Encapsulation of Cells within Microporous Annealed Particle (MAP) Scaffold for a Tissue Engineered Brain Tumor Microenvironment

A Technical Report submitted to the Department of Biomedical Engineering

Presented to the Faculty of the School of Engineering and Applied Science

University of Virginia • Charlottesville, Virginia

In Partial Fulfillment of the Requirements for the Degree

Bachelor of Science, School of Engineering

Zoe Apsel

Spring, 2023

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On my honor as a University Student, I have neither given nor received unauthorized aid on this assignment as defined by the Honor Guidelines for Thesis-Related Assignments

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Abstract

Glioblastoma is the most malignant and common type of primary brain tumor. A deeper understanding of the tumor microenvironment may help lead to improved treatments for the cancer. In this project, we aimed to engineer an *in vitro* tumor microenvironment using microporous annealed particle (MAP) scaffold, a type of hydrogel-based system composed of granular particles. For our proof-of-concept experiments, we encapsulated non-neural cells within gel particles using microfluidic devices. Our overall goal was to optimize the encapsulation protocol to improve non-neural cell viability due to their greater proliferation than neural cells. We investigated potential factors of the encapsulation process affecting cell viability, including collection and purification methods, cell concentration, and temperature of hot plate. Gel particles were imaged using a confocal microscope and fluorescence imaging, with different stains representing live and dead cells and the particles themselves. We found that collecting in cell culture media, lowering the temperature of the hot plate holding the collection beaker, and removing hexanes from the purification process all led to improved viability immediately post-encapsulation. A cell concentration of 8e6 cells/mL was found to have a lower rate of decrease in viability compared to 2e6 cells/mL. Future work will include encapsulating neural cells, co-culture encapsulations, and ultimately investigating glioblastoma behavior once added to the scaffold.

Introduction

Glioblastoma, also known as Glioblastoma multiforme (GBM), is the most malignant type of brain tumor, with less than 5% of patients surviving five years after diagnosis.[1] It is also the most common type of malignant primary brain tumor with an incidence of 3.23 in 100,000 in the US annually, and with a median survival of 15 months.[2] The cause for GBM is unknown in most patients. Additionally, early detection is not currently available; magnetic resonance imaging is the most sensitive tool for detecting GBM, but once the tumor is able to be identified via imaging, it is already advanced.[3]

The current standard treatment for GBM is maximal surgical resection followed by radiation therapy and concomitant temozolomide. Even with decades of research on various therapies, the outcomes have not significantly improved. The tumorigenesis of the disease needs to be better understood in order to develop more effective treatment options. Modeling diseases *in vitro* offers high levels of control, yet many animal models are not able to accurately recapitulate the same conditions found in humans.[4] Tissue engineering offers synthetic models with high manipulability and specificity as a solution.

The tumor cells can stimulate certain cellular responses and direct physical change in the host tissue in a region known as the tumor microenvironment (TME).[5] It is essential to understand the complexity of the TME in order to better understand how GBM progresses. Through recruiting non-malignant cells, tumor cells can avoid certain treatments, overcome oxygen and nutrient deficiency, and survive in acidic conditions. By better understanding how the TME directs tumorigenesis, more precise and effective therapies can be developed and new targets for treatments can be found.

One of the characteristic elements of glioblastoma behavior is its invasiveness into surrounding brain tissue. This along with the sensitive nature of brain tissue makes it incredibly difficult to study and/or treat glioblastoma *in vivo*. As a result, in vitro models have become vital to researching glioblastoma behavior and possible (non-surgical) treatment options. In the past five years, the vast majority of *in vitro* GBM models being studied and developed have been 2D cell culture models.[6] In the past five years, the vast majority of glioblastoma models that have had papers published have been 2D cell culture models. However, it was found that glioblastoma-derived cancer stem cells (GSCs) more closely resemble the structure of glioblastoma in vivo when developed in 3D, due to the tumor microtubules that support cell invasion and proliferation, the brain specific extracellular matrix (ECM), and the closer to in vivo behavior shown in 3D cultures compared to 2D cultures.[7] Additionally, recent

developments have shown 3D models could more closely replicate the TME which is important for including factors such as immune responses and microglia facilitated tumor growth in glioblastoma treatment studies. Despite the importance of mimicking the TME, some 3D models do not include certain aspects such as the blood brain barrier, substrate simulating extracellular matrix, and/or interactions with non-tumor cells.[8] The hydrogel-based system created in this technical project could make up for what other models lack. By creating a 3D model that focuses on simulating the tumor microenvironment, our system accounts for these shortcomings of current models. This is done by including ECM components, such as collagen and laminin, and non-tumor cells that both interact with glioblastoma cells and interact with each other as part of the blood-brain barrier, namely endothelial cells and astrocytes. Additionally, the plug and play design of the hydrogel and cell encapsulation allows for more customization of the TME, so the system could be adjusted to match specific conditions desired by the investigator or to elicit certain behavior from the glioblastoma cells.

Currently, there is minimal literature involving the encapsulation of cells within hydrogel particles, with the majority of existing studies involving encapsulation for the purpose of drug and/or cell delivery rather than an *in vitro* tissue model. While there is additional literature about cell encapsulation within hydrogels for more similar purposes, it is primarily using macro samples rather than particles.

This application of microporous annealed particle (MAP) hydrogel with cell encapsulation is a novel approach to creating hydrogel scaffolds for *in vitro* tissue models. It improves on previous approaches as the use of microfluidic devices to create particles allows for more even and controlled distribution of cells across the system, as well as the possibility for creating heterogeneity across the sample which improves complexity and multifunctionality.[9]

The main focus of this research was to improve the viability of encapsulated cells within the microgel building blocks of MAP scaffold. Two primary parameters were tested for the effect on cell viability: the use of hexanes to remove oil in purification, and the initial cell concentration used.

Hexanes were included in the original purification protocol for MAP gel without cell encapsulation, as such it became part of the initial protocol for cell encapsulated gel. When added to the gel-oil mixture, the hexanes chemically react with the fluorinated oil and a foamy layer forms to decrease the density of the oil solution such that it can then be removed more easily than manually removing the more dense oil from the bottom of the gel mixture. Some literature suggests that hexanes could be slightly cytotoxic, so the inclusion of hexanes in the purification was investigated to determine if this possible cytotoxicity had an impact on cell viability.[10]

It was thought in previous experimentation with this protocol that a higher cell concentration contributed to increased viability. The target cell concentration from this testing was 8E6 cells/ml. In order to verify these findings, a low cell concentration and high cell concentration were tested and compared for viability over time.

Materials and Methods

PDMS Device Preparation

An SU-8 wafer photomask was used to create the microfluidic devices. The PDMS and curing agent were mixed in a 10:1 ratio and poured over the wafer, which included photomasks for four devices. This was left in a vacuum to remove bubbles and cure. Once cured, the devices were cut out of the mold using a razor blade and lifted using a spatula. The individual devices were then cut apart with a razor blade, and a 1.5 mm biopsy punch was used to create the inlet and outlet holes, as seen in Figure 1. One glass slide was used per device; tape was used to remove dust from the devices and slides.

The glass slides and devices were plasma-treated; once removed from the plasma cleaner the design side of the device was placed against the glass slide and bonded. The devices were then surface-treated by filling the devices with a PFOCTS-fluorinated oil solution (NOVEC), allowing the devices to

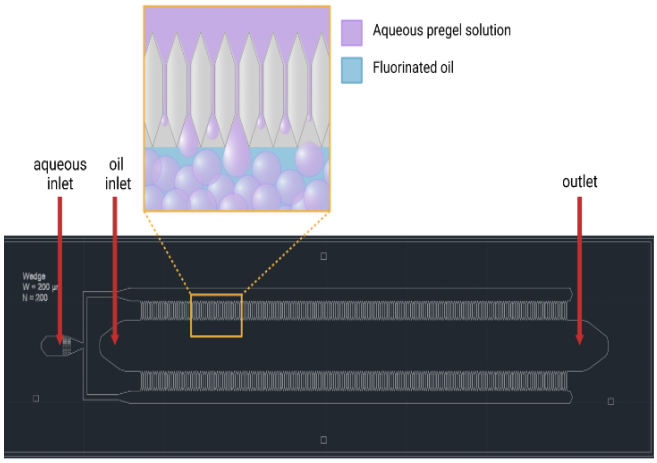

Fig 1. Microfluidic device design.[11]

sit for a few minutes, and aspirating out the treatment. The devices were flushed with fluorinated oil to ensure all surface treatment was removed.

Gel preparation

The 2.5 wt% PEG-VS gel precursor solution was prepared by first reconstituting a 2.6 mg aliquot of arginylglycylaspartic acid (RGD) with 1336.2 µL of sterile triethanolamine (TEOA) solution at a pH of 7.8. This was then used to reconstitute a 10 mg aliquot of polyethylene glycol dithiol (PEG-VS) with 668.8 µL of solution.[12] Next a 9 mg aliquot of matrix metalloproteinase (MMP) was dissolved with 455.4 µL of the media corresponding to the cell type used (both human dermal fibroblasts and beta cells were used separately throughout the course of this study). Added to the MMP aliquot was 60.7 µL of collagen IV solution, 91.1 µL of laminin solution, and 1.5 µL of Alexa Fluor 488 C5 maleimide. The number of cells determined to achieve the desired cell concentration was then isolated in a cell pellet by centrifuging the determined volume of cell and media mixture in a 1.5 mL Eppendorf tube and removing the supernatant with a pipette. 125 µL each of the RGD/PEG-VS solution and MMP/extracellular matrix protein solution were added to the cell pellet to resuspend it.[13] Then the gel precursor was added to a 1 mL syringe with 400 µL of NOVEC oil, and transferred to the syringe pumps to begin encapsulation.

Encapsulation procedure

A syringe filled with 2% surfactant solution was attached to the oil inlet of the device via Tygon and Polyetheretherketone (PEEK) tubing. It was placed on a syringe pump set to flow at 3 mL/hr, and the oil channel was primed. The gel precursor syringe was attached to the aqueous inlet and set to flow at 2 mL/hr, and the aqueous channels were primed. The syringe pumps were started simultaneously; the cells were encapsulated and particles formed as the gel precursor flowed from the side channels into the center channel, as seen in Figure 2. The surfactant solution aided in particle formation and shape. The particles and surfactant solution flowed through the device to the outlet, which was attached to a tubing spiral on a hot plate. The spiral aided in holding the spherical shape of the particles, and the hot plate was used to prevent cold shock to the cells. The hot plate was set to approximately 32 degrees celsius to avoid overheating the cells and keep the temperature close to body temperature. Once the gel particles flowed through the spiral, they were dispensed into a beaker with cell media and a stir bar to prevent clumping. After the gel precursor syringe had been completely emptied, the flow rate of the surfactant solution was increased to 5 mL/hr to ensure no clumping occurred and all particles moved through the device and spiral.

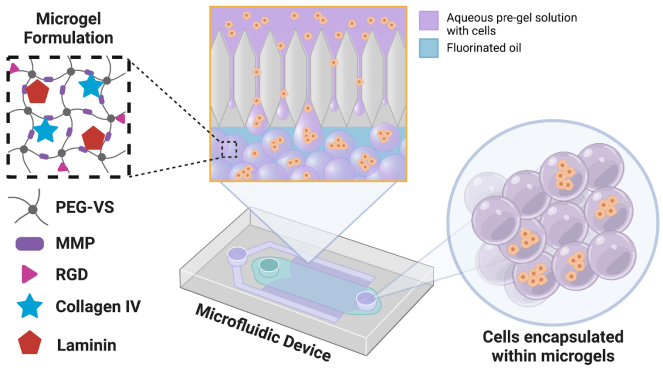

Fig 2. Visualization of encapsulation process and MAP scaffold components.[11]

Purification procedure

Multiple variations of the purification procedure were tested and altered for ease of use, reduced gel waste, and testing the use of hexanes, the following was the final iteration of the purification procedure. Once both the gel and surfactant syringes had emptied, the beaker containing the particles and surfactant-oil solution was poured over a 70 µm cell strainer. The cell strainer was then flipped over a 50 mL conical tube and media washes were performed to transfer the particles from the strainer into the tube. The tube was centrifuged at 300 x g for five minute increments to separate the gel from the media and oil layers. The oil was pipetted out until there was no visible oil layer, with subsequent media washes and centrifuging. If there were any large clumps or particles, a 300 µm cell strainer was used to increase the number of monodisperse particles. The particles were then plated in a 96-well plate with additional media and placed in an incubator until imaged.

Imaging

Immediately before imaging, the encapsulated cells were stained using a blue/red cell viability kit. The gels were imaged in 24-hour increments, with three wells being imaged at once. Viability was quantified using ImageJ particle counter analysis in which the three color channels of the gel, live, and dead cells were separated then analyzed.

Results

As seen in Figure 3, the overall average viability immediately post-encapsulation was around 80%, close to the cell viability immediately before encapsulation. The viability decreased to approximately 70% 24 hours after encapsulation, remaining steady until 96 hours after encapsulation, where it decreased to approximately 55%.

It was found that hexanes did decrease short-term viability when compared with only media washes, as shown in Figure 4, suggesting some cytotoxic effects during the purification protocol. However, the long-term viability of the particles purified with hexanes did not decrease as much as the particles purified with media washes only, as seen in Figure 5.

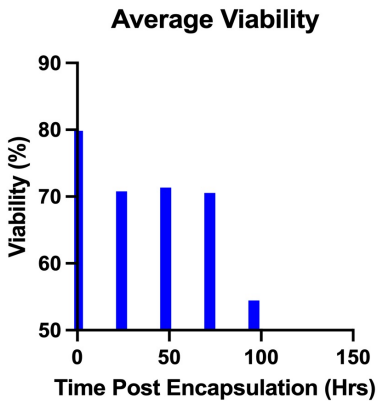

Fig. 3. Average cell viability over time post-encapsulation.

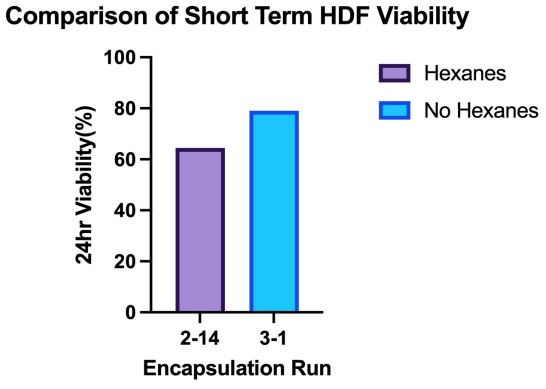

Fig. 4. Comparison of short-term cell viability post-encapsulation based on hexane use.

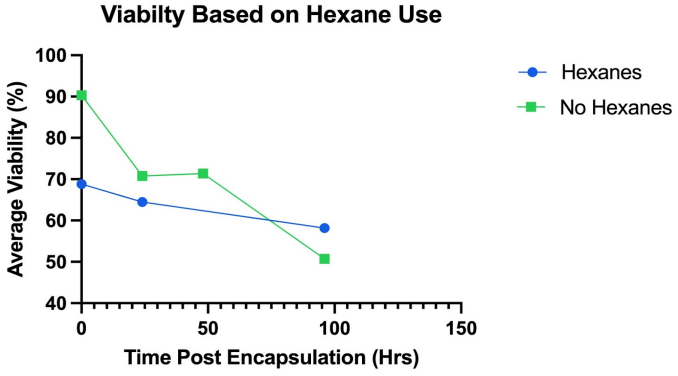

Fig 5. Comparison of average cell viability over time post-encapsulation based on hexane use.

Two cell concentrations were tested over the entire 96 hours, 2E6 cells/mL and 8E6 cells/mL. While the lower cell concentration gel has a higher starting viability, likely due to a greater starting viability, it has a much larger decrease in viability over the 96 hour period, 39.59% viability decrease, than the higher cell concentration gel, 10.65% viability decrease as seen in Figure 6.

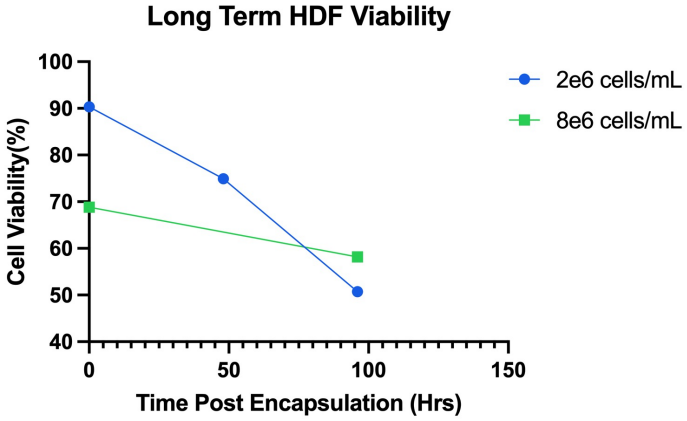

Fig. 6. Long term cell viability based on initial cell concentration for encapsulation.

Discussion

The average viability of cells exhibited two notable drops over the 96 hr period examined: one in the 24 hours after encapsulation, and one between 72-96 hours after encapsulation. The first drop in cell viability most likely demonstrates a cause of cell death related to the stress experienced by the cells during encapsulation procedure. This is to be expected as the cells go through additional stresses by being removed from incubator conditions, and put through the shear and other mechanical stresses involved in the encapsulation process. This could also include the cytotoxic effect on short term viability shown in Figure 4. However, this effect did not appear to continue in the long term, as shown in Figure 5, and would not have demonstrated the second drop in viability between 72-96 hours. The second viability drop could have been due to the lack of change in media after plating the gel. This aligns with the need to change human dermal fibroblast media (the cells used for the majority of cell encapsulation runs) every 3-4 days. This could also include the effects of a low cell concentration, demonstrated by the stark drop in viability over the 96 hour period.

Though we were unable to encapsulate neural cells, we optimized the encapsulation procedure to improve short-term viability of non-neural cells so that future work could use the same protocol for neural cells. Further testing could be done to better quantify the effects of each of the parameters tested on viability, and determine other factors that could influence viability. Changing media after the gel particles are plated could potentially improve long-term viability. Other factors that could affect long-term viability could also be investigated. Co-cultures of different neural cell types, and ultimately glioblastoma behavior could be investigated in order to match the brain tumor microenvironment.[14] Gel components could be varied to better match the stiffness of brain tissue.

Although the impact of the collection fluid on the cell viability was not quantified, it was noticed that using media as the collection fluid made purification more efficient. This is primarily due to the large amounts of oil that would have to be separated from the gel solution during purification, extending the purification process. Additionally, it was thought that collecting in media made it easier to wash oil from the surface of the gel particles, leaving less residual oil following media washes. Part of further improvement and optimization of the purification process should include testing the short and long term impacts on viability of using cell media, fluorinated oil, or an alternative fluid in the collection beaker. Viability would likely be the main parameter to test the effect of the collection fluid as there was no noticeable difference between fluids in the clumping of particles when circulating in the collection beaker.

The future system would use MAP scaffold with encapsulated neural cells to mirror the TME creating a 3D in vitro model to study glioblastoma. The system would be made by resuspending astrocytes, microglia, and endothelial cells in gel precursor and run through a microfluidic device with oil-based surfactant to create uniform microparticles with evenly dispersed cells. Due to the plug and play

nature of this system, the parameters are highly manipulatable allowing them to be adjusted for the desired traits such as matching the gels mechanical properties (stiffness) to that of brain tissue. Additionally, the customizable hydrogel system could make up for the aforementioned pitfalls of current in vitro models. First, the blood brain barrier issue could be solved by the use of astrocytes and endothelial cells in hydrogel which has been successfully used to recreate the blood brain barrier for models of other cancers. Second, the gel particles will be seeded with ECM proteins such as laminin and collagen to help with cell viability and simulate extracellular matrices. Third, the encapsulation of the gel with neural cells, specifically astrocytes, microglia, and endothelial cells allows for important interactions between tumor and non-tumor cells that are key in the TME. Moreover, the customizability of the system could allow for the addition of other cell types in the future should it be deemed beneficial to the promotion of TME interaction.

End Matter

Author Contributions and Notes

D.R.G. and C.R. designed research, Z.A. and L.W.G. performed research.
The authors declare no conflict of interest.

Acknowledgments

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