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Modification of poly(L-Homoallylglycine) for the Synthesis of Functional Polypeptides

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Modification of poly(L-Homoallylglycine) for the Synthesis of Functional Thioethers

A dissertation submitted in partial satisfaction of the

requirements for the degree of Doctor of Philosophy

in Chemistry

by

Pesach Perlin

2019

ABSTRACT OF THE DISSERTATION

Modification of poly(L-Homoallylglycine)

for the Synthesis of Functional Polypeptides

by

Pesach Perlin

Doctor of Philosophy in Chemistry

University of California, Los Angeles, 2019

Professor Timothy J. Deming, Chair

Synthetic polypeptides have demonstrated great potential for a variety of biomaterial applications due to their ability to self-assemble into ordered structures. The ability to design polypeptides with a variety of functionalities is necessary for tailoring the solubility, conformational properties, and environmental responses of polypeptide-based materials. Post polymerization modification of reactive residues has emerged as a desirable method for the design of functional polypeptides due to straightforward monomer synthesis and purification and the potential to synthesize a variety of functional polypeptides from a single reactive polypeptide precursor. This dissertation describes the development and subsequent modification of poly(L-homoallylglycine), a soluble, α-helical alkene bearing polypeptide.

L-Homoallylglycine *N*-carboxyanhydride monomers were synthesized and used to prepare poly(L-homoallylglycine) polypeptides with controllable lengths of up to 245 residues. These polypeptides were modified under mild conditions via UV initiated thiol-ene chemistry to give a variety of α-helical water soluble thioether containing polypeptides. These derivatives

were able to undergo a conformational change from α-helix to random coil upon thioether oxidation or alkylation. Incorporation of L-homoallylglycine residues into block copolypeptides with L-methionine residues allowed for the synthesis of block copolypeptides with separate ordered segments of sulfoxide residues and sulfonium residues.

The thiol-ene reactivity of poly(L-homoallylglycine) was then utilized to synthesize polypeptides containing N-methylaminooxy functionality. The solubility, conformation, and reactivity of these poly(L-Homoallylglycine) derived polypeptides was compared to more hydrophilic N-methylaminooxy polypeptides synthesized via a functional monomer approach. The unique reactivity of N-methylaminooxy groups with non-protected reducing sugars facilitated the straightforward synthesis of glycopolypeptides which possessed good aqueous solubility and were stable at pH 7.4 for one week. This post polymerization modification technique shows promise as a potential strategy for the synthesis of proteoglycan mimics.

The thiol-ene chemistry of poly(L-homoallylglycine) was proven to be highly versatile, but the resulting thiol-ene conjugates often have limited aqueous solubility due to their long hydrophobic side chains. Therefore, poly(L-homoallylglycine) was oxidized to an epoxide bearing polypeptide, poly(5,6-epoxy-L-norleucine) and modified with thiols under basic conditions to synthesize a variety of β-hydroxy thioether containing polypeptides with considerably higher aqueous solubility than previously synthesized thiol-ene conjugates. Diethylene glycol thiol modified poly(5,6-epoxy-L-norleucine) derivatives displayed lower critical solution temperature properties in water that could be modulated by varying polypeptide concentration, polypeptide composition, and could be switched off through oxidation of their thioether groups.

The dissertation of Pesach Perlin is approved.

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University of California, Los Angeles

2019

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List of Abbreviations

AcOH	glacial acetic acid
Boc	*tert*-butoxycarbonyl
Boc-mao Ehc	poly(2-(N-Boc-N-methylaminooxy)ethyl-L-homocysteine)
Boc-mao-Ehc^{M}	poly(2-(N-methylaminooxy)ethyl-L-homocysteine methyl sulfonium)
Boc-mao-Ehc^{O}	poly(2-(N-methylaminooxy)ethyl-L-homocysteine sulfoxide)
Boc-mao-Etn	poly(6-(2-N-Boc-N-methylaminooxy)ethylthio)-L-norleucine)
Boc-mao-Etn^{O}	poly(6-(2-N-Boc-N-methylaminooxy)ethylthio)-L-norleucine sulfoxide)
br	broad
CSA	(+/-) camphorsulfonic acid
d	doublet
DART-MS	direct analysis in real time mass spectrometry
dd	doublet of doublets
ddq	doublet of doublet of quartets
dq	doublet of quartets
dt	doublet of triplets
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCM	dichloromethane
DI	deionized
DMF	N,N-dimethylformamide
DMPA	2,2-dimethoxy-2-phenylacetophenone
DP	degree of polymerization

eq	equivalents
Enl	poly(5,6-epoxy-L-norleucine)
R-Enl	poly(5,6-epoxy-L-norleucine) thiol-epoxy conjugate
Et_2O	diethyl ether
EtOAc	ethyl acetate
G^{HA}	poly(L-homoallyglycine)
R-G^{HA}	poly(L-homoallyglycine) thiol-ene conjugate
Hag	L-homoallylglycine
HFIP	1,1,1,3,3,3-hexafluoroisopropanol
hv	ultraviolet light irradiation
K	poly(L-lysine·HCl)
KOtBu	potassium tert-butoxide
M : I	monomer to initiator ratio
mao-Ehc^{O}	poly(2-(N-methylaminooxy)ethyl-L-homocysteine sulfoxide)
mao-Ehc^{M}	poly(2-(N-methylaminooxy)ethyl-L-homocysteinemethyl sulfonium)
mao-Etn^{O}	poly(6-(2-N-methylaminooxy)ethylthio)-L-norleucine sulfoxide)
Met	L-methionine
M	poly(L-methionine)
m	multiplet
MeCN	acetonitrile
MeI	methyl iodide
MeOH	methanol
MWCO	molecular weight cutoff

NCA	N-carboxyanhydride
PEG-NCO	α-methoxy-ω-isocyanoethyl-poly(ethylene glycol)
p	pentet
q	quartet
rotovap	rotary evaporation
RT	room temperature
s	singlet
t	triplet
TBHP	tert-butyl hydroperoxide
TMS	trimethylsilane
TMSCl	trimethylsilylchloride
TEA	triethylamine
TFA	trifluoracetic acid
THF	tetrahydrofuran

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1. Kaneko, T.; Asif Ali, M.; Captain, I.; Perlin, P.; Deming, T.J., Polypeptide gels incorporating the exotic functional aromatic acid 4-amino-L-phenylalanine. *Polym. Chem.* **2018**, 9, 3466-3472.

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3. Wollenberg, A.L.; Perlin, P.; Deming, T.J., Versatile N-methylaminooxy functionalized polypeptides for preparation of neoglycoconjugates. *Biomacromolecules* **2019**, 20, 1756-1764.

Chapter 1: Functional Polypeptides: Synthesis and Applications

1.1 Polypeptide Synthesis

Proteins and peptides play vital roles in every living organism. These naturally occurring molecules are composed of various amino acids combined in unique and precise sequences.[1] They are involved in many physiological and biological processes including transport of substances through membranes, intracellular signaling, and playing a role in organisms' immune system as antibodies. Therefore, proteins and peptides have been of major interest to biologists and chemists alike for well over a century, inspiring a variety of biological and chemical methods to synthesize and isolate these macromolecules.[2]

Biological protein synthesis involves utilizing organisms' cellular machinery to synthesize complex, monodisperse polypeptides and can be divided into two categories: cloning a DNA sequence from an organism which encodes a natural protein, and designing a DNA sequence to encode an originally designed amino acid sequence tailored for specific structure and function.[3] The DNA sequences are then inserted into cells in the form of plasmids, where proteins expressed and synthesized *de novo* from their amino acid monomers.[4]

Cloning naturally occurring DNA sequences has been utilized to synthesize enzymes which are useful for a variety of chemical processes[5], as well as naturally occurring protein-based materials.[6] However, the scope of this method is restricted by what is readily available in nature. Synthesis of non-natural proteins has been used to design proteins with discrete structural motifs, such as β-sheets[7] and α-helices,[8] allowing for complex protein structure activity relationship studies.[9] This technique can also be applied to incorporate non-canonical amino acids into proteins which can then be subsequently chemically modified to modulate activity or label the protein for observation *in vivo*.[10]

Though recombinant DNA based synthetic techniques are useful for synthesizing highly complex polypeptides with precise sequence control, there are notable drawbacks to these methods. If modifications to a sequence are required, a new gene must be synthesized. Biosynthesized proteins often require intensive purification protocols or modification of the amino acid sequence for purification via affinity chromatography.[4] This methodology can also be intolerant of non-canonical amino acids, resulting in low yields.[11]

Chemical polypeptide synthesis aims to avoid these pitfalls, though it is difficult to rival the length and structural complexity of biosynthesized proteins.[4] The two most common chemical polypeptide synthesis methods are solid phase peptide synthesis (SSPS) and N-carboxyanhydride (NCA) polymerization.[12] Solid phase peptide synthesis (Figure 1.1) is a process involving immobilization of a growing peptide chain on a solid resin, stepwise addition of N-protected amino acid monomers through use of a coupling agent (current methods use triazoles such as HOBt or HBTU), and cleavage from the resin to release the synthesized polypeptide.[13]

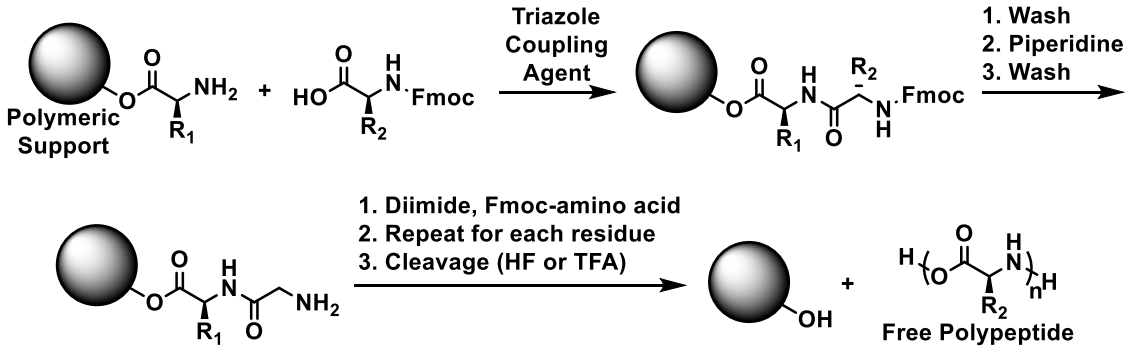

Figure 1.1 Solid-phase peptide synthesis. Fmoc = fluorenylmethoxycarbonyl.

Though this method produces polypeptides with sequence control rivaling biosynthetic methods and is highly compatible with non-canonical amino acids, it is not practical for preparation of large polypeptides (100 residues or greater).[12] Each residue addition requires at

least four steps, and additional protecting group considerations must be made for amino acids with reactive side chains.[1] This process suffers from low atom economy and sequence defects due to incomplete coupling and deprotections steps.[13] NCA polymerization is currently the most efficient method for synthesizing high molecular weight polypeptides. This technique involves the ring opening polymerization of cyclic anhydride derivatives of amino acid to synthesize polypeptides (Figure 1.2).

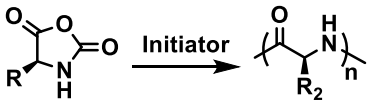

Figure 1.2 Ring-opening polymerization of NCA monomers.

Benefits to NCA polymerization include the potential for high molecular weight, high atom economy, simple deprotection, and facile isolation.[12] Though NCA polymerization does not have the same precision in sequence control of SPPS, current initiation methods allow for the synthesis of multiblock copolypeptides of defined size and sequence that give potential for highly precise structure and function.[14]

1.2 NCA Polymerization

NCA polymerization has been the most common method for large scale preparation of high molecular weight polypeptides for over 70 years.[15] Until the late 1990's, materials synthesized through this procedure were primarily homopolymers, random copolymers, and other architectures which lacked the sequence specificity and low dispersity desirable for use in material applications.[12, 14, 16] The limitations of NCA polymerization were due to initiation methods that gave poor control leading to deleterious side reactions. However, the development of controlled NCA polymerization methods has allowed for the synthesis of high molecular weight polypeptides with low dispersity and high complexity that have facilitated the design of a variety of polypeptide-based materials.[14, 17]

There are two common methods for the preparation of NCA's: the Leuchs method and the Fuchs-Farthing method (Figure 1.3)[18]. The Leuchs method involves cyclization of N-alkoxycarbonyl amino acid halides.[19] The acid halide is typically generated in situ via use of a halogenating agent such as PX_3, PX_5 (X = Cl, Br), dichloromethyl methyl ether, or Ghosez reagent.[16, 20, 21] The Fuchs-Farthing method, on the other hand is used to synthesize an NCA directly from a free amino acid via treatment with phosgene.[22, 23]

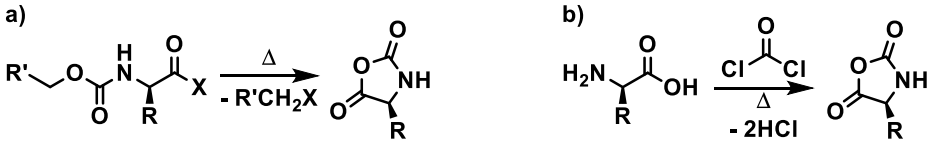

Figure 1.3 Common methods of NCA synthesis. a) Leuchs method, X = Cl, Br. b) Fuchs-Farthing method.

Due to the high volatility of phosgene, many later procedures use liquid diphosgene[24] and solid triphosgene.[25] Though the Leuchs method typically leads to more impurities than the Fuchs-Farthing method, both synthetic methods can be used to produce pure NCAs after purification steps including precipitation, crystallization, or a recently developed purification method involving anhydrous column chromatography.[26] Therefore, the method used should be chosen based on the desired NCA. If synthesizing an NCA from a free amino acid, the Fuchs-Farthing method is preferable. However, some amino acids require protection of the alpha-amino group during their synthesis, making the Leuchs method more convenient.[27]

The three most prominent methods of controlled NCA polymerization are amine-initiated, transition metal initiated, and silane initiated polymerizations.[17] Though traditional amine-initiated polymerizations showed poor control, techniques such as polymerization under vacuum[28], and polymerization under low temperature[29] were developed to reduce side reactions, allowing for good length control and low polydispersity. The mechanism for this

process involves nucleophilic attack by the amine to open the ring and subsequent loss of CO_2 (Figure 1.4).

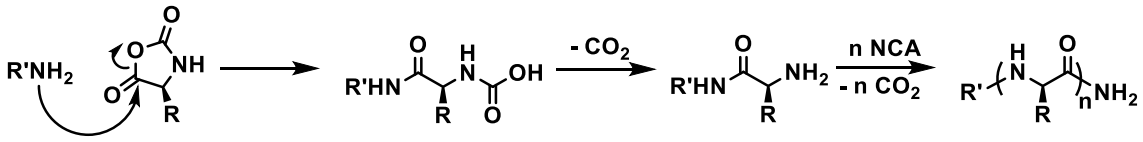

Figure 1.4 Amine-initiated mechanism for NCA polymerization.

Transition metal initiation allows for living NCA polymerization with control of chain lengths and low dispersity at a considerably higher polymerization rate than controlled amine-initiated polymerizations, allowing for facile synthesis of complex block copolypeptides.[12] This method involves the use of electron-rich zerovalent complexes of Cobalt, Nickel, Iron, Ruthenium, or Platinum.[30] The mechanism (Figure 1.5) begins with oxidative addition of the metal across the anhydride bond followed by subsequent loss of carbon monoxide.[31] Addition of a second monomer unit followed by loss of CO_2 then occurs to form a six-membered metallocycle. The propagating species was found to be a five-membered amido-amidate metallocycle, which is formed by subsequent monomer addition followed by CO_2 loss and proton migration.[12] The zerovalent complex $Co(PMe_3)_4$ has been shown to be very reactive during initiation, giving good initiation and propagation rate for NCA polymerization.[17]

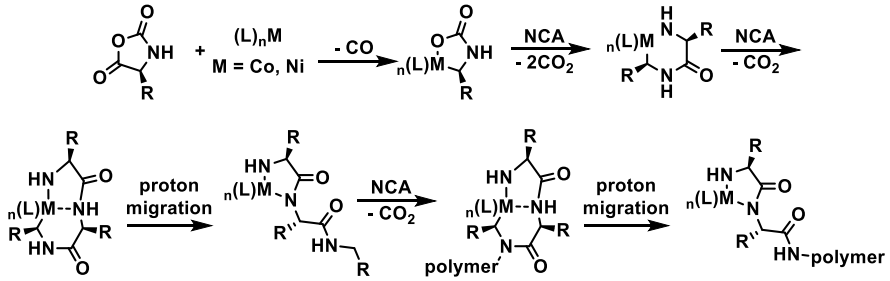

Figure 1.5 Zerovalent metal-initiated mechanism for NCA polymerization.

A third distinct method for the ring opening polymerization of NCA monomers was developed utilizing hexamethyldisilazane (HMDS).[32] Unlike traditional amines, HMDS allows for

controlled polymerization at room temperature and standard pressure.[17] HMDS and similar N-TMS amines are thought to open the NCA to yield a TMS-carbamate active chain-end[12] that reduces side reactions in a similar fashion to group-transfer polymerization of vinyl monomers (Fig 1.6).[33] These initiators can be used in a similar fashion as zerovalent metal initiators to synthesize block copolypeptides, but polymerize at a considerably slower rate.[17]

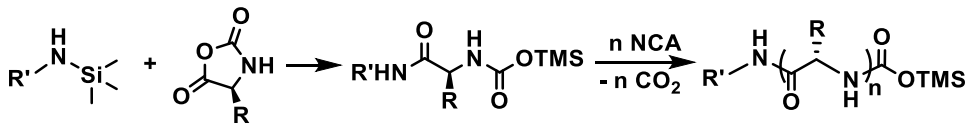

Figure 1.6 N-TMS amine-initiated mechanism for NCA polymerization.

A notable difference between NCA polymerization and the other methods of polypeptide synthesis is the lack of precise sequence control.[4] However, the controlled polymerization techniques discussed in this section allow for the synthesis of block copolypeptides with segments of controlled length. This allows for regional control of polypeptide conformation and solubility, similar to what is seen in proteins with more complex amino acid sequences, allowing for the design of a variety of functional materials which will be discussed in depth in the following section.[12]

1.3 Polypeptide Materials for Biomedical Applications

The versatile functionality of proteins is due to their ability to form ordered structures through hydrogen bonding within the protein backbones and non-covalent interactions involving the amino acid side chains. The ability of NCA derived polypeptides to form defined structures makes them highly attractive for material applications. The ability to form organized self-assembled structures and biodegradability of polypeptide materials has led to their use in a variety of applications including drug delivery, gene delivery, and tissue engineering.[34]

The solubility, conformation, and length of the individual polypeptide segments determine the type of assembly formed. The conformations commonly employed in the design

of polypeptide materials are α-helices, β-sheets, and random coils.[35] The flexibility of random coils prevent aggregation allowing for facile solvation of the peptide backbone, and facilitate assembly under mild conditions due their high hydrophilicity.[36] α-helices, on the other hand, are stiff and rod-like. Helical polypeptides are prone to dense packing, which is beneficial in the assembly of supramolecular materials.[35] Due to their powerful interchain H-bonding, β-sheets are insoluble in nearly all solvents, with the exception of solvents such as TFA and concentrated aqueous salts such as LiBr, which also denature the chain structure.[37] However, the strong association of β-sheets is sometimes beneficial if a strong physically crosslinked system is desired.[38]

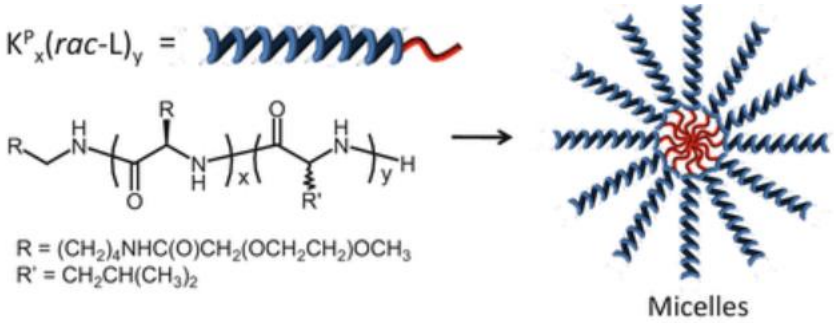

Figure 1.7 Block copolypeptide micelles with α-helical hydrophilic shell and disordered hydrophobic core. Reprinted with permission from Ref. 12. Copyright 2013 Springer Nature.

NCA polymerization techniques have allowed for the design of amphiphilic block copolypeptides, which have been shown to be able to assemble into various biomedically relevant structures including micelles, vesicles, and hydrogels.[35] Polypeptide-containing micelles typically consist of a flexible polyethylene glycol hydrophilic shell with a rigid hydrophobic polypeptide core and are utilized for the encapsulation of hydrophobic drugs.[34, 39] However, the Deming group was able to design micelles compromised solely from polypeptides by using a disordered racemic hydrophobic poly(Leucine) segment to facilitate spherical core packing in the presence of a rigid helical poly(N_ε-2-[2-(2-methoxyethoxy)ethoxy]acetyl-L-lysine)

hydrophilic segment, K^P (Figure 1.7).[40] They were able to load the anticancer drug Campothecin into the micelles with 76% efficiency, illustrating their potential as a drug delivery vehicle.[12]

Vesicles are of high interest in the field of drug delivery due to their ability to load both hydrophilic and hydrophobic cargos.[41] Polymer derived vesicles have a variety of advantages over lipid-based vesicles due to their increased stability and tunable functionality/permeability.[42] The Deming lab has designed a variety of vesicles assembled from block copolypeptide amphiphiles.[12] The first contained the same α-helical hydrophobic K^P residues used in the block copolypeptides micelles described above, but also utilized a homochiral poly(L-leucine) hydrophobic segment, since anisotropic packing is required for bilayer assembly. Due to the rigidity of the helical block copolypeptide, a denaturant (TFA) was required in the annealing process.[43] In subsequent work, they discovered that hydrophilic segments with disordered conformations were most desirable for assembly of vesicles, as demonstrated with poly(L-lysine)-block-poly(L-leucine) block copolypeptides ($K_{60}L_{20}$). The flexibility of the hydrophilic segments eliminated the need for a denaturant during annealing and allowed for control of vesicle diameter via extrusion.[36] The ability to control vesicle diameter allows for design of vesicles of optimal size (10 nm to 200 nm) to maximally exploit the enhanced permeability and retention effect via blood circulation, making this type of block copolypeptide vesicle ideal for cancer drug delivery.[44] Polypeptides containing a cationic poly(L-arginine) hydrophilic segment ($R_{60}L_{20}$) assembled into vesicles that showed strong cell penetrating ability and were able to deliver DNA into mammalian cells for transfection.[45] However, these vesicles exhibited a degree of cytotoxicity, which was subsequently reduced through incorporation of non-ionic, disordered poly(L-methionine sulfoxide) segments.[46]

Hydrogels are materials that are useful in the fields of tissue engineering and cell delivery. The characteristic of hydrogels that makes them desirable for these applications is

their highly hydrated and porous structure that mimics extracellular matrices.[12] Polypeptides with long hydrophilic disordered segments and short helical hydrophobic segments are capable of forming injectable, shear thinning physical hydrogels.[35] The Deming lab has designed a variety of hydrogels using this motif that have been used for a variety of applications.[12, 38, 47] Hydrogels with cationic poly(L-lysine) segments have been used to study spinal cord injury in mice and rats through localized delivery of growth factors that can facilitate axon regrowth.[48, 49] Non-ionic hydrogels containing antifouling poly(L-methionine sulfoxide) hydrophilic segments were shown to be an effective, non-toxic medium for the culture of neural stem/progenitor cells, and may prove to be a useful NSPC delivery vehicle for treatment of central nervous system injuries.[47]

1.4 Post-polymerization Modification of Polypeptides

The ability to incorporate a diverse array of functionalities into polypeptides is vital for varying conformation, solubility, stimuli response, and cellular recognition.[27, 50] Though the 20 natural amino acids contain a variety of functional groups, the incorporation of unnatural amino acids is often necessary to achieve a variety of desired material properties.[51]

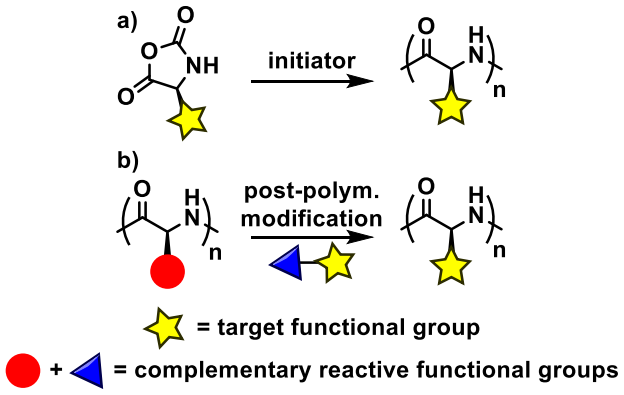

Figure 1.8 Synthetic approaches to functional polypeptides with unnatural residues.

There are two common methods for the synthesis of functional polypeptides containing unnatural residues: side chain modified (SCM) NCA approach and post-polymerization

modification (PPM) approach (Figure 1.8). Both methods have advantages and drawbacks that must be considered when planning a polypeptide synthesis. The SCM NCA approach guarantees full functionalization of the desired polypeptide segment.[27] However, functional NCA's often require complex synthesis and tedious purification, and a new monomer must be synthesized when a different structure or functionality is desired.[52] PPM approaches may sometimes suffer from incomplete functionalization, but often have simple synthetic protocols and allow the ability to introduce a variety of functional groups and structures without the need to synthesize multiple monomers.[27] Post-polymerization chemistry can be optimized to obtain complete or near complete functionality, making PPM synthetic methods highly desirable for the synthesis of functional polypeptide materials.[53]

The most common methods of post-polymerization modification involve "click" type reactions.[27] Defined by their high reaction rates, and lack of side products[54], click reactions allow for high degrees of polymer functionalization under mild conditions without the formation of macromolecular impurities.[27] Some reactions utilized for PPM of polypeptides include amidation[55], azide-alkyne cycloaddition,[21] thiol-x chemistry,[56, 57] alkene oxidation chemistry[58], and thioether alkylation chemistry.[53] Some examples of reactive polypeptides that have been employed for PPM are shown in Figure 1.9.

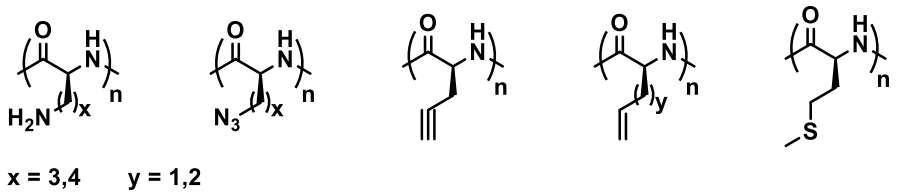

Figure 1.9 Selected polypeptides containing reactive handles for PPM.

A highly effective handle for post-polymerization modification is the thioether group. The thioether group is present in the natural amino acid methionine, and can undergo reactions that can modify its charge, conformational preference, functionality, and reactivity (Figure 1.10).[51]

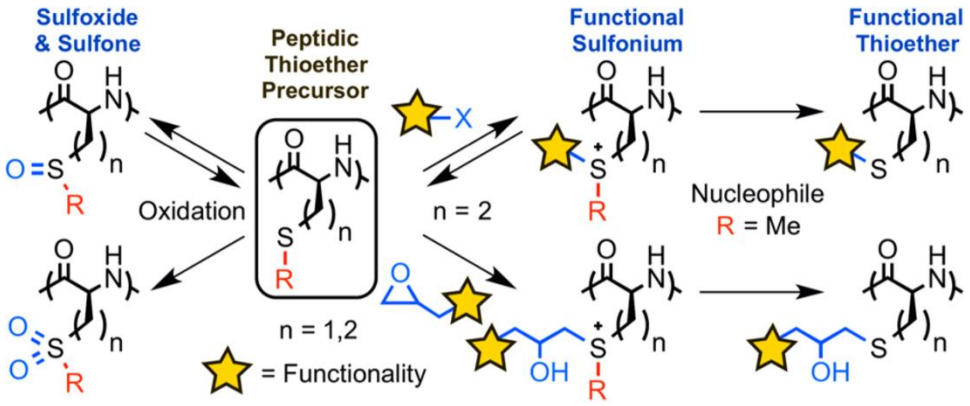

Figure 1.10 PPM of thioether functionalities in polypeptides. Reprinted with permission from Ref. 51. Copyright 2017 American Chemical Society.

Thioethers can undergo oxidation or alkylation reactions after polymerization.[51] Alkylation of thioether groups in polypeptides with reactive alkyl halides/triflates[53] or epoxides[59] give water solubilizing, cationic sulfonium salts that can have varied functionality based on the alkylating agent used. The resulting sulfonium containing polypeptides can be further modified a demethylation reaction to yield water soluble functional thioethers.[60] Thioethers can also undergo oxidation to sulfoxides or sulfones depending on reaction conditions. Poly(L-methionine sulfoxide) is disordered in aqueous conditions and highly water soluble while Poly(L-methionine sulfone) is α-helical with poor water solubility, likely due to strong interactions between sulfone groups.[61] Functional thioether containing polypeptides can also be synthesized via post-polymerization thiol-ene chemistry and can be subsequently alkylated or oxidized to potentially generate a large variety of materials.[57]

1.5 Stimuli-responsive Polypeptides

Advances in NCA polymerization techniques and innovative functionalization strategies have allowed for the synthesis of a variety of stimuli-responsive polypeptide materials. Stimuli-responsive “smart” polypeptides have potential applications in tissue engineering, drug delivery, and biodiagnostics.[62] “Smart” polypeptides undergo chemical changes, conformational changes

and/or phase separation in response to a variety of stimuli including environmental cues (temperature, pH), biological cues (enzymes/proteins), and chemical cues such as redox.[63]

Temperature is regarded as a highly useful stimulus in the design of "smart" polypeptide materials. It is highly exploitable for drug delivery due to the difference between room temperature and physiological temperature.[64] Thermoresponsive polymers in solution commonly undergo phase transitions at an upper or lower critical solution temperature (UCST or LCST).[65] The Deming lab has synthesized a variety of thermoresponsive polypeptide materials. Oligoethylene glycol (EG) functionalized poly(L-homocysteine) derivatives synthesized via PPM of L-methionine residues were shown to allow high variability of LCST depending on the type of EG functionality used.[66] Nonionic hydrogels containing thermoresponsive EG-L-Glutamate residues were liquids at room temperature and formed gels under physiological conditions. This material was utilized to increase survival of grafted neural stem cells 3-fold *in vivo* compared to standard media.[67]

Polypeptides that respond to changes in pH are desirable due to the lower pH of tumor tissue (pH 5.7-6.8)[68] and intracellular compartments such as endosomes and lysosomes (pH 4.5-6.5)[69] compared to physiological pH (pH 7.4). Polypeptide materials with polyelectrolyte segments containing L-lysine and/or L-glutamate residues have been utilized in the design of pH responsive polypeptides due to pH driven solubility changes.[62] An interesting application of these residues was demonstrated by the Lecommandoux group. pH responsive vesicles were synthesized from poly(L-glutamic Acid)$_{15}$-block-poly(L-lysine) block copolypeptides. These vesicles showed a pH dependent assembly mechanism. Under acidic conditions, the vesicles contained a neutral hydrophobic poly(L-glutamic acid) membrane layer and a cationic hydrophilic poly(L-lysine) corona, while basic conditions led to formation of vesicles with a neutral hydrophobic poly(L-lysine) membrane layer and anionic poly(L-glutamic acid) corona.[70]

Enzyme-responsive materials are attractive for drug delivery, since many enzymes are overexpressed in specific diseases, and many are found only intracellularly. These qualities facilitate targeted drug release from enzyme-responsive nanocarriers.[71] Matrix metalloproteinases (MMPs) are enzymes that are highly oversecreted by metastatic cancer cells. Vesicles with a poly(L-lysine) corona conjugated to antifouling PEG chains via an MMP cleavable peptide linker (PLGLAG) were synthesized by Kim and coworkers. Secreted MMP enzymes cleave between the glycine and leucine residues of the linker and release the PEG chains when the vesicle is in proximity to tumor cells, promoting preferential uptake.[72] An example of utilizing an enzyme response to trigger intracellular drug release was demonstrated by the Deming lab. Poly(L-methionine sulfoxide) was utilized as a hydrophilic segment for nonionic polypeptide vesicles. These chains were acted on by intracellular methionine sulfoxide reductase (MSR) enzymes to reduce hydrophilic methionine sulfoxide residues to hydrophobic methionine residues, leading to vesicle disruption and cargo release.[61]

Redox chemistry is involved in many physiological processes. The intracellular environment is inherently reductive due to an abundance of glutathione[62], while increased production of reactive oxygen species (ROS) is seen in a variety of diseased cells and tissues.[73] Naturally occurring reduction and oxidation processes can be exploited for the design of stimuli responsive materials.[71] Nanostructures with disulfide crosslinked cores have been utilized for reduction-triggered drug delivery[74] and gene delivery[75] applications. Polypeptides containing residues with thioether functionality can undergo a helix to coil transition under oxidative conditions, which is accompanied by an increase in solubility.[76] This transformation can be utilized to design oxidation-responsive materials such as hydrogels that degrade upon exposure to reactive oxygen species.[73]

The post-polymerization methodologies discussed in subsequent chapters allow for the design of a variety of stimuli-responsive polypeptide materials.

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Chapter 2: Synthesis and Thiol-ene Modification of poly(L-Homoallylglycine)

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2.1 Abstract

Homoallylglycine *N*-carboxyanhydride, Hag NCA, monomers were synthesized and used to prepare polypeptides containing Hag segments with controllable lengths of up to 245 repeats. Poly(L-homoallylglycine), **G^{HA}**, was found to adopt an α-helical conformation, which provided good solubility in organic solvents and allowed high yield functionalization of its alkene side-chains *via* radical promoted addition of thiols. The conformations of these derivatives were shown to be switchable between α-helical and disordered states in aqueous media using thioether alkylation or oxidation reactions. Incorporation of **G^{HA}** segments into block copolymers with poly(L-methionine), **M**, segments provided a means to orthogonally modify thioether side-chains different ways in separate copolypeptide domains. This approach allows preparation of functional polypeptides containing discrete domains of oxidized and alkylated thioether containing residues, where chain conformation and functionality of each domain can be independently modified.

2.2 Introduction

There has been considerable recent interest in the development of methods to selectively introduce functional tags into peptide, protein, and polypeptide sequences.[1-3] Among these, the thiol–ene reaction has been used extensively,[4] since it can provide modifications with high yields and high functional group selectivity. For polypeptides, many unnatural alkene containing residues have been employed for subsequent thiol–ene modification (Figure 2.1).[5] In these examples, the side-chain structures of these alkene amino acid residues have substantial

influence on resulting polypeptide chain lengths, conformations, solubility, and consequently the efficiency of thiol–ene conjugations. We sought to optimize the design of alkene containing residues to enable robust polypeptide and block copolypeptide synthesis, high efficiency in subsequent thiol–ene modifications, and control of chain conformations.

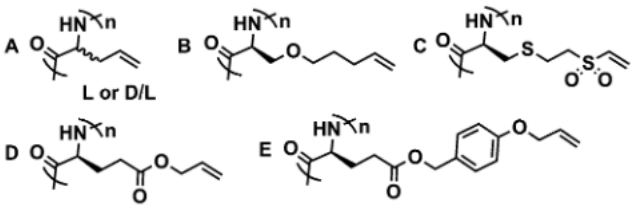


Figure 2.1 (A-E) Alkene containing homopolypeptides used for thiol-ene conjugation.

The simplest alkene containing polypeptides used for thiol–ene functionalization are based on allylglycine (Figure 2.1A). Both poly(L-allylglycine) and poly(DL-allylglycine) have been prepared and were found to adopt β-sheet conformations, which result in aggregation and limit the ability to prepare high molecular weight chains.[6, 7] Consequently, efficient thiol–ene functionalization of these polymers was restricted to samples with short chain lengths (i.e. typically <20 residues), and often required incorporation of comonomers or segments (i.e. PEG) to promote solubility.[8, 9] Related polypeptides have been prepared based on alkene functionalized serine[10] and cysteine[11] residues (Figure 2.1B and C) that also adopt β-sheet conformations leading to poorly controlled chain aggregation, which would be problematic for downstream use as segments in block copolypeptide assemblies.

Additional studies have utilized functionalized glutamate esters as components for preparation of alkene containing polypeptides (Figure 2.1D and E).[12-14] These polypeptides have the advantage of adopting α-helical conformations, which possess good solubility and allow formation of high molecular weight chains. Homopolypeptides up to 100 residues long were prepared and could be efficiently modified with different thiols yielding α-helical derivatives. While this strategy is useful for preparation of homopolypeptides, the labile side-chain ester

linkages would be problematic in preparation of multifunctional copolypeptides.[15] Also, this strategy only allows for preparation of polypeptides with α-helical conformations, which cannot be switched due to their long, hydrophobic side-chains.[12-14] Polypeptides with conformations that can be switched reversibly under mild conditions are desirable for use in development of self-assembled materials such as nanocarriers and hydrogels that can actively respond to biological and chemical cues.

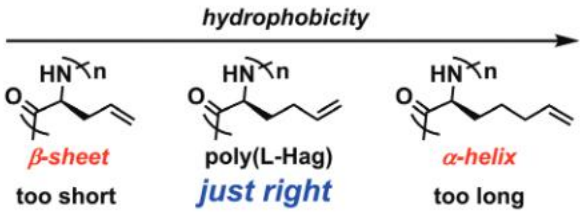


Figure 2.2 Comparison of allyl, homoallyl, and pentenyl glycine homopolypeptides.

To take full advantage of the thioether functionality introduced by thiol–ene conjugation, we sought to develop polypeptides containing alkene side-chains of minimal length so that modifications of product thioether groups would induce switchable chain conformations.[16] Further, to enable preparation of soluble, high molecular weight chains, α-helical conformations were desired for the initial alkene bearing polypeptides. While poly(allylglycine)s are known to form β-sheets, it has also been reported that poly(L-pentenylglycine) adopts an α-helical conformation (Figure 2.2).[6, 7] While poly(L-pentenylglycine) has not been used for thiol–ene conjugation, its hydrophobic side-chains might be too long to allow conformational switching, similar to the glutamate derivatives described above. Since single carbon homologation of side-chain functional groups in β-sheet forming polypeptides can result in polypeptides that adopt α-helical conformations, such as homologation of cysteine to homocysteine,[17] we hypothesized that the intermediate side-chain length of Hag would be sufficiently long to stabilize α-helical conformations in **G^{HA}** and yet be short enough to allow introduced thioether groups to influence

chain conformations (Figure 2.2). Notably, Hag has also been utilized as an artificial residue for efficient thiol–ene modification in proteins.[18, 19]

Consequently, we sought to develop procedures for preparation of new NCA monomers of L-Hag and rac-Hag, and synthesize their corresponding new homopolypeptides (Figure 2.3). To enhance the ability to prepare multifunctional polypeptides with stimulus responsive conformations, we also sought to prepare block copolypeptides of Hag with L-methionine, Met. Specifically, we aimed to utilize Hag residues as “masked” precursors of thioether groups, which could be functionalized orthogonally to the thioether groups in Met residues. The goal of this approach being the preparation of block copolypeptides where discrete domains can be functionalized and conformationally switched independent of one another.

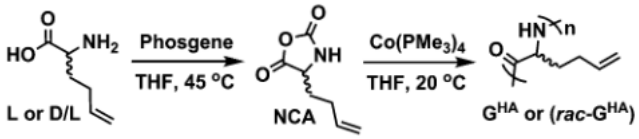


Figure 2.3 Synthesis of homoallylglycine NCAs and polypeptides.

2.3 Results and Discussion

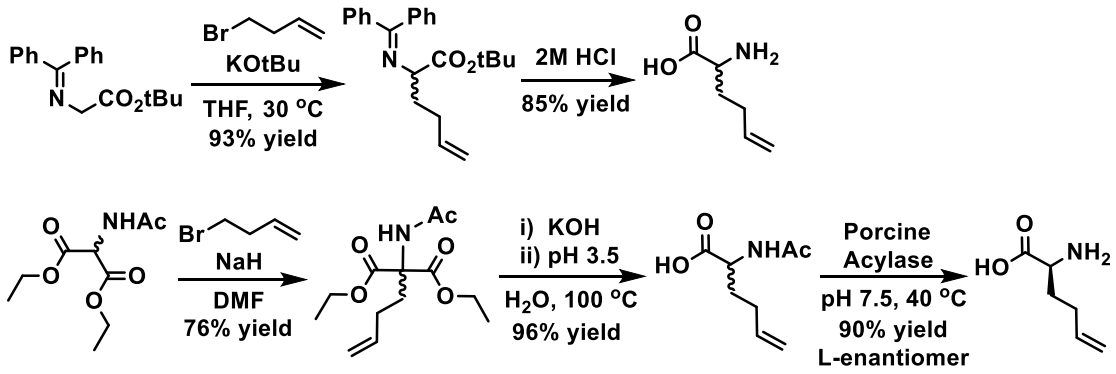


Figure 2.4 Synthetic schemes for homoallylglycine amino acids.

For NCA monomer preparation, the Hag and *rac*-Hag amino acid precursors were prepared following literature methods (Figure 2.4).[18-22] *rac*-Hag was obtained by alkylation of diphenylimino glycine tert-butyl ester, which gave the free amino acid directly upon hydrolysis of

the protecting groups. Hag was prepared by alkylation of diethyl acetamidomalonate, followed by ester deprotection and decarboxylation to give *N*-acetyl-*rac*-Hag. This racemic mixture was readily resolved by enantioselective hydrolysis catalyzed by porcine acylase to give multigram quantities of Hag (Figure 2.5), which possessed an enantiomeric excess of >99% suitable for preparation of NCAs and polypeptides with high optical purity.

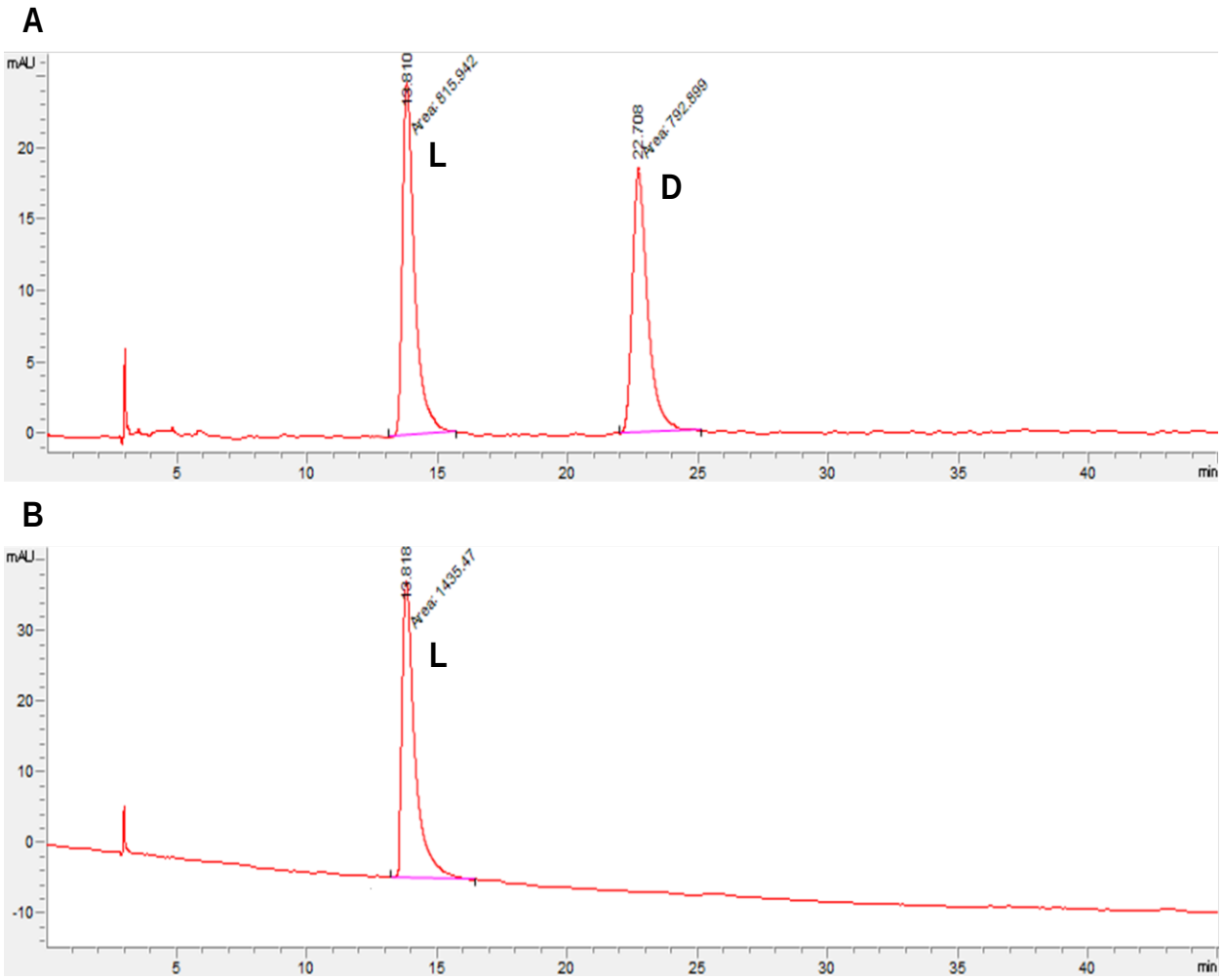


Figure 2.5 Chiral HPLC Chromatograms of *N*-(carbobenzyloxy)-DL-homoallylglycine (A) and *N*-(carbobenzyloxy)-L-homoallylglycine (B). Amino Acids were N-protected to give solubility in mobile phase.

Analysis of both Hag and *rac*-Hag matched literature data.[18-22] Hag and *rac*-Hag were each subsequently treated with phosgene under standard conditions to obtain the corresponding NCAs that were obtained as high purity colorless solids after column chromatography and recrystallization (Figure 2.6).[23]

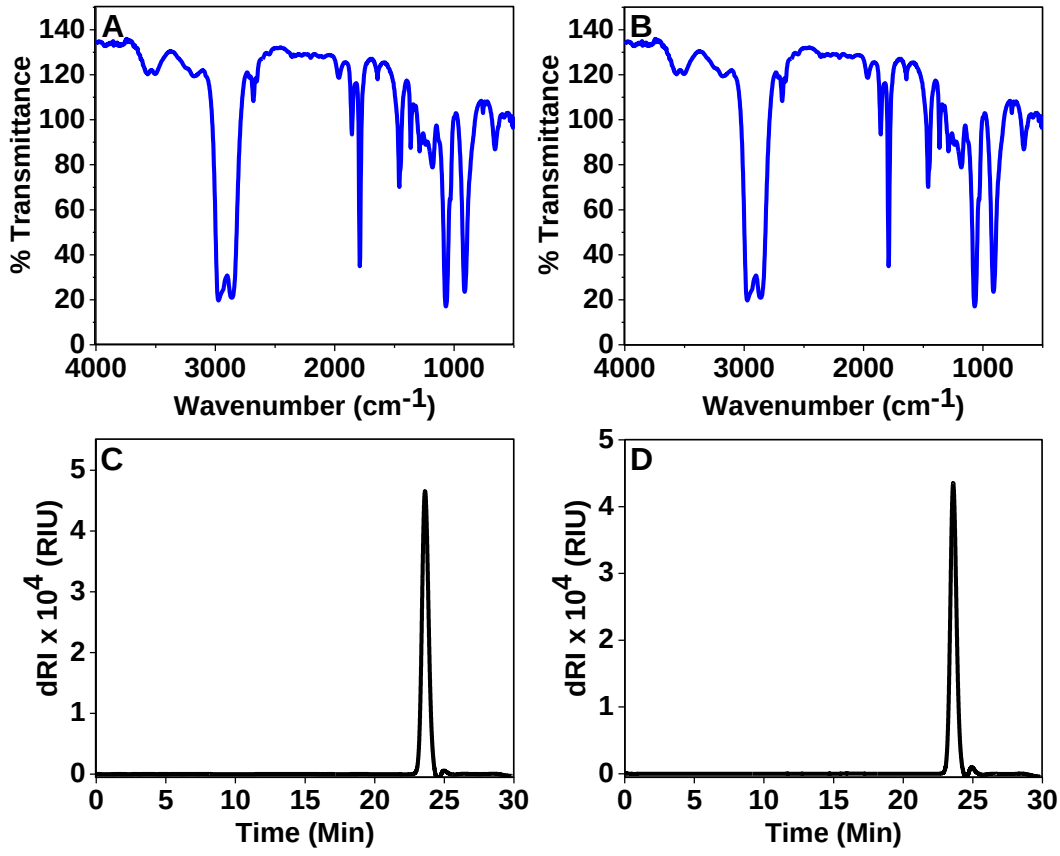


Figure 2.6 FTIR and GPC data for Hag NCA and *rac*-Hag NCA. TOP: FTIR Spectrum of Hag NCA (A) and *rac*-Hag NCA (B) (50 mg/mL in THF). Note NCA bands at 1856 and 1790cm^{-1}, and alkene stretch at 1642cm^{-1}. BOTTOM: SEC chromatogram of Hag NCA (C) and *rac*-Hag NCA (D) in 0.5% (w/w) KTFA in HFIP. RIU = arbitrary refractive index units.

Homopolymerizations of Hag and *rac*-Hag NCAs at different monomer to initiator (M : I) ratios were conducted using $Co(PMe_3)_4$ initiator in THF.[24] While Hag NCA polymerizations

rapidly went to completion and remained homogeneous up to M : I = 80 (Figure 2.7A), the *rac*-Hag NCA polymerizations did not go to completion above M : I = 20 (Figure 2.8). By FTIR we observed the poly(DL-homoallylglycine), (***rac*-G^{HA}**), forms β-sheet aggregates during polymerization that likely inhibit chain growth (Figure 2.9).[6, 7] On the contrary, **G^{HA}** homopolymers were found to be highly α-helical in organic solvents (Figure 2.7B), which promoted good solubility and enabled the synthesis of polymers up to 245 residues long.

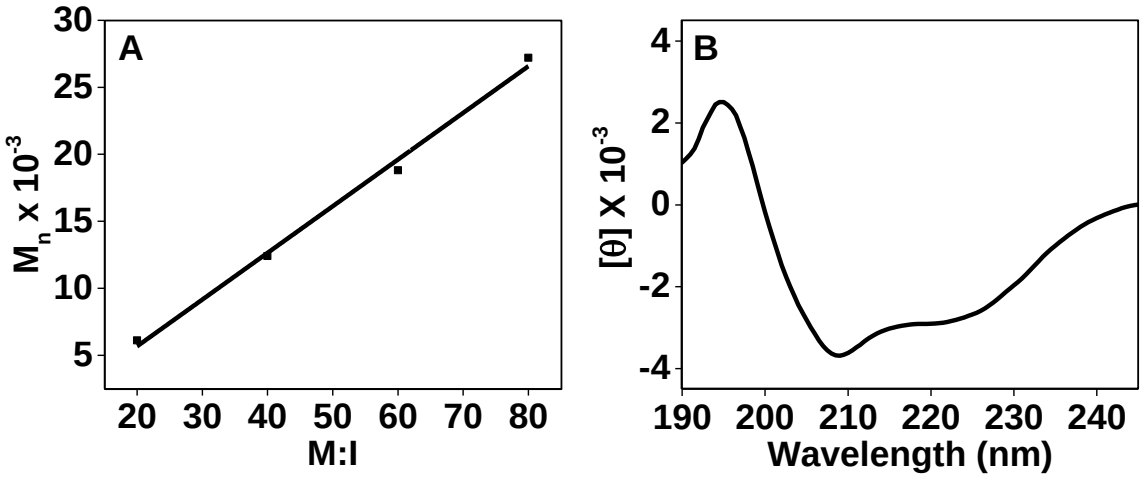


Figure 2.7 Synthesis and properties of poly(L-homoallylglycine), **G^{HA}**. (A) Number average molecular weight (M_n) of **G^{HA}** plotted as a function of monomer to initiator ratio (M : I) at complete monomer conversion using $Co(PMe_3)_4$ in THF (r^2 = 0.9874). M_n values were determined by ^{1}H NMR analysis of PEG end-capped polymers. (B) Circular dichroism spectrum of **G^{HA}** in 15 : 1 : 2 cyclohexane : MeCN : IPA (0.5 mg/mL) at 20 °C. Molar ellipticity reported in deg cm^2 dmol^{-1}.

Analysis of chain lengths at different M : I showed linear chain growth during Hag NCA polymerization, an indicator of controlled polymerization (Figure 2.7A). GPC analysis of **G^{HA}** samples, derivatized using thiol–ene reactions to improve solubility (Figure 2.10), gave unimodal peaks with dispersities of ca. 1.1–1.2, confirming the formation of uniform polymers.

M:I	Monomer Conversion	DP	M_w/M_n	Yield (%)
20	Complete	44	1.13	96
40	Incomplete	N/A	N/A	87
60	Incomplete	N/A	N/A	65
80	Incomplete	N/A	N/A	63

Figure 2.8 Synthesis of **(*rac*-G^{HA})** at different monomer to initiator ratios (M:I) using $Co(PMe_3)_4$ initiator in THF at 20 °C. DP = number average degree of polymerization. Yield is the total isolated yield of purified polypeptide. N/A = Not determined due to incomplete polymerization reaction.

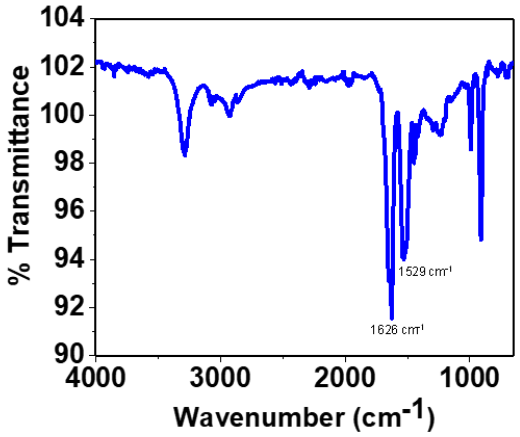


Figure 2.9 Solid state FTIR of ***rac*-G^{HA}_{43}**. The Amide I and Amide II bands at 1626 and 1529 cm^{-1}, respectively, are characteristic of a β-Sheet conformation.

To further test the ability of Hag NCA to undergo controlled polymerization, diblock copolypeptides with Met NCA were prepared (Figure 2.11). Block copolypeptides of defined sequence and composition were obtained in excellent yields, and GPC analysis of derivatized copolymers (vide infra) showed uniform chain length distributions with low dispersity (Figure 2.10).

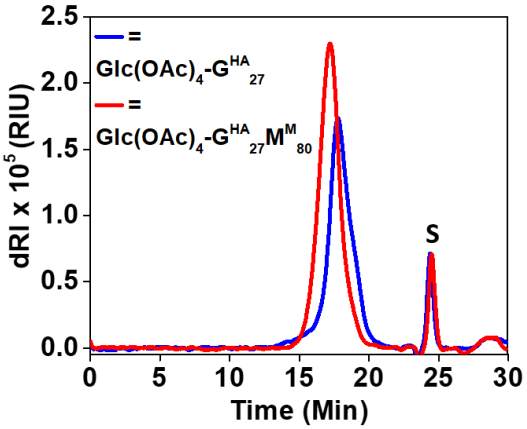


Figure 2.10 GPC chromatograms of derivatized homo and diblock polypeptides **Glc(OAc)$_4$-G$^{HA}_{27}$** (blue) and **Glc(OAc)$_4$-G$^{HA}_{27}$M$^{M}_{80}$** (red) in HFIP containing 0.5% (w/w) KTFA. S = solvent. RIU = arbitrary refractive index units.

Compositions		First segment			Diblock copolymer			
1st monomer	2nd monomer	M_n	DP	M_w/M_n	M_n	DP	M_w/M_n	Yield (%)
20 Met NCA	10 Hag NCA	6600	50	1.27	9200	74	1.32	99
10 Hag NCA	30 Met NCA	12 800	27	1.14	27 400	107	1.18	99

Figure 2.11 Synthesis of diblock copolypeptides using $Co(PMe_3)_4$ in THF at 20 °C. First and second monomers were added stepwise to the initiator. Molecular weight and Dispersity determined by ^{1}H NMR and GPC of derivatized polypeptides.

After successful polymerization of Hag NCA, the reactivity of **G^{HA}** with a variety of thiols was evaluated. Toward the goal of obtaining water soluble derivatives, oligoethylene glycol and monosaccharide thiols were chosen for these studies (Figure 2.12). Under optimized conditions, near quantitative thiol–ene functionalization of Hag residues was obtained for all thiols (see experimental).[18, 19] For comparison, thiol–ene functionalization of (***rac*-G^{HA}**) was also attempted using similar conditions (see experimental). While >90% thiol conjugation efficiency could be obtained on short (***rac*-G^{HA}**) chains, these derivatives exhibited poor water solubility due to the

formation of β-sheet structures (Figure 2.13). Contrary to this result, all thiol–ene derivatives of **G^{HA}** were found to possess good water solubility, except for **mEG$_3$-G^{HA}**, which was soluble in organic solvents.

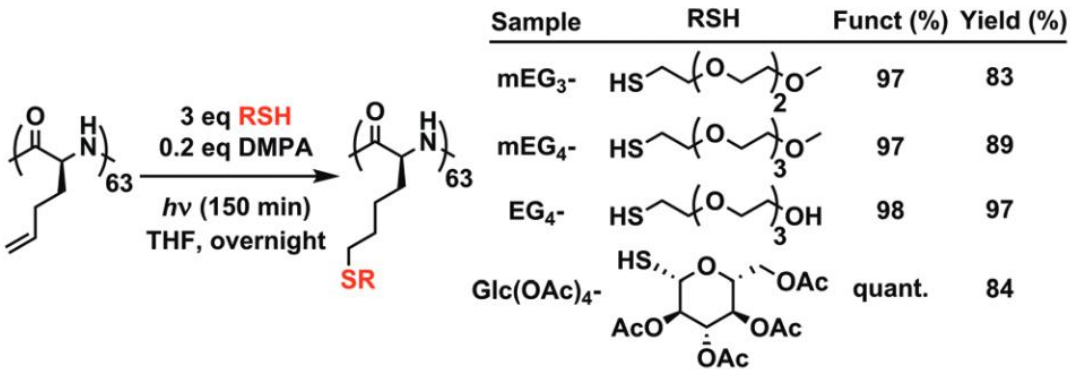


Figure 2.12 Thiol–ene modification of **G^{HA}** (4 mg/mL) in THF with UV irradiation followed by overnight stirring at ambient temperature. Funct = percentage of side-chain modification. Yield = total isolated yield of purified polypeptide. Quant. = quantitative.

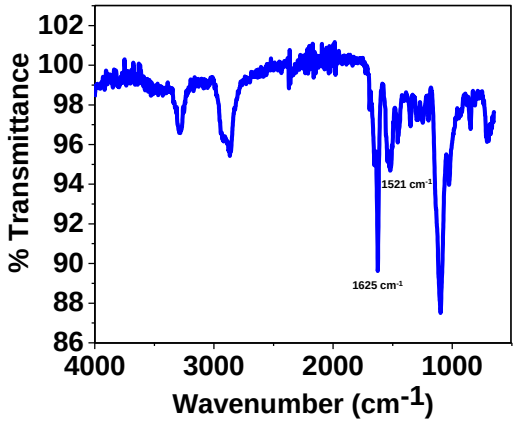


Figure 2.13 Solid state FT-IR of **mEG$_4$-(*rac*-G^{HA})$_{43}$.** The Amide I and Amide II bands at 1625 and 1521 cm^{-1}, respectively, are characteristic of a β-Sheet conformation.

Aqueous solutions of **G^{HA}** derivatives analyzed by circular dichroism (CD) spectroscopy were found to primarily adopt α-helical conformations, similar to the parent **G^{HA}** (Figure 2.14A). α-Helical content was found to be greatest (ca. 100% α-helix) for the **EG$_4$-G^{HA}** sample, which

contained side-chains with greatest hydrophilicity. The methoxy terminated oligoethylene glycol and glycosylated samples (**mEG$_3$-G^{HA}**, **mEG$_4$-G^{HA}**, and **Glc-G^{HA}**) possessed diminished minima at 208 and 222 nm, yet retained partial helical content (49 to 71% α-helix). The addition of hydrophilic thiols to **G^{HA}** was found to be an efficient means to obtain water soluble, α-helical polypeptides with high degrees of functional modification.

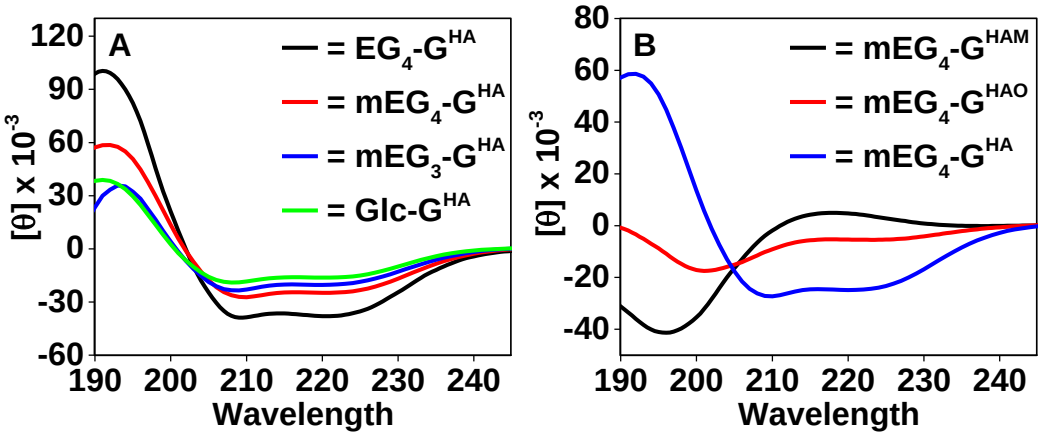


Figure 2.14 Circular dichroism spectra of functionally modified **G$^{HA}_{63}$** samples**.** (A) Thiol–ene adducts **mEG$_3$-G^{HA}** (blue), **mEG$_4$-G^{HA}** (red), **EG$_4$-G^{HA}** (black), **Glc-G^{HA}** (green). All samples in DI water except **mEG$_3$-G^{HA}** in THF. (B) Parent **mEG$_4$-G^{HA}** (blue, 71% α-helix) and its sulfonium (black, 0% α-helix) and sulfoxide (red, 22% α-helix) derivatives in DI water. All samples (0.5 mg/mL) analyzed at 20 °C. Molar ellipticity reported in deg cm^2 dmol^{-1}. Percent α-helical content of polypeptides was estimated using the formula % α-helix = 100 × (−[θ]$_{222}$ + 3000)/39000), where [θ]$_{222}$ is the measured molar ellipticity at 222 nm.[25]

Since functionalized **G^{HA}** contain thioether linkages, there is potential for additional secondary modification of the polypeptide side-chains via selective alkylation or oxidation reactions.[16] To examine the feasibility of such modifications and test their effects on polymer properties, **mEG$_4$-G^{HA}** was reacted separately with either iodomethane or tert-butylhydroperoxide (TBHP) to obtain the methylated derivative, **mEG$_4$-G^{HAM}**, or oxidized

derivative, **mEG$_4$-G^{HAO}**, respectively (Figure 2.15). These reactions gave high yields of the fully modified polypeptides, which retained the water solubility of the precursor **mEG$_4$-G^{HA}**. CD analysis of **mEG$_4$-G^{HAM}** and **mEG$_4$-G^{HAO}** in water revealed that both modifications destabilized the α-helical conformation of the parent **mEG$_4$-G^{HA}** (Figure 2.14B), similar to results obtained for alkylation and oxidation of thioether containing **M** chains even though the thioether groups in **mEG$_4$-G^{HAM}** are two bonds further removed from the peptide backbone compared to Met residues.[16] The degree of conformational disruption was greater for **mEG$_4$-G^{HAM}**, likely due to the introduction of charged groups as compared to the non-ionic sulfoxides in **mEG$_4$-G^{HAO}**. This ability to switch between α-helical and disordered conformations in **mEG$_4$-G^{HA}** polypeptides is a desirable feature that has not been demonstrated in thiol–ene derivatives of other alkene containing polypeptides.

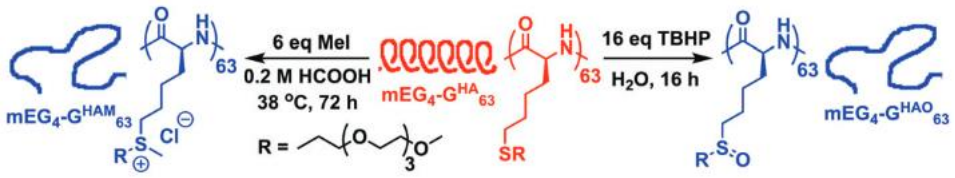


Figure 2.15 Conformational changes induced by thioether alkylation or oxidation of **mEG$_4$-G$^{HA}_{63}$**.

To illustrate how **G^{HA}** segments can be used in conjunction with other polypeptide segments to obtain chains with discrete modified thioether domains, we sought to prepare diblock copolypeptides containing both sulfoxide and sulfonium functionality in separate segments (Figure 2.16). Independent control over placement of bio-inert segments, i.e. sulfoxide,[26, 27] and segments that may promote cell uptake, i.e. sulfonium,[28] is needed for continued development of multifunctional biomaterials. While both sulfoxide and sulfonium groups can be introduced into **M** homopolymers, there is no means to control placement of these groups as they will be statistically distributed along the chains. In our experience, due to

limited solubility of **M** in suitable reaction media, precise control over partial oxidation or partial alkylation of **M** chains is challenging. Hence, methodology for facile installation of sulfoxide and sulfonium functionality in discrete segments within copolypeptide sequences would be valuable.

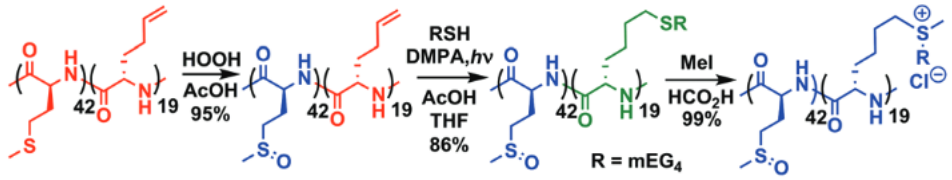


Figure 2.16 Synthesis of diblock copolypeptide $\mathbf{M^{O}_{42}mEG_4\text{-}G^{HAM}_{19}}$ that contains discrete sulfoxide and sulfonium domains. Percent yields are total isolated yields of purified copolypeptides.

To demonstrate the feasibility of such modifications, a block copolymer of Met and Hag, $\mathbf{M_{42}G^{HA}_{19}}$ prepared as described above, was subjected to a sequence of selective reactions (Figure 2.16). Hydrophobic, α-helical $\mathbf{M_{42}G^{HA}_{19}}$ was first oxidized at Met residues to give the amphiphilic copolymer $\mathbf{M^{O}_{42}G^{HA}_{19}}$ containing disordered hydrophilic poly(L-methionine sulfoxide), $\mathbf{M^{O}}$, segments.[26, 27] The thiol mEG_4SH was then selectively added to the Hag residues via radical coupling in acidic media, which is beneficial for thiol–ene conjugation and also prohibits undesirable reduction of sulfoxides by thiols. The resulting copolymer, $\mathbf{M^{O}_{42}mEG4\text{-}G^{HA}_{19}}$, now became fully hydrophilic, but retained α-helical conformations in the $\mathbf{mEG4\text{-}G^{HA}}$ domains. The thioether groups in this copolymer were then selectively alkylated using iodomethane, taking advantage of the resistance of $\mathbf{M^{O}}$ residues toward alkylation under these conditions.[29] The resulting sulfoxide-sulfonium diblock copolypeptide, $\mathbf{M^{O}_{42}mEG4\text{-}G^{HAM}_{19}}$, was water soluble and both segments were now conformationally disordered in water. In addition to successful selective functional modification of each copolypeptide domain, the respective thioether modifications also allowed independent conformational switching of each segment (Figure 2.17).

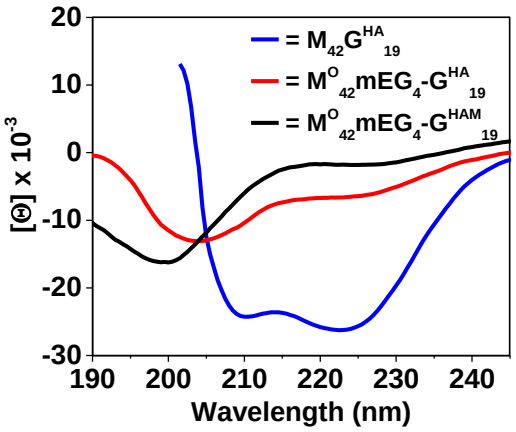


Figure 2.17 Circular dichroism spectra of $\mathbf{M_{42}G^{HA}_{19}}$ and its derivative block copolypeptides at 0.1 mg/mL, 20 °C. $\mathbf{M_{42}G^{HA}_{19}}$ (blue, in THF), $\mathbf{M^{O}_{42}mEG_4\text{-}G^{HA}_{19}}$ (red, in DI water, 25% α-helix), $\mathbf{M^{O}_{42}mEG_4\text{-}G^{HAM}_{19}}$ (black, in DI water, 12% α-helix). The derivative $\mathbf{M^{O}_{42}G^{HA}_{19}}$ possessed poor solubility in both organic and aqueous solvents and was not analyzed.

2.4 Conclusion

The efficient polymerization of Hag NCA, good solubility of $\mathbf{G^{HA}}$ allowing preparation of high molecular weight homo- and copolymers, facile modification of Hag residues with thiols, and ability to further modify the thioether products provide a number of attractive features supporting utilization of Hag residues in peptidic materials. Beyond what has been achieved in previous alkene containing polypeptides, the example process in Figure 2.16 shows how incorporation of Hag residues into polypeptides can be used to differentially modify discrete segments in an orthogonal manner and also modulate polypeptide chain conformations.

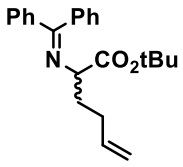
2.5 Experimental

2.5.1 Materials and Methods

Unless specified, all post-polymerization modification chemistry was performed in glass vials under ambient atmosphere. Thiol-ene reactions were performed in 1 dram screw top glass vials capped with septa to allow sparging. An Exo Terra Repti-Glo 5.0 compact fluorescent tropical terrarium lamp was used as the ultraviolet light source. Small molecule chemistry was performed in heat-dried glassware under a nitrogen atmosphere, unless otherwise specified. THF and hexanes were degassed with dinitrogen and passed through an alumina column before use. All other reagents and solvents were used as received. Unless otherwise specified, all reactions were performed at ambient temperature (ca. 20 °C). In-house deionized water was used for all aqueous chemistry and dialysis unless otherwise specified. Circular dichroism (CD) spectroscopy was performed on samples prepared using deionized water filtered through a Millipore Milli-Q Biocel A10 filter system unless otherwise specified. CD spectra were collected using 0.1 or 0.5 mg/mL solutions of polypeptide on an Olis DSM 10 spectrophotometer using a 0.1 cm path length quartz cuvette. Percent α-helical content of polypeptides was estimated using the formula % α-helix = 100 × (−$[\theta]_{222}$ + 3000)/39000), where $[\theta]_{222}$ is the measured molar ellipticity at 222 nm. Thin-layer chromatography was performed with EMD gel 60 F254 plates (0.25 mm thickness) and spots were visualized using a UV lamp or $KMnO_4$ stain. Silicycle Siliaflash G60 silica (60-200 μm) was used for all column chromatography. Silica used for chromatographic purification of NCA monomers was dried under vacuum at 250 °C for 48 hours and then stored in a dinitrogen filled glovebox. Compositions of mobile phases used for chromatography and compositions of solvent systems used for circular dichroism are given in volume percents. Dialysis was performed with regenerated cellulose tubing obtained from Spectrum labs. NMR spectra were recorded on a Bruker AV400 instrument with chemical shifts reported relative to the deuterated solvent used. Infrared spectra of solution samples were

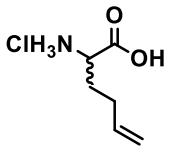
collected using a Perkin Elmer 1605 FT-IR Spectrophotometer. Solid state FTIR spectra were collected using a Perkin Elmer Spectrum One FT-IR Spectrophotometer with a Perkin Elmer Universal ATR Sampling Accessory. DART-MS spectra were collected on a Thermo Exactive Plus MSD (Thermo Scientific) equipped with an ID-CUBE ion source at the low desorption setting and a Vapur Interface (IonSense). Both the source and MSD were controlled by Excalibur v. 3.0. Analytes were dissolved 1 mg/mL in 1:3 THF:MeCN and spotted onto OpenSpot sampling cards (IonSense). Ionization was accomplished using He plasma with no additional ionization agents. Mass calibration was carried out using Pierce LTQ Velos ESI (+) and (-) Ion calibration solutions (Thermo Fisher Scientific). Tandem gel permeation chromatography/light scattering (GPC/LS) was performed at 25 °C using an SSI Accuflow Series III pump equipped with Wyatt DAWN EOS light scattering and Optilab REX refractive index detectors. Separations were achieved using 100 Å and 1000 Å PSS-PFG 7 µm columns at 30 °C with 0.5% (w/w) KTFA in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) as eluent and sample concentrations of 10 mg/ml. Enantiomeric excess of L-Hag was measured against a racemic standard using an Agilent 1260 Infinity HPLC. Separations were achieved using a Chiralpak AD-H 5 µm column with 10% isopropanol in hexanes as eluent and sample concentrations of 1 mg/ml. Melting points were measured using a DigiMelt MPA160 SRS melting point apparatus with a ramp rate of 5 °C/minute.

2.5.2 Synthesis of Amino Acids and NCA Monomers

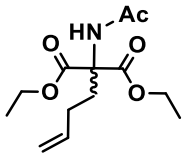


***N*-(Diphenylmethylene)-DL-homoallylglycine *tert*-butyl ester.** This compound was synthesized by following a previously reported method.[1] A 10% THF solution of potassium *tert*-butoxide (1.9 g, 17 mmol) was added dropwise to a 10% THF solution of *N*-

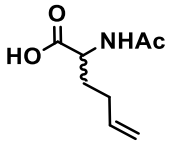
(diphenylmethylene)-glycine *tert*-butyl ester (2.5 g, 8.5 mmol) at -70 °C (EtOAc/liquid nitrogen bath). After 15 minutes, 2.0 mL (20 mmol) 3-butenyl bromide was added via syringe. The reaction was let warm to ambient temperature then transferred to a 30 °C water bath and let stir overnight. The reaction was then let cool to ambient temperature and quenched with 30 mL of saturated ammonium chloride solution. The resulting layers were partitioned in a separatory funnel, and the aqueous layer was then extracted with 4 x 25 mL DCM. Combined organic extracts were washed with brine, dried with anhydrous sodium sulfate, and volatiles were removed under reduced pressure. The resulting oil was purified by silica gel chromatography with 8 % EtOAc in hexanes as mobile phase. Solvent was removed under reduced pressure to yield the product as a pale yellow oil (2.8 g, 93% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 7.68-7.56 (br m, 2H), 7.52-7.29 (br m, 6H), 7.21-7.12 (br m, 2H), 5.80-5.66 (dq, J = 27.2, 6.1 Hz, 1H), 5.00-4.86 (br m, 2H), 3.93, (t, J = 6.2 Hz, 1H) 2.15-1.93 (br m, 4H), 1.44 (s, 9H).



DL-Homoallylglycine hydrochloride, *rac*-Hag. This compound was synthesized by following a previously reported method.[20] *N*-(Diphenylmethylene)-DL-homoallylglycine *tert*-butyl ester (1.72 g) was suspended in 27.5 mL of 2 M aqueous HCl, and stirred rapidly at ambient temperature for 3 hours. The suspension became a clear solution in 60 minutes and a yellow oil began to separate. Organic byproducts were removed by washing the reaction mixture with 3 x 20 mL DCM. The aqueous layer was then concentrated under reduced pressure and further concentrated under high vacuum to give the product as a white solid (654 mg, 85% yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 5.82-5.71 (br m, 1H), 5.08-5.01 (dq, J = 17.2, 1.6 Hz, 1H) 5.01-4.96 (dd, J = 11.8, 8.8 Hz, 1H), 3.87 (t, J = 12.5 Hz, 1H), 2.14-2.06 (q, J = 7.0 Hz, 2H), 2.01-1.81 (br m, 2H). Spectral data were consistent with previously published results.[1]

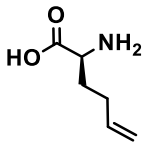


Diethyl 2-homoallyl-2-acetamidomalonate. This compound was synthesized by following a previously reported method.[21] A solution of diethyl 2-acetamidomalonate (45 g, 210 mmol, 1.0 eq) in DMF (300 mL) was prepared in a round bottom flask and capped with a septum. Separately, a suspension of 60 % NaH (9.1 g, 230 mmol, 1.1 eq) in DMF (100 mL) was prepared in a Schlenk flask and stirred in an ambient temperature water bath under N_2 atmosphere. The malonate solution was slowly cannula transferred into the NaH suspension (caution: exothermic). The resulting mixture was stirred 10 min. 3-Butenyl bromide (23 mL, 230 mmol, 1.1 eq) was then added in one portion via syringe. The mixture was stirred for 16 h on a 60 °C oil bath, and then quenched with AcOH (2 mL) and concentrated by rotary evaporation, then using high vacuum. The residue was dissolved in Et_2O (250 mL) and washed with H_2O (2 x 200 mL), and then the organic phase was dried over $MgSO_4$ and concentrated by rotary evaporation. This crude material was purified by column chromatography (25-35% EtOAc/hexanes) to provide diethyl 2-homoallyl-2-acetamidomalonate as a pale yellow solid (43 g, 76% yield). R_F = 0.32; 25% EtOAc/hexanes. ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 6.79 (s, 1 H), 5.75 (ddq, *J* = 17.1, 10.3, 6.5 Hz, 1 H), 5.03 (dq, *J* = 17.1, 1.7 Hz, 1 H), 4.97 (dq, *J* = 10.2, 1.5 Hz, 1 H), 4.25 (q, *J* = 7.1 Hz, 4 H), 2.45 (m, 2 H), 2.04 (s, 3 H), 1.92 (m, 2 H), 1.25 (t, *J* = 7.0, 6 H).



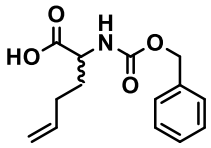
***N*-acetyl-DL-homoallylglycine.** This compound was synthesized by following a previously reported method.[21] Diethyl 2-homoallyl-2-acetamidomalonate (13 g, 49 mmol) was suspended in aqueous 1 N KOH (2.6 eq). The mixture was refluxed with vigorous stirring. The initially

biphasic mixture became homogenous after *ca*. 30 min. Heating was maintained for 3 h in total. The mixture was adjusted to pH 3.5 (temperature compensated) with conc. HCl. The mixture was then refluxed for an additional 1 h. The pH was then adjusted again back to pH 3.5 using conc. HCl (*Note:* the pH rises as the decarboxylation progresses). The mixture was again refluxed for an additional 2 h then allowed to cool to ambient temperature. The solution was then concentrated to about 2/3 its original volume by rotary evaporation. The resultant amber mixture was treated with activated charcoal (12 mL of powder). After stirring for 10 min, the suspension was filtered. The colorless filtrate was adjusted to pH 2.0 with conc. HCl. This mixture was then extracted with 4 x 80 mL of EtOAc. The organic extracts were dried over Na_2SO_4 and concentrated by rotary evaporation to provide *N*-acetyl-DL-homoallylglycine (7.7 g, 96% yield) as a colorless solid. ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 5.89 (ddq, *J* = 17.2, 10.3, 6.8 Hz, 1 H), 5.12 (dq, *J* = 17.2, 1.7 Hz, 1 H), 5.06 (m, 1 H), 4.35 (dd, *J* = 9.5, 4.7 Hz, 1 H), 2.21 (m, 2 H), 2.06 (s, 3 H), 2.01 (m, 1 H), 1.86 (m, 1 H).



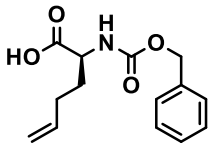
L-Homoallylglycine. This compound was synthesized by following a previously reported method.[21] A 300 mM aqueous solution of *N*-acetyl-DL-homoallylglycine (46 g, 290 mmol) containing 5 mM KH_2PO_4 was prepared in a Schlenk flask. The solution was adjusted to pH 7.5-8.0 with aqueous 4 N KOH. The mixture was placed in a 40 °C oil bath and degassed by stirring under N_2 for 15 min. Porcine amino acylase (4.2 U per mmol of *N*-acetyl-DL-homoallylglycine) was added and the reaction mixture was gently stirred. At selected time points 50 µL aliquots were removed and analyzed using a ninhydrin colorometric assay.[21] The pH of the solution was periodically measured and if necessary readjusted to pH 7.5-8.0 by addition of 4 N KOH. When

the ninhydrin assay showed no further increase in amino acid concentration, the resolution reaction was terminated by briefly heating the mixture to 60 ^{o}C followed by stirring with activated charcoal (72.5 mL of powder). The mixture was vacuum filtered to yield a colorless filtrate, which was adjusted to pH 1.5 with conc. HCl, and then washed with EtOAc (3 x 490 mL). The aqueous phase was applied onto a Dowex® 50WX8 column (490 mL) and desalted by flushing the column with H_2O until the eluent reached neutral pH followed by desorption of the amino acid by eluting with aqueous 1.0 N NH_3. L-Homoallylglycine was recovered as a colorless solid after rotary evaporation followed by lyophilization (15.3 g, 92% yield based on amount of *N*-acetyl-L-homoallylglycine). ^{1}H NMR (400 MHz, 0.5 % TFA-d/D_2O, 25 °C): δ 5.80 (m, 1 H), 5.09 (m, 1 H), 5.02 (m, 1 H), 4.02 (m, 1 H), 2.15 (m, 2 H), 2.0 (m, 2 H). $[\alpha]_D^{24}$ = +27.4 (c 1.00, 1 N HCl). Spectral data were consistent with previously published results.[21]



***N*-(Carbobenzyloxy)-DL-homoallylglycine.** DL-Homoallylglycine hydrochloride (104 mg, 0.64 mmol) was dissolved in DI water (9.0 mL). Sodium bicarbonate (586 mg, 7.0 mmol) was added to the resulting solution and the reaction mixture was let stir at room temperature for 10 minutes. The reaction was then cooled to 0 °C and benzyl chloroformate (144 mg, 0.84 mmol) was added dropwise. The reaction was let warm to room temperature overnight with vigorous stirring. The reaction mixture was transferred to a separatory funnel and washed with 4 x 6 mL Et_2O. The aqueous layer was then brought to pH 2 with 3M HCl and extracted with 4 x 5 mL EtOAc. The EtOAc extracts were dried with Na_2SO_4, decanted, concentrated under reduced pressure and further concentrated under vacuum to give a white solid (79.4 mg, 48% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 7.42-7.28 (br m, 5H), 6.02-5.68 (br m, 1H), 5.32-4.92 (br m, 5H), 4.51-

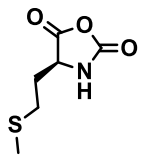
4.22 (br m, 1H), 2.22-2.10 (q, J = 7.1 Hz, 2H), 2.10-1.72 (br m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ 177.3, 156.1, 136.6, 136.1, 128.6, 128.3, 128.2, 116.1, 67.2, 53.3, 31.5, 29.4. MP = 104.0-105.2 °C.



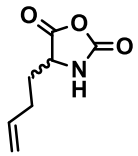
***N*-(Carbobenzyloxy)-L-homoallylglycine.** L-Homoallylglycine (58 mg, 0.46 mmol) was dissolved in 1M sodium bicarbonate (5 mL) and let stir for 10 minutes. The reaction was then cooled to 0 °C and benzyl chloroformate (108 mg, 0.63 mmol) was added dropwise. The reaction was let warm to room temperature overnight with vigorous stirring. The reaction mixture was transferred to a separatory funnel and washed with 4 x 3 mL Et_2O. The aqueous layer was then brought to pH 2 with 3M HCl and extracted with 4 x 2 mL EtOAc. The EtOAc extracts were dried with Na_2SO_4, decanted, concentrated under reduced pressure and further concentrated under vacuum to give a white solid (90 mg, 75% yield). ^1H NMR (400 MHz, $CDCl_3$, 25 °C): δ 7.40-7.29 (br m, 5H), 6.06-5.65 (br m, 1H), 5.37-4.88 (br m, 5H), 4.52-4.18 (br m, 1H), 2.25-2.09 (q, J = 7.1 Hz, 2H), 2.09-1.71 (br m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ 177.3, 156.1, 136.6, 136.1, 128.6, 128.3, 128.2, 116.1, 67.2, 53.3, 31.6, 29.4. MP = 69.3-71.8 °C.

Amino acid *N*-carboxyanhydride (NCA) monomer syntheses. NCA monomers were prepared using a previously established method.[23] ***Caution! Phosgene is extremely hazardous and all manipulations must be performed in a well-ventilated chemical fume hood with proper personal protection and necessary precautions taken to avoid exposure.*** After evaporation of crude reaction mixtures to dryness using a Schlenk manifold, crude products, in sealed Schlenk flasks under vacuum, were brought into a dinitrogen filled

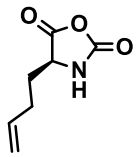
glovebox for purification. The method of purification varied for each monomer, as detailed below.



L-Methionine *N*-carboxyanhydride, Met NCA. Purified via column chromatography using 33% THF in hexanes. Spectral data were consistent with previously published results.[23]



DL-Homoallylglycine *N*-carboxyanhydride, *rac*-Hag NCA. Purified via column chromatography using 33 % THF in hexanes. DL-Homoallylglycine hydrochloride (590 mg) was used to prepare *rac*-Hag NCA, which was obtained as a colorless solid (408 mg, 73% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 6.40 (s, 1H), 5.84-5.71 (br m, 1H), 5.18-5.08 (m, 2H), 4.37-4.31 (m, 1H), 2.33-2.20 (q, J = 6.9 Hz, 2H), 2.13-2.03 (m, 1H), 1.93 (pent, J = 7.1 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ 169.7, 153.0, 135.6, 117.3, 57.1, 30.8, 29.1. DART-MS m/z = 154.05 $[M - H]^-$ (calcd for $C_7H_8O_3N$: 154.05). MP = 63.8-65.4 °C.



L-Homoallylglycine *N*-carboxyanhydride, Hag NCA. Purified via column chromatography using 33% THF in hexanes, followed by 2x recrystallization from THF/hexanes at -35 °C. L-Homoallylglycine (460 mg) was used to prepare Hag NCA, which was obtained as a white solid

(370 mg, 66% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 6.51 (s, 1H), 5.85-5.70 (br m, 1H), 5.18-5.07 (m, 2H), 4.37-4.31 (br m, 1H), 2.33-2.20 (br m, 2H), 2.13-2.03 (m, 1H), 1.93 (pent, J = 7.2 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ 169.7, 153.0, 135.5, 117.4, 57.1, 30.8, 29.2 DART-MS m/z = 154.05 $[M - H]^-$ (calcd for $C_7H_8O_3N$: 154.05). MP = 41.6-43.1 °C.

2.5.3 Homopolymerizations of Hag and *rac*-Hag NCAs

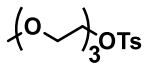
General Procedure for Polymerization of Hag NCA. All polymerization reactions were performed in a dinitrogen filled glove box using anhydrous solvents. To a solution of Hag NCA in THF (164 mg, 1.05 mmol, 25 mg/mL) was added a solution of $Co(PMe_3)_4$ in THF (970 µL, 52.5 µmol). Once mixed, the reaction was let stand at ambient temperature for 1-2 h, and completion of polymerization was confirmed by FTIR. Polymerization at all different monomer to initiator feed ratios used were found to be complete within 2 h. The completed reaction was removed from the glove box and 1-2 drops of 3 M HCl were added. The polymer was then precipitated with DI water, centrifuged at 3000 rpm, and the supernatant was discarded. The pellet was washed 3 times with DI water and then lyophilized to yield poly(L-homoallylglycine), $\mathbf{G^{HA}}$, as a white solid (105 mg, 95% yield). ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 5.87-5.70 (dq, J = 17.7, 14.2 Hz, 1H), 5.17-5.01 (br m, 2H), 4.74-4.70 (dd, J = 8.4, 7.0 Hz, 1H), 2.28-2.08 (br m, 2H), 2.07-1.87 (br m, 2H).

General procedure for polymerization of *rac*-Hag NCA. All polymerization reactions were performed in a dinitrogen filled glove box using anhydrous solvents. To a solution of *rac*-Hag NCA in THF (173 mg, 1.11 mmol, 25 mg/mL) was added a solution of $Co(PMe_3)_4$ in THF (1.00 mL, 55.0 µmol). Once mixed, the reaction was let stand at ambient temperature for 2 h, and completion of polymerization was confirmed by FTIR. Only polymerizations with M:I ratios up to 20:1 were found to go to completion within 2 hours, polymerizations at higher M:I ratios did not go to completion even after many hours. After 2 hours, the reaction was removed from the glove box and 1-2 drops of 3 M HCl were added. The polymer was then precipitated with DI

water, centrifuged at 3000 rpm, and the supernatant was discarded. The pellet was washed 3 times with DI water and then lyophilized to yield poly(DL-homoallylglycine), **(*rac*-G^{HA})**, as a white solid (100 mg, 85% yield). ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 5.92-5.72 (br m, 1H), 5.17-5.01 (br m, 2H), 4.74 (br s, 1H), 2.34-2.13 (br m, 2H), 2.13-1.89 (br m, 2H).

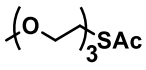
General procedure for endcapping of polypeptides with PEG chains. The general procedure for polymerization of Hag NCA was followed. Once the reaction was determined to be complete by FTIR, a solution of α-methoxy-ω-isocyanoethyl-poly(ethylene glycol), PEG-NCO (MW = 1000 Da, 4 eq per $Co(PMe_3)_4$) in THF was added to the polymerization mixture inside a dinitrogen filled glovebox. The reaction was let stand overnight, and then removed from the glovebox. 1-2 drops of 3M HCl were then added and the reaction was precipitated with water, centrifuged at 3000 rpm and the supernatant was discarded. The pellet was washed 3 times with DI water to remove unconjugated PEG-NCO, and the resulting pellet was then lyophilized to yield PEG-endcapped polypeptide as a white solid. To determine the molecular weight of the polypeptides (M_n), ^{1}H NMR spectra were obtained in deuterated trifluoroacetic acid (TFA-d) or deuterated chloroform ($CDCl_3$) containing 1-2 drops of TFA-d. Similar to procedures described in literature,[30] the integral of the alkene proton resonance of **G^{HA}** at 5.17-5.01 ppm was compared to the integral of the polyethylene glycol resonance at δ 4.01 (TFA-d) or δ 3.64 ($CDCl_3$/ TFA-d) to obtain **G^{HA}** lengths.

2.5.4 Thiol-ene modification of G^{HA} and (*rac*-G^{HA}) homopolypeptides

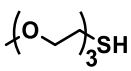


p-Toluenesulfonyl triethylene glycol methyl ether. This compound was synthesized using a previously reported procedure.[31] H_2O (10.0 mL) was added to triethylene glycol monomethyl ether (3.21 g, 19.5 mmol) and NaOH (1.56 g, 39.0 mmol). Tosyl chloride (2.89 g, 20.5 mmol) was dissolved in THF (10.0 mL) and added dropwise over ice with stirring. The reaction was let

warm to ambient temperature overnight. Diethyl ether (20.0 mL) was then added to the reaction and the mixture was taken up into a separatory funnel and partitioned. The aqueous layer was washed with diethyl ether (3 x 5.00 mL) then the combined organic fractions were washed with H_2O (3 x 20.0 mL). The organic layer was dried with anhydrous sodium sulfate, decanted and evaporated to dryness to give the product as a clear oil (4.08 g, 79% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 7.81-7.77 (d, J = 8.3 Hz, 2H), 7.35-7.31 (d, J = 8.0 Hz, 2H), 4.17-4.13 (m, 2H), 3.70-3.48 (br m, 10H), 3.36 (s, 3H), 2.44 (s, 3H).

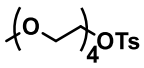


2-[2-(2-Methoxyethoxy)ethoxy]ethanethioacetate. This compound was synthesized using a previously reported procedure.[32] p-Toluenesulfonyl triethylene glycol methyl ether (3.80 g, 14.2 mmol) and potassium thioacetate (2.02 g, 17.7 mmol) were dissolved in a round bottom flask containing acetone (150 mL). A reflux condenser was added and the reaction was stirred vigorously overnight at 50 °C. Solvent was removed via vacuum and the resulting mixture was dissolved in a biphasic mixture of 30 mL DCM and 30 mL H_2O, taken up into a separatory funnel, and partitioned. The aqueous layer was washed with 2 x 30 mL DCM and combined organics were washed with 10 mL brine. The organic layer was dried with anhydrous sodium sulfate, decanted, and evaporated to dryness to give the product as a yellow oil (2.64 g, 90% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 3.66-3.52 (br m, 10H), 3.37 (s, 3H), 3.09 (t, J = 6.5 Hz, 2H), 2.33 (s, 3H).

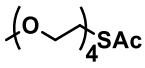


2-[2-(2-Methoxyethoxy)ethoxy]ethanethiol, mEG$_3$SH. This compound was synthesized using a previously reported procedure.[33] Methanol (20.0 mL) and conc. HCl (1.50 mL) were added to a sealed pressure tube containing 2-[2-(2-methoxyethoxy)ethoxy]ethanethioacetate (2.06 g,

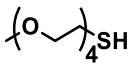
10.0 mmol). The reaction mixture was stirred at 100 °C for 3 hours. The reaction was let cool to ambient temperature and then H_2O (20.0 mL) was added. This mixture was extracted with 3x 15.0 mL DCM. Combined organic extracts were then washed with 3x 15.0 mL H_2O and 1x 15 mL brine, dried with anhydrous sodium sulfate, decanted, and evaporated to dryness to give the product as a yellow oil (1.37 g, 83% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 3.68-3.53 (br m, 10H), 3.38 (s, 3H), 2.73-2.66 (dt, J = 8.2, 6.5 Hz, 2H), 1.58 (t, J = 8.2 Hz, 1H).



p-Toluenesulfonyl tetraethylene glycol methyl ether. The procedure for synthesis of p-toluenesulfonyl triethylene glycol methyl ether was followed. Tetraethylene glycol monomethyl ether (4.23 g) was used to prepare the product, obtained as a clear oil (4.18 g, 66% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 7.83-7.76 (d, J = 8.2 Hz, 2H), 7.36-7.30 (d, J = 8.1 Hz, 2H), 4.15 (t, J = 9.7 Hz, 2H), 3.70-3.51 (br m, 14H), 3.37 (s, 3H), 2.44 (s, 3H).

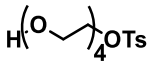


2-[2-[2-(2-Methoxyethoxy)ethoxy]ethoxy]ethanethioacetate. The procedure for synthesis of 2-[2-(2-methoxyethoxy)ethoxy]ethanethioacetate was followed. p-toluenesulfonyl tetraethylene glycol methyl ether (2.74 g) was used to prepare the product, obtained as a dark yellow oil (2.00 g, 90% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 3.67-3.52 (br m, 14H), 3.37 (s, 3H), 3.08 (t, J = 6.5 Hz, 2H), 2.33 (s, 3H).

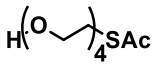


2-[2-[2-(2-Methoxyethoxy)ethoxy]ethoxy]ethanethiol, mEG$_4$SH. The procedure for synthesis of 2-[2-(2-methoxyethoxy)ethoxy]ethanethiol was followed. 2-[2-[2-(2-methoxyethoxy)ethoxy]ethoxy]-ethanethioacetate (1.31 g) was used to prepare the product,

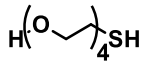
obtained as a pale yellow oil (851 mg, 78% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 3.67-3.49 (br m, 14H), 3.35 (s, 3H), 2.71-2.61 (m, 2H), 1.57 (t, J = 8.2 Hz, 1H).



Tetraethylene glycol monotosylate. This compound was synthesized using a previously reported procedure.[34] Tetraethylene glycol (23.4 g, 120. mmol) was added to 100 mL round bottom flask and dissolved in THF (4.3 mL). NaOH (727 mg, 18.2 mmol) was added as solution in 4.3 mL H_2O. Tosyl chloride (2.23 g, 12.0 mmol) was dissolved in THF (14.0 mL) and added dropwise over ice with stirring. The reaction was stirred at 0 °C for 3 hours then diluted with ice water (70 mL). The mixture was extracted with 3x 45 mL DCM. The combined organic fractions were washed with 2 x 130 mL DI water, dried with anhydrous sodium sulfate, decanted, and evaporated to dryness to give the product as a pale yellow oil (3.69 g, 87% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 7.82-7.77 (d, J = 8.3 Hz, 2H), 7.36-7.30 (dd, J = 8.5 Hz, 0.58 Hz, 2H), 4.16 (t, J = 4.8 Hz, 2H), 3.80-3.50 (br m, 14H), 2.44 (s, 3H) 2.17 (br s, 1H).

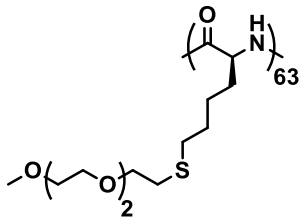


Tetraethylene glycol monothioacetate. The procedure for synthesis of 2-[2-(2-methoxyethoxy)-ethoxy]ethanethioacetate was followed. Tetraethylene glycol monotosylate (3.69 g) was used to prepare the product, further purification (column chromatography with 100 % ethyl acetate) was required to obtain the product as a yellow oil (1.76 g, 57% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 3.74-3.55 (br m, 14H), 3.09 (t, J = 6.5 Hz, 2H), 2.33 (s, 3H) 2.29 (br s, 1H).

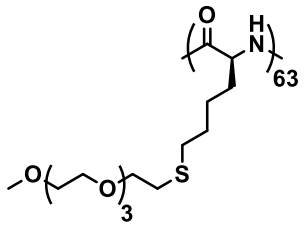


1-Mercapto-11-hydroxy-3,6,9-trioxaundecane, EG_4SH. The procedure for synthesis of 2-[2-(2-methoxyethoxy)ethoxy]ethanethiol was followed. Tetraethylene glycol monothioacetate (200 mg) was used to prepare the product, obtained as a pale yellow oil (95 mg, 58% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 3.75-3.55 (br m, 14H), 2.71-2.61 (dt, J = 8.1 Hz, J = 6.5 Hz, 2H), 2.11 (br s, 1H), 1.62 (t, J = 8.2 Hz, 1H).

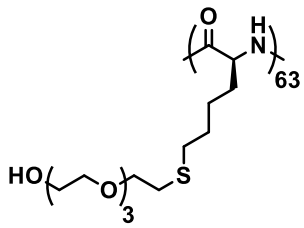
General procedure for thiol-ene modification of G^{HA}. A sample of G^{HA} (*ca*. 7 mg) and DMPA (0.2 eq per G^{HA} residue) were placed in a 1 dram screw top vial. THF was then added to give a 4 mg/mL polymer concentration. The desired thiol was then added via micropipette (3 eq per Hag residue) and the solution was degassed by sparging with N_2 for 10 minutes. The vial was then covered with parafilm and the solution was irradiated with UV light for 2.5 hours (Exo Terra Reptile Lamp) and let stir overnight. The solution was then transferred to a 2000 MWCO dialysis bag and dialyzed against methanol for 24 hours with one change of dialysate, followed by dialysis in water for 24 hours with one water change. The dialyzed polymer was lyophilized to dryness to give the product as a white solid.



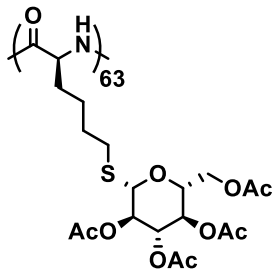
mEG_3-G^{HA}_{63}. The general procedure for thiol-ene modification of G^{HA} was followed. G^{HA}_{63} (6.5 mg) and mEG_3SH (3 eq per Hag residue) were used to prepare the product, obtained as a white solid (14.1 mg, 83% yield). ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 4.89-4.53 (br m, 1H), 4.22-3.77 (br m, 10H), 3.75-3.02 (br m, 5H), 3.02-2.60 (br m 2H), 2.23-1.43 (br m, 6H).



mEG$_4$-G$^{HA}_{63}$. The general procedure for thiol-ene modification of **G^{HA}** was followed. **G$^{HA}_{63}$** (7.5 mg) and mEG$_4$SH (3 eq per Hag residue) were used to prepare the product, obtained as a white solid (16.3 mg, 89% yield). ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 4.99-4.58 (br m, 1H), 4.25-3.80 (br m, 14H), 3.80-2.55 (br m, 7H), 2.31-1.46 (br m, 6H).

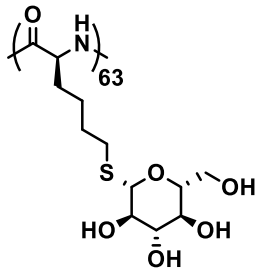


EG$_4$-G$^{HA}_{63}$. The general procedure for thiol-ene modification of **G^{HA}** was followed. **G$^{HA}_{63}$** (7.5 mg) and EG$_4$SH (3 eq per Hag residue) were used to prepare the product, obtained as a white solid (16.3 mg, 89 % yield). ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 4.95-4.50 (br m, 1H), 4.27-3.60 (br m, 14H), 3.59-2.54 (br m, 4H), 2.20-1.43 (br m, 6H).

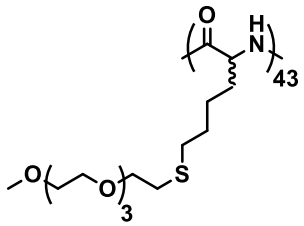


Glc(OAc)$_4$-G$^{HA}_{63}$. The general procedure for thiol-ene modification of **G^{HA}** was followed. **G$^{HA}_{63}$** (10.8 mg) and 1-Thio-β-D-glucose tetraacetate (3 eq per Hag residue) were used to prepare the product, obtained as a white solid (38.6 mg, 84% yield). ^{1}H NMR (400 MHz, $CDCl_3$/TFA-d, 25

°C): δ 5.32 (t, J = 9.3 Hz, 1H), 5.15 (t, J = 9.6 Hz, 1H), 5.03 (t, J = 9.6, 1H), 4.59-4.48 (br d, J = 10. Hz, 1H), 4.43 (br s, 1H), 4.24 (br s, 2H), 3.83-3.70 (br d, J = 9.8 Hz, 1H), 2.75-2.55 (br d, J = 6.8 Hz, 2H), 2.20-1.97 (br m, 12H), 1.78-1.17 (br m, 6H).



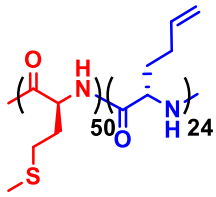
Glc-G$^{HA}_{63}$. This deprotection follows a previously reported method that was used for a different glycopolymer.[35] **Glc(OAc)$_4$-G$^{HA}_{63}$** (10 mg) was dissolved in 1:2 DCM:MeOH (1 mL). An aqueous solution of hydrazine monohydrate (65 % (w/w), 4 eq per acetyl group) was added via syringe and the mixture was stirred. Shortly after the addition of hydrazine monohydrate, a white precipitate formed. The reaction was let stir overnight, after which 1-2 drops of acetone were added to quench excess hydrazine. Et_2O (2 mL) was added, the product was centrifuged at 3000 rpm, and the supernatant was discarded. The pellet was washed with ether (2 mL). Solvent was removed via vacuum and the product was dissolved in DI water, transferred to a 2000 MWCO dialysis bag, and dialyzed for 48 hours with 2 water changes daily. The dialyzed polymer was lyophilized to dryness to give the product as a white solid (6.2 mg, quantitative yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.48-4.34 (br d, J = 9.1 Hz, 1H), 3.95 (br s, 1H), 3.82-3.71 (br d, J = 11.4 Hz, 1H), 3.66-3.54 (br m, 1H), 3.44-3.25 (br m, 3H), 3.25-3.15 (br m, 1H), 2.67 (br s, 2H), 2.04-1.09 (br m, 6H).



mEG$_4$-(*rac*-G^{HA})$_{43}$. The general procedure for thiol-ene modification of **G^{HA}** was followed. **(*rac*-G^{HA})$_{43}$** (8.0 mg) and mEG$_3$SH (6 eq per *rac*-Hag residue) were used to prepare the product, obtained as a white solid (16.3 mg, 68% yield). (93% functionalization, determined by comparison of alkene 2H integral (5.17 ppm) to the integral from the oligoethylene glycol group (4.37-3.82)). ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 5.05-4.53 (br m, 1H), 4.37-3.82 (br m, 14H), 3.82-2.59 (br m, 7H), 2.30-1.50 (br m, 6H).

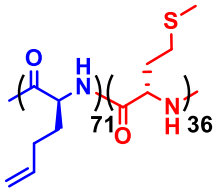
2.5.5 Synthesis and Modification of Diblock Copolypeptides

Example Diblock Copolypeptide Syntheses



Poly(L-methionine)$_{50}$-*block*-poly(L-homoallylglycine)$_{24}$, M$_{50}$G$^{HA}_{24}$. All polymerization reactions were performed in a dinitrogen filled glove box using anhydrous solvents. To a solution of Met NCA in THF (98.0 mg, 550 µmol, 50 mg/mL) was added a solution of Co(PMe$_3$)$_4$ in THF (515 µL, 28 µmol). Once mixed, the reaction was let stand at ambient temperature for 1 hour and completion of polymerization was confirmed by FTIR. An aliquot (203 µL) was removed from the reaction and endcapped with PEG-NCO to determine DP of the first block (found DP = 50) (see method above). To the remaining polymerization solution was added a solution of Hag NCA in THF (39.8 mg, 2.60 µmol, 50 mg/mL). The reaction was let stand for 1 hour and completion of polymerization was confirmed by FTIR. The reaction was removed from

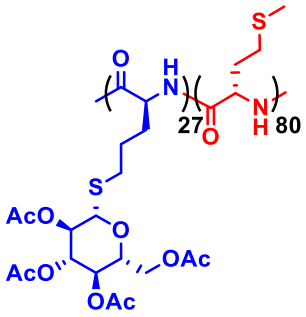
the glove box and 1-2 drops of 3 M HCl were added. The polypeptide was then precipitated with DI water, centrifuged at 3000 rpm, and the supernatant was discarded. The pellet was washed 3 times with DI water and then lyophilized to yield poly(L-methionine)$_{50}$-*block*-poly(L-homoallylglycine)$_{24}$, **M$_{50}$G$^{HA}_{24}$** (composition determined by ^{1}H NMR), as a white solid (94.4 mg, 99% yield). ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 5.92-5.72 (br m, 1H), 5.19-5.05 (br m, 2H), 5.00-4.88 (br m, 2H), 4.79-4.70 (m, 1H), 2.90-2.64 (br m, 4H), 2.40-2.13 (br m 12H), 2.10-1.90 (br m, 2H).



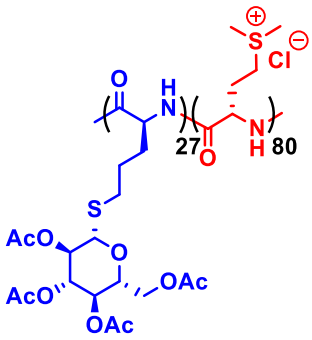
Poly(L-homoallylglycine)$_{71}$-*block*-poly(L-methionine)$_{36}$, G$^{HA}_{71}$M$_{36}$. All polymerization reactions were performed in a dinitrogen filled glove box using anhydrous solvents. To a solution of Hag NCA in THF (20.1 mg, 130 μmol, 25 mg/mL) was added a solution of Co(PMe$_3$)$_4$ in THF (119 μL, 6.47 μmol). Once mixed, the reaction was let stand at ambient temperature for 1 hour and completion of polymerization was confirmed by FTIR. An aliquot (370 μL) was removed and endcapped with PEG-NCO to determine DP of the first block (found DP = 71). To the remaining polymerization solution was added a solution of Met NCA in THF (6.83 mg, 39.0 μmol, 50 mg/mL). The reaction was let stand for 1 hour and completion of polymerization was confirmed by FTIR. The reaction was removed from the glove box and 1-2 drops of 3 M HCl were added. The polymer was then precipitated with DI water, centrifuged at 3000 rpm, and the supernatant was discarded. The pellet was washed 3 times with DI water and then lyophilized to yield Poly(L-homoallylglycine)$_{71}$-*block*-poly(L-methionine)$_{36}$, **G$^{HA}_{71}$M$_{36}$** (composition determined by ^{1}H NMR) as a white solid (13.4 mg, 97% yield). ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 5.92-

5.72 (br m, 2H), 5.19-5.05 (br m, 4H), 5.00-4.88 (br m, 1H), 4.81-4.65 (br m, 2H), 2.90-2.66 (br m, 2H), 2.36-2.13 (br m, 9H), 2.10-1.90 (br m, 4H).

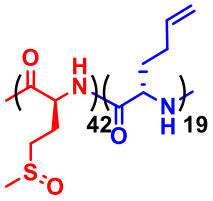
Modifications of Diblock Copolypeptides



Glc(OAc)$_4$-G$^{HA}_{27}$M$_{80}$. **G$^{HA}_{27}$M$_{80}$** (14.4 mg), 1-Thio-β-D-glucose tetraacetate (8 eq per Hag residue) and DMPA (2.92 mg, 0.4 eq per Hag residue) were placed in a 1 dram screw top vial. THF was then added to give a copolymer concentration of 10 mg/mL. The solution was then degassed via sparging with N_2 for 10 minutes. The vial was then covered with parafilm and the solution was irradiated with UV light for 2.5 hours (Exo Terra Reptile Lamp) and let stir overnight. The solution was then transferred to a 2000 MWCO dialysis bag and dialyzed against methanol for 24 hours with one change of dialysate, followed by dialysis against water for 24 hours with one water change. The dialyzed polymer was lyophilized to dryness to give the product as a white solid (20.4 mg, 82% yield). ^{1}H NMR (400 MHz, $CDCl_3$/TFA-d, 25 °C): δ 5.34 (t, J = 9.2 Hz, 1H), 5.18 (t, J = 9.6 Hz, 1H), 5.05 (t, J = 9.6, 1H), 4.61-4.50 (br d, J = 9.6 Hz, 1H), 4.43 (br s, 1H), 4.26 (br s, 5H), 3.83-3.70 (br d, J = 10.1 Hz, 1H), 2.80-2.47 (br m, 8H), 2.33-2.01 (br m, 27H), 1.78-1.21 (br m, 6H).

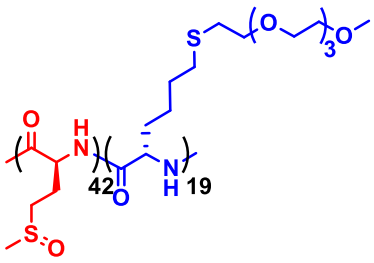


Glc(OAc)$_4$-G$^{HA}_{27}$M$^{M}_{80}$. Glc(OAc)$_4$-G$^{HA}_{27}$M$_{80}$ (11.7 mg) was suspended in DI water (1.2 mL). Methyl iodide was added via syringe (6 eq per Met residue), the reaction was then covered and stirred vigorously for 72 hours. The reaction mixture was transferred to a 2000 MWCO dialysis bag and dialyzed against 0.1 M NaCl for 24 hours, followed by DI water for 24 hours with 2 water changes daily. The dialyzed polymer was then lyophilized to dryness to give the product as a translucent white solid (13.0 mg, 95% yield). ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 5.80-5.59 (br m 1H), 5.58-5.41 (br m, 1H), 5.41-5.28 (br m, 1H), 5.25-4.94 (br m, 3H), 4.94-4.80 (br m, 1H), 4.80-4.61 (br m, 1H), 4.61-4.41 (br m, 2H), 4.19-3.98 (br m, 1H), 3.97-3.50 (br d, J = 45.2 Hz, 6H), 3.23-3.04 (br d, J = 5.8 Hz, 18H), 2.99-2.49 (br m, 8H), 2.37-2.25 (br m, 12H), 2.10-1.37 (br m, 6H).

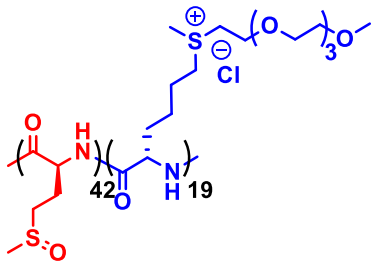


M$^{O}_{42}$G$^{HA}_{19}$. A solution of hydrogen peroxide : acetic acid in DI water (3 %:1.5 % (v/v), 2.61 mL) was added to a vial containing **M$_{42}$G$^{HA}_{19}$** (31.3 mg). The suspension was stirred vigorously overnight, briefly vortexed after 18 hours and let stir for 6 more hours. The reaction was quenched with 1-2 drops of aqueous saturated sodium thiosulfate solution and then transferred to a 2000 MWCO dialysis bag. The reaction mixture was dialyzed against water for 48 hours

with two water changes daily. Lyophilization yielded the product as a white, fluffy solid (32.2 mg, 95% yield). ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 5.88-5.72 (br m, 1H), 5.18-5.06 (br m, 2H), 5.05-4.86 (br m, 2H), 4.80-4.64 (br m, 1H), 3.59-3.15 (br m 5H), 3.05-2.95 (br d, J = 7.9 Hz, 7H), 2.81-2.41 (br m , 5H), 2.34-1.89 (br m, 4H).



M^{O}_{42}mEG$_4$-G^{HA}_{19}. **$M^{O}_{42}G^{HA}_{19}$** (8.0 mg), and DMPA (1.9 mg, 0.4 eq per Hag residue) were placed in a 1 dram screw top vial. A mixture of 25% THF in AcOH was then added to give a polymer concentration of 4 mg/mL. mEG$_4$SH was then added via micropipette (10 eq per Hag residue) and the solution was degassed via sparging with N_2 for 10 minutes. The vial was then covered with parafilm and the solution was irradiated with UV light for 2.5 hours (Exo Terra Reptile Lamp) and let stir overnight. The solution was then transferred to a 2000 MWCO dialysis bag and dialyzed against methanol for 24 hours with one change of dialysate, followed by dialysis in water for 24 hours with one water change. The dialyzed polymer was lyophilized to dryness to give the product as a white solid (10.3 mg, 86% yield). ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 5.08-4.89 (br m, 2H), 4.85-4.60 (br m, 1H), 4.30-3.79 (br m, 14H), 3.76-3.22 (br m, 9H), 3.07-3.00 (br d, J = 8.1 Hz, 6H), 2.99-2.65 (br m , 4H), 2.65-2.43 (br m, 2H), 2.18-1.49 (br m, 6H).



$M^{O}_{42}mEG_4\text{-}G^{HAM}_{19}$. **$M^{O}_{42}mEG_4\text{-}G^{HA}_{19}$** (7.7 mg) was suspended in 0.2 M formic acid (520 µL). Methyl iodide was added via syringe (6.6 eq per mEG_4-Hag residue), the reaction was then covered and stirred vigorously for 72 hours. The reaction mixture was transferred to a 2000 MWCO dialysis bag and dialyzed against 0.1 M NaCl for 24 hours, followed by DI water for 24 hours with 2 water changes daily. The dialyzed polymer was then lyophilized to dryness to give the product as a translucent white solid (8.3 mg, 99% yield). ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 5.08-4.86 (br m, 2H), 4.83-4.61 (br m, 1H), 4.24-3.80 (br m, 14H), 3.76-3.56 (br m, 5H), 3.14-3.06 (br s, 3H), 3.04-2.97 (br d, J = 7.9 Hz, 6H), 2.83-2.63 (br m , 2H), 2.60-2.42 (br m, 2H), 2.28-1.56 (br m, 6H).

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Chapter 3: Synthesis of Neoglycoconjugates via Post-Polymerization modification of *N*-methylaminooxy functionalized polypeptides

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3.1 Abstract

The preparation and characterization of a new set of well-defined polypeptides containing *N*-methylaminooxy side-chain functionality is described. These functional groups enabled the direct coupling of polypeptides with a variety of unmodified reducing saccharides in water to give neoglycopolypeptides in high yields. The use of different polypeptide scaffolds resulted in neoglycoconjugates with tunable chain conformations, hydrophobicity, and charge. These new neoglycopolypeptides were also found to be stable in aqueous media at pH 7.4 and 37 °C for 1 week. The combination of straightforward synthesis using unmodified saccharides, high yields of saccharide conjugation, and conjugate stability makes these polypeptides attractive candidates for development of degradable glycoprotein mimics.

3.2 Introduction

Natural glycoproteins possess abundant biological functionality as well as structural and physical properties that enable their ability to provide a cellular support in the extracellular matrix.[1] Because of the large size and complexity of glycoproteins, it is desirable to obtain structurally defined synthetic glycoprotein mimics for the study of cell–substrate interactions and for regenerative medicine biomaterial applications.[2, 3] While many polymeric neoglycoconjugates have been reported, many rely on nonpeptidic backbones,[4-6] require tedious multistep syntheses,[7-12] or utilize chemically modified saccharide building blocks,[13-16] where many are limited to conjugation of simple monosaccharides. To better mimic glycoproteins in biomaterial

applications, use of degradable peptidic polymer backbones and incorporation of complex oligosaccharides from natural glycoprotein components are preferred.[2, 3] Further, to allow facile preparation of a variety of glycoprotein mimics for analysis of structure–property relationships, straightforward and robust synthetic methods are needed. Here, we describe the preparation of a new set of well-defined polypeptides containing *N*-methylaminooxy side-chain functionality, which enables direct coupling with unmodified reducing saccharides in water to give neoglycopolypeptides in high yields. The ability to react different saccharides to these polypeptides to produce a variety of stable conjugates provides a useful means for preparation of well-defined, highly functional neoglycoconjugates.

There has been much recent activity toward preparation of neoglycopolypeptides. These methods are based on two strategies: (i) ring-opening polymerization of glycosylated amino acid *N*-carboxyanhydride (NCA) monomers[7-12] or (ii) post-polymerization conjugation of saccharides to reactive precursor polypeptides.[13-16] Both of these methods are able to provide polypeptides with high degrees of glycosylation, and many examples have been reviewed. The glycosylated NCA route provides advantages of 100% saccharide functionalization and the ability to utilize natural glycoside linkages (e.g., to serine residues). The main disadvantage of this method is the need for multistep monomer synthesis, use of protecting groups, and tedious monomer purification. Consequently, use of this methodology for incorporation of complex oligosaccharides containing additional functionality, for example, sulfonate and carboxylate groups often present in glycoproteins, is challenging. Post-polymerization neoglycoconjugate formation offers the advantage of simple and often more readily prepared polypeptide chains but conjugations can suffer from incomplete saccharide functionalization.[13-16] Most of the reported methods also require use of saccharides that have been modified to contain reactive functionality, such as azide, alkyne, thiol, epoxide, alkyl halide, or isothiocyanate groups.[13-16] These modifications require additional synthetic steps, and also result in incorporation of

unnatural linkages such as triazole groups into the neoglycoconjugates that may affect biological properties.

On the basis of these studies, we sought to develop a facile method for neoglycopolypeptide synthesis utilizing a post-polymerization conjugation strategy, but wanted to improve conjugation to allow incorporation of more complex saccharides, and remove the need for saccharide chemical modification. Our strategy was focused on use of the oxime ligation,[17, 18] a chemoselective and efficient method that has been used in many different polymer systems for post-polymerization functionalization, including neoglycoconjugate formation.[4-6, 19-21] While oxime ligation has not been used to prepare neoglycopolypeptides,[22] it has been utilized to prepare neoglycoconjugates in short peptide and peptoid sequences.[23-26] A significant advantage in the use of aminooxy-functionalized peptides and polymers is their ability to react directly with unmodified reducing saccharides. Another important feature is that while saccharide conjugation to aminooxy groups typically results in ring-opened saccharides, the use of *N*-alkylaminooxy or *N*-alkoxyamino groups results in ring closed saccharide conjugates that better mimic natural glycan presentation.[27] Using this knowledge, Godula's laboratory recently showed that *N*-methylaminooxy-functionalized polyacrylamides can be used to prepare neoglycoconjugates with a variety of unmodified complex oligosaccharides derived from glycoproteins.[4-6] Here, we build upon this work by development of new water-soluble polypeptides containing side-chain *N*-methylaminooxy functionality. The conjugation of saccharides to these polypeptides was evaluated, and physical properties of neoglycoconjugates with different side-chain structures were compared. These new polypeptides were found to enable straightforward preparation of neoglycopolypeptides with high degrees of saccharide conjugation in aqueous media.

3.3 Results and Discussion

A *N*-methylaminooxy-functionalized amino acid derived from L-homoserine has been previously reported for preparation of neoglycoconjugates in peptides.[24, 25] While this residue may be amenable for polypeptide synthesis, we initially chose to develop a new *N*-methylaminooxy-functionalized derivative of L-homocysteine because previous work suggests that the resulting polypeptide would possess good solubility during polymerization, and the side-chain thioether groups would allow further modification by oxidation or alkylation to adjust polymer properties.[28] The target 2-(*N*-Boc-*N*-methylaminooxy)ethyl-L-homocysteine amino acid was prepared in two steps from readily available L-methionine in high yield. Subsequent phosgenation in the presence of acid scavengers gave the desired monomer, 2-(*N*-Boc-*N*-methylaminooxy)ethyl-L-homocysteine NCA, Boc-mao-Ehc NCA, as an oil that was purified by chromatography on dried silica (Figure 3.1).[29]

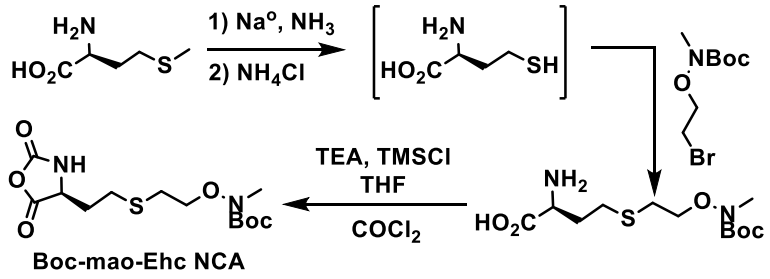


Figure 3.1 Synthesis of Boc-mao-Ehc NCA.

Boc-mao-Ehc NCA was found to polymerize readily in THF to complete monomer conversion using $Co(PMe_3)_4$ initiator (Figure 3.2).[30] Homopolypeptide chain lengths were controlled by adjustment of monomer to initiator ratios enabling preparation of poly(2-(*N*-Boc-*N*-methylaminooxy)ethyl-L-homocysteine)$_n$, **Boc-mao-Ehc$_n$**, with degrees of polymerization up to 145 with low dispersity (Figure 3.3). Subsequent deprotection of a model sample, **Boc-mao-Ehc$_{70}$**, under a variety of acidic conditions was found to yield only intractable cross-linked solids.

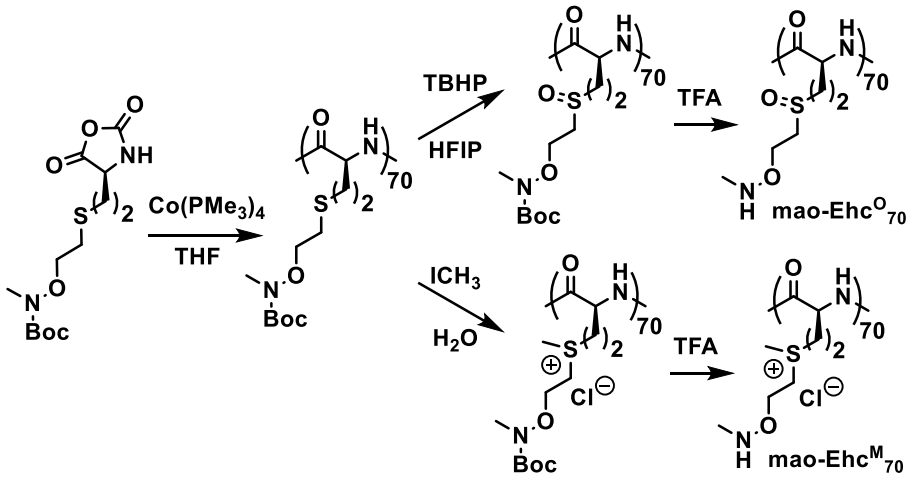


Figure 3.2 Synthesis of **mao-Ehc$^{O}_{70}$** and **mao-Ehc$^{M}_{70}$**.

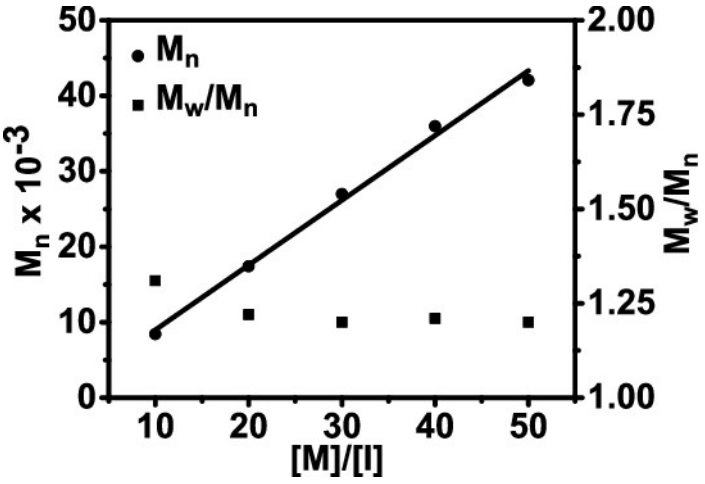


Figure 3.3 Molecular weight (M_n, circles) and dispersity (M_w/M_n, squares) of **Boc-mao-Ehc$_n$** as a function of monomer to initiator ratio ([M]/[I]) using $(PMe_3)_4Co$ in THF at 20 °C. M_n values were calculated via end group analysis using 1H NMR integrations, and GPC/MALS was utilized to determine M_w/M_n.

We suspect that protonation of free *N*-methylaminooxy groups formed during acidic deprotection results in sulfur mustard type chemistry where *N*-methylhydroxylamine can eliminate, yielding electrophilic cyclic sulfonium intermediates that can be attacked by *N*-methylaminooxy groups of other chains to give crosslinks (Figure 3.4).[31]

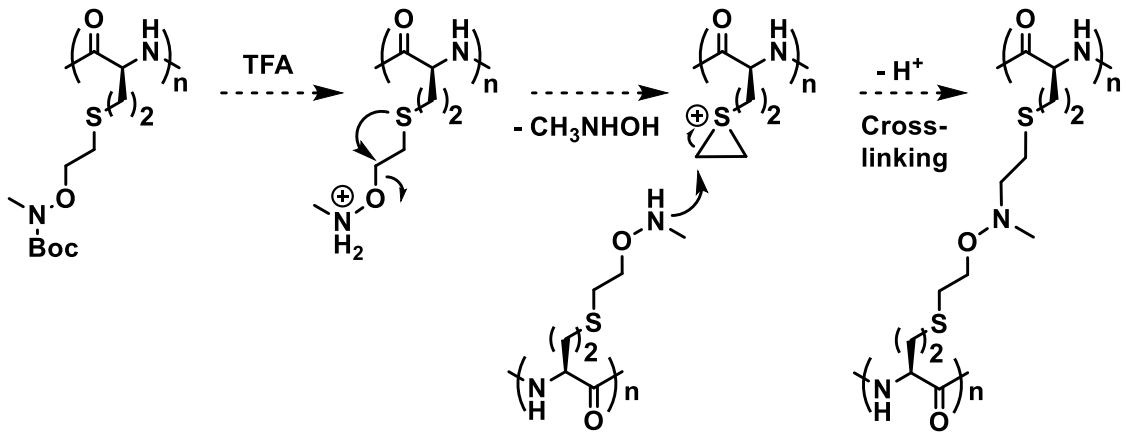


Figure 3.4 Possible crosslinking side reaction during TFA deprotection of **Boc-mao-Ehc$_{70}$**.

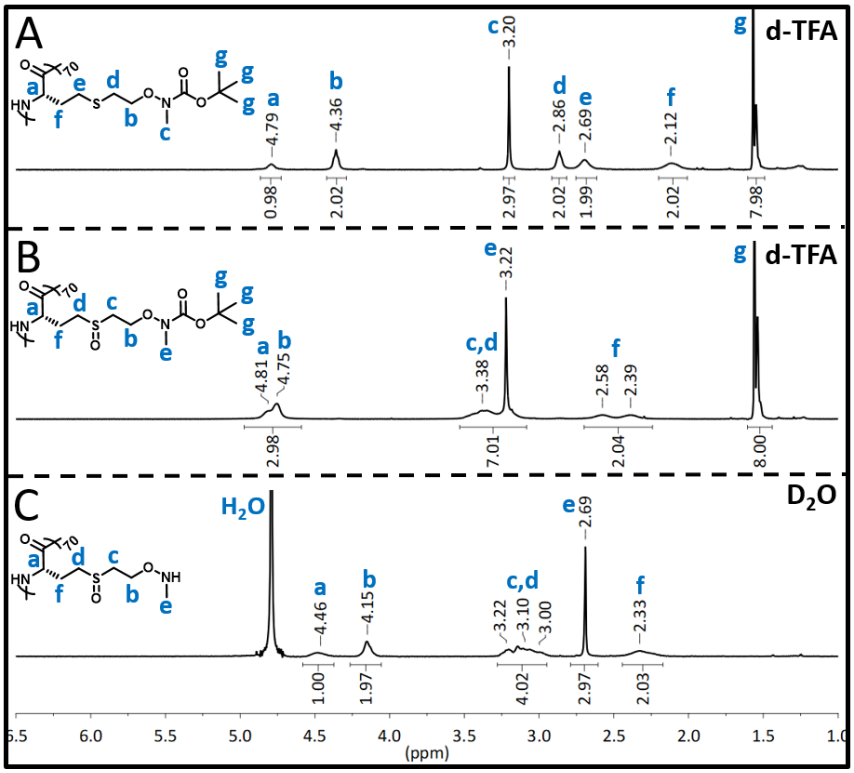


Figure 3.5 ^{1}H NMR spectra overlay of A) **Boc-mao-Ehc$_{70}$** B) **Boc-mao-Ehc$^{O}_{70}$ and** C) **mao-Ehc$^{O}_{70}$**.

To circumvent this issue, **Boc-mao-Ehc$_{70}$** was first oxidized or methylated before deprotection to give the more polar derivatives poly(2-(*N*-Boc-*N*-methylaminooxy)ethyl-L-homocysteine sulfoxide)$_{70}$, **Boc-mao-Ehc$^{O}_{70}$**, or poly(2-(*N*-Boc-*N*-methylaminooxy)ethyl-L-

homocysteine methyl sulfonium)$_{70}$, **Boc-mao-Ehc$^{M}_{70}$**, respectively (Figure 3.2, Figure 3.5, Figure 3.6).

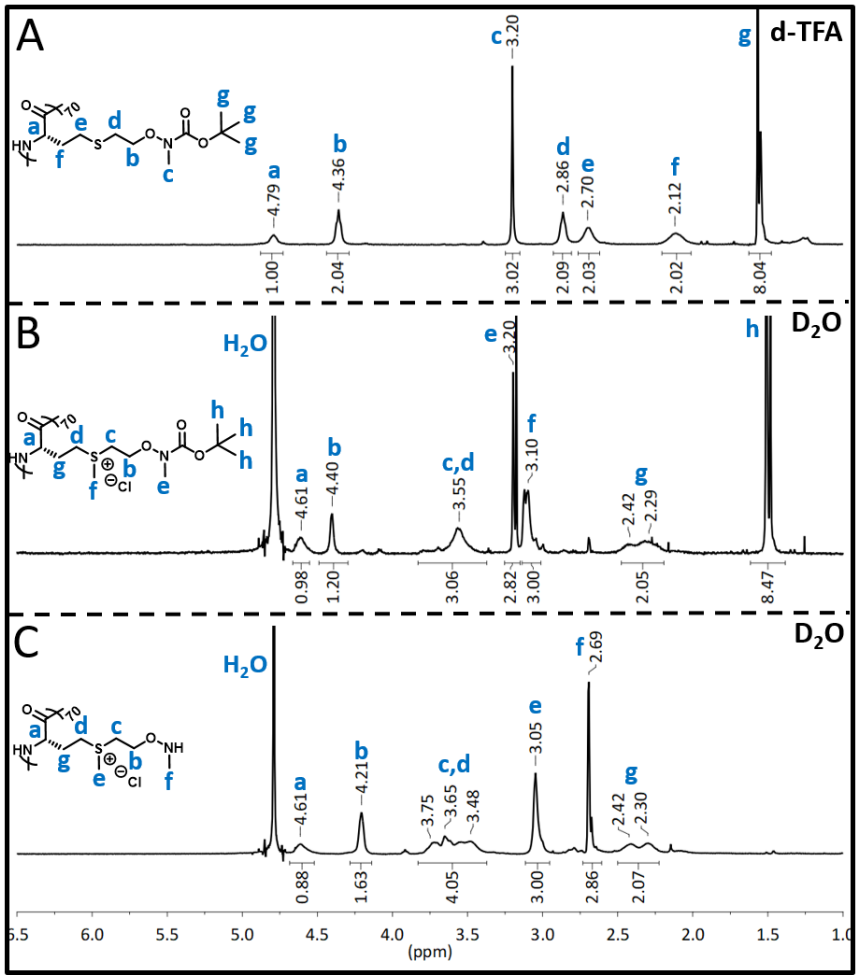


Figure 3.6 ^{1}H NMR spectra overlay of A) **Boc-mao-Ehc$_{70}$** B) **Boc-mao-Ehc$^{M}_{70}$ and C) mao-Ehc$^{M}_{70}$.**

Both of these reactions gave essentially quantitative modifications of the parent thioether groups, similar to previously described results on related polypeptides.[28] Subsequent deprotection of **Boc-mao-Ehc$^{O}_{70}$** and **Boc-mao-Ehc$^{M}_{70}$** using trifluoroacetic acid gave the desired water-soluble, *N*-methylaminooxy-functionalized poly(2-(*N*-methylaminooxy)ethyl-L-homocysteine sulfoxide)$_{70}$, **mao-Ehc$^{O}_{70}$**, and poly(2-(*N*-methylaminooxy)ethyl-L-homocysteine methyl sulfonium)$_{70}$, **mao-Ehc$^{M}_{70}$**, without cross-linking (Figure 3.2, Figure 3.5, Figure 3.6).

With these *N*-methylaminooxy-functionalized polypeptides in hand, one nonionic and one cationic, we also prepared a homologous polypeptide using a different synthetic route. This target polypeptide, poly((6-(2-*N*-methylaminooxy)ethylthio)-L-norleucine sulfoxide)$_{70}$, **mao-Etn$^{O}_{70}$**, was prepared by coupling poly(L-homoallylglycine)$_{70}$, **G$^{HA}_{70}$**, with a *N*-methylaminooxy containing thiol, followed by oxidation and deprotection as described above (Figure 3.7, Figure 3.8, Figure 3.9).[32]

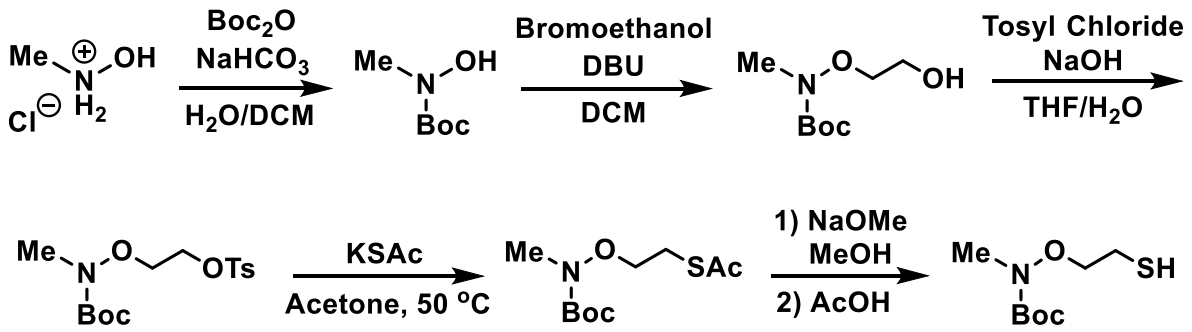


Figure 3.7 Synthesis of 2-(*N*-Boc-*N*-methylaminooxy)ethyl mercaptan**.**

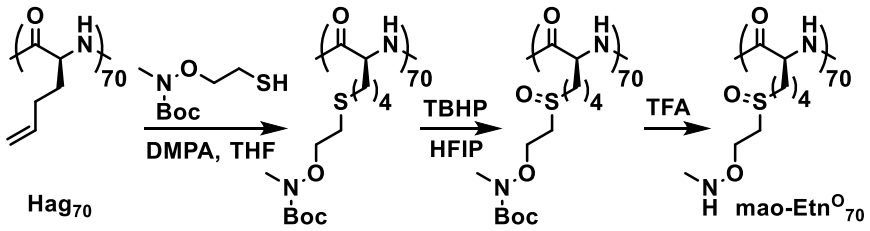


Figure 3.8 Synthesis of **mao-Etn$^{O}_{70}$**.

In **mao-Etn$^{O}_{70}$**, the terminal *N*-methylaminooxy groups are two additional methylenes further from the polypeptide backbone compared with **mao-Ehc$^{O}_{70}$**. This difference was expected to lead to greater side-chain hydrophobicity and α-helical conformational stability in water for **mao-Etn$^{O}_{70}$**. To compare aqueous solution properties of these polypeptides, they were analyzed using CD spectroscopy.

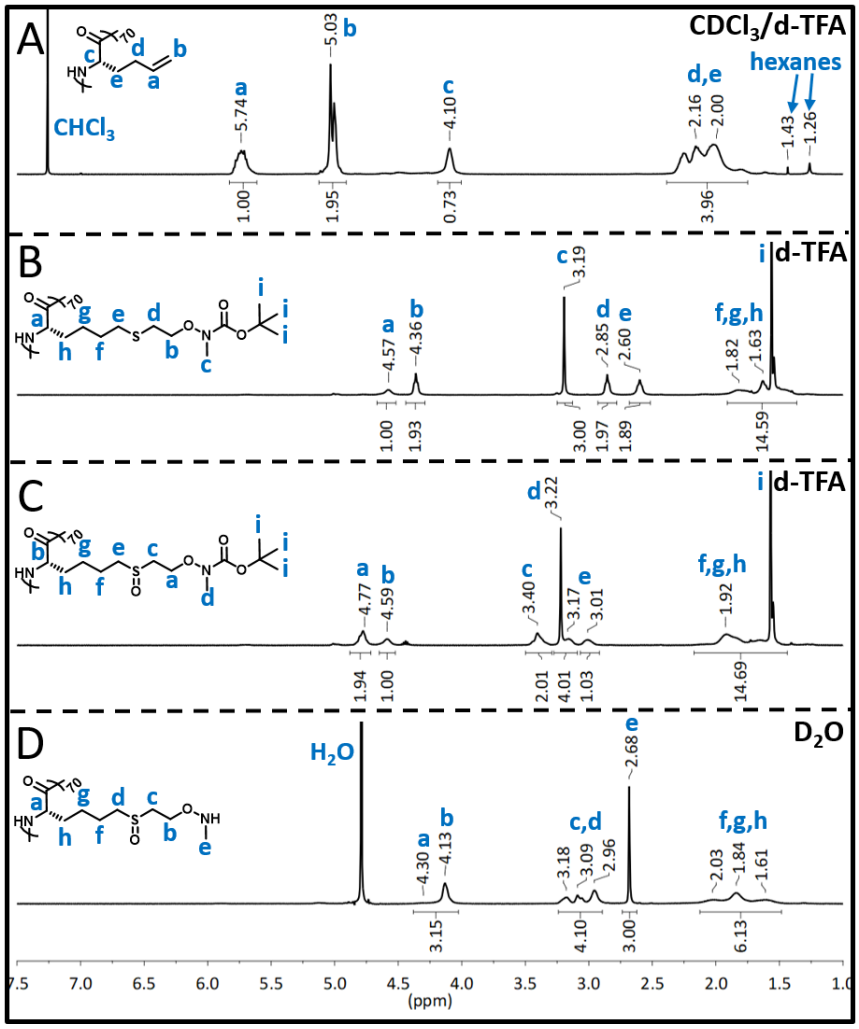


Figure 3.9 ^{1}H NMR spectra overlay of A) **G$^{HA}_{70}$**, B) **Boc-mao-Etn$_{70}$**, C) **Boc-mao-Etn$^{O}_{70}$**, and D) **mao-Etn$^{O}_{70}$**.

First, **mao-Ehc$^{O}_{70}$** solutions were prepared in aqueous buffers ranging in pH from 2 to 9. Analysis of these solutions using CD spectroscopy showed that the chains primarily adopt disordered conformations across this pH range, yet a subtle change occurs between pH 3 and 4 where the chains appear to adopt a partial α-helical conformation above pH 4 (Figure 3.10A).[33] Measurement of zeta potential for solutions of **mao-Ehc$^{O}_{70}$** as a function of pH revealed a transition from positive to slightly negative values between pH 3 and 5, indicative of a change from protonated to nonprotonated *N*-methylaminooxy groups over this pH range (Figure 3.10B).

This transition correlates well with the expected pKa range of *N*-methylaminooxy groups (ca. 3.65–4.75)[34] and was corroborated by analysis of other control samples that included nonionic poly(L-methionine sulfoxide) (**M$^{O}_{70}$**), pH invariant cationic poly(*S*-methyl-L-methionine sulfonium) (**M$^{M}_{70}$**), and poly(L-lysine·HCl)$_{70}$ (**K$_{70}$**) that undergoes a cationic to uncharged transition at a much higher pH (pKa ca. 9–10) (3.10B).[28, 35] These results suggest that **mao-Ehc$^{O}_{70}$** is uncharged above pH ~ 5 where it also retains good water solubility, which contrasts sharply with K$_{70}$ that precipitates readily upon neutralization.

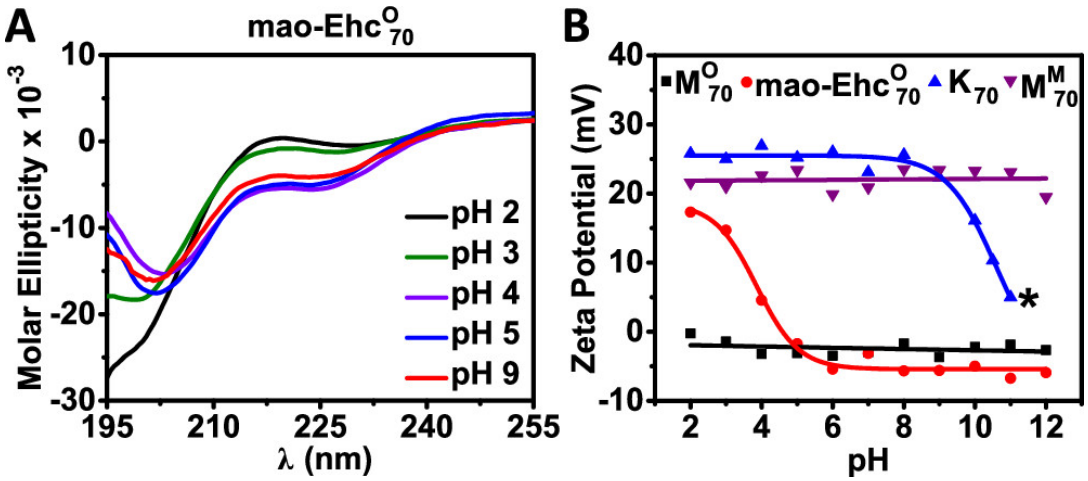


Figure 3.10 (A) CD spectra of **mao-Ehc$^{O}_{70}$** at 20 °C and 0.1 mg/mL concentration in aqueous buffers containing 100 mM phosphate and 10 mM Tris adjusted to pH 2 (black), 3 (green), 4 (purple), 5 (blue), or 9 (red) using HCl (0.1 M) or NaOH (0.1 M). (B) Zeta potentials as a function of pH for aqueous solutions of **mao-Ehc$^{O}_{70}$** (red circles), **M$^{O}_{70}$** (black squares), **M$^{M}_{70}$** (purple down triangles), and **K$_{70}$** (blue up triangles). * = **K$_{70}$** was observed to precipitate above pH 11.

To further analyze the chain conformation of uncharged **mao-Ehc$^{O}_{70}$**, CD analysis was performed in water containing NaOH and increasing concentrations of methanol. As the methanol fraction increased, chains transitioned to α-helical conformations, up to a plateau of ca. 60% helicity (Figure 3.11). Increased α-helical content in methanol likely reflects the weaker

solvation of sulfoxide groups by this solvent compared with water, which is known to bind sulfoxides strongly and disrupt H-bonding as previously observed in $\mathbf{M}^{O}$ polypeptides.[28]

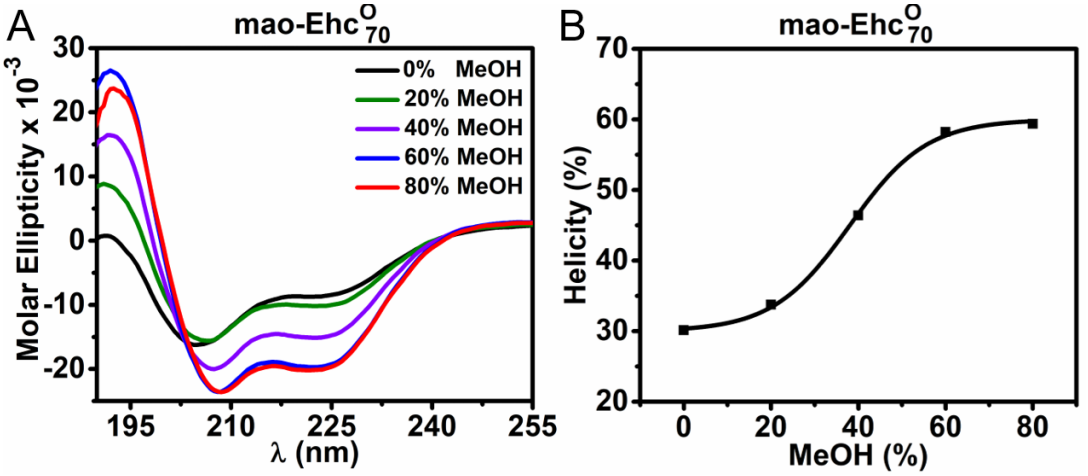


Figure 3.11 A) Circular dichroism spectra of **mao-Ehc$^{O}_{70}$** dissolved in aqueous solvent mixtures containing NaOH and methanol. Concentrations of methanol ranged from 0 to 80 volume %, and concentration of NaOH was 2 x 10^{-5} M. B) The α-helical content (% helicity) as a function of methanol content was calculated using molar ellipticity values at 222 nm from the circular dichroism spectra in A.

To evaluate how the side-chain length affects properties, a CD spectrum of **mao-Etn$^{O}_{70}$** was collected at pH 2 for comparison to data obtained under identical conditions for **mao-Ehc$^{O}_{70}$**. At this pH, **mao-Etn$^{O}_{70}$** displays higher α-helical content compared with **mao-Ehc$^{O}_{70}$** likely because of its longer hydrophobic tether (Figure 3.12). However, the increased hydrophobicity of **mao-Etn$^{O}_{70}$** also impacts solubility such that it only possesses good water solubility in its protonated form below pH of ca. 4–5. CD spectroscopy was also used to study the conformation of cationic **mao-Ehc$^{M}_{70}$**. Because this polypeptide is charged independent of pH, it was found to always adopt a disordered conformation similar to most highly charged polypeptides in water (Figure 3.13).[28]

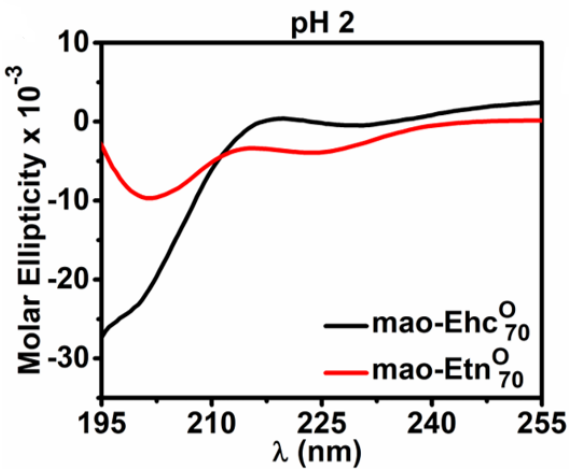


Figure 3.12 Circular dichroism spectra of **mao-Ehc$^{O}_{70}$** (black) and **mao-Etn$^{O}_{70}$** (red) at pH 2. All polypeptides were dissolved in an aqueous buffer containing 100 mM phosphate and 10 mM Tris, and the pH was adjusted using 0.1 M HCl.

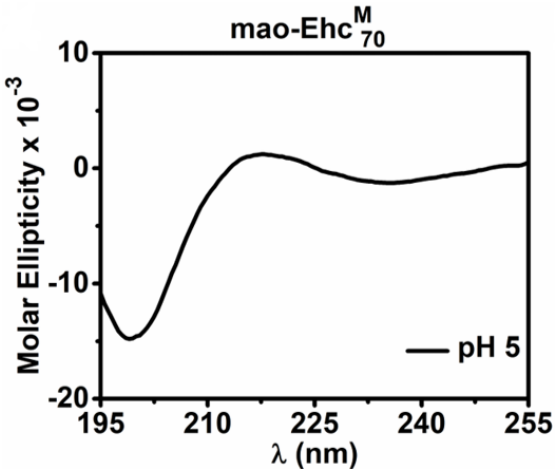
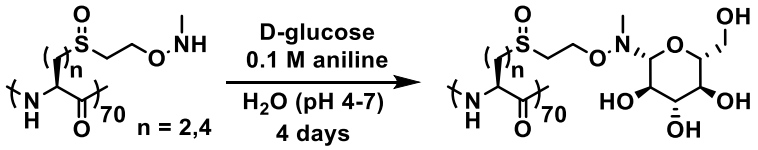


Figure 3.13 Circular dichroism spectrum of **mao-Ehc$^{M}_{70}$** at 20 °C and 0.1 mg/mL concentration in aqueous buffer containing 100 mM phosphate and 10 mM Tris adjusted to pH 5 using HCl (0.1 M).

The three *N*-methylaminooxy-functionalized polypeptides were next evaluated for their ability to conjugate a model monosaccharide, D-glucose, under different conditions. First, nonionic sulfoxide containing **mao-Ehc$^{O}_{70}$** and **mao-Etn$^{O}_{70}$** were reacted with D-glucose in the

presence of aniline catalyst in water,[36, 37] where pH, D-glucose concentration, and temperature were varied (Figure 3.14).

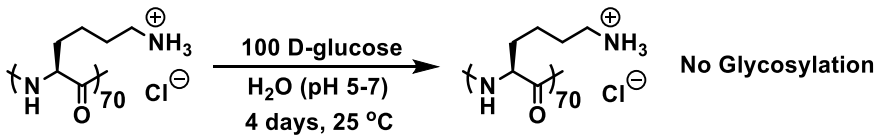


Entry	n	Polypeptide Concentration (mg/mL)	Glucose (eq)	Buffer (pH)	Temperature (°C)	Saccharide Conjugation (%)	Yield (%)
1	2	5	50	4	25	78	100
2	2	5	50	5	25	81	100
3	2	5	50	6	25	86	89
4	2	5	50	7	25	83	90
5	2	5	5	7	25	18	100
6	2	5	10	7	25	31	100
7	2	5	100	7	25	89	90
8	2	5	500	7	25	93	99
9	2	5	5	7	50	65	79
10	2	5	10	7	50	70	98
11	2	5	100	7	50	90	92
12	2	5	500	7	50	93	100
13	4	1.5	50	4	50	80	86*
14	4	1.5	50	5	50	83	97
15	4	1.5	50	6	50	79	86
16	4	1.5	50	7	50	60	79
17	4	1.5	500	5	50	93	100
18	4	1.5	500	7	50	96	99

Figure 3.14 Isolated and saccharide conjugation yields for reactions of **mao-Ehc$^{O}_{70}$** (n = 2) or **mao-Etn$^{O}_{70}$** (n = 4) with D-glucose. Experiments with **mao-Etn$^{O}_{70}$** were performed at lower polypeptide concentrations due to low solubility. All reaction conditions as shown in Eq 4, except * = without aniline. Reaction conditions shown in equation.

Entries 1–4 and 13–16 in Figure 3.14 show that pH had little effect on saccharide conjugation yields after 4 days for **mao-Ehc$^{O}_{70}$**, and a modest effect on yields with **mao-Etn$^{O}_{70}$** possibly because of the diminished solubility of this polypeptide at higher pH. Although lower pH accelerates the coupling reaction, it also accelerates the hydrolysis of the product, leading to

equilibrium levels of saccharide conjugation that are less than quantitative. As such, we found it useful to perform conjugations mainly at pH 7 to disfavor hydrolysis of the neoglycoconjugates and obtain high levels of saccharide functionalization (ca. 80–90%).[27, 38] As a control experiment to highlight the significance of the *N*-methylaminooxy functionality for neoglycoconjugate formation, attempts were also made to conjugate D-glucose to poly(L-lysine·HCl)$_{70}$ under similar conditions. For all conditions attempted, no glycoconjugate formation was observed for poly(L-lysine·HCl)$_{70}$, showing that glycoconjugation is selectively efficient for *N*-methylaminooxy functionality (Figure 3.15).

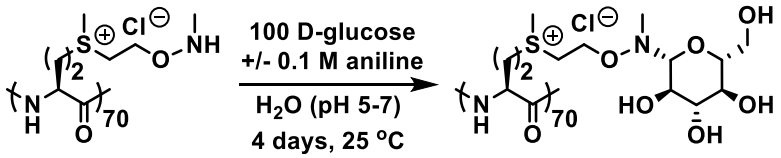


Aniline (0.1 M)	pH	Saccharide Conjugation (%)	Yield (%)
-	5	0	99
+	7	0	96

Figure 3.15 Isolated and saccharide conjugation yields for reactions of poly(L-lysine·HCl)$_{70}$, **K$_{70}$**, with D-glucose. Reaction conditions shown in equation.

At pH 7, saccharide conjugation is slow even in the presence of aniline, so the equivalents of D-glucose per *N*-methylaminooxy group were increased from 5 to 500 (Entries 5-8 and 17-18). A large excess of glucose was found to give high levels of saccharide conjugation for both **mao-Ehc$^{O}_{70}$** and **mao-Etn$^{O}_{70}$**, although this strategy may not be practical for conjugation of expensive saccharides. Changing the temperature of **mao-Ehc$^{O}_{70}$** conjugations from 25 to 50 °C was also found to be an effective way to increase saccharide conjugation yields when using fewer equivalents of D-glucose (Entries 9-12), and may be a better strategy

for conjugation of expensive saccharides. For comparison to the non-ionic polypeptides discussed above, cationic **mao-Ehc$^{M}_{70}$** was also reacted with D-glucose under a select set of conditions (Figure 3.16). At pH 7, significantly lower saccharide conjugation to **mao-Ehc$^{M}_{70}$** (37%) was observed under identical conditions used for **mao-Ehc$^{O}_{70}$** (89%; Figure 3.15, Entry 7). However, at pH 5 with aniline catalyst, high levels of saccharide conjugation to **mao-Ehc$^{M}_{70}$** (93%) were readily obtained.



Aniline	Buffer (pH)	Saccharide Conjugation (%)	Yield (%)
-	5	47	99
+	5	93	93
+	7	37	91

Figure 3.16 Isolated and saccharide conjugation yields for reactions of **mao-Ehc$^{M}_{70}$** with D-glucose. Reaction conditions shown in equation.

To evaluate physical properties of the neoglycopolypeptide conjugates, **Glc-mao-Ehc$^{O}_{70}$** and **Glc-mao-Etn$^{O}_{70}$** were chosen as model samples for CD spectroscopy analysis. CD spectra of **Glc-mao-Ehc$^{O}_{70}$** collected at pH 5 and 9 were nearly identical (Figure 3.17A) and suggest that this polypeptide adopts highly disordered conformations over this pH range. Increased solvation of this glucose-functionalized polypeptide in water as compared to **mao-Ehc$^{O}_{70}$** may explain the loss of partial α-helical content observed in the parent sample. CD spectra of **Glc-mao-Etn$^{O}_{70}$** collected at pH 5 and 9 showed that this polypeptide also adopted partial α-helical conformations with the helical content increasing with pH (Figure 3.17B).[33] The longer hydrophobic tether in **Glc-mao-Etn$^{O}_{70}$** likely helps to stabilize the α-helical conformation in these

polypeptides despite the addition of polar glucose groups. As expected for a polyelectrolyte, cationic **Glc-mao-Ehc$^{M}_{70}$** was found to be disordered between pH 5 and 9, similar to parent **mao-Ehc$^{M}_{70}$** (Figure 3.18).[39]

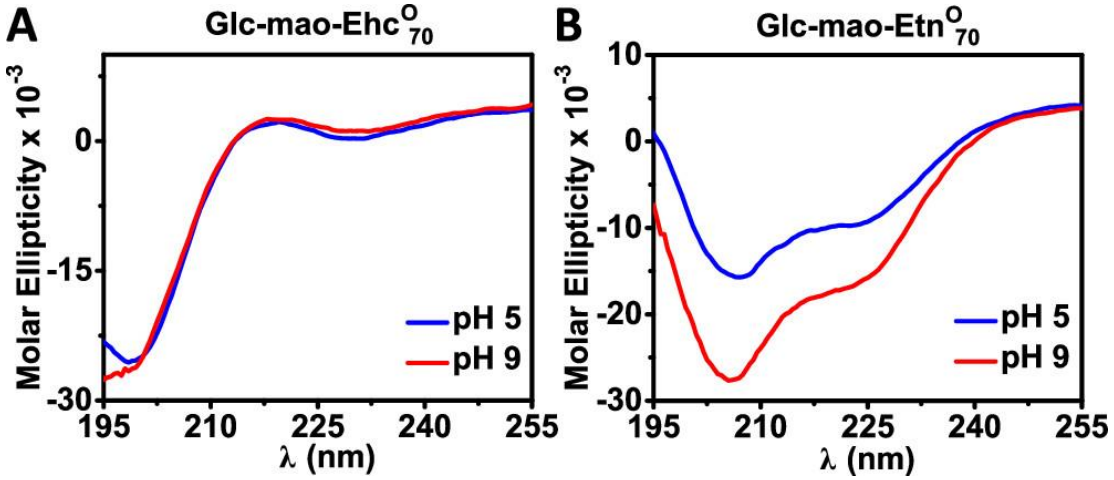


Figure 3.17 CD spectra of (A) **Glc-mao-Ehc$^{O}_{70}$** and (B) **Glc-mao-Etn$^{O}_{70}$** at 20 °C. Samples were prepared at 0.1 mg/mL concentration in aqueous buffers containing 100 mM phosphate and 10 mM Tris adjusted to pH 2 (black), 5 (blue), or 9 (red) using HCl (0.1 M) or NaOH (0.1 M).

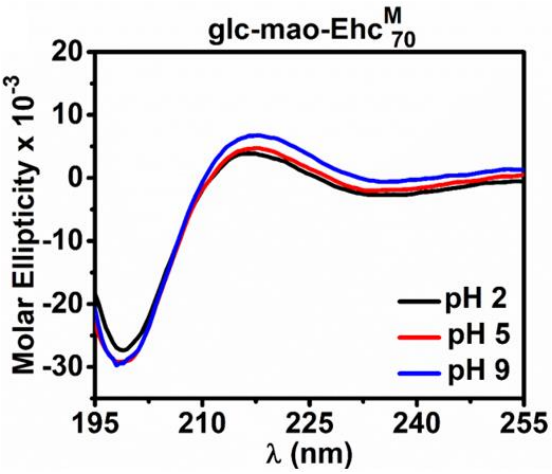


Figure 3.18 Circular dichroism spectra of **glc-mao-Ehc$^{M}_{70}$** at 20 °C and 0.1 mg/mL concentration in aqueous buffers containing 100 mM phosphate and 10 mM Tris adjusted to pH 2 (black), 5 (blue), or 9 (red) using HCl (0.1 M) or NaOH (0.1 M).

Stability of model neoglycoconjugates at different pH in water was also evaluated. Aqueous solutions of **Glc-mao-Ehc$^{O}_{70}$**, **Glc-mao-Etn$^{O}_{70}$**, and **Glc-mao-Ehc$^{M}_{70}$** (all greater than 90% glycosylation) were individually dialyzed at 37 °C for 1 week at pH of either 5.5 or 7.4 (Figure 3.19). Subsequent analysis of the samples by ^{1}H NMR was used to quantify the loss of D-glucose from each sample.

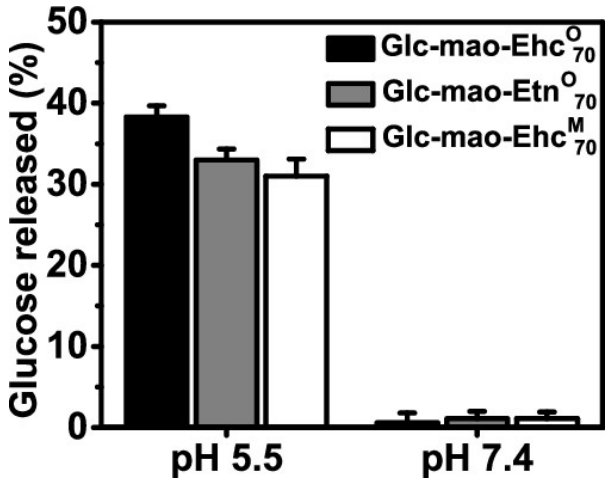


Figure 3.19 Release of free D-glucose from **Glc-mao-Ehc$^{O}_{70}$** (black), **Glc-mao-Etn$^{O}_{70}$** (gray) and **Glc-mao-Ehc$^{M}_{70}$** (white) at pH 5.5 (200 mM phosphate) and 7.4 (200 mM phosphate). All samples were incubated at 37 °C for 1 week. Error bars are consistent with standard deviations of n = 3.

Because acid is known to catalyze both glycoconjugate formation and hydrolysis, and free D-glucose was removed during reactions, all samples were partially deglycosylated at pH 5.5.[40] Although the degree of deglycosylation (ca. 30–40%) was comparable for these samples, the more polar **Glc-mao-Ehc$^{O}_{70}$** showed greater loss of D-glucose compare with less polar **Glc-mao-Etn$^{O}_{70}$**. The cationic neoglycoconjugate **Glc-mao-Ehc$^{M}_{70}$** was also found to be more stable at pH 5.5 compared with **Glc-mao-Ehc$^{O}_{70}$**, where the nearby cationic sulfonium group in Glc-**mao-Ehc$^{M}_{70}$** may be hindering *N*-glycoside protonation, which is the first step in hydrolysis. All

three samples showed high neoglycoconjugate stability at pH 7 for 1 week at 37 °C, which makes them promising for use in downstream biological studies.

3.4 Conclusion

A set of *N*-methylaminooxy-functionalized polypeptides has been prepared and characterized. These new functional polypeptides are water soluble and were found to react with unmodified reducing saccharides to form neoglycoconjugates in high yields under a variety of aqueous conditions. While *N*-methylaminooxy-functionalized and related polymers have been reported, this approach has not been previously demonstrated in synthetic polypeptides. The variation of polypeptide scaffolds resulted in neoglycoconjugates with different chain conformations, hydrophobicity, and charge. The combination of straightforward synthesis, high yields of saccharide conjugation, and conjugate stability makes these polypeptides attractive candidates for use as degradable glycoprotein mimics.

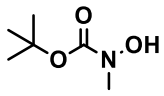
3.5 Experimental

3.5.1 Materials and Methods

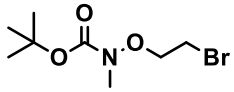
THF, hexanes, and DCM were dried by purging with nitrogen and passage through activated alumina columns prior to use. TEA and TMSCl were purified by distillation and stored over 3 Å molecular sieves. $Co(PMe_3)_4$, L-methionine NCA, and N_ε-Z-L-lysine NCA monomers were prepared according to literature procedures.[29, 30, 35] Poly(L-methionine sulfoxide)$_{70}$ (M^O_{70}), poly(*S*-methyl-L-methionine sulfonium)$_{70}$ (M^M_{70}), and poly(L-lysine·HCl)$_{70}$ (K_{70}) were prepared according to literature procedures.[39, 41] All other chemicals were purchased from commercial suppliers and used without further purification unless otherwise noted. Selecto silica gel 60 (particle size 0.032– 0.063 μm) was used for flash column chromatography. FTIR measurements were taken using a Perkin Elmer RX1 FTIR calibrated using polystyrene film. ^{1}H NMR spectra were acquired on a Bruker ARX 400 spectrometer. GPC/MALS was performed at

25 °C using an SSI Accuflow Series III pump equipped with Wyatt DAWN EOS light scattering and Optilab REX refractive index detectors. Separations were achieved using 100 Å and 1000 Å PSS-PFG 7 µm columns at 30 °C with 0.5% (w/w) KTFA in HFIP as eluent and sample concentrations of 10 mg/mL. Zeta potential data were collected using a Zetasizer NanoZS. Samples of **mao-Ehc$^{O}_{70}$**, **M$^{O}_{70}$**, **M$^{M}_{70}$**, and **K$_{70}$** were dissolved at 5–15 mg/mL in filtered (0.45 µm) water containing 20 mM NaCl. Polypeptide solutions were adjusted to pH 2.0 using filtered (0.45 µm) aqueous 1.0 M HCl. The solutions were titrated to pH 12 with filtered (0.45 µm) aqueous 1.0 M NaOH. Aliquots (1 mL) were removed from the solutions at specific pH values. Pyrogen free DI water was obtained from a Millipore Milli-Q Biocel A10 purification unit. Dialysis was conducted using regenerated cellulose dialysis tubing (Spectrum Labs, MWCO 2000 Da). All CD spectra were collected using an OLIS RSM CD spectrophotometer (OLIS, USA) using conventional scanning mode. Samples were characterized by recording spectra (185-260 nm) within a quartz cuvette of 0.1 cm path length. Samples of **mao-Ehc$^{O}_{70}$**, **mao-Ehc$^{M}_{70}$**, **mao-Etn$^{O}_{70}$**, **glc-mao-Ehc$^{O}_{70}$, glc-mao-Ehc$^{M}_{70}$** and **glc-mao-Etn$^{O}_{70}$** were prepared at concentrations of 0.1 mg/mL in aqueous buffers containing 100 mM phosphate and 10 mM Tris. The buffered solutions were adjusted to pH 2.0, 3.0, 4.0, 5.0, and 9.0 using HCl (0.1 M) or NaOH (0.1 M). Percent α-helical content of polypeptides was estimated using the formula % α-helix = 100 × (−$[\theta]_{222}$ + 3000)/39000), where $[\theta]_{222}$ is the measured molar ellipticity at 222 nm. Mass spectrometry data was obtained by dissolving the sample in acetonitrile (50 ng/mL) and analysis in negative mode using a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with Dionex Ultimate 3000 RSLCnano System. Resolving power was 70,000 at m/z 200. Multiple spectra (50) were averaged to improve signal to noise.

3.5.2 Synthesis of small molecules

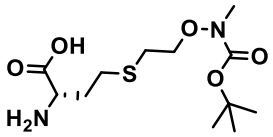


***N*-Boc-*N*-methylhydroxylamine.**[26] Sodium bicarbonate (10.9 g, 130 mmol, 2.3 eq) was slowly added to a stirring mixture of *N*-methylhydroxylamine hydrochloride (6.00 g, 72 mmol, 1.3 eq) in DI water (78 mL) and methylene chloride (60 mL). The reaction was cooled in an ice bath and di-tert-butyl dicarbonate (12.50 g, 57 mmol, 1.0 eq) was slowly added. The reaction was allowed to come to room temperature and stirred for 16 hours. It was then quenched with concentrated sodium bicarbonate in water (18 mL). The product was extracted from water (100 mL) using 4 x ethyl acetate (100 mL). The organic fractions were combined and dried with anhydrous sodium sulfate followed by concentration under reduced pressure to yield a yellow oil. The crude product was further purified using column chromatography (10% ethyl acetate/hexanes to 30% ethyl acetate/hexanes) to give a colorless oil (7.08 g, 83.9% yield). 1H NMR (400 MHz, CDCl3, 25 °C): δ 3.15 (s, 3H), 1.46 (s, 9H). 13C NMR (100 MHz, CDCl3, 25 °C): δ 157, 81.9, 37.9, 28.4.

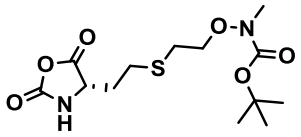


2-(*N*-Boc-*N*-methylaminooxy)ethyl bromide. Adapted from a procedure for synthesis of the unsubstituted aminooxy derivative.[42] DBU (4.05 mL, 27 mmol, 1.0 eq) was slowly added to a solution of *N*-Boc-*N*-methylhydroxylamine (4.00 g, 27 mmol, 1.0 eq) in 1,2-dibromoethane (16.8 mL). The reaction was allowed to stir at ambient temperature for 16 hours and went from a clear colorless solution to a yellow suspension. The product was extracted from water (200 mL) using 4 x ethyl acetate (200 mL). Organic fractions were combined and dried with anhydrous sodium sulfate followed by concentration under reduced pressure to yield a yellow oil. The crude product was further purified using column chromatography (15% ethyl acetate/hexanes) to give

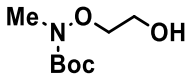
a yellow oil. Finally, the product was vacuum distilled to yield a colorless oil (3.51 g, 50.8% yield). 1H NMR (400 MHz, CDCl3, 25 °C): δ 4.13 (t, J = 6.3 Hz, 2H), 3.51 (t, J = 6.3 Hz, 2H), 3.11 (s, 3H), 1.49 (s, 9H). 13C NMR (100 MHz, CDCl3, 25 °C): δ 157, 81.9, 74.1, 37.5, 28.4, 28.39.



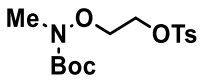
2-(*N*-Boc-*N*-methylaminooxy)ethyl-L-homocysteine. Synthesis of the L-homocysteine derivative was accomplished by adapting a procedure from literature.[43] L-methionine (0.94 g, 6 mmol, 1.0 eq) was dissolved in liquid ammonia (30 mL) and sodium metal (0.57 g, 24 mmol, 4.0 eq) was added in small pieces until the solution was heterogeneous and a blue color persisted for 15 minutes. The blue color was then quenched by adding ammonium chloride (1.0 g, 42 mmol, 7 eq) until the reaction mixture was colorless and homogenous. At this point, tert-Butyl 2-bromoethoxy(methyl)carbamate (1.59 g, 6 mmol, 1.0 eq) was slowly added and the reaction was allowed to stir for 1 hour. The ammonia was removed under reduced pressure to yield a crude white solid. The white solid was dissolved in a minimal amount of DI water (15 mL) and 3 M HCl (5 mL) was added until the pH was between 6 and 7. The water was removed under reduced pressure to yield a crude white solid which was contaminated with salt (60% salt wt/wt) and used without further purification (5.75 g (2.30 g without salt), 100% yield). 1H NMR (400 MHz, D2O, 25 °C): δ 4.15 (t, J = 6.2 Hz, 2H), 3.93 (dd, J = 7.0, 5.5 Hz, 1H), 3.23 (s, 3H), 2.92 (t, J = 6.2 Hz, 2H), 2.80 (t, J = 7.6 Hz, 2H), 2.32-2.14 (m, 2H), 1.56, (s, 9H). 13C NMR (100 MHz, D2O, 25 °C): 174, 157, 83.8, 72.7, 53.9, 35.9, 30.4, 29.1, 27.6, 27.2.



2-(*N*-Boc-*N*-methylaminooxy)ethyl-L-homocysteine *N*-carboxyanhydride, Boc-mao-Ehc NCA. To a solution of crude 2-(*N*-Boc-*N*-methylaminooxy)ethyl-L-homocysteine (3.0 g (ca. 1.2 g amino acid), 3.3 mmol, 1.0 eq) in dry THF (30 mL) in a Schlenk flask was added TEA (0.92 mL, 6.6 mmol, 2.0 eq) and TMSCl (0.84 mL, 6.6 mmol, 2.0 eq) via syringe. The reaction was stirred under N_2 at room temperature for 1 hour. Upon addition of TEA and TMSCl, precipitation of TEA·HCl was observed. A solution of 15% (w/v) phosgene in toluene (4.7 mL, 6.6 mmol, 2.0 eq) was then added via syringe and the reaction was stirred under N_2 at 50 °C for 2 hours. Caution! Phosgene is extremely hazardous and all manipulations must be performed in a well-ventilated chemical fume hood with proper personal protection and necessary precautions taken to avoid exposure. After 2 hours, the reaction was cooled and evaporated to dryness then transferred into a N_2 filled glovebox. In the fume hood, the condensate in the Schlenk line vacuum traps was treated with 50 mL of concentrated aqueous NH_4OH to neutralize residual phosgene. In the glove box, THF was added to the crude product and the insoluble salts were removed by vacuum filtration and were washed with THF. The filtrate was evaporated under reduced pressure to yield an orange/red oil that was purified by passing it through vacuum dried silica using 10% THF/hexanes to 30% THF/hexanes.[29] Fractions containing the NCA were combined and concentrated under reduced pressure to yield a pale yellow oil (0.73 g, 67% yield). ^{1}H NMR (400 MHz, CDCl3, 25 °C): δ 7.58-7.46 (bs, 1H), 4.57 (ddd, J = 9.2, 4.1, 0.8 Hz, 1H), 4.10 (m, 1H), 4.01 (m, 1H), 3.12 (s, 3H), 2.92 (m, 2H), 2.74 (t, J = 5.6 Hz, 2H), 2.22 (m, 1H), 2.06 (m, 1H), 1.50 (s, 9H). ^{13}C NMR (100 MHz, CDCl3, 25 °C): δ 170, 157, 152, 82.6, 74.1, 56.3, 36.9, 31.3, 29.9, 28.4, 28.1. FTIR: NCA bands at 1857, 1787 and Boc bands at 1730 and 1705 cm^{-1}. MS m/z = 333.1128 $[M-H]^-$ (calcd 333.1120 for $C_{13}H_{21}O_6N_2S$).

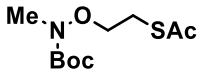


2-(*N*-Boc-*N*-methylaminooxy)ethanol. Adapted from a procedure for synthesis of the unsubstituted aminooxy derivative.[44] *N*-Boc-*N*-methylhydroxylamine (6.45 g, 44 mmol, 1.0 eq) and 2-bromoethanol (9.23 mL, 130 mmol, 3.0 eq) were dissolved in dry DCM (50 mL). The solution was cooled in an ice bath and then DBU was added dropwise (6.51 mL, 44 mmol, 1.0 eq). The reaction was allowed to come to room temperature and was stirred for 72 hours. 50 mL DCM was added to the reaction mixture and the resulting solution was washed with DI water (3 x 80 mL). The organic layer was dried with anhydrous sodium sulfate, decanted and evaporated to dryness. The crude residue was purified by column chromatography using 7:3 hexane:ethyl acetate until elution of the product began, then 1:1 hexane:ethyl acetate until product had eluted. The fractions were evaporated to dryness to give a clear oil (3.14 g, 38% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 3.89 (sext, *J* =1.8, 2H), 3.76-3.42 (br m, 3H), 3.09 (s, 3H), 1.49 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ 158, 82.3, 75.4, 59.5, 36.6, 28.2.

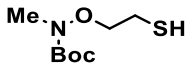


2-(*N*-Boc-*N*-methylaminooxy)ethyl tosylate. Adapted from a procedure for synthesis of a related molecule.[45] A solution of NaOH (1.23 g, 31 mmol, 2.7 eq) in DI water (15 mL) was added to a flask containing 2-(*N*-Boc-*N*-methylaminooxy)ethanol (2.83 g, 15 mmol, 1.3 eq). Tosyl chloride (2.19 g, 12 mmol, 1.0 eq) was dissolved in THF (15 mL) and added dropwise at 0 °C. The reaction was allowed to come to room temperature overnight. Ether was then added (30 mL) and the reaction mixture was partitioned. The aqueous layer was washed with ether (3 x10 mL) and the combined organics were washed with DI water (3 x 30 mL). The organic layer was dried with anhydrous sodium sulfate, decanted and evaporated to dryness. The crude residue was purified by column chromatography using 7:3 hexane:ethyl acetate. The desired fractions

were combined and evaporated to dryness to give the product as a clear oil (4.37 g, 74% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 7.83-7.77 (m, 2H), 7.35 (d, *J* = 8.0 Hz, 2H), 4.21 (q, *J* = 3.1 Hz, 2H), 4.03 (q, *J* = 3.1 Hz, 2H), 3.01 (s, 3H), 2.45 (s, 3H), 1.46 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ 157, 145, 133, 130, 128, 81.8, 71.7, 67.2, 37.3, 28.2, 21.6.



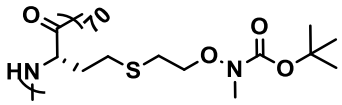
***S*-(2-(*N*-Boc-*N*-methylaminooxy)ethyl) ethanethioacetate.** Adapted from a procedure for synthesis of a related molecule.[46] 2-(*N*-Boc-*N*-methylaminooxy)ethyl tosylate (3.03 g, 9 mmol, 1.0 eq) was dissolved in acetone (140 mL). Potassium thioacetate (1.47 g, 13 mmol, 1.4 eq) was added to the resulting solution. A condenser was placed on the flask and the reaction was let stir at 50 °C overnight. Acetone was then removed via vacuum and DCM (35 mL) and water (35 mL) was used to transfer the crude residue to a separatory funnel. The aqueous layer was washed with DCM (2 x 35 mL) and the combined organics were washed with brine. The organic layer was dried with anhydrous sodium sulfate, decanted and evaporated to dryness to give the product as a yellow oil (2.19 g, 86% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 3.95 (t, *J* = 6.3 Hz, 2H), 3.13 (t, *J* = 6.3 Hz, 2H), 3.08 (s, 3H), 2.34 (s, 3H), 1.49 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ 195, 157, 81.5, 72.6, 37.0, 30.5, 28.3, 27.4.



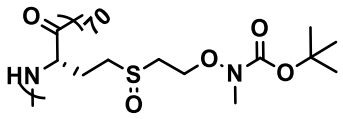
2-(*N*-Boc-*N*-methylaminooxy)ethyl mercaptan. Adapted from a procedure for deprotection of a related molecule.[47] A solution of *S*-(2-(*N*-Boc-*N*-methylaminooxy)ethyl) ethanethioacetate (1.12 g, 5 mmol, 1.0 eq) in methanol (20 mL) was degassed for 30 minutes via sparging with N_2. NaOMe in methanol (30 wt%) was then added via syringe (2.51 mL, 11 mmol, 2.2 eq). The reaction was let stir under nitrogen atmosphere for 1 hour, after which degassed acetic acid was added (0.92 mL). The reaction mixture was diluted with DI water (60 mL), transferred to a separatory funnel and extracted with DCM (4 x 50 mL). The organic layer was dried with

anhydrous sodium sulfate, decanted and evaporated to dryness to give the product as a yellow oil (0.819 g, 88% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 3.96 (t, *J* = 6.5, 2H), 3.10 (s, 3H), 2.73 (sext, *J* = 4.23, 2H), 1.65 (t, *J* = 8.3, 1H), 1.49 (s, 9H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ 157, 81.6, 75.7, 37.0, 28.3, 22.7.

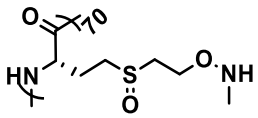
3.5.3 Synthesis of mao-Ehc$^{O}_{70}$, mao-Ehc$^{M}_{70}$, and mao-Etn$^{O}_{70}$



poly(2-(*N*-Boc-*N*-methylaminooxy)ethyl-L-homocysteine)$_{70}$, Boc-mao-Ehc$_{70}$. All polymerization reactions were performed in a N_2 filled glove box using anhydrous THF. To prepare **Boc-mao-Ehc$_{70}$** at ca. 100 mg scale, a solution of $Co(PMe_3)_4$ (3.6 mg, 0.010 mmol, 1.0 eq) in THF (20 mg/mL) was quickly added to a solution of Boc-mao-Ehc NCA (100 mg, 0.30 mmol, 30 eq) in THF (50 mg/mL) at 20 °C. After ca. 60 minutes, complete consumption of NCA was confirmed by FTIR spectroscopy. In order to determine the length of Boc-mao-Ehc$_{70}$, a small aliquot (200 µL) of the polymerization mixture was removed for end-group analysis (see SI). The reaction mixture was then removed from the glove box and the polypeptide was precipitated into DI water (100 mL), collected using centrifugation, and dried under reduced pressure to yield an off white solid with yields ranging from 85 to 90%. ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 4.87-4.73 (br m, 1H), 4.42-4.29 (br m, 2H), 3.20 (s, 3H), 2.92-2.81 (br m, 2H), 2.75-2.61 (br m, 2H), 2.20-2.01 (br m, 2H), 1.57 (s, 8H). FTIR: polypeptide bands at 3288, 1652, and 1548 cm^{-1}.

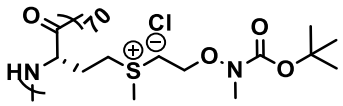


poly(2-(*N*-Boc-*N*-methylaminooxy)ethyl-L-homocysteine sulfoxide)$_{70}$, Boc-mao-Ehc$^{O}_{70}$. The oxidation of thioether groups was performed before removal of the *N*-Boc groups. To convert thioether groups to sulfoxides, a volume of 70 wt. % TBHP (32 eq per residue) was added to a solution of **Boc-mao-Ehc$_{70}$** in HFIP (20 mg/mL). The reaction was stirred at room temperature for 48 hours and then it was transferred to a 2000 MWCO dialysis bag and dialyzed against: (i) pyrogen free deionized milli-Q water (3.5L) containing sodium thiosulfate (1.2 g, 2.16 mM) for 2 days to neutralize residual peroxide, (ii) pyrogen free milli-Q water (3.5 L) for 2 days to remove residual sodium thiosulfate. For each step above, dialysate was changed every 12 hours. Within the first couple hours of dialysis, the polypeptide precipitated within the dialysis bag. The contents of the dialysis bag were removed and freeze dried to yield dense white solids with average yields of 90 to 95%, and complete conversion of thioether groups to sulfoxide groups. ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 4.97-4.59 (br m, 3H), 3.53-3.28 (br m, 4H), 3.22 (s, 3H), 2.69-2.47 (br m, 1H), 2.47-2.26 (br m, 1H), 1.56 (s, 8H).

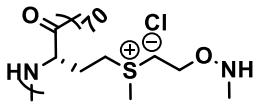


poly(2-(*N*-methylaminooxy)ethyl-L-homocysteine sulfoxide)$_{70}$, mao-Ehc$^{O}_{70}$. The removal of N-Boc groups was performed after oxidation of the thioether groups. To remove the *N*-Boc groups, **Boc-mao-Ehc$^{O}_{70}$** was dissolved in TFA (20 mg/mL) and allowed to stir at room temperature for 2 hours. The reaction mixture was then transferred to a 2000 MWCO dialysis bag and dialyzed against: (i) pyrogen free milli-Q water (3.5L) acidified to pH 2 with HCl for 2 days to aid cobalt ion removal/counter ion exchange, and (ii) pyrogen free milli-Q water (3.5 L) for 2 days to remove residual HCl. For each step above, dialysate was changed every 12

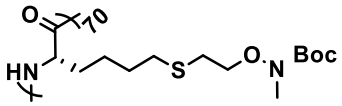
hours. The polypeptide solution remained clear throughout dialysis. The contents of the dialysis bag were removed and then freeze dried to yield the polypeptide as a white fluffy solid (95% yield) with > 99% removal of Boc groups. ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.58-4.37 (br m, 1H), 4.26-4.06 (br m, 2H), 3.28-2.95 (br m, 4H), 2.69 (s, 3H), 2.44-2.17 (br m, 2H).



poly(2-(*N*-Boc-*N*-methylaminooxy)ethyl-L-homocysteine methyl sulfonium)$_{70}$, Boc-mao-Ehc$^{M}_{70}$. The alkylation of thioether groups was performed before removal of the *N*-Boc groups. To convert thioether groups in Boc-mao-Ehc$_{70}$ to S-methyl sulfonium groups, iodomethane (5 eq per thioether group) was added to a 20 mg/mL suspension of Boc-mao-Ehc$_{70}$ in 0.1 M phosphate buffer (pH 7). The reaction was covered with aluminum foil to protect iodomethane from light and the suspension was vigorously stirred for 48 hours at room temperature. Afterwards, the reaction mixture was transferred to a 2000 MWCO dialysis bag and dialyzed against: (i) pyrogen free deionized milli-Q water (3.5 L) containing NaCl (7 g, 35 mM) for 2 days to facilitate counterion exchange, (ii) pyrogen free milli-Q water (3.5 L) for 2 days to remove residual NaCl. For each step above, dialysate was changed every 12 hours. Within the first couple hours of dialysis, the polypeptide became soluble. The contents of the dialysis bag were removed and freeze dried to yield a dense white solid (93% yield), and complete conversion of thioether groups to S-methyl sulfonium groups. ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.66-4.55 (br m, 1H), 4.49-4.30 (br m, 1H), 3.83-3.37 (br m, 3H), 3.25-3.15 (br m, 3H), 3.14-3.01 (br m, 3H), 2.46-2.19 (br m, 2H), 1.61-1.38 (br m, 8H).

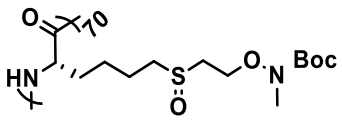


poly(2-(*N*-methylaminooxy)ethyl-L-homocysteine methyl sulfonium)$_{70}$, mao-Ehc$^{M}_{70}$ The removal of **Boc-mao-Ehc$^{M}_{70}$** was performed after alkylation of the thioether groups. To remove the N-Boc groups, **Boc-mao-Ehc$^{M}_{70}$** was dissolved in TFA (20 mg/mL) and allowed to stir at room temperature for 2 hours. The reaction mixture was then transferred to a 2000 MWCO dialysis bag and dialyzed against: (i) pyrogen free deionized milli-Q water (3.5 L) containing NaCl (7 g, 35 mM) for 2 days to facilitate counterion exchange, (ii) pyrogen free milli-Q water (3.5 L) for 2 days to remove residual NaCl. For each step above, dialysate was changed every 12 hours. The polypeptide solution remained clear throughout dialysis. The contents of the dialysis bag were removed and then freeze dried to yield the polypeptide as a dense white solid (96% yield) with > 99% removal of Boc groups. ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.68-4.52 (br m, 1H), 4.28-4.14 (br m, 2H), 3.83-3.37 (br m, 4H), 3.12-2.95 (br m, 3H), 2.73-2.61 (br m, 3H), 2.50-2.23 (br m, 2H).

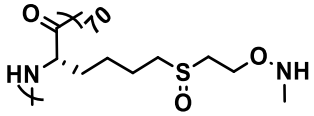


poly(6-(2-N-Boc-*N*-methylaminooxy)ethylthio)-L-norleucine)$_{70}$, Boc-mao-Etn$_{70}$. poly(L-homoallylglycine)$_{70}$, **G$^{HA}_{70}$**, (11 mg), and DMPA (5 mg, 0.02 mmol, 0.2 eq per residue) were placed in a 1 dram screw top vial. THF was then added to give a 4 mg/mL resulting polymer concentration. 2-(*N*-Boc-*N*-methylaminooxy)ethyl mercaptan (82 mg, 0.39 mmol, 4 eq per residue) was then added via micropipette and the solution was degassed via sparging with N_2 for 10 minutes. The vial was then sealed with parafilm and the solution was irradiated with UV light for 2.5 hours (Exo Terra Reptile Lamp) and let stir overnight. The solution was then transferred to a 2000 MWCO dialysis bag and dialyzed against methanol for 24 hours with one

change of dialysate, followed by dialysis in water for 24 hours with one water change. The solution was lyophilized to dryness to give the product as a white solid (31 mg, 95% yield). ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 4.66 (br s, 1H), 4.44 (d, J = 5.4, 2H), 3.28 (s, 3H), 2.94 (t, J = 5.1, 2H), 2.78-2.58 (br m, 2H), 2.24-1.27 (br m, 15H).



poly(6-(2-*N*-Boc-*N*-methylaminooxy)ethylthio)-L-norleucine sulfoxide)$_{70}$, Boc-mao-Etn$^O_{70}$. The oxidation of thioether groups was performed before removal of *N*-Boc groups. **Boc-mao-Etn$^O_{70}$** (81 mg) was dissolved in HFIP (5.0 mL). 70% TBHP in water was then added (1.14 mL, 8 mmol, 32 eq per residue). The solution was let stir overnight and then dialyzed against water for 48 h with two water changes daily. The resulting solution was lyophilized to dryness to give the product as a sticky, white solid (84 mg, 99% yield). ^{1}H NMR (400 MHz, TFA-d, 25 °C): δ 4.95-4.76 (br m, 2H), 4.67 (br s, 1H), 3.56-3.39 (br m, 2H), 3.35-3.16 (br m, 4H), 3.09 (br s, 1H), 2.30-1.52 (br m, 15H).



poly(6-(2-*N*-methylaminooxy)ethylthio)-L-norleucine sulfoxide)$_{70}$, mao-Etn$^O_{70}$. The removal of *N*-Boc groups was performed after oxidation of thioether groups. **Boc-mao-Etn$^O_{70}$** (31 mg) was dissolved in TFA (2.0 mL). The reaction was let stir for 3 hours at room temperature and then dialyzed against a pH 2 HCl solution (24 hr, 2 water changes), then against deionized water (24 hr, 2 water changes). The resulting solution was lyophilized to dryness to give the product as a white solid (13 mg, 62% yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.40-3.97 (br m, 3H), 3.30-2.84 (br m, 4H), 2.68 (s, 3H), 2.18-1.45 (br m, 6H).

General procedure for endcapping of polypeptides with PEG chains. The general procedure for polymerization of Boc-mao-Ehc NCA was followed. Once the reaction was determined to be complete by FTIR, a solution of α-methoxy-ω-isocyanoethyl-poly(ethylene glycol), PEG-NCO (MW = 1000 Da, 4 eq per $Co(PMe_3)_4$) in THF was added to the polymerization mixture inside a dinitrogen filled glovebox. The reaction was let stand overnight, and then removed from the glovebox. 1-2 drops of 3M HCl were then added and the reaction was precipitated with water, centrifuged at 3000 rpm and the supernatant was discarded. The pellet was washed 3 times with DI water to remove unconjugated PEG-NCO, and the resulting pellet was then lyophilized to yield PEG-endcapped polypeptide as a white solid. To determine the molecular weight of the polypeptides (M_n), ^{1}H NMR spectra were obtained in deuterated trifluoroacetic acid (TFA-d) or deuterated chloroform ($CDCl_3$) containing 1-2 drops of TFA-d. Similar to procedures described in literature,[48] the integral of the **Boc-mao-Ehc$_n$** resonance at δ 3.19 was compared to the integral of the polyethylene glycol resonance at δ 3.92 to obtain **Boc-mao-Ehc$_n$** lengths.

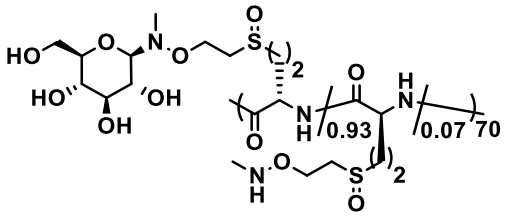
3.5.4 Glycosylation of mao-Ehc$^O_{70}$, mao-Ehc$^M_{70}$, and mao-Etn$^O_{70}$

Procedure A (saccharide-mao-Ehc$^O_{70}$). A sample of **mao-Ehc$^O_{70}$** was dissolved at 5 mg/mL in a 0.1 M buffer (acetate pH 4-6, phosphate pH 7) with or without 0.1 M aniline. Upon complete dissolution of mao-Ehc$^O_{70}$, D-Glucose (5-500 eq) was added to the reaction mixture. The reaction was adjusted to the appropriate temperature (4-50 °C) and stirred for 1 to 4 days. The reaction mixture was then transferred to a 2000 MWCO dialysis bag and dialyzed against pyrogen free milli-Q water (3.5L) for 1 day to remove ions and excess saccharide. Dialysate changes were performed every 12 hours. The resulting solution was removed and freeze dried to yield a white fluffy solid with isolated yields ranging from 80 to 100%, and saccharide conjugation yields ranging from 15 to 95%. The degree of saccharide conjugation was quantified by comparing ^{1}H NMR integrals of the *N*-methyl resonances of unconjugated

methylaminooxy groups (2.69 ppm) with the saccharide conjugated methylaminooxy groups (2.72-2.80 ppm).[38]

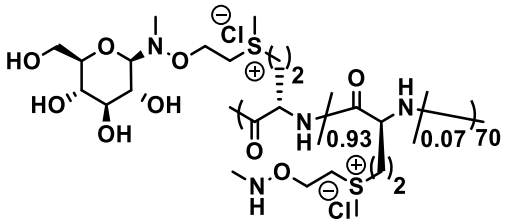
Procedure B (glc-mao-Ehc$^{M}_{70}$). A sample of **mao-Ehc$^{M}_{70}$** was dissolved at 5 mg/mL in a 0.1 M buffer (acetate pH 4-6, phosphate pH 7) with or without 0.1 M aniline. Upon complete dissolution of mao-Ehc$^{M}_{70}$, D-glucose (100 eq) was added to the reaction mixture. The reaction was adjusted to 25 °C and stirred for 4 days. The reaction mixture was then transferred to a 2000 MWCO dialysis bag and dialyzed against (i) pyrogen free milli-Q water (3.5L) containing NaCl (7.0 g, 35mM) for 1 day to facilitate counterion exchange (ii) pyrogen free milli-Q water (3.5L) for 1 day to remove excess NaCl. Dialysate changes were performed every 12 hours. The resulting solution was removed and freeze dried to yield a white fluffy solid with isolated yields ranging from 91 to 99%, and saccharide conjugation yields ranging from 37 to 93%. The degree of saccharide conjugation was quantified by comparing ^{1}H NMR integrals of the *N*-methyl resonances of unconjugated methylaminooxy groups (2.69 ppm) with the saccharide conjugated methylaminooxy groups (2.82 ppm).[38]

Procedure C (glc-mao-Etn$^{O}_{70}$). A sample of **mao-Etn$^{O}_{70}$** was dissolved at 1.5 mg/mL in a 0.1 M buffer (acetate pH 4-6, phosphate pH 7) with or without 0.1 M aniline to give a turbid solution. The D-glucose (50-500 eq) was then added to the reaction mixture. The reaction was stirred at 50 °C for 1 to 4 days. The reaction mixture was then transferred to a 2000 MWCO dialysis bag and dialyzed against pyrogen free milli-Q water (3.5L) for 1 day to remove ions and excess saccharide. Dialysate changes were performed every 12 hours. The resulting solution was removed and freeze dried to yield a white fluffy solid with isolated yields ranging from 70 to 100%, and saccharide conjugation yields ranging from 60 to 100%. The degree of saccharide conjugation was quantified by comparing ^{1}H NMR integrals of the *N*-methyl resonances of unconjugated methylaminooxy groups (2.69 ppm) with the saccharide conjugated methylaminooxy groups (2.80 ppm).[38]



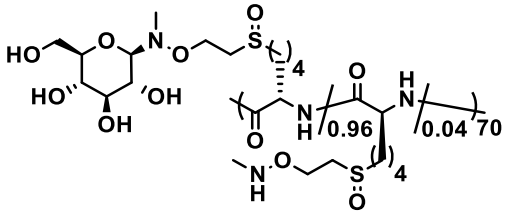
glc-mao-Ehc$^{O}_{70}$ (procedure A).

^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.60-4.46 (br m, 5.9H), 4.28-4.09 (br m, 14.9H), 3.97-3.88 (br d, *J* = 12 Hz, 5.0H), 3.80-3.71 (br m, 4.9H), 3.61-3.47 (br m, 9.4H), 3.47-3.32 (br m, 9.4H), 3.31-2.90 (br m, 13.8H), 2.81 (s, 13.8H), 2.69 (s, 1.0H, 2.39-2.14 (br m, 8.9H).



Glc-mao-Ehc$^{M}_{70}$ (procedure B).

^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.67-4.52 (br m, 8H), 4.36-4.18 (br m, 20H), 4.00-3.88 (br m, 7H), 3.85-3.31 (br m, 64H), 3.19-2.95 (br m, 21H), 2.92-2.73 (br m, 20H), 2.73-2.62 (br m, 1H), 2.56-2.19 (br m, 13H).



glc-mao-Etn$^{O}_{70}$ (procedure C).

^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.38-4.07 (br m, 16H), 3.98-3.86 (br m, 3.9H), 3.81-3.68 (br m, 3.9H), 3.59-3.46 (br m, 7.4H), 3.45-3.30 (br m, 8.2H), 3.28-3.13 (br m, 3.8H), 3.14-3.02 (br m, 4.2H), 3.02-2.85 (br m, 8.3H), 2.84-2.72 (br m, 13H), 2.70-2.65 (br m, 1H), 2.06-1.71 (br m, 16.4H), 1.72-1.43 (br m, 11.8H).

3.5.5 Stability of neoglycoconjugates

Samples of **glc-mao-Ehc$^{O}_{70}$**, **glc-mao-Ehc$^{M}_{70}$**, and **glc-mao-Etn$^{O}_{70}$** (each having > 90% saccharide functionalization) were dissolved in a 0.2 M phosphate buffer (1.0 mg/mL) at either pH 5.5 or 7.4. These solutions were then transferred to 2000 MWCO dialysis bags and dialyzed at 37 °C against 200 mL of a 0.2 M phosphate buffer at either pH 5.5 or 7.4. Samples were dialyzed for 1 week with dialysate changes every day. After 1 week the contents of the dialysis bags were collected and lyophilized. ^{1}H NMR spectra of the resulting solids were obtained and the integrals of N-methyl group resonances at 2.80 and 2.69 ppm were compared to determine the relative amounts of glucose neoglycoconjugate residues and free methylaminooxy residues, respectively.[38] This data was used to calculate the percentage of glucose released from the polypeptide neoglycoconjugates after 1 week. Error bars are consistent with the standard deviation for n = 3.

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Chapter 4: Synthesis and Applications of poly(5,6-epoxy-L-norleucine)

4.1 Abstract

An epoxide-bearing α-helical polypeptide, poly(5,6-epoxy-L-norleucine), **EnI** as synthesized via post-polymerization mCPBA oxidation of poly(L-homoallylglycine). **EnI** was found to be soluble in polar organic solvents and allowed high yield conjugation reactions with a variety of functional thiols under basic conditions. Thiol conjugates of **EnI** displayed high aqueous solubility and partially disordered conformations due to the presence of hydroxyl groups close to the polypeptide backbone. **EnI** conjugates with diethylene glycol derived thiols showed LCST properties in water, which could be modulated by varying concentration, varying polypeptide composition, and through oxidation of polypeptide thioether groups.

4.2 Introduction

Functional polypeptides have been shown to be useful for a variety of biomedical applications.[1] However, the development of polypeptides with complex biomimetic functionality is a continuing challenge. Post-polymerization modification of polypeptides has emerged as an advantageous method of introducing functionality, because it avoids the tedious synthetic procedures often associated with functional monomers and allows for the synthesis of a diverse array of functional polypeptides from a single monomeric precursor.[2, 3] Residues in polypeptides containing an alkene functionality have been utilized for post-polymerization modification via thiol-ene chemistry and alkene oxidation chemistry.[2, 4, 5] Recently, Deming and coworkers reported the synthesis of poly(L-homoallylglycine) (**G^{HA}**), an alkene-bearing α-helical polypeptide that could be modified efficiently under mild conditions using thiol-ene chemistry.[6] However, the resulting thioethers often lacked aqueous solubility due to long hydrophobic side chains (Figure 4.1), and the chemistry performed best in organic solvent systems, which limited

the polarity of usable thiols.[6, 7] Therefore, a method of synthesizing functional polypeptides with higher aqueous solubility was desired. The presence of hydroxyl groups on polypeptide sidechains is known to increase the aqueous solubility of polypeptides.[8] For this reason, an epoxide functional polypeptide approach for the synthesis of water soluble **G^{HA}** derived thioethers was envisioned (Figure 4.2).

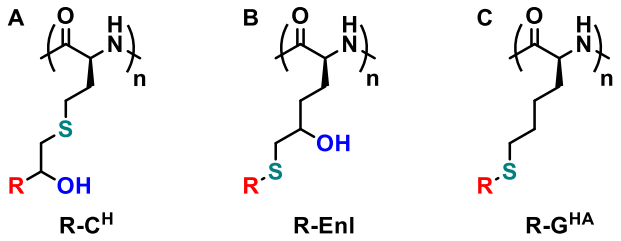


Figure 4.1 Structural variants of functional polypeptides containing thioether side-chain groups. A) Poly(S-alkyl-L-homocysteine), **R-C^{H}**, synthesized via epoxide alkylation and demethylation of poly(L-methionine). B) Thiol modified poly(5,6-epoxy-L-norleucine), **R-EnI**, via nucleophilic ring-opening. C) Thiol modified poly(L-homoallylglycine), **R-G^{HA}**, via thiol-ene conjugation.

Polypeptides containing epoxide functionality were reported by Daly and coworkers, but no subsequent modification was attempted.[9] However, previous studies involving the ring opening of epoxide bearing polymers with thiols under basic conditions have shown the chemistry to be regioselective, and highly efficient under mild conditions.[10-12] Therefore, this methodology could be readily applied for the synthesis of thioether containing polypeptides with functional side chains.

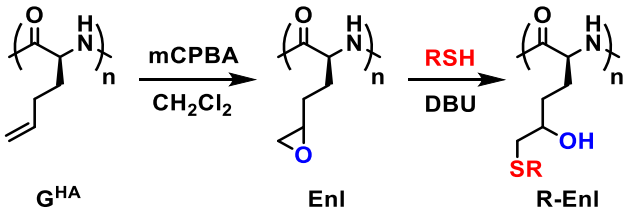


Figure 4.2 Oxidation of poly(L-homoallylglycine), **G^{HA}**, to give poly(5,6-epoxy-L-norleucine), **EnI**, for synthesis of thiol functionalized polypeptides, **R-EnI**.

The Deming lab previously synthesized epoxide derived thioether containing polypeptides by reacting poly(L-methionine) (**M**) residues with oligoethylene glycol (OEG) epoxides to generate sulfonium salts then using sulfonium dealkylation chemistry to generate functional thioether containing polypeptides. The resulting polypeptides were water-soluble with thermoresponsive solution properties.[8, 13] **EnI** derived thioether containing polypeptides have long hydrophobic side chains, but their solubilizing hydroxyl groups are closer to their backbones (Figure 4.1). We predicted that this structural difference may lead to differences in conformation, solubility, and thermoresponsive properties compared to **M** derived β-hydroxy thioethers.

We predicted that ring opening of an epoxide-bearing polypeptide would proceed readily with thiols of varied polarity and functionality under mild conditions. We also hypothesized that OEG derived thiols could be utilized to give polypeptides with thermoresponsive properties similar to what has been reported in other OEG functionalized polypeptide systems.[14-16] Therefore, poly(5,6-epoxy-L-norleucine) (**EnI**) was synthesized and subsequently reacted with a variety of thiols under basic conditions. The solubility, conformational features, and thermoresponsive properties of the resulting conjugates were investigated.

4.3 Results and Discussion

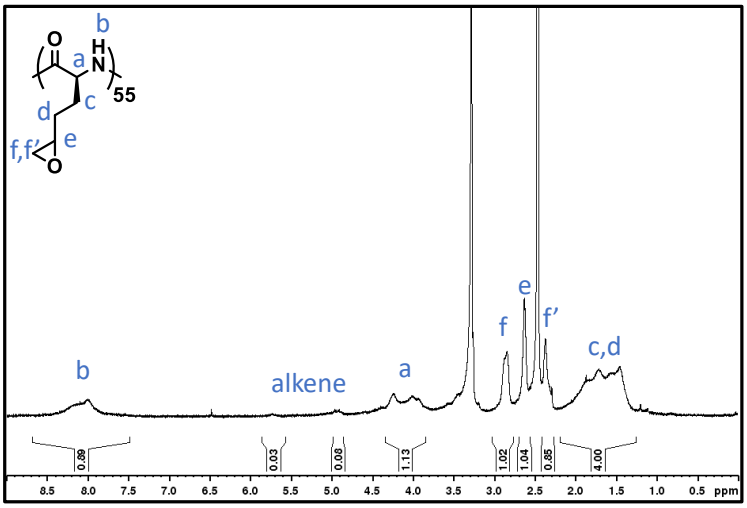


Figure 4.3 ^{1}H NMR Spectrum of **EnI$_{55}$** (400 MHz, DMSO-d$_6$, 25 °C). 95% consumption of alkene groups based on integration of alkene resonances.

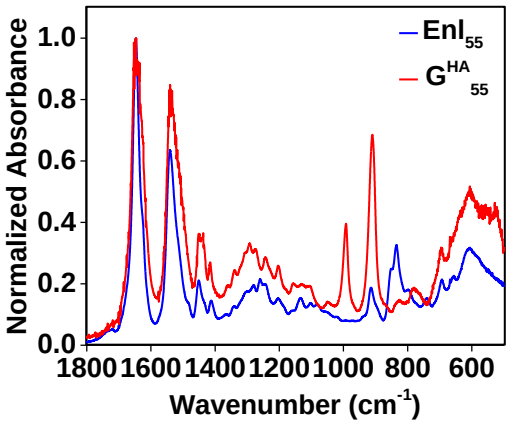


Figure 4.4 Normalized ATR-FTIR absorption (1800-500 cm^{-1}) of **G$^{HA}_{55}$** (red) and **EnI$_{55}$** (blue). **EnI$_{55}$** band at 835 cm^{-1} is consistent with an epoxide ring deformation.

EnI$_{55}$ was synthesized via post-polymerization mCPBA oxidation of **G$^{HA}_{55}$**.[9] Near complete disappearance of alkene resonances was observed via ^{1}H NMR (Figure 4.3). The formation of the epoxide functional groups was confirmed using ATR-FTIR and ^{13}C CP-MAS NMR (Figure 4.4, 4.5).[17, 18] The normalized ATR absorbances of **G$^{HA}_{55}$** and **EnI$_{55}$** were

compared (Figure 4.4). The FTIR spectra of **Enl$_{55}$** contained an absorbance at 835 cm^{-1}, which is in a known stretching frequency range for epoxide ring deformation.[18, 19] GPC analysis of **Enl$_{55}$** showed uniform chain length distribution with low dispersity (Figure 4.6).

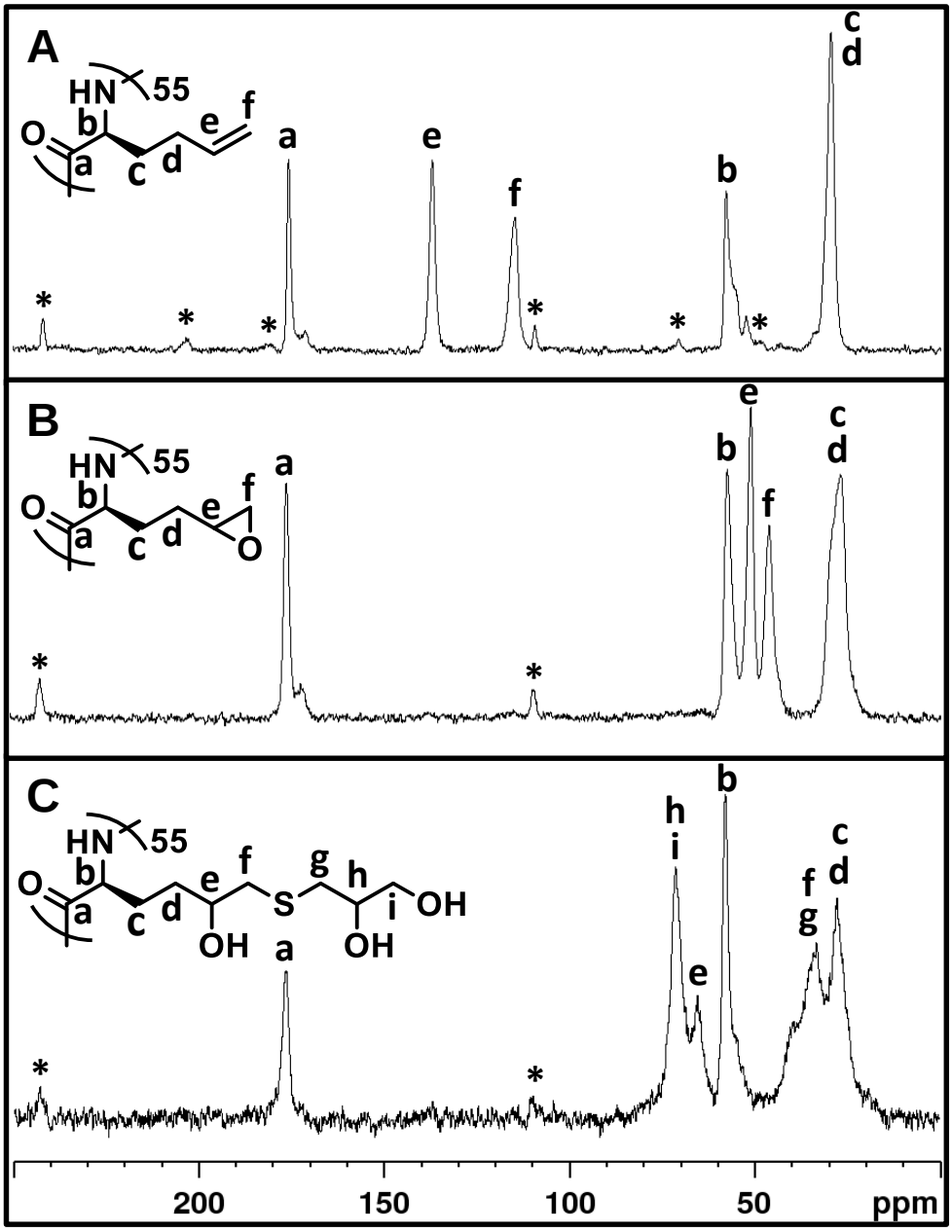


Figure 4.5 ^{13}C CP-MAS NMR spectra overlay of A) **G$^{HA}_{55}$** B) **Enl$_{55}$** and C) **GL-Enl$_{55}$**. Asterisks indicate spinning sidebands.

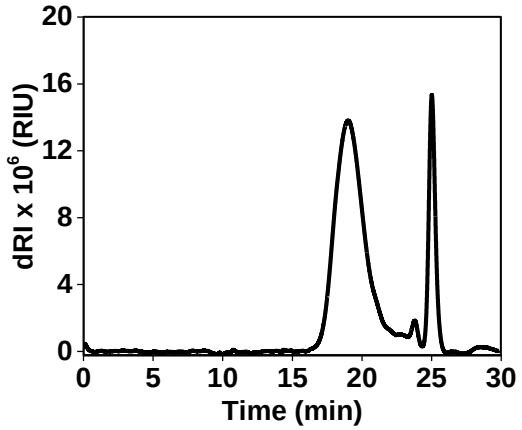


Figure 4.6 GPC Chromatogram of **EnI_{55}** in 0.5% (w/w) KTFA in HFIP at 10 mg/mL. RIU = arbitrary refractive index units, M_w/M_n= 1.26.

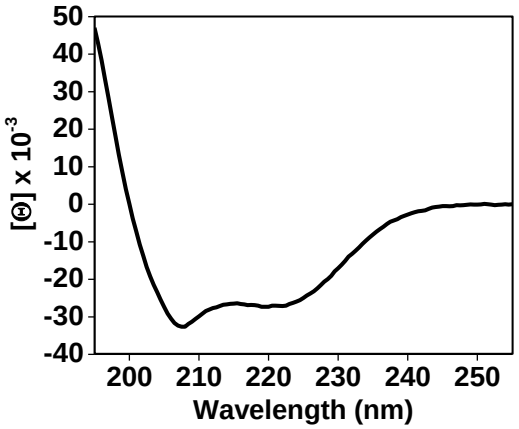
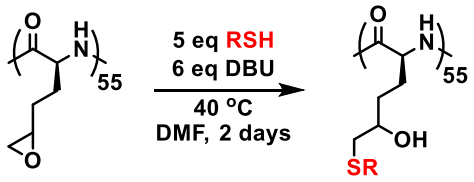


Figure 4.7 Circular dichroism spectrum of **EnI_{55}** in HFIP at 0.1 mg/mL, 20 °C.



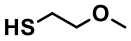
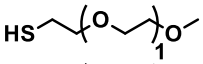
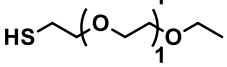
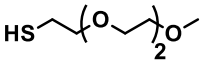
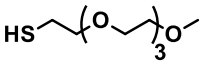
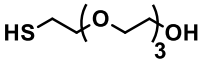
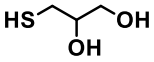
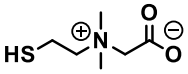
Sample	RSH	Funct (%)	Yield (%)
mEG_1-Enl		100	100
mEG_2-Enl		98	98
eEG_2-Enl		100	73
mEG_3-Enl		92	82
mEG_4-Enl		95	100
EG_4-Enl		100	88
GL-Enl		96	75
CB-Enl*		93	97

Figure 4.8 Base-catalyzed thiol-epoxy chemistry of **Enl_{55}**. Yield is total isolated yield of fully functionalized polypeptide. * = H_2O/room temp, 10 eq thiol.

After confirming the oxidation of alkene to epoxide, the conformation of **Enl_{55}** was studied via CD spectroscopy (Figure 4.7). **Enl_{55}** displayed a predominately α-helical conformation, which demonstrated that chirality of the polypeptide backbone was maintained during the course of the oxidation.[20] The reactivity of **Enl_{55}** was then examined by treating the polypeptide with a variety of thiols under basic conditions (Figure 4.8). OEG derived thiols were chosen for initial **Enl_{55}** conjugation studies, due to their solubilizing properties, ease of synthesis, and the known thermoresponsive properties of OEG containing polypeptides.[6, 8, 13] Reactions with thiols derived from ethylene glycols of varying oligomer lengths proceeded with high functionalization under mild conditions. Successful thiol conjugation was confirmed via ^{1}H NMR, ATR-FTIR, and ^{13}C CP-MAS NMR (Figure 4.5, 4.9). 2-methoxyethane-1-thiol modified poly(5,6-epoxy-L-norleucine)$_{55}$ (**mEG_1-Enl_{55}**) was chosen for ATR-FTIR analysis due to its

structural simplicity (Figure 4.9). The disappearance of the epoxy C-O band at 835 cm^{-1} demonstrated that ring opening of **EnI_{55}** under basic conditions went to completion.[17, 18] GPC analysis of **mEG_4-EnI_{55}** showed a significant increase in molecular weight, while the polypeptide maintained the uniform weight distribution and low dispersity of its **EnI_{55}** precursor (Figure 4.10).

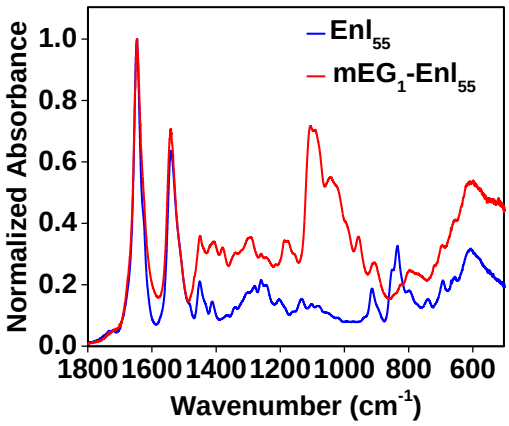


Figure 4.9 Normalized ATR-FTIR absorption (1800-500 cm-1) of **EnI_{55}** (blue) and **mEG_1-EnI_{55}** (red). Note the disappearance of epoxide ring deformation band at 835 cm^{-1} after the thiol-epoxy reaction.

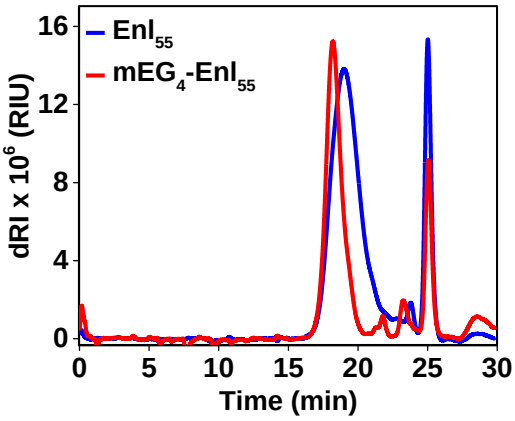


Figure 4.10 GPC Chromatograms of **EnI_{55}** (blue, Mw/Mn= 1.26) and **mEG_4-EnI_{55}** (red, Mw/Mn= 1.27) in HFIP containing 0.5 % (w/w) KTFA at 10 mg/mL.

As expected, thiol-epoxy ring-opening products of **EnI** showed considerably higher aqueous solubility than thiol-ene modified $\mathbf{G^{HA}}$ polypeptides. $\mathbf{G^{HA}}$ was water insoluble when modified with 2-[2-(2-methoxyethoxy)ethoxy]ethanethiol (mEG_3SH).[6] However, the ring-opening conjugate of **EnI** demonstrated good aqueous solubility (> 10 mg/mL) when modified with the same thiol. This notable increase in solubility was likely due to the addition of side chain hydroxyl groups in **EnI**, which are not present in the $\mathbf{G^{HA}}$ thiol-ene conjugate (Figure 4.1).[8]

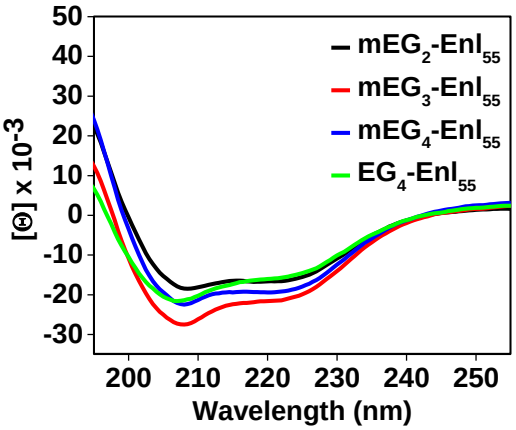


Figure 4.11 Circular dichroism spectra of $\mathbf{mEG_2\text{-}EnI_{55}}$ (black, 50% α-helix), $\mathbf{mEG_3\text{-}EnI_{55}}$ (red, 63% α-helix), $\mathbf{mEG_4\text{-}EnI_{55}}$ (blue, 57% α-helix), and $\mathbf{EG_4\text{-}EnI_{55}}$ (green, 48% α-helix) polypeptides in DI water at 0.1 mg/mL, 20 °C.

The conformational properties of water soluble **EnI** OEG conjugates ($\mathbf{EG_n\text{-}EnI}$) were analyzed using circular dichroism spectroscopy (Figure 4.11). $\mathbf{EG_n\text{-}EnI}$ polypeptides displayed partially disordered conformations in aqueous solution, which was assumed to be due to hydration of their side chain hydroxyl groups.

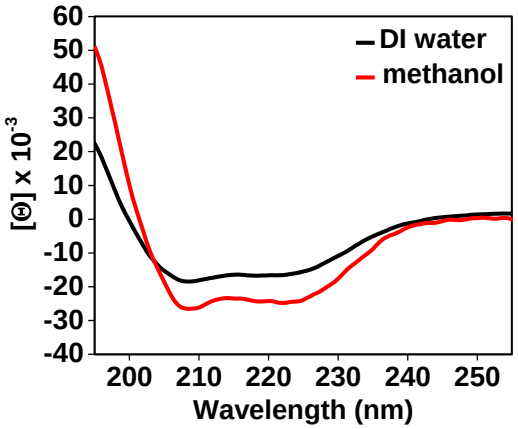


Figure 4.12 Circular dichroism spectra of **mEG$_2$-EnI$_{55}$** at 20 °C in DI water (black, 50% α-helix) and methanol (red, 72% α-helix)**.**

In order to check the effects of water hydration, the circular dichroism spectrum of **mEG$_2$-EnI$_{55}$** was collected in methanol and compared to its spectrum in water (Figure 4.12). The increased α-helical content in methanol confirmed the effect of aqueous solvation on **EnI** derivatives. **EG$_n$-E** polypeptides were found to be significantly more disordered than related, previously synthesized **G^{HA}** and **M** derived functional polypeptides (Figure 4.13).[6, 8]

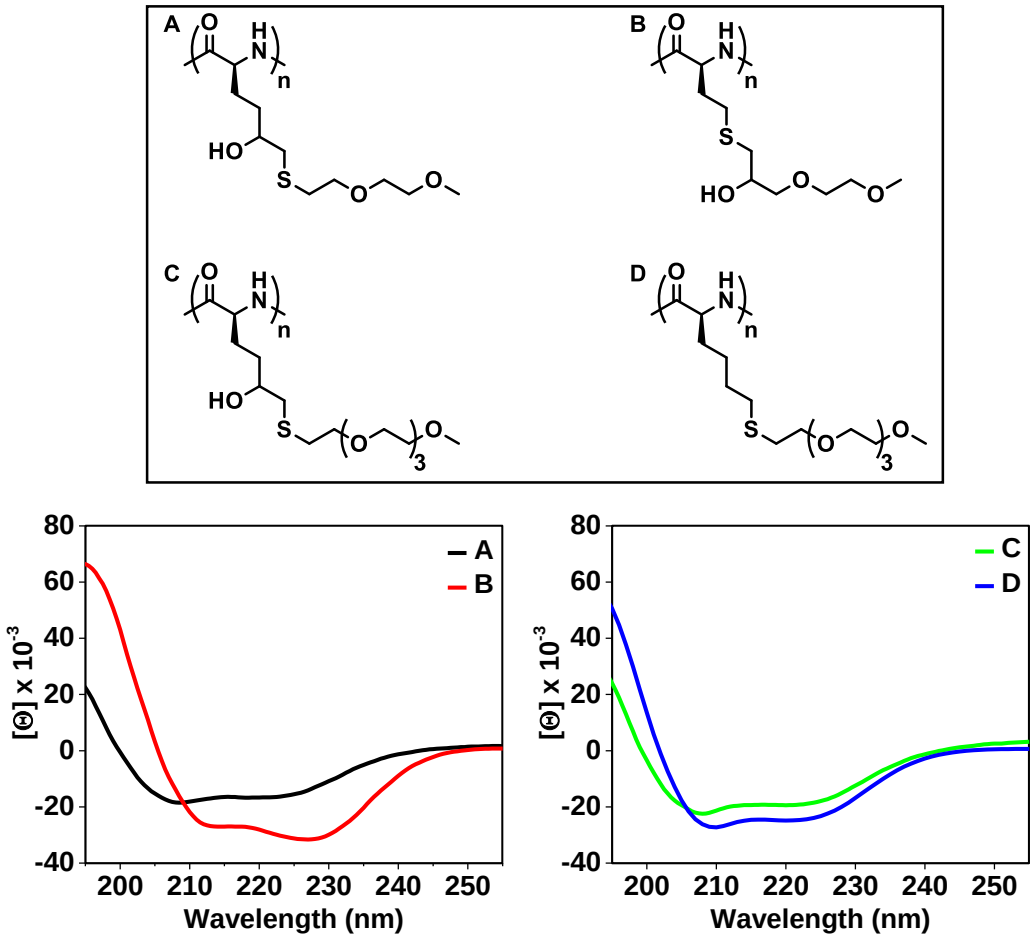


Figure 4.13 Conformational Comparison of **EnI** derivatives to previously reported OEG thioether containing polypeptides. Circular Dichroism spectra were collected in DI water at 20 °C. A) **mEG$_2$-EnI** (black, 0.1 mg/mL, 50% α-helix), B) **mEG$_1$-C^H** (red, 0.5 mg/mL, 84% α-helix), C) **mEG$_4$-EnI** (green, 0.1 mg/mL 57% α-helix), D) **mEG$_4$-G^{HA}** (blue, 0.5 mg/mL 71% α-helix).

After characterizing **EG$_n$-EnI** polypeptides, the reactivity of **EnI$_{55}$** with more polar thiols was examined. Thioglycerol was utilized due to its commercial availability and water solubilizing effect by addition of hydrogen bond donors to the polypeptide. Thioglycerol functionalized **EnI$_{55}$** (**GL-EnI$_{55}$**) was found to have high aqueous solubility for a nonionic polypeptide, readily dissolving to give solutions up to 90 mg/mL in water. **EnI** could also be readily functionalized with thiols in aqueous media, as demonstrated by its reaction with water soluble *N,N*-dimethyl-

cysteamine-carboxybetaine (CB-SH). However, **CB-EnI$_{55}$** was found to possess poor aqueous solubility near neutral pH, likely due to cooperative ion pairing, which is known to occur with betaine-containing zwitterionic polymers.[21, 22] However, this polypeptide is soluble under acidic conditions where the carboxylate is neutralized, displaying a disordered conformation at pH 2 in water (Figure 4.14).

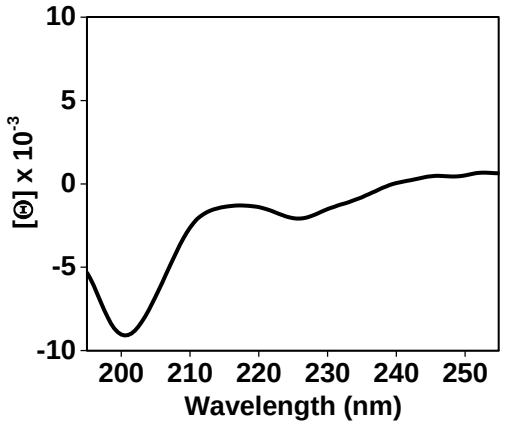


Figure 4.14 Circular Dichroism spectrum of **CB-EnI$_{55}$** at pH 2. Polypeptide was dissolved at 0.1 mg/mL in an aqueous buffer containing 100 mM phosphate and 10 mM Tris, and the pH was adjusted using 0.1 M HCl.

After demonstrating the versatility of thiol addition to **EnI**, we sought to investigate whether **EG$_n$-EnI$_{55}$** polypeptides had similar thermoresponsive properties as previously reported oligoethylene glycol functionalized polypeptides.[13, 15] Out of the 6 oligoethylene glycol conjugates of **EnI** that were prepared, only **mEG$_2$-EnI$_{55}$** and **etEG$_2$-EnI$_{55}$** demonstrated thermoresponsive behavior in water. **mEG$_1$-EnI$_{55}$** was found to be insoluble in water at all temperatures studied, due to its mostly hydrophobic side chain, while **mEG$_3$-EnI$_{55}$**, **mEG$_4$-EnI$_{55}$**, and **EG$_4$-EnI$_{55}$** were soluble at all temperatures studied. The thermoresponsive properties of **EnI** conjugates were highly sensitive to oligoethylene glycol length, with longer oligoethylene glycol groups giving fully water soluble polypeptides assisted by the proximity of their hydroxyl groups to their polypeptide backbones (Figure 4.1).

The thermoresponsive behavior of **EG_2-EnI** conjugates were studied in greater detail. **$etEG_2$-EnI_{55}** solutions possessed a cloud point below room temperature and were not analyzed further. The cloud point of **mEG_2-EnI_{55}** solutions was measured via UV-Vis spectroscopy at different concentrations (Figure 4.15).

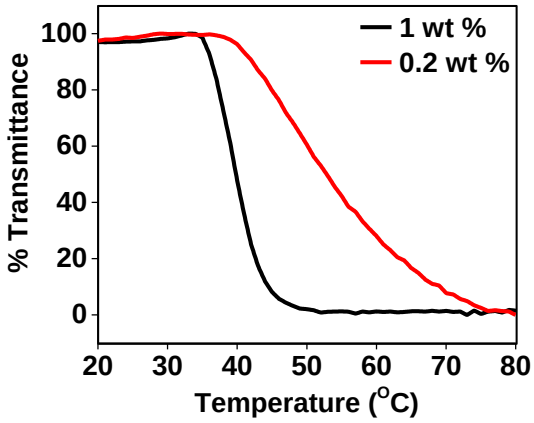


Figure 4.15 Concentration dependence of **mEG_2-EnI_{55}** heating curve in water. 1% w/w solution in DI water (black) shows a sharp transition with a cloud point temperature of 40 °C, while 0.2% w/w solution in DI water (red) has a broader transition and higher cloud point temperature (53 °C).

Increasing polypeptide concentration facilitated intermolecular aggregation, which led to a sharper phase transition that occurred at a significantly lower temperature.[14, 23] However, even at 1% w/w, the cloud point temperature of **mEG_2-EnI_{55}** was beyond the physiological temperature range. Therefore, we sought to further modulate the cloud point temperature of **mEG_2-EnI_{55}** through the incorporation of less soluble **$etEG_2$-EnI** residues (Figure 4.16). A 1:1 statistical copolypeptide of **mEG_2-EnI** and **$etEG_2$-EnI** was synthesized by reacting **EnI_{55}** with a 1:1 ratio of 2-(2-methoxyethoxy)ethane-1-thiol (mEG_2SH) and 2-(2-ethoxyethoxy)ethane-1-thiol ($etEG_2SH$) under basic conditions.

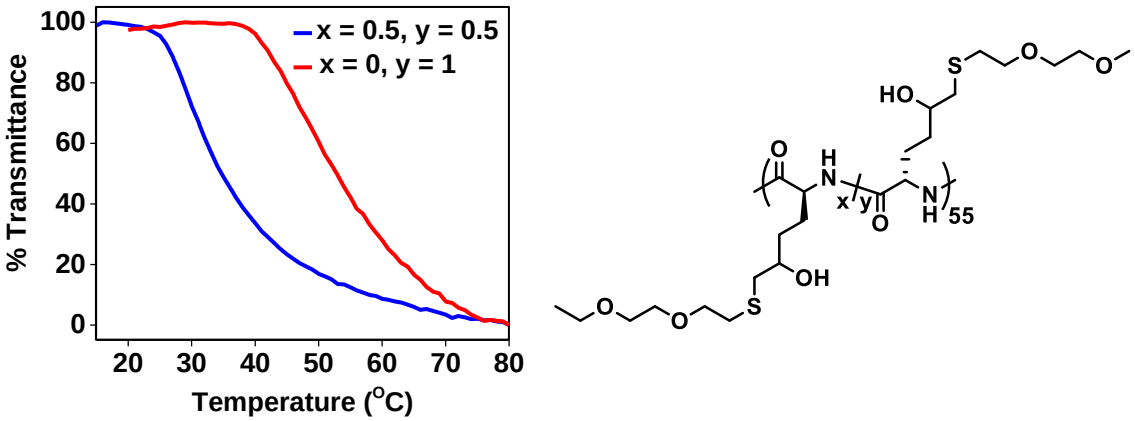


Figure 4.16 Modulation **of mEG$_2$-EnI$_{55}$** cloud point temperature at 0.2 % w/w through incorporation of **etEG$_2$-EnI** residues (x = **etEG$_2$-EnI**, y = **mEG$_2$-EnI**). **mEG$_2$-EnI$_{55}$** (red, x = 0, y = 1) exhibits a cloud point temperature of 53 °C. However, a 1:1 statistical copolymer of **etEG$_2$-EnI** and **mEG$_2$-EnI** (blue, x = 0.5, y = 0.5) exhibits a physiologically relevant cloud point temperature (34 °C).

The increased helicity and lower solubility of previously synthesized oligoethylene glycol-homocysteine (**OEG-C^H**) derivatives allowed for higher tunability of cloud point temperature, while the closer proximity of hydroxyl groups to the backbones of **EnI** derived thioethers led to higher solubility and therefore less adjustment of cloud point temperature (Figure 4.1).[13] Though the high solubility of **EnI** conjugates made tuning their cloud points difficult, the incorporation of **etEG$_2$-EnI** residues into **mEG$_2$-EnI** polypeptides allowed enough tunability to provide a physiologically relevant cloud point temperature (34 °C).

Previously, **OEG-C^H** polypeptides demonstrated a loss of ordered secondary structure and loss of thermoresponsive properties upon oxidation of their thioethers to sulfoxides.[13] We predicted that **mEG$_2$-EnI$_{55}$** and **etEG$_2$-EnI$_{55}$** would respond similarly to oxidation, therefore we oxidized these polypeptides to their corresponding sulfoxides, **mEG$_2$-EnI$^O_{55}$** and **etEG$_2$-EnI$^O_{55}$** (Figure 4.17).

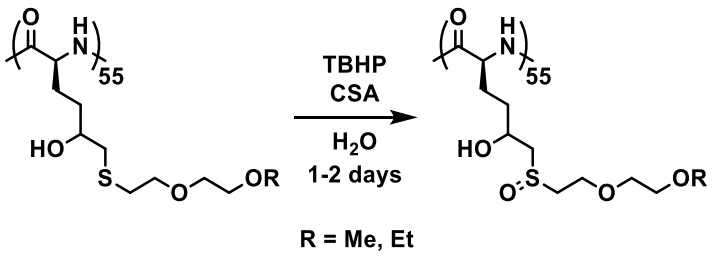


Figure 4.17 Oxidation of **mEG$_2$-EnI$_{55}$** and **etEG$_2$-EnI$_{55}$**. **etEG$_2$-EnI$_{55}$** required longer reaction time due to increased hydrophobicity.

mEG$_2$-EnI$^O_{55}$ and **etEG$_2$-EnI$^O_{55}$** exhibited partially disordered conformations, but not as disordered as previously synthesized **OEG-C^{HO}** polypeptides (Figure 4.18, 4.19).[24] Both polymers displayed good aqueous solubility and neither polymer showed thermoresponsive behavior in water after oxidation. Therefore, oxidation of **EnI** derived thioether residues to their corresponding sulfoxides was an effective method of switching off LCST properties, similar to **OEG-C^H** polypeptides.[13] Since sulfoxides can be readily reduced back to thioethers, reversible switching of thermoresponsive behavior in water may be possible.[25, 26]

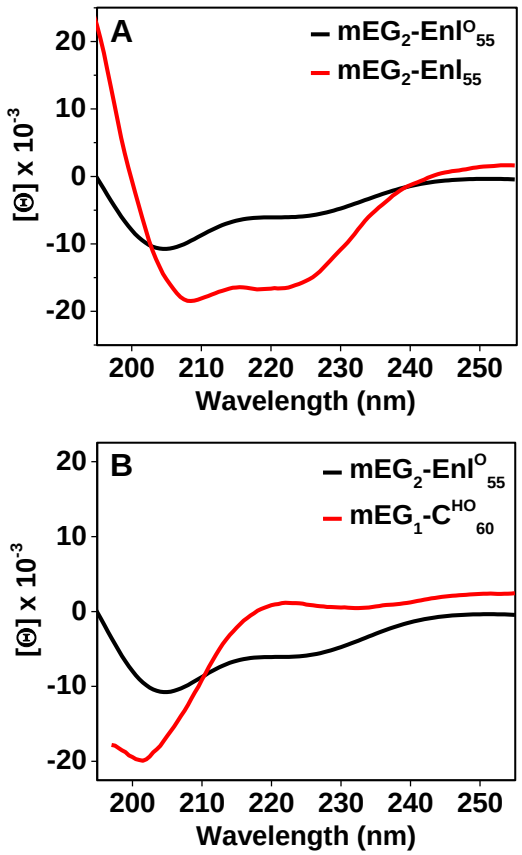


Figure 4.18 A) Comparison of circular dichroism spectra for **mEG$_2$-Enl$_{55}$** (red, 50% α-helix) and **mEG$_2$-Enl$^{O}_{55}$** (black, 23% α-helix) showing change in chain conformation after oxidation. Circular dichroism spectra collected at 0.1 mg/mL in DI water at 20 °C. B) Comparison of circular dichroism spectra for **mEG$_2$-Enl$^{O}_{55}$** (black, 0.1 mg/mL, 23% α-helix) and **mEG$_1$-C$^{HO}_{60}$** (red, 0.25 mg/mL, 5% α-helix).

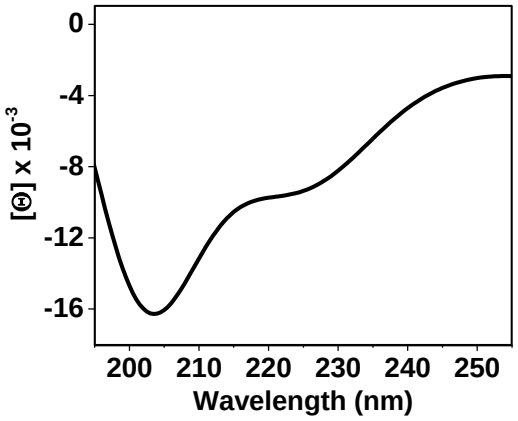


Figure 4.19 Circular dichroism spectrum of **etEG$_2$-EnlO$_{55}$** in DI water at 0.1 mg/mL, 20 °C (32% α-helix).

4.4 Conclusion

A new epoxide functionalized polypeptide was synthesized and then modified by reaction with thiols to furnish polypeptides with a variety of functional groups tethered with thioether linkages. These **R-Enl** polypeptides displayed higher aqueous solubility and more disordered chain conformations compared to structurally related, previously reported polypeptides, due to the proximity of water solubilizing side-chain hydroxyl groups to the polypeptide backbones. Thiol conjugation to **Enl** was found to be an effective method of synthesizing a variety of water-soluble non-ionic polypeptides. Though the high water solubility of **Enl** derivatives made the design of thermoresponsive polypeptides challenging, **EG$_2$-Enl** polypeptides demonstrated thermoresponsive behavior that was modified by altering sample concentration and polypeptide composition, and could be switched off by oxidizing the thioether side-chains. The versatility and high water solubility of **Enl** derivatives gives them potential for a variety of biomaterial applications.

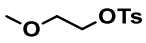
4.5 Experimental

4.5.1 Materials and Methods

Unless specified, all post-polymerization modification chemistry was performed in glass vials under ambient atmosphere. Thiol-epoxy reactions were performed in 1 dram screw top glass vials capped with septa to allow sparging. Small molecule chemistry was performed in heat-dried glassware under a nitrogen atmosphere, unless otherwise specified. THF and hexanes were degassed with dinitrogen and passed through an alumina column before use. All other reagents and solvents were used as received. Poly(L-homoallylglycine)$_{55}$, OEG-thiols (except 2-(2-methoxyethoxy)ethane-1-thiol and 2-(2-ethoxyethoxy)ethane-1-thiol), and *N,N*-dimethyl-cysteamine-carboxybetaine (CB-SH) have been previously reported and synthesized by known procedures.[6, 27] Unless otherwise specified, all reactions were performed at ambient temperature (ca. 20 °C). In-house deionized water was used for all aqueous chemistry and dialysis unless otherwise specified. Circular dichroism (CD) spectroscopy was performed on samples prepared using deionized water filtered through a Millipore Milli-Q Biocel A10 filter system unless otherwise specified. CD spectra were collected using 0.1 mg /mL solutions of polypeptide on either an Olis DSM 10 spectrophotometer or a JASCO J-715 spectrophotometer using a 0.1 cm path length quartz cuvette. Percent α-helical content of polypeptides was estimated using the formula % α-helix = 100 × (−$[\theta]_{222}$ + 3000)/39000), where $[\theta]_{222}$ is the measured molar ellipticity at 222 nm.[28] Thin-layer chromatography was performed with EMD gel 60 F254 plates (0.25 mm thickness) and spots were visualized using a UV lamp or $KMnO_4$ stain. Silicycle Siliaflash G60 silica (60-200 μm) was used for all column chromatography. Silica used for chromatographic purification of NCA monomers was dried under vacuum at 250 °C for 48 hours and then stored in a dinitrogen filled glovebox. Compositions of mobile phases used for chromatography and compositions of solvent systems used for circular dichroism are given in volume percents. Dialysis was performed with regenerated cellulose tubing obtained

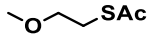
from Spectrum labs. NMR spectra of solution samples were recorded on a Bruker AV400 instrument with chemical shifts reported relative to the deuterated solvent used. Solid state ^{13}C Cross Polarization-Magic Angle Spinning NMR was conducted on a Bruker AV III HD instrument with a magnetic field of 14.1 T. 10 mg of $\mathbf{G^{HA}_{55}}$, 10 mg of $\mathbf{Enl_{55}}$, and 50 mg of $\mathbf{GL\text{-}Enl_{55}}$ were used for acquisition of spectra. The contact time was 1.5 ms with a recycle delay of 5 s. The sample spinning rate was 10 kHz. Solid state FTIR spectra were collected using a Thermo Scientific Nicolet iS5 FT-IR spectrophotometer with an ID7 ATR Sampling Accessory. Cloud point temperature measurements were recorded on an HP 8453 spectrophotometer equipped with an Agilent 8909A temperature controller. Tandem gel permeation chromatography/light scattering (GPC/LS) was performed at 25 ^{o}C using an SSI Accuflow Series III pump equipped with Wyatt DAWN EOS light scattering and Optilab REX refractive index detectors. Separations were achieved using 100 Å and 1000 Å PSS-PFG 7 µm columns at 30 ^{o}C with 0.5% (w/w) KTFA in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) as eluent and sample concentrations of 10 mg/ml.

4.5.2 Thiol Synthesis

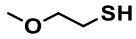


2-methoxyethyl 4-methylbenzenesulfonate. This compound was synthesized using a procedure for a related molecule.[6] H_2O (7.00 mL) was added to 2-methoxyethanol (2.26 g, 29.7 mmol) and NaOH (2.37 g, 59.4 mmol). Tosyl chloride (5.95 g, 31.2 mmol) was dissolved in THF (7.00 mL) and added dropwise over ice with stirring. The reaction was let warm to ambient temperature overnight. Diethyl ether (14.0 mL) was then added to the reaction and the mixture was taken up into a separatory funnel and partitioned. The aqueous layer was washed with diethyl ether (3 x 3.50 mL) then the combined organic fractions were washed with H_2O (3 x 14.0 mL). The organic layer was dried with anhydrous sodium sulfate, decanted and evaporated to dryness to give the product as a clear oil (4.64 g, 69% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25

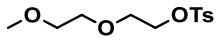
°C): δ 7.80 (d, J = 8.3 Hz, 2H), 7.34 (d, J = 8.0, 2H), 4.18-4.13 (m, 2H), 3.60-3.54 (m, 2H), 3.30 (s, 3H), 2.44 (s, 3H).



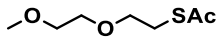
***S*-(2-methoxyethyl) ethanethioate.** This compound was synthesized using a procedure for a related molecule.[6] 2-methoxyethyl 4-methylbenzenesulfonate (2.04 g, 8.86 mmol) and potassium thioacetate (1.26 g, 11.1 mmol) were dissolved in a round bottom flask containing acetone (54.0 mL). A reflux condenser was added and the reaction was stirred vigorously overnight at 50 °C. Solvent was removed via vacuum and the resulting mixture was dissolved in a biphasic mixture of 16.0 mL DCM and 16.0 mL H_2O, taken up into a separatory funnel, and partitioned. The aqueous layer was washed with 2x 16.0 mL DCM and combined organics were washed with 6.0 mL brine. The organic layer was dried with anhydrous sodium sulfate, decanted, and evaporated to dryness to give the product as a yellow oil (0.370 g, 31% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 3.51 (t, J = 6.3 Hz, 2H), 3.36 (s, 3H), 3.09 (t, J = 6.2 Hz, 2H), 2.34 (s, 3H).



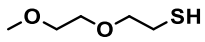
2-methoxyethane-1-thiol. Adapted from a procedure for deprotection of a related molecule.[29] Methanol (7.50 mL) and conc. HCl (0.600 mL) were added to a sealed pressure tube containing *S*-(2-methoxyethyl) ethanethioate (0.320 g, 2.38 mmol). The reaction mixture was stirred at 100 °C for 3 hours. The reaction was let cool to ambient temperature and then H_2O (7.50 mL) was added. This mixture was extracted with 3x 6.00 mL DCM. Combined organic extracts were then washed with 3x 6.00 mL H_2O and 1x 6.00 mL brine, dried with anhydrous sodium sulfate, decanted, and rotovapped (30 °C, 400 mbar) to give the product as a yellow oil (0.200 g, 95% yield). Spectral data agreed with previously published results.[30]



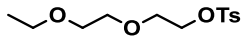
2-(2-methoxyethoxy)ethyl 4-methylbenzenesulfonate. The procedure for the synthesis of 2-methoxyethyl 4-methylbenzenesulfonate was followed. Diethylene glycol monomethyl ether (5.1 g) was used to prepare the product, obtained as a clear oil (9.00 g, 77% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 7.81-7.75 (m, 2H), 7.33 (dd, J = 8.6 Hz, 0.60 Hz), 4.20-4.11 (m, 2H), 3.71-3.43 (br m, 6H), 3.33 (s, 3H), 2.43 (s, 3H).



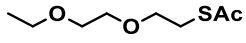
***S*-(2-(2-methoxyethoxy)ethyl) ethanethioate.** The procedure for the synthesis of *S*-(2-methoxyethyl) ethanethioate was followed. 2-(2-methoxyethoxy)ethyl 4-methylbenzenesulfonate (8.97 g) was used to prepare the product, obtained as a yellow oil (5.50 g, 94% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 3.65-3.49 (br m, 6H), 3.34 (s, 3H), 3.10 (t, J = 6.5 Hz, 2H), 2.33 (s, 3H).



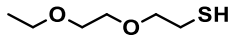
2-(2-methoxyethoxy)ethane-1-thiol. This compound was synthesized using a procedure for a related molecule.[6] Methanol (20.0 mL) and conc. HCl (1.50 mL) were added to a sealed pressure tube containing *S*-(2-(2-methoxyethoxy)ethyl) ethanethioate (2.02 g, 11.3 mmol). The reaction mixture was stirred at 100 °C for 3 hours. The reaction was let cool to ambient temperature and then H_2O (20.0 mL) was added. This mixture was extracted with 3x 15.0 mL DCM. Combined organic extracts were then washed with 3x 15.0 mL H_2O and 1x 15 mL brine, dried with anhydrous sodium sulfate, decanted, and evaporated to dryness to give the product as a yellow oil (1.18 g, 77% yield). Spectral data agreed with previously published results.[31]



2-(2-ethoxyethoxy)ethyl 4-methylbenzenesulfonate. The procedure for the synthesis of 2-methoxyethyl 4-methylbenzenesulfonate was followed. Diethylene glycol monoethyl ether (2.02 g) was used to prepare the product, obtained as a clear oil (3.57 g, 82% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 7.82-7.76 (m, 2H), 7.33 (d, J = 8.0 Hz, 2H), 4.19-4.14 (m, 2H), 3.71-3.66 (m, 2H), 3.60-3.55 (m, 2H), 3.53-3.45 (m, 4H), 2.44 (s, 3H), 1.19 (t, J = 7.0 Hz, 3H).

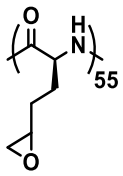


***S*-(2-(2-ethoxyethoxy)ethyl) ethanethioate.** The procedure for the synthesis of *S*-(2-methoxyethyl) ethanethioate was followed. 2-(2-ethoxyethoxy)ethyl 4-methylbenzenesulfonate (2.25 g) was used to prepare the product, obtained as a yellow oil (1.26 g, 84% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 3.66-3.45 (br m, 8H), 3.10 (t, J = 6.5 Hz, 2H), 2.33 (s, 3H), 1.21 (t, J = 7.0 Hz, 3H)



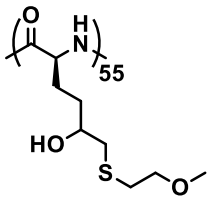
2-(2-ethoxyethoxy)ethane-1-thiol. The procedure for the synthesis of 2-(2-methoxyethoxy)ethane-1-thiol was followed. *S*-(2-(2-ethoxyethoxy)ethyl) ethanethioate (1.25 g) was used to prepare the product, obtained as a clear oil (0.740 g, 76% yield). ^{1}H NMR (400 MHz, $CDCl_3$, 25 °C): δ 3.63-3.46 (br m, 8H), 2.67 (dt, J = 8.2 Hz, 6.5 Hz, 2H), 1.56 (t, J = 8.2 Hz, 1H), 1.18 (t, J = 7.0 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, 25 °C): δ 72.9, 70.3, 69.7, 66.6, 24.2, 15.1.

4.5.3 Post-polymerization Chemistry



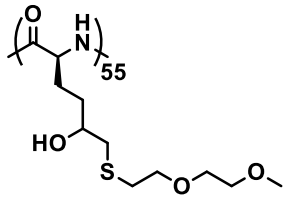
poly(5,6-epoxy-L-norleucine)$_{55}$, Enl$_{55}$. G$^{HA}_{55}$ (45.2 mg) was dissolved in DCM (5 mg/mL). m-CPBA was added to the solution (210.0 mg, 2.5 eq). The reaction was let stir for 2 days then transferred to a 2000 MWCO dialysis bag. The reaction mixture was dialyzed against methanol for 1 day, then DI water for 1 day, with 2 dialysate changes daily. The dialyzed polymer was lyophilized to dryness, yielding a white solid (41.5 mg, 81% yield). ^{1}H NMR (400 MHz, DMSO-d6, 25 °C): δ 8.68-7.50 (br m, 1H), 4.36-3.85 (br m, 1H), 3.03-2.78 (br m, 1H), 2.70-2.56 (br m, 1H), 2.42-2.28 (br m, 1H), 2.20-1.26 (br m, 4H) ^{13}C NMR (CP-MAS, 25 °C): δ 175.8, 57.3, 50.9, 46.2, 26.9.

General Procedure for base-catalyzed thiol-epoxy ring opening of Enl. **Enl** was dissolved in DMF to give a 3 mg/mL solution in a 1 dram screw top vial. 5 equivalents of thiol per residue were added and the solution was degassed for 10 minutes. 6 equivalents of DBU (50mg/mL in DMF, degassed) were added to the reaction mixture via syringe and the reaction was let stir under nitrogen at 40 °C for 2 days. The reaction mixture was dialyzed against DI water (2000 MWCO) for 2 days with 2 water changes daily. The dialyzed polymer was lyophilized to dryness, yielding a white solid.

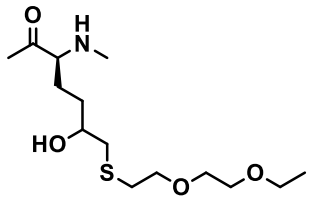


mEG$_1$-Enl$_{55}$. The general procedure for base-catalyzed thiol-epoxy ring opening of **Enl** was followed. **Enl$_{55}$** (5.0 mg) and 2-methoxyethane-1-thiol (5 eq per **Enl** residue) were used to

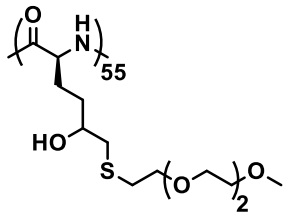
prepare the product, obtained as a white solid (8.5 mg, 100% yield). ^{1}H NMR (400 MHz, d-TFA, 25 °C): δ 5.04-4.38 (br m, 1H), 4.20-3.98 (br m, 1H), 3.91 (br t, J = 5.9 Hz, 2H), 3.68-3.51 (br m, 3H), 3.15-2.60 (br m, 4H), 2.32-1.76 (br m, 4H).



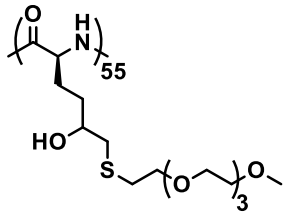
mEG$_2$-EnI$_{55}$. The general procedure for base-catalyzed thiol-epoxy ring opening of **EnI** was followed. **EnI$_{55}$** (2.5 mg) and 2-(2-methoxyethoxy)ethane-1-thiol (5 eq per **EnI** residue) were used to prepare the product, obtained as a white solid (5.0 mg, 98% yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.27-3.87 (br m, 1H), 3.79-3.43 (br m, 7H), 3.27 (s, 3H), 2.80-2.44 (br m, 4H), 2.15-1.26 (br m, 4H)



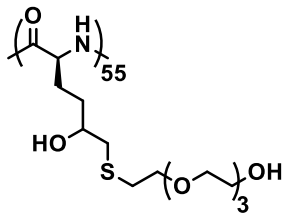
etEG$_2$-EnI$_{55}$. The general procedure for base-catalyzed thiol-epoxy ring opening of **EnI** was followed. **EnI$_{55}$** (6.3 mg) and 2-(2-ethoxyethoxy)ethane-1-thiol (5 eq per **EnI** residue) were used to prepare the product, obtained as a white solid (9.9 mg, 73% yield). ^{1}H NMR (400 MHz, d-TFA, 25 °C): δ 5.10-4.46 (br m, 1H), 4.21-3.69 (br m, 9H), 3.37-2.58 (br m, 4H), 2.43-1.76 (br m, 4H), 1.41 (t, J = 7.1, 3H).



mEG$_3$-EnI$_{55}$. The general procedure for base-catalyzed thiol-epoxy ring opening of **EnI** was followed. **EnI$_{55}$** (2.6 mg) and 2-[2-(2-methoxyethoxy)ethoxy]ethanethiol (5 eq per **EnI** residue) were used to prepare the product, obtained as a white solid (5.1 mg, 82% yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.32-3.87 (br m, 1H), 3.79-3.31 (br m, 11H), 3.30-3.20 (br s, 3H), 2.82-2.40 (br m, 4H), 2.12-1.33 (br m, 4H).

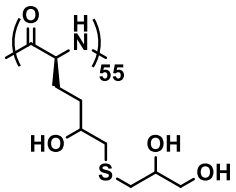


mEG$_4$-EnI$_{55}$. The general procedure for base-catalyzed thiol-epoxy ring opening of **EnI** was followed. **EnI$_{55}$** (2.0 mg) and 2-[2-[2-(2-methoxyethoxy)ethoxy]ethoxy]ethanethiol (5 eq per **EnI** residue) were used to prepare the product, obtained as a white solid (5.5 mg, 100% yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.27-3.88 (br m, 1H), 3.79-3.47 (br m, 15H), 3.27 (s, 3H), 2.81-2.45 (br m, 4H), 2.11-1.32 (br m, 4H).

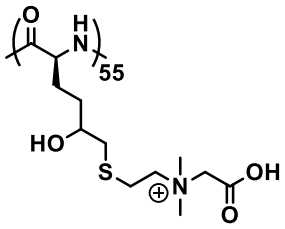


EG$_4$-EnI$_{55}$. The general procedure for base-catalyzed thiol-epoxy ring opening of **EnI** was followed. **EnI$_{55}$** (2.0 mg) and 1-mercapto-11-hydroxy-3,6,9-trioxaundecane (5 eq per **EnI** residue) were used to prepare the product, obtained as a white solid (4.6 mg, 88% yield). ^{1}H

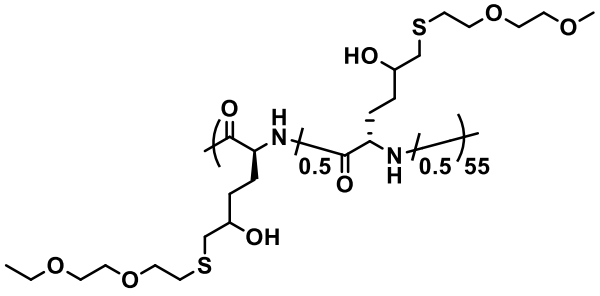
NMR (400 MHz, D_2O, 25 °C): δ 4.38-3.82 (br m, 1H), 3.81-3.17 (br m, 15H), 2.94-2.26 (br m, 4H), 2.07-1.30 (br m, 4H).



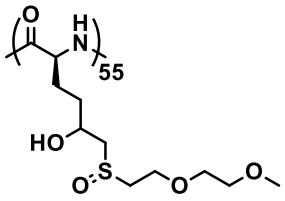
GL-Enl$_{55}$. The general procedure for base-catalyzed thiol-epoxy ring opening of **Enl** was followed. **Enl$_{55}$** (4.3 mg) and 1-thioglycerol (5 eq per **Enl** residue) were used to prepare the product, obtained as a white solid (6.0 mg, 75% yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.29-3.91 (br m, 1H), 3.81-3.62 (br m, 2H), 3.62-3.51 (br m, 1H), 3.50-3.42 (m, 1H), 2.82-2.28 (br m, 4H), 2.17-1.31 (br m 4H).



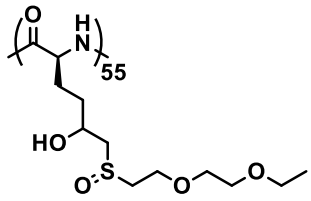
CB-Enl$_{55}$. Enl$_{55}$ (2.5 mg) and *N,N*-dimethyl-cysteamine-carboxybetaine (36 mg, 10 eq per **Enl** residue) were added to a 1 dram screwtop vial. DI water (420 µL) was added and the reaction mixture was sonicated to give a fine suspension. DBU was then added (40 µL, 10.5 eq) and the reaction was stirred vigorously for 48 hr to give a turbid solution, which was transferred to a 2000 MWCO dialysis tube. The reaction mixture was dialyzed against DI water for 2 days with 2 water changes daily then lyophilized to dryness, yielding a white solid (5.6 mg, 97% yield). ^{1}H NMR (400 MHz, 10% DCl in D_2O, 25 °C): δ 2.60-2.21 (br m, 3H), 2.00-1.68 (br m, 3H), 1.46-1.19 (br m, 6H), 1.12-0.95 (br m, 2H), 0.90-0.64 (br m, 2H), 0.02- -0.52 (br m, 4H).



(etEG$_2$- Enl$_{0.5}$-*stat*-mEG$_2$- Enl$_{0.5}$)$_{55}$. The general procedure for base-catalyzed thiol-epoxy ring opening of **Enl** was followed. **Enl$_{55}$** (5.2 mg), 2-(2-ethoxyethoxy)ethane-1-thiol (2.5 eq per ENL), and 2-(2-methoxyethoxy)ethane-1-thiol (2.5 eq per **Enl** residue) were used to prepare the product, obtained as a white solid (8.8 mg, 80% yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): δ 4.29-3.86 (br m, 1H), 3.82-3.36 (br m, 8H), 3.26 (s, 1.5H), 2.83-2.42 (br m, 4H), 2.24-1.29 (br m, 4H), 1.08 (t, J = 7.0, 1.5H).



mEG$_2$-Enl$^O_{55}$. This compound was synthesized using a procedure for a related polypeptide.[1] **mEG$_2$-Enl$_{55}$** (7.5 mg) and CSA (1.3 mg) were dissolved in DI water (0.88 mL). An aqueous solution of TBHP (70 % (w/w), 16 eq per **mEG$_2$-Enl$_{55}$** residue) was then added. The reaction was let stir overnight. The reaction mixture was then transferred to a 2000 MWCO dialysis bag and dialyzed against DI water for 48 hours with 2 water changes daily. The dialyzed polymer was lyophilized to dryness to give the product as a white solid (6.6 mg, 83% yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): 4.25-4.10 (br m, 1H), 4.06-3.95 (br m, 1H), 3.89-3.73 (br m, 2H), 3.64-3.47 (br m, 4H), 3.26 (s, 3H), 3.20-2.77 (br m, 4H), 2.09-1.44 (br m, 4H).



etEG$_2$-EnI^O$_{55}$. This compound was synthesized using a procedure for a related polypeptide.[1] **etEG$_2$-EnI$_{55}$** (7.4 mg) and CSA (1.2 mg) were dissolved in DI water (0.87 mL). An aqueous solution of TBHP (70 % (w/w), 16 eq per **etEG$_2$-EnI$_{55}$** residue) was then added. The reaction was let stir 48 hr. The reaction mixture was then transferred to a 2000 MWCO dialysis bag and dialyzed against DI water for 48 hours with 2 water changes daily. The dialyzed polymer was lyophilized to dryness to give the product as a white solid (7.1 mg, 91% yield). ^{1}H NMR (400 MHz, D_2O, 25 °C): 4.29-4.09 (br m, 1H), 4.07-3.95 (br m, 1H), 3.91-3.75 (br m, 2H), 3.65-3.52 (br m, 4H), 3.48 (q, J = 7.1, 2H), 3.22-2.77 (br m, 4H), 2.01-1.48 (br m, 4H), 1.08 (t, J = 7.0, 3H).

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