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Synthesis of 10, 20, and 30-Length Hex-poly{benzyl-L-glutamic acid)

Marisa Emily Thompson

Synthesis of 10, 20, and 30-Length Hex-poly(benzyl-L-glutamic acid)

By

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Date

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Marisa Thompson
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May 5, 2020
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Abstract

Huntington's disease is a fatal genetic disease that causes deterioration of nerve cells in the brain over the course of 10 to 30 years. Huntington's disease and some other neurodegenerative diseases are caused by a mutation that also causes long repeating chains of the amino acid poly(glutamine). The role that these chains play in the experience and progression of Huntington's disease is not well known, thus research into the behavior of poly(glutamine) is needed to better understand the disease on a molecular level. Hex-poly(benzyl-L-glutamic acid), the precursor to poly(glutamine) capped with hexylamine, with chain lengths of 10, 20, and 30 residues were made by synthesizing and polymerizing benzyl-protected glutamic acid N-carboxyanhydride. The polymers with chain lengths of 10 and 20 residues were analyzed using 1D ^{1}H NMR spectroscopy and the resulting spectra confirmed the identities of the polymers as hex-poly(benzyl-L-glutamic acid).

Future work will include the synthesis of 40-length polymers and the conversion of all hex-poly(benzyl-L-glutamic acid) to hex-poly(glutamine). Once enough of the polymer has been synthesized, research will be performed into the interactions between poly(glutamine) and cells.

Introduction

Poly(glutamine) chains that are 35 to 40 residues long are present in individuals afflicted with Huntington's disease and other poly(glutamine) expansion diseases. These neurodegenerative diseases generally begin in adulthood, progress over the course of 10 to 30 years, and are all currently fatal.[1] The time of disease onset depends on the length of the poly(glutamine) chains present, with longer chains leading to an early onset of disease.[2] Research on poly(glutamine) is needed to identify the structural changes that occur in poly(glutamine) during aggregation; whether these aggregates play a role in the development of the disease or whether they are a harmless side-effect or even a defense mechanism; and, if Huntington's and other poly(glutamine) diseases are caused by these aggregates, what reactive pathways lead from poly(glutamine) chains of these lengths to neural dysfunction.[1,3] With research into these aspects of Huntington's and other poly(glutamine) diseases, it may be possible to find a method of slowing or curing these ailments. However, in order to perform such research, poly(glutamine) chains of the proper lengths are needed.

Synthesis of poly(amino acid)s with a high molecular weight, such as poly(glutamine) chains with the desired lengths, was at one point a difficult process. However, conversion of amino acids to amino acid N-carboxyanhydrides followed by the polymerization of this NCA has proven a reliable, controllable method of synthesizing poly(amino acid)s with high molecular weights and a high product yield.[4,5]

The synthesis of hex-poly(glutamine), poly(glutamine) that is capped by the initiator hexylamine, is performed by first synthesizing benzyl-protected glutamic NCA and then performing a ring-opening polymerization reaction to form hex-poly(benzyl-L-glutamic acid). It

is necessary to use benzyl-protected glutamic acid to ensure that the carboxylic acid on the side chain does not react in the synthesis of NCA.

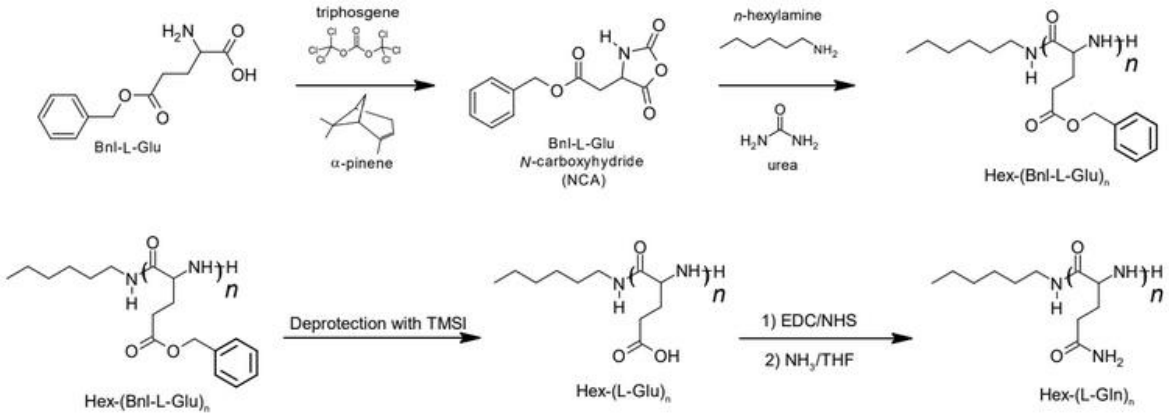

Figure 1: Reaction scheme for the synthesis of hex-poly(glutamine).[6]

Following polymerization, a deprotection reaction yields poly(L-glutamic acid), and esterification and reaction with ammonia converts the poly(L-glutamic acid) into poly(glutamine). The reaction scheme is shown in figure 1. Modification of the amount of hexylamine used in step two of the reaction process allows for control of the length and molecular weight of the synthesized polymer.

Materials and Methods

The synthesis of benzyl protected poly(glutamic acid) was performed by synthesizing NCA, purifying the NCA twice, performing the polymerization reaction to form benzyl protected poly(glutamic acid), purifying the polymerized benzyl protected poly(glutamic acid), and lyophilizing the protected polymer. In order to confirm the identity of the synthesized polymer, samples were prepared for NMR analysis. To convert the Hex-poly(benzyl-L-glutamic acid) to useable hex-poly(glutamine), a deprotection reaction would be performed.

Synthesis of NCA

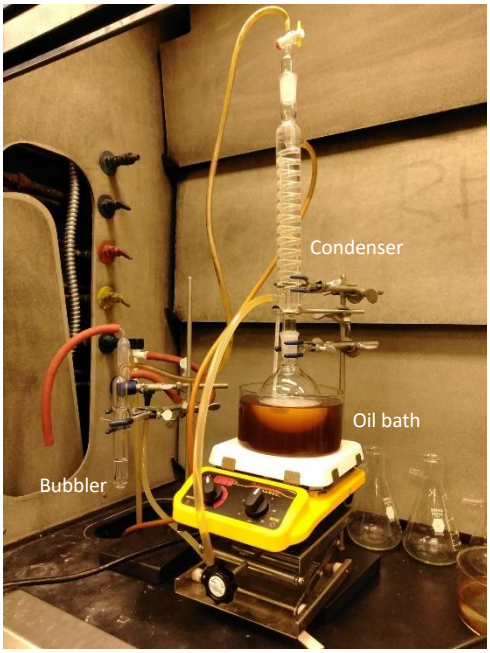

Figure 1: Apparatus for the synthesis of NCA.[7]

The synthesis apparatus was set up as shown in figure 2. The oil bath was allowed to heat up to 110 °C and that temperature was held constant throughout the synthesis of the NCA. A small amount of sodium hydroxide (KOH) was placed in a 50 mL beaker and dissolved in water. This solution was poured into the bubbler, taking care not to add more KOH solution than the volume of the top section of the bubbler. This KOH solution neutralizes any acid that is formed in the synthesis and might escape through the top of the condenser. Gloves were changed after the chance of coming into contact with water. The synthesis of NCA is very sensitive to water and all procedures must be performed anhydrously (without contact with water). Because water can cause NCA to act as a nucleophile, accidental exposure of the reaction or NCA to water before polymerization could trigger premature, uncontrolled polymerization of the NCA.[Habraken]

Two grams of a benzyl glutamate (bnz-glu) were added into a 250 mL round-bottom flask. Alpha (α)-pinene in a ratio of 6.1 times the number of moles of bzn-glu, or 6.891 g of α-pinene, was measured into a glass vial. The α-pinene acts as a scavenger for the hydrochloric acid that is created in the synthesis of NCA, ensuring the acid does not cause any unwanted reactions.[Habraken] This α-pinene was added to the round-bottom flask with the bnz-glu. Triphosgene in a ratio of 2.1 times the number of moles of bnz-glu, or 5.25 g of triphosgene, was measured into a small beaker.* Under a fume hood, 30 mL of ethyl acetate (EA) was added to the beaker containing the triphosgene, and this was stirred until the triphosgene was completely dissolved.

For the synthesis of the 10-length hex-poly(glutamic acid), 2.0102 g of bnz-glu, 6.8981 g of α-pinene, and 5.4072 g of triphosgene were used; for the synthesis of the 20-length hex-poly(glutamic acid), 2.0086 g of bzn-glu, 6.9033 g of α-pinene, and 5.35 g of triphosgene were used; and for the synthesis of the 30-length hex-poly(glutamic acid), 2.0085 g of bzn-glu, 7.0035 g of α-pinene, and 5.4340 g of triphosgene were used.

One hundred milliliters of EA was added to the round-bottom flask containing the bnz-glu and α-pinene. The dissolved triphosgene solution was then added to this flask as well, and the beaker was rinsed three times with a total of approximately 70 mL of EA.

The round-bottom flask containing the mixture was connected to the condenser apparatus and the oil bath was raised to submerge the portion of the flask that contained reactants. The reaction was allowed to proceed with stirring for two to four hours or until there were no white

*Caution! Triphosgene is a highly caustic substance that can cause serious damage to the eyes and respiratory system. Use personal protective equipment when handling this substance.

particles visible in the solution. It was possible to determine that the reaction was proceeding based upon the gas being released from the reaction flask and escaping as bubbles through the KOH solution in the bubbler.

All glassware that touched triphosgene was rinsed with acetone in the fume hood before washing with soap and water in the sink.

First Purification of NCA

After allowing the bnz-glu/triphosgene solution to react for two to four hours, the solution was allowed to cool and then filtered through a glass fritted filter into another round-bottom flask using vacuum filtration. EA was used to rinse the flask and filter. Using a rotovap that was set at 40 °C, the EA was evaporated off of the filtered solution until only a small amount of cloudy liquid remained. The flask was removed from the rotovap and to it was added approximately 25 to 30 mL of EA and this was stirred until the solution was clear. While stirring continuously, hexane was added by parts until the solution became cloudy and crystals formed.

After crystal formation, the flask was sealed with a rubber septum and placed in the freezer overnight or until the second purification could be performed. If the second purification was not to be performed the following day then two or three 20 mL aliquots of hexane were added at intervals (either throughout the next day or across a few days) to encourage continued crystallization. When removed from the freezer, care was taken to wipe any ice crystals off of the outside of the flask and the septum to avoid accidental contamination of the crystals with water.

Second Purification of NCA

The flask was removed from the freezer and the crystals filtered off using a glass frit filter and vacuum filtration and the flask was rinsed with hexane to remove as many crystals as possible. Following filtration, the crystals were scraped off of the filter and into a prepared 250

Figure 2: Sealed Schlenk flask attached to the Schlenk line.[8]

mL Schlenk flask, which was then sealed with a rubber septum and connected to the Schlenk line with a rubber hose as seen in figure 3. All valves between the Schlenk line and the hose and the hose and the flask were closed. The vacuum connected to the Schlenk line was turned on and the valve between the line and the hose to the flask was opened with the blue marking facing downwards to vacuum any air that might contain water or other impurities out of the hose. The valve between the hose and the flask was then very slowly opened to vacuum the air out of the flask without vacuuming any of the crystals out of the flask. The crystals were allowed to dry under vacuum for 15 to 20 minutes.

After allowing the crystals to dry, the valves to the vacuum were closed and the argon gas flow was turned on. The valve between the line and the hose was opened with the blue marking facing upwards to allow the hose to fill with argon. It can be determined that the hose is full when argon bubbles are again exiting the bubbler. The valve between the hose and the flask was then opened to let a small amount of argon into the flask and then quickly shut when the mineral oil in the bubbler began to rise too high. If the mineral oil rises too high up the bubbler then it can enter the hose from the bubbler to the Schlenk line and even the Schlenk line itself, which could potentially contaminate a reaction. The valve between the hose and the flask was opened and closed to allow argon into the flask in small amounts until the flask was full and argon bubbles were again exiting the bubbler. This procedure was repeated each time the flask needed to be filled with argon.

Once the flask was filled with argon, the valves were both closed and the argon flow was turned off so that argon would not be wasted and the Schlenk flask was disconnected from the Schlenk line. When the flask is full of argon the septum can be removed and the argon, which is heavier than air, will remain in the flask and minimize the amount of air that comes into contact with the NCA crystals. While stirring vigorously, just enough EA (approximately 6-8 pipettes) was added to flask to dissolve the NCA crystals. A little less than half as much hexane as ethyl acetate was slowly added to the solution, with care not to add enough hexane that crystals formed. If too much hexane had been added and crystals had begun to form, more EA would be added to dissolve these crystals.

A 1:1 solution of hexane and EA was prepared and the NCA solution in the flask was filtered into another prepared 250 mL Schlenk flask using a glass frit filter and vacuum filtration and rinsed with the 1:1 hexane/EA solution. The new Schlenk flask containing the filtered NCA solution was sealed and placed in an ice bath, taking extreme care not to allow any water into the Schlenk flask, and connected to the Schlenk line. The valve between the vacuum and the hose was opened and the hose was vacuumed out but the flask was not. The argon was then turned on and the hose was filled with argon. The flask was then filled with argon and the septum was removed from the flask.

Hexane was added to the flask by 20 mL aliquots every five minutes until crystals formed. These crystals were filtered off using a glass frit and vacuum filtration and the flask was rinsed with hexane to ensure as many crystals as possible were obtained. After filtering, the crystals were scraped from the filter into a weigh boat and their mass was obtained. They were then placed in another prepared 250 mL Schlenk flask and allowed to dry under vacuum for 10-

15 minutes. Following drying, the hose and flask were filled with argon before closing all valves and turning the argon off and the crystals were stored under argon overnight.

Schlenk flasks were cleaned by allowing them to rest in a large beaker of dichloromethane (DCM) for a few minutes and then washed with soap and water. Filters were cleaned by placing the filters over Erlenmeyer flasks in the sinks, adding a small amount of sodium hydroxide pellets in the filter, followed by a small amount of hydrogen peroxide. This reaction was allowed to proceed until bubbling ceased and the filters were then rinsed with deionized water and vacuumed out.

Polymerization

Urea was measured out and ground with a mortar and pestle before being allowed to dry in a prepared 100 mL Schlenk flask under vacuum for 15 minutes or overnight. Urea is used in the polymerization to ensure uninterrupted polymerization and prevent precipitation of the growing polymer. The amount of urea needed for the polymerization is proportional to the amount of dimethylformamide (DMF) used as a solvent and was measured out according to the calculations shown below.

$$Mass\ of\ Urea = volume\ of\ DMF\ (mL) \times \frac{1\ L}{1000\ mL} \times \frac{0.2\ moles}{1\ L} \times \frac{60\ g}{1\ mole}$$

$$Volume\ of\ DMF\ (mL) = moles\ of\ NCA\ synthesized \times \frac{1\ L}{0.1\ moles} \times \frac{1000\ mL}{1\ L}$$

After drying the urea, the flask was filled with argon, the needed amount of DMF was measured and added to the flask along with a stir bar, the flask was resealed, and the mixture was stirred to dissolve the urea. After the urea was completely dissolved, the solution was placed under vacuum while stirring continued. This degases the solution, removing air that was

dissolved in the solution. The solution was kept stirring under vacuum until it no longer bubbled. The valves to the vacuum were then closed and the flask was filled with argon.

The urea solution was then poured into the flask containing NCA that had been stored under argon and this was stirred until the NCA crystals were completely dissolved. Hexylamine is a nucleophile that acts as the initiator of the polymerization. The volume of hexylamine was measured out and added to the flask containing the NCA solution using a volumetric pipette. The volume of hexylamine needed was found according to the calculations below.

$$Volume\ Hexylamine\ (\mu L) = \frac{moles\ of\ NCA}{Desired\ length\ of\ polymers} \times \frac{101.19\ g}{1\ mole} \times \frac{1000\ \mu L}{0.77\ g}$$

For the synthesis of the 10-length polymer 72.39 μL of hexylamine were used, for the synthesis of the 20-length polymer 27.93 μL of hexylamine were used, and for the synthesis of the 30-length polymer 24.26 μL of hexylamine were used.

Following the addition of hexylamine, the valves between the reaction flask and the Schlenk line were close, the argon flow to the line was turned off, and the reaction was allowed to proceed with stirring. The polymerization reaction creates carbon dioxide and thus the flask must be vented every few hours and this can be used as a method to ascertain whether or not the reaction is occurring.

To check for the creation of carbon dioxide and vent the reaction flask, the hose to the reaction flask (but not the reaction flask) was vacuumed out and then filled with argon. The flow of argon to the hose was turned off but the valve between the hose and the Schlenk line was left open. After the bubbles in the bubbler had ceased, the valve between the reaction flask and the hose was opened, releasing carbon dioxide. If bubbles appear in the bubbler then carbon dioxide was present in the flask and the reaction was proceeding. The valve between the reaction flask and the hose was closed and the argon flow was resumed. The valve between the hose and the

Schlenk line was turned to vacuum out the hose. In quick succession, the valve between the flask and the hose was opened to the vacuum, the valve between the hose and the line was turned from vacuum to argon flow, and the valve between the flask and the hose was closed. The flask was then filled with argon. The procedure was repeated approximately every three hours for the first day of polymerization, and two or three times on the second day of polymerization and every day until the reaction no longer creates carbon dioxide, indicating that the reaction has ceased. The reaction generally takes two to three days.

Purification of Hex-poly(benzyl-L-glutamate)

When the reaction had stopped forming carbon dioxide, the flask was filled with argon and removed from the Schlenk line. The contents of the Schlenk flask were transferred to a 250 mL round-bottom flask and rinsed with DMF and the solvent was evaporated off using the rotovap. Approximately 100 mL of tetrahydrofuran (THF) was added to the flask containing the polymer and this was sealed with a glass stopper and stirred for approximately 40 minutes to dissolve the polymer and precipitate the urea. Some urea precipitation should happen almost immediately after adding the THF to the flask.

While the polymer solution was stirring, an apparatus for dialysis was prepared. An approximately 15 mL pre-treated dialysis bag with pores one half the expected size of the polymer or less was soaked in a beak of deionized (DI) water for 10 minutes. The bag was closed on the bottom using a metal and plastic clip. After stirring for 40 minutes, the polymer solution was filtered using vacuum filtration and the urea that was filtered off was discarded. It was possible to be certain that only urea was filtered off by taking a small sample of the solid filtrate and dissolving it in water. If the crystals dissolve then only urea is present. The filtrate was pipetted into the dialysis bag and the flask was rinsed with about 10-15 mL of THF. The bubbles

were coaxed out of the bag, the top of the bag was sealed with a plastic clip, and foam flotation devices were attached to the top of the dialysis bag.

The polymer solution was allowed to dialyze in 5 liters of DI water for two to three days, changing the water twice a day.

Lyophilization

Following dialysis, the polymer solution was poured into a 250 mL beaker and the dialysis bag was cut into three pieces and rinsed with distilled water to ensure that as much product as possible was removed. The mixture of polymer and water was flash frozen using liquid nitrogen, the beaker was covered with a Kimwipe, placed in a lyophilization container, and lyophilized for two to three days or until all the water was sublimated away, leaving the polymer as a powder.

After lyophilization, the powdered benzyl-protected poly(glutamic acid) was weighed and placed in a labeled vial. The lyophilizer was drained and the glassware cleaned.

Analysis of Samples using NMR

The 10-length and 20-length polymers were analyzed using NMR. To prepare the samples for analysis, 7 mg of each polymer was measured out and dissolved in approximately 0.75 mL of DMSO-d_6. A small amount of glass wool was tamped into two glass pipettes. These acted as filters to ensure that no large particles or aggregates end up in the NMR sample that could cause irregular NMR results. The pipettes were placed in NMR tubes and the polymer solutions were filtered through them and into the NMR tubes. The tubes were sealed until NMR analysis could be performed. A simple 1D ^{1}H NMR spectrum was taken for both the 10-length and the 20-length polymers.

Results and Discussion

The mass of NCA obtained in the synthesis of the 10-length polymer was 1.373 g and the percent yield of the NCA was 65.36%. The mass of NCA obtained in the synthesis of the 20-length polymer was 1.06 g and the percent yield of the NCA for the 20-length synthesis was 50.21%. The mass of NCA obtained in the synthesis of the 30-length polymer was 1.379 g and the percent yield of the NCA for the synthesis of the 30-length polymer was 65.38%.

The masses of the 10-length, 20-length, and 30-length hex-poly(benzyl-L-glutamic acid) were 1.1788 g, 0.8695 g, and 1.1076 g, respectively. The percent yields were 93.28%, 91.20%, and 87.37%, respectively.

The NMR spectra of the 10-length and 20-length hex-poly(benzyl-L-glutamate) are shown in figures 4 and 5, respectively. The peak at 2.5 is the solvent peak for DMSO-d_6 that has been incompletely deuterated and the peak at 3.33 could be due to water.

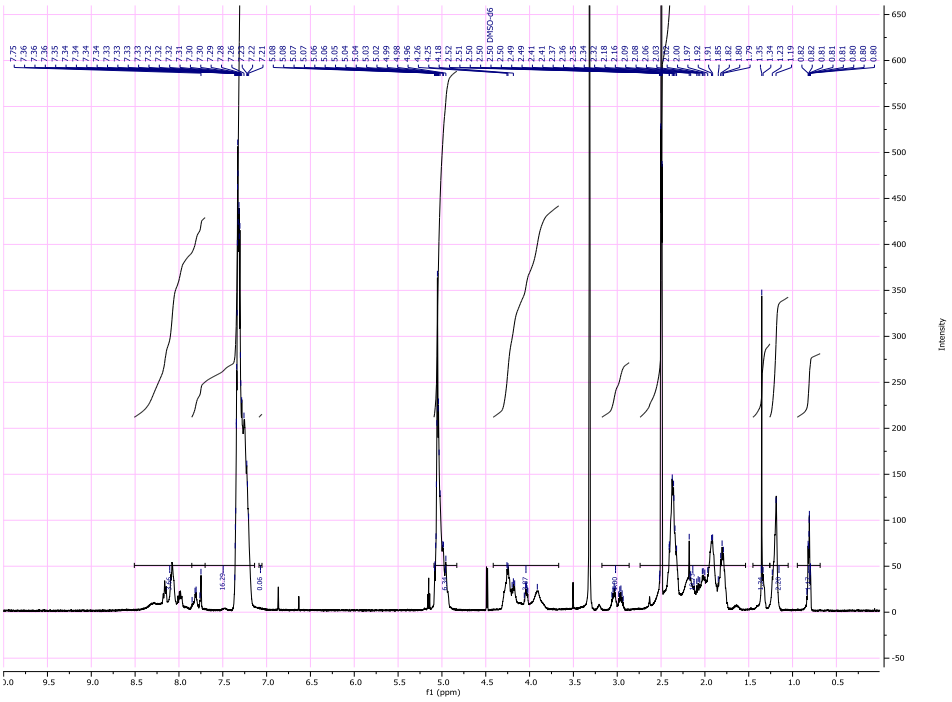

Figure 4: NMR spectrum of the 10-length hex-poly(benzyl-L-glutamic acid) in DMSO.

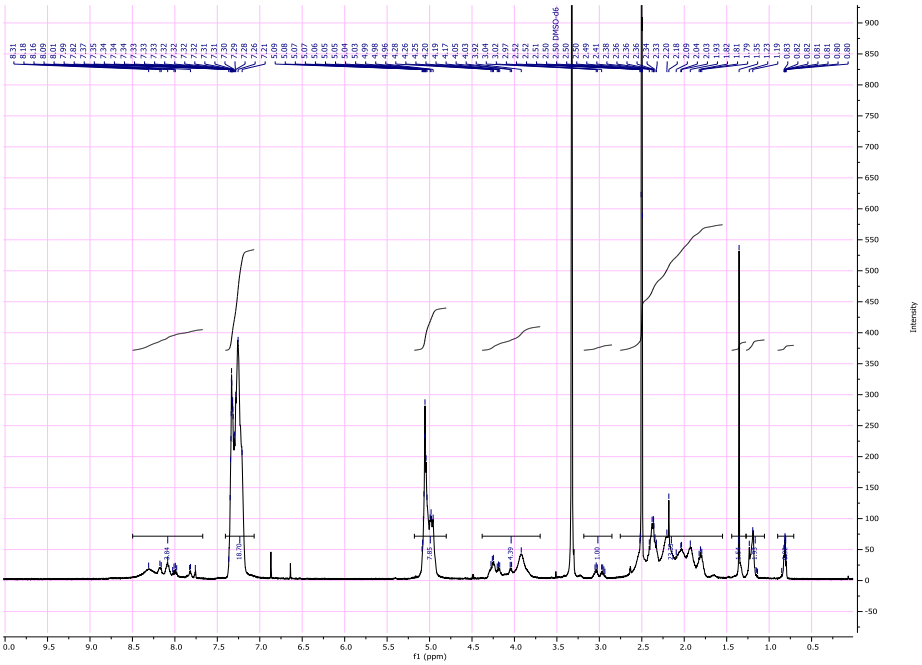

Figure 5: NMR spectrum of the 20-length hex-poly(benzyl-L-glutamic acid) in DMSO.

The NMR spectra matched up well with a spectrum taken by previous researchers of 30-length PEG-poly(benzyl-L-glutamic acid) that is shown in figure 6. This confirmed the identities of the polymers as hex-poly(benzyl-L-glutamic acid).

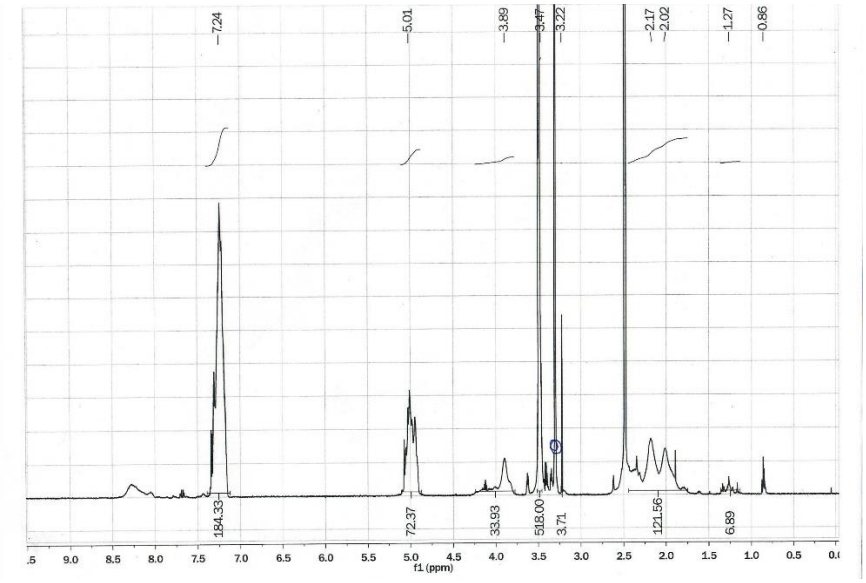

Figure 6: NMR spectrum of PEG-poly(benzyl-L-glutamic acid) in DMSO from previous research.[9]

Future Work

The ultimate goal of the synthesis of hex-poly(benzyl-L-glutamic acid) was to convert it to hex-poly(glutamine). In order to do this, a deprotection reaction in which trimethylsilyl iodide (TMSI) removes the protecting benzyl group, resulting in hex-poly(L-glutamic acid). Following this, the hex-poly(L-glutamic acid) would be converted to hex-poly(L-glutamine) by coupling 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) with N-hydroxysuccinimide (NHS) and then followed by ammonia in THF.

In the future, more batches of hex-poly(benzyl-L-glutamic acid) will be synthesized, including batches of the 40-length polymer, and converted to hex-poly(glutamine). The synthesized hex-poly(glutamine) will be used to research how the polymer interacts with cells.

Conclusion

Chains of poly(glutamine) that are 35 residues long and above are characteristic of a certain kind of neurovegetative diseases, such as Huntington's disease. Research on poly(glutamine) is needed to determine the role that these aggregates have in the neurodegeneration of those suffering from these diseases. Synthesis of poly(glutamine) with desired chain lengths is possible by synthesizing and polymerizing benzyl-protected glutamic acid NCA and then deprotecting the hex-poly(benzyl-L-glutamic acid) to form hex-poly(glutamine).

Hex-poly(benzyl-L-glutamic acid), the precursor to hex-poly(glutamine), with chain lengths of 10, 20, and 30 residues were synthesized using this method. The 10 and 20-length polymers were analyzed using 1D ^{1}H NMR spectroscopy and the resulting spectra matched closely with what was expected and with spectra from previous syntheses of PEG-poly(benzyl-L-glutamic acid), confirming their identities. The masses of the 10, 20, and 30-length hex-poly(benzyl-L-glutamic acid) synthesized were 1.1788 g, 0.8695 g, and 1.1076 g, respectively, and the percent yields were 93.28%, 91.20%, and 87.37%.

Future work will include the synthesis of 40-length polymers and the conversion of all hex-poly(benzyl-L-glutamic acid) to hex-poly(glutamine). Once enough of the polymer has been synthesized, research will be performed into the interactions between poly(glutamine) and cells.

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David Cook <dac0010@uah.edu>

Re: Final Honors Capstone Report

Carmen Scholz <scholzc@uah.edu> Tue, May 5, 2020 at 3:09 PM
To: Marisa Thompson <met0021@uah.edu>, William Wilkerson <wilkerw@uah.edu>, David Cook <dac0010@uah.edu>, Leslie Foster <john.foster@uah.edu>

Dear Marisa,
I approve your Honors Thesis. Congratulations!!!!! Good work!!!! Please submit your thesis now.
I hope I have you back in the lab soon, hopefully research labs will open again soon. As you point out in your conclusion, there is work to do.
Dean Wilkerson, Mr. Cook and Dr. Foster are copied on this email.

Congratulations on your Graduation, even though the official graduation fell victim to this crisis, once we are back to some normalcy we will have a graduation party for you and Jonathan in the lab.

Stay safe!

CS

On Tue, May 5, 2020 at 2:17 PM Marisa Thompson <met0021@uah.edu> wrote:

> Dr. Scholz,
>
> Here is my final capstone thesis. Once you approve it, please email approval back to me and copy the Department Chair, the Honors Dean at wikerw@uah.edu, and David Cook at dac0010@uah.edu. If you would like to sign it then you can but it is not required. After receiving your email of approval I can upload my thesis to the submission portal.
>
> Thank you so much,
> Marisa Thompson

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Dr. Carmen Scholz
Professor of Chemistry
University of Alabama in Huntsville