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Biodegradable Poly(Ester Urea's) Scaffold Sponges Containing Platelet-Rich Plasma For Targeted Tendon-Bone Fixation

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ABSTRACT

In this report the synthesis and characterization of L-valine and L-isoleucine poly(ester ureas) (PEUs) for future use in the synthesis of biodegradable PEUs sponges will be discussed. The method of monomer and polymer synthesis will be explained along with the characterization of the monomer and polymer products. This characterization will be done by the use of Nuclear Magnetic Resonance (NMR) and Size Exclusion Chromatography (SEC). NMR is a quick and convenient method for determining structure and purity of the monomer and polymer products. SEC gives the number average molecular weight, which is the statistical average molecular weight of all the polymer chains in a sample (Mn), the weight average molecular weight (Mw), and the Polydispersity Index (PDI), which is the Mw/Mn. Further characterization will be performed using Thermogravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC). The data from the TGA provides degradation temperature, while the date from the DSC provides glass transition temperature. These values are of great importance since the application of the polymers will be in the body at a temperature of 37 °C. This report is only the first of three steps that will follow and are explained in the introduction.

INTRODUCTION

Every year, around 200,000 Americans undergo shoulder surgery related to repair of the rotator cuff. An additional 400,000 Americans have surgery for related rotator cuff tendonitis or for partial tears (1). These procedures can be done by arthroscopic or open surgery, and involve reattachment of the torn rotator cuff by suturing the tissues back together. Recovery from these surgeries is often tedious and requires long-term physical therapy (2).

After surgery, if the severity of the tear was large, the incidence of re-tear is quite high. In 2012, large rotator cuff repairs re-tore at a rate of 57% (3). Various techniques are used for the repair of

the rotator cuff; with a subset of the patient population having a lower incidence of re-tear. Studies have found significantly lower re-tear rates for double-row repairs when compared with single-row repairs for all tears greater than 1 cm (4). Even with the advances in suturing and repair techniques the re-tear problem is still prevalent.

The increasing interest in alternative or supplemental treatments have produced viable options such as platelet-rich plasma (PRP) injections and decellularized biological scaffolds. PRP therapy is of clinical interest because the highly concentrated platelet solutions have been associated with stimulating tissue repair and regeneration (5). The decellularized biological scaffold acts as a tissue bridge between tendon and bone, as well as a platform for aligned cellular growth and collagen assembly (6).

This project proposes the use of biodegradable poly(ester ureas) (PEUs) sponges utilizing tunable mechanical properties and chemistry. This PEU sponge combined with PRP could help enhance the tendon-bone healing time and reduce the rate of re-tear. Our hypothesis is that L-valine or L-isoleucine based PEU will allow for retention of PRP for sustained activation of PRP growth factor release.

EXPERIMENTAL SECTION

NMR

NMR spectra were obtained using a Varian NMRS 300. Chemical shifts are reported in ppm and referenced to the chemical shifts of the residual solvent resonances (DMSO 2.50 ppm).

Materials for Monomer Synthesis. Reagents: 1,6-hexandiol, 1,8-octanediol, 1,10-decandiol, 1,12-dodecandiol, L-valine, L-isoleucine, p-toluenesulfonic acid, and toluene used in the monomer synthesis were purchased from Sigma-Aldrich and used as received. A 2 L round-bottom flask(RBF), dean-Stark trap, condenser, thermometer, hot plate with magnetic stirring capabilities, magnetic stir bar, and an oil bath capable of withstanding temperatures well above 140 °C were used.

Synthesis of PEU Monomer.

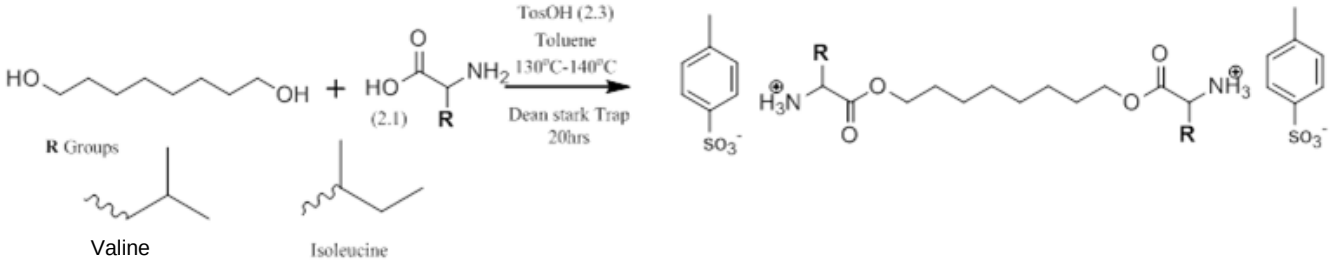

FIGURE 1: *Reaction conditions and reactants for monomer synthesis*

First, 1 equivalent of a diol, 2.1 equivalents of amino acid, 2.3 equivalents of p-toluenesulfonic acid, and 41.4 equivalents of toluene and a magnetic stir bar were combined in a 2 L RBF. An oil bath was prepared and placed on a hot plate with magnetic stirring capabilities. The temperature of the oil was brought to 130 °C – 140 °C and the round bottom flask was placed into the oil bath. A Dean-Stark trap and condenser were added to the top of the RBF so refluxing could take place. Water is a bi-product of the reaction and collected in the dean-stark trap to assist the reaction to completion. Insulating padding was then wrapped around the entire reaction unit to prevent heat loss. The reaction was run for 48 hours, followed by three rounds of purification. The monomer product was dissolved in 500 mL of boiling water then left to recrystallize. Once recrystallization had completed the monomer produced was filtered using a porcelain filter, 125

mm Whatman filter paper, and 1 L filter flask. This procedure was repeated three times followed by on round of hot filtering to ensure purity. The monomer product was then packaged into various 50 mL Falcon tubes and set in the freezer for 2 days followed by an additional 2 days on the lyophilizer to ensure all water was removed. The product was then ready for use in polymer synthesis reactions.

Materials for Polymer Synthesis. Sodium carbonate (Na_2CO_3), triphosgene, and chloroform used in the polymer synthesis were purchased from Sigma-Aldrich and used as received. Monomer previously synthesized was used in the polymer synthesis as well. For example, 1-Ile-6 monomer (1-Ile-6M) was used in the synthesis of the 1-Ile-6 polymer (1-Ile-6P). A 2 L 3-neck RBF, pressure equalizing glass drip funnel, large steel bowl (that can fit the RBF along with water to half submerge RBF), thermometer, and glass separatory funnel were used in the polymer synthesis.

Synthesis of PEU Polymer.

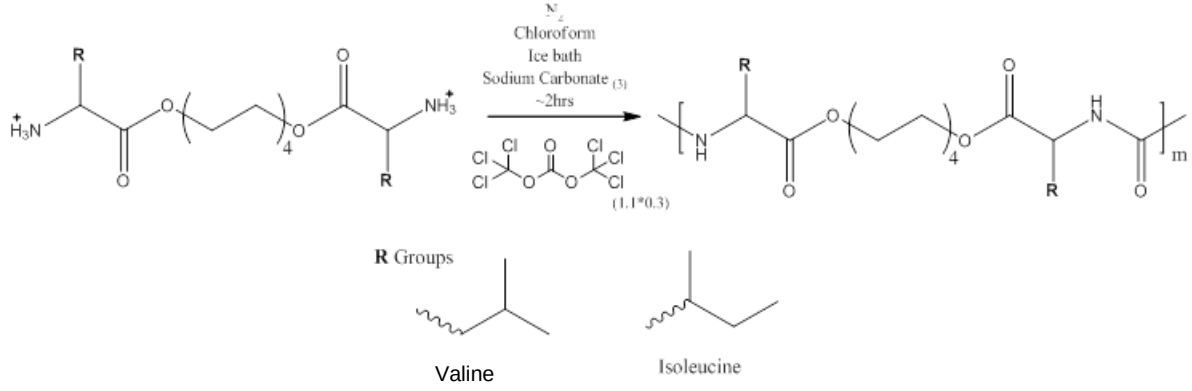

FIGURE 2: *Reaction conditions and reactants for polymer synthesis*

First, 1 equivalent of monomer, 3 equivalents of Na_2CO_3, and 16 equivalents of water are added to the 3-neck RBF. The pressure equalizing glass drip funnel was placed in one of the opening on the top of the RBF while the third and final opening was corked with a thermometer. The RBF was placed into a steel bowl containing 35 °C water and a mechanical stirrer was connected to the top. The solution was mechanically stirred for 30 minutes. Once the 30 minutes had ended the steel bowl was removed and filled with a water, ice, and salt mixture. The solution's temperature was brought down to 0 °C while mechanical stirring was taking place. While the solution was cooling, the first addition of 0.4 equivalents of triphosgene in 2.5 equivalents of chloroform was prepared. Once the solution had reached 0 °C, the triphosgene solution was added to the drip funnel and added into the monomer solution with the use of air pressure to make the addition as quick as possible. The temperature was maintained as close to 0 °C as possible and mechanically stirred for 30 minutes. The second addition of 0.2 equivalents of triphosgene in 0.3 equivalents of chloroform was then prepared. Upon completion of the 30 minutes the second addition of triphosgene was added to the drip funnel and added to the reaction one drip every 5 seconds until the all of the triphosgene solution had been added. The reaction was left to stir while still keeping the temperature as close to 0 °C as possible for 2 hours. A 4 L beaker of boiling water was prepared for precipitation of the polymer. The contents were them poured into a separatory funnel. Two layers formed inside the separatory funnel, the top layer containing water and the by-products of the reaction and the bottom layer containing chloroform and the polymer product. The bottom layer was slowly dripped into the boiling water. Since the polymer was insoluble in water, it coagulated on the surface. The boiling water was also hot enough to boil off any remaining chloroform. Once the entire bottom layer had been added to the boiling water, the polymer was removed from the boiling water with forceps. The

polymer was cut while still hot and placed into 50 mL falcon tubes. This product was then frozen and set on the lyophilizer for two days to remove any excess solvent. The polymer product was now ready for characterization.

Thermogravimetric Analysis (TGA). Degradation temperatures for each of the polymer samples was found using the following procedures; The samples were loaded into the TGA apparatus and the temperature was ramped at 10 °C/min to 600 °C. The degradation temperature can be deduced by the initial drop in weight % shown on the graphs produced by the TA Universal Analysis software. Three separate samples were run.

Differential Scanning Calorimetry (DSC). Glass transition temperatures for each of the polymer samples were found using the following procedure; the samples were equilibrated at 0 °C, 0 °C was held for 2 minutes, followed by temperature ramping at 10 °C/min to 100 °C. 100°C was held for 2 minutes, which marked the end of cycle 1. The sample was then ramped at 10 °C/min to -20 °C. -20 °C was held for 2 minutes, which marked the end of cycle two. The first two cycles were performed to ensure the polymer had all air removed that could skew the results of the pervious cycle, which gives the actual glass transition temperature. The final cycle was run by ramping at 10 °C/min to 100 °C. The glass transition temperature was deduced from the small change in heat flow shown on the graph produced by the TA Universal Analysis software. Three separate samples were run.

RESULTS

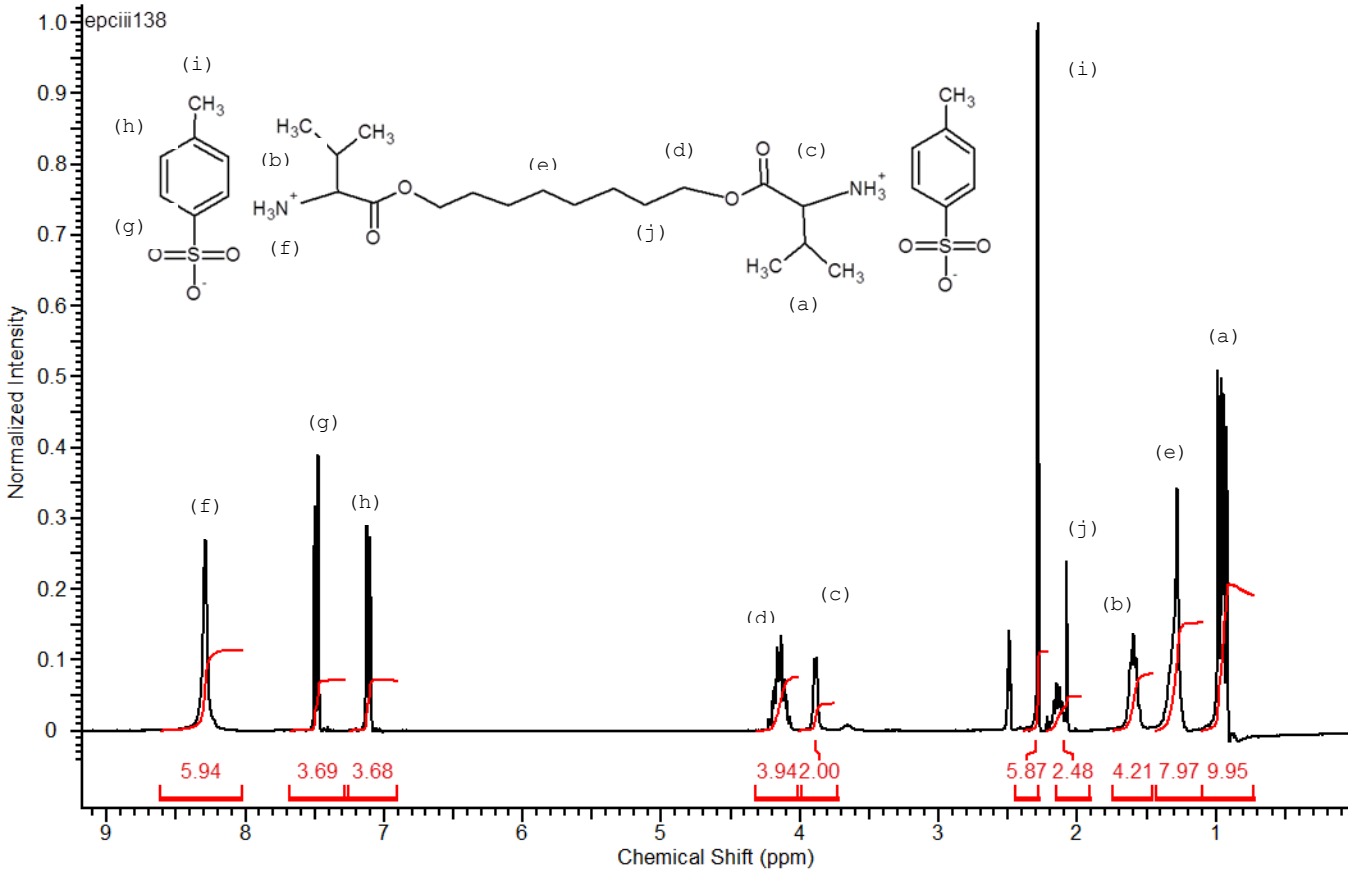

FIGURE 3: *^{1}H NMR Spectra for 1-Val-8M*

Within the ^{1}H NMR of the 1-Val-8M, key peaks appear at δ = 8.39 the six protons attached to the amine groups of each end, δ = 7.48 and δ = 7.08 the eight aromatic protons of the benzene rings, δ = 4.25 and δ = 3.88 the six protons from the two carbons surrounding the ester groups, δ = 2.50 the DMSO solvent peak, δ = 2.37 the protons of the methyl substituents of the benzene rings, δ = 2.12 the proton corresponding to (j) in ***FIGURE 3,*** δ = 1.60 the protons of the apex carbons in the valine side chains, δ = 1.29 the protons of the of the central carbons of the main carbon chain, δ = 0.93 the protons of each arm of the valine side chains.

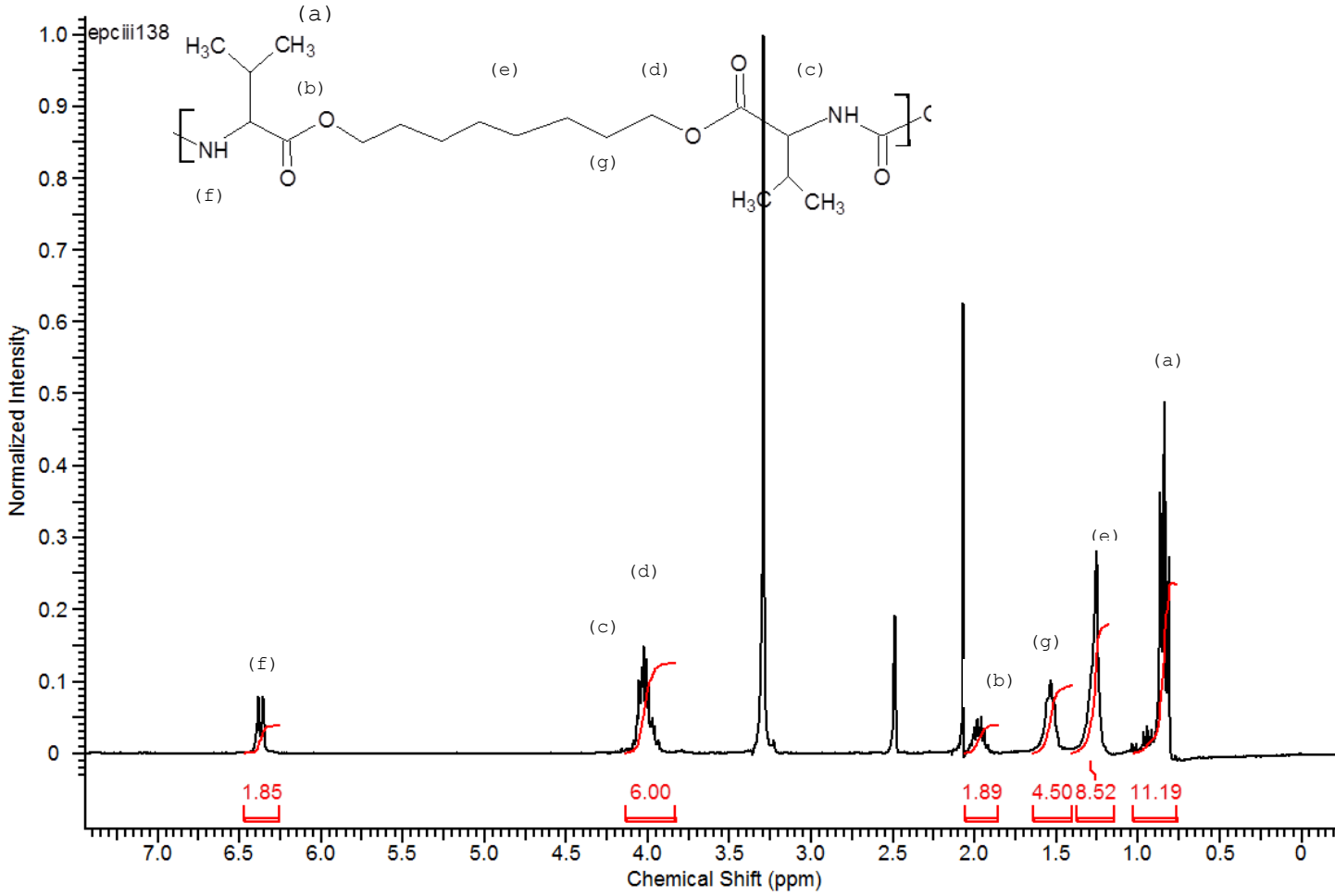

FIGURE 4: *^{1}H NMR Spectra for 1-Val-8P*

Within the ^{1}H NMR of the 1-Val-8P, key peaks appear at δ = 6.42 the two protons of the amine groups, δ = 4.15 the six protons from the two carbons surrounding the ester groups, δ = 3.33 a HDO peak from the DMSO solvent, δ = 2.50 the DMSO solvent peak, δ = 2.22 the acetone peak, δ = 1.99 the protons of the apex carbons in the valine side chains, δ = 1.64 the proton corresponding to (g) in ***FIGURE 4,*** δ = 1.29 the protons of the central carbons of the main carbon chain, δ = 0.73 the protons of each arm of the valine side chains.

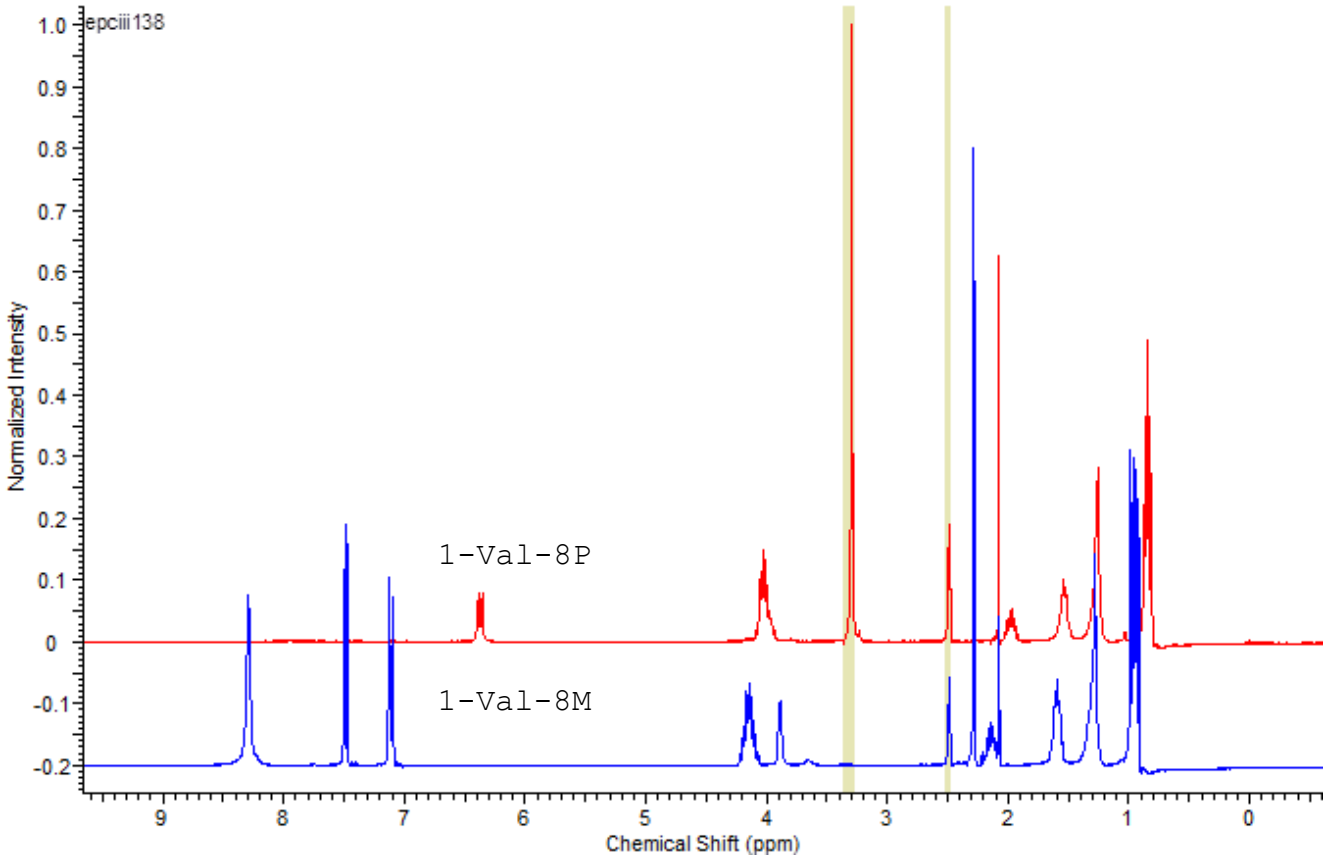

FIGURE 5: *Comparison of 1-Val-8M and 1-Val-8P* ^{1}*H NMR Spectra*

When looking at the comparison between the ^{1}H NMR spectra for 1-Val-8M and 1-Val-8P, like with all monomers and polymers of this study, the disappearance of the p-Toluenesulfonic acid peaks ($\delta = 7.48$ and $\delta = 7.08$) is desired in the polymer products. The loss of the positive charge on the amine group cause a shit and reduction in intensity of the amine peak ($\delta = 8.38$ to $\delta = 6.42$). The loss of splitting on the peak corresponding to the protons surrounding the ester and the addition of the water peak are also observed.

SEC Results

Polymer	Mn (D)	Mw (D)	PDI (Mw/Mn)
1-Ile-6P	41,443	79,755	1.924
1-Ile-8P	29,162	55,203	1.893
1-Val-6P	25,823	51,347	1.988
1-Val-8P	70,182	118,101	1.683

Table 1: *SEC Results for chosen Polymers for next step in the project*

SEC graphical results are shown in **Appendix B,** for the 1-Ile-6P and 1-Val-6P graphs, one of the detectors was broken while the samples were run. The single peak showing a uniform distribution is still visible.

TGA Results

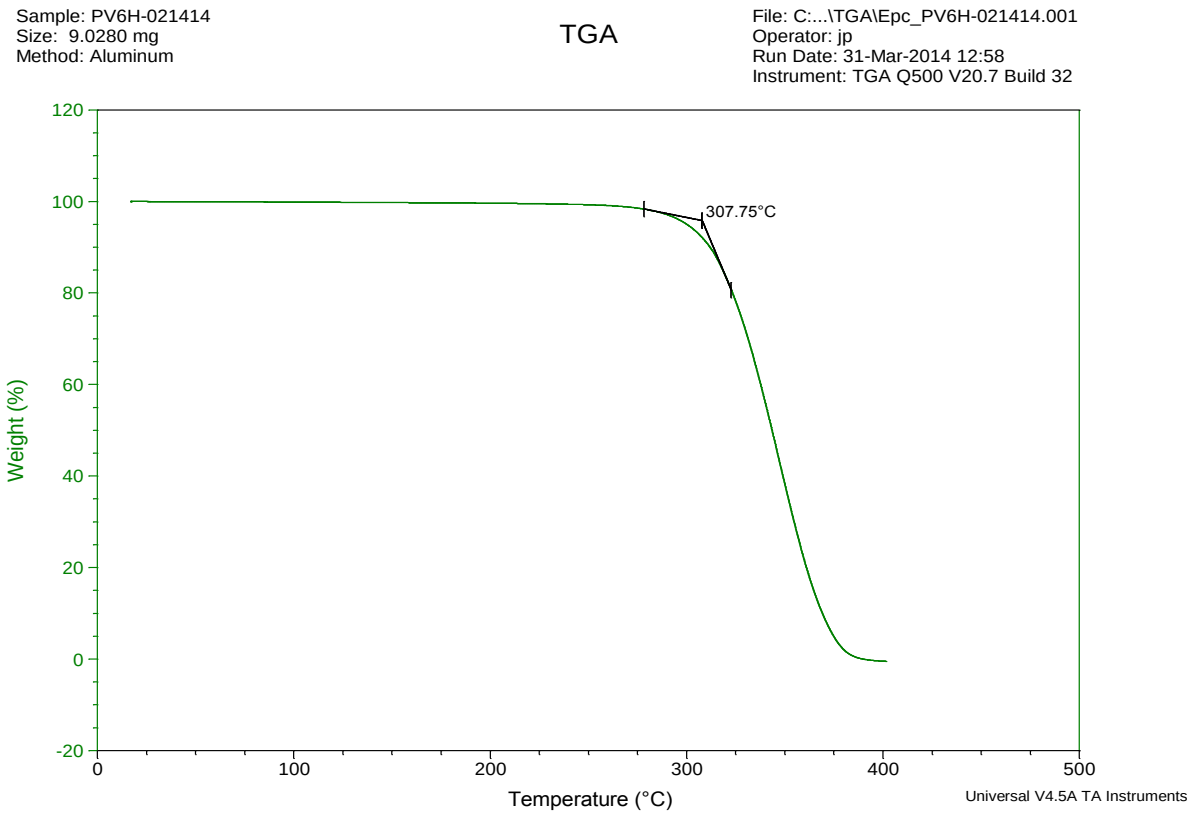

FIGURE 6: *Sample 1 1-Val-6P Degradation Temperature*

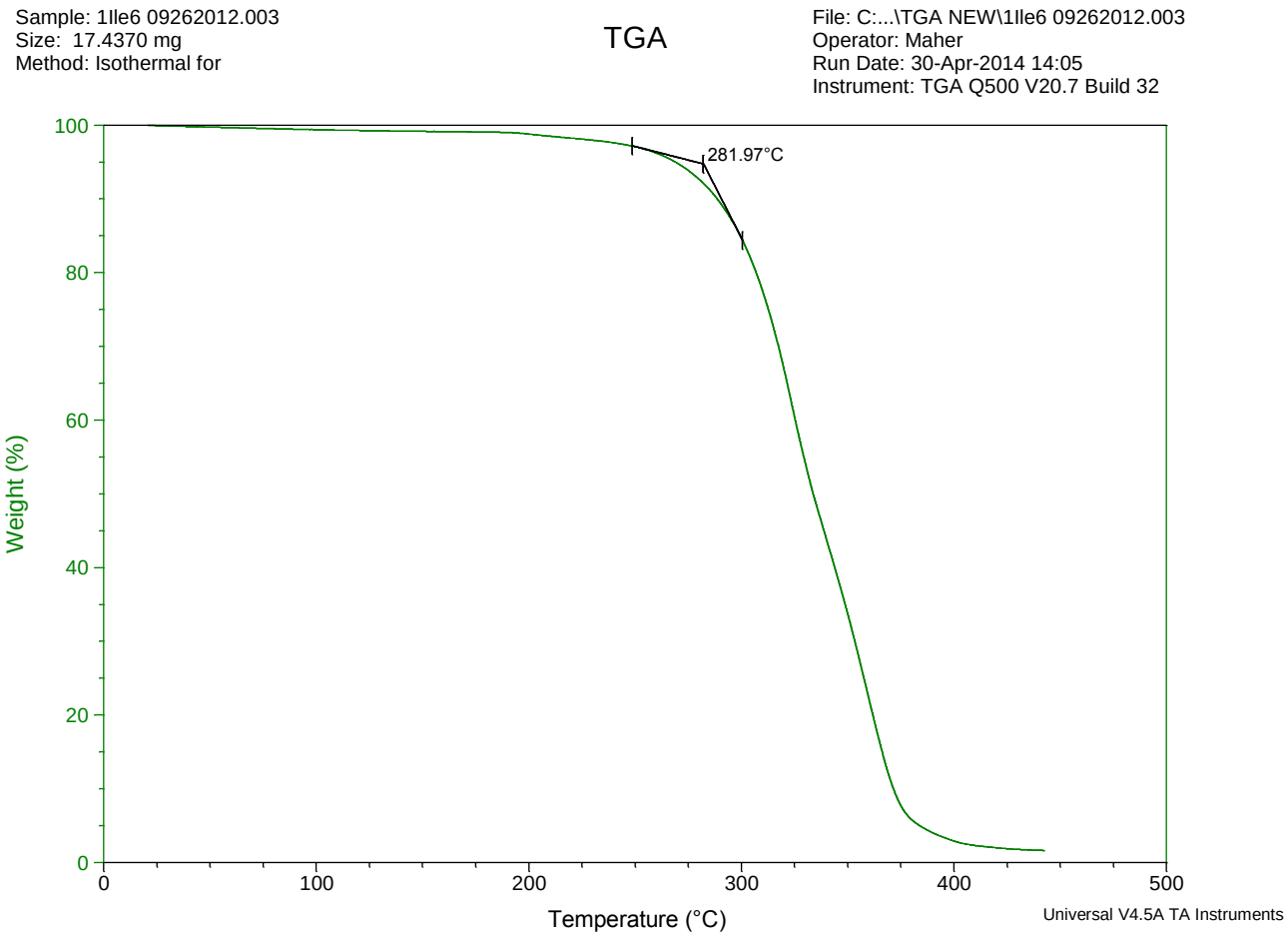

FIGURE 7: *Sample 1 1-Ile-6P Degradation Temperature*

DSC Results

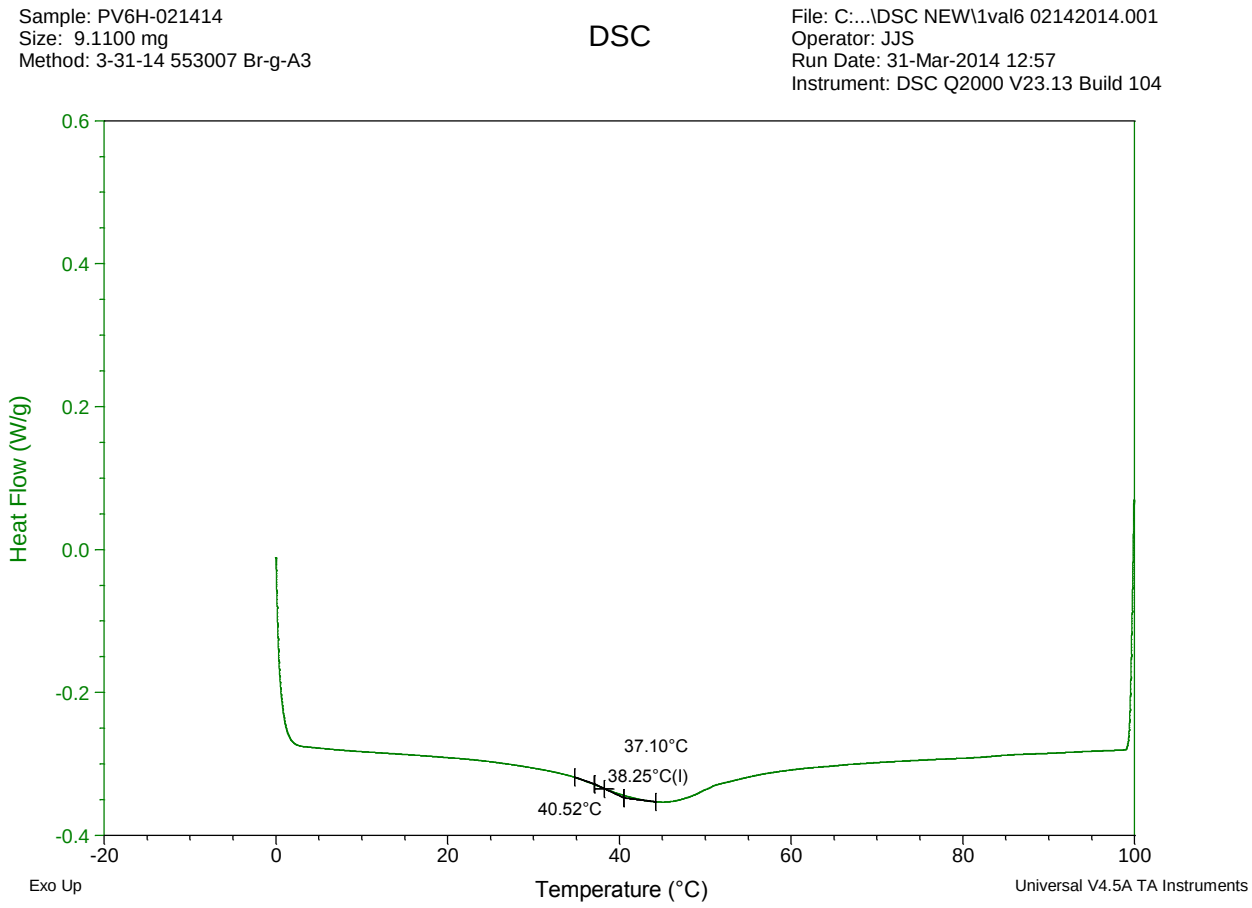

FIGURE 8: *Sample 1 1-Val-6P Glass Transition Temperature*

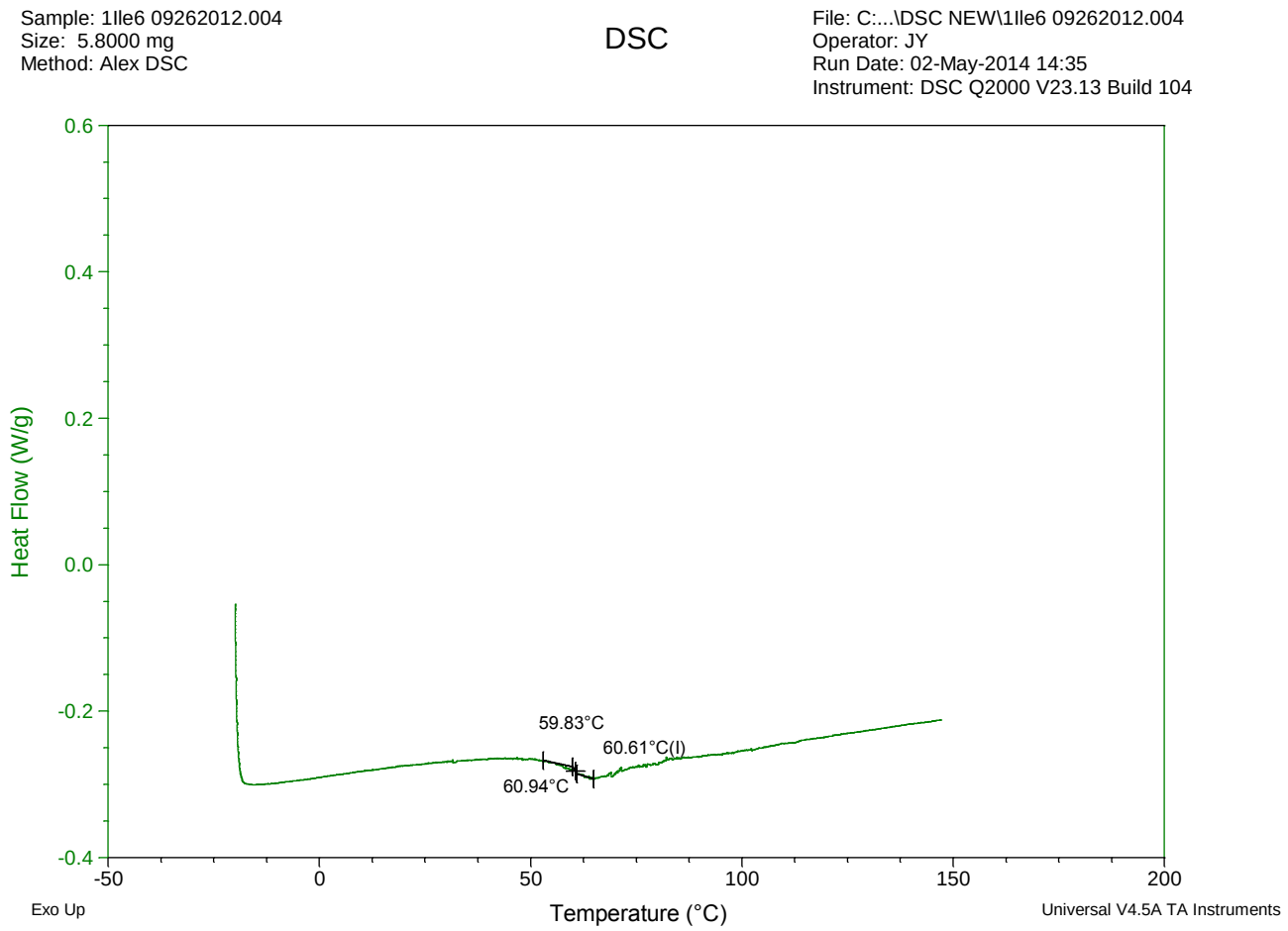

FIGURE 9: *Sample 1 1-Ile-6P Glass Transition Temperature*

The remaining graphs for the first samples degradation and glass transition temperatures of the polymers are shown in the **Table 1** are shown in **Appendix D.**

Polymer	Tg (°C) 1st	Tg (°C) 2nd	Tg (°C) 3rd	Avg Tg	Td (°C) 1st	Td (°C) 2nd	Td (°C) 3rd	Avg Td
1-Ile-6P	62	62	61	62	278	276	280	278
1-Ile-8P	43	44	43	43	295	294	290	293
1-Ile-10P	24	25	25	25	271	269	275	272
1-Ile-12P	16	15	15	15	217	208	212	212
1-Val-6P	62	63	62	62	307	302	279	296
1-Val-8P	48	41	47	45	314	310	312	312
1-Val-10P	41	41	42	41	299	297	295	297
1-Val-12P	31	30	31	31	180	178	184	181

TABLE 2: *Summary of TGA and DSC characterization results*

The graphs produced by the TA Universal Analysis software for sample 1 for each polymer are provided above. Only one graph was shown for each polymer for the degradation and glass transition temperatures to avoid redundancy. All three samples results for each polymer are shown above in ***TABLE 2***. The results for each sample were averaged for a tangible value.

DISCUSSION

NMR and SEC were done on the samples chosen to move forward with in the project. As shown in **FIGURE 3-5** and **APPENDIX C,** the samples run through NMR were shown to have the representative peaks that were desired for each polymer sample. Each peak was labeled, along with a molecular representation corresponding to each peak. The SEC results are shown in **TABLE 1** and **APPENDIX B,** the main focus of these results is to observe a singular approximately uniform peak. This ensures that you have only polymer in your sample with no residual, unreacted monomer. The values for Mn, Mw, and PDI are also given by the SEC software. The most relevant data for our project is the Mw. Our desired Mw for each polymer sample is approximately 50 Kilo Daltons (KD) or above. 50 KD is the assumed entanglement point for our polymers. If polymers are not above their entanglement point, their properties are inconsistent. This would cause a large amount of problem when these polymers are processed in industry or other for use in other applications. Using the Mn and Mw, (software calculations are

shown in **APPENDIX E)** the PDI for each polymer sample was deduced. Our polymers, as seen in **TABLE 1,** have a PDI of around 1.5-2. The PDI gives an idea about the homogeneity of a polymer. Synthetic polymers tend to have a PDI above one, while natural polymers have a value closer to 1. The reason our polymers have a value closer to 2 is the method of polymerization. When using interfacial polymerization, it is very difficult to impossible to ensure that all of your polymer chains grow at the same rate because the random movement of the solution as triphosgene is added. Therefore a PDI of 2 with the use of Interfacial polymerization is acceptable. In respect to our project this value has little relevance compared to the Mw. Once the purity of each sample was ensured by the use NMR and GPC, the values shown in ***TABLE 2*** were found using the TA Universal Analysis apparatuses and software. The goal of this step in the project was to find a polymer that had physical properties that would hold up in various processing techniques such as; spin-coating, salt-leeching, and 3D-printing as well as when used in applications within the human body. Since the average temperature of the human body is approximately 37 °C, any polymer that would leave its native state below this temperature would not be applicable to our project. Observing the degradation temperatures found using TGA, all samples had degradation temperatures above 180 °C. Since this value is far above the needed temperature for processing, the worry of any of the polymers degrading is of no concern**.** Furthermore, when observing the values obtained from the DSC, which gives values for glass transition temperature, there are a few concerns that arise. For each amino acid based polymer containing chain lengths above 8 carbons, the values for the glass transition temperature is either below or very close to the human body temperature of 37 °C. If these polymers were to be applied in the human body they could structurally change and lose their intended functionality.

CONCLUSION

Results obtained from this study indicate the removal of the 1-Ile-10P, 1-Ile-12P, 1-Val-10P, and 1-Val12P from further use in this project. On the other hand, the 1-Ile-6, 1-Ile-8, 1-Val-6, and 1-Val-8 contain the desired properties to move forward in this project. The next step will be the use of these polymers in spin coating for Quartz Crystal Microbalance (QCM) use and mechanical testing and characterization. Hopefully one of these polymers will give the appropriate physical and mechanical properties to move forward with the PRP and PRP growth Factor retention studies. Ultimately, the goal is to design a PEU sponge combined with PRP that could help enhance the tendon-bone healing time and reduce the rate of re-tear after rotator cuff surgeries.

ACKNOWLEDGEMENTS

I want to start off by thanking the Goodyear Polymer Center for giving us undergraduate students the opportunity to assist in the research goals of their faculty and to gain vital experience that will help us in our future endeavors. I want to thank my Honors Project Sponsor, Dr. Matthew Becker for giving me the opportunity to join his research group. He has been nothing but supportive of me and all of the fellow researchers in our group. Also the guidance he has given me with my hopeful future as an orthopedic surgeon has been more than I could have ever asked for. I would also like to thanks PhD student Erin Childers. I have been assisting Erin with her research for the past two years and have learned so much from her. As with all members of Dr. Becker's research group she has been nothing but supportive of me and has helped me with any problems I have had. I am forever thankful for her support. Lastly, I want to thank my Honors Faculty Advisor, Dr. Claire Tessier as well as my two project readers, Dr. Brian Bagatto and Dr. Thomas Leeper for their time and contribution to this paper.

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APPENDICES

Appendix A:

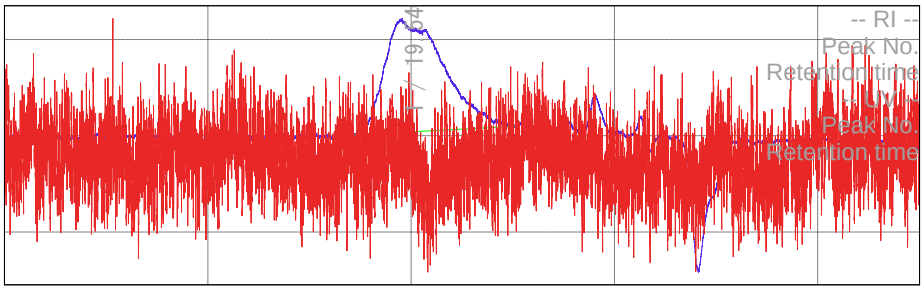
Safety Considerations

Chemical safety is of the upmost importance when synthesizing the monomer and polymer products in this experiment. Triphosgene, p-Toluenesulfonic acid, Toluene, and Chloroform were all used in either the monomer or polymer synthesis. Skin contact, eye contact, ingestion, and inhalation all needed to be avoided with these compounds. If the substances were exposed to any mucous openings, the area needed to be flushed with water for 15 min. Medical attention needed to be sought if irritation occurred. Special attention was maintained during the use of triphosgene because of its extremely high toxicity if inhaled or ingested and its reactivity with water. Phosgene gas is produced on the addition of triphosgene with water and is highly toxic and could result in death with inhalation. Safety glasses and gloves were required at all times when in the laboratory. Shorts and open-toed shoes were prohibited, and hair and loose clothing needed to be secured. Once chemicals were used, they needed to be stored by compatibility. Disposable of chemicals needed to be in the proper container: non-halogenous vs halogenous waste containers. If a spill occurred, it needed to be absorbed with an inert dry material and placed in the appropriate waste disposal container. Awareness is vital for proper safety around toxic chemicals.

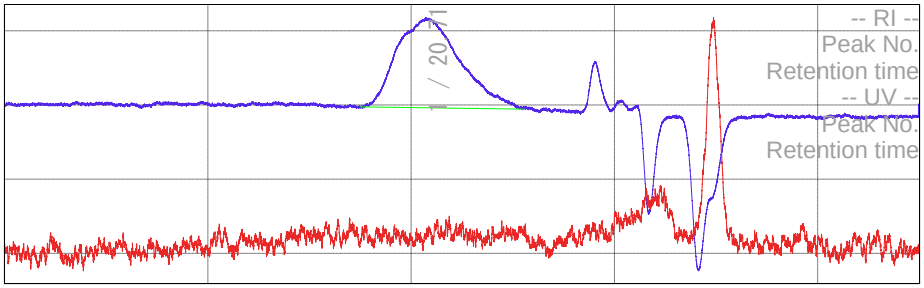
Appendix B:

SEC Results

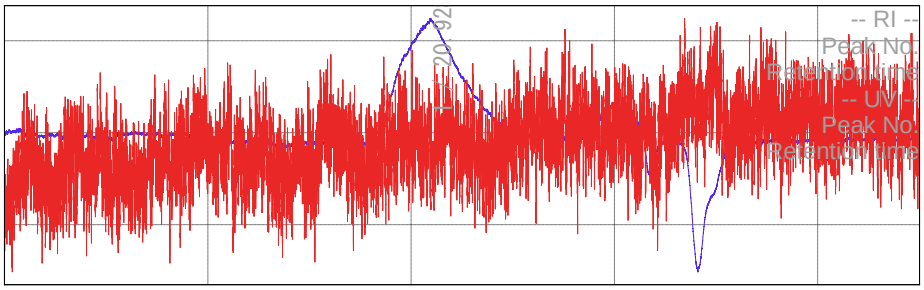
As mentioned previously under **TABLE 1,** for the 1-Ile-6P and 1-Val-6P graphs, one of the detectors were broken (unrelated to our desired results) while the samples were run. The single peak showing a uniform distribution is still visible at 20 min.



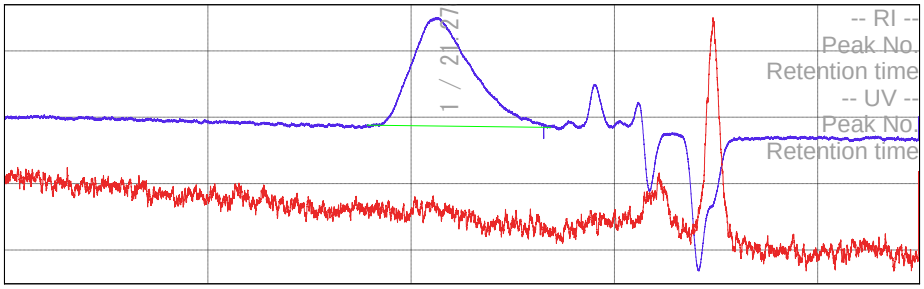
SEC. 1-Ile-6P



SEC. 1-Ile-8P



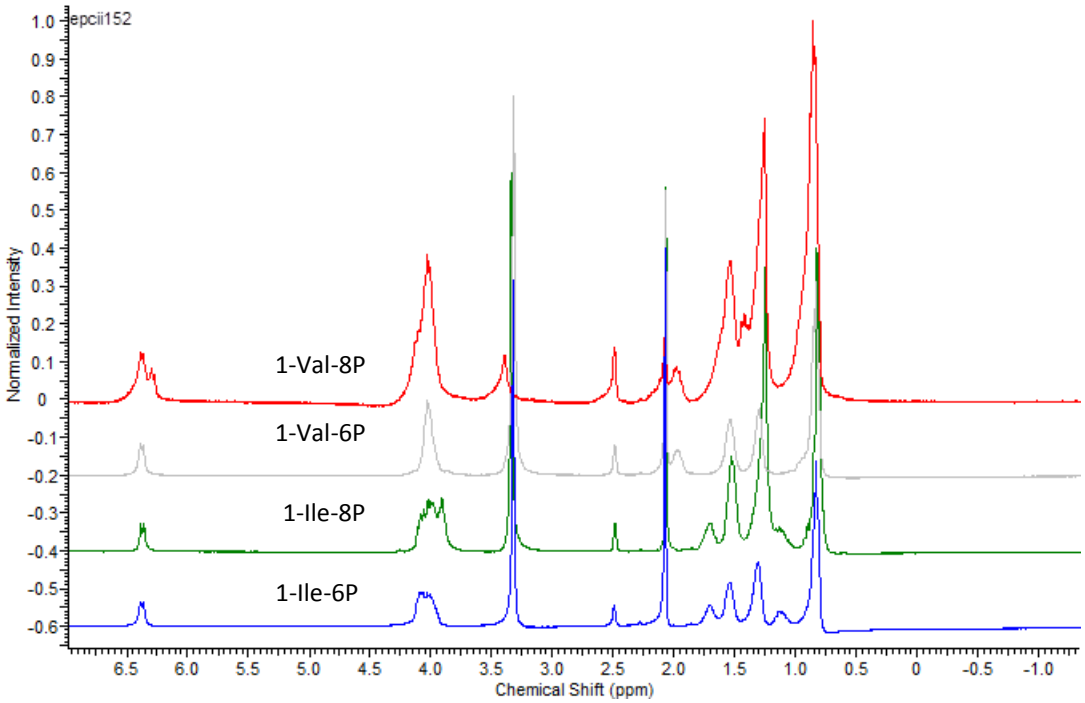
SEC. 1-Val-6P



SEC. 1-Val-8P

Appendix C:

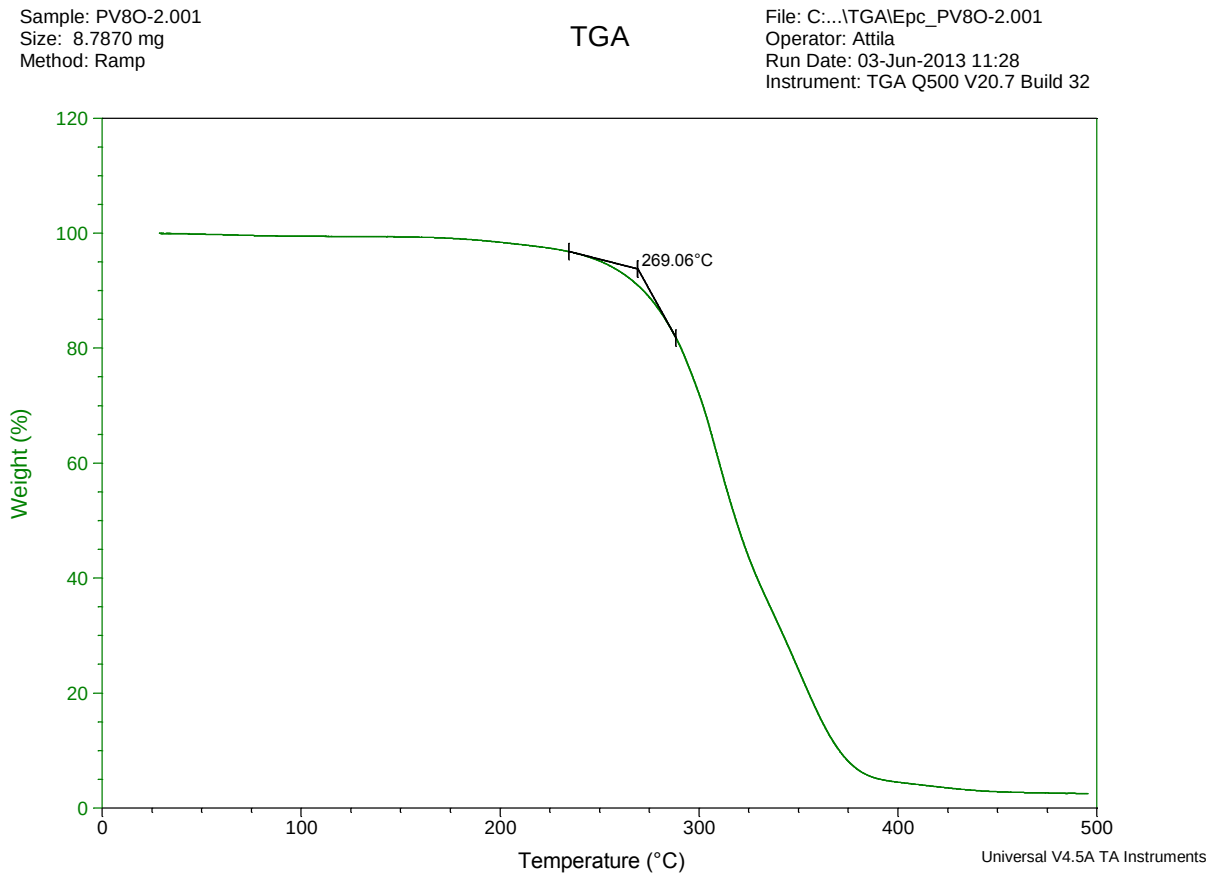
Polymer NMR Results



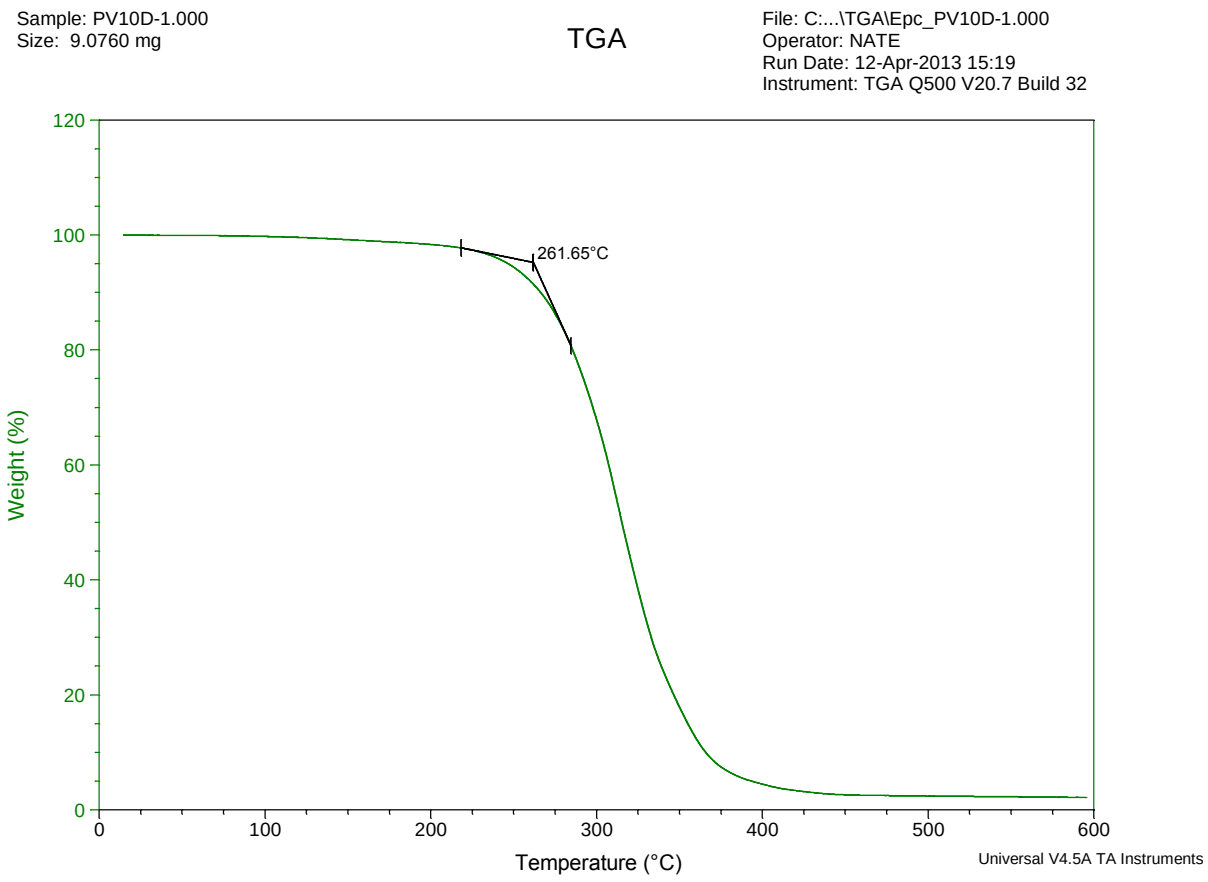
Comparison of ^{1}H NMR Spectra for chosen polymer products

Appendix D:

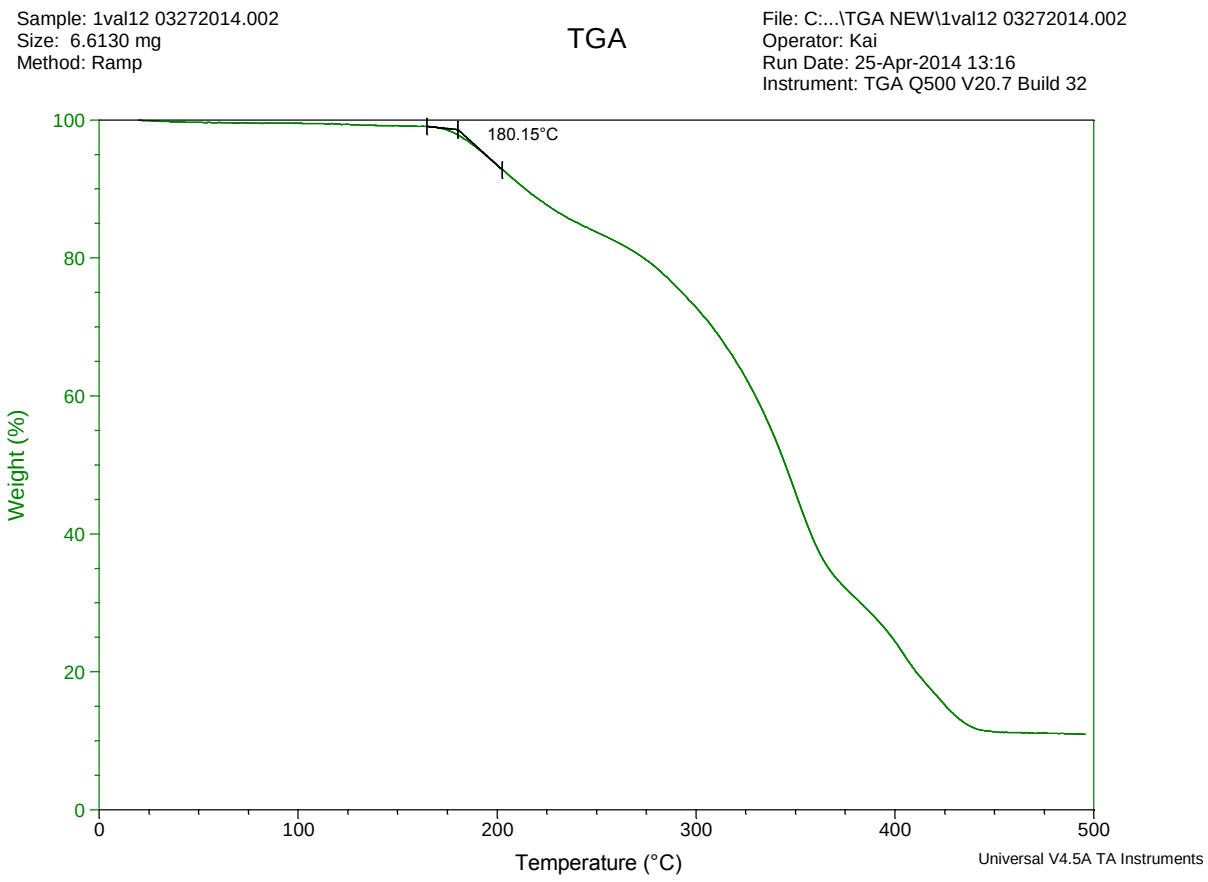
TGA Results



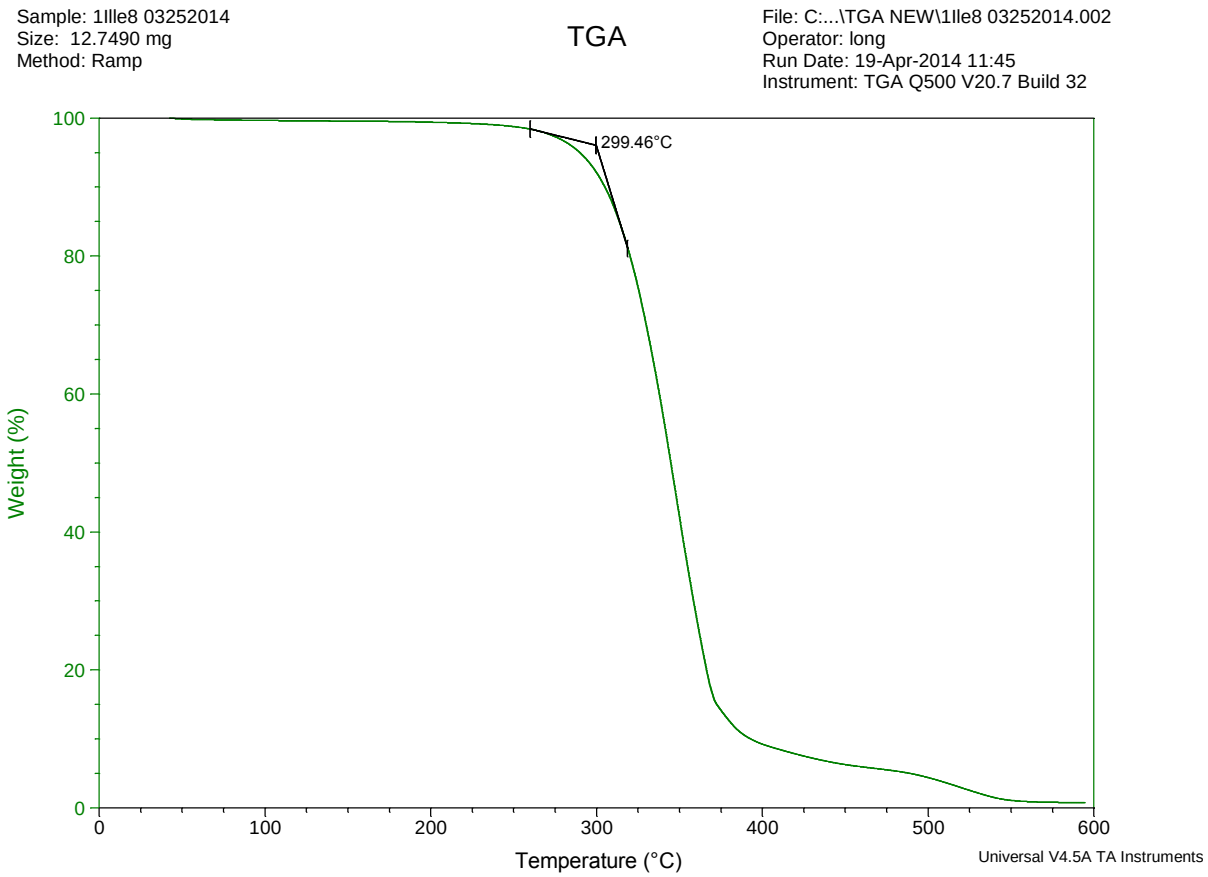
Sample 1 1-Val-8P Degradation Temperature



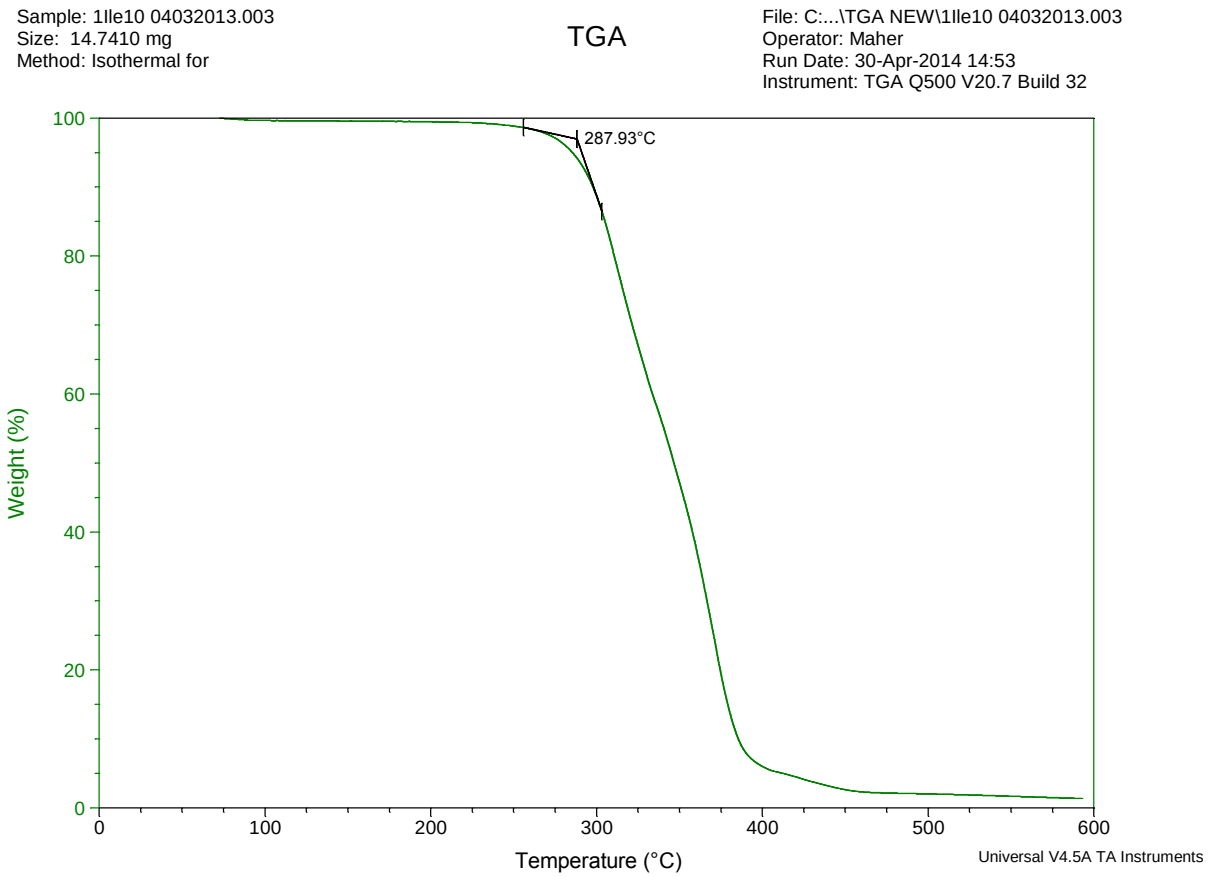
Sample 1 1-Val-10P Degradation Temperature



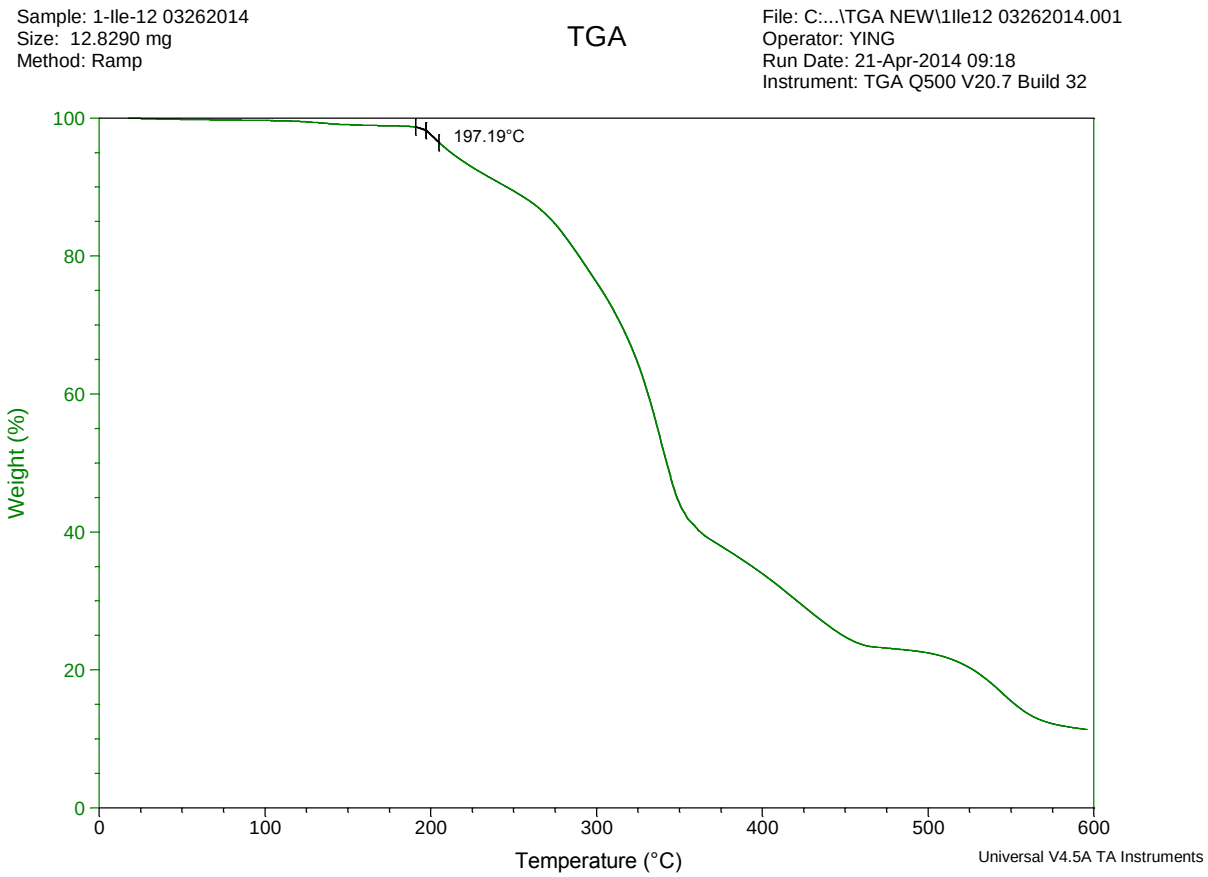
Sample 1 1-Val-12P Degradation Temperature



Sample 1 1-Ile-8P Degradation Temperature

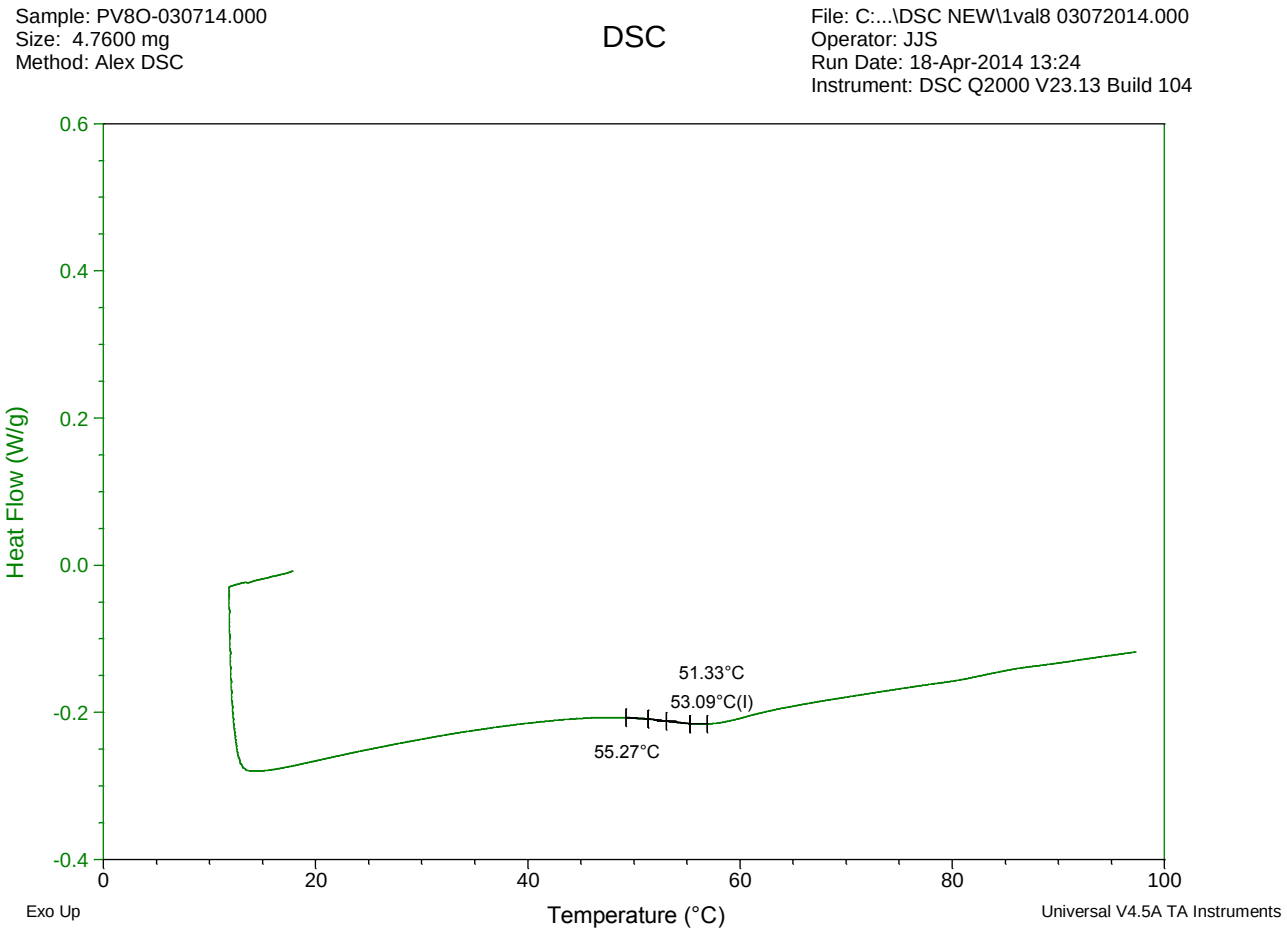


Sample 1 1-Ile-10P Degradation Temperature

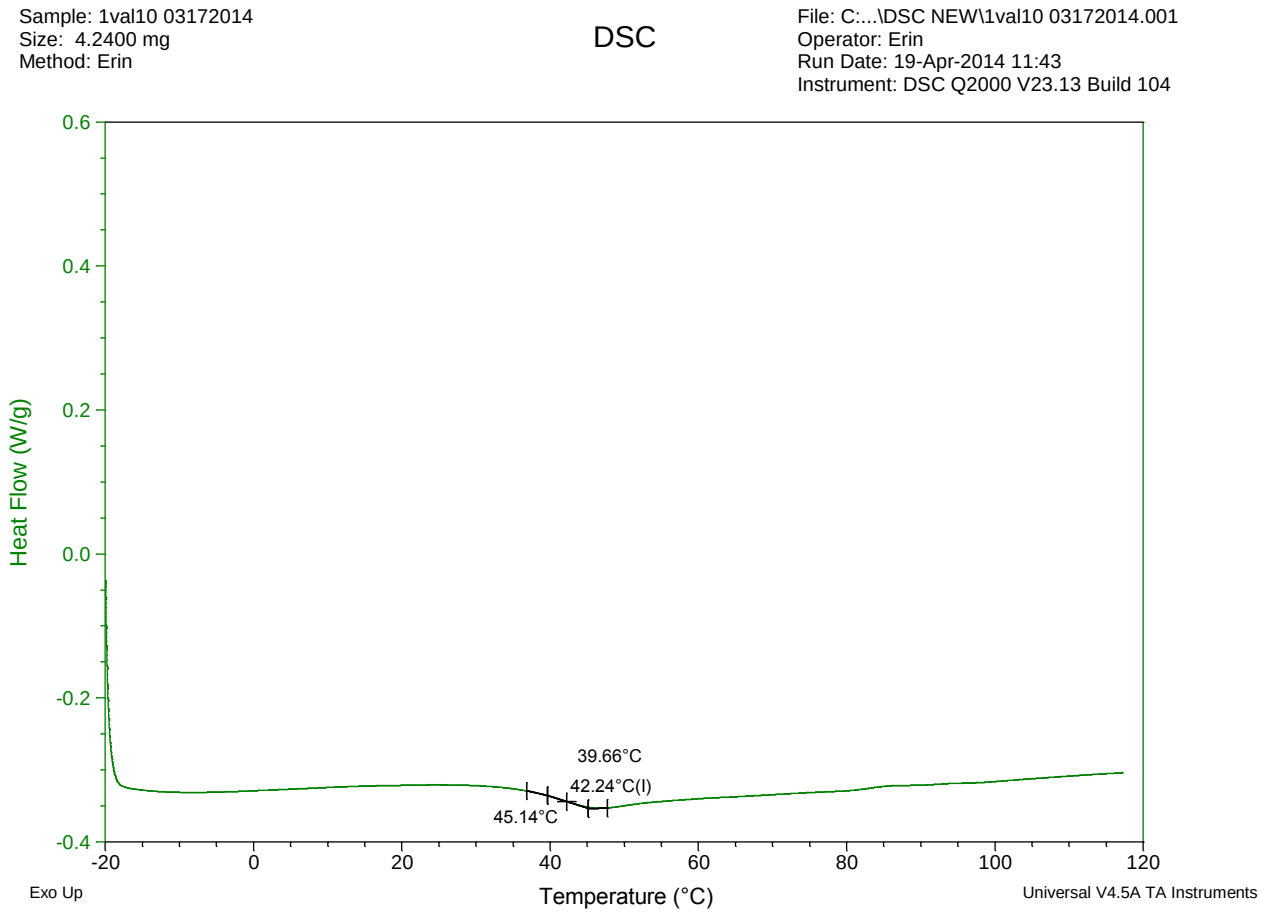


Sample 1 1-Ile-12P Degradation Temperature

DSC Results



Sample 1 1-Val-8P Glass Transition Temperature



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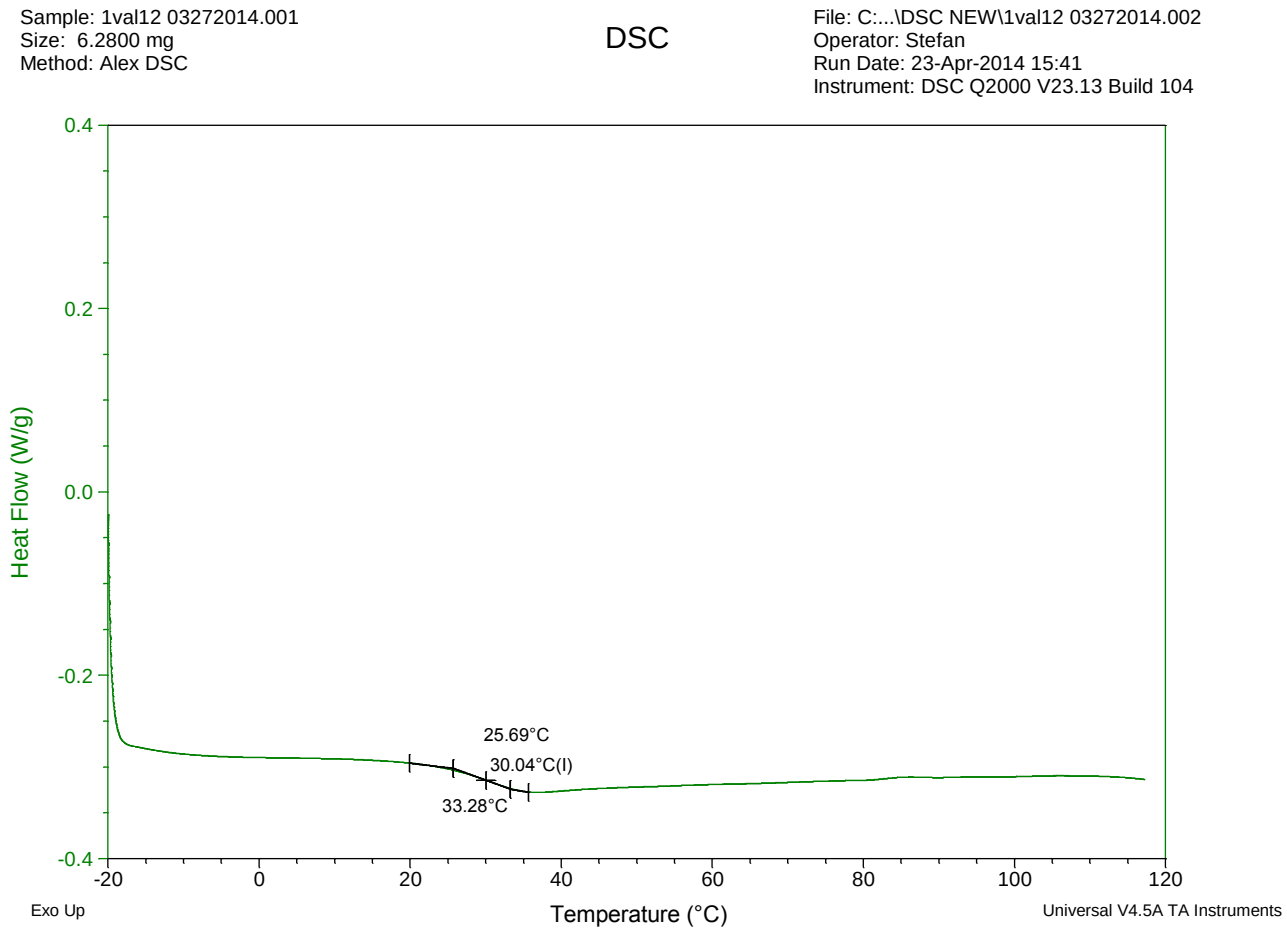

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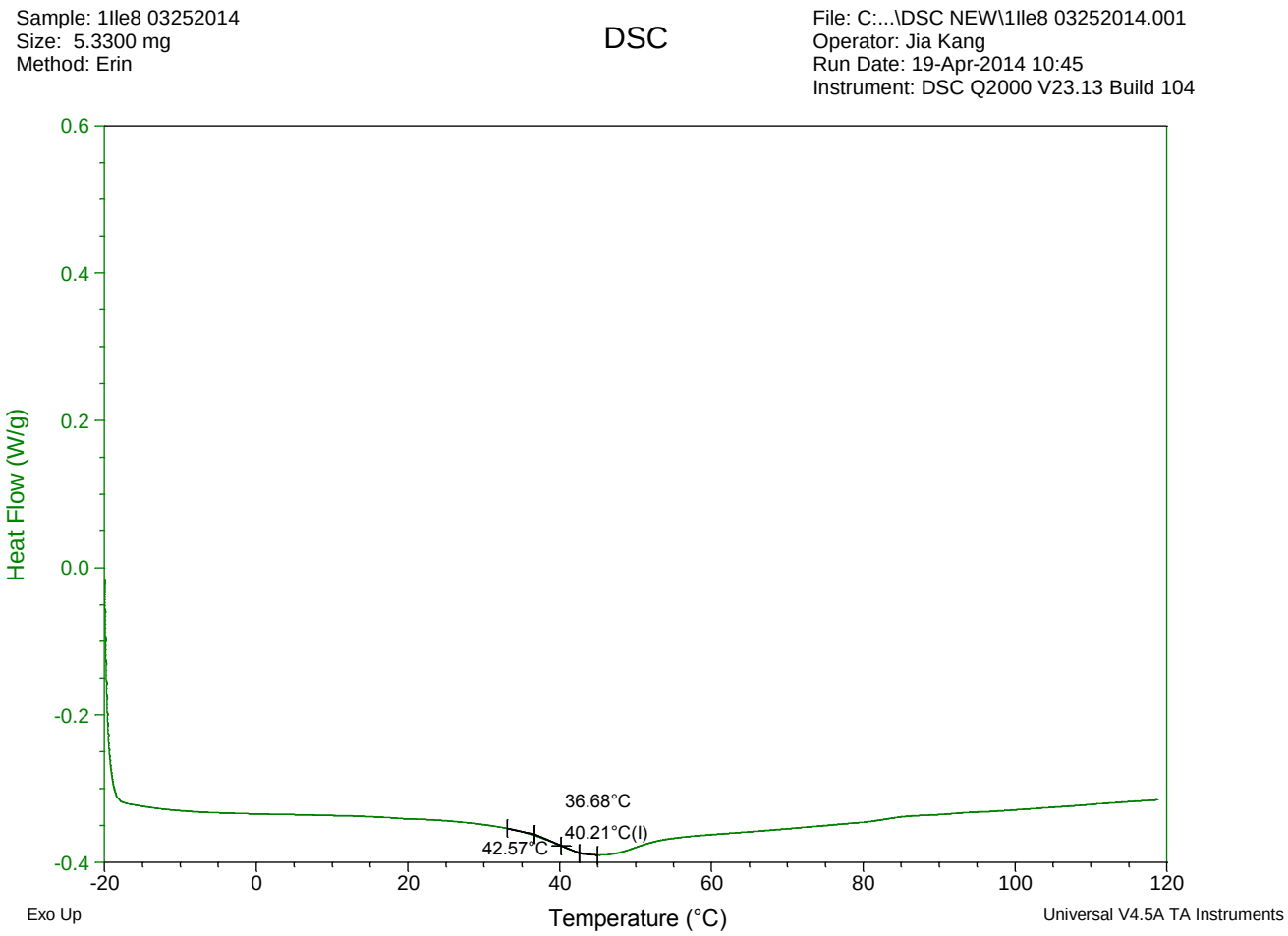
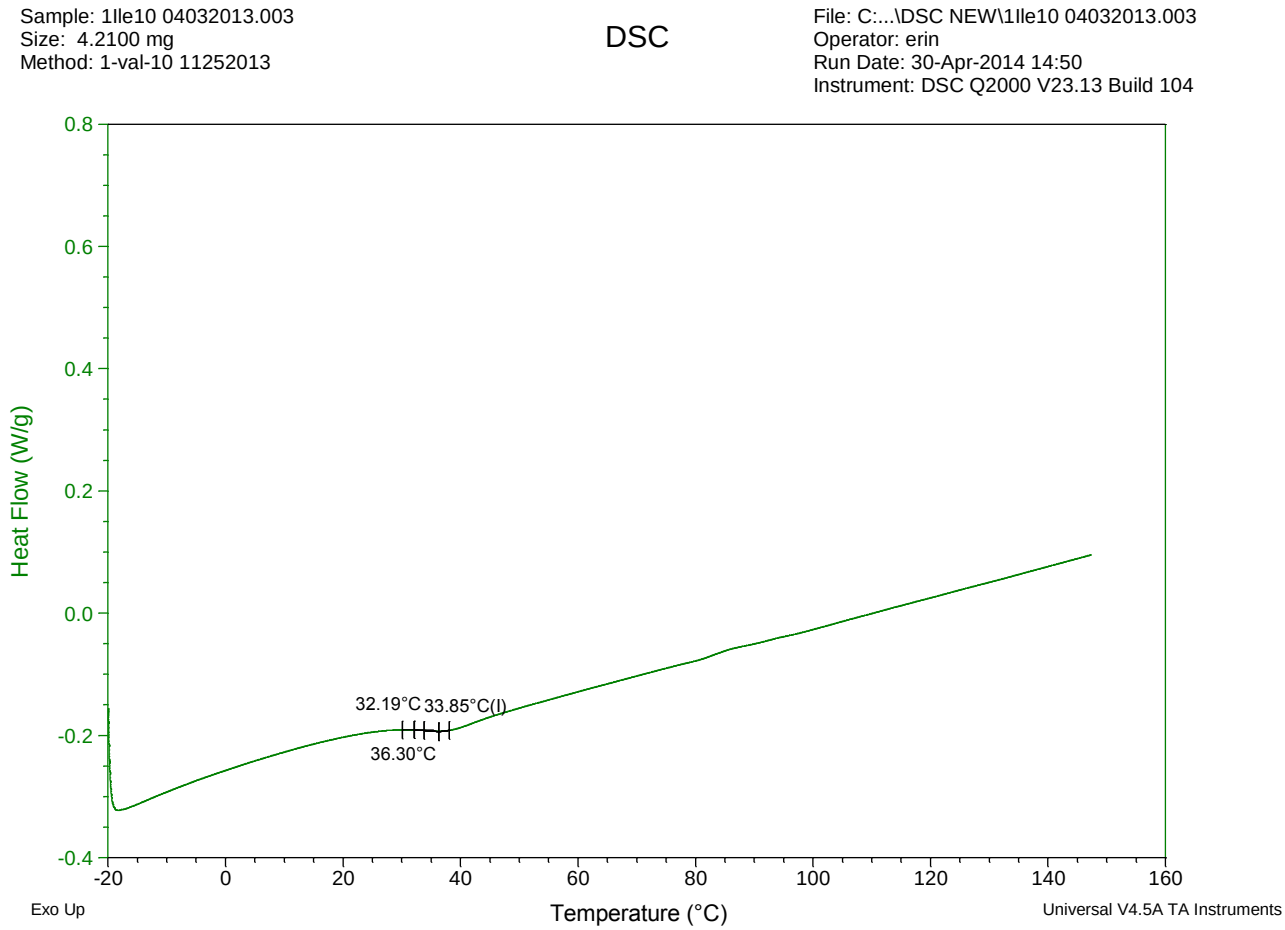
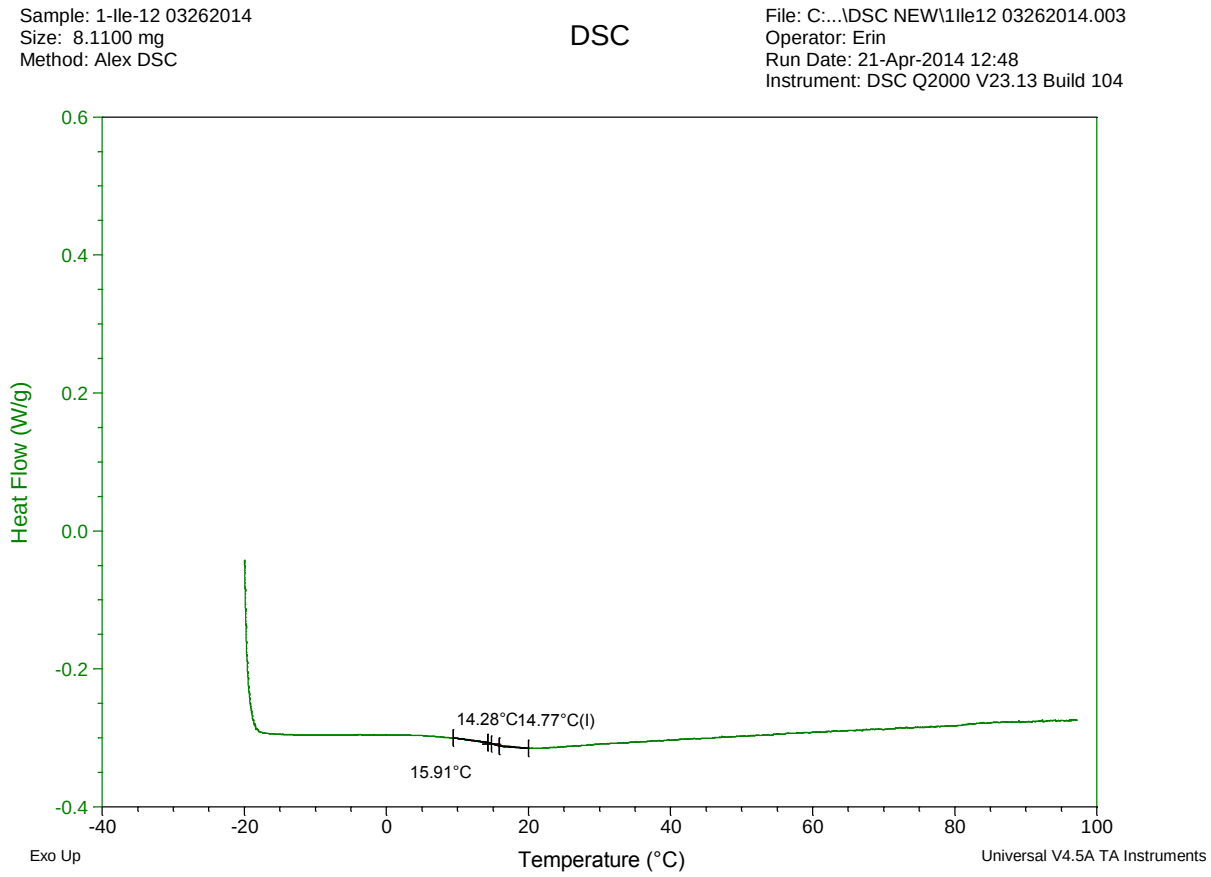


Sample 1 1-Ile-8P Glass Transition Temperature



Sample 1 1-Ile-10P Glass Transition Temperature



Sample 1 1-Ile-12P Glass Transition Temperature

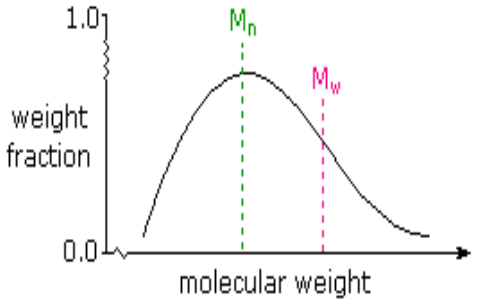
Appendix E:

SEC Software PDI Calculation

$M_n = \sum n_i M_i$ n_i = mole fraction of chains with molecular weight M_i

$M_w = \sum w_i M_i$ w_i = weight fraction of chains with molecular weight M_i

note $w_i = \frac{n_i M_i}{M_n}$



M_n and M_w in a typical sample of polydispersed macromolecules