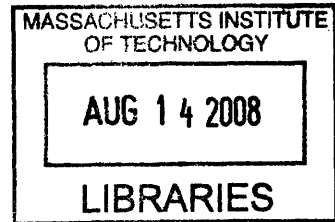


Scalable Production of Cellular Aggregates for the Differentiation of Embryonic Stem Cells into Cardiac Muscle

by

Joshua Garvin



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JOSHUA GARVIN

Submitted to the Department of Mechanical Engineering
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ABSTRACT

Embryonic stem (ES) cells have the potential to treat many diseases, such as heart disease, diabetes, and Parkinson's disease. However, large numbers of desired differentiated or progenitor cells must be generated from ES cells for many regenerative medicine applications to be successful. Current methods of culture used in the laboratory either cannot be scaled-up to produce sufficiently large numbers of cells or do not consistently produce aggregates of uniform size. In this study, novel methods for aggregating and encapsulating embryonic stem cells were investigated. Latex microspheres and the β TC3 cell line were used in place of ES cells during the development of the methods. Microspheres and cells were encapsulated in an alginate solution coated with poly-L-lysine using an established drip method and a novel fluorinated oil "floating drop" method. Results from these experiments demonstrate that both methods can be used for encapsulating and growing cells. However aggregation, an important aspect for the directed differentiation of ES cells, only occurred using the "floating drop" method, and this method was used to encapsulate a predetermined number of cells in capsules of a specified size. The "floating drop" method has the advantage that culture media can be changed during cell culture to increase the duration of experiments without transferring the aggregates to culture flasks and can potentially be scaled up to produce large numbers of encapsulations.

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I. INTRODUCTION

Cell microencapsulation is a recent technology that has a large potential for medicine and drug delivery. In this process, capsules which have a semi-permeable polymer membrane are able to protect the encapsulated cells from the immune system. The thin polymer membrane allows for nutrients essential to the cells metabolism to diffuse in and waste products to diffuse out.

For embryonic stem cells, aggregation is essential for the generation of many cell types. The methods used now in the laboratory to aggregate the cells and induce embryoid body formation do not generate enough embryonic stem cells to be useful in regenerative medicine or cannot be sufficiently controlled to dictate the cell number or shape of the embryoid bodies. These methods include the hanging drop and liquid suspension culture.¹ Clinical medicine and technology based on stem cells is limited by the insufficient size of cells masses. Scientists now try to control the differentiation of embryonic stem cells into heart muscle cells, blood cells, or nerve cells by changing the chemical composition of the culture medium, altering the surface of the culture dish, or modifying the cells by inserting genes.²

In this study, stem cell aggregation will be combined with cell microencapsulation. Liquid filled capsules containing a cell suspension will be made as a new way for the scalable production of cellular aggregates for stem cell differentiation. Encapsulation and microfluidic technologies will be used to perfect the method of encapsulating, and hopefully in the future, the methods developed will be applied to encapsulating stem cells and finally, differentiating embryonic stem cells inside these capsules.

In order to achieve microencapsulation and aggregation, established methods will be used and a new floating drop method will be developed. The established method that will be used involves making capsules using alginate and calcium chloride (CaCl_2) and subsequently adding a layer of poly-L-lysine (PLL) around the capsule to increase the durability of the capsule. The floating drop method involves first placing a measured drop composed of cell media and a predetermined number of cells on perfluoro-compound FC-70 (fluorinated oil), and waiting for the cells to settle into a single aggregate. Following this, the drop will be combined with another measured drop of alginate in order for the aggregate and alginate to mix. Lastly, this mixture will be combined with a measured drop of CaCl_2 to gel the mixture and form a capsule.

Latex microspheres as well as βTC3 mouse pancreatic cells will be used to perfect each method and ensure both encapsulation and aggregation occurs. Also, the growth of the βTC3 cells will be documented over time in an incubator.

The latex microspheres will first be tested using each method following by βTC3 cells. The rationale for this is to gradually increase the complexity of the experiments. It was determined that microspheres are the most simplistic yet still realistic particles to aggregate and encapsulate because they do not need to be cared for like a living cell, yet their dimensions like size and shape are similar to actual stem cells. It was then decided that βTC3 cells were ideal to use after the microspheres because they are more complex than microspheres and less complex than actual stem cells. They would serve a bridge in order to apply the developed methods to stem cells. Their dimensions are similar to stem cells and since they are cancerous, pancreatic, mouse cells, they have fast growth rates which will allow more data to be collected than non cancerous cells. Also because they are live cells, the effects of cellular interaction during aggregation and growth, which is essential to forming embryoid bodies, will be observed. The

next step in complexity for these experiments would be using actual embryonic stem cells, which this study does not encompass.

II. BACKGROUND

Stem Cells

Stem cells are a type of cell that is characterized by three properties: they are unspecialized, they have the ability to renew themselves through cycles of cell division and they have the ability to differentiate into various cell types with specialized functions upon being induced.² The last stated property is called potency which is the latent ability of the stem cell to differentiate into different cell types. The different types of potency's are totipotent, in which stem cells can differentiate into embryonic and extraembryonic stem cells, pluripotent, in which stem cells can differentiate into cells of the germ layers, multipotent, in which stem cells can differentiate into a related family of cells, and unipotent, in which the stem cells can only produce one cell type.³

In mammals, there are two main stem cell types, embryonic and adult. Embryonic stem cells are found in blastocysts, also known as embryos. Embryonic stem cells are pluripotent and can develop into nearly every cell found in the body if they are induced to do so. They are grown in laboratories either on gelatin or fibroblasts depending on whether the embryonic stem cells are mouse or human, and require the presence of various growth factors. If, after a period of 6 months or more, the embryonic stem cells have grown but not differentiated, they can be deemed pluripotent.² Other embryonic stem cell tests include identifying surface markers found on undifferentiated cells, observing the chromosomes to ensure the number hasn't changed, differentiating the cells to form specific types of cells as well as other methods.² Embryonic

stem cells can be stimulated to differentiate into different types of cells by culturing the cells in various chemically composed conditioned medium, altering the surface of the cell culture dish, or by inserting specific genes into the embryonic stem cells.

Under certain conditions, embryonic stem cells can proliferate for months and remain undifferentiated. If they are allowed to aggregate together, differentiation takes place and they form embryoid bodies. Embryoid bodies are ball-like aggregates of cells. Through initial contact with other embryonic stem cells, the embryoid bodies differentiate spontaneously.⁴ As the differentiation within the embryoid body continues to take place, internal structures like parietal endoderms, also called yolk sacs, and visceral endoderms form.⁵ Cardiomyocytes, or heart muscle cells, are also formed in the embryoid body and pump essential nutrients throughout it. The drawback of spontaneous differentiation within the embryoid body is that differentiation into specific cell types can't be efficiently controlled by growth factors and other means.⁴

The second type of stem cell, the adult stem cell, like the embryonic stem cell is undifferentiated but is not derived from blastocysts or embryos. They are derived from sources such as bone marrow, liver, neural tissues, skin, muscle as well as other tissues and organs.² As such, most of these adult stem cells are limited in their potency and can only differentiate into the specialized cell types of the organ or tissue from where they came. Nearly all adult stem cells are multipotent although recently a few pluripotent adult stem cells have been discovered in place such as umbilical cord blood.² The pluripotent ability of adult stem cells is called plasticity. Adult stem cells are defined by the ability to divide and create another cell like itself as well as creating a cell more specialized than itself. Adult stem cells are identified in several ways including labeling the cells with a molecular marker in tissue and determining the

differentiated cell they produce, removing the cells from one mammal, marking them, and putting them back in another mammal to determine whether they proliferate and grow their tissue of origin, or finally growing the cells in a cell culture, adding genes or growth factors and observing what type of cells they can become.²

Stem Cells in Medicine

The interest in stem cells over the past decade is a result in a greater belief in their ability to be used in regenerative medicine and clinical applications. Currently, adult stem cell therapies exist but embryonic stem cell uses and therapies have not been created, although there is a wide scope in which many researchers believe they can be used in the future. Adult stem cells are currently used for patients who need a bone marrow transplant used to treat leukemia as well as various other blood disorders using stem cells from the bone marrow, peripheral blood, and umbilical cord blood.² Adult stem cells are useful in regenerative medicine because they can be isolated from a patient and have the ability to repopulate damaged tissue in a living mammal. Since the adult stem cells come from the patient, there is not possibility for rejection. Other current adult stem cell therapies include treating autoimmune diseases, cerebral palsy, type 2 diabetes, heart failure, multiple sclerosis, osteoarthritis, degenerative joint disease, Parkinson's disease, rheumatoid arthritis, stroke and many other ailments.⁶

Potential embryonic stem cell therapies focus on adult stem cells. Differentiated human embryonic stem cells could be used to test new drugs. Human embryonic stem cells have the ability to differentiate into any type of cell, so a wide range of drugs for various ailments of the body have the potential to be tested. Other potential uses of human embryonic stem cells include cell-based therapies. These therapies include differentiating cells to grow into specialized cells,

tissue and potentially organs for patients in need of transplant. Human embryonic stem cells are a prospective renewable source of cells that can be used to treat spinal cord injury, burns, Alzheimer's diseases, type 1 diabetes and heart disease. Human embryonic stem cells can also be used to transport bioactive gene products to deliver a therapeutic protein.⁴

Stem Cells into Cardiac Muscle

In this study, methods of aggregating and encapsulating stem cells were developed with the potential of being used for cardiac muscle to treat heart disease. Many forms of heart disease occur because cell types, usually cardiomyocytes, lose functionality. Adult cardiomyocytes have limited regenerative capacity and therefore can't regenerate myocardium, the muscular tissue of the heart, once a heart attack occurs. Eventually, the left ventricle dilates which leads to progressive heart failure.⁵ currently, research has focused on the replacement of the myocardium by implanting myogenic cells but clinical applications of this have been hindered due to an insufficient number of cells for tissue implantation.⁵

Most research into using human embryonic stem cells focuses on applying it to cell replacement therapy in which diseased, dead, or scarred tissue is replaced. Once any significant myocardial cell death occurs from disease, infection or other conditions, the performance of the myocardial cells is worsened which will ultimately lead to heart failure. Current promising research into solving heart degeneration and heart failure deals with cellular cardiomyopathy where new tissue is grown for the damaged or missing portions of the heart. This tissue grafting is done using various types of cells including skeletal myoblasts, fibroblasts, fetal and neonatal cardiomyocytes, mouse embryonic stem cells, and bone marrow. All of these cells have proven to grow heart tissue and improve the performance of the heart once they have differentiated.⁵

The limiting factor in this approach has also been the inability to obtain large quantities of human cardiomyocytes.

The most common method used for inducing differentiation of embryonic stem cells into cardiac muscle requires an initial aggregation of cells to form embryoid bodies. Within the formed embryoid bodies, cardiomyocytes tissue as well as other cell types can be identified by their heart-like beating. Various factors influence the ability of embryonic stem cells to differentiate into cardiomyocytes including the cell lines used, the number of cells allowed to aggregate, the length of time the embryoid bodies are cultivated, the type of cell culture media used and growth factors.⁵

Several hurdles block the clinical applications of using human embryonic stem cells to be used for heart regeneration including development of strategies for directing the stem cells to differentiate into cardiomyocytes, creating selection protocols to allow pure populations of cardiomyocytes to be generated for transplantation, increasing the yield from stem cell differentiation to numbers which can be used for clinical applications and developing methods to prevent the graft cells from being rejected by the immune system.⁵ This study hopes to address the last two issues through a promising new method of scaling up the production of stem cell aggregates and encapsulating them with a polymer which potentially will not get reject by the immune system.

A typical heart attack results in the death of up 1 billion cardiomyocytes. Additionally, if tissue grafting were to be used to repair the damaged heart, the majority of the transplanted cells do not survive cell grafting. Therefore, any method which hopes to replace the damaged cardiomyocytes of a heart needs to produce a few billion cardiomyocytes to be a useful application.⁵

Current Encapsulation Methods

There are several encapsulation methods used today to encapsulate cells or some other core. Most of the encapsulation taking place today for biotechnical purposes that use polymers use microencapsulation, as opposed to macro encapsulation. Microencapsulation of cells has a few advantages over macro-encapsulation and conventional suspension culture. First, microcapsules are usually spherical in shape and have a better surface-to-volume ratio than macro-capsules, which are usually hollow fibers or diffusion chambers. Microcapsules can be made to allow important nutrients and oxygen to diffuse inside while allowing cell derived waste products like carbon dioxide to diffuse out of the permeable membrane. By being so small in size, microcapsules have a better chance of getting past immune defenses and not being rejected because there are thousands of them as compared to a single large one. Microcapsules can be directly implanted into a patient without large scale surgeries taking place.⁷ Microcapsules also have the advantage over suspension culture of being able to achieve higher cell densities and resistance to most forces created by agitation and aeration.⁸

Currently, microcapsules used in clinical research are made up of a variety of hydrogels including alginate, agar, agarose, gellan gum, chitosan, PLL, acrylate copolymers and synthetic polymers. The most prominent and widely used encapsulation materials used are alginate and PLL. This is because encapsulation can occur in room temperature conditions and involve non-toxic components that are suitable to use for mammalian cells. Further, alginate is one of the most abundant naturally occurring polymers so it can be used and studied extensively.⁹ Alginate-PLL encapsulation can take place in a variety of ways. The actual capsule or bead is

always formed by cross-linking the alginate with an oppositely charged counterion, usually Ba^{2+} or Ca^{2+} . Ca^{2+} cross-linked microcapsules have a disadvantage compared to Ba^{2+} cross-linked microcapsules because those microcapsules can be liquefied by chelators, resulting in a liquid core. Once these alginate capsules are formed, a coat of PLL or other polyelectrolytes such as polyethylene imine, polybrene or polyethylene glycol, is applied to the capsule. After this, the core is usually liquefied, and then a second or third coat of alginate is added to promote longevity of the microcapsule.⁷

In order for the beads to be formed, cells are first suspended and mixed in an alginate solution for a period of time. This solution is forced through a nozzle like a syringe and droplets are formed upon exiting the nozzle. These droplets fall into a NaCl solution containing Ba^{2+} or Ca^{2+} which cross-links with the alginate to form capsules.

Other methods of drop formation include using a coaxial air jet in which a solution of alginate and cells are forced through a nozzle surrounded by a jet of air. This is usually used to form capsules that are small and uniform in size, although the size can be varied by changing the air flow. Additionally, vibrations are often incorporated into the jet flow, causing the beads to fall at timed intervals. This causes smaller drop sizes and greatly limits the size distribution of the beads. Other techniques and devices used to make drops are using a three-channel bead generator. In this model, solid beads with a liquid core are able to be formed directly from the generator. Air is forced out of the outermost nozzle, cells by themselves or a combination of low cells and alginate are forced out of the inner nozzle and alginate with a greater viscosity (weight by volume) is forced out of the central nozzle.⁷

Other novel methods of cell encapsulation include microinjecting cells into capsules. In this method, macro-capsules are automatically injected with cells to avoid cellular stress and

heterogeneous distribution of cells in the capsules. Another method of encapsulating cells involves coating a core particle with a hydrogel then degrading the core particle resulting in an empty capsule. Following this, cells are injected into the hollow hydrogel.¹⁰ Many of these new and established methods have benefits and drawbacks when applied to encapsulating stem cells for clinical application, which usually include inability to scale up or to produce a specific size range of embryoid bodies.

Current Embryoid Body Formation Methods

There are various methods currently in use today that promote embryoid body formation of stem cells. These standard methods include the hanging drop method, liquid suspension, static suspension, and methylcellulose culture.¹¹ All of these methods are culture systems in which stem cells are allowed to aggregate to form embryoid bodies. The most popular of these methods is the hanging drop method. In this system, cells mixed in media or another liquid are placed onto the lid of a Petri dish in volumes of 15-20 μ L. The lid is then flipped upside down and placed in an incubator. Over a period of time, the stem cells aggregate at the bottom of the drop into a three-dimensional structure and begin differentiating into an embryoid body.¹ The drawbacks of this method are that the media in which the cells are in can't be changed and the number of embryoid bodies can't be scaled up because of limited space on a Petri dish.

In suspension culture, embryoid bodies are formed by introducing stem cells onto a differentiation medium and the cells aggregate spontaneously into three dimensional structures. This method can produce hundreds to thousands of embryoid bodies, however the shape and size of them can't be controlled. Also, embryoid bodies have a tendency of agglomerating, meaning various embryoid bodies grow together producing irregular shaped cell masses.¹ An ideal

method of aggregating stem cells would be able to produce specific sized embryoid bodies on a large scale.

Other new methods of generating embryoid bodies are using rotary suspension cultures. In this method, stem cells are placed in a differentiation medium then placed in a rotary suspension culture which is attached to an orbital rotary shaker. Spheroid embryoid bodies are formed in the orbital rotary shaker more efficiently because of the constant circular motion. Multiple Petri dishes can be placed on the rotary platform at the same time, allowing more embryoid bodies to be made faster.¹

Recently, spinner flask, slow-turning vessel, and high-aspect rotating vessel bioreactors have used the principle of rotary motion to make embryoid bodies. The circular motion not only improves the transfer of nutrients to the forming embryoid bodies but also influences aggregation and the size of the embryoid bodies.¹ These methods also suffer from not being able to precisely control the size of the embryoid bodies.

Specific Aims

Embryonic stem cells, both human and adult, have the potential to be used for a wide variety of applications in medicine and biotechnology, only a few of which are in use today. The main barriers which prevent embryonic stem cells from being used more for clinical trials in these areas are the inability to scale up embryoid body formation using established methods as well as the inability for these methods to be automated or process-controlled.⁹ Further, conventional methods of embryoid body formation lack the ability to reproducibly generate homogeneous cell populations. This research is a mostly qualitative approach in the formation and analysis of the experiments. It is hoped that through this research, it will be shown that

potentially, an easy and effective method to form embryoid bodies from embryonic stem cells has been developed. Specific aims are as follows:

1. Utilize alginate-PLL encapsulation materials in an established “falling drop” method to encapsulate particles. Alginate mixed with particles of microspheres or β TC3 cells were dropped into beakers containing CaCl_2 and were then coated with a layer of PLL. The resulting appearance and size of the capsules were documented as well as the aggregation behavior of the particles.

2. Develop a new floating drop method to aggregate and encapsulate particles.

2a. Use the floating drop method with alginate-PLL encapsulation materials to encapsulate particles. A drop of alginate mixed with particles of microspheres or β TC3 cells were dropped into a Petri dish containing fluorinated oil. The particles were allowed to aggregate for a period of time then a droplet of CaCl_2 was combined with it to gel the alginate/particle mixture followed by a droplet of PLL. The appearance of the capsules as well as the aggregation behavior of the particles was documented.

2b. Use the floating drop method with cell culture media combined with alginate-PLL encapsulation materials to first aggregate, then encapsulate particles. A drop of DMEM cell culture media with particles of β TC3 cells were allowed to aggregate for a period of time on fluorinated oil. This mixture was then combined with a drop of alginate followed by a drop of CaCl_2 to encapsulate the β TC3 cells. The aggregation behavior of the cells as well as the appearance of the capsules was documented.

III. METHODS AND MATERIALS

Falling Drop Protocol

The materials use for this protocol include sodium alginate (Sigma-Aldrich, St. Louis, MO) solutions prepared in concentrations of 0.15%, 0.33%, 1.5%, and 3.3% weight by volume. The sodium alginate was made by dissolving alginate in Ca-free krebs and letting the solution sit overnight on a heated magnetic stir plate at a temperature of 50°C. 100mM of CaCl₂, 2H₂O, .1% PLL, hepes (Sigma-Aldrich, St. Louis, MO), 310mM of Ca-free krebs, krebs, and 55mM sodium citrate solutions were made. Table 1 shows the materials used to make 100mM of CaCl₂, 2H₂O, 310mM of Ca-free krebs, krebs.

100mM CaCl ₂ , 2H ₂ O		Hepes		Ca-Free Krebs 310mM		Krebs 25mM	
Material	Mass (g)	Material	Mass (g)	Material	Mass (g)	Material	Mass (g)
CaCl, 2H ₂ O	1.47	NaCl	0.771	NaCl	0.789	NaCl	0.777
KCl	0.015	KCl	0.035	KCl	0.035	KCl	0.035
Hepes	0.238	MgCl ₂ , 6H ₂ O	0.024	Hepes	0.596	KH ₂ PO ₄	0.016
		Hepes	0.596	KH ₂ PO ₄	0.016	MgSO ₄ , 7H ₂ O	0.03
				MgSO ₄ , 7H ₂ O	0.03	Hepes	0.596
						CaCl, 2H ₂ O	0.037

Table 1: Composition of 100mM of CaCl₂, 2H₂O, 310mM of Ca-free krebs and krebs. The masses given are the amount of each material needed to make 100mL of the various media for microencapsulation. All materials were bought from Sigma-Aldrich, St. Louis, MO.

Before encapsulation, alginate was mixed with either red dyed microspheres (Estapor, Fishers, IN: 0.1µm diameter), green microspheres (Estapor, Fishers, IN: 9.85µm diameter), or βTC3 (ATTC, Manassas, VA) in a 15mL tube for various experiments. This tube was centrifuged for 6 seconds to allow for an adequate mixing of the particles and alginate. Next, 1mL of the resulting mixture was sucked inside a 1mL syringe. For earlier experiments, a 20 or 22 gauge needle was placed on the end of the syringe and the droplets were formed by pushing

on the plunger and extruding out droplets of the alginate-particle solution. For later experiments, droplets were formed by connecting the 1mL syringe to a vacuum pipet-aid (Drummond Scientific Co., Broomall, PA) and switching the settings to push air instead of vacuum in order to make the droplets smaller. The pipet-aid had variable air flow so the droplet size could be changed. The syringe plunger was pushed and the alginate-particle mixture was extruded from the end of a 22 gauge needle. For all experiments, the capsules were formed by allowing the droplets to fall into a beaker containing a 20mL bath of CaCl_2 . Figure 1 below shows the setup of the experiments using only a syringe and a syringe with a pipet-aid.

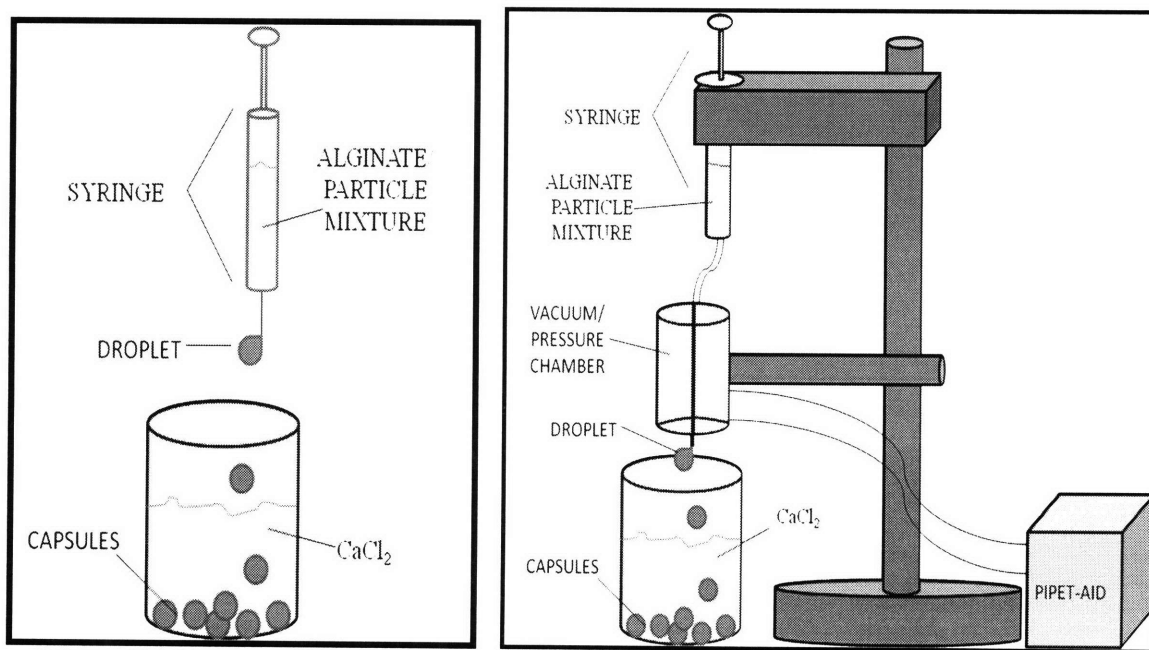


Figure 1: Diagram of the falling drop experiment. On the left, only a syringe is used to produce the capsules. This method produces larger capsules than the method on the right, in which a syringe is connected to a pipet-aid. The pipet-aid pumps air into a chamber to produce air pressure and the fast moving air exits the bottom of the chamber around the needle and over the droplet. The added force on the droplet makes it fall from the needle sooner, as a smaller droplet, than it would from just gravity alone.

The capsules were allowed to polymerize in the CaCl_2 solution for 15-30 min before the CaCl_2 was removed using a pipette and the formed capsules were washed with saline or hepes

for 3 minutes. Following this, the hepes was removed with a pipette and 15mL of PLL was added to the capsules. The PLL coated the capsules for 10 minutes to add a shell in order to increase the durability of the capsules. The PLL was removed from the beaker with a pipette and the capsules were washed with 15mL of saline for 3 minutes. The saline was removed using a pipette and a 15mL of 10-times diluted sodium alginate solution was added to the beaker to further strengthen the outer shell of the capsules. The diluted alginate solution was removed after 5 minutes using a pipette and the capsules were washed with 15mL of saline. The chelation, or liquefaction, of the calcium-alginate core was performed by adding sodium citrate from 10 minutes to an hour. Sodium citrate was used to remove the calcium in the core and replacing it with sodium, in the process liquefying the core. Finally, the sodium citrate was removed with a pipette and the capsules were washed with saline. The capsules were ultimately suspended in a Petri dish containing a saline solution to allow the capsules to be observed. During all incubation periods, the capsules were gently stirred.

Floating Drop Protocol

The materials used for this protocol include perfluoro-compound FC-70 (“fluorinated oil,” Sigma-Aldrich, St. Louis, MO), 0.33% and 1.5% sodium alginate weight by volume, DMEM (ATTC, Manassas, VA), and 100mM of CaCl₂, 2H₂O. These materials were prepared in the same manner as those materials from the falling drop experiments.

For the first experiments with the floating drop method, 25mL fluorinated oil was poured into a Petri dish. This amount was decided by the constraints imposed by the key dimensions of the experiment. The fluorinated oil had to be deep enough so that a drop floating on it would not touch the bottom of the Petri dish, which wouldn’t allow for proper aggregation of the particles.

However it couldn't be so deep that it would spill over the edges if it was agitated or carried to be viewed under a microscope. Following this, alginate was combined with red dyed microspheres or green microspheres in a small 2mL tube for various experiments. This tube was centrifuged for 6 seconds to allow for an adequate mixing of the microspheres and alginate. Next, the resulting mixture was sucked inside a pipette tip and a single drop was placed onto the fluorinated oil using a pipette with a connected tip. The drop sizes used were between .5 μ L and 2 μ L. The drops were left undisturbed for a period of time on the fluorinated oil in order for the microspheres to aggregate on the bottom of the drop. Next, drops of CaCl₂ were added on both sides of alginate drop. The drops were brought close together using the tip of a micropipette and once they were close enough, the attractive forces between them allowed them to combine instantaneously. The CaCl₂ was allowed to polymerize the sodium-alginate mixture for 10 minutes before the formed capsule was gently removed with a pipette tip and placed inside of a Petri dish containing 20mL of DMEM. The Petri dish was placed inside of an incubator and the cells were allowed to grow. Initially, the aggregation step would only last for 15-30 minutes. This is because the charges on the drop of fluorinate oil would make the drop move towards the walls of the Petri dish and once it made contact with the walls, the experiment had to be repeated. The tip of the micropipette was used to keep the drops away from the walls as best as possible by placing the tip near the drop or into the fluorinated oil near the drop to attract the drop towards the tip. The 15-30 minute aggregation time proved to be an insufficient amount of time for the cells to aggregate.

The next experiments followed the same procedures but used β TC3 cells instead of microspheres. In these experiments, a predetermined amount of cells were added to 5mL of DMEM which was placed in a 15mL tube. The amount of cells added was such that there would

be 500 cells per specified volume of DMEM. The number of cells was found from using a cytometer. The cell/DMEM mixture was dropped into the fluorinated oil and allowed to aggregate for 24-72 hours prior to adding a drop of alginate next to it and allowing them to combine. Some of the drops still moved towards the walls but several experiments were performed at the same time, larger square Petri dishes were used, and agitation was kept to a minimum so that the cells could actually aggregate for longer periods of time. The cell/DMEM drops were allowed to mix for 20 minutes with the alginate which was followed by adding drops of CaCl_2 to both sides of the floating drop. The CaCl_2 drops and the cell/DMEM drop were combined using the tip of a micropipette. Figure 2 below shows the setup of the floating drop experiments.

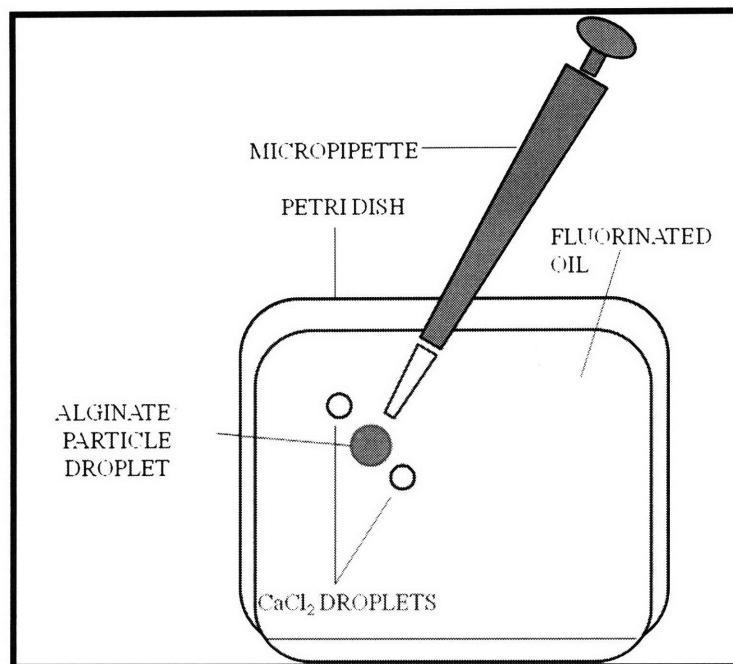


Figure 2: Diagram of the floating drop experiment. During the experiments, drops of microspheres or β TC3 cells mixed with alginate or DMEM, were allowed to aggregate on fluorinated oil. Larger droplets were dropped onto the fluorinated oil while smaller droplets were placed onto the fluorinated oil because the surface tension binding it to the micropipette tip was much greater than gravity pulling it down.

Cell Culture

All cell cultures were incubated in a humidified 37°C atmosphere. The beta cell lines were maintained in a gelatin coated T-75 flask in DMEM. The cells were split and passaged every 3 days when they are approximately 50% confluent. The DMEM was aspirated out and replaced with fresh media every 3 to 4 days when the cells were not in use. In preparation to split the cells, 0.05% trypsin/0.53mM EDTA was pre-warmed in a water bath held a constant 37°C. The media was aspirated out inside a biological safety cabinet and the cells were washed with 3mL of PBS. 1.5mL of the pre-warmed trypsin solution was added to the flask, and the flask was placed inside of an incubator for 7 minutes to active the trypsin. After this, 3mL of DMEM was added to the flask and the resulting mixture was aspirated up and down to disperse the cells. This trypsin, DMEM, and cell mixture was placed in a 15mL tube and centrifuged for 3 minutes at 300 RCF. Following this, the supernatant was aspirated out leaving the cells at the bottom of the tube and 1mL of DMEM was added to the cells. The cells were dispersed by aspirating up and down. The cells were then replated on the gelatin coated T-75 flask with a splitting ratio of 1:4 and 25mL of DMEM was added to the flask. The flask was returned to the incubator until the cells were 50% confluent or another experiment needed to be performed. Passages 15-23 were used for the experiments.

IV. RESULTS AND DISCUSSION

Falling Drop

The initial experiments were performed using either the red microspheres or the green microspheres with the falling drop protocol using needle and syringe. Figures 3 and 4 show pictures of one of the first capsules made using red microspheres.

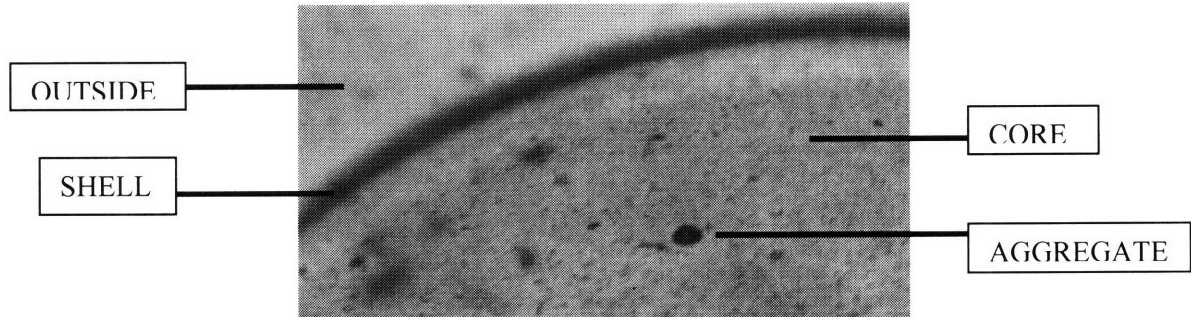


Figure 3: Core and shell of a capsule made using red microspheres and 1.5% alginate. The alginate and microsphere mixture was allowed to polymerize in CaCl_2 for 15 minutes and the formed capsules were treated with citrate for 7 minutes. A small aggregate has formed in the capsule but overall, the microspheres are spread out inside the capsule. Note all pictures were taken from the top down because of equipment limitations.

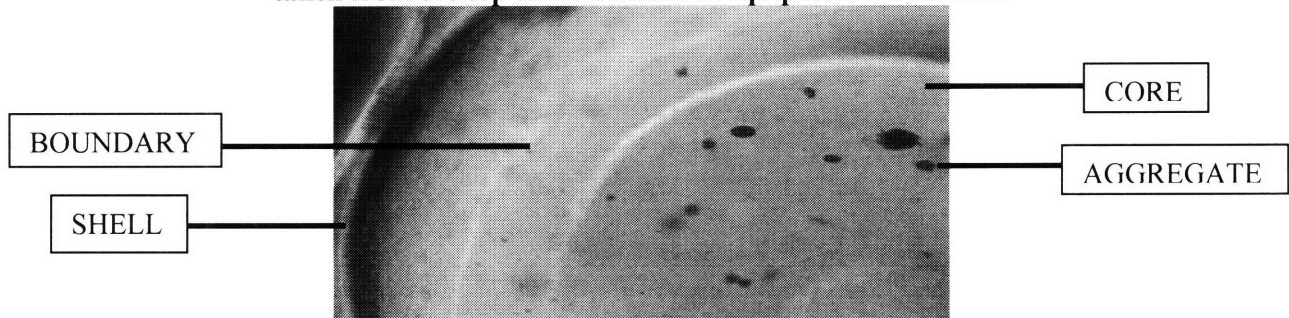


Figure 4: Core and shell of a capsule using red microspheres and 3.3% alginate. The alginate and microsphere mixture was allowed to polymerize in CaCl_2 for 15 minutes and the formed capsules were treated with citrate for 7 minutes. Small aggregates have formed in the core of the capsule. Also of note is that the core is liquefied like in the previous figure. The core is a gel-ball inside the capsule and a liquid portion between this ball and the shell of the capsule.

Figures 3 and 4 show the early results of the capsules using the red microspheres mixed with the alginate. In both figures, the same protocol was used except Figure 3 shows a capsule made using 1.5% alginate and Figure 4 shows a capsules using 3.3% alginate. In both figures the layers of PLL and alginate can be seen near the edge. In Figure 4, there is a red gel-like core which is visible. This is a result of the sodium citrate solution not completely liquefying the core of the capsule. The red microspheres are in both this inner core and the outer liquefied solution surrounding it; however in the central core small aggregates have formed. There was not significant aggregation of the microspheres in either one of these capsules meaning that in general, the microspheres were spread out inside the capsules. It was determined that

microspheres should aggregate inside the capsules because sedimentation of the microspheres was observed when left alone inside a 1.5mL vial filled PBS.

When the ratio of alginate to microspheres was low or when a low weight to volume percentage of alginate was used to make capsules, the capsules appeared deformed with tails or lemon shaped, and often striations appeared on the outside of the capsules. Figure 5 is an example of a lemon-shaped capsule with striations formed using the falling drop method with a needle and syringe.

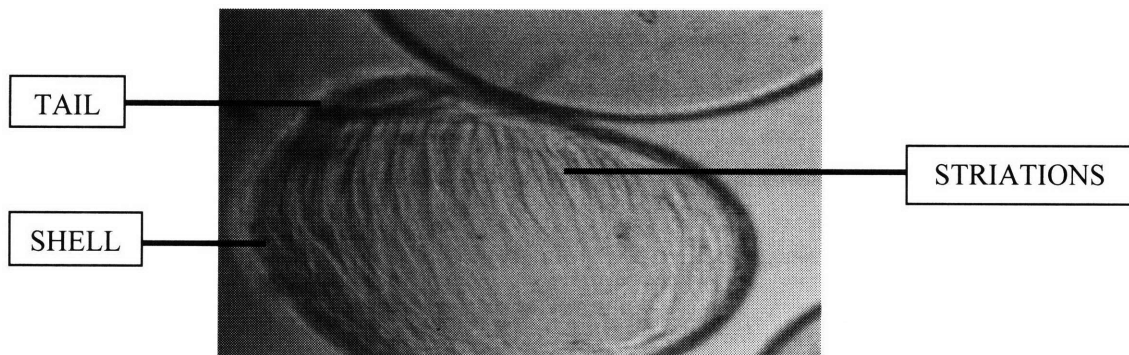


Figure 5: Non-spherical shaped capsule with striations. Red microspheres mixed with 1.5% alginate were polymerized for 15 minutes in a CaCl_2 solution and sodium citrate chelation was performed for 30 minutes. A microsphere to alginate ratio of 1:10 was used, however it was hypothesized that the vial of microspheres wasn't mixed adequately, so very few microspheres were suctioned into the micropipette tip resulting in a small number of microspheres being encapsulated.

Figure 5 shows that when low weight by volume alginate solutions was used, combined with long chelation periods, striations occurred. The striations are a signal that the alginate/PLL shell of the capsule is in a weakened state and about rupture. These types of capsules would not survive long periods of incubation or could not be used in clinical applications because they aren't stable. The tails shown in Figure 5 occurred because the droplets were too large and the concentration of alginate was too low. It was found through various sets of experiments that a higher concentration of alginate would produce rounder capsules because they polymerize more

quickly upon falling into the CaCl_2 bath and have a greater surface tension. It was observed that using a syringe and a needle to manually produce droplets of an alginate and microsphere mixture produced generally large capsules of but with varying size. This is because the droplet size is dependent on the rate of which the plunger on the syringe is pushed down and the alginate/particle mixture is extruded. Further, the smallest needle that could be used with the alginate, due to its high viscosity, was 22 gauge.

Because the microspheres were not settling and aggregating even after they were incubated for long periods of time in sodium citrate, it was hypothesized that larger microspheres and smaller, spherical capsules would help the microspheres settle to the bottom and aggregate. Green microspheres with a diameter of almost 100 times larger than the red microspheres were used instead of the red microspheres. Also, in order to make smaller droplets and therefore smaller capsules, a pipet-aid was used. Figures 6 and 7 show the capsules made with green microspheres.

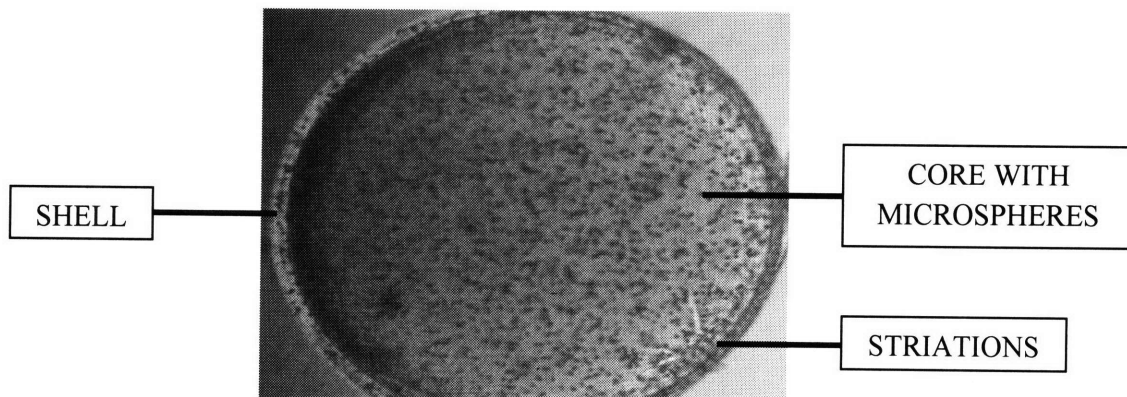


Figure 6: Capsule made with green microspheres and 1.5% alginate. The alginate and microsphere mixture was allowed to polymerize in CaCl_2 for 30 minutes and the formed capsules were treated with citrate for 15 minutes. Small striations appear on the weakest part of the capsule, which is the last part of droplet to enter the CaCl_2 .

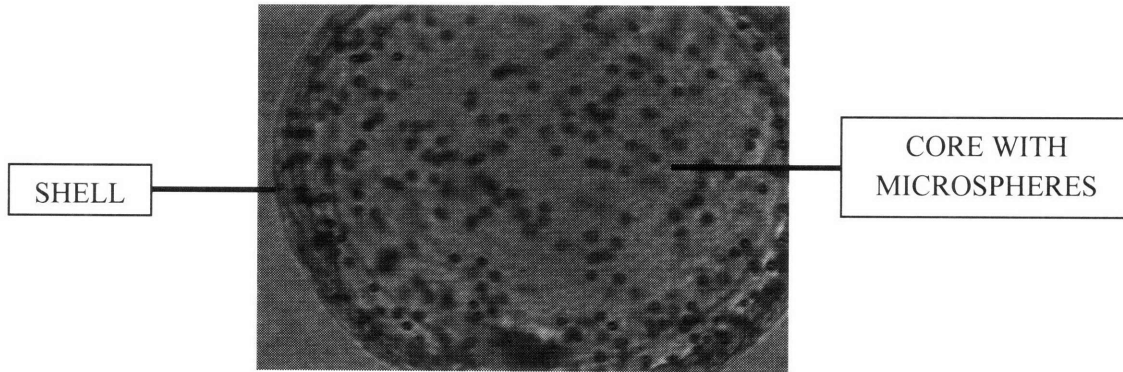


Figure 7: Capsule made with green microspheres and 3.3% alginate. The alginate and microsphere mixture was allowed to polymerize in CaCl_2 for 30 minutes and the formed capsules were treated with citrate for 15 minutes. No striations appear on the shell wall.

Figures 6 and 7 show that spherical capsules were made using the pipet-aid to extrude the alginate and microsphere mixture instead of using a syringe and needle. As the concentration of alginate was increased, the amount of striations decreased meaning the capsules were more stable and less prone to rupturing. This is also a result of using a pipet-aid because small diameter capsules were able to be made. Table 2 below shows the long and short dimensions of the 10 measured capsules. The capsules were measured using the scale on the microscope.

Bead #	Long Length (mm)	Short Length (mm)
1	1.07	1.05
2	0.45	0.43
3	1.11	1.11
4	1.16	1.14
5	0.78	0.74
6	0.68	0.58
7	1.28	1.28
8	0.54	0.54
9	0.97	0.95
10	0.83	0.83
AVG	0.89	0.86

Table 2: 10 measurements of capsules made using 1.5% alginate. The long and short lengths denote the widest and skinniest portions of the capsules. This table shows that the capsules were around .9mm in diameter and were mostly spherical as determined by the closeness of the long length and short length values.

With regards to aggregation, similar results were found using the green microspheres as were found using the red microspheres. The microspheres did not appear to aggregate even after long periods of incubation with sodium citrate. The microspheres are spread out with most of them located near the shell of the capsules. The falling drop experiment was repeated using β TC3 cells instead of microspheres. It was hypothesized that live cells may behave differently than microspheres and may have a natural affinity for each other that would allow them to aggregate. Figure 8 below shows the result of the falling drop experiment using β TC3 cells

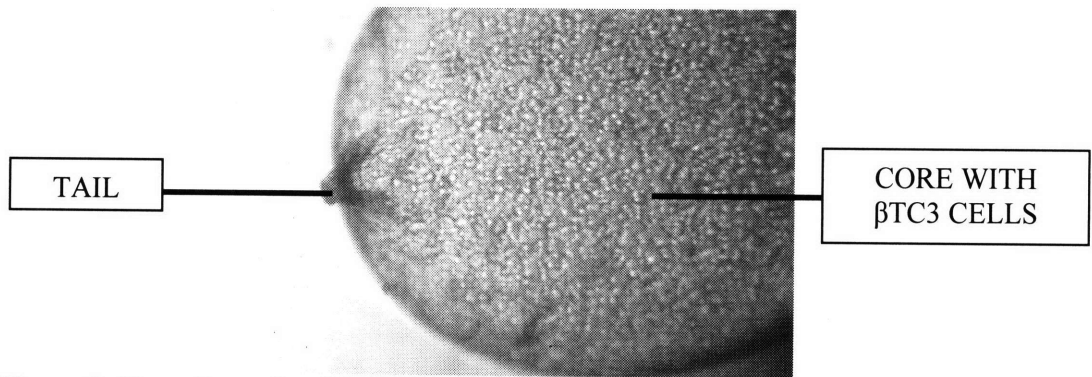


Figure 8: Capsule made with β TC3 cells and 1.5% alginate. The alginate and β TC3 mixture was allowed to polymerize in CaCl_2 for 30 minutes and the formed capsules were treated with sodium citrate for 2 hours. In the batch of capsules made under these conditions, about 50% ruptured due to the long citrate treatment. The above capsule is one of the few that didn't rupture. In the figure, a distinctive tail can be seen because the capsule was made large so as to perhaps increase the aggregation behavior.

Figure 8 shows an interesting behavior noted for nearly all the microcapsules made with β TC3 cells. It was hypothesized that if the core was sufficiently liquefied, the cells would have an easier time aggregation since there would be less resistance to their movement. No matter how long the capsules were given the citrate treatment to liquefy their core, the cells seemed to stay along the shell. There was no aggregation behavior noted in the capsules made. It appeared that the cells had an affinity for the PLL which caused them to position themselves near or on the shell of the capsule.

The final results of using the falling drop protocol are that encapsulation using this method is an effective way of making capsules that can survive for several days in saline solution without rupturing. Hundreds of small diameter capsules are able to be made in a few minutes using a coaxial nozzle such as the one used for with the pipet-aid. However, the aggregation of the microspheres and cells, which is essential when working with stem cells, didn't take place. Even after the chelation of the core of the capsules for extended periods, the cells or microspheres didn't appear to aggregate into a ball like three dimensional structure. It was hypothesized that the microspheres and cells have an attraction to the PLL which forces them towards the shell of the alginate capsule, not enabling them to aggregate together. Additionally, it was determined that the concentration of alginate used may be too viscous for aggregation to take place. Even when 1.5% alginate was used to make capsules, neither the microspheres nor the pancreatic cells completely aggregated. If complete aggregation doesn't occur if this method is applied to human embryonic stem cells, several embryoid bodies of varying dimensions will grow and agglomeration is likely to take place. It was determined that using this method of aggregation and encapsulation to form embryoid bodies can't work in its present form.

Floating Drop

The fluorinated oil method was developed subsequently as a way for the microspheres to aggregate completely before they are encapsulated. It was determined that using alginate and CaCl_2 is the best way to make capsules that can survive several days in saline solution but the falling drop method in its present form couldn't be used because the encapsulated particles didn't aggregate. The first experiments involved mixing green microspheres with 0.33% alginate in 2:1 or 3:1 ratios of alginate to microspheres. It was hypothesized that the lower densities and

viscosities of lower concentrations of alginate might not hinder the aggregation of the particles. The mixed drops were placed onto a Petri dish containing fluorinated oil and the microspheres were allowed to settle in the alginate. The settling behavior of the microspheres was documented at various time intervals. Figure 9 shows the settling and aggregation of the microspheres using this method.

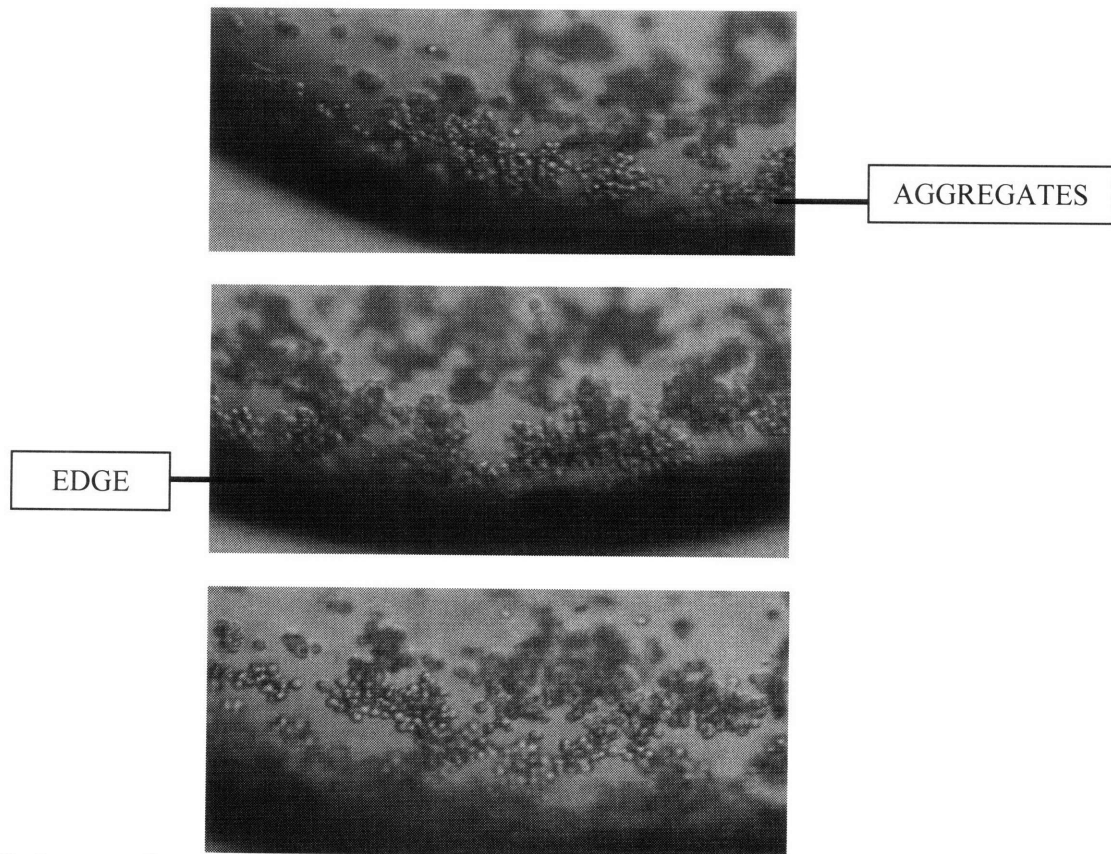


Figure 9: Aggregation of the green microspheres in .33% alginate after 30 minutes. The three pictures are three different views of the same $2\mu\text{L}$ drop. These pictures clearly show a bunch of aggregates being formed near the edges of the drop. Note that the entire droplet could not be shown because of the equipment limitations of the Nikon camera used.

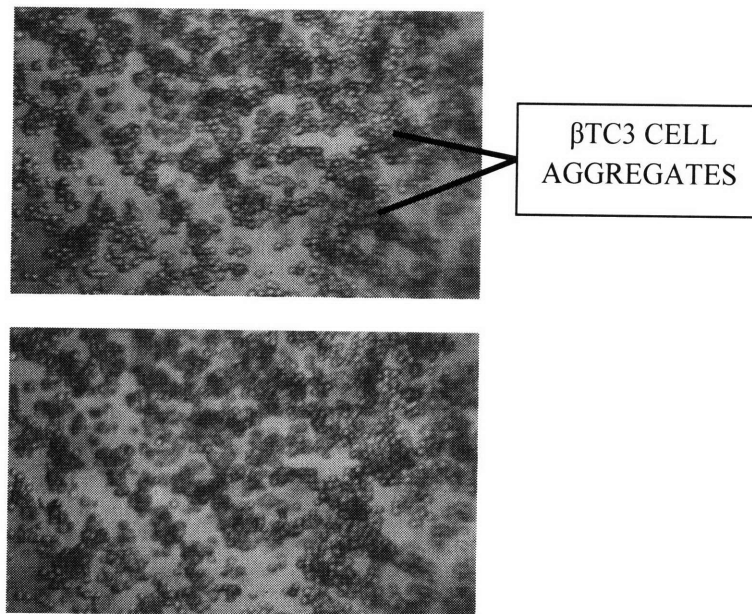
Figure 9 shows that microsphere aggregation took place in the alginate drops. The microspheres aggregated in separate sections, but not as one solid body. However this aggregation showed that the microspheres, and potentially cells, could aggregate in alginate

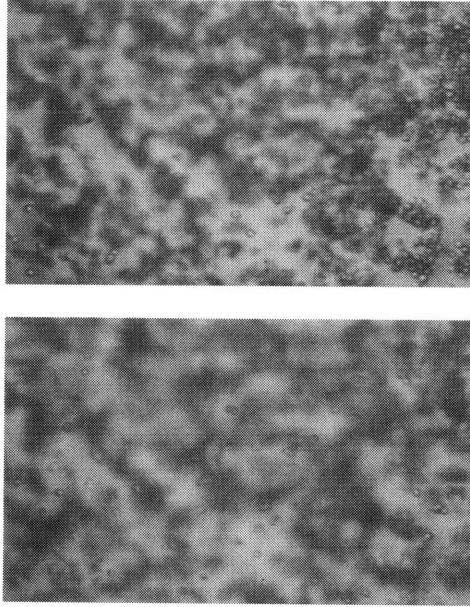
given enough time. The aggregation time in this experiment was cut short because the drop hit the side of the Petri dish and it wasn't possible to remove it from the side.

The next step was to perform the entire floating drop protocol to ensure that capsules can in fact be formed. The capsules that were made using the floating drop method were not spherical but were irregularly shaped due to the way in which the alginate and CaCl₂ drops interacted. As soon as the alginate/microsphere mixture and the CaCl₂ touched, the alginate gelled along the contact surface, and since a drop of CaCl₂ was placed on each side of the alginate drop, the alginate gelled to form a non-spherical shape.

The floating drop experiment was repeated but with 1.5% alginate with an alginate to β TC3 cell ratio of 1:2. Note this ratio is the ratio of cells from an approximately 35% confluent T-75 flask after the cells had been split and replated. The drop, which was 2 μ L, was allowed to settle for approximately an hour. Figure 10 shows the settling of the cells.

Bottom of the Drop





Top of the Drop

Figure 10: β TC3 cell distributions in a capsule from the bottom of the capsule to the top of the capsule. After the cells were allowed to aggregate for an hour, the drop was polymerized and encapsulated using CaCl_2 for 10 minutes. The drop was removed from the Petri dish containing fluorinated oil and placed in one containing a saline solution.

Figure 10 shows that the β TC3 cells did form separate aggregates and that most of these aggregates were settled on the bottom. Figure 10 was generated by changing the focus of the microscope and taking pictures of the drop from the bottom to the top. From the top of the set of pictures in Figure 10 to the bottom, the first picture (labeled Bottom of the Drop) shows that the β TC3 cells were mostly on the bottom and the last picture (labeled Top of the Drop) in Figure 10 shows that very cells few were located at the top because there are very few cells in focus. This shows that cells might be able to form a complete aggregate alginate although the viscosity of the alginate might hinder this process. Figure 10, as well as other floating drops experiments, shows that the cells are aggregating in a layer on the bottom, but not as a single ball of aggregates. Figure 11 below shows the aggregation being viewed in the experiments compared with the aggregation that is desired.

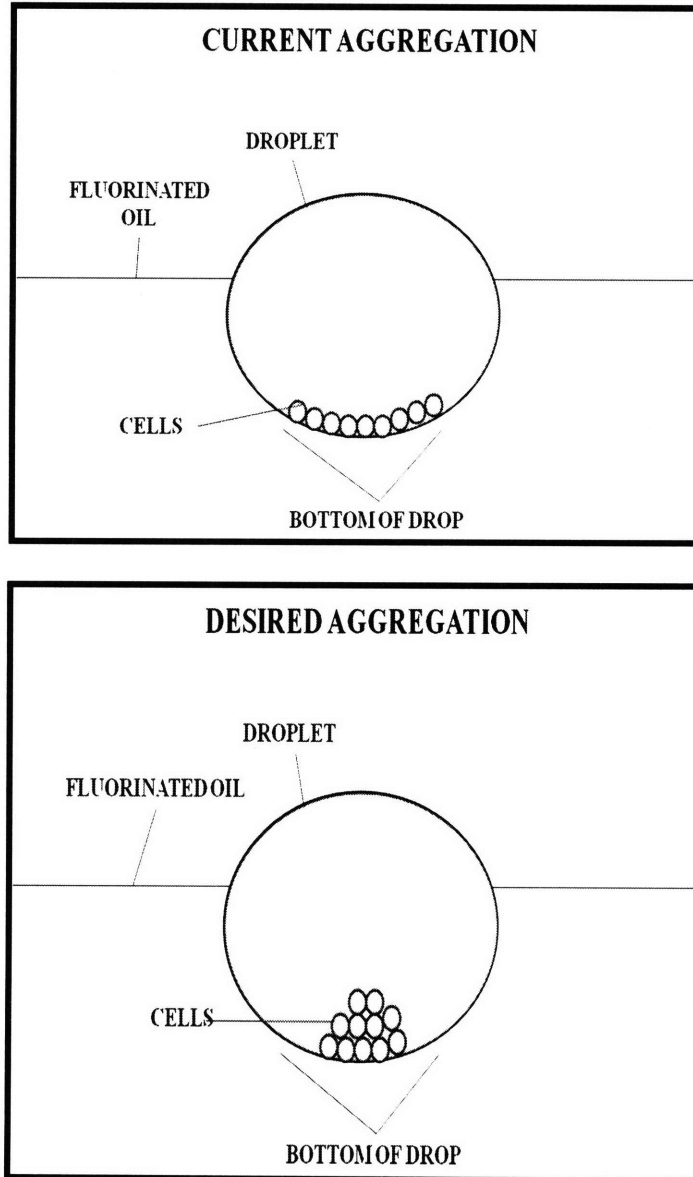


Figure 11: Diagrams of the fluorinated oil experiment from the side. The top picture shows the aggregation that is currently taking place inside the alginate droplet and the bottom picture shows the aggregation that is desired, a single aggregate.

Figure 11 shows that the current form and shape that the cells taking when aggregating is not the desired ball-like aggregate. It was hypothesized that the viscosity of the alginate even when it is not gelled the liquefied, hinders the cells from completely aggregating. Further, Figure 11 shows how the drops deformed when in fluorinated oil. Most of the drop is below the surface of the fluorinated oil because the specific gravity of fluorinated oil is 1.94, meaning its

density is slightly greater than the alginate solutions'. The bottom of the drop in Figure 11 therefore is flat and not rounded. As the droplet size gets bigger, the bottom of the drop gets flatter which also might influence the aggregation behavior.

It was hypothesized that the number of β TC3 cells inside each drop might also influence the ability of the cells to form complete aggregates. and in fact, have too many cells may lead to not allow a complete aggregation to take place. Note that chelation of the inside of the capsule is no longer needing using this method because aggregation takes place before encapsulation.

Following these experiments, β TC3 mouse cells were used to determine the amount of time it would take for human embryonic stem cells to completely aggregate to form a single aggregate. The first few experiments were performed by mixing cells with DMEM, placing a drop on fluorinated oil and letting them aggregate. Figure 12 shows the result of this procedure after 45 minutes. Note that the number of cells is greater than 10,000. This preliminary experiment was to observe if aggregation takes place inside the DMEM drop. Cell number would be counted and controlled for in later experiments.

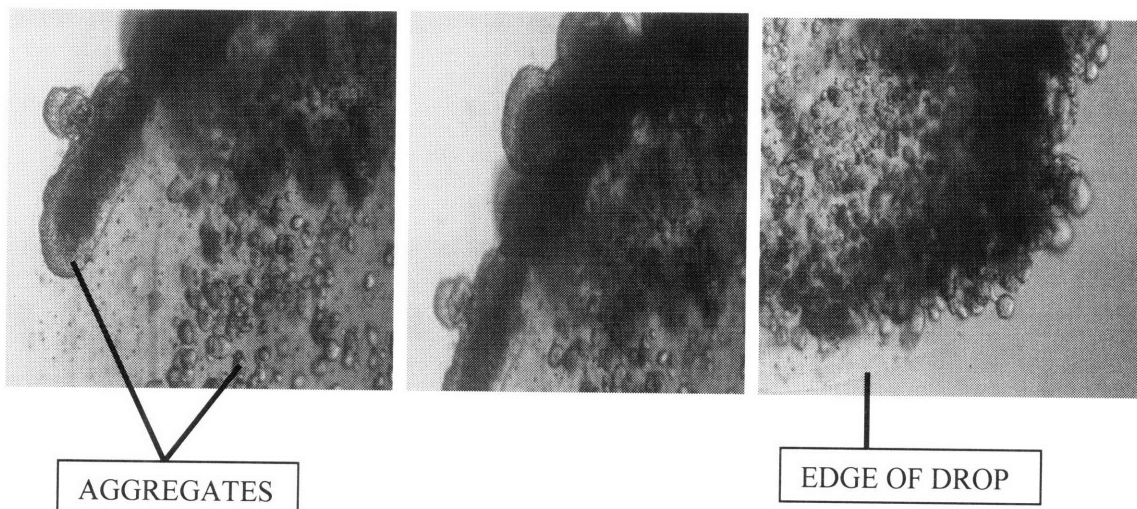


Figure 12: 3 different views of β TC3 cells aggregating in a 15 μ L drop of DMEM on fluorinated oil for 45 minutes. The Petri dish was lifted out of the incubator causing much of the DMEM to evaporate. Many dense aggregates of various size and shape can be seen.

Figure 12 shows that several aggregates of cells formed during the procedure. There are aggregates of various size and shape inside the drop. It was determined that the number of cells was still too many to allow aggregation to take place and therefore the next trials were going to take place with a relatively small number of cells. Figure 12 also shows that the droplet partially evaporated on the fluorinated oil. The Petri dish was placed inside of a larger closed container containing water. This was done to add humidity to the environment inside the container so the drop wouldn't evaporate, but this was insufficient. It was decided that all future experiments should be placed inside an incubator to prevent evaporation.

After 45 minutes, the floating drop protocol by combining a 15 μ L drop of alginate with the DMEM droplet and polymerizing the resulting mixture for 10 minutes to encapsulate the cells. The capsule was placed in a Petri dish containing DMEM and left in an incubator. The cells grew for 2 weeks inside the incubator. An experiment performed at the same time as this one using DMEM also grew for 3 weeks before it become contaminated. Figure 13 shows the cell growth at 2 weeks. Again, greater than 10,000 cells were using in this experiment.

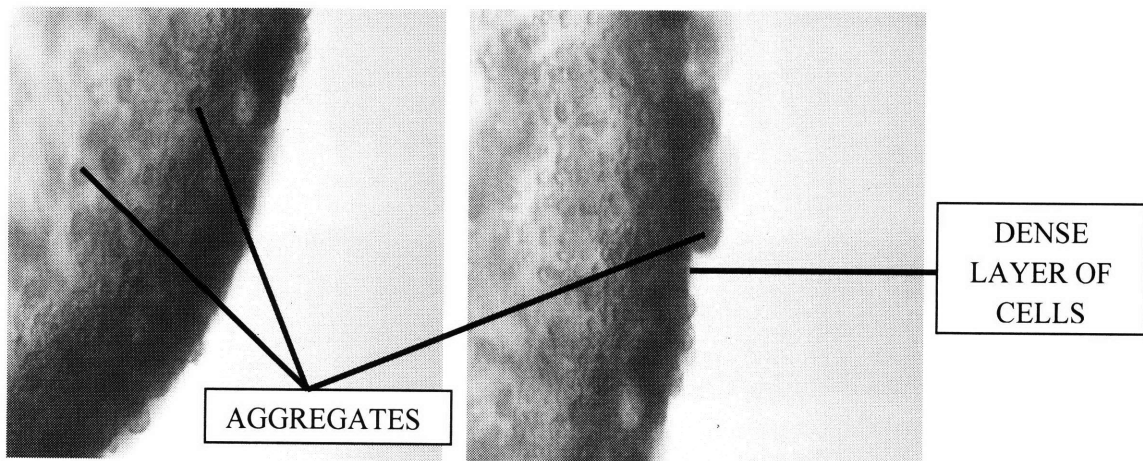


Figure 13: 2 different views of β TC3 cells aggregating in a 15 μ L drop of DMEM on fluorinated oil for 45 minutes. The figure shows the growth of the capsule after two weeks. A dense layer of cells can be seen around the edges of the capsule. This layer is a result of the capsules aggregating at the bottom of at the DMEM drop. This view is of the capsule on its side.

Later experiments with β TC3 cells used 500 cells. A cytometer was used to determine the dilution factor needed of the cells taken from a T-75 flask to yield 500 cells per specified drop size. Figure 14 shows the aggregation of a $5\mu\text{L}$ drop after various periods of time.

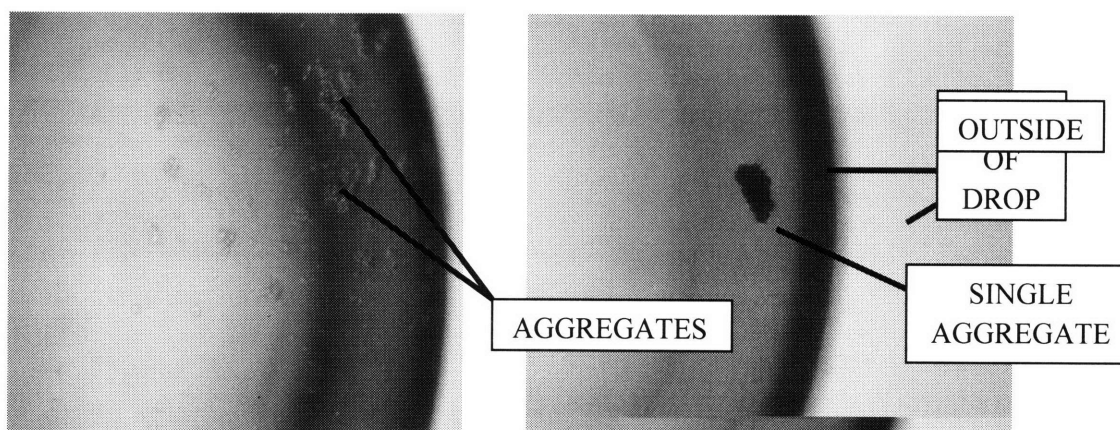


Figure 14: Aggregation of a $5\mu\text{L}$ drop after 24 hours (left picture) and 96 hours (right picture). After 24 hours, the 500 cells formed several aggregates all located on one side of the cell. The experiment was carefully placed back in an incubator and check again after 3 days. A single aggregate composed of all 500 cells was formed.

Note that the total time it took the aggregate to form is not exactly 96 hours. It was decided that several observations of the drop, which would involve much agitation, might cause the experiment to fail, therefore the experiment was left alone for 3 days before it was observed again. The shape of the aggregate is elongated even though it was expected that the shape would be circular which happens when a hanging drop experiment is performed under similar conditions. Previous experiments before this used 500 cells in $20\mu\text{L}$ which also resulted in single aggregates but after a longer period of time. It was determined that a smaller drop would yield a faster aggregation time because it would have a rounder bottom. A smaller drop would still be elongated but not to the same degree because it is smaller, therefore it would have greater curvature at the bottom.

Aggregation of β TC3 cells using the floating drop method was successful, but using these current methods, only one experiment can occur at a time and often, the droplets hit the wall, ending the experiment. Although the capsules formed using this method are irregularly shaped, they can be made small enough to be used in clinical applications. In order to scale up the experiment, it was determined that utilizing dielectrophoresis by using a pc board with electrodes would not only keep the droplets in place but would also undoubtedly enable several dozen embryoid bodies of varying size to be grown on the same Petri dish.¹² Due to the limited time of this experimental investigation, trials using dielectrophoresis could not be investigated.

V. CONCLUSION

In this study, a potential new method to grow embryoid bodies was created which addressed several issues hindering established methods including the ability to grow numerous embryoid bodies while being able to control for the size of them. This was accomplished by using the new floating drop method in which floating droplets of DMEM mixed with a predetermined number of β TC3 cells on fluorinated oil. These droplets were able to form a single aggregate on the fluorinated oil and the droplets were also encapsulated using alginate and CaCl_2 . This method is also beneficial in that the media in which the cells are growing is able to be changed by using a pipette and sucking out the old media then subsequently adding new media to the drop. This cannot be done using existing methods of generating embryoid bodies. Further, since this new floating drop method is able to be scaled up by using dielectrophoresis, it may be able to help solve the current problems of using human embryonic stem cells to make cardiomyocytes. Finally, since the embryoid bodies can be encapsulated using alginate, a

biodegradable polymer which is not likely to be rejected by an immune system, this method has the potential to be used for clinical applications.

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