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Functionalized Methionine Polypeptides And Their Self Assembly

A thesis submitted in partial satisfaction

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by

Robin Higgins

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ABSTRACT OF THE THESIS

Functionalized Methionine Polypeptides

And Their Self Assembly

by

Robin Higgins

Master of Science in Chemistry

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Professor Timothy J. Deming, Chair

The synthesis and modification of polypeptides from NCAs is described. Diblocks containing a hydrophilic segment of poly(Met) and a hydrophobic segment of *rac*-Leu were self-assembled into micelles. The poly(Met) was either oxidized to the sulfoxide or alkylated to the carboxymethylated sulfonium.

Additionally, a diblock containing poly(Met) and PEG was synthesized. The poly(Met) segment was alkylated with bromo methyl boronic acid, and the polymers bound covalently and reversibly to compounds that contain diol groups.

The thesis of Robin Higgins is approved.

Neil Garg

Heather Maynard

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University of California, Los Angeles

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I'd like to thank the only form of radiation that bakes you cookies, gramma-rays, as well as my actual grandmother Louise White.

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N/A

List of Abbreviations

° degrees
aq. aqueous
C Celsius
DLS dynamic light scattering
DMF *N,N*-dimethylformamide
eq equivalent
EtOAc ethyl acetate
g gram(s)
h hour(s)
Hex hexanes
FTIR Fourier transform infrared spectroscopy
IDUA alpha-L-iduronidase
L liter
m milli
µ micro
Me methyl
M NCA methionine *N*-carboxyanhydride
min minute(s)
mol mole(s)
NCA *N*-carboxyanhydride
NMR nuclear magnetic resonance
p para
pH hydrogen ion concentration in aqueous solution
ppm parts per million
***rac*-L NCA** racemic leucine *N*-carboxyanhydride
RT room temperature
TFA trifluoroacetic acid
THF tetrahydrofuran
TEM transmission electron microscopy
UV ultraviolet

Thesis Introduction

Synthetic polypeptides have been widely studied for decades because of their similarity to naturally occurring peptides and proteins. [1] These constructed polypeptides have many applications in the biomedical field, [2] and offer advantages simpler polymers do not have such as the ability to mimic secondary structures of proteins.

In the 1940s, initial studies on the polymerization of α-amino acid-N-carboxy-anhydride (NCA) monomers was first reported, [3] and in the 1990s, Deming and co-workers discovered that transition metal complexes could initiate these ring opening polymerizations (ROP) and provide controlled chain lengths and low polydispersities.[4]

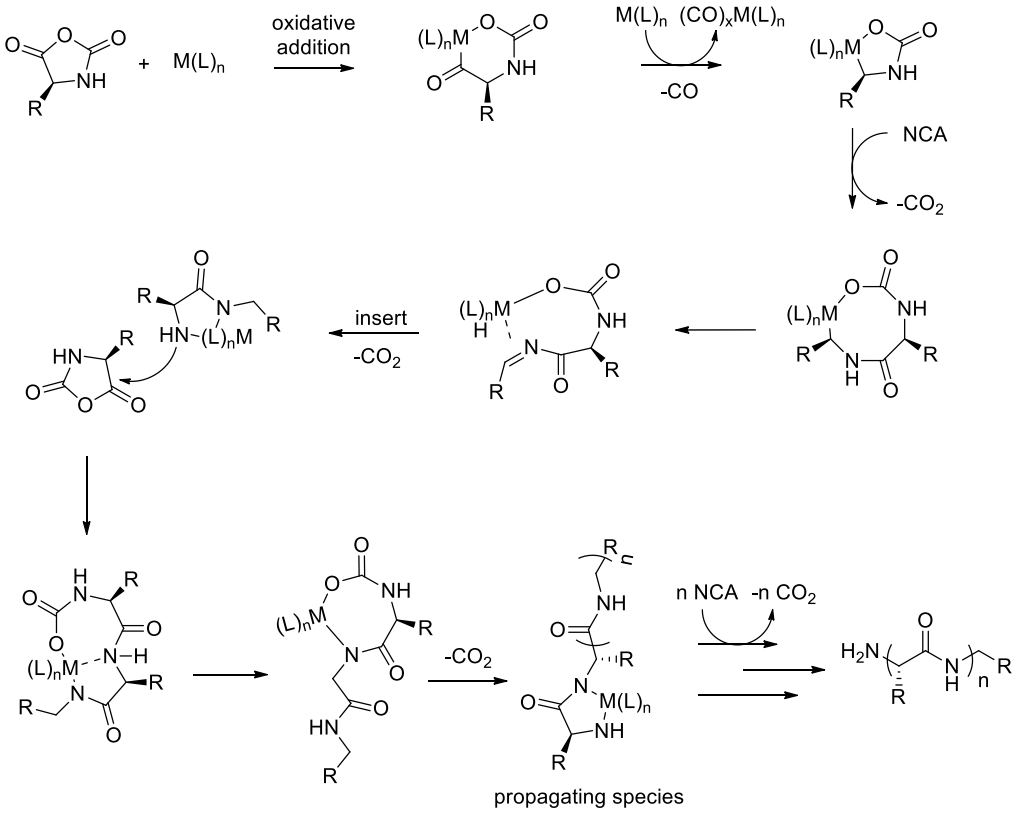


Figure x.1: The transition metal initiated polymerization of NCAs.

Until recently, the predominant purification technique of NCAs has been recrystallization.[5] Although there were some other methods available such as sublimation,[6] exposure to activated charcoal columns,[7] and washing NCA solutions in ethyl acetate with water and aqueous biocarbonate at 0 ^{o}C,[8] these procedures all had significant limitations or drawbacks. Recrystallization removes impurities and works for a wide variety of NCAs with high melting points, however, NCAs which have low melting points or which can only be isolated as oils cannot be purified via this method. [9]

We have previously reported the purification of NCAs using anhydrous flash column chromatography.[10] This purification method is commonly used by synthetic organic chemists, and is fast and inexpensive.[11] Flash chromatography can be used to purify a wide range of NCAs, including those which are unable to crystallize. NCAs which were purified via this method were able to form high molecular weight polypeptides without needing any other purification steps.[10]

Since the discovery that NCAs could be effectively purified using flash column chromatography, we have polymerized many NCAs that were previously unable to be purified.[12] In particular, our lab has discovered a wide range of modifications that can be performed on poly(Met), which has been difficult to prepare since M NCA is difficult to crystallize. We have alkylated poly(Met) to form sulfoniums containing amide, ester, propargyl and benzyl functionalities.[12] These chemoselective alkylations, along with previously known methionine reactions such as oxidation to the sulfoxide,[13] make poly(Met) a versatile polymer which can be modified to have many properties.

x.2 References

(1) Woodward, R.B.; Schramm, C.H. *J. Am. Chem. Soc.* **1947**, *69*, 1551-1552.

(2) Afsaneh, L.; Samuel, J.; Kwon, G, *Advanced Drug Delivery Reviews* **2002**, *54*, 169–190.

(3) Kricheldorf, H. R. *Angew. Chem., Int. Ed.* **2006**, *45*, 5752-5784.

(4) Deming T.J. *Nature* **1997**, *390*, 386.

(5) Kricheldorf, H. R. α-Amino acid-N-carboxyanhydrides and Related Materials; Springer-Verlag: New York, 1987.

(6) Waley, S. G.; Watson, J. *Proc. R. Soc. A* **1949**, *199*, 499-517.

(7) Noguchi, J.; Tokura, S.; Nishi, N. *Angew Makromol. Chem.* **1972**, *22*, 107-131.

(8) Poche, D. S.; Moore, M. J.; Bowles, J. L. *Synth Commun.* **1999**, *29*, 843-854.

(9) Boiteau, L.; Collet, H.; Lagrille, O.; Taillades, J.; Vayaboury, W.; Gianai, O.; Schue, F.; Commeyras, A. *Polym. Int*. **2002**, *51*, 1037-1040.

(10) Kramer, J.; Deming, T.J., *Biomacromolecules* **2010**, *11*, 3668-3672.

(11) Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem*. **1978**, *43*, 2923-2925.

(12) Kramer, J.; Deming, T.J., *Biomacromolecules* **2012,** *13*, 1719-1723.

(13) Shechter, Y.; Burstein, Y., *Biochemistry*, **1975**, *14 (20)*, 4497-4503.

Modified Methionine Based Micelles with Hydrophobic Racemic Leucine Cores

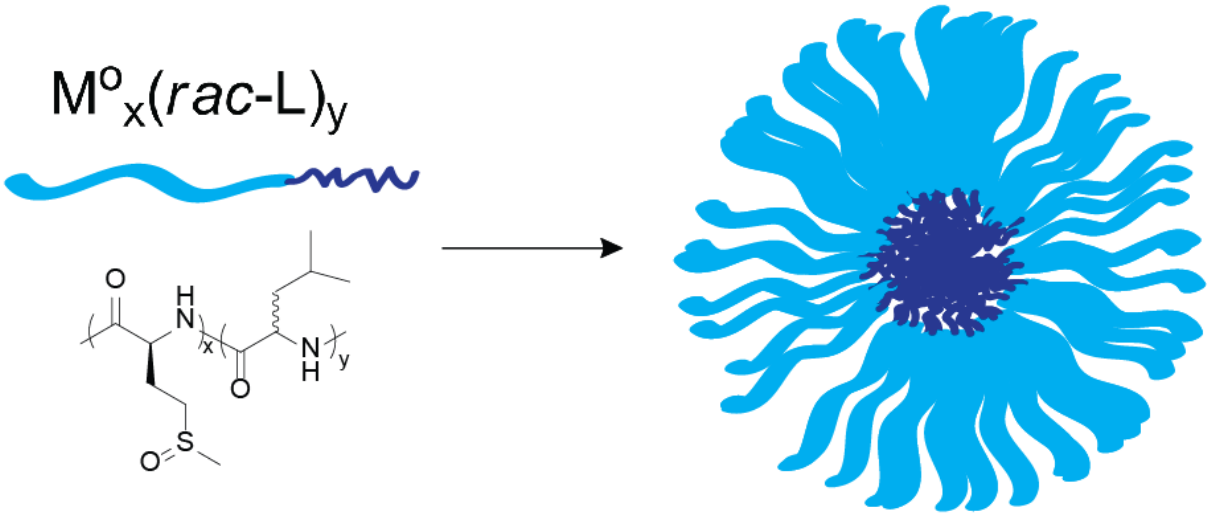


Figure 1.1: Schematic showing the self-assembly of $M^{O}_{x}(rac\text{-}L)_{y}$ micelles.

1.1 Abstract

The synthesis of modified methionine and racemic leucine diblock copolymers is described, as well as their self-assembly into micelles. Both poly((sodium carboxymethyl)-L-methionine sulfonium chloride)$_x$-*block*-poly(*rac*-leucine)$_y$ and poly(L-methionine sulfoxide)$_x$-*block*-poly(*rac*-leucine)$_y$ ($M^{C}_{x}(rac\text{-}L)_{y}$ and $M^{O}_{x}(rac\text{-}L)_{y}$, respectively) were synthesized. Both diblock copolymers were able to form micelles when the hydrophobic segment was 15-40 mol% of the overall composition.

The diblocks were found to self-assemble into micelles with diameters of approximately 30-50nm as determined by TEM. Micelles were formed when the polymers were directly dissolved in H_20 as well as when a variety of solvent annealing conditions were used. The micelles were found to be stable under many conditions including exposure to acid, salt, and surfactant. The

drug loading properties of the micelles were tested, using camptothecin and pyrene as model drugs. Drug loading efficiency was quantified using fluorescence.

1.2 Introduction

There is a great amount of interest in being able to deliver drugs to a specific part of the body rather than dispersing them throughout[1]. One of the methods being researched to achieve this goal is the Enhanced Permeability and Retention (EPR) effect, which shows that large macromolecules accumulate in tumor blood vessels more quickly than healthy blood vessels[2].

Micelles are not only the correct size to utilize the EPR effect (10-50nm diameter), but they also contain a hydrophobic core, which allows for the encapsulation of drugs which often have poor solubility in water. Much research has been done on poly(ethylene glycol)-*block*-(poly-L-amino acid) micelles[1], however these micelles do not have the advantages of a polypeptide corona. A polypeptide outer layer can take on secondary structure as found in proteins, and may more closely mimic biological structures. Polypeptides may also be modified using a variety of reactions[3], allowing the hydrophilic portion of the micelles to have a range of properties, while keeping the hydrophobic portion intact.

We have synthesized block copolypeptides containing two different types of modified methionine hydrophilic segments, both which formed micelles. They proved to be stable under many different conditions and with further development could be effective drug carriers.

1.3 Results and Discussion

Our lab has recently reported a technique for the improved purification of Met NCA[4]. We have also reported that Met NCA undergoes living polymerization when treated with $(PMe_3)_4Co$ initiator in THF for a few hours at room temperature. The resulting polymers have been shown to have narrow polydispersity indices (M_w/M_n) and controllable chain lengths[3]. The length of the chains is measured by quantitative end-capping using isocyanate terminated PEG (M_n = 2000 Da) followed by end-group analysis using ^1H NMR[5].

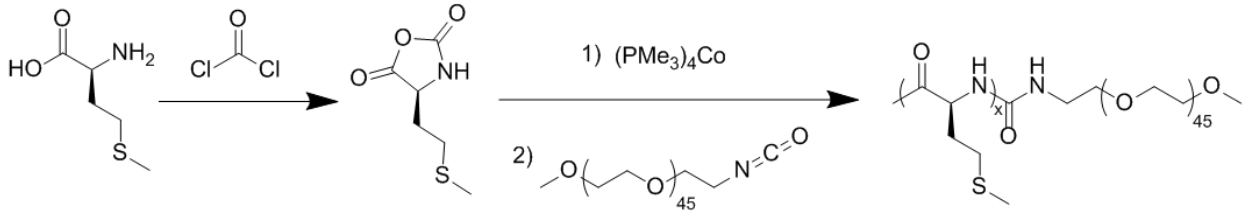


Figure 1.2: Preparation of Met NCA, Met NCA polymerization, and poly(Met) end-capping.

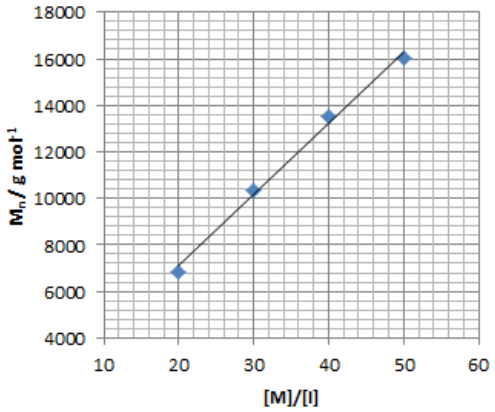


Table 1.1: Molecular Weight (M_n) as a function of monomer to initiator ratio [M]/[I] for poly(Met) made using $(PMe_3)_4Co$ in THF at 20 ^oC.

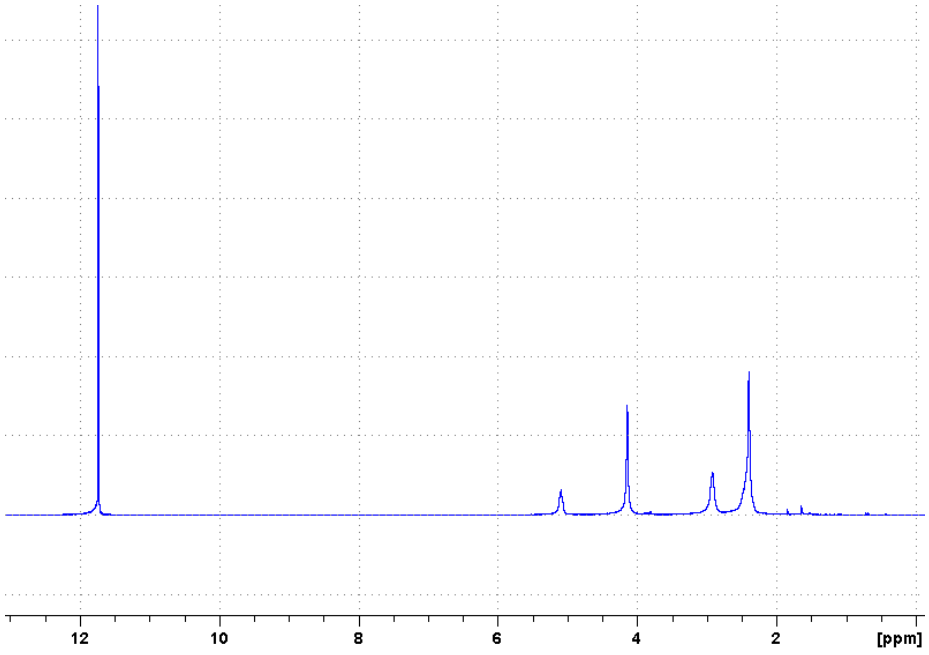


Figure 1.3: ^{1}H NMR of end-capping product, $M_{71}PEG_{45}$

We have also reported that Met NCA can form statistical copolymers with other NCAs such as Z-lysine.[3] We now report that in addition to these NCAs, that we can also form block copolymers with racemic leucine NCA. The diblocks were formed by creating a living poly(L-methionine) macroinitiator, and then adding the racemic leucine NCA. A small aliquot of the methionine macroinitiator was removed and end-capped with isocyanate terminated PEG (M_n = 2000 Da). The length of the racemic leucine segment was determined by comparing resonances of the known length methionine segment to leucine resonances in the ^{1}H NMR spectrum. The leucine segments showed quantitative addition in proportion to the amount added.

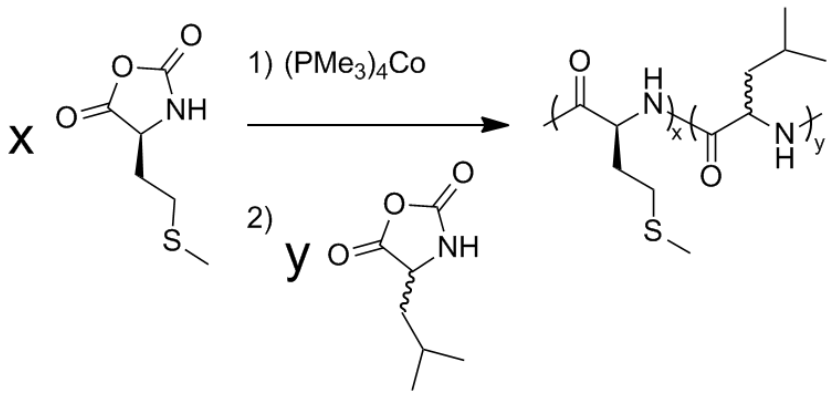


Figure 1.4: The synthesis of M_x(*rac*-L)$_y$.

Monomer[a]	M_n[b]	DP[c]
30 *rac*-L NCA	3,627	31
19 *rac*-L NCA	2,457	21
12 *rac*-L NCA	1,170	10

Table 1.2: Polymerization data for poly(*rac*-L), prepared using living M_{52} macroinitiator at 20 ^{o}C in THF. [a] Number shows equivalents of monomer per living poly(L-methionine)$_{52}$ macroinitiator. [b] Molecular weight of poly(*rac*-L) after polymerization as determined by ^{1}H NMR. [c] DP = number average degree of polymerization of poly(*rac*-L) by ^{1}H NMR.

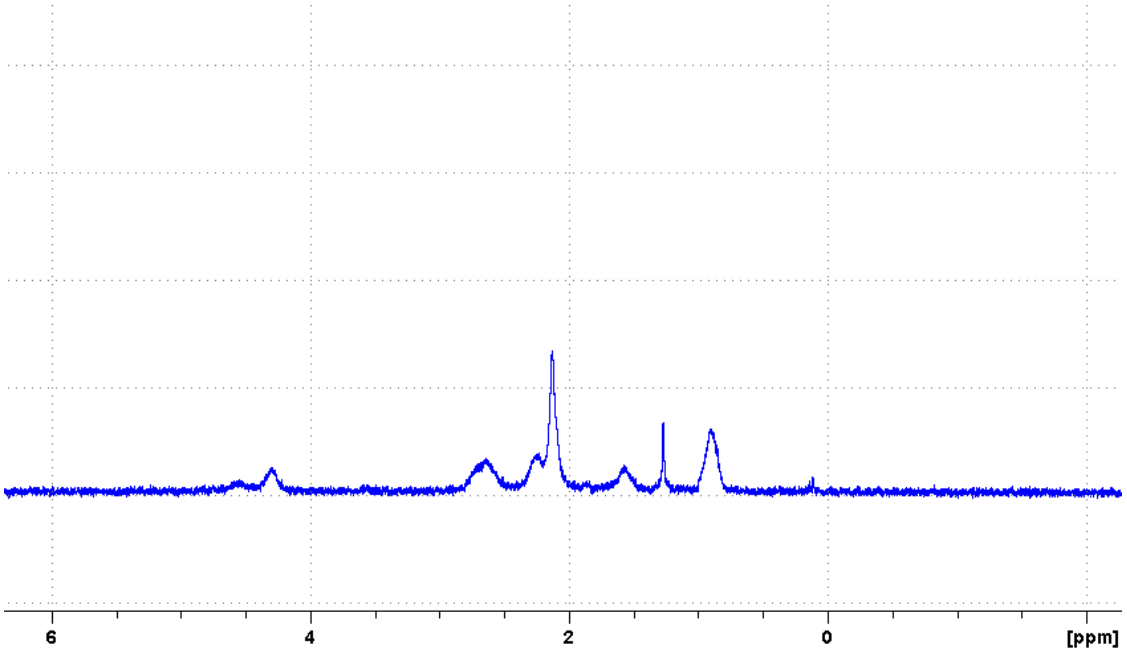
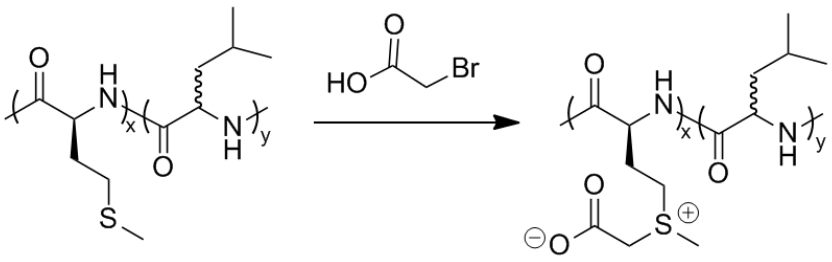


Figure 1.5: ^{1}H NMR of M_{52}(*rac*-L)$_{19}$

In the past, our lab has shown that poly(Met) side chains can be easily alkylated with a wide variety of reactants via a "click" type process[3]. We have also shown that these reactions are chemoselective, and can be performed in the presence of other functional groups within a given polypeptide. In our initial study here, the M_{50}(*rac*-L)$_7$ samples were carboxymethylated, leaving the *rac*-L untouched.



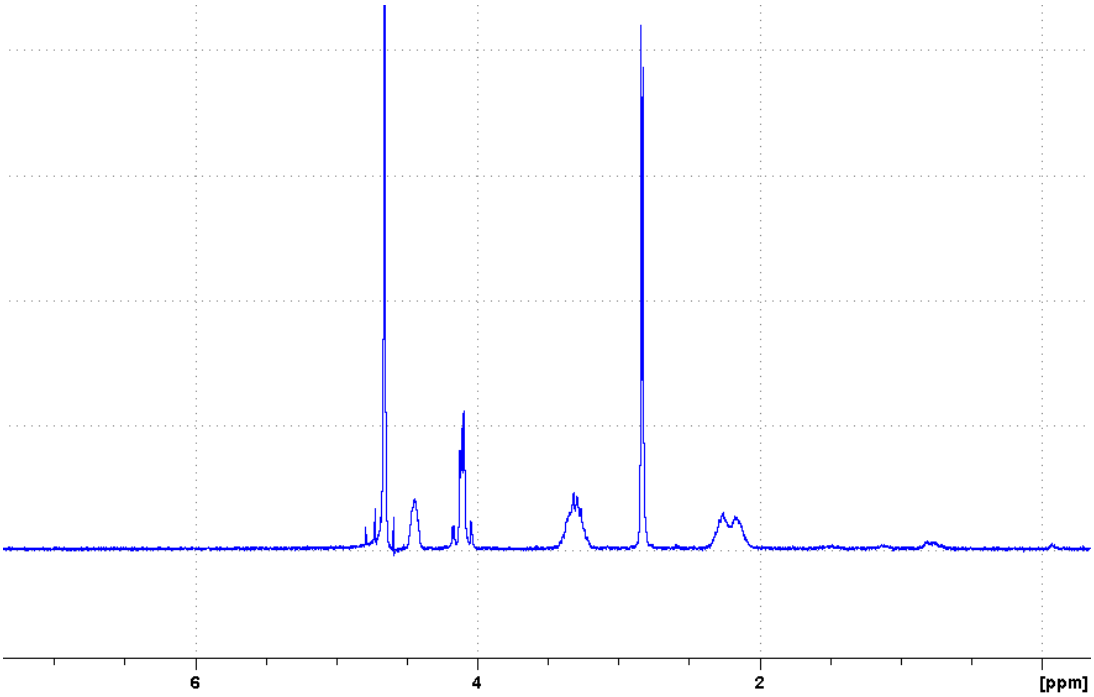
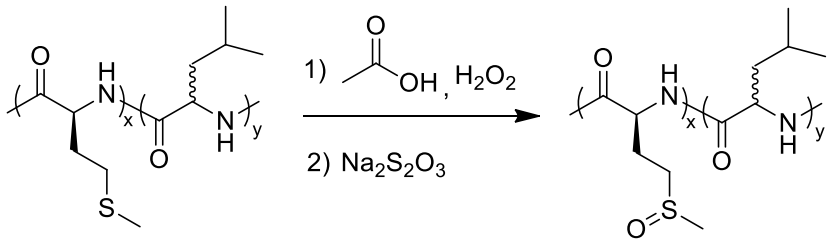


Figure 1.6: The synthesis and ^{1}H NMR of $M^{C}_{50}(rac\text{-}L)_{7}$.

The oxidation of methionine on poly(Met) to the sulfoxide has been known for some time[6]. The $M_{71}(rac\text{-}L)_{31}$. diblocks were oxidized to the sulfoxide according to known procedures.



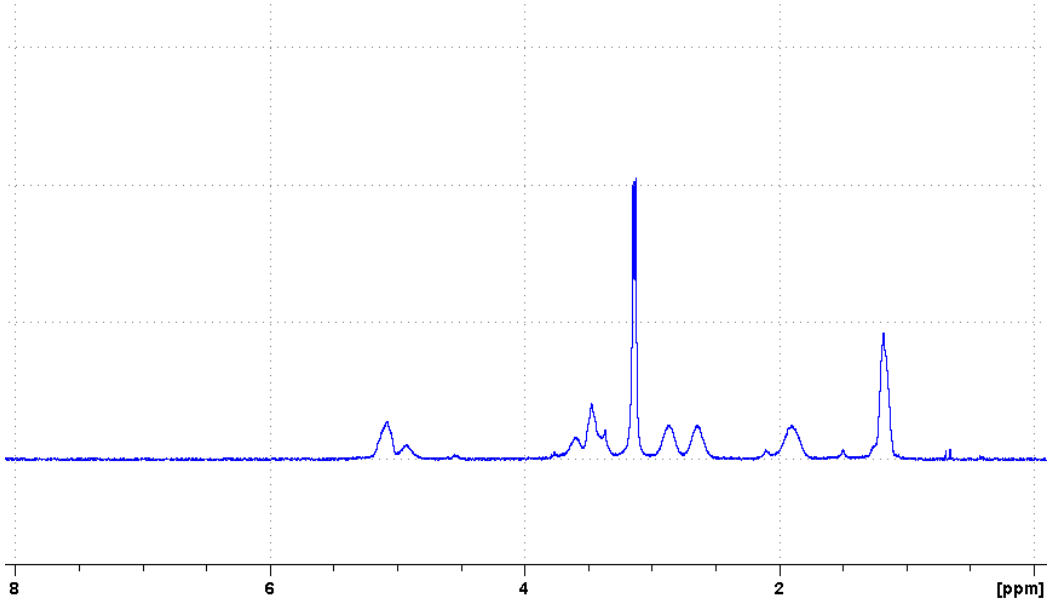
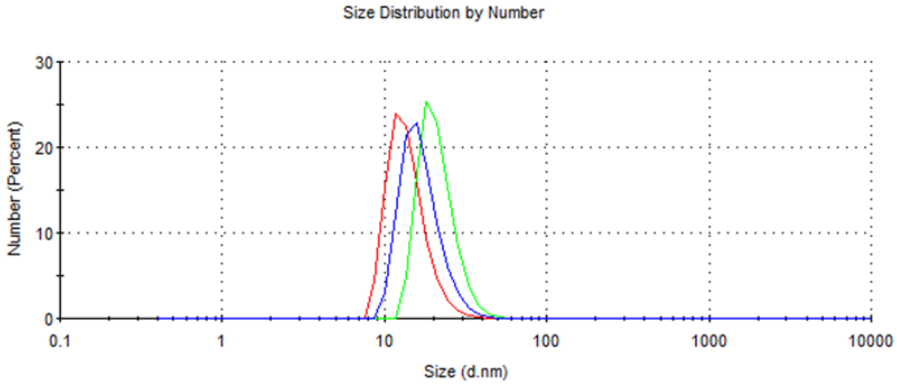
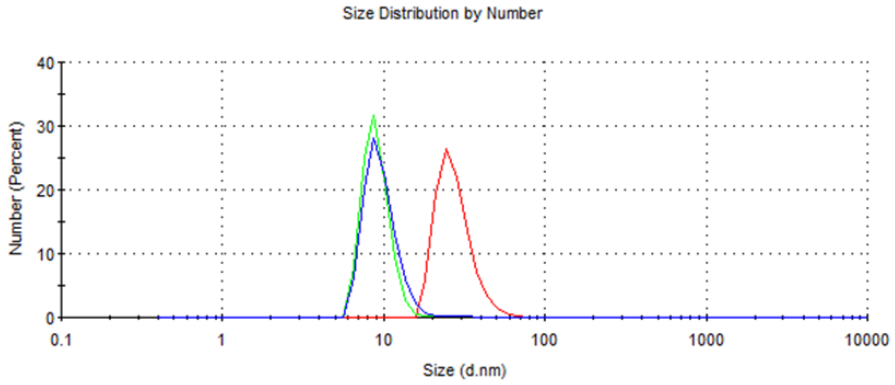


Figure 1.7: The synthesis and ^{1}H NMR of M^{O}_{71}(*rac*-L)$_{31}$.

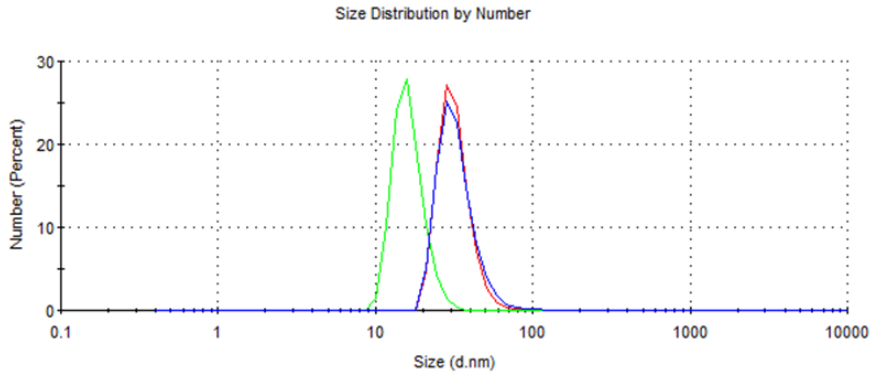
Once the polymers were modified, the methionine segments became water soluble, making the diblocks amphiphilic, and thus capable of self assembly in water. Based on previous research[1], the optimal length of diblock copolypeptides to form micelles was in the range of 60 and 100 residues, composed of 10-50 mol% of hydrophobic residues, and 90-50 mol% hydrophilic. Three different lengths were synthesized, M_{60}(*rac*-L)$_{15}$, M_{60}(*rac*-L)$_{21}$, and M_{60}(*rac*-L)$_{39}$. These diblocks were self assembled first by direct addition of solid polymer to water, and analyzed via Dynamic Light Scattering (DLS) to see if any samples formed assemblies in the micellular size range (10-50nm diameter). Both the carboxymethylated and the oxidized samples, of all compositions formed micellular sized assemblies.



$\mathbf{M^{O}_{60}(\mathit{rac}\text{-}L)_{15}}$ Avg. diameter: 17nm PDI: 0.50



$\mathbf{M^{O}_{60}(\mathit{rac}\text{-}L)_{21}}$ Avg. diameter: 16 nm PDI: 0.46

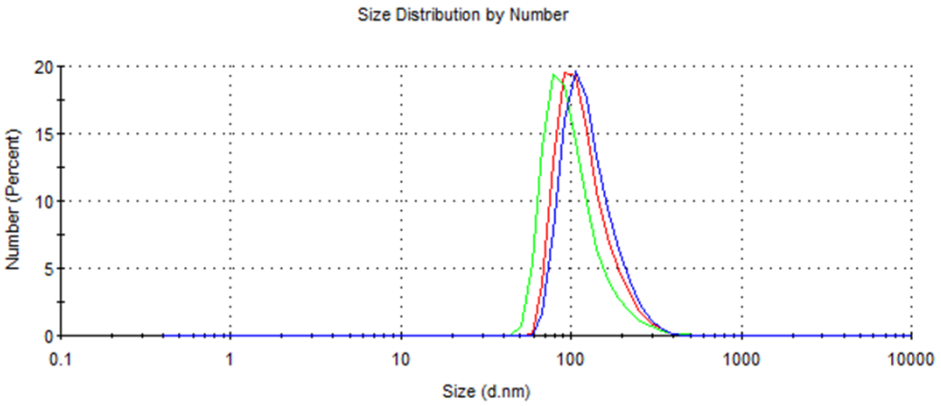


$\mathbf{M^{O}_{60}(\mathit{rac}\text{-}L)_{39}}$ Avg. diameter: 33 nm PDI: 0.38

Figure 1.8: Direct dissolution DLS data of $M^{O}_{60}(\mathit{rac}\text{-}L)_{x}$ at three different leucine lengths at 0.2

mg/mL. Three scans were taken for each sample. The average diameter is determined by an approximation from the time it takes for the sample to scatter light. Although each peak is from the same sample, scattering time varies marginally on each run, making the average diameters slightly different.

All three samples showed diameters consistent with micelle formation, however the polydispersities of the assemblies formed by direct dissolution were higher than desired. For DLS, a good PDI for monodisperse micelles is ≤ 0.05[7]. A PDI of 1.0 is the highest possible and implies a large distribution in assembly size. Different self-assembly techniques were tried, as well as various filtration methods to obtain lower polydispersities. The most effective technique proved to be solvent annealing procedures where the polymer was dissolved in either a single or a mixture of organic solvents and while stirring, a small amount of DI water was added dropwise. This mixture was stirred overnight, then dialyzed for 24 hours against DI water to remove the organic solvent, and was finally filtered through a 400nm pore size filter.



M^{O}_{60}(*rac*-L)$_{21}$ Avg. diameter: 120 nm PDI: 0.18

Figure 1.9: Solvent annealing DLS data of M^{O}_{60}(*rac*-L)$_{21}$ at 0.2 mg/mL in H_2O

This technique produced micelles with a lower PDI, but with diameters that were larger than expected. To confirm that spherical micelles had indeed formed, TEM images of the assemblies were taken. These images confirmed that the assemblies created through solvent

annealing were micelles and did have the expected diameter of 30-50 nm, and that the larger diameter seen in DLS was possibly due to micelle aggregation in suspension.

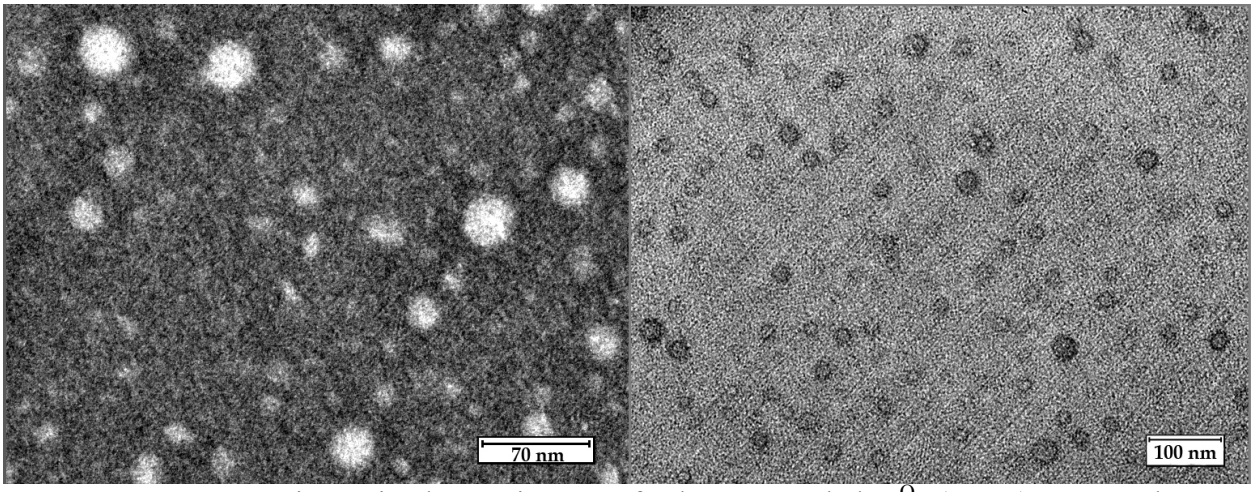


Figure 1.10: Negative stained TEM images of solvent annealed $M^{O}_{60}(rac\text{-}L)_{21}$ at 1 and 0.2 weight % respectively. Uranyl acetate stain applied for 20 seconds at 0.1 mg/mL.

To test the stability of the assemblies and check for aggregation, the solvent annealed micelles were exposed to a variety of conditions designed to disrupt any existing aggregation. The aqueous assemblies were exposed to Triton X-100 or SDS surfactant at concentrations up to 10 mg/mL, 1% by volume TFA, 10% by volume EtOH, 0.2 M NaCl, or extrusion.

Condition	Avg. Diameter (nm)	PDI
Triton X-100, 0.001 wt%/vol	25	0.277
SDS, 0.001M	26	0.281
10 mg/mL in H_2O	17	0.406
TFA, 1% by volume	62	0.277
EtOH, 10% by volume	130	0.319
NaCl, 0.2 M	61	0.283
Extrusion, 50 nm	37	0.252

Table 1.3: Solvent annealed $M^{O}_{60}(rac\text{-}L)_{21}$ at 0.2mg/mL* in various conditions. *except row 3, where the micelles were at 10 mg/mL.

These tests confirmed that the solvent annealed micelles were indeed in the range we had found by TEM, and that the micelles were also stable to a wide range of conditions. While the PDIs were higher than after the initial solvent annealing, they were within acceptable ranges.

The drug loading capabilities of the micelles were also tested. Camptothecin and pyrene were chosen as test molecules because they are hydrophobic and they are easily quantified via fluorometry[8]. A wide variety of drug loading methods were tried, however, none of the methods yielded significant drug retention.

Loading Technique	Pyrene Loaded(%)	CPT Loaded(%)
Direct Dissolution	24	1
Solvent Annealing	7	1

Table 1.4: Pyrene and camptothecin drug loading quantified via fluorescence. $M^{O}_{60}(rac\text{-}L)_{21}$ micelles prepared either by direct dissolution or solvent annealing. Loaded refers to the amount of drug encapsulated in the micelles after loading and dialysis.

1.4 Conclusion

We have synthesized two novel types of modified methionine based micelles. These micelles were formed by the self-assembly of diblock copolypeptides consisting of a hydrophobic racemic poly(leucine) segment and a hydrophilic modified poly(methionine) segment. The diblocks were shown to form micelles when the hydrophobic segment ranged from fifteen to forty mole percent of the overall composition. The hydrophobic poly(methionine) segment was modified to give either a sulfoxide or a carboxymethyl sulfonium hydrophilic segment.

Micelles were formed by self-assembly either through direct dissolution or solvent annealing. The solvent annealing method provided micelles with a lower polydispersity compared to direct dissolution.

Micelle stability was tested under a wide variety of conditions. The micelles showed stability while under exposure to acid, ethanol, surfactant, salt, extrusion, and when formulated at high concentrations. While the drug loading of the micelles was not in an optimal range, different loading techniques and hydrophobic drugs may be tested in the future.

1.5 Experimental

1.5.1 Materials and Methods

Reactions were done in oven-dried glassware under a nitrogen atmosphere using anhydrous solvents, any exceptions will be stated. THF, Hexanes, and DMF were purified by first being purged with dry nitrogen, then passed through activated alumina columns to remove water. Water was purified by taking in-house deionized water (18 MΩ-cm) and putting it through a Millipore Milli-Q Biocel A10 purification unit. Every commercial reagent was used as received, with no further purification unless mentioned otherwise. $(PMe_3)_4Co$ initiator was prepared using literature procedures.

The temperature of the reactions was controlled by an IKA magnetic temperature modulator, and all reactions were performed at room temperature (approximately 20 ^oC). Thin-layer chromatography (TLC) was performed using EMD gel 60 F254 precoated plates (0.25 mm). Visualization was completed using UV light, and when stain was required, phosphomolybdic acid or anisaldehyde was used. All flash column chromatography was done using Selecto silica gel 60 (particle size 32-63 μm). ^1H NMR spectra are reported relative to deuterated solvent signals, and recorded using Bruker spectrometers (at 300 MHz).

FTIR (Fourier Transform Infrared) samples were prepared on sodium chloride plates as films, and spectra were recorded on a Perkin Elmer RX1 FTIR spectrometer.

Extrusion of micelles was performed using an Avanti Mini Extruder with different pore size Whatman Nuclelopore Track-Etched polycarbonate (PC) membranes.

Dynamic Light Scattering (DLS) experiments were performed using a Malvern Zetasizer Nano ZS model Zen 3600 (Malvern Instruments Inc, Westborough, MA.) A total scattering intensity of

approximately 1 x 10^5 cps was targeted. The autocorrelation data was fitted using the CONTIN algorithm to determine the diameters of suspended assemblies. Measurements were taken at 0.2 mg/mL unless otherwise indicated. Low volume (400 μL) disposable cuvettes were used. The protein pre-setting was used for all measurements, and all data were reported using number weighting.

All Transmission Electron Microscopy (TEM) was performed using 300 mesh Formvar/ carbon coated copper grids (Ted Pella). The grids containing sample were imaged with a Tungsten filament CM120 transmission electron microscope at 120kV. All samples were analyzed with the computer program EMMENU4. Uranyl acetate was used to stain all samples, ranging from 0.1-2 weight %. Samples were at 0.1 mg/mL.

1.5.2 Preparation Methods

General Preparation of NCAs: Amino acid was added to a Schlenk flask and air was removed by placing the flask under vacuum for five minutes then refilling with dinitrogen and repeating three times. Dry THF was added via syringe (0.15 M) to the Schlenk flask. A solution of phosgene in toluene (20% (w/v), 3 equiv) is added via syringe. Warning! Phosgene is extremely hazardous and every interaction with it must be done in a well-ventilated chemical fume hood with appropriate personal protection and necessary precautions taken to avoid exposure. The reaction was stirred under N_2 at 45 ^oC for a total of 3 hrs. By 1.5 hours the amino acid should be completely dissolved and the solution should be clear. The reaction was stirred for 1.5 hours after all amino acid dissolves to ensure completion. After 3 hours, the reaction is evaporated to dryness, then transferred to a dinitrogen filled glove box. 50 mL of concentrated aqueous

NH_4OH was added to the condensate in the Schlenk line vacuum traps to neutralize residual phosgene.

Purification of racemic leucine (*rac*-L) NCA: NCA was purified by recrystallization 3x with minimal toluene in hexanes in a dinitrogen filled glove box.

Purification of L-methionine (Met) NCA: NCA was purified by silica chromatography in a dinitrogen filled glove box by loading the column with hexanes, and running the column from 20% to 40% EtOAc in hexanes. For 2.5g scale, a 1" x 4" column was used, and fractions 1-15 were collected.

General Procedure for Polymerization of NCAs: All of the polymerization reactions were performed in a dinitrogen filled glove box. NCA was measured out, and dry THF (50 mg/mL) was added and the NCA dissolved. A $(PMe_3)_4Co$ solution was prepared in THF (30 mg/mL) and added rapidly via syringe in one aliquot to the NCA solution. The reaction was conducted at room temperature and progress was monitored by removing small aliquots and analyzing them via FTIR. The reactions were usually complete after one hour. Small aliquots of the completed polymerizations were removed for end-capping experiments. Once reactions were complete, they were removed from the dinitrogen filled glove box and dried to a film. The polymers were washed with 0.2 mg/mL acidic water, centrifuged, and the water removed. They were washed with neutral water twice at 0.2 mg/mL, then dried under reduced pressure to yield white solids.

Preparation of Isocyanate Terminated poly(ethylene glycol): Amine terminated PEG (1.0g, 0.5 mmol, MW=2000, Nanocs) was added to a Schlenk flask and air was removed by placing the flask under vacuum for five minutes then refilling with dinitrogen and repressurizing three

times. Dry THF was added via syringe (25mL) to the Schlenk flask. A solution of phosgene in toluene (20% (w/v), 3 equiv) was added via syringe. The reaction was stirred under N_2 at room temperature for a total of 16 hrs. After 16 hours, the reaction was evaporated to dryness, then transferred to a dinitrogen filled glove box. 50 mL of concentrated aqueous NH_4OH was added to the condensate in the vacuum traps to neutralize residual phosgene. The isocyanate was purified by dissolving in minimal THF and then precipitating into hexanes. It was recovered as a white powdery solid.

General procedure for endcapping poly(methionine) with poly(ethylene glycol) and molecular weight determination by endgroup analysis:

Polymers were prepared by the general procedure described above. When the reactions were completed via FTIR monitoring, a small portion was removed and a solution of isocyanate terminated PEG (30 mg/mL in THF, 3 equiv per $(PMe_3)_4Co$)) was added. The reaction turned from a pale orange to a dark green. The reaction was left overnight at room temperature to ensure completion, then removed from the dry box. The solution was dried down to a film, then washed to remove all unconjugated PEG with two portions of water at pH 2 at 0.2 mg/mL, followed by 2 portions of deionized water at 0.2 mg/mL. The PEG end-capped polymers were lyophilized and obtained as white solids. To determine the polypeptide lengths (M_n), ^1H NMR spectra were obtained. It has been found that end-capping is quantitative when excess isocyanate is used, and integrations were calibrated using the polyethylene glycol chemical shift at δ 3.64.

Poly(L-methionine)$_{60}$-*block*-poly(*rac*-leucine)$_{10-40}$: All of the polymerization reactions were performed in a dinitrogen filled glove box. L-methionine-N-carboxyanhydride (M NCA) was dissolved in THF (50 mg/mL) and placed in a 20 mL scintillation vial. A $(PMe_3)_4Co$ solution

was prepared in THF (30 mg/mL) and added rapidly via syringe. The reaction was conducted at room temperature and progress was monitored by removing small aliquots and analyzing them via FTIR. The reactions were usually polymerized by one hour. Small aliquots of the completed polymerizations were removed for end-capping experiments. To the remainder of the polymerization reaction with active chain ends, a solution of racemic-leucine-N-carboxyanhydride (*rac*-L NCA) dissolved in THF was added (50mg/mL) via syringe. The vial was sealed and left in the glove box to react for 4 hours to give the diblock copolypeptides M_{60}(*rac*-L)$_{15\text{-}40}$. FTIR was used to confirm complete consumption of NCA. Outside of the dry box, the PEG end-capped polypeptide was isolated and purified by previously described methods. The average composition was determined by ^{1}H NMR integrations of PEG end-capped polymer and diblock copolymers.

Poly(L-methionine sulfoxide)$_{60}$-*block*-poly(*rac*-leucine)$_{10\text{-}40}$: Outside of the dry box, a sample of M_{60}(*rac*-L)$_{15\text{-}40}$ was added to a 20 mL scintillation vial. A solution of 1% AcOH in 30% H_2O_2 (1mL) was added to the vial, which was sealed and allowed to react for 20 min at 0 ^oC. It was then slowly warmed to room temperature for another 20 min. The sample was quenched with saturated sodium thiosulfate and transferred to a 2000 MWCO dialysis bag and dialyzed against water for 2 days with frequent water changes. The solution was lyophilized to dryness to yield a white solid. Yield = 80%.

Poly(S-(carboxymethyl)-L-methionine sulfonium)$_{60}$-*block*-poly(*rac*-leucine)$_{10\text{-}40}$: A 20 mL scintillation vial was charged with a sample of M_{60}(*rac*-L)$_{15\text{-}40}$. Bromoacetic acid was added in a threefold molar excess per methionine residue. H_2O was added to give a 10 mg/mL suspension. The reaction was covered in aluminum foil and stirred for 48 hours. The reaction mixture was

then dialyzed against 0.1M NaCl for 24 hours, followed by H_2O for 48 hours with frequent water changes. The solution was lyophilized to dryness to yield a white solid. Yield = 80%.

Direct dissolution self-assembly of poly(L-methionine sulfoxide)$_{60}$-*block*-poly(*rac*-leucine)$_{10-40}$ and poly((sodium carboxymethyl)-L-methionine sulfonium chloride)$_{60}$-*block*-poly(*rac*-leucine)$_{10-40}$: A 20mL scintillation vial was charged with M^{O}_{60}(*rac*-L)$_{15-40}$ or M^{C}_{60}(*rac*-L)$_{15-40}$. H_2O (2.5 mg/mL) was added. The vial was sonicated for 20 min, and filtered through a disposable 400nm filter. The suspension was diluted with filtered water for any experiments which were performed at lower concentrations.

Solvent annealing self-assembly of poly(L-methionine sulfoxide)$_{60}$-*block*-poly(*rac*-leucine)$_{10-40}$ and poly((sodium carboxymethyl)-L-methionine sulfonium chloride)$_{60}$-*block*-poly(*rac*-leucine)$_{10-40}$): A 20mL scintillation vial was charged with M^{O}_{60}(*rac*-L)$_{15-40}$ or M^{C}_{60}(*rac*-L)$_{15-40}$. A 50:50 THF: DMSO mixture (5 mg/mL) was added to the polymer and the suspension was stirred for 5 min. H_2O (2.5 mg/mL) was added dropwise, while the suspension stirred. The vial was left stirring with no cap for 16 hours, then transferred to a dialysis bag and dialyzed against DI water for 24 hours. The suspension was then filtered through a disposable 400 nm filter. The suspension was diluted with filtered water for any experiments which required it at a lower concentration.

Drug Loading: Fluorescence was measured on a Jobin-Yvon Horiba Spectramax-3 fluorimeter. Standard curves were made for both pyrene and camptothecin in 50:50 ethanol. For pyrene, the emission spectrum was recorded between 350-450nm with excitation at 333 nm. For

camptothecin, the emission spectrum was recorded between 400-500 nm, with excitation of 350 nm. All spectra was taken with an integration time of 1 second per 0.5 nm. Stock solutions of pyrene were made in chloroform at 2.0 mg/mL and stock solutions of camptothecin were made with 75:25 chloroform:methanol at 2.0 mg/mL. For direct dissolution, 63 μL of a stock solution was added to 0.5 mL of micelle suspension (0.5 wt. %), which was then covered in foil and allowed to stir overnight. The suspension was then dialyzed for 24 hours to remove the organic solvents, filtered through a 450 nm filter to remove unencapsulated, precipated cargo, followed by washing with 0.5 mL Millipore water and the filtrates combined. For solvent annealing, 63 μL of suspension was added to 2.5 mg lyophilized powder. The suspension was stirred, and 1 mL of H_2O was added dropwise, and the suspension was then covered in foil and stirred overnight. The suspension was dialyzed for 24 hrs to remove the organic solvents, filtered through a 450 nm filter to remove unencapsulated, precipated cargo. The precipitate was washed with 0.5mL Millipore water and the filtrate combined.

1.6 References

(1) Afsaneh, L.; Samuel, J.; Kwon, G, *Advanced Drug Delivery Reviews* **2002**, *54*, 169–190.

(2) Matsumura, Y.; Maeda, H., *Cancer Res.* **1986**, *46*, 6387-6392.

(3) Kramer, J.; Deming, T.J., *Biomacromolecules* **2012,** *13*, 1719-1723.

(4) Kramer, J.; Deming, T.J., *Biomacromolecules* **2010**, *11*, 3668-3672.

(5) Brzezinska, K. R.; Curtin, S. A.; Deming, T. J. *Macromolecules* **2002**, *35,* 2970-2976.

(6) Shechter, Y.; Burstein, Y., *Biochemistry*, **1975**, *14 (20)*, 4497-4503.

(7) Malvern Instruments Limited, *Inform White Paper*, **2011**, 1-6.

(8) Hanson, J.; Li, Z.; Deming, T.J., *Macromolecules* **2010**, *43,* 6268-6269.

CHAPTER 2

Phenyl Boronic Acid Modified Polypeptides

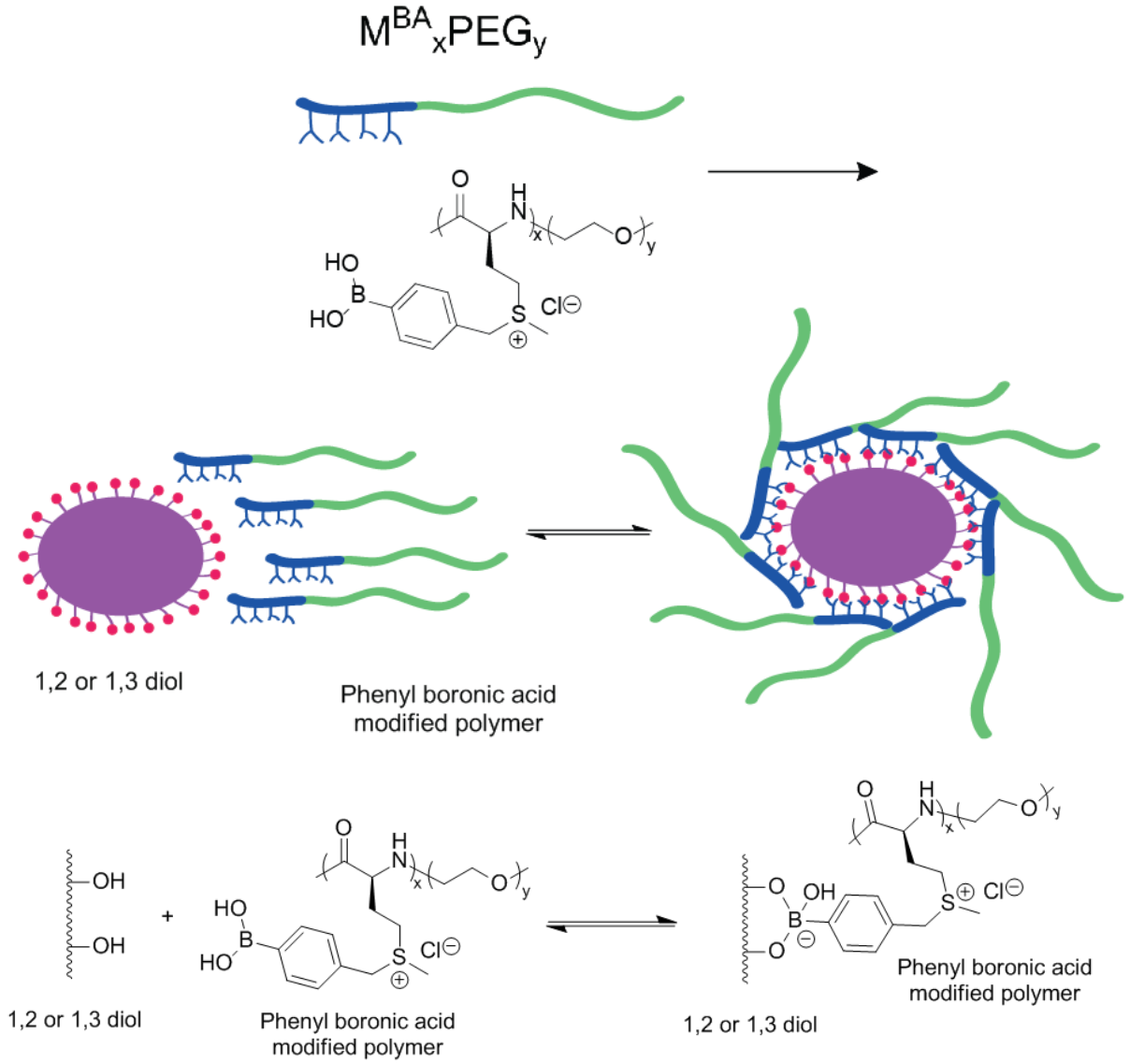


Figure 2.1: A schematic showing the covalent bonding of phenyl boronic acid modified polymers to a glycosylated substrate.

2.1 Abstract

We have synthesized poly(boroxyphenylmethyl)-L-methionine sulfonium-*block*-poly(ethylene glycol) ($M^{BA}_xPEG_y$) block copolymers. These water soluble polymers with boronic acid functionality bind covalently and reversibly to compounds that contain diol groups.

In particular, we have found that our polymers bind to glycosylated structures including cysteine polypeptides modified with carbohydrates, and iduronidase, a glycosylated enzyme.

2.2 Introduction

Boronic acids are not naturally occurring, but have been known since the late 1800s.[1] Recently they have received much attention in biomedical research because of their ability to reversibly covalently bind to diols.[2] In aqueous environments, boronic acids are in equilibrium between neutral, trigonal boron, and anionic tetrahedral boron. When boronic acids are in the tetrahedral geometry, they can form stable, reversible covalent linkages with 1,2- or 1,3-diols.[3] Attaching the boronic acid to a phenyl ring allows for the pK_a for the transition between trigonal and tetrahedral forms to be near physiological pH.[2]

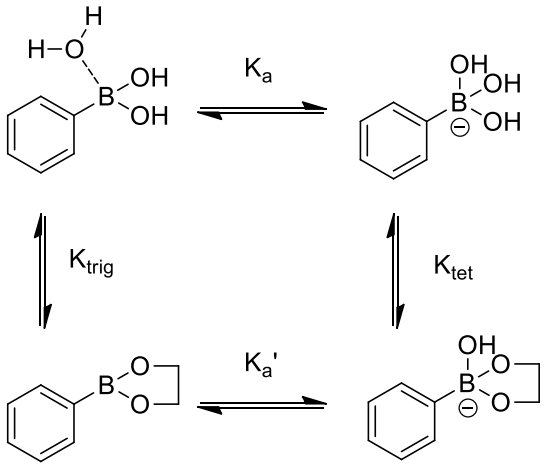


Figure 2.2: A schematic showing phenyl boronic acid in equilibrium between its trigonal and

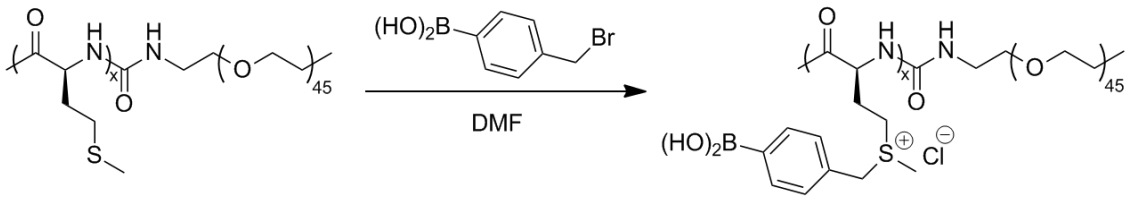
tetrahedral forms as well as its binding to diols. K_a and K_a' are the rates at which the boronic acids convert between tetrahedral and trigonal, K_{tet} and K_{trig} are the rates at which the boronic acid binds to diols. $K_{tet} > K_{trig}$.

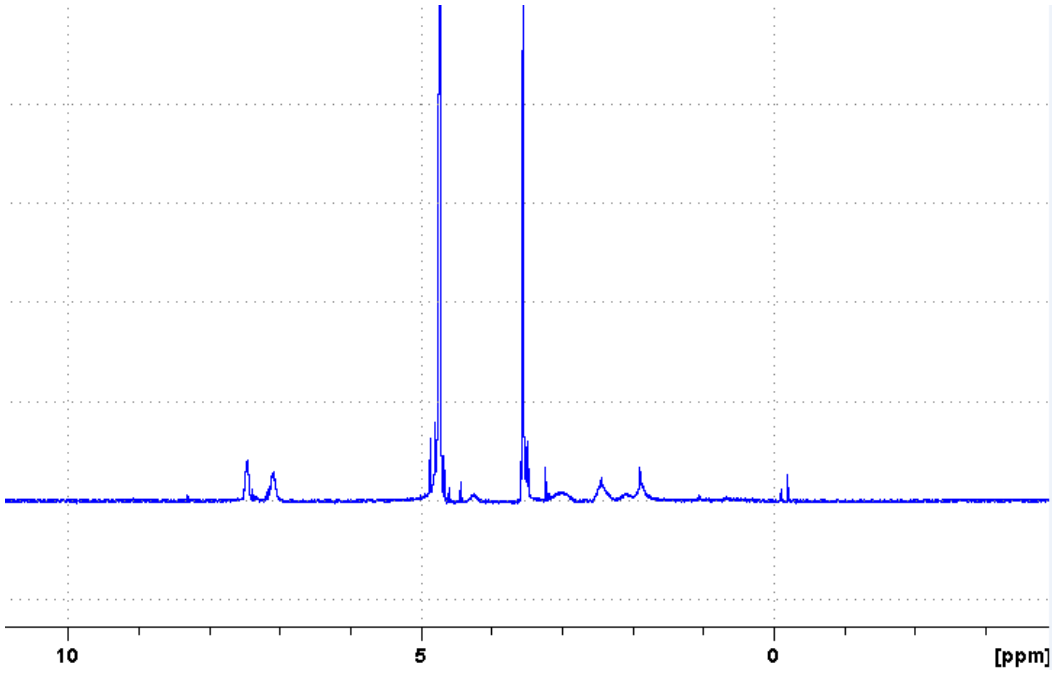
This reversible binding property has led to biomedical research interest including applications with lipase and HIV inhibition, as well as saccharide detection.[2] Because boronic acids can bind to diols, any polymer that is functionalized with boronic acid moieties will also bind to diols. In this way, diblock copolymers with one boronic acid binding segment and one hydrophilic segment can bind to and encapsulate macromolecules containing diol groups.

2.3 Results and Discussion

Poly(L-methionine)$_{33}$-*block*-poly(ethylene glycol)$_{45}$ copolymers ($M_{33}^{o\text{-}BA}PEG_{45}$ and $M^{p\text{-}BA}PEG$) were synthesized using a solvent mixture of DMF and THF to more easily obtain short methionine segment lengths. Addition of DMF to $(PMe_3)_4Co$ initiated NCA polymerizations in THF increases initiator efficiency, and yields shorter chain lengths. Short methionine segments were desired since only a few boronic acid moieties are required per chain to bond with diols, and so that the longer PEG_{45} segment can act as an efficient solubilizing agent. The PEG segment was added by traditional end-capping procedures.[4]

The poly(L-methionine)$_{33}$-*block*-poly(ethylene glycol)$_{45}$ diblocks were alkylated with either (4-(bromomethyl)phenyl)boronic acid or (2-(bromomethyl)phenyl)boronic acid. Routine alkylation procedures were used for both modifications,[5] and both resulting derivatives were found to be soluble in water.


(HO)2B
Br
DMF
(HO)2B
S
Cl
45


10
5
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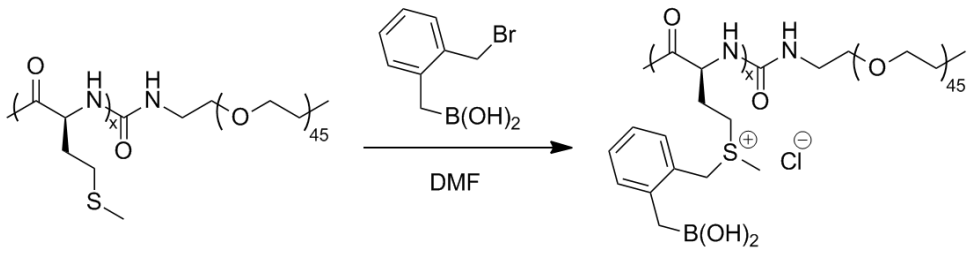
Br
B(OH)2
DMF
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Cl
B(OH)2
45

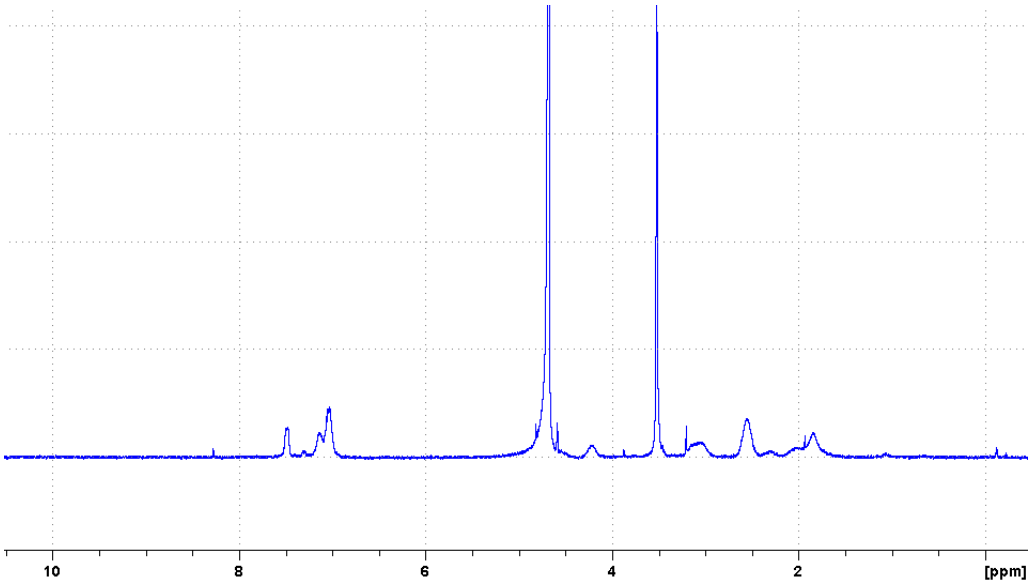


Figure 2.3: Synthesis and ^{1}H NMR spectra of $M^{o\text{-}BA}PEG_{45}$ and $M^{p\text{-}BA}PEG_{45}$.

Both ortho and para $M^{BA}{}_{33}PEG_{45}$ solutions were analyzed using DLS at a pH ranging from 6-8 to determine which pH resulted in the least aggregation. The ortho polymer had the least aggregation at pH 7, the para at pH 8. At these respective pHs both polymers (0.1 mg/mL) had count rates of under 100 kcps, which is considered to be a low aggregation rate.[7]

In an initial study, the $M^{BA}{}_{33}PEG_{45}$ polymers were combined with a glycosylated polymer previously synthesized in our lab[6]. At 0.1 mg/mL, at either pH 7 or 8, poly(α-gal-C^{O2})$_{80}$ had DLS count rates of under 140 kps.

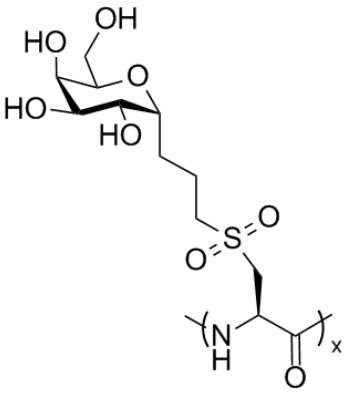
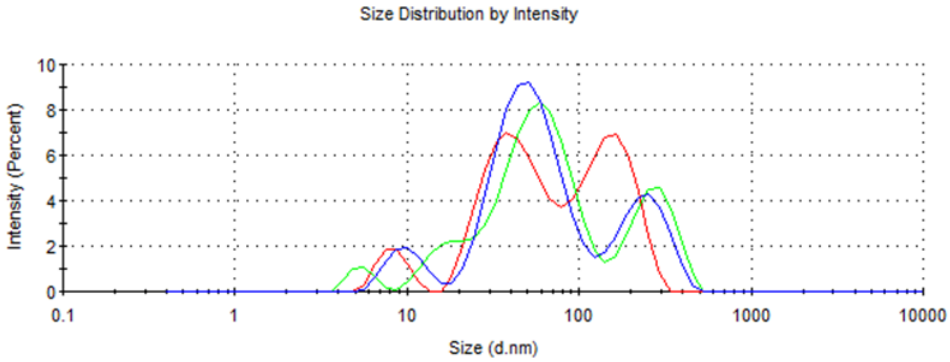
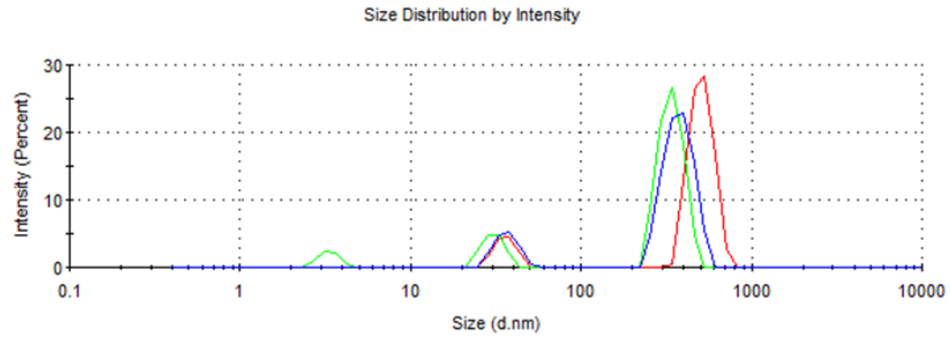


Figure 2.4: The structure of poly(α-gal-C^{O2})



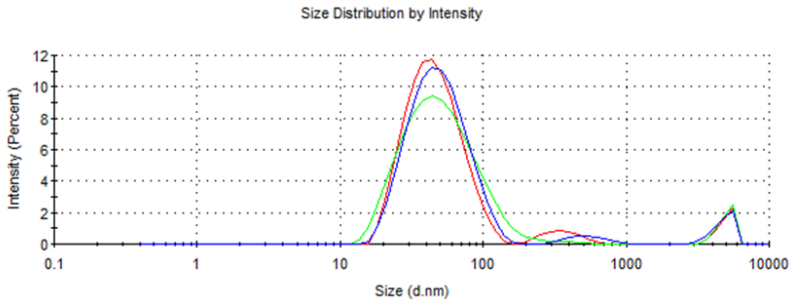
$M^{o\text{-}BA}{}_{33}PEG_{45,}$ pH 7, 0.1 mg/mL Z-avg: 130 nm PDI: 0.30 Count Rate: 98 kcps



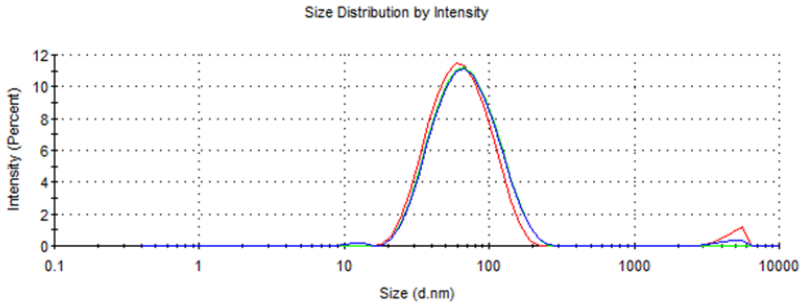
Poly(α-gal-C^{O2})$_{80,}$ pH 7, 0.1 mg/mL Z-avg: 1,000 nm PDI: 0.80 Count Rate: 130 kcps

Figure 2.5: The DLS measurements of both $M^{o\text{-}BA}{}_{33}PEG_{45}$ and poly(α-gal-C^{O2})$_{80}$

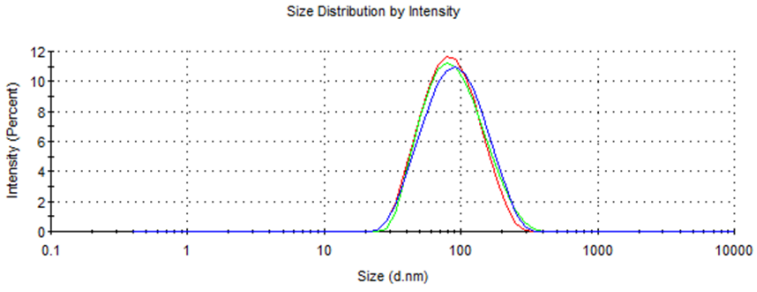
The $M^{o\text{-}BA}{}_{33}PEG_{45}$ polymer and poly(α-gal-C^{O2})$_{80}$ were combined at 2:1, 1:1, and 1:2 ratios of boronic acid to sugar moieties, respectively, at pH 7 and 0.1 mg/mL for both concentrations.



2:1 poly(α-gal-C^{O2})$_{80}$: $M^{o\text{-}BA}{}_{33}PEG_{45}$ pH 7, sample at 0.1 mg/mL Z-avg: 48 nm PDI: 0.36 Count Rate: 120 kcps



1:1 poly(α-gal-C^{O2})$_{80}$: $M^{o\text{-}BA}{}_{33}PEG_{45}$ pH 7, sample at 0.1 mg/mL Z-avg: 60 nm PDI: 0.21 Count Rate: 130 kcps



1:2 poly(α-gal-C^{O2})$_{80}$: $M^{o\text{-}BA}{}_{33}PEG_{45}$ pH 7, sample at 0.1 mg/mL Z-avg: 78 nm PDI: 0.19 Count Rate: 330 kcps

Figure 2.6: DLS of mixtures of poly(α-gal-C^{O2})$_{80}$ and $M^{o\text{-}BA}{}_{33}PEG_{45}$ at pH 7

Even when there were twice as many sugars as boronic acid groups, there was significant complexation between the two polymers. Whereas the poly(α-gal-C^{O2})$_{80}$ solution alone contained some particles with Z-average diameters of over a micron, few large aggregates were present in the mixture at this ratio. When there were an even number of sugar and boronic acid groups, the PDI (0.21) was lower than either polymer on its own. At twice as many boronic acid groups per sugar, the PDI was significantly less at 0.19, and the count rate was 330 kcps, or three times the individual count rates indicating more particles which scatter more light. TEM imaging confirmed that each separate polymer solution contained a few small aggregates, whereas the same concentration of a mixture both polymers showed many larger aggregates.

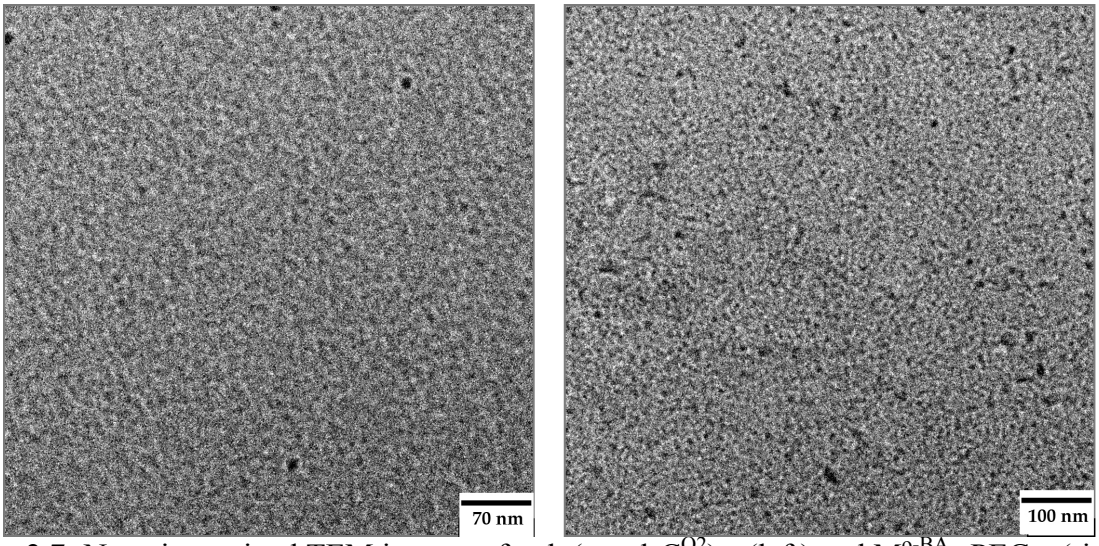


Figure 2.7: Negative stained TEM images of poly(α-gal-C^{O2})$_{80}$ (left) and $M^{o\text{-}BA}{}_{33}PEG_{45}$ (right) at pH 7, 0.1 mg/mL.

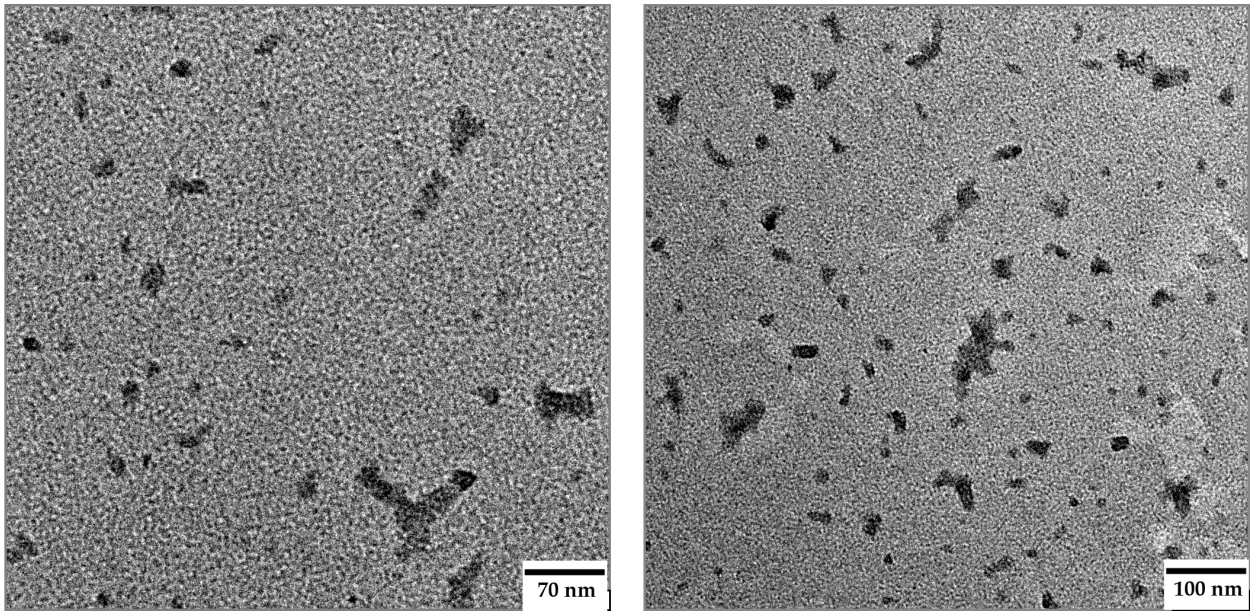


Figure 2.8: Negative stained TEM images of poly(α-gal-C^{O2})$_{80}$ and $M^{o\text{-}BA}{}_{33}PEG_{45}$ combined at a 1:2 ratio at pH 7, solution at 0.1 mg/mL.

The complexing properties of $M^{o\text{-}BA}{}_{33}PEG_{45}$ and $M^{p\text{-}BA}{}_{33}PEG_{45}$ were also tested by mixing with the glycosylated enzyme alpha-L-iduronidase (IDUA) at neutral pH. IDUA is an enzyme in the metabolic pathway that controls the degradation of heparin sulfate and dermatan sulfate.[8] By coating the enzyme with the $M^{BA}PEG$ polymers, the PEG segment of the polymer was be exposed to the solvent. This has a solubilizing effect on the enzyme, possibly allowing better cellular uptake. The enzyme was combined with each polymer in the ratio of 1 enzyme per 20 polymer strands, which is approximately one enzyme glycosylation site for every 100 boronic acid moieties.

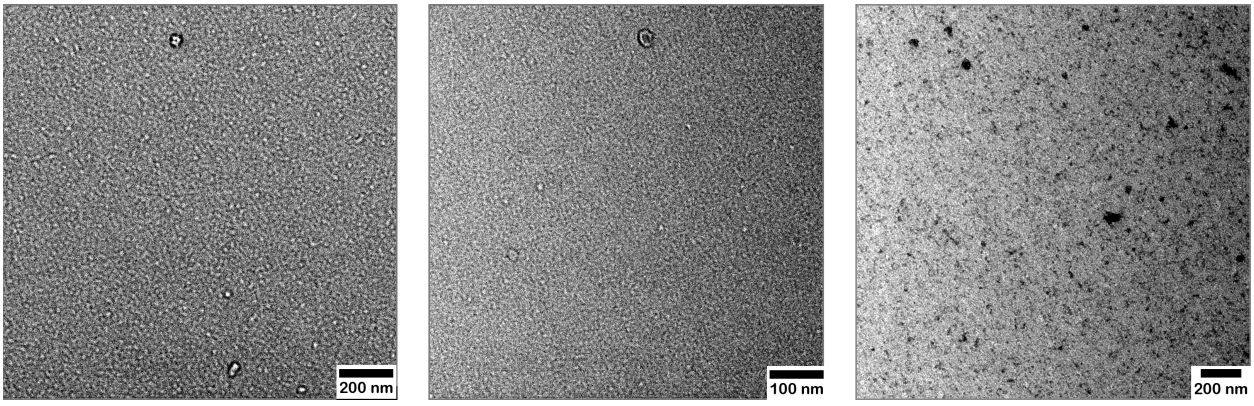


Figure 2.9: Negative stained TEM images from left to right: $M^{o\text{-}BA}{}_{33}PEG_{45}$ at 0.18 mg/mL, $M^{p\text{-}BA}{}_{33}PEG_{45}$ at 0.18 mg/mL, and IDUA at 0.07 mg/mL, all at pH 7.4.

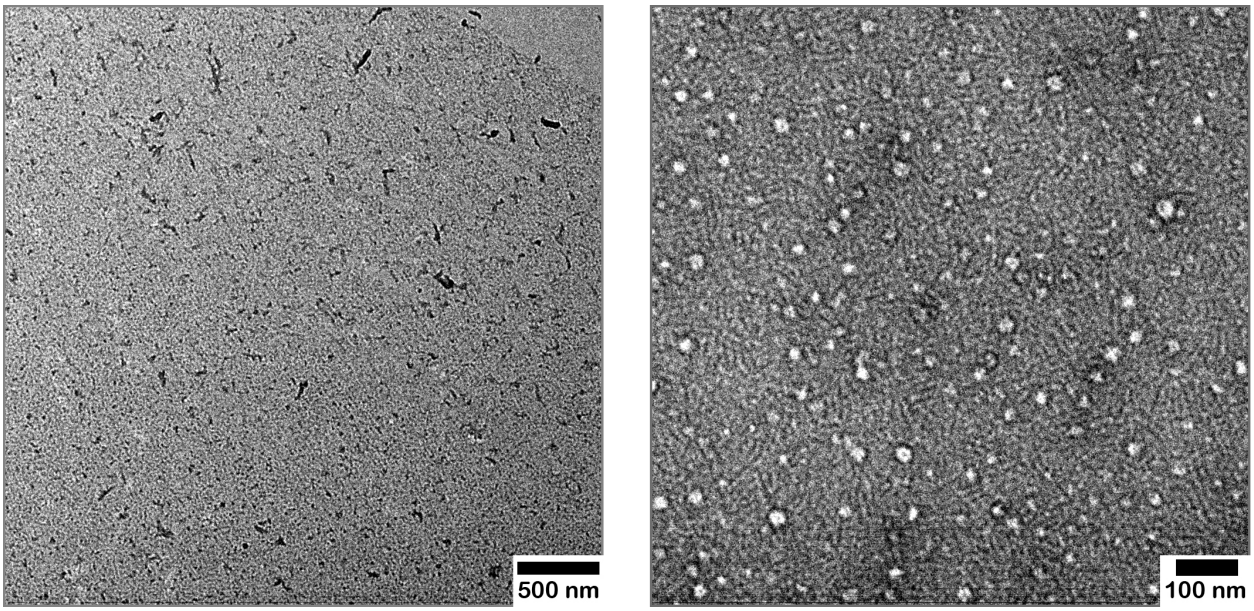


Figure 2.10: Negative stain TEM images. An aqueous mixture consisting of 1 enzyme: 20 polymer strands with IDUA at 0.07 mg/mL and $M^{o\text{-}BA}{}_{33}PEG_{45}$ at 0.18 mg/mL (left), and a mixture of 1 enzyme: 20 polymer strands with IDUA at 0.07 mg/mL and $M^{p\text{-}BA}{}_{33}PEG_{45}$ at 0.18 mg/mL (right). All samples at 7.4 pH.

Both of the boronic acid modified copolymers showed small amounts of aggregation at 0.18 mg/mL. Iduronidase showed more significant aggregation at 0.07 mg/mL, which was mostly due to particles in the less than 50 nm size range. When iduronidase was combined with $M^{o\text{-}BA}{}_{33}PEG_{45}$, it showed the formation of worm shaped particles which were not present in either of the starting reagents. When iduronidase was combined with $M^{p\text{-}BA}{}_{33}PEG_{45}$, it formed monodisperse spherical particles of approximately 10-20 nm. Additionally, in the $M^{p\text{-}BA}{}_{33}PEG_{45}$ mixture the unordered, random aggregates seen in the enzyme only sample were non-existent. This suggested that the boronic acid polymers were indeed interacting with the enzyme and complexing it in aqueous solutions.

2.4 Conclusion

Poly(methionine)-block-poly(ethylene glycol) diblock copolymers were synthesized and the poly(methionine) segments were modified to contain phenyl boronic acid moieties. These modified polymers were combined with polyol containing substances to form reversible, covalently bonded complexes. The suspensions of the diblock with glycosylated poly(cysteine) formed large aggregates which were not present in the precursors.

The diblock copolymers were also combined with the glycosylated enzyme iduronidase, and were found via TEM to complex the protein. Binding the polymers to the sugar sites on the enzyme using boronic acids allowed us to add a hydrophilic poly(ethylene glycol) coating to the enzyme. In the future, these materials could be combined with additional proteins, viruses, or other structures that have biomedical uses.

2.5 Experimental

2.5.1 Materials and Methods

See chapter one experimental

(Poly(*S*-(boroxyphenylmethyl)-L-methionine sulfonium chloride),)-*block*-poly(ethylene glycol): A 20mL scintillation vial was charged with $M_{17}PEG_{45}$. Bromo methyl boronic acid was added in 3x excess. 10 mg/mL of DMF per mg of polymer was added. The reaction was covered in foil and stirred for 48 hours. The reaction mixture was then dialyzed against 0.1M NaCl for 24 hours, and H_2O for 48 hours with frequent water changes. The solution was lyophilized to dryness to yield a white solid. Yield is 80%. Provide spectral data.

Phosphate Buffers: Sodium phosphate buffers were made at pHs 6, 7, and 8 by titrating sodium phosphate di basic and sodium phosphate mono basic. All buffers were made using a pH meter at 10 mg/mL and diluted to 0.2 mg/mL for DLS measurements.

2.6 References

(1) Frankland, E.; Duppa, B.F., *Justus Liebigs Annalen der Chem* **1860**, *115(3),* 319e22.

(2) Cambre, J.; Sumerlin, B., *Polymer* **2011**, *52*, 4631-4643.

(3) Springsteen, G.; Binghe, W., *Tetrahedron* **2002**, *58(26),* 5291-5300.

(4) Brzezinska, K. R.; Curtin, S. A.; Deming, T. J. *Macromolecules* **2002**, *35,* 2970-2976.

(5) Kramer, J.; Deming, T.J., *Biomacromolecules* **2012,** *13*, 1719-1723.

(6) Kramer, J.; Deming, T.J., *J. Amer. Chem. Soc.,* **2012,** *134*, 4112-4115.

(7) Malvern Instruments Limited, *Inform White Paper*, **2011**, 1-6.

(8) Hopwood, J., *Am. J. Hum. Genet.*, **1990**, *47(5)*, 802-8