ALGINATE CORE-SHELL SCAFFOLDS FOR CAR T CELL MANUFACTURING

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ALGINATE CORE-SHELL SCAFFOLDS FOR CAR T CELL MANUFACTURING

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To my beloved parents, whom without none of this would be possible. To my sister and brother in law, for their sincere encouragement and support. To my little nephew, Mehdi, who brings me joy and is the happiness of my life.

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LIST OF SYMBOLS AND ABBREVIATIONS

CAR	Chimeric Antigen Receptor
GMP	Good Manufacturing Practices
FDA	Food and Drug Administration
PBMC	Peripheral Blood Mononuclear Cells
NK cells	Natural Killer cells
MOPSO	3-Morpholino-2-hydroxypropanesulfonic acid
HA	Hemagglutinin
PLO	Poly-L-ornithine
PLL	Poly-L-lysine
PBS	Phosphate-buffered Saline
G	α-l-guluronic acid
M	β-d-mannuronic acid

SUMMARY

Cancer patients with advanced-stage disease develop resistance to traditional therapies such as chemotherapy and radiation therapy, leading to the necessity of a novel technology that is both specific and efficient to the patient's cancer. Genetically engineered T cell receptors can recognize and bind to antigens expressed on tumor cells and kill them. As a result, T cell immunotherapies have been established as a new strategic cancer therapy. The promising success of T cell immunotherapies for treatment of cancer and other diseases demonstrate the need for scalable manufacturing processes for product commercialization. Unlike traditional chemotherapies, cell-based immunotherapies are composed of living entities, and hence they are different in their development, properties and regulatory pathways in comparison to traditional drugs, which are simple chemicals. Current T cell manufacturing techniques are complicated processes that do not account for the complexity of the lymph nodes, where T cells expand rapidly in response to disease. Consequently, it is difficult to produce enough cells for quality control assays and meet the Good Manufacturing Practices (GMP) guidelines. Thus, we hypothesize that creating a novel microenvironment that can mimic the lymph nodes will enhance expansion of T cells and allow this promising treatment to reach more patients in the clinic. To do so, we proposed using alginate which has been widely used for cell encapsulation. We prepared and optimized alginate core-shell scaffolds to provide an environment for close cell-cell contact and communication as well as protect T cells against stress for CAR T cell signaling and expansion. After expansion, the scaffolds were dissolved through both enzymatic and physical dissociation. This method will eventually allow the modified T cells to be placed in a bioreactor, allowing this technique to be commercialized by companies in the cell manufacturing industry.

CHAPTER 1

INTRODUCTION

1.1 Chimeric Antigen Receptor Therapy

Surgery, chemotherapy and radiation therapy have been the foundation of cancer treatments for a long time. However, many cancers grow resistant to these treatments and remain refractory[1]. A new groundbreaking cancer treatment known as Chimeric Antigen Receptor (CAR) T cell therapy has shown great promise in treating lymphoblastic leukemia, as well as other diseases. These receptors, known as CARs, can recognize and attach to antigens on tumor cells and destroy them[2]. Currently there are 200 ongoing T cell therapy clinics and 2 CAR T cell therapies have been FDA approved[3].

The starting material of CAR T cell therapy is T cells, which are responsible for killing cells infected by pathogens. For this therapy, the cancer patient's blood is collected at a clinic. The blood contains peripheral blood mononuclear cells (PBMCs), and these cells consist of 3 different types of lymphocytes: T cells, B cells, and NK cells.

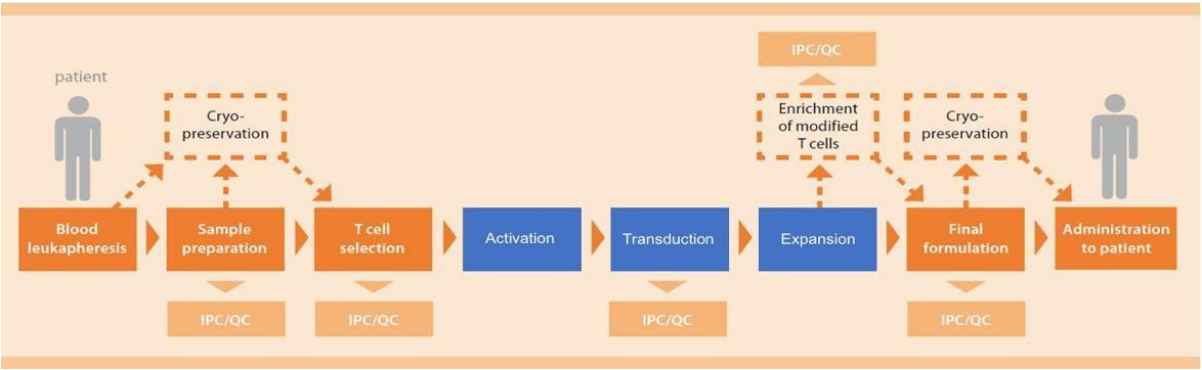

Figure 1: Workflow for gene engineered T cell production[4]: blue boxes indicate scope of this project.

The T cells are isolated from the PBMC suspension, activated using antibodies and transduced by a lentiviral vector to produce synthetic receptors on their surface. The modified T cells are expanded in a variety of ways (current manufacturing techniques focus mainly on anti-CD3 and anti-CD28 T cell activation) to reach the minimum cell density

needed for an effective treatment dose. After final formulation, CAR T cells are administered back into the patient's body[4].

1.1.1 Manufacturing Limitations of CAR T cell Therapy

Although this treatment has been proven to be promising, T cell therapies are severely constrained by manufacturing limitations in quality and quantity. Prior to receiving T cell therapy treatment, the majority of patients undergo chemotherapy and radiation therapy, which results in bone marrow lymphodepletion and exhaustion, hence limiting the T cell count of the patient[5]. This leads to a limited T cell count and thus, would be difficult to use for quality control, safety testing, and storage, given time and cost constraints. Further, as cell therapies are composed of living entities, slight alterations in stimuli may lead to considerable changes in cells. This creates a complex process for clinical manufacturing of CAR T cells and requires an optimization of T cell expansion methods.

Current expansion methods use suspension bioreactors that require large amounts of cytokines and media and unlike lymph nodes, maintain large distances between cells, limiting communication[6]. A strategy to overcome this problem is to create a novel microenvironment that has the ability to mimic the lymph nodes in order to provide high yielding expansion of T cells for multiple dose distributions[7]. The lymph node provides an optimal environment for T cell clustering, which increases cell signaling and proliferation. The extracellular matrix surrounding the lymph node contains pro-survival mechanical cues, which have significant effects on cell proliferation, differentiation and death[8]. Current T cell suspension methods lack these characteristics, so in order to capture these key components, we mimicked the lymph node microenvironment by preparing and optimizing core-shell scaffolds for enhance CAR T cell attachment, growth and eventually manufacturing.

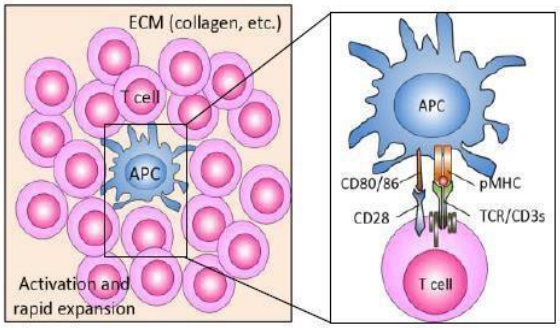

Figure 2: Lymph nodes (in vivo).

1.1.2 Developing a Novel Microenvironment for T cell Manufacturing

The lymph node microenvironment can be mimicked using core-shell scaffolds to achieve high cell density, enhance signalling, and protect cells from high shear environment. Alginate was chosen over other candidates for preparing the core-shell scaffolds due to its reverse spherification, low cost, non-toxicity, ease of use and stability at room temperature[9].

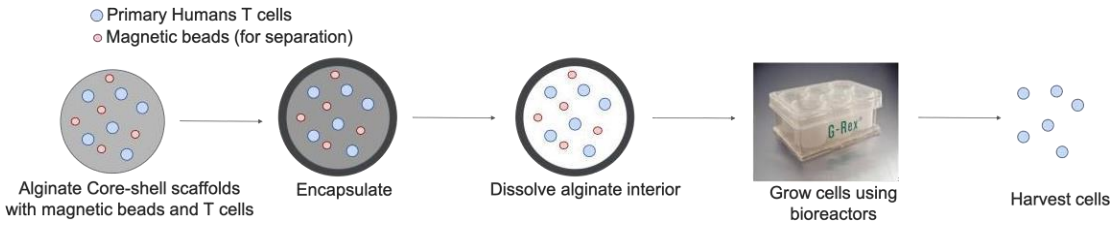

Figure 3: Illustration of the process of encapsulating, expanding and harvesting T cells.

The goal is to enhance signaling and expansion of T cells by using alginate scaffolds to protect against stress as well as provide an environment for close cell-cell contact and communication. After T cell expansion, the scaffolds will be dissolved through both enzymatic and/or physical dissociation. The T cells will eventually be placed in a bioreactor, making this technique favorable for commercial manufacturing of CAR T cells in the biotechnology industry.

CHAPTER 2

METHODS AND MATERIALS

2.1 Primary Human T cell Activation

A vial containing naïve T cells was retrieved from liquid nitrogen and thawed to 37C. 1ml of cells were added to a conical tube containing 8ml of thaw media, which was prepared according to manufacturer's instructions (CTL Anti-Aggregate Wash™). To ensure that all of the vial content had been transferred, the vial was washed with 1ml of thaw media and then transferred to the conical tube. The suspension was centrifuged at 200g for 10 minutes and was then aspirated down to 100ul. Using a pipette, the remaining suspension was resuspended in 1ml of TexMACS media and counted using trypan or AO/PI staining. The final cell density was calculated and resuspended to be 5e6/ml in TexMACS media.

Primary human T cells were activated using Anti-Biotin MACSiBead particles, which were loaded with biotinylated anti CD3 and CD28 antibodies. To do so, 5ul of loaded MACSiBeads per 1e6 PBMCs were added to 500ul of TexMACS media. The media was centrifuged at 400g for 5 min and then aspirated down. Finally, the naïve T cells (obtained from the previous step) were transferred to the MACSiBeads tube and mixed thoroughly using a pipette.

2.2 Primary Human T cell Expansion

In order to create the alginate scaffolds, an electrostatic droplet generator was set up as shown in Figure 4, and a solution consisting of sodium chloride, 0.4% NovaMatrix Pronova SLG20 alginate, Pierce Anti-HA Magnetic Beads (purchased from Thermo Fisher

Scientific and used for keeping the scaffolds in the solution during washing steps) and activated human primary T cells were loaded into a 5ml syringe. The optimal height and voltage for the scaffolds were determined to be 6cm and 10kV, respectively[10]. After turning on the voltage, the alginate solution was sprayed in the form of small droplets into a calcium chloride solution and crosslinked. Using an EasySep Magnet, the scaffolds were then washed with a buffer consisting of sodium chloride and MOPSO (purchased from Sigma-Aldrich) to remove excess calcium chloride. The scaffolds were then coated with 1ml of either Poly-L-lysine or Poly-L-ornithine solution (purchased from Sigma-Aldrich and Fisher Scientific, respectively) for 20 minutes in order to create an exterior shell. To ensure that the alginate scaffolds have a liquid interior, 20ml of a buffer solution containing sodium nitrate was added and the resulting solution was vortexed for 10 minutes. The encapsulated cells were transferred to a T25 flask and incubated at 37C and expanded for 2 weeks.

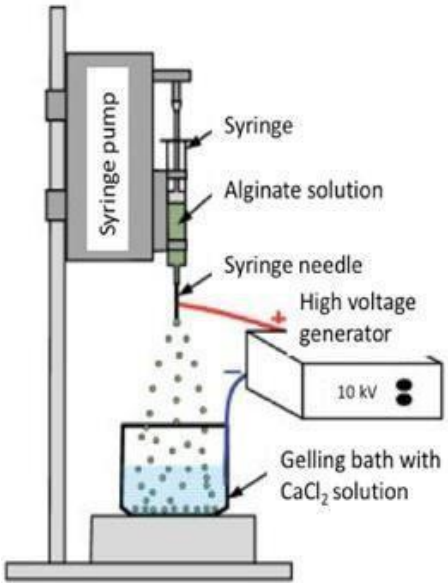

Figure 4: Experiment set up of electrostatic droplet generator for creating core shell scaffolds[11].

2.3 Primary Human T cell Harvesting

After expansion, the scaffolds were dissolved using enzymatic/mechanical methods. The first method consisted of alginate lyase (purchased from Sigma-Aldrich) and trypsin for enzymatic dissociation of alginate and trypsinization of PLL. To determine the effects of alginate lyase and trypsin on the scaffolds' dissolution, 4 runs were designed. To prepare the first run, which was the control, 1ml of sodium chloride was transferred to a microcentrifuge tube. For the second run, 1ml of trypsin was added to a separate microcentrifuge tube. In the third run, 0.5mg of alginate lyase was added to 1ml of sodium chloride and the solution was vortexed. For the last run, 0.5 mg of alginate lyase was transferred to 1 ml of trypsin and mixed properly.

For the second method, the scaffolds were dissolved using a combination of alginate lyase and a physical dissociator named gentleMACS. gentleMACS is a semi-automated device designed for standardized dissociation of tissues into single-cell suspensions. The device setting was as follows:

Figure 5: gentleMACS Dissociator.

a) Temperature on (set to 37C)
b) Loop 5x
c) Spin 2000 rpm, 15 seconds
d) Spin 20rpm, 2 minutes
e) End loop
f) End

The obtained dissolved scaffolds were filtered using a cell strainer to remove any left-over alginate or magnetic beads and cell viability was determined using trypan staining and a cell counter (Countess, Thermo Fisher).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Alginate Core-shell Optimization

To determine maximum cell fold change in different alginate G/M (ß-D-mannuronic acid, poly α-L-guluronic acid, respectively) compositions (1 and 1.5) and viscosities (100 and 300mPa*s), initial growth studies were performed in cell culture plates using Jurkats, an immortalized cancer cell line for which its expansion is independent of activation. In comparison to the control group (PBS), it was observed that 0.4% alginate G/M 1.5 contained the highest Jurkat fold change, whilst 1.2% G/M 1.5 had the lowest fold change (Figure 6).

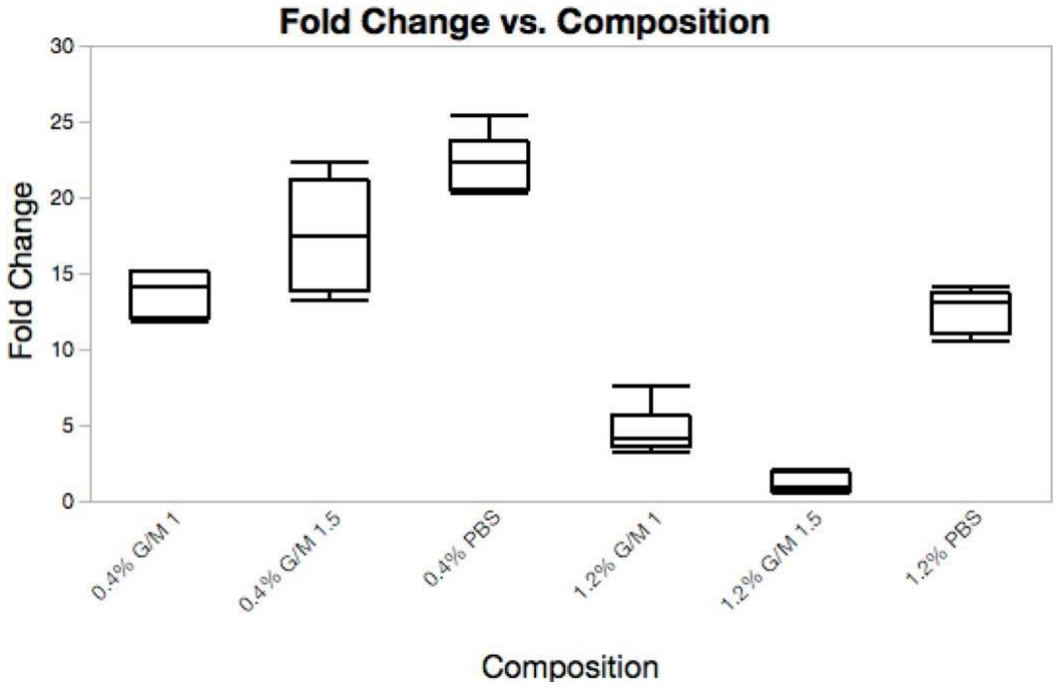

Figure 6: Initial growth studies in different alginate G/M compositions (1 and 1.5) were assessed using Jurkats.

To assess cell growth in Anti-HA magnetic beads, which contained sodium azide and TWEEN 20 that are toxic to cells, a dose response curve was used to find the relationship between cell growth and the magnetic beads' dilution factor. The dose response curve on

day 1 did not appear to significantly change between concentrations (Figure 7), but on day 2, the curve showed that the viability of cells dramatically increased between 0.0195 and 0.039 dilution factor (Figure 8). We concluded that the concentration diluted to 0.00195 would not be detrimental for Jurkat viability.

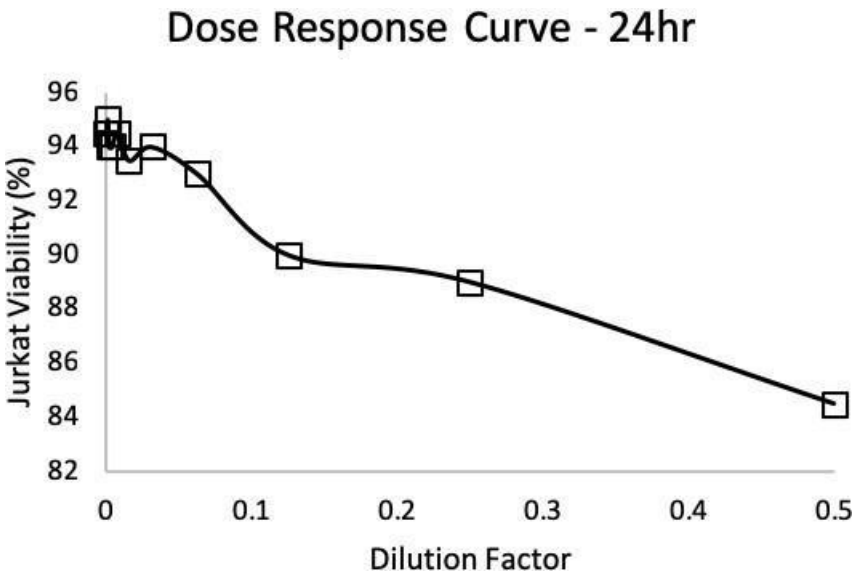

Figure 7: Dose response curve on day 1.

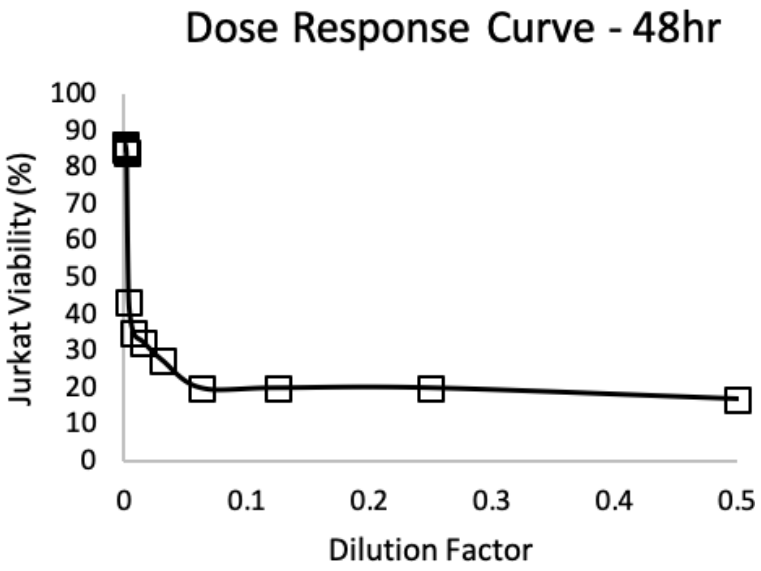

Figure 8: Dose response curve on day 2.

3.2 T cell Expansion in Alginate Core-shell Scaffolds

Jurkats were expanded in PLO and PLL core-shell scaffolds (Figure 9), and process conditions that were found successful for Jurkat expansion were applied to T cell expansion. To determine growth of T cells in scaffolds, the encapsulated primary human T cells were imaged every two days using a phase-contrast light microscope. It appears that by day 4 the Jurkats and T cells have grown and expanded in the scaffolds.

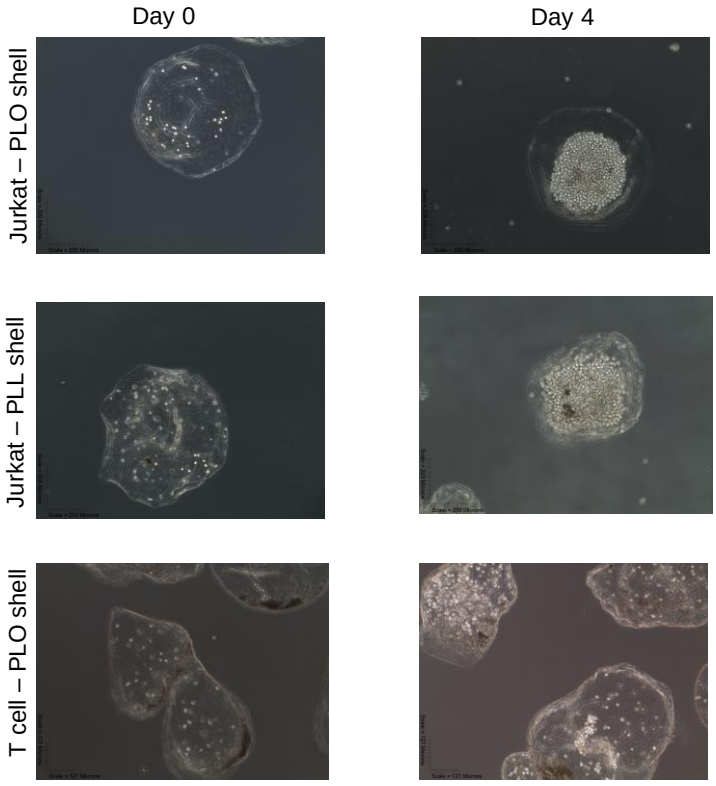

Figure 9: Encapsulated T cells and Jurkats in core-shell scaffolds.

Diffusion in Jurkat-PLO alginate scaffolds was qualitatively assessed by Propidium Iodide and Calcein AM staining. The stained scaffolds were imaged using RFP and GFP filters on confocal microscopy (Figure 10). The red staining represents dead cells whilst the green staining represents live cells.

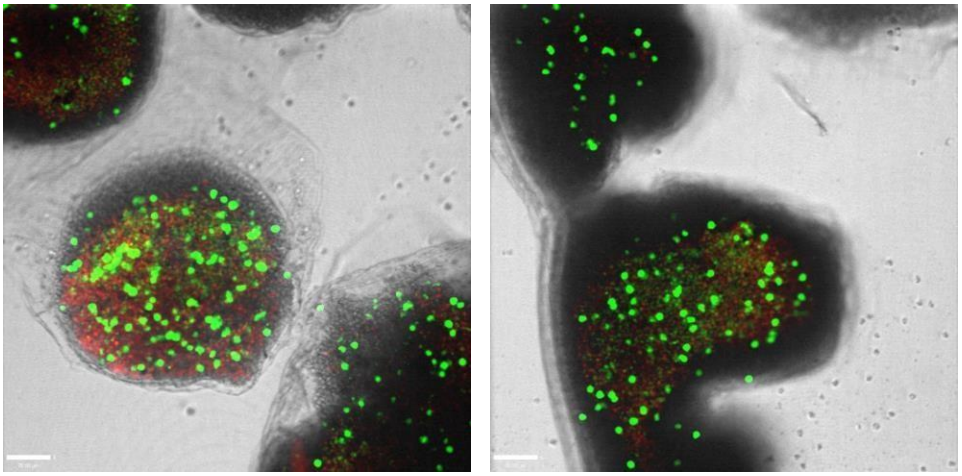

Figure 10: Viability was determined by live/dead assay staining.

3.3 Enzymatic Dissolution of PLO and PLL Alginate Core-shell Scaffolds

To harvest the encapsulated cells from the core-shell scaffolds, a comparison study for enzymatic dissolution rate was carried out between PLL-alginate scaffolds and PLO-alginate scaffolds. Initially, the PLL-alginate scaffolds and PLO-alginate scaffolds were dissolved using trypsin. As PLL-alginate scaffolds were shown to dissolve at a much faster rate, they were chosen as the preferred scaffolds for encapsulating cells (Figure 11). However, none of the scaffolds were dissolved within less than a day.

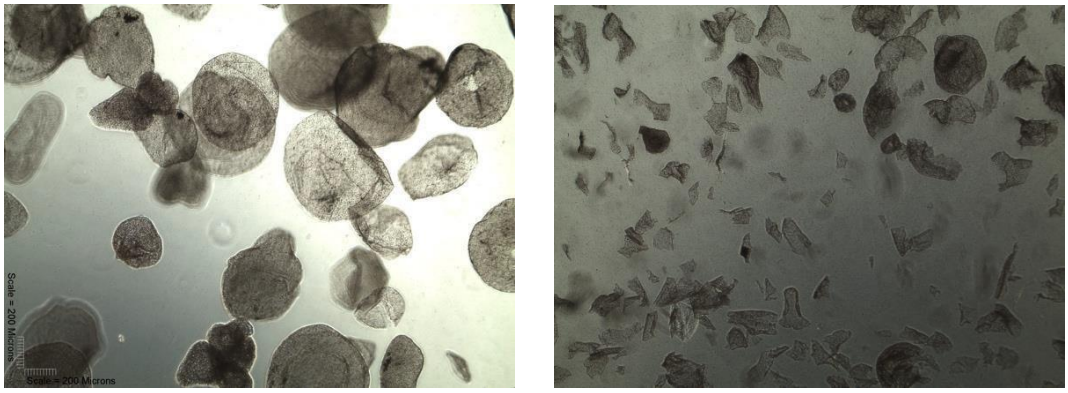

Figure 11: PLL-alginate scaffolds. Left: Day 0, Right: Day 3

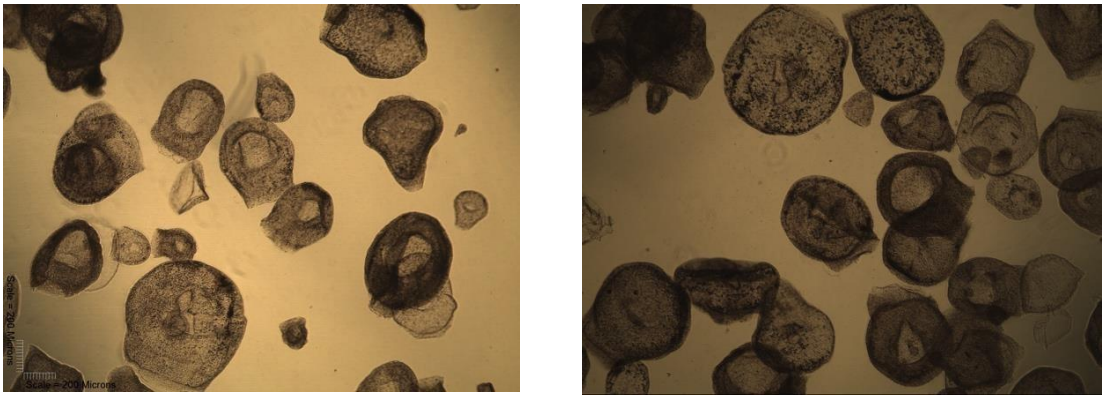

Figure 12: PLO-alginate scaffolds. Left: Day 0, Right: Day 3

In order to choose the optimal alginate lyase concentration, a growth study on Jurkats was carried out and the cell viability was measured every 10 minutes (Figure 13). As shown, cell viability did not decrease in 0.5mg/ml Alginate Lyase.

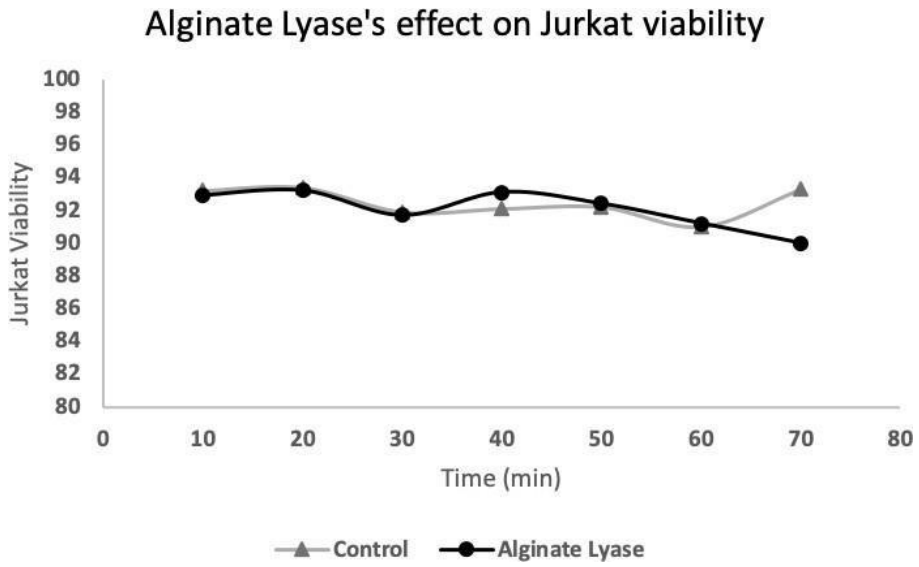

Figure 13: Grey trendline: Jurkat viability in PBS (control), black trendline: Jurkat viability in 0.5mg/ml alginate lyase. Viability was measured every 10 minutes.

Additionally, it was found that a pH of 8 and a temperature of 37C was successful for alginate lyase's activity on the PLL-alginate scaffolds (Figure 14).

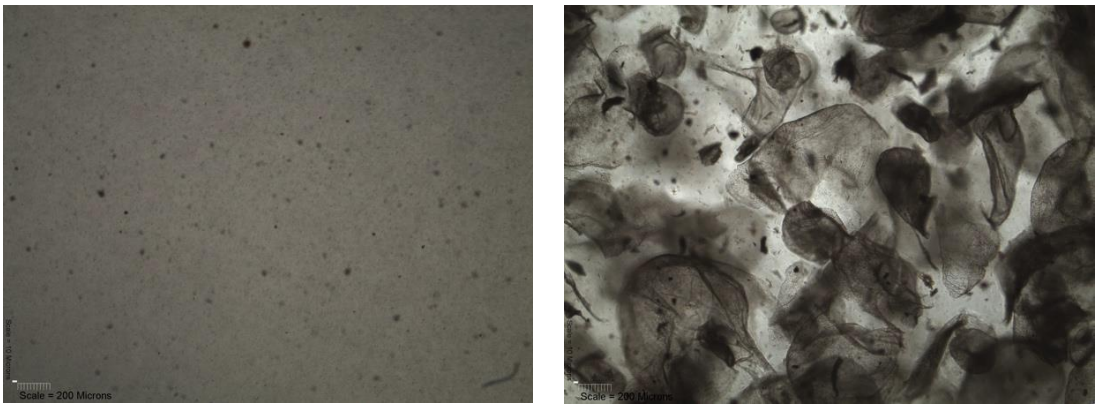

Figure 14: Left: Alginate scaffolds dissolved using alginate lyase (control). Right: PLL-alginate scaffolds dissolved using alginate lyase with pH of 8 and temperature of 37C. Imaged after 1 hour.

The combination of trypsin and alginate lyase for dissolving PLL-alginate scaffolds was tested and imaged as depicted in Figure 15. Further dissolution experiments were conducted on scaffolds containing Jurkat cells and although the majority of the scaffolds were dissolved, the cell viability that was measured was extremely low (data not shown).

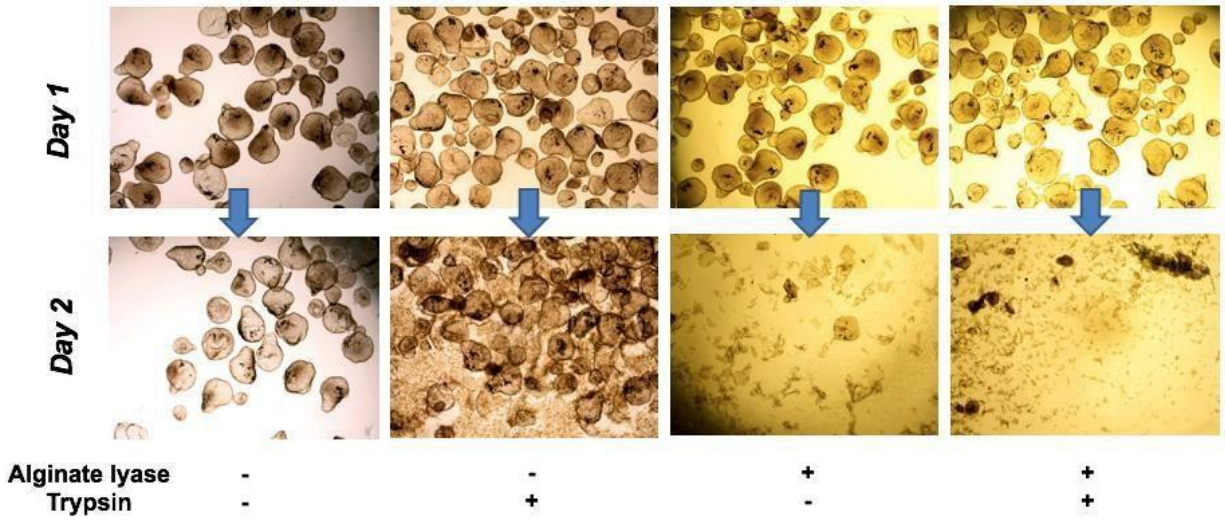

Figure 15: PLL-alginate scaffolds dissolved using trypsin and alginate lyase.

3.4 Physical Dissolution of PLO and PLL Alginate Core-shell Scaffolds

The physically dissociated PLL-alginate scaffolds can be observed in Figure 16. The scaffolds were dissolved using gentleMACS. A second dissolution experiment involving encapsulated T cells was carried out and the measured viability was observed to significantly decrease by 70 %.

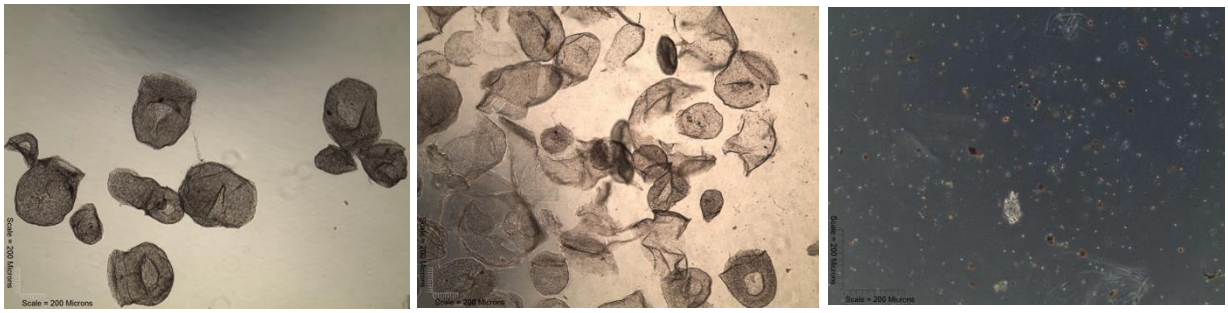

Figure 16: PLL-alginate scaffolds before (left) and after (right) physical dissociation. Far right: Liberated T cells floating in a suspension of dissolved alginate and magnetic beads.

A combination of alginate lyase and gentleMACS was tested on the alginate scaffolds and the suspension was filtered using a cell strainer (Figure 17). The optimal method of dissolving all the scaffolds whilst maintaining a high cell viability is yet to be determined.

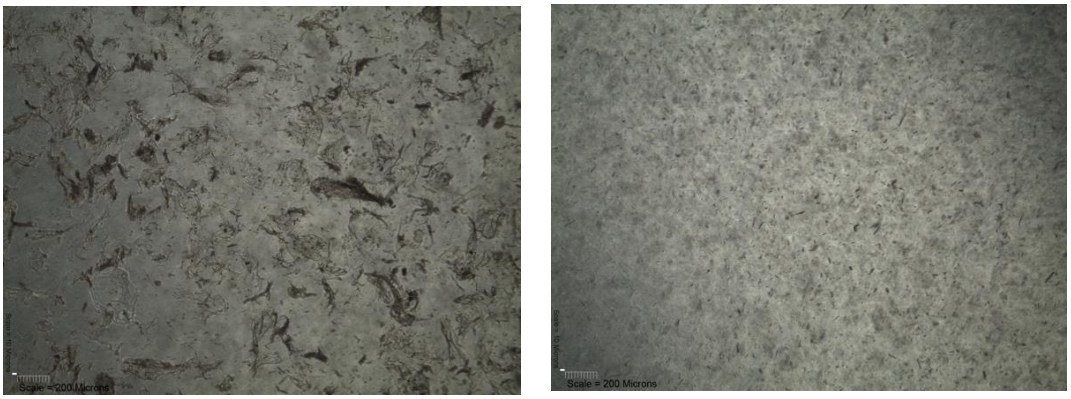

Figure 17: Left: core-shell scaffolds dissolved through enzymatic and physical dissociation. Right: final suspension filtered using a 200um cell strainer to remove undissolved scaffolds.

CHAPTER 4

CONCLUSION AND FUTURE WORK

The three major aims for this project was to encapsulate, expand and harvest T cells. Alginate core-shell scaffolds provided an environment for close cell-cell communication, resulting in expansion of Jurkats and T cells. Alginate lyase and gentleMACS had the ability to dissolve the majority of the scaffolds, however, enzyme concentration and duration of dissociation must be adjusted to prevent cell death. Once liberation of scaffolds has been successful, an experiment involving primary human T cells will be conducted to assess phenotype and growth, and to eventually be placed in a bioreactor for cell manufacturing. As this is a manufacturing-based project, future experiments will be carried out to determine optimal process parameters, including needle height and weight, electric field and flow rate during electrospray and the effect of alginate types on scaffolds' shape and size in order to achieve consistent, manufacturable results.

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