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Development and Characterization of Adhesive Hydrogels for Stem Cell Culture

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Development and Characterization of Adhesive Hydrogels for Stem Cell Culture

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Abstract

Pluripotent embryonic stem cells (ESCs) offer the unique capacity to differentiate into almost any cell type and have sweeping implications in both basic research and clinical applications. However, unregulated differentiation can cause issues, preventing ESCs from entering clinical research. In order to maximize ESC growth, three dimensional culturing has been utilized in order to have results more similar to *in vivo* conditions. In the case of alginate scaffolds, cell adhesion sites are missing from the matrix, leading to differentiation. We propose that the inclusion of adhesive polymer to the alginate scaffold will increase cell attachment and maintain pluripotency. The polymer N[3-Dimehylamino propyl methacrylamide] DMAPMA has been shown, in a screening of 66 monomers, to increase cell adhesion and proliferation. By adding a methacrylic acid (MAA) group to the polymer, it readily dissolves in alginate and can form microstrands (200 μm) for 3D adhesive mouse ESC cell culture. This study compared four growth conditions: alginate solid core, alginate liquefied core, DMAPMA-MAA modified alginate solid core and modified liquefied core, in terms of ESC growth and gene expression of pluripotent markers by qPCR analysis. It was found that the DMAPMA-MAA-modified liquefied alginate microstrands support the best organized, high density ESC growth while maintaining the best pluripotent marker Oct4 expression.

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1.   Introduction

Pluripotent embryonic stem cells (ESCs) offer a viable and practical solution to the issues facing biological researchers today. Though ESCs show promise in the treatment of diseased models, proper control over differentiation and pluripotency expression is still needed before clinical applications can be sought. Embryonic stem cells (ESCs) are indefinitely self-renewing and possess the aptitude to differentiate into a variety of cell types [1-3]. In vivo, these cells can form functional units and tissues through self-assembly [4-8]. Though self-assembly has been comprehensively examined at the molecular level, it is important to understand how these individual cells can self-assemble on a cellular level in order to further the field of tissue engineering [9]. There has been extensive work into the culturing of ESCs on nano-fibrous mats in order to mimic the effects of the extracellular matrix, but 3D culturing systems to promote cell attachment through entrapment remains a largely unexamined aspect of research for examining maintained pluripotency in ESC cell culturing.

Two dimensional (2D) stem cell culturing can misrepresent in vivo responses [10]. Therefore, there is a necessity for three dimensional (3D) cell culturing in addition to the need for implantable scaffolds. The benefits of 3D stem cell culturing over traditional 2D methods exist by allowing for *in vivo*-like cell to cell connections, signaling and organization which will better model the cell growth and tissue function in living organisms.

This concept is exceedingly important in the case of ESCs as it is believed that the stem cell niche contributes to the maintained pluripotency and directed differentiation. The stem cell niche is the environment comprised of cellular, non-cellular, physical and chemical features that regulate cell fate [11]. These factors include micro- and nano- topography, surface stiffness, soluble factors such as hormones proteins and growth factors [12]. The extracellular matrix

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offers the ability to control many of these physical needs *in vivo* [13]. *In vitro*, these conditions are difficult to meet without the spatial relationships offered through 3D culturing techniques.

Alginate hydrogel is one of the most promising biomaterials for 3D cell culture. Alginate is derived from a family of polysaccharides found in the cell walls of brown algae. It is biocompatible, FDA-approved and is currently used in the treatment of burns, dental and orthopedic molding, and cell culturing. Due to its versatility in structure from its district G/M block make up, alginate can be extremely flexible in its use in cell culturing. The hydrogel formed from the alginate has virtually no effect to stem cell proliferation and function and alginate hydrogels are stable in the same environments that promote cell growth. This implies that alginate would be an effective scaffold to promote stem cell propagation [14]. Traditionally, alginate hydrogel microbeads or microcapsules have been used for cell culture, cell delivery and therapy. However, microbeads and microcapsules typically have larger diameters, around 500 μm to above 1 mm [15, 16, 17]. This is larger than the optimal distance for the transfer of oxygen, nutrients and waste products to and from cells [18]. Microstrands offer an elegant solution to the issue of a viable 3D cell culturing method. Typically, these microstrands are fabricated from alginate and create functional units that are easily reproduced with relatively small diameters (100-200μm) and mimic the morphologies and functions of living tissues. These microtubes are implantable and have even been shown to regulate the blood glucose in diabetic mice [19].

Microstrands are currently being used for the culture of different types of mammalian progenitor and differentiated cells as well as for the culturing of bacterial cells [9,10,19,20,21]. These groups are using microtubes in attempt to create a 3D scaffold that mimics tubular structures in vivo [9,10,19,20,21]. These scaffolds can be used in cell immobilization or

modified to affect the growth environment. A better understanding and characterization of these microtubes would aid in the research of stem cells and other types of cell culturing as well as advance the field of tissue engineering.

In this study we aim to provide a 3D scaffold for maintained growth of pluripotent ESCs. Due to the fact that 2D ESC culturing can misrepresent *in vivo* responses due to a lack of cell to cell connection, 3D culturing through utilization of hydrogel microenvironment would be the optimal condition. The hydrogels are formed by the crosslinking of sodium alginate through the use of calcium ions which has virtually no effect on cell proliferation and function [9]. The issue arises from alginate's inherent lack of cell adhesions sites. Adhesion sites are critical for the maintenance of ESC pluripotency [23]. In 2D, gelatin coated flasks give anchorage points which dissuade differentiation. In microstrands, without the inclusion of binding sites, cells aggregate. This aggregation leads to ESC differentiation which is undesirable for the maintenance of a pluripotent line. Differentiated ESCs do not behave as pluripotent ESCs would and would create specious results. This could hinder the ability of alginate to provide a 3D scaffold for pluripotent stem cell culture on a more general level.

We propose that by incorporating a polyamide into the alginate gel matrix, cell differentiation can be halted. The polyamide DMAPMA was found to better maintain stemness of a pluripotent line when cultured in 2D [22]. This was due to an increase in cell adhesion and hydrophilicity of the polyamide modified surface. By incorporating a methacrylic acid (MAA) group the polymer could dissolve in alginate. Solutions were made by dissolving the polyamide in deionized water at a pH of 7 and then incorporating the alginate at 1.5%. Through this method, we were able to successfully incorporate the adhesive polymer into the gel matrix. The further liquid modification was accomplished by coating the strands in poly-L-lysine (PLL) and liquefying the

core of the microstrand with sodium citrate. Initial results have shown variant growth patterns between the unmodified and polyamide modified microstrands. We see an oriented "cobblestone" pattern in the liquified modifed microstrand that suggests the adhesion of the cell growths to the inner wall of the microstrand.

2. Materials and Methods

2.1 Mouse ESC Culture

Mouse CCE ESCs were obtained from StemCell Technologies, Inc. (Vancouver, Canada). Mouse ESCs were cultured in gelatin coated tissue culture flasks in a cell culture incubator at 37˚C, 5% CO_2. The cells were maintained in an undifferentiated state and cultured in growth media consisting of Dulbecco's Modified Eagle's Medium (DMEM 4.5 g/l D-glucose), with added 15% (v/v) fetal bovine serum (FBS), 100μg/ml streptomycin, 100 U/ml penicillin, 0.1mM non-essential amino acids , 2mM L-Glutamate, 1mM Sodium pyruvate, 10 ng/ml, 0.1 mM Monothioglycerol (Sigma-Aldrich, St. Louis, MO), and recombinant leukemia inhibitory factor (LIF) (StemCell Technologies, Vancouver Canada).
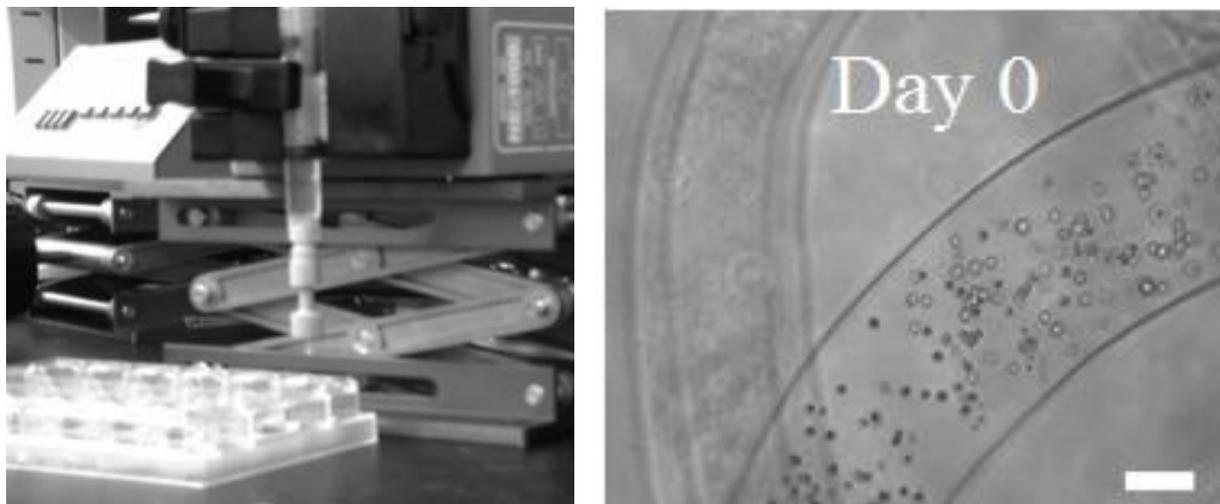

Figure 1. *Fabrication Setup: left, Syringe pump loaded with alginate-cell solution over 50mM calcium chloride bath. Right, fabricated alginate microstrand with encapsulated ESCs. Scale bar equal to 100μm*

2.2 Fabrication of Polymer Modified and Unmodified Alginate

The alginate solutions were prepared with powdered sodium alginate (Sigma Aldrich). Sodium alginate was added to a 0.9% sodium chloride solution. This created an overall 1.5% sodium alginate solution with a viscosity of 100-300cP in in 2% solution at 25˚C. To create the polymer modified solution, the polyamide (DMAPMA-MAA, Sigma Aldrich) was dissolved in a 0.9% sodium chloride solution at a pH of 7. The solution was shaken in a 37˚C rocking incubator for 24 hours, until dissolved. If solution remained undissolved after 24 hours, it was titrated with 1M HCl in order to reach a pH of 7 before it was incubated for another 24 hour period. After the DMAPMA-MAA fully dissolved into the 0.9% NaCl, the alginate powder was added to create a 1.5% alginate solution to form the modified alginate solution. The solution was allowed to stir over night.

2.3   Alginate Microstrand Fabrication

The microstrands were formed by suspending ESCs in either the modified or unmodified alginate solution. The ESCs were seeded in the alginate solutions at a concentration of $1x10^6$ cells/mL. The cell suspension was loaded into a 3 mL syringe and placed into syringe pump. Once the pump was inverted, the solution was flown through 100 μm silica-capillary tubing at a constant rate provided by the syringe pump (NE-1000 New Era Pump Systems) at a rate of 0.1 mL/min. The alginate cell solution crosslinks in the presence of calcium ions and forms a hydrogel upon entering the 24-well plate containing the 50 mM calcium chloride bath (Figure 1). The strands were allowed to form in the well for 3 minutes. The tip was occasionally shaken in order to prevent clogging of the silica capillary tubing. In order to assure the formation of the microstrands, an inverted microscope (Nikon eclipse TS100) was used to image the wells. In the case of the alginate and modified alginate microstrands with solid cores, the calcium chloride

was removed and replaced with growth media. The microstrands with liquefied cores were coated in 0.1% PLL (Sigma-Aldrich) after the removal of the calcium chloride. This is a positively charged polymer that adheres to the negatively charged sodium alginate surface. This prevents the microstrands from being dissolved during the liquefying step. Coating took place over the course of 10 minutes; all the while the microstrands were subjected to constant rocking. Once the tubes were coated, the PLL was removed and 1.6% sodium citrate was introduced for 2 minutes. This dissolved the core of the microstrand while the outside retained its form due to the semi-permeable PLL shell. This created the liquid core of the microenvironment in both the modified and unmodified alginate microstrands. The microstrands were maintained for a week of growth in ESC pluripotency media, observed, and then prepared for subsequent assays. Initial success criteria were based on the ability of the microstrands to not dissolve on contact with the sodium citrate and for cellular growth to continue throughout the entire week in both the modified and unmodified strands in the solid and liquid core growth conditions.

2.4 Rheology Analysis

Measurements were recorded on a TA Discovery rheometer at Rensselaer Polytechnic Institute during a TA Rheology workshop. The samples were prepared by making 5 mL of solid alginate and modified alginate microstrands. The calcium chloride was removed and the microstrands were placed in 15 mL conical tubes for transport. 0.9% NaCl was added to the microstrands to keep them osmotically balanced. At the time of the measurement, 1 ml of microstrands was used for rheology data. The measurements were taken by Dr. Madhu Namani.

2.5 Quantitative Polymerase Chain Reaction

RNA was extracted from the samples using a combination of TRIzol® homogenization and chloroform phase separation (Life Technologies, Carlsbad, CA). This was then followed by isolation using an RNeasy kit (Qiagen, Valencia, CA). The RNA samples were then reverse transcribed using a first-strand cDNA synthesis kit (Invitrogen, Grand Island, NY).Quantitative real-time PCR (qPCR) analysis was performed using a StepOnePlus™ Real Time PCR system (Applied Biosystems, Foster City, CA). Samples were reported using a SYBR® green I PCR master mix. Expression levels were normalized to the housekeeping gene GAPDH. The relative quantitation of Oct4 in addition to a transcriptional regulator highly expressed in pluripotent mouse ESCs, Nanog were examined using the comparative Ct method.

3.   Results and Discussion

3.1   DMAPMA-MAA Microstrand fabrication

To demonstrate the feasibility of an adhesively modified alginate microstrand we dissolved the DMAPMA-MAA in deionized water (Mili-Q, EMD Millipore, Darmstadt Germany), PEO and alginate, without ESCs. It was found that the DMAPMA-MAA did not affect the alginate's ability to crosslink in calcium chloride. The microstrands were optically and physically similar under the same fabrication conditions.

In the next trial, ESCs were used to appropriately tune the growth condition. It was found that the initial protocol for modified alginate did not bolster cell growth and, due to the lack of visibility, obscured cells from view by brightfield imaging (Figure 2a). Initial trials have shown vast improvement of cellular proliferation in the microstrands by decreasing the amount of

polyamide used from 1% to 0.5%. Subsequently, the elimination of PEO, which was a part of the original protocol, has increased visibility and cellular growth of the week long maintenance one week period (Figure 2).

The lower concentration of polyamide coupled with the addition of sodium chloride shows a much clearer growth pattern of the ESCs. The removal of polyethylene oxide improves visibility and also appeared to increase confluency of the microstrand. The original incorporation of polyethylene oxide was for stability needed in electrospinning alginate. Though there was initial concern over the solubility of DMAPMA-MAA after the omission of the PEO, It was deemed unnecessary for the low flow rates of microstrand fabrication.

Over the week long growth experiments there was a clear difference between ESCs growing in the modified versus unmodified alginate microstrands (Figure 3). There was also an obvious, though predicted, variance between cells cultured in the solid and liquefied microenvironments. Alginate solid microstrands showed patches of cellular growth indicative of distinct colonies
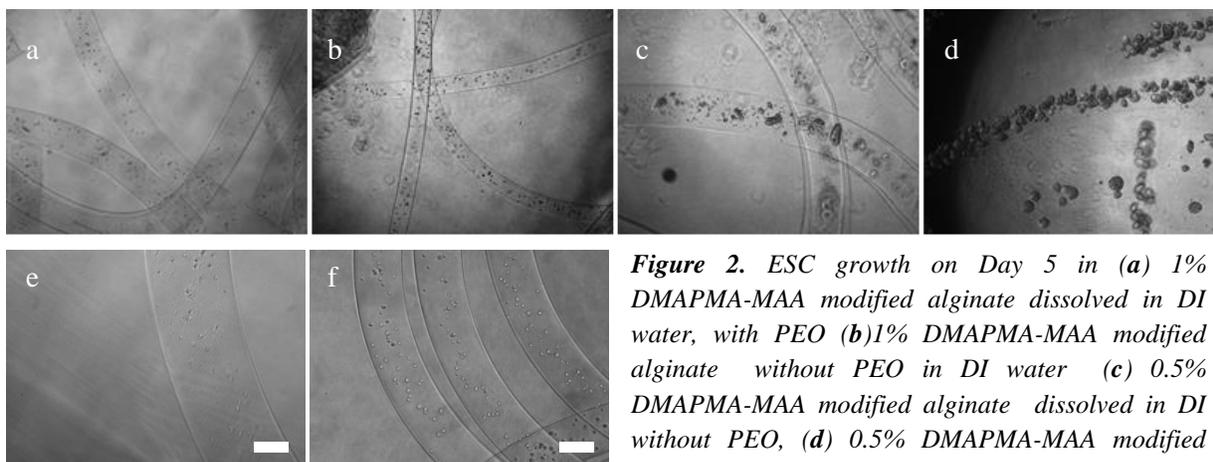

Figure 2. *ESC growth on Day 5 in (**a**) 1% DMAPMA-MAA modified alginate dissolved in DI water, with PEO (**b**)1% DMAPMA-MAA modified alginate without PEO in DI water (**c**) 0.5% DMAPMA-MAA modified alginate dissolved in DI without PEO, (**d**) 0.5% DMAPMA-MAA modified alginate dissolved in 0.9% NaCl no PEO.(**e**)DMAPMA microstrands with PEO (**f**) DMAPMA microstrands without PEO. Both images were taken on day 0 of cell growth. Scale bar is equal to 100 μm.*

forming around a few progenitor cells trapped in the gel matrix. As these the colonies grew, they joined together to make larger aggregates. In the liquefied unmodified microstrands, these cells showed uniform growth along the length of the strand. The fluid environment appeared to have enabled less entrapment and faster growth-rates. The solid modified microstrands had cell clusters that were very similar to the patterns observed in the unmodified strands though there were more clear patterns of orientation observed in the modified alginate solid strands. The major difference between the two conditions was where the clusters were located. In the unmodified solid strands the cluster formed closely along the center axis whereas the modified strands were more sporadically aligned, with many cell clusters adhering to the outside edge of
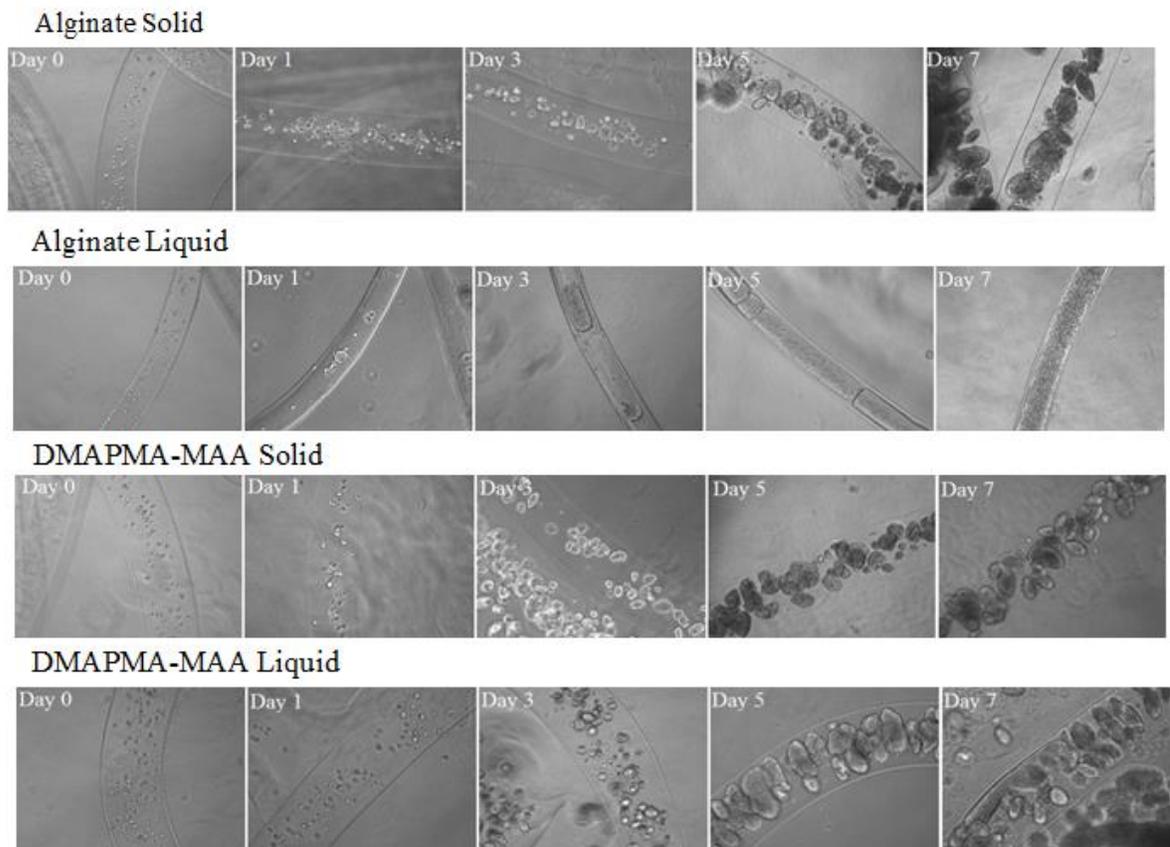

Figure 3. Time course for mouse ESCs grown in alginate and DMAPMA-MAA/Alginate microstrands over a seven day period **Top Row:** Alginate Solid microstrands. **Second Row:** Alginate Liquefied Microstrands. **Third Row:** DMAPMA-MAA/alginate solid microstrands. **Bottom Row:** DMAPMA-MAA alginate liquefied microstrands.

the microstrand. Again we see differences between the solid and liquefied strands, this time in the modified alginate microstrands, though the most marked difference is in alignment of the ESCs in the liquid microenvironment. The structures form a 'cobblestone' pattern which is orientated perpendicularly to the length of the modified microstrand. Rather than only adhere to other cells, there appears to be adhesion to the walls of the modified alginate microstrand (Figure 3). This adhesion could potentially increase the pluripotency of the ESC in the matrix as creating a scaffold that promotes adhesion between fabricated structure and ESC has shown to increase pluripotency of the culture [22].

3.2 Elasticity of Alginate Hydrogel Microstrands

The opportunity arose to test the different rheological properties of the modified and unmodified alginate hydrogels. It was found that DMAPMA-MAA modified solid alginate microstrands had a higher storage and loss modulus as compared to the unmodified alginate microstrands. This translates into a higher complex viscosity of the modified alginate
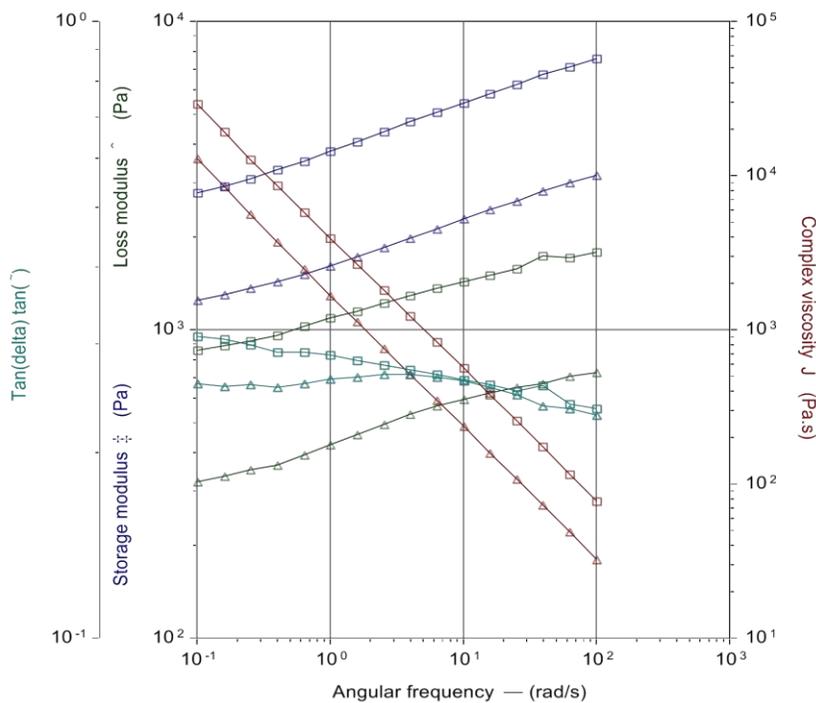

Figure. 4 *Measurements taken on a TA Discovery Rheometer. The triangle represents the data from alginate solid microstrands and the square represents data from the DMAPMA-MAA modified microstrands*

under an increasing angular frequency of the rheometer. This could have interesting implications on the unique pluripotency maintenance and differentiation potential the microstrands exhibit in this modified growth environment. It has become increasingly important to characterize mechanical factors of cellular scaffolds as these factor impact the fate of differentiating cell lines [13, 24]. The variance of microstrand physical properties may also lead to the differences observed between cellular growth patterns in the modified and unmodified microstrands.

3.3 Quantitative Polymerase Chain Reaction Analysis of Pluripotent Stem Cell Markers
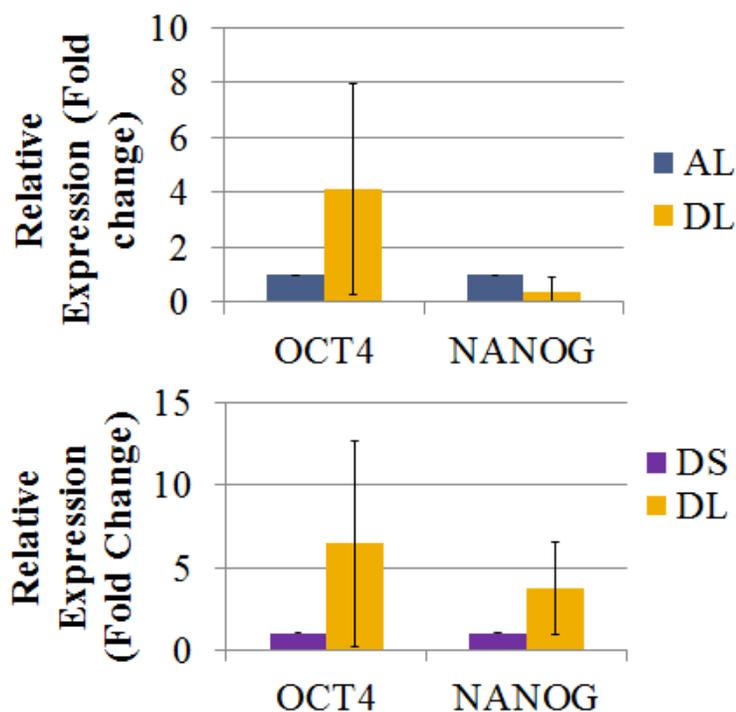

Figure 5. qPCR results for Top: ESCs grown within alginate liquefied microstrands (AL) and DMAPMA-MAA/Alginate liquefied microstrands (DL) and Bottom: DL microstrands compared to DMAPMA-MAA/Alginate solid microstrands (DS)).

We assessed the pluripotency through quantitative polymerase chain reaction (qPCR) analysis of pluripotent stem cell markers. Oct4 and Nanog, these traditional ESC pluripotency markers were used to establish the stemness of the culture after the 7 day culture period (Figure 5). Nanog, a transcription factor found in pluripotent stem cells, and octamer binding protein 4 (Oct4) acted as a transcription factors to maintain stem cell pluripotency. GAPDH was probed as the housekeeping gene. In comparing the alginate liquid core microstrands to the modified alginate liquid core microstrands, it was found that there was an increase in the in Oct4 expression which would suggest that there was an increase in pluripotency in the modified

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microstrands. Conversely, we also observed a decrease in Nanog expression which seems to contradict the Oct4 results. This decrease is believed to be caused by an issue during the transferring process from one PCR plate to another. This data needs to be repeated further as Nanog and Oct4 expression are directly related and evidence has been gathered which supports Nanog and Oct4 work in tandem to regulate stem cell pluripotency [24]. More qPCR trials are needed to be run to further investigate this discrepancy. The trial run between the modified alginate microstrands with solid cores and the modified microstrands with liquefied cores demonstrated that there is higher expression of both Oct4 and Nanog in the liquefied strand.

3.4   Troubleshooting Alginate Microstrand Liquefaction and Fabrication

Issues arose in the fabrication and liquefaction steps after the alginate supplier Sigma Aldrich changed alginate source. Production of alginate is difficult as it requires precise control over G/M blocks which can change the gelling properties of the alginate.   After the alginate manufacturer changed, it was found that the initial protocol would cause the microstrands to dissolve on contact with the 1.6% sodium citrate after coating with the PLL. Additionally, there were issues in making the strands in a consistent manner. This issue had arisen in the past year
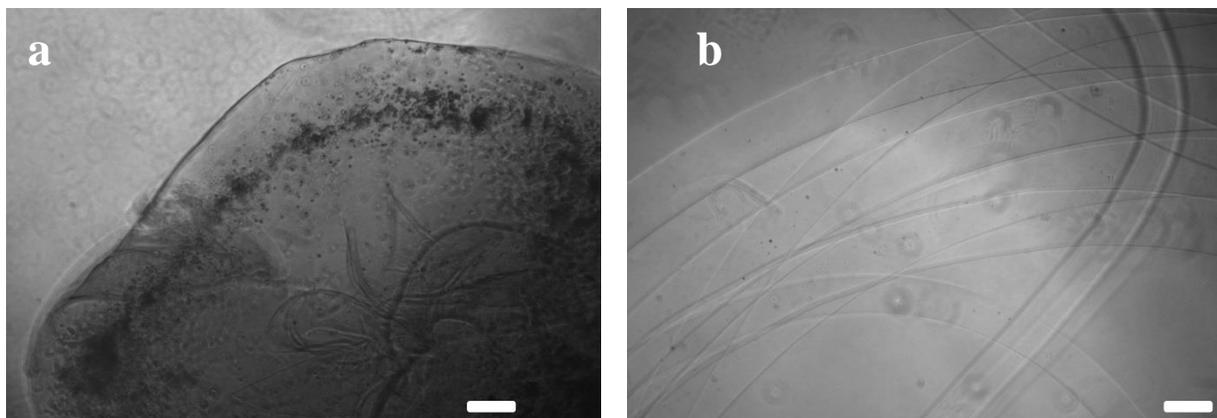

Figure 6. a) globular alginate formation from old syringe pump b) uniform microstrand production from the syringe pump. Scale bar equal to 200μm.

and had slowly deteriorated with time. Large globules would form rather than the 200μm strands which were anticipated (Fig. 6a).

The microstrand liquefaction problem was tackled by initially decreasing the dissolve time from 3 minutes to 2 minutes. It was noted that though the timing was reduced, the strands were fully dissolving on contact with the 1.6% sodium citrate. To combat this, the concentration of PLL was stepped up from 0.05% to 0.1%. This way the semi-porous shell could be formed more readily. It was found that the increase in PLL concentration did not stop the microstrands from dissolving so the time was stepped back to 2 minutes, with only four wells subjected to the sodium citrate at one time. This made it easier on the operator to remove the sodium citrate without taking up the microstrands. Using this protocol, microstrands with liquefied cores could be fabricated with the new alginate.

To address the issue of microstrand formation, we first examined the need to change the calcium chloride concentration. We believed that a higher concentration would encourage better crosslinking. We then examined different alginate concentrations asides form the 1.5% in order to have a more similar viscosity to the 100-300cP of the original alginate. After many trials of changing the concentration of alginate and calcium chloride solutions it was decided that calcium chloride cross linking and alginate concentration should be kept the same as in previous studies. Additionally, changing the different concentrations did not seem to have a consistent impact on the fabrication of the microstrands. We changed tactics and examined different flowrates and different silica capillary tips. As with the previous trials, microstrands and globules would form in an unrepeatable and inconsistent manner. It was found that the irregularity of the syringe pump was causing the globules to form opposed to the microstrands. A new syringe pump was

purchased and was found to create microstrands similar to the initial experiments before the hardware issues.

4. Conclusions and Future Directions

We were successfully able to incorporate DMAPMA-MAA into the alginate solution to create adhesive alginate hydrogels for stem cell growth. These microstrands supported cell proliferation over the one-week growth period and appeared to affect the pluripotency of the ESCs. Issues that arose from the new alginate and pump have been extensively examined and corrected. Additional quantitative comparisons of pluripotency in ESCs grown in modified and unmodified microstrands are needed to further prove maintenance of pluripotency in modified alginate microstrands. This will be done through the use of standard biological assays such as immunocytochemistry (ICC), quantitative polymerase chain reaction (qPCR) and Western Blot analysis. For qPCR and ICC, the study will utilize traditional ESC pluripotency markers such as Oct-4 and Nanog. This will allow for a quantitative comparison of week-long growth in modified versus unmodified strands based on the expression of these pluripotency markers. Success would be determined through positive expression of these pluripotency markers in the modified strand compared to the negative strands in reference to the control (GAPDH). As of now, this section has remained largely untested. Longer term goals of the project would be to run the experiment without LIF to assist in pluripotency maintenance and see if cell adhesion alone can promote cell stemness. It would be an exciting area for more quantitative examination, and hopefully the project is continued in the coming years to test highly efficient pluripotent stem cell expansion and directed stem cell differentiation.

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