Encapsulation of Cells in Microporous Annealed Particle Hydrogel for Type 1 Diabetes Treatment

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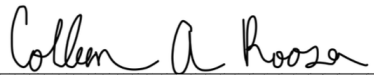
On our honor as University Students, we have neither given nor received authorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

Signed 

Colleen Roosa, PhD Candidate, Capstone Mentor

Signed 

Dr. Don Griffin, Assistant Professor of BME, Capstone Advisor

Authors: Jonathan Daniel, Alessia Randazzo

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Jonathan Daniel[1], Alessia Randazzo[1], Colleen Roosa[1], Don Griffin[1]
[1] Biomedical Engineering Department, University of Virginia

Abstract

Treatment for Type 1 Diabetes (T1D) is coming to reach the cusp of change. For many years and to this day, external insulin injections have been the standard of treatment for patients with T1D. However, because of the "price gouging" that insulin has undergone over the last two decades, insulin is becoming an increasingly unaffordable treatment option for many individuals around the country (Knox, 2020; Monier, 2019). Furthermore, strict daily glucose monitoring and insulin injections are not viable treatments for all patients. A plausible alternative is to develop a cell transplant of insulin producing cells to restore endogenous insulin secretion. Implementation of this treatment is limited by the persistent inflammatory response at the injection site, which results in high cell mortality. Therefore, to protect cells from the inflammatory blood response, we propose a solution to develop a cell transplant treatment that encapsulates dissociated beta cells within the microgel-building blocks of microporous annealed particle (MAP) gel. These encapsulated cells will then be injected into the body and serve as an efficacious treatment for T1D. The annealed MAP gel will provide support as a bioinert scaffold which has been shown to promote growth and integration into the body and protection from an inflammatory response (Griffin et al., 2015; Pruett et al., 2021). The results from the experiments illustrate successful encapsulation of the cells. Encapsulation efficiency reached 78% and cell viability post-encapsulation was 93%. Future experiments will focus on retaining high viability and functionality following storage in frozen conditions.

Introduction

Clinical Disease Focus:

Type 1 Diabetes (T1D) is a chronic autoimmune disorder resulting in affected individuals incapable of producing insulin for the body. This typically results in high levels of glucose in the body. The cause of this insufficient insulin in the body is because the body's immune system attacks most, if not all, of the insulin-producing pancreatic β cells (Atkinson et al., 2014). T1D cases represent 5-10% of all diabetes cases in the world. Although T1D can be diagnosed to individuals of any age, it is most commonly identified during childhood. Diabetes is also among the most commonly recognized and diagnosed diseases. As of 2018, there are 1.6 million Americans living with T1D and 64,000 new cases are diagnosed every year. Unfortunately, the rates of T1D continue to rise as it is projected that by 2050, there will be close to 5 million individuals living with T1D (JDRF, 2020). In America, the prevalence is 3.9 individuals out of every 10,000 and the incidence is 20 individuals out of 100,000 (Mobasseri et al., 2020). T1D, and Diabetes overall, is particularly worrisome because in the long-term, having diabetes greatly increases the risk for heart disease, nerve damage, and kidney damage. Accordingly, $1 out of every $4 in US health care costs is spent on caring for people with diabetes. This comes out to be $237 billion spent each year solely on direct medical costs (American Diabetes Association, 2018). These numbers emphasize the importance and high impact of the disease.

Limitations of Predominant Treatment: Insulin

Fortunately, the predominant treatment for T1D has been known to be effective. The most common treatment plan for individuals for T1D is to take injections of insulin. These shots exogenously restore insulin to its proper levels in the body. The frequency of these injections vary, but most commonly, individuals will have to take these injections daily for the rest of their lives. However, there is one significant downside to utilizing insulin as a treatment: insulin costs are incredibly expensive. It has been established that insulin has been undergoing price gouging for the past two decades (Knox, 2020). This is when a seller increases the prices of goods, services, or commodities to a level much higher than is considered reasonable or fair. As a result of price gouging,

pharmaceutical companies price a vial of insulin at ~$350 which is 30,000% more than the initial cost at patenting. On average, a patient has to spend $1500 a month on buying insulin. With the average monthly salary only being $4,125, it is evident that the financial burden insulin places on an individual or household is tremendous (Lazarus, 2021). In fact, reports show that close to 25% of individuals in need of insulin ration or choose not to buy it entirely because of the cost burden (Monier, 2019). The inability to afford insulin is the leading cause for ketoacidosis, which can be life-threatening if untreated for large periods of time (Tseng et al., 2020). In total, it has been reported that individuals with T1D in America cumulatively spend $211 billion more on healthcare costs than those without (Sussman et al., 2020). This clearly illustrates a clinical unmet need for cost-saving preventive treatments. In addition, there are several short and long term complications involved with daily insulin injections. These include allergic reactions to the injection site, weight gain, an increased risk for heart disease and cancer, and a higher rate of hypoglycemic events. Lastly, insulin regimens can be impractical for many individuals. The need to take daily doses of insulin and having to continuously monitor blood glucose levels for young patients and/or older patients is difficult. These age groups can lack responsibility or the capability to be responsible for such tasks, often relying on those around them to supervise. This is something not everyone has access to. Therefore, there is an increasingly important clinical need to develop a long-term treatment for T1D that can restore endogenous insulin secretion.

Limitations of Alternative Treatment:Cell Transplant

Transplanting insulin producing cells (i.e., islet cells) is a promising alternative to external insulin injections. In the current clinically approved cell transplants, insulin producing cells can be isolated from the pancreases of cadavers and injected into the body via the portal vein (Rao, 2022). These transplanted cells will then start producing insulin and help restore endogenous insulin secretion. However, there are several key factors that prohibit this form of treatment from advancing to consistent clinical application. First and foremost, clinical studies have shown many instances of failure of the transplant and rejection issues because the transplantation site is highly inflammatory (Rother and Harlan, 2004). This subsequently results in an immune response and islet cell mortality. To prevent such a response, patients were given immunosuppressive medication. While this did improve the success of the delivery, the inflammation would resume once patients were off of the immunosuppression. It is not advisable for diabetic patients to be under immunosuppressive medication for too long because their immune system is already compromised. This opens up a host of dangerous viral and bacterial situations. In addition, a large sum of islet cells are lost immediately after transplantation. This is partly caused by insufficient oxygen diffusion to the large islet cluster. Lastly, healthy donor islet cells are extremely rare and difficult to access. There are only 1000 pancreas transplants performed per year because the need for a healthy organ to match precisely (Rao, 2022). Therefore, there is not only a need to find an alternative to insulin, but a need to develop a treatment that will restore endogenous insulin secretion efficiently and safely.

Proposed Solution: Encapsulate dissociated beta cells within Microporous Annealed Particle (MAP) gel

Hypothesis: We hypothesize that encapsulating dissociated beta cells within MAP gel and injecting them into the body will be an effective cell transplant that can withhold the inflammatory response and integrate into the body.

MAP gel is a highly tunable, novel hydrogel that will serve as a protective scaffold for the dissociated beta cells to integrate into the host body. By encapsulating the beta cells within the MAP microspheres, they will have minimal exposure to the inflammatory host environment until well integrated into the body. MAP gel is particularly well-suited for this role as it is injectable. Unlike many hydrogels, MAP gel can be injected utilizing a syringe, and subsequently annealed using low-intensity UV light. This allows for maximum surface area adherence and a minimally invasive procedure for the patient. Secondly, MAP gel is regarded as bioinert. This means that once injected into the body, there is minimal risk for any inflammatory response. However, any resulting immune response that may occur will result in minimal damage to the transplanted cells as they are encapsulated within the microspheres. MAP gel is also porous (Figure 1). The porosity of MAP gel allows for the release of insulin to secrete out of the microspheres, as well as enhanced growth of vascularity and tissue into the scaffold. This leads to accelerated integration of the scaffold into the host body. Lastly MAP gel can be tuned to degrade at 6 months. This means that it can degrade as the cells integrate into the body. Therefore, MAP gel is an effective hydrogel scaffold to encapsulate dissociated beta cells while providing enhanced protection and

growth with the host body. To attain this treatment, the project was broken down into three specific aims that represented important milestones towards the development of the treatment.

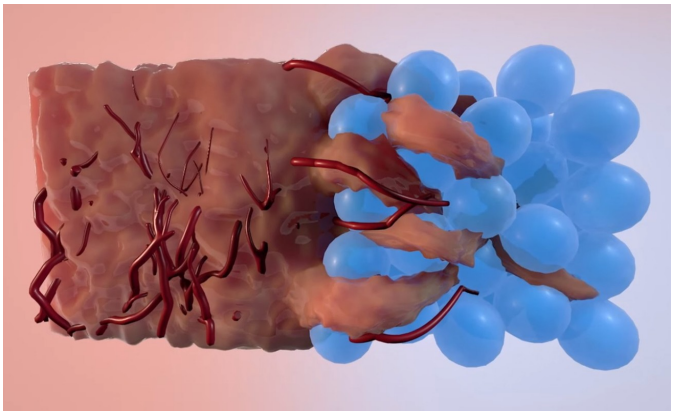

Figure 1: *A visual representation of MAP microspheres integrating into the body and promoting the growth of local vasculature. The blue microspheres represent the MAP gel scaffold and the rest of the material is tissue and vascularity. This is a cartoon depiction on the microscopic level.*

Aim 1: Encapsulation of cells within MAP using microfluidics

Encapsulation of cells within the microgel building-blocks of MAP gel has not yet been successfully attempted in the Griffin Lab. Therefore, the focus of this aim was to develop a comprehensive and reproducible methodology to efficiently encapsulate cells while ensuring they remain viable (Figure 2). The viability of the cells was identified by dividing the live cells on a representative image by the total number of cells on the same image (Equation 1). The goal was to consistently achieve a cell viability of 70% or more. Encapsulation efficiency can be calculated by dividing the number of microspheres containing cells by the total number of microspheres in the same representative image (Equation 2). The goal was to achieve at least 80% efficiency. An added metric we later implemented was cell cluster encapsulation efficiency. This is defined as the number of microspheres that have more than 5 cells encapsulated (Equation 3). The cell cluster encapsulation efficiency is calculated utilizing the same method. The goal was to achieve 60% clustering efficiency or greater. Having a high cluster efficiency is important for dissociated islets, and stem-cell derived beta cells typically require cell aggregation *in vitro* prior to implantation. This facilitates biochemical and biomechanical cell interactions that are necessary for cell differentiation, survival, and function.

(Shembekar et al., 2016)

Figure 2: *A real-time annotated image of a t-junction microfluidic device encapsulating cells. The gel and cell solution flows down the top channel. It meets at the "t junction" where two oil channels meet. This pinches off the gel solution, encapsulating cells in the gel. Adjusting the flow rates can lead to single cell encapsulation or cell clustering. This is the main microfluidic design for the experiments in this project.*

Cell Viability:

$$\frac{Total\ Live\ Cells}{(Total\ Live\ Cells + Total\ Dead\ Cells)} \quad (Eq.\ 1)$$

Encapsulation Efficiency:

$$\frac{Total\ Microspheres\ with\ Encapsulated\ Cells}{Total\ Microspheres} \quad (Eq.\ 2)$$

Cluster Encapsulation Efficiency:

$$\frac{Total\ Microspheres\ with\ Encapsulated\ Cell\ Clusters}{Total\ Microspheres} \quad (Eq.\ 3)$$

Aim 2: Designing methodology to store MAP encapsulated cells

To ensure the applicability and feasibility of this future T1D treatment, it is important to ensure the treatment can be stored for extended periods of time without losing functionality. This is because clinical translation demands that treatments are capable of being sent across different clinical settings and still be useful and effective. Accordingly, our devised cell transplant needs to be able to be stored and transported for extended periods of time and remain functional. Accordingly, the best practice would be to freeze the treatment and thaw when needed. Therefore, the focus of this aim was to develop a comprehensive and reproducible methodology to freeze and thaw the encapsulated cells, while maintaining a cell viability of over 70%.

Aim 3: Evaluating cell functionality post-encapsulation and post-frozen storage

Most significantly, after reaching high viability rates of cells after encapsulation and storage, we need to ensure

that the beta cells perform their necessary function and release insulin. Accordingly, the relevant functional tests will be performed.

Materials and Methods

Throughout the experiments, a series of optimizations have been performed to create the ideal and most reproducible methodology. The most optimized methodology will be delved further into.

Aim 1: For the microfluidic setup, there is one outlet phase and two inlet phases: the MAP scaffold polymer backbone and oil surfactant phase (Figure 3). The MAP scaffold polymer backbone consists of a matrix metalloproteinase (MMP) crosslinker component, the tripeptide motif consisting of Arginine, Glycine, and Aspartate (RGD) for cell attachment, a biotin-maleimide motif, and 4-arm polyethylene glycol vinyl sulfone (PEG-VS). Following encapsulation, streptavidin conjugated to a fluorophore was added which will bind to the biotin in the precursor solution and allow for visualization. The RGD and PEG-VS was resuspended in sterile triethanolamine (TEOA) buffer at pH of 7.8 and the MMP resuspended in sterile DI water. The oil surfactant phase is composed of a 5% Picosurf surfactant diluted in fluorinated NOVEC oil to a final concentration of 1%. This oil surfactant is crucial for the formation of the microparticles and prevention of aggregation. The precursor solution formulation was combined with the desired concentration of cells and flowed through one inlet of the microfluidic device. The oil surfactant solution flowed through the other inlet. As the two phases flow through the device, microparticles formed and were collected out of the outlet channel and into a 20 μm Pluriselect filter on top of a 50 mL conical tube, so that the oil drains through the filter and the microgels are retained on top. The optimized flow rates for cell encapsulation were found to be 3mL/hr for the oil phase and 2mL/hr for the precursor solution. In addition, the optimized cell density was ~8 million cells/mL for encapsulation. These metrics provide the highest levels of encapsulation and clustering.

The murine and human fibroblasts are obtained from commercial cell banks and are thawed from existing vials and cultured according to protocol.

Following the microfluidic encapsulation process, the encapsulated cells in the MAP scaffold were submerged in media and placed in the incubator for the MAP to fully gel. After the incubation period, the encapsulated cells and the gel were purified thoroughly to get rid of all of the oil phase. This was done through a series of media and 0.1% pluronic (gentle detergent to remove oil) buffer washes. The gel and cells can be then transferred to a conical tube for storage or a well-plate for imaging. Quantitative analysis was then performed to identify percent encapsulation and viability. The viability is measured utilizing a "LIVE/DEAD; Cell Imaging Kit (488/570)" from Thermo Fisher. This assay fluorescently labels live cells and dead cells under a fluorescent microscope. The percent encapsulated was quantified by doing an ImageJ analysis using a fluorescent confocal microscope. Since the encapsulated cells in the MAP scaffold are mixed with biotin, following encapsulation, streptavidin conjugated to a fluorophore is added. This causes the scaffold and the cells within to fluoresce under the fluorescent microscope. By counting the cells and the microspheres, the aforementioned formulas can be used to generate percent viability, percent encapsulation, and percent cluster encapsulation efficiency. To optimize viability in this workflow, it is important to ensure all work is as sterile as possible and that things move efficiently and smoothly.

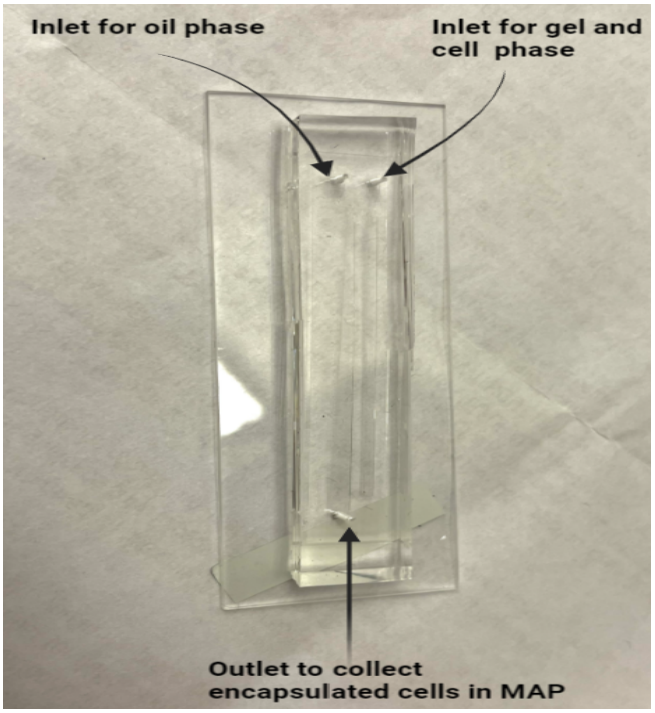

Figure 3: *The 20 μm microfluidic device that is utilized in the encapsulation runs. The inlet on the left is where the oil/surfactant solution goes. The inlet on the right is for the gel and cell solution. The outlet on the bottom is to collect the final solution.*

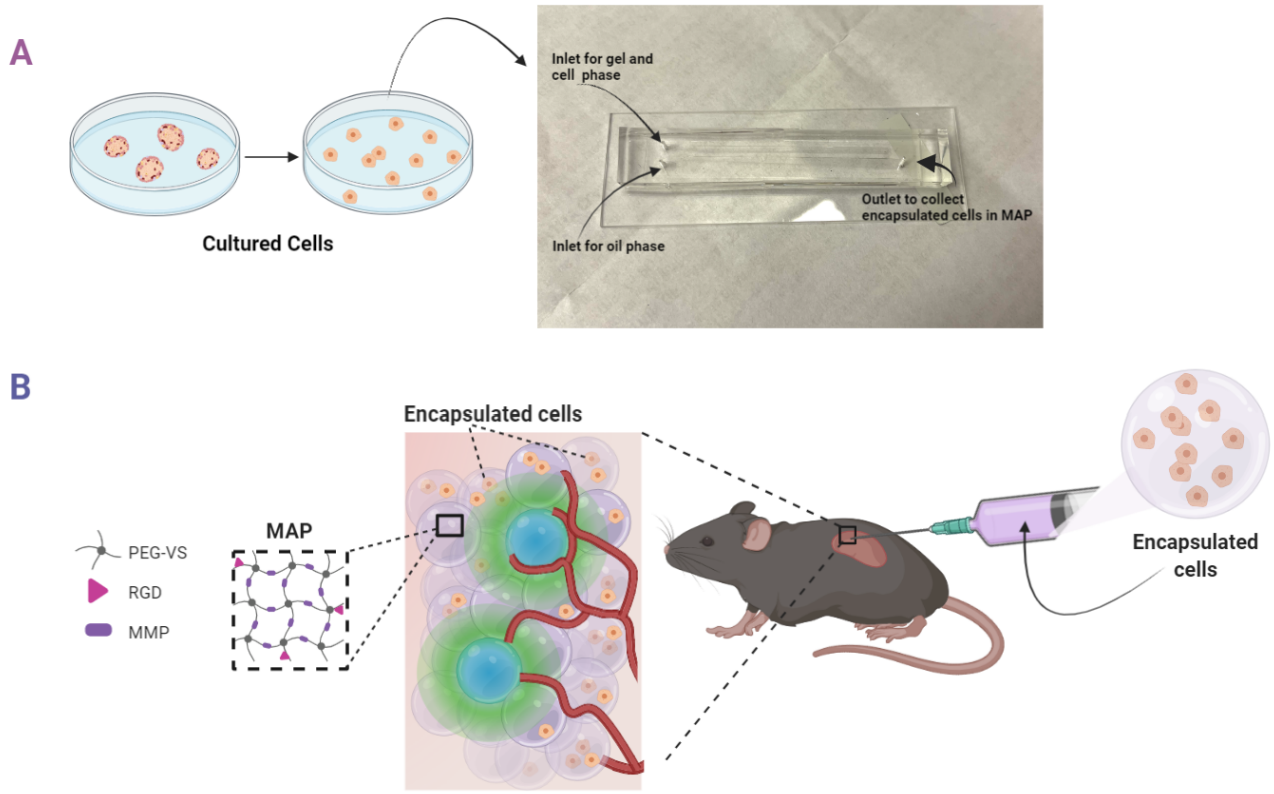

Figure 4: *Diagram representing the overall methodology and workflow of the project. (A) Cells are cultured and maintained until the target cell density is hit. These cells will be collected and flown through the microfluidic device with the MAP precursor solution, encapsulating the cells within MAP microspheres. (B) The MAP precursor solution contains PEG-VS, RGD, and MMP which effectively create a supportive mesh and network for the cells to grow in and for the hydrogel to integrate into the body. This final solution will then be injected into mice to evaluate efficacy and safety.*

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Aim 2: The focus of this methodology is to provide a safe and efficient way to freeze and thaw cells to ensure viability remains high. The viability is measured using live/dead stain imaging. The MAP scaffold with encapsulated cells was put in a 10% solution of dimethyl sulfoxide (DMSO) freezing medium. Because cells contain water, cells were frozen at a slow, controlled rate to prevent crystallization. To thaw, the vial was put into a 37℃ bead bath for 2-3 minutes. The encapsulated cells and gel were put into transwell plates, and then, as the cryoprotectant is toxic to cells, the cells undergo a thorough purification process to remove any excess DMSO. After applying the live/dead stain, these transwell plates were subsequently imaged utilizing a Confocal microscope. Utilizing this methodology, a comprehensive imaging study of live/dead staining was performed with 2 hour, 48 hour, and 96 hour timepoints.

Aim 3: This will be the focus of our studies in the future. The methods to evaluate the functionality of the cells post-encapsulation begins with an *in vitro* study. This study will be a glucose-stimulated insulin secretion (GSIS) assay to conclude whether the beta cells are at least 70% viable, and that they release at least 8.4mIU insulin per mL of beta cells (Spero et al., 2017). This is the recommended amount to be released to achieve healthy levels in the body. Insulin will be quantified by relocating beta cells from a low-glucose concentration media to a high-glucose concentration media and measuring the produced glucose level with an insulin ELISA. These can progress to the rat models and experimentation if positive results are attained.

A schematic diagram of the entire methodology used is shown in Figure 4.

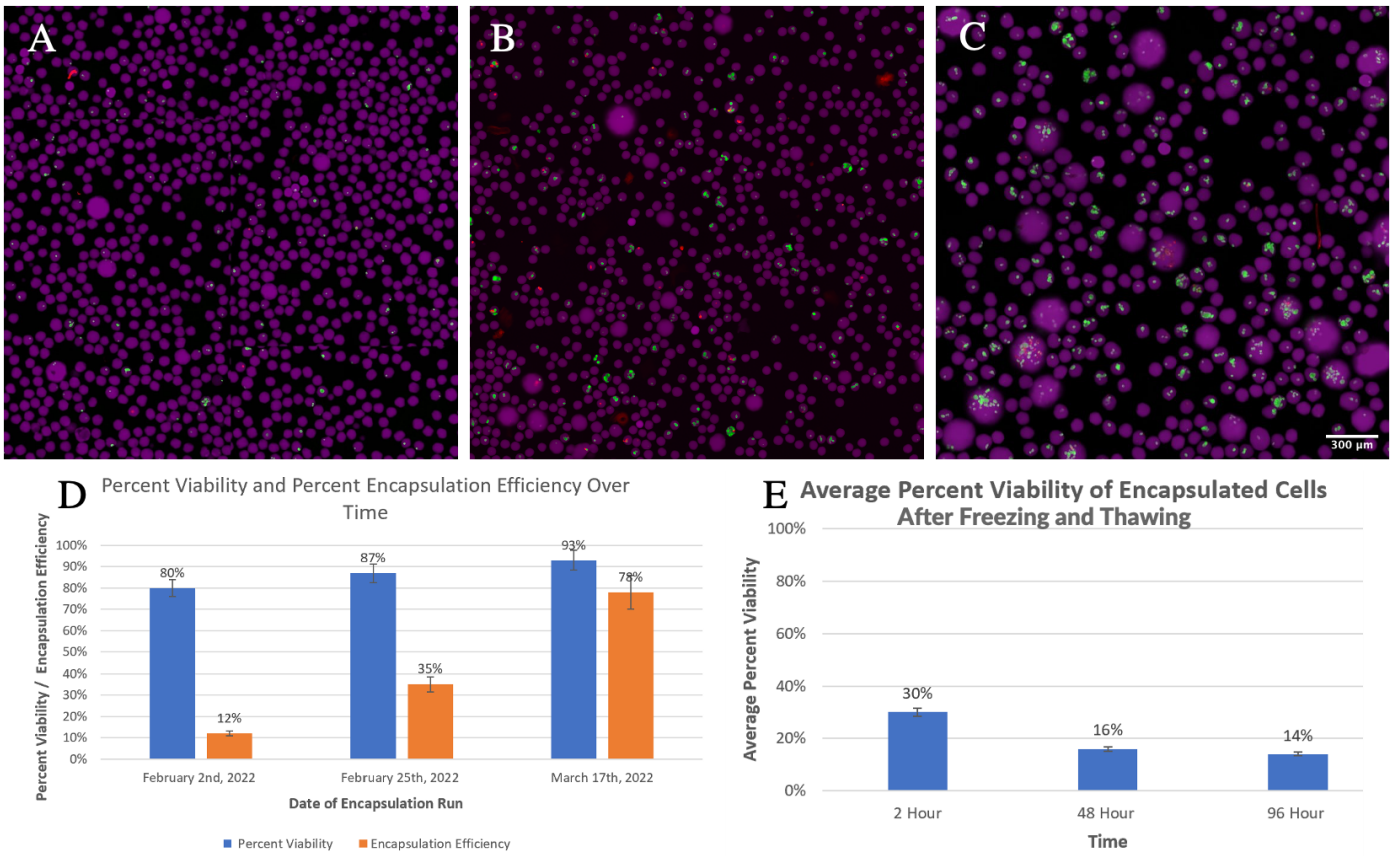

Figure 5: *(A) Encapsulation run from February 2, 2022. Imaging shows a high degree of uniformity in size of MAP microspheres (purple), but less than optimal levels of encapsulation of human dermal fibroblast cells (green). (B) Encapsulation run from February 25, 2022, showing higher viability and an increase in encapsulation efficiency. (C) Encapsulation run from March 17th, 2022, showing the highest levels of encapsulation and clustering rates compared to previous runs; scale bar, 300 µm. (A-C) uses the same cell type and color scheme for cells and microspheres. (D) Graphical summary of the live-dead study and encapsulation efficiency immediately after encapsulation runs from A-C. Standard deviation bars are shown. (E) Graph of the cell viability 2, 48, and 96 hours following the freezing and thawing protocol (Methodology). Standard deviation bars are shown.*

Results

The two main significant measures used to quantify progress was encapsulation viability, the percentage of live cells over the total number of cells (Equation 1), and encapsulation efficiency, the percentage of microspheres with encapsulated cells over total microspheres (Equation 2). Representative images are displayed in chronological order to emphasize resulting progress after adaptations and improvements made to the encapsulation methodology (Figure 5A-C).

Cell viability

Using a 20 µm microfluidic device, cell viability levels reached a maximum of above 90% immediately after encapsulation (Figure 5D). After cells were frozen and then thawed, viability was measured using a live-dead assay 2 hours, 48 hours, and 96 hours post-thaw (Figure 5E). These results showed a reduction in viability after 4 days from 30% to 14%, though viability remained relatively constant for the latter two days.

Encapsulation efficiency

Encapsulation rates reached a maximum of 78% immediately after cell encapsulation (Figure 5D). The two-fold increase in encapsulation from the February 25th to March 17th runs occurred after heavily increasing cell density numbers from 1-2 million cells/mL to 8 million cells/mL. More clusters of cells were found in the microspheres, as well. With greater

cell-to-cell interactions, more clustering is generally thought to provide a longer lifespan and greater success of treatment via providing the necessary biochemical and biomechanical cell interactions to promote cell differentiation, survival, and proper functioning.

Discussion

We have successfully been able to encapsulate a variety of cell types, including mouse embryonic fibroblasts and human dermal fibroblasts, as a proof of concept for beta cell encapsulation. A maximum 78% encapsulation efficiency and 93% cell viability have been achieved immediately after encapsulation. The current freezing and thawing protocol allows a 14% cell viability of cells after 4 days. Potential limitations to this protocol are the formation of ice crystals as a result of inefficient freezing, or too much exposure to cytotoxic DMSO from using too high of a concentration or leaving the cells to thaw for too long. Future steps include modifying the storage protocol to mitigate ice crystal formation and cell exposure to cytotoxic DMSO freezing medium.

The encapsulation protocol will be adapted to using 40 µm microfluidic devices to achieve higher rates of clustered encapsulation and theoretically, higher rates of viability. Once optimal levels are achieved, the process will transition to using beta cells. Whole islets will be isolated from the pancreases of healthy C56BL/6 mice and then dissociated using Flow activated cell sorting (FACS) to purify the beta cell population. Beta cells will then be encapsulated in MAP gel to evaluate their functionality after freezing and thawing. This will be quantified via a glucose-stimulated insulin secretion (GSIS) assay.

Conclusion

This study has revealed that using MAP gel for encapsulation can potentially serve as a precedent for noninvasively transporting various cell types, proteins, and molecules into the body. MAP gel has the ability to protect its contents from an inflammatory response, which broadens the possibilities for contents for which it can serve as a vessel. Its natural degradation also reduces the chance of toxicity of treatment. Both of these behaviors are advantageous properties to have in a biomaterial and will improve the safety and wellbeing of patients when this translates to a clinical setting. MAP gel's intention to be used as a one-time treatment also makes it superior to daily doses of insulin and periodic beta cell transplants. Successfully encapsulating insulin-producing beta cells and retaining their functionality after freezing and thawing will provide support for using this therapeutic for the treatment of Type 1 Diabetes.

End Matter

Author Contributions and Notes

The authors declare no conflict of interest.

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