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The production and characterization of chitosan based microgels using a novel microfluidic device to promote cell survival

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Executive Summary

Problem Statement: The purpose of this project is to synthesize and characterize perfluorocarbon modified methacrylamide chitosan (MACF) microgels produced with the use of a capillary-based microfluidic device. Since cell survival and development is highly related to particles physical properties, analysis of microgels set out to determine the particle's physical properties. I hypothesize that the viscosity of the MACF polymer as well as the amount of photo initiator influence the physical properties of synthesized particles and as a result can generate particles with various features for cell culture in terms of shape, size, charge and swelling abilities. These microgels will be used later to provide space between cells and to improve gas transport chemically to enhance the exchange of nutrients into and out of 3D cell structures. Previously, the Leipzig Laboratory proved that MACF hydrogel dressing has the ability to sustain the delivery of oxygen and enhance the partial pressure of oxygen at the wound area thereby increasing the rate of wound healing. [11] Utilizing these data, I want to use MACF in the form of microgels to maintain local oxygenation for cells.

Statements of Quantitative Results: The characterization parameters of overall morphology, size, stability, and swelling provide crucial insight into the feasibility of using these microgels for cell culture applications. I was successful in making spherical shaped microparticles with a diameter of ~3 to 10 μm. From dynamic light scattering (DLS) measurements, it was found that at higher levels of photo initiator resulted in larger particle sizes. In addition, when the polymer is more viscous (3 wt%) I achieved significantly smaller particles compared to those when 2 wt% MACF was used. Zeta potential results showed that increasing the amount of photo initiator as well as polymer viscosity caused negative charges, associated with F groups, to shift inward. The positive charges, associated with amine groups of chitosan, shift outward resulting in a positive

surface charge. Swelling studies verified that particles made with the low viscosity polymer solutions displayed a significantly higher swelling ratio than those particles made with the higher viscosity polymer solution.

Definitive Conclusions: The initial hypothesis for this study was that polymer viscosity and the amount of photo initiator can affect the size, shape, surface charge, and swelling behavior of MACF microparticles produced with the microfluidic technique. This hypothesis was validated through the characterization of particle size, charge, and swelling behavior. However, in order to make a conclusion on which particles formulation is preferred for future application, their influence on cell survival, proliferation, function and phenotype should be studied – which is outside the scope of this honor's thesis. Conclusions about microparticle morphology can be made in confirming that the microfluidic device produces spherical microgels with defined size. These particles have different surface charges and swelling behaviors, which can be used to improve transportation of gas and nutrients into and out of tissue structures which is the most important challenge in making in vitro models.

Recommendations: Most research on microfluidics should focus on testing various particle generation methods or factors to improve their uniformity. Any small change in flow rates of polymer and oil phases, viscosities of solutions and amount of photo inhibitor can greatly affect the final size of microparticles. Therefore, future research should begin by designing an experiment to test the effects of these three factors on microparticle size for various polymers. Since our polymer is photo curable, different amounts of photo initiators influence the formation of particles and their size. Zeta potential values were close to neutral indicating low stability of particles and therefore a tendency for microgels to aggregate. Synthesized microgels do not have long term stability and should be used for experiments shortly after being produced. Factors that

affect zeta potential include pH of the medium, ionic strength, concentration of any additives/surfactants, and temperature. These factors should be taken into consideration when attempting to reproducing results.

Broader Implications of Work: Undergraduate research challenges individuals to apply critical thinking skills in practical situations. Students reason through a process, gain the ability to integrate theory and practice, analyze data and develop the skills to interpret results. Undergraduate students learn to balance collaborative and individual work while potentially providing clarification of a career path. The research described in this paper will aid in the overall objective to research whether chitosan based microgels surrounding spheroids will create a healthy living environment for cell cultures. This method, by virtue of its simplicity and use of novel materials, will facilitate access to long-lasting in vitro models and will provide a potential alternative to current synthetic hydrogels for growth and the expansion of functional 3D cell structures.

Introduction

It has been proved by many studies that hydrogels are excellent models for mimicking natural extracellular matrix (ECM) and can manipulate stem cell behaviors and organization [1]. Due to this reason hydrogels, and in particular microgels are of crucial importance in tissue engineering and regenerative medicine [2]. Based on this knowledge I aim to produce fluorinated methacrylamide chitosan (MACF) microgels with well-defined shape and size using a microfluidic technique. I have hypothesized that different amounts of photo initiator and polymer solution weight percentage can affect the properties of MACF microgels produced by a microfluidic device. The generation of monodisperse particles is essential for biomedical application. Because of their size, which is similar to the cell size, upon contact with cell microparticles can alter the cell's response. Therefore having monodisperse particles will lead to reliable cumulative results, the main challenge in making microparticles. Aiming to overcome this challenge, several factors should be taken into consideration when making particles using microfluidic technique. Droplet size, velocity, and frequency can be controlled by controlling the flow rates, inlet pressures and altering the phase viscosities and orifice size. [17] Therefore, the primary goal of this honors project is to analyze microgels produced from MACF using a microfluidic device as well as determine ideal characteristics that will achieve the primary goal described above.

Background

In recent years, polymeric biomaterials have received attention for their potential to enhance the biocompatibility of devices used in drug delivery and tissue engineering. Microgels are comprised of a solvent swollen polymer network and are colloidal in shape with dimensions that range from tens of nanometers to many micrometers. These features allow microgels to

interface with cellular and subcellular domains. [10] Chitosan is a linear cationic copolymer of b-(1-4)-linked 2-acetamido-2-deoxy-b-D- glucopyranose and 2-amino-2-deoxy-b-D-glycopyranose that is derived from chitin. Due to the presence of amine groups, chitosan is positively charged in acidic solutions and can form a gel network via ionic interactions. Another advantage of the amine group is that it can be modified physically and chemically for tissue engineering, drug delivery, and wound-healing applications. In the Leipzig Laboratory, chitosan is functionalized with perfluorocarbons (PFCs). One of the most significant properties of PFC materials is their affinity to dissolve large volumes of respiratory gases, such as oxygen. These gas molecules are able to fill the molecular spaces in the PFC liquid without any chemical reactions taking place. Considering this unique property, PFCs have attracted a lot of attention in biomedical applications especially in oxygen delivery. In a study previously completed by students within the Leipzig Laboratory, a new class of perfluorocarbon-modified chitosan biomaterials (MACF) was introduced. MACF was found to have great oxygen uptake and release properties for wound healing applications. [13]

There are a number of factors that influence microgel viability in the application of biofabrication. Such factors include porosity, charge, segment density, amphiphilicity, size, degradation, and softness. [10] It is crucial to fabricate microparticles with the desired shape and size to minimize uncontrolled variability in cell responses. According to Duncanson and coworkers, conventional emulsification techniques to produce microparticles that have a large variation in size, structure, and wide range of encapsulation efficiencies. [12] For this reason, microfluidics is an ideal technique for fabrication of advanced microparticles that can be then modified for different applications. [12] The microfluidic technique offers a rapid and efficient way to fabricate monodisperse polymeric particles with precise size and morphology. It allows

control over structure and size of particles which is important for bioengineering applications. [14] Microfluidics controls fluids at the microscale and generates a wide range of polymer particles, such as solid, porous, and hollow particles with well-defined structure and morphology. Using light allows more control on microdroplet generation. In different studies, UV-induced gelation of the droplets has been used to generate microgels with various features. Two different categories of microfluidic devices have been identified for the production of micron-range droplets: microchannel-based and capillary-based systems. Current micro-devices have to be fabricated through micro and nano-fabrication technologies in a clean laboratory environment. This increases cost and limits microfluidic development for larger scale industrial applications. [8] Capillary-based microfluidic devices can be assembled within a few minutes and have a simple fabrication process. [15] In one study, Choi and coworkers utilized microfluidic techniques to produce poly (ethylene glycol) microspheres hexadecane. Span 80 and PEG-diacrylate plus photo initiators were chosen as the dispersed and continuous phases respectively. Finally, using intensified UV light, PEG microgels were rapidly solidified with a very short exposure time. [16] The use of a microfluidic device to produce monodispersed microgels is further supported by Lapierre and coworkers. In their study, researchers produced monodisperse porous polymer beads utilizing a capillary-based microfluidic device. PolyHIPE emulsions were used as the dispersed phase while fluorinated oil containing 2% Picosurf surfactant served as the continuous phase. Droplets from the microfluidic device were polymerized by UV polymerization and collected in a plastic vile. The capillary-based microfluidic device described by Lapierre and coworkers allows quick and cheap development of an easy to use and reusable platform for monodisperse emulsion droplet generation. [8] The above studies support that capillary-based microfluidic devices produce microgels with monodispersity and controlled

shape. [8] For these reasons, the capillary-based microfluidic device described by Lapierre and coworkers was used for the development of microgels characterized in this study.

Experimental Methods

Preparation of Fluorinated Methacrylamide Chitosan (MACF)

The process used to prepare fluorinated methacrylamide chitosan (MACF) is as follows [7]. First, pentadecafluorooctanoyl chloride (PDFOC) modified chitosan is produced through a mixture of 10g 3% W/V and 2% V/V CH_3COOH/H_2O chitosan solution in 100 mL round bottom flask. The flask is then stirred at 60 RPM using a stirrer. 0.374 mL of 97% PDFOC liquid with 1.122 mL of methanol is mixed to make an emulsion. This emulsion is then added to the chitosan solution and stirred for 48 hours after the addition is completed. The solution is transferred into a dialysis membrane with distilled (DI) water where it remains for three days with three DI water changes per day. Dialyzed product is then collected in 50 mL tubes and frozen. It is then kept on freeze dryer and lyophilized for three days. The freeze dried product is then weighted and redissolved in Acetic acid at 3 wt%. Methacrylic anhydride is added based on the density of liquid chitosan PFC. Once combined in a vial, the mixture is placed on a stirring plate for 12 hours. The solution is then transferred to dialysis membranes in distilled water where it sits on a stir plate for three days. Distilled water is changed three times a day. Solution is transferred to 50 mL c-tubes and then put in the freeze dryer to remove water. For this purpose, samples are placed into the freezer until frozen. Once frozen, the c-tubes are placed in the freeze dryer for approximately two days. Before producing the microgels, the MACF must be redissolved in distilled water to the desired wt%. The chemical reactions for the methacrylation and fluorination on chitosan based polymer to produce MACF polymer is pictured in Figure 1.

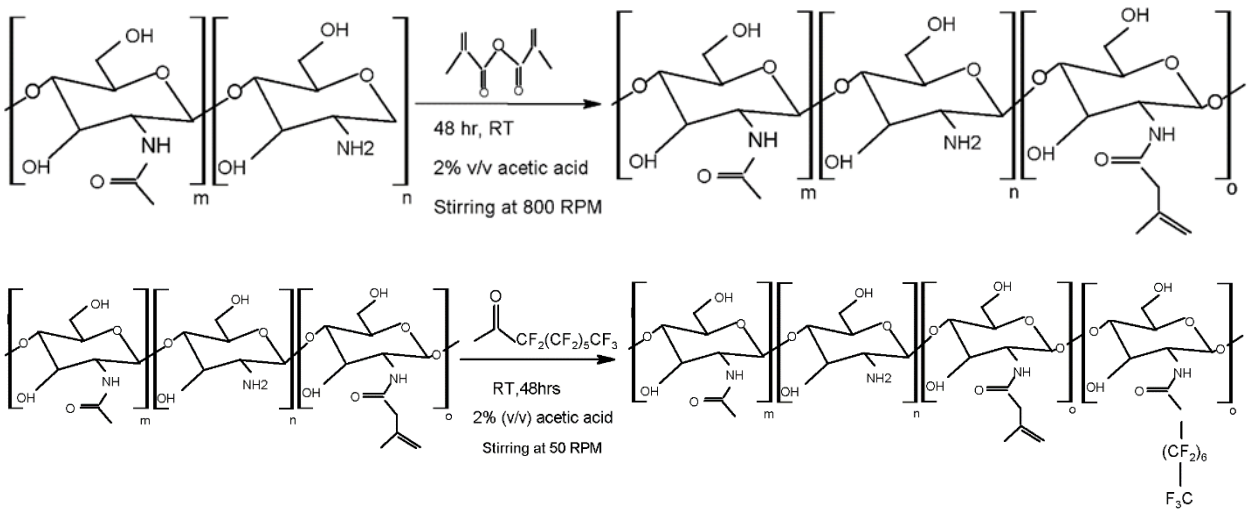

Figure 1: Methacrylation (upper) and fluorination (lower) on chitosan based polymer to produce MACF polymer

Making the flow focusing microfluidic microdroplet generator device:

In order to create microparticles using MACF, a microfluidic device that generates emulsion droplets enclosed in an oil or aqueous environment was created based on the protocol reported by Lapierre and coworkers [8]. The microfluidic device in this project was fabricated with conventional and inexpensive components. This device, pictured in Figure 2 and Appendix A, allows hydrodynamic focusing in a microchannel with two sheath flows. The first step involves creating the mould of the inner part of a micropipette tip using rubber. Two PTFE tubes are placed inside the micropipette tip to create spaces for inlet streams of polymer and oil. The rubber mould sets for 24 hours before the micropipette tip and PTFE tubes are to be removed. After the mould is fully cured, two new PTFE tubes are inserted into their proper places. The two tubes are then connected to two syringe pumps that will deliver the dispersed and continuous phase liquids. One 2 mm long PTFE tube is inserted into a silicon tube and a hole is punched between the PTFE and silicon tubes to create a nozzle. Silicon tubing is placed inside a new micropipette tip while the PTFE tube delivering the dispersed phase is placed in the silicon tube,

next to the nozzle. The PTFE tube delivering the continuous phase will fill the pipette tip, and finally; the pipette tip is inserted into the mould. For this study, the continuous phase is a mixture of oil and surfactant (Span80) while the dispersed phase is MACF dissolved in distilled water. Pictured below you can see a schematic of a microcapillary based microfluidic device:

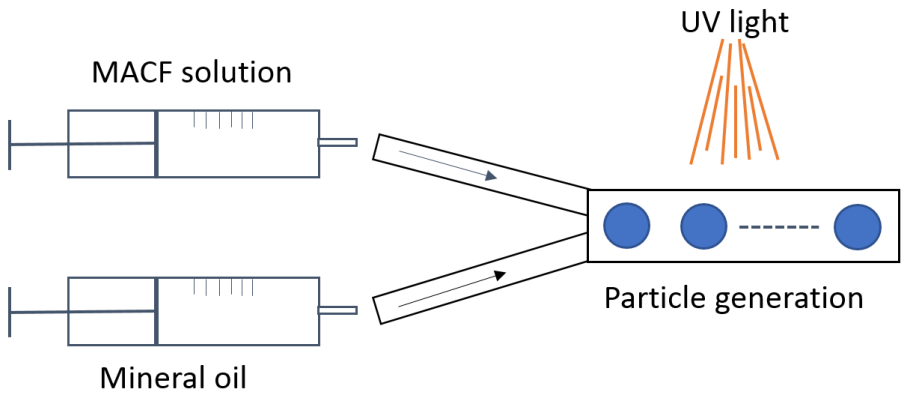

Figure 2: Microcapillary based microfluidic device. Design based on protocol reported by Lapierre and coworkers [8]

Design of experiments:

The concentration of polymer solutions (MACF) and the flow rate of both polymer and mineral oil should be taken into consideration for fabrication of microgels. Work performed by the previous graduate student on this project studied the effect of different flow rates on the size of MACF particles. Based on her findings, the ratio of oil to polymer material was adjusted on a 2:1 (1.5 µL/min polymer flow rate and 3 µL/min oil flow rate). Therefore, I aimed to study the effects of two other factors: polymer solution weight percentage and photo-initiator concentration. These two factors were varied at two levels each to produce microgels using the microdroplet generator described above;

Flow rates: Polymer: 1.5 µl/min

Oil: 3 µl/min

Table 1: Summary of factors and levels examined

Factors	Levels
Polymer solution wt%	2, 3 (wt%)
Photo-initiator (v/w%, µl/g)	1, 3 (µl/g)

Table 2: Summary of experiment and their parameters

Experiment Number	Level A	Level B
Run 1	2 (wt%)	1 (µl/g)
Run 2	2 (wt%)	3 (µl/g)
Run 3	3 (wt%)	1 (µl/g)
Run 4	3 (wt%)	3 (µl/g)

Two levels, high and low values, for each of these parameters were chosen based on previous work done with MACF hydrogels in the Leipzig Laboratory. Using Minitab, all possible combinations of these two parameters were varied to create four different experiments. After carrying out the experiments, microgels produced were analyzed in terms of shape, size, surface charge, and swelling behavior. Microgels were produced utilizing these parameters with the microdroplet generator and then combined with a photo-initiator and subjected to UV exposure to create microgels. Microgels were then washed with chloroform and stored in the fridge for further analysis.

Characterization of microgels:

After creating microgels, morphology and shape of the particles was characterized using scanning electron microscopy (SEM). In SEM, signals created from a focused beam of high energy electrons interact to reveal a samples external morphology. [9] Microgels analyzed by SEM were put in deionized water and dried flat on tin foil overnight. These dried microgel samples were then transferred to a slide and examined.

Next, the hydrodynamic size was studied with dynamic light scattering (DLS). DLS uses a light source such as a laser. As light hits the particle, the wavelength of incoming light is altered and this change is related to particle size. [5] Zeta potential, or surface charge measurement which is an indication of particle stability, was measured by the electrophoretic mobility of the sample as it moves across one electrode to the other. The use of DLS and zeta potential results were determined within the same machine and therefore have identical preparation methods. Microgel samples were diluted in a 1X PBS solution. 60 µL of the dilute solution were then transferred into a cuvette. The cuvette was then placed into a Mobius zeta potential and DLS detector (Wyatt Technology Corporation). Zeta potential is then calculated from mobility by using a model, the most common of which is the Smoluchowski model. [5] Zeta potential and particle radius was recorded multiple times for four samples. Overall, three batches of samples from each group were prepared for size and surface charge analysis. From this, we are able to run statistical analysis on the data.

Other properties measured include swelling. Swelling was determined by measuring four microgel samples initial weight in 50ml tubes. For the swelling test, 1mL of 1X PBS was added to each sample. The tubes were then put inside an incubator shaker for one hour at 37°C and 100 RPM. After an hour, the samples were centrifuged for 10 minutes at 1500 RPM to allow for phase separation. Liquid at the top and solid at the bottom. Liquid from the top was pipetted of and the weight of the polymer that remained was measured. The swelling capacity (g/g) was measured by dividing the weight of the swollen particles (W_s) by weight of the initial dried sample (W_d). This equation can be found in Appendix C, Equation 1 of the Appendices. In order to complete a statistical analysis on the results this procedure was repeated on three samples from each of the four groups.

Data and Results

Scanning electron microscope (SEM)

The morphology of microgels was studied using SEM.

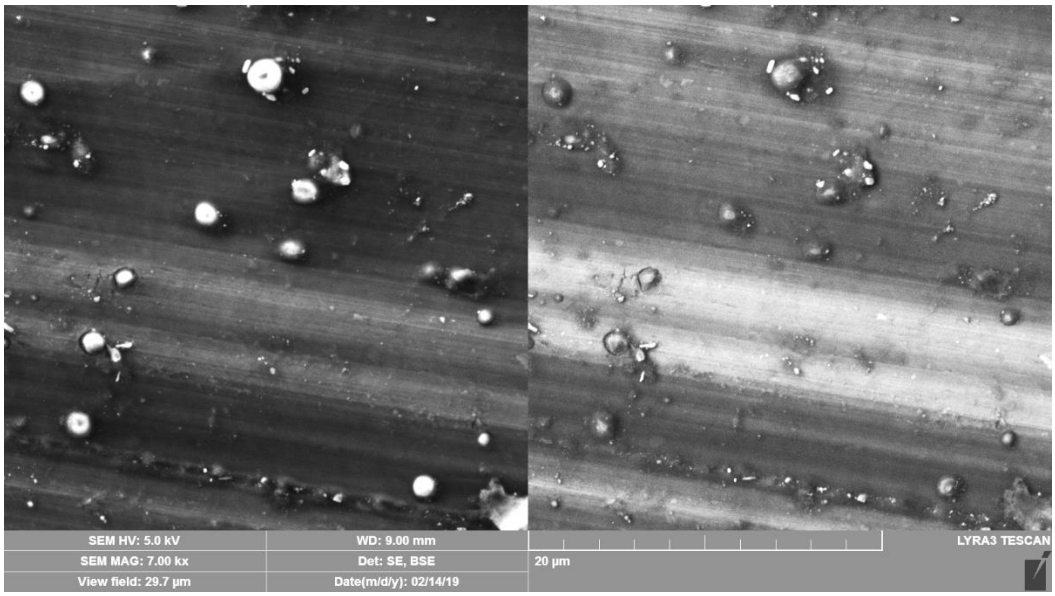

Figure 3: SEM secondary electron (left) and backscattered electron (right) images of MACF microgels prepared based on design 1 at 7 kx magnification

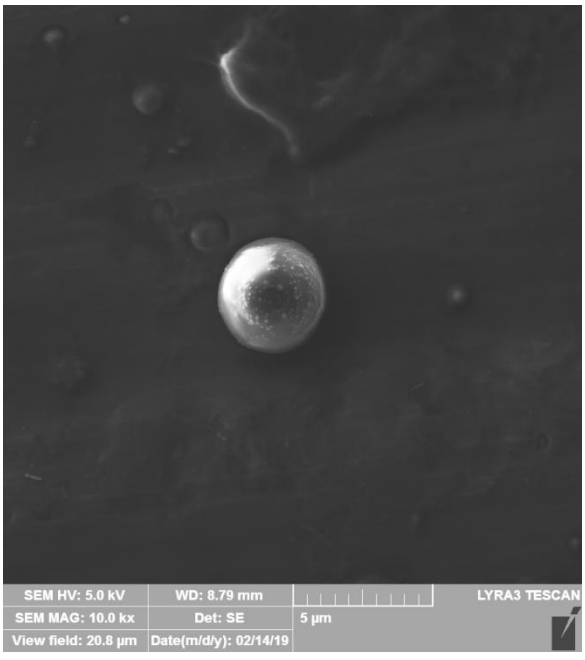

Figure 4: SEM secondary electron images of a single microgel prepared based on design 1 at 10 kx magnification

Surface charge analysis

Möbius® Zeta Potential Analyzer was used to measure the zeta potential. The results are shown in Figure 5.

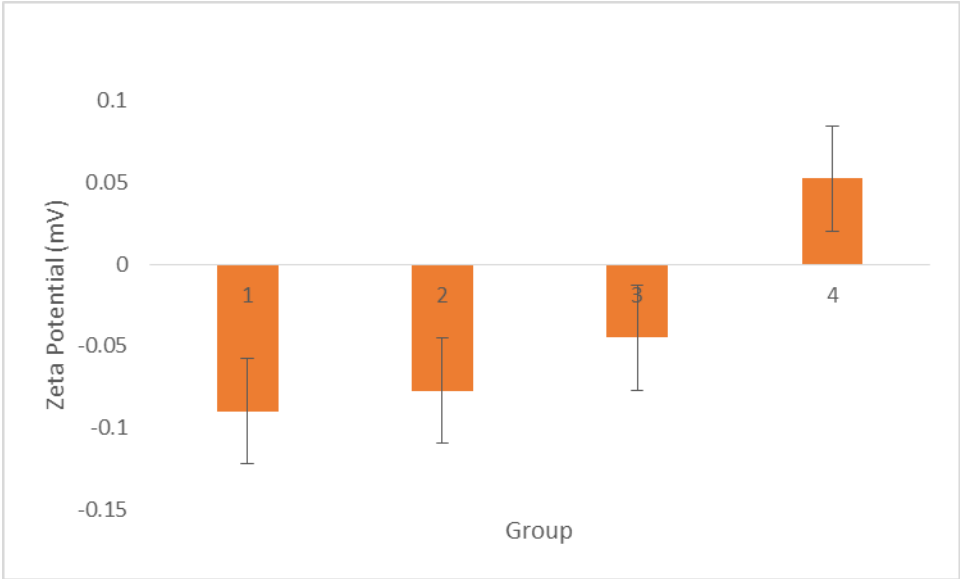

Figure 5: Average zeta potential values for the four groups of particles determined through the use of Möbius® Zeta Potential Analyzer (mean ± SD, n = 3, 4)

Table 3: Statistical analysis of average zeta potential

Tukey's multiple comparisons test	Significant?	Summary	Adjusted P Value
1 vs. 2	No	ns	0.9975
1 vs. 3	No	ns	0.9044
1 vs. 4	No	ns	0.1960
2 vs. 3	No	ns	0.9604
2 vs. 4	No	ns	0.2589
3 vs. 4	No	ns	0.4872

Table 4: Average zeta potential and standard deviation values

Group	Average Zeta Potential (mV)	Standard Deviation
1	-0.09	0.044
2	-0.0775	0.145
3	-0.045	0.058
4	0.0525	0.096

Hydrodynamic diameter of microgels

Möbius® Zeta Potential Analyzer (Wyatt Technology Corporation), the same instrument used to measure zeta potential, measured hydrodynamic diameter of microgels. The DLS detector offers instantaneous size measurements for particles ranging from nano to sub-micron in size. There were significant differences between the sizes of microgels. However, microgels from group 1 and 3 were the only samples that did not show any significant differences in term of size and had approximately the same size.

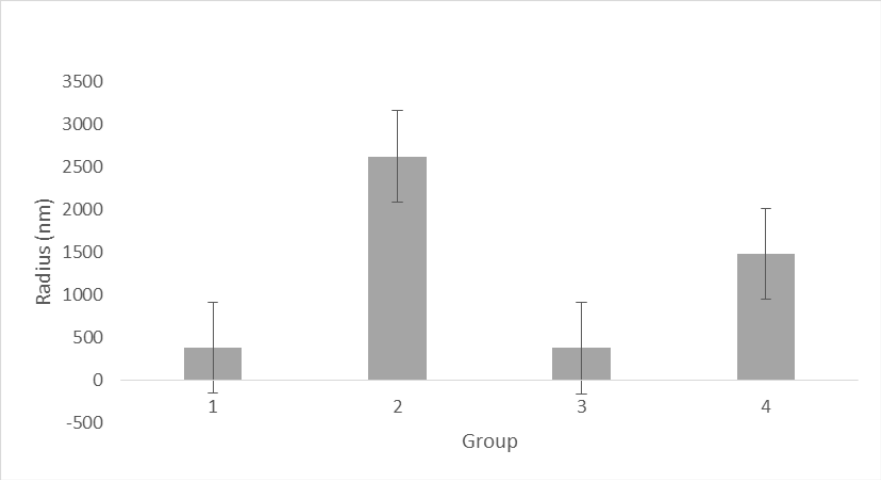

Figure 6: Average radius for the four groups of particles determined through DLS (mean ± SD, n = 3, 4)

Table 5: Statistical analysis of average radius

Tukey's multiple comparisons test	Significant?	Summary	Adjusted P Value
1 vs. 2	Yes	****	<0.0001
1 vs. 3	No	ns	>0.9999
1 vs. 4	Yes	***	0.0002
2 vs. 3	Yes	****	<0.0001
2 vs. 4	Yes	***	0.0002
3 vs. 4	Yes	***	0.0002

Table 6: Average radius and standard deviation values

Group	**Average Radius (nm)**	**Standard Deviation**
1	387.43	131.91
2	2625.85	199.26
3	381.88	55.04
4	1484.78	240.97

Swelling Testing

The swelling capacities of the particles decreased after increasing the polymer weight percentage. Based on the results chitosan particles made with 2 wt% MACF solution swell by as much as 10 times its original weight in 1 h while microgels generated from 3wt% polymer solution swell to lesser extent around 3 times its starting dry weight.

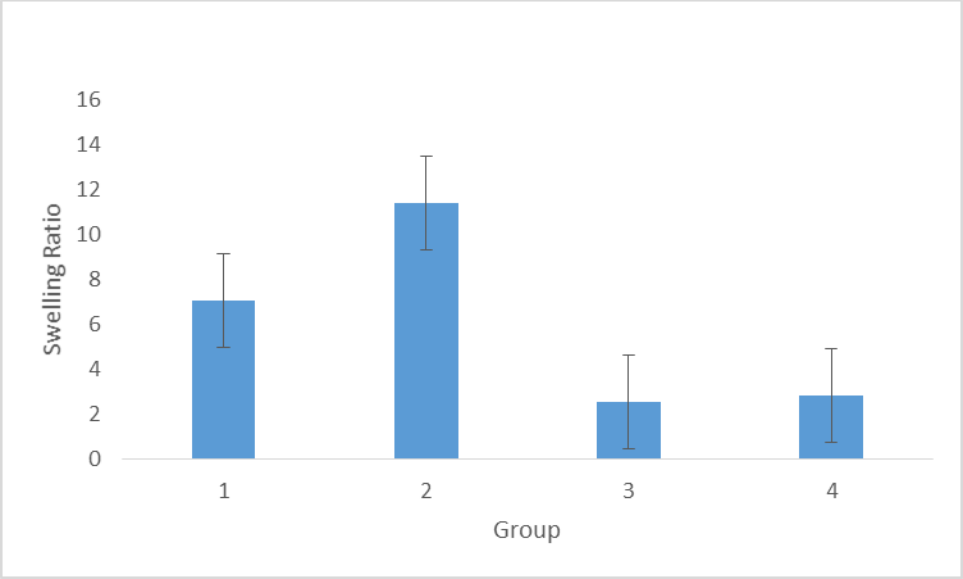

Figure 7: Average swelling ratio for the four groups of particles (mean ± SD, n = 3, 4)

Table 7 Statistical analysis of swelling ratio

Tukey's multiple comparisons test	Significant?	Summary	Adjusted P Value
1 vs. 2	No	ns	0.0609
1 vs. 3	No	ns	0.0541
1 vs. 4	No	ns	0.0687
2 vs. 3	Yes	**	0.0012
2 vs. 4	Yes	**	0.0014
3 vs. 4	No	ns	0.9981

Table 8: Swelling ratio and standard deviation values

Swelling Ratio	A	B	C	Average	Standard Deviation
Group 1	7.24	6.73	7.16	7.04	0.28
Group 2	8.08	14.84	11.27	11.39	3.38
Group 3	3.06	2.41	2.24	2.57	0.43
Group 4	2.48	3.56	2.40	2.81	0.65

Discussion and Analysis

SEM detects two types of signals, backscattered (BSE) and secondary electrons (SE), to form an image. [9] Figure 3 compares images formed by both backscattered and secondary

electrons. The left side of Figure 3 displays secondary electrons, giving insight into the topography of the microgels surface. On the right side, backscattered electrons mirror the left image distinguishing different phases. Figure 4 displays a single microgel utilizing secondary electron signals. Ultimately, Figures 3 and 4 provide sufficient evidence to support that the microgels are spherical in shape. Studies completed by both Lapierre and coworkers as well as Choi and coworkers utilized SEM to characterize microgels produced through capillary-based microfluidic devices. Both studies concluded particles produced through the use of the microfluidic device were rigid spherical shells. [15] Original SEM analysis proved difficult as dried microgels clumped to one another. Unable to determine a single microgels shape, preparation protocol was altered. Dried microgel particles were rewet in DI water and pipetted onto tin foil. The particles were dried on the tin foil and transferred onto carbon black tape to be examined through SEM.

Zeta potential is significant because its value can be related to stability of emulsions. [5] Nano and microparticles have a surface charge that attracts a thin layer of ions of opposite charge to the particle surface. This double layer of ions travels with the nanoparticle as it diffuses throughout the solution. The electric potential at the boundary of the double layer is known as the zeta potential. Zeta potential typically ranges from +100mV to -100mV, with values greater than +30 mV or less than -30 mV considered to have a high degree of stability. Particles with neutral or relatively low zeta potential values tend to coagulate or flocculate leading to poor physical stability. Figure 5 displays average zeta potential values that are presented in Table 4. Based on this data, a surfactant would need to be added to provide further electrostatic repulsions to prevent agglomeration. [5] Analyzing zeta potential results, a trend can be seen as the amount of photo initiator and polymer viscosity is increased. Group 4 particles, with higher photo

initiator and polymer viscosity values, have a positive average zeta potential while Groups 1-through have a negative average zeta potential as seen in Figure 5. This difference is accounted for by two counteracting forces: van der Waals, and electrical double layer. [5] Negative charges, associated with F groups, shift inward while positive charges, associated with amine groups of chitosan, shift outward resulting in a positive surface charge for particles with increased photo initiator and polymer viscosity values. The opposite is true for Groups 1-3.

Figure 6 is a graphical representation of radius values determined through DLS. The data presented in Table 6 shows a large variation in microgel size ranging from 381.88 to 2626 nm. It can be concluded that at higher levels of photo initiator larger particles were produced. The opposite is true when increasing polymer viscosity. In Figure 6, the particles produced at a higher viscosity were smaller than those produced at the lower viscosity. A high standard deviation among all four groups tested indicates that microgel radius was spread out over a wide range of values. The requirement for microgel size varies depending on the intended application, but particle size must remain consistent.

Figure 7 is a graphical representation of swelling ratio data found in Table 8. The data presented, shows a large variation in average swelling ratio ranging from 11.39 to 2.57. Particles produced at lower viscosities, groups 1 and 2, displayed a higher swelling ratio than particles made with the higher viscosity polymer solution.

Conclusion

Four methods utilized to characterize the microgels produced by the microfluidic device confirmed polymer viscosity and amount of photo initiator greatly influenced particle size, swelling, and charge. It appears that when the amount of photo-initiator increases, the size of the particles becomes larger. I account for this by claiming that the increased photo initiator brings

more polymer chains together resulting in the formation of larger particles. In terms of polymer weight percentage, based on the results I conclude that when the polymer used has a greater weight percentage (3 wt%) significantly smaller particles are produced compared to particles produced using 2 wt% MACF. Results provided insight into how increasing factors impact physical properties of microgels. Research should continue to focus on testing various microgel generation methods and factors to improve particle uniformity. In the future, it is recommended influence on cell survival, proliferation, function and phenotype be studied to determine which particle is best for producing spheroids.

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Appendices

Appendix A: Microfluidic Device

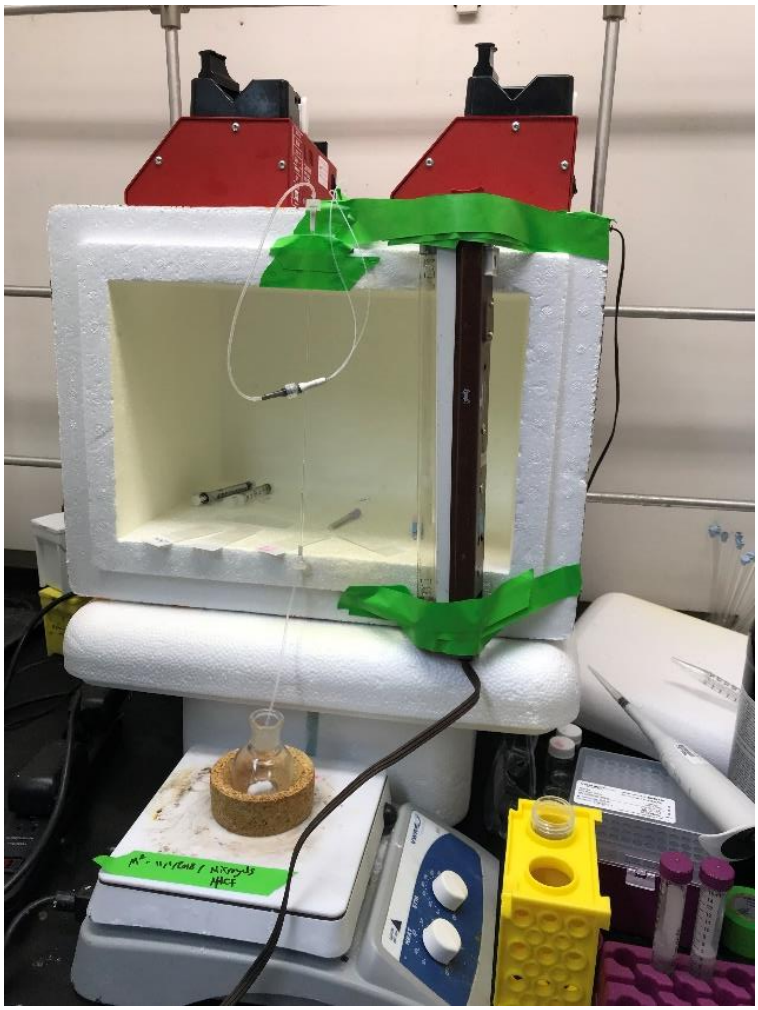

Figure 8: Microfulidic Device used to produce MACF microgels

Appendix B: Raw Data

Table 9: Zeta Potential results for the four groups of microgels tested

	Trial	Zeta Potential (mV)
Group 1	1	-0.12
	2	-0.11
	3	-0.04

	Trial	Zeta Potential (mV)
Group 2	1	-0.25
	2	-0.11
	3	-0.05
	4	0.1

	Trial	Zeta Potential (mV)
Group 3	1	-0.12
	2	0.01
	3	-0.01
	4	-0.06

	Trial	Zeta Potential (mV)
Group 4	1	-0.03
	2	0.19
	3	0.01
	4	0.04

Table 10: Radius measurements from the four groups of microgels tested

	Trial	Radius (nm)
Group 1	1	289.8
	2	335
	3	537.5

	Trial	Radius (nm)
Group 2	1	2652.5
	2	2472.5
	3	2897.6
	4	2480.8

	Trial	Radius (nm)
Group 3	1	426.8
	2	351.7
	3	429.5
	4	319.5

	Trial	Radius (nm)
Group 4	1	1600.2
	2	1196.4
	3	1394.1
	4	1748.4

Table 11: Weight of dry particles

Dry (Md)	A (g)	B (g)	C (g)
Group 1	0.9993	0.9962	0.9968
Group 2	0.9845	0.988	0.9976
Group 3	0.9868	0.9954	0.9876
Group 4	0.9846	0.9977	0.9952

Table 12: Weight of particles after soaking in 1X PBS for an hour

After Swelling (Ms)	A (g)	B (g)	C (g)
Group 1	1.0716	1.0632	1.0682
Group 2	1.064	1.1346	1.11
Group 3	1.0170	1.0194	1.0097
Group 4	1.009	1.0332	1.0191

Appendix C: Calculations

Swelling (%): $\frac{M_s - M_d}{M_d} \times 100$

M_d – Molecular weight for dry particles

M_s – Molecular weight for swollen particles

Equation 1: Utilized to determine the swelling ratio of particles produced

Standard Deviation (σ): $\sqrt{\frac{\Sigma\,(x-\bar{x})^2}{n}}$

Equation 2: Utilized to determine standard deviation