SIZE CHARACTERISTICS OF ELECTROSPRAYED POLYETHYLENE GLYCOL BEADS FOR PRODUCING TISSUE ENGINEERING SCAFFOLDS

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ABSTRACT

Size Characteristics of Electrosprayed Polyethylene Glycol Beads For Producing Tissue Engineering Scaffolds

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Submersion Electrospraying involves the injection of a polymer solution with photo-initiator into an immiscible liquid under an electric field. This document will reveal the changes in size and yield percentage of poly(ethylene glycol) hydrogel beads created with submersion electrospraying and UV light photo-polymerization. The varied physical parameters include: polymer chain molecular weight, intensity of the electric field applied, cross-linker used, the distance from the syringe to the electrical grounding ring, the flowrate from the syringe, and the gauge size of the syringe needle. Once size from each physical parameter was characterized, bead sizes from 0.712mm to 0.150mm were used to produce tissue engineering scaffolds, and their effects on retention of PBS were characterized during degradation.

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NOMENCLATURE

PBS	Phosphate Buffered Saline
PEG	Poly(ethylene glycol)
DT	Di-Thiol
NB	Norbornene
ECM	Extra Cellular Matrix
PDMS	Poly(dimethylsiloxane)
BG	Bulk Gel
McP	Microparticle

CHAPTER I

INTRODUCTION

One of the key challenges in tissue engineering is to manufacture biologically active scaffolds from synthetic materials. Hydrogels are leading candidates for engineering these tissue scaffolds because of their unique compositional and structural similarities to the natural extracellular matrix (ECM) [1]. Hydrogels can also be manufactured into porous scaffolds which provide enhanced nutrient and waste transport to allow vascularization as well as promote rapid ingrowth of cells [2] A prevalent challenge in biologically active or cell-instructive hydrogels is that the pores are typically on the nanoscale which creates a restrictive barrier that must be degraded for cell spreading, migration, and deposition of new ECM and tissue. This hurdle can be overcome by using hydrogel microspheres as building blocks for these scaffolds, as this approach results in the formation of inherently microporous structures. However, there is a critical need to develop a scalable method to create the microparticles that will comprise the scaffolds and control their properties.

There are currently four major methods involving liquid-liquid two-phase systems for the production of microspheres: emulsion polymerization, suspension polymerization, dispersion polymerization, and precipitation polymerization. Suspension polymerization, also called bead polymerization, is characterized by radical initiation within each monomer-rich droplet and with more than one radical per droplet at any time [3] [4]. Suspension polymerization also differs from emulsion polymerization since its droplets are greater than 1 μm [5]. Emulsion techniques are the most popular group of a variety of different techniques that have been studied for the manufacture of polymeric microparticles [6]. During an emulsion fabrication, molecules are introduced into a polymer solution, dispersed and eventually emulsified to form microspheres

that are dried after solvent removal. Precipitation polymerization utilizes differences in the monomer and polymer solubility to produce microparticles [2]. Larger polymer chains typically have less solubility within the same solvent as compared to smaller chains [7]. If surfactants or other stabilizers are added, then the precipitation polymerization is deemed dispersion polymerization [8]. Unfortunately, these traditional microsphere fabrication methods typically suffer from a lack of batch-to-batch reproducibility which can significantly hinder the development of clinically translatable scaffolds [9].

In contrast to the aforementioned methods, microfluidic and lithographic synthesis methods offer greater control over particle polydispersity [10]. Both require a microfluidic device typically made from polydimethylsiloxane (PDMS) and allow improved control over particle structure and complexity as compared to the previously mentioned batch schemes [11]. Emulsion based microfluidic systems employ multiple phases including aqueous, oil, and crosslinker which then meet in a junction geometry for droplet formation [10]. After the droplets are formed, chemical or ionic crosslinking occurs. Crosslinking via photoinduced polymerization is characteristic of flow lithography-based microfluidic systems [12].

Photolithography, imprint lithography, and flow lithography comprise the three major lithographic fabrication methods. In all three methods, a template of some sort is required. This template allows the user to tune the size and morphology of the resulting microgels [10]. For photolithography the template is employed as a photomask for UV polymerization. In imprint lithography, the template is used as a mold to hold the precursor solution during UV photopolymerization [10]. Lastly, flow lithography utilizes the UV light source to photopolymerize microgels within a flow channel [10]. Although these methods offer the user

an improved level of control and reproducibility, they are also hindered by a slower synthesis speed and poor manufacturing throughput.

Recently, submersion electrospraying has emerged as a time effective and reproducible technique to manufacture polymer microparticles. Submersion electrospraying is a simple process which has the potential to create microparticles with a narrow size distribution and high yield percentage [13]. The submersion electrospraying apparatus is comprised of a high voltage source, syringe pump, syringe with flat-ended needle submerged in mineral oil, and a grounded ring. The size, yield percent, and stiffness of each batch can be tuned using the physical parameters of the electrospray setup, the cross-linker used, the molecular weight of the PEG-NB used, and any bioactive molecules included within the polymer solution.

Currently, there is a critical knowledge gap regarding the effects of electrospraying parameters on the properties of microparticles produced by this fabrication technique. To address this gap, this research seeks to accurately characterize the effects of the different parameters involved during the submersion electrospray fabrication of these hydrogel microparticles and the scaffolds they comprise. The following work will provide future studies with an accurate, repeatable, and expedient method to fabricate tunable microparticles which will in turn allow the creation of tunable hydrogel scaffolds for tissue engineering.

CHAPTER II

METHODS

Thiol-ene Click Chemistry

The poly(ethylene glycol) (PEG) hydrogel microparticles in this project were crosslinked using the thiol-ene click chemistry reaction. The hydrogel microparticles were crosslinked using 5kD, 10kD, and 20kD PEG-norbornene (PEG-Nb) **(Figure 1)** and a crosslinker with two thiol groups [14]. The crosslinkers used were PEG-Dithiol (PEG-Dt), Tryptophan K12 (KCGPQGIWGQCK), Proline C11 (CGPQGPAGQCR), and Alanine K12 (KCGPQGIAGQCK). The variety of peptide crosslinkers allowed for variation in degradation rate. The photoinitiator was Lithium phenyl-2,4,6-trimethylbenzoylphosphinate (LAP) due to its relatively quick polymerization rate and cytocompatibility [15] CGRGDS was incorporated into the hydrogel precursor solution to act as a cell adhesive site after attachment to any free norbornene groups.

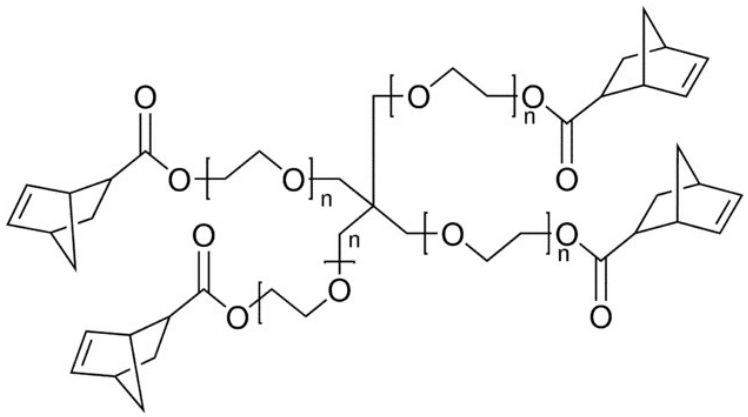


Figure 1 [16] Structure of 4-arm Poly(ethylene glycol) norbornene

Submerged Electrospraying

The submerged electrospraying was carried out on a benchtop apparatus as shown in **Figure 2a**. The hydrogel precursor solution filled syringe with an interchangeable tip was mounted onto a syringe pump to allow gauge adjustment and flowrate adjustment, respectively. A DC high voltage source was connected to the injection syringe and a grounded ring which were both submerged into a reaction beaker filled with mineral oil/Span 80 (0.4%w/v) as depicted in **Figure 2b**. Span 80 was included within the submersion mineral oil as a surfactant. An Omnicure Series 2000 mercury arc lamp equipped with a 365nm wavelength filter was used to produce the UV light needed for crosslinking. The syringe pump was set to a specific flowrate and allowed to run, injecting the hydrogel precursor solution into the reaction beaker. The reaction beaker was irradiated with 365nm wavelength light at an intensity of 60mW/cm^2 during the injection and for 3 minutes afterwards in order to crosslink the resulting PEG hydrogel beads. Additionally, an adjustable lab jack was incorporated into the setup in order to allow height variability.

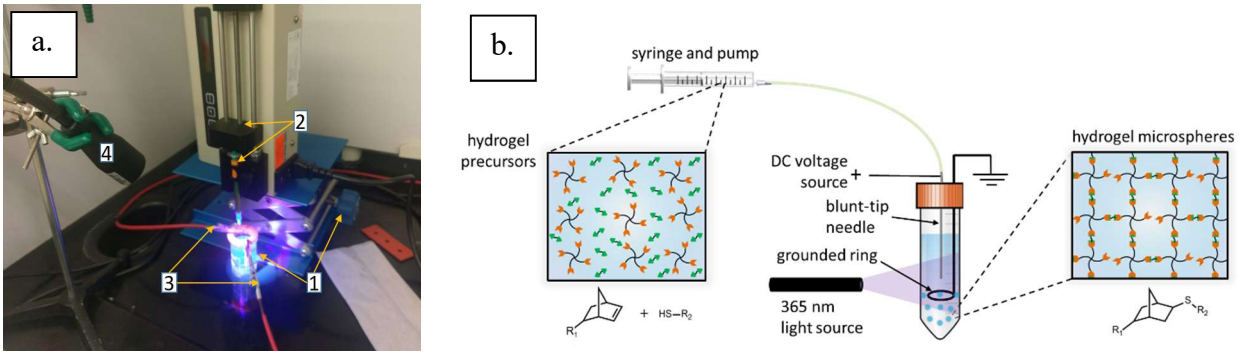


Figure 2a The benchtop submerged electrospray apparatus: 1. adjustable lab jack and reaction beaker 2. syringe and syringe pump 3. High voltage source clamps 4. UV light source **Figure 2b** General submerged electrospray setup depicting the reaction beaker and the hydrogel precursor solution before and after reaction

After fabrication, the microparticle and oil mixture was added to a 50 mL conical tube along with 5 mL of PBS. The mixture was then subject to centrifugation at 4.4g for 5 minutes.

The organic supernatant was then removed via vacuum aspiration. The microparticles then underwent 3 wash cycles which included the addition of 5 mL of PBS followed by 5 minutes of centrifugation at 4.4g. Finally, the microparticles were allowed to sit in chilled PBS (10 °C) for at least 24 hours in order to facilitate swelling.

Microparticle Size Characterization

After swelling, excess PBS was removed from the microparticle mixture within the conical tube. The microparticles were then transferred from the conical tube onto a glass slide via spatula and imaged using 4x light microscopy. Imagej was then used to measure the diameter of the microparticles in the image.

Scaffold Swelling Characterization

50 μL of polymer precursor solution was pipetted into a silicone disk mold and irradiated at 10mW/cm^2 for 3 minutes in order to create the bulk gel disks (BG disk). Preswollen microparticles approximately 250 μm in size were placed into the disk mold along with 2 μL of PEG-dt and 0.8 μL of LAP and irradiated at 10mW/cm^2 for 3 minutes to create the microparticle scaffold disks (McP disk). Both the scaffold and gel were measured before and after 24 hours of swelling using a 1x stereoscope.

Degradation

To test the effects of peptide crosslinker on degradability, cylindrical bulk gels were prepared using 50 μL precursor solution which was irradiated 365nm wavelength light at an intensity of 10 mW/cm^2 for 3 minutes. In addition, microparticle scaffolds were prepared using electrosprayed microparticles, which were preswollen, by adding 2 μL of PEG-dt and 0.8 μL of LAP and performing the same UV light treatment. The gels were then placed onto cell strainers within a 6-well plate. Each well was filled with 10 mL of 0.2 mg/mL Collagenase B PBS to

completely submerge the gel. The plate was then heated to 37 °C. The mass remaining was determined by removing the plate from the heat source, removing the cell strainer from the plate, drying the cell strainer, and weighing the cell strainer/gel combination at each time point. The peptide crosslinkers used were Proline C11 (CGPQGPAGQCR), Alanine C11 (CGPQGIAGQCR) and Tryptophan K12 (KCGPQGIWGQCK)

CHAPTER III

RESULTS & DISCUSSION

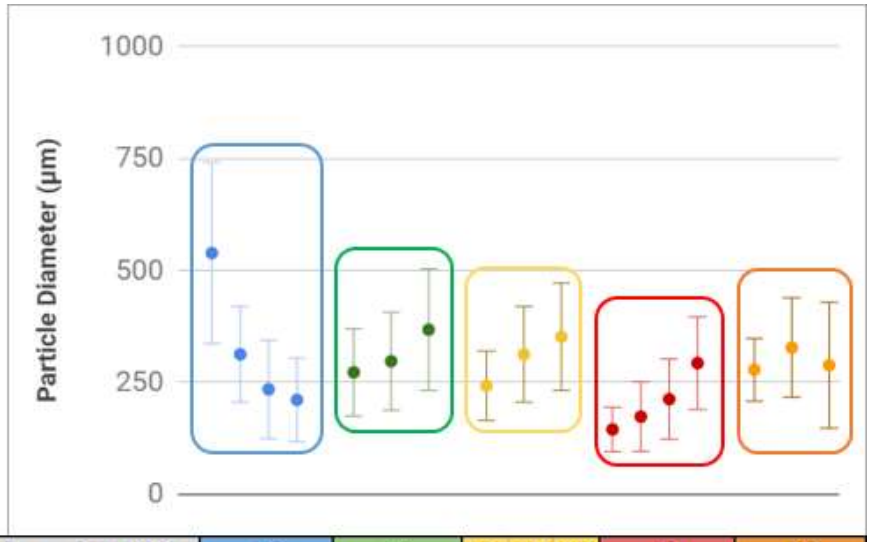
Submerged Electrospraying

The diameters of the hydrogel microparticles crosslinked with PEG-dt are shown in **Figure 3**. As expected, the diameter decreased as the voltage was increased. The electrified field creates an electrostatic force inside the droplet of hydrogel precursor which then competes with the surface tension of the droplet [9]. Ergo, the greater the electric field, the smaller the microparticle. This phenomenon was established by Lord Rayleigh in 1882 [17]. Once the voltage was increased past 6 kV, however, there was a considerable drop off in yield as well as no significant change in the diameter of these beads. At 8kV there was also a thin white film that appeared between the aqueous phase and the organic phase after centrifugation. This film and drop off in yield percentage suggests that there is another factor that is impeding fabrication of smaller particles when the voltage is raised to this extent.

The influence of an electric field upon radical polymerization has been studied extensively. However, an electric field's effect upon a reaction is highly unique depending on the reaction occurring [18], and there is little to no research done on the effect of an electric field on PEG-Nb thiol-ene click chemisty. In this case it could be possible that the increase in applied electric field impedes the free radical polymerization. This phenomenon combined with the greater molecular weight of the longer PEG-Nb chains could hinder the polymerization reaction enough so that not all or even none of the reagents are used within the photopolymerization time (5 minutes). The same thin white film was observed within a 5kD PEG-Nb reaction beaker at a higher voltage (10kV) which yielded no microparticles. Another reason for the drop off in yield percentage is the fact that the centrifugal force used was not great enough to separate the beads

from the organic phase due to their small size (<200 μm) and were discarded during the wash cycles. This yield percentage explanation holds true for the 1 and 3 mL/hr flowrate variable microparticles due to their size and extremely low yield percentage.

Additionally, **Figure 3** shows that microparticle diameter increased as the gauge size of the needle was increased. This increase is due to the fact that the syringe serves as the focal point for the droplet creation as well as acts as the last point of contact with the charged metal. The same trend could be said for the PEG-Nb molecular weight increase. The increase in diameter due to this parameter is the result of a higher molecular weight between crosslinks, which leads to greater swelling and, thus, greater particle diameter. The distance from the needle tip to the grounding ring did not show a difference in the microparticle size. However, it did show that polydispersity within the sample is positively influenced by this distance.



Molecular weight (kDa)	10	10	**5 10 20**	10	10
Flowrate (mL/hr)	12	12	12	**1 3 6 12**	12
Voltage (kV)	**2 4 6 8**	4.5	4	4.5	4.5
Distance (mm)	16	16	16	16	0 16 32
Needle Gauge	22	**26 22 18**	22	22	22

Figure 3 Particle Diameter with varying Voltage, Needle Gauge size, PEG-Nb Molecular Weight, Distance from needle tip to grounding ring, and Flowrate

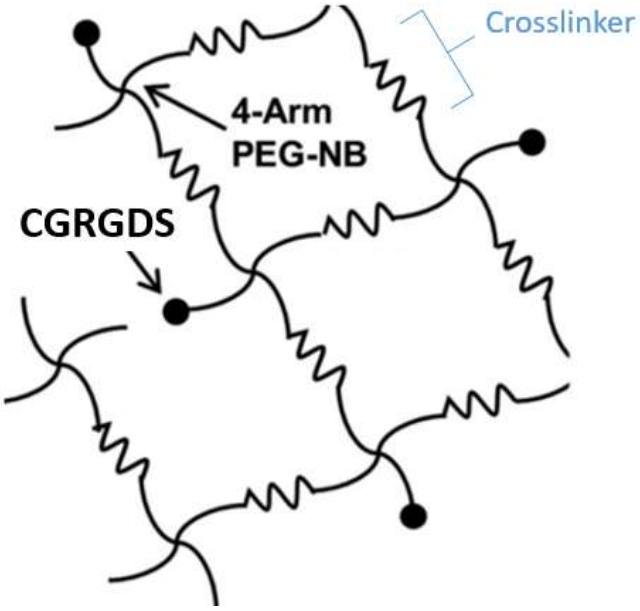


Figure 4 [19] Resulting Crosslinked network

As **Figure 4** would suggest, the length of the crosslinker used also has a profound effect on the size of the microparticles. Increasing the length of the crosslinker used will in turn increase the diameter of the swelled microparticles. The three different crosslinkers tested here were PEG-dt, KCGPQGIWGQCK, and CGPQGIAGQCR with molecular weights 3.4kD, 1.3kD, and 1.1kD respectively. The voltage used to fabricate the beads with the peptide crosslinkers was 3.5kV whereas the voltage to fabricate the PEG-dt crosslinked beads was 4kV. However, there is still a distinct difference in size between the peptide crosslinked beads and the PEG-dt beads as shown in **Figure 5**. These circumstances show that the length of the crosslinker significantly changes the diameter of the resulting swelled microparticle.

Although there is a slight difference in molecular weights between the Tryptophan K12 peptide and the Alanine K12 peptide, there is little to no difference in their resulting beads' diameters. This is as expected since the point of connection to the PEG-Nb arms is at the cysteine residues, and the number of residues between the two cysteines is the same for each

peptide sequence. The number of residues between the two cysteines is a more accurate characterization of the length of the peptide crosslinker rather than the molecular weight of the entire peptide sequence. For the PEG-dt crosslinked beads, the molecular weight of the PEG-dt is a more descriptive characteristic for the length of the crosslinker.

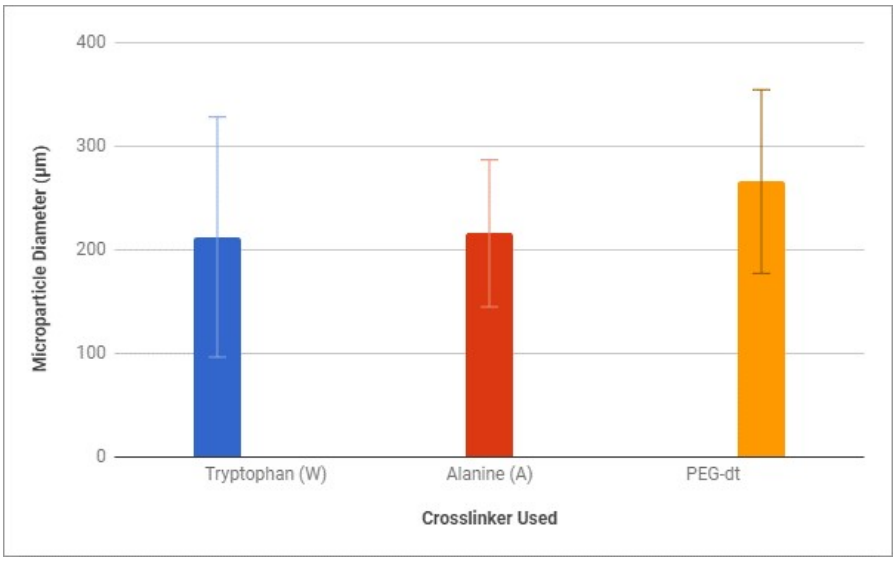


Figure 5 Microparticle Diameter vs. Crosslinker used. Tryptophan (KCGPQGIWGQCK), Alanine (CGPQGIAGQCR), PEG-dt

The swelling of bulk gels was compared to the swelling of microparticle scaffolds. As expected, the microparticle scaffolds had no change from their original size because the particles used to fabricate them had already reached equilibrium swelling. In contrast, the bulk gel exhibited a dramatic increase in mass as well as diameter as shown in **Figure 6** and **Figure 7**.

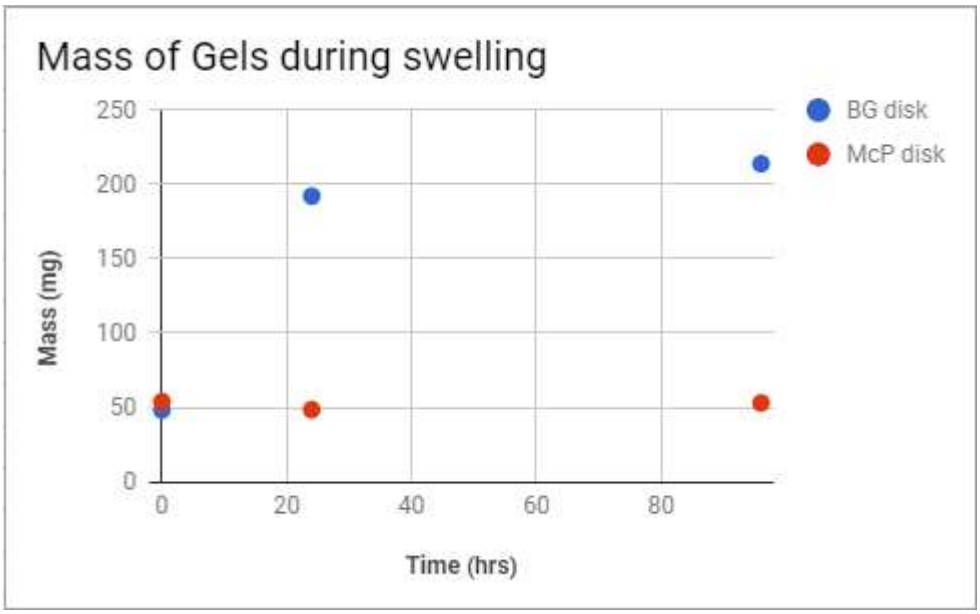


Figure 6 Mass of Gel disks before and after swelling

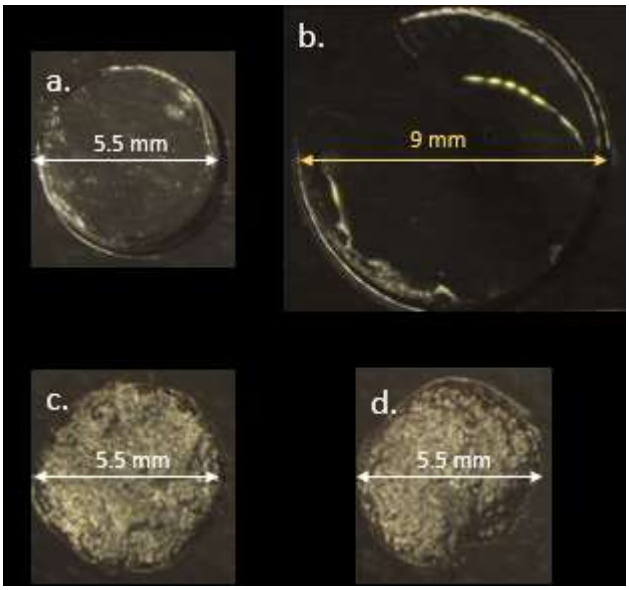


Figure 7 Diameter of Gel disks before and after swelling; a. bulk gel before swelling, b. bulk gel after swelling, c. microparticle scaffold before swelling, d. microparticle scaffold after swelling

Degradation

Figure 8 shows the mass remaining of the bulk gels of the three different peptide strands. Expectedly, the proline C11 had the longest degradation time. Surprisingly, the Tryptophan K12 strand has shown a longer degradation time than the Alanine C11. This is most likely due to the greater steric hindrance from the tryptophan residue as compared to the alanine in the other peptide sequence.

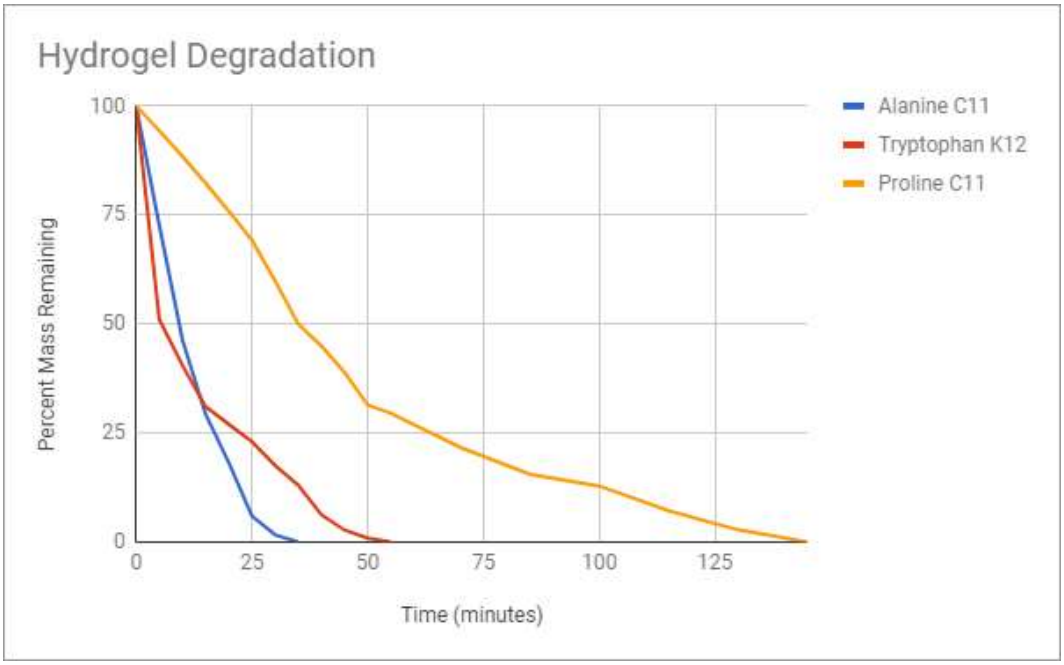


Figure 8 Hydrogel degradation of bulk gels crosslinked with Proline C11 (CGPQGPAGQCR), Alanine C11 (CGPQGIAGQCR) and Tryptophan K12 (KCGPQGIWGQCK)

CHAPTER IV

CONCLUSION

Submerged electrospraying is a viable method for fabricating microparticles. While it does not offer the precision of microfluidics based methods, its simplicity, reproducibility, and scalability are highly attractive for manufacturing hydrogel microspheres that can be used to produce tissue engineering scaffolds. Also, the degradation of the peptide crosslinked bulk gels and the swelling profile of the microparticle scaffolds illustrates that the microparticle scaffolds could offer tunable degradation while not increasing in size during implantation. The thiol-ene click chemistry is also a viable coupling method for polymerizing these microspheres for most voltages and precursor polymer chain molecular weights. However, it was observed that higher precursor polymer molecular weights must be coupled with a lower threshold for the electric field voltage in order to have an acceptable yield.

Nevertheless, submerged electrospraying appears to be a promising method for manufacturing tissue mimetic hydrogel microspheres. Moreover, these microspheres can be used to fabricate tissue engineering scaffolds with tunable properties for the host tissue they are supporting and their encapsulated cells. Future studies should be conducted on the relationship between the thiol-ene click chemistry and the electric field applied in order to accurately characterize the threshold for each. Rheology measurements should also be carried out on the peptide crosslinked hydrogels to further characterize their properties.

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