 1 mL of .25% trypsin to samples and put incubator for 5 minutes
8. Look under microscope to make sure cells lifted off microcarriers
9. Add 3-5 mL of medium
10. Centrifuge at 1000 rpm for 5 minutes
11. Aspirate media
12. Resuspend microcarriers in 450 uL of medium
13. In 1.5 mL eppendorf mix 40 uL trypan blue and 40 uL of resuspended cell solution
14. Inject 10 uL of trypan blue/cells into hemocytometer
15. Micropipette 40 uL trypan blue (counting dye) to Eppendorf tube
16. Micropipette 40 ul of well-mixed cell solution to same Eppendorf tube and micropipette up and down to mix
17. Sterilize hemocytometer with ethanol and wipe with kim wipe to dry
18. Place glass slide over hemocytometer grid
19. Micropipette 10 uL of well-mixed dye and cell solution into groove of hemocytometer under the glass slide. The solution should spread out over the grid surface by capillary action

20. Take hemocytometer to microscope at 10x magnification
21. Count cells in areas 1, 3, 5, 7, and 9 only show in Figure 1 and record.
22. Use Equation 1 to calculate cells/ml in sample
23. Multiply number determined in equation by the number of 450 uL. This gives you the total number of cells you have in the sample
24. Next multiply the total amount of medium in the culture (200 mL to begin) and divide by the sample size initially taken from the bottle (3 mL) to get total number of cells in culture
25. Repeat three times to average cell count

C. T47D Breast Cancer Cell Culture Protocol

(Highlighted text shows changes made to initial protocol)

Preparation for Culture

1. Make medium: RMPI 1640 + 10% FBS + 1% Pen Strep
2. Silianize 500 mL bottels to be used in culture to prevent unwanted cell adhesion
3. Place 2.9 of microcarriers in silianized bottle with 30 mL of DI water and autoclave for 30 minutes at 121°C
4. Autoclave micropipette tips, eppendorf tubes, and magnetic stir bars

Lifting the Cells of a Culture Flask

1. Sterilize all materials and hood with ethanol before using
2. Remove medium from T75 flasks containing cells
3. Add 5 mL of sterile PBS without Ca^{2+}or Mg^{2+} to rinse away any extra medium and remove from flask
4. Add 2.5 mL of .05% trypsin per flask, swirl to coat bottom, and put in incubator for 5 min
5. Add 7.5 mL of medium to deactivate trypsin
6. Transfer solution of media and cells (10mL) to a centrifuge tube from each flask
7. Spin in centrifuge at 1000 rpm for 5 minutes
8. Aspirate off liquid media to leave cell pellet
9. Resuspend in 10-13 mL of media and mix well

Counting Cells from Flask

1. In 1.5 mL eppendorf mix 40 uL trypan blue and 40 uL of resuspended cell solution
2. Inject 10 uL of trypan blue/cells into hemocytometer
3. Micropipette 40 uL trypan blue (counting dye) to Eppendorf tube
4. Micropipette 40 ul of well-mixed cell solution to same Eppendorf tube and micropipette up and down to mix
5. Sterilize hemocytometer with ethanol and wipe with kim wipe to dry
6. Place glass slide over hemocytometer grid
7. Micropipette 10 uL of well-mixed dye and cell solution into groove of hemocytometer under the glass slide. The solution should spread out over the grid surface by capillary action
8. Take hemocytometer to microscope at 10x magnification
9. Count cells in areas 1, 3, 5, 7, and 9 only of Figure 1
10. Use Equation 1 to calculate the number of cells/mL
11. Multiply number determined in equation by the number of mL used to resuspend cells. This gives you the total number of cells you have
12. Repeat three times to average cell count
13. Clean hemocytometer with ethanol

Seeding the Cells

1. Add remaining resuspended medium/cell mixture to silanized bottles with sterile stir bar
2. Add enough medium to total 60 mL
3. Place bottles in incubator on shaker plate and spin at 80 rpm which is the slowest speed where the microcarriers lifted the bottom of the bottle and evenly mixed

Maintaining Culture

1. For the first 3-6 hours alternate spinning both collagen-coated and non-coated microcarriers for 3 minutes and 30 minutes off
2. Spin culture continuously
3. After a 24 hour attachment period at 140 mL to give a total of 200 mL
4. Replace 1/2 – 2/3 of cell medium every day

Counting Cells from Microcarrier Culture

1. Remove 3 mL sample from culture and put in 15 mL centrifuge tube
2. Let microcarriers settle
3. Aspirate off medium
4. Add 5 mL of PBS
5. Let microcarriers settle
6. Aspirate off PBS
7. Add 1 mL of .05% trypsin to samples and put incubator for 5 minutes
8. Add 3-5 mL of medium
9. Centrifuge at 1000 rpm for 5 minutes
10. Aspirate media
11. Resuspend microcarriers in 450 uL of medium
12. In 1.5 mL eppendorf mix 40 uL trypan blue and 40 uL of resuspended cell solution
13. Inject 10 uL of trypan blue/cells into hemocytometer
14. Micropipette 40 uL trypan blue (counting dye) to Eppendorf tube
15. Micropipette 40 ul of well-mixed cell solution to same Eppendorf tube and micropipette up and down to mix
16. Sterilize hemocytometer with ethanol and wipe with kim wipe to dry
17. Place glass slide over hemocytometer grid
18. Micropipette 10 uL of well-mixed dye and cell solution into groove of hemocytometer under the glass slide. The solution should spread out over the grid surface by capillary action
19. Take hemocytometer to microscope at 10x magnification
20. Count cells in areas 1, 3, 5, 7, and 9 only show in Figure 1 and record.
21. Use Equation 1 to calculate cells/ml in sample
22. Multiply number determined in equation by the number of 450 uL. This gives you the total number of cells you have in the sample
23. Next multiply the total amount of medium in the culture (200 mL to begin) and divide by the sample size initially taken from the bottle (3 mL) to get total number of cells in culture
24. Repeat three times to average cell count

D. Data Sheet for Cell Counts

Table 2: Calculated values for 99T cell culture.

Days	0	2	7	11
	99T coated			
Count 1		2	149	12
Count 2		2	202	5
Count 3		3	164	12
Count avg		2.333333	171.6667	9.666667
Total cells	1.95E+07	1.24E+06	2.06E+07	1.15E+06
Total std		3.08E+05	3.28E+06	4.84E+05
	99T non-coated			
Count 1		0	0	11
Count 2		2	2	4
Count 3		2	0	10
Count avg		1.333333	0.666667	8.333333
Total cells	1.87E+07	7.11E+05	7.99E+04	9.99E+05
Total std		6.16E+05	1.38E+05	4.54E+05

Table 3: Calculated values for T47D cell culture.

Days	0	1	2	3	4	5	6
	T47D coated						
Count 1		211	588	702	136	300	263
Count 2		157	424	619	254	318	317
Count 3		211	347	564	182	251	280
Count avg		193	453	628.3333333	190.6666667	289.6666667	286.6666667
Cells/mL	5885700	2610132	17503920	23793224	14292678	10633084	10522960
Total std		611068	6893006	3812613	3230708	1883529	98986542
	T47D non-coated						
Count 1		174	197	250	226	163	7
Count 2		202	138	224	253	187	28
Count 3		175	152	224	274	115	8
Count avg		183.6666667	162.3333333	232.6666667	251	155	14.33333333
Cells/mL	5299200	2483908	6272560	8810435	9310694	5629848	520610
Total std		214829	1191174	568429	911176	1388234	448584