Organic Synthesis and Characterization of Poly(L-glutamine)

Allana Rae Schafer

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Organic Synthesis and Characterization of Poly(L-glutamine)

by

Allana Rae Schafer

An Honors Capstone

submitted in partial fulfillment of the requirements

for the Honors Diploma

to

The Honors College

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April 23, 2021

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Allana Schafer

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4/3/21

Date
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Abstract

In the United States, 1 in every 10,000 people are affected by the neurodegenerative disorder, Huntington’s disease. This incurable, hereditary disease is caused by a mutation in one region of the Huntingtin protein (HTT) made up of CAG trinucleotide repeat units which code for the amino acid glutamine \((L\text{-Gln})\), sometimes abbreviated as Q. The severity of the disease correlates to the amount of glutamine repeat units found in the protein, which sheaths neural tissue. To understand the physical, plaque-building properties of this protein, poly\((L\text{-Gln})\) must be synthesized at controlled chain lengths to mimic this region in the naturally occurring protein. Direct synthesis of poly\((L\text{-Gln})\) has proven to be difficult due to low reactivity, lack of control over chain length, and degradation during deprotection. By comparison, another amino acid, glutamic acid \((L\text{-Glu})\), has a very similar structure and polymerizes easily at controlled lengths. Formation of poly\((L\text{-Gln})\) through a polymer-analogous conversion of poly\((L\text{-Glu})\) was investigated using four different substitution approaches: EDC/NHS followed by \(\text{NH}_3/\text{THF}\), EDC/NHS followed by \(\text{NH}_4\text{OH/H}_2\text{O}\), EDC/NHS followed by TFE and \(\text{NH}_3/\text{THF}\), and finally EDC/NHS followed by \(p\text{-nitrophenol and NH}_3/\text{THF}\). Polymers investigated had chain lengths between 10-40 repeat units, thereby covering the protein lengths from normal to pathological structures. Results show that this indirect synthesis of poly\((L\text{-Gln})\) proves to be successful using the EDC/NHS coupling followed by ammonia in THF conversion, based upon the spectra acquired using \(^1\text{H-NMR}\) and Fourier-transform infrared spectroscopy (FTIR). Additionally, characterization techniques including Differential Scanning Calorimetry (DSC), Thermogravimetric analysis (TGA), Gel Permeation Chromatography (GPC), MALDI TOF, and Wide-Angle X-ray Scattering (WAXS) were utilized to determine degree of polymerization and polymer crystallinity.
1. Introduction

Neurodegenerative diseases are characterized as disorders that progressively degenerate the structure and function of the central or peripheral nervous system. Common examples of neurodegenerative diseases include Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease. The contributing causes of these disorders include individual genetics as well as environmental factors that increase susceptibility. Because neurodegenerative disorders ultimately result in neuron death, affected individuals experience loss of motor control, memory, speech, and intelligence as a result of disruption in brain cell communication. There is no known cure for neurodegenerative disorders because the mechanism of progression is complex and not yet fully understood.\textsuperscript{1,2}

Huntington’s disease is a neurodegenerative disease that causes progressive loss of motor control due to deterioration of the basal ganglia, a group of neurons in the brain that aid in movement. This genetic disorder is caused by an expansion of cytosine-adenine-guanine (CAG) trinucleotide repeat units, which code for the amino acid glutamine, in the HTT gene that codes for the huntingtin protein. Normal huntingtin protein functions include chemical signaling, vesicle transportation, protein binding, and cell destruction, or apoptosis. Although the exact mechanism by which the huntingtin protein works within the body is not fully understood, these functions are known to be associated with normal protein operation. Individuals affected by Huntington’s disease experience loss of these functions as a result of protein misfolding and aggregation. Huntingtin protein aggregates form inclusion bodies in the neurons of affected individuals, and the role of the aggregates is highly debated in the field. Some scientists speculate that these inclusion bodies contribute to the neurodegenerative nature of the disease.
while others theorize that these inclusion bodies are a protective response from the body that prolongs neuronal cell death.\textsuperscript{2-4}

The range of the huntingtin protein that includes the CAG trinucleotide repeat units has been investigated thoroughly such that normal and diseased proteins can be distinguished once specific protein lengths are obtained. It is well known that the amount of glutamine repeats coded by the CAG units directly correlates to the severity of disease. Individuals carrying 6 to 25 glutamine repeat units in this segment of the protein are unaffected by Huntington’s disease and experience normal huntingtin protein function. While individuals in this range are not directly affected by the disease, the higher the number of glutamine repeat units the more likely it is that the mutated version of the gene will be passed down to offspring. Individuals with 36 or more glutamine repeat units in this segment of the protein are considered to be in the disease range with a higher number of repeat units contributing to disease intensity as shown in Figure 1.\textsuperscript{3,4}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{huntingtin_glutamine_units.png}
\caption{Huntingtin Protein Glutamine Unit Illustration}
\end{figure}
To better understand protein behavior, a biomimetic polymer containing glutamine repeat units in several lengths, ranging from unaffected to disease lengths, was synthesized and characterized. Synthesizing poly(L-glutamine) by direct methods has been attempted in the past and found to be difficult due to lack of control over degree of polymerization as well as purification complications causing polymer degradation. To mitigate these issues, the poly(amino acid) was synthesized indirectly using a polymer analogous side chain conversion of the carboxylic acid end chain in poly(L-glutamic acid) to an amide group. Once polymer samples of four lengths were obtained with degree of polymerization ranging from 10-40, characterization techniques were utilized including $^1$H-NMR, FTIR, DSC, GPC, MALDI-TOF, and WAXS to explore degree of polymerization and crystallinity.
2. Experimental Methods

2.1. Polymer Synthesis

In polymer synthesis of poly(amino acid)s, the general procedure includes synthesizing an N-carboxyanhydride (NCA) activated monomer, conducting a ring-opening polymerization, deprotecting to remove protecting groups, purifying out solvents and by-products, and finally, in this indirect synthesis scheme, converting functional side groups from carboxylic acid to amide functional groups through investigation of esterification reactions.\(^5\)

2.1.1. NCA Synthesis

Well-defined poly(L-glutamic acid) samples were synthesized by first creating the activated monomer used in ring-opening polymerization. Commercially available benzyl-L-glutamate (2.00 g, 8.42 mmol) was dissolved in distilled ethyl acetate and reacted with excess triphosgene (5.25 g, 17.70 mmol) and α-pinene (6.89 g, 50.58 mmol) to form benzyl-L-glutamate N-carboxyanhydride (NCA) as shown in the reaction schematic in Figure 2. This anhydrous reaction took place at approximately 110°C and refluxed for about 3-4 hours. Potassium hydroxide solution was used in the bubbler to prevent any harmful phosgene vapors from escaping the apparatus.\(^6\) After reaction completion, the reaction solution was filtered into a round bottom flask to remove unreacted particles before removing the ethyl acetate solvent using a rotary evaporator. Fresh ethyl acetate was used to redissolve the NCA and small amounts of hexanes were added slowly to aid in the precipitation of NCA crystals.\(^7\) Once crystals precipitated out, the flask was kept in the freezer and small aliquots of hexanes were added every few hours to maximize crystal formation. Vacuum filtration was used to isolate crystals for
further purification. Crystals were redissolved using ethyl acetate and a small amount of hexanes was added. The solution was filtered again and washed with a 50:50 ethyl acetate:hexane mixture. The dissolved crystals were put under argon and placed in an ice bath while small aliquots of hexanes were added to assist in recrystallization. NCA crystals were isolated using vacuum filtration and placed under vacuum on the Schlenk line to remove residual solvent.

![Figure 2: NCA Synthesis Schematic](image)

2.1.2. Polymer Synthesis

Poly(amino acid) synthesis proceeded through a ring-opening polymerization mechanism as illustrated in Figure 3. The initiator used for polymerization of the benzyl-L-glutamate NCA was n-hexylamine. Prior to polymer synthesis, the urea was dried under vacuum to remove any excess moisture which could halt the reaction. The urea (0.72 g, 6.02 mmol) was dissolved using anhydrous dimethylformamide (60.19 mL, 6.02 mmol) as a solvent, and this mixture was degassed using vacuum on the Schlenk line to remove any dissolved gasses that might interfere with the polymerization reaction. The urea/DMF mixture was then added to the NCA under
argon. The n-hexylamine initiator was then pipetted into the reaction flask which was purged periodically to remove carbon dioxide and to relieve vessel pressure. The reaction time required was dependent on the polymer length of the sample which was controlled by manipulating the initiator to monomer ratio as shown in Table 1.  

<table>
<thead>
<tr>
<th>Degree of Polymerization</th>
<th>n-hexylamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>79.09 μL, 0.602 mmol</td>
</tr>
<tr>
<td>20</td>
<td>39.56 μL, 0.301 mmol</td>
</tr>
<tr>
<td>30</td>
<td>26.37 μL, 0.201 mmol</td>
</tr>
<tr>
<td>40</td>
<td>19.78 μL, 0.151 mmol</td>
</tr>
</tbody>
</table>

Note: Based on 1.5g, 6.02 mmol of benzyl-L-glutamate NCA

Once reaction completion was achieved, the DMF was evaporated off using a rotary evaporator, and the polymer sample was purified further by precipitating out excess urea using tetrahydrofluoran (THF) followed by vacuum filtration. THF solvent was removed using the rotary evaporator and the leftover polymer sample was then purified using dialysis methods. The pore size of the dialysis bag was chosen based on the molecular weight of the sample to prevent the sample from diffusing out of the bag. After at least 24 hours of dialysis, the polymer sample was then flash frozen with liquid nitrogen and the water was sublimated out using a lyophilizer, or freeze-drying machine, to obtain solid sample.
2.1.3. Polymer Deprotection

To remove the benzyl protecting group, trimethylsilyl iodide (TMSI), a powerful deprotecting agent was used. The dried polymer sample (0.5 g, 0.223 mmol) was dissolved using anhydrous dichloromethane (DCM) and the measured amount of TMSI (539.3 μL, 3.79 mmol) was added to the reaction flask which was refluxed under argon at about 45°C overnight. A small amount of HCl was added to the reaction flask and the polymer was dialyzed for a few days to remove undesired products and solvent. The polymer sample was purified further using acetone washes followed by centrifugation which was repeated multiple times until the polymer became a white color as opposed to the yellow color that results from using TMSI. The polymer was then freeze-dried using lyophilization. The deprotection reaction is shown below in Figure 4.

* Stoichiometric amounts based on hex-poly(benzyl-L-glu)$_{10}$ with an estimated molecular weight of 2243 g/mol.
2.1.4. Esterification Study

Formation of poly(L-glutamine) through a polymer-analogous conversion of poly(L-Glutamic acid) was investigated using four different substitution approaches: EDC/NHS followed by NH$_3$/THF, EDC/NHS followed by NH$_4$OH/H$_2$O, EDC/NHS followed by TFE and NH$_3$/THF, and finally EDC/NHS followed by p-nitrophenol and NH$_3$/THF.$^{11-13}$ For the EDC/NHS coupling followed by NH$_3$/THF, polymer sample (0.100 g, 0.0718 mmol) was dissolved in DMF, and a heat gun was used to aid in solubility. EDC (0.275 g, 1.44 mmol) and NHS (0.165 g, 1.44 mmol) were measured and dissolved in DMF before adding them to the polymer mixture and allowing adequate time for stirring. DMF was removed using the rotary evaporator. Ammonia in THF (3.6 mL, 1.44 mmol) was then added to the remaining mixture and allowed to react overnight at room temperature. Dialysis was used as a purification step before lyophilization. For the EDC/NHS coupling followed by NH$_4$OH in H$_2$O, a similar procedure was followed, but solubility became an issue when the NH$_4$OH/H$_2$O was added to the sample. For the

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$^\dagger$ Stoichiometric amounts based on hex-poly(L-glu)$_{10}$ with an estimated molecular weight of 1392.5 g/mol.
EDC/NHS coupling followed by trifluoroethanol (TFE) and NH$_3$/THF, the TFE was added after the EDC/NHS coupling reaction occurred, followed by NH$_3$ in THF, and allowed to react overnight before dialysis. A similar procedure was conducted for EDC/NHS coupling followed by $p$-nitrophenol and NH$_3$ in THF. Because of the solubility constraints, the EDC/NHS coupling followed by NH$_4$OH/H$_2$O reaction and the EDC/NHS coupling followed by TFE or $p$-nitrophenol and NH$_3$/THF reactions were not pursued further. The EDC/NHS coupling followed by NH$_3$/THF esterification reaction illustrated in Figure 5 was used to make the polymer samples used for later characterization.

![Figure 5: Conversion Reaction Schematic](image)

### 2.2. Polymer Characterization

To better understand the structure of the synthesized polymer, several characterization techniques were performed. FTIR and $^1$H-NMR were used to confirm structure and end chain functional groups as well as estimate the degree of polymerization of each sample. DSC and TGA studies were conducted to explore the thermal properties of the polymer samples. WAXS was used to determine crystalline structure of the polymer samples. GPC and MALDI TOF were used to determine polydispersity and to estimate degree of polymerization.$^{8,14,15}$ FTIR, DSC, WAXS, and TGA were all solid-state characterization techniques that used minimal sample
whereas $^1$H-NMR, GPC, and MALDI TOF required the polymer to be dissolved in solvents to make solutions. The recovery of polymer sample depended on the technique used, but often times the samples were not recoverable which posed a limitation for the study of polymer structure and behavior.

The spectra acquired from $^1$H-NMR studies of the deprotected poly($L$-glutamic acid) and poly($L$-glutamine) polymers were compared to determine the change in functional groups as a result of the esterification study. This data was acquired using a 500 MHz NMR spectrometer. Additionally, the degree of polymerization was estimated for each sample to confirm polymer lengths. The $^1$H-NMR solvent used for the starting material, poly($L$-glutamic acid), was trifluoroacetate while the $^1$H-NMR solvent used for the end product, poly($L$-glutamine), was DMSO. The use of different solvents to perform $^1$H-NMR was due to a change in solubility of the polymer sample after the amide conversion. FTIR spectra were obtained using a Bruker VERTEX 70 IR spectrometer for the starting and end material in a comparison study to determine the change in carbonyl group peaks which would indicate a successful shift from carboxylic acid to amide end chain groups.

Because TGA and DSC analysis requires solid state polymer sample, little preparation was necessary to conduct the study of the polymer thermal properties. Instrumentation used for this experimentation included a Mettler Toledo DSC 822 machine. For this study, TGA was used primarily to determine the thermal stability of the polymer samples to establish the temperature range and heating rate needed for the DSC study. Approximately 5-10 mg of sample was necessary to perform DSC experiments. First the samples were heated from 25°C to 100°C at a heating rate of 5 K/min and then were held at 100°C for 10 minutes. The polymer samples were cycled twice with these parameters to develop DSC curves for each length of polymer.
The WAXS study was performed using a Bruker D4 Endeavor diffractometer with diffraction patterns recorded from 1 to 80° with a step of 0.01° at a 2 second counting time. The study was used to create a diffraction pattern of the polymer samples to determine the crystallinity of the polymer chains. Background signals were subtracted to obtain well defined diffraction patterns.

GPC was carried out using a Viscotek GPCmax VE 2001 with a Viscotek VE 3580 IR detector. Poly(methyl methacrylate) standards were used for GPC calibration. Polymer samples were dissolved in either THF or DMF at a concentration of 5 mg/mL. The column was maintained at 25°C while the detector was maintained at 35°C during experimentation. The flow rate was set to 1 mL/min.

MALDI-TOF mass spectrometry was conducted using a Bruker Autoflex III Smartbeam with the utilization of flexAnalysis software for data analysis. The polymer samples were dissolved in DMF at a concentration of 5 mg/mL and mixed with a 20 mg/mL solution of DCTB (trans-2-[3-(4-tert-Butylphenyl)-2-methyl-2-propenylidene]malononitrile) in THF and a 20 mg/mL solution of lithium trifluoroacetate in THF at a ratio of 100:10:1 for matrix, analyte, and salt, respectively.
3. Results and Discussion

3.1. $^1$H-NMR and FTIR

The following $^1$H-NMR spectra show the structure confirmation of the end product, poly(L-glutamine), with assigned peaks as shown in Figure 6. The spectra were analyzed using MestreNova software, and the methyl peak, represented in Figure 6 as a, was used to normalize the protons by setting this peak integration to 3. By doing this, the proton represented by h in the repeat unit of the polymer gave a good estimation of degree of polymerization when integrated. This technique of characterization gave confirmation after the deprotection and amide conversion reactions that the structure did in fact change resulting in shifted peaks in the sample spectra, but it should be noted that other methods of characterization are necessary to form a final conclusion about structure. The diminishing aromatic peak confirmed the level of deprotection while the new peaks appearing around 7-8 ppm were suspected of being from the protons in the new amide functional group. Polymer samples that did not go through successful completion of the deprotection elicited an additional aromatic peak as shown in Figure 7.

Figure 6: $^1$H-NMR Data for Poly(L-Gln)$_{30}$
Figure 7: $^1$H-NMR Data of Incomplete Deprotection of Poly($L$-Gln)$_{20}$

The NMR data shown in Figure 6 resulted from the poly($L$-glutamine)$_{30}$ sample and Figure 7 resulted from the poly($L$-glutamine)$_{20}$ sample, although spectra were collected for all four polymer lengths which can be found in Appendix A. To further confirm the successful reaction converting poly($L$-glutamic acid) to poly($L$-glutamine), FTIR studies were performed on the deprotected polymer and the final product to compare. Since the only change in structure between these two molecules was a change in terminal functional group in the side chain, finding conclusive evidence proved to be difficult. Referencing Figure 8, a comparison in the IR spectra of the two polymers showed a shift in the carbonyl region (~1700 cm$^{-1}$) designating a change in the type of carbonyl functional group. This shift from 1605 cm$^{-1}$ to 1650 cm$^{-1}$ was indicative of the conversion from a carboxylic acid functional group to an amide functional group as a result of the esterification study which can be seen in the reaction schematic of Figure 5. The samples used to produce the data represented in Figure 8 were poly($L$-glutamic acid)$_{10}$ and poly($L$-glutamine)$_{10}$. 
3.2. TGA and DSC

As previously mentioned, the main purpose of conducting TGA was to find the appropriate parameters, heating rate and temperature, used in the DSC experiments performed on the available polymer samples. This was done to preserve the samples as much as possible due to low sample amounts available for testing. Because of limited sample amount, TGA and DSC were not performed on poly(L-glutamine)$_{40}$ to preserve the sample for other characterization techniques of interest. From the analysis of the DSC curves shown in Figure 9, none of the polymer samples were shown to produce results indicating a melting temperature or glass transition temperature, thermal characteristics that are associated with crystallinity. This result is in line with DSC curves suggestive of amorphous polymer samples. Because of this, the polymer samples were considered to be amorphous but WAXS testing was used to further confirm this result.
3.3. GPC

Although multiple experiments were run using GPC, no usable data was received because of the low solubility of the polymer in the THF and DMF solvents. Once the samples were dissolved in solvent, they were filtered before being placed in the GPC which is likely when most of the sample was filtered out due to low solubility. Another possible explanation for no elution detection could be that the peaks of the sample were overshadowed by the solvent peaks. Future experimentation would have to be explored to find the best parameters and solvents for running successful GPC to obtain usable data.

3.4. MALDI-TOF

Much like GPC, the exact parameters and materials necessary to produce usable data were quite elusive which made this experimentation difficult. The matrices and salts tested with
the poly(L-glutamine) samples included DCTB and LiTFA as well as dithranol and KTFA, but both of these methods failed to provide usable results due to the inability of the matrix and salt to fragment and ionize the sample. Later work done by the Kressler group showed that DHB (2,5-dihydroxybenzoic acid) worked as an appropriate matrix for the poly(L-glutamic acid) and poly(L-glutamine) samples.

3.5. WAXS

Because limited samples were brought to the Kressler lab group for characterization, there was an insufficient amount of poly(L-glutamine)$_{40}$ to perform x-ray scattering but all other lengths were tested. The results of the diffraction peaks are shown in Figure 10 below. As shown in the figure, an amorphous halo exists for all three samples in the range of $5^\circ < 2\theta < 35^\circ$. The three sharp peaks shown in the data can be attributed to the film used to prepare the samples for testing. This result indicated that the samples are not crystalline and instead are considered to be amorphous confirming the results found from DSC.

![Figure 10: WAXS Diffraction Pattern for Poly(L-Gln)$_{10-30}$ Samples](image-url)

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*Figure 10: WAXS Diffraction Pattern for Poly(L-Gln)$_{10-30}$ Samples*
4. Conclusion

The ultimate goal of this research was aimed at synthesizing poly(L-glutamine) samples with degrees of polymerization including 10, 20, 30, and 40 repeat units by utilizing an indirect synthesis route. By first synthesizing well-defined poly(L-glutamic acid) samples, the polymer length could be controlled and the deprotection could be conducted without polymer degradation. From the results of this synthesis and characterization research of poly(L-glutamine), it has become evident that the samples synthesized may have been a mixture of glutamine, glutamic acid, and benzyl-L-glutamate repeat units. This copolymer likely resulted from incomplete deprotection and potentially incomplete conversion. The $^1$H-NMR spectra also indicate that impurities or byproducts could still remain in the samples showing that further purification techniques are necessary. Since the samples maintained chain length after the amidation reaction, this synthesis scheme could be fine-tuned to provide more complete conversion in the future. Additionally, the deprotection reaction was proven incomplete in samples such as the 20 length, based on $^1$H-NMR results, which could be improved upon by running kinetic studies of the deprotection reaction to find optimal conversion whilst maintaining chain length. By manipulating reaction time, reactant concentration, and reaction temperature, the optimal amide conversion and deprotection reaction conditions could improve on the final product by producing a homopolymer of poly(L-glutamine) in the desired lengths. Once the synthesis of this poly(amino acid) is improved upon and higher polymer yield is obtained, studies can be performed to better understand the aggregation behavior of poly(L-glutamine) which in turn may lead to a better understanding of how the biological huntingtin protein behaves in individuals with Huntington’s disease.
References


Appendix A: Additional $^1$H-NMR Data for Poly($L$-Gln)$_{10}$ and Poly($L$-Gln)$_{40}$
Appendix B: Unsuccessful MALDI-TOF Results

PolyQ10, 47% laser

PolyQ10, 80% laser

PolyQ20, 47% laser

PolyQ20, 80% laser