UNIVERSITY OF CALIFORNIA

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Functional Polypeptides for Biomedical Applications

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in Chemistry

by

Jessica R. Kramer

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ABSTRACT OF THE DISSERTATION

Functional Polypeptides for Biomedical Applications

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Doctor of Philosophy of Chemistry

University of California, Los Angeles, 2012

Professor Timothy J. Deming, Chair

Synthetic polypeptides have shown great promise as materials for biotechnology and medicine, with applications in tissue engineering, drug delivery, and as therapeutics. Despite significant advances in the preparation of well-defined polypeptides and the development of self-assembling materials, a need remains for a broader scope of polypeptides with functionalities that mimic the complexity and function of post-translationally modified natural proteins. The display of functionalities that have therapeutic effects, target materials to specific tissues, passivate the immune response, or have stimuli responsive behavior, are highly desirable yet generally require complex and inefficient synthetic approaches.

This dissertation reports several distinct advances in the preparation of a wide variety of highly functional polypeptide materials. Progress in both the polymerization of functionalized NCA monomers and the post-polymerization modification of polypeptides is described. A new method of NCA purification was developed, and has allowed access to diverse monomers with functionalities previously unattainable due to impurities that impeded polymerization. This purification technique was applied to the synthesis of glycosylated NCAs, which yielded the first living polymerizations of glycosylated NCAs and gave access to glycopolypeptides with unique properties and conformations. The display of sugar functionalities from synthetic polymers is an area of great interest due to the many attractive properties imparted upon the parent material, such as non-ionic water solubility, biological targeting, and shielding of the polypeptide from proteases. These glycopolypeptides were used to explore the effect of conformation on self-assembly and ligand binding, and to develop vesicles with potential medical applications in targeted drug delivery. In addition, a new conjugation technique for facile post-polymerization modification of polypeptides is described. This conjugation utilizes the unique chemistry of the natural amino acid methionine to allow chemoselective introduction of a wide variety of functional groups via alkylation. We investigated the stability of various alkylated methionines to thiolysis, and developed a simple method to attach and later remove different groups. Finally, work performed during an NSF-IGERT funded internship at HRL Laboratories Inc. is described. Surface modification of carbon foam anodes for microbial fuel cell applications was explored, and the use of thermogravimetric analysis as technique to evaluate biofilm formation was developed.

The dissertation of Jessica R. Kramer is approved**.**

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University of California, Los Angeles

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LIST OF ABBREVIATIONS

°	degrees
Ac	acetyl, acetate
ACS	American Chemical Society
aq.	aqueous
Bn	benzyl
C	Celsius
calcd	calculated
CBz, Z	carbobenzyloxy
CD	circular dichroism
d	doublet
DCM	dichloromethane
DIC	differential interference contrast
DLS	dynamic light scattering
DMF	*N,N*-dimethylformamide
eq	equivalent
ESI	electrospray ionization
EtOAc	ethyl acetate
g	gram(s)
h	hour(s)
Hex	hexanes
HRMS	high resolution mass spectroscopy
Hz	hertz

IPA	isopropanol
FTIR	Fourier transform infrared spectroscopy
J	coupling constant
λ	wavelength
L	liter
LSCM	laser scanning confocal microscopy
m	Multiplet, milli
m/z	mass to charge ratio
μ	micro
Me	methyl
MHz	megahertz
min	minute(s)
mol	mole(s)
mp	melting point
MS	molecular sieves
NCA	*N*-carboxyanhydride
NMR	nuclear magnetic resonance
p	para
pH	hydrogen ion concentration in aqueous solution
ppm	parts per million
q	quartet
RT	room temperature
s	singlet
t	triplet

TBAF	tetrabutyl ammonium fluoride
TBDMS	*tert*-butyldimethylsilyl
Tf	trifluoromethanesulfonyl (trifyl)
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TEM	transmission electron microscopy
TMS	trimethylsilyl
UV	ultraviolet

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2. Kramer, J. R.; Deming, T. J. General method for purification of α-amino acid-*N*-carboxyanhydrides using flash chromatography. *Biomacromolecules* **2010**, *12*, 3668–3672.

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4. Kramer, J. R.; Deming, T. J. Preparation of Multifunctional and Multireactive Polypeptides via Methionine Alkylation. *Biomacromolecules* **2012**, *13*, 1719-1723.

5. Kramer, J. R.; Soukiazian, S.; Mahoney, S.; Hicks-Garner, J. Microbial Fuel Cell Biofilm Characterization with Thermogravimetric Analysis on Bare and PEI Surface Modified Carbon Foam Anodes *J. Power Sources* **2012**, *210*, 122-128.

6. Deming, T. J.; Kramer, J. R. Preparation Of Functionalized Polypeptides, Peptides, And Proteins By Alkylation Of Thioether Groups. (2012) U.S. Patent Application No. 615,809 Washington, DC: U.S. Patent and Trademark Office.

PRESENTATIONS

1. Kramer, J. R.; Deming, T. J. Glycopolypeptides by Ring-Opening Polymerization. Poster presentation, International Center for Materials Research US-Argentina Workshop on Nanomaterials, Bariloche, Argentina March 15-17, 2009.

2. Kramer, J. R.; Deming, T. J. Glycopolypeptides by Ring-Opening Polymerization. Poster presentation, 239th ACS National Meeting, San Francisco, CA, United States, March 21–25, 2010.

3. Kramer, J. R.; Deming, T. J. Well-defined Glycopolypeptides via Polymerization of Glycosylated N-carboxyanhydrides. Poster presentation, 241st ACS National Meeting, Anaheim, CA, United States, March 27–31, 2011.

4. Kramer, J. R.; Deming, T. J. Glycopolypeptides via Living Polymerization of Glycosylated-L-Lysine *N*-Carboboxyanhydrides. Oral presentation, 241st ACS National Meeting, Anaheim, CA, United States, March 27–31, 2011.

5. Kramer, J. R.; Deming, T. J. Glycopolypeptides via Living Polymerization of Glycosylated-L-Lysine N Carboboxyanhydrides. Poster presentation, ACS, Division of Organic Chemistry Graduate Research Symposium, Santa Barbara, CA, United States, July 14-17, 2011.

6. Kramer, J. R.; Deming, T. J. Glycopolypeptides via Living Polymerization of Glycosylated *N*-Carboboxyanhydrides. Oral presentation, 242st ACS National Meeting, San Deigo, CA, United States, March 25–39, 2012.

CHAPTER ONE

Biomedical Applications of Synthetic Polypeptides

1.1 Introduction

Peptides and proteins are present throughout the living world and perform diverse tasks from the catalysis of complex chemical reactions to sophisticated structural functions.[1] These natural biopolymers possess such remarkable abilities due to the precisely controlled sequences and compositions of their constituent amino acid monomers, which allow linear chains to assemble into complex, highly ordered structures. Similarly, synthetic polypeptide mimics of these biopolymers share the ability to self-assemble into complex structures.[2] There has been great interest in developing such materials for applications in biotechnology and medicine, which is the motivation for the current work.

Many synthetic polymers investigated for use in biotechnology and medicine are readily prepared with controlled chain length and have demonstrated biocompatibility (e.g. polyethylene glycol and poly(lactic acid)-(glycolic acid) copolymers), yet their simplicity hinders the utility and scope of these materials. Peptide based polymers have many advantages over conventional synthetic polymers as they possess the ability to cooperatively hydrogen bond into stable secondary structures in solution.[3] Combined with hydrophobic, electrostatic and dipolar interactions, these secondary structures can drive the formation of complex architectures from relatively simple linear materials. In addition, these materials are readily obtained from simple amino acid precursors and are generally biodegradable via natural proteases.

1.2 Preparation of Polypeptides by Transition Metal Catalysis

Polypeptides can be prepared by recombinant DNA and solid phase techniques, however, the fastest and most economical route to high molecular weight material is the ring opening polymerization of α-amino acid *N*-carboxyanhydride (NCAs). (Figure 1.1) These monomers have been known since the 1950's, however poor control over chain length, amino acid composition and sequence, and chain-end functionality limited their utility.[4] In the late 1990's, Deming and co-workers described the development of transition metal catalysts that allow the living polymerization of NCAs.[5] (Figure 1.2) This versatile polymerization reaction tolerates a wide variety of both natural and synthetic amino acid monomers, and yields well-defined polypeptides with controlled molecular weights and low polydispersities. In addition, high molecular weights, block sequences, and end group control can be readily achieved.

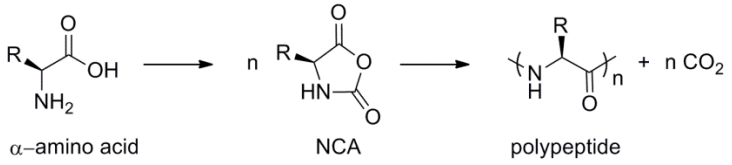

Figure 1.1 Preparation and polymerization of NCAs

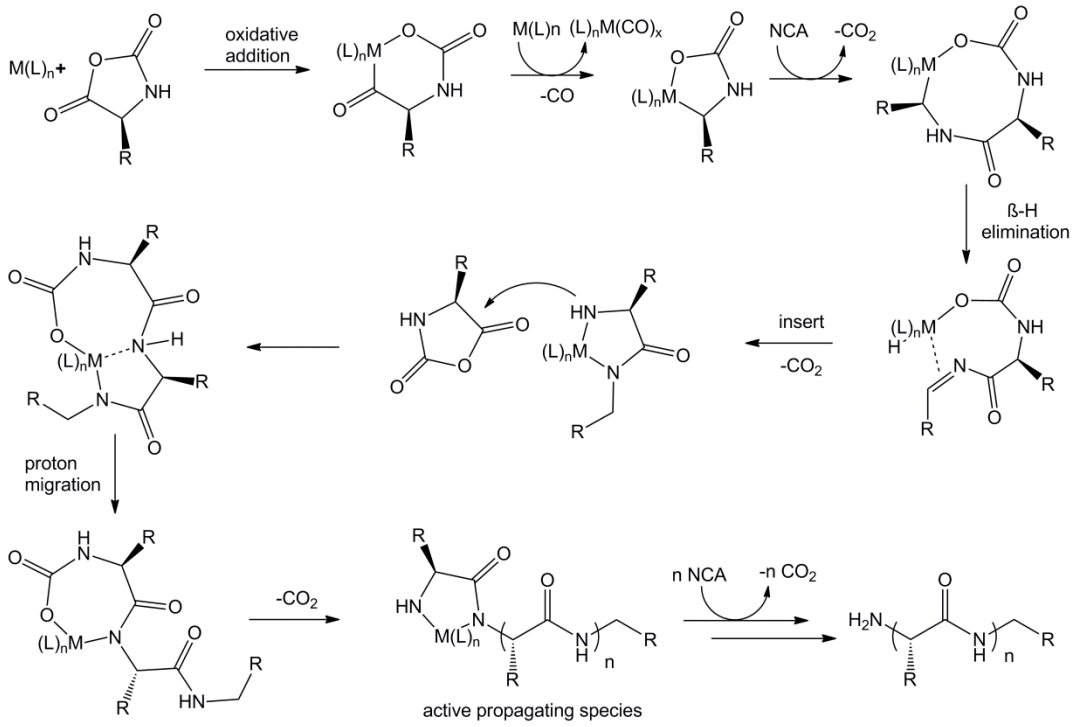

Figure 1.2 Mechanism of transition metal catalyzed NCA polymerization.

1.3 Biomedical Applications of Block Copolypeptides

Using transition metal catalyzed NCA polymerization chemistry, Deming and coworkers developed hydrophilic-hydrophobic diblock copolypeptides that self-assemble in aqueous solution to form therapeutically useful structures such as vesicles[6] and hydrogels[7], with applications in tissue engineering, drug delivery, and as therapeutics. (Figure 1.3) Hydrogels are a class of materials that have significant promise for use in cell culturing, and soft tissue and bone engineering due to their well-hydrated, porous structure that can mimic natural extracellular matrices. These polypeptide hydrogels have been shown to be well tolerated in the central nervous system in rodent models.[8] Membrane based structures such as vesicles, have important biomedical applications in the encapsulation and stabilization of sensitive materials such as therapeutic drugs or genes for more effective delivery *in vivo*. These structures are thought to

form due to hydrogen bonding and hydrophobic interactions from within the hydrophobic block, and are solubilized into different morphologies due to the longer hydrophilic segment.

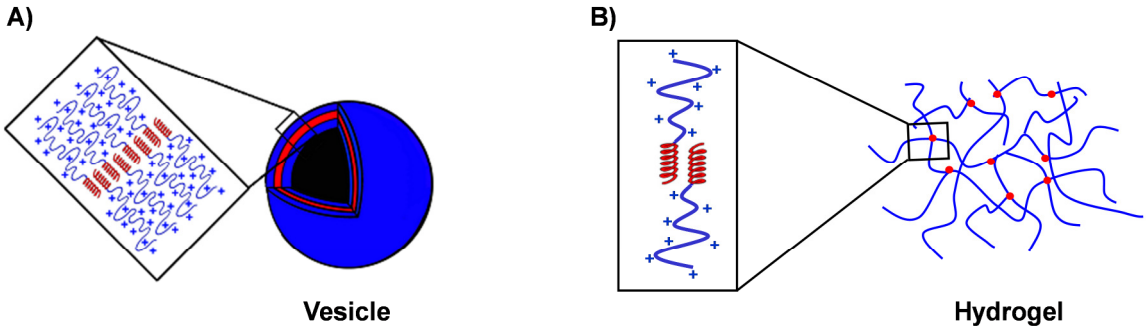

Figure 1.3 Hydrophilic-hydrophobic diblock copolypeptide A) vesicle and B) hydrogel.

Despite these significant advances in the preparation of well-defined polypeptides and the development of self-assembling materials, there remains a need for a broader scope of polypeptides with functionalities that mimic the complexity and function of post-translationally modified natural proteins. The display of functionalities that target materials to specific tissues, passivate the immune response, or have therapeutic effects, are highly desirable yet require more complex synthetic approaches and present new challenges.

There also remains a need to develop non-ionic, water-soluble polypeptides with greater biocompatibility than the polyelectrolytes (e.g. poly(L-lysine), poly(L-aspartic acid), poly(L-glutamic acid)) used in pioneering studies.[9] Polyelectrolytes, which are highly charged at neutral pH, can be problematic due to electrostatic interaction and precipitation with other charged macromolecules found in bodily fluids.[10] Vesicles based on highly cationic polypeptides such as poly(L-lysine) have shown undesirable toxic effects in cytoxicity studies.[11] In addition, these simple polyelectrolytes are of limited functionality for targeting and drug delivery.

Homoarginine based vesicles have been shown to be internalized by cells via endocytosis, yet appear to be trapped in the early endosome and recycled back to the cell surface before delivering their payload.[12] Studies have shown that vesicles based on anionic poly(L-glutamic acid) have low toxicity, yet are not able to enter cells.[13]

The display of sugar functionalities from synthetic polymers, or glycosylation, is an area of great recent interest due to the many attractive properties imparted upon the parent material. Glycosylation enhances aqueous solubility without the addition of charged groups that may non-specifically adsorb biomolecules. Indeed, glycosylation of polymeric drug and gene carriers has been shown to lower cytotoxicity.[14] Glycosylation of these materials may also provide a method of targeting to specific cells and organs because both glycoproteins and glycoside receptors have been implicated in disease states from viral infection to tumor cell metastasis.[15] Glycosylated peptides and proteins are ubiquitous in nature and display a wide range of biological functions including mediation of recognition events, protection from proteases, and lubrication in eyes and joints.[16] Similarly, synthetic glycopolypeptides are also expected to show great potential as biomedical materials (e.g. scaffolds for tissue repair and drug carriers), as well as serve as valuable tools for probing carbohydrate-protein interactions.

1.4 Routes to Highly Functional Polypeptides

There are two routes to prepare glycosylated and other highly functional polypeptides: 1) the polymerization of a functionalized monomer, and 2) the chemical conjugation of functional groups to existing polypeptides, or post-polymerization modification. Both routes have advantages and disadvantages that warrant careful consideration and the nature of the desired functional group must be considered. The functionalized monomer route has the distinct advantage of producing well-defined materials with up to 100% functionalization based simply

on monomer feed ratios, while post-polymerization modifications may be difficult to characterize and achieving high functionalization is often not possible to due steric factors. Post-polymerization modification however, is advantageous since it avoids the need to prepare many different functional monomers and individually optimize their polymerization conditions.

1.5 Advances in Functional Polypeptide Synthesis Described in the Dissertation

This dissertation reports several distinct advances in the preparation of a wide variety of highly functional polypeptide materials by both the functionalized monomer route, and the post-polymerization modification route. Chapter two describes a new method of NCA monomer purification that now allows access to diverse functional monomers previously inaccessible due to impurities that impeded polymerization. Chapters three and four describe how this purification technique was applied to the synthesis of a variety of glycosylated NCAs which yielded the first living polymerizations of glycosylated NCAs and gave access to glycopolypeptides with unique properties and conformations. The utility of these glycopolypeptides is exemplified in chapter five for both fundamental research into the effect of conformation on self-assembly and ligand binding, as well as potential medical applications in drug delivery. Chapters six and seven focus on the development of a new conjugation technique that allows facile post-polymerization modification of polypeptides with a wide variety of functional groups using the unique chemistry of the natural amino acid methionine. Chapter six describes the chemoselective alkylation of methionine with diverse functional groups using different methods. Chapter seven describes studies on reversible methionine alkylation, which can be used to attach and later remove a desired functional group. Finally, work performed during and NSF IGERT funded internship at HRL Laboratories Inc. is described in chapter eight. Surface modification of carbon foam anodes

for microbial fuel cell applications was explored, and the use of thermogravimetric analysis to evaluate biofilm formation was developed.

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CHAPTER TWO

General Method for Purification of α-Amino acid-N-carboxyanhydrides Using Flash Chromatography

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Kramer, J. R.; Deming, T. J. *Biomacromolecules* **2010**, 12, 3668–3672.

2.1 Abstract

We describe the application of flash column chromatography on silica gel as a rapid and general method to obtain pure α-amino acid-*N*-carboxyanhydride (NCA) monomers, the widely used precursors for synthesis of polypeptides, without the need for recrystallization. This technique was effective at removing all common impurities from NCAs, and was found to work for a variety of NCAs, including those synthesized using different routes, as well as those bearing either hydrophilic or hydrophobic side chains. All chromatographed NCAs required no further purification and could be used directly to form high molecular weight polypeptides. This procedure is especially useful for preparation of highly functional and low melting NCAs that are difficult to crystallize, and consequently to polymerize. This method solves many long-standing problems in NCA purification and provides rapid access to NCAs that were previously inaccessible in satisfactory quality for controlled polymerization. This method is also practical in that it requires less time than recrystallization and often gives NCAs in improved yields.

2.2 Introduction

The controlled synthesis of polypeptides of defined chain lengths and complex architectures has been actively pursued for many years.[1,2] Recently, much progress has been made in the development of polymerization initiators and reaction conditions to achieve this goal.[3,4] However, less attention has been paid to the purification of the α-amino acid-*N*-carboxyanhydride (NCA) monomers, which in many cases is the limiting factor in controlled polypeptide synthesis and commercial development of synthetic polypeptides.[5] The need to obtain NCAs in high purity has been challenging scientists for over 50 years,[6] and many approaches have been taken to solve this problem. Special preparation methods or purification steps are often employed to obtain NCAs sufficiently pure so that they can be crystallized, where recrystallization is most often the ultimate step required to prepare pure NCAs.[7] Here, we describe the use of flash column chromatography on silica gel as a rapid and general method to obtain pure NCAs without the need for recrystallization. While this procedure simplifies purification of a wide range of NCAs, it is especially useful for preparation of highly functional and low melting NCAs that are difficult to crystallize, and consequently to polymerize.

NCAs were first reported over 100 years ago by Hermann Leuchs,[2] and have been widely used for the synthesis of polypeptides since the 1940s.[1] Over 200 different NCAs have been synthesized,[8] and a wide variety of polypeptides have been prepared that contain diverse structures and functionalities. NCAs are most often synthesized by either treatment of α-amino acids with phosgene (Fuchs-Farthing method),[7,9,10] or the reaction of *N*-alkyloxycarbonyl-amino acids with halogenating agents (Leuchs method) (Figure 2.1).[11,12] Triphosgene,[13] diphosgene,[14] and di-*tert*-butyltricarbonate[15] have also been used as phosgene substitutes, but these reagents have low volatility and any excess must be later removed by purification. Di-*tert*-

butyltricarbonate, aside from having low reactivity, also generates *tert*-butyl alcohol which may interfere with NCA polymerization.[15] NCAs synthesized with phosgene contain byproducts including HCl,[6] HCl-amino acid salts,[16] 2-isocyanatoacyl chlorides[17], and *N*-chloroformyl amino acids.[17] NCAs prepared via the Leuchs method contain byproducts including HCl, HBr, alkyl halides as well as any excess, and byproducts, of low volatility halogenating agents.[6] For use in the controlled synthesis of polypeptides, NCAs must be free of these electrophilic contaminants that can quench or inhibit chain growth. Chloride ions can also act polymerization initiators in dimethylformamide and must be removed.[18]

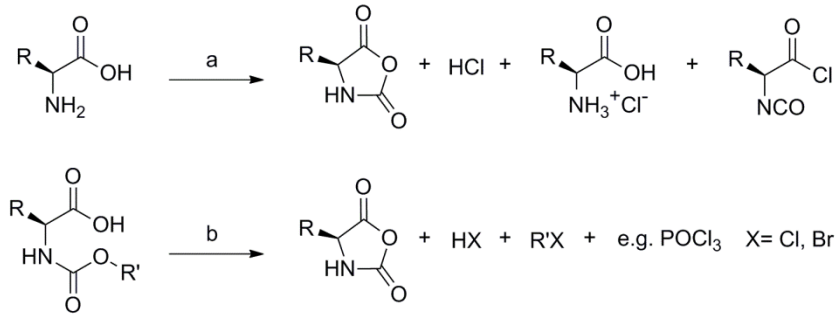


Figure 2.1 Preparation of NCAs and resulting byproducts. a) Phosgene b) Various halogenating reagents (e.g. PBr_3, $POCl_3$, PCl_5, $SOCl_2$, α,α-dichloromethylmethyl ether).

NCAs are most readily purified by repeated crystallization under anhydrous conditions.[19] There have been reports of NCA purification by sublimation, but this procedure is limited to a few, select NCAs and is accompanied by low yields due to thermal polymerization.[20,21] Recrystallization is useful for purification of many common NCAs that are high-melting solids, yet multiple recrystallizations are usually required to remove traces of impurities. Oily impurities, such as residual diphosgene, alkyl halides, and 2-isocyanatoacyl chlorides hinder NCA crystallization, and some form of initial purification must be used before the NCA can be crystallized.[22] A number of NCA purification procedures have been developed. α-Pinene or limonene has been added to NCA preparations to consume HCl,[5] and although this greatly assists

the scale-up of NCA synthesis, removal of the alkyl hydrochloride that is formed can be an issue. Washing NCA reaction mixtures in ethyl acetate with water and aqueous bicarbonate at 0 ºC has also been used to remove HCl and HCl-amino acid salts.[23] This procedure has been found to work well for some amino acids. However, difficult to separate emulsions can form with long-chain γ-alkyl-glutamate NCAs,[23] and this procedure can also introduce an initiator, water, into NCAs leading to premature polymerization. A second phosgenation or "rephosgenation" of NCAs has been proposed to eliminate hard to remove HCl-amino acid salts from NCAs,[16] yet excess phosgene can lead to 2-isocyanatoacyl chloride formation with aliphatic amino acids. Columns of activated charcoal,[24] zeolites,[25] or urea,[26] with or without Ag_2O as a chloride scavenger, have also been used to purify NCA solutions, yet NCA polymerization during purification limits this procedure.

While these methods are able to remove some impurities found in NCA preparations, further purification, most often recrystallization, is required to obtain NCAs suitable for controlled polypeptide synthesis. NCA recrystallizations are often slow and tedious, where considerable expertise is needed to determine solvent mixtures and compositions that will encourage NCA crystallization and separation from impurities. While some common NCAs (e.g. N_ε-carbobenzyloxy-L-lysine NCA, *Z*-lys NCA, or γ-benzyl-L-glutamate NCA, Bn-Glu NCA) can be sufficiently purified after two recrystallizations, many NCAs, typically those with more polar or complex functionality (e.g. N_ε-2-[2-(2-methoxyethoxy)ethoxy]acetyl-L-lysine NCA, EG_2-Lys NCA), require an arduous process of 6 or more recrystallizations that can lead to low yields.[12,27] Furthermore, some NCAs can only be isolated as oils (e.g. γ-alkyl-L-glutamate NCAs with long *n*-akyl chains[23] or have low melting points (e.g. L-methionine NCA, Met NCA), and either cannot be recrystallized or recrystallize only with great difficulty.[14,28,29] As more complex NCA

monomers are developed to create polypeptides with new functionalities, the need for a method to purify NCAs that does not rely on recrystallization has become increasingly urgent. Recently, many novel NCAs have been reported that contain alkyne groups for click chemistry reactions,[30,31] carbohydrates,[32] and oligoethylene glycol segments,[33] yet many of these monomers could not be recrystallized or adequately purified. In order to obtain high purity, non-crystalline glycosylated NCAs in our own lab,[34] we investigated the use of flash column chromatography on silica gel for NCA purification.

Flash column chromatography is widely used by synthetic organic chemists to purify a desired product from a variety of impurities including salts, inorganic compounds, and other organic molecules.[35,36] Chromatography has many advantages in that it is fast, separation can be optimized easily, the silica gel is reasonably inexpensive, and most importantly works well for purification of both crystalline and non-crystalline compounds. This method has not been explored much in the past and has rarely been used for the general purification of NCAs, likely due to concerns over NCA stability towards silica gel. We are aware of only one example where silica gel chromatography was used to isolate the NCAs of L-asparagine and L-glutamine prior to purification by recrystallization.[37] Here, chromatography was used since the lack of protecting groups on these amino acids led to many side-products, leading to low yields of NCAs (*ca.* 30 %). We have found that under anhydrous conditions, a wide range of NCAs can be purified using silica gel chromatography, and were all subsequently capable of forming high molecular weight polypeptides without the need for additional purification (i.e. recrystallization).

2.3 Results and Discussion

Silica gel chromatography was used to purify NCAs prepared by both the phosgene method and by halogenation of *Z*-protected amino acids (Table 2.1). Bn-Glu, *Z*-Lys and Met NCAs were prepared by treatment with phosgene. *Z*-EG_2-Lys[12] and *Z*-Glc-Lys[34] were prepared as previously reported and the corresponding NCAs were prepared by treatment of the *Z*-protected amino acids with α,α-dichloromethylmethyl ether.[38] Each NCA was loaded onto a column of silica gel and eluted using a solvent mixture whose composition was varied based on compound polarity. Passage through a silica gel column proved effective at separating the NCAs from side-products such as benzyl chloride and other amino acid based compounds as evidenced by TLC (Figure 2.2), FTIR, and ^{1}H and ^{13}C NMR. It is also notable that chromatographic purification was not associated with any loss in NCA or polypeptide yield, and in some cases improved NCA yields were obtained compared to recrystallized monomers. The NCAs obtained by chromatography were also found to be stable upon storage at -20 ºC under N_2 for over 1 year.

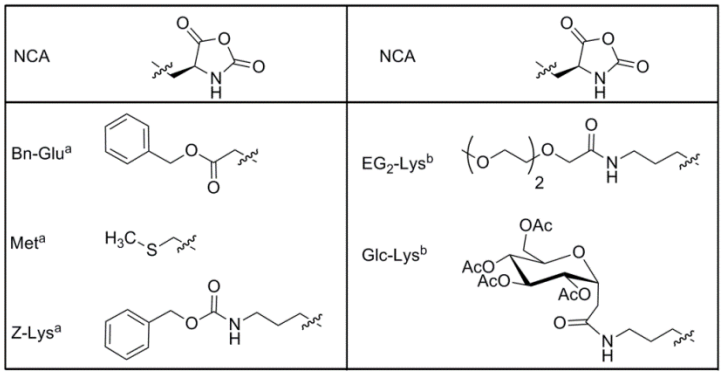


Table 2.1 NCA monomers purified by column chromatography. (a) NCA prepared from the corresponding amino acid using $COCl_2$, THF, 50 ^{o}C. (b) NCA prepared from the corresponding *N*-carbobenzyloxy amino acid using Cl_2CHOCH_3, CH_2Cl_2, 45 ^{o}C.

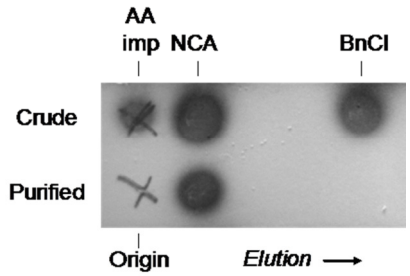


Figure 2.2 Image of plate used for thin layer chromatography of Glc-Lys NCA before (Crude) and after (Purified) column purification. Image has been rotated so that solvent flow is from left to right. Origin is where compounds were initially spotted onto plate (marked with "X"s). AA imp are amino acid containing impurities. Image has been cropped for clarity, but shows all data. TLC plate was stained for visualization of compounds using 20% phosphomolybic acid in ethanol with heat.

While NMR and FTIR spectroscopy were able to show that chromatographically purified NCAs had few impurities, these techniques are unable to detect small amounts (< 1 %) of impurities that will severely hamper controlled NCA polymerization. A classic method to evaluate the suitability of NCAs for preparation of polypeptides is to attempt polymerization at a high monomer to initiator (M:I) ratio (i.e. 200:1) under anhydrous conditions using carefully purified solvents.[6] The complete consumption of NCA under these conditions indicates that the amount of electrophilic impurities is sufficiently low so that their termination of chains is insignificant. To compare the quality of NCAs purified by chromatography versus recrystallization, polymerizations of Bn-Glu and *Z*-Lys NCAs were conducted at M:I ratios of 300 to 1 using $(PMe_3)_4Co$[39] initiator in THF at room temperature. This initiator was chosen since it has been shown to promote living polymerization of NCAs and we have extensive experience in its use.[40] As impurities in NCAs will adversely affect any known NCA initiator, the $(PMe_3)_4Co$ data here is relevant to research using other methods for NCA polymerization. Both polymerizations of chromatographed NCAs went to completion (as determined by FTIR and polymer isolation), while both polymerizations of recrystallized samples were quenched by

impurities and reached only *ca.* 50-60 % conversion. This study showed that the purity of the chromatographed NCAs was significantly higher than recrystallized samples. To better quantitate impurity levels, the *Z*-lys NCAs from above were analyzed for chloride impurities using ICP-MS. Recrystallized *Z*-Lys NCA was found to contain 1.8 ppm chlorine, while chromatographed *Z*-Lys NCA was found to contain only 0.35 ppm. Likewise, crude Met NCA oil was found to contain 207 ppm chlorine, and after chromatography this level dropped to 0.36 ppm. Although the values for chromatographed NCAs fall below the instrumental limit for accurate detection (0.7 ppm), they confirm the level of chloride impurities in these samples is very low.

Polymerization studies to compare chain lengths using chromatographed NCAs versus recrystallized NCAs were also performed (Table 2). Bn-Glu, *Z*-Lys, and EG_2-Lys NCAs, each purified by both methods, were separately polymerized at M:I ratios of 50 to 1, and their resulting chain lengths were compared. All monomers were completely consumed under these conditions and were able to form polypeptides with low polydispersities in high yields. However, it was seen that chromatographed NCAs always gave shorter chains compared to those purified by recrystallization. Larger chain lengths in these polymerizations are indicative of initiator quenching at the beginning of the polymerizations caused by electrophilic impurities (e.g. HCl) in the NCA. All polymers had higher than theoretical molecular weights, which is partially due to the known incomplete efficiency of $(PMe_3)_4Co$ initiation in THF.[40] Although not all of the $(PMe_3)_4Co$ pre-initiator is able to generate active chains, this system is able to produce low polydispersity polypeptides of controlled chain lengths, and has been used to prepare numerous well-defined block copolypeptides.[3,4] As such, the absence of chain-breaking side reactions makes this a useful system to probe low levels of impurities in NCAs that might be masked by

the abundance of side-reactions that occur in uncontrolled polymerizations. Here, it was seen that the amounts of initiator quenching impurities were significantly lower in the chromatographed NCAs compared to recrystallized counterparts. Recrystallized NCAs of high purity could also be obtained by additional recrystallization (i.e. 8 times vs. 2 times), yet this is a time consuming and tedious process that also lowers yields. From this study, it can be seen that chromatographic purification of NCAs has a distinct advantage in minimizing initiator deactivation and chain quenching during polymerization, which is important for the synthesis of well-defined homo and block copolypeptides using any initiating system.

Monomer[a]	Yield (%)[b]	M_n[c] GPC/LS	M_n[d] Calcd.	M_w/M_n[c]	Yield (%)[e]
50 Lys[f]	93	49,660	13,100	1.12	97
50 Lys[g]	92	109,600	13,100	1.12	98
50 Glu[f]	97	37,800	10,950	1.17	94
50 Glu[g]	90	62,070	10,950	1.12	96
50 EG_2-Lys[f]	61	28,770	14,400	1.16	91
50 EG_2-Lys[g]	67	53,890	14,400	1.08	93
50 Glc-Lys[f]	62	57,260	25,025	1.23	91
50 Met[f,h]	91	–	–	–	ND[i]

Table 2.2 Polymerization data for NCAs purified by chromatography compared to recrystallization. (a) Number indicates equivalents of monomer per $(PMe_3)_4Co$. Polymerizations run in THF at room temperature under N_2. (b) Isolated yield of NCA. (c) Observed molecular weight and polydispersity index after polymerization as determined by GPC/LS. (d) Theoretical polymer molecular weight. (e) Total isolated yield of polypeptide. (f) Monomer purified by silica gel chromatography. (g) Monomer purified by two recrystallizations. (h) Polymer not characterized by GPC due to insolubility in DMF. (i) ND = not determined.

To highlight the potential of chromatographic NCA purification for the development of new biopolymer materials, we have conducted polymerization studies on Met NCA. This monomer is infrequently used since it is difficult to purify, yet poly-L-methionine has interesting fiber forming properties.[24] Homo and copolymerizations of Met NCA, usually used as a non-crystallizable syrup,[41,42] have been previously reported,[43,44] yet controlled polymerization has

never been demonstrated. Met NCA purified by chromatography, obtained as a white solid, was successfully polymerized using $(PMe_3)_4Co$ in THF at M:I ratios up to 200 to 1 with complete conversion to polymer. However, molecular weight measurement of the poly-L-methionine was obstructed by its insolubility in common organic solvents, where its α-helical chains tend to aggregate to form gels.[45] To enable molecular weight measurement, Met NCA was copolymerized with *Z*-Lys NCA to form statistical copolypeptides that were soluble in DMF and could be analyzed by GPC/LS. Since the polymerizations went to 100% monomer conversion, and did not aggregate in non-solvents for poly-L-methionine, it is likely that blocky copolymers were not formed. To check for chain-breaking side reactions during these copolymerizations, experiments to verify molecular weight control were performed. Polymerization of equimolar mixtures of Met NCA and Z-Lys NCA at different M:I ratios gave copolypeptides whose lengths (M_n) increased linearly with M:I stoichiometry, and which possessed narrow chain length distributions (M_w/M_n) (Figure 2.3). These data show the first instance where well-defined methionine containing copolypeptides have been prepared, and open the possibility for using methionine building block in new biopolymer materials.

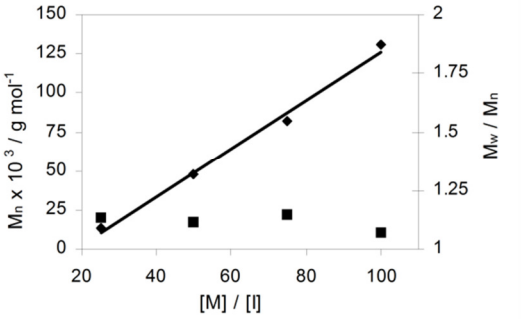


Figure 2.3 Molecular weight (M_n, ♦) and polydispersity index (M_w/M_n, ■) of equimolar statistical copolymerizations of Met NCA and *Z*-Lys NCA as functions of monomer to initiator ratio ([M]/[I]) using $(PMe_3)_4Co$ in THF at 20 °C.

2.4 Conclusion

In summary, we have established that silica gel column chromatography is a useful general method for rapid and straightforward purification of NCA monomers. This method solves many long-standing problems in NCA purification and provides rapid access to NCAs that were previously inaccessible in satisfactory quality for controlled polymerization. This technique was effective at removing all common impurities from NCAs, and was found to work for a variety of NCAs, including those synthesized using different routes, as well as those bearing either hydrophilic or hydrophobic side chains. All chromatographed NCAs required no further purification and could be used directly to form high molecular weight polypeptides. This method is also practical in that it requires less time than recrystallization and often gives NCAs in improved yields. This procedure is especially useful for the purification and polymerization of monomers that cannot be recrystallized, or crystallize only with difficulty. Synthetic polypeptides are being developed and studied for many diverse uses, and this methodology greatly simplifies the purification of NCA monomers, which should increase the accessibility to and synthetic possibilities for these important materials.

2.5 Experimental

2.5.1 Materials and Methods.

Unless stated otherwise, reactions were conducted in oven-dried glassware under an atmosphere of nitrogen using anhydrous solvents. Hexanes, THF, and diethyl ether were purified by first purging with dry nitrogen, followed by passage through columns of activated alumina. EtOAc was freshly distilled from CaH_2. All commercially obtained reagents were used as received without further purification unless otherwise stated. $(PMe_3)_4Co$ was prepared according to

literature procedures.[46] Reaction temperatures were controlled using an IKA magnetic temperature modulator, and unless stated otherwise, reactions were performed at room temperature (RT, approximately 23 °C). NMR spectra were recorded on Bruker spectrometers at 500 MHz for ^{1}H and at 125 MHz for $^{13}C\{^{1}H\}$ NMR. Fourier Transform Infrared spectroscopy (FTIR) samples were prepared as thin films on NaCl plates and spectra were recorded on a Perkin Elmer RX1 FTIR spectrometer. Tandem gel permeation chromatography/light scattering (GPC/LS) was performed on a SSI Accuflow Series III liquid chromatograph pump equipped with a Wyatt DAWN EOS light scattering detector and Wyatt Optilab rEX refractive index (RI) detectors. Separations were achieved using 10^5, 10^4, and 10^3 Å Phenomenex Phenogel 5 mm columns using 0.10 M LiBr in DMF as the eluent at 60 °C. All GPC/LS samples were prepared at concentrations of 5 mg/mL. Inductively coupled plasma-mass spectrometry (ICP-MS) samples were run on an Agilent 7500ce instrument in helium collision gas mode.

2.5.2 General Preparative Methods

General Preparation of NCAs by Phosgenation of α-amino acids. To a solution of amino acid in dry THF (0.15 M) in a Schlenk flask was added a solution of phosgene in toluene (20 % (w/v), 2 equiv) via syringe. *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. The reaction was stirred under N_2 at 50 ^{o}C for 3 hrs. The reaction was evaporated to dryness and transferred to a dinitrogen filled glove box. The condensate in the vacuum traps was treated with 50 mL of concentrated aqueous NH_4OH to neutralize residual phosgene.

General Preparation of NCAs from *N*-carbobenzyloxy α-amino acids. To a solution of *N*-carbobenzyloxy α-amino acid in dry CH_2Cl_2 (0.05M) in a Schlenk flask under N_2 was added α,α-dichloromethylmethyl ether (1.5 equiv) and the solution was refluxed for 36 hours. The reaction was evaporated to dryness under reduced pressure and transferred to a dinitrogen filled glove box.

General Procedure for Silica Chromatography of NCAs. Selecto silica gel 60 (particle size 0.032–0.063 mm) was heated to 150 ^{o}C under vacuum for 48 hrs before use. Thin-layer chromatography (TLC) was conducted with EMD gel 60 F254 precoated plates (0.25 mm) and visualized using a combination of UV, potassium permanganate, and phosphomolybdic acid staining. Inside a dinitrogen filled glove box, silica was slurry packed into a glass column fitted with a glass filter frit. NCA was dissolved in a minimal amount of solvent, loaded onto the column, and eluted with additional solvent. Fractions were collected and analyzed by TLC for the presence of NCA. All fractions containing NCA were combined and condensed under reduced pressure.

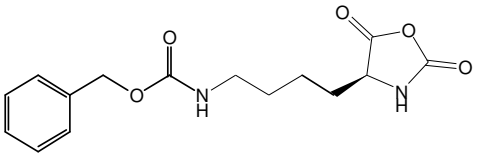
General Procedure for Recrystallization of NCAs. Inside a dinitrogen filled glove box, NCAs were dissolved in dry THF and recrystallized via solvent diffusion under a layer of dry hexanes at RT. After 16 hours, the mother liquor was poured off and solids were dried under vacuum.

General Procedure for Polymerization of NCAs. All polymerization reactions were performed in a dinitrogen filled glove box. To a solution of NCA in dry THF (50 mg/mL) was rapidly added via syringe, a solution of $(PMe_3)_4Co$ in dry THF (20 mM). The reaction was stirred at RT and polymerization progress was monitored by removing small aliquots and analysis by FTIR. Polymerization reactions were generally complete within 1 hour. Aliquots were removed for

GPC analysis immediately upon polymerization completion. Reactions were precipitated from THF into hexanes and polymers were collected by centrifugation and dried under reduced pressure to yield white solids. (91-99 % yield).

General Procedure for Preparation of Statistical Copolymers of Methionine and *Z*-Lysine. In the drybox, Met NCA and *Z*-Lys NCA were mixed in equimolar amounts and dissolved in THF (100 mg/mL). General polymerization procedures were followed as described above. Reactions were precipitated from THF into hexanes and polymers were collected by centrifugation and dried under reduced pressure to yield white solids. (88-94% yield).

2.5.3 Experimental Procedures for Preparation and Purification α-Amino Acid NCAs



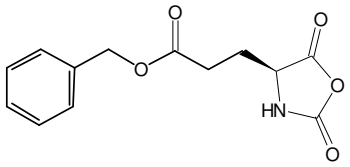
***Z*-Lys NCA.** NCA was prepared from N_{ε}-*Z*-L-lysine according to the general procedure for phosgenation of α-amino acids (1.00 g, 3.57 mmol).

Crystallization procedure: *Z*-Lys NCA was dissolved in 4 mL of THF and recrystallized by diffusion gradient layering under 16 mL hexanes. Process was repeated for a total of two recrystallizations to yield 0.997g (91.5%) of the product as a fine white powder.

Chromatography procedure: *Z*-Lys NCA was dissolved in a minimal amount of EtOAc and loaded onto a 2.4 x 10 cm silica column slurry packed with 50% hexanes in EtOAc. The compound was eluted with a gradient from 50%-80% EtOAc in hexanes. Ten 15 mL fractions were collected and analyzed by TLC. Fractions containing NCA were combined and condensed to give 1.02 g of the product as a white solid (93.3%). Rf = 0.26, 40% hexanes in EtOAc, Rf =

0.38 in Et_2O, black spot with PMA stain. ^1H NMR (500 MHz, $CDCl_3$, 25 ^oC): δ 7.41-7.29 (m, 5H), 6.93 (broad s, 1H), 5.09 (s, 2H), 4.99-4.89 (broad s, 1H), 4.26 (dd, *J* = 5.2, 5.2, 1H), 3.27-3.14 (m, 2H), 2.01-1.91 (m, 1H), 1.87-1.77 (m, 1H), 1.60-1.35 (m, 4H), $^{13}C\{^1H\}$ NMR (125 MHz, $CDCl_3$, 25 ^oC): δ 169.8, 156.8, 152.5, 136.3, 128.5, 128.1, 127.9, 66.8, 57.4, 40.1, 30.4, 29.0, 21.3.

Molecular weights for poly *Z*-Lys by GPC/LS in 0.1M LiBr in DMF were calculated using the dn/dc value of 0.123.[47]



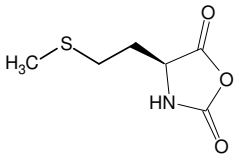
Bn-Glu NCA: NCA was prepared from γ-benzyl-L-glutamate according to the general procedure for phosgenation of α-amino acids (1.00 g, 4.22 mmol).

Crystallization procedure: Bn-Glu NCA was dissolved in 3 mL of THF and recrystallized by diffusion gradient layering under 15 mL hexanes. Process was repeated for a total of two recrystallizations to yield 1.00 g (90.0%) of the product as fine, colorless needles.

Chromatography procedure: Bn-Glu NCA was dissolved in a minimal amount of EtOAc and loaded onto a 2.4 x 10 cm silica column slurry packed with 50% hexanes in EtOAc. The compound was eluted with a gradient from 50-75% EtOAc in hexanes. Ten 15 mL fractions were collected and analyzed by TLC. Fractions containing NCA were combined and condensed to give 1.08 g of the product as a white solid (97.1%). Rf = 0.52, 40% hexanes in EtOAc, Rf = 0.2 in 20% Et_2O in hexanes, black spot with PMA stain. ^1H NMR (500 MHz, $CDCl_3$, 25 ^oC): δ 7.42-7.32 (m, 5H), 6.45 (broad s, 1H), 5.14 (s, 2H), 4.37 (dd, *J* = 6.1, 6.1, 1H), 2.60 (t, *J* = 6.8, 2H),

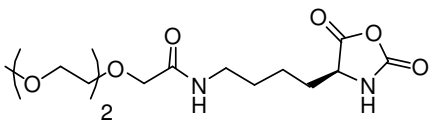
2.33-2.29 (m, 1H), 2.17-2.07 (m, 1H); $^{13}C\{^{1}H\}$ NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ 172.3, 169.2, 151.5, 135.1, 128.6, 128.5, 128.3, 67.0, 56.9, 26.8.

Molecular weights for poly Bn-Glu by GPC/LS in 0.1M LiBr in DMF were calculated using the dn/dc value of 0.104.[47]



Met NCA: NCA was prepared from L-methionine according to the general procedure for phosgenation of α-amino acids (2.00 g, 13.4 mmol).

Chromatography procedure: Crude Met NCA, a yellow oil, was dissolved in a minimal amount of 50% EtOAc in hexanes and loaded onto a 2.4 x 12 cm silica column slurry packed with 20% EtOAc in hexanes. The NCA was eluted with a gradient from 20-50% EtOAc in hexanes. Ten 15 mL fractions were collected and analyzed by TLC. Fractions containing NCA were combined and condensed to give 2.11 g (91.1%) of the product as a colorless viscous liquid that spontaneously crystallized upon standing. Rf = 0.45, 40% EtOAc in hexanes, Rf = 0.29 in 25% hexanes in Et_2O, black spot with PMA stain. ^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 6.68 (broad s, 1H), 4.49 (dd, *J* = 7.2, 4.7 1H), 2.74-2.64 (m, 2H), 2.34-2.24 (m, 1H), 2.16-2.04 (m, 1H), 2.12 (s, 3H); $^{13}C\{^{1}H\}$ NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ 169.4, 152.2, 56.6, 30.0, 29.9, 15.0.

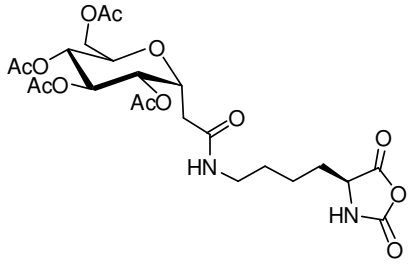


EG_2-Lysine NCA: NCA was prepared from N_ε-2-[2-(2-methoxyethoxy)ethoxy]acetyl-N_α-Z-L-lysine[2] according to the general procedure for preparation of NCAs from *N*-carbobenzyloxy α-amino acids (1.07 g, 2.44 mmol).

Crystallization procedure: Crude NCA, a yellow oil, was dissolved in 3 mL of THF and recrystallized by diffusion gradient layering under 15 mL hexanes. Colorless crystalline material was scraped away and separated from yellow solids that formed at the flask bottom. This fractionation process to separate crystalline NCA from yellow impurities was repeated a total of four times. The combined colorless NCA fractions were then combined and recrystallized two more times using the same THF/hexanes mixture. Yield after two recrystallizations, 0.501 g (67%). Yield of NCA after six total recrystallizations, 0.235 g (29.0%), as fine, colorless needles

Chromatography procedure: NCA was dissolved in a minimal amount of EtOAc and loaded onto a 2.4 x 7 cm silica column slurry packed with EtOAc and eluted with EtOAc. Ten 10 mL fractions were collected and analyzed by TLC. Fractions containing NCA were combined and condensed to give 0.538 g of the product as a white solid (66.3%). Rf = 0.4, THF, Rf = 0.15 in EtOAc, yellow spot in $KMnO_4$. ^1H NMR (500 MHz, $CDCl_3$, 25 ^oC): δ 7.55 (broad s, 1H), 7.32 (broad s, 1H), 4.29 (dd, *J* = 7.2, 4.3 1H), 4.0 (q, *J* = 16.3, 2H), 3.72-3.65 (m, 6H), 3.59-3.55 (m, 2H), 3.38 (s, 1H), 3.31 (q, *J* = 6.5, 2H) 2.74-2.64 (m, 2H), 2.06-1.96 (m, 1H), 1.87-1.7724 (m, 1H), 1.67-1.54 (m, 2H), 1.52-1.42 (m, 2H); $^{13}C\{^1H\}$ NMR (125 MHz, $CDCl_3$, 25 ^oC): δ 170.9, 170.2, 152.1, 71.6, 70.6, 70.3, 70.0, 69.6, 58.7, 57.4, 37.6, 30.4, 28.1, 21.0.

Molecular weights for poly EG_2-Lys by GPC/LS in 0.1M LiBr in DMF were calculated using the dn/dc value of 0.123.[12]



Glc-Lysine NCA: NCA was prepared from *N*-carbobenzyloxy-L-lysine-1-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside)[34] according to the general procedure for preparation of NCAs from *N*-carbobenzyloxy α-amino acids (0.596 g, 0.913 mmol).

Chromatography procedure: NCA was dissolved in a minimal amount of EtOAc and loaded onto a 2.4 x 6 cm silica column slurry packed with 50% hexanes in EtOAc. The compound was eluted with a gradient from 50-75% EtOAc in hexanes. Ten 10 mL fractions were collected and analyzed by TLC. Fractions containing NCA were condensed to give 0.294 g of the product as a white solid (59.2%). Rf = 0.25, 40% hexanes in EtOAc, black spot with PMA stain. ^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 7.41 (s, 1H), 6.38-6.32 (m, 1H), 5.22 (dd, *J* = 8.3, 8.3, 1H), 5.07 (dd, *J* = 8.5, 5.2, 1H), 4.99 (dd, *J* = 8.3, 8.3, 1H) 4.66-4.60 (m, 1H), 4.33 (dd, *J*(H,H)= 6.6, 4.7, 1H), 4.20 (d, *J* = 4.2, 2H) 4.0-3.95 (m, 1H), 3.41-3.32 (m, 1H), 3.22-3.14 (m, 1H), 2.65 (dd, *J* = 15.5, 10.0, 1H), 2.48 (dd, *J* = 15.5, 4.2, 1H), 2.09 (s, 3H), 2.05 (s, 3H), 2.04 (s, 6H), 2.02-1.92 (m, 2H), 1.60-1.53 (m, 2H), 1.51-1.38 (m, 2H); ^{13}C{^{1}H} NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ 170.8, 170.4, 169.9, 169.6, 169.5, 169.4, 152.3, 69.7, 69.6, 69.4, 69.3, 68.2, 62.0, 57.4, 38.8, 34.0, 30.8, 28.6, 21.6, 20.57, 20.47, 20.46.

Molecular weights for poly Glc-Lys by GPC/LS in 0.1M LiBr in DMF were calculated using the dn/dc value of 0.054.[34]

ICP-MS: Inductively coupled plasma-mass spectrometry (ICP-MS) samples were prepared by diluting NCA samples (5-15 mg) in 200 μL of concentrated OPTIMA grade HNO_3. Samples were hydrolyzed at 95 ^{o}C for 2 hrs in open vessels in a dust-free environment. The remaining liquid was diluted to 2.0 mL with 2% OPTIMA HNO_3 and the samples were analyzed on an Agilent 7500ce ICP-MS in helium collision gas mode relative to a 50 ppb scandium internal standard.

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CHAPTER THREE

Glycopolypeptides Via Living Polymerization of Glycosylated-L-lysine-*N*-carboxyanhydrides

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3.1 Abstract

The preparation of new glycosylated-L-lysine-N-carboxyanhydride (glyco-K NCA) monomers is described. These monomers employ *C*-linked sugars and amide linkages to lysine for improved stability without sacrificing biochemical properties. Three glyco-K NCAs were synthesized, purified, and found to undergo living polymerization using transition metal initiation. These are the first living polymerizations of glycosylated NCAs, and were used to prepare well-defined, high molecular weight glycopolypeptides and block and statistical glycocopolypeptides. This methodology solves many long-standing problems in the direct synthesis of glycopolypeptides from *N*-carboxyanhydrides relating to monomer synthesis, purification and polymerization, and gives polypeptides with 100% glycosylation. These long chain glycopolypeptides have potential to be good mimics of natural high molecular weight glycoproteins.

3.2 Introduction

Glycosylated peptides and proteins are ubiquitous in nature and display a wide range of biological functions including mediation of recognition events, protection from proteases, and lubrication in eyes and joints.[1] Similarly, synthetic glycopolypeptides are also expected to show great potential as biomedical materials (e.g. scaffolds for tissue repair and drug carriers), as well as serve as valuable tools for probing carbohydrate-protein interactions.[2,3] Although block and hybrid copolypeptides capable of forming vesicles,[4,5] fibrils,[6] and other structures[7,8] are readily prepared by amino acid *N*-carboxyanhydride (NCA) polymerization,[9] the synthesis of well-defined glycopolypeptide materials has been challenging. Even though *O*-linked glyco-serine NCAs have been known for some time,[10,11] their synthesis is inefficient, and their polymerization gives only short, oligomeric products where chain growth is likely inhibited by steric and H-bonding interactions between the sugar substituents and the NCA rings.[12] An improved synthesis of *O*- and *S*-linked glyco-serine as well as glyco-threonine NCAs was recently reported, however these monomers were not sufficiently purified to allow polymerization.[13] To overcome these difficulties in synthesis, purification, and polymerization of glycosylated NCAs, we have prepared new glycosylated-L-lysine NCA (glyco-K NCA) monomers that employ *C*-linked sugars and amide linkages to lysine[14] for improved stability without sacrificing biochemical properties. Three different glyco-K NCAs were synthesized, purified, and found to undergo living polymerization using transition metal initiation.[15] These are the first living polymerizations of glycosylated NCAs allowing preparation of well-defined, high molecular weight glycopolypeptides and glycopolypeptide containing block copolymers.

Aside from direct polymerization of glycosylated NCAs, other strategies to prepare glycopolypeptides rely primarily on the addition of sugars to existing polypeptides.

Glyconamidated polypeptides were prepared by reaction of D-gluconolactone or lactobionolactone with poly(L-lysine),[16,17] or by coupling of β-D-galactosylamine to glutamic acid residues,[18] to attach carbohydrates via amide linkages. Also using polypeptide precursors, glycopolypeptide synthesis has been reported using either copper catalyzed azide-alkyne[19,20] or thiol-ene[21] click chemistry, respectively. While promising, these methods can suffer from incomplete sugar functionalization,[21] presentation of sugars in non-native forms (i.e. ring-opened),[16,17] or incorporation of triazole groups[19,20] that may limit biological uses. Consequently, we have focused on the glycosylated NCA approach to obtain well-defined glycopolypeptides with 100% glycosylation, as well as the ability to incorporate glycosylated residues either as random or blocky sequences. We designed glycosylated-L-lysine monomers since many derivatives of lysine NCAs are readily synthesized and polymerized to high molecular weights (>100,000 Da),[22] and since acid and base stable linkages can be readily incorporated to prevent deglycosylation. We chose a glycosylated-L-lysine structure which has been used for glycopeptide synthesis[14] and employs *C*-linked sugars and amide linkages to lysine. Although these are non-native linkages, it is well known that *C*-linked glycopeptides can bind targets with nearly equal affinity and conformation as native *O*-linked analogues[23,24] and are widely utilized when stable glycoprotein mimetics are desired.[25]

3.3 Results and Discussion

The sugar-lysine conjugates were prepared using β-D-glucose (glc), β-D-mannose (man), and β-D-galactose (gal). *C*-allylation of glc, man, and gal pentaacetates was accomplished according to literature procedures followed by isolation of the pure α anomers (Figure 3.1, see Experimental section 3.5).[14] Purification of anomers at this stage in the synthesis is crucial for

the ultimate preparation of optically pure, glyco-K NCA monomers. Mixtures of anomers would lead to polypeptides containing different sugar configurations, which could make analysis of their properties difficult. Both α- and β-anomers are common in glycopeptides and glycoproteins.[26] Although our methodology allows use of either anomer, we chose to use the α-anomers here since they were easily obtained in high purity and natural glycopeptides utilize both α- and β-anomers[27]. The allyl sugar derivatives were oxidized to carboxylic acids, converted to *N*-hydroxysuccinimide (NHS) esters, and then coupled to N_ε-carbobenzyloxy-L-lysine (N_ε-Z-L-lysine) to give the desired conjugates (Figure 3.1).[14]

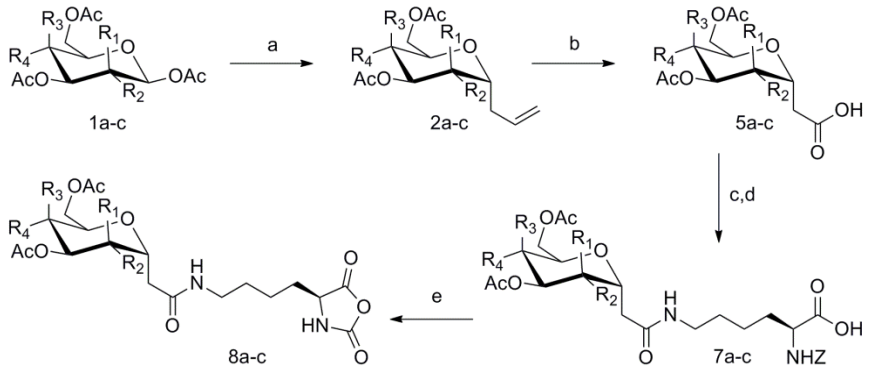


Figure 3.1 Synthesis of glyco-K NCA monomers. Reagents and conditions: a) allyltrimethylsilane, BF_3-Et_2O, MeCN (55 – 75% yield); b) $NaIO_4$, $RuCl_3 \cdot 3H_2O$, MeCN:CCl_4:H_2O (72 – 92% yield); c) NHS, DCC, THF; d) ε-*Z*-L-Lys-OH, $NaHCO_3$ (75 – 81% yield for c and d); (e) Cl_2CHOMe, DCM, 50 ^oC (59 – 64% yield). 8a = α-gal-K NCA (R_1, R_4 = H; R_2, R_3 = OAc). 8b = α-glc-K NCA (R_1, R_3 = H; R_2, R_4 = OAc). 8c = α-man-K NCA (R_1, R_4 = OAc; R_2, R_3 = H).

The preparation of glyco-K NCAs was accomplished using Cl_2CHOCH_3[22c] followed by purification by precipitation and flash chromatography to give NCAs suitable for polymerization. All three glyco-K NCAs were obtained with acceptable yields (60-95%) in each step. Furthermore, all three monomers were found to polymerize efficiently using $(PMe_3)_4Co$[28]

initiator in THF at room temperature (Figure 3.2),[15] giving polymers in excellent yields (Table 3.1).

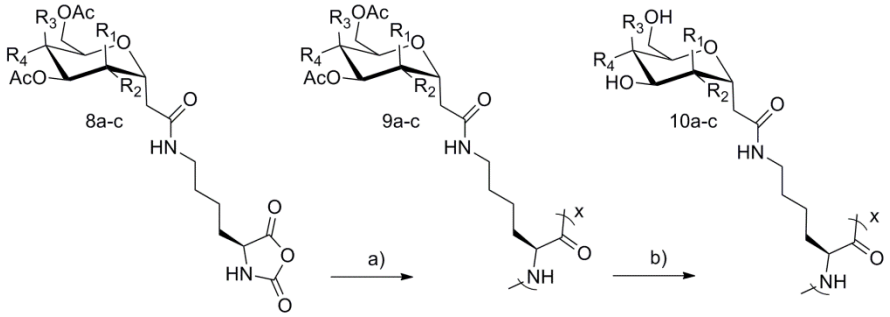


Figure 3.2 Polymerization of glyco-K NCAs and glycopolypeptide deprotection. Reagents and conditions: a) $(PMe_3)_4Co$, THF; b) $NH_2NH_2{\cdot}H_2O$, MeOH. 10a = poly(α-gal-K) (R_1, R_4 = H; R_2, R_3 = OH). 10b = poly(α-glc-K) (R_1, R_3 = H; R_2, R_4 = OH). 10c = poly(α-man-K) (R_1, R_4 = OH; R_2, R_3 = H).

Monomer[a]	M_n[b]	M_w/M_n[b]	DP[c]	yield (%)[d]
10 α-man-K NCA	15,870	1.21	32	91
25 α-man-K NCA	36,920	1.14	74	95
50 α-man-K NCA	81,340	1.19	163	89
75 α-man-K NCA	121,300	1.11	242	94
100 α-man-K NCA	158,600	1.14	317	92
25 Glyco-K NCAs[e]	47,470	1.23	95	88
50 Glyco-K NCAs[e]	84,710	1.07	169	94

Table 3.1 Synthesis of glycopolypeptides using $(PMe_3)_4Co$ in THF at 20 °C. [a] Number indicates equivalents of monomer per $(PMe_3)_4Co$. [b] Molecular weight and polydispersity index (as determined by GPC/LS). [c] DP = number average degree of polymerization from GPC/LS. [d] Total isolated yield of glycopolypeptide. [e] Statistical terpolymers prepared from a 1:1:1 mixture of α-man-K:α-gal-K:α-glc-K NCAs.

To check for chain-breaking side reactions during polymerizations, experiments to verify molecular weight control and extension of active chains were performed. Variation of monomer

to initiator ratios for each glyco-K NCA gave glycopolypeptides whose lengths increased linearly with stoichiometry, and which possessed narrow chain length distributions (M_w/M_n). Data for the α-D-mannose-L-lysine monomer (α-man-K NCA) are shown in Figure 3.3a, and data for the other two monomers are given in the Experimental section 3.5. Good chain length control was obtained and soluble homoglycopolypeptides could be prepared with degrees of polymerization (DP) greater than 300 residues, significantly larger than chains prepared from other glycosylated NCAs (DP < 50).[12a] Compared to shorter chains, long chain glycopolypeptides are expected to be much better mimics of natural high molecular weight glycoproteins. We found that the polymers had higher than theoretical molecular weights, calculated from monomer to initiator stoichiometry, which is due to the known incomplete efficiency of $(PMe_3)_4Co$ initiation in THF.[29] In any case, reproducible and precisely controlled glycopolypeptide lengths were readily prepared. Chain extension experiments of active chains to prepare diblock copolymers (Table 3.2, Experimental section 3.5) all proceeded in high conversion to yield predictable compositions, and with no evidence of inactive chains by GPC analysis (Figure 3.3b). Diblock copolymers were prepared by combining different glyco-K NCAs, as well as by combination of glyco-K NCAs with conventional NCAs regardless of order of monomer addition. In addition to block copolymers, equimolar mixtures of the three glyco-K NCAs were also copolymerized to yield statistical ternary glycocopolypeptides of controllable chain length (Table 3.1). Overall, these data show that the glyco-K NCAs were able to undergo living polymerization when initiated with $(PMe_3)_4Co$, similar to conventional NCAs,[15] and this chemistry enabled preparation of the first examples of glycosylated diblock copolypeptides.

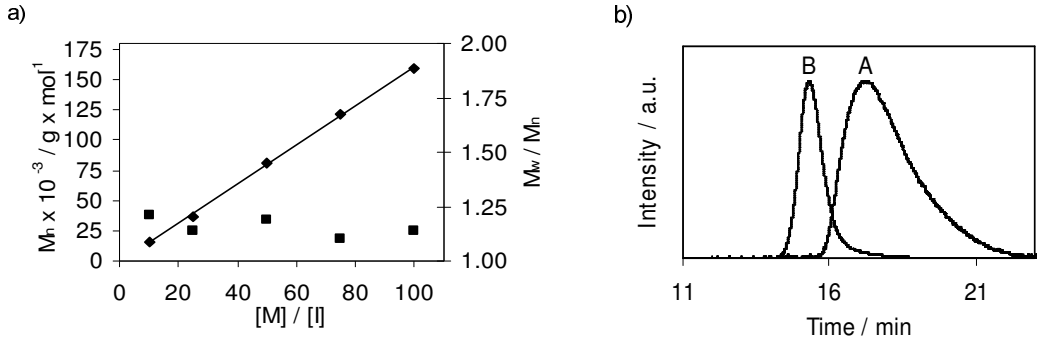


Figure 3.3 a) Molecular weight (M_n, ♦) and polydispersity index (M_w/M_n, ■) of poly(α-man-K) as functions of monomer to initiator ratio ([M]/[I]) using $(PMe_3)_4Co$ in THF at 20 °C. b) GPC chromatograms (normalized LS intensity versus elution time in arbitrary units (a.u.)) of glycopolypeptides after initial polymerization of α-man-K NCA to give a poly(α-man-K)$_{68}$ homoglycopolypeptide (A); and after chain extension by polymerization of a-gal-K NCA to give a poly(α-man-K)$_{68}$-*b*-poly(α-gal-K)$_{200}$ diblock glycopolypeptide (B).

		1st segment[b]			diblock copolymer[c]			
1st monomer[a]	2nd monomer[a]	M_n	M_w/M_n	DP	M_n	M_w/M_n	DP[d]	yield (%)[e]
30 Lys NCA	90 α-man-K NCA	18,100	1.15	69	124,000	1.01	281	93
30 α-man-K NCA	90 Lys NCA	34,090	1.23	68	86,820	1.07	269	99
30 α-man-K NCA	90 α-gal-K NCA	34,090	1.23	68	134,200	1.12	268	91

Table 3.2 Synthesis of diblock copolypeptides using $(PMe_3)_4Co$ in THF at 20 °C. [a] First and second monomers added stepwise to the initiator; number indicates equivalents of monomer per $(PMe_3)_4Co$. Lys NCA = N_ε-*Z*-L-lysine-*N*-carboxyanhydride.[30] [b] Molecular weight and polydispersity index after polymerization of the first monomer (as determined by GPC/LS). [c] Molecular weight and polydispersity index after polymerization of the second monomer. [d] Total degree of polymerization of diblock glycopolypeptide. [e] Total isolated yield of diblock glycopolypeptide.

It is noteworthy that polymerizations of glyco-K NCAs proceeded efficiently at room temperature to yield polymers with DP > 150 in less than 3 hours, as compared to 3-6 days for polymerization of *O*-linked glyco-serine NCAs (DP < 25) using amine initiators.[12a] The sluggish polymerization of *O*-linked glyco-serine NCAs is thought to be due to either the proximity of the bulky peracetylated sugar to the NCA ring, or H-bonding of acetyl carbonyls with NH groups of

NCAs.[12b,c] Although we also see ^{1}H NMR evidence for H-bonding in our glyco-K NCAs (see Experimental section 3.5), the increased tether length between sugar and NCA likely weakens this interaction and removes steric hindrance allowing efficient polymerization. However, the influence of H-bonding is not negligible, since the glyco-K NCAs were found to polymerize 2 to 5 times more slowly than other lysine NCA derivatives. As all of the acetyl protected poly(glyco-K)s were found to be soluble in THF, it appears that there are no significant interchain H-bonding interactions in the polymers. Furthermore, all three homoglycopolypeptides were found to have good water solubility after deprotection using $NH_2NH_2{\cdot}H_2O$ in MeOH. To investigate the solution conformations of these samples, circular dichroism (CD) spectra of the poly(glyco-K)s were measured in deionized water after purification and removal of all residual cobalt impurities by extensive dialysis against deionized water. All three glycopolypeptides were found to be α-helical in water at room temperature, with characteristic minima at 208 and 222 nm indicating greater than 88% helicity[31] (Figure 3.4a, see Experimental section 3.5). Poly-L-lysine inherently prefers to be α-helical when uncharged,[32] and the bulky sugar groups are positioned far enough away from the backbone to avoid perturbing this conformation. Measurement of CD spectra from 4 to 90 ºC revealed that the α-helical conformation of poly(α-gal-K) was gradually disrupted as temperature was increased. This behavior is likely due to disruption of amide H-bonding by interactions with water molecules.[33] These disordered polypeptides remained water soluble and their a-helical conformations were completely regained upon cooling, showing this process is reversible. Ternary glycocopolypeptides containing randomly placed sugars also were found to be α-helical (see Methods section 3.5), indicating that the poly-L-lysine backbone is directing the chain conformation rather than any specific interactions between sugars. This result

allows the display of a diverse variety of sugars off of ordered α-helical polypeptide scaffolds, potentially useful for diagnostic applications.

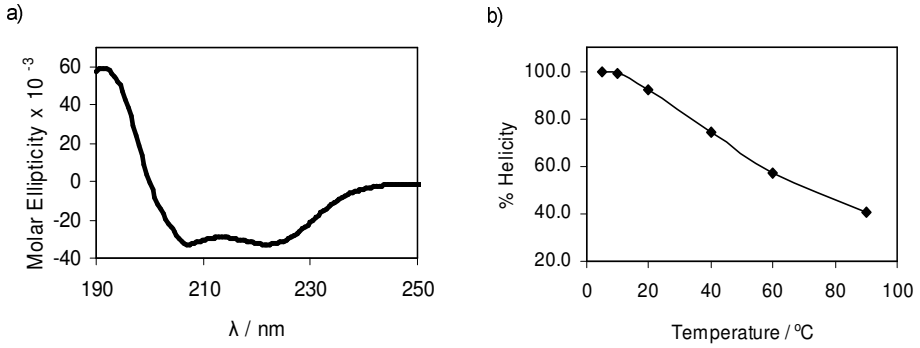


Figure 3.4 a) Circular dichroism spectrum of poly(α-gal-K) at 20 °C, 92% α-helical. (**10a**). b) Percent of α-helical content (% Helicity, calculated using molar ellipticity at 222 nm) of poly(α-gal-K) as a function of temperature. M_n = 90,040 Da, 0.25 mg/mL in deionized water, pH 7. Molar ellipticity is reported in millidegrees·cm^2·dmol^{-1}.

3.4 Conclusion

We have prepared new glycosylated lysine NCAs that undergo living polymerization to give well-defined, high molecular weight homoglycopolypeptides and block and statistical glycocopolypeptides. This system solves many long-standing problems in the direct synthesis of glycopolypeptides from NCAs relating to monomer synthesis, purification and polymerization, and is advantageous in that polypeptides with 100% glycosylation are easily obtained. These water soluble glycopolypeptides have potential to impart functionality and improve biocompatibility in copolypeptide materials, such as hydrogels for tissue engineering and vesicles for drug delivery, as well as for preparation of structurally defined sugar presenting polymers for glycomics research.

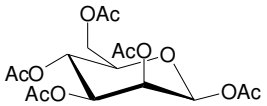
3.5 Experimental

3.5.1 Materials and Methods

Unless stated otherwise, reactions were conducted in oven-dried glassware under an atmosphere of nitrogen using anhydrous solvents. Hexanes, THF, DCM, and DMF were purified by first purging with dry nitrogen, followed by passage through columns of activated alumina (or 4Å molecular sieves for DMF). EtOAc and MeCN were freshly distilled from CaH_2. Deionized water (18 MΩ-cm) was obtaining by passing in-house deionized water through a Millipore Milli-Q Biocel A10 purification unit. All commercially obtained reagents were used as received without further purification unless otherwise stated. Reaction temperatures were controlled using an IKA magnetic temperature modulator, and unless stated otherwise, reactions were performed at room temperature (RT, approximately 20 °C). Thin-layer chromatography (TLC) was conducted with EMD gel 60 F254 precoated plates (0.25 mm) and visualized using a combination of UV, anisaldehyde, and phosphomolybdic acid staining. Selecto silica gel 60 (particle size 0.032–0.063 mm) was used for flash column chromatography. ^{1}H NMR spectra were recorded on Bruker spectrometers (at 500 MHz) and are reported relative to deuterated solvent signals. Data for ^{1}H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity, coupling constant (Hz) and integration. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet and br, broad ^{13}C NMR spectra were recorded on Bruker Spectrometers (at 125 MHz). Data for ^{13}C NMR spectra are reported in terms of chemical shift. High-resolution mass spectrometry (HRMS) was performed on a Micromass Quatro-LC Electrospray spectrometer with a pump rate of 20 μL/min using electrospray ionization (ESI). All Fourier Transform Infrared (FTIR) samples were prepared as thin films on

NaCl plates and spectra were recorded on a Perkin Elmer RX1 FTIR spectrometer and are reported in terms of frequency of absorption (cm^{-1}). Tandem gel permeation chromatography/light scattering (GPC/LS) was performed on a SSI Accuflow Series III liquid chromatograph pump equipped with a Wyatt DAWN EOS light scattering (LS) and Optilab rEX refractive index (RI) detectors. Separations were achieved using 10^5, 10^4, and 10^3 Å Phenomenex Phenogel 5 µm columns using 0.10 M LiBr in DMF as the eluent at 60 °C. All GPC/LS samples were prepared at concentrations of 5 mg/mL. Circular dichroism spectra were recorded on an OLIS RSM CD spectrophotometer running in conventional scanning mode.

3.5.2 Experimental Procedures for Preparation of Glyco-K NCAs

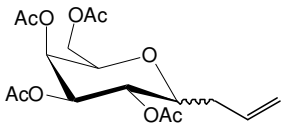


Mannose pentaacetate (1c)[34]

To a solution of β-D-mannose (3.06 g, 17.0 mmol) in pyridine (15 mL, 186 mmol, 11 equiv) at 0 ^oC under N_2 was added acetic anhydride (16 mL, 170 mmol, 10 equiv). The reaction mixture was sealed and kept at –20 ^oC for 17 hours. The reaction was slowly poured into ice water (100 mL) and then extracted with ethyl acetate (3 x 150 mL). The organic layer was washed with saturated $NaHCO_3$ until then evolution of gasses ceased (3 x 150 mL), and then washed with water (2 x 100 mL), then brine (1 x 150 mL). The organic phase was dried over Na_2SO_4 and condensed to yield 6.49g of **1** (97.7%) as a glassy solid. Physical data obtained for **1c** were in agreement with literature values.[34]

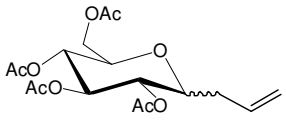
General Procedure for Allylation of Galactose, Glucose, and Mannose Pentaacetate[35,36]

To a solution of pentaacetate **1a**, **b**, or **c** (7.50 g, 19.2 mmol) in dry acetonitrile (40 mL) at 0 ^{o}C under N_2 was added allyltrimethylsilane (9.20 mL, 57.6 mmol, 3 equiv). Boron trifluoride etherate (13.6 mL, 96.1 mmol, 5 equiv) was then added dropwise over fifteen minutes. The reaction mixture was stirred for 2 hours at 0 ^{o}C and then was allowed to warm to room temperature as stirring continued an additional 14 hours for **1a,b** and 72 hours for **1c**. The reaction was slowly poured into saturated $NaHCO_3$ (150 mL) and stirred until the evolution of gases ceased. The mixture was extracted with CH_2Cl_2 (3 x 100 mL), washed with brine, and dried over Na_2SO_4. Following evaporation of the solvent under reduced pressure, the crude residue was purified by flash chromatography (35-45% EtOAc in hexanes) to give *C*-glycosides **2a-c** as clear oils (55-75% yield). Physical data obtained for **2a-c** were in agreement with literature values.[35,36]



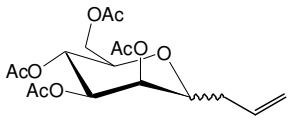
1-Allyl-2,3,4,6-tetra-*O*-acetyl-α,β-D-galactopyranoside (2a)

(75% yield, *α:β* ca. 11:1 by ^{1}H NMR)



1-Allyl-2,3,4,6-tetra-*O*-acetyl-α,β-D-glucopyranoside (2b)

(74% yield, *α:β* ca. 10:1 by ^{1}H NMR). Anomers were separated by crystallization from $CHCl_3$ and hexanes to yield pure *α*-**2b**.



1-Allyl-2,3,4,6-tetra-*O*-acetyl-α,β-D-mannopyranoside (2c)

(55% yield, *α:β* ca. 3:1 by ^{1}H NMR).

Separation of alpha and beta anomers of 2a,c:

Isolation of pure **α-2a,c** has been accomplished as shown in Figure 3.5. In addition, isolation of pure **α-2a** has been accomplished by recrystallization (methanol/diethyl ether) of compound 3a shown in Figure 3.5, followed by acetylation with acetic anhydride and pyridine as previously described.

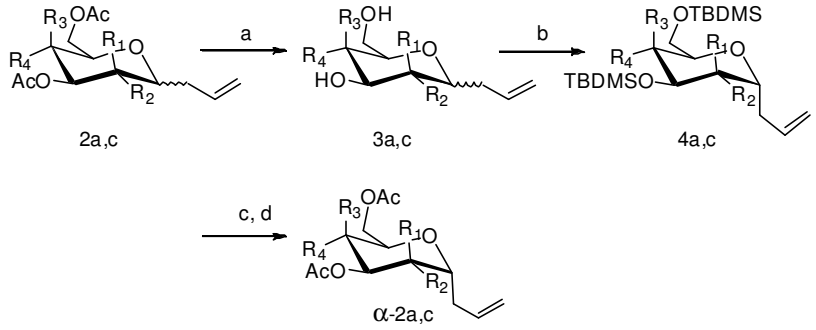
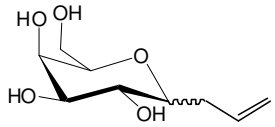


Figure 3.5 Purification of alpha anomers of **2a,c**. Reagents and conditions: a) NaOMe, MeOH; b) TBDMSOTf, DIPEA, DCM; c) TBAF, DMSO, 60 ^oC; d) Ac_2O, Pyridine. **2a** = α-gal-K NCA (R_1, R_4 = H; R_2, R_3 = OAc). **2c** = α-man-K NCA (R_1, R_4 = OAc; R_2, R_3 = H). **3a** = α-gal-K NCA (R_1, R_4 = H; R_2, R_3 = OH). **3c** = α-man-K NCA (R_1, R_4 = OH; R_2, R_3 = H). **4a** = α-gal-K NCA (R_1, R_4 = H; R_2, R_3 = OTBDMS). **4c** = α-man-K NCA (R_1, R_4 = OTBDMS; R_2, R_3 = H).

General Procedure for Deacetylation of 1-Allyl-2,3,4,6-tetra-*O*-acetyl-α,β-D-galacto- and manno- pyranosides[35,36]

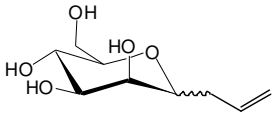
To a solution of **2a** or **2c** (5.15 g, 13.8 mmol) in methanol (50 mL) was added 0.5 M sodium methoxide in methanol (2.8 mL, 1.38 mmol, 0.1 equiv). The reaction was stirred for 4 hours at

room temperature. The reaction mixture was neutralized by stirring with Dowex 50W-X8 [H^+] resin for 30 min, filtered, and concentrated to yield white solid **3a** or **3c** (93-98% yield). **3a,c** were used directly with no further purification.



1-Allyl-α-D-galactopyranoside (3a)

(98% yield).

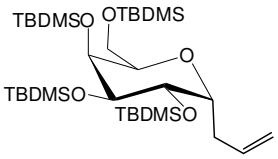


1-Allyl-α-D-mannopyranoside (3c)

(93% yield).

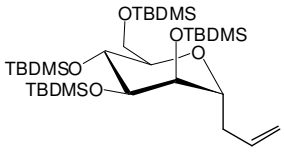
General Procedure for TBDMS protection of *C*-Allyl-galacto- and manno-pyranosides[35,36]

To a solution of **3a** or **3c** (2.02 g, 9.89 mmol) in dry CH_2Cl_2 (100 mL) under N_2 was added DIPEA (8.61 mL, 49.5 mmol, 5 equiv) followed by TBDMSOTf (10.0 mL, 43.5 mmol, 4.4 equiv). The reaction was stirred 16 hours at room temperature, then diluted with CH_2Cl_2 (200 mL) and washed with saturated $CuSO_4$ (3 x 150 mL), then water (2 x 100 mL), and brine (100 mL). The organic phase was dried over Na_2SO_4, and following evaporation of the solvent under reduced pressure, the crude residue was purified by flash chromatography (1.5% EtOAc in hexanes). The α and β anomers of **4a** and **4c** were separated as clear oils. (89-91% yield, combined α, β).



1-Allyl-2,3,4,6-tetra-*O*-*tert*-butyldimethylsilyl-α-D-galactopyranoside (4a)

(91% combined yield, ***α*-4a** and ***β*-4a**). ^{1}H NMR (500 MHz, $CDCl_3$, 25°C): δ 5.93-5.76 (m, 1H), 5.14 (dd, *J* = 28.7, 2.9 1H), 5.03 (dd, *J* = 17.3, 2.6 1H), 4.25-4.14 (m, 2H), 3.96-3.82 (m, 2H), 3.76-3.67 (m, 2H), 3.48 (d, *J* = 5.9, 1H), 2.44-2.31 (m, 1H), 2.18-2.06 (m, 1H), 0.921 (s, 9H), 0.899 (s, 18 H), 0.883 (s, 9H), 0.0937-0.0131 (m, 24H); ^{13}C NMR (125 MHz, $CDCl_3$, 25°C): δ 136.3, 115.7, 81.3, 78.9, 78.0, 72.1, 71.9, 61.4, 36.3, 26.9 26.4, 26.2, 26.0, 25.8, 26.7, 19.3, 18.6, 18.2, 18.0, -2.1, -3.0, -3.3, -3.7, -4.1, -4.6, -5.3, -5.4, -5.8; FTIR (DCM): 3052, 3016, 2982, 2954, 2928, 2884, 2854, 2304, 1472, 1420, 1264 cm^{-1}; HRMS-ESI (*m/z*) $[M + Na]^+$ Calcd for $C_{33}H_{72}O_5Si_4Na$, 683.43; found 683.44.



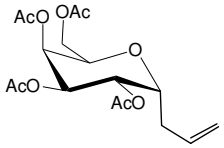
1-Allyl-2,3,4,6-tetra-*O*-*tert*-butyldimethylsilyl-α-D-mannopyranoside (4c)

(89% combined yield, ***α*-4c** and ***β*-4c**). ^{1}H NMR (500 MHz, $CDCl_3$, 25°C): δ 5.99-5.88 (m, 1H), 5.10 (dd, *J* = 13.6, 5.5 1H), 5.03 (dd, *J* = 10.3, 1.8 1H), 3.94-3.98 (m, 2H), 3.83-3.74 (m, 3H), 3.66 (t, *J* = 7.2, 1H), 2.53-2.44 (m, 1H), 2.10-1.99 (m, 1H), 0.924-0.895 (m, 24 H), 0.883 (s, 9H), 0.116-0.0213 (m, 24H); ^{13}C NMR (125 MHz, $CDCl_3$, 25°C): δ=136.6, 116.1, 82.4, 79.3, 79.1, 72.4, 71.1, 61.6, 36.1, 26.9 26.5, 26.2, 26.0, 25.9, 26.8, 19.3, 18.6, 18.1, 18.0, -2.0, -2.9, -3.3, -3.7, -4.0, -4.6, -5.3, -5.3, -5.8; FTIR (DCM): 3050, 3016, 2981, 2950, 2926, 2883, 2855, 2302,

1471, 1423, 1265 cm^{-1}; HRMS-ESI (*m/z*) $[M + Na]^+$ Calcd for $C_{33}H_{72}O_5Si_4Na$, 683.43; found 683.43.

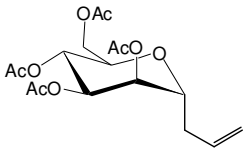
General Procedure for TBDMS Deprotection and Acetylation of 1-Allyl-2,3,4,6-tetra-*O*-*tert*-butyldimethylsilyl-α-D-galacto- and manno-pyranosides[35,36]

To a solution of ***α*-4a** or ***α*-4c** (10.62 g, 16.1 mmol) in dry DMSO (100 mL) under N_2 was added 1.0 M TBAF in THF (70.8 mL, 70.8 mmol, 4.4 equiv) and the reaction stirred for 3 hours at 60 ^oC. The reaction was cooled to room temperature, pyridine (14.3 mL, 177 mmol, 11 equiv) and acetic anhydride (15.2 mL, 161 mmol, 10 equiv) were added, and stirring was continued for 16 hours. The reaction was slowly poured into ice water (200 mL) and then extracted with ethyl acetate (3 x 200 mL). The organic layer was washed with saturated $NaHCO_3$ until then evolution of gasses ceased (3 x 200 mL), and then washed with water (2 x 200 mL), and brine (1 x 200 mL). The organic phase was dried over Na_2SO_4 and condensed to yield ***α*-2a** or ***α*-2c** as white solids (89-90%). Physical data obtained for ***α*-2a,c** were in agreement with literature values. [35,36]



1-Allyl-2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranoside (*α*-2a)

(90% yield over 2 steps).

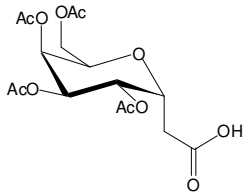


1-Allyl-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside (*α*-2c)

***α*-2c** was recovered as a clear glassy solid (89% yield over 2 steps).

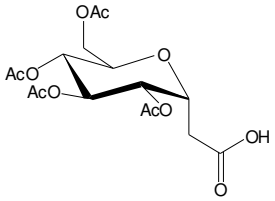
General Procedure for Oxidation of 1-Allyl-2,3,4,6-tetra-*O*-acetyl-α,β-D-galacto-, gluco-, and manno- pyranosides[35,36]

To a solution of ***α*-2a**, **b**, or **c** (0.755 g, 2.03 mmol) in acetonitrile (8 mL), carbon tetrachloride (8 mL), and water (12 mL) was added first $NaIO_4$ (1.95 g, 9.13 mmol, 4.5 equiv) followed by $RuCl_3$ (21.2 mg, 0.08 mmol, 4 mol%,). The reaction was stirred at room temperature for 3 hours then transferred to a separatory funnel. After the addition of water (50 mL) the mixture was extracted with CH_2Cl_2 (3 x 50mL), and the combined organic phase was washed with brine (50 mL) and dried over Na_2SO_4. Following evaporation of the solvent under reduced pressure, the crude dark brown oil was passed through a silica plug (100% EtOAc) to give **5a**, **b**, and **c** as white solids. (72-92% yield). Physical data obtained for **5a-c** were in agreement with literature values. [35,36]



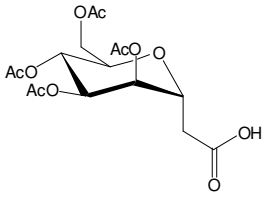
2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl acid (5a)

(92% yield).



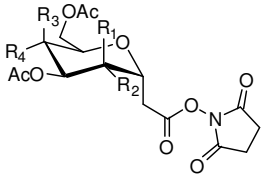
2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl acid (5b)

(85% yield).



2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl acid (5c)

(72% yield).



gal R_1,R_4= H R_2,R_3= OAc
glc R_1,R_3= H R_2,R_4= OAc
man R_1,R_4= OAc R^2,R_3= H

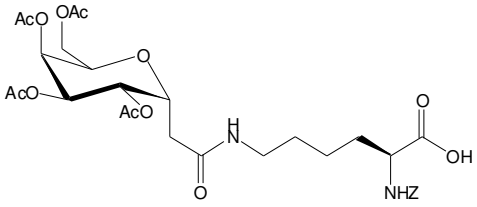
General Procedure for Preparation of *N*-hydroxysuccinimide esters, 6a, b, and **c from 2,3,4,6-tetra-*O*-acetyl-α-D-galacto-, gluco-, and manno- pyranosyl acids**

To a solution of **5a**, **b**, or **c** (1.21 g, 3.10 mmol) in dry THF (50 mL) at 0 ^{o}C under N_2 was added *N*-hydroxysuccinimide (0.392 g, 3.41 mmol, 1.1 equiv) followed by DCC (0.704 g, 3.41 mmol, 1.1 equiv). The reaction was sealed and kept at 4 ^{o}C for 16 hours. Crystalline dicyclohexylurea (DCU) was filtered off and washed with cold, dry THF. The filtrate was concentrated under reduced pressure, dissolved in dry THF (15 mL) and cooled to –20 ^{o}C for 30 minutes. The

insoluble DCU was again filtered off and the process was repeated until the THF was free of solids (a total of 3 times). **6a**, **b**, and **c** were obtained as white crystalline solids in quantitative yield and were used directly with no further purification.

General Procedure for Amide Couplings of *C*-linked Glycoconjugates

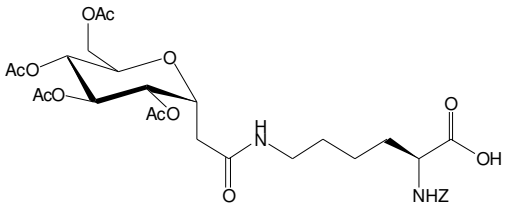
To a solution of **6a**, **b**, or **c** (1.51 g, 3.10 mmol) in THF (30 mL) was added N_{ε}-Z-L-lysine (1.30 g, 4.65 mmol, 1.5 equiv) and $NaHCO_3$ (0.417 g, 4.96 mmol, 1.6 equiv). Water was added in small portions (4 x 1 mL) until the reaction cleared and all materials dissolved. The reaction was stirred for 3 hours at room temperature and then condensed to half the original volume under reduced pressure. The reaction mixture was then acidified to pH 2 by dropwise addition of 6M HCl, and extracted with CH_2Cl_2 (3 x 50 mL). The organic layers were combined and washed with water (2 x 50 mL) and brine (1 x 200 mL), dried over Na_2SO_4, and condensed. The crude residue was purified by flash chromatography (5% MeOH, 1% AcOH in EtOAc) to give **7a**, **b**, and **c** as white crystalline solids. (75-81% yield).



***N*-Carbobenzyloxy-L-lysine-1-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranoside) (7a)**

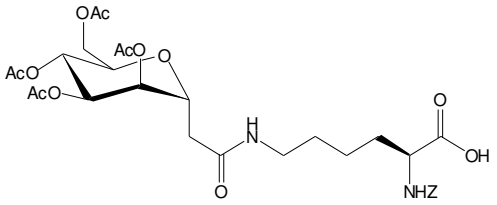
(77% over 2 steps). ^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 7.39-7.33 (m, 5H), 6.60 (s, 1H), 5.79 (d, *J* = 5.7, 1H), 5.43 (s, 1H), 5.29 (dd, *J* = 8.5, 4.7, 1H), 5.19 (dd, *J* = 8.6, 3.0, 1H), 5.13 (s, 2H), 4.73-4.65 (m, 1H), 4.37 (d, *J* = 4.5, 1H), 4.28 (dd, *J* = 8.8, 8.8, 1H), 4.24-4.19 (m, 1H), 4.16 (dd, *J* = 11.0, 4.3, 1H) 3.31-3.20 (m, 2H), 2.63 (dd, *J* =15.4, 9.7), 2.48 (dd, *J* = 15.3, 3.8, 1H) 2.13 (s,

3H), 2.08-2.04 (m, 9H), 1.94-1.85 (m, 1H), 1.80-1.72 (m, 1H), 1.60-1.50 (m, 2H), 1.46-1.34 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ 170.7, 170.2, 170.0, 169.8, 169.6, 156.2, 136.1, 128.4, 128.0, 127.8, 69.2, 68.8, 67.8, 67.7, 67.6, 66.9, 61.1, 53.4, 39.2, 34.1, 31.7, 28.6, 22.2, 20.6, 20.5, 20.5, 20.5; FTIR (DCM): 2930, 1746, 1716, 1654, 1508, 1458, 1370, 1220 cm^{-1}; HRMS-ESI (*m/z*) $[M + Na]^+$ Calcd for $C_{30}H_{39}N_2O_{14}Na$, 674.23; found 675.24 $[M + H]^+$ Calcd for $C_{30}H_{40}N_2O_{14}$, 652.64; found 653.26.



***N*-Carbobenzyloxy-L-lysine-1-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside) (7b)**

(75% over 2 steps). ^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 7.37-7.28 (m, 5H), 6.40 (s, 1H), 5.71 (d, *J* = 6.6, 1H), 5.24 (dd, *J* = 8.4, 8.4, 1H), 5.10 (s, 2H), 5.07 (dd, *J*(H,H)=8.6, 5.3, 1H), 4.97 (dd, *J* = 8.4, 8.4, 1H), 4.66-4.60 (m, 1H), 4.41-4.30 (m, 1H), 4.20 (dd, *J* = 12.2, 5.1, 1H), 4.13 (dd, *J* = 13.3, 1.4, 1H), 4.01-3.92 (m, 1H), 3.29-3.18 (m, 2H), 2.63 (dd, *J* = 15.4, 10.0, 1H), 2.48 (dd, *J* = 15.4, 4.1, 1H), 2.06 (s, 3H), 2.03 (s, 3H), 2.01 (s, 6H), 1.91-1.82 (m, 1H), 1.78-1.68 (m, 1H), 1.56-1.48 (m, 2H), 1.44-1.32 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ 170.8, 170.0, 169.7, 169.5, 169.4, 156.2, 136.1, 128.4, 128.1, 127.9, 69.8, 69.7, 69.4, 69.2, 68.2, 66.9, 62.0, 53.4, 39.2, 33.9, 31.8, 28.6, 22.1, 20.6, 20.6, 20.5; FTIR (DCM): 2932, 1746, 1710, 1650, 1510, 1454, 1360, 1222 cm^{-1}; HRMS-ESI (*m/z*) $[M + Na]^+$ Calcd for $C_{30}H_{39}N_2O_{14}Na$, 674.23; found 675.26 $[M + H]^+$ Calcd for $C_{30}H_{40}N_2O_{14}$, 652.64; found 653.28.



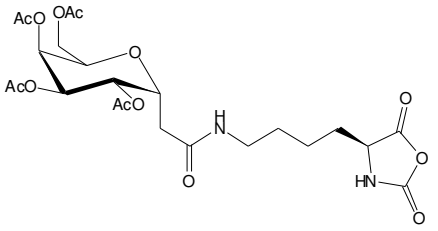
***N*-Carbobenzyloxy-L-lysine-1-(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranoside) (7c)**

(81% over 2 steps). ^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 7.40-7.34 (m, 5H), 6.48 (s, 1H), 5.77 (d, *J* =7.0, 1H), 5.29 (dd, *J* = 6.6, 3.7, 1H), 5.18-5.12 (m, 4H), 4.46-4.36 (m, 3H), 4.23 (dd, *J* = 12.1, 3.9, 1H), 4.04 (dd, *J* = 10.2, 6.3, 1H), 3.37-3.18 (m, 2H), 2.61 (dd, *J* = 15.1, 9.0, 1H), 2.55 (dd, *J* = 15.1, 4.6, 1H), 2.12-2.08 (m, 12H), 1.96-1.84 (m, 1H), 1.83-1.73 (m, 1H), 1.62-1.50 (m, 2H), 1.49-1.37 (m, 2H) ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ 170.7, 170.0, 169.7, 169.4, 156.2, 136.1, 128.4, 128.1, 127.9, 71.8, 69.1, 67.9, 67.3, 66.8, 61.7, 53.4, 39.1, 36.9, 31.7, 28.7, 22.1, 20.6, 20.6; FTIR (DCM): 2932, 1746, 1710, 1654, 1512, 1454, 1368, 1220 cm^{-1}; HRMS-ESI (*m/z*) $[M + Na]^+$ Calcd for $C_{30}H_{39}N_2O_{14}Na$, 674.23; found 675.27 $[M + H]^+$ Calcd for $C_{30}H_{40}N_2O_{14}$, 652.64; found 653.28.

General procedure for preparation of glyco-K NCAs

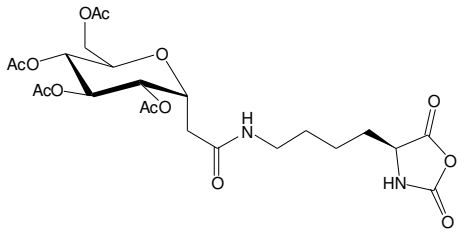
To a solution of **7a**, **b**, or **c** (0.788 g, 1.21 mmol) in CH_2Cl_2 (30 mL) under N_2 was added α,α-dichloromethylmethyl ether (0.163 mL, 1.81 mmol, 1.5 equiv) and the solution refluxed for 36 hours. The reaction was evaporated to dryness under reduced pressure. In a dinitrogen filled glove box, the crude residue was dissolved in anhydrous THF (3 mL) and precipitated into hexanes (30 mL) three times and then purified by flash chromatography under N_2 (65-85% EtOAc in hexanes) followed by solvent evaporation to yield the glyco-K NCAs **8a**, **b**, and **c** as white crystalline solids (59-64% yield). Silica gel was dried at 150 ^{o}C under vacuum for 48 hours before use.

Evidence for H-bonding between an acetyl carbonyl of a sugar with the NH group of the NCA was observed by ^{1}H NMR for NCA compounds **8a** and **8b**. In NCAs **8a** and **8b,** the two methylene protons on the lysine sidechain α to the amide bond each yield a distinct multiplet. The same protons are observed as a single multiplet for the acid compounds **7a**,**b**. This shift likely indicates the loss of free rotation due to a rigid H-bonded structure. This splitting pattern was not observed for α-man-K compounds **7c** and **8c**, which may indicate the acetate groups in these compounds are in configurations less able to H-bond to the NH group.



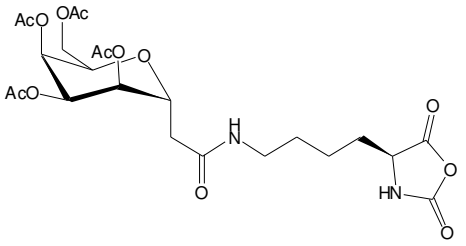
2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl-L-lysine-*N*-carboxyanhydride (α-gal-K NCA, 8a)

(64% yield). ^{1}H NMR (500 MHz, $CDCl_3$, 25°C): δ 7.55 (s, 1H), 6.44 (t, *J* = 5.5, 1H), 5.40 (dd, *J* = 3.1, 3.1, 1H), 5.26 (dd, *J* = 11.1, 2.4, 1H), 5.15 (dd, *J* = 8.7, 3.2, 1H), 4.69-4.64 (m, 1H), 4.35-4.27 (m, 2H), 4.20-4.14 (m, 1H), 4.09 (dd, *J* = 11.5, 5.4, 1H), 3.37-3.29 (m, 1H), 3.25-3.17 (m, 1H), 2.60 (dd, *J* = 15.4, 9.8, 1H), 2.44 (dd, *J* = 15.4, 4.3, 1H), 2.12 (s, 3H), 2.07 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 2.01-1.93 (m, 2H), 1.61-1.41 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$, 25 °C): δ 170.9, 170.2, 169.93, 169.89, 169.87, 169.7, 152.3,69.1, 68.9, 67.7, 67.6, 66.9, 61.1, 57.4, 38.6, 34.2, 30.8, 28.5, 21.5, 20.7, 20.62, 20.61, 20.5; FTIR (THF): 2680, 1854, 1790, 1752, 1678, 1458, 1374 cm^{-1}; HRMS-ESI (*m/z*) $[M + Na]^+$ Calcd for $C_{23}H_{32}N_2O_{13}Na$, 567.50; found 567.17 $[M + H]^+$ Calcd for $C_{23}H_{32}N_2O_{13}$, 545.52; found 545.19.



2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-L-lysine-*N*-carboxyanhydride (α-glc-K NCA, 8b)

(62% yield). ^{1}H NMR (500 MHz, $CDCl_3$, 25^{o}C): δ 7.41 (s, 1H), 6.38-6.32 (m, 1H), 5.22 (dd, *J* = 8.3, 8.3, 1H), 5.07 (dd, *J* = 8.5, 5.2, 1H), 4.99 (dd, *J* = 8.3, 8.3, 1H) 4.66-4.60 (m, 1H), 4.33 (dd, *J*(H,H)= 6.6, 4.7, 1H), 4.20 (d, *J* = 4.2, 2H) 4.0-3.95 (m, 1H), 3.41-3.32 (m, 1H), 3.22-3.14 (m, 1H), 2.65 (dd, *J* = 15.5, 10.0, 1H), 2.48 (dd, *J* = 15.5, 4.2, 1H), 2.09 (s, 3H), 2.05 (s, 3H), 2.04 (s, 6H), 2.02-1.92 (m, 2H), 1.60-1.53 (m, 2H), 1.51-1.38 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ 170.8, 170.4, 169.9, 169.6, 169.5, 169.4, 152.3, 69.7, 69.6, 69.4, 69.3, 68.2, 62.0, 57.4, 38.8, 34.0, 30.8, 28.6, 21.6, 20.57, 20.47, 20.46; FTIR (THF): 2680, 1850, 1788, 1752, 1676, 1458, 1360 cm^{-1}; HRMS-ESI (*m/z*) $[M + Na]^+$ Calcd for $C_{23}H_{32}N_2O_{13}Na$, 567.50; found 567.19 $[M + H]^+$ Calcd for $C_{23}H_{32}N_2O_{13}$, 545.52; found 545.21.



2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-L-lysine-*N*-carboxyanhydride (α-man-K NCA, 8c)

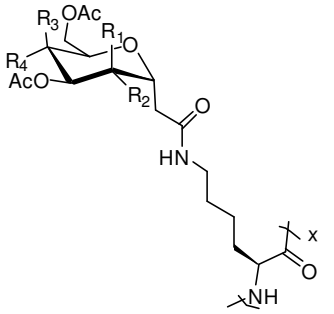
(59% yield). ^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 7.49 (s, 1H), 6.40 (t, *J* = 3.8, 1H), 5.23 (dd, *J* = 7.6, 3.2, 1H), 5.15 (dd, *J* = 9.9, 5.7, 1H), 5.14 (d, *J* = 14.2, 1H) 4.40-4.29 (m, 3H), 4.18 (dd, *J*

= 12.1, 4.0, 1H), 3.98-3.93 (m, 1H), 3.30-3.23 (m, 2H), 2.57 (d, *J* = 7.0, 2H), 2.09 (s, 3H), 2.08 (s, 3H), 2.07 (s, 3H), 2.05 (s, 3H), 1.99-1.91 (m, 1H), 1.85-1.75 (m, 1H), 1.61-1.38 (m, 4H) ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^oC): δ 170.7, 170.4, 170.3, 169.9, 169.4, 169.3, 152.2, 71.6, 69.2, 68.1, 67.1, 61.8, 57.4, 38.7, 37.0, 30.9, 28.6, 21.6, 20.71, 20.66, 20.63, 20.58; FTIR (THF): 2680, 1848, 1786, 1744, 1676, 1458, 1364 cm^{-1}; HRMS-ESI (*m/z*) $[M + Na]^+$ Calcd for $C_{23}H_{32}N_2O_{13}Na$, 567.50; found 567.18 $[M + H]^+$ Calcd for $C_{23}H_{32}N_2O_{13}$, 545.52; found 545.20.

Experimental Procedures for Preparation of Glycopolypeptides

General procedure for polymerization of glyco-K NCAs

All polymerization reactions were performed in a dinitrogen filled glove box. To a solution of glyco-K NCA **8a**, **b**, or **c** in THF (100 mg/mL) was rapidly added a solution of $(PMe_3)_4Co$ in THF (20 mM) via syringe. The reaction was stirred at 20 ºC and polymerization progress was monitored by FTIR. Polymerizations with monomer to initiator ([M]/[I]) ratios below 50:1 were generally complete within 3 hours, while reactions with [M]/[I] ratios above 50:1 were generally complete within 6-8 hours. Aliquots were removed for GPC/LS analysis immediately upon polymerization completion. Reactions were dried under reduced pressure, diluted with minimal EtOAc, and precipitated into hexanes. Polymers were collected by centrifugation and dried under reduced pressure to yield the poly(glyco-K)s **9a**, **b**, or **c** as white solids (88-99% yield).



9a gal R_1,R_4= H R_2,R^3= OAc
9b glc R_1,R_3= H R_2,R_4= OAc
9c man R_1,R_4= OAc R_2,R_3= H

Poly-2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl-L-lysine (9a)

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 5.57-5.16 (br m, 3H), 4.88-4.67 (br s, 1H), 4.35-3.94 (br m, 3H), 3.39-2.96 (br s, 2H), 2.87-2.35 (br d, 2H) 2.18-1.89 (br m, 12H), 1.81-1.30 (br m, 6H); FTIR (film in THF): 3272, 2968, 2854, 1750, 1650, 1540, 1456, 1366 cm^{-1}.

Poly-2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-L-lysine (9b)

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 5.41-4.99 (br m, 3H), 4.83-4.68 (br s, 1H), 4.36-4.19 (br s, 1H), 4.16-3.95 (br s, 2H), 3.93-3.79 (br s, 1H), 3.34-3.07 (br s, 2H), 2.86-2.48 (br s, 2H), 2.28-1.81 (br m, 12H), 1.77-1.31 (br m, 6H); FTIR (THF): 3276, 2973, 2852, 1750, 1650, 1540, 1458, 1359 cm^{-1}.

Poly-2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-L-lysine (9c)

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 5.34-5.05 (br m, 3H), 4.50-4.25 (br s, 2H), 4.21-3.96 (br m, 2H), 3.34-3.06 (br s, 2H), 2.88-2.68 (br s, 1H), 2.66-2.48 (br s, 1H), 2.23-1.81 (br m, 12H), 1.76-1.27 (br m, 6H); FTIR (THF): 3271, 2971, 2850, 1750, 1650, 1540, 1451, 1364 cm^{-1}.

General Procedure for Preparation of Diblock Glycocopolypeptides

All polymerization reactions were performed in a dinitrogen filled glove box. To a solution of the first NCA monomer (30 equiv) in THF (100 mg/mL) was rapidly added a solution of $(PMe_3)_4Co$ (1 equiv) in THF (20 mM) via syringe. The reaction was stirred at RT and polymerization progress was monitored by FTIR. Upon consumption of monomer, a solution of the second NCA monomer (90 equiv) in THF (100 mg/mL) was added via syringe. Polymerization progress was monitored by FTIR and was typically complete within 3 hours. Aliquots were removed for GPC/LS analysis immediately upon polymerization completion of either the initial segment or the diblock copolymer. Reactions were dried under reduced pressure, diluted with minimal EtOAc, and precipitated into hexanes. Polymers were collected by centrifugation and dried under reduced pressure to yield the copolymers as white solids (88-99% yield).

Poly(α-man-K)$_{68}$-*block*-Poly(Z-Lys)$_{201}$

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 7.26-7.19 (br s, 1H), 5.35-4.89 (br m, 3H), 4.55-3.77 (br m, 4H), 3.37-2.99 (br m, 2H), 2.92-2.43 (br m, 2H), 2.16-1.92 (br m, 7H), 1.76-1.16 (br m, 6H); FTIR (THF): 3286, 2964, 2850, 1750, 1722, 1650, 1544, 1458, 1364 cm^{-1}.

Poly(Z-Lys)$_{69}$-*block*-Poly(α-man-K)$_{207}$

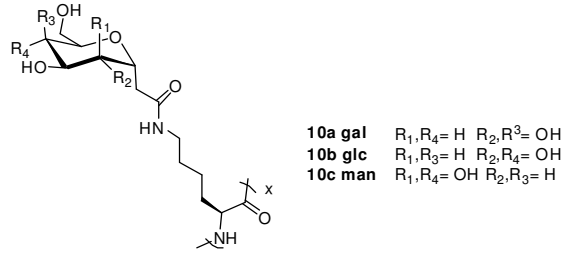
^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 7.26-7.20 (br s, 4H), 5.63-4.86 (br m, 3.5H), 4.59-4.26 (br m, 0.5H), 4.20-3.75 (br m, 1H), 3.31-2.92 (br s, 2H), 2.84-2.48 (br m, 0.5H), 2.20-1.95 (br m, 3H) 1.90-1.16 (br m, 6H); FTIR (THF): 3271, 2970, 2850, 1746, 1718, 1654, 1544, 1460, 1366 cm^{-1}.

General Procedure for Preparation of Statistical Glyco-K Ternary Copolymers

In a dinitrogen filled glove box, α-man-K NCA, α-glc-K NCA, and α-gal-K NCA were mixed in equimolar amounts and dissolved in THF (100 mg/mL). General polymerization procedures were then followed as previously described. Reactions were dried under reduced pressure, diluted with minimal EtOAc, and precipitated into hexanes. Polymers were collected by centrifugation and dried under reduced pressure to yield the copolymers as white solids (88-94% yield).

General glycopolypeptide deprotection procedure

To a solution of acetyl protected poly(glyco-K) in methanol (10 mg/mL) was added hydrazine monohydrate (20 equiv) and the reaction was stirred overnight at room temperature. Reactions were quenched by addition of drops of acetone and then condensed to half the original volume under reduced pressure. The mixture was then diluted with water and transferred to dialysis tubing (2000 Da molecular weight cutoff) and the sample was dialyzed against deionized water for 3 days, with water changes once every two hours for the first 8 hours, and then twice per day thereafter. Dialyzed polymers were lyophilized to dryness to yield poly(glyco-K)s **10a**, **b**, or **c** as white fluffy solids (70-80% yield).



10a gal R_1,R_4= H R_2,R^3= OH
10b glc R_1,R_3= H R_2,R_4= OH
10c man R_1,R_4= OH R_2,R_3= H

Poly-α-D-galactopyranosyl-L-lysine (Poly(α-gal-K), 10a)

^{1}H NMR (500 MHz, D_20, 25 ^{o}C): δ 4.57-4.45 (br s, 1H), 4.11-3.96 (br s, 3H), 3.89-3.81 (br s, 1H), 3.81-3.58 (br m, 4H), 3.30-3.13 (br m, 2H), 2.81-2.64 (br m, 1H), 2.63-2.53 (br m, 1H), 2.11-1.75 (br s, 2H), 1.74-1.33 (br m, 4H).

Poly-α-D-glucopyranosyl-L-lysine (Poly(α-glc-K), 10b)

^{1}H NMR (500 MHz, D_20, 25 ^{o}C): δ 4.55-4.46 (br s, 1H), 4.13-3.96 (br s, 3H), 3.89-3.57 (br m, 4H), 3.29-3.13 (br s, 2H), 2.77-2.64 (br m, 1H), 2.63-2.53 (br m, 1H), 2.10-1.76 (br s, 2H), 1.67-1.33 (br m, 4H).

Poly-α-D-mannopyranosyl-L-lysine (Poly(α-man-K), 10c)

^{1}H NMR (500 MHz, D_20, 25 ^{o}C): δ 4.35-4.26 (br s, 1H), 3.91-3.60 (br m, 5H), 3.58-3.49 (br m, 1H), 3.24-3.01 (br s, 2H), 2.79-2.67 (br m, 1H), 2.53-2.37 (br m, 1H), 2.02-1.69 (br s, 2H), 1.63-1.28 (br m, 4H).

Poly(α-man-K)-*stat*-Poly(α-glc-K)-*stat*-Poly(α-gal-K)

^{1}H NMR (500 MHz, D_20, 25 ^{o}C): δ 4.41-4.31 (br s, 1H), 4.16-3.99 (br m, 1H), 3.96-3.65 (br m, 5H), 3.64-3.55 (br m, 1H), 3.28-3.12 (br s, 2H), 2.83-2.44 (br m, 2H), 2.06-1.71 (br s, 2H), 1.66-1.30 (br m, 4H).

Glycopolypeptide dn/dc Calculations

For accurate mass estimation by GPC/LS, the dn/dc of protected glycopolypeptides **9a,b** was measured. Glycopeptide dn/dc values were calculated directly from GPC/LS measurements, assuming 100% mass recovery and using the template provided in Astra 5.3.2.21 software. Values for dn/dc were also measured off-line in batch mode. Seven standard solutions of glycopeptides with concentrations ranging from 0.125 to 3.00 mg/mL were injected directly into the Wyatt Optilab rEX RI detector. The refractive indices were plotted against concentration and the slope of the resulting line gave the dn/dc value. This process was completed for both **9a** and **9b.** The dn/dc values obtained from GPC/LS and off-line were in good agreement and similar for different glycopolypeptides, and so the averaged value of 0.054 was used for all subsequent GPC/LS glycopolypeptide analysis.

Polymerization of α-glc-K NCA and α-gal-K NCA at different monomer to initiator ([M]/[I]) ratios

Monomer[a]	M_n[b]	M_w/M_n[b]	DP	yield (%)[c]
10 α-glc-K NCA	14,930	1.44	30	99
25 α-glc-K NCA	28,340	1.24	57	93
50 α-glc-K NCA	57,260	1.23	114	91
75 α-glc-K NCA	81,850	1.23	164	97
100 α-glc-K NCA	122,500	1.14	245	97
10 α-gal-K NCA	47060	1.19	94	99
25 α-gal-K NCA	90,040	1.20	180	87
50 α-gal-K NCA	194,200	1.15	388	95
100 α-gal-K NCA	325,600	1.09	651	98

Table 3.3 Molecular weight (M_n) of poly(α-glc-K) and poly(α-gal-K) as a function of monomer to initiator ratio ([M]/[I]) using $(PMe_3)_4Co$ in THF at 20 °C. [a] Number indicates equivalents of monomer per $(PMe_3)_4Co$. [b] Molecular weight and polydispersity index after polymerization (as determined by GPC/LS). [c] Total isolated yield of glycopolypeptide.

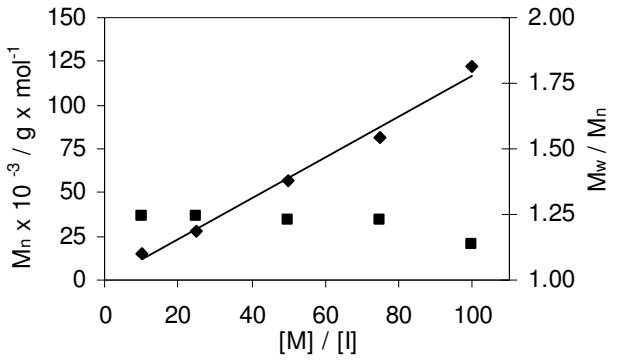


Figure 3.6 Molecular weight (M_n, ♦) and polydispersity index (M_w/M_n, ■) of poly(α-glc-K) as a function of monomer to initiator ratio ([M]/[I]) using $(PMe_3)_4Co$ in THF at 20 °C.

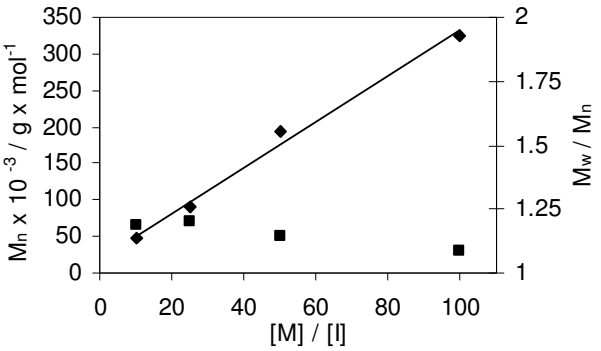


Figure 3.7 Molecular weight (M_n, ♦) and polydispersity index (M_w/M_n, ■) of poly(α-gal-K) as a function of monomer to initiator ratio ([M]/[I]) using $(PMe_3)_4Co$ in THF at 20 °C.

Circular Dichroism of Glycopolypeptides

Following purification by dialysis, solutions of glycopolypeptides **10a-c** were filtered through 0.45 μm PVDF syringe filters and then lyophilized to dryness. CD (190–250 nm) spectra were recorded in a cuvette of 0.1 cm path length with samples prepared at concentrations of 0.25 to 1.0 mg/mL using deionized water. All spectra were recorded as an average of 3 scans. The spectra are reported in terms of molar ellipticity [θ] (deg·cm^2·dmol^{-1}). The formula used for calculating molar ellipticity, [θ], was [θ] = (θ x 100 x M_W)/(c x l) where θ is the experimental ellipticity in millidegrees, M_W is the average molecular weight of a residue in g/mol, c is the peptide concentration in mg/ml; and l is the cuvette pathlength in cm. The percent α-helical content of the glycopeptides was calculated using the formula % α-helix = (-$[\theta]_{222nm}$ +3000)/39000) where $[\theta]_{222nm}$ is the measured molar ellipticity at 222 nm.[31]

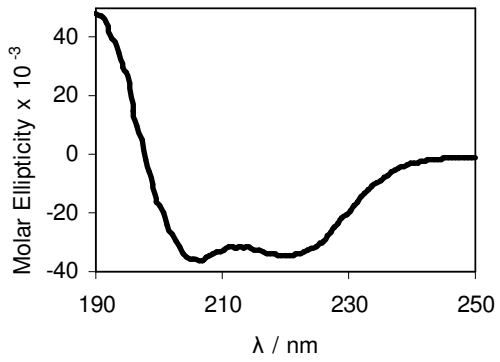


Figure 3.8 Circular dichroism spectrum of poly(α -glc-K) (**10b**). M_n = 81,850 Da, 0.4 mg/mL in deionized water, pH 7, 20 °C, 95% α -helical. Molar ellipticity is reported in degrees^{-3}·cm^2·dmol^{-1}.

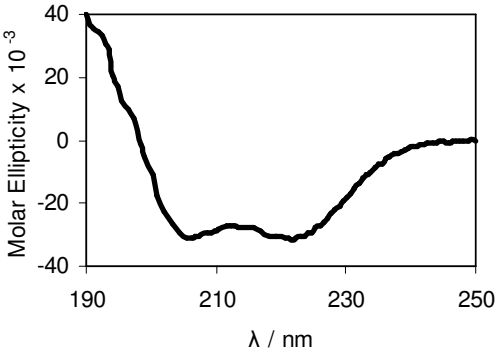


Figure 3.9 Circular dichroism spectrum of poly(α-man-K) (**10c**). M_n = 81,340 Da, 1.0 mg/mL in deionized water, pH 7, 20 °C, 88% α-helical. Molar ellipticity is reported in degrees^{-3}·cm^2·dmol^{-1}.

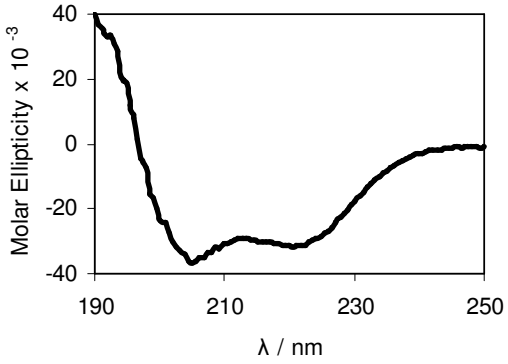


Figure 3.10 Circular dichroism spectrum of statistical ternary glycocopolypeptide prepared from a 1:1:1 mixture of α-man-K, α-gal-K, α-glc-K NCAs. M_n = 84,710 Da, 0.4 mg/mL in water, pH 7, 20 °C, 88% alpha helical. Molar ellipticity is reported in degrees^{-3}·cm^2·dmol^{-1}.

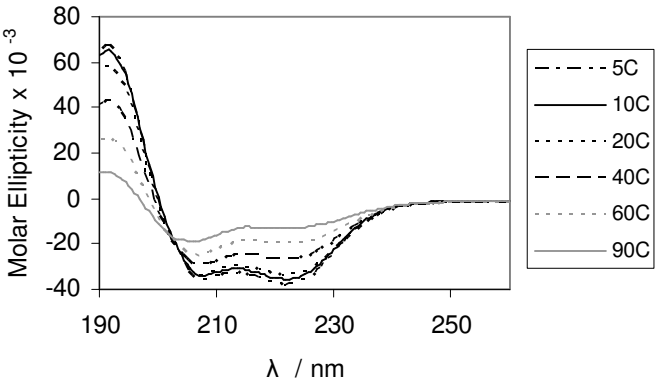


Figure 3.11 Circular dichroism spectra of poly(α-gal-K) at various temperatures in water (increasing temperature). M_n = 90,040 Da, 0.25 mg/mL in deionized water, pH 7. Molar ellipticity is reported in millidegrees·cm^2·dmol^{-1}.

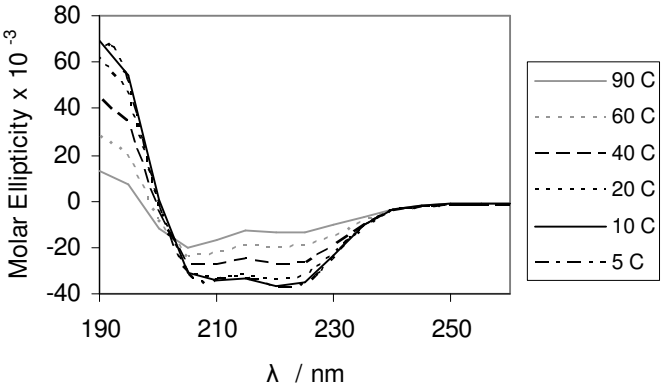


Figure 3.12 Circular dichroism spectra of poly(α-gal-K) at various temperatures in water (decreasing temperature). M_n = 90,040 Da, 0.25 mg/mL in deionized water, pH 7. Molar ellipticity is reported in millidegrees·cm^2·dmol^{-1}.

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CHAPTER FOUR

Glycopolypeptides with a Redox Triggered Helix to Coil Transition

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4.1 Abstract

Conformation switchable glycopolypeptides were prepared by the living polymerization of glycosylated L-cysteine-*N*-carboxyanhydride (glyco-C NCA) monomers. These new monomers were prepared in high yield by coupling of alkene-terminated C-linked glycosides of D-galactose or D-glucose to L-cysteine using thiol-ene "click" chemistry, followed by their conversion to the corresponding glyco-C NCAs. The resulting glycopolypeptides were found to be water soluble and α-helical in solution. Aqueous oxidation of the side-chain thioether linkages in these polymers to sulfone groups resulted in disruption of the α-helical conformations without loss of water solubility. The ability to switch chain conformation and remain water soluble is unprecedented in synthetic polymers, and allows new capabilities to control presentation of sugar functionality in subtly different contexts.

4.2 Introduction

The development of stimuli responsive polymers has received much recent interest. These materials are promising since they aim to mimic adaptive biological systems, trigger release of therapeutics, or amplify signals in biosensors.[1] Polymers have been prepared that are

responsive to light,[2] pH,[3] temperature,[4] or oxidation,[5] where the responsive component typically undergoes a transition between hydrophobic and hydrophilic states.[6] Polypeptide and protein materials offer an additional stimulus response mechanism since they adopt ordered chain conformations (e.g. α-helices) that can be disrupted under mild conditions. Thermal responsive elastin mimetics[7] and pH responsive synthetic polypeptides[8] have both taken advantage of ordered/disordered conformation transitions to drive the formation or disruption of aqueous self-assemblies such as vesicles and micelles. However, these conformational changes act primarily as a solubility switch, and thus do not replicate the more subtle structural changes found in biological systems, such as in motor proteins[9] and membrane proteins involved in signal transduction,[10] where bulk phase separation does not occur. Addressing this challenge, we have developed new glycopolypeptides that undergo α-helix to coil transitions upon oxidation and possess unprecedented good water solubility in both conformational states. A fine energetic balance was achieved by careful placement of oxidizable thioether groups combined with solubilizing and functional sugar moieties to give polymers with switchable, soluble conformations potentially useful for applications in sensing, diagnostics, and biomimetics.

The ability of simple synthetic homopolypeptides, such as poly-L-lysine or poly-L-glutamate, to undergo helix/coil transitions in aqueous solution is well known.[11] In these polymers, the transition occurs upon protonation or deprotonation of the side-chain functional groups where the charged polymers are disordered and the neutral forms adopt stable α-helices. The resulting uncharged, α-helical polypeptides are sparingly soluble in water and precipitate above micromolar concentrations, essentially making these helix/coil transitions a solubility switch.[11] Numerous conformation switchable synthetic polypeptides have been reported, including those that respond to pH,[12] light,[13] or sugar binding,[14] but none has good water

solubility in both the ordered and disordered conformational states. Polypeptides that undergo helix/coil transitions and remain soluble in organic solvents are known,[15] but these are not amenable to biomimetic studies or biotechnological applications. To obtain polypeptides that can undergo a conformational change and remain water soluble, we prepared glycopolypeptides based on L-cysteine residues. The key design features in these polymers are the incorporation of thioether linkages and stable 100% glycosylation via use of *C*-linked glycosides. The polar glycosides provide excellent non-ionic, pH and buffer tolerant water solubility, and add the potential for selective binding to biological targets. The thioether groups provide an oxidation sensitive switch from less-polar thioether to highly polar sulfone groups.

4.3 Results and Discussion

We had previously prepared stable glycopolypeptides based on L-lysine residues, using amide linkages to attach different monosaccharides.[16] These polymers displayed high α-helical contents in aqueous media and only became partially disordered when heated to ~ 90 ºC. L-Lysine is an inherently helicogenic residue,[11] which combined with the ability of side-chain amide groups to cooperatively H-bond, favored formation of stable α-helices in this polymer. To obtain glycopolypeptides with lower helical stability, we chose to build off of L-cysteine, since the presence of the α-heteroatom weakens formation of α-helices.[11] Synthetic glycopolypeptides using *O*- and *S*-linked glycoside derivatives of L-serine and L-cysteine have been prepared,[17] yet this approach is not practical due difficulties both in monomer synthesis and polymerization, and these materials also do not allow for conformational switching. In our design, we used the method of Dondoni to attach the alkene-terminated *C*-linked glycosides of D-galactose and D-glucose to L-cysteine using thiol-ene "click" chemistry,[18] followed by conversion to the corresponding new α-amino acid-*N*-carboxyanhydride (glyco-C NCA) monomers (Figure 4.1).

Although these monomers use non-native linkages, it has been well demonstrated that *C*-linked glycopeptides can bind targets with nearly equal affinity and conformation as native *O*-linked analogues,[19] and have been widely utilized when stable glycoprotein mimetics are desired.[20] Our approach allows formation of the glycosylated amino acids in high yield, is amenable to a variety of sugars, and imparts these residues with many desirable features for polymer formation. The unbranched, thioether containing side-chains are long and hydrophobic and hence should favor α-helix formation in water, the spacing of sugars away from the backbone should relieve steric crowding and assist NCA polymerization, and the incorporation of thioether groups allows for facile post-polymerization stimulus response via oxidation.

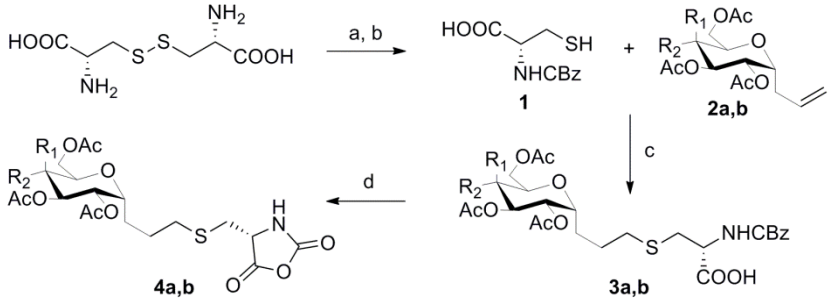


Figure 4.1 Synthesis of glyco-C NCA monomers. Reagents and conditions: (a) CBz-Cl, THF:H_2O 1:9, NaOH (98% yield); (b) PPh_3, THF:H_2O 9:1 (98% yield); (c) **2a** or **2b**, 2,2-dimethoxy-2-phenylacetophenone, DMF, 365 nm (94% yield) (d) Cl_2CHOMe, DCM, 50 °C (63-67% yield). **4a** = α-gal-C NCA (R_1 = OAc; R_2 = H). **4b**= α-glc-C NCA (R_1 = H; R_2 = OAc).

The glyco-C NCA monomers were found to polymerize efficiently using $(PMe_3)_4Co$ initiator in THF at room temperature (Figure 4.2), giving side-chain protected polymers in excellent yields (see Experimental section 4.5).[21] Variation of monomer to initiator ratios for each glyco-C NCA gave glycopolypeptides whose lengths increased linearly with stoichiometry, and which possessed narrow chain length distributions (M_w/M_n). Data for the α-D-glucose-L-cysteine monomer (α-glc-C NCA) are shown in Figure 4.3, and data for the α-D-galactose-L-cysteine monomer (α-gal-C NCA) and statistical copolymers are given in the Experimental

section 4.5. Soluble, 100% glycosylated high molecular weight polypeptides were prepared with reproducible and precisely controlled chain lengths up to *ca.* 200 residues long, which is difficult to achieve using post-polymerization glycosylation strategies.[22] Chain extension experiments using sequential monomer additions to prepare diblock copolymers (Table 4.1, see Experimental section 4.5) all proceeded in high conversion to yield predictable compositions, and with no evidence of inactive chains by GPC analysis (Figure 4.3). Overall, these data show that the glyco-C NCAs were able to undergo living polymerization when initiated with $(PMe_3)_4Co$, similar to conventional NCAs.[21] Furthermore, both poly(α-glc-C) and poly(α-gal-C) were found to have good water solubility after removal of protecting groups (Figure 4.2).

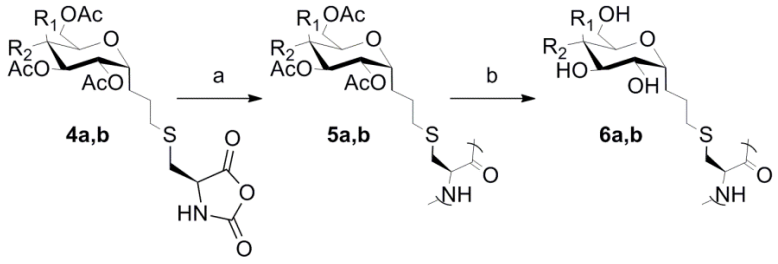


Figure 4.2 Polymerization of glyco-C NCAs and glycopolypeptide deprotection. Reagents and conditions: (a) $(PMe_3)_4Co$, THF (98% yield); (b) $NH_2NH_2 \cdot H_2O$, (95% yield). For **4a** and **5a**: R_1 = OAc; R_2 = H. For **4b** and **5b**: R_1 = H; R_2 = OAc. For **6a** (poly(α-gal-C)): R_3 = OH; R_4 = H. For **6b** (poly(α-glc-C)): R_3 = H; R_4 = OH.

		1st segment[b]			diblock copolymer[c]			
1st monomer[a]	2nd monomer[a]	M_n	M_w/M_n	DP	M_n	M_w/M_n	DP[d]	yield (%)[e]
25 Lys NCA	100 α-glc-C NCA	15,980	1.15	61	131,880	1.01	305	97
25 α-glc-C NCA	100 Lys NCA	27,550	1.09	58	88,330	1.03	290	96

Table 4.1 Synthesis of diblock copolypeptides using (PMe3)4Co in THF at 20 °C. [a] First and second monomers added stepwise to the initiator; number indicates equivalents of monomer per (PMe3)4Co. Lys NCA = *N*ε-CBz-L-lysine-*N*-carboxyanhydride. [b] Molecular weight and polydispersity index after polymerization of the first monomer (as determined by GPC/LS). [c] Molecular weight and polydispersity index after polymerization of the second monomer (as determined by GPC/LS and 1H NMR). [d] Total degree of polymerization of diblock glycopolypeptide. [e] Total isolated yield of diblock glycopolypeptide.

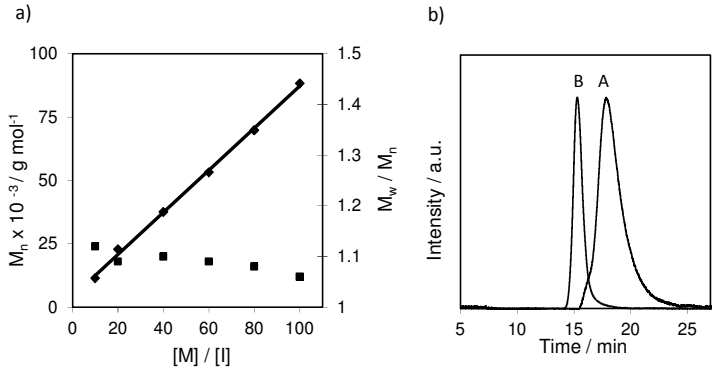


Figure 4.3 a) Molecular weight (Mn, ♦) and polydispersity index (Mw/Mn, ■) of poly(α-glc-C) as functions of monomer to initiator ratio ([M]/[I]) using $(PMe_3)_4Co$ in THF at 20 °C. b) GPC chromatograms (normalized LS intensity in arbitrary units (a.u.) versus elution time) of glycopolypeptides after initial polymerization of α-glc-C NCA to give a poly(α-glc-C)$_{58}$ homoglycopolypeptide (**A**); and after chain extension by polymerization of N_ε-CBz-L-lysine-N-carboxyanhydride to give a poly(α-glc-C)$_{58}$-*b*-poly(CBz-Lys)$_{290}$ diblock glycopolypeptide (**B**).

To investigate solution conformations, circular dichroism (CD) spectra of the poly(glyco-C)s were measured in PBS buffer (pH = 7.4) after purification and removal of all residual cobalt ions by extensive dialysis against deionized water. Both poly(α-glc-C) and poly(α-gal-C) were found to be soluble in both DI water and PBS buffer at 20 ºC (> 50 mM), and gave CD spectra

with characteristic minima at 208 and 222 nm indicating predominantly α-helical conformations (Figure 4.4, see Experimental section 4.5).[23] CD analysis of a thin film of poly(α-gal-C) cast from a solution in DI water also showed that this polymer is predominantly α-helical in the solid-state (see Experimental section 4.5). The thioether groups in these polymers can be oxidized to either sulfoxide or sulfone functionalities, both of which have increased polarity. A similar change of polarity has also been used by Hubbell who oxidized thioether groups in synthetic copolymers to disrupt vesicular assemblies.[5b] Complete oxidation of our poly(glyco-C)s using hydrogen peroxide/acetic acid gave quantitative conversions to the sulfone derivatives, poly(glyco-C^{O2})s, by NMR analysis. To confirm these structures, we also prepared a fully characterized sulfone monomer (α-gal-C^{O2} NCA), and polymerized it to obtain a polymer that showed properties identical to oxidized poly(α-gal-C) samples (Figures 4.5, 4.6).

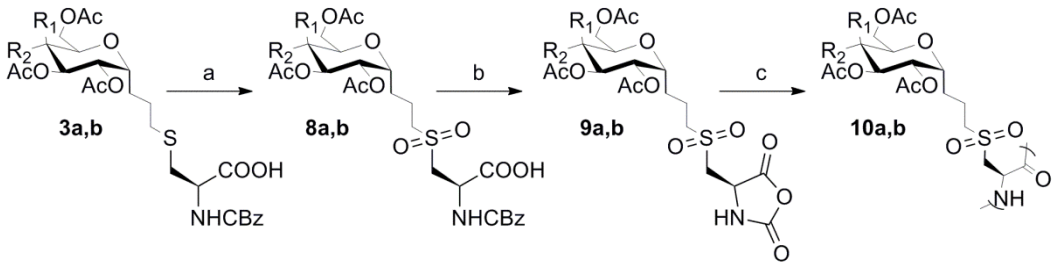


Figure 4.4 Synthesis and polymerization of glyco-C^{O2} NCAs. Reagents and conditions: (a) H_2O_2, AcOH (99% yield); (b) Cl_2CHOMe, DCM, 50 °C (71% yield); (c) $(PMe_3)_4Co$, THF. For **3a, 8a, 9a** (α-gal-C^{O2} NCA), and 10a (poly(α-gal-C^{O2})): R_1 = OAc; R_2 = H. For **3b, 8b, 9b** (α-glc-C^{O2} NCA), and **10b** (poly(α-glc-C^{O2})): R_1 = H; R_2 = OAc.

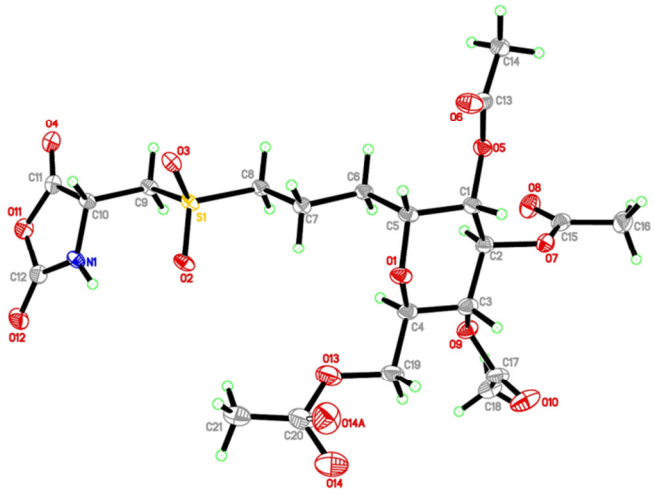


Figure 4.5 X-ray crystal structure of α-Gal-C^{O2} NCA

The oxidized poly(glyco-C^{O2})s also had good solubility in DI water and PBS buffer at 20 ºC (> 50 mM), yet their CD spectra showed loss of the α-helical minima at 208 and 222 nm. Instead, the spectra were characteristic of random coil conformations (Figure 4.8),[11,23] and showed that oxidation of the thioether groups to sulfones in poly(glyco-C)s was able to destabilize the α-helices and cause a transition to disordered conformations. Examination of the corresponding sulfoxide, i.e. poly(α-gal-C^{O}), prepared by milder oxidation of poly(α-gal-C), revealed that this functionality with intermediate polarity is not sufficient to destabilize the α-helical conformation (see Experimental Section). The increased polarity of the sulfone groups likely drives the helix to coil transition by interacting strongly with solvent water molecules, which can disrupt hydrophobic packing of the polypeptide side-chains and increase steric crowding around the backbone to destabilize the α-helical conformation. This substantial change in chain conformation from such small molecular changes in these samples suggests that even subtle shifting in the balance of forces that dictate chain conformation can have large effects. This hypothesis was further validated by preparation and study of the homologous sample

poly(α-gal-C^H), based on L-homocysteine residues, where the thioether group is separated from the peptide backbone by one additional methylene unit (Figure 4.6, 4.7). Poly(α-gal-C^H) is similar to poly(α-gal-C) in that both are water soluble and α-helical, however, upon oxidation to the corresponding polysulfone, poly(α-gal-C^{HO2}) chains remain α-helical (Figure 4.8). The addition of methylene units that move the sulfur atoms just one bond further from the peptide backbone is enough to negate any conformation switching when the sulfur is oxidized. Hence, it seems the poly(glyco-C)s have an optimized structure that puts the α-helical and coil states close enough energetically to allow unprecedented switching from soluble α-helices to soluble disordered conformations.

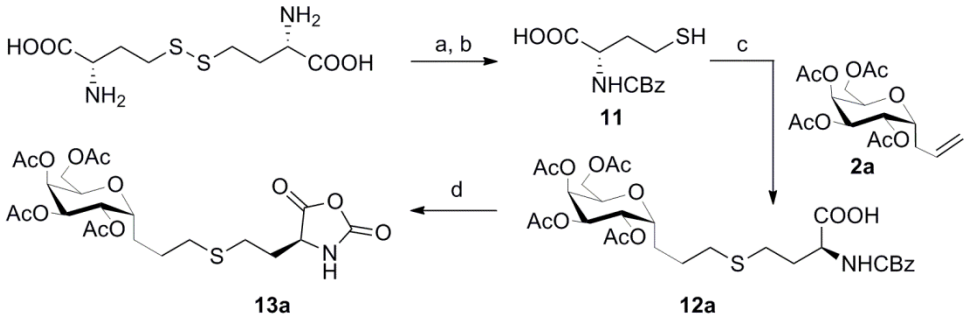


Figure 4.6 Synthesis of α-gal-C^H NCA monomer. Reagents and conditions: (a) CBz-Cl, THF:H_2O 1:9, NaOH (95% yield); (b) PPh_3, THF:H_2O 9:1 (98% yield); (c) **2a**, 2,2-dimethoxy-2-phenylacetophenone, DMF, 365 nm (93% yield); (d) Cl_2CHOMe, DCM, 50 °C (69% yield).

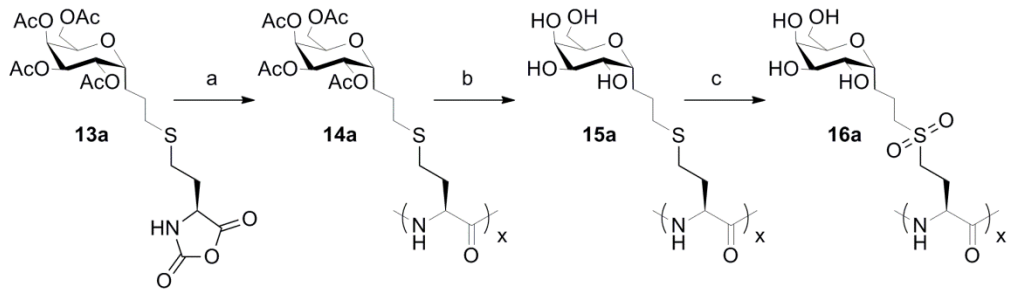


Figure 4.7 Polymerization of α-gal-C^{H} NCA; and glycopolypeptide deprotection and oxidation. Reagents and conditions: (a) $(PMe_3)_4Co$, THF (98% yield); (b) $NH_2NH_2 \cdot H_2O$, (95% yield); (c) H_2O_2, H_2O-AcOH (98% yield). **15a** = poly(α-gal-C^{H}), **16a** = poly(α-gal-C^{HO2}).

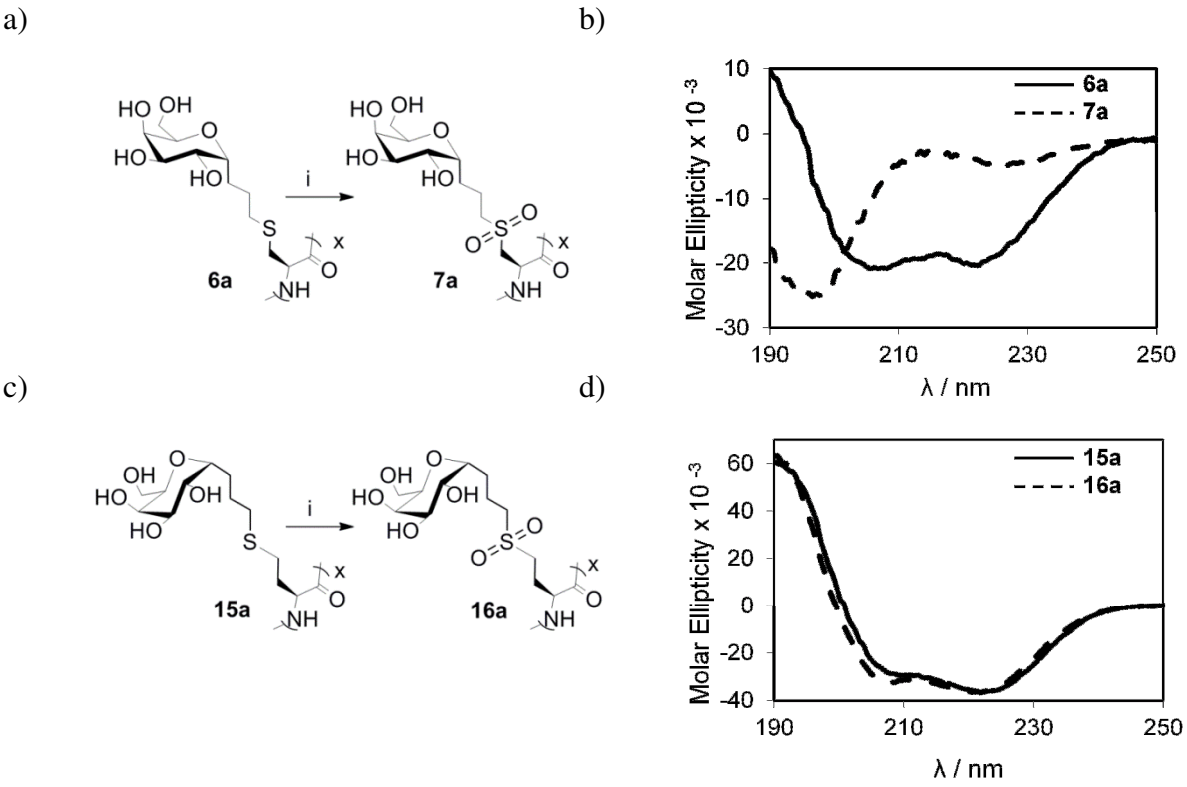


Figure 4.8 Circular dichroism spectra and corresponding structures of parent and oxidized glycopolypeptides. a) Conversion of poly(α-gal-C) **6a** to poly(α-gal-C^{O2}) **7a**. b) Circular dichroism spectra of poly(α-gal-C)$_{108}$ M_n = 33,200 Da (solid line), and poly(α-gal-C^{O2})$_{108}$ M_n = 36,650 Da (dashed line). c) Conversion of poly(α-gal-C^{H}) **15a** to poly(α-gal-C^{HO2}) **16a**. d) Circular dichroism spectra of poly(α-gal-C^{H})$_{88}$ M_n = 28,280 Da (solid line) and poly(α-gal-C^{HO2})$_{88}$ M_n = 31,100 (dashed line). All samples analyzed at concentrations of 0.5 mg/mL in PBS buffer at 20 ^{o}C. Molar ellipticity is reported in degrees·cm^2·dmol^{-1}. i = H_2O_2, H_2O-AcOH (98% yield).

4.4 Conclusions

While other synthetic homopolypeptides undergo triggered helix/coil transitions coupled with a change in water solubility, the incorporation of sugars in our polymers imparts good water solubility for both conformers. The solubility of both conformations is advantageous since it allows for the presentation of sugar functionalities in different contexts in solution, which may be used to tune of their interactions with biomolecules and biological surfaces in different applications.[24] Also, oxidative switching of the chain conformations occurs in aqueous solution, making the helix to coil transition amenable for use in devices in vitro, or in aqueous self-assembled materials. The oxidation of thioether groups in proteins is known to occur naturally, and the consequent perturbed folding can serve as a signal for the oxidized protein to be degraded by the proteasome.[25] In a similar manner, the poly(glyco-C)s may also be able to sense oxidative environments in biological systems and respond to them by changing conformation.

4.5 Experimental

4.5.1 Materials and Methods

Unless stated otherwise, reactions were conducted in oven-dried glassware under an atmosphere of nitrogen using anhydrous solvents. Hexanes, THF, DCM, and DMF were purified by first purging with dry nitrogen, followed by passage through columns of activated alumina (or 4Å molecular sieves for DMF). EtOAc and MeCN were freshly distilled from CaH_2. Deionized water (18 MΩ-cm) was obtaining by passing in-house deionized water through a Millipore Milli-Q Biocel A10 purification unit. All commercially obtained reagents were used as received without further purification unless otherwise stated. Reaction temperatures were controlled using

an IKA temperature modulator, and unless stated otherwise, reactions were performed at room temperature (RT, approximately 20 °C). Thin-layer chromatography (TLC) was conducted with EMD gel 60 F254 precoated plates (0.25 mm) and visualized using a combination of UV, anisaldehyde, and phosphomolybdic acid staining. Selecto silica gel 60 (particle size 0.032–0.063 mm) was used for flash column chromatography. ^{1}H NMR spectra were recorded on Bruker spectrometers (at 500 MHz) and are reported relative to deuterated solvent signals. Data for ^{1}H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity, coupling constant (Hz) and integration. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet and br, broad. ^{13}C NMR spectra were recorded on Bruker Spectrometers (at 125 MHz). Data for ^{13}C NMR spectra are reported in terms of chemical shift. High-resolution mass spectrometry (HRMS) was performed on a Micromass Quatro-LC Electrospray spectrometer with a pump rate of 20 μL/min using electrospray ionization (ESI). All Fourier Transform Infrared (FTIR) samples were prepared as thin films on NaCl plates, spectra were recorded on a Perkin Elmer RX1 FTIR spectrometer, and are reported in terms of frequency of absorption (cm^{-1}). Tandem gel permeation chromatography/light scattering (GPC/LS) was performed on a SSI Accuflow Series III liquid chromatograph pump equipped with a Wyatt DAWN EOS light scattering (LS) and Optilab rEX refractive index (RI) detectors. Separations were achieved using 10^{5}, 10^{4}, and 10^{3} Å Phenomenex Phenogel 5 □m columns using 0.10 M LiBr in DMF as the eluent at 60 °C. All GPC/LS samples were prepared at concentrations of 5 mg/mL. Circular dichroism spectra were recorded on an OLIS RSM CD spectrophotometer running in conventional scanning mode.

4.5.2 Polymerization Data

Polymerization of Glyco-C NCAs

Monomer[a]	M_n[b]	M_w/M_n[c]	DP[d]	yield (%)[e]
10 α-glc-C NCA	11,400	1.11	24	95
20 α-glc-C NCA	22,800	1.09	48	99
40 α-glc-C NCA	37,525	1.10	79	94
60 α-glc-C NCA	53,200	1.09	112	98
80 α-glc-C NCA	69,825	1.08	147	99
100 α-glc-C NCA	88,350	1.06	186	97

Table 4.2 Molecular weight (M_n) of poly(α-glc-C) as a function of monomer to initiator ratio ([M]/[I]) using $(PMe_3)_4Co$ in THF at 20 °C. [a] Number indicates equivalents of monomer per $(PMe_3)_4Co$. [b] Molecular weight after polymerization as determined by ^1H NMR endgroup analysis. [c] Polydispersity index after polymerization as determined by GPC/LS. [d] DP = number average degree of polymerization by ^1H NMR endgroup analysis. [e] Total isolated yield of glycopolypeptide.

Monomer[a]	M_n[b]	M_w/M_n[c]	DP[d]	yield (%)[e]
10 α-gal-C NCA	9,975	1.12	21	99
20 α-gal-C NCA	19,475	1.08	41	95
40 α-gal-C NCA	34,200	1.03	72	96
60 α-gal-C NCA	48,925	1.09	103	97
80 α-gal-C NCA	62,700	1.08	132	99
100 α-gal-C NCA	84,550	1.09	178	97

Table 4.3 Molecular weight (M_n) of poly(α-gal-C) as a function of monomer to initiator ratio ([M]/[I]) using $(PMe_3)_4Co$ in THF at 20 °C. [a] Number indicates equivalents of monomer per $(PMe_3)_4Co$. [b] Molecular weight after polymerization as determined by ^1H NMR by endgroup analysis. [c] Polydispersity index after polymerization as determined by GPC/LS. [d] DP = number average degree of polymerization by ^1H NMR by endgroup analysis. [e] Total isolated yield of glycopolypeptide.

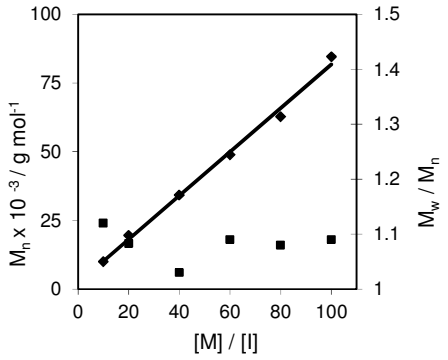


Figure 4.9 Molecular weight (M_n, ♦) and polydispersity index (M_w/M_n, ■) of poly(α-gal-C) as functions of monomer to initiator ratio ([M]/[I]) using $(PMe_3)_4Co$ in THF at 20 °C.

Statistical copolymerization of equimolar α-glc-C and Lys NCAs at different monomer to initiator ([M]/[I]) ratios

	Monomer[a]	M_n[b]	M_w/M_n[c]	DP[d]	yield (%)[e]
10	α-glc-C/Lys NCA	23,100	1.18	63	97
50	α-glc-C/Lys NCA	39,580	1.16	107	92
75	α-glc-C/Lys NCA	58,320	1.13	158	98
100	α-glc-C/Lys NCA	80,870	1.14	218	96

Table 4.4 Molecular weight (M_n) of poly(α-glc-C)-*stat*-poly(CBz-Lys) prepared from copolymerization of an equimolar mixture of α-glc-C and Lys NCAs as a function of monomer to initiator ratio ([M]/[I]) using $(PMe_3)_4Co$ in THF at 20 °C. [a] Number indicates equivalents of monomer per $(PMe_3)_4Co$. [b] Molecular weight as determined by GPC/LS. [c] Polydispersity index after polymerization as determined by GPC/LS. [d] DP = number average degree of polymerization. [e] Total isolated yield of glycopolypeptide copolymers.

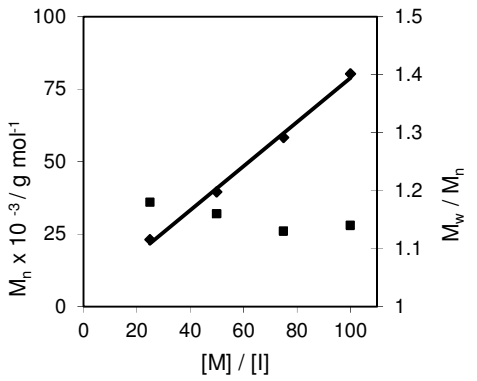


Figure 4.10 Copolymerization of an equimolar mixture of α-glc-C and Lys NCAs. Molecular weight (M_n, ♦) and polydispersity index (M_w/M_n, ■) of poly(α-glc-C)-*stat*-poly(CBz-Lys) as functions of monomer to initiator ratio ([M]/[I]) using $(PMe_3)_4Co$ in THF at 20 °C.

4.5.3 Check for Chain Cleavage Upon Oxidation

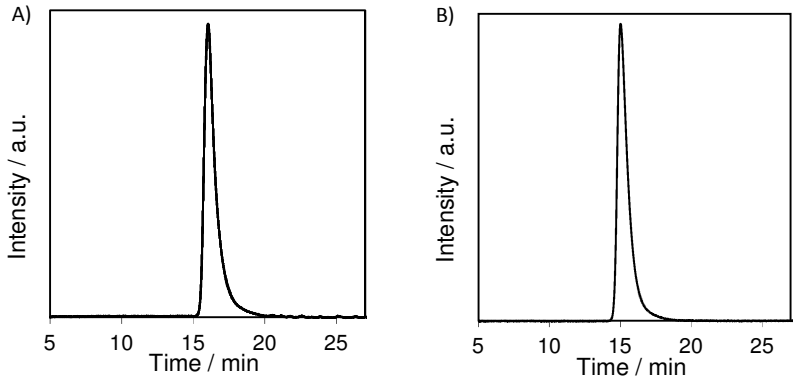


Figure 4.11 GPC chromatogram (normalized LS intensity in arbitrary units (a.u.) versus elution time in minutes) of A) poly(α-gal-C) **5a**, M_w/M_n = 1.09; and B) poly(α-gal-C) **5a** after treatment with 5% acetic acid and 10% H_2O_2 in DI water, 38 ^oC, 16 hours, M_w/M_n = 1.10.

4.5.4 X-ray crystal structure analysis of α-Gal-C^{O2} NCA

α-Gal-C^{O2} NCA, **9a**, was crystallized by vapor diffusion of hexanes into a THF solution of **9a**. Analysis was performed using a Bruker APEX II CCD-array single crystal X-ray diffractometer.

Data was obtained with graphite-monochromated Mo Kα (λ = 0.71073 Å) radiation. Crystal data are given in Table S4.

	9a
color, habit	colorless plate
T, K	100(2)
crystal system	monoclinic
Z	2
a, Å	12.622(3)
b, Å	5.2414(14)
c, Å	22.052(6)
α, deg	90
β, deg	93.864(3)
γ, deg	90
V, Å3	1455.5(7)

Table 4.5 Crystallographic data for α-gal-C^{O2} NCA, **9a**.

4.5.5 Circular Dichroism of Glycopolypeptides

Circular Dichroism spectra (190–250 nm) were recorded in a quartz cuvette of 0.1 cm path length with samples prepared at concentrations of 0.25 to 1.0 mg/mL using Millipore deionized water. All spectra were recorded as an average of 3 scans. The spectra are reported in units of molar ellipticity [θ] (deg·cm^2·dmol^{-1}). The formula used for calculating molar ellipticity, [θ], was [θ] = (θ x 100 x M_W)/(c x l) where θ is the experimental ellipticity in millidegrees, M_W is the average molecular weight of a residue in g/mol, c is the peptide concentration in mg/mL; and l is the cuvette pathlength in cm. The percent α-helical content of the glycopeptides was estimated using the formula % α-helix = 100x(-$[\theta]_{222nm}$ +3000)/39000) where $[\theta]_{222nm}$ is the measured molar ellipticity at 222 nm.[26] Using this estimation, poly(α-glc-C)$_{97}$ and poly(α-gal-C)$_{108}$ were greater then 60% α-helical in deionized water, 20 ^{o}C; and the homologous poly(α-gal-C^H)$_{88}$ was *ca.* 100% α-helical in deionized water, 20 ^{o}C. The partially oxidized sulfoxide polymer poly(α-

gal-C^{O})$_{108}$ was *ca.* 50% α-helical in deionized water, 20 °C. The fully oxidized sulfone polymers, poly(α-glc-C^{O2})$_{97}$ and poly(α-gal-C^{O2})$_{108}$, were less than 15% α-helical in deionized water, 20 °C; and the oxidized homologous poly(α-gal-C^{HO2})$_{88}$ was *ca.* 100% α-helical in deionized water, 20 °C.

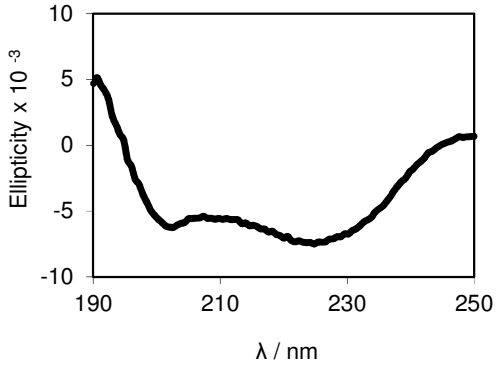


Figure 4.12 Circular dichroism spectrum of poly(α-gal-C)$_{108}$ M_n = 33,199 Da, prepared as a thin film cast from a 0.5 mg/mL solution in deionized water, 20 °C. Spectrum shows sample adopts an α-helical conformation in the solid state. Since sample is a solid film, molar ellipticity could not be calculated. Ellipticity is reported in degrees·cm^2.

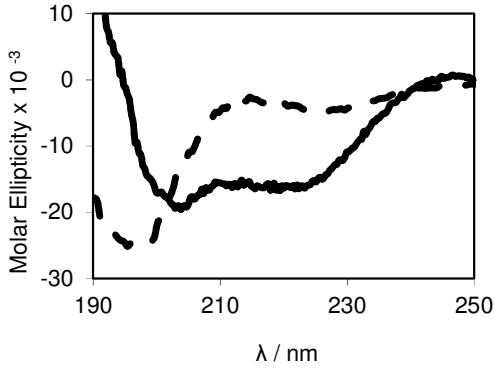


Figure 4.13 Circular dichroism spectra of poly(α-glc-C)$_{97}$ M_n = 29,810 Da (solid line), and poly(α-glc-C^{O2})$_{97}$ M_n = 32,920 Da (dashed line) in PBS buffer, 0.5 mg/mL, 20 °C.

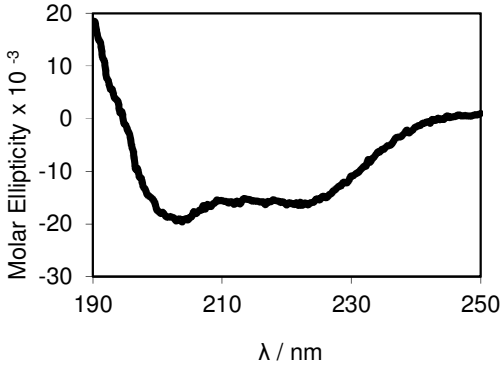
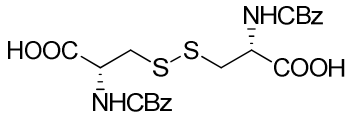


Figure 4.14 Circular dichroism spectrum of poly(α-gal-C^{O})$_{108}$ M_n = 34,920 Da in PBS buffer, 0.5 mg/mL, 20 °C.

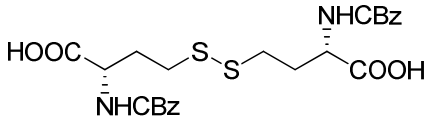
4.5.6 Experimental Procedures



***N*-CBz-L-Cystine**

L-Cystine (6.00 g, 25.0 mmol) was dissolved in 9:1 water:THF (60 mL). 6M NaOH in water was added dropwise until pH 10 was reached. The cloudy suspension was cooled in an ice bath and CBz-Cl (8.79 mL, 62.4 mmol, 2.5 equiv) was added dropwise over 15 min via syringe. The pH was monitored and kept at pH 10 by addition of drops of 6M NaOH. The mixture was stirred for 3 hours as the ice bath expired. Water was added (100 mL) and the reaction was made basic to pH 10, transferred to a separatory funnel, and the aqueous phase was washed with Et_2O (3 x 100 mL). Concentrated HCl was added dropwise with stirring until the mixture reached pH 2, with formation of a white precipitate. The solids were extracted with ethyl acetate (3 x 150 mL), and the combined organic phases were washed with pH 2 water (2 x 100 mL) followed by brine (100 mL), and dried with $MgSO_4$. The solution was filtered and condensed to 12.4 g of a white solid, 98% yield. No further purification required.

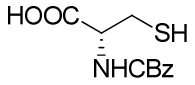
^{1}H NMR (500 MHz, CD_3OD, 25 ^{o}C): δ 7.34-7.24 (m, 10H), 5.07 (s, 4H), 4.51-4.48 (m, 2H), 3.24 (dd, *J* = 13.9, 4.2, 2H), 2.98 (dd, *J* = 13.8, 9.2, 2H); ^{13}C NMR (125 MHz, CD_3OD, 25 ^{o}C): δ172.4, 157.0, 136.6, 127.9, 127.4, 127.3, 66.2, 53.1, 39.6, ; FTIR (DCM): 3053, 2986, 2305,1721, 1622, 1581, 1480, 1422, 1350, 1335 cm^{-1}; HRMS-ESI (*m/z*) [M + H]$^{+}$ Calcd for $C_{22}H_{24}N2O_8S_2$, 508.10; found 509.11.



_N_-CBz-L-Homocystine

N-CBz-L-Homocystine was prepared from homocystine (1.0 g, 3.73 mmol) according to the procedure for *N*-CBz-L-cystine, and was recovered as a white solid (1.90 g, 95%).

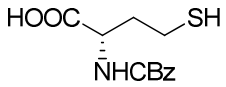
^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 7.35-7.29 (m, 5H), 6.21 (d, *J* = 7.4, 1H), 5.26 (dd, *J* = 9.0, 9.0, 1H), 5.12 (s, 2H), 5.02 (dd, *J* = 9.8, 5.1, 1H), 4.93 (dd, *J* = 8.9, 8.9, 1H), 4.78-4.73 (m, 1H), 4.21 (dd, *J* = 12.2, 5.5, 1H), 4.16-4.12 (m, 1H), 4.11-4.07 (m, 1H), 3.84-3.80 (m, 1H), 3.67 (d, *J* = 3.3, 2H), 3.09-3.01 (m, 1H), 2.06 (s, 3H), 2.03 (s, 3H), 2.01 (s, 6H), 2.00-1.84 (m, 2H), 1.82-1.72 (m, 1H), 1.62-1.53 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ 171.2, 170.2, 169.9, 169.6, 156.1, 135.7, 128.5, 128.3, 128.0, 71.6, 70.0, 69.9, 68.9, 68.5, 67.4, 62.1, 53.8, 53.3, 49.7, 23.8, 20.6, 20.6, 20.6, 20.5, 17.6; FTIR (DCM): 2930, 1746, 1716, 1654, 1508, 1458, 1370, 1220 cm^{-1}; HRMS-ESI (*m/z*) [M + H]$^{+}$ Calcd for $C_{24}H_{28}N_2O_8S_2$, 536.13; found 537.14.



_N_-CBz-L-Cysteine, 1

_N_-CBz-L-Cystine (19.5 g, 38.4 mmol) was dissolved in THF:H_2O 10:1 (300 mL) and PPh_3 (11.1 g, 42.2 mmol, 1.1 equiv) was added. The mixture was stirred at room temperature for 16 hours. The THF was evaporated with rotary evaporation, then water was added (300 mL), and the reaction was made basic to pH 10 with 6M NaOH. The aqueous phase was extracted with EtOAc (4 x 100 mL) to remove $OPPh_3$ and excess PPh_3. Concentrated HCl was then added dropwise with stirring until the mixture reached pH 2, with formation of a white precipitate. The solids were extracted using ethyl acetate (3 x 150 mL), and the combined organic phases were washed with pH 2 water (100 mL) followed by brine (100 mL), and dried with $MgSO_4$. The solution was filtered and condensed to 19.2 g of 1 as a white solid, in 98% yield. No further purification required.

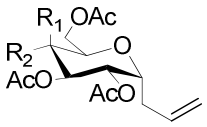
^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 9.89 (br s, 1H), 7.39-7.31 (m, 5H), 5.76 (d, J = 7.1, 1H), 5.14 (s, 2H), 4.75-4.71 (m, 1H), 3.11-3.05 (m, 1H), 3.02-2.97 (m, 1H), 1.47 (t, J = 9.0, 1H) ; ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ174.3, 155.8, 135.7, 128.5, 128.1, 120.0, 67.4, 54.9, 26.8; FTIR (DCM): 3419, 3053, 2986, 2305, 1721, 1510, 1422, 1335 cm^{-1}; HRMS-ESI (*m/z*) $[M + H]^+$ Calcd for $C_{11}H_{13}NO_4S$, 255.06; found 255.07.



_N_-CBz-L-Homocysteine, 11

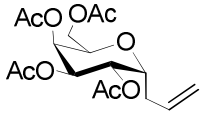
_N_-CBz-L-Homocysteine was prepared from _N_-CBz-L-homocystine (1.90 g, 3.54 mmol) according to the procedure for **1** and was recovered as a white solid (1.87 g, 98%).

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 10.2 (br s, 1H), 7.36-7.31 (m, 5H), 5.54 (s, 1H), 5.11 (s, 2H), 4.59-4.55 (m, 1H), 2.61-2.55 (m, 2H), 2.19-2.12 (m, 1H), 2.04-1.96 (m, 1H), 1.54 (t, *J* = 8.7, 1H); ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ176.3, 156.3, 135.8, 128.2, 128.0, 127.9, 67.3, 52.5, 36.4, 20.6; FTIR (DCM): 2930, 1746, 1716, 1654, 1508, 1458, 1370, 1220 cm^{-1}; HRMS-ESI (*m/z*) $[M + H]^+$ Calcd for $C_{12}H_{15}NO_4S$, 269.07; found 270.08.



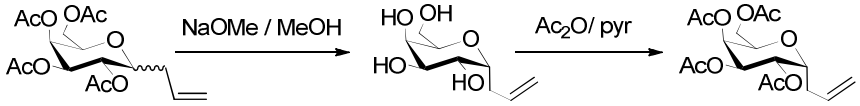
Example Procedure for Allylation of Galactose and Glucose Pentaacetates, 2a-b[27]

To a solution of D-galactose pentaacetate (3.00 g, 7.69 mmol) in dry acetonitrile (30 mL) at 0 ^{o}C under N_2 was added allyltrimethylsilane (2.45 mL, 15.4 mmol, 2 equiv). TMSOTf (1.39 mL, 7.68 mmol, 1 equiv) was then added dropwise. The reaction mixture was stirred for 16 hours as the ice bath expired. The reaction was monitored by TLC (40% EtOAc in hexanes, anisaldehyde stain). After 16 hours allyltrimethylsilane was added (1.23 mL, 7.69 mmol, 1 equiv) and the reaction stirred a further 4 hours. The reaction was slowly poured into saturated $NaHCO_3$ (100 mL) and stirred until the evolution of gases ceased. The mixture was extracted with CH_2Cl_2 (3 x 75 mL), washed with water (100 mL) then brine (100 mL), and dried over $MgSO_4$. Following evaporation of the solvent under reduced pressure, the crude residue was purified by flash chromatography (35% EtOAc in hexanes) to give 2.37 g of *C*-glycoside 2a as a glassy solid (83 % yield, *α:β* ca. 11:l by ^{1}H NMR). Physical data obtained were in agreement with literature values.[27, 28]

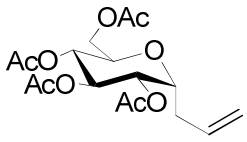


1-Allyl-2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranoside, α-2a

Separation of α and β anomers:

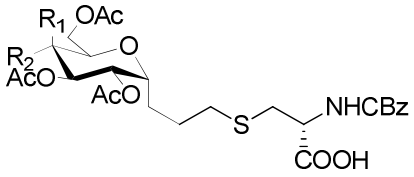


To a solution of **2a** *α:β* 11:l (5.15 g, 13.8 mmol) in methanol (50 mL) was added 0.5 M sodium methoxide in methanol (2.8 mL, 1.38 mmol, 0.1 equiv). The reaction was monitored by TLC. After 4 hours at room temperature, the reaction mixture was neutralized by stirring with Dowex 50W-X8 [H^+] resin for 30 min, filtered, and concentrated to give 2.81 g of a white solid (quantitative yield). The residue was crystallized from methanol/diethyl ether by dissolution in minimum boiling methanol with addition of ether until the cloud point, followed by slow cooling to -20 ^oC. Pure alpha anomer crystals were collected by filtration, 2.19 g (78%). The pure alpha sugar was reacetylated by treatment with 15 mL of acetic anhydride and 15 mL of pyridine followed by stirring overnight at room temperature. The reaction was slowly poured into ice water (200 mL) and then extracted with ethyl acetate (3 x 200 mL). The organic layer was washed with saturated $NaHCO_3$ until the evolution of gasses ceased (2 x 200 mL), and then washed with water (1 x 200 mL), and brine (1 x 200 mL). The organic phase was dried over $MgSO_4$ and condensed to 4.01 g of ***α*-2a** as a white solid (97 %). Physical data obtained were in agreement with literature values.[28, 29]



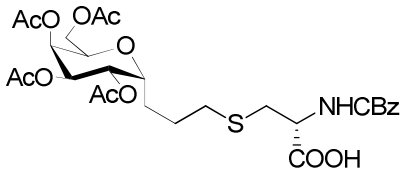
1-Allyl-2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside, α-2b

2b was prepared from glucose pentacetate (2.50 g, 6.41 mmol) according to the procedure for **2a**, (1.93 g, 81% yield, *α:β* ca. 10:1 by ^{1}H NMR). Anomers were separated by crystallization from minimum boiling $CHCl_3$ with addition of hexanes until the cloud point, followed by slow cooling to -20 ^{o}C to yield α-2b 1.67 g (70% after crystallization).



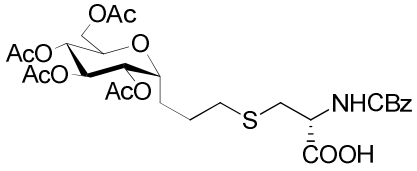
Example Procedure for Thiol-ene Coupling to Prepare Glyco-C Conjugates

N-CBz-L-Cysteine **1** (6.40 g, 25.4 mmol, 2.5 equiv) was dissolved in DMF (50 mL). **2a** (3.78 g, 10.2 mmol, 1 equiv) and 2,2-dimethoxy-2-phenylacetophenone (0.789 g, 3.04 mmol, 0.15 equiv) were added. The reaction was vacuum purged and backfilled with N_2 three times. The flask was then subjected to 365 nm light (handheld UV lamp with filter removed) for 1 hour. Water was added (500 mL) and the mixture extracted with EtOAc (3 x 150 mL). The combined organic layers were washed with water (3 x 150 mL), followed by brine (100 mL), dried with $MgSO_4$, filtered, and condensed to a yellow oil. The crude reaction was chromatographed on silica, 45% EtOAc in hexanes with 1% AcOH until all excess thiol was flushed off the column, followed by 60% EtOAc in hexanes with 1% AcOH, to give 5.99 g of 3a as a white solid (94% yield).



***N*-Carbobenzyloxy-L-cysteine-1-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranoside), 3a**

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 8.93 (br s, 1H), 7.35-7.29 (m, 5H), 5.75 (d, *J* = 7.9, 1H), 5.38 (dd, *J* = 2.7, 2.7, 1H), 5.23 (dd, *J* = 9.4, 5.0, 1H), 5.18 (dd, *J* = 9.4, 3.2, 1H), 5.11 (s, 2H), 4.63-4.59 (m, 1H), 4.26 (dd, *J* = 12.8, 9.0, 1H), 4.21-4.16 (m, 1H), 4.05-4.02 (m, 2H), 3.07-2.96 (m, 2H), 2.65-2.59 (m, 2H), 2.56-2.50 (m, 2H), 2.11 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 2.01 (s, 3H), 1.79-1.63 (m, 2H), 1.62-1.47 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ174.0, 170.9, 170.1, 170.1, 170.0, 155.8, 135.9, 128.4, 128.1, 128.0, 71.5, 68.3, 67.9, 67.8, 67.5, 67.1, 61.5, 53.4, 34.1, 32.1, 25.0, 24.2, 20.7, 20.6, 20.6, 20.5; FTIR (DCM): 3054, 2986, 2305, 1748, 1720, 1641, 1506, 1421, 1320 cm^{-1}; HRMS-ESI (*m/z*) $[M + Na]^+$ Calcd for $C_{28}H_{36}NO_{13}SNa$, 649.18; found 650.18. $[M + H]^+$ Calcd for $C_{28}H_{37}NO_{13}S$, 627.20; found 628.21.

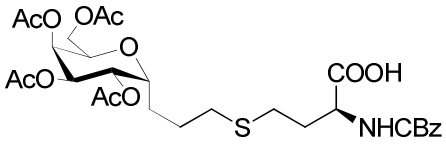


***N*-Carbobenzyloxy-L-cysteine-1-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranoside), 3b**

3b was prepared from **α-2b** (1.67 g, 4.48 mmol) according to the procedure for **3a**, and recovered as a white solid (2.67 g, 95%).

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 8.36 (br s, 1H), 7.35-7.29 (m, 5H), 5.71 (d, *J* = 7.7, 1H), 5.29 (dd, *J* = 9.1, 9.1, 1H), 5.12 (s, 2H), 5.04 (dd, *J* = 9.5, 5.7, 1H), 4.98 (dd, *J* = 8.6, 8.6, 1H), 4.64-4.59 (m, 1H), 4.23 (dd, *J* = 12.2, 5.2, 1H), 4.18-4.13 (m, 1H), 4.09 (dd, *J* = 12.1, 2.4, 1H), 3.83-3.80 (m, 1H), 3.08-3.03 (m, 1H), 3.00-2.96 (m, 1H), 2.66-2.61 (m, 1H), 2.57-2.51 (m, 1H),

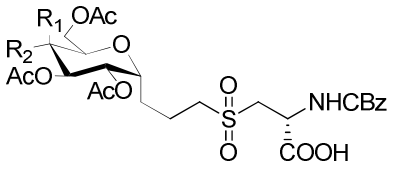
2.07 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 2.02 (s, 3H), 1.85-1.71 (m, 1H), 1.71-1.53 (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ170.9, 170.2, 169.9, 169.5, 155.8, 128.4, 128.2, 128.0, 71.9, 70.3, 70.2, 68.7, 68.6, 67.1, 62.2, 53.3, 32.0, 24.7, 23.9, 20.6, 20.6, 20.6, 20.5, ; FTIR (DCM): 3055, 2986, 2303, 1748, 1721, 1640, 1508, 1421, 1320 cm^{-1}; HRMS-ESI *m/z*) $[M + Na]^+$ $C_{28}H_{36}NO_{13}SNa$, 649.18; found 650.19. $[M + H]^+$ Calcd for $C_{28}H_{37}NO_{13}S$, 627.20; found 628.20.



***N*-Carbobenzyloxy-L-homocysteine-1-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranoside), 12a**

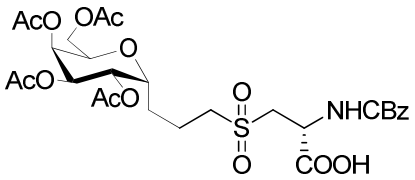
12a was prepared from **11** (1.87 g, 6.94 mmol) and **2a** (1.03 g, 2.78 mmol) and according to the procedure for **3a** and was recovered as a white solid (1.66 g, 93%).

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 10.4 (br s, 1H), 7.34-7.25 (m, 5H), 5.71 (d, *J* = 7.7, 1H), 5.39-5.45 (m, 1H), 5.27-5.30 (m, 2H), 5.16 (dd, *J* = 9.5, 3.2, 1H), 5.07 (s, 2H), 4.52-4.44 (m, 1H), 4.24-4.14 (m, 2H), 4.05-3.98 (m, 2H), 2.60-2.45 (m, 4H), 2.21-2.00 (m, 14H), 1.84-1.47 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ176.7, 170.8, 170.2, 170.0, 170.0, 156.2, 136.0, 128.4, 128.1, 127.9, 71.7, 68.2, 67.8, 67.5, 67.0, 61.5, 53.4, 52.9, 32.0, 31.3, 27.6, 24.9, 24.3, 20.7, 20.7, 20.6, 20.5; FTIR (DCM): 2930, 1746, 1716, 1654, 1508, 1458, 1370, 1220 cm^{-1}; HRMS-ESI (*m/z*) $[M + Na]^+$ Calcd for $C_{29}H_{38}NO_{13}SNa$, 663.20; found 664.21, $[M + H]^+$ Calcd for $C_{29}H_{39}NO_{13}S$, 641.21; found 642.22.



Example Procedure for Oxidation of Glyco-C Conjugates, 8a-b

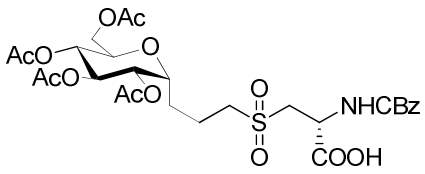
3a (0.550 g, 0.876 mmol) was dissolved in glacial acetic acid (5 mL) and hydrogen peroxide (0.5 mL, 4.38 mmol, 5 equiv of a 30 % aqueous solution) was added. The reaction was stirred at 50 ^{o}C for 6 hours. The reaction was cooled to room temperature and 1 M sodium thiosulfate was added (10 mL). After 15 min, the reaction was diluted with water (100 mL) and extracted with EtOAc (3 x 50 mL). The combined organic phases were washed with 10% aqueous bicarbonate (50 mL), water (50 mL), and brine (50 mL), dried with $MgSO_4$, and condensed to 0.575 g of **8a** as a white solid (99%). No further purification required.



***N*-Carbobenzyloxy-L-cysteine sulfone-1-(2,3,4,6-tetra-*O*-acetyl-α-D-galacto-pyranoside) 8a**

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 7.38-7.29 (m, 5H), 6.15 (d, *J* = 7.3, 1H), 5.39 (dd, *J* = 2.8, 2.8, 1H), 5.22 (dd, *J* = 9.0, 4.8, 1H), 5.17 (dd, *J* = 9.0, 3.2, 1H), 5.13 (s, 2H), 4.78-4.73 (m, 1H), 4.31 (dd, *J* = 12.4, 9.1, 1H), 4.23-4.16 (m, 1H), 4.07-4.02 (m, 2H), 3.70-3.65 (m, 2H), 3.11-3.05 (m, 2H), 2.09 (s, 3H), 2.06 (s, 3H), 2.05 (s, 3H), 2.02 (s, 3H), 2.00-1.92 (m, 2H), 1.86-1.76 (m, 1H), 1.59-1.50 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ 171.2, 171.1, 170.1, 170.1, 170.0, 156.0, 135.7, 128.5, 128.2, 128.0, 71.7, 68.4, 68.3, 67.8, 67.4, 67.4 61.4, 53.8, 53.4, 49.8, 24.3, 20.7, 20.6, 20.6, 20.5, 17.9; FTIR (DCM): 2930, 1746, 1716, 1654, 1508, 1458, 1370, 1220

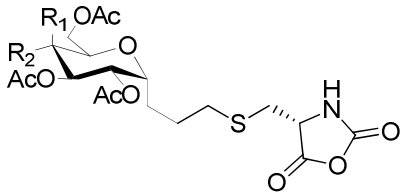
cm^{-1}; HRMS-ESI (*m/z*) [M + Na]$^+$ Calcd for $C_{28}H_{36}NO_{15}SNa$, 681.17; found 682.18. [M + H]$^+$ Calcd for $C_{28}H_{37}NO_{15}SNa$, 659.19; found 660.20.



***N*-Carbobenzyloxy-L-cysteine sulfone-1-(2,3,4,6-tetra-*O*-acetyl-α-D-gluco-pyranoside) 8b**

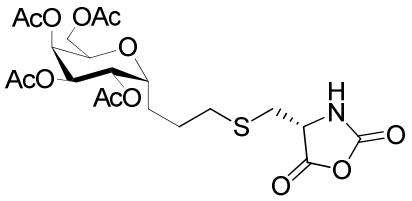
8b was prepared from **3b** (0.456 g, 0.727 mmol) according to the procedure for **8a**, and recovered as a white solid (0.478 g, 99%).

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 7.35-7.29 (m, 5H), 6.21 (d, *J* = 7.4, 1H), 5.26 (dd, *J* = 9.0, 9.0, 1H), 5.12 (s, 2H), 5.02 (dd, *J* = 9.8, 5.1, 1H), 4.93 (dd, *J* = 8.9, 8.9, 1H), 4.78-4.73 (m, 1H), 4.21 (dd, *J* = 12.2, 5.5, 1H), 4.16-4.12 (m, 1H), 4.11-4.07 (m, 1H), 3.84-3.80 (m, 1H), 3.67 (d, *J* = 3.3, 2H), 3.09-3.01 (m, 1H), 2.06 (s, 3H), 2.03 (s, 3H), 2.01 (s, 6H), 2.00-1.84 (m, 2H), 1.82-1.72 (m, 1H), 1.62-1.53 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ 171.2, 170.2, 169.9, 169.6, 156.1, 135.7, 128.5, 128.3, 128.0, 71.6, 70.0, 69.9, 68.9, 68.5, 67.4, 62.1, 53.8, 53.3, 49.7, 23.8, 20.6, 20.6, 20.6, 20.5, 17.6; FTIR (DCM): 2930, 1746, 1716, 1654, 1508, 1458, 1370, 1220 cm^{-1}; HRMS-ESI (*m/z*) [M + Na]$^+$ Calcd for $C_{28}H_{36}NO_{15}SNa$, 681.17; found 682.17. [M + H]$^+$ Calcd for $C_{28}H_{37}NO_{15}S$, 659.19; found 660.20.



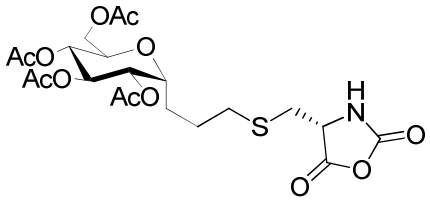
Example Procedure for Preparation of Glyco-C NCAs, 4a-b

To a solution of **3a** (1.57 g, 2.50 mmol) in dry CH_2Cl_2 (50 mL) in a Schlenk flask under N_2 was added α,α-dichloromethylmethyl ether (0.44 mL, 5.00 mmol, 2 equiv). The solution was refluxed under N_2 for 36 hours, then evaporated to dryness under reduced pressure and transferred to a dinitrogen filled glove box. The crude yellow oil was dissolved in 15 mL of dry THF and precipitated into 150 mL of dry hexanes to remove benzyl chloride. The resulting white solids were purified by silica chromatography[30] with a gradient of 55% to 65% dry THF in dry hexanes to give 0.818 g of **4a** as a white solid (63%). High purity NCA was also obtained without column chromatography by performing an aqueous workup of the reaction mixture followed by repeated precipitation according to the following procedure. After the 36 hours of reflux, the reaction mixture was cooled in an ice bath and diluted with 4 ^{o}C DCM. The solution was washed with 2 small portions of 4 ^{o}C 10% $NaHCO_3$ in DI water, followed by 2 portions of DI water. The organic phase was dried with $MgSO_4$, filtered, and evaporated to give solid NCA. The washed NCA was brought into the glove box, diluted with dry THF and precipitated into dry hexanes. Solids were collected by centrifugation and this precipitation was repeated a total of three times.



2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl-L-cysteine-*N*-carboxyanhydride (α-gal-C NCA) 4a

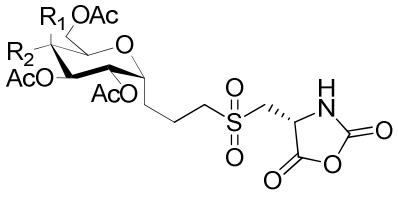
^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ6.63 (s, 1H), 5.40 (dd, *J* = 2.8, 2.8, 1H), 5.24 (dd, *J* = 9.1, 4.9, 1H), 5.18 (dd, *J* = 9.1, 3.2, 1H), 4.55-4.52 (m, 1H), 4.38 (dd, *J* = 11.3, 7.0 1H), 4.23-4.19 (m, 1H), 4.06-4.03 (m, 1H), 3.98 (dd, *J* = 11.4, 5.7, 1H), 3.12 (dd, *J* = 14.1, 2.6, 1H), 2.86 (dd, *J* = 14.2, 1.9, 1H), 2.71-2.58 (m, 1H), 2.13 (s, 3H), 2.08 (s, 3H), 2.08 (s, 3H), 2.03 (s, 3H), 1.83-1.60 (m, 3H), 1.55-1.49 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ 171.1, 169.9, 169.9, 169.8, 168.2, 151.5, 71.3, 68.3, 68.0, 67.7, 67.2, 61.1, 57.6, 33.5, 32.5, 25.0, 24.3, 20.8, 20.7, 20.6, 20.5; FTIR (DCM): 3560, 3496, 3052, 2984, 1858, 1790, 1746, 1420, 1368, 1294 cm^{-1}; HRMS-ESI negative ion mode (*m/z*) $[M- H]^-$ Calcd for $C_{21}H_{29}NO_{12}S$, 519.14; found 518.13. Melting point: 183-185 ^{o}C.



2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-L-cysteine-*N*-carboxyanhydride (α-glc-C NCA) 4b

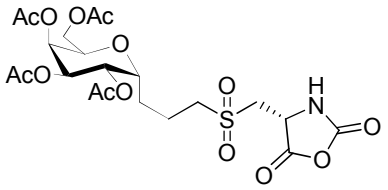
4b was prepared from **3b** (0.660 g, 1.05 mmol) according to the procedure for **4a** and was recovered as a white solid (0.366 g, 67%).

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ6.49 (s, 1H), 5.29 (dd, *J* = 8.9, 8.9, 1H), 5.05 (dd, *J* = 9.2, 5.7, 1H), 4.96 (dd, *J* = 8.9, 8.9, 1H), 4.53 (dd, *J* = 6.7, 3.6, 1H), 4.26 (dd, *J* = 12.1, 5.1, 1H), 4.20-4.16 (m, 1H), 4.12 (dd, *J* = 12.2, 2.8, 1H), 3.85-3.82 (m, 1H), 3.10 (dd, *J* = 14.3, 3.5, 1H), 2.87 (dd, *J* = 14.3, 6.9, 1H), 2.70-2.60 (m, 1H), 2.10 (s, 3H), 2.07 (s, 3H), 2.05 (s, 3H), 2.03 (s, 3H), 1.90-1.82 (m, 1H), 1.77-1.56 (m, 3H); ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ170.9, 170.0, 169.7, 169.5, 168.1, 151.4, 71.8, 70.1, 70.0, 69.0, 68.6, 62.4, 57.7, 33.6, 32.5, 24.9, 24.0, 20.7, 20.6, 20.6, 20.6; FTIR (THF): 3557, 3496, 2964, 2856, 2678, 1956, 1854, 1790, 1752, 1462, 1364 cm^{-1}; HRMS-ESI negative ion mode (*m/z*) $[M - H]^-$ Calcd for $C_{21}H_{29}NO_{12}S$, 519.14; found 518.14. Melting point: 189-191 ^{o}C.



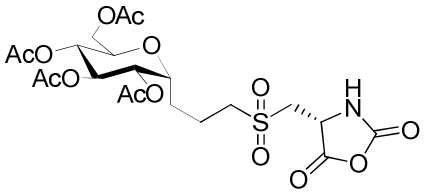
Example Procedure for Preparation of Glyco-C^{O2} NCAs, 9a-b

To a solution of **8a** (0.575 g, 0.876 mmol) in dry CH_2Cl_2 (18 mL) in a Schlenk flask under N_2 was added α,α-dichloromethylmethyl ether (0.156 mL, 1.75 mmol, 2 equiv). The solution was refluxed for 36 hours, then evaporated to dryness under reduced pressure and transferred to a dinitrogen filled glove box. The crude yellow oil was dissolved in 8 mL of dry THF and crystallized by layering underneath 16 mL dry hexanes. Yield after 3 recrystallizations, 0.343 g of white needles **9a** (71%).



2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl-L-cysteine sulfone-*N*-carboxyanhydride (α-gal-C^{O2} NCA), 9a

^{1}H NMR (500 MHz, CD_3CN, 25 ^oC): δ 7.01 (s, 1H), 5.40 (d, *J* = 2.3, 1H), 5.21-5.16 (m, 2H), 4.93 (d, *J* = 7.6, 1H), 5.41 (dd, *J* = 11.0, 7.6, 1H), 4.23-4.22 (m, 1H), 4.08-4.05 (m, 1H), 4.00 (dd, *J* = 11.6, 4.7, 1H), 3.66 (dd, *J* = 13.2, 1H), 3.48-3.43 (m, 1H), 3.24-3.23 (m, 2H), 2.12 (s, 3H), 2.09 (s, 3H), 2.07 (s, 3H), 2.04 (s, 3H), 1.92-1.83 (m, 3H), 1.65-1.59 (m, 1H); ^{13}C NMR (125 MHz, CD_3CN, 25 ^oC): δ 171.1, 169.9, 169.9, 169.8, 167.4, 150.9, 68.7, 68.3, 67.6, 67.6, 67.1, 61.0, 53.6, 53.1, 51.8, 24.6, 20.7, 20.6, 20.6, 20.5, 18.0; FTIR (THF): 3562, 3494, 2966, 2850, 2676, 1956, 1850, 1792, 1750, 1458, 1364 cm^{-1}; HRMS-ESI negative ion mode (*m/z*) $[M - H]^-$ Calcd for $C_{21}H_{29}NO_{14}S$, 551.13; found 550.12. Melting point: 198-200 ^oC.

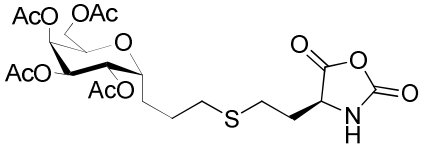


2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-L-cysteine sulfone-*N*-carboxyanhydride (α-glc-C^{O2} NCA), 9b

9b was prepared from **8b** (0.478 g, 0.727 mmol) according to the procedure for **9a**, and was recovered as a white needles. Yield after three recrystallizations, 0.280 g (70%).

^{1}H NMR (500 MHz, CDCN, 25 ^oC): δ 5.23 (dd, *J* = 8.8, 8.8, 1H), 5.23 (dd, *J* = 8.8, 8.8, 1H), 4.95 (dd, *J* = 9.1, 5.5, 1H), 4.89 (dd, *J* = 8.7, 8.7, 1H), 4.77 (dd, *J* = 7.1, 3.6, 1H), 4.19 (dd, *J* =

12.1, 6.3 1H), 4.13-4.09 (m, 1H), 4.05 (dd, *J* = 12.1, 2.7, 1H), 3.89-3.85 (m, 1H), 3.63-3.60 (m, 2H), 3.50 (dd, *J* = 15.0, 7.1, 1H), 3.16 (t, *J* = 7.8, 2H), , 2.01 (s, 3H), 1.99 (s, 3H), 1.98 (s, 3H), 1.97 (s, 3H), 1.80-1.70 (m, 3H), 1.65-1.59 (m, 1H); ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ 170.3, 169.7, 169.6, 169.5, 168.6, 151.2, 71.3, 69.8, 69.3, 69.1, 68.4, 62.0, 53.6, 52.4, 52.3, 23.6, 19.9, 19.8, 19.8, 19.8, 17.4; FTIR (THF): 3562, 3495, 2964, 2850, 2677, 1955, 1854, 1790, 1752, 1457, 1365 cm^{-1}; HRMS-ESI negative ion mode (*m/z*) [M - H]$^-$ Calcd for $C_{21}H_{29}NO_{14}S$, 551.13; found 550.13. Melting point: 199-201 ^{o}C.



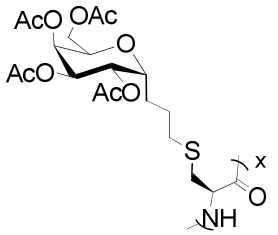
2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl-L-homocysteine-*N*-carboxyanhydride (α-gal-C^{H} NCA) 13a

13a was prepared from **12a** (1.66 g, 2.58 mmol) according to the procedure for **4a** and was recovered as a white solid (0.951 g, 69%).

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ7.34 (s, 1H), 5.38 (dd, *J* = 2.8, 2.8, 1H), 5.23-5.15 (m, 2H), 4.47 (dd, *J* = 6.2, 5.9, 1H), 4.32 (dd, *J* = 12.5, 3.5, 1H), 4.24-4.20 (m, 1H), 4.05-3.99 (m, 2H), 2.67 (t, *J* = 6.8, 2H), 2.62-2.48 (m, 2H), 2.27-2.19 (m, 1H), 2.12-1.99 (m, 13H), 1.87-1.46 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ 171.1, 170.0, 169.9, 169.9, 169.8, 152.0, 71.5, 68.3, 67.8, 67.7, 67.4, 61.1, 56.4, 31.3, 30.9, 27.4, 24.9, 24.3, 20.8, 20.7, 20.6, 20.5; FTIR (DCM): 3560, 3496, 3052, 2984, 1858, 1790, 1746, 1420, 1368, 1294 cm^{-1}; HRMS-ESI negative ion mode (*m/z*) [M - H]$^-$ Calcd for $C_{22}H_{31}NO_{12}S$, 533.16; found 532.15.

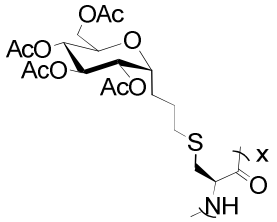
General Procedure for Polymerization of NCAs:

All polymerization reactions were performed in a dinitrogen filled glove box. To a solution of NCA in THF (50 mg/mL) was rapidly added, via syringe, a solution of $(PMe_3)_4$ Co in THF (30 mg/mL). The reaction was stirred at RT and polymerization progress was monitored by FTIR. Polymerization reactions were generally complete within 3 hours. Aliquots were removed for GPC/LS analysis immediately upon polymerization completion. Reactions were removed from the drybox and precipitated into hexanes. Solids were collected by centrifugation and washed with 2 portions of water at pH 2 (HCl), followed by DI water. The polymers were lyophilized to yield white solids. (95-99% yield).



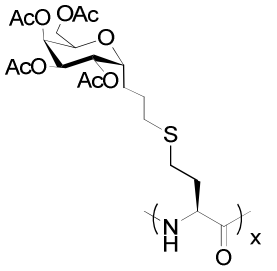
Poly-2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl-L-cysteine, 5a

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^oC): δ 5.35 (br s, 1H), 5.19-5.16 (br m, 2H), 4.25-4.13 (br m, 2H), 4.07-3.98 (br m, 2H), 3.07-2.82 (br m, 2H), 2.55 (br s, 2H), 2.07 (br s, 3H), 2.05-1.95 (br m, 9H), 1.84-1.64 (br m, 2H), 1.59-1.45 (br m, 2H); FTIR (film in THF): 3566, 3492, 3282, 2964, 2852, 2678, 1958, 1752, 1654, 1522, 1458, 1366 cm^{-1}.



Poly-2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl-L-cysteine, 5b

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 5.30 (br s, 1H), 5.12-4.95 (br m, 2H), 4.29-3.82 (br m, 5H), 3.13-2.95 (br m, 2H), 2.62 (br s, 2H), 2.04 (br m, 12H), 1.92-1.53 (br m, 4H); FTIR (film in THF): 3554, 3488, 2962, 2850, 2678, 1958, 1748, 1658, 1524, 1462, 1366 cm^{-1}.



Poly-2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl-L-homocysteine, 14a

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 5.34 (br s, 1H), 5.20 (br s, 2H), 4.22 (br s, 2H), 4.07 (br s, 3H), 2.77-2.49 (br m, 4H), 2.12-2.00 (br m, 14H), 1.87-1.50 (br m, 4H); FTIR (film in THF): 3565, 3494, 3281, 2964, 2853, 2678, 1958, 1749, 1651, 1542, 1458, 1366 cm^{-1}.

General Procedure for Determination of Poly(glyco-C) Molecular Weight by Endgroup Analysis:[31]

The general procedure for polymerization of glyco-C NCAs was followed. Upon completion of the reaction as observed by FTIR, aliquots were removed for GPC/LS analysis, and then a solution of isocyanate terminated PEG (see below) in THF (3 equiv per $(PMe_3)_4Co$) was added. The reaction immediately turned from pale orange to green. The reaction was stirred overnight

at room temperature and then precipitated into hexanes. Solids were collected by centrifugation and washed to remove all unconjugated PEG with 2 portions of water at pH 2, followed by 2 portions of water. The PEG end-capped polymers were lyophilized to yield white solids (95-99% yield). To determine polypeptide molecular weights (M_n), ^{1}H NMR spectra were obtained. Since it has been shown that end-capping is quantitative when excess isocyanate is used,[31] integrations were calibrated using the polyethylene glycol chemical shift found at δ 3.64 (see example in spectral data section).

Preparation of Isocyanate Terminated PEG: To a solution of amine terminated PEG (1.0 g, 0.500 mmol, MW= 2000, Nanocs) in dry THF (25 mL) in a Schlenk flask was added a solution of phosgene in toluene (0.50 mL, 1.00 mmol, 20 % (w/v) in toluene, 2 equiv) via syringe. *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.* The reaction was stirred under N_2 at room temperature for 16 hrs then evaporated to dryness and transferred to a dinitrogen filled glove box. The condensate in the vacuum traps was treated with 50 mL of concentrated aqueous NH_4OH to neutralize residual phosgene. The isocyanate was precipitated from minimal THF into 1:1 Et_2O:hexanes and was recovered as 1.01 g of a white solid (99%).

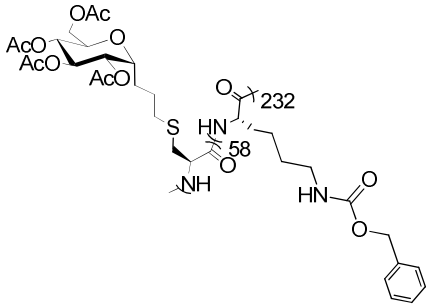
Glycopolypeptide *dn/dc* Calculations

Glycopeptide *dn/dc* values were calculated directly from GPC/LS measurements, assuming 100% mass recovery and using the template provided in Astra 5.3.2.21 software. The *dn/dc*

values obtained were similar for glycopolypeptides **5a** and **5b**, and so the averaged value of 0.071 was used for all subsequent GPC/LS glycopolypeptide analysis.

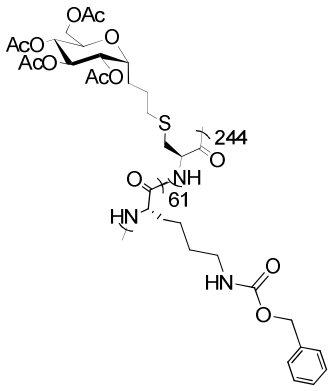
General Procedure for Preparation of glyco-C Containing Diblock Copolymers:

The general procedure for polymerization of glyco-C NCAs was followed. Upon completion of polymerization of the first NCA as observed by FTIR, aliquots were removed for GPC/LS analysis, and then a solution of the second NCA was added (50 mg/mL in THF). The reaction was monitered by FTIR. Aliquots were removed for GPC/LS analysis immediately upon polymerization completion. The polymerization solution was precipitated into hexanes. Solids were collected by centrifugation and washed with 2 portions of water at pH 2, followed by 2 portions of DI water. The polymers were lyophilized to yield white solids. (95-99 % yield).



Poly(α-glc-C)$_{58}$-*block*-Poly(Z-Lys)$_{232}$

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 7.28 (br s, 5H), 5.51 (br s, 1H), 5.31 (br s, 0.5H), 5.16-4.99 (br m, 3.5H), 4.34-3.84 (br m, 3H), 3.15 (br s, 3 H), 2.66 (br s, 1H), 2.25-1.28 (br m, 19H); FTIR (THF): 3560, 3504, 3282, 2966, 2854, 2678, 1958, 1750, 1720, 1648, 1542, 1462, 1364 cm^{-1}.

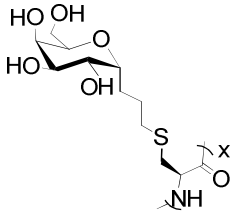


Poly(Z-Lys)$_{61}$-*block*-Poly(α-glc-C)$_{244}$

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 7.27 (br s, 1.25H), 5.34 (br s, 1H), 5.15-5.0 (br m, 3.5H), 4.34-3.88 (br m, 6H), 3.23-2.98 (br m, 2.5 H), 2.66 (br s, 2H), 2.26-1.39 (br m, 19H); FTIR (THF): 3564, 3505, 3282, 2964, 2854, 2682, 1958, 1749, 1720, 1650, 1541, 1460, 1362 cm^{-1}.

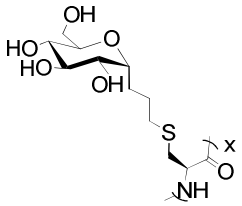
General Poly(glyco-C) Deprotection Procedure:

To a solution of acetylated poly(glyco-C) in DCM:methanol 1:2 (10 mg/mL) was added hydrazine monohydrate (20 equiv) and the reaction was stirred overnight at room temperature. The product was observed as a white precipitate. Reactions were quenched by addition of drops of acetone. Et_2O was added and the solids collected by centifugation (99% yield). The solids were taken up with water and transferred to bags of 2000 molecular weight cutoff dialysis tubing and dialyzed against Millipore water for 3 days, with water changes twice per day. Dialyzed polymers were lyophilized to dryness to yield white fluffy solids. (80% yield after dialysis)



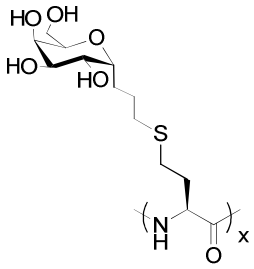
Poly-α-D-galactopyranosyl-L-cysteine (Poly(α-gal-C)), 6a

^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ 4.12-3.91 (br m, 4H), 3.86-3.67 (br m, 4H), 3.15-2.92 (br m, 2H), 2.55 (br s, 2H), 1.88-1.54 (br m, 4H); FTIR (nujoll mull): 3320 br, 2716, 1636, 1534 cm^{-1}.



Poly-α-D-glucopyranosyl-L-cysteine (Poly(α-glc-C)), 6b

^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ 4.00 (br s, 1H), 3.87-3.79 (br m, 1H), 3.76-3.59 (br m, 4H), 3.50 (br s, 1H), 3.41-3.31 (br m, 1H) 3.11-2.88 (br m, 2H), 2.66 (br s, 2H) 1.96-1.53 (br m, 4H). FTIR (nujoll mull): 3320 br, 2717, 1635, 1531 cm^{-1}.

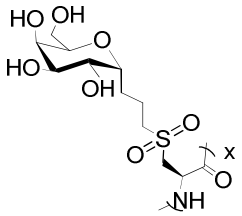


Poly-α-D-galactopyranosyl-L-homocysteine (Poly(α-gal-C^H)), 15a

^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ 4.35-4.25 (br m, 1H), 4.09-3.92 (br m, 3H), 3.82-3.65 (br m, 4H), 3.02-2.79 (br m, 2H), 2.39-2.05 (br 2, 2H), 1.85-1.57 (br m, 4H). FTIR (nujoll mull): 3322 br, 2718, 1647, 1539 cm^{-1}.

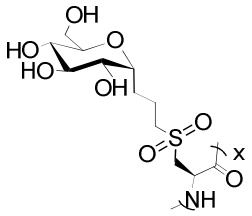
General Poly(glyco-C) Oxidation to Sulfone:

A poly(glyco-C) sample was dissolved in a solution of 5% acetic acid and 10% H_2O_2 in DI water (20 mg/mL), and the reaction was heated to 38 ^oC for 16 hours. Drops of 1M sodium thiosulfate were added, and then the reaction was transferred to 2000 molecular weight cutoff dialysis tubing, and dialyzed against Millipore water for 3 days, with water changes twice per day. Dialyzed polymers were lyophilized to dryness to yield white fluffy solids (80% yield after dialysis).



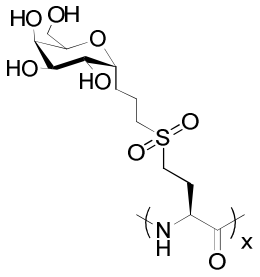
Poly-α-D-galactopyranosyl-L-cysteine-sulfone (Poly(α-gal-C^{O2})), 7a

^1H NMR (500 MHz, D_2O, 25 ^oC): δ 4.13-3.67 (br m, 10H), 3.39 (br s, 2H), 2.06-1.72 (br m, 4H).



Poly-α-D-glucopyranosyl-L-cysteine-sulfone (Poly(α-glc-C^{O2})), 7b

^1H NMR (500 MHz, D_2O, 25 ^oC): δ 4.29-3.60 (br m, 8H), 3.55-3.25 (br m, 4H), 2.06-1.72 (br m, 4H).

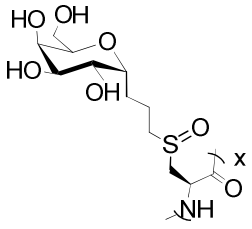


Poly-α-D-galactopyranosyl-L-homocysteine-sulfone (Poly(α-gal-C^{HO2})), 16a

^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ 4.42-4.33 (br m, 1H), 4.08 (br s, 1H), 4.03-3.94 (br m, 2H), 3.81-3.68 (br m, 4H), 3.46-3.23 (br m, 2H), 2.59-2.35 (br m, 2H), 2.05-1.71 (br m, 6H).

General Poly(glyco-C) Oxidation to Sulfoxide:

A poly(glyco-C) sample was dissolved in a solution of 1% acetic acid and 1% H_2O_2 in DI water (20 mg/mL), and the reaction heated to 38 ^oC for 6 hours. Drops of 1M sodium thiosulfate were added, and then the reaction was transferred to 2000 molecular weight cutoff dialysis tubing, and dialyzed against Millipore water for 3 days, with water changes twice per day. Dialyzed polymers were lyophilized to dryness to yield white fluffy solids (85% yield after dialysis).



Poly-α-D-galactopyranosyl-L-cysteine-sulfoxide (Poly(α-gal-C^O)), 17a

^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ 4.37-4.23 (br m, 1H), 4.10 (br s, 1H), 4.02-3.96 (br m, 2H), 3.85-3.69 (br m, 3H), 3.64-3.21 (br m, 3H), 3.06 (br s, 2H), 2.02-1.71 (br m, 4H).

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CHAPTER FIVE

Glycopolypeptide Conformations in Block Copolymer Assemblies Influence Both Nanoscale Morphology and Ligand Binding Properties

5.1 Abstract

We describe the preparation and assembly of glycosylated amphiphilic block copolypeptides, where the hydrophilic glycosylated segments are either α-helical or disordered. In this study, glycosylated amphiphilic diblock copolypeptides were prepared using poly(α-D-galactopyranosyl-L-lysine), poly(α-gal-K), and poly(α-D-galactopyranosyl-L-cysteine), poly(α-gal-C^{O2}), hydrophilic segments, which are known to be fully α-helical (> 90% at 20 deg C) and fully disordered in water, respectively. We found that block copolypeptides containing galactosylated hydrophilic segments of either α-helical or disordered conformation give different assembly morphologies, where the disordered glycopolypeptide segments favor vesicle formation and present sugar residues that more effectively bind biological targets.

5.2 Introduction

There has been considerable recent interest in the development of multifunctional, nanoscale carriers for targeted delivery of therapeutics.[1-3] To precisely control the nanostructured morphology of these delivery vehicles, including shape (e.g. spherical micelles, cylindrical micelles, discs, or vesicles) and internal structure (e.g. spotted, segmented, or core-shell), the assembly of amphiphilic block copolymers in aqueous solution has been highly useful.[4,5] However, the incorporation of multifunctionality (e.g. for cellular targeting, uptake, or intracellular release of cargos),[6-8] can often perturb the self-assembly process, leading to different

morphologies with altered stabilities. We are pursuing the development of block copolypeptide based drug carriers since they are resorbable materials possessing the ordered chain conformations of proteins, which provide an additional means to direct nanostructure independent of many other parameters, such as amino acid composition.[9-11] Here, we describe the preparation and assembly of glycosylated amphiphilic diblock copolypeptides, where the hydrophilic glycosylated segments adopt either α-helical or disordered chain conformations. These distinct glycopolypeptide conformations were found to significantly impact both block copolymer self-assembly as well as the ability of sugar residues in these assemblies to bind to biological targets. These results show how careful choice of polypeptide chain conformations can be used to direct assembly of nanocarriers into desired morphologies and simultaneously enhance their bioactive functionality.

Glycosylation of polymeric drug and gene carriers has been shown to lower cytotoxicity, enhance aqueous solubility, and provide targeting to specific cells and organs.[12,13] It is also well known in biology that the way in which sugar functional groups are presented greatly affects their ability to bind targets and signal cells.[14] We recently reported the preparation of fully glycosylated, high molar mass synthetic polypeptides, which are water soluble and mimic the structures of naturally occurring glycoproteins.[15,16] A key feature of these glycopolymers is that their chain conformations are readily controlled, either by choice of peptide backbone or by selective oxidation of side-chain functional groups, such that fully α-helical or fully disordered chains can be obtained. We have now incorporated these glycopolypeptides as hydrophilic segments in amphiphilic diblock copolypeptides to study their aqueous self-assembly and evaluate the properties of the resulting nanostructures. While much is known about how different

chain conformations of hydrophobic polypeptide segments influence nanoscale morphology, little is known about the corresponding role played by hydrophilic polypeptide conformations in self-assembly.[17-20] This has been difficult to study since hydrophilic polypeptide segments presenting similar functionality but differing only in conformation are rare. Here, we have found that block copolypeptides containing galactosylated hydrophilic segments of either α-helical or disordered conformation give different assembly morphologies, where the disordered glycopolypeptide segments favor vesicle formation and present sugar residues that more effectively bind to biological targets.

Previously, we and others reported that amphiphilic diblock copolypeptides containing α-helical hydrophobic segments assemble in water to form spherical, unilamellar vesicles ranging in diameter from tens of nanometers to tens of microns.[18,19,21-25] The rod-like conformations of these hydrophobic segments were found to favor side-by-side packing resulting in lamellar vesicle membranes, while samples with disordered hydrophobic segments were found to pack into spherical micelles,[26,27] similar to other, conformationally disordered, synthetic block copolymers.[5] Block copolypeptides containing hydrophilic segments with either disordered or α-helical conformations in combination with α-helical hydrophobic segments have been found to give vesicular assemblies under certain conditions.[18,19,21-25] However, it has been difficult to unequivocally determine the role of the hydrophilic chain conformation in directing nanostructure, since there have been significant differences between the disordered and α-helical hydrophilic segments in these materials (e.g. ionic vs. nonionic). For example, both nonionic poly(*N*ε-2-(2-(2-methoxyethoxy)ethoxy)acetyl-L-lysine)$_{100}$-*b*-poly(L-leucine)$_{20}$ ($K^{P}_{100}L_{20}$) with an α-helical hydrophilic segment and ionic poly(L-homoarginine·HCl)$_{60}$-*b*-poly(L-leucine)$_{20}$ ($R^{H}_{60}L_{20}$) with a disordered hydrophilic segment can be assembled into micron sized vesicles in

water.[18,19] Some ionic vesicles can be neutralized by adjustment of pH, which results in a transition from disordered to α-helical conformation, but the uncharged, α-helical polypeptide segments are sparingly soluble in water and precipitate above micromolar concentrations.[28,29] Likewise, $K^{P}_{100}L_{20}$ samples with nonionic, disordered hydrophilic segments have been prepared using racemic K^{P} residues, yet inter-chain H-bonding interactions in the resulting disordered segments limit their aqueous solubility as well.[18] Consequently, there remains a need for fully α-helical and disordered hydrophilic polypeptide segments that display similar functionality to determine how hydrophilic chain conformation can be used to direct nanoscale assembly and control presentation of polypeptide functionality.

5.3 Results and Discussion

In this study, glycosylated amphiphilic diblock copolypeptides were designed to incorporate poly(α-D-galactopyranosyl-L-lysine), poly(α-gal-K),[15] and poly(α-D-galactopyranosyl-L-cysteine sulfone), poly(α-gal-C^{O2}),[16] hydrophilic segments, which are known to be fully α-helical (> 90% at 20 ^{o}C) and fully disordered in water, respectively. The precursor galactosylated amino acid *N*-carboxyanhydride (α-gal-K NCA and α-gal-C NCA) monomers[15,16] were used to prepare diblock copolymers containing galactose bearing hydrophilic segments ca. 65 residues long connected to α-helical hydrophobic oligoleucine segments ca. 20 residues long, i.e. $(\alpha\text{-gal-K})_{65}L_{20}$ and $(\alpha\text{-gal-C})_{65}L_{20}$ (Figure 5.1). These chain lengths were chosen to encourage assembly into vesicles, which are desirable nanostructures that can encapsulate both hydrophilic and hydrophobic cargos,[6-8] and are based on optimized compositions determined for other vesicle forming diblock copolypeptides.[19, 22-25] Synthesis of these copolypeptides using $(PMe_3)_4Co$ initiator in THF yielded samples with narrow chain length distributions and desired compositions,[30] and removal of protecting groups gave the galactosylated amphiphilic block

copolymers (Table 5.1).[15,16] Since poly(α-gal-C) is partially α-helical in water, we oxidized the thioether linkages in these segments to the corresponding sulfones to produce copolypeptides with fully disordered poly(α-gal-C^{O2}) hydrophilic chains (Figure 5.1).[16] Circular dichroism analysis of the block copolymers confirmed that $(\alpha\text{-gal-K})_{65}L_{20}$ is predominantly α-helical in water, and that $(\alpha\text{-gal-C}^{O2})_{65}L_{20}$ is predominantly disordered in water (Figure 5.2).

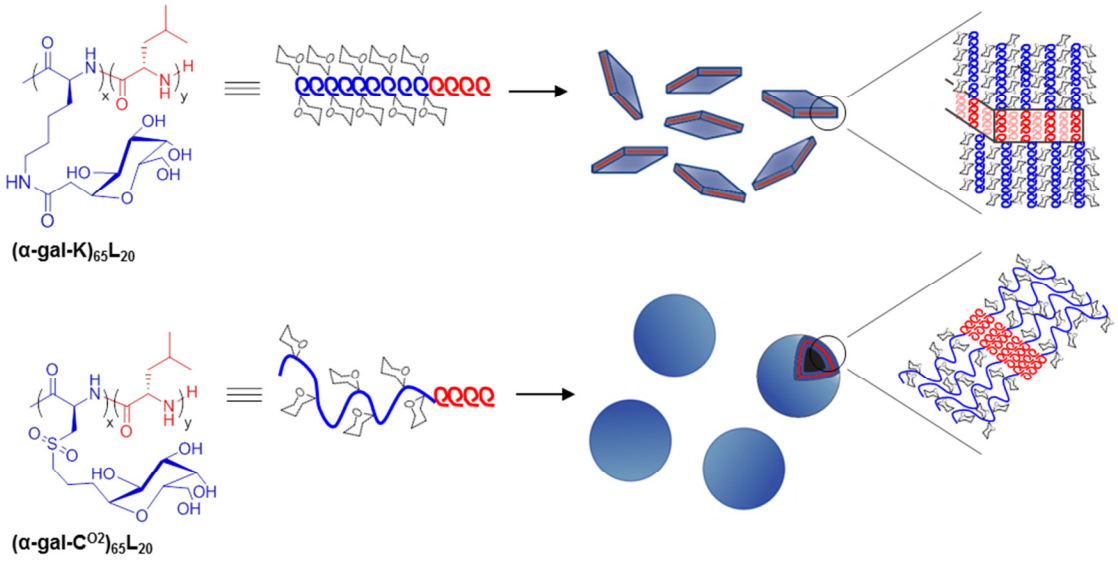

Figure 5.1 Schematic showing structures of amphiphilic glycosylated diblock copolypeptides and observed self-assemblies.

Block Copolypeptide	M_n[a]	M_w/M_n[a]	Found Composition[b]	Yield (%)[c]	Self-Assembled Structure[d]
$(\alpha\text{-gal-C})_{65}L_{20}$	30,910	1.09	$(\alpha\text{-gal-C})_{65}L_{22}$	95	V
$(\alpha\text{-gal-K})_{65}L_{20}$	33,130	1.07	$(\alpha\text{-gal-K})_{67}L_{23}$	99	P, A

Table 5.1 Characterization and properties of $(\alpha\text{-gal-C})_{65}L_{20}$ and $(\alpha\text{-gal-K})_{65}L_{20}$ diblock copolypeptides. [a] Hydrophilic segment lengths (number average molecular weight, M_n, for α-gal-K, and α-gal-C segments) and polydispersities (M_w/M_n) determined using gel permeation chromatography and ^{1}H NMR. [b] Calculated using ^{1}H NMR. [c] Total isolated yield of diblock glycopolypeptide. [d] Structures observed visually using optical microscopy (V = vesicle, A = irregular aggregate, P = plate).

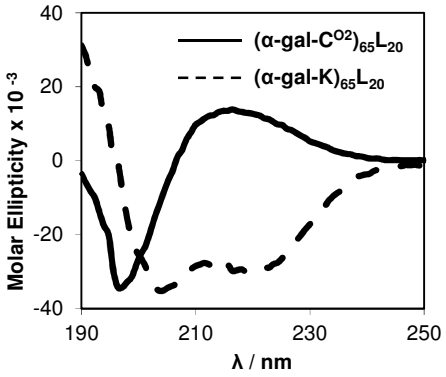

Figure 5.2 Circular dichroism spectra of glycosylated diblock copolypeptides. Samples are (α-gal-C^{O2})$_{65}L_{20}$ (solid line) and (α-gal-K)$_{65}L_{20}$ (dashed line), 0.2 mg/mL in deionized water. Molar ellipticity is reported in millideg·cm^2·dmol^{-1}.

Attempts were made to assemble vesicles from the galactose containing copolypeptides using mixed solvent annealing, which has been found to assist formation of ordered nanostructures in many other block copolypeptide systems.[18,21] The hydrophilic chain conformations of the galactosylated block copolypeptides were found to strongly influence their self-assembly in water. The sample with a disordered hydrophilic segment, (α-gal-C^{O2})$_{65}L_{20}$, gave exclusively vesicles with diameters ranging from hundreds of nanometers to a few microns in diameter (Figure 5.3A). The vesicular morphology of the (α-gal-C^{O2})$_{65}L_{20}$ assemblies was confirmed by labeling their hydrophobic domains with $DiOC_{18}$ dye and imaging thin slices through suspensions of the samples using laser scanning confocal microscopy (LSCM), which revealed their membrane structure and hydrophilic interior (Figure 5.3C). The ability of these vesicles to encapsulate hydrophilic cargos was also shown by their retention of Texas Red labeled dextran (Mn = 3000 Da) after removal of unencapsulated cargo by dialysis (Figure 5.3D). The (α-gal-C^{O2})$_{65}L_{20}$ vesicles could also be extruded to obtain low polydispersity nanovesicles with average diameters of 140 nm (PDI = 0.060), which are a desirable size range for use as circulating nanocarriers (Figure 5.3E,F).[6-8]

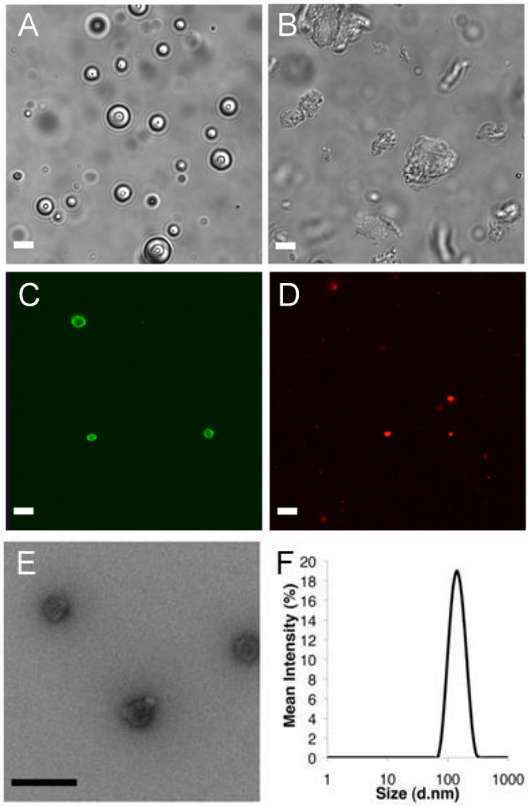

Figure 5.3 Imaging of glycosylated block copolymer self-assemblies. DIC images of A) (α-gal-C^{O2})$_{65}L_{20}$ vesicle suspension and B) (α-gal-K)$_{65}L_{20}$ plates and aggregrates. LSCM images of C) (α-gal-C^{O2})$_{65}L_{20}$ vesicles containing $DiOC_{18}$ dye and D) Texas Red labeled dextran encapsulated within (α-gal-C^{O2})$_{65}L_{20}$ vesicles. E) TEM image of extruded (α-gal-C^{O2})$_{65}L_{20}$ vesicles. F) DLS of extruded (α-gal-C^{O2})$_{65}L_{20}$ vesicles. White scale bars = 5 μm, black scale bar = 200 nm.

In contrast to the results above, the highly α-helical sample, (α-gal-K)$_{65}L_{20}$ gave nearly no vesicles and instead an abundance of micron sized irregular aggregates and some platelike objects were observed (Figure 5.3B). Similar aggregation behavior has been observed previously in block copolypeptides containing either shorter hydrophilic or longer hydrophobic segment lengths, i.e. lower hydrophilic volume fractions.[18,21] Their inability to form vesicles is likely due to the smaller hydrophilic content of these samples not being able to effectively solubilize and stabilize the assemblies against further aggregation. The rod-like nature of the α-helical hydrophilic segments in (α-gal-K)$_{65}L_{20}$ also likely acts to stiffen any membranes formed, leading

to rigid sheet-like membranes that lack the flexibility needed to accommodate vesicle curvature.[18] The presence of different hydrophilic chain conformations in $(\alpha\text{-gal-K})_{65}L_{20}$ and $(\alpha\text{-gal-C}^{O2})_{65}L_{20}$ thus significantly altered their self-assembled morphologies, where the desired vesicle structures were favored by the disordered segments in $(\alpha\text{-gal-C}^{O2})_{65}L_{20}$. The flexibility in the poly(α-gal-C^{O2}) segments also allows better mixing with water, essentially increasing their hydrophilicity, compared to the conformationally rigid poly(gal-K) chains. The more open structure of solvated disordered poly(α-gal-C^{O2}) segments should also partially frustrate packing of the rigid hydrophobic oligoleucine segments, making the vesicle membranes themselves more dynamic, flexible and able to accommodate curvature.[21]

Although $(\alpha\text{-gal-K})_{65}L_{20}$ and $(\alpha\text{-gal-C}^{O2})_{65}L_{20}$ self-assemble into different structures in water, they were both designed to present the same α-D-galactosyl functionality. To determine how the different glycopolypeptide conformations affect presentation and bioactivity of their pendant galactose units, we separately incubated $(\alpha\text{-gal-K})_{65}$ and $(\alpha\text{-gal-C}^{O2})_{65}$ homopolymers with lectins in a precipitation assay. We chose ricinus communis agglutinin (RCA_{120}) for the polymer binding lectin since it is known to specifically and selectively bind to galactosyl groups, and concanavalin A (Con A) as a control lectin that binds mannosyl and glucosyl, but not galactosyl, groups.[14] When the galactosyl-polypeptides were incubated with RCA_{120}, turbidity of both solutions was found to increase rapidly as expected from aggregation due to lectin binding (Figure 5.4).[14] Neither glycopolymer solution became turbid when incubated with Con A, indicating the interactions with RCA_{120} are specific binding interactions between this lectin and the galactosyl groups of the polymers.

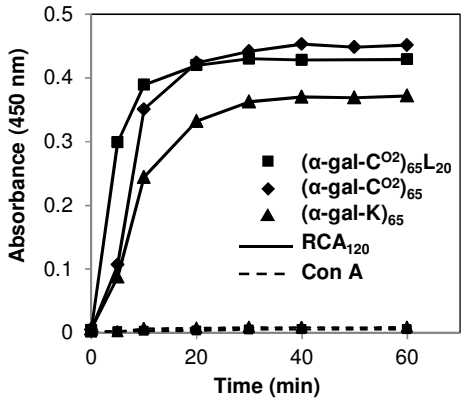

Figure 5.4 Lectin binding of glycopolypeptides versus time. Turbidity (absorbance at 450 nm) of (α-gal-K)$_{65}$ (▲), (α-gal-C^{O2})$_{65}$ (♦), or (α-gal-C^{O2})$_{65}$L$_{20}$ vesicles (■) when mixed with lectin RCA$_{120}$ (solid lines) or Con A (dashed line), in PBS buffer. Glycopeptide concentration = 3.3 mM.

Visible absorbance measurements (450 nm) were used to quantify mixture turbidity, which has been shown to correlate with the concentration of sugar groups available for lectin binding.[31] Notably, at equivalent sugar concentrations, the disordered (α-gal-C$_{O2}$)$_{65}$ polymer gave rise to increased turbidity when mixed with RCA$_{120}$ compared to the α-helical (α-gal-K)$_{65}$ polymer, which indicates the disordered (α-gal-C^{O2})$_{65}$ is more effective at binding to the lectin (Figure 5.4). Similarly, (α-gal-C^{O2})$_{65}$L$_{20}$ vesicle suspensions mixed with RCA$_{120}$ also became turbid, to roughly the same extent as the (α-gal-C^{O2})$_{65}$ homopolymer, indicating the disordered glycopolymer segments remain effective in binding biological targets when incorporated into nanoscale vesicles. Comparison of the ability of helical and disordered glycopolymers to bind to lectins has been studied previously, but with mixed results.[32,33] In one study, glycopolymers based on helical polyisocyanide and flexible polyacrylamide backbones were compared.[32] Similar to our findings, the disordered polyacrylamide was found to bind more efficiently to the lectin, but the analysis was complicated by use of different polymer backbones and unknown

helical content of the polyisocyanide.[34] Another study compared glycosylated poly(lysines), where the enantiomerically pure (α-helical) and racemic (disordered) polymers were found to bind to lectins with nearly equal affinity.[33] In this study, the α-helical contents of the ordered polymers ranged from 30 to 62%, indicating considerable disorder, which may explain why little difference in lectin binding between samples was observed. Our results here show that a significant difference in lectin binding for α-helical versus disordered polypeptides does exist when using ordered samples with high (>90%) α-helical content, which can pack the sugar residues densely around the chain.

The (α-gal- C^{O2})$_{65}L_{20}$ vesicles were also found to be minimally cytotoxic (Figure 5.5), as compared to cationic polypeptide vesicles such as $R^{H}_{60}L_{20}$.[19] This low cytotoxicity, even at high concentrations, make these vesicles attractive for development as biofunctional drug carriers with controlled nanostructure.

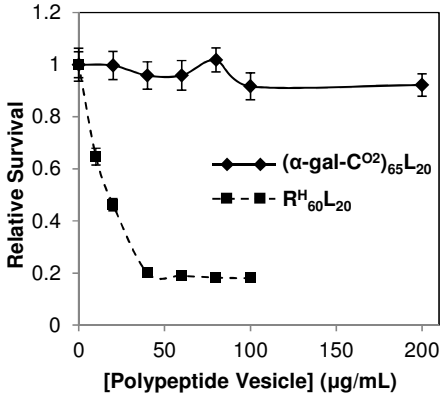

Figure 5.5 Relative survival of HeLa cells incubated for 5 hours with copolypeptide vesicles determined using MTS assay. Samples are (α-gal-C^{O2})$_{65}L_{20}$ (solid line) and $R^{H}_{60}L_{20}$ (dashed line).

5.4 Conclusions

Overall, we have found that the chain conformation of hydrophilic polypeptide segments

can play a significant role in dictating both structure and function in self-assembled nanostructures. The use of hydrophilic segments with disordered conformations in amphiphilic diblock copolypeptides was found to be particularly effective both for formation of vesicular assemblies as well as presentation of functionality in an accessible, active form. The low cytotoxicity, biological targeting capability, and nanoscale size make these vesicles attractive for development as biofunctional drug carriers with controlled nanostructure.

5.5 Experimental

5.5.1 Materials and Methods

Unless stated otherwise, reactions were conducted in oven-dried glassware under an atmosphere of nitrogen using anhydrous solvents. Hexanes, THF, DCM, and DMF were purified by first purging with dry nitrogen, followed by passage through columns of activated alumina. Deionized water (18 MΩ-cm) was obtaining by passing in-house deionized water through a Millipore Milli-Q Biocel A10 purification unit. All commercially obtained reagents were used as received without further purification unless otherwise stated. Reaction temperatures were controlled using an IKA temperature modulator, and unless stated otherwise, reactions were performed at room temperature (RT, approximately 20 °C). Thin-layer chromatography (TLC) was conducted with EMD gel 60 F254 precoated plates (0.25 mm) and visualized using a combination of UV, anisaldehyde, and phosphomolybdic acid staining. Selecto silica gel 60 (particle size 0.032–0.063 mm) was used for flash column chromatography. ^{1}H NMR spectra were recorded on Bruker spectrometers (at 500 MHz) and are reported relative to deuterated solvent signals. Data for ^{1}H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity, coupling constant (Hz) and integration. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet and br, broad. ^{13}C NMR spectra were recorded on Bruker

Spectrometers (at 125 MHz). Data for ^{13}C NMR spectra are reported in terms of chemical shift. High-resolution mass spectrometry (HRMS) was performed on a Micromass Quatro-LC Electrospray spectrometer with a pump rate of 20 μL/min using electrospray ionization (ESI). All Fourier Transform Infrared (FTIR) samples were prepared as thin films on NaCl plates, spectra were recorded on a Perkin Elmer RX1 FTIR spectrometer, and are reported in terms of frequency of absorption (cm^{-1}). Tandem gel permeation chromatography/light scattering (GPC/LS) was performed on a SSI Accuflow Series III liquid chromatograph pump equipped with a Wyatt DAWN EOS light scattering (LS) and Optilab rEX refractive index (RI) detectors. Separations were achieved using 10^5, 10^4, and 10^3 Å Phenomenex Phenogel 5 μm columns using 0.10 M LiBr in DMF as the eluent at 60 °C. All GPC/LS samples were prepared at concentrations of 5 mg/mL.

5.5.2 Experimental Procedures

The preparation of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl-L-lysine-*N*-carboxyanhydride[15] (α-gal-K NCA), 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl-L-cysteine-N-carboxyanhydride[16] (α-gal-C NCA), L-leucine N-carboxyanhydride (Leu NCA)[38], and $(PMe_3)_4Co$[39] have been previously reported.

Preparation of Glycosylated Diblock Copolypeptides:

All polymerization reactions were performed in a dinitrogen filled glove box. To a solution of α-gal-K NCA or α-gal-C NCA (1 equiv) in THF (50 mg/mL) was rapidly added, via syringe, a solution of $(PMe_3)_4$ Co in THF (0.05 equivs, 30 mg/mL). The reaction was stirred at RT and polymerization progress was monitored by FTIR. Polymerization reactions were generally complete within 3 hours. Immediately upon polymerization completion, aliquots were removed

for GPC/LS and endgroup analysis[36] using 1K MW isocyanate terminated PEG. A solution of Leu NCA in THF (0.33 equivs, 50 mg/mL) was added and the polymerization was monitored by FTIR. Polymerization reactions were generally complete within 3 hours. After complete consumption of NCA, reactions were removed from the drybox and precipitated into hexanes. Solids were collected by centrifugation and washed with 2 portions of water at pH 2 (HCl), followed by DI water. The polymers were lyophilized to yield white solids. (95-99% yield).

Molecular Weight Determination:

The degree of polymerization (DP) of the first block, poly(α-gal-C) or poly(α-gal-K), was determined by ^{1}H nmr integrations of the aliquot end-capped with PEG. Integrations were calibrated using the polyethylene glycol chemical shift found at δ 3.64, and the polypeptide DP's were found to be $(\alpha\text{-gal-C})_{65}$ and $(\alpha\text{-gal-K})_{67}$. Polydispersities were determined by GPC/LS, M_w/M_n for $(\alpha\text{-gal-C})_{65}$ = 1.09 and M_w/M_n for $(\alpha\text{-gal-K})_{67}$ =1.07. The DP of the second block was determined by ^{1}H nmr integrations calibrated using the DP of the first block. Final polypeptide compositions were determined to be $(\alpha\text{-gal-C})_{65}L_{22}$ and $(\alpha\text{-gal-K})_{67}L_{23}$.

Poly(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl-L-cysteine)$_{65}$-*b*-(leucine)$_{22}$:

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 5.39 (s, 65H), 5.26-5.17 (m, 128H), 4.27-4.02 (m, 359.5H), 3.16-2.98 (m, 132H), 2.61 (s, 126H), 2.15-1.96 (m, 893H), 1.89-1.52 (m, 882H), 0.97-0.84 (m, 136H). FTIR (thin film, THF): 3568, 3492, 3284, 2966, 2851, 2678, 1955, 1752, 1651, 1524, 1457, 1366 cm^{-1}.

Poly(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl-L-lysine)$_{67}$-*b*-(leucine)$_{23}$:

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 5.42 (s, 67H), 5.34-5.04 (m, 200H), 4.75 (s, 71H), 4.31-3.80 (m, 396H), 3.19 (s, 225H), 2.76-2.36 (m, 257H), 2.2-1.26 (m, 1274H), 0.98-0.84 (m, 137H). FTIR (thin film, THF): 3272, 2966, 2853, 1752, 1654, 1541, 1450, 1365 cm^{-1}.

Glycosylated Diblock Copolypeptide Deprotection Procedure:

To a solution of acetylated (α-gal-C)$_{65}$L$_{22}$ or (α-gal-K)$_{67}$L$_{23}$ in DCM:methanol 1:2 (10 mg/mL) was added hydrazine monohydrate (4 equivs/ OH group). The reactions were stirred overnight at room temperature. The product was observed as a white precipitate. Reactions were quenched by addition of drops of acetone. Et_2O was added and the solids collected by centrifugation (99% yield). The solids were taken up with water and transferred to 2000 molecular weight cutoff dialysis tubing and dialyzed against Millipore water for 3 days, with water changes twice per day. Dialyzed polymers were lyophilized to dryness to yield white fluffy solids. (80% yield after dialysis)

Poly(α-D-galactopyranosyl-L-cysteine)$_{65}$-*b*-(leucine)$_{22}$; (α-gal-C)$_{65}$L$_{22}$:

^{1}H NMR (500 MHz, d-TFA, 25 ^{o}C): δ 4.69 (s, 65H), 4.54-4.05 (m, 308H), 3.21-2.61 (m, 233H), 2.04-1.57 (m, 314H), 1.27 (s, 21H), 0.99-0.89 (m, 150H).

Poly(α-D-galactopyranosyl-L-lysine)$_{67}$-*b*-(leucine)$_{23}$; (α-gal-K)$_{67}$L$_{23}$:

^{1}H NMR (500 MHz, D_2O, 25 ^{o}C): δ 4.35 (s, 67H), 3.91-3.81 (m, 148H), 3.67 (s, 59H), 3.63-3.43 (m 257H), 3.05 (s, 135H), 2.66-2.38 (m, 153H), 1.88-1.08 (m, 552H), 0.85-0.71 (m, 61H).

Oxidation of (α-Gal-C)$_{65}$L$_{22}$:

(α-Gal-C)$_{65}$L$_{22}$ was dissolved in a solution of 5% acetic acid and 10% H_2O_2 in DI water (20 mg/mL), and the reaction was heated to 38 ^{o}C for 16 hours. A few drops of 1M sodium thiosulfate were added, and then the reaction was transferred to 2000 molecular weight cutoff dialysis tubing, and dialyzed against Millipore water for 3 days, with water changes twice per day. Dialyzed copolypeptides were lyophilized to dryness to yield poly(α-D-galactopyranosyl-L-cysteine sulfone)$_{65}$-*b*-(leucine)$_{22}$ ((α-gal-C^{O2})$_{65}$L$_{22}$) as a white fluffy solid (80% yield after dialysis). ^{1}H NMR (500 MHz, d-TFA, 25 ^{o}C): δ 4.75 (s, 65H), 4.49 (s, 155H), 4.36-4.06 (m, 242H), 3.49 (s, 133H), 2.39-1.94 (m, 268H), 1.89-1.64 (m, 132H), 1.35 (s, 24H), 1.08-0.92 (m, 153H).

Circular Dichroism of Diblock Glycopolypeptides:

Circular dichroism spectra were recorded on an OLIS RSM CD spectrophotometer running in conventional scanning mode. Spectra (190–250 nm) were recorded in a quartz cuvette of 0.1 cm path length with samples prepared using Millipore deionized water. All spectra were recorded as an average of 3 scans. The spectra are reported in units of molar ellipticity [θ] (deg·cm^2·dmol^{-1}). The formula used for calculating molar ellipticity, [θ], was [θ] = (θ x 100 x M_W)/(c x l) where θ is the experimental ellipticity in millidegrees, M_W is the average molecular weight of a residue in g/mol, c is the peptide concentration in mg/mL; and l is the cuvette pathlength in cm. The percent α-helical content of the glycopeptides was estimated using the formula % α-helix = 100x(-[θ]$_{222nm}$ +3000)/39000) where [θ]$_{222nm}$ is the measured molar ellipticity at 222 nm.[37]The calculated helicity of (α-gal-K)$_{67}$L$_{23}$ was 94%.

Preparation of Diblock Glycopolypeptide Assemblies:

Solid (α-gal-C^{O2})$_{65}L_{22}$ or (α-gal-K)$_{67}L_{23}$ was dispersed in THF to give a 1% (w/v) suspension. The suspension was placed in a bath sonicator for 30 minutes to evenly disperse the polypeptide and reduce large particulates. An equivalent amount of millipore water was then added to give a 0.5 % (w/v) suspension. The suspension became clear as the solution was mixed by vortexing. The mixture was then dialyzed (2,000 MWCO membrane) against millipore water overnight with 3 water changes. Vesicular assemblies were also obtained via slow evaporation of the THF.

Differential Interference Microscopy (DIC):

Assembled copolypeptide suspensions of (α-gal-C^{O2})$_{65}L_{22}$ or (α-gal-K)$_{67}L_{23}$ (0.5 % (w/v)) were visualized on glass slides with a spacer between the slide and the coverslip (double-sided tape or Secure Seal Imaging Spacer, *Grace Bio-labs*) allowing the structures to be minimally disturbed during focusing. The samples were imaged using a Zeiss Axiovert 200 DIC/Fluorescence Inverted Optical Microscope.

Extrusion of Vesicle Assemblies:

A 0.2 % (w/v) aqueous (α-gal-C^{O2})$_{65}L_{22}$ vesicle suspension was extruded using an Avanti Mini-Extruder. Serial extrusion of vesicle suspensions were performed through Whatman Nuceleopore Track-Etched polycarbonate (PC) membranes with decreasing filter pore sizes: 3 times through a 1.0 μm filter, 3 times through 0.4 μm filter, and 3 times through 0.2 μm filter. The PC membranes and filter supports are soaked in millipore water for 10 minutes prior to extrusion.

Dynamic Light Scattering (DLS) of Extruded Vesicles:

A 0.2 % (w/v) solution of extruded (α-gal-C^{O2})$_{65}L_{22}$ vesicles was placed in a disposable cuvette and analyzed with the Malvern Zetasizer Nano ZS model Zen 3600 (Malvern Instruments Inc, Westborough, MA). A total scattering intensity of approximately 1 x 10^5 cps was targeted. The autocorrelation data was fitted using the CONTIN algorithm to determine the diameters of suspended assemblies.

Laser Scanning Confocal Microscopy (LSCM) of Fluorescently Labeled Vesicles:

LSCM images of (α-gal-C^{O2})$_{65}L_{22}$ vesicle suspensions were taken on a Leica Inverted TCS-SP1 MP-Inverted Confocal and Multiphoton Microscope equipped with an argon laser (476 and 488 nm blue lines), a diode (DPSS) laser (561 nm yellow-green line), and a helium-neon laser (633 nm far red line). Suspensions of the fluorescently labeled copolypeptides (0.5 % (w/v)) were visualized on glass slides with a spacer between the slide and the cover slip (Secure Seal Imaging Spacer, *Grace Bio-labs*) allowing the self-assembled structures to be minimally disturbed during focusing. Imaging of an xy plane with an optical z-slice showed that the assemblies were water filled, unilamellar vesicles.

Transmission Electron Microscopy (TEM) of Extruded Vesicles:

Extruded (α-gal-C^{O2})$_{65}L_{22}$ vesicle suspensions were diluted to 0.1 % (w/v). Samples (4 μL) were placed on a 300 mesh Formvar/carbon coated copper grid (Ted Pella) and allowed to remain on the grid for 60 seconds. Filter paper was used to remove the residual sample. One drop of 2 % (w/v) uranyl acetate (negative stain) was then placed on the grid for 90 seconds, and subsequently removed by washing with drops of millipore water and removing the excess liquid

with filter paper. The grids were allowed to dry before imaging with JEM 1200-EX (JEOL) transmission electron microscope at 80 kV.

Encapsulation of Texas Red Labelled Cyclodextran in Vesicles:

Vesicles composed of poly(α-gal-C^{O2})$_{65}$-*b*-(Leu)$_{22}$ were prepared as previously described, except the aqueous phase contained 0.125 mg/mL Texas red-cyclodextrin (3000 Da). Vesicle solutions were dialyzed in 8000 MWCO tubing overnight to remove unencapsulated Texas red-cyclodextrin. As a control, pre-formed (α-gal-C^{O2})$_{65}$-*b*-(Leu)$_{22}$ vesicles were incubated overnight with a 0.125 mg/mL Texas red-cyclodextrin solution and then dialyzed in 8000 MWCO tubing overnight. The samples were then imaged by DIC and confocal microscopies as previously described. No fluorescence was observed in the pre-formed vesicles incubated with Texas red-cyclodextrin.

Evaluation of Carbohydrate-Lectin Binding by Turbidity:

Ricinus Communis Agglutinin I (RCA_{120}) was purchased from Vector labs, Conconavalin A (Con A) was purchased from Sigma-Aldrich. Lectin solutions were prepared at a concentration of 2 mg/mL in 10 mM phosphate, 0.15 M NaCl, pH 7.8. Lectin solutions (600 μL) were transferred to cuvettes and baseline measurements were taken. Solutions of poly(α-gal-K)$_{67}$, poly(α-gal-C^{O2})$_{65}$, and poly(α-gal-C^{O2})$_{65}$-*b*-(Leu)$_{22}$ vesicles were prepared at a concentration of 1 mg/mL in DI water, and 60 μL of each solution was added to the cuvettes containing either RCA_{120} or Con A. Final glycopeptide concentrations were 3.3 mM. The solutions were gently mixed and absorbance spectra were recorded at various time points.

MTS Cell Proliferation Assay:

The MTS cell proliferation assay (CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay) was used to quantify any cytotoxic effects of the poly(α-gal-C^{O2})$_{65}$-*b*-(Leu)$_{22}$ vesicle suspensions. HeLa cells were seeded at a density of $4x10^4$ cells per cm^{-2} on a 96-well plate prior to the experiment. At the start of the experiment, the cell culture medium was aspirated, and the cells were incubated in medium containing the polypeptide vesicles for 5 h in a 37 ^oC humidified atmosphere with 5% CO_2. The incubation medium was the same as the cell culture medium except for the absence of FBS, penicillin, and streptomycin. Following the 5 h incubation period, the medium containing polypeptide vesicles was aspirated. Fresh medium containing 20% MTS was then added to the cells. The cells were placed back into a CO_2 incubator for 1 h and then the absorbance at 490 nm (A_{490}) was measured with an Infinite F200 plate reader (Tecan Systems Inc., San Jose, CA, USA). The background absorbance was read at 700 nm (A_{700}) and subtracted from A_{490}. The relative survival of the cells at each polypeptide concentration was quantified by taking the ratio of the (A_{490}–A_{700}) values and comparing between the experimental and control cells.

5.6 References

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CHAPTER SIX

Multifunctional and Multireactive Polypeptides via Methionine Alkylation

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6.1 Abstract

We report the development of a new "click" type reaction for polypeptide modification based on the chemoselective alkylation of thioether groups in methionine residues. The controlled synthesis of methionine polymers and their alkylation by a broad range of functional reagents to yield stable sulfonium derivatives are described. These "methionine click" functionalizations are compatible with deprotection of other functional groups, use an inexpensive, natural amino acid that is readily polymerized and requires no protecting groups, and allow the introduction of a diverse range of functionality and reactive groups onto polypeptides.

6.2 Introduction

The post-polymerization modification of polymers to introduce a diverse range of functional groups has received much recent attention.[1,2] This approach, where different molecules are conjugated to reactive side-chain groups in a polymer, is advantageous since it avoids the need to prepare many different functional monomers and individually optimize their polymerization conditions. Due to the need for highly efficient side-chain conjugations and compatibility of reactive groups with polymerization, "click" type reactions are typically

employed.[1-3] We are interested in the preparation of side-chain functionalized polypeptides as these can mimic the diversity of post-translationally modified proteins found in biology, and may be useful for medical applications.[4] Reactive precursor polypeptides have been prepared that contain side-chain alkyne,[5-8] alkene,[9-13] azide,[14] or thiol[15] groups for modification using thiol/ene, thiol/yne or azide/alkyne click chemistry. However, these methods require the use of expensive unnatural amino acids or prior introduction of unnatural functional groups or protecting groups onto polypeptide side chains, adding substantial cost, and often use linkages (i.e. esters)[5-14] that are unstable to deprotection conditions necessary to create copolypeptides containing other functional residues (e.g. lysine, glutamic acid). Here, we report the development of a new "click" type reaction for polypeptides that utilizes the unique reactivity of the thioether group found in the natural amino acid methionine. Since it is not protonated at low pH, methionine is the most reactive nucleophile present in peptides and proteins under acidic conditions.[16-19] We have found that methionine can undergo chemoselective, broad scope, highly efficient alkylation reactions in homo- and copolypeptides yielding stable sulfonium derivatives. These "methionine click" functionalizations are compatible with deprotection of other functional groups, use an inexpensive, natural amino acid that is readily polymerized and requires no side-chain modification or protecting groups, and allow the introduction of a diverse range of functionality and reactive groups onto polypeptides.

Due to its role in biology, the alkylation of thioether groups in methionine has been studied for some time, with pioneering studies being done by Toennies in the 1940s.[20,21] The majority of work in this area has employed simple alkylating agents, such as iodomethane to give the naturally occurring, dietary supplement *S*-methyl-methionine,[22,23] as well as iodoacetic acid to form soluble derivatives and probe the active sites of proteins.[24-26] There are some reports on

use of other activated alkyl halides to alkylate methionine, including those containing alkene and alkyne groups, yet no reactions were performed on the resulting sulfonium derivatives.[27-31] Katchalski was the first to alkylate poly(L-methionine), poly(Met), preparing both methyl and carboxymethyl sulfonium derivatives from the corresponding alkyl bromides and poly(Met) in neat formic acid.[32] These polysulfoniums were found to be stable, water soluble, and were studied for their conformational and polyelectrolyte behavior. In spite of the simplicity of this reaction, which gives quantitative alkylation with no side-products, there have been few further studies on these materials.[33,34] More significantly, there have been no reports on the use of any other functional reagents for alkylation of poly(Met). This lack of activity may be due to traditional difficulties in synthesis of poly(Met),[35,36] as the monomer, L-methionine *N*-carboxyanhydride (Met NCA), is difficult to purify by standard methods, and the living homopolymerization of Met NCA has not been demonstrated. Realizing that the alkylation of methionine residues has potential to be much broader in scope, we sought to develop the synthesis of well-defined poly(Met), and subsequently expand its alkylation chemistry as a means to create polypeptides containing diverse and reactive functionality.

6.3 Results and Discussion

Our lab recently reported a method for straightforward preparation of Met NCA in high yield and high purity,[37] which enables the further development of methionine polymers (Figure 6.1). We also showed that statistical copolymers of equimolar Met NCA with N_{ε}-CBZ-L-lysine *N*-carboxyanhydride (Z-Lys NCA) could be prepared with controlled chain lengths and narrow chain length distributions using $(PMe_3)_4Co$ initiator in THF. We now report that homopolymerizations of Met NCA also go to completion within a few hours at room temperature, yet molecular weight analysis of these chains was prohibited by aggregation of

poly(Met).[38] To determine chain lengths, Met NCA was polymerized at different monomer to $(PMe_3)_4Co$ initiator ratios, and after complete monomer consumption, active chains were end-

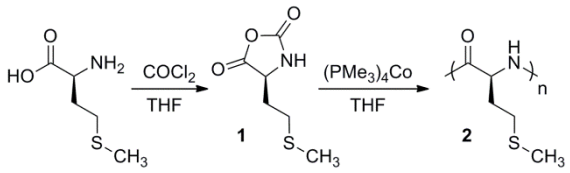


Figure 6.1 Preparation and polymerization of Met NCA

capped with isocyanate terminated PEG (M_n = 2000 Da).[39] Compositional analysis of purified, end-capped polymers by ^1H NMR gave average poly(Met) chain lengths that increased linearly with stoichiometry (Figure 6.2). Although chain length distributions of these poly(Met) samples could not be obtained, GPC analysis of an alkylated poly(Met) (*vide infra*) was found to possess a narrow polydispersity index (M_w/M_n) of 1.14, indicating the parent poly(Met)s are also well-defined (Figure 6.2) Poly(Met) was prepared in high yield with precisely controlled chain lengths up to over 400 residues long, and could also be prepared as statistical and diblock copolymers with other amino acids (See Experimental section 6.5). Overall, these data show that Met NCA, similar to other NCAs,[40,41] is able to undergo living polymerization when initiated with $(PMe_3)_4Co$.

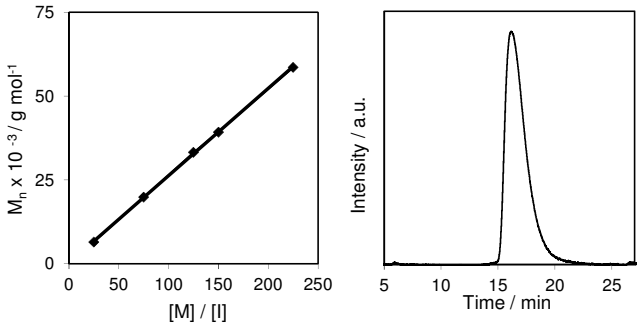
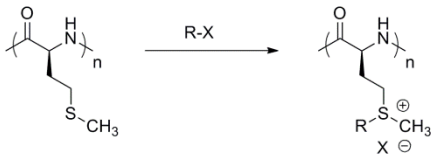


Figure 6.2 (a) Molecular weight (M_n, ♦) as a function of monomer to initiator ratio ([M]/[I]) for poly(Met) prepared by polymerization of Met NCA using $(PMe_3)_4Co$ in THF at 20 °C. (b) GPC chromatogram (normalized LS intensity versus elution time in arbitrary units (a.u.)) of glycopolypeptide **15**, M_w/M_n = 1.14.

Although α-helical poly(Met) has low solubility in many solvents, DCM and TFA being notable exceptions, it is soluble enough in a variety of different media to allow facile alkylation. This property provides a significant advantage over poly(L-cysteine), which forms insoluble β-sheets that prohibit preparation of long chains, as well as make high yield modification (i.e. alkylation) of the free thiol groups challenging.[15] For initial studies poly(Met) was reacted with a variety of alkylating reagents in either DMF, deionized water, or 0.2 M aqueous formic acid. We observed, similar to previously reported reactions on methionine amino acid,[27-31] that only activated alkyl bromides and iodides were able to react efficiently with poly(Met) under mild conditions (Figure 6.3). The significant exception in this comparison was allyl bromide/iodide, which has been reported to alkylate methionine amino acid,[42] yet was found by us to react only sluggishly with poly(Met) (*vide infra*). In addition to the previously described products **3** and **4**,[32] these direct alkylation reactions were used to prepare a number of new sulfonium derivatives of poly(Met). Various haloacetyl derivatives reacted readily and quantitatively to generate polysulfoniums bearing amide (**5**), ester (**6**) and active ester (**7**) functionalities, where the latter

may be useful for further derivatization with nucleophiles such as primary amines. Propargylic and benzylic/pseudo-benzylic halides also reacted efficiently with poly(Met), and allow the introduction of a variety of useful functional groups into polypeptides. This method for introduction of alkyne functionality (**8**) is straightforward and is more economical than other routes to install this click reactive group onto polypeptides.[5-8] The facile formation of pyridine containing sulfoniums (**9**, **10**) allows incorporation of this basic functionality that is otherwise difficult to introduce in polypeptides. Phenyl boronic acid containing polypeptides (**11**) have also been of interest for their sugar-binding abilities,[43,44] and now can be readily prepared with high degrees of incorporation in a single step.



R-X	Product	Yield
ICH_3	**3**	99%[a]
Br, O, OH	**4**	97%[a]
Br, O, NH_2	**5**	99%[a]
Br, O, OMe	**6**	96%[b]
I, O, OPNP	**7**	98%[c]
Br	**8**	99%[a]
Br, NHCl	**9** (*ortho*)	97%[a]
	10 (*meta*)	96%[a]
Br, $B(OH)_2$	**11**	99%[a]

Figure 6.3 Alkylation of poly(Met). Reagents and Conditions: R-X in DMF, H_2O, or 0.2 M aqueous formic acid, 20 ^oC. Yield is total isolated yield of completely functionalized polypeptide. [a] product was dialyzed against 0.1 M aqueous NaCl to give X = Cl. [b] X = Br. [c] X = I.

In contrast to the results above, unactivated alkyl halides, especially those without adjacent multiple bonds, reacted either sluggishly or not at all with poly(Met) under similar conditions. This result is not surprising since in all earlier work on methionine alkylation using amino acids, peptides or proteins, there are no reports of sulfonium formation using unactivated reagents.[16-19,27-31,42] To further expand the scope of poly(Met) alkylation, we explored different means to increase reactivity of unactivated alkyl halides. Silver tetrafluoroborate is known to promote thioether alkylation in small molecules,[45,46] and we found that addition of this reagent to alkylations in acetonitrile promoted the complete reaction of poly(Met) with unactivated alkyl halides (e.g. haloethyl compounds) (Figure 6.4). This strategy allowed the introduction of an even wider variety of reactive and functional groups onto poly(Met) giving additional new reactive and functional polypeptides. In addition, use of silver salts now facilitated the facile incorporation of alkene functionality (**12**) onto polypeptides, which is useful for a variety of further modifications including thiol/ene click reactions.[9,10] Ethylene glycol derivatives (**13**) were added to impart the water solubilizing and passivating properties of PEG.[47,48] Reactive ketone groups, useful for conjugation in aqueous environments,[49] were introduced in a single step using the 1,3-dioxolane derivative (**14**) that deprotects to give the water soluble polyketone during acidic workup. Glycopolypeptides, of interest as mimics of natural glycoproteins,[50,51] often require many synthetic steps, especially for preparation of longer chains with high sugar content. Using iodoethyl glycosides, high molecular weight and well defined, fully glycosylated polypeptides (**15**) were readily prepared in excellent yield by poly(Met) alkylation.

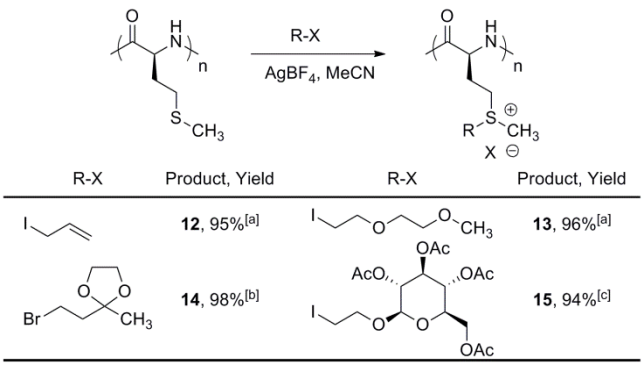


Figure 6.4 Alkylation of poly(Met) using $AgBF_4$. Reagents and Conditions: R-X, $AgBF_4$, MeCN, 50 ^oC. Yield is total isolated yield of completely functionalized polypeptide. [a] product was dialyzed against 0.1 M aqueous NaCl to give X = Cl. [b] product was dialyzed against aqueous HCl at pH = 2 with 0.1 M NaCl to give the polyketone, 14a, and X = Cl. [c] X= BF_4.

Since the use of silver salts may not be desirable in some applications, we also explored functionalization of poly(Met) with alternative reactive reagents. Alkyl triflates are known to be powerful alkylating agents,[52] and, although these have not been used to alkylate methionine in amino acids, peptides or proteins, we found that these react efficiently and cleanly with poly(Met). Functional alkyl triflates were prepared in a straightforward manner from a variety of hydroxyalkyl compounds. These reagents reacted efficiently with poly(Met) in organic solvents under mild conditions to give the fully alkylated polymers (Figure 6.5). Due to the significant difference in reactivity between alkyl triflate and bromide, this method also allowed incorporation of alkyl bromide functionality onto poly(Met) (**16**). This electrophilic functionality can be readily modified by subsequent reaction with different nucleophiles, such as amines or thiols. We reacted polysulfonium **16** with aminomethane sulfonic acid, which gave quantitative incorporation of sulfonate functionality (**16a**) that may be useful in mimicking sulfonated biopolymers (Figure 6.6). Alkyl triflate modification of poly(Met) was also found to be a facile method to introduce the azide functional group (**17**), which gave polypeptides bearing the click

reactive counterpart to the alkyne groups introduced above. Other functional groups that required silver salts for introduction onto poly(Met) could also be introduced by use of the corresponding alkyl triflates. Thus, ether (**18**) and glycoside (**19**) functionalities were added to poly(Met) via the corresponding alkyl triflates. Removal of the acetyl protecting groups from the sugars in polypeptide **19** gave a water soluble glycopolypeptide with no signs of any degradation (see SI). In general, all of the above poly(Met) alkylations were found to cause no polypeptide chain cleavage, and gave polysulfoniums that were stable in a variety of media, at different pH (2 to 10), at elevated temperature (80 ºC), and after storage for more than 3 months (see Experimental section 6.5).

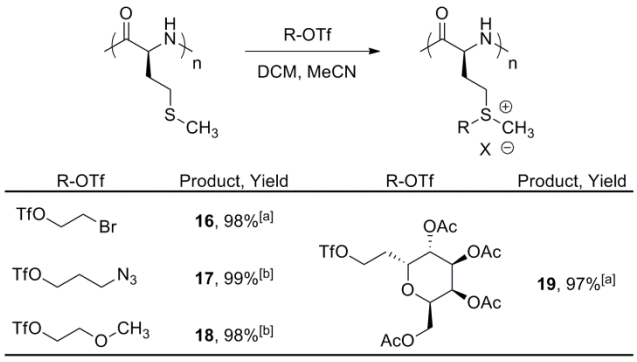


Figure 6.5 Alkylation of poly(Met) using alkyl triflates. Reagents and Conditions: R-OTf, DCM/MeCN, 20 ^{o}C. Yield is total isolated yield of completely functionalized polypeptide. [a] X = OTf. [b] product was dialyzed against 0.1 M aqueous NaCl to give X = Cl.

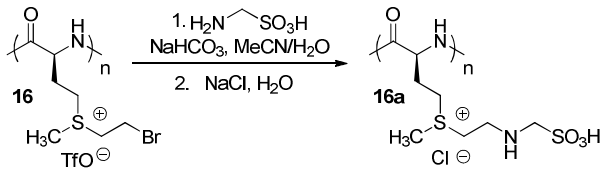


Figure 6.6 Preparation of highly sulfonated polypeptides.

The results above confirm that alkylation of poly(Met) is a highly efficient process for preparation of functionalized polypeptides. Many of the polysulfoniums reported here are the first examples of methionine derivatives bearing these reactive or functional groups, and, compared with other methods, these reactions are also very cost effective and straightforward ways to introduce these groups onto polypeptides. We have shown that alkylation of poly(Met) is broad in scope, high yielding, occurs readily, can use equimolar reagents, has a single reaction trajectory, gives stable products, and purification of products is facile: characteristics that define "click" type reactions.[3] For methionine alkylation to be considered a "click" reaction, it must also be chemoselective.[3] In peptides, polypeptides, and proteins, there can be many nucleophilic functional groups that react with alkylating reagents.[42] Of these, the cysteine thiol group is most widely utilized in protein and peptide alkylation reactions,[53] as it typically gives single products, in contrast to lysine amines and histidine imidazoles, which give multiple alkylation products. It is noteworthy that in water the thiol, amine, and imidazole functional groups are highly reactive with alkylating agents at near neutral pH and above, but their reactivity diminishes greatly with decreasing pH.[42,54] This trend is due to protonation of all these nucleophilic amino acids at low pH, which greatly decreases their reactivity. Since methionine is more resistant to protonation, it is consequently the only highly reactive nucleophilic amino acid at pH < 3.[16-19,27-31] To highlight this chemoselectivity and show its utility for selective alkylation of methionine in polypeptides, we prepared a statistical copolymer of methionine and lysine and studied its alkylation (Figure 6.7). We chose lysine as a competing nucleophile since it is the most abundant nucleophile found in proteins, it is more widely used in synthetic polypeptides compared to histidine or cysteine, and it is known to compete with thiol and imidazole groups in protein alkylations.[42,53] As is also true for cysteine and histidine polypeptides, the synthesis of poly(L-lysine) requires the use of

protecting groups, and methionine alkylation needs to be compatible with and orthogonal to deprotection chemistry to be useful as a "click" reaction. Here we found that the methionine residues of our copolymer could be alkylated with propargyl bromide either before or after lysine deprotection to give the same final product. These results show that methionine sulfonium groups are stable to deprotection reactions (see Experimental section 6.5), and alternatively that methionine residues can be alkylated chemoselectively at pH 2.0 in the presence of a fourfold excess of free amine groups (Figure 6.7). Confirming this selectivity, a control reaction of propargyl bromide with pure poly(L-lysine) under identical conditions gave no alkylation products (see Experimental section 6.5). All the alkyne groups in the copolymer prepared above were then quantitatively conjugated with azide terminated PEG chains, showing that alkyne reactivity is not compromised and that the sulfonium groups are stable and useful for further polypeptide modifications.

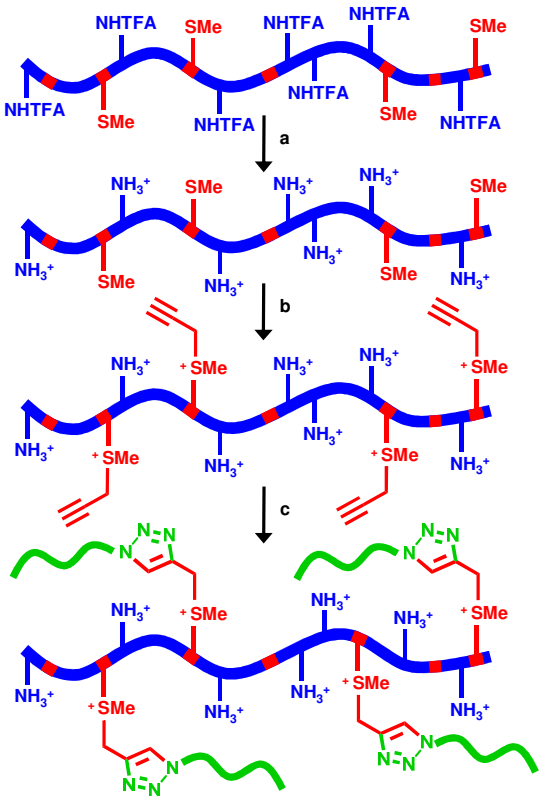


Figure 6.7 Schematic showing chemoselective alkylation of methionine residues in the presence of excess amine groups, followed by clicking of PEG-N_3 to the alkylated methionine residues. Initial copolymer is poly[(N_ε-TFA-L-lysine)$_{0.8}$-*stat*-(Met)$_{0.2}$]$_{206}$, where 0.8 and 0.2 are mole fractions of lysine and methionine residues, respectively. Reagents and conditions: (a) K_2CO_3, MeOH, H_2O (99%); (b) propargyl bromide, 0.2M formic acid (94%); (c) α-methoxy-ω-azidoethyl-poly(ethylene glycol) (M_n = 1,000 Da), $CuSO_4$, ascorbic acid, PMDETA, H_2O (95%).

6.4 Conclusion

In summary, the alkylation of methionine residues in polypeptides has all the features of a "click" reaction,[2] and consequently is an attractive means for preparation of functionalized and side-chain reactive polypeptides. Aside from the examples given here, these methods should also be applicable to a variety of other alkylating reagents and thioether containing residues, such as *S*-alkyl cysteines. In comparison to other methods for installation of functional and click reactive groups onto polypeptides, the starting material methionine is substantially less expensive than

the side chain modified or unnatural amino acids typically employed, and poly(Met) is readily prepared with controlled and high molecular weights, which makes these "methionine click" reactions attractive for large-scale use. Facile incorporation of other click-reactive functional groups (e.g. alkyne, azide, or alkene) by methionine alkylation also allows for further chemoselective modification of polypeptides. Such "secondary click" strategies, as shown in Figure 6.7, allow methionine alkylation to also utilize the broad diversity of reagents already developed and available for existing click conjugation methods.[1,2]

6.5 Experimental

6.5.1 Materials and Methods

Unless stated otherwise, reactions were conducted in oven-dried glassware under an atmosphere of nitrogen using anhydrous solvents. Hexanes, THF, DCM, and DMF were purified by first purging with dry nitrogen, followed by passage through columns of activated alumina (or 4 Å molecular sieves for DMF). MeCN was freshly distilled from CaH_2. Deionized water (18 MΩ-cm) was obtaining by passing in-house deionized water through a Millipore Milli-Q Biocel A10 purification unit. All commercially obtained reagents were used as received without further purification unless otherwise stated. Reaction temperatures were controlled using an IKA magnetic temperature modulator, and unless stated otherwise, reactions were performed at room temperature (RT, approximately 20 °C). Thin-layer chromatography (TLC) was conducted with EMD gel 60 F254 precoated plates (0.25 mm) and visualized using a combination of UV, anisaldehyde, and phosphomolybdic acid staining. Selecto silica gel 60 (particle size 0.032–0.063 mm) was used for flash column chromatography. ^{1}H NMR spectra were recorded on Bruker spectrometers (at 500 MHz) and are reported relative to deuterated solvent signals. Data for ^{1}H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity, coupling

constant (Hz) and integration. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet and br, broad. ^{13}C NMR spectra were recorded on Bruker spectrometers (at 125 MHz). Data for ^{13}C NMR spectra are reported in terms of chemical shift. High-resolution mass spectrometry (HRMS) was performed on a Micromass Quatro-LC Electrospray spectrometer with a pump rate of 20 μL/min using electrospray ionization (ESI). All Fourier Transform Infrared (FTIR) samples were prepared as thin films on NaCl plates and spectra were recorded on a Perkin Elmer RX1 FTIR spectrometer and are reported in terms of frequency of absorption (cm^{-1}). Tandem gel permeation chromatography/light scattering (GPC/LS) was performed on a SSI Accuflow Series III liquid chromatograph pump equipped with a Wyatt DAWN EOS light scattering (LS) and Optilab rEX refractive index (RI) detectors. Separations were achieved using 10^5, 10^4, and 10^3Å Phenomenex Phenogel 5 μm columns using 0.10 M LiBr in DMF as the eluent at 60 °C. All GPC/LS samples were prepared at concentrations of 5 mg/mL.

6.5.2 Polymerization Data

Monomer[a]	M_n[b]	DP[c]	yield (%)[d]
25 Met NCA	6,450	49	98
75 Met NCA	19,830	151	96
125 Met NCA	33,220	253	97
150 Met NCA	39,260	299	96
225 Met NCA	58,560	446	99

Table 6.1 Polymerization data for poly(Met), prepared with $(PMe_3)_4Co$ in THF at 20 °C, using polyethylene glycol (M_n = 2000 Da) endgroup analysis. [a] Number indicates equivalents of monomer per $(PMe_3)_4Co$. [b] Molecular weight of poly(Met) after polymerization as determined by ^1H NMR. [c] DP = number average degree of polymerization of poly(Met) by ^1H NMR. [d] Total isolated yield of polypeptide.

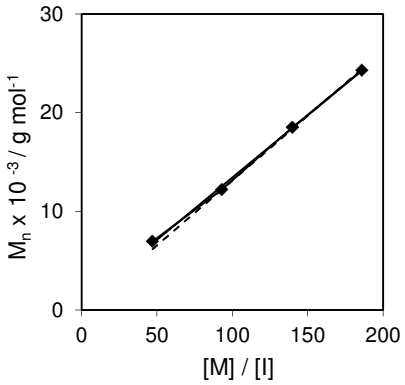


Figure 6.8 Molecular weight (M_n, ♦, solid line) of poly(Met) as a function of ratio of monomer to living poly(Z-L-lysine) macroinitiator ([M]/[I]) in THF at 20 °C. Expected molecular weight (dashed line) calculated based on monomer to macroinitiator stoichiometry. Macroinitiator was prepared by polymerization of Z-Lys NCA using $(PMe_3)_4Co$ in THF, M_n = 24,370 and M_w/M_n = 1.17 determined by GPC/LS.

Monomer[a]	M_n[b]	DP[c]	yield (%)[d]
47 Met NCA	6,960	53	91
93 Met NCA	12,210	93	94
140 Met NCA	18,510	141	90
186 Met NCA	24,290	185	95

Table 6.2 Polymerization data for poly(Met), prepared using living poly(Z-L-lysine)$_{93}$ macroinitiator in THF at 20 °C. [a] Number indicates equivalents of monomer per living poly(Z-L-lysine)$_{93}$ macroinitiator. [b] Molecular weight of poly(Met) after polymerization as determined by ^{1}H NMR. [c] DP = number average degree of polymerization of poly(Met) by ^{1}H NMR. [d] Total isolated yield of polypeptide.

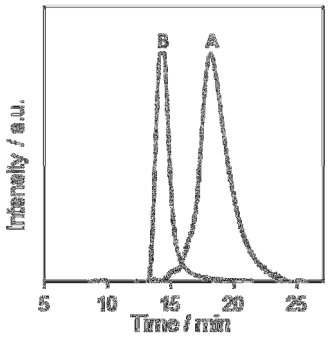
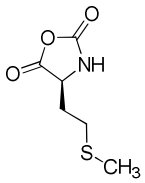


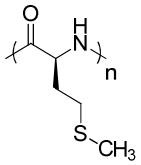
Figure 6.9 GPC chromatograms (normalized LS intensity in arbitrary units (a.u.) versus elution time) of polypeptides: (A) after initial polymerization of N_{ε}-CBz-L-lysine-*N*-carboxyanhydride to give poly(CBz-Lys)$_{67}$; (B) after chain extension by polymerization of Met NCA to give poly(CBz-Lys)$_{67}$-*b*-poly(Met)$_{72}$ diblock copolypeptide.

6.5.3 Experimental Procedures



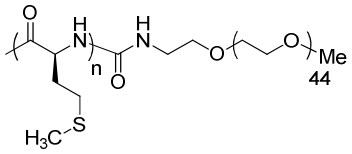
L-Methionine-*N*-carboxyanhydride (Met NCA), 1

To a solution of L-methionine (2.00 g, 13.4 mmol) in dry THF (0.15 M) in a Schlenk flask was added a solution of phosgene in toluene (26.8 mmol, 20 % (w/v), 2 equiv) via syringe. ***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*. The reaction was stirred under N_2 at 50 ^{o}C for 3 hrs, then evaporated to dryness and transferred to a dinitrogen filled glove box. The condensate in the vacuum traps was treated with 50 mL of concentrated aqueous NH_4OH to neutralize residual phosgene. Crude Met NCA, a yellow oil, was purified by column chromatography[37] in 20% THF in hexanes to give 2.11 g (91 %) of the product as a colorless viscous liquid that spontaneously crystallized upon standing. Spectral data was in agreement with previously published results.[37]



Poly(L-methionine), poly(Met), 2, General Procedure for Polymerization of Met NCA

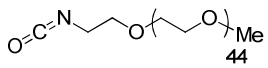
All polymerization reactions were performed in a dinitrogen filled glove box. To a solution of Met NCA in dry THF (50 mg/mL) was rapidly added, via syringe, a solution of $(PMe_3)_4Co$[55] in dry THF (20 mM). The reaction was stirred at room temperature and polymerization progress was monitored by removing small aliquots for analysis by FTIR. Polymerization reactions were generally complete within 1 hour. Reactions were removed from the drybox and HCl (2 equiv per $(PMe_3)_4Co$, 6M in H_2O) was added to the solution, which turned a blue-green color. After 10 min stirring, poly(Met) was collected by precipitation into acidic water (pH 3, HCl, >10x the reaction volume), followed by centrifugation. The white precipitate was washed with two portions of DI water and then lyophilized to yield poly(Met) as a fluffy white solid (99 % yield). ^{1}H NMR (500 MHz, d-TFA, 25 ^{o}C): δ 5.07 (br s, 1H), 2.90 (br s, 2H), 2.48-2.29 (m, 5H).



General Procedure for Endcapping of Polymethionine with poly(ethylene glycol) and Molecular Weight Determination by Endgroup Analysis[39]

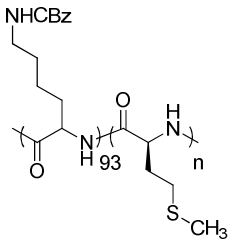
The general procedure for polymerization of Met NCA was followed. Upon completion of the reaction, as confirmed by FTIR, a solution of α-methoxy-ω-isocyanoethyl-poly(ethylene glycol), PEG-NCO, (see below) in THF (3 equiv per $(PMe_3)_4Co$) was added to the polymerization

reaction in a dinitrogen filled glove box. The reaction immediately turned from pale orange to green. The reaction was stirred overnight at room temperature and then was removed from the glove box and HCl (2 equiv per $(PMe_3)_4Co$, 6M in H_2O) was added to the solution, which turned a blue-green color. After 10 min stirring, endcapped poly(Met) was collected by precipitation into water (pH 3, HCl, >10x the reaction volume), followed by centrifugation.The white solids were washed with 3 portions of DI water to remove all unconjugated PEG-NCO. The PEG endcapped polymers were then isolated by lyophilization as white solids (95-99% yield). To determine poly(Met) molecular weights (M_n), ^1H NMR spectra were obtained. Since it has been shown that end-capping is quantitative for $(PMe_3)_4Co$ initiated NCA polymerizations when excess isocyanate is used,[39] integrations of methionine resonances versus the polyethylene glycol resonance at δ 3.64 could be used to obtain poly(Met) lengths.



Preparation of α-methoxy-ω-isocyanoethyl-poly(ethylene glycol)

To a solution of α-methoxy-ω-aminoethyl-poly(ethylene glycol), PEG-NH_2 (1.0 g, 0.500 mmol, M_n = 2000 Da, Nanocs) in dry THF (25 mL) in a Schlenk flask was added a solution of phosgene in toluene (0.50 mL, 1.00 mmol, 20 % (w/v) in toluene, 2 equiv) via syringe. ***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.* The reaction was stirred under N_2 at room temperature for 16 h then evaporated to dryness and transferred to a dinitrogen filled glove box. The condensate in the vacuum traps was treated with 50 mL of concentrated aqueous NH_4OH to neutralize residual phosgene. The isocyanate was precipitated from minimal THF into 1:1 Et_2O:hexanes and was recovered as 1.01 g of a white solid (99%), no further purification.

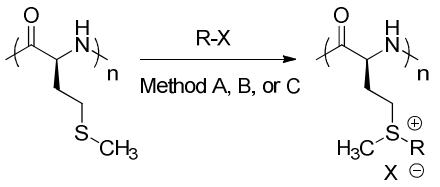


General Procedure for Polymerization of Met NCA using Living Poly(Z-L-lysine) Macroinitiator

Inside a dinitrogen filled glove box, a solution of Z-Lys NCA[56] in dry THF (0.15 M) was prepared. A solution of $(PMe_3)_4Co$ in dry THF (20 mM) was rapidly added via syringe. After 45 min, the polymerization reaction was complete as determined by FTIR. An aliquot of poly(Z-L-lysine) was removed and analyzed by GPC/LS (M_n = 24,370, M_w/M_n = 1.17, DP = 93). To a solution of Met NCA in dry THF (50 mg/mL) was rapidly added via syringe, a solution of the living poly(Z-L-lysine) macroinitiator, poly(Z-lysine)$_{93}$, in dry THF (0.15 M). The reaction was stirred at room temperature and polymerization progress was monitored by FTIR. Polymerization reactions were generally complete within 1 hour. Reactions were removed from the drybox and HCl (2 equiv per $(PMe_3)_4Co$, 6M in H_2O) was added to the solution, which then turned a blue-green color. After 10 min stirring, copolymers were collected by precipitation into acidic water (pH 3, HCl, >10x the reaction volume), followed by centrifugation. The precipitates were washed with two portions of DI water and then lyophilized to yield the poly(Z-L-lysine)$_{93}$-*block*-poly(Met)$_n$ block copolymers as fluffy white solids (99 % yield).

To determine polymer molecular weights by ^{1}H NMR spectra, all poly(Z-L-lysine)$_{93}$-*block*-poly(Met)$_n$ samples were first oxidized to poly(Z-L-lysine)$_{93}$-*block*-poly(L-methionine sulfoxide)$_n$ to improve their solubility.[57] Poly(Z-L-lysine)$_{93}$-*block*-poly(Met)$_n$ samples were suspended in 30% H_2O_2 in water with 1% AcOH and stirred for 30 min at 0 ^oC. The reactions

were quenched with drops of 1M sodium thiosulfate in water and then transferred to 2000 MWCO dialysis bags, and dialyzed against DI water for 48 hours with water changes twice per day. The contents of the dialysis bags were then lyophilized to dryness. Integrations of methionine resonances versus the resonances of the poly(Z-lysine)$_{93}$ benzyl groups found at δ 7.30 and 5.18 were used to obtain poly(Met) lengths.



Poly(Met) Alkylation using activated R-X (Method A)

Poly(Met) was suspended in either DMF, water, or 0.2 M aqueous formic acid (10 mg/mL). Alkyl halide (3 eq per methionine residue) was added. 1.1 eq alkyl halide per methionine can also be used with an increased reaction time of 72 hours to give identical products. The reaction mixture was covered with foil and stirred at room temperature for 48 hours. The reaction was then diluted 2x with water, transferred to a 2000 MWCO dialysis bag, and dialyzed against 0.10 M NaCl for 24 hours, followed by DI water for 48 hours with water changes twice per day. Dialysis against NaCl serves to exchange counterions so that only chloride is present. The contents of the dialysis bag were then lyophilized to dryness to give the product as a white solid.

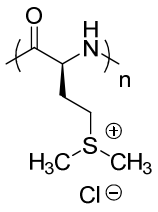
Poly(Met) Alkylation using R-OTf (Method B)

Poly(Met) was dissolved in dry DCM (10 mg/mL). Alkyl triflate (2 eq per methionine residue) was added. The reaction mixture was stirred at room temperature for 48 hours. White precipitate was observed after 24 hours in all cases. After 24 hours, MeCN was added to give a 1:1

MeCN:DCM mixture to solubilize the polymer, and the resulting solution was stirred for 24 more hours. The reaction was precipitated with ether to remove excess alkyl triflate and then evaporated to dryness to give the product as a white solid. The product can then be dispersed in water, transferred to a 2000 MWCO dialysis bag, and then dialyzed against 0.10 M NaCl for 24 hours, followed by DI water for 48 hours with water changes twice per day. Dialysis against NaCl serves to exchange counterions so that only chloride is present.

Poly(Met) Alkylation using unactivated R-X (Method C)

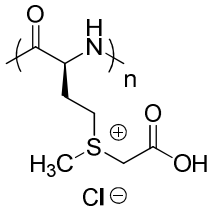
Poly(Met) was suspended in dry MeCN (10 mg/mL). Alkyl halide (1.1 eq per methionine residue) was added, followed by a solution of $AgBF_4$ in MeCN (50 mg/mL, 1 equiv). The reaction mixture was covered with foil and stirred at 50 ^oC for 24 hours under N_2. A yellow precipitate was observed in all cases. The reaction was centrifuged to remove the precipitate, and polymer isolated by precipitation with ether and evaporation to dryness to give the product as a white solid. The product can then be dispersed in water, transferred to a 2000 MWCO dialysis bag, and then dialyzed against 0.10 M NaCl for 24 hours, followed by DI water for 48 hours with water changes twice per day. Dialysis against NaCl serves to exchange counterions so that only chloride is present.



Poly(*S*-methyl-L-methionine sulfonium chloride), 3

Prepared from poly(Met) and methyl iodide using method A in either DMF, water, or 0.2 M aqueous formic acid.

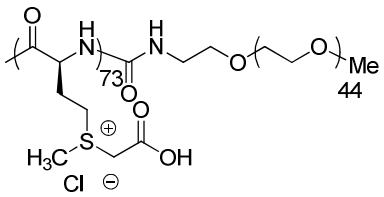
^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ 3.46 (br s, 2H), 2.98 (br m, 6H), 2.44-2.33 (br m, 1H), 2.32-2.20 (br m, 1H).



Poly(*S*-carboxymethyl-L-methionine sulfonium chloride), 4

Prepared from poly(Met) and either iodoacetic acid or bromoacetic acid using method A in either DMF, water, or 0.2 M aqueous formic acid.

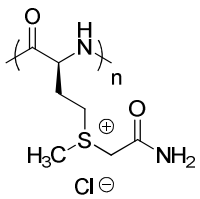
^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ 4.56 (br s, 1H), 4.29-4.15 (br m, 2H), 3.53-3.33 (br m, 2H), 3.00-2.93 (br d, 3H), 2.37-2.33 (br m, 1H), 2.32-2.22 (br m, 1H).



Poly(*S*-carboxymethyl-L-methionine sulfonium chloride)$_{73}$-*block*-poly(ethylene glycol)$_{44}$

Prepared from poly(Met)$_{73}$-*block*-poly(ethylene glycol)$_{44}$ and bromoacetic acid using method A in water. ^{1}H NMR integrals were calibrated using the polyethylene glycol resonance found at δ 3.72 in D_2O. Polypeptide chain length after alkylation was in agreement with the length observed before alkylation, indicating no degradation of the polypeptide chains occurs during aklylation.

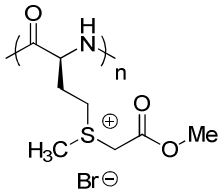
^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ 4.58 (br s, 68H), 3.72 (br s, 176H), 3.52-3.36 (br m, 148H), 2.97 (br d, *J*= 6.6,219H), 2.46-2.35 (br m, 73H), 2.35-2.23 (br m, 73H).



Poly(*S*-carbamidomethyl-L-methionine sulfonium chloride), 5

Prepared from poly(Met) and bromoacetamide using method A in either DMF, water, or 0.2 M aqueous formic acid.

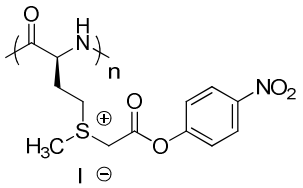
^{1}H NMR (500 MHz, D_2O, 25 ^{o}C): δ 4.70-4.63 (br m, 1H), 3.68-3.50 (br m, 2H), 3.12-3.06 (d, 3H), 2.53-2.43 (br m, 1H), 2.39-2.30 (br m, 1H).



Poly(*S*-(carboxymethyl methyl ester)-L-methionine sulfonium bromide), 6

Prepared from poly(Met) and methyl bromoacetate using method A in DMF. The reaction was stirred for 5 days at room temperature, then isolated by precipitation with ether.

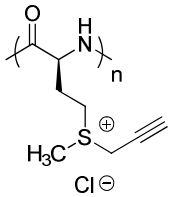
^{1}H NMR (500 MHz, d-TFA, 25 ^{o}C): δ 4.85 (br s, 1H), 3.82 (s, 3H), 3.38 (br s, 2H), 2.90 (s, 3H), 2.76 (br s, 2H).



Poly(*S*-(carboxymethyl *p*-nitrophenyl ester)-L-methionine sulfonium iodide), 7

Prepared from poly(Met) and *p*-nitrophenyl iodoacetate using method A. The reaction was performed in dry DMF, stirred for 5 days at room temperature, then isolated by precipitation with ether.

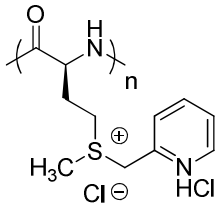
^{1}H NMR (500 MHz, d-DMSO, 25 ^{o}C): δ 8.21 (br s, 2H), 7.36 (br s, 2H), 4.35(br s, 1H), 3.60 (br s, 2H), 2.64 (br s, 2H), 2.46 (s, 3H), 2.00-1.75 (br m, 2H).



Poly(*S*-propargyl-L-methionine sulfonium chloride), 8

Prepared from poly(Met) and propargyl bromide using method A in either DMF, water, or 0.2 M aqueous formic acid.

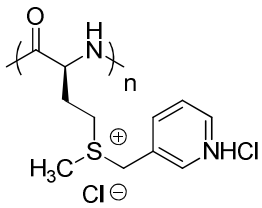
^{1}H NMR (500 MHz, d-TFA, 25 ^{o}C): δ 5.17 (br s, 1H), 4.66-4.45 (m 1H), 4.42-4.30 (m, 1H), 3.93-3.65 (br m, 2H), 3.12 (s, 3H), 3.01-2.55 (br m, 2H), 2.35 (s, 1H).



Poly(*S*-(2-pyridylmethyl hydrochloride)-L-methionine sulfonium chloride), 9

Prepared from poly(Met) and 2-(bromomethyl)pyridine hydrochloride using method A in DI water.

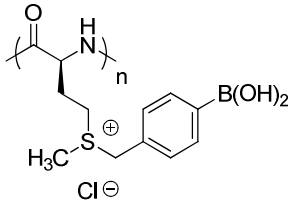
^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ 8.52 (s, 1H), 7.89 (m, 1H), 7.55 (m, 1H), 7.46 (s, 1H), 4.60 (s, 1H), 3.61-3.38 (m, 2H), 2.89 (s, 3H), 2.46-2.20 (br m, 2H).



Poly(*S*-(3-pyridylmethyl hydrochloride)-L-methionine sulfonium chloride), 10

Prepared from poly(Met) and 3-(bromomethyl)pyridine hydrochloride using method A in DI water.

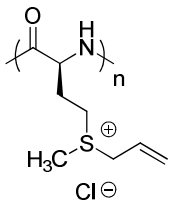
^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ 8.59 (s, 2H), 7.97 (s, 1H), 7.53 (s, 1H) , 4.54 (s, 1H), 3.53-3.01 (m, 2H), 2.87 (s, 3H), 2.45-2.18 (br m, 2H).



Poly(*S*-(4-boroxyphenylmethyl)-L-methionine sulfonium chloride), 11

Prepared from poly(Met) and 4-(bromomethyl)phenylboronic acid using method A in DMF.

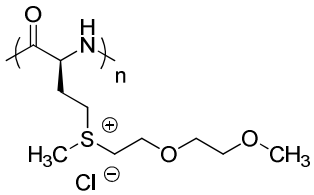
^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ 7.68 (br s, 2H), 7.37 (br s, 2H), 4.71-4.60 (br m, 1H), 4.55 (br s, 2H), 3.45-3.22 (br m, 2H), 2.82-2.71 (br m, 3H), 2.38-2.12 (br m, 2H).



Poly(*S*-allyl-L-methionine sulfonium chloride), 12

Prepared from poly(Met) and allyl iodide (prepared from commercially available allyl chloride using the Finkelstein reaction[58]) using method C.

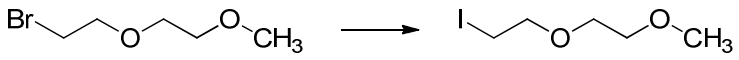
^{1}H NMR (500 MHz, d-TFA, 25 ^oC): δ 5.94-5.86 (br m, 1H), 5.81-5.71 (br m, 2H), 4.93 (br s, 1H), 4.18-4.11 (br m, 1H), 4.01-3.95 (br m, 1H), 3.60-3.41 (br m, 2H), 2.89 (br s, 3H), 2.63 (br s, 1H), 2.52-2.42 (br s, 1H).



Poly(*S*-(2-(2-methoxyethoxy)ethyl)-L-methionine sulfonium chloride), 13

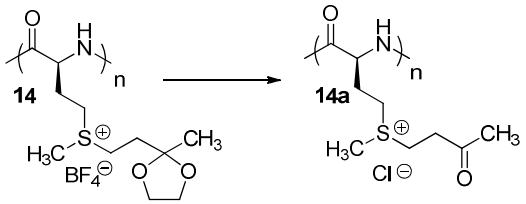
Prepared from poly(Met) and 1-iodo-2-(2-methoxyethoxy)ethane using method C. After reaction completion and removal of silver iodide by centrifugation, the polymer was precipitated from solution with ether and collected by centrifugation. The white solids where taken up in water and transferred to a 2000 MWCO dialysis bag, and dialyzed against 0.10 M NaCl for 24 hours, followed by DI water for 48 hours with water changes twice per day. The contents of the dialysis bag were then lyophilized to dryness to give the product as a white solid.

^{1}H NMR (500 MHz, d-TFA, 25 ^{o}C): δ 4.67 (br s, 1H), 3.95 (br s, 1H), 3.84-3.56 (br m, 9H), 3.48 (br s, 3H), 2.88 (s, 3H), 2.52 (br s, 1H), 2.35 (br s, 1H).



1-Iodo-2-(2-methoxyethoxy)ethane was prepared from commercial 1-bromo-2-(2-methoxyethoxy)ethane using the Finkelstein reaction. 1-Bromo-2-(2-methoxyethoxy)ethane (0.100 g, 0.546 mmol, 1 eq) was dissolved in acetone (5 mL, dried over $MgSO_4$) and sodium iodide (0.246 g, 1.64 mmol, 3 eq) was added. The reaction was covered with foil and stirred at 40 ^{o}C for 16 hours under N_2. The reaction was evaporated to dryness by rotary evaporation. The residue was taken up into 75 mL EtOAc and washed with 25 mL of 0.1 M sodium thiosulfate, and 2 x 50 mL of brine. The organic phase was dried over $MgSO_4$ and condensed by rotary evaporation to give 0.125 g of 1-iodo-2-(2-methoxyethoxy)ethane as a pale yellow oil (99%).

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 3.73 (t, *J* = 7.0, 2H), 3.63-3.62 (m, 2H), 3.54-3.52 (m, 2H), 3.36 (s, 3H), 3.24 (t, *J* = 7.0, 2H); ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ71.9, 71.8, 70.0, 59.0, 2.6. HRMS-ESI *m/z*) $[M + H]^+$ Calcd for $C_5H_{12}IO_2$, 230.98; found 230.98.

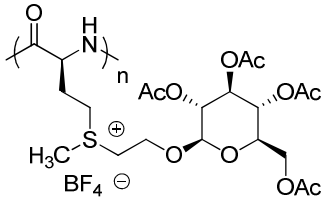


Poly(*S*-(3-oxybutyl)-L-methionine sulfonium chloride), 14a

Prepared from poly(Met) and bromoethyl-2-methyl-1,3-dioxolane using method C. After reaction completion and removal of silver iodide by centrifugation, the polymer was precipitated from solution with ether and collected by centrifugation (99% yield). Deprotection to give the

ketone groups was accomplished during dialysis (2000 MWCO tubing) against 2M HCl with 0.10 M NaCl for 24 hours, followed by DI water for 48 hours with water changes twice per day. The contents of the dialysis bag were then lyophilized to dryness to give the product **14a** as a white solid. (98%)

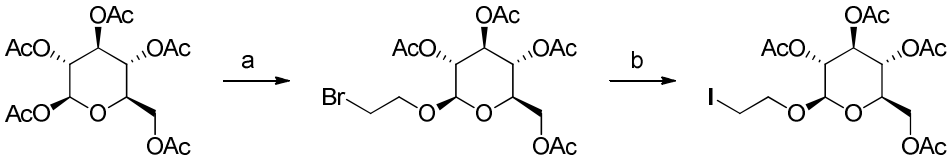
^{1}H NMR (500 MHz, D_2O, 25 ^{o}C): δ 4.63-4.44 (br m, 1H), 3.65-3.38 (br m, 4H), 3.27 (br s, 2H), 2.99 (s, 3H), 2.64 (br s, 1H), 2.41 (br s, 1H), 2.28 (s, 3H).^{19}F NMR (400 MHz, D_2O, 25 ^{o}C): no signals.



Poly(*S*-2-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)oxyethyl-L-methionine sulfonium tetrafluoroborate), 15

Prepared from poly(Met) and 1-iodo-2-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)ethane using method C.

^{1}H NMR (500 MHz, d-TFA, 25 ^{o}C): δ 5.60-5.53 (br m, 1H), 5.44-5.26 (br m, 1H), 4.86 (br s, 1H), 4.46 (br s, 3H), 4.24 (br s, 1H), 4.03 (br s, 1H), 3.90 (br s, 1H), 3.58 (br s, 2H), 3.39 (br s, 2H), 3.03 (d, J = 21.5, 3H), 2.59 (br s, 1H), 2.37-2.13 (br m, 13H).^{19}F NMR (400 MHz, d-DMAC, 25 ^{o}C): δ -79.5.

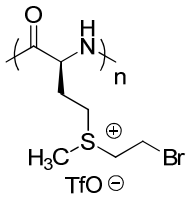


a) 2-bromoethanol, BF_3-OEt_2, DCM; b) NaI, acetone

Preparation of 1-iodo-2-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)ethane:

1-Bromo-2-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)ethane was prepared from commercial glucose pentaacetate according to a literature method[59], and then converted to 1-iodo-2-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)ethane using the Finkelstein reaction. 1-Bromo-2-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)ethane (1.17 g, 2.56 mmol, 1 eq) was dissolved in acetone (30 mL, dried over $MgSO_4$) and sodium iodide (1.15 g, 7.68 mmol, 3 eq) was added. The reaction was covered with foil and stirred at 40 ^oC for 16 hours under N_2. The reaction was evaporated to dryness by rotary evaporation. The residue was taken up into 75 mL EtOAc and washed with 25 mL of 0.1 M sodium thiosulfate, 50 mL of water, and 50 mL of brine. The organic phase was dried over $MgSO_4$ and condensed by rotary evaporation to give 1.26 g of 1-iodo-2-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)ethane (98%).

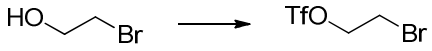
^1H NMR (500 MHz, $CDCl_3$, 25 ^oC): δ 5.06 (dd, *J* = 9.5, 9.5, 1H), 4.92 (dd, *J* = 9.6, 9.6, 1H), 4.88 (dd, *J* = 8.7, 8.7, 1H), 4.46 (d, *J* = 7.9, 1H), 4.11 (dd, *J* = 12.0, 4.9, 1H), 4.00-3.92 (m, 2H), 3.67-3.52 (m,2H), 3.19-3.06 (m, 2H), 1.93 (s, 3H), 1.92 (s, 3H), 1.87 (s, 3H), 1.85 (s, 3H); ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^oC): δ170.2, 169.8, 169.2, 169.0, 100.4, 72.4, 71.6, 70.8, 70.2, 68.1, 20.6, 20.5, 20.4, 2.2; HRMS-ESI *m/z*) $[M + H]^+$ $C_{16}H_{24}IO_{10}$,502.03; found 502.03.



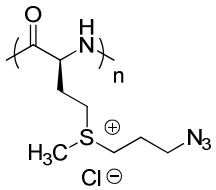
Poly(*S*-(2-bromoethyl)-L-methionine sulfonium triflate), 16

Prepared from poly(Met) and 2-bromoethyltriflate (prepared from commercially available 2-bromoethanol) using method B.

^{1}H NMR (500 MHz, d-TFA, 25 ^{o}C): δ 4.89 (br s, 1H), 4.06-3.95 (br m, 1H), 3.89-3.53 (br m, 5H), 3.07 (br s, 3H), 2.61 (br s, 1H), 2.43 (br s, 1H).^{19}F NMR (400 MHz, d-DMAC, 25 ^{o}C): δ -79.3.



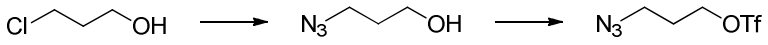
2-Bromoethyltriflate was prepared using a modified literature procedure.[60] 2-Bromoethanol (0.5 g, 4.00 mmol, 0.284 mL, 1 eq) was dissolved in dry DCM (15 mL) and dry pyridine was added (0.380 g, 4.80 mmol, 0.387 mL, 1.2 eq) and cooled to 0 ^{o}C under N_2. Triflic anhydride (1.24 g, 4.40 mmol, 0.740 mL, 1.1 eq, previously distilled over P_2O_5) was added dropwise and the reaction stirred for 20 min. The reaction was diluted with 100 mL of EtOAc and washed with 2 x 50 mL of water at pH 3 (HCl) to remove pyridine and pyridine salts, followed by 50 mL of 10% aqueous bicarbonate, and finally 25 mL of brine. The organic phase was dried over $MgSO_4$ and condensed by rotary evaporation at 25 ^{o}C to give 1.02 g of 2-bromoethyltriflate as a clear oil (99%), which was stored at -20 ^{o}C under N_2 and used with no furthur purification. Spectral data was in agreement with previously published results.[60]



Poly(*S*-(3-azidopropyl)-L-methionine sulfonium chloride), 17

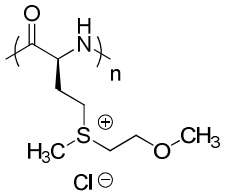
Prepared from poly(Met) and 3-azidoproyl-1-triflate (prepared from commercially available 3-chloro-1-propanol) using method B. Upon reaction completion, polymer was precipitated from solution with ether and collected by centrifugation. The white solids where taken up in water and transferred to a 2000 MWCO dialysis bag, and dialyzed against 0.10 M NaCl for 24 hours, followed by DI water for 48 hours with water changes twice per day. The contents of the dialysis bag were then lyophilized to dryness to give the product as a white solid.

^{1}H NMR (500 MHz, d-TFA, 25 ^{o}C): δ 4.88 (br s, 1H), 3.74-3.33 (m, 6H), 3.01 (s, 3H), 2.62 (br s, 1H), 2.43 (br s, 1H), 2.17 (br s, 2H).^{19}F NMR (400 MHz, D_2O, 25 ^{o}C): no signals.



3-Chloro-1-propanol (0.250 g, 2.64 mmol, 1 eq) was dissolved in DMSO (5 mL) andNaN_3 was added (0.198 g, 2.91 mmol, 1.1 eq) and the mixture heated to 60 ^{o}C for 48 hours under N_2. The reaction was diluted with 100 mL of water and the product was extracted with ether (3 x 50 mL). The combined organic phases were washed with water, then brine, dried over magnesium sulfate and condensed to 0.246g of a clear oil (92%). Spectral data was in agreement with previously published results.[61] 3-azido-1-propanol (0.246 g, 2.43 mmol, 1 eq) was dissolved in dry DCM (15 mL) and dry pyridine was added (0.231 g, 2.92 mmol, 0.235 mL, 1.2 eq) and the mixture cooled to 0 ^{o}C under N_2. Triflic anhydride (0.754 g, 2.67 mmol, 0.449 mL, 1.1 eq, previously distilled over P_2O_5) was added dropwise and the reaction stirred for 20 min. The reaction was

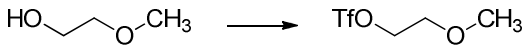
diluted with 100 mL of EtOAc and washed with 2 x 50 mL of water at pH 3 (HCl) to remove pyridine and pyridine salts, followed by 25 mL of 10% aqueous bicarbonate, followed by brine. The organic phase was dried over $MgSO_4$ and condensed by rotary evaporation at 25 ^oC to give 0.538 g of 3-azidopropyl-1-triflate as a clear oil (95%). The triflate was used directly with no furthur purification.



Poly(*S*-(2-methoxyethyl)-L-methionine sulfonium chloride), 18

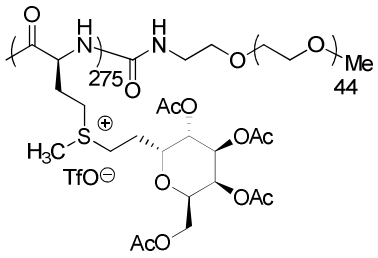
Prepared from poly(Met) and 2-methoxyethyl triflate (prepared from commercially available 2-methoxyethanol) using method B. Upon reaction completion, polymer was precipitated from solution with ether and collected by centrifugation. The white solids where taken up in water and transferred to a 2000 MWCO dialysis bag, and dialyzed against 0.10 M NaCl for 24 hours, followed by DI water for 48 hours with water changes twice per day. The contents of the dialysis bag were then lyophilized to dryness to give the product as a white solid.

^1H NMR (500 MHz, D_2O, 25 ^oC): δ 4.58 (br s, 1H), 3.94 (br s, 2H), 3.74-3.35 (br m, 7H), 3.01 (d, J = 5.7, 3H), 2.38 (br s, 1H), 2.26 (br s, 1H).^{19}F NMR (400 MHz, D_2O, 25 ^oC): no signals.



2-methoxyethanol (0.500 g, 6.57 mmol, 0.518 mL, 1 eq) was dissolved in dry DCM (15 mL) and dry pyridine was added (0.623 g, 7.88 mmol, 0.635 mL, 1.2 eq) and the mixture cooled to 0 ^oC under N_2. Triflic anhydride (2.04 g, 7.23 mmol, 1.22 mL, 1.1 eq, previously distilled over P_2O_5)

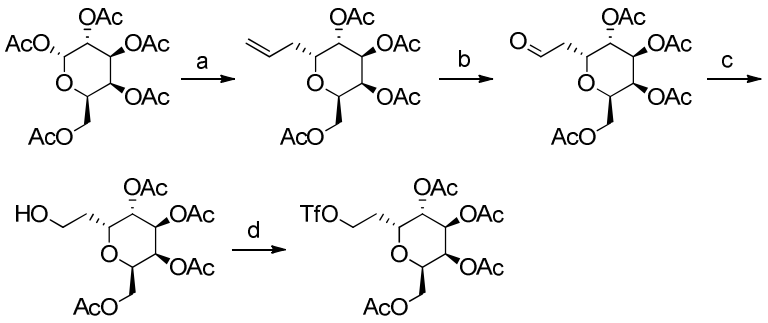
was added dropwise and the reaction stirred for 20 min. The reaction was diluted with 100 mL of EtOAc and washed with 2 x 50 mL 1 M NaCl at pH 3 (HCl) to remove pyridine and pyridine salts, followed by 25 mL of brine. The organic phase was dried over $MgSO_4$ and condensed by rotary evaporation at 25 ^oC to give 1.33 g of 2-methoxyethyl triflate as a clear oil (97%). The triflate was used directly with no furthur purification.



Poly(*S*-2-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)ethyl-L-methionine sulfonium triflate)-*block*-PEG$_{44}$,[19]

Prepared from poly(Met)-*block*-poly(ethylene glycol)$_{44}$ and 2-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)ethyl triflate using method B. ^1H NMR integrals were calibrated using the polyethylene glycol resonance found at δ 3.72 in D_2O. Polypeptide chain length after alkylation was in agreement with the length observed before alkylation.

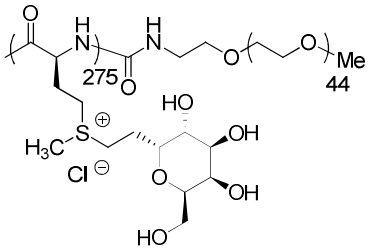
^1H NMR (500 MHz, d-TFA, 25 ^oC): δ 5.65 (br s, 282H), 5.45-5.41 (br m, 553H), 4.85 (br m, 567H), 4.62-4.28 (br m, 1124H), 3.78 (s, 176H), 3.75-3.40 (br m, 1110H), 3.07 (d, *J* = 23.5 840H), 2.67 (br s, 548H), 2.43 (br s, 544H), 2.27-2.18 (br m, 3260H).^{19}F NMR (400 MHz, d-DMAC, 25 ^oC): δ -79.1.



a) allyltrimethylsilane, BF_3-OEt_2, MeCN; b) O_3, DCM; PPh_3; c) $NaBH_4$, MeOH; d) $(Tf)_2O$, pyridine, DCM

Preparation of 2-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)ethyl triflate:

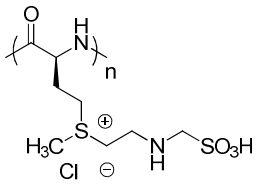
2-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)ethanol was prepared using previously published procedures via allylation of galactose pentaacetate,[62,63] followed by ozonolysis,[64] and reduction of the aldehyde.[65] 2-(2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl)ethanol (20.5 mg, 0.054 mmol, 1 eq) was dissolved in dry DCM (1.5 mL), dry pyridine was added (5.2 mg, 0.065 mmol, 5.3 µL, 1.2 eq), and the mixture cooled to 0 ^oC under N_2. Triflic anhydride (16.9 mg, 0.060 mmol, 10.1 µL, 1.1 eq, previously distilled over P_2O_5) was added and the reaction stirred for 20 min. The reaction was diluted with 50 mL of EtOAc and washed with 2 x 20 mL of water at pH 3 (HCl) to remove pyridine and pyridine salts, followed by 20 mL of 10% aqueous bicarbonate, and finally 20 mL of brine. The organic phase was dried over $MgSO_4$ and condensed by rotary evaporation at 25 ^oC to give 2-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)ethyl triflate as a clear oil (28 mg, >99% yield) which was used directly with no furthur purification.



Poly(*S*-2-(α-D-galactopyranosyl)ethyl-L-methionine sulfonium chloride)-*block*-poly(ethylene glycol)$_{44}$

To a solution of poly(S-2-(2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl)ethyl-L-methionine sulfonium triflate)-*block*-poly(ethylene glycol)$_{44}$ in methanol (10 mg/mL) was added hydrazine monohydrate (2 equiv per OAc group) and the reaction stirred overnight at room temperature. The reaction was quenched by addition of a few drops of acetone. Et_2O was added and the white solids were collected by centifugation, (99% yield). To exchange counterions, the solids were taken up with water and transferred to 2000 molecular weight cutoff dialysis tubing and dialyzed against 0.10 M NaCl for 24 hours, followed by DI water for 48 hours with water changes twice per day. The contents of the dialysis bag were then lyophilized to dryness to give the product as a white solid (88% yield).

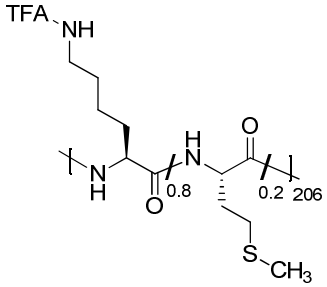
^{1}H NMR (500 MHz, D_2O, 25 ^{o}C): δ 4.58-4.41 (br m, 640H), 4.16 (br s, 275H), 4.02-3.96 (br m, 287H), 3.94 (br s, 270H), 3.77-3.70 (br m, 870H), 3.66 (s, 177H), 3.53-3.30 (br m, 1095H), 2.96 (br s, 817H), 2.63-2.47 (br m, 580H), 2.41-1.96 (br m, 617H).^{19}F NMR (400 MHz, D_2O, 25 ^{o}C): no signals.



Poly-*S*-(4-sulfonato-3-azabutyl)-L-methionine sulfonium chloride, 16a

A solution of amino methane sulfonic acid (3 eq) in saturated $NaHCO_3$ (25 mg/ml, pH 10) was added to a solution of poly-*S*-2-bromoethyl-L-methionine sulfonium triflate in MeCN (20 mg/mL). The reaction was stirred overnight at room temperature, then transferred to a 2000 MWCO dialysis bag, and dialyzed against 0.10 M NaCl for 24 hours, followed by DI water for 48 hours with water changes twice per day. The contents of the dialysis bag were then lyophilized to dryness to give a white solid.

^{1}H NMR (500 MHz, D_2O, 25 ^{o}C): δ 4.60 (s, 1H), 3.74 (m, 2H), 3.55-3.40 (m, 6H), 3.04 (s, 3H), 2.48-2.24 (m, 2H). ^{19}F NMR (400 MHz, D_2O, 25 ^{o}C): no signals.

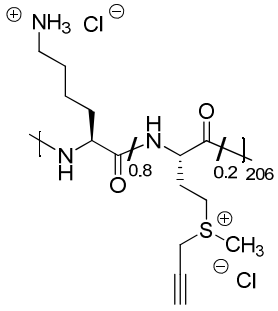


Poly[(*N*-ε-L-TFA-lysine)$_{0.8}$-*stat*-(L-Methionine)$_{0.2}$]$_{206}$, 20

Inside a dinitrogen filled glove box, a solution of $N_ε$-TFA-L-lysine-N-carboxyanhydride, TFA-Lys NCA, (440 mg, 1.64 mmol, 8 eq) and Met NCA (71.7 mg, 0.409 mmol, 2 eq) in dry THF (0.15 M) was prepared. A solution of $(PMe_3)_4Co$ in dry THF (20 mM) was rapidly added via syringe (18.6 mg, 0.051 mmol, 0.025 eq). After 45 min, the polymerization reaction was complete as determined by FTIR. An aliquot was removed and analyzed by GPC/LS (M_n =

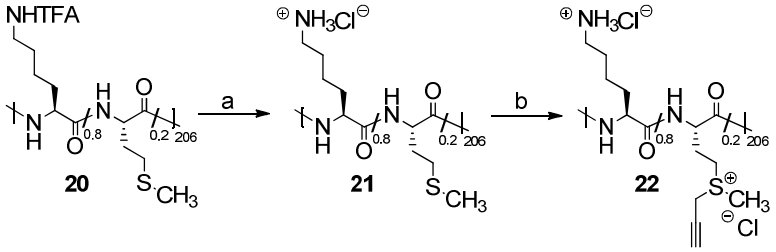
46,180, PDI=1.07, DP = 206). The polymerization was removed from the drybox and HCl (2 eq per $(PMe_3)_4Co$, 6M in H_2O) was added to the solution, which turned a blue-green color. After 10 min stirring, the copolymer was collected by precipitation into acidic water (pH 3, HCl, >10x the reaction volume), followed by centrifugation. The white precipitate was washed with two portions of DI water and then lyophilized to give the copolymer as a fluffy white solid (99 % yield).

^{1}H NMR (500 MHz, d-TFA, 25 ^{o}C): δ 4.63 (br s, 1H), 3.48 (br s, 2H), 2.46-1.37 (br m, 9H). ^{19}F NMR (400 MHz, MeOD, 25 ^{o}C): -75.3.



Poly[(L-lysine·HCl)$_{0.8}$-*stat*-(*S*-propargyl-L-methionine sulfonium chloride)$_{0.2}$]$_{206}$, 22

Route 1: Deprotection of lysine residues followed by methionine aklylation



a) K_2CO_3, MeOH, H_2O; b) propargyl bromide, 0.2 M aqueous formic acid.

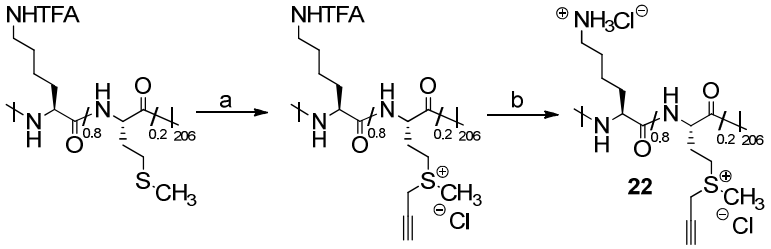
Poly[(N_ε-TFA-L-lysine)$_{0.8}$-*stat*-L-methionine)$_{0.2}$]$_{206}$ was dispersed in methanol:water, 9:1 (20 mg/mL) and K_2CO_3 (2 eq per lysine residue) was added. The reaction was stirred for 8 hours at

50 °C and then the methanol was removed by rotory evaporation. The remaining solution was then diluted with water and transferred to a 2000 MWCO dialysis bag, and dialyzed against 0.10 M NaCl at pH 3 (HCl) for 24 hours, followed by DI water for 48 hours with water changes twice per day. The contents of the dialysis bag were then lyophilized to dryness to give a white solid, **21**.

^{1}H NMR (500 MHz, D_2O, 25 °C): δ 4.53-4.45 (m, 0.25H), 4.32 (br s,1H), 3.03-2.99 (m, 0.45H), 2.10-1.97 (m, 1.18H), 1.85-1.67 (br m, 4H), 1.52-1.37 (br m, 2H).

The resulting poly[(L-lysine·HCl)$_{0.8}$-*stat*-(L-methionine)$_{0.2}$]$_{206,}$ **21,** was next reacted with propargyl bromide using alkylation method A in 0.2 M aqueous formic acid to give the final product, **22**.

Route 2: Methionine alkylation followed by deprotection of lysine residues



a) propargyl bromide, DMF; b) K_2CO_3, MeOH, H_2O.

Poly[(N_ε-TFA-L-lysine)$_{0.8}$-*stat*-(L-methionine)$_{0.2}$]$_{206}$ was dissolved in DMF (20 mg/mL) and propargyl bromide was added (3 eq per methionine residue). The solution was covered with foil and stirred for 48 hours at room temperature. The polymer was precipitated with ether and the solids collected by centrifugation. The solids were washed with 2 more portions of ether and then

dried under high vacuum. Poor solubility of this copolymer prevented collection of a meaningful NMR spectrum.

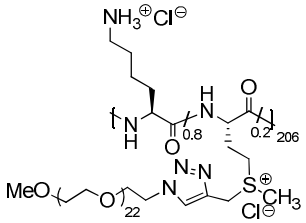
The TFA protecting groups of the resulting poly[(N_ε-TFA-L-lysine)$_{0.8}$-*stat*-(*S*-propargyl-L-methionine sulfonium chloride)$_{0.2}$]$_{206}$ were next removed using the same procedure as described under **Route 1** above to give the final product, **22**.

The ^{1}H NMR spectra of the final alkylated copolymers prepared using either of the two routes above were found to be identical.

^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ 4.67-4.56 (m, 0.25H), 4.33 (br s, 1H), 3.60-3.41 br m, 0.48H), 3.20-2.95 (m, 3.24H), 2.47-2.22 (m, 0.77 H), 1.90-1.67 (br m, 4H), 1.50 (br s, 2H). ^{19}F NMR (400 MHz, D_2O , 25 ^oC): no signals.

Reactivity of poly(L-lysine·HCl) with propargyl bromide under acidic conditions

As a control experiment, poly(L-lysine·HCl) was dissolved in 0.2M aqueous formic acid (20 mg/mL). Propargyl bromide was added (2 eq) and the reaction was covered with foil and stirred for 36 hours. The reaction was transferred to a 2000 MWCO dialysis bag, dialyzed against DI water for 72 hours with water changes twice per day, and then lyophilized to give a white solid. ^{1}H NMR spectrum was identical to the starting poly(L-lysine·HCl), no alkylation was observed.



Poly[(L-lysine·HCl)$_{0.8}$-*stat*-(*S*-(1-poly(ethylene glycol)$_{44}$-1,2,3-triazolylmethyl-L-methionine sulfonium chloride)$_{0.2}$]$_{206}$, 23

$CuSO_4$ (0.05 eq per alkyne) was dissolved in water and sodium ascorbate (0.25 eq per alkyne) was added followed by PMDETA (0.1 eq per alkyne). The dark blue solution was stirred under N_2 for 15 min and then added to a solution of poly[(L-lysine·HCl)$_{0.8}$-*stat*-(S-propargyl-L-methionine sulfonium chloride)$_{0.2}$]$_{206}$ and α-methoxy-ω-azidoethyl-poly(ethylene glycol) (1.5 eq per alkyne, M_n = 1000 Da, Sigma Aldrich). The solution was degassed by placing under partial vacuum and backfilling with N_2. The reaction was stirred for 24 hours at room temperature and then transferred to an 8000 MWCO dialysis bag. The reaction was dialyzed against 0.10 M NaCl for 24 hours, followed by DI water for 72 hours with water changes twice per day. The contents of the dialysis bag were then lyophilized to dryness to give the product as a white solid (93% yield, 100% pegylation as determined by NMR).

^{1}H NMR (500 MHz, D_2O, 25 ^{o}C): δ 4.56-4.49 (m, 0.25 H), 4.32 (br s, 1H), 3.72 (s, 22 H), 3.67-3.62(m, 0.48 H), 3.54-3.49 (m, 0.25H), 3.40 (s, 0.48H), 3.02 (br s, 2.75 H), 2.64-2.52 (m, 0.49 H), 1.88-1.64 (br m, 4H), 1.47 (br s, 2H).

TFA-Lysine NCA

To a solution of N_ε-TFA-L-lysine (1.00 g, 4.13 mmol) in dry THF (0.15 M) in a Schlenk flask was added a solution of phosgene in toluene (8.26 mmol, 20 % (w/v), 2 eq) via syringe.

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*. The reaction was stirred under N_2 at 50 ^oC for 3 hrs. The reaction was evaporated to dryness and transferred to a dinitrogen filled glove box. The condensate in the vacuum traps was treated with 50 mL of concentrated aqueous NH_4OH to neutralize residual phosgene. The NCA was purified by recrystallization in dry THF/hexanes to give 0.940 g (85 % yield) of the product as a white solid. Spectral data was in agreement with previously published results.[66]

Stability of poly(Met) sulfonium salts

In general, poly(Met) sulfonium salts prepared in this study were stable for >3 months when stored as solids at room temperature (poly(*S*-(2-bromoethyl)-L-methionine sulfonium triflate) was the only sample stored at -20 ^oC). Aqueous solutions of poly(*S*-methyl-L-methionine sulfonium chloride), **3**, or poly(*S*-carboxymethyl-L-methionine sulfonium chloride), **4**, (10 mg/mL) were subjected to various conditions to evaluate stability. Solutions of these polymers were heated at 80 ^oC for 16 hours, or stored for 3 hours at pH 2 (HCl), pH 10 (NaOH), or in 0.5M NaCl. Solutions of **3** or **4** (10 mg/mL) in water, DMF, PBS buffer, or DMEM cell culture media were stable for >1 week at room temperature. ^1H NMR spectra of samples after being subjected to each of these various conditions were identical to the starting materials and no polymer precipitation was observed.

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CHAPTER SEVEN

Chemoselective "Click and Release" Functionalization of Polypeptides via Reversible Methionine Alkylation

7.1 Abstract

The ability to easily attach and later remove a desired functional group from a peptide has many applications in biological assays, peptide and protein labeling, drug delivery, and stimuli responsive materials. Currently, few methods exist to attach and later release a functional group from peptides without complicated synthetic routes, harsh conditions, and in aqueous solution. Widely used "click" reactions (i.e. Cu catalyzed or strain promoted azide alkyne cycloadditions, radical thiol-ene reactions etc) are generally not reversible. To overcome this challenge, we have developed a simple, rapid, and reversible "click" type method of functionalization for peptides based on reversible alkylation of the natural amino acid methionine. These functionalizations quantitatively add and remove a desired group, are chemoselective, and can be performed in aqueous solution.

7.2 Introduction

"Click" type reactions have received much attention in recent years due to simplicity of design, the robust and chemoselective nature of the reactions, and wide functional group tolerance. Copper catalyzed or strain promoted azide-alkyne cycloadditions, radical thiol-ene couplings, and thiol-maleimide Michael additions, are currently popular methods to attach a desired functional group to polymeric materials, peptides, proteins, and even small molecules. While undoubtedly useful, these reactions are generally not reversible without complicated synthetic routes and harsh conditions. The ability to easily attach, and then later remove, a

desired functional group from a peptide has many applications in biological assays, peptide and protein labeling, drug delivery, and stimuli responsive materials. Here, we report the development of a simple, rapid, and reversible "click" type method of functionalization for peptides and polypeptides based on the reversible alkylation of methionine.

The Deming lab recently demonstrated that methionine will undergo chemoselective, broad scope, highly efficient alkylation reactions in homo- and copolypeptides yielding stable sulfonium derivatives.[1] These "methionine click" functionalizations use an inexpensive, natural amino acid that is readily polymerized, and requires no side-chain modification or protecting groups. The alkylation reactions can be performed in aqueous solution and allow the quantitative introduction of a diverse range of functionality and reactive groups onto polypeptides.

While many sulfonium salts are stable to a range of pH, salt, and temperature conditions,[1] the reaction of electrophilic sulfonium groups with nucleophiles has been reported. Phenyl dialkyl[2] and benzylic[3] sulfonium salts are known to efficiently alkylate a variety of nucleophiles, and have been used as reagents in organic synthesis. In peptide synthesis, the formation of benzylic methionine sulfonium salts is problematic during deprotection of residues that generate potent electrophilic alkylating species (i.e. benzyl and carbobenzyloxy groups). Treatment of these undesired sulfoniums with 2-mercaptopyridine has been reported remove the benzylic impurities and regenerate methionine.[4] Reversible alkylation of acetamido methionine sulfonium salts has also been described in two studies by Naider and co-workers.[5,6] A methionine containing tripeptide was alkylated with acetamido alkyl halides and then treated with various nucleophiles. The sulfonium salts were reportedly stable to hydroxyl and amine nucleophiles, but treatment with thiols resulted in cleavage of the sulfonium group and regeneration of methionine. Based on these reports, we sought to investigate the potential for reversible methionine

alkylation to be utilized as a rapid, simple, and efficient method to attach and later remove useful functional group from peptides and polypeptides. (Figure 7.1)

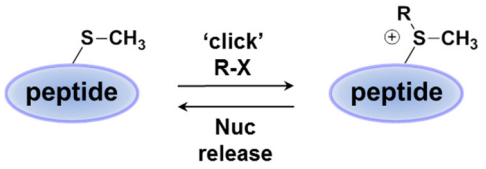


Figure 7.1 Click and release functionalization of peptides using the amino acid methionine.

7.3 Results and Discussion

In this study, statistical copolymers of 80% lysine and 20% methionine were used as water-soluble scaffolds to study the reversible alkylation of methionine residues. The $(K_{0.8}M_{0.2})_n$ polypeptides were prepared via established methods[1] using TFA-Lys and Met *N*-carboxyanhydrides and transition metal catalysis. Degrees of polymerization were either 40 or 120. The lysine residues were deprotected with standard methods and the methionine residues were then chemoselectively alkylated at low pH where lysine is protonated and not nucleophilic. We alkylated methionine with methyl, carboxymethyl, acetamido, benzylic, and propargylic groups to test a range of functionalities for susceptibility to nucleophilic attack and thiolysis. (Figure 7.2) To take advantage of the many azide and alkyne reagents that have been developed for Cu catalyzed or strain promoted azide alkyne cycloadditions, we designed several alkylating reagents that are amenable to further functionalization. All the sulfonium groups we tested were stable in aqueous solution at neutral pH, with 0.1 M NaCl, and in PBS buffer for greater than 2 weeks, and were stable for greater than 3 months when stored as dry solids at room temperature.

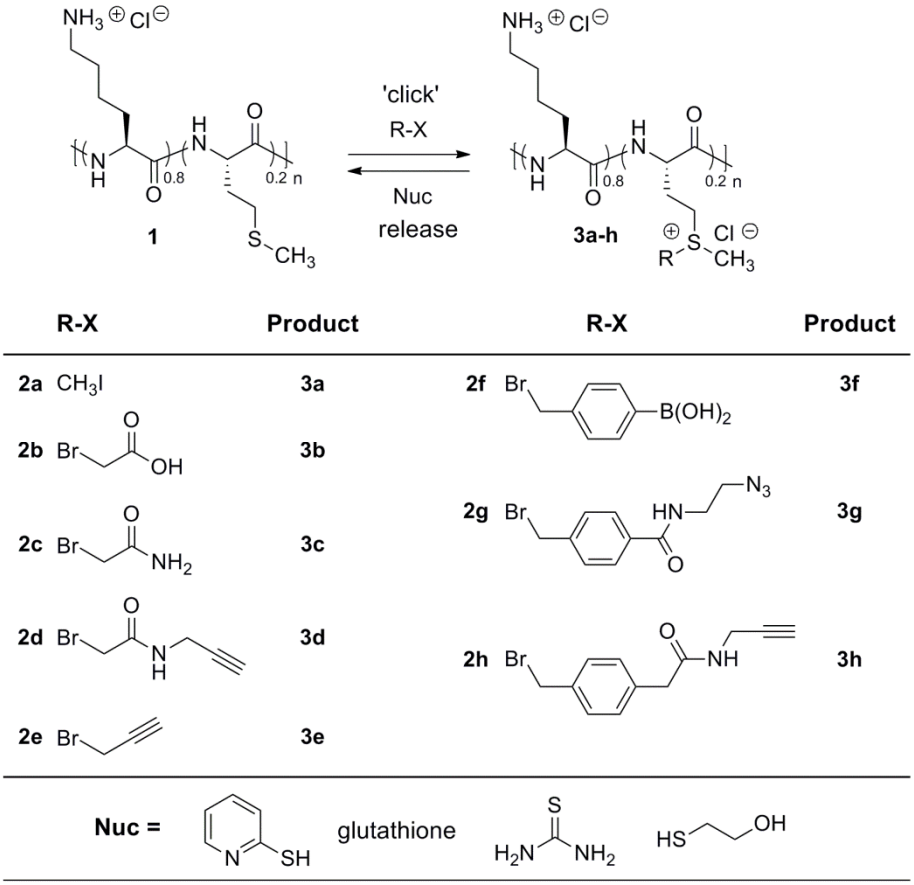


Figure 7.2 Alkylation of $(K_{0.8}M_{0.2})_n$ copolypeptides and treatment with thiol nucleophiles.

We treated the alkylated $(K_{0.8}M_{0.2})_n$ polypeptides with several thiol compounds to study the stability of the sulfonium groups. (Figure 7.2) When treated with 0.1 M thiol nucleophiles in PBS buffer (pH 7.4), all the sulfoniums we tested were either stable to the nucleophile, or regenerated methionine as the exclusive reaction product. Benzylic and propargylic sulfoniums regenerated methionine fastest, while amido sulfoniums were moderately labile. (Figure 7.3 and Experimental section 7.5.3) Methyl and carboxymethyl groups were stable to all thiol nucleophiles in the conditions tested.

Reversible alkylation of methylated methionine has previously been proposed as a method of protection to shield methionine residues from oxidation during protein synthesis.[7] In

this report, methionine was methylated and incorporated into short peptides. The resulting sulfoniums were reportedly stable to aminolysis, methanolic ammonia, HBr/acetic acid, and secondary amines, however, treatment with excess mercaptoethanol and triethylamine in DMF for 16 hours at room temperature regenerated methionine. Nucleophilic attack on methyl sulfoniums has also been shown in ring strained systems with thiolates,[8] and in enzyme catalyzed methyl transfer from *S*-adenosyl methionine, an important biological methylating agent for DNA, RNA, and proteins.[9] In our $(K_{0.8}M_{0.2})_n$ copolypeptides, however, the methyl sulfonium group was inert to thiolysis in aqueous PBS buffer at 37 ^oC for several days. In addition, we have previously shown that both the methyl and carboyxmethyl sulfonium salts of homopoly(methionine) are stable for 16 hours in boiling water, in aqueous solution at pH 2 (HCl) and pH 10 (NaOH), and with 0.1M NaCl. The carboxymethyl sulfonium group has been reported to be labile in proteins when activated by interactions with other charged residues,[10] or by boiling in strong acid,[11] however this group seems to be quite stable in both methionine homopolypeptides and $(K_{0.8}M_{0.2})_n$, which is in agreement with a previous report by Naider and co-workers.[5]

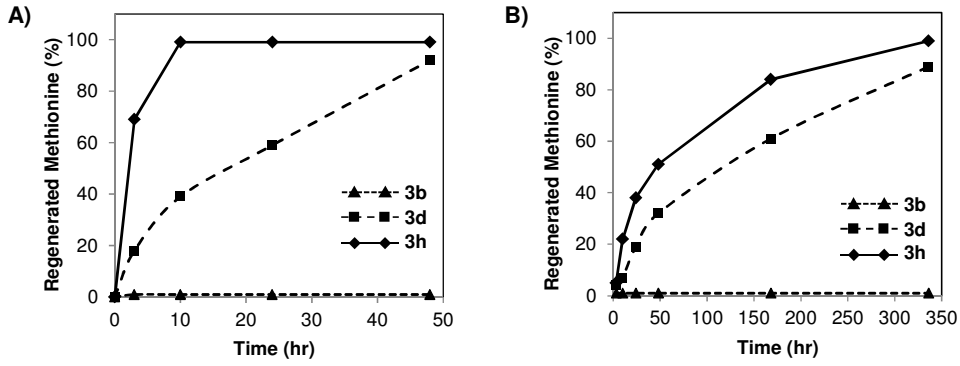


Figure 7.3 Rates of regeneration of methionine from various alkylated $(K_{0.8}M_{0.2})_{40}$ copolypeptides using A) 2-mercaptopyridine or B) glutathione; 0.1 M thiol in PBS buffer, 37 ^oC.

We found that 2-mercaptopyridine (2-PySH), thiourea, and mercaptoethanol cleave sulfonium groups and regenerate methionine at similar rates, while glutathione, a much more sterically hindered thiol, cleaved sulfonium groups much more slowly. (Figure 7.3 and experimental section 7.5.3) We chose to do further studies with 2-PySH as the thiol nucleophile because this compound quickly regenerates methionine, but will not reduce cysteine disulfide bonds (see Experimental section 7.5.2) and thus is more likely to be compatible with peptides and proteins. To investigate the mechanism of sulfonium cleavage by 2-PySH, alkylated $(K_{0.8}M_{0.2})_n$ **3h** was treated with 0.1 M 2-PySH in PBS buffer at 37 ^oC for 24 hours. The reaction was extracted with organic solvent and the by-products were analyzed by NMR and mass spectrometry. The sulfide shown in Figure 7.4 was isolated and compared to an authentic sample (see Experimental section 7.5.5), indicating the cleavage reaction proceeds by nucleophilic attack at the benzylic position, the most electrophilic carbon.

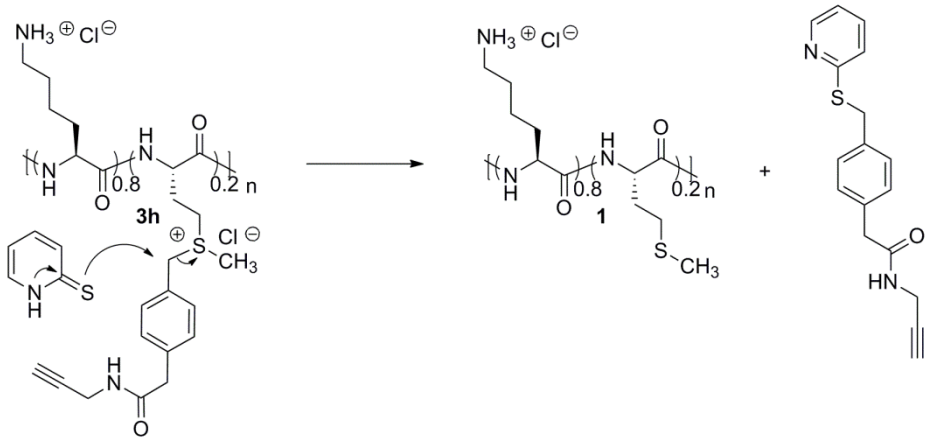


Figure 7.4 Proposed mechanism of sulfonium thiolysis and structure of isolated reaction by-product.

We prepared several different benzylic sulfonium derivatives, **3f**, **3g**, and **3h**, and all were equivalent in susceptibility to thiolysis by 2-PySH, which indicates that benzylic linkers may be

useful for reversible attachment of diverse functionalities onto methionine containing peptides. Simple alkyl and carboxymethyl sulfonium groups do not appear to be electrophilic enough to react with thiol nucleophiles in the mild conditions we tested, therefore these groups may be useful for permanent attachment of desired functional groups.

Alkylation of methionine with cyanogen bromide is known to cause hydrolysis of peptide bonds C-terminal to methionine residues in peptides and proteins.[12] To investigate whether alkylation of methionine or cleavage of sulfonium groups causes any cleavage of the parent polypeptide chain, a statistical copolymer of 50% CBz-lysine and 50% methionine was used to study the reversible alkylation of methionine residues by gel permeation chromatography (GPC). (Figure 7.5) This polypeptide was prepared via established methods from CBz-Lys and Met *N*-carboxyanhydrides and transition metal catalysis[1] and endcapped with poly(ethylene glycol)$_{22}$ (PEG) isocyanate.[13] The PEG group serves as an NMR handle for an additional check for chain cleavage. The methionine residues were alkylated with the benzylic alkyne reagent **2h**, and then treated with 2-PySH. The polypeptides were analyzed by GPC and NMR at each stage and molecular weights were in agreement with expected values, indicating that no chain cleavage occurred. (Figure 7.6)

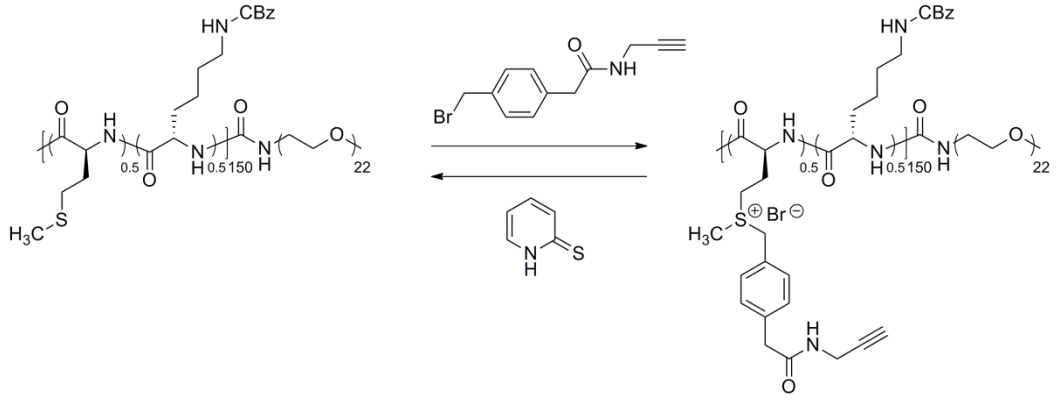


Figure 7.5 Alkylation of a 50/50 statistical copolymer of CBz-lysine and methionine residues followed by cleavage of the sulfonium group.

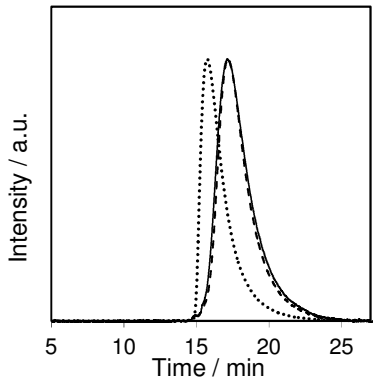


Figure 7.6: GPC chromatograms (normalized LS intensity versus elution time in arbitrary units (au)) of polypeptides after initial copolymerization of Met and CBz-Lys NCAs to give $(Met_{0.5}CBz\text{-}K_{0.5})_{150}$, (——), alkylation with 4-bromomethyl-*N*-propargyl-phenylacetamide **3h** to give polysulfonium (·····), and after cleavage of the sulfonium to give the parent $(Met_{0.5}CBz\text{-}K_{0.5})_{150}$ (- - - -).

As previously mentioned, azide-alkyne cycloadditions have found broad applicability in molecular and polymer functionalization as well as in chemically orthogonal ligations to study biological systems. The triazole cycloaddition product, however, is not readily reversed into the parent precursors with simple methods. The cycloreversion of a poly(methyacrylate) linked triazole by high intensity ultrasound in a Suslick cell was recently reported,[14] however this

method requires sophisticated equipment and has not been tested on any other substrates. In some cases, it would be advantageous to attach and later remove the many azide and alkyne reagents that have been developed for these "click" reactions. To this end, we investigated a "double click and release" strategy where azide or alkyne groups can be attached to methionine via reversible benzyl sulfonium linkage, "click" functionalized with a compatible group, and then removed by cleavage of the sulfonium linkage. (Figure 7.7)

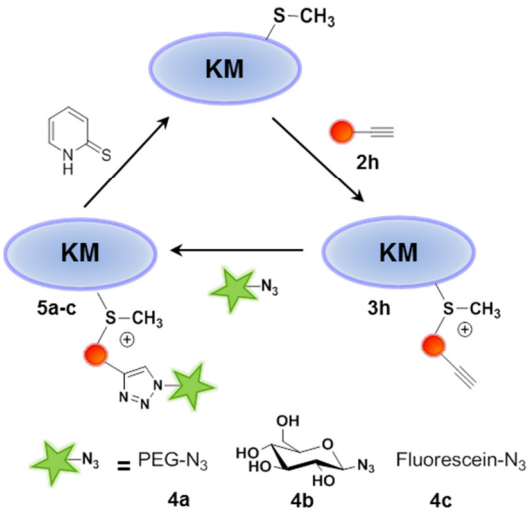


Figure 7.7 "Double click and release" strategy.

As proof of concept, alkyne functionalized $(K_{0.8}M_{0.2})_n$ **3h**, was reacted with azide functionalized poly(ethylene glycol) (MW= 1,000), **4a**, glucose, **4b**, or fluorescein **4c**. (Figure 7.7) Copper catalyzed click reactions proceeded smoothly in water with standard methods giving complete conversion to the triazole products. Fluorescence spectra were taken of **3h**, after copper click functionalization with **4c** to generate **5c,** and after thiolysis to regenerate **1**. (Figure 7.8) The fluorescence spectra clearly indicate methionine can be reversibly alkylated with a fluorescent tag using our benzyl sulfonium linkers. We also investigated click reactions on the propargyl

derivative **3e**, however, reactions were sluggish and increasing the copper catalyst loading resulted in premature sulfonium cleavage.

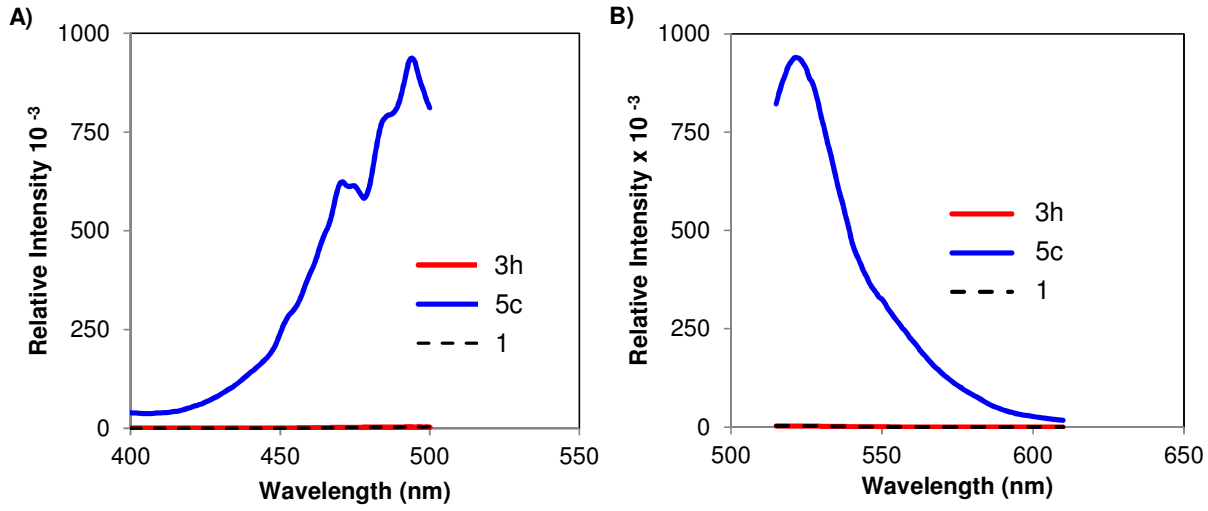


Figure 7.8 Release of fluorescein from polypeptide **5c** to give parent polypeptide **1** A) absorbance and B) emission spectra.

To demonstrate our chemoselective "click and release" methodology on a more complex system, we alkylated PHCKRM, a natural antioxidant peptide. This peptide contains all of the highly reactive naturally occurring amino acid nucleophilic groups and is a good model for selective alkylation. Alkylation reactions with PHCKRM and **2h** were performed in 0.2 M formic acid (pH 2.4) or sodium carbonate buffer (pH 9.2), and crude reaction products were analyzed by NMR and mass spectrometry. Alkylations in acidic solution resulted in a single alkylation product at the methionine residue, whereas reactions in basic solution resulted in several products with molecular weights corresponding to di- and tri- alkylations. (Figure 7.8 and Experimental section 7.5.4). As a control, cysteine, histidine, and lysine amino acids were separately incubated with excess benzyl bromide at pH 2.4 and no reaction occurred. PHCKRM alkylated at pH 2.4 was treated with 0.1 M aqueous 2-PySH at 37 ^{o}C for 24 hours, and after

extraction with organic solvent to remove excess 2-PySH and sulfonium cleavage by-products, the aqueous solution was analyzed by NMR and mass spectrometry. Thiolysis with 2-PySH resulted in a single product that was spectroscopically identical to the parent PHCKRM. (Figure 7.8 and Experimental section 7.5)

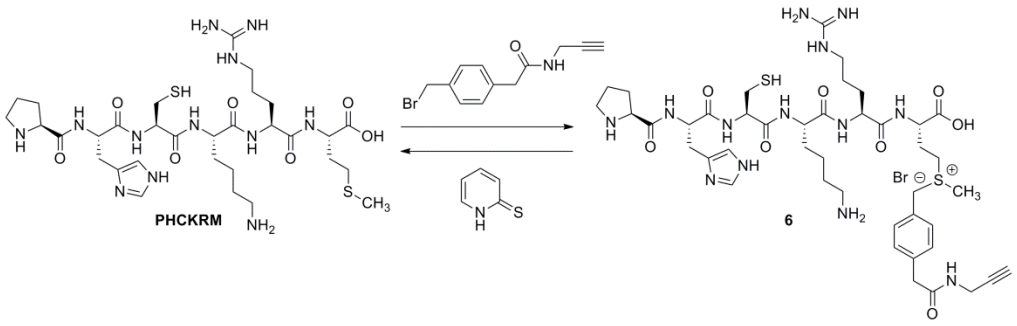


Figure 7.9 Click and release on PHCKRM

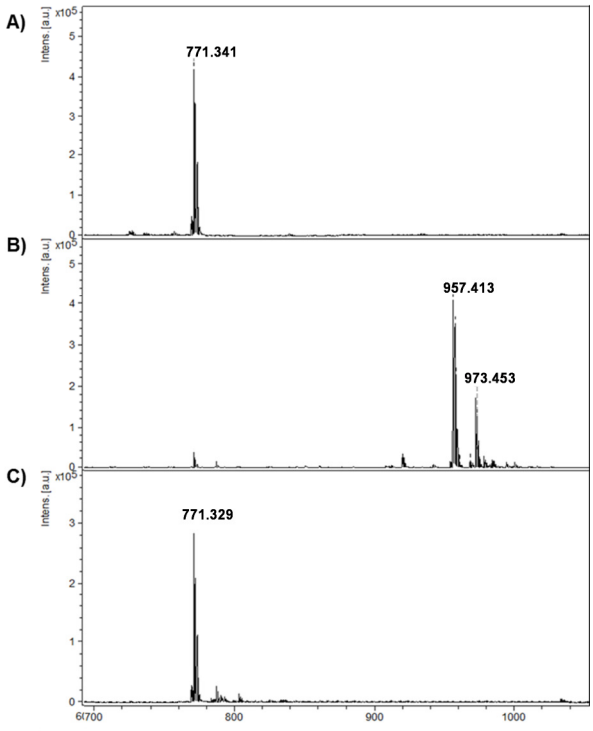


Figure 7.10 MALDI mass spectrums of A) PHCKRM, B) PHCKRM alkylated with **2h**, and C) PHCKRM regenerated by sulfonium cleavage using 2-mercaptopyridine.

7.4 Conclusions

In summary, we have developed a simple, rapid, and reversible "click" type method of functionalization for peptides based on reversible alkylation of the natural amino acid methionine. These functionalizations quantitatively add and remove diverse functional groups, are chemoselective at low pH, and can be performed in aqueous solution. Methionine can be alkylated with groups that are inert or susceptible to thiolysis, providing opportunities to conveniently engineer complexity and responsiveness into peptides. This methodology has many applications in biological assays, peptide and protein labeling, drug delivery, and stimuli responsive materials.

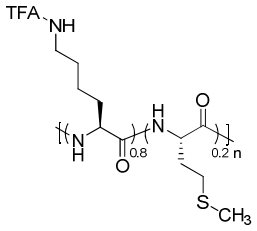
7.5 Experimental Section

7.5.1 Materials and Methods

Unless stated otherwise, reactions were conducted in oven-dried glassware under an atmosphere of nitrogen using anhydrous solvents. Hexanes, THF, DCM, and DMF were purified by first purging with dry nitrogen, followed by passage through columns of activated alumina. Deionized water (18 MΩ-cm) was obtaining by passing in-house deionized water through a Millipore Milli-Q Biocel A10 purification unit. All commercially obtained reagents were used as received without further purification unless otherwise stated. Reaction temperatures were controlled using an IKA magnetic temperature modulator, and unless stated otherwise, reactions were performed at room temperature (RT, approximately 20 °C). Thin-layer chromatography (TLC) was conducted with EMD gel 60 F254 precoated plates (0.25 mm) and visualized using a combination of UV, anisaldehyde, and phosphomolybdic acid staining. Selecto silica gel 60 (particle size 0.032–0.063 mm) was used for flash column chromatography. 1H NMR spectra were recorded on Bruker spectrometers (at 500 MHz) and are reported relative to deuterated

solvent signals. Data for ^{1}H NMR spectra are reported as follows: chemical shift (δ ppm), multiplicity, coupling constant (Hz) and integration. Splitting patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet and br, broad. ^{13}C NMR spectra were recorded on Bruker spectrometers (at 125 MHz). Data for ^{13}C NMR spectra are reported in terms of chemical shift. High-resolution mass spectrometry (HRMS) was performed on a Micromass Quatro-LC Electrospray spectrometer with a pump rate of 20 μL/min using electrospray ionization (ESI). All Fourier Transform Infrared (FTIR) samples were prepared as thin films on NaCl plates and spectra were recorded on a Perkin Elmer RX1 FTIR spectrometer and are reported in terms of frequency of absorption (cm-1). Tandem gel permeation chromatography/light scattering (GPC/LS) was performed on a SSI Accuflow Series III liquid chromatograph pump equipped with a Wyatt DAWN EOS light scattering (LS) and Optilab rEX refractive index (RI) detectors. Separations were achieved using 105, 104, and 103Å Phenomenex Phenogel 5 μm columns using 0.10 M LiBr in DMF as the eluent at 60 °C. All GPC/LS samples were prepared at concentrations of 5 mg/mL.

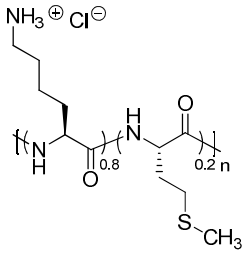
7.5.2 Experimental Procedures



General Procedure for Preparation of Poly[(N_ε-trifluoroacetyl-L-lysine)$_{0.8}$-*stat*-(L-methionine)$_{0.2}$]$_n$

Inside a dinitrogen filled glove box, a solution of N_ε-TFA-L-lysine-N-carboxyanhydride[1] (TFA-Lys NCA), (616 mg, 2.30 mmol, 4 eq) and L-methionine-*N*-carboxyanhydride (Met NCA)[15] (100

mg, 0.573 mmol, 1 eq) in dry THF (0.15 M) was prepared. A solution of $(PMe_3)_4Co$[16] in dry THF (20 mM) was rapidly added via syringe (26.0 mg, 0.0714 mmol, 0.025 eq). After 45 min, the polymerization reaction was complete as determined by FTIR. An aliquot was removed and analyzed by GPC/LS. The polymerization was removed from the drybox and HCl (2 eq per $(PMe_3)_4Co$, 6M in H_2O) was added to the solution, which turned a blue-green color. After 10 min stirring, the copolymer was collected by precipitation into acidic water (pH 3, HCl, >10x the reaction volume), followed by centrifugation. The white precipitate was washed with two portions of DI water and then lyophilized to give the copolymer as a fluffy white solid (595mg, 99 % yield). Spectra data was in agreement with previous reports.[1]



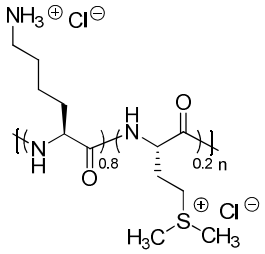
General Procedure for Deprotection of TFA-Lysine, Poly[(L-lysine·HCl)$_{0.8}$-*stat*-(L-methionine)$_{0.2}$]$_n$, ($K_{0.8}M_{0.2}$)$_n$, 1

Poly[(N_ε-trifluoroacetyl-L-lysine)$_{0.8}$-*stat*-L-methionine)$_{0.2}$]$_n$ was dispersed in methanol:water, 9:1 (20 mg/mL) and K_2CO_3 (2 eq per lysine residue) was added. The reaction was stirred for 8 hours at 50 ^oC and then the methanol was removed by rotory evaporation. The remaining solution was acidified to pH 3 with 6M HCl, and transferred to 2000 MWCO dialysis tubing. The polypeptide was dialyzed at pH 4 (HCl) for 24 hours, followed by DI water for 48 hours with water changes twice per day. The contents of the dialysis tubing were then lyophilized to dryness to give poly[(L-lysine·HCl)$_{0.8}$-*stat*-(L-methionine)$_{0.2}$]$_n$, ($K_{0.8}M_{0.2}$)$_n$ as a white solid. (82% yield) ^{1}H NMR

(500 MHz, D_2O, 25 ^oC): δ 4.51 (s, 1H), 4.32 (s, 4H), 3.02 (m, 8H), 2.66-2.52 (m, 2H), 2.16-1.97 (m, 5H), 1.88-1.66 (m, 16H), 1.46 (s, 8H).

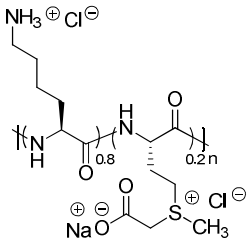
General Procedure for Alkylation of $(K_{0.8}M_{0.2})_n$

$(K_{0.8}M_{0.2})_n$ was dissolved in 0.2 M formic acid (10 mg/mL) and the electrophile (1.5 eqv per methionine residue) was added. The reaction mixture was covered with foil and stirred at room temperature for 48 hours. The reaction was then transferred to 2000 MWCO dialysis tubing and dialyzed against 0.10 M NaCl for 24 hours to exchange all counterions to chloride, followed by dialysis against DI water for 48 hours with water changes twice per day. The contents of the dialysis tubing were then lyophilized to dryness to give the product as a white solid.



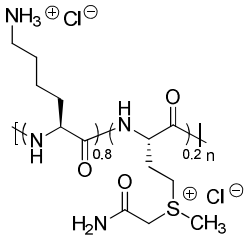
Poly[(L-lysine·HCl)$_{0.8}$-*stat*-(*S*-methyl-L-methionine sulfonium chloride)$_{0.2}$]$_n$, 3a

Polysulfonium **3a** was prepared from $(K_{0.8}M_{0.2})_n$ and methyl iodide according to the general procedure for alkylation of $(K_{0.8}M_{0.2})_n$, (88% yield). ^1H NMR (500 MHz, D_2O, 25 ^oC): δ ^1H NMR (500 MHz, D_2O, 25 ^oC): δ 4.56 (s, 1H), 4.28 (s, 4H), 3.39 (s, 2H), 3.04-2.89 (m, 12H), 2.37-2.16 (m, 2H), 1.84-1.60 (m, 16H), 1.45 (s, 8H).



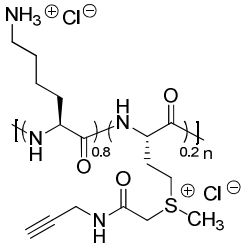
Poly[(L-lysine·HCl)$_{0.8}$-*stat*-(*S*-(sodium carboxymethyl)-L-methionine sulfonium chloride)$_{0.2}$]$_n$, 3b

Polysulfonium **3b** was prepared from $(K_{0.8}M_{0.2})_n$ and bromoacetic acid according to the general procedure for alkylation of $(K_{0.8}M_{0.2})_n$, (85% yield). ^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ ^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ 4.56 (s, 1H), 4.26 (s, 4H), 3.46-3.28 (m, 2H), 3.04-2.84 (m, 12H), 2.37-2.14 (m, 2H), 1.85-1.59 (m, 16H), 1.42 (s, 8H).



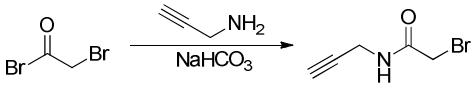
Poly[(L-lysine·HCl)$_{0.8}$-*stat*-(*S*-(acetamido)-L-methionine sulfonium chloride)$_{0.2}$]$_n$, 3c

Polysulfonium **3c** was prepared from $(K_{0.8}M_{0.2})_n$ and bromoacetamide according to the general procedure for alkylation of $(K_{0.8}M_{0.2})_n$, (85% yield). ^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ ^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ 4.55 (s, 1H), 4.24 (s, 4H), 3.46-3.25 (m, 2H), 3.02-2.83 (m, 12H), 2.35-2.13 (m, 2H), 1.84-1.58 (m, 16H), 1.41 (s, 8H).



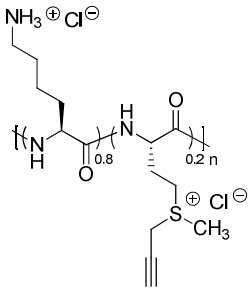
Poly[(L-lysine·HCl)$_{0.8}$-*stat*-(*S*-(*N*-propargyl-acetamido)-L-methionine sulfonium chloride)$_{0.2}$]$_n$, 3d

Polysulfonium **3d** was prepared from $(K_{0.8}M_{0.2})_n$ and *N*-propargyl-bromoacetamide **2d** according to the general procedure for alkylation of $(K_{0.8}M_{0.2})_n$, (85% yield). ^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ 4.62 (s, 1H), 4.32 (s, 4H), 4.06 (s, 2H), 3.64-3.46 (m, 2H), 3.07-2.97 (m, 12H), 2.69 (s, 1H), 2.44-2.20 (m, 2H), 1.88-1.62 (m, 16H), 1.47 (s, 8H).



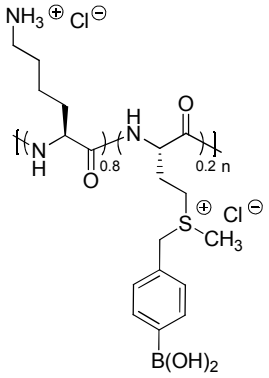
Preparation of 2-bromo-*N*-(prop-2-ynyl)acetamide, 2d:

N-propargyl-bromoacetamide was prepared from bromoacetylbromide according to a modified literature procedure.[17] Propargyl amine (0.166 mL, 2.60 mmol, 1.05 eq) was added dropwise to a solution of K_2CO_3 (0.358 g, 2.60 mmol, 1.05eq) and bromoacetyl bromide (0.500 g, 2.48 mmol, 1eq) in CH2Cl2 (20 mL) at 0 ºC. The resulting solution was allowed to reach rt and stir for 4 hours. The reaction was filtered, the filter cake rinsed with CH_2Cl_2, and the filtrate was evaporated to a brown solid, which was recrystallized from THF and hexanes to give 2-bromo-*N*-(prop-2-ynyl)acetamide (0.313 g, 72%). Spectral data was consistent with literature values.



Poly[(L-lysine·HCl)$_{0.8}$-*stat*-(*S*-propargyl-L-methionine sulfonium chloride)$_{0.2}$]$_n$, 3e

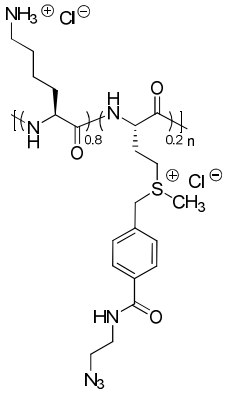
Polysulfonium **3e** was prepared from $(K_{0.8}M_{0.2})_n$ and propargylbromide according to the general procedure for alkylation of $(K_{0.8}M_{0.2})_n$, (92% yield). ^{1}H NMR (500 MHz, 2% d-TFA in D_2O, 25 ^oC): δ 4.44 (s, 1H), 4.15 (s, 6H), 3.31 (s, 2H), 2.89-2.82 (m, 16H), 2.26-2.15 (m, 3H), 2.08 (s, 1H), 1.70-1.48 (m, 265H), 1.30 (s, 13H).



Poly[(L-lysine·HCl)$_{0.8}$-*stat*-(*S*-(4-boroxyphenylmethyl)-L-methionine sulfonium chloride)$_{0.2}$]$_n$, 3f

Polysulfonium **3f** was prepared from $(K_{0.8}M_{0.2})_n$ and 4-(bromomethyl)phenylboronic acid according to the general procedure for alkylation of $(K_{0.8}M_{0.2})_n$. (89% yield)

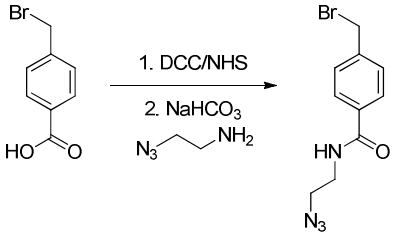
^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ 7.88-7.74 (m, 2H), 7.54-7.41 (m, 2H), 4.56 (s, 1H), 4.30 (s, 4H), 3.43-3.22 (m, 2H), 2.99 (m, 9H), 2.85-2.71 (m, 3H), 2.18 (s, 2H), 1.85-1.59 (m, 16H), 1.44 (s, 8H).



Poly[(L-lysine·HCl)$_{0.8}$-*stat*-(*S*-(α-methyl-(*N*-azidoethyl)-*p*-toluamide)-L-methionine sulfonium chloride)$_{0.2}$]$_n$, 3g

Polysulfonium **3g** was prepared from $(K_{0.8}M_{0.2})_n$ and α-bromomethyl-(*N*-azidoethyl)-*p*-toluamide **2g** according to the general procedure for alkylation of $(K_{0.8}M_{0.2})_n$, except that α-bromomethyl-(*N*-azidoethyl)-*p*-toluamide was added as a 25 mg/mL solution in ethanol. (87% yield)

^{1}H NMR (500 MHz, D_2O, 25 ^{o}C): δ 7.91-7.307 (m, 4H), 4.47 (s, 1H), 4.19 (s, 3H), 3.58-3.11 (m, 4H), 2.89 (s, 1H), 2.74 (s, 1H), 2.25-1.96 (m, 2H), 1.78-1.11 (m, 23H).

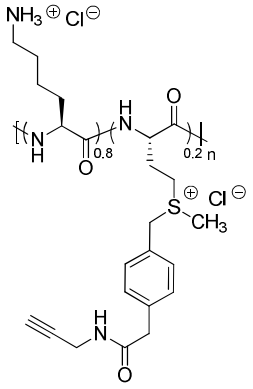


Preparation of α-bromomethyl-(*N*-azidoethyl)-*p*-toluamide, 2g:

The NHS ester of α-bromomethyl-*p*-toluic acid was prepared according to a literature procedure.[18] α-Bromomethyl toluic acid (0.140 g, 0.651 mmol, 1 eq) was dissolved in DMF/ethylacetate 1/1 (5 mL). NHS (0.0787 g, 0.684 mmol, 1.05 eq) then DCC (0.141 g, 0.684 mmol, 1.05 eq) were added. A white precipitate formed within 10 minutes. The reaction was stirred for 2 hours, filtered, the filter cake was washed with ethyl acetate, and the filtrate was

condensed to a white solid. The crude NHS ester was redissolved in DMF (5 mL) and K_2CO_3 was added (0.105 g, 0.716 mmol, 1.1 eq) followed by 2-azidoethylamine[19] (0.0654 g, 0.716 mmol, 1.1 eq). The reaction was stirred for 4 hours, then diluted with water (200 mL). The product was extracted with 3 portions of ethyl acetate (50 mL), the combined organic layers were washed with water and brine, dried over sodium sulfate, and condensed. The pale yellow solid was chromatographed on silica in 5% methanol in benzene to give α-bromomethyl-(*N*-azidoethyl)-*p*-toluamide, 0.496 g (84%).

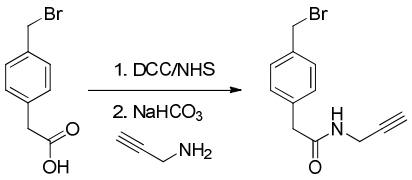
^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 7.74 (d, *J* = 8.0, 2H), 7.43 (d, *J* = 8.0, 2H), 6.70 (s, 1H), 4.49 (s, 2H), 3.61-3.58 (m, 2H), 3.53 (t, *J* = 5.5, 2H). ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ 167.0, 141.3, 133.9, 129.2, 127.4, 50.7, 39.4, 32.2. 282.01 C10H11BrN4O



Poly[(L-lysine·HCl)$_{0.8}$-*stat*-(*S*-(4-(*N*-propargyl)-acetamidophenylmethyl)-L-methionine sulfonium chloride)$_{0.2}$]$_n$, 3h

Polysulfonium **3h** was prepared from $(K_{0.8}M_{0.2})_n$ and 4-bromomethyl-*N*-propargyl-phenylacetamide **2h** according to the general procedure for alkylation of $(K_{0.8}M_{0.2})_n$, except that 4-bromomethyl-*N*-propargyl-phenylacetamide was added as a 25 mg/mL solution in ethanol, (92% yield). ^{1}H NMR (500 MHz, D_2O, 25 ^{o}C): δ 7.39-7.24 (m, 4H), 4.48 (s, 1H), 4.19 (s, 4.5H),

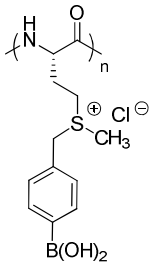
3.85 (s, 2H), 3.57-3.48 (m, 2H), 3.34-3.13 (m, 2H), 2.90 (m, 9H), 2.73-2.61 (m, 3H), 2.50 (s, 1H), 2.23-2.03 (m, 2H), 1.74-1.48 (m, 18H), 1.34 (s, 9H).



Preparation of 4-bromomethyl-*N*-propargyl-phenylacetamide, 2h:

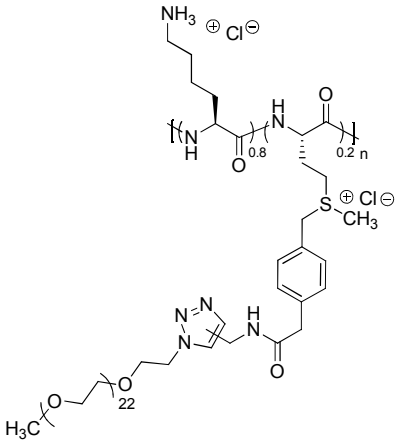
4-Bromomethyl-phenylacetic acid (0.509 g, 2.22 mmol, 1 eq) was dissolved in dry THF (20 mL). NHS (0.258 g, 2.24 mmol, 1.01 eq) then DCC (0.463 g, 2.24 mmol, 1.01 eq) were added. A white precipitate formed within 10 minutes. The reaction was stirred for 2 hours, filtered, the filter cake was washed with THF, and the filtrate was condensed to a white solid. The crude NHS ester was redissolved in DMF (20 mL) and K_2CO_3 was added (0.307 g, 2.22 mmol, 1 eq) followed by propargyl bromide (0.149 mL, 2.33 mmol, 1.05 eq). The reaction was stirred for 4 hours, then diluted with water (200 mL). The product was extracted with 3 portions of ethyl acetate (50 mL), the combined organic layers were washed with water and brine, dried over sodium sulfate, and condensed. The pale yellow solid was chromatographed on silica in 5% methanol in benzene to give 4-bromomethyl-*N*-propargyl-phenylacetamide, 0.496 g (84%).

^{1}H NMR (500 MHz, MeOD, 25 ^{o}C): δ 7.38 (d, *J* = 8.2, 2H), 7.28 (d, *J* = 8.2, 2H), 4.55 (s, 2H), 3.95 (d, *J* = 2.5, 2H), 3.51 (s, 2H), 2.58 (t, *J* =2.6, 1H). ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ 170.0, 137.0, 134.5, 129.8, 129.6, 79.2, 71.6, 43.0, 32.9, 29.3. HRMS-ESI (m/z) $[M + H]^+$ $C_{12}H_{13}BrNO$, calcd for 266.01; found 266.02.



Poly(*S*-(4-boroxyphenylmethyl)-L-methionine sulfonium chloride)$_n$, 7

The preparation of polysulfonium **7** has been previously described.[1] Spectral data was in agreement with the literature. (92% yield)

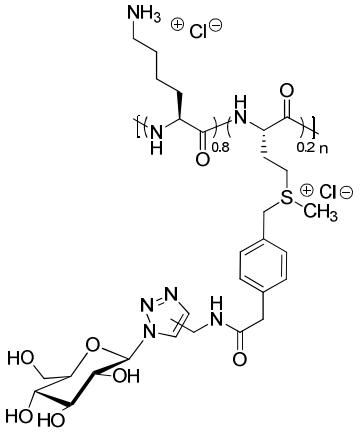


Copper(I)-catalyzed Azide-Alkyne Cycloaddition of azide terminated polyethyleneglycol with sulfonium 3h:

Polysulfonium **3h** was dissolved in water (5 mg/mL) and azide terminated polyethyleneglycol, purchased from Sigma, MW=1,000, (1.2 eq/alkyne) was added. The solution was degassed by bubbling N_2 through the solution for 20 minutes and then stirred under N_2. Separately, a solution of Cu(I) was prepared by addition of sodium ascorbate (0.5 eqv / alkyne) to a degassed solution of Cu(II)SO_4 (0.1 eqv / alkyne) and pentamethylethylenediamine (0.1 eqv / alkyne). The solution turned dark blue. The Cu(I) solution was transferred to the azide/alkyne solution via syringe. The reaction was stirred at room temperature for 48 hours and then transferred to 8000 MWCO

dialysis tubing and dialyzed against 0.10 M NaCl for 24 hours, followed by dialysis against DI water for 72 hours with water changes twice per day. The contents of the dialysis tubing were then lyophilized to dryness to give the product, **5a**, as a white solid. (95% yield)

^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ 7.92 (s, 1H), 7.46-7.18 (m, 4H), 4.27 (s, 4H), 4.00-3.42 (m, 152 H), 3.20 (s, 2H), 2.97 (s, 8H), 2.77 (s, 3H), 2.53 (m, 2H), 2.19 (m, 2H), 1.83-1.59 (m, 16 H), 1.42 (s, 8H).



Copper(I)-catalyzed Azide-Alkyne Cycloaddition of β-D-glucopyranosyl azide with sulfonium 3h:

Polysulfonium **3h** was dissolved in water (5 mg/mL) and β-D-glucopyranosyl azide, purchased from Carbosynth, (1.2 eq/alkyne) was added. The solution was degassed by bubbling N_2 through the solution for 20 minutes and then stirred under N_2. Separately, a solution of Cu(I) was prepared by addition of sodium ascorbate (0.5 eqv / alkyne) to a degassed solution of $Cu(II)SO_4$ (0.1 eqv / alkyne) and pentamethylethylenediamine (0.1 eqv / alkyne). The solution turned dark blue. The Cu(I) solution was transferred to the azide/alkyne solution via syringe. The reaction was stirred at room temperature for 48 hours and then transferred to 2000 MWCO dialysis tubing and dialyzed against 0.10 M NaCl for 24 hours, followed by dialysis against DI water for 48

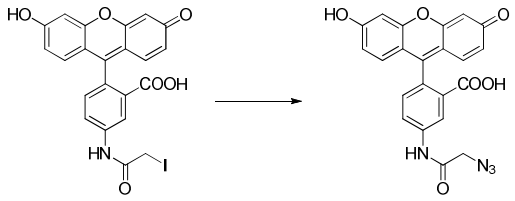
hours with water changes twice per day. The contents of the dialysis tubing were then lyophilized to dryness to give the product, **5b**, as a white solid. (95% yield)

^{1}H NMR (500 MHz, D_2O, 25 ^oC): δ 8.13 (s, 1H), 7.49-7.31 (m, 4H), 5.73 (s, 1H), 4.59 (s, 1H), 4.49 (s, 2H), 4.30 (s, 5.5H), 4.00-3.86 (m, 2H), 3.81-3.58 (m, 7H), 3.41 (s, 1H), 3.29 (s, 1H), 3.01 (s, 11H), 2.84-2.72 (m, 3H), 2.35-2.15 (m, 22H), 1.45 (s, 11H).

Click and release of fluorescein from 3h:

Polysulfonium **3h** was dissolved in water (5 mg/mL) and 5-azidoacetamido-fluorescein **4c**, (0.02 eq/alkyne) was added. The solution was degassed by bubbling N_2 through the solution for 20 minutes and then stirred under N_2. Separately, a solution of Cu(I) was prepared by addition of sodium ascorbate (0.5 eqv / alkyne) to a degassed solution of Cu(II)SO_4 (0.1 eqv / alkyne) and pentamethylethylenediamine (0.1 eqv / alkyne). The solution turned dark blue. The Cu(I) solution was transferred to the azide/alkyne solution via syringe. The reaction was covered with foil and stirred at room temperature for 48 hours and then transferred to 2000 MWCO dialysis tubing and dialyzed against 0.10 M NaCl for 24 hours, followed by dialysis against DI water for 48 hours with water changes twice per day. The contents of the dialysis tubing were then lyophilized to dryness to give the product, **5c**, as a white solid. (95% yield)

Preparation of 5-azidoacetamido-fluorescein:



5-iodoacetamido-fluorescein (5 mg, 9.70 μmol) was dissolved in DMSO (1 mL). NaN_3 (1.89 mg, 29.1 μmol, 3 equivs) was added. The reaction was covered with foil and stirred at 50 ^oC for 8 hours. Water was added (20 mL) and the mixture was extracted with 3 portions of 1/1 ethyl acetate/isopropanol (10mL each). The combined organic phases were washed with 2 small portions of brine, dried with magesium sulfate, and condensed to give **4c** as an orange solid (4.1 mg, 99%)

General Procedure for Cleavage of Methionine Sulfonium Salts to Regenerate $(K_{0.8}M_{0.2})_n$

Alkylated $(K_{0.8}M_{0.2})_n$ was dissolved in 0.1 M nucleophile (2-mercaptopyridine, thiourea, mercaptoethanol, or glutathione) in PBS buffer, pH 7.4 and stirred at 37 ^oC. At varied timepoints, an aliqout of the reaction was removed and transferred to 2000 MWCO dialysis tubing. Samples were dialyzed against 0.10 M NaCl for 24 hours to exchange all counterions to chloride, followed by dialysis against DI water for 48 hours with water changes twice per day. Reactions with glutathione were dialyzed against 0.10 M NaCl at pH 3 for 24 hours to break up electrostatic interactions between glutathione and the polypeptide, followed by dialysis against DI water for 48 hours with water changes twice per day. The contents of the dialysis tubing were then lyophilized to dryness.

^{1}H NMRs of the products of cleavage reactions of polysulfoniums **3a** and **3b** were found to be identical to the respective starting materials, no regeneration of methionine was observed. Thiolysis products of polysulfoniums **3c-h** and **5a-c** gave products with ^{1}H NMRs identical to the parent polypeptide $(K_{0.8}M_{0.2})_n$. Thiolysis of homopolysulfonium **7** with 2-mercaptopyridine gave only polymethionine by ^{1}H NMR.

Reactivity of 2-Mercaptopyridine with Disulfide bonds

2-Mercaptopyridine (2.42 mg, 0.220 mmol, 1.1 eqv) was added to a solution of cystine (48.0 mg, 0.200 mmol, 1 eqv) in DI water:MeCN 2:1 (2 mL). The solution was stirred for 24 hours at 37 ^{o}C. Water was added (3 mL) and the aqueous solution was extracted with EtOAC (3 x 5 mL). The aqueous phase was lyophilized to dryness and analyzed by ^{1}H NMR and ^{13}C NMR. Spectral data was identical to that of an authentic sample of cystine, no disulfide reduction was observed.

Realkylation of Regenerated Methionine:

$(K_{0.8}M_{0.2})_n$, recovered from the cleavage reaction of polysulfonium **3h** with mercaptopyridine, was treated with propargylbromide according to the general procedure for alkylation of $(K_{0.8}M_{0.2})_n$. Spectral data for this polypeptide was identical to the data previously described for polysulfonium **3e**.

7.5.3 Thiolysis data with various thiol nucleophiles and sulfonium salts

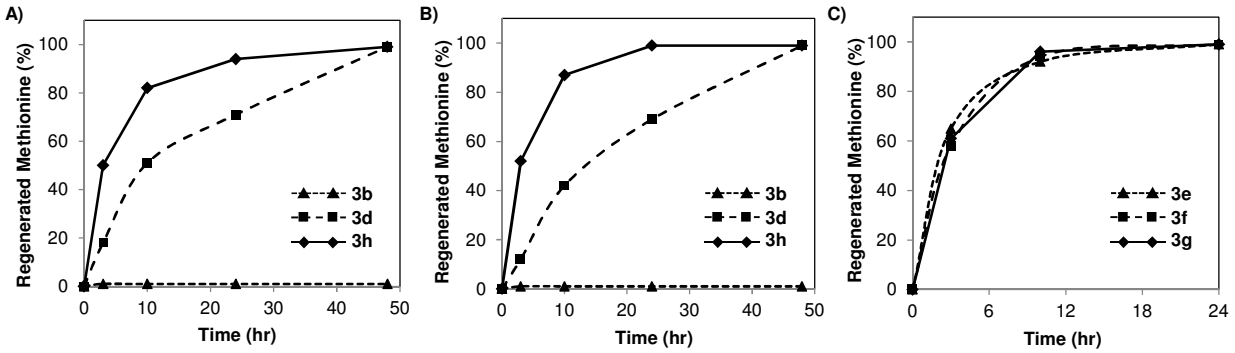
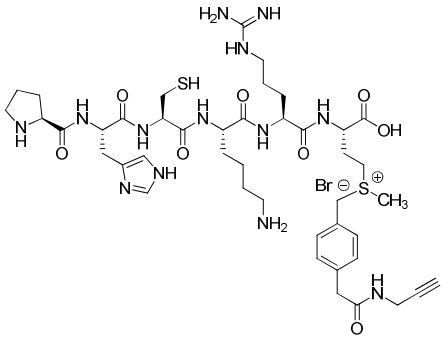


Figure 7.11 Regeneration of methionine from polypeptides using A) 0.1 M 2-mercaptoethanol, B) 0.1M thiourea, C) 2-mercaptopyridine, 37 ^{o}C, PBS buffer.

7.5.4 Click and Release on PHCKRM



Alkylation of PHCKRM at pH 2.4:

PHCKRM was purchased from Bachem. PHCKRM (2.0 mg, 2.59 µmol, 1.0 eq) was dissolved in 0.2M formic acid (0.5 mL) and 4-bromomethyl-*N*-propargyl-phenylacetamide **2h** (0.76 mg, 2.85 µmol, 1.5 eq) was added as a 25 mg/mL solution in ethanol. The reaction was stirred for 48 hours and then extracted with 3 portions of ethyl acetate. A sample of the remaining aqueous solution was analyzed by mass spectrometry and the remainder was lyophilized to yield a white

solid. The alkylated peptide was separated from excess 4-bromomethyl-*N*-propargyl-phenylacetamide by HPLC in MeCN/H_2O with 1 % TFA, to give 2.27 mg of **6**, 92% yield.

Alkylation of PHCKRM at pH 8.3:

PHCKRM (1.0 mg, 1.30 µmol, 1.0 eq) was dissolved in carbonate buffer (0.25 mL) pH 8.3 and 4-bromomethyl-*N*-propargyl-phenylacetamide **2h** (0.068 mg, 2.58 µmol, 2.0 eq) was added as a 25 mg/mL solution in ethanol. The reaction was stirred for 48 hours and then extracted with 3 portions of ethyl acetate. A sample of the remaining aqueous solution was analyzed by mass spectrometry and the remainder was lyophilized to yield a white solid.

Cleavage of Sulfonium from Alkylated PHCKRM

Alkylated PHCKRM **6** (2.27 mg, 2.37 µmol, 1.0 eq) was dissolved in DI water and 2-mercaptopyridine (0.78 mg, 7.11 µmol, 3 eq) was added. The solution was stirred for 24 hours at room temperature and then extracted with 4 portions of ethyl acetate. A sample of the remaining aqueous solution was analyzed by mass spectrometry and the remainder was lyophilized. ^{1}H NMR was found to be identical to the original peptide PHCKRM.

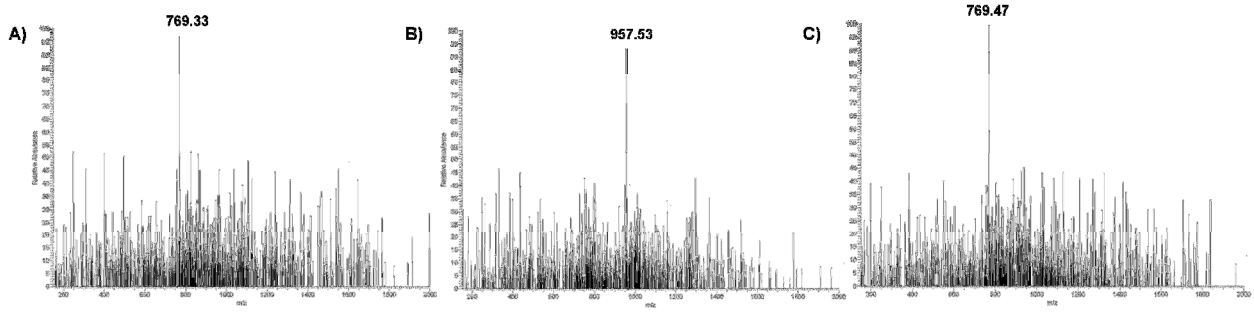


Figure 7.12 ESI-MS of A) PHCKRM; B) PHCKRM alkylated at pH 2.4; and C) PHCKRM regenerated after treatment with 2-mercaptopyridine; negative ionization.

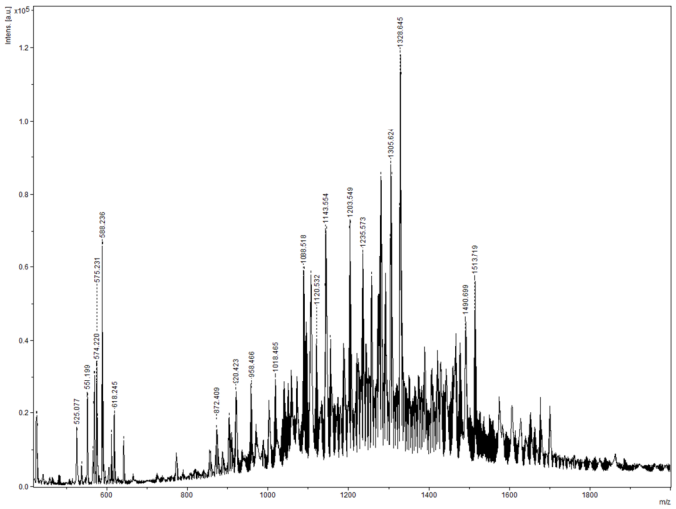


Figure 7.13 MALDI-MS of PHCKRM alkylated at pH 8.3.

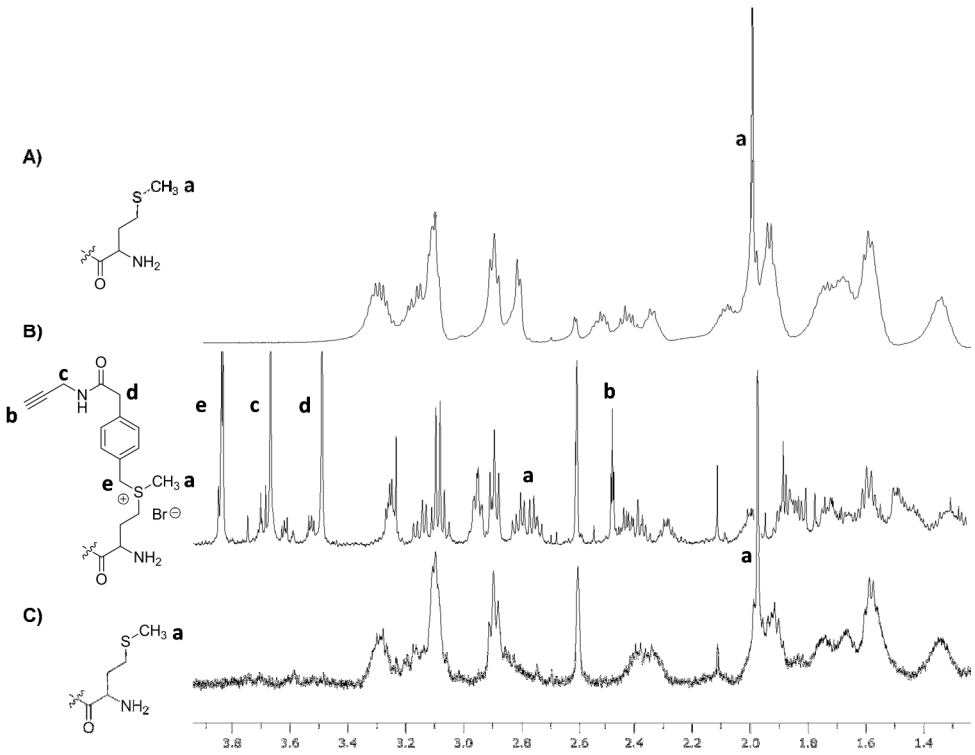


Figure 7.14 ^{1}H NMR spectra of A) PHCKRM, B) alkylated PHCKRM **6**, and C) PHCKRM regenerated after treatment with 2-mercaptopyridine.

Reactivity of Cysteine, Histidine, and Lysine with Benzyl Bromide Under Acidic Conditions

As a control experiment, the reactivity of *N*-α-CBz-cysteine, *N*-α-CBz-histidine, or *N*-α-CBz lysine to an electrophile was tested.

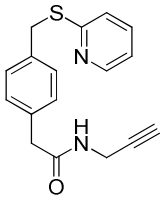
Cysteine: *N*-α-CBz-cysteine (50.0 mg, 0.196 mmol, 1 eq) was dissolved in 1:1 THF:0.2M aqueous formic acid (2 mL), ~pH 2.4. Benzyl bromide (67.0 mg, 0.392 mmol, 2 eq) was added and the reaction was covered with foil and stirred for 48 hours at room temperature. The reaction was diluted with water (30 mL), made basic with 2M NaOH, and extracted with diethyl ether (3 x 15 mL). The combined diethyl ether extracts were dried over magnesium sulfate and condensed to a clear oil. The aqueous phase was made acidic with concentrated HCl and extracted with EtOAc (3 x 15 mL). The EtOAC extracts were pooled, washed with brine, dried over magnesium sulfate, and condensed to a white solid. ^{1}H NMR of the diethyl ether extract was found to contain only benzyl bromide, and the EtOAc extract contained only *N*-α-CBz-cysteine. No alkylation occured at pH 2.4.

Histidine: *N*-α-CBz-histidine (55.5 mg, 0.192 mmol, 1 eq) was dissolved 1:1 THF:0.2M aqueous formic acid (2 mL), pH 2.4. Benzyl bromide (65.7 mg, 0.384 mmol, 2 eq) was added and the reaction was covered with foil and stirred for 48 hours at room temperature. The reaction was extracted with diethyl ether (3 x 15 mL). The combined diethyl ether extracts were dried over magnesium sulfate and condensed to a clear oil. The aqueous phase was lyophilized to dryness. ^{1}H NMR of the diethyl ether extract was found to contain only benzyl bromide, and ^{1}H NMR of the aqueous portion contained only *N*-α-CBz-histidine. No alkylation occured at pH 2.4.

Lysine: *N*-α-CBz-lysine (0.050 mg, 0.178 mmol, 1 eq) was treated with benzyl bromide (61.0 mg, 0.357 mmol, 2 eq) as previously described for *N*-α-CBz-histidine. ^{1}H NMR of the diethyl

ether extract was found to contain only benzyl bromide, and ^{1}H NMR of the aqueous portion contained only *N*-α-CBz-lysine. No alkylation occured at pH 2.4.

7.5.5 Investigation of the mechanism of thiolysis

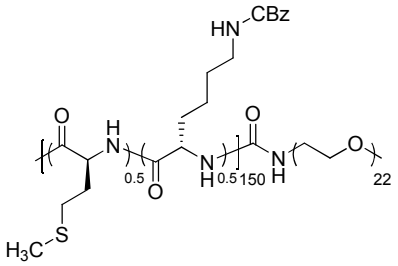


Isolation of methionine regeneration by-product:

Polysulfonium **3h** was treated with 0.1M mercaptopyridine in PBS buffer for 24 hours at room temperature. The reaction was extracted with 3 portions of ethyl acetate, and the combined organic extractions were condensed by rotary evaporation. The residue was purified by flash chromatography on silica (5% methanol in benzene) and was found to contain only excess mercaptopyridine and the reaction product pictured above. This structure was confirmed by preparation of an authentic sample by reaction of mercaptopyridine (1.5 eq) with 4-bromomethyl-*N*-propargyl-phenylacetamide **2h** (1 eq) and K_2CO_3 (1.5 eq) in DMF for 16 hours. Spectral data was identical to the product isolated from the polypeptide reaction.

^{1}H NMR (500 MHz, $CDCl_3$, 25 ^{o}C): δ 8.46 (d, *J* = 4.9, 1H), 7.48 (dd, *J* = 8.4, 7.0 1H), 7.40 (d, J = 8.0, 2H), 7.18 (d, J = 8.2, 2H), 7.16 (s, 1H), 7.00 (dd, J = 6.9, 5.4 1H), 5.54 (s, 1H), 4.44 (s, 2H), 4.00 (dd, *J* = 5.3, 2.5 2H) , 3.56 (s, 2H), 2.17 (t, *J* = 2.5, 1H). ^{13}C NMR (125 MHz, $CDCl_3$, 25 ^{o}C): δ 170.5, 158.5, 149.4, 137.6, 136.0, 133.0, 129.7, 129.6, 122.2, 119.7, 79.3, 71.6, 43.2, 33.9, 29.3. HRMS-ESI (m/z) $[M + H]^+$ $C_{17}H_{16}N_2OS$, calcd for 296.10; found 296.10.

7.5.6 Experiments to Check for Chain Breakage During Alkylation or Thiolysis:

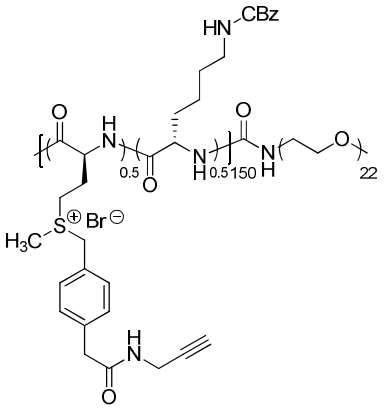


Poly[(N_ε-carbobenzyloxy-L-lysine)$_{0.5}$-*stat*-(L-methionine)$_{0.5}$]$_{150}$-*block*-poly(ethylene glycol)$_{22}$, 8

Inside a dinitrogen filled glove box, a solution of N_ε-carbobenzyloxy-L-lysine-N-carboxyanhydride[20] (Cbz-Lys NCA), (25 mg, 0.0817 mmol, 1 eq) and Met NCA(14.3 mg, 0.0817 mmol, 1 eqv) in dry THF (0.15 M) was prepared. A solution of $(PMe_3)_4Co$ in dry THF (20 mM) was rapidly added via syringe (1.5 mg, 4.09 μmol, 0.025 eq). After 45 min, the polymerization reaction was complete as determined by FTIR. An aliquot was removed and analyzed by GPC/LS. A solution of α-methoxy-ω-isocyanoethyl-poly(ethylene glycol)[1] in THF (12.3 mg, 0.012 mmol, 3 equiv per $(PMe_3)_4Co$) was added to the polymerization reaction. The reaction immediately turned from pale orange to green and was stirred overnight at room temperature. The reaction was was removed from the glove box and HCl (2 equiv per $(PMe_3)_4Co$, 6M in H_2O) was added to the solution, which turned a blue-green color. After 10 min stirring, the PEG-endcapped polypeptide was collected by precipitation into water (pH 3, HCl, >10x the reaction volume), followed by centrifugation.The white solids were washed with 3 portions of DI water to remove all unconjugated PEG-NCO, collected by centrifugation, and lyophilized to give 44 mg of a white solid (99% yield). Since it has been shown that end-capping is quantitative for $(PMe_3)_4Co$ initiated NCA polymerizations when excess isocyanate is used[13],

integrations of polypeptide resonances versus the polyethylene glycol resonance at δ 3.64 could be used to obtain polypeptide lengths. M_n = 29,490, PDI=1.14, DP = 150.

^{1}H NMR (500 MHz, $CDCl_3$ with 1% d-TFA, 25 ^{o}C): δ 8.16 (br s, 2H), 7.31 (s, 5H), 5.11 (s, 2H), 4.19 (s, 1H), 3.95 (s, 1H), 3.75 (s, 1.18), 3.14 (s, 2H), 2.74-2.47 (m, 2H), 2.31-1.77 (m, 7 H), 1.60-1.29 (br m, 4H).



Poly[(N_ε-carbobenzyloxy-L-lysine)$_{0.5}$-*stat*-(S-(4-*N*-propargyl-acetamidophenylmethyl)-L-methionine sulfonium bromide)$_{0.5}$]$_{150}$-*block*-poly(ethylene glycol)$_{22}$, 9

8 (44.1 mg, 1.45 μmol) was dissolved in DMF (3 mL). 4-Bromomethyl-*N*-propargyl-phenylacetamide **2h** (28.9 mg, 0.109 mmol, 2 eqv per methionine residue) was added. The reaction mixture was covered with foil and stirred at room temperature for 48 hours. Diethyl ether was added (20 mL) and the white precipitate was collected by centrifugation. The precipitate was washed with 2 portions of dichloromethane and then dried under high vacuum to give 77.4 mg (99%). A solution of the alkylated polypeptide was prepared in 0.1M LiBr in DMF (5 mg/mL) and analyzed by GPC. ^{1}H NMR showed no change in the ratio of PEG to polypeptide. M_n = 53,750; PDI=1.18.

^{1}H NMR (500 MHz, d-TFA, 25 ^{o}C): δ 7.59-7.35 (m, 4H), 7.26 (s, 5H), 6.78 (br s, 1H), 5.15 (s, 2H), 4.80 (s, 1H), 4.65 (s, 2H), 4.52 (s, 1H), 4.15-3.98 (m, 3H), 3.86 (s, 4.02), 3.71 (s, 2H), 3.19 (s, 2H), 3.00 (s, 2H), 2.83-2.55 (m, 2H), 2.25-2.0 (m, 4H), 1.99-1.68 (m, 4H), 1.67-1.30 (m, 4H).

Cleavage of Sulfonium group from 9

9 (77.4 mg, 1.45 μmol) was dissolved in DMF (3 mL). 2-Mercaptopyridine (12.0 mg, 0.109 mmol, 2 eqv per methionine residue) was added. The reaction mixture was stirred at room temperature for 24 hours. Diethyl ether was added (20 mL) and the white precipitate was collected by centrifugation. The precipitate was washed with 2 more portions of diethyl ether and then dried under high vacuum to give 43.9 mg (98%). A solution of the polypeptide was prepared in 0.1M LiBr in DMF (5 mg/mL) and analyzed by GPC. GPC and ^{1}H NMR spectra were identical to the parent polypeptide **8**.

7.5.7 General stability of Polysulfoniums:

Polysulfoniums **3d** and **3h** were dissolved in PBS buffer (10 mg/mL) and allowed to sit at room temperature for 2 weeks and then transferred to 2000 MWCO dialysis tubing. The solutions were then dialyzed against DI water for 24 hours, and then the contents of the dialysis tubing were lyophilized to dryness. ^{1}H NMR spectras were identical to spectras of the parent polysulfoniums. Homopolymers of sulfoniums **3a** and **3b** have been previously shown to be stable in water, DMF, PBS buffer, or DMEM cell culture media for >1 week at room temperature, in water at 80 ^{o}C for 16 hours, and at pH 2 (HCl) or pH 10 (NaOH) for >3 hours.[1] All polysulfoniums described have been found to be stable for >6 months when stored as dry powders at room temperatures.

7.6 References

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CHAPTER EIGHT

Microbial Fuel Cell Biofilm Characterization with Thermogravimetric Analysis on Bare and PEI Surface Modified Carbon Foam Anodes

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Kramer, J. R.; Soukiazian, S.; Mahoney, S.; Hicks-Garner, J. *J.Power Sources* **2012**, *210*, 122-128.

8.1 Abstract

Thermogravimetric analysis (TGA) of microbial biofilms on bare and polyethyleneimine (PEI) surface modified carbon foam is described. PEI-modified carbon foam was incorporated into a microbial fuel cell (MFC) as the anode, inoculated with electrogenic bacteria, and the voltage and power outputs were monitored over five weeks. The results were compared to MFCs containing unmodified carbon foam anodes. Biofilm formation was investigated by scanning electron microscopy and TGA. TGA is presented as a new method to assess the relative amounts of biofilm on an electrode surface.

8.2 Introduction

The energy field has recently developed a strong interest in renewable and alternative energy sources that are capable of reducing dependence on petroleum based energy. Microbial fuel cells (MFCs) are bio-electrochemical energy systems that provide new opportunities for environmentally friendly energy production to help meet the growing demand for energy. In

MFCs, microorganisms oxidize organic materials and produce electrons that can be harnessed and routed through an electrical circuit where they can perform work.[1-4] While the energy density of MFCs is currently on par with traditional hydrogen based proton exchange membrane fuel cells, the power derived from MFCs remains relatively low.[5] Despite the relatively low power output, MFCs have successfully been used to power small scale buoys equipped with environmental monitoring devices (sediment/benthic MFCs),[6-7] demonstrating the benefit of their application in remote and unattended environments. The most promising initial large-scale application appears to be in the area of wastewater treatment where MFCs have been used to remove organic matter from waste streams while generating electricity.[8-10] MFC development is still at an early stage, and there is tremendous potential to increase power output through electrochemical, microbiological, and systems engineering improvements. With such improvements, the application space for this technology will broaden tremendously.

One strategy to increase MFC power output is optimization of the anode material with consideration of the surface area, porosity, and affinity for the microbes. In MFC systems, the electrical current scales with both the available surface area and the amount of metabolically active microbial biofilm.[11] Increasing the electrode surface area available for biofilm formation can result in increased power output, however, porosity and fluid flow inside the anode must be considered.[11-12] The electrode structure must allow for circulation of biological media (a mixture of water, food/fuel, trace nutrients, etc.) to the entire microbial population, and diffusion of waste byproducts away from the biofilm. If fuel and waste cannot be transported throughout the biofilm, cell viability and current production will be variable. The anode material requires an ability to accommodate a ~40μm thick biofilm comprised of electrogenic bacteria typically 1-2

μm in length,[11] with adequate room for fluid flow. Affinity of electrogenic bacteria to the anode material will also impact the rate of biofilm formation, and subsequently current production.[11]

Carbon foam anodes address the surface area and porosity requirements of MFC anodes by combining extensive three-dimensional surface area with porosity that provides voids for biofilm growth and fuel/waste circulation. Electrogenic bacteria are naturally attracted to materials that can serve as an acceptor or sink for the electrons generated as part of their metabolic cycle.[13] Conductive carbon in such forms as graphitic cloth, vitreous carbon foam, and carbon felt, has proven capable of supporting bacterial biofilms in laboratory MFCs while conducting current.[10,11,14]

Though research has demonstrated that electrogenic bacteria readily couple with and colonize on conductive carbon anodes, straightforward and readily available methods to characterize these biofilms are lacking. Confocal microscopy is often used for precision analysis of biofilm properties, but the technique requires expensive equipment as well as considerable time and training to obtain quality data. Thermogravimetric analysis (TGA) is an analytical tool which measures the rate and amount of mass loss of a sample as its temperature is raised. TGA is routinely used to analyze materials ranging from small molecules to polymers, to determine physical characteristics such as decomposition temperature, or levels of inorganic or organic components. TGA is also useful in observing the mass effect of surface functionalization of a material. The technique is simple, rapid, inexpensive, and requires little training. Adhesion of a bacterial biofilm to an MFC anode is analogous to surface modification and affects the mass of the anode. We have investigated the use of TGA as an analytical tool to observe trends in biofilm formation on carbon foam anode surfaces. We have correlated the trends in TGA data to

our observations of cell voltages and the appearance of biofilms by scanning electron micrographs (SEM).

We have also investigated the use of carbon foam anodes that have been covalently surface modified with polyethyleneimine (PEI) in an effort to enhance the affinity of a mixed colony of electrogenic bacteria to the electrode. Currently, there has been little work done to increase the rate of biofilm formation or biofilm stability on MFC anode surfaces by enhancing attraction of the bacteria to the anode. Branched PEI is a polycationic organic polymer that has a high density of primary, secondary, and tertiary amino groups. PEI has been extensively used as a nonviral DNA transfection agent both in cell culture applications and *in vivo*.[15-16] PEI has also been used as an attachment factor for culturing both eukaryotic[17-18] and prokaryotic[19] cell lines. The cationic polymer is an effective bacterial flocculating agent and has been used to aid construction of both artificial biofilms[20] and artificially constructed microbial consortia[21] on electrode surfaces. Goncalves and Govind recently reported that PEI coating a gold electrode surface improved the biofilm formation of wastewater bacteria.[22] In this case, electrode surfaces were coated with PEI, polylysine, or collagen, and biofilm formation was compared to untreated electrodes. The PEI coated surface was reported to give the highest increase in biofilm spread. It is noteworthy that in this study, biofilm growth was observed via changes in electrode impedance and capacitance, noting that confocal microscopy is often time consuming and difficult. Given the reported benefits of PEI electrode coating on wastewater bacterial biofilm formation, we applied this to an MFC.

Herein, the MFC application of carbon foam anodes, PEI-modified carbon foam anodes, and biofilm characterization with TGA are described. Carbon foam anodes were covalently surface modified with PEI, then characterized by infrared spectroscopy (IR), TGA, and SEM.

Biofilm formation was observed on PEI-modified versus unmodified carbon anodes in functional MFCs. Cell voltages and power densities were observed over five weeks, and biofilms were then examined by TGA and SEM. To our knowledge, this is the first application of anode surface modification with PEI in an MFC application, and the first use of TGA as a method for evaluating biofilm formation.

8.3 Results and Discussion

8.3.1 Preparation of PEI-modified carbon foam anodes

Carbon foam anodes for MFC applications were surface modified with branched PEI to investigate the polymer's effect on biofim formation. Various forms of carbon, such as nanotubes,[24-25] and graphitic nanofibers,[26] have been surface oxidized using heat and acid to bear carboxylic acid groups. The reported procedures were adapted for carbon anodes in this publication, which we oxidized by heating in nitric acid. Treatment with PEI under standard peptide coupling conditions with aqueous NHS and EDC, allowed formation of covalent amide bonds with the carbon surface (Figure 8.1). This method of anode surface modification is rapid, simple, and applicable to the covalent attachment of any polymer or small molecule with a free amine.

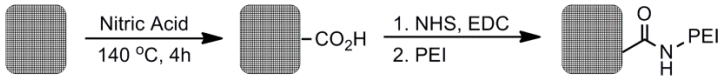

Figure 8.1 Surface modification of carbon foam anodes with polyethyleneimine (PEI).

8.3.2 Characterization of PEI-modified carbon foam anodes

Anodes were examined by FT-IR and TGA before surface modification, after oxidation to bear carboxylic acid groups, and finally after attachment of PEI. The FT-IR spectrum of the oxidized

carbon anodes revealed two new absorptions with maxima at 1730 cm^{-1} and 1620 cm^{-1} when compared to the unmodified anodes (Figure 8.2). These absorptions can be attributed to the carbonyls of the acid groups. It was also noteworthy that after oxidation the carbon anodes became much more hydrophilic and could be immersed in water. The unoxidized anodes are highly hydrophobic and float in water. The FT-IR spectrum of the PEI-modified anodes also revealed two absorptions in the carbonyl region, with maxima at 1730 cm^{-1} and 1577 cm^{-1}. The slight downshift in absorbance from 1620 cm^{-1} to 1577 cm^{-1} is characteristic of amide bonds (Figure 8.2 inset). Peaks observed between 3500 and 3300 cm^{-1} in both modified anode spectrums are most likely due to water adhered to the hydrophilic surfaces.

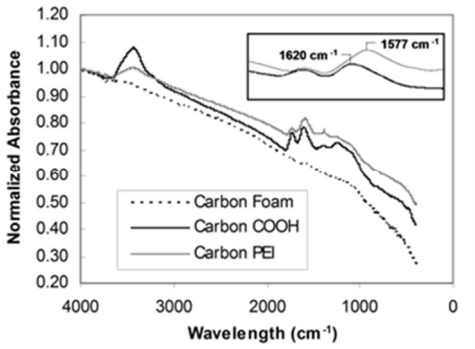

Figure 8.2 IR spectrum of anodes before modification (Carbon Foam), after oxidation (Carbon COOH), and after PEI modification (Carbon PEI).

Anodes were also examined by TGA before surface modification, after oxidation to bear carboxylic acid groups, and finally after attachment of PEI. Distinct changes in overall mass loss were observed for both the acid and PEI-modified anodes (Figure 8.3). Water adhered to the anode surfaces is likely to have contributed to mass losses from 25 to 100 ^{o}C and so only losses between 100 ^{o}C and 1000 ^{o}C were considered significant. The unmodified carbon foam showed a 16.06 % total weight loss from 100-1000 ^{o}C. A slight increase to 17.96 % was observed for the

carboxylic acid modified carbon, whereas a significant increase to 22.46 % loss was observed for the PEI-anodes. Two distinct mass loss events were observed during PEI-anode heating, 3.75 % from 100-200 ^{o}C and 7.99 % from 200-375 ^{o}C. The distinct changes in TGA profiles can be attributed to the increased amount of volatile organic matter in the PEI chains and support the success of the surface modification chemistry.

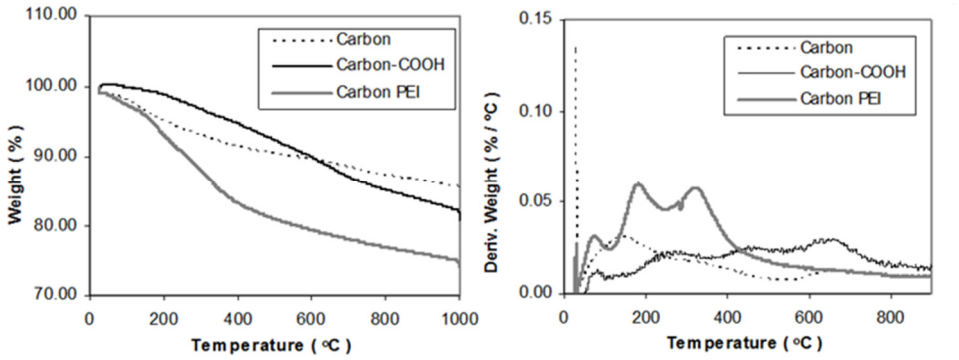

Figure 8.3 TGA spectrum of anodes before modification (Carbon), after oxidation (Carbon-COOH), and after PEI modification (Carbon PEI).

PEI-modified anodes were examined by SEM and compared to unmodified anodes (Figure 8.4 A, B). Imaging revealed that the foam anode surface was coated with a film, presumably the polymer coating. Visible film formation was an unexpected result of the PEI modification, however, it is plausible given the highly charged nature of the polymer. The cationic polymer is likely to have a large hydrodynamic radius due to inter-chain charge repulsion, which could cause the polymer to swell, resulting in the observed film.

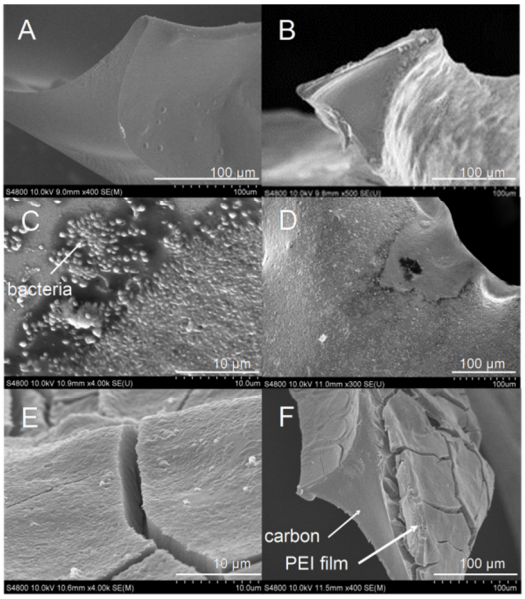

Figure 8.4 Scanning Electron Microscopy Images (SEM) of unmodified carbon foam anode (A), PEI-modified carbon foam anode (B), unmodified carbon anode after 5 weeks in an MFC (C,D), PEI-modified anode after 5 weeks in an MFC (E,F).

8.3.3 PEI-modified carbon anodes in MFC applications

To observe if PEI surface modification has an effect on biofilm formation, modified anodes were incorporated into MFCs and inoculated with a mixed colony of anaerobic digester bacteria. For comparison, MFCs containing unmodified carbon foam anodes were simultaneously inoculated with the same anaerobic bacteria sample. MFC cell voltages and power outputs were monitored over a period of approximately five weeks. Acetate was used as the oxidant and fuel source to initiate the bacterial metabolic cycle. Ferricyanide was used as the reductant and therefore the cell voltages reported are the difference in the oxidation potential of acetate and the reduction potential of ferricyanide, minus any contribution from internal cell resistance.[23] As expected, the voltage of the fuel cell with the unmodified anode gradually increased from 0.18V to 0.58V over a 40 day period (Figure 8.5). However, the voltage of the system with the PEI-modified anode did not increase within the observation period.

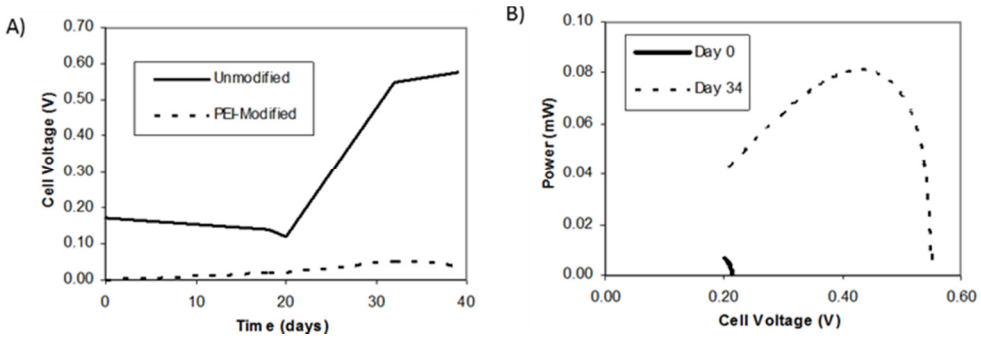

Figure 8.5 (A) Fuel cell voltages of PEI-modified and unmodified carbon foam anodes. A 500Ω resistor was placed in series with the anode and cathode at day 20. (B) Unmodified MFC polarization curve, PEI-modified MFC curve not pictured due to low voltage. Electron donor: 10 mM acetate. Electron acceptor: 50 mM potassium ferricyanide.

Figure 8.5 shows the change in power output over the same 40 day period. A substantial increase in power output was observed for the MFC containing the unmodified anode. The fuel cell's peak power output after approximately four weeks of biofilm growth was 0.08 mW at 0.45 V, characteristic of metabolically active bacteria populating the MFC anode surface. The power output of the MFC with the PEI-modified anode was negligible, which is expected given the low cell voltage. This data indicates that the bacteria originally inoculated into the MFCs were metabolically active and capable of biofilm formation, but that PEI modification either does not support biofilm growth, or that the PEI coating prevents electrochemical observation of the biofilm.

8.3.4 Characterization of Biofilms on Anode Surfaces

Biofilms on the carbon foam anodes and PEI-modified carbon foam anodes were examined by both SEM and TGA after five weeks in MFCs. Carbon foam anodes are known to support biofilm growth[10,11,14] and thicker anode biofilms have been associated with higher MFC power

outputs.[11] Though SEM is not capable of measuring depth, the technique is useful for a qualitative examination of the presence and quality of the biofilms. SEM imaging of the unmodified carbon anode surface clearly revealed bacterial populations (Figure 8.4). Common electrogenic bacteria such as Geobacter and Shewanella species are typically on the order of 1 μm in length. At the 10 μm scale (Figure 8.4 C), rod-like individual bacteria were observed populating the anode surface. At the 100 μm scale (Figure 8.4 D), the unmodified carbon foam anode surface appeared to be covered with a layer of bacteria and seemed to support a healthy biofilm. Although there appeared to be some structures on the PEI-anode after use in an MFC (Figure 8.4 E, F), it was not clear whether they were live bacteria composing a biofilm. The presence of a biofilm is possible but the structures are not as uniformly rod-like as those observed on the unmodified anode, and could be cellular debris instead of live bacteria. It is difficult to measure depth with SEM so it is unclear whether the film on the anode surface is thicker after use in the MFC, which could indicate the presence or absence of a biofilm. In the MFC environment, electrostatic interactions with negatively charged organic compounds in the buffered nutrient media and debris from the charged bacterial cell wall are likely to further contribute to film formation. Further work is underway to elucidate the nature of the PEI coating.

In an effort to quantitate the differences in biofilm formation, we investigated TGA as a method to observe differences in the mass loss profiles. To our knowledge, this is the first application of TGA in biofilm characterization. Biofilm thickness is typically characterized by confocal microscopy,[10] which is a highly effective method, but requires expensive equipment and considerable training to obtain quality images. TGA is widely used for materials characterization, requires little training and sample preparation, and yields rapid results.

After five weeks in functional MFCs, anodes were removed from the fuel cells and subjected to TGA analysis. A comparison of the mass loss profiles of PEI-anodes versus the unmodified anodes before and after use in the MFCs, revealed significant differences. Only mass loss events above 100 ^{o}C were considered significant due to the likely presence of surface adhered water. In the case of the unmodified carbon anodes before and after use in the MFC, a 5% change in mass loss was observed between 100 ^{o}C and 200 ^{o}C in the TGA weight loss profile (Figure 8.6). This is a temperature range where bacterial organic matter is likely to decompose and burn, and thus this difference is attributed to a bacterial biofilm. The TGA data is in agreement with our observations by SEM, as well as our electrochemical data. An active biofilm in direct contact with the carbon foam surface should create a potential difference between the anode and the cathode and generate current, such as observed.

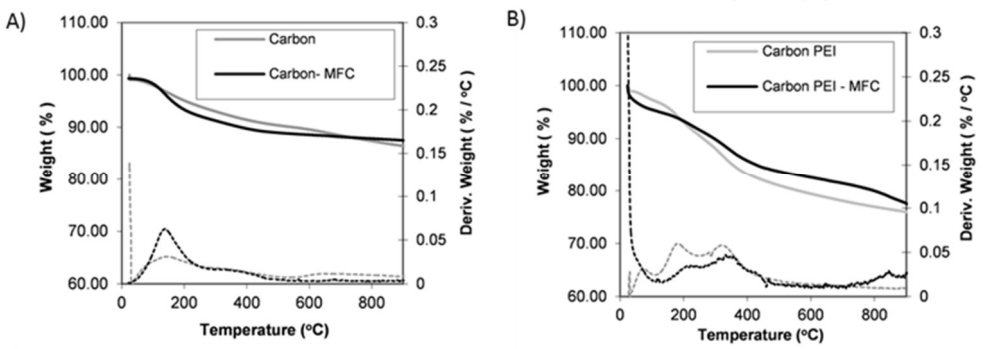

Figure 8.6 (A) TGAs of unmodified carbon foam anode before and after use in an MFC. (B) TGAs of PEI-modified carbon foam anode before and after use in an MFC. Solid lines (—) are % weight loss and dashed lines (---) are the derivative of this loss with respect to temperature.

A 5 % mass loss correlates to approximately 4 mg of dry bacterial biofilm, in the unmodified MFC. Taking 1.06 g cm^{-3} as an average density of a wet cell[10] and estimating that the dry mass accounts for roughly 30 % of the total cell mass, the cumulative cell volume is calculated to be 0.0133 cm^{3}. If this volume was distributed uniformly over the surface of the

unmodified anode (15 cm^2) the biofilm thickness would be about 9 μm. A biofilm of 9 μm is within the range expected given the current and power output of the MFC.

The mass loss profile of the PEI-anode after five weeks in the MFC displayed no new events when compared to the profile before use in the MFC (Figure 8.6). The same two mass loss events, centered around 175 ^oC and 325 ^oC, were observed in both profiles. No significant change in mass loss was observed between 100 ^oC and 200 ^oC as in the unmodified case, suggesting the lack of a substantial biofilm for the PEI-modified case. The overall mass loss was slightly less (1.9%) after use in the MFC than before, which is most likely due to loss of material that was electrostatically adsorbed, but not covalently linked, to the anode surface (e.g. anions to the PEI chains). The buffered anolyte solution is likely to break up such interactions over time.

The lack of MFC voltage and power output, together with the TGA data, seems to indicate that the PEI prevented the formation of a healthy biofilm. Given the previously reported success with PEI coatings for wastewater bacterial biofilm formation[22] this result was surprising. One explanation for the lack of power is that the PEI coating prevented direct contact with the anode surface and consequently prevented electron transfer from the bacteria to the anode. As previously noted, the biofilm must be in electrical contact with an electron sink for current generation. It is inconclusive from the SEM images whether a biofilm had grown on top of the PEI coating, but appears unlikely. The bacteria may have initially adhered and populated the surface of the PEI-anode, but were not in contact with the carbon foam anode and as a result no voltage and power output was generated. Another explanation is that high charge density of the PEI has a toxic effect on the bacteria, however PEI toxicity is generally associated with free polymer in solution, not PEI immobilized on a surface.[17] Another likely possibility for the difference in effect of PEI between our study and Goncalves and Govind's study is that the

wastewater bacterial composition was very different from ours and contained strains that flourish on the PEI surface. The bacterial composition of wastewater is likely to be highly variable even between samples collected from the same treatment plant depending on the nutrient composition of the water, the time of year and other factors. In addition, Goncalves and Govind's study involved gold anodes rather than carbon, and the branching density of the polyethyleneimine used in their study was not reported.

To further verify the correlation between TGA data, electrochemical observations, and biofilm formation; we set up an identical fuel cell as in the unmodified carbon case previously described. The cell, JKAD7, was inoculated with the same anaerobic digester bacteria samples collected from wastewater. Fuel cell power output was monitored over 36 days and the anode was subsequently removed from the cell and subjected to TGA analysis.

Similar to the results previously described for the carbon anode, both the voltage and power output of the fuel cell gradually increased over time. (Figure 8.7) The fuel cell's peak power output after approximately four weeks of biofilm growth was 0.09 mW at 0.46 V, characteristic of metabolically active bacteria. Anodes were removed from the fuel cells and subjected to TGA analysis as previously described. (Figure 8.8) A 5.4 % change in mass loss from before and after use in the MFC was observed between 100 ^{o}C and 350 ^{o}C, with the majority of this loss occurring between 200 ^{o}C and 350 ^{o}C. This is in good agreement with the data reported in Figure 8.6. The slightly higher mass loss for JKAD7 when compared to the first unmodified carbon MFC correlates to the slightly higher voltage and power output of JKAD7. The mass loss for JKAD7 occurred over a higher temperature range than for the anode shown in Figure 8.6, despite the two anodes having been exposed to identical conditions. This difference is likely due to variance in the morphologies of the biofilms on the two anodes. It is known that

bacterial biofilms commonly form fibrilar and mushroom like structures.[27] The types of structures composing the biofilm and their densities are likely to affect the temperature that mass losses occur.

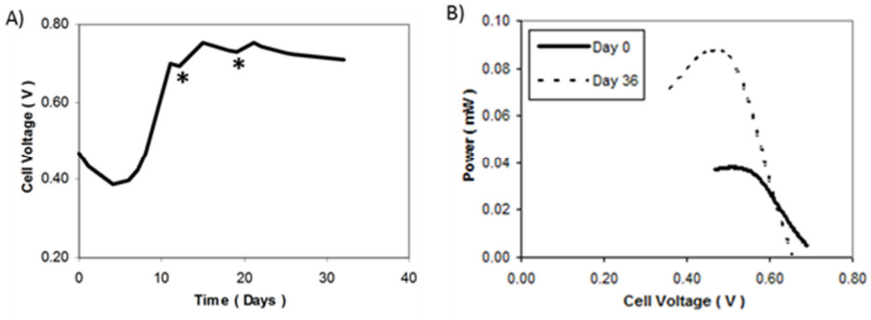

Figure 8.7 (A) Fuel cell voltages of MFC JKAD7. A 170Ω resistor was placed in series with the anode and cathode at day 12. Star (*) indicates dates fresh acetate media was added to the cells. (B) MFC JKAD7 polarization curves. Electron donor: 10 mM acetate. Electron acceptor: 50 mM potassium ferricyanide.

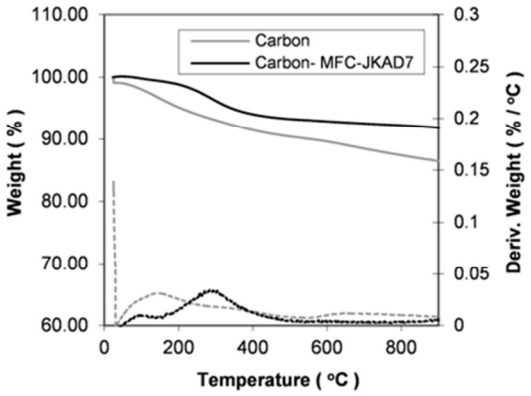

Figure 8.8 TGAs of the carbon foam anodes before and after use in an MFC. Solid lines (—) are % weight loss and dashed lines (---) are the derivative of this loss with respect to temperature.

Based on our data, TGA appears to be a valid new method for observing trends in biofilm formation on anodes for MFC applications. Clear differences were observed between the PEI-modified anode and the two unmodified anodes before and after application in an MFC. Furthermore, the data was in agreement with the SEM images and electrochemical observations. While analysis of the TGA data in PEI-modified case was complicated by electrostatic interactions, the method appears to be useful for a semi-quantitative assessment of biofilms. The method lacks the precision of confocal microscopy, but it is simple, rapid, inexpensive, and demonstrates trends in biofilm formation.

8.4 Conclusions

In conclusion, we have successfully conjugated PEI unto the surface of carbon foam anodes for MFC applications. We also propose TGA as a useful new tool for observing trends in biofilm thickness. Our PEI conjugation method is rapid, simple, and applicable to surface modification with any compound containing a free amine. We have found that in MFC applications, PEI does not facilitate increased power output. Two possibilities for this observation are that the biofilm formation was retarded by the PEI or that the PEI prevented electron transfer to the anode. Biofilms were characterized by both SEM and TGA, and appeared more dense on the unmodified carbon. Based on our observations, TGA is a useful new tool for simple and rapid observation of trends in biofilm thickness.

8.5 Experimental

8.5.1 General Materials and Methods

All chemicals were purchased from commercial sources unless otherwise noted. Water was purified in a Nanopure(R) water system.

8.5.2 Electrogenic microorganisms

A mixed colony was obtained as anaerobic digester sludge from the Tapia Wastewater Treatment Facility (Malibu, CA). The colony was propagated in a biological medium containing 10mM acetate (electron donor) and 40mM fumarate (electron acceptor).

8.5.3 Microbial fuel cell assembly

The MFCs used in these experiments were constructed from two pieces (7 x 7 x 1.1 cm) of machined acrylic glass, one having the anode compartment with dimensions 1.9 x 1.9 x 0.8 cm, and the other having the cathode compartment with dimensions 3.2 x 3.2 x 0.8 cm. Two pieces of butyl rubber, each with a one inch square opening in the middle, served as the gaskets. A piece of exchange membrane (Nafion® 117) soaked in 0.5M H_2SO_4 was placed between the rubber gaskets to electrically separate the anode and cathode compartments while permitting proton transport.

Reticulated vitreous carbon foam (20 ppi density, 3% density) from ERG Materials and Aerospace Corp., Oakland, CA, was used as the anode and a 3.0 x 3.0 cm piece of graphite cloth (0.3 mm thick GC-14, Electrolytica, Amherst, NY) was used as the cathode. A piece of platinum (Pt) mesh pressed to the carbon foam anode and a piece of Pt wire woven through the cloth were used to provide external electrical contact to the electrodes. The wire and mesh were secured outside of the cell to an electrical post with a screw and washer. All MFC materials were sterilized prior to use by autoclave, bleach, or ethanol treatment, and the cell components were assembled under sterile water.

8.5.4 Microbial fuel cell operation

Assembled MFCs were inoculated with 20mL of propagated anaerobic digester solution, which was added anaerobically to 200 mL of media that was continuously circulated through the anode

chamber at a flow rate of 20 mL min^{-1}. Anolyte (media) and catholyte were contained in sealed 250 mL bottles and continuously circulated through the anode and cathode compartments at a rate of 20 mL min^{-1} using a peristaltic pump. The anolyte consisted of anaerobic freshwater media[23] with 10 mM acetate as the electron donor, which was continuously purged with an 80/20 mixture of N_2/CO_2. A solution of 50 mM potassium ferricyanide in TRIS buffer served as the electron acceptor.[23] After the bacteria attached to the surface of the anode (as evidenced by the cell voltage) the anode and cathode were connected through a 500 ohm resistor.

8.5.5 Anode surface modification procedure

Carbon foam anodes were surface oxidized by immersion in concentrated nitric acid followed by heating for 4 hours at 140 ^oC. Anodes were carefully removed from the acid solution and washed with copious distilled water until neutral. The carboxylic acid functionalized anodes were then submerged in 25 mL of an aqueous solution of 100 mM N-hydroxysuccinimide (NHS) and 100mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride. Anodes were incubated for 4 hours to allow formation of NHS esters, and then 1 mL of a 50 wt% solution of branched PEI (Mn= 1200, Sigma-Aldrich) was added. Anodes were incubated in this solution overnight at room temperature and then removed from the solution and washed with copious distilled water.

8.5.6 Infrared spectroscopy (IR) and thermogravimetric analysis (TGA)

IR samples were prepared by first oven drying at 120 ^oC overnight to remove water, and then grinding with dry KBr at 1 wt% sample. Samples were pressed into thin discs and analyzed on a Nicolet 6700 FT-IR spectrophotomer under N_2 with a dry KBr background. TGA samples (10-15 mg) were analyzed on TA Instruments Q500, thermogravimetric analyzer, at a heating rate of 15 ^oC min^{-1} in an atmosphere of N_2.

8.5.7 Scanning electron micrograph imagng (SEM)

Samples were prepared for SEM imaging by drying overnight at 75^{o}C in atmosphere oven. Prior to imaging, an electronically conductive coating was applied to the sample to reduce charging in the SEM. Images were taken on a Hitachi S-4800.

8.5.8 Electrochemical characterization

Fuel cell voltages were monitored periodically with a high impedance multimeter. Current–voltage curves were generated using a Solartron SI 1287 Electrochemical Interface.

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