Spring 2018

Degradability of Poly(ethylene) Based Microgels and Methods of Improving High Polydispersity Index

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Honors Research Project Report

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Abstract

In order to improve upon the utilization of poly(ethylene glycol) (PEG) microgel-based scaffolds in drug delivery applications, this work explores the use of degradable and non-degradable PEG for tunable scaffolds. Previously, issues concerning the required uniformity in microgel size and low polydispersity index have been encountered. As such, methods of refining polymer synthesis, microgel fabrication, and microgel size separation were explored. Overall, no solution has been found to correct the issue for the microgels were still observed to have high polydispersity index regardless of varied troubleshooting alterations.

Introduction

The utilization of tunable materials is highly attractive for the purpose of drug delivery applications. Poly(ethylene glycol) (PEG) is studied for tissue engineering scaffolds due to its nontoxic, water soluble, and low immunogenicity (1). Microgels that formed from PEG are micron-sized hydrogel particles that are easy to inject once fabricated. Because the PEG microgels have customizable mechanical properties and are created in mild precipitation reaction conditions, they are applicable for in vivo purposes and are formed with information shape and size, signifying a low polydispersity index, due to the nature of the precipitation reaction (2). PEG can be synthesized to include an acrylated block copolymer with poly(lactic acid) (PLA) (PLA-b-PEG-b-PLA), which in turn creates degradable PEG microgels (3). In fabricating microgels with mixed ratio polyethylene glycol diacrylate (PEG-DA) and polyethylene glycol-lactic acid- diacrylate (PEG-LA-DA), one is able to create PEG microgel-based scaffolds with controllable degradation (4).

Background Information

In previous studies, non-degradable PEG-DA and degradable PEG-LA-DA were used to fabricate non-degradable and degradable microgels. These microgels were combined at a variation of different ratios in order to create varied degrees of degradability in microgel-based scaffolds (5). Due to the 3D nature and configuration of the scaffold, and its future application as a drug delivery vehicle, it is essential that the microgel precipitation reaction provides microgels with low PDI, as well uniform size, shape, and swelling, which all affect the assembly of microgels into the 3D scaffold.

While synthesizing the PEG-DA and PEG-LA-DA polymers, there have been recent issues regarding the polydispersity index (PDI) of the microgels fabricated using the PEG-LA-DA polymer. Despite the examination of the chemical configuration of the polymers and the characterizations of the fabricated microgel, the cause of the issue could not be determined.

Project Objectives and Goals

The overreaching goal of this project is to create polyethylene glycol (PEG) microgel-based scaffolds with tunable degradability. The microgel-based scaffolds are created utilizing degradable and non-degradable microgels mixed at various ratios. Two different polymers are synthesized for the fabrication of the microgels, including polyethylene glycol diacrylate (PEG-DA) for the non-degradable microgels and polyethylene glycol-lactic acid-diacrylate (PEG-LA-DA) for the degradable microgels. The purpose of this project is to allow for the tunable variation of microgel-based scaffolds based on particular needs in the drug delivery application.

The foremost objective is to determine and learn of the cause and solutions to the issue

causing the high polydispersity index (PDI) in microgels that are being formed from the PEG-LA-DA. This step is crucial in being able to create the ideal PEG microgel-based scaffolds due to the fact that changes in such characteristics would alter the assembly and configuration of the microgels into a 3D scaffold.

Methods and Procedures

Synthesis of PLA-PEG-PLA, PEG-LA-DA, PEG-DA

The synthesis of PLA-b-PEG-b-PLA triblock copolymer through the use of polyethylene glycol (PEG) with the molecular weight of 3 kDa is a prerequisite for the synthesis of PEG-lactic acid-diacrylate. Lactide was recrystallized in ethyl acetate and placed in a 40°C vacuum oven for 24 hours to ensure that all water and residual ethyl acetate has been removed. To minimize the addition of water added to the reaction, all glassware is dried in a 125°C oven. After each step, the reaction is evacuated and inerted with argon. 20 grams of lyophilized 3 kDa PEG was placed in a round bottom flask and melted at a temperature between 180- 190°C. Once the PEG melted, the 4.8 g lactide was added. After the lactide melted, 15.1 μL stannous octoate (Tin(II) 2-ethylhexanoate) is added. The reaction is then stirred under vacuum at a temperature of 180-190°C. After 4 hours, the temperature is lowered to 160°C for 2 hours. After the 2 hour period, the reaction is cooled to room temperature. Once the polymer is dried, it is dissolved in a minimal amount of dichloromethane (DCM). It is then precipitated in chilled anhydrous ether, filtered to collect the polymer. The polymer is then left to dry in a chemical hood, and is further dried in a vacuum desiccator (6).

The acrylation of the PLA-PEG-PLA to achieve PEG-LA-DA and the acryaltion of PEG to achieve PEG-DA follows the same protocol. Either the synthesized PLA-PEG-PLA or the PEG is lyophilized to remove any residual water in the polymer. All glassware is dried to ensure that they are dry, and each step required the evacuation and inertion to prevent water from factoring into the reaction. A dried round bottom flask was evacuated and interted, and while the argon was flowing, the PEG (respective to PEG-LA-DA and PEG-DA) was added. Anhydrous DCM was added to dissolve the PEG. Triethylamine (TEA) was slowly added to the reaction, and the reaction was left to mix for 5 minutes. In a drop-wise fashion, the acryloyl chloride was added. The solution was left to reaction overnight while stirring on ice. Next, the reaction undergoes a phase separation by placing the reacted solution in a 250 mL separatory funnel. DCM was added to wash out the round bottom flask, and 2M potassium carbonate (K_2CO_3) was added to the separatory funnel so that it flowed down the side of the glassware. The funnel was stopped and shook vigorously for n5 second intervals (where n is integer 1, 2, 3…) until no more gas was released in venting. The separatory funnel was covered in parafilm and left to separate by gravity. The organic phase, which contained the acrylated PEG and was the lowest phase, was drained into a 1000 mL beaker. The solution was mixed with magnesium sulfate ($MgSO_4$), filtered to remove the $MgSO_4$, and then precipitated out with diethyl ether. The final product was left to dry overnight and then moved to dry under vacuum. It was then crushed to a fine powder and stored in -20°C freezer (2, 7).

Altering Synthesis of Polymer

In order to solve the issue of inconsistent microgel size and large PDI, the synthesis of the polymers were altered in troubleshooting. These methods of troubleshooting included changing synthesis reaction times and temperatures, as well as dialyzing and filtering the polymers at each step. The initial temperature for the PLA-PEG-PLA reaction was 180- 190°C for 4 hours. Troubleshooting syntheses were changed to temperatures of 160°C for 1 hour, 160°C for 3 hours, 140°C for 8 hours, and 120°C for 20 hours. Because the reaction essentially reaches completion before the temperature drop, this was the point that was varied in experimentation.

In altering the synthesis with dialysis of polymers, synthesized batches of PLA-PEG-PLA and PEG-LA-DA were dialyzed using Slide-A-Lyzer Dialysis Cassettes that had a 2K molecular weight cutoff. This was done in attempt to remove any unreacted lactic acid which falls under the molecular weight cutoff. The polymer was diluted with DI water and injected into the cassette membranes. The dialysis cassettes were submerged in DI water and left for a period of 24 hours. The solutions with the polymer were removed from within the cassette membranes and placed in 50 mL centrifuge tubes. These were lyophilized in order to dry the polymer and remove the water to obtain the solid form of the polymer.

Additionally, the PLA-PEG-PLA and PEG-LA-DA polymers were centrifugally filtered through the use of centrifugal filters with a molecular weight cutoff of 1K. This cutoff was appropriate in attempt to remove the lactic acids that were not reacted in the PEG chains. Diluted PEG in DI water were added to the centrifugal filters and centrifuged at 5000rpm for 20 minutes. The filtrate and residue were both collected and lyophilized to obtain the solid forms.

Fabrication of Microgels

Two different types of microgels were fabricated via a precipitation reaction, including PEG-DA and PEG-LA-DA microgels. For a single 1x microgel reaction, 5 grams of 3K PEG-DA or PEG-LA-DA polymer was dissolved in a PBS, TEOA, and HCl 25 buffer μL and heated to 37°C. In order to fabricate degradable microgels, PEG-LA-DA polymer was used, and to fabricate non-degradable microgels, PEG-DA polymer was used. A 1.5M sodium sulfate solution is made and heated to 37°C. In preheated tubes containing 127.5 μL and 10 μL of 0.5% Irgacure 2959, 25 μL of the PEG solution is added. 1 μL of tetrathiol is then added and the solution is vortexed to mix. 87.5 μL of the 1.5M Na_2SO_4 is added, and mixed by pipetting the solution up and down. The addition of the salt solution causes the initial precipitation of the PEG diacrylate. The solution is then crosslinked under a Cure Spot UV light (3.2 mW/cm2, 365 nm) for 30 seconds. The crosslinking allows for the permanent precipitation of microgels that consist of polyacrylate backbone and crosslinked PLA-b-PEG-b-PLA chains. The completed solution was washed five times in DI H_2O to remove any residual salt (2).

Altering Fabrication of Microgels

The alteration of the microgel fabrication protocol was completed in order to determine whether the issue of the inconsistent sizing and large PDI was due to the fabrication techniques and whether making changes to the protocols would rectify the issue. The following steps were added or adjusted variables whilst troubleshooting the PEG-LA-DA microgel fabrication: changed Irgacure concentration, changed salt type and concentration, changed reaction

temperature, changing time period before crosslinking, adding period of sonication, and altering the ratio of PEG-LA-DA to PEG-DA.

Altered Irgacure Concentration: The regular protocol calls for .5% Irgacure. In troubleshooting, Irgacure concentrations were altered to include 1% Irgacure. Because the Irgacure serves as the photoinitiator, it was thought that in altering the concentration, it may be that the microgels crosslink and photopolymerize at a faster rate to avoid uneven photopolymerization, which may contribute to inconsistent microgel diameter sizes.

Altered Salt Concentration: In changing salt concentration, the microgels were fabricated using sodium sulfate solution concentrations of 2.25M, 2.5M, 2.75M, 3M, rather than the regular 1.5M called for in the protocol.

Altered Microgel Reaction Temperature: The reaction and solutions of microgel fabrication is kept at 37°C. The temperature of the reaction was altered to 21°C, 25°C, 29°C, and 33°C.

Altered Time Period Before Crosslinking: In the regular protocol, the microgel reaction is crosslinked under UV light immediately after the salt solution has been added and mixed. In troubleshooting, the microgel solution were vortexed after adding the sodium sulfate solution. The solutions sat for 0.5, 1, 2, and 3 minutes before crosslinking. Immediately before crosslinking the solutions, they were vortexed again.

Addition of Sonication: The purpose of sonication is to apply sound energy to agitate particles in a solution. This is not a step in the regular protocol, but was added in attempt to further mix the microgel solution and break up any potential clumps that resulted in uneven precipitation that could cause large diameters in microgels. Solutions were sonicated for 1

minute before and after adding the salt solution. In addition, microgel solutions were sonicated prior to crosslinking for 2, 3, 4, 5, and 9 minutes.

Altering PEG-LA-DA to PEG-DA Ratio: Ideally, non-degradable and degradable microgels used for scaffolds are combined after the fabrication of each is complete. The tunability of the scaffolds is determined by the ratio of the two types of microgels added to create the scaffold. In attempt to limit the possibility of uneven hydrophobicity in PEG-LA-DA microgel fabrication, mixed ratios of PEG-LA-DA and PEG-DA polymers were added to the initial PEG solutions prior to microgel fabrication. These PEG-LA-DA to PEG-DA ratios included 2:0, 1.9:0.1, 1.5:0.5, 1:1, 0.5:1.5, and 0:2 respectively.

Separation Techniques

Because the issue of large, inconsistent diameters and a large PDI persisted, techniques to separate larger microgels from those that were the ideal size were explored. These included separating the microgels through vacuum filtration, centrifugal filtration, dextran density gradients, and size exclusion.

Vacuum Filtration: In attempt to filter out smaller microgels from the microgels with larger diameters, microgels were fabricated and filtered utilizing a Buchner funnel. 90 mm Whatman 50 filter paper was set up and pre-wet with DI water. Microgels in DI water were placed above the filter paper and a quick vacuum at 200 mmHg was set to pull the microgels.

Centrifugal Filtration: Centrifugal filters with a molecular weight cutoff of 1K were used to filter microgels diluted in water. Both the filtrate and residue were collected and imaged to determine whether there was a difference in microgel diameters.

Dextran Density Gradient: According to previous publication (WENDA), microgels made with PEG-LA-DA have a density of 1.025 g/cm^3. As such, the microgels are expected to fall within the 8 and 9% dextran solutions. Dextran density solutions were created ranging from 0.5 to 10% which corresponds with densities between 1.008 to 1.022 g/cm. Gradients were created in centrifuge tubes using these solutions, and microgels were added to the gradients. The gradients were then centrifuged at 2000 rpm for 5 minutes. The microgels within each layer were extracted, washed, and imaged.

Size exclusion: To mimic size exclusion chromatography, microgels diluted in DI water were added to a separatory funnel contained 2mm glass beads. Every 2mL of solution that passed through was collected and examined.

Analysis

Nuclear Magnetic Resonance

In order to analyze all synthesized polymers, polymer samples are were dissolved in deuterated chloroform. The prepared samples were examined utilizing Varian Nuclear Magnetic Resonance (NMR) Spectroscopy 300 MHz for ^{1}H NMR. In order to analyze the obtained spectra, the peaks at 1.5 ppm, 5.59 ppm, and 6.27 ppm are integrated. The PEG peak is set to a reference of 1. The peak at 1.5 ppm reveals the number of lactic acid subunits, while the peaks at 5.59 ppm, and 6.27 ppm reveal the percent acrylation (4).

Microgel Imaging

Fabricated microgels were imaged using a Zeiss inverted microscope and Axiovision software. The microgel sample was prepared by adding 30 μL of diluted microgel sample in water to a glass slide. A cover slip is then placed on the droplet of sample, and pressed down to remove excess liquid. At 100x DIC objective in oil, the microgels are observed under the microscope and images are obtained using Zen (Zeiss) software. Using this software, the diameters of each microgel are measured.

Polydispersity Index

After obtaining the diameter measurement of the microgels, the radius of each microgel is then calculated, as well as the volume and volume squared of the spherical microgels. The polydispersity index (PDI) of the microgels was calculated using the following formula (8):

$$PDI = \frac{\frac{\sum V_i^2}{\sum V_i}}{\frac{\sum V_i}{N}}$$

where $\sum V_i^2$ is sum of all volume squared values, $\sum V_i$ is sum of all volume values of the microgels, and N is the total number of microgels (9).

Results

Polymer Syntheses and Alterations

Past tests on successful batches of PEG-LA-DA show that the number of lactide groups on both ends, calculated by the lactide result peak divided by the integrated PEG result peak, resulted in 7. Current tests, reveal that the number of lactide groups total on both ends consistently range from 4.0-5.7. Besides this and lower percent completions, there are no apparent differences between past successful batches of PEG-LA-DA and troubleshooting batches.

Microgels fabricated from troubleshooting batches of PEG-LA-DA have no significant difference ($p<0.05$) between the microgel diameter averages. The PDIs have all been shown to be too high, for the ideal value is that which is close to 1. While they are all too high, there is no consistent PDI value the microgel batches cohere to.

Alteration of Microgel Fabrication and Separation Techniques

Despite the broad variety of variables altered in the fabrication of the microgels, none of them proved to create ideal-sixes microgels with low PDI. While the mixed ratio of PEG-LA-DA to PEG-DA resulted in microgels with lower PDI and more consistent diameter sizes, the mixing of degradable and non-degradable polymers prior to the fabrication of microgels, rather than mixing after microgels are created, would limit the possibilities of scaffold degradability. For all microgel diameter averages, standard deviation, and PDIs, refer to Appendix C.

Future Direction

Because no solution or fix to the problem of inconsistent, large microgel diameters and large PDI has yet to be found, future work includes continued troubleshooting with both the syntheses of the polymers and the separation techniques used on PEG-LA-DA microgels. One option includes completing a filtration and/or dialysis on the polymers at each consecutive step in order to remove any impurities or unreacted chemicals. Synthesizing PEG-LA-DA from dialyzed PLA-PEG-PLA, and then dialyzing the resulting PEG-LA-DA, is the next step.

Additional future work includes refining a size exclusion chromatography technique. In improving this technique, one is more likely to ensure that the larger microgels (by either diameter or molecular weight) will elute out before the smaller, more ideal sized microgels. This will also for a tighter separation, which may result in a more precise separation technique.

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Appendices

Appendix A: Fabrication Illustrations

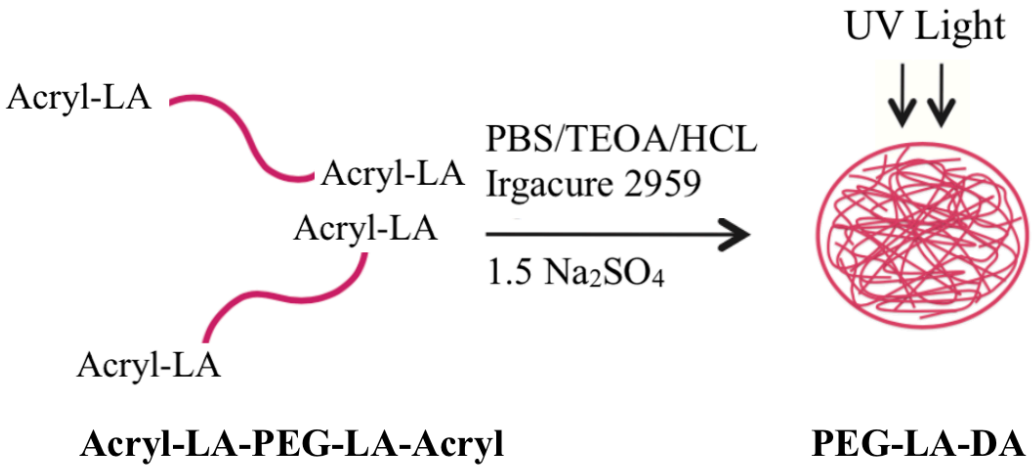

Figure 1: Illustrated cartoon of the fabrication of PEG-LA-DA degradable microgels. PEG-LA-DA polymer is mixed with PBS/TEOA/HCl buffer, .5% Irgacure, and 1.5M sodium sulfate solution. The solution is crosslinked under a UV light. Note: for use with scaffolds, tetrathiol is added to PEG-LA-DA solution as well.

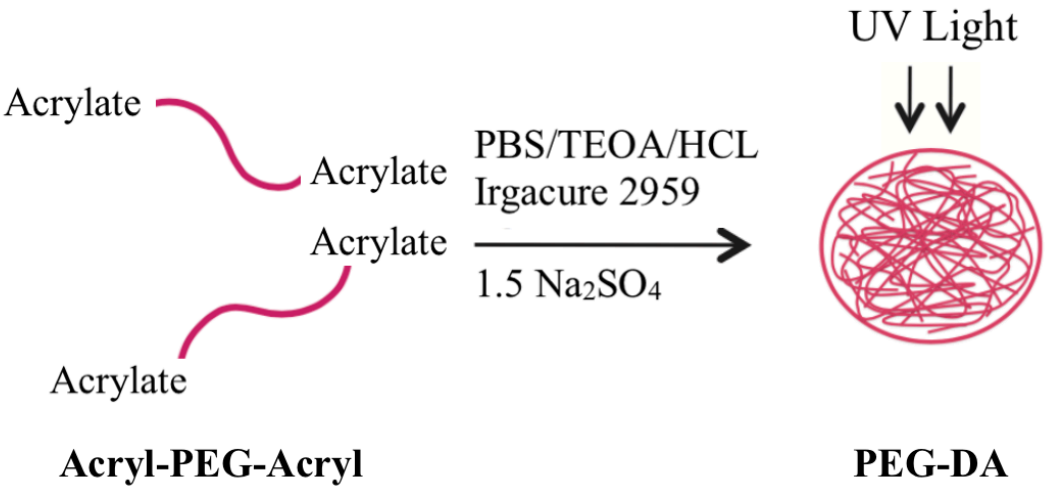

Figure 2: Illustrated cartoon of the fabrication of PEG-DA non-degradable microgels. PEG-DA polymer is mixed with PBS/TEOA/HCl buffer, .5% Irgacure, and 1.5M sodium sulfate solution. The solution is crosslinked under a UV light. Note: for use with scaffolds, tetrathiol is added to PEG-DA solution as well.

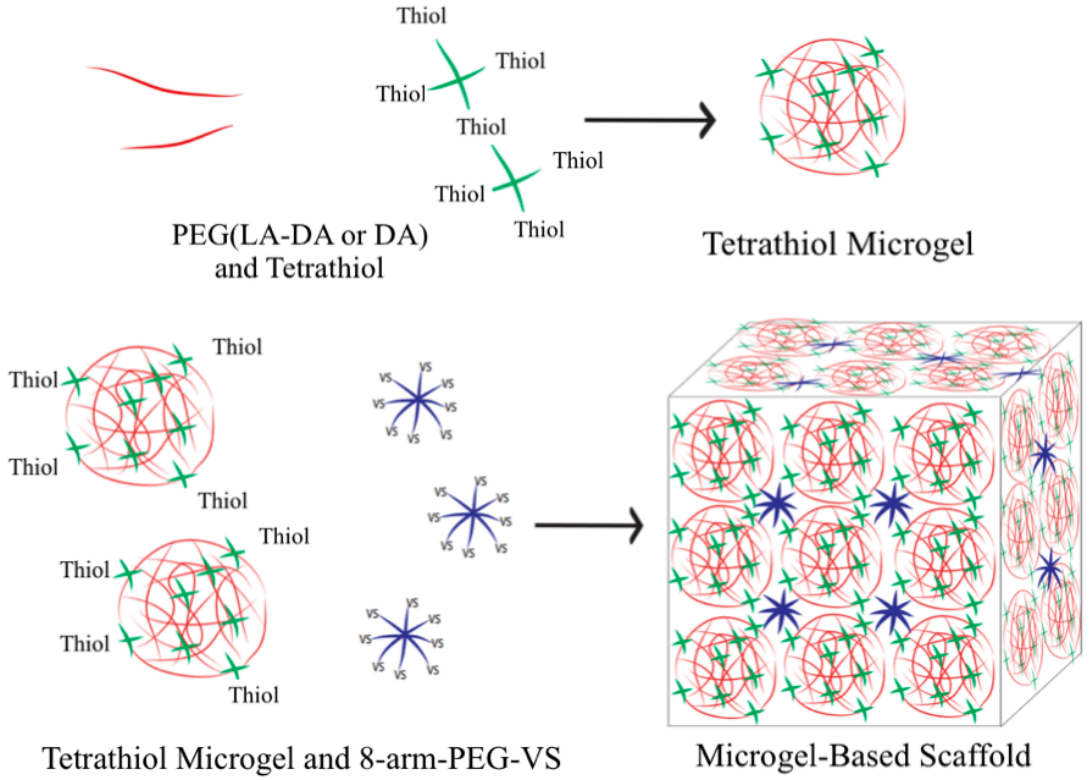

Figure 3: Illustrated cartoon depicted the creation of a microgel- based scaffold. PEG-LA-DA for degradable, and PEG-DA for non-degradable, polymer is mixed with tetrathiol and created as depicted in Fig. 1 and 2. Fabricated microgels are mixed with 8-arm-PEG-VS to assemble the scaffold.

Appendix B: NMR Spectra and Analysis

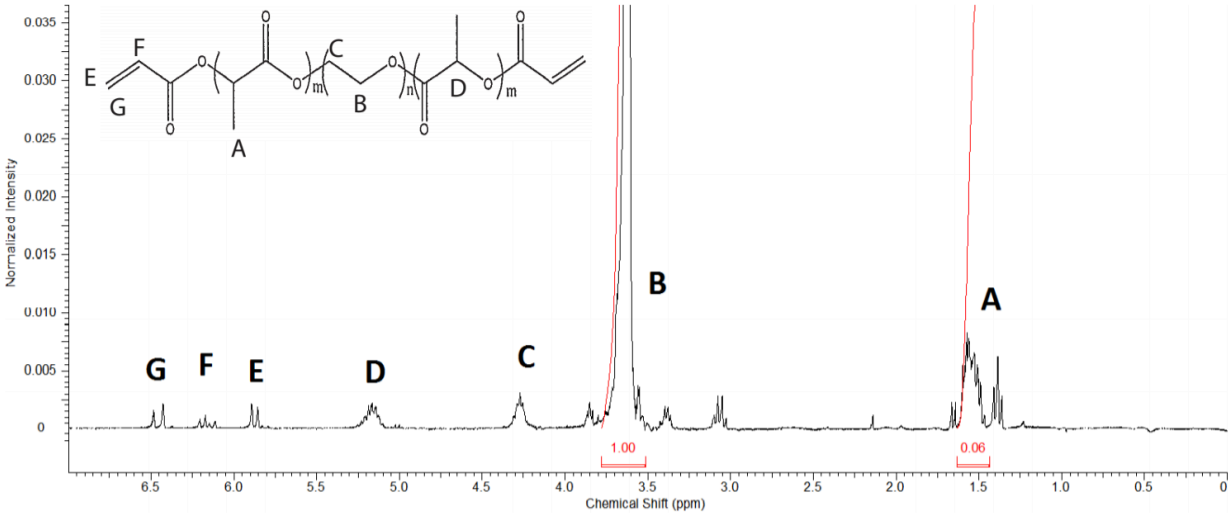

Figure 4: PEG-LA-DA NMR spectra with labeled shifts corresponding to chemical structure.

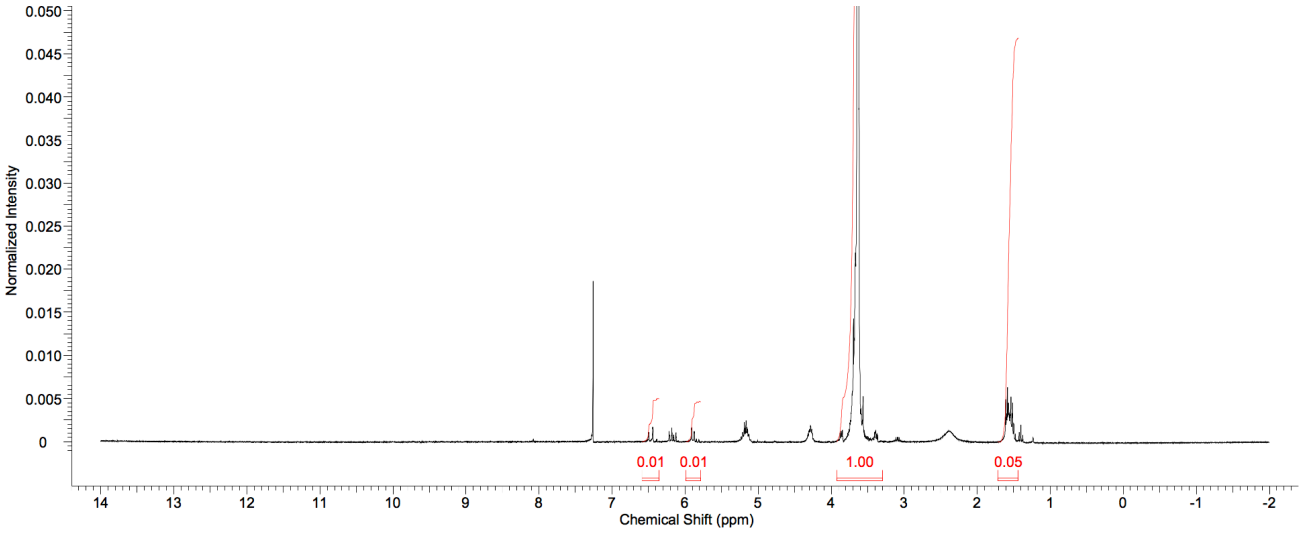

Figure 5: Example of obtained 3K PEG-LA-DA NMR spectra with integrated peaks.

PEG MW	3000		
# mers	67.77272727		
# protons	271.0909091		
1.5 peak theoretical protons/lactide	3		
1.5 peak actual	0.05306385		
3.6 signal	1		
3.6 result (signal/#protons)	0.003688799		
1.5/3.6 result (# protons expt)	14.38512914365		
# lactide groups total (both ends)	4.795043047886		
6.27 signal	0.00520362	5.59 signal	0.00563508
6.27/3.6 result	1.410654253593	5.59/3.6 result	1.52761915
# protons theoretical	2	# protons theoretical	2
Percent Completion	70.53271267965	Percent Completion	76.3809575

Table 1: Example of NMR analysis corresponding to NMR in Fig. 5.

Appendix C: Microgel Separation Methods and Size Characterization

Dextran Density Percent	0.5%	1%	2%	3%	4%	5%	6%	7%	8%	9%	10%
Density (g/cm^3)	1.008	1.010	1.012	1.014	1.016	1.018	1.020	1.022	1.024	1.026	1.028

Table 2: Dextran density percents used in the density gradient and corresponding density in (g/cm^3)

	Microgel Type	Average Diameter (μm)	Standard Deviation	PDI	n
	Regular Protocol	2.1033	2.1477	30.8160	10
Altered Irgacure Concentration	1% Irgacure	1.5054	2.1024	46.0819	6
Altered Salt Concentration	2.25M sodium sulfate	5.7839	6.7594	41.9092	4
	2.5M sodium sulfate	8.7373	8.6807	24.1454	4
	2.75M sodium sulfate	9.5715	9.0921	10.9481	3
	3M sodium sulfate	11.9162	8.6477	15.0245	3
Altered Microgel Reaction Temperature	21°C	No microgels formed			2
	25°C	No microgels formed			2
	29°C	No microgels formed			2
	33°C	No microgels formed			2
Altered Time Period Before Crosslinking	0.5 minute prior to crosslink	3.7814	4.9514	13.0757	2
	1 minute prior to crosslink	2.0619	2.3049	14.0927	2
	2 minutes prior to crosslink	3.0046	5.1613	13.7242	2
	3 minutes prior to crosslink	3.2678	4.4897	8.3508	2
Addition of Sonication	before sodium sulfate	2.2334	2.4653	13.6774	3
	after sodium sulfate	2.0297	2.2539	11.8774	3
	2 minutes prior to crosslink	11.1675	3.8768	8.1514	1
	3 minutes prior to crosslink	11.9804	8.8906	5.4846	1
	4 minutes prior to crosslink	8.5433	6.9775	17.8417	1
	5 minutes prior to crosslink	5.0239	2.4432	1.8718	1
	9 minutes prior to crosslink	No microgels formed			1
Altering PEG-LA-DA to PEG-DA Ratio	2 to 0	1.9501	1.9213	25.3698	3
	1.9 to 0.1	2.2597	3.4413	25.5391	3
	1.5 to 0.5	1.5557	4.7629	0.97149	3
	1 to 1	1.8521	3.0006	71.0358	3
	0.5 to 1.5	1.6109	1.2609	0.30526	3
	0 to 2	1.3873	0.2025	1.1966	3

Table 3: Microgel size characterization from experiments that involved altering the fabrication of the microgels. Note: n refers to the number of batches/ trials competed.

	Microgel Type	Average Diameter (μm)	Standard Deviation	PDI	n
Dextran Density Gradient	0.5%	4.4501	5.4697	7.7640	2
	1%	No microgels retrieved			2
	2%	3.7028	2.8129	4.4682	2
	3%	9.3817	8.7849	8.9206	2
	4%	1.6728	1.3321	19.3557	4
	5%	1.5922	1.3651	16.5418	3
	6%	2.1053	2.5145	30.2098	3
	7%	1.8003	1.2717	11.0902	5
	8%	1.5850	0.9970	27.0608	4
	9%	2.4664	2.8507	25.0904	4
Vacuum Filtration	90 mm Whatman 50 filter paper	1.5047	1.6094	13.9714	3
Centrifugal Filtration	1K MW CO	2.4331	2.5844	18.6096	3
Size Exclusion	First 2mL	2.8221	1.9991	8.3862	1
	Second 2mL	2.5381	1.6281	8.8616	1
	Third 2mL	3.6838	3.1026	9.7101	1
	Fourth 2mL	2.8598	2.8192	9.5692	1
	Fifth 2mL	2.7860	2.4954	12.0866	1
	Sixth 2mL	2.4631	2.7012	20.9374	1
Dialyzed PEG-LA-DA	Dialyzed PEG-LA-DA following regular protocol	2.4387	2.0888	26.0776	2

Table 4: Microgel size characterization from experiments that involved altered synthesis of PEG-LA-DA and from techniques of separation after fabrication of microgels.