

Polymers to Modulate Host-Microbe Interactions

by

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Abstract

Chemical interactions between microbes and hosts both support and destroy life. Recognition of self- and non-self, destruction of pathogens, and recruitment of symbionts are all largely mediated by multivalent interaction between macromolecules. Multivalent binding occurs when multiple receptors on one surface engage multiple ligands on another. Analogous to protein-small molecule recognition, wherein precise arrangement of atoms in ligand and receptor create complementarity, multivalent ligands and receptors gain specificity based on the three-dimensional arrangement of both targets. Cells control multivalent systems by displaying ligand-receptor pairs on polymers such as proteins. By controlling polymer properties such as size, shape, and ligand density, life utilizes multivalent chemistry to accomplish key cellular and organismal functions such as proliferation and adaptive immunity. In particular, multivalent recognition is critical to the maintenance of host-microbe symbiosis and pathogenesis. Mucins, the massive glycoprotein structural components of mucus, feature multivalent displays of oligosaccharide which specifically bind microbial adhesins recruiting them to mucosal barriers. Mucin structure has proven critical to their ability to attract symbionts, repel pathogens, and control microbial virulence. Additionally, adaptive immunity hinges on multivalent recognition of pathogenic epitopes for precise identification and elimination of harmful microbes. The structure of protein and carbohydrate antigens have profound influences on the ability of the immune system to recognize and destroy pathogens. Similar to structure-activity relationships for small molecules, synthetic polymers can be systematically tuned to perturb and enhance these systems. However, it is only with the advent of living polymerizations, such as the ring opening metathesis polymerization, that sufficient control over polymer structure has enabled precise structure-activity investigations for multivalent interactions. To better understand how chemical structure influences host-microbe relationships, I have synthesized precisely defined and highly tunable synthetic polymers to mimic the structure and anti-virulence properties of mucin and the immunogenicity of natural antigens. To assist in the transfer of this knowledge to practical applications, I have developed methodology for functionalization of degradable polymers whose structures can be readily controlled. This research has resulted in tools to better understand host-microbe symbiosis and pathogenesis. It is my sincere hope that they will contribute to a brighter future for all life.

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List of Abbreviations in Order of Appearance

PTS	proline, threonine, serine
PRR	pattern recognition receptor
DC	dendritic cell
DC-SIGN	dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
APC	antigen presenting cell
BC	B cell
BCR	B cell receptor
MHC	major histocompatibility complex
TCR	T cell receptor
TC	T cell
ROMP	ring opening metathesis polymerization
Ctx	cholera toxin
NMR	nuclear magnetic resonance
FT-IR	Fourier transform infrared
IC ₅₀	concentration of substrate at which half of the target enzyme/receptor is bound given a fixed concentration of enzyme/receptor
AFM	atomic force microscopy
SANS	small angle neutron scattering
DCM	dichloromethane
MeOH	methanol
ACN	acetonitrile
THF	tetrahydrofuran
DMSO	dimethylsulfoxide

DMF	N,N-dimethylformamide
NHS	N-hydroxysuccinimide
EDCI	1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
MWCO	molecular weight cutoff
Ether	diethyl ether
EtOAc	ethyl acetate
Fmoc-Cl	9-fluorenylmethoxycarbonyl chloride
NMM	N-methylmorpholine
FC	flash Chromatography
TLC	thin layer chromatography
ESI	electrospray ionization
GPC	gel permeation chromatography
IL10	interleukin 10
B10	bovine gamma globulin interleukin 10 conjugate vaccine
TLR7	toll like receptor 7
IgY	immunoglobulin Y
BGG	bovine gamma globulin
CatD	cathepsin D
T _{reg}	regulatory T cells
DP	degree of polymerization
SPPS	solid-phase peptide synthesis
TFA	trifluoroacetic acid

LC-MS	liquid chromatography-mass spectrometry
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
R _f	relative mobility
LAH	lithium aluminum hydride
TMSCl	chlorotrimethylsilane
AcOH	acetic acid
NaOAc	sodium acetate
EtOH	ethanol
MALDI	matrix-assisted laser desorption/ionization
TOF	time of flight
ELISA	enzyme-linked immunosorbent assay
PPM	post-polymerization modification
ROP	ring opening polymerization
LRP	living radical polymerization
RAFT	reversible addition-fragmentation transfer
CuAAC	click azide-alkyne cycloaddition
ADBCOA	azadibenzocyclooctyne acid
BIBB	α -bromoisobutyryl bromide
BCy ₂ Cl	chlorodicyclohexylborane
[cat]	catalyst concentration
Conv	conversion
M _n ^{GPC}	number average molecular weight determined by gel permeation chromatography
PDI	polydispersity index

DNP	2,4-dinitrophenol
AF488	AlexaFluor 488
GAG	glycosaminoglycan
ATD	artificial transduction domain
s	singlet
d	doublet
dd	doublet of doublets
ddd	doublet of doublet of doublets
t	triplet
dt	doublet of triplets
ddt	doublet of doublet of triplets
quart	quartet
pent	pentet
m	multiplet
ABX ₂	AB quartet
HRESI-MS	high resolution electrospray ionization mass spectrometry
SEC	size-exclusion chromatography
HPLC	high performance liquid chromatography
M _n	number average molecular weight
M _w	weight average molecular weight
rt	room temperature
eq	equivalents
wrt	with respect to

Chapter 1: Introduction – Host-Microbe Interactions

1.1 *Host-Microbe Symbiosis and Pathogenesis are Mediated by Multivalency*

Thousands of millennia ago, phylum Cnidaria evolved the capacity to produce and secrete mucin gels.^{1,2} These gels form the ecological niche for a vast array of symbiotic microflora and, by maintaining spatial segregation between host and microbe, mucus quite probably enabled evolution of the vast majority of animal phyla (Figure 1.1).¹⁻⁷

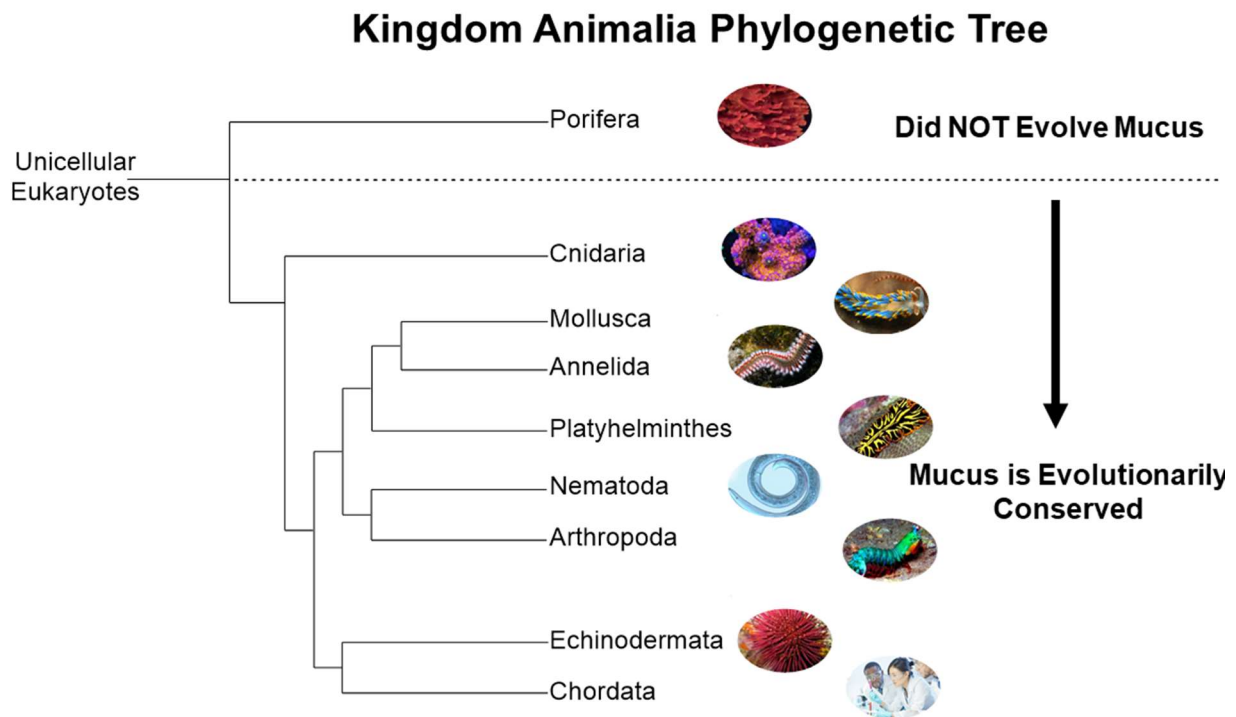


Figure 1.1 Phylogenetic tree of Kingdom Animalia. The evolutionary ancestor to Cnidaria evolved mucus likely setting the stage for an explosion of diversity whereas Poriferan evolution has remained relatively stagnant.

By tapping the immense genetic diversity of microbes, animal hosts have rendered themselves extraordinarily adaptable. Humans, for instance, possess remarkably flexible gut microbiota which allow them to switch between purely vegetarian and carnivorous lifestyles at will.⁸⁻¹⁰ It is now known that symbionts' contribution to host biology is far

reaching having been implicated in myriad processes ranging from immune development to cognition.^{11–14} Nonetheless, infectious disease claimed the lives of over 5.6 million people in 2016 making microbes the third leading cause of death worldwide.¹⁵ Human hosts, therefore, must carefully manage their relationships with microbial life and possess two principle features for doing so: mucus and the immune system.^{4,5,12,16–18}

The Physical and Chemical Structure of Secretory Mucin

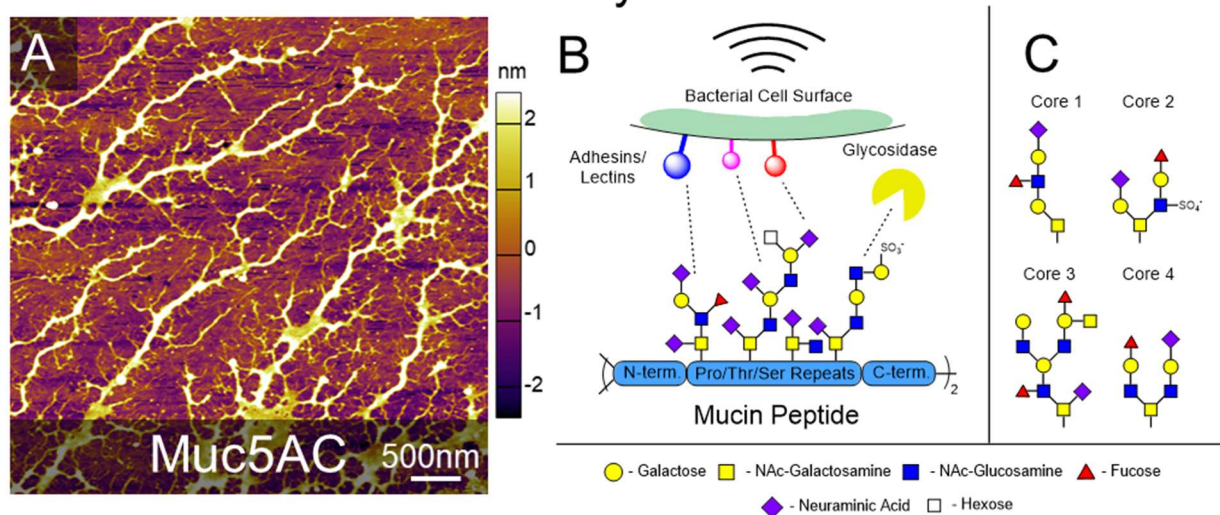


Figure 1.2 A) Atomic force microscopy image of natively purified porcine salivary mucin Muc5AC showing extended conformation and oligomerization in yellow-white. B) Microbial cells can interact with mucin-linked oligosaccharides via multivalent interactions with cell-surface adhesins and chemosensory arrays. Mucin-linked glycans can also be digested by microbial glycosidases for metabolic use. C) The four major core *O*-oligosaccharide structures found on mucins with abridged glycan symbol code below.

Just as flowers have evolved to attract their cognate pollinators, the glycan structures of mucus have evolved to recruit symbionts. Mucus gains function from a densely glycosylated polypeptides known as mucins (Figure 1.2).¹⁹ These glycoproteins are over 5000 amino acids in length and possess three domains: N- and C-terminal

domains and a central proline-serine-threonine-repeat (PTS) domain which constitutes approximately half of the sequence.^{19–22} The absolute structure of the termini is unknown; however, our data and others indicate they are globular and serve as oligomerization points for assembly of mucins into crosslinked networks (Figure 1.2).^{23,24} The PTS domain is composed of highly repetitive stretches rich in proline, serine, and threonine which adopt extended conformations, particularly once glycosylated.^{25–27} Mucin's biochemical function derives primarily from *O*-glycosylation of the PTS domain.^{28–31} Up to 80% of a mucin's impressive 2.5–7.2 MDa molecular weight consists of oligosaccharides appended to serines and threonines in the central domain of the protein.^{28,32–34} Between 100–260 unique oligosaccharide structures have been detected in a single sample of mucin.^{28,35–37} These glycans are both large^{35–37} and densely packed^{38–40} which imparts mucin with an extended, micron-scale structure^{23,26,27,41} that precludes protein-protein contacts across the bulk of its backbone.^{23,42,43} Consequently, the vast majority of mucin-microbe recognition is mediated by carbohydrates (Figure 1.2) and mucin glycosylation is well known to drive the composition of the enteric microflora.^{4,5,13,44–46}

Paradoxically, however, binding between a lone carbohydrate and its lectin receptor is generally weak (i.e. $K_a \approx 0.1\text{--}10\text{mM}$)^{47–49} and lectins often exhibit poor specificity for monovalent sugars.^{50–53} Additionally, oligosaccharide biosynthesis is template independent. Thus, the fidelity of complex carbohydrate assembly is sensitive to the cellular concentration of monosaccharide precursors and the expression level, regulation, substrate specificity, and kinetics of glycan processing machinery.^{54,55} One may then predict that carbohydrates, and by extension mucins, should be incapable of highly targeted interactions between cells. Nonetheless, protein-carbohydrate

recognition triggers numerous vital processes including proliferation,⁵⁶ apoptosis,⁵⁷ chemotaxis,⁵⁸ and adaptive immunity.⁵⁹

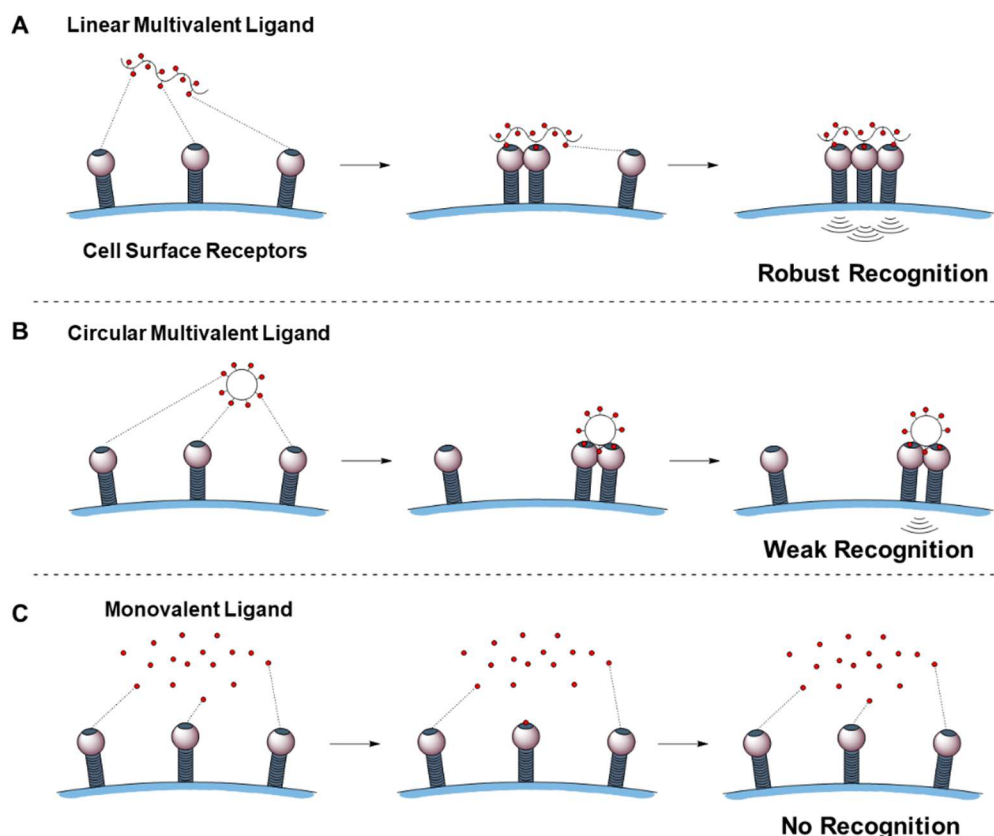


Figure 1.3 Model system wherein multivalent display of a generic ligand (red circles) on linear scaffolds (A) yields more robust binding and signaling than when the same ligand is presented on a circular scaffold (B). (C) Monovalent interactions between ligands and receptors evolved for multivalent recognition are often weak and promiscuous.

By utilizing multivalent binding modes for most non-metabolic protein-carbohydrate interactions, nature overcomes the limitations of monovalent glycans.^{60,61} Multivalent binding occurs when receptors on one surface are engaged by multiple ligands on another (Figure 1.3).⁶² By summing weak and promiscuous monovalent interactions, multivalent binding yields collectively robust yet kinetically labile recognition.^{62,63} These interactions hinge not only on correct pairing of receptor and ligand but also on the arrangement of each in space (Figure 1.3).^{64–66} Consequently, the

architecture to which a ligand or receptor is attached becomes an information carrier. Factors such as the mechanical stiffness,⁶⁷ flexibility,⁶⁸ and three-dimensional shape⁶⁴ of a multivalent scaffold can dramatically influence its binding specificity. For this reason, both mucins⁵ and the immune system⁶⁹ leverage the unique properties of multivalency to precisely identify and influence cells.

Reversible, multivalent binding between mucin-linked oligosaccharides and microbial adhesins^{46,70,71} enforces a spatial equilibrium of microbes at mucus barriers. Those symbionts which can selectively adhere to mucin-linked glycans while avoiding excretion or epithelial invasion are positively selected for.^{3,5,72} Additionally, it has been suggested that mucins have evolved to downregulate microbial virulence traits thereby promoting coexistence between otherwise incompatible microbes and hosts.^{18,73,74} Glycosylation is critical to this function and mucins likely attenuate pathogenicity by specifically engaging microbial signaling pathways via lectins.^{30,44,45} Once established, symbiotic microbes possessing the requisite glycosidases and metabolism to digest mucin-linked oligosaccharides gain an additional survival advantage.^{4,75-77} Having secured themselves in this ecological niche, symbionts can act in concert with the immune system to exclude colonization by pathogens.⁷⁸⁻⁸⁰

Immune cells at the intestinal epithelium then face the daunting challenge of distinguishing food, symbiont, pathogen, and host. Pattern recognition receptors (PRRs) have evolved to recognize conserved elements of foreign organisms such as lipopolysaccharide⁸¹ and single-stranded RNA.⁸² The intestinal lumen is rich in these components and, depending on the ligand and an immune cell's programming, PRRs

can induce both tolerogenic and inflammatory responses.^{83–86} This sets balance for the enteric immune system and promotes colonization of the intestines while maintaining epithelial integrity.⁸⁷ Once damage occurs, however, inflammation is rapidly invoked

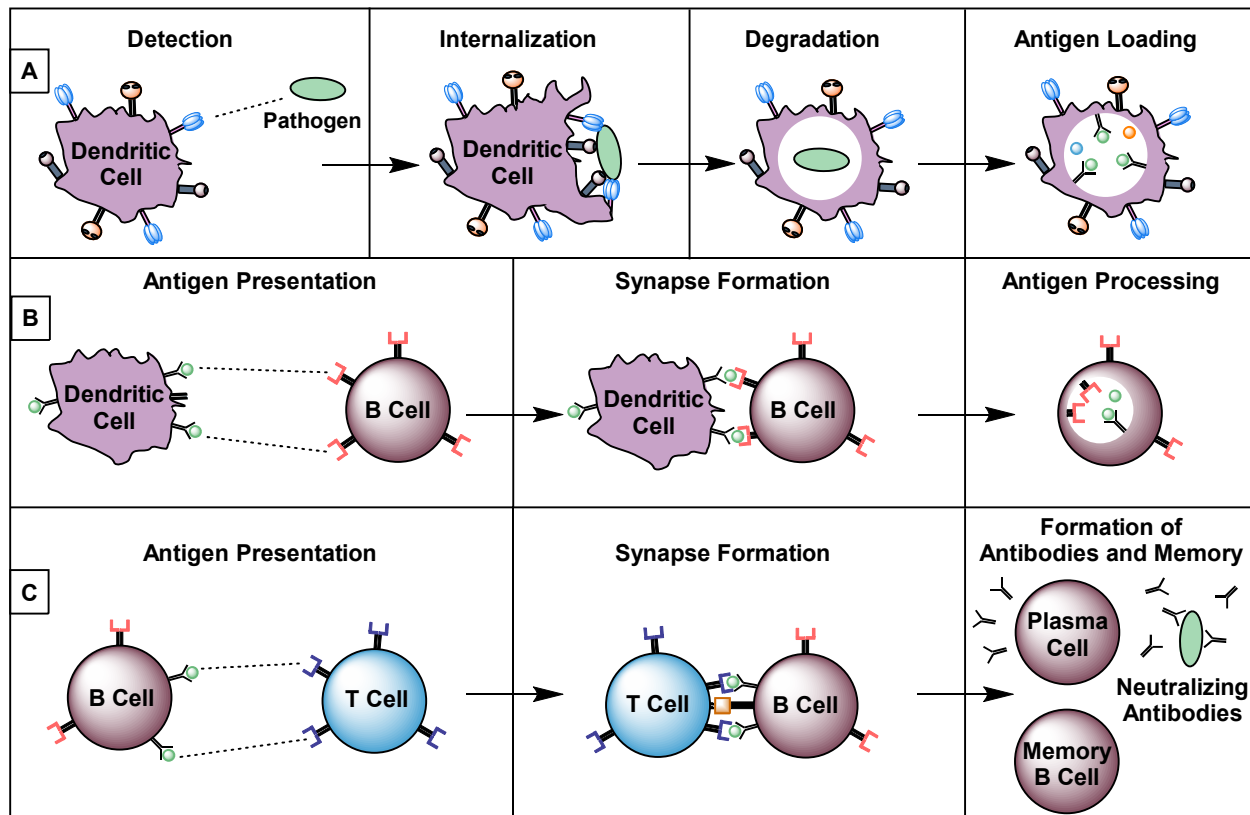


Figure 1.4 A) Antigen presenting cells such as dendritic cells can recognize and internalize pathogens using multivalent sensory arrays leading to B) Presentation of antigens on MHCII complexes for display to B cells. B cells with sufficient affinity for antigen can take the epitope up for C) Display of the antigen to T cells to induce differentiation and production of antibodies.

while the adaptive immune system evolves antibodies that specifically bind and mark the pathogen for destruction.^{84,88–91} This feat, known as adaptive immunity, emerges from intricate multivalent interactions between pathogens and host cells (Figure 1.4).

Multivalent sensory arrays enable high fidelity identification of pathogens.

Dendritic cells (DCs), among others, possess several membrane-bound receptors such as Dectin-1 and DC-SIGN which multivalently bind pathogen-associated epitopes.^{92–94}

Crucially, such receptors can differentially crosslink, signal, and internalize antigen in response to various ligand architectures which allows them to tune immune responses to specific microbial threats.^{93,95-97} Moreover, antigen presenting cells (APCs) can utilize multivalency to prioritize uptake, degradation, and processing of a pathogen's surface-associated macromolecules.^{93,98} This arises from the ability of large multivalent ligands to cluster membrane-bound receptors allowing cells to identify surface-associated patterns.^{93,96,99} These are critical targets for antibodies due to their accessibility. APCs utilize their multivalent hardware to prioritize antigens which will maximize antibody efficacy. For instance, of the ~900 proteins synthesized by *Chlamydia trachomatis* 719 can raise antibodies in humans but only 3% form the immuno-dominant antigens recognized by patients' sera.¹⁰⁰

It appears that B cells (BCs), those cells ultimately responsible for evolution of antibodies and immunological memory, rely on multivalency to accomplish this.^{69,101} BCs possess a unique cell-surface receptor known as the B cell receptor (BCR) which forms the basis of neutralizing antibodies. This receptor's antigen binding region undergoes purposeful mutation¹⁰² which can increase affinity for a newly encountered antigen. BCs must measure their BCR's antigen affinity to ensure robust antibody formation. Since the forces involved in lone protein-ligand binding are infinitesimal, BCs utilize multivalency to reliably 'estimate' the affinity of their BCR for antigen. This occurs through physical stripping of macromolecular membrane components from APC's and pathogens by the collective action of multiple BCRs.^{98,103} High affinity BCRs result in higher uptake of antigen.¹⁰⁴ The newly acquired epitopes are then loaded onto major histocompatibility complexes (MHCs) and transported to the cell surface for further multivalent interaction.^{104,105} Antigen-loaded MHCs next bind to T cell receptors

(TCRs) on the surface of T cells (TCs).¹⁰⁶ A higher density of multivalent MHC-TCR contacts prolongs BC-TC interaction^{106–108} resulting in transfer of pro-proliferatory signals to the BC^{106,107} encouraging those BCs with high affinity BCRs to amplify and further hone their affinity via mutation.^{109,110} After multiple rounds of selection, only those B cells with the highest affinity BCR's remain from which to derive neutralizing antibodies and immunological memory.

Thus, animals evolved to utilize the exquisite precision of multivalent binding to mediate microbial symbiosis and pathogenesis. In stark contrast to human use of antibiotics, mucins and immune systems have been stably employed for some billion years to combat microbial invasion. By contrast, it is now clear that even the latest generations of small molecule antibiotics serve to accelerate the evolution of drug resistance.¹¹¹ Moreover, traditional vaccine formulations have proven effective against only a handful of organisms.¹¹² Therefore, it is critical that the chemistry of multivalent recognition is explored and leveraged to advance human health. To this end, I have synthesized polymers whose morphology and activity mimic that of mucin, synthetic multivalent antigens for coccidiosis treatment in poultry farms, and degradable polymers whose structures can be tailored to interact precisely with immune cells. These systems offer systematically tunable scaffolds with which to further investigate the molecular basis of host-microbe symbiosis and pathogenesis. It is my hope that this research will contribute to more stable strategies by which to manage health and infectious disease.

Chapter 2: Stereochemical Control Yields Mucin Mimetic Polymers

Contributions: Experimental design, polymer and ligand synthesis, cholera toxin binding assays, and atomic force microscopy were performed by Austin Kruger and Dr. Spencer Brucks with assistance from Deborah Wen and Dayanne Carvalho. The tungsten alkylidene was synthesized by Dr. Tao Yan. 2D NMR was performed and interpreted by Dr. Bruce Adams. Natively purified mucins were isolated by Dr. Gerardo Cárcamo-Oyarce. Small angle neutron scattering was performed and interpreted by Yuan Wei and Professor Michael Hore.

2.1 *Abstract*

Mucins are high molecular weight *O*-glycoproteins that constitute mucus barriers. Their high levels of glycosylation cause these macromolecules to adopt linear structures that can block microbial virulence phenotypes. While it is clear that glycosylation is critical to their function, the precise characteristics of mucins that give rise to their advantageous biological activities are unknown. To better understand mucin activity, our goal was to mimic the morphology, glycosylation, and virulence inhibition of mucin with a glycopolymer scaffold whose structure can be systematically varied. We selected ring-opening metathesis polymerization (ROMP) for its functional group tolerance and its exquisite control over polymer structure. Depending on the catalyst, ROMP reactions of norbornene derivatives can give rise to polymers containing predominantly *cis* or *trans* alkenes. Based on conformational analysis, we postulated that *cis*- rather than *trans*-poly(norbornene) glycopolymers would adopt linear structures that mimic mucins. Using atomic force microscopy, we compared our synthetic polymers to natively purified Muc2, Muc5AC, and Muc5B mucins and confirmed that *cis*-polymers adopt extended, mucin-mimetic conformations. The *cis*-polymers retained this structure in solution as evidenced by small-angle neutron scattering. Consistent with mucin's linear morphology, *cis*-glycopolymers were more potent binders of a bacterial virulence factor, cholera toxin, than were *trans*-polymers or natural mucins. These findings provide valuable guidance for the design of well-defined polymers that mimic mucin properties including their ability to inhibit virulence pathways.

2.2 Introduction

All known life relies on the precise transmission of information through sugars.¹¹³ However, interactions between individual carbohydrates and their protein receptors, known as lectins, rank among the weakest and least specific in cells.⁶⁵ Nature resolves this paradox by utilizing multivalency. Multivalent binding occurs when multiple ligands on one surface simultaneously engage multiple receptors on another.⁶² When grafted to proteins or synthetic polymers, glycans take on unique three dimensional arrangements. These arrangements can then bind multiple copies of their target lectin with amplified avidity and specificity.^{62,68,114,115} Given the intrinsic difficulty of systematically altering and analyzing glycosylation,¹¹⁶ mechanistic studies on protein-carbohydrate interactions often employ synthetic mimics of natural glycopolymers.⁶⁵ For instance, a recent study determined that cholera toxin, a pentameric lectin secreted by *Vibrio cholerae*, binds multivalently to synthetic linear glycopeptides more tightly than to globular displays.⁴⁸ This observation has direct consequences for the toxin's activity at intestinal mucus barriers.^{27,117–119}

Studies of this nature have greatly benefitted from the ring-opening metathesis polymerization (ROMP) of norbornene derivatives. ROMP's versatility and functional group tolerance enable precise tuning of glycopolymer structure. Subsequent lectin binding analysis allows one to correlate polymer structure with affinity.^{58,96,120,121} This approach has revealed that lectins can exhibit astonishing sensitivity towards features such as glycopolymer size, functionalization density, and architecture.¹²² By tuning these factors in nature, cells utilize carbohydrate chemistry to trigger key cellular and organismal functions including proliferation, apoptosis, chemotaxis, and adaptive immunity.^{56–59}

Evidence now points to the critical role that protein-carbohydrate interactions play in host-microbe symbiosis. Pioneering studies by Ribbeck and co-workers show that mucin, the glycoprotein structural component of mucus, has the remarkable ability to suppress microbial virulence traits.^{18,31,74,123} Moreover, mucin serves as a trap for microbial toxins, a shield against microbial invasion, and a food source for symbiotic bacteria.^{3,4,51,75,124,125} While it is clear that mucin *O*-glycosylation is necessary for these functions,^{30,44,76,125–127} how glycan identity and presentation affect them is largely unknown. We hypothesize that synthetic mimics of mucin could elucidate the mechanisms by which these fascinating biomolecules operate.

As exhaustively glycosylated peptides, mucins are forced to adopt extended linear conformations stretching hundreds of nanometers (Figure 2.1).^{20,21,25,27–29,32,35,39,128} While the resulting viscoelastic properties of mucins have previously been approximated with synthetic polymers, these scaffolds do not feature bottlebrush displays of mucin-linked glycans.^{129–133} This glycan presentation is critical to recapitulate since perturbing mucin glycosylation *in vivo* renders the intestinal microflora extremely pathogenic.^{30,44,126,127,134} To better understand how mucin domesticates microbes, glycopolymers that more closely mimic the structure of mucin are required.

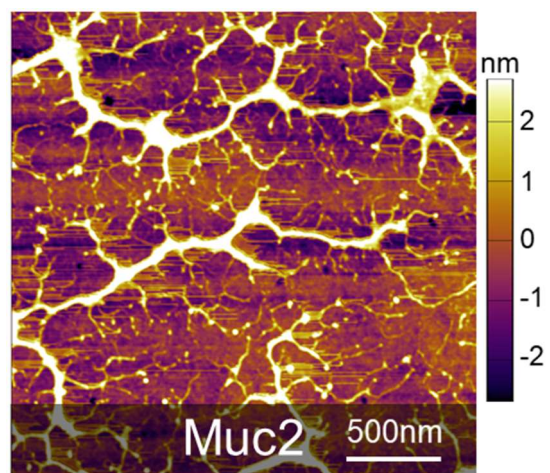


Figure 2.1 Atomic force microscopy image of natively purified porcine salivary mucin Muc5AC showing extended conformation in yellow-white.

Using conformational analysis, we hypothesized that cis-poly(norbornenes) may adopt a linear, extended structure relative to the more commonly employed trans-poly(norbornenes) (Figure 2.2). Though many studies have focused on developing stereocontrolled ROMP,^{135–141} the literature is relatively devoid of how backbone geometry influences polymer morphology and function. A high degree of allylic strain is evident in the cis-dimer but markedly reduced in the trans-dimer (Figure 2.2). Thus, we surmised that formation of hairpin turns in cis-poly(norbornene) chains would be extremely unfavorable whereas trans-polymer chains could readily double back on themselves.

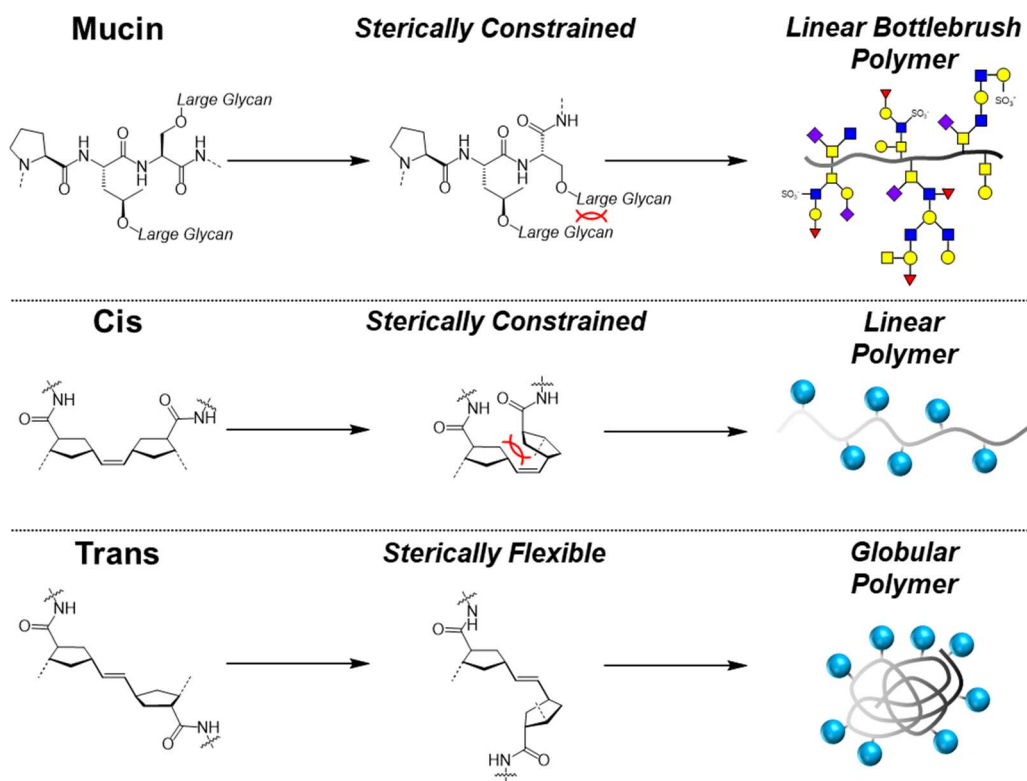


Figure 2.2 Mucin glycopeptides adopt linear bottlebrush structures due to steric repulsion between adjacent glycan chains. We hypothesized that allylic strain in cis-poly(norbornene) could perform a similar function and yield linear mucin mimetic glycopolymers. Trans-poly(norbornene) possesses a high degree of conformational flexibility and may readily form globular aggregates.

Based on this analysis, we postulated that cis-polymers would adopt an extended conformation that mimics mucin. In contrast, the trans-polymer chains have enhanced flexibility allowing the polymer to coil. Indeed, a recent study showed that prototypical trans poly(norbornene) glycopolymers form globular structures.⁹⁶ Moreover, the poly(norbornene) backbone is hydrophobic and when functionalized with a hydrophilic ligand the trans-polymer could adopt conformations in which large swathes of hydrophobic backbone are exposed to solvent. In water, this could induce globule formation to minimize solvent contact with the backbone.

To test this hypothesis, we synthesized glycopolymers from cis- and trans-poly(norbornene) to determine whether cis-polymers would more closely mimic the extended morphology of mucin. Using galactose-functionalized poly(norbornenes) we demonstrate that cis-polymers more faithfully recapitulate mucin's extended structure and toxin-binding properties. To further support our mechanistic hypothesis, we modified the polymer backbone to generate intermediate morphologies and found that the resulting binding avidities for cholera toxin matched our predictions. These synthetic mucin mimics address a critical need for probing the biological activity of mucins through faithful recapitulation of mucin structure. We expect these results to guide design of mucin mimetic polymers for further investigations of host-microbe symbiosis and pathogenesis.

2.3 *Results and Discussion*

To determine if the differences between cis- and trans-poly(norbornene) structure is relevant in a mucin-mimetic context, we tailored our polymers for inhibition of cholera toxin (Ctx). Ctx is a virulence factor produced by the bacterium *Vibrio*

cholerae and was selected as the model lectin for this study based on several criteria. First, previous studies have shown that Ctx prefers binding to linear versus globular galactose conjugates setting precedent for expected differences in activity based on structure.^{48,142} Second, there exist well-established protocols for assaying galactose-functionalized materials as Ctx inhibitors.^{142–144} Finally, Ctx must penetrate the intestinal mucus barrier before it can intoxicate cells so its interaction with mucin-linked galactose is of clinical relevance.^{51,145}

To generate our Ctx inhibitors, we employed a post-polymerization functionalization strategy based on succinimidyl ester monomer **1** (Figure 2.3).

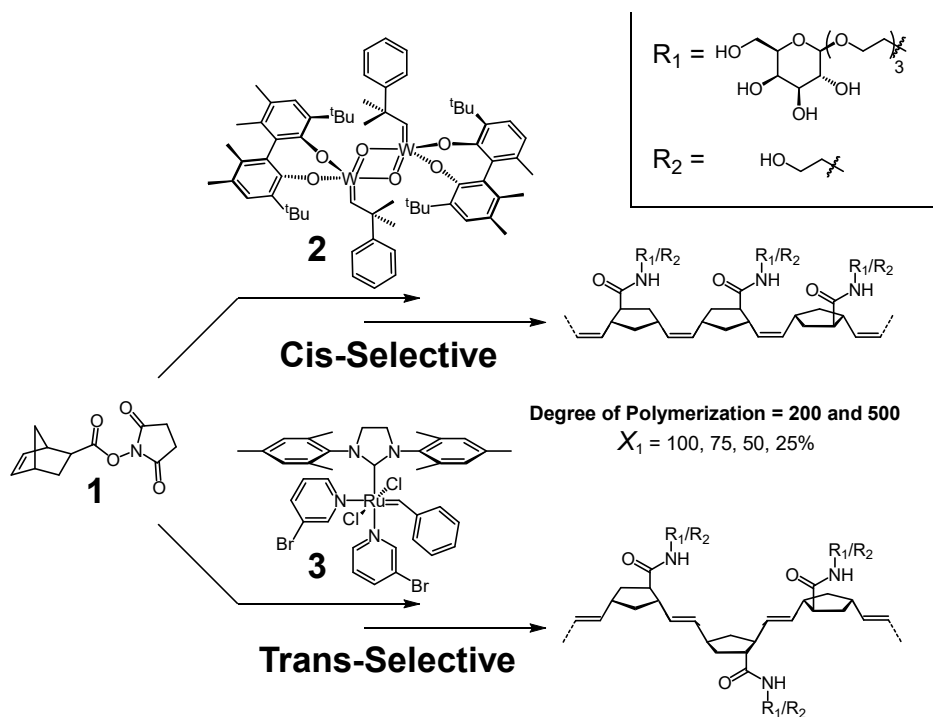


Figure 2.3 Galactose functionalized cis- and trans-poly(norbornenes) were synthesized using either tungsten catalyst **2** or ruthenium catalyst **3** respectively followed by post-polymerization functionalization with linker-functionalized galactose and ethanolamine.

In brief, exo-norbornene carboxylic acid was esterified with *N*-hydroxysuccinimide generating amine-reactive monomer **1**.¹⁴⁶ This monomer was then polymerized with either the Schrock tungsten catalyst **2**,¹³⁸ or the Grubbs' 3rd generation ruthenium

catalyst **3**,¹⁴⁷ yielding cis- or trans-polyolefins, respectively. A combination of 2D NMR and FT-IR spectra confirmed that tungsten complex **2** yielded a polymer with primarily cis-olefins whereas polymers generated with **3** appear exclusively trans- (see Experimental Details).¹³⁸ The degree of polymerization was controlled by monomer to catalyst ratio and confirmed by ¹H NMR end-group analysis.^{121,147} To more closely mimic the high molar mass of mucin, large polymers with a degree of polymerization of 200 and 500 were targeted.^{28,32} Following polymerization, the materials were grafted with various percentages of D-galactose and backfilled with ethanolamine to furnish candidate substrates for Ctx binding (Figure 2.3).¹⁴⁸ Though previous studies have demonstrated that Ctx prefers binding to sparsely galactosylated materials,^{48,121,144} an optimal galactose density has not been established for poly(norbornene). Thus, we generated a panel of glycopolymers between 25-100% galactose functionalization (Figure 2.3).

We next tested the affinity of these materials for Ctx and compared them to purified native secretory mucins. Based on a recently reported fluorescence-based Ctx inhibition assay, we determined IC₅₀ values as the concentration of galactose residues at which half of the toxin was bound by the polymer (Figure 2.4). Briefly, fluorescein-labeled Ctx¹⁴⁴ was pre-incubated with a dilution series of polymer ligand or mucin and added to a plate coated with the lectin's purported native ligand, GM1 ganglioside. Fluorescence was quantified and the resulting concentration-dependent inhibition curve was used to assess the extent of either polymer or mucin binding. Inhibition assays with the 200mers consistently demonstrated that the cis-polymers had IC₅₀'s five- to ten-fold lower than those obtained for the corresponding trans-polymers indicating a significant preference for cis-polymer binding (Figure 3A). Once galactose incorporation was

reduced below 50% the trans-polymers were no longer sufficiently soluble to obtain an IC_{50} , whereas the cis- analogues not only remained soluble but were the best inhibitors tested. Indeed, all cis-materials proved more soluble in buffer than their trans-counterparts (Experimental Details Table 2.5). The observed binding enhancement by decreasing functionalization mirrors previous studies on glycan-lectin interactions and is believed to arise by minimizing steric occlusion of epitopes.^{48,115} The longer 500mer polymers demonstrated similar trends in ctx binding and solubility but with higher variability (Experimental Details Figure 2.2). As such, we used the 200mer scaffold for all subsequent studies.

We wished to compare the activity of these synthetic glycopolymers to native secretory mucins. We purified Muc2 (intestinal mucin), Muc5AC (salivary mucin), and

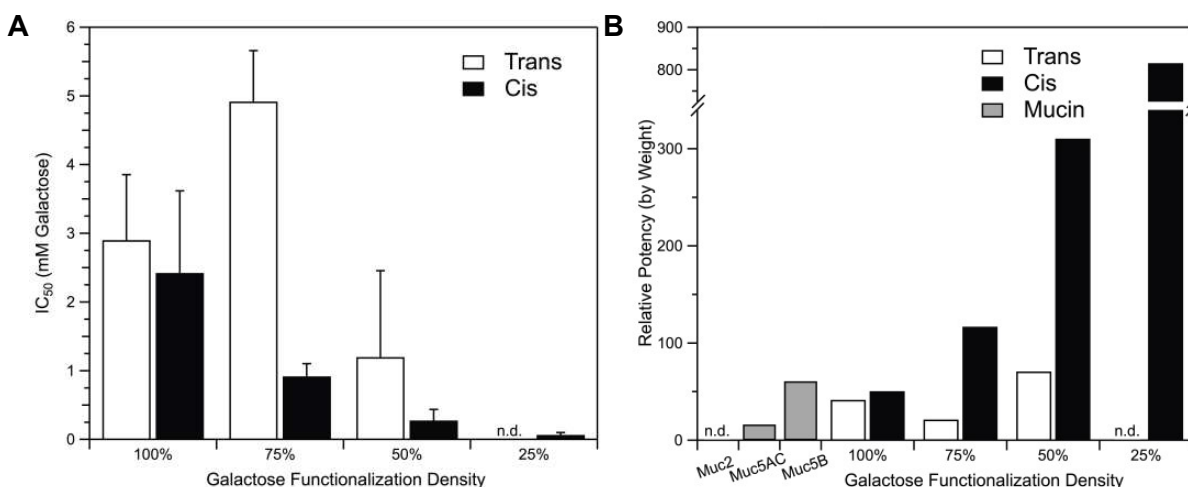


Figure 2.4 A) Inhibition of cholera toxin binding to GM1 ganglioside by galactose functionalized cis- and trans-poly(norbornene) 200mers. Polymers are listed by their percent galactose functionalization. Inhibition data are reported as the concentration of polymer with respect to galactose at which cholera toxin binding to GM1 ganglioside was reduced to half of its maximum value (IC_{50}). n.d. represents compounds with an IC_{50} greater than their maximum solubility in buffer. B) Relative inhibitory potency towards cholera toxin by weight percent for galactose functionalized cis- and trans-poly(norbornene) 200mers, porcine Muc2, Muc5AC, and Muc5B relative to monomeric galactose.

Muc5B (gastric mucin) from porcine tissue samples and assessed Ctx inhibition by the aforementioned assay. As all three mucins are heavily glycosylated with an unknown percentage of galactose, a galactose concentration could not be quantified. Instead, here we determined the IC_{50} as a function of weight percentage and normalized to the measured inhibition of monomeric D-galactose ($IC_{50} = 310 \text{ mM}$ and 5.3 wt%) to yield a relative potency (Figure 2.4B). Excitingly, we found that some purified mucins are able to inhibit Ctx in this assay, with Muc5B as the most potent while Muc2 was insufficiently soluble in buffer for an IC_{50} value to be determined. These data suggest that purified gastric mucin and salivary mucin can serve as efficient decoy receptors for Ctx. When compared to our synthetic mucin mimics, however, it is immediately evident that native mucins are far less potent inhibitors. We ascribe this preferential binding to the fact that our mucin mimetic polymers were specifically designed to have terminal galactose residues for Ctx binding whereas native mucins possess a diversity of glycans.

To ensure that Ctx binding was galactose-dependent and that the observed differences in solubility were due to polymer architecture, we generated glucamine functionalized poly(norbornenes). Glucamine is an open-chain sugar and should exhibit no affinity for Ctx. Gratifyingly, neither the cis- nor trans- glucamine polymer were able to bind Ctx and the cis-polymer again exhibited much higher water solubility compared to its trans-analogue (Experimental Details Table 2.5). Together with the Ctx inhibition results, these data suggest that stereochemical control of the glycopolymer backbone can modulate polymer structure and solubility and drive glycan-lectin binding in mucin-mimetic interactions.

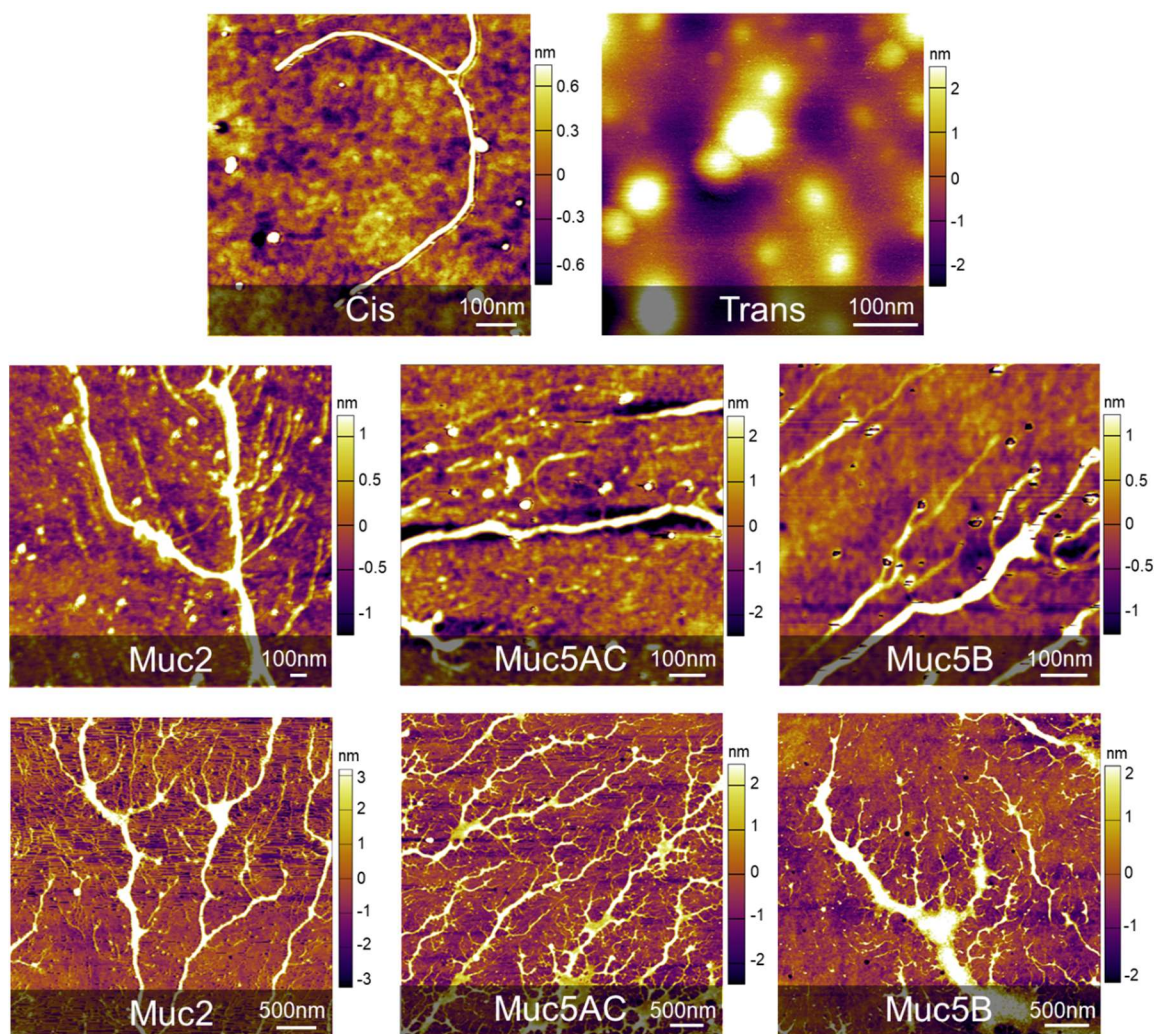


Figure 2.5 Atomic force microscopy images of 50% galactose functionalized cis- and trans-poly(norbornene) 200mers and natively purified porcine intestinal mucin (Muc2), salivary mucin (Muc5AC), and respiratory mucin (Muc5B).

To determine whether the enhanced affinity of cis-polymers for Ctx was correlated with structural differences we characterized our cis- and trans-glycopolymers by atomic force microscopy (AFM) and small-angle neutron scattering (SANS). We compared cis- and trans- 200mers that were 50% functionalized with galactose since both of these polymers were sufficiently soluble to yield an IC_{50} but the cis- material was nearly an order of magnitude more potent. We prepared dilute (10nM) solutions of these glycopolymers and drop-cast them on silicon wafers for visualization by AFM.

Distinct morphologies were observed for the cis- and trans- polymers (Figure 2.5). Most cis-polymers adopted extended linear structures extending over 100nm or formed part of an intertangled network (Experimental Details Figure 2.3). The networks likely arose from linear chain entanglement as solvent evaporated from the silicon wafer.¹⁴⁹ By contrast, the trans-polymers were always found in spherical- globules ranging in size from 10-100 nm in diameter and about 4 nm in height. We similarly prepared samples of Muc2, Muc5AC, and Muc5B and for each found the governing morphology to be an extended bottlebrush structure. We imaged the mucin networks over several microns and observed hitherto unseen capillary-like features. We ascribe these images to the high quality of mucin used in this study which was carefully isolated to ensure minimal degradation and denaturation.^{73,150} While all three mucins formed extended, micron-scale bottlebrushes we also observed a unique condensed morphology in Muc5B (Experimental Details Figure 2.4). Thus, the extended linear structures, exclusively found in the cis-polymer, are mucin mimetic.

To confirm that the morphology observed in the solid state translated to solution phase-we analyzed cis-/trans-50%-Gal-200mers by small-angle neutron scattering (SANS) in water (Figure 2.6). SANS enables determination of the bulk solution structure of polymers composed of light elements by fitting mathematical

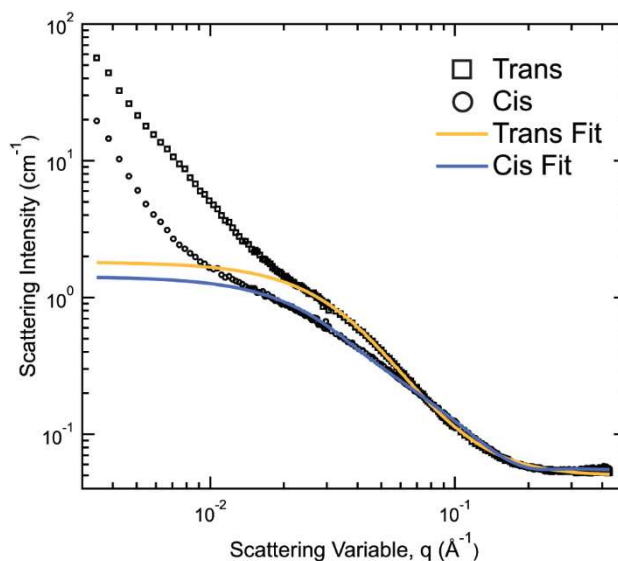


Figure 2.6 Small-angle neutron scattering (SANS) data for 50% galactose functionalized a) cis-poly(norbornene) and b) trans-poly(norbornene).

models to neutron scattering data. The cis-polymers were best fit to a model of rigid cylinders confirming their solution structure as free, extended polymer chains. By contrast, trans-polymers best fit a globular model confirming that they were coiling in solution. These data indicate cis- and trans-polymers retain the conformations observed in solid state when presented for lectin binding in solution.

Finally, to confirm that conformational restriction and backbone hydrophobicity drive poly(norbornene) conformation, we modified the backbone alkenes by syn-dihydroxylation and hydrogenation (Figure 2.7A). From conformational analysis, we predicted that dihydroxylated polymers would gain a minimal amount of rotational freedom relative to the parent trans-polymer but would have significantly reduced hydrophobicity. We therefore anticipated that syn-dihydroxylation would afford materials with a more cis-like morphology, solubility, and potency towards Ctx. Trans-200mers 50% functionalized with galactose readily underwent near quantitative dihydroxylation with OsO₄ and *N*-methylmorpholine *N*-oxide as evidenced by ¹H NMR. In line with our predictions, the dihydroxy-polymer was far more water soluble than its parent polymer (Experimental Details Table 2.6). These materials were then assayed for Ctx inhibition and imaged by AFM (Figure 2.7B and 2.7C). The dihydroxylated polymer adopted a compact cylindrical structure intermediate between the morphology of the trans- and cis-polymers. We were pleased to find that this material gave IC₅₀'s intermediate between the cis- and trans-polymers suggesting that aggregation of trans-polymers into globules was largely responsible for their lower affinity. We expected that hydrogenation of the backbone would increase chain flexibility and thereby promote further globule formation.

are only observed at lower functionalization densities. AFM, however, revealed that the hydrogenated polymer now adopted giant globular structures $>1\text{ }\mu\text{m}$ in diameter (Figure 2.7C). Thus, chemical as well as stereochemical manipulation of poly(norbornene) backbones has significant effects on polymer structure and function.

In sum, we synthesized cis-, trans-, dihydroxy-, and tetrahydro-poly(norbornenes) functionalized with galactose to evaluate their mucin mimetic properties. We assessed their ability to bind the bacterial toxin Ctx, and established that solubility, activity, and morphology in these poly(norbornene) systems are governed by allylic strain and hydrophobic interactions in the polymer backbone. Our results suggest that cis- or dihydroxy-poly(norbornenes) are appropriate for approximating the extended linear morphology of native mucin. We expect these findings to form a foundation for exploring the structure-activity relationships between mucin and microbial symbiosis and pathogenesis.

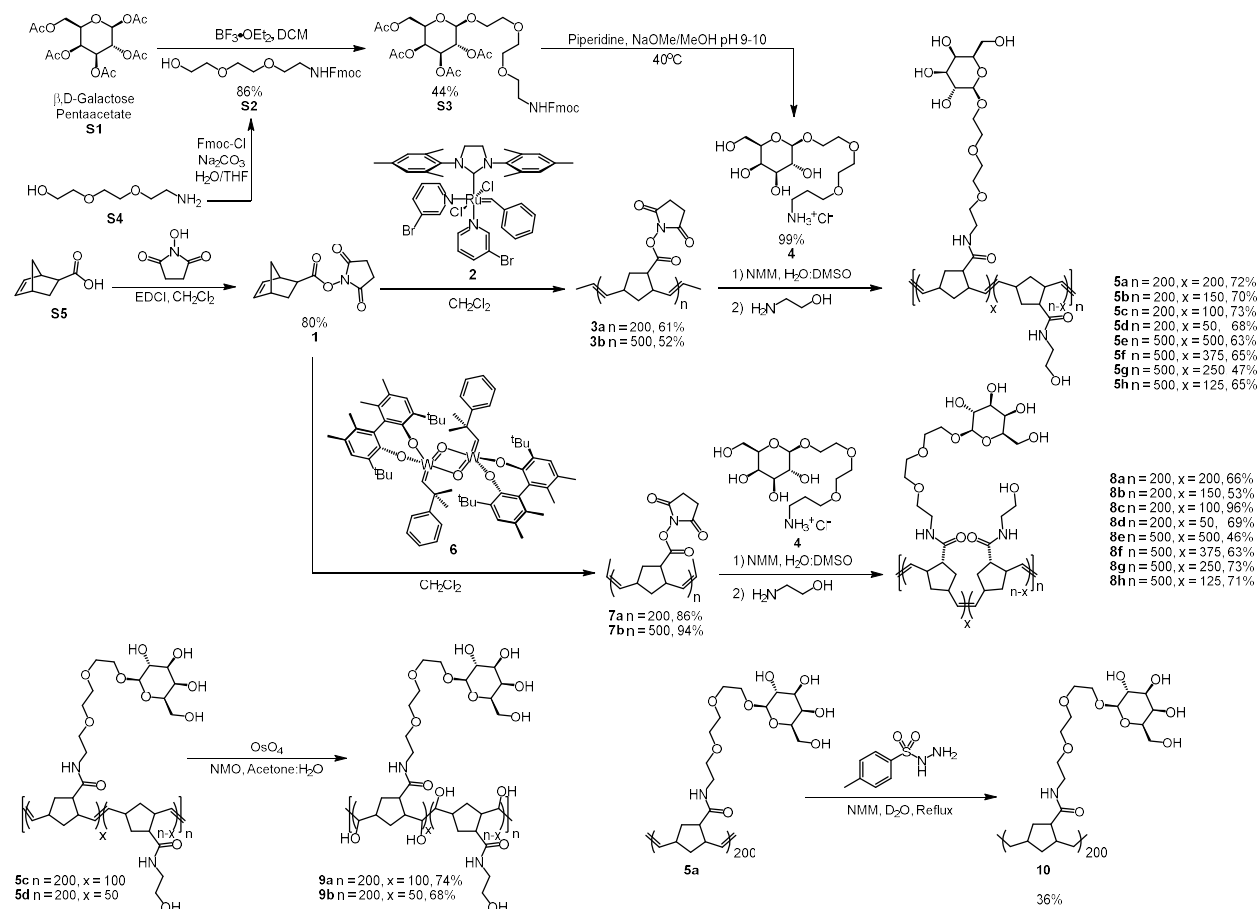
2.4 *Experimental Details*

Materials. Dichloromethane (DCM), Methanol (MeOH), Acetonitrile (ACN), Tetrahydrofuran (THF), Dimethylsulfoxide (DMSO), N,N-dimethylformamide (DMF) Boron Trifluoride Diethyl Etherate, β ,D-Galactose Pentaacetate, Exo-Norbornene-5-Carboxylic Acid, Sodium Carbonate, N-Hydroxysuccinimide (NHS), 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI), Sodium Metal, Grubbs' II Catalyst, Ethanolamine, Amberlyst A26 hydroxide form resin, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES), Sodium Chloride, Magnesium Chloride Hexahydrate, Calcium Chloride Dihydrate, Manganese Chloride, Disodium Hydrogen Phosphate, Potassium Dihydrogen Phosphate, Potassium Chloride, Cholera Toxin B Subunit – Fluorescein Isothiocyanate Conjugate (Ctx), D-Glucamine, Whatman Nylon 0.44 μ m cutoff membrane filters, Whatman PTFE 0.22 μ m cutoff syringe filters, Amberlyst A26 Hydroxide Form Resin, and 10,000 molecular weight cutoff (MWCO) cellulose dialysis tubing were purchased from Millipore Sigma (Burlington, MA). Diethyl Ether (Ether), Hexanes, Chloroform, Acetone, Ethyl Acetate (EtOAc), 9-fluorenylmethoxycarbonyl chloride (Fmoc-Cl), Piperidine, and N-methylmorpholine (NMM), and Ultra-Flat 100mm silicon wafers (Orientation <100>) manufactured by Ted Pella Inc. were purchased from Fisher Scientific (Hampton, NH). Dry solvents were obtained using a solvent purification system by Pure Process Technology. Flash chromatography (FC) was performed using SiliaFlash Irregular Silica Gel P60 from Silicycle and analytical thin layer chromatography (TLC) was performed using Siliaplate TLG-R10014B-323 with F254 fluorescence indicator. GM-1 ganglioside was purchased from Carbosynth LLC (Compton, Berkshire, United Kingdom). Dow Corning Costar 3577 Flat Black High-Binding Flat Bottom 384-well plates and Bovine Serum Albumin were purchased from

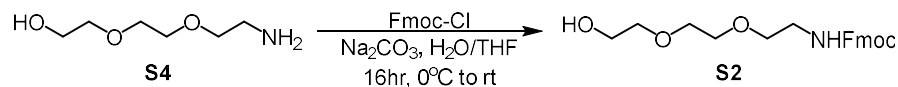
Thermo Fisher Scientific (Waltham, MA). AC160TS-R3 atomic force microscopy probes (7nm silicon tip, f(kHz) 200-400, k(N/m) 8.4-57) by Asylum Research Probes were purchased from Oxford Instruments. Porcine Muc2 and Muc5B intestinal and gastric mucins were isolated and purified as previously described.⁷³ Porcine Muc5AC was isolated and purified as previously described.¹⁵⁰

Instrumentation. All nuclear magnetic resonance (NMR) spectra were obtained using a Bruker 600 MHz Avance Neo spectrometer equipped with a 5mm helium-cooled QCI-F cryoprobe or a Bruker 500 MHz Avance Neo spectrometer equipped with a BBFO SmartProbe. Non-analytical mass spectra were acquired using an Advion Expression CMS in positive ESI mode. Fourier transform-infrared spectroscopy was performed using a Bruker Alpha II spectrometer equipped with a Diamond Crystal Attenuated Total Reflectance device. Gel permeation chromatography (GPC) was performed using an Agilent 1260 Infinity HPLC equipped with a dual Agilent PL1110-6500 columns using a mobile phase of 25mM LiBr in DMF with a flow rate of 1mL/min at 60°C. Elution was monitored by a Wyatt Optilab T-rEX differential refractive index detector and a Wyatt Dawn Heleos II multi-angle light scattering detector. Additional GPC measurements were taken with a Tosoh EcoSEC HLC-8320 equipped with dual SuperH3000 columns running a chloroform mobile phase at 1ml/min. Fluorescence measurements were made using a TECAN M1000 plate reader. Atomic force microscopy (AFM) was performed using a Cypher S microscope by Asylum Research (Oxford Instruments).

Galactose Ligand and Polymer Synthesis



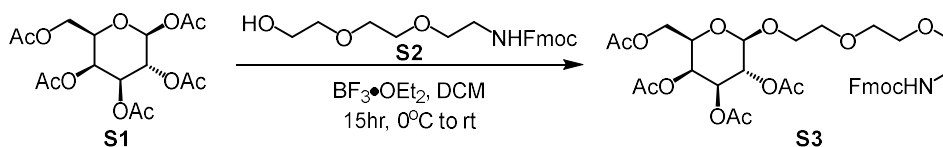
Experimental Details Scheme 1.1 Full synthetic route for galactose-functionalized cis-, trans-, dihydroxy-, and tetrahydro-poly(norbornenes).



Compound S2: Known compound **S2** was synthesized by modification of the procedure for compound B2 in the cited reference.¹⁵² [2-(2-(2-aminoethoxy)ethoxy)ethanol] **S4** was obtained as a colorless to cloudy orange viscous oil used without further purification. The reagent bottle is weighed and the oil subsequently poured into the reaction vessel followed by 2x 5mL rinses with H_2O each added to the reaction vessel to

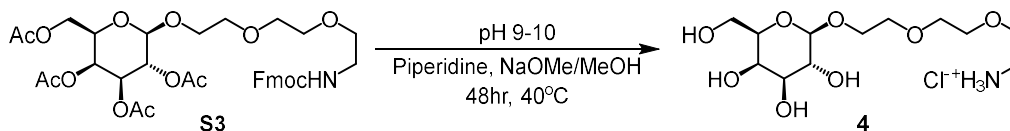
ensure complete addition. The container is then dried and weighed to determine the actual mass of **S4** in the reaction. This value is then used to calculate stoichiometry and the Na_2CO_3 solution is simply prepared with 10mL less H_2O than would be necessary to give the final desired concentration. Procedure: Add 9.936g of **S4** (66.6mmol, 2.5 equiv.) to a 500mL round bottom flask equipped with a stir bar. Rinse the PEG bottle with 2x 5mL portions H_2O and add each rinse to the reaction vessel. Prepare a solution of 17.65g Na_2CO_3 (167mmol, 1 equiv.) in 179mL H_2O . Dissolve the PEG chain in the Na_2CO_3 solution and cool to 0°C . In a 250mL beaker prepare a solution of 17.23g Fmoc-Cl (66.6mmol, 1 equiv.) in 72mL THF and add the chloroformate solution to the reaction vessel and allow the ice bath to melt as the reaction proceeds overnight. After stirring overnight, the reactant solution is clouded by white precipitate and will resolve to a cloudy white aqueous phase (bottom layer) and a champagne colored organic phase (top layer) on standing. Remove the THF under reduced pressure. Extract the product with 3x 200mL portions CH_2Cl_2 , combine the organic phases, dry over MgSO_4 , and concentrate under reduced pressure. Purify the crude oil by flash chromatography using 600mL silica gel and a solvent gradient of 4L 25% EtOAc: CH_2Cl_2 followed by 2L 10% MeOH: CH_2Cl_2 . Product is generally obtained as a champagne colored viscous oil with $R_f = 0.18$ on neat ethyl acetate, however, the material once solidified to a white to grey waxy solid after extensive drying under high vacuum and cooling to -20°C overnight. Note that the compound streaks heavily on the column despite the optimistic TLC profile obtained with neat ethyl acetate and while product appears simple to isolate from the high R_f impurity they elute extremely closely on the column. Also note that the spots appear better behaved by TLC in EtOAc:Hexanes, however, this solvent system results in dramatic streaking leading to poor resolution and even more excessive solvent

volumes required to elute product. It is also noteworthy that the crude material obtained from the extraction may be employed in the subsequent glycosylation step with no further purification, however, this leads to less reproducible yields. Average Yield: 86%. Minimum Yield: 81%. Maximum Yield: 99%. $^1\text{H-NMR}$ (600 MHz, Chloroform-*d*) δ 7.78 (d, $J = 7.5$ Hz, 2H), 7.63 (d, $J = 7.6$ Hz, 2H), 7.42 (t, $J = 7.5$ Hz, 2H), 7.33 (t, $J = 7.5$ Hz, 2H), 5.60 (s, 1H), 4.45 (d, $J = 6.8$ Hz, 2H), 4.24 (brt, $J = 6.8$ Hz, 1H), 3.82 – 3.22 (m, 13H).



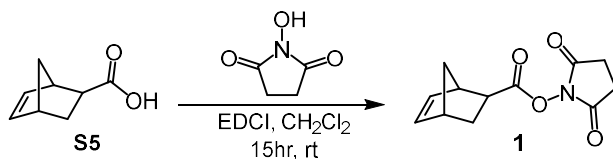
Compound S3: Compound **S3** was synthesized according to a modified procedure for preparation of compound 35 in the cited reference.¹⁵³ To a flame dried N_2 -flushed 500mL pear shaped flask equipped with a stir bar add 5.03g β ,D-Galactose Pentaacetate **S1** (12.9mmol, 1 equiv.), dissolve in 103mL dry CH_2Cl_2 , add 4.78g **S2** (12.9mmol, 1 equiv.), and cool to 0°C . If the present batch of **S2** failed to solidify it may be dried over high vacuum for 24 hours directly prior to reaction to remove residual moisture and a stock solution of 512mg/mL **S2** in dry CH_2Cl_2 prepared. In this instance the sugar-acetate would be dissolved in 93.7mL dry CH_2Cl_2 and 9.34mL of the **S2** stock solution (4.78g, 12.9mmol, 1 equiv.) added to the reactant solution to arrive at the appropriate total volume of CH_2Cl_2 . Once the reactant solution has cooled to 0°C add dropwise over 5 minutes 4.77mL boron trifluoride diethyl etherate (38.6mmol, 3 equiv.) and allow the ice bath to melt as the reaction proceeds overnight at room temperature. Note that the quality of boron trifluoride diethyl etherate impacts the yield of the transformation and

if the Lewis acid appears brown or is suspected to contain moisture it should be doubly distilled by shortpath apparatus and stored under N_2 prior to use. By morning, the reactant solution is a dark brown or colorless solution (no correlation between reaction color and yield was observed). Concentrate the reactant solution and partition the concentrate between 115mL each EtOAc and saturated aq. $NaHCO_3$. Swirl the biphasic mixture to an emulsion in the flask while open to air to allow the initial evolution of CO_2 to escape before transferring to a separatory funnel and washing with a further 1x 115mL portion saturated aq. $NaHCO_3$, 2x 115mL portions H_2O , dry over $MgSO_4$, and concentrate. Note that performing the extraction with CH_2Cl_2 as the organic phase yields emulsions which take considerable time and brine to resolve. Purify the material by FC using 400mL silica and a solvent gradient of 3L 60% EtOAc:Hexanes followed by 1L EtOAc. **S3** is a cloudy faintly green tinted oil which forms a white crystalline foam on drying over high vacuum with $R_f=0.20$ on 60% EtOAc:Hexanes. Average Yield: 44%. Minimum Yield: 30%. Maximum Yield: 56%. ^1H NMR (600 MHz, Chloroform-*d*) δ 7.79 (d, $J = 7.6$ Hz, 2H), 7.63 (d, $J = 7.5$ Hz, 2H), 7.42 (t, $J = 7.5$ Hz, 2H), 7.34 (td, $J = 7.5, 1.1$ Hz, 2H), 5.39 (d, $J = 3$ Hz, 2H), 5.23 (abq, $J = 10.5, 8.0$ Hz, 1H), 5.03 (dd, $J = 10.4, 3.4$ Hz, 1H), 4.54 (d, $J = 8$ Hz, 1H), 4.42 (d, $J = 7.1$ Hz, 2H), 4.25 (t, $J = 7.1$ Hz, 1H), 4.15 (dtd, $J = 17.9, 11.5, 6.6$ Hz, 2H), 3.98 (dt, $J = 10.9, 4.3$ Hz, 1H), 3.89 (t, $J = 6.4$ Hz, 1H), 3.80 – 3.24 (m, 12H), 2.21 – 1.94 (m, 12H).

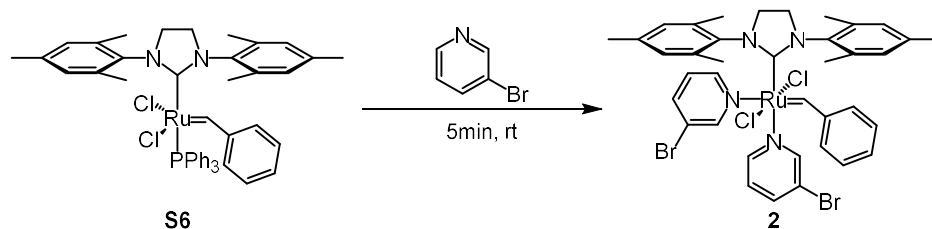


Compound 4: Compound **4** was synthesized according to a modified procedure for compound **37** in the cited reference.¹⁵³ To a 500mL round bottom flask equipped with a stir bar add 5.06g of protected galactoside **S3** (7.21mmol, 1 equiv.). In a 24mL vial prepare a solution of 1M NaOMe in MeOH by addition of 230mg sodium metal to 10mL MeOH. Using this solution, titrate at least 140mL MeOH to pH 9-10 by pH paper. Dissolve the protected galactoside **S3** in 131mL of the pH 9-10 NaOMe/MeOH solution, add 1.42mL piperidine (14.4mmol, 2 equiv.), heat to 40°C, and allow the reaction to proceed for 48 hours after which the reactant solution is a light champagne color. After stirring for 48 hours, concentrate the reactant solution. Partition the concentrate between 40mL each H₂O and EtOAc, wash with a further 2x 40mL portions EtOAc, basify the aqueous phase to pH 11 by pH paper with 1-3g Amberlyst A26 Hydroxide Form Resin, wash with 3x 40mL portions CH₂Cl₂ (note that the Amberlyst resin must be physically present within the separatory funnel to aid in piperidine removal), transfer the aqueous phase to a 500mL round bottom flask and remove the residual CH₂Cl₂ under reduced pressure. Pass the aqueous phase over a 0.22µm syringe filter, neutralize by pH paper with 1-10mL 1M HCl, and lyophilize. Product is a light yellow resinous crystalline solid. The product is generally obtained with residual piperidine contaminant and is extremely hygroscopic. Consequently, the mass of product generally exceeds that of a theoretical 100% yield, however, the material is still readily grafted in the desired stoichiometry to ROMP polymers without accounting for these impurities. Average Yield: ≥99%. Minimum Yield: 97%. Maximum Yield: ≥99%. ¹H NMR (600 MHz,

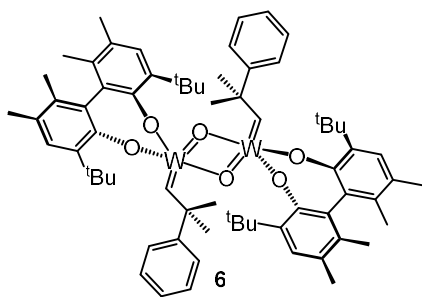
Deuterium Oxide) δ 4.35 (d, J = 7.9 Hz, 1H), 4.02 (dt, J = 11.4, 4.0 Hz, 1H), 3.85 (d, J = 3.5 Hz, 1H), 3.80 – 3.74 (m, 1H), 3.74 – 3.64 (m, 12H), 3.62 (dd, J = 7.8, 4.3 Hz, 1H), 3.58 (dd, J = 9.9, 3.4 Hz, 1H), 3.46 (abq, J = 9.9, 7.9 Hz, 1H).



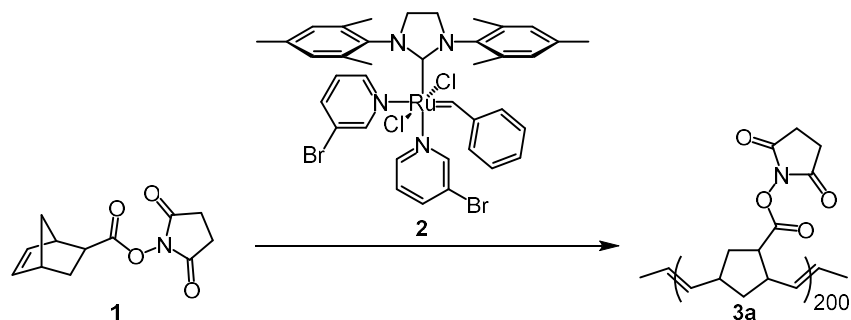
Compound 1: Compound **1** was synthesized according to a modified procedure for compound 15-NHS in the cited reference.¹⁵⁴ To a flame dried N₂ flushed 100mL round bottom flask add 2g Exo-Norbornene-5-Carboxylic Acid **S5** (14.5mmol, 1 equiv.), 2.33g N-Hydroxysuccinimide (20.2mmol, 1.4 equiv.), 3.05g 1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide•HCl (15.9mmol, 1.1 equiv.), dissolve in 24.1mL dry CH₂Cl₂, and allow the reaction to proceed overnight before concentrating. The reactant solution remains clear and colorless throughout the reaction. Partition the concentrate between 90mL each EtOAc and H₂O, extract the aqueous phase with a further 2x 90mL portions EtOAc, combine the organic phases, wash with 2x 45mL portions saturated NH₄Cl, 2x 90mL portions saturated NaHCO₃, 1x 90mL portion brine, dry over MgSO₄, and concentrate. Product is an off-white solid. Average Yield: 80%. Minimum Yield: 73%. Maximum Yield: 88%. ¹H NMR (600 MHz, Chloroform-*d*) δ 6.23 (dd, J = 5.7, 3.0 Hz, 1H), 6.17 (dd, J = 5.7, 3.1 Hz, 1H), 3.32 – 3.24 (m, 1H), 3.02 (m, 1H), 2.86 (d, J = 6.6 Hz, 5H), 2.53 (ddd, J = 9.0, 4.5, 1.4 Hz, 1H), 2.07 (dt, J = 12.0, 3.8 Hz, 1H).



Compound 2: Catalyst **2** was synthesized according to a modified literature procedure for compound **6** in the cited reference.¹⁵⁵ To a 24mL vial add 200mg Grubbs' II catalyst **S6** (236μmol, 1 equiv.), 223μL 3-Bromopyridine (2.33mmol, 10 equiv.), and stir to a bright green paste over 5 minutes with a glass rod. To this paste add 4mL Hexanes and cool to 4°C in a refrigerator overnight after which the supernatant has taken on a faint yellow-green color. After cooling overnight, collect the catalyst by filtration over a Büchner funnel and rinse the filtrate with 3x 4mL portions Hexanes. Allow the catalyst to air dry overnight before storage. Product is a bright green powder which is best stored under N₂ for periods of more than a few weeks. Average Yield: 73%. Minimum Yield: 54%. Maximum Yield: 88%.

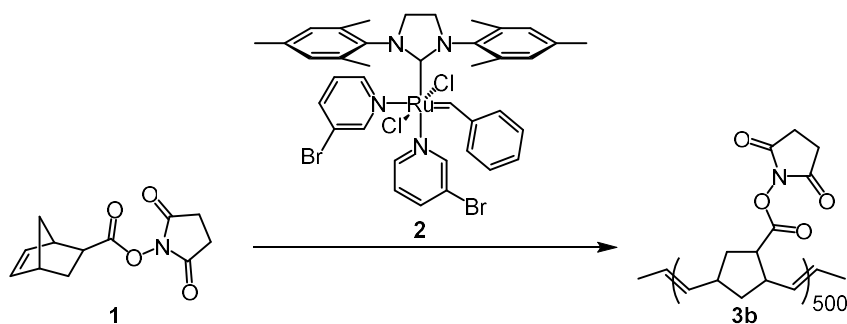


Compound 6: Catalyst **6** was synthesized according to the procedure for compound *rac*-2a in SI the cited reference.¹³⁸



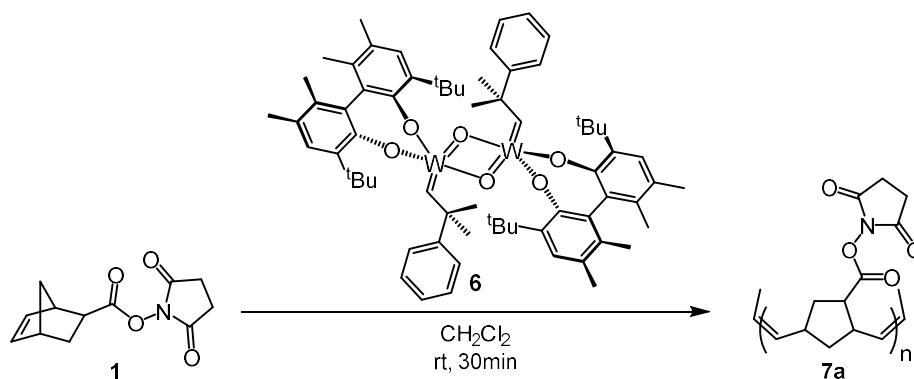
Polymer 3a: Polymers **3a** was synthesized according to a modified procedure for polymer **2** in the cited reference.¹²¹ To a flame dried N₂ flushed 25mL round bottom flask add 500mg NHS-monomer **1** (2.13mmol, 200 equiv.), dissolve in 6.1mL dry CH₂Cl₂, and cool to -15°C in a brine slush. In a flame dried N₂ flushed 6mL vial prepare a solution of 9.41mg/mL Grubbs' III catalyst **2** in dry CH₂Cl₂, add 1mL of the catalyst stock (10.6μmol, 1 equiv.) as rapidly as possible via syringe to the monomer solution and allow the polymerization to proceed for 15 minutes. The catalyst solution is a rich, deep green in color which will decay to a brown-black if left to stand for 5-10 minutes. This color is indicative of catalyst decomposition in solutions containing only the catalyst. On addition to monomer, the solution will rapidly take on a yellow-brown color which is associated with initiation and propagation. Thus it is advisable to employ the catalyst solution immediately upon dissolution. After stirring for 15 minutes remove the reaction from the brine slush, quench by addition of 500μL Ethyl Vinyl Ether, and allow the solution to stir for 30 minutes as it warms to room temperature and turns a deep brown-black in color. Add the reactant solution dropwise to 160mL Diethyl Ether, centrifuge, and decant. Allow the precipitate to air dry for at least 2 hours before redissolving in 8mL CH₂Cl₂ and repeating the precipitation process once more. Product is a white solid

as obtained from precipitation which concentrates to a pink-brown tinted clear film from CH₂Cl₂. DP = 230 by ¹H NMR end group analysis where the phenyl signal from 7.43-7.20ppm is integrated for 5H and the integral of the signal at 3.37ppm is measured to determine DP. Đ = 1.003 Average Yield: 61%. Minimum Yield: 43%. Maximum Yield: 70%. ¹H NMR (600 MHz, Methylene Chloride-*d*₂) δ 7.43-7.20 (m, 5H) 5.68 – 5.24 (brm, 1073H, overlap with solvent), 3.37 (brm, *J* = 229H), 3.01 – 2.63 (brm, 993H), 2.26 (brs, 172H), 2.16 – 1.96 (brm, 165H), 1.82 (brm, 181H), 1.29 (brm, 207H).



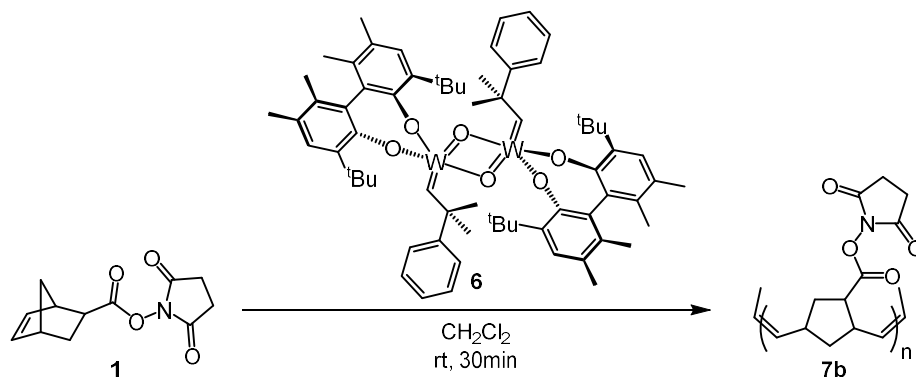
Polymer 3b: Polymers **3b** was synthesized according to a modified procedure for polymer 2 in the cited reference.¹²¹ To a flame dried N₂ flushed 5mL round bottom flask add 200mg NHS-monomer **1** (850μmol, 500 equiv.), dissolve in 2.35mL dry CH₂Cl₂, and cool to -15°C in a brine slush. In a flame dried N₂ flushed 6mL vial prepare a solution of 3.14mg/mL Grubbs' III catalyst **2** in dry CH₂Cl₂, add 480μL of the catalyst stock (1.70μmol, 1 equiv.) as rapidly as possible via syringe to the monomer solution and allow the polymerization to proceed for 15 minutes. The catalyst solution is a rich, deep green in color which will decay to a brown-black if left to stand for 5-10 minutes. This color is indicative of catalyst decomposition in solutions containing only the catalyst. On addition to monomer, the solution will rapidly take on a yellow-brown color which is

associated with initiation and propagation. Thus it is advisable to employ the catalyst solution immediately upon dissolution. After stirring for 15 minutes, remove the reaction from the brine slush, quench by addition of 200 μ L Ethyl Vinyl Ether, and allow the solution to stir for 30 minutes as it warms to room temperature. Add the reactant solution dropwise to 80mL Diethyl Ether, centrifuge, and decant. Allow the precipitate to air dry for at least 2 hours before dissolving in 4mL CH₂Cl₂ and repeating the precipitation process once more. Product is a white solid as obtained from precipitation which concentrates to a pink-brown tinted clear film from CH₂Cl₂. DP = 405-550 by ¹H NMR end group analysis where the phenyl signal at 7.43-7.21ppm is integrated for 5H and the integral of the signal at 3.35ppm is measured to determine DP. $\bar{D} = 1.09$. Average Yield: 52%. Minimum Yield: 43%. Maximum Yield: 60%. ¹H NMR (600 MHz, Methylene Chloride-*d*₂) δ 7.43-7.21 (m, 5H), 5.75 – 5.12 (brm, 1485H), 3.35 (brm, 551H), 2.81 (brm, 2457H), 2.40 – 2.16 (brm, 431H), 2.04 (brm, 405H), 1.86 (brm, 700H), 1.49 – 1.09 (brm, 479H).

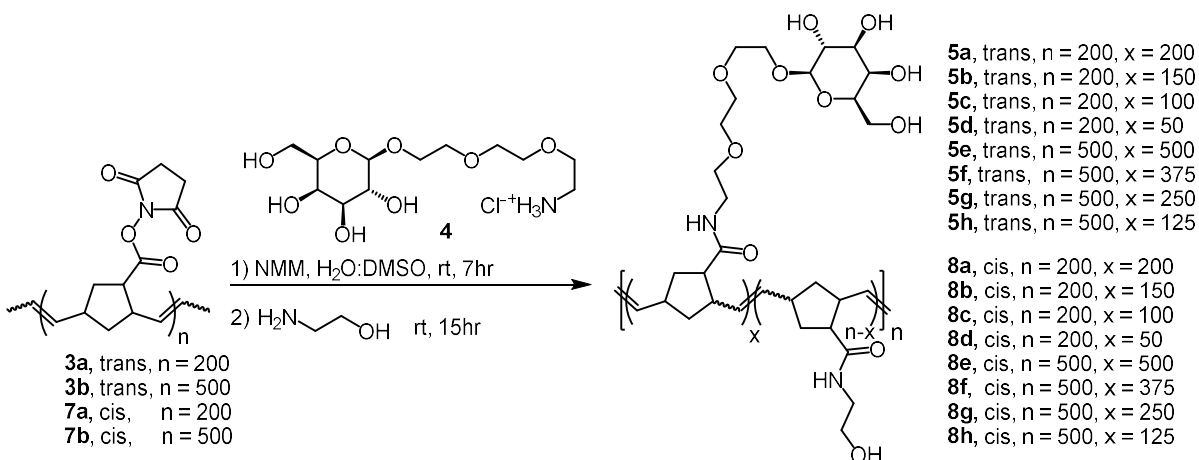


Polymer 7a: Polymer **7a** was synthesized according to the modified procedure for entry 8 (main text) or entry 9 (SI) in the cited reference.¹³⁸ All procedures for carrying out cis-polymerization are performed in a dry N₂ filled glovebox. To a 24mL vial

equipped with a stir bar, add 250mg NHS-Monomer **1** (1.06mmol, 150 equiv.) and dissolve in 3-5mL dry CH₂Cl₂. In a 24mL vial, prepare a solution of 9.7mg (7.1μmol, 1 equiv.) catalyst **6** in 2-4mL dry CH₂Cl₂. The catalyst forms a bright orange-yellow solution. Note that the rate of propagation for catalyst **6** is greater than initiation and so, at low DP, it typically yields polymers of greater DP than targeted. To compensate for this, low DP reactions are set up using lower equivalents of monomer to catalyst than theoretically required to yield a polymer of a particular length. Here, a ratio of monomer to catalyst of 150:1 was found to yield approximately 200mer by ¹H NMR end group analysis. Add the catalyst solution rapidly to the monomer solution and allow the reaction to proceed for 30 minutes before removing from the glovebox. The reactant solution rapidly takes on a burnt-orange color after initiation. Add the reactant solution dropwise to 40mL MeOH, centrifuge, and decant. Product is a white rock-like solid as obtained from precipitation. DP = 230 by ¹H NMR end group analysis where the phenyl signal at 7.44-7.13ppm is integrated for 5H and the integral of the signal at 1.82ppm is taken to determine DP. PDI = 2.06 . Average Yield: 86%. Minimum Yield: 86%. Maximum Yield: 86%. ¹H NMR (600 MHz, Methylene Chloride-*d*₂) δ 7.44-7.13 (m, 5H) 5.58 – 5.12 (brm, 731H, overlap with solvent), 3.45 (brm, 663H), 2.81 (brm, 1100H), 2.42 – 1.99 (brm, 440H), 1.82 (brm, 227H), 1.70 – 1.32 (brm, 163H), 1.25 (brm, 239H).



Polymer 7b: Polymer **7b** was synthesized according to the modified procedure for entry 8 (main text) or entry 9 (SI) in the cited reference.¹³⁸ All procedures for carrying out cis-polymerization are performed in a dry N₂ filled glovebox. To a 24mL vial equipped with a stir bar, add 250mg NHS-Monomer **1** (1.06mmol, 500 equiv.) and dissolve in 3-5mL dry CH₂Cl₂. In a 24mL vial, prepare a solution of 2.9mg (2.13μmol, 1 equiv.) catalyst **6** in 2-4mL dry CH₂Cl₂. The catalyst forms a bright orange-yellow solution. Add the catalyst solution rapidly to the monomer solution and allow the reaction to proceed for 30 minutes before removing from the glovebox. The reactant solution rapidly takes on a burnt-orange color after initiation. Add the reactant solution dropwise to 40mL MeOH, centrifuge, and decant. Product is a white rock-like solid as obtained from precipitation. DP = 550 by ¹H NMR end group analysis where the phenyl signal at 7.48-7.12ppm is integrated for 5H and the integral of the signal at 1.81ppm is taken to determine DP. PDI = 1.64. Average Yield: 94%. Minimum Yield: 90%. Maximum Yield: 96%. ¹H NMR (600 MHz, Methylene Chloride-*d*₂) δ 7.48 – 7.12 (m, 5H), 5.37 (brm, 1991H, overlap with solvent), 3.51 – 3.00 (brm, 1325H), 2.81 (brm, 2748H), 2.38 – 2.01 (brm, 1096H), 1.81 (brm, 546H), 1.25 (brm, 560H).

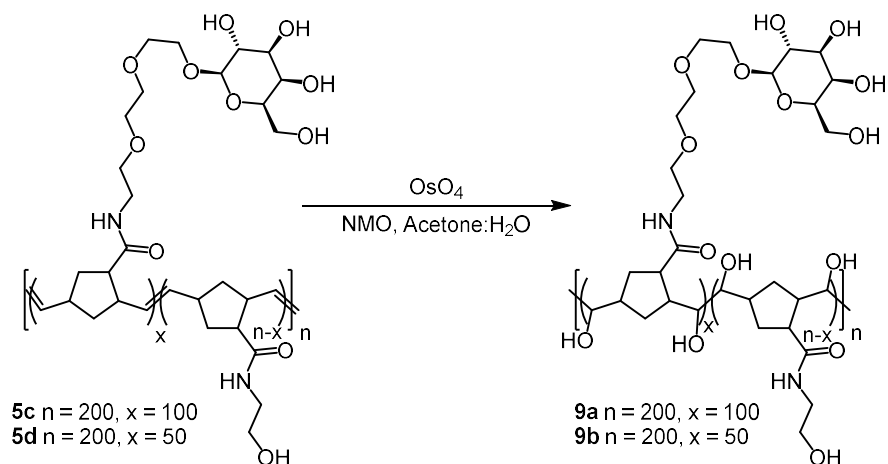


Polymers 5a-h and 8a-h: To a 6mL vial equipped with a stir bar add 40mg cis- or trans-poly(Norbornene-2-Succinimidyl Ester) 200mer or 500mer **3a**, **3b**, **7a**, or **7b** (200mer: 848nmol, 1 equiv.) (500mer: 340nmol, 1 equiv.). Prepare a solution of 7.7% (v/v) H₂O:DMSO containing 172μL/mL N-methylmorpholine (NMM). Using a portion of this solution, prepare a solution of 83.8mg/mL Galactoside **4**. Based on the desired extent of galactose incorporation, gel the polymer in the specified volume of NMM/aq. DMSO solution according to the table below, add the corresponding volume of Galactoside stock, and allow the reaction to proceed for 7 hours. The reaction maintains the light champagne color of Galactoside **4** throughout. After stirring for 7 hours, add 64μL Ethanolamine (200mer functionalization: 1.06mmol, 1250 equiv.) (500mer functionalization: 1.06mmol, 3100 equiv.) and allow the reaction to proceed overnight. After stirring overnight, transfer the reactant solution to a 13cm length of 10,000 molecular weight cutoff cellulose dialysis tubing and dialyze against 4L deionized water for 2 days with 4 water changes per day. On the final water change, ensure the dialysis water is pH = 7 by pH meter by titration with a few hundred microliters 100mM NaOH

or HCl. Pass the contents of the dialysis cassette over a 0.45 μ M nylon membrane filter and lyophilize. Products are fluffy white solids. The 50% and 25% functionalized materials are occasionally obtained in substantially higher functionalization percentages than implied by stoichiometry. This is thought to occur due to precipitation of polymer chains bearing low densities of galactoside **4** which are subsequently filtered resulting in a polymer solution rich in higher densities of galactoside **4**. The extent of galactose incorporation in polymers **5a**, **5b**, **5e**, **5f**, **5g**, **8a**, **8b**, **8c**, **8e**, **8f**, and **8g** is determined by ^1H NMR in D_2O where the vinyl protons at 5.17ppm are integrated for 400H or 1000H depending on DP and the integral of the anomeric signal at 4.33ppm is measured to determine extent of galactose functionalization. A representative NMR annotation of polymer **8c** in D_2O is provided below. The ^1H NMR spectra for polymers **5a**, **5b**, **5e**, **5f**, **5g**, **8a**, **8b**, **8c**, **8e**, **8f**, and **8g** are essentially identical save for the integrations of the anomeric signal at 4.33ppm and the PEG region from 3.91-3.43 ppm. Polymers **5c**, **5d**, **5h**, **8d**, and **8h** are not sufficiently soluble in D_2O to obtain reliable integrals and so are taken in MeOD with a few drops D_2O . For polymers **5c**, **5d**, **8d**, and **8h** the vinyl protons at 5.34ppm are integrated for 400H or 1000H depending on DP and the integral of the anomeric signal at 4.34ppm is measured to determine extent of functionalization with galactose. The ^1H NMR spectra for polymers **5c**, **5d**, **5h**, **8d**, and **8h** are essentially identical save for the integral of the anomeric signal at 4.34ppm and the PEG region from 3.96-3.46ppm. Polymer **8c** ^1H NMR (600 MHz, Deuterium Oxide, $\text{D}_1 = 10$) δ 5.17 (brm, 400H), 4.33 (apparent d, $J = 7.6$ Hz, 95H), 3.99 (m, 99H), 3.91 – 3.43 (brm, 1751H), 3.43 – 0.67 (brm, 2164H). Polymer **5d** ^1H NMR (600 MHz, Methanol- d_4 , $\text{D}_1 = 10$) δ 5.47 – 5.17 (brm, 400H), 4.34 (m, 50H), 4.07 (m, 45H), 3.90 (apparent s, 46H), 3.84 – 3.49 (brm, 796H), 3.33 (brm, 906H, overlap with solvent),

3.07 (brm, 275H), 2.71 (brm, 194H), 2.57 – 2.36 (brm, 201H), 1.94 (brm, 434H), 1.78 – 1.43 (brm, 309H), 1.39 – 1.01 (brm, 234H).

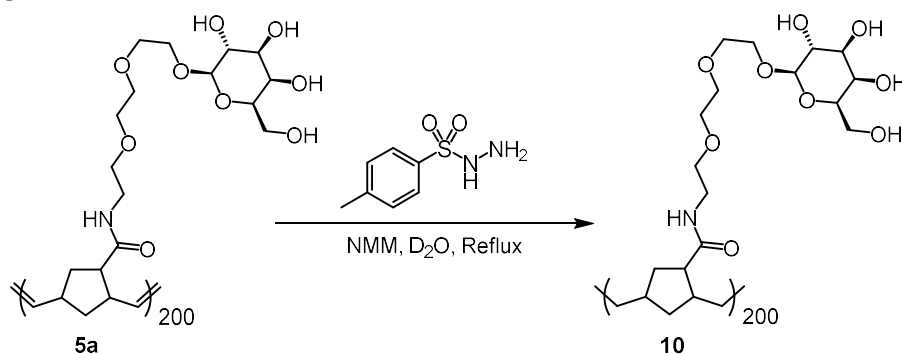
Experimental Details Table 2.1 Stock solution volumes, mmol of galactoside 3 and equivalents per polymer, and yields for galactose functionalization of succinimidyl ester modified poly(norbornene)s.							
Starting Polymer	Product	Volume NMM-H ₂ O:DMSO Stock (μL)	Volume 3 Stock (μL)	μmol 3	Equiv. 3 (200mer)	Equiv. 3 (500mer)	Yield
3a	5a	0	3140	757	892	2226	72%
3a	5b	0	531	128	151	376	70%
3a	5c	179	352	84.8	100	249	73%
3a	5d	355	176	42.4	50	125	68%
3b	5e	0	3140	757	892	2226	63%
3b	5f	0	531	128	151	376	65%
3b	5g	179	352	84.8	100	249	47%
3b	5h	355	176	42.4	50	125	65%
7a	8a	0	3140	757	892	2226	66%
7a	8b	0	531	128	151	376	53%
7a	8c	179	352	84.8	100	249	96%
7a	8d	355	176	42.4	50	125	69%
7b	8e	0	3140	757	892	2226	46%
7b	8f	0	531	128	151	376	63%
7b	8g	179	352	84.8	100	249	73%
7b	8h	355	176	42.4	50	125	71%



Polymers 9a and 9b: To a 24mL vial equipped with a stir bar add either 21.8mg polymer **5c** (355nmol, 1 equiv.) or 21.5mg polymer **5d** (440nmol, 1 equiv.), 29.1mg N-methylmorpholine-N-oxide (248 μmol , 700 equiv. with respect to **5c**) or 36.1mg N-methylmorpholine-N-oxide (308 μmol , 700 equiv. with respect to **5d**) respectively, and dissolve in 1.13mL or 1.4mL 50% vol./vol. Acetone:H₂O respectively. In a 24mL vial prepare a solution of 6.24mg/mL OsO_4 in 50% vol./vol. Acetone:H₂O and add 290 μL of the OsO_4 stock (7.12 μmol , 20 equiv.) to the reaction with **5c** or 359 μL of the OsO_4 stock (8.81 μmol , 20 equiv.) to the reaction with **5d** and allow the reaction to proceed overnight. Note that the reactant solution can drastically vary in appearance between reactions with identical results. This behavior is typical of OsO_4 dihydroxylations and should not be immediately taken as an indication of failure or success of the transformation. The reaction was in one case observed to remain colorless for 1-2 hours after OsO_4 addition and was a clear dusty brown color by morning. Another iteration gave large volumes of fluffy white globular precipitate rapidly on OsO_4 addition which took on a green-brown color while the supernatant remained colorless. By morning, this

precipitate had congealed to a clear, yellow, viscous gel which formed a ring around the stir bar's path. This precipitate was transferred to the dialysis tubing where it readily dissolved on dilution with H₂O and proved to be product. In yet another reaction the solution rapidly took on a light brown color and by morning there were small flakes of jet black precipitate. In each case near quantitative dihydroxylation was achieved. After stirring overnight, transfer the reactant solution to a 13cm length of 10,000 molecular weight cutoff dialysis tubing and dialyze against 4L H₂O for 2 days with 4 water changes per day ensuring neutral pH on the final exchange by titrating the dialysis water to pH = 7 by pH meter using a few hundred μ L 100mM NaOH or HCl. Pass the contents of the dialysis tube over a 0.45 μ m nylon membrane filter and lyophilize. Products are fluffy very faintly brown tinted solids which collapse to a brown gelatinous speck immediately on addition of water implying residual contamination with OsO₄ before rapidly dissolving. Average Yield: 71%. Minimum Yield: 68%. Maximum Yield 74%. Average Olefin Conversion: 95%. Minimum Olefin Conversion: 93%. Maximum Olefin Conversion: $\geq$ 95%. Polymer **9a** ¹H NMR (600 MHz, Deuterium Oxide, D₁ = 10) δ 4.35 (d, *J* = 7.9 Hz, 53.8H) 4.00 (m, 54H), 3.84 (apparent d, *J* = 3.4 Hz) 3.80 – 3.13 (brm, 2218zH), 2.43 (brm, 800H), 2.07 – 0.99 (brm, 1290H). Polymer **9b** ¹H NMR (600 MHz, Deuterium Oxide) δ 4.35 (apparent d, *J* = 7.9 Hz, 99H), 4.05 – 3.93 (m, 105H), 3.84 (m, 113H), 3.81 – 2.91 (brm, 2979H), 2.80 – 2.15 (brm, 800H), 1.53 (brm, 1548H).

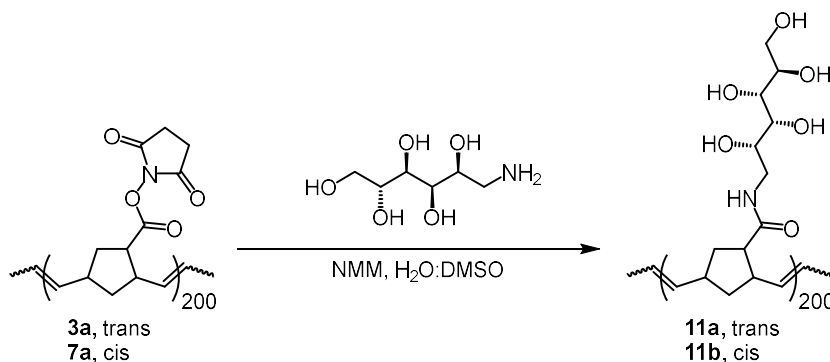
Polymer 10



Polymer 10: To a flame dried N₂ back-filled 5mL round bottom flask equipped with a reflux condenser add 9mg (104nmol, 1 equiv.) polymer **5a**, 38.8mg (208μmol, 2000 equiv.) para-toluenesulfonyl hydrazide, dissolve in 2.7mL D₂O (deuterated solvent is employed such that conversion may be checked after each reaction period), add 27.5μL (250μmol, 2400 equiv.) NMM, and allow the reaction to reflux overnight. After refluxing overnight, assess conversion by NMR setting the anomeric proton at 4.33ppm to 1H and integrating the olefin at 5.4-4.98ppm. Maximum conversion is approximately 85% olefin reduction under these conditions and is achieved by daily addition of 38.8mg (208μmol, 2000 equiv.) para-toluenesulfonyl hydrazide and 27.5μL (250μmol, 2400 equiv.) NMM followed by 12-24hr reflux period and conversion assessment by NMR. Generally, 4 total charges of hydrazide and base are required to reach maximum conversion. If NMM is omitted from the reaction it becomes non-reproducible and appears that tosylate addition across the alkene competes with olefin reduction rendering off-white precipitate in the reaction and dialysis step. Successful transformations remain clear and colorless throughout the reaction and purification. Once the reaction has completed by NMR, transfer the reactant solution to a 10,000 MWCO dialysis cassette and dialyze against 4L H₂O for 3 days with 4 water changes per

day ensuring neutral pH on the final exchange by titration with a few hundred μL 100mM NaOH or HCl. Pass the contents of the dialysis cassette over a 0.44 μm nylon membrane filter and lyophilize. Product is a fluffy white solid. Average Yield: 36%. Minimum Yield: 26%. Maximum Yield: 46%. Average Olefin Conversion: 81%. Minimum Olefin Conversion: 75%. Maximum Olefin Conversion: 86%. ^1H NMR (600 MHz, Deuterium Oxide, D₁ = 10) δ 5.53-4.90 (brs, 49H), 4.33 (apparent d, J = 7.7 Hz, 200H), 4.00 (m, 210H), 3.92 – 3.09 (m, 3611H), 3.04 – 0.50 (brm, 2217H).

Polymers 11a and 11b



Polymers 11a-b: To a 4mL vial equipped with a stir bar add 15mg trans- or cis- poly poly(Norbornene-2-Succinimidyl Ester) 200mer **3a** or **7a**, respectively (320 nmol, 1 equiv.) Prepare a solution of 7.7% (v/v) H₂O:DMSO and add 2.6 mL (0.025M) to dissolve polymers. Add 58mg of D-glucamine (5 equiv.), inject 175 μL NMM (25 equiv)m and allow the reaction to stir overnight at room temperature. After stirring transfer to a 2,000 molecular weight cutoff cellulose membrane dialysis tubing and dialyze against 4L deionized water for 2 days with at least 3 exchanges of water per day. Filter the dialysis membrane contents through a 0.45 μm nylon membrane filter and lyophilize. Product is an off-white solid and functionalization density is determined by ^1H NMR in

D₂O where the vinyl protons at 5.44ppm are integrated for 400H and compared to the triplet integration at 2.54ppm. Polymer **11a** ¹H NMR (600 MHz, D₂O, D1=5) δ 5.43 (brn, 400H), δ 3.84-3.66 (brn, 1400H), δ 3.37 (brn, 200H), δ 3.09 (brn 350H), δ 2.54 (brn, 400H), δ 2.03 (brn, 350H), δ 1.70 (brn 200H), δ 1.22 (brn 200H).

Fluorescence Inhibition Assay – Cholera Toxin B Subunit FITC Conjugate

The fluorescence inhibition assay described below is based on that reported in the cited reference.¹⁴⁴ Here a model assay featuring polymers **7e** (trans-100%-Galactose-500mer), **7b** (trans-75%-Galactose-200mer), and **10b** (cis-75%-Galactose-200mer) is described. To adapt the procedure to the other materials in this study, only the polymer dilution series need be altered based on the maximum solubility of the polymer in question.

Cholera Toxin B Subunit FITC Conjugate Stock Solution Preparation

Dissolve a 500μg aliquot of cholera toxin B subunit FITC conjugate (ctx) in 8mL HEPES to give a 62.5 μg/mL stock solution and store in an opaque container to protect from light at room temperature. The manufacturer claims the material is stable in solution for up to 3 days at room temperature.

Polymer Dilution Series

Dissolve 2x 3mg aliquots of polymer **7e** (13.9nmol each, 6.95μmol with respect to galactose each) in 369μL HEPES each to give an 18.75mM stock solution with respect to galactose. Dissolve 2x 3mg aliquots of polymer **7b** (40.6nmol each, 6.09μmol with

respect to galactose each) in 323 μ L HEPES each to give an 18.75mM stock solution with respect to galactose. Dissolve 2x 3mg aliquots of polymer **10b** (40.6nmol each, 6.09 μ mol with respect to galactose each) in 323 μ L HEPES each to give an 18.75mM stock solution with respect to galactose. Prepare the dilution series according to the tables below.

Experimental Details Table 2.2 Polymer 5a dilution series				
Solution	Concentration with respect to galactose in stock	Concentration with respect to galactose in assay	Solution and volume to be diluted	Volume HEPES used to dilute
1	18.75mM	15mM	738 μ L – 1	0 μ L
2	15mM	12mM	160 μ L – 1	40 μ L
3	11.3mM	9mM	120 μ L – 1	80 μ L
4	7.53mM	6mM	80 μ L – 1	120 μ L
5	3.77mM	3mM	80 μ L – 1	320 μ L
6	1.88mM	1.5mM	200 μ L – 5	200 μ L
7	942 μ M	750 μ M	200 μ L – 6	200 μ L
8	471 μ M	375 μ M	200 μ L – 7	200 μ L
9	235 μ M	188 μ M	200 μ L – 8	200 μ L
10	118 μ M	94 μ M	200 μ L – 9	200 μ L
11	58.8 μ M	47 μ M	200 μ L – 10	200 μ L
12	29.4 μ M	23.5 μ M	200 μ L – 11	200 μ L

Experimental Details Table 2.3 Polymer 5e Dilution Series				
Solution	Concentration with respect to galactose in stock	Concentration with respect to galactose in assay	Solution and volume to be diluted	Volume HEPES used to dilute
1	18.75mM	15mM	738μL – 1	0μL
2	15mM	12mM	160μL – 1	40μL
3	11.3mM	9mM	120μL – 1	80μL
4	7.53mM	6mM	80μL – 1	120μL
5	3.77mM	3mM	80μL – 1	320μL
6	1.88mM	1.5mM	200μL – 5	200μL
7	942μM	750μM	200μL – 6	200μL
8	471μM	375μM	200μL – 7	200μL
9	235μM	188μM	200μL – 8	200μL
10	118μM	94μM	200μL – 9	200μL
11	58.8μM	47μM	200μL – 10	200μL
12	29.4μM	23.5μM	200μL – 11	200μL

Experimental Details Table 2.4 Polymer 8b Dilution Series				
Solution	Concentration with respect to galactose in stock	Concentration with respect to galactose in assay	Solution and - volume to be diluted	Volume HEPES used to dilute
1	18.75mM	15mM	738μL - 1	0μL
2	15mM	12mM	160μL - 1	40μL
3	11.3mM	9mM	120μL - 1	80μL
4	7.53mM	6mM	80μL - 1	120μL
5	3.77mM	3mM	80μL - 1	320μL
6	1.88mM	1.5mM	200μL - 5	200μL
7	942μM	750μM	200μL - 6	200μL
8	471μM	375μM	200μL - 7	200μL
9	235μM	188μM	200μL - 8	200μL
10	118μM	94μM	200μL - 9	200μL
11	58.8μM	47μM	200μL - 10	200μL
12	29.4μM	23.5μM	200μL - 11	200μL

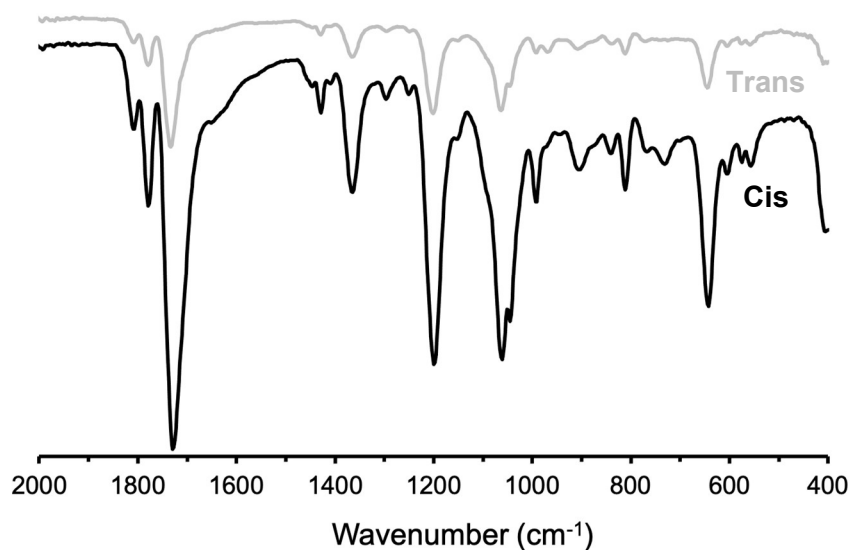
Plate Preparation and Inhibition Assay

Prepare at least 20mL of a 1mg/mL solution of GM1 ganglioside in PBS, add 50μL of the GM1 solution to each well of a Corning 3577 384-well, high binding, flat bottom, flat black plate, and place on a shaker overnight. After shaking overnight, decant and rinse each well with 3x 50μL portions PBS. Prepare at least 40mL of a 1 wt.% solution of bovine serum albumin in PBS, add 100μL to each well, place the plate on a shaker for 1 hour, decant, and wash each well with 1x 100μL portion PBS. To a 200μL aliquot of each

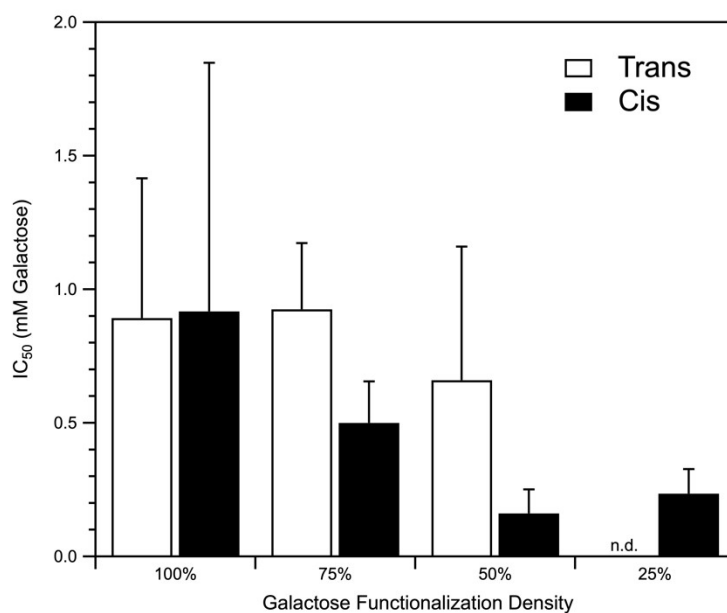
polymer dilution add 50µL of the 62.5µg/mL ctx stock, cover with foil, and gently shake on a shaker for 2 hours. After shaking, distribute 50µL of each polymer dilution + ctx stock into the desired wells and incubate the plate at 37°C for 30 minutes. After incubation, decant the plate and rinse each well with 3x 50µL portions HEPES. Analyze fluorescence on a TECAN M1000 plate reader with excitation at 485nm, emission at 528nm, and a standardized gain of 247.

Experimental Details Table 2.5 Solubility data for cis- and trans- galactose functionalized poly(Norbornene) 200mers and 500mers and cis- and trans-glucamine functionalized poly(Norbornene) 200mers. All concentration values are reported on a per galactose basis.		
% functionalization, Sugar, and Polymer Length	Cis Solubility (mM with respect to Galactose)	Trans Solubility (mM with respect to Galactose)
100%-Gal-200mer	280	55
75%-Gal-200mer	125	41
50%-Gal-200mer	100	6
25%-Gal-200mer	65	0.7
100%-Gal-500mer	95	31
75%-Gal-500mer	8	7.5
50%-Gal-500mer	8	7.5
25%-Gal-500mer	0.65	<0.00006
100%-GlcNH ₂ -200mer	≥200	1.6

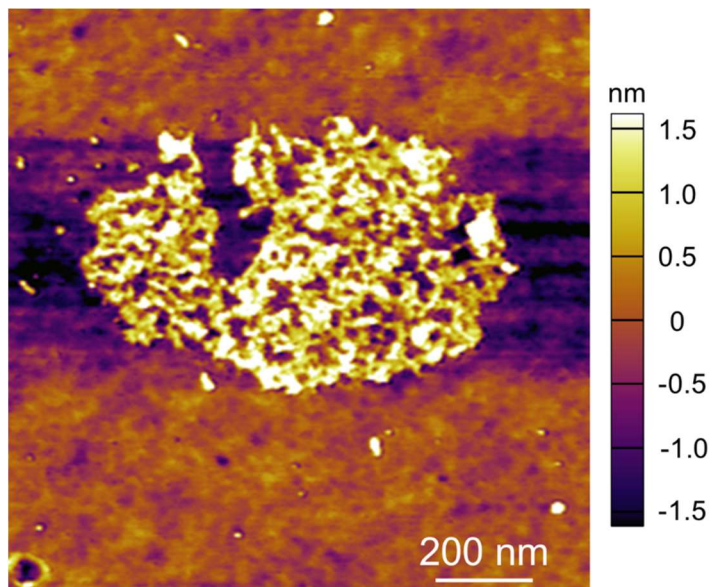
Experimental Details Table 2.6 Solubility data for dihydroxylated and hydrogenated galactose functionalized poly(Norbornene) 200mers. All concentration values are reported on a per galactose basis.	
Backbone Modification, % Functionalization, Sugar, and Polymer Length	Solubility (mM with respect to Galactose)
Tetrahydro-100%-Gal-200mer	45
Dihydroxy-50%-Gal-200mer	88
Dihydroxy-25%-Gal-200mer	54



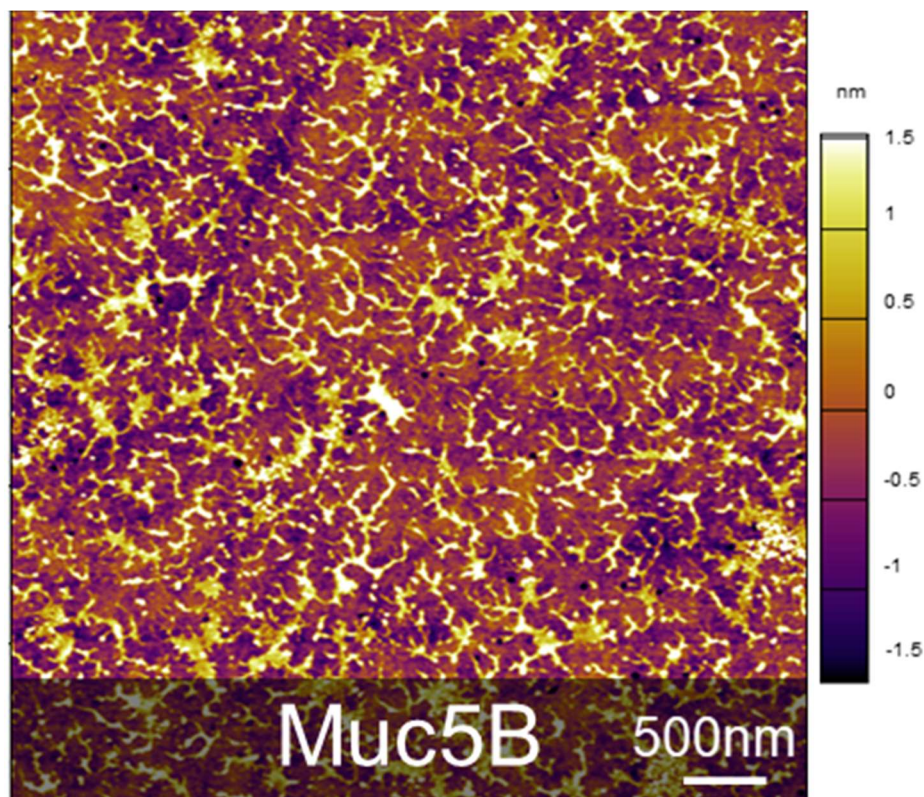
Experimental Details Figure 2.1 Fourier transform-infrared spectroscopy of cis- (black) and trans-(gray) 50% galactose-functionalized 200mers. The cis polymer exhibits a shoulder at $\sim 1650\text{ cm}^{-1}$ and a signal at $\sim 733\text{ cm}^{-1}$ characteristic of cis olefins which are absent in the trans spectrum.



Experimental Details Figure 2.2 Binding activity of cis- and trans- galactose-functionalized 500mers against Ctx. Polymers are listed by their percent galactose functionalization. Activity is shown as the half-maximal inhibitory concentration (IC₅₀) based on galactose concentration. N.d. represents compounds with an IC₅₀ greater than their maximum solubility. Error bars represent the standard deviation of biological replicates.



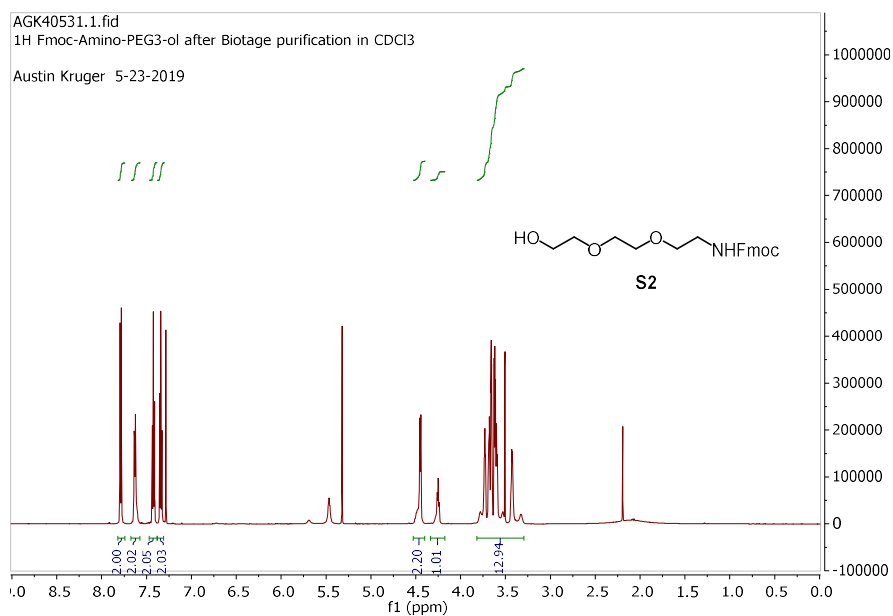
Experimental Details Figure 2.3 Atomic force micrograph of 50% galactose functionalized cis-(poly norbornene) 200mer forming intertangled network of polymer chains.



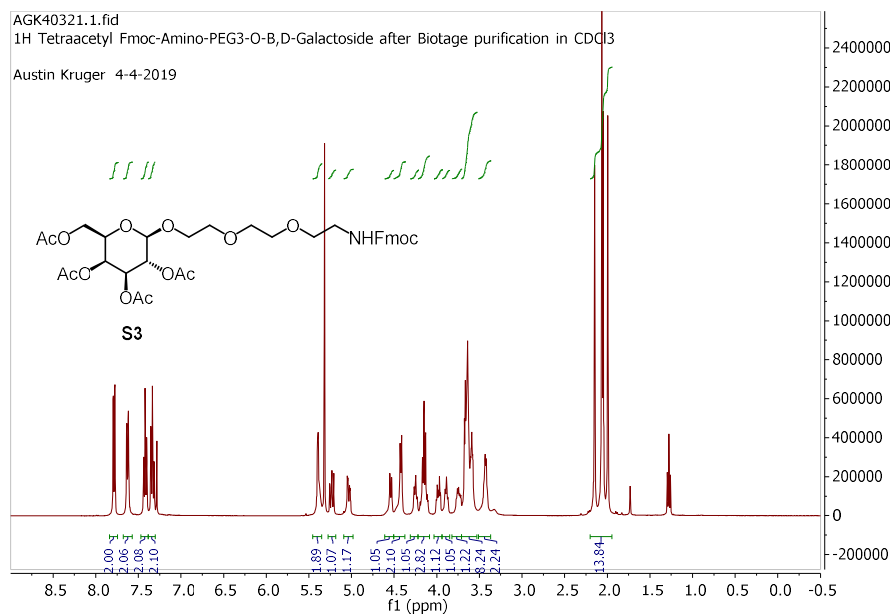
Experimental Details Figure 2.4. Atomic force micrograph of porcine Muc5B showing compact structures observed in some locations on the silicon chip.

^1H NMR Spectra

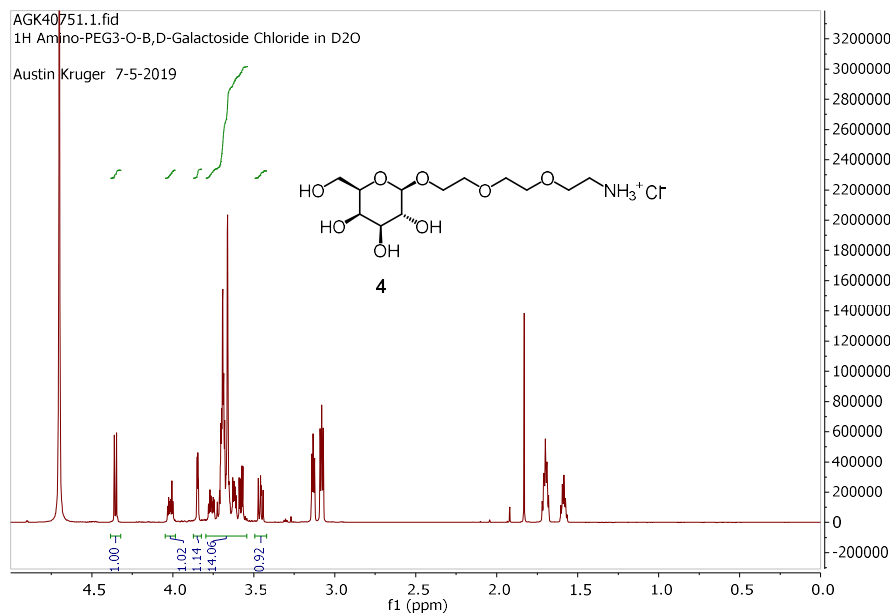
Compound S2



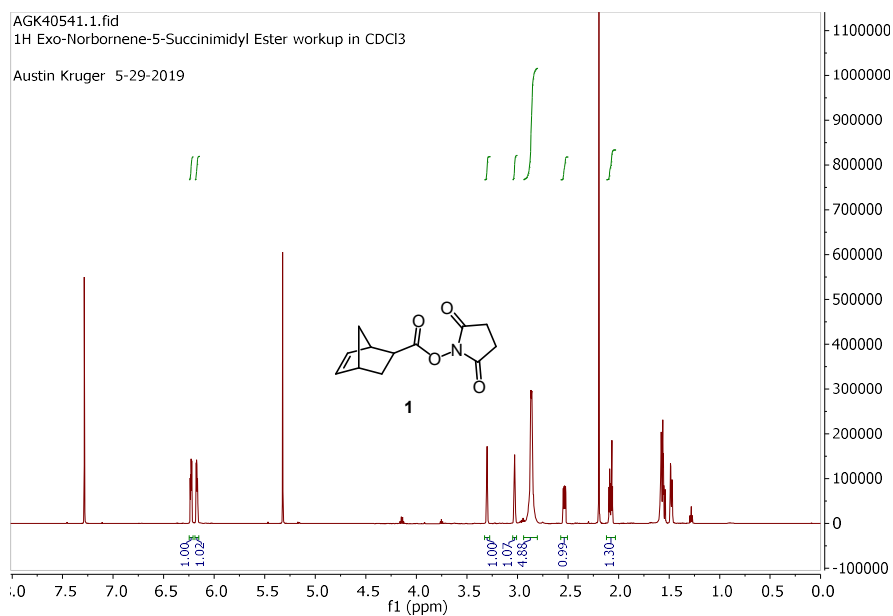
Compound S3



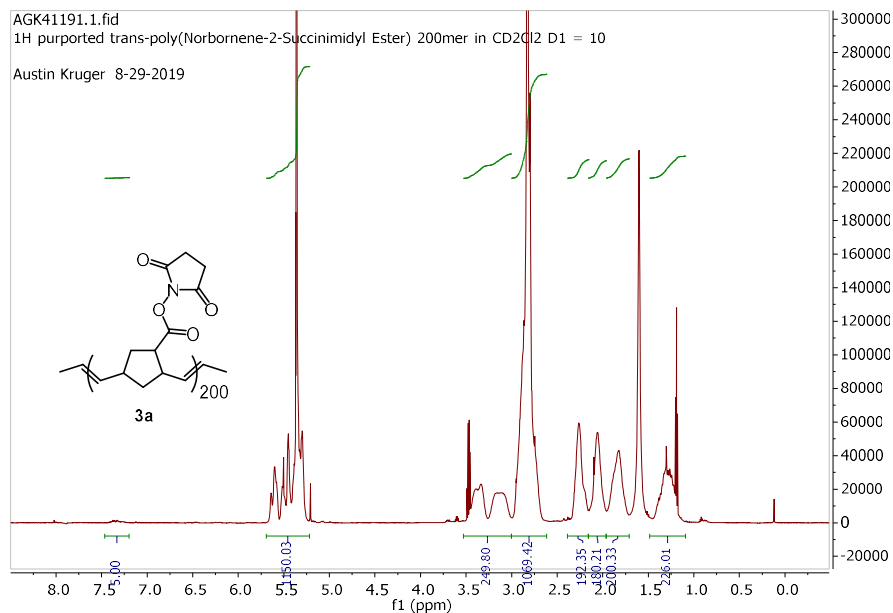
Compound 4



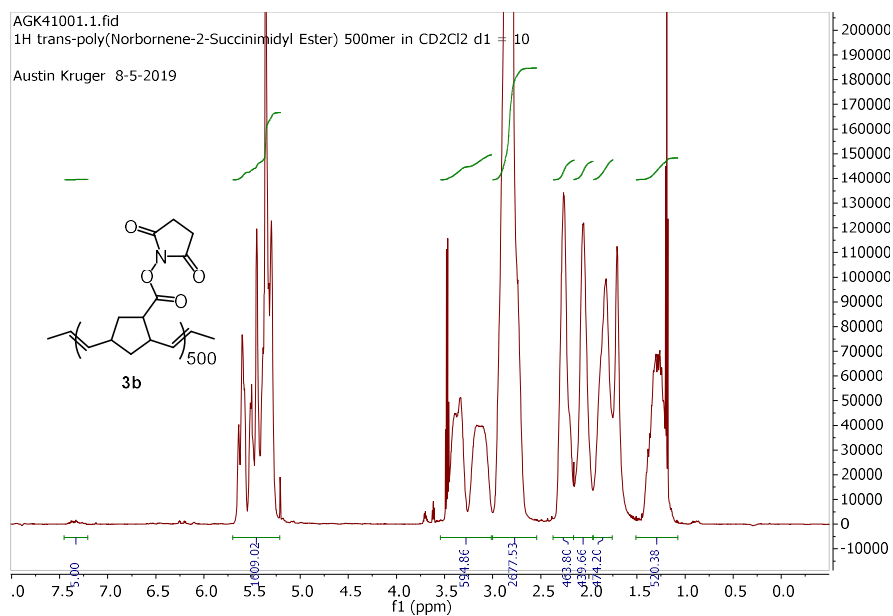
Compound 1



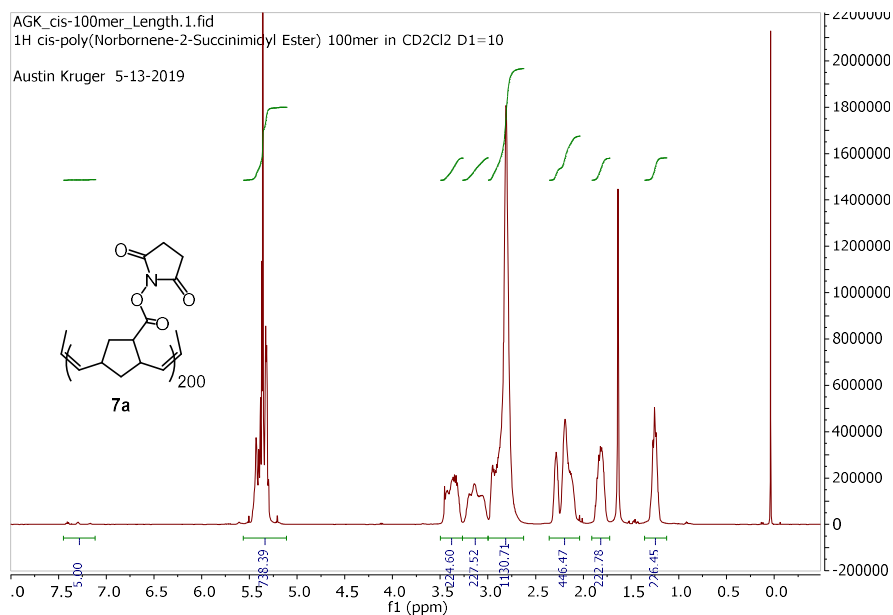
Polymer 3a



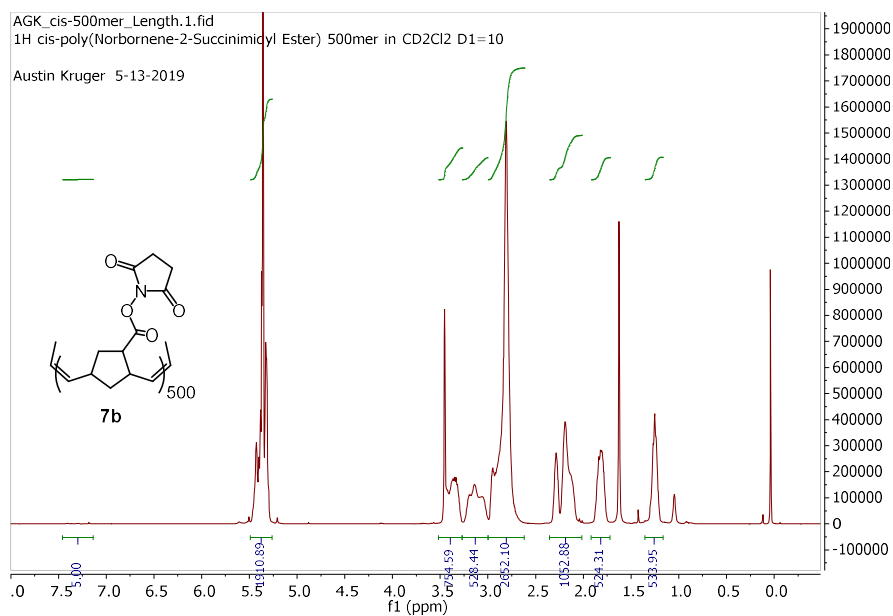
Polymer 3b



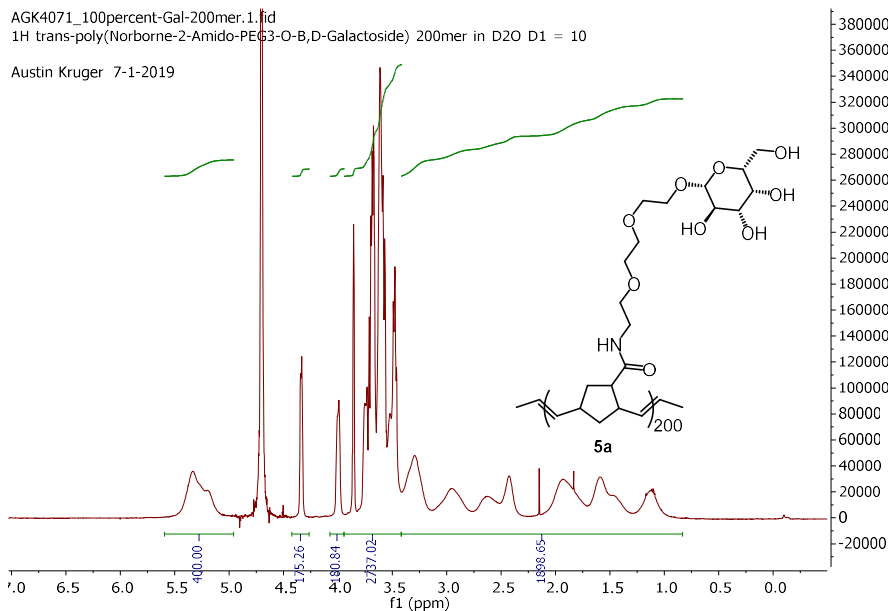
Polymer 7a



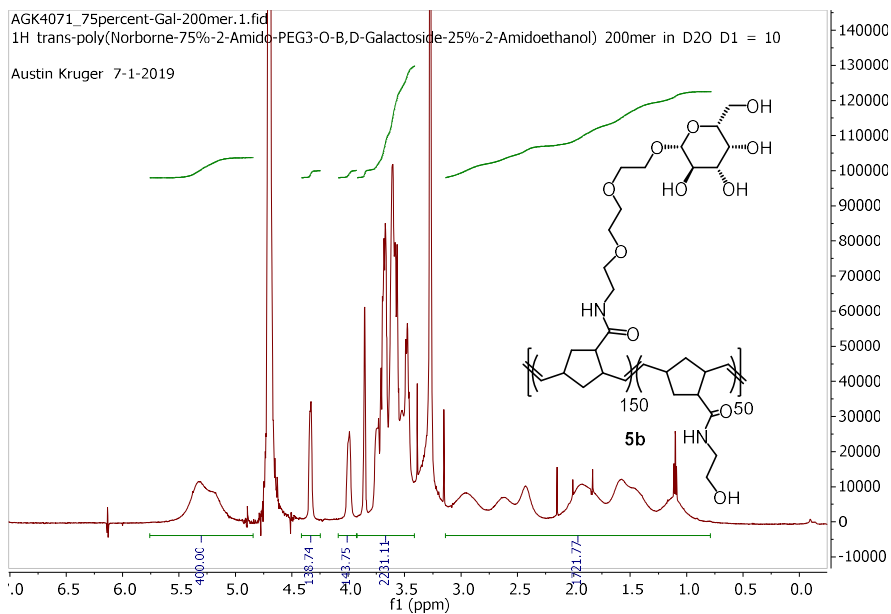
Polymer 7b



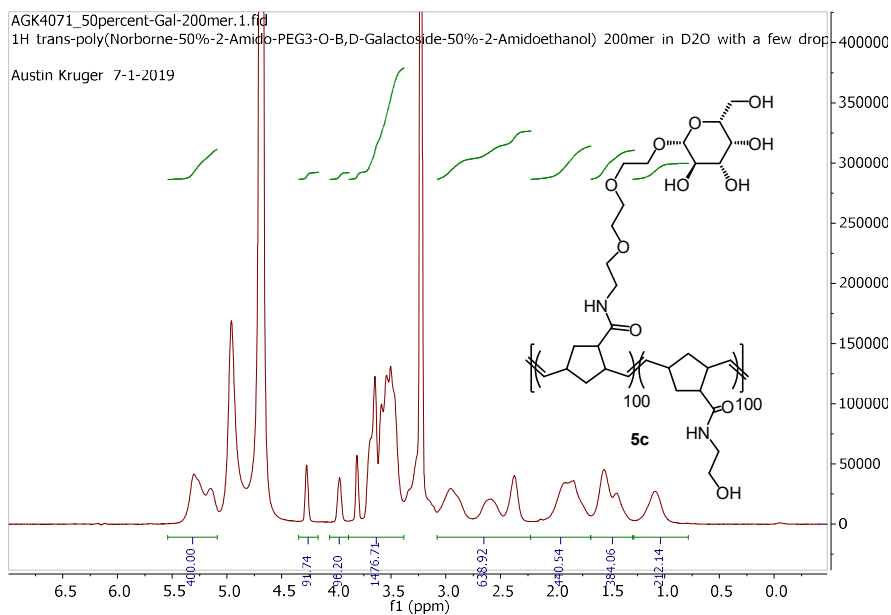
Polymer 5a



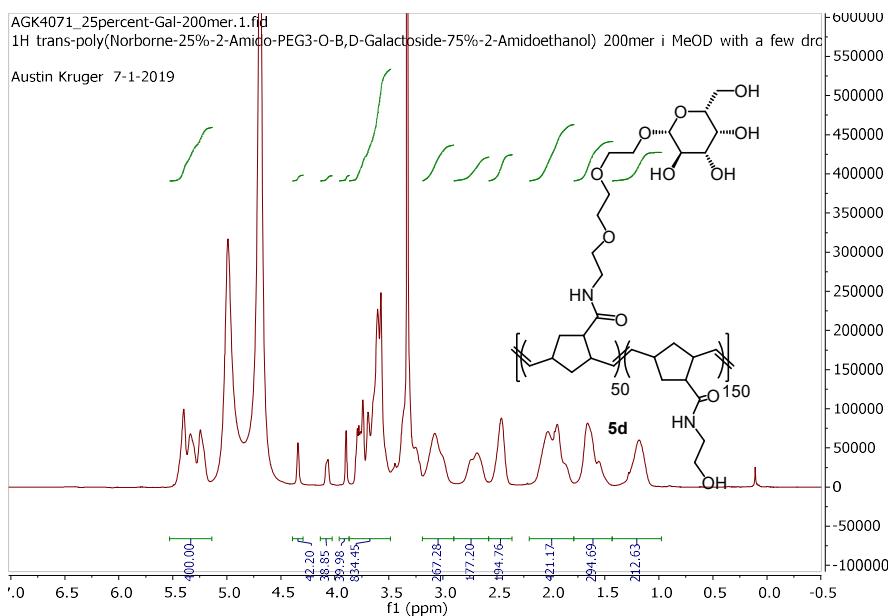
Polymer 5b



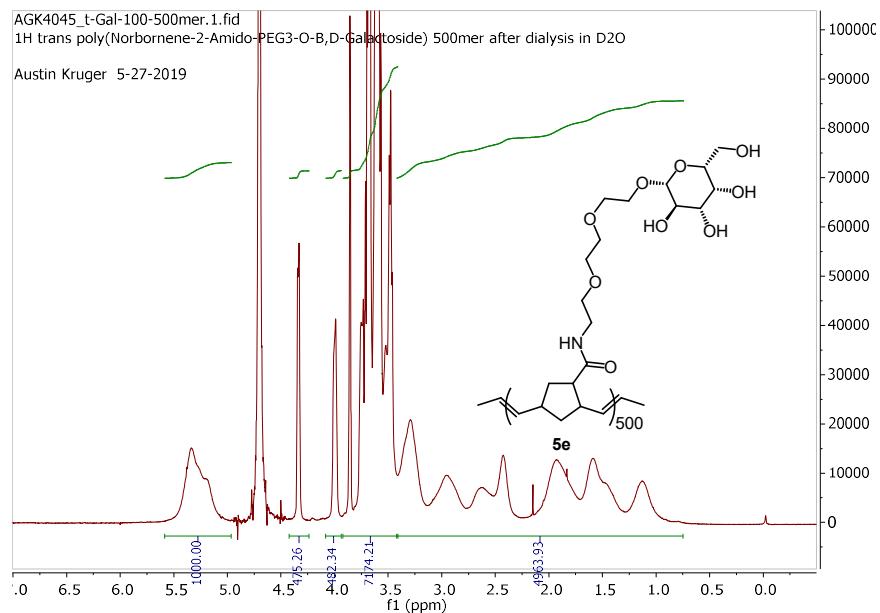
Polymer 5c



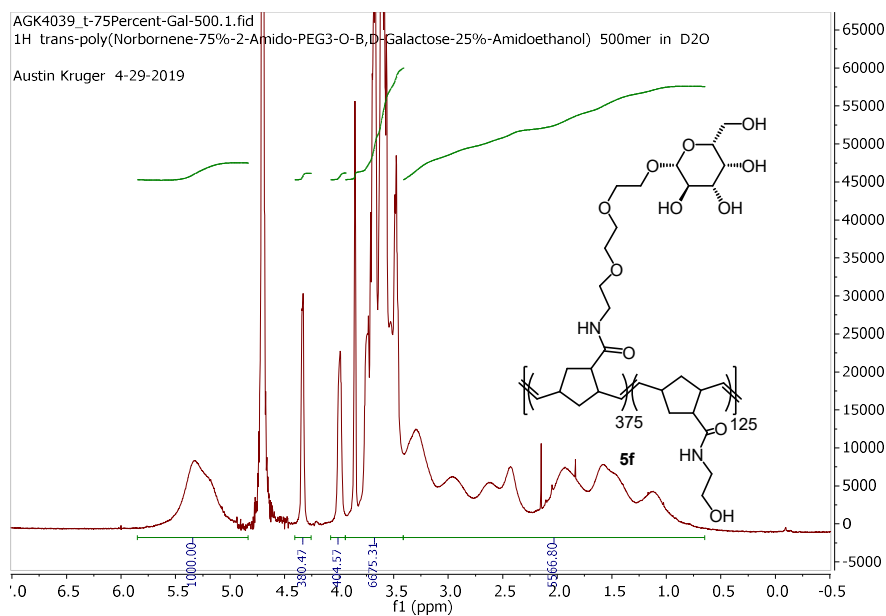
Polymer 5d



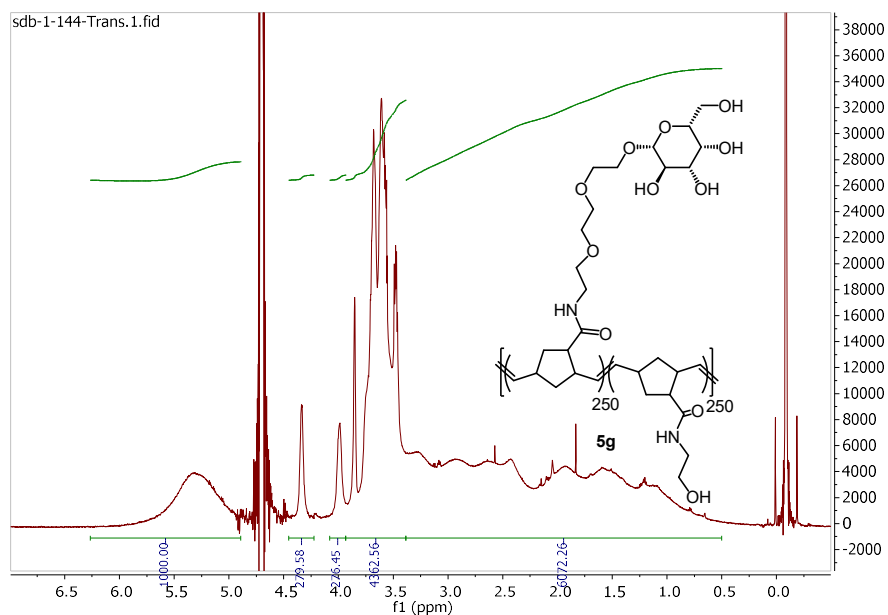
Polymer 5e



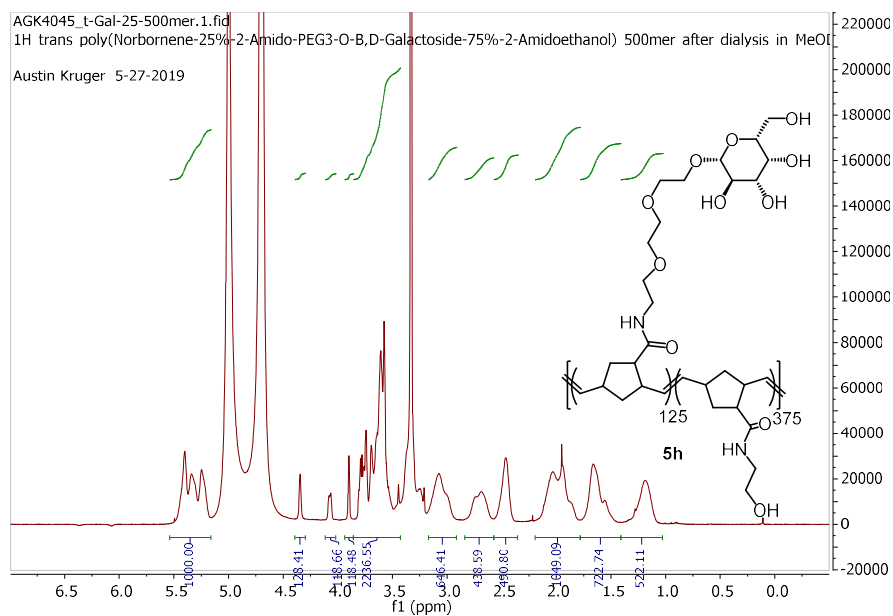
Polymer 5f



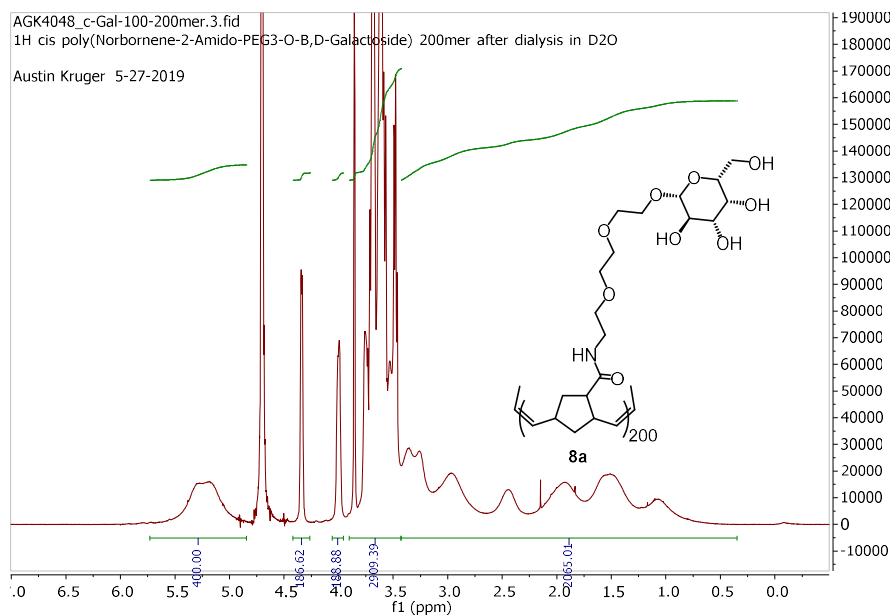
Polymer 5g



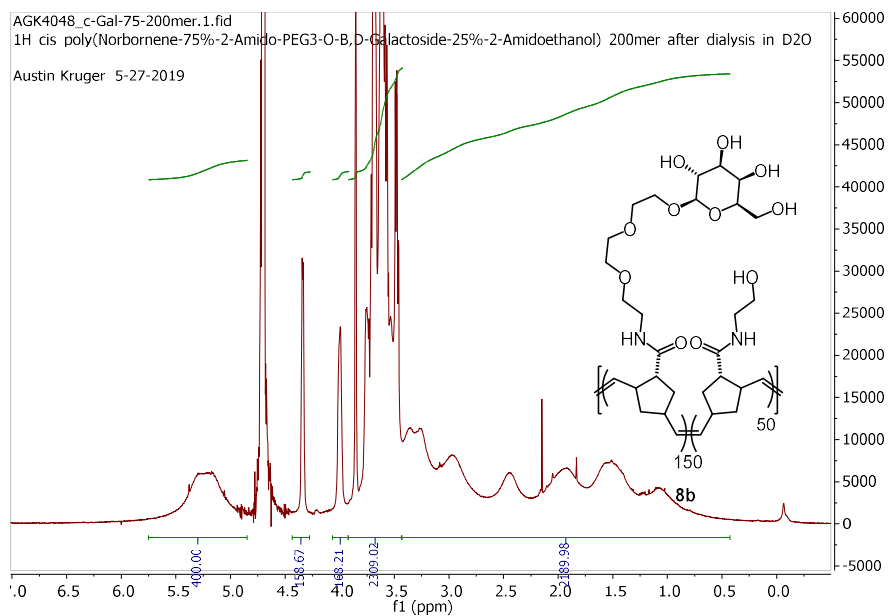
Polymer 5h



Polymer 8a



Polymer 8b



Polymer 8c

AGK41211_c-50p-Gal-200mer.2.fid
1H cis-poly(Norbornene-50%-2-Amido-PEG3-O-B,D-Galactoside-50%-2-Amidoethanol) 200mer
in D2O D1 = 10
Austin Kruger 9-3-2019

Chemical structure of the polymer repeat unit (8c) is shown, featuring a norbornene backbone with amide linkages to a PEG3 chain, which is further linked to a B,D-galactoside unit and an amidoethanol group.

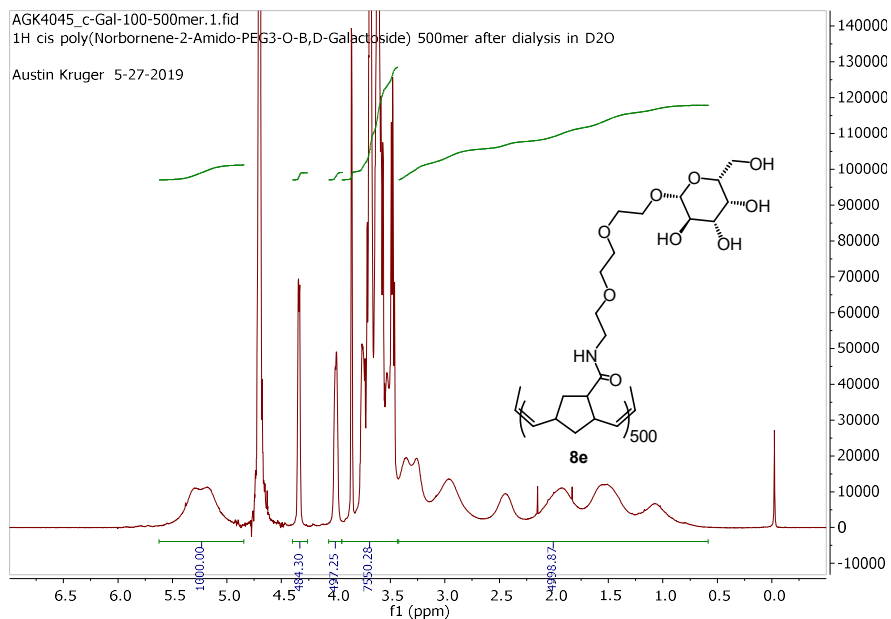
Polymer 8d

AGK4048_c-Gal-25-200mer.1.fid
1H cis poly(Norbornene-25%-2-Amido-PEG3-O-B,D-Galactoside-75%-2-Amidoethanol) 200mer after dialysis in D2O
Austin Kruger 5-27-2019

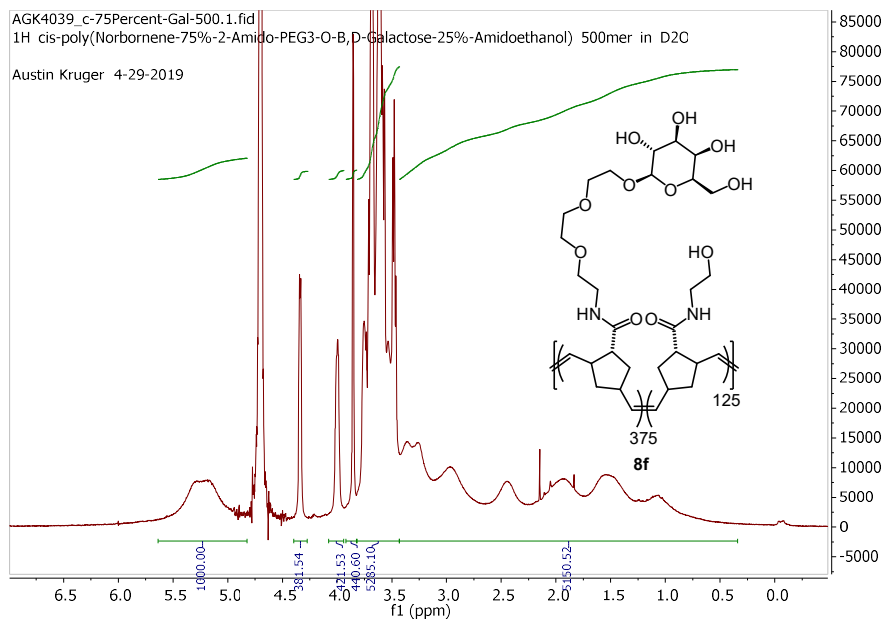
Chemical structure of the repeating unit of Polymer 8d is shown, featuring a Norbornene backbone with a 2-amido-PEG3-O-B,D-Galactoside-2-amidoethanol side chain. The structure is labeled 8d.

Integration values (from left to right): 359.77, 44.42, 36.38, 38.08, 766.87, 288.76, 325.38, 284.17, 227.47, 200.00.

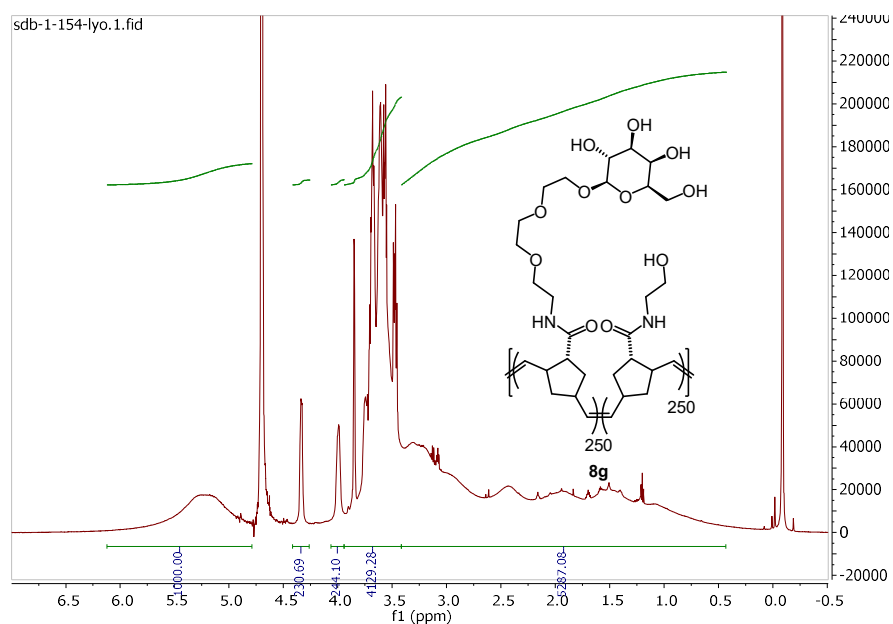
Polymer 8e



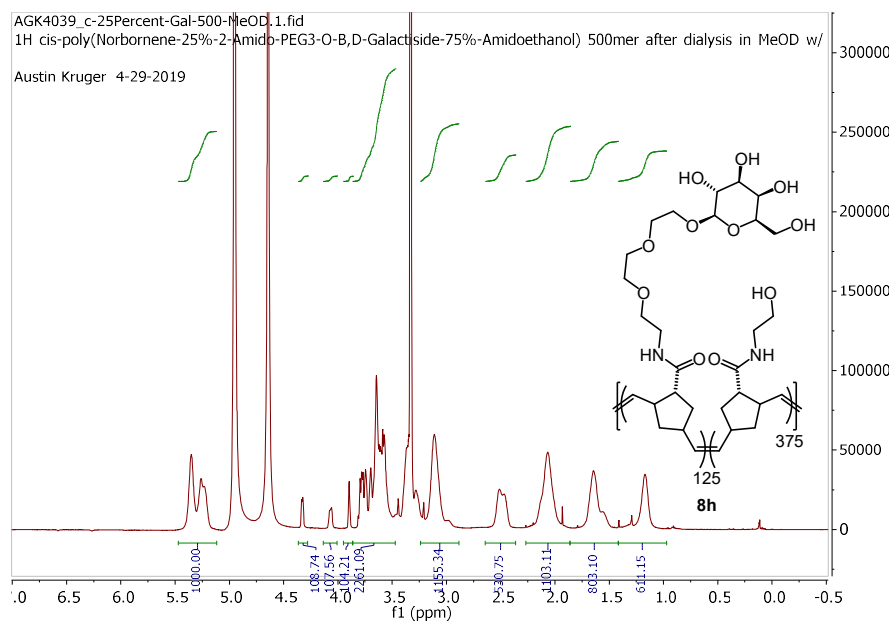
Polymer 8f



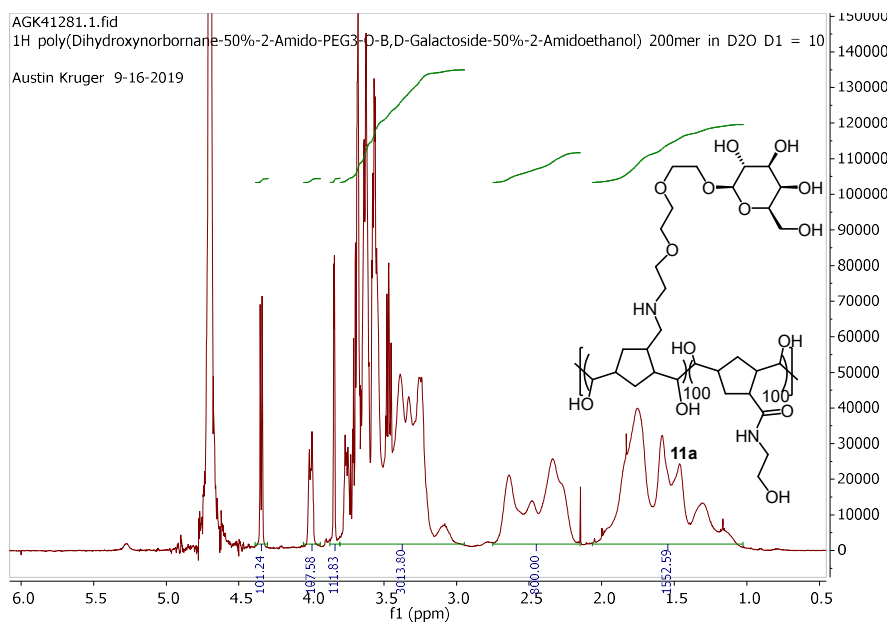
Polymer 8g



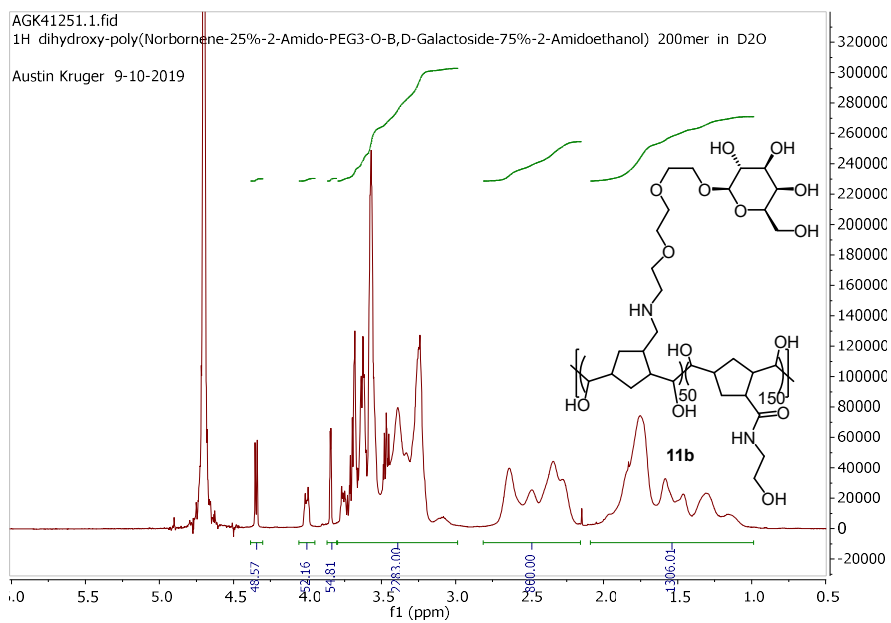
Polymer 8h



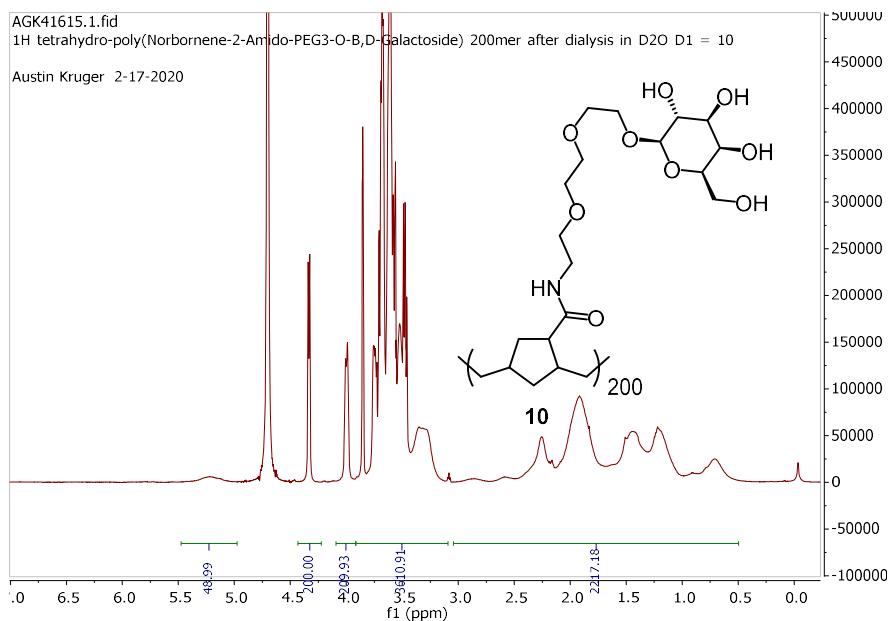
Polymer 9a



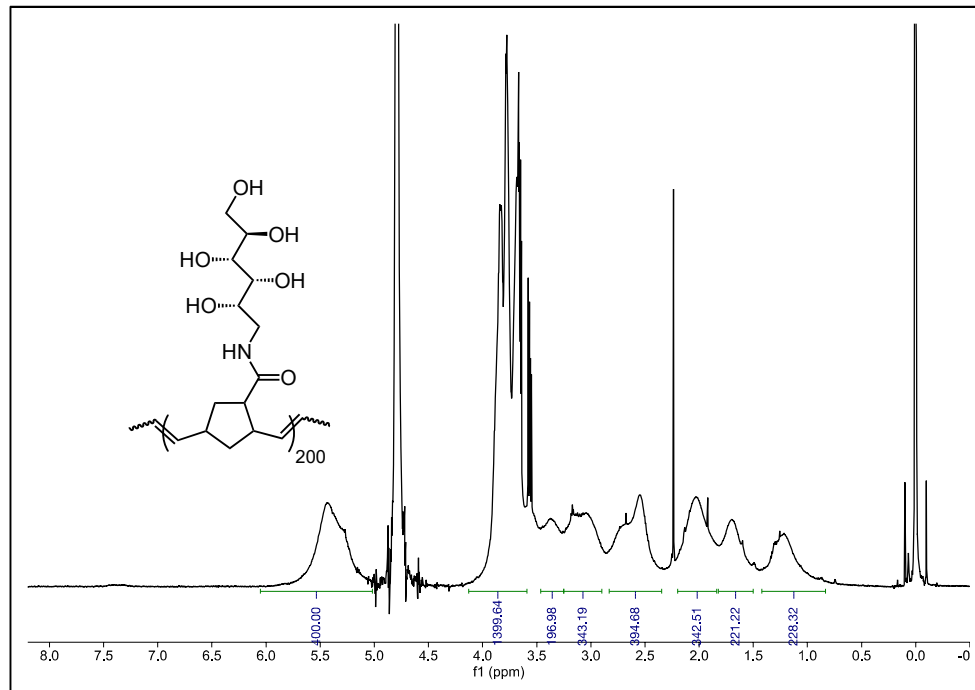
Polymer 9b



Polymer 10

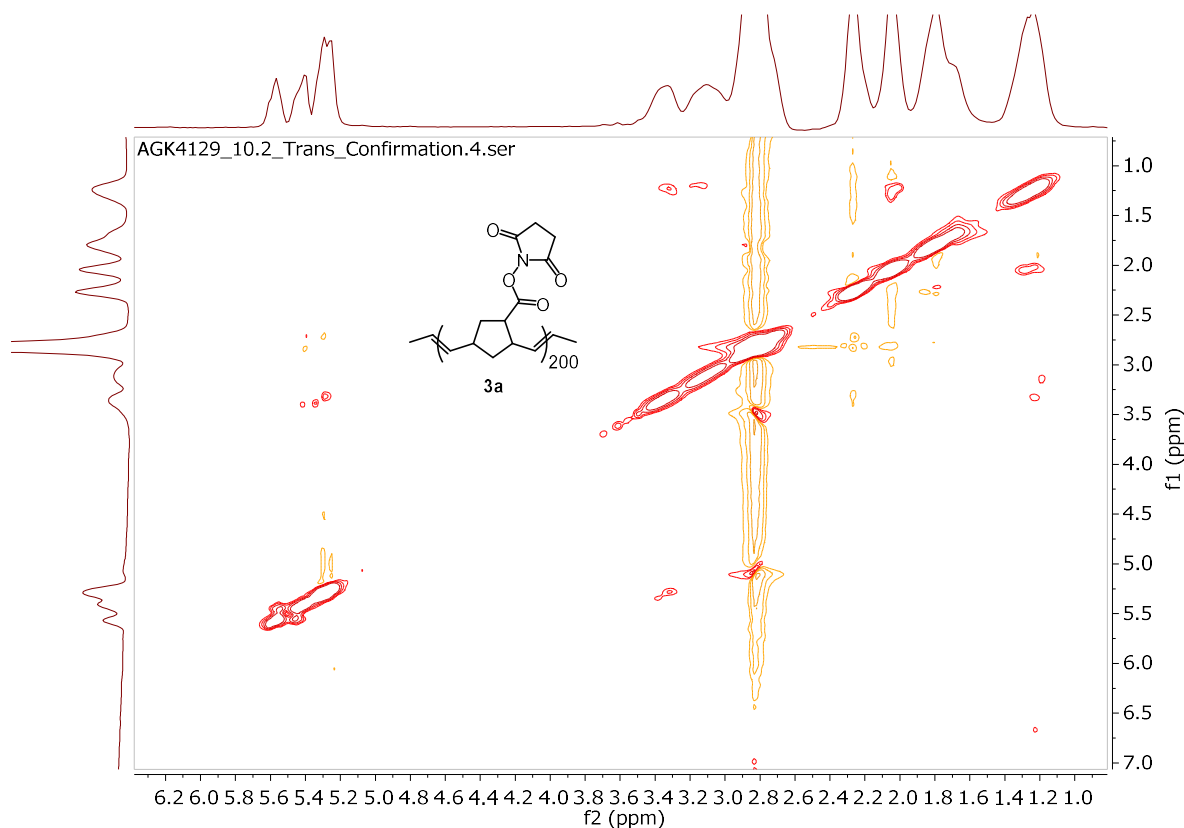


Polymer 11a

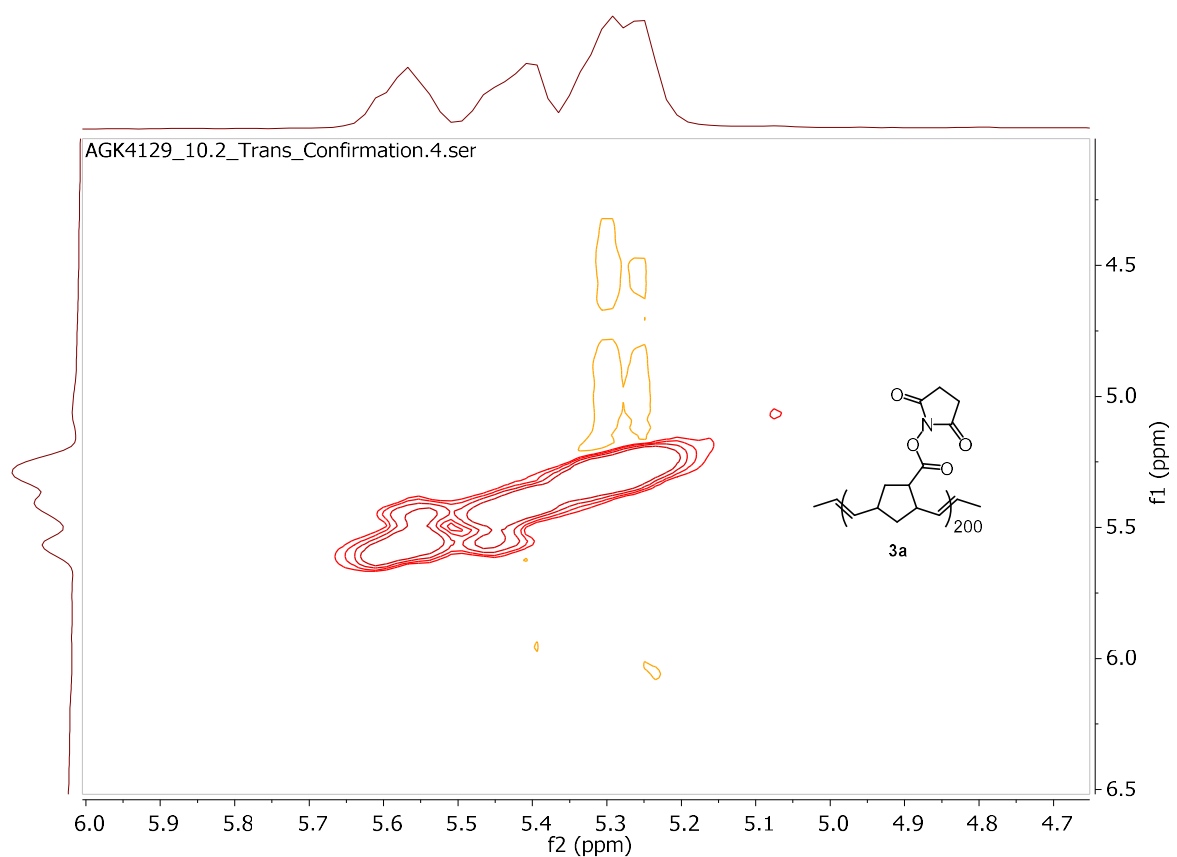


2D NMR Spectra

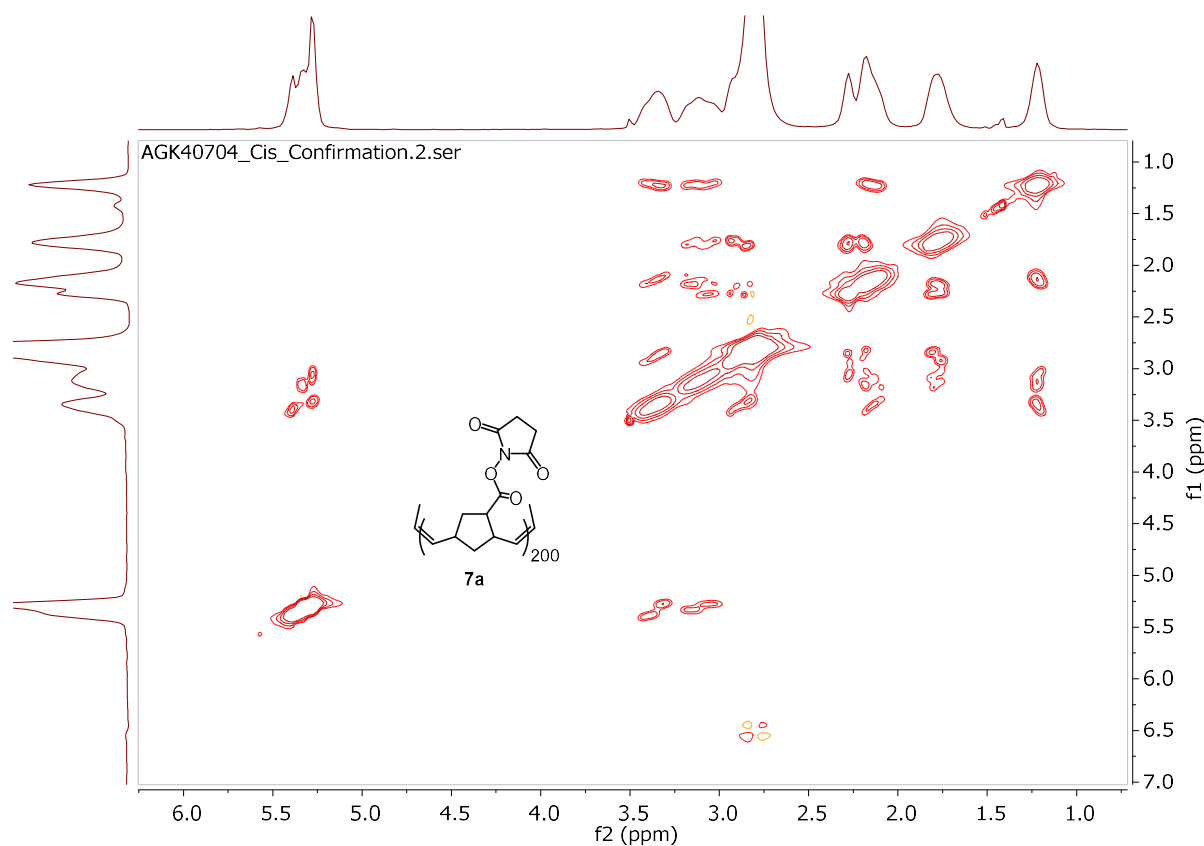
Polymer 3a TOCSY spectrum. The spectrum was acquired with a mixing time of ~7msec and shows only cross-peaks between spins actually coupled to one another. In this experiment, the magnitude of cross-peaks between spins is proportional to the magnitude of coupling between them. It can be concluded that polymer **3a** contains an abundance of trans-alkenes due to the strong cross-peak in the vinyl region (zoom-in on following page).



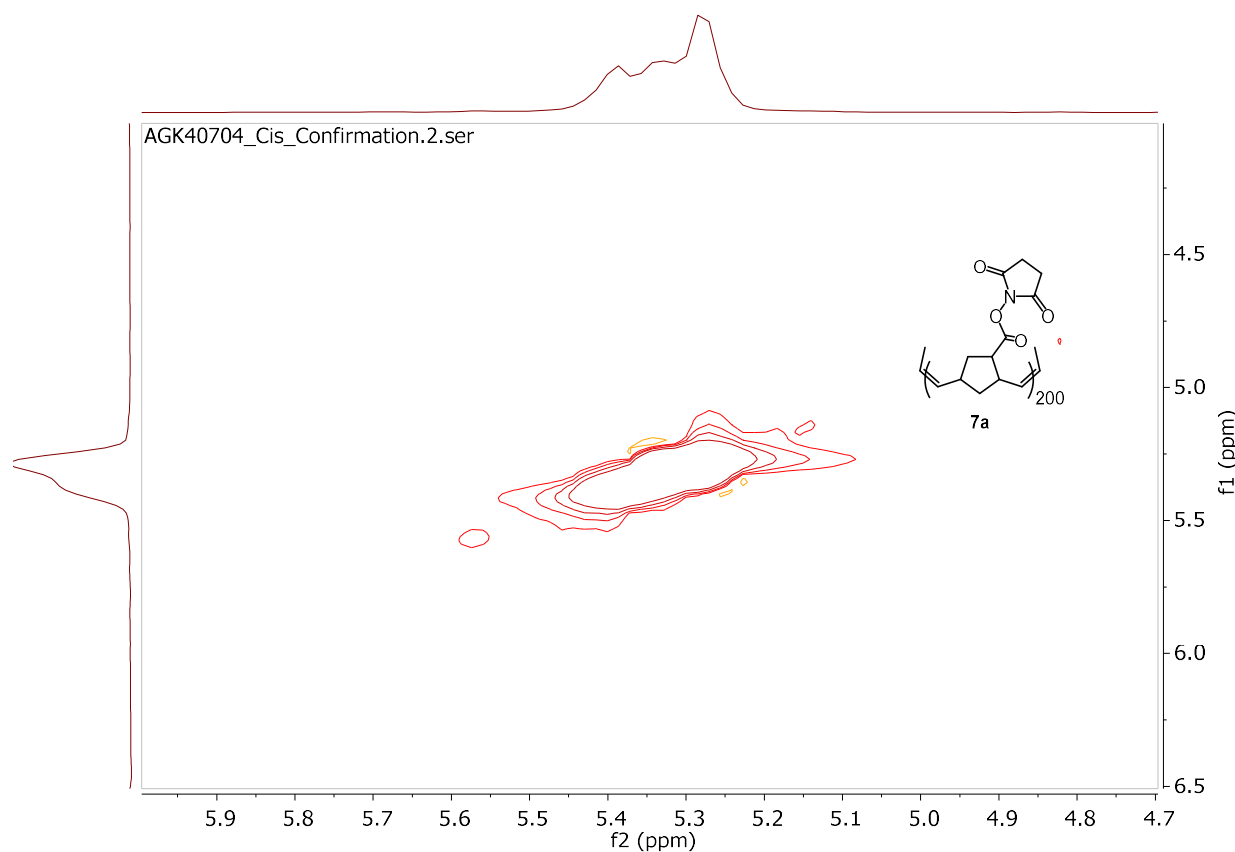
Polymer 3a TOCSY spectrum showing the vinyl region. The spectrum was acquired with a mixing time of ~7msec and shows only cross-peaks between spins actually coupled to one another. In this experiment, the magnitude of cross-peaks between spins is proportional to the magnitude of coupling between them. It can be concluded that polymer **3a** contains an abundance of trans-alkenes due to the strong cross-peak in the vinyl region.



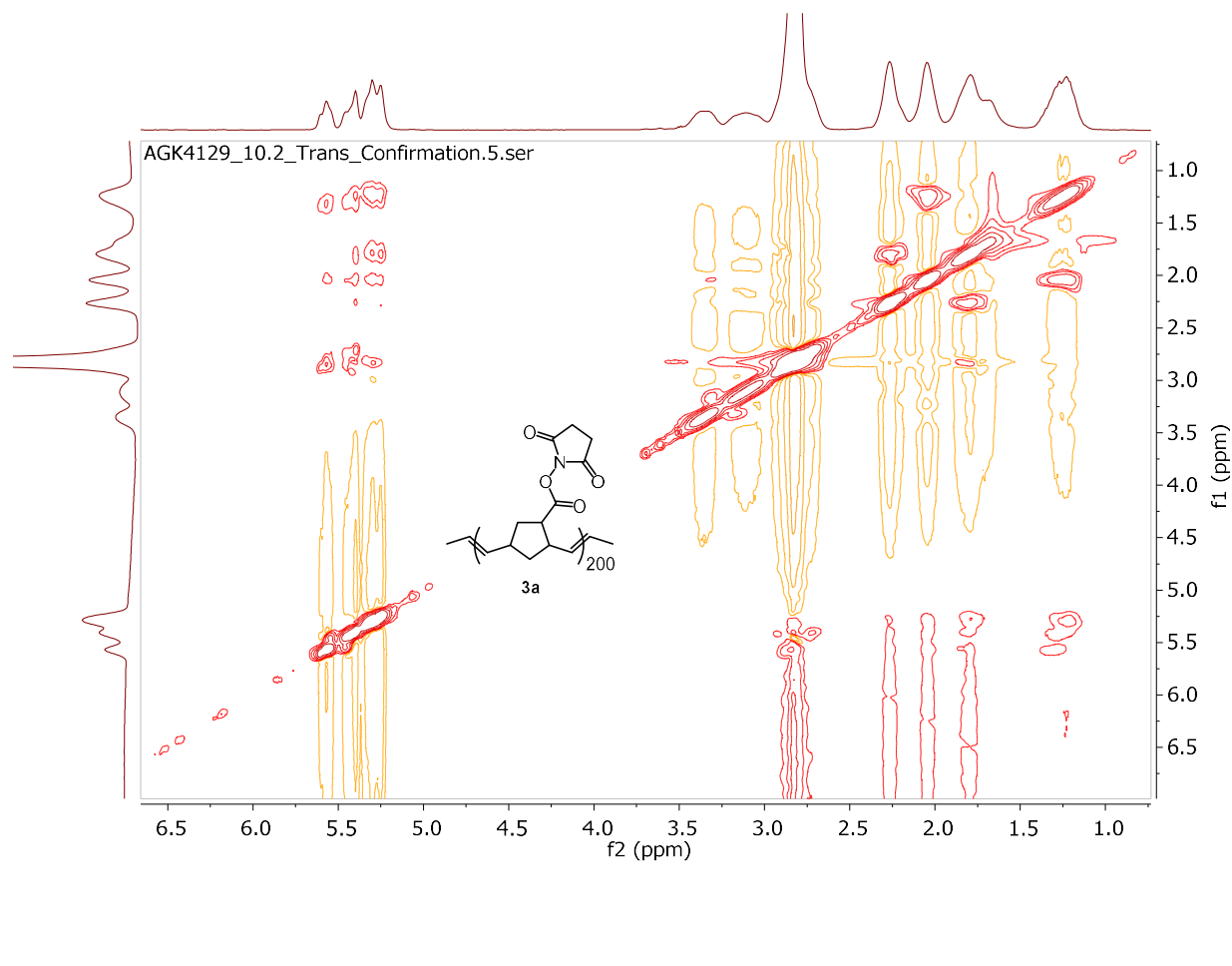
Polymer 7a TOCSY spectrum. The spectrum was acquired with a mixing time of ~7msec and shows only cross-peaks between spins actually coupled to one another. In this experiment, the magnitude of cross-peaks between spins is proportional to the magnitude of coupling between them. It can be concluded that polymer **7a** contains an abundance of cis-alkenes due to the lack of cross-peaks in the vinyl region (see following page).



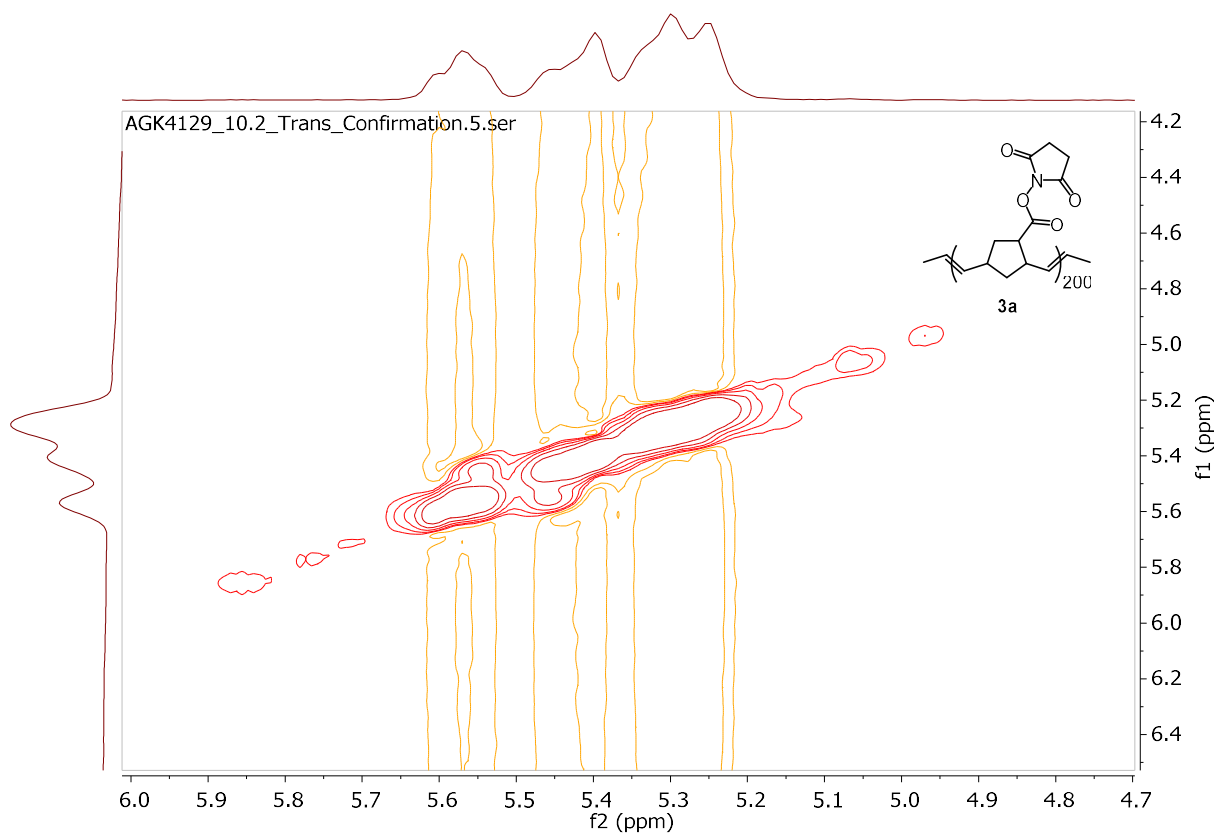
Polymer 7a TOCSY spectrum vinyl region. The spectrum was acquired with a mixing time of ~7msec and shows only cross-peaks between spins actually coupled to one another. In this experiment, the magnitude of cross-peaks between spins is proportional to the magnitude of coupling between them. It can be concluded that polymer **7a** contains an abundance of cis-alkenes due to the lack of cross-peaks in the vinyl region.



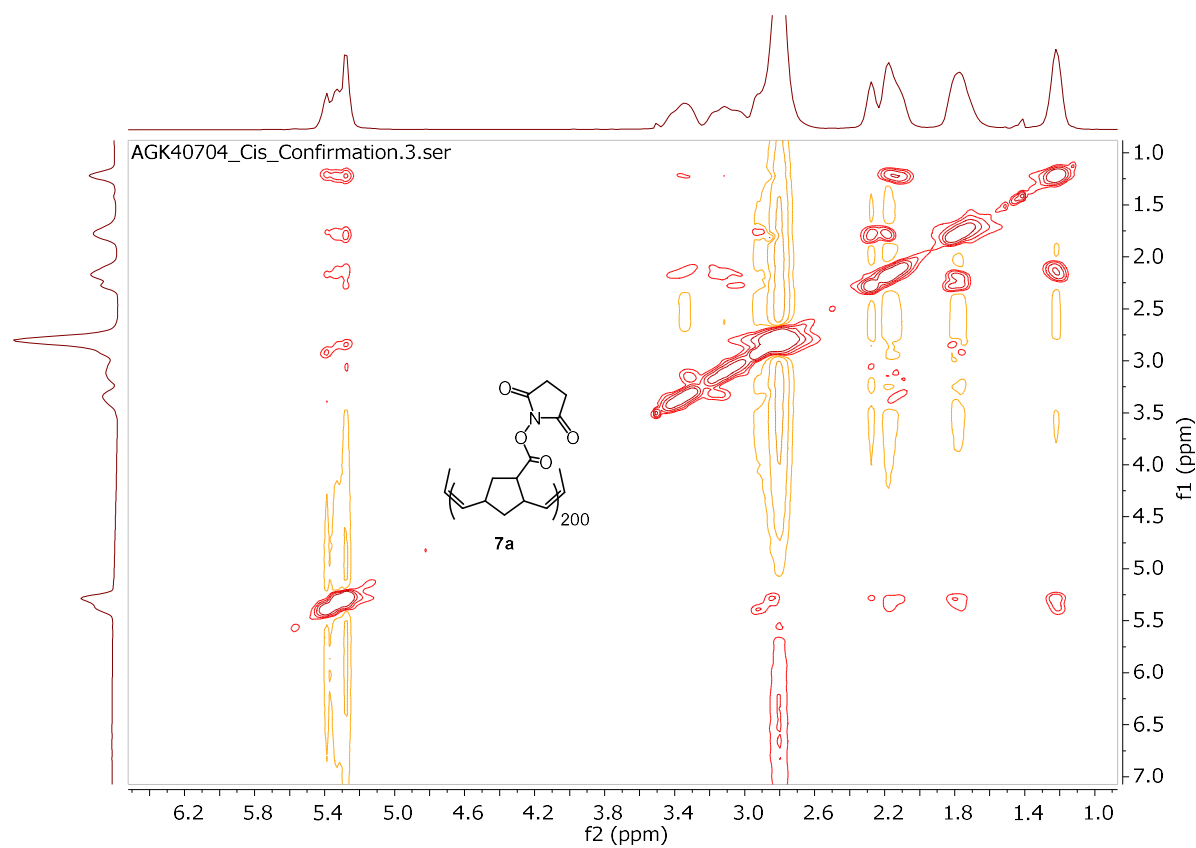
Polymer 3a NOESY spectrum. Note that the vinyl peaks exhibit little to no cross-peaks with one another consistent with trans orientation of the vinyl protons and a lack of cis olefins (zoom-in on next page).



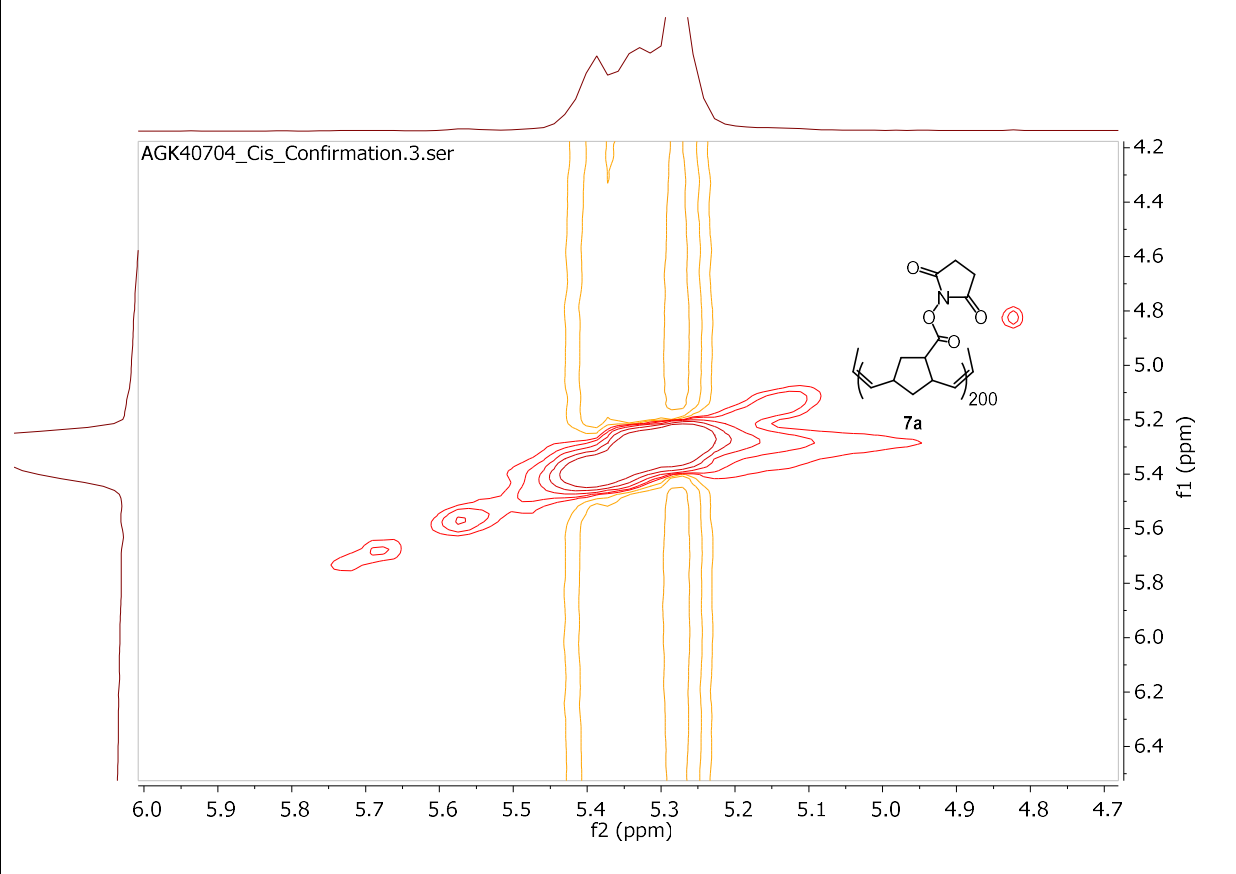
Polymer 3a NOESY spectrum showing vinyl region. Note that the vinyl peaks exhibit little to no cross-peaks with one another consistent with trans orientation of the vinyl protons and a lack of cis olefins.



Polymer 7a NOESY spectrum. Note that the vinyl peaks form a truncated box-like shape indicating cross-peaks within the signal consistent with cis orientation of the vinyl protons (zoom-in on next page).



Polymer 7a NOESY spectrum. Note that the vinyl peaks form a truncated box-like shape indicating cross-peaks within the signal consistent with cis orientation of the vinyl protons.



Chapter 3: Polymer Vaccines to Augment Agricultural Antimicrobials

Contributions: Ligand and polymer synthesis performed by Cassie M. Jarvis and Austin G. Kruger. Hen immunizations performed by Maria K. Arendt-Dashek. Biochemical experiments performed by Maria K. Arendt-Dashek, Natalie Schmidt, and Cassie M. Jarvis. Research designed by Cassie M. Jarvis, Maria K. Arendt-Dashek, Mark E. Cook and Laura L. Kiessling.

3.1 Abstract

The world's agricultural poultry flocks are greatly threatened by lethal coccidiosis induced by protozoan *Eimeria spp.* Like many human pathogens, these microbes upregulate host-expression of interleukin 10 (IL10) in the digestive tract suppressing the immune system and enhancing pathogenicity. Administering anti-IL10 antibodies in chicken feed can reverse this effect and is unlikely to result in antimicrobial resistance since it does not target microbial factors. Currently, anti-IL10 IgY antibodies are extracted from the eggs of hens which have been vaccinated using a bovine gamma globulin IL10 protein subunit vaccine (B10). These antibodies are nearing cost-parity with anti-coccidial drugs and, in addition to avian coccidiosis, a cost-effective source of anti-IL10 antibodies may enable resistance-recalcitrant treatment of several human pathogens. While promising, limited structural control is available over protein subunit vaccines making their optimization challenging and it is unclear how B10 interacts with the chicken immune system or how it should be altered to improve cost-effectiveness of antibody production. To address these issues, we synthesized a panel of well-defined polymer vaccines via the ring opening metathesis polymerization carrying combinations of B cell, T cell, and toll-like receptor 7 (TLR7) epitopes. While our polymers did not prime anti-IL10 responses in chickens, our simplest materials functionalized only with B cell epitopes outperformed all protocols tested when utilized as boosters. These results suggest that well-characterized, low-cost polymer vaccines can increase anti-IL10 IgY secretion in laying hens and provide insight into overcoming tolerogenic immune responses.

3.2 Introduction

Despite the remarkable success of vaccines,^{156,157} their development remains an unparalleled challenge^{158,159} with the World Health Organization recognizing only 25 that are both broadly available and effective.¹¹² Vaccines have been augmented by the discovery of small molecule antimicrobials which temporarily stymied the toll microbes exact on human and animal life, however, widespread use of these compounds garners resistance rendering them ineffective.^{111,160} In 2015 an estimated 100,000 tons of antimicrobial drugs were administered to animals¹⁶¹ surpassing human consumption 7-fold.¹⁶² Consequently, farmers and their microbial symbionts are routinely exposed to antibiotics leading to large populations of human-associated, antibiotic-resistant bacteria.¹⁶³ Novel strategies to combat microbial invasion are thus required to ensure animal welfare^{164,165} and avoid a post-antibiotic era.

The health¹⁶⁶ and profitability^{167,168} of agricultural poultry flocks are greatly threatened by coccidiosis, an intestinal infection caused by protozoan *Eimeria* species. These protozoa localize in the digestive tract where they upregulate host expression of interleukin 10 (IL10),^{169–171} an immunosuppressive cytokine,^{172,173} leading to enhanced pathogenicity (Figure 3.1).^{174,175} Exploitation of IL10's role in dampening the immune response is a general strategy utilized by numerous human pathogens¹⁷³ including *Mycobacterium tuberculosis*¹⁷⁶ and HPV.¹⁷⁷ Inhibition of IL10 signaling could protect against these infections^{178,179} and, in the case of coccidiosis, is equally effective as antimicrobial treatment.¹⁸⁰

Several studies have demonstrated complete restoration of weight gain and feed conversion in *Eimeria* infected chickens fed low concentrations of anti-IL10 antibodies

(340µg antibody per kg feed).^{170,175,180} By targeting a host factor rather than the pathogen itself, this mode of therapy is far less susceptible to the evolution of resistance than traditional vaccines and antibiotics (Figure 3.1A).¹⁸¹

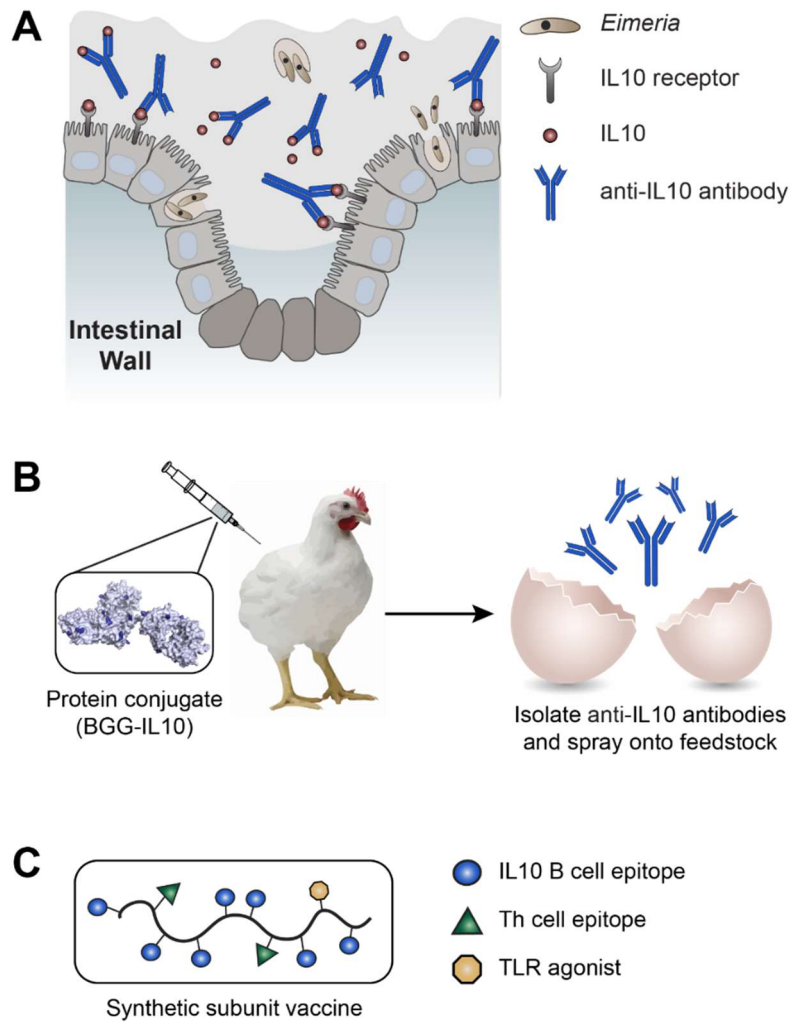


Figure 3.1 A) *Eimeria* spp. infect the digestive tract of chickens and upregulate IL10, an immunosuppressive cytokine, to enhance pathogenicity. Neutralizing IL10 with antibodies reduces mortality and weight loss equal to antimicrobial therapy. B) Hens vaccinated with a bovine gamma globulin IL10 conjugate secrete large conjugates of anti-IL10 antibodies into their eggs which can neutralize IL10 when sprayed onto feed. C) Synthetic conjugate vaccines can have tuned structures to maximize immune response and antibody production.

Therapeutic regulation of IL10 could establish a long-term solution for fighting several critical pathogens across the animal kingdom. The principle challenge of this approach is the sustainable acquisition of antibodies.^{182,183}

Chickens can secrete an incredible 150-300mg of immunoglobulin Y antibodies (IgY) into each egg.^{183,184} These antibodies are particularly stable and can even survive the harsh digestive tracts of birds and mammals while neutralizing their targets.^{181,185} Remarkably, hens immunized with an IL10 protein conjugate vaccine, wherein a portion of the IL10 peptide is linked to a bovine gamma globulin (BGG) carrier, yielded egg antibodies specific for IL10 that are only about twice the cost of anti-coccidial drugs (Figure 3.1B).^{170,175} Given that IL10 is a self-antigen, the ability of this simple vaccine construct to elicit antibodies is especially promising. While protein conjugate vaccines can enable immunization against some recalcitrant targets (e.g. self-epitopes,^{170,175,186} tumors,¹⁸⁷ immune-evasive pathogens¹⁸⁸) they suffer from limited tuneability. By contrast, an immense amount of structural control is available over synthetic polymers enabling systematic engagement of distinct immune pathways and cells (Figure 3.1C).

To explore how antigen structure affects anti-IL10 secretion in laying hens, we synthesized a panel of polymer vaccines bearing B cell, T cell, and TLR epitopes via the ring opening metathesis polymerization (ROMP). We then compared the ability of each polymer to raise IL10-specific IgY antibodies in the eggs of vaccinated hens versus the standard BGG-IL10 vaccination protocol. Surprisingly, we found that the simplest polymer construct, the polymer bearing only the B cell epitope, was the most effective vaccine booster. Our results suggest that low-cost, low-complexity polymer vaccines can lengthen IgY titer persistence in chickens.

Next we turned to generation of our immune epitopes. In addition to the previously utilized chicken IL10 amino acid sequence (VLPRAMQT) which elicited antigen-specific B cells in chickens,^{170,175} we chose to construct an elongated IL10 fragment consisting of 15 amino acids (Figure 3.3).

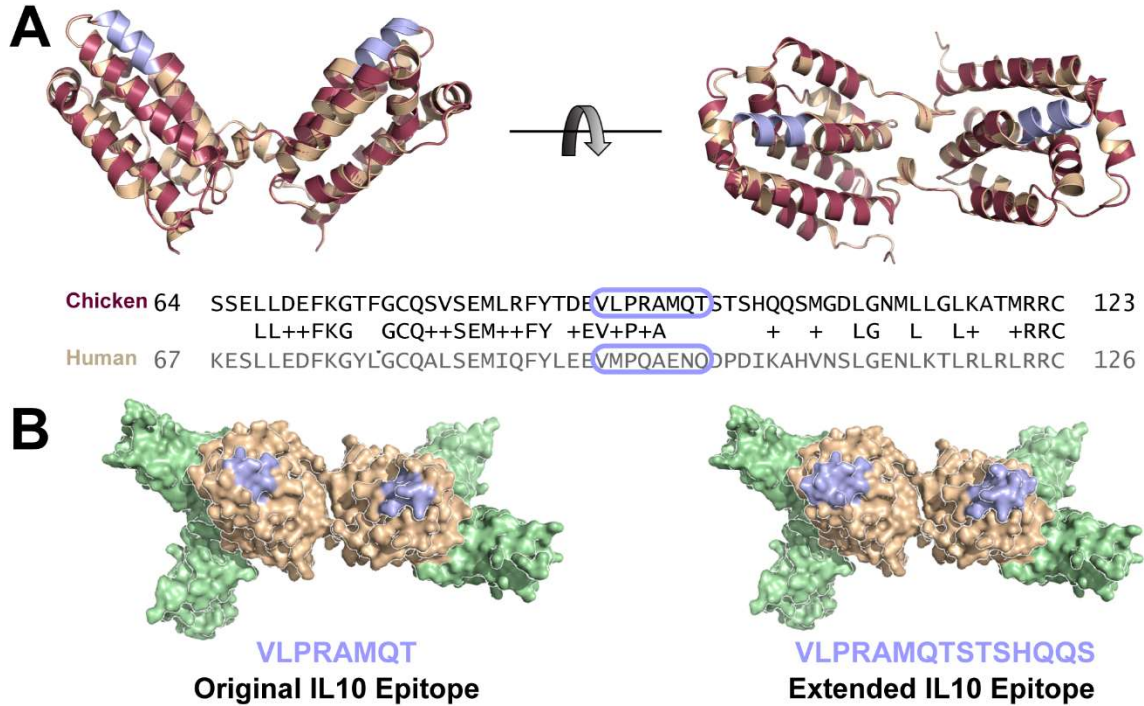
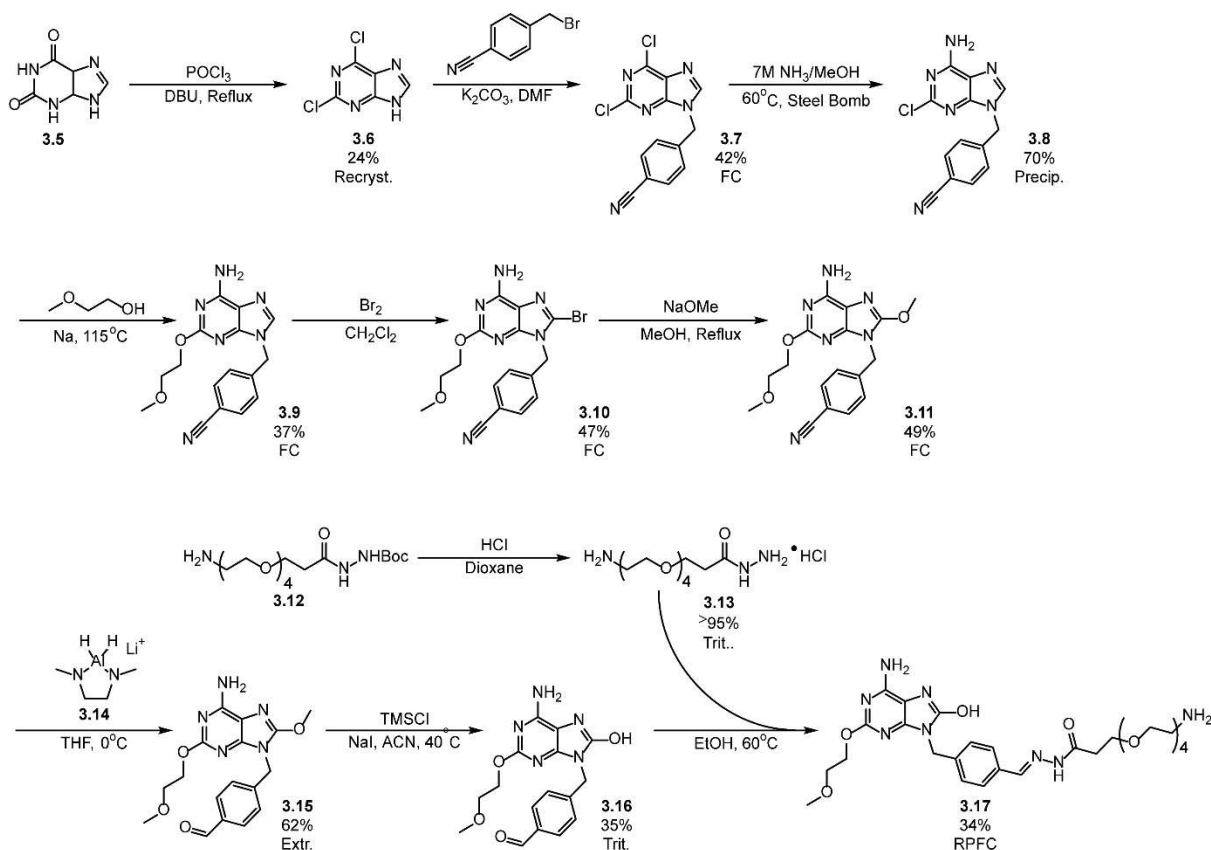


Figure 3.3. A) Chicken IL10 shown in red (homology model produced using SWISS-MODEL^{189,190}) rendered over human IL10 (PDB ID: 2H24) showing the IL10 8mer epitope used here and previously^{170,175} in addition to sequence alignment. B) Space-filling model of human IL10 (wheat) in complex with the IL10 receptor (green) showing short and extended IL10 15mer epitopes (purple). The original IL10 8mer fragment was selected using antigen prediction software¹⁷⁵ which is limited by data pools enriched in short amino acid sequences.¹⁰¹ More comprehensive structural data suggests that B cell epitopes are generally 12-22 amino acids in length and we hoped that our elongated IL10 sequence would enhance immunogenicity.^{191,192}

In addition to targeting B cells, we sought to recruit T cell help with our polymer constructs by including cathepsin D (CatD) cleavable peptides (Figure 3.2). CatD is an endocytic peptidase which we previously leveraged for *in cellulo* liberation of peptide antigens to facilitate synapse formation between B and T cells.¹²¹ Once cleaved, the peptide antigen can be bound by a major histocompatibility complex (MHC) and transported to the cell surface of an antigen presenting cell¹⁹³ to prime follicular helper T cells.¹⁹⁴ We envisioned that polymers functionalized with B and T cell epitopes would elicit more robust germinal centers^{121,195} thus yielding increased antibody secretion in eggs analogous to results obtained in mammals.¹⁹⁶ To test this hypothesis, we included two CatD-cleavable T cell epitopes in our polymer panel. Our antigen prediction software indicated that the IL10 B cell epitope is recognized by T cells. Therefore we included a CatD releasable version of our short IL10 fragment to enhance T cell priming with our target antigen. Since the IL10 sequence had not been fully validated as a T cell epitope, we wished to ensure that T cell help was recruited by including a vetted T cell antigen. A peptide sequence from influenza hemagglutinin which was previously reported to recruit T cell help in birds was therefore included on a subset of the polymers.^{197–199}

Finally, we hypothesized that stimulation of an innate immune receptor would enhance antibody titers against IL10. We selected a novel toll-like receptor 7 (TLR7) agonist, UC-1V150,^{200–202} to append to our polymers based on a few criteria. First, TLR7 stimulation with a commercially available agonist has boosted immune responses in a number of chicken studies.^{202–204} Since IL10 is a self-epitope, we reasoned that a high degree of innate immune stimulation may be required to overcome tolerogenic

responses against the peptide. UC-1V150 derivatives are highly potent TLR7 ligands^{200,205–207} making them particularly appealing for this application.



Scheme 3.1. Synthesis of TLR7 agonist UC-1V150-hydrazide conjugate for linkage to polymer vaccines.

Since TLR7 localizes to endosomes, our polymer vaccine strategy enables targeted delivery of UC-1V150 to its receptor within antigen-primed cells^{121,196,200,208} further enhancing potency²⁰⁹ while limiting aberrant inflammation.²¹⁰ We synthesized a hydrazide conjugate of UC-1V150 according to a modified literature route²⁰⁰ (scheme 3.1) and tethered it to our polymers via the primary amine (Figure 3.2). Once delivered to endosomes, the acid-labile hydrazide is cleaved^{200,211} allowing the agonist to bind and initiate TLR7 signaling.

Immune epitopes were then grafted to succinimidyl ester polymer **3.4** using stoichiometric control and sequential addition to yield a panel of precisely defined polymer vaccines. Based on previous studies^{121,200,212} and the solubility profile of our peptides, we incorporated approximately 30mol% loading of B cell epitopes, ~5mol% T cell epitopes, and ~5mol% TLR7 agonist in the respective constructs (Figure 3.2). As structural complexity rose these materials became increasingly challenging to

characterize. To ensure that each epitope was grafted in the desired ratio a combination of ^1H and ^{19}F NMR was utilized to

determine the structure of the polymer vaccines. By incorporating differentially fluorinated phenylalanines into each peptide the ratio of

each epitope to the trifluoromethyl endcap at the polymer terminus could be measured (Figure 3.5).

Similarly, the integral of backbone alkenes to benzyl signals from UC-1V150 could be taken to determine the extent of agonist conjugation.

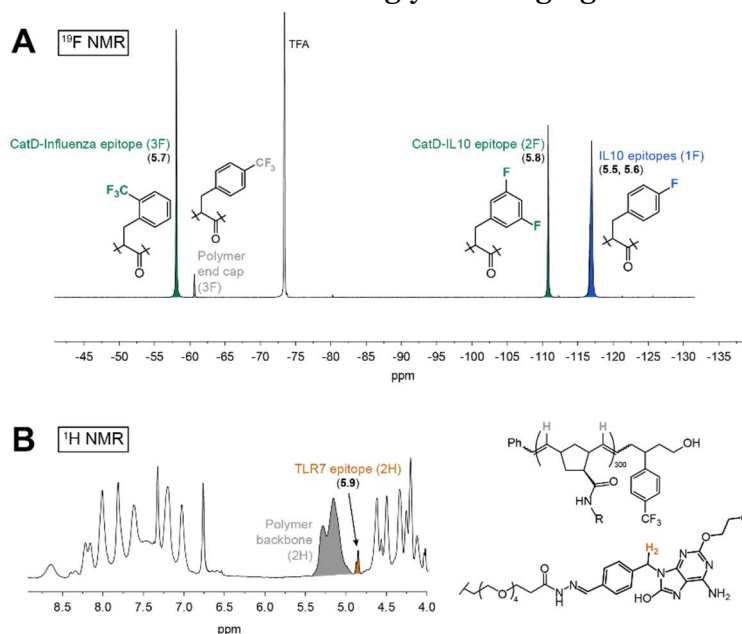


Figure 3.4. A) Each peptide epitope in the study incorporated a distinct fluorophenylalanine derivative whose ^{19}F NMR signal was integrated relative to backbone $-\text{CF}_3$ endcap to determine functionalization percent. B) The extent of TLR7 agonist incorporation was measured by the ratio of backbone alkene to the ligand's benzylic protons by ^1H NMR.

3.4 Vaccination Protocol and Results

Our vaccination scheme was based on a previously employed protocol wherein primary injections of three birds per group are followed by booster injections on day 21 (Figure 3.5).^{170,175} Dosage was based on the concentration of IL10 peptide administered (300µg) given intramuscularly as 1mL formulations in 50% v/v PBS/Freund's Complete or Incomplete Adjuvant for primary and secondary injections respectively. The bovine gamma globulin (BGG) carrier was used as negative control in these trials and did not elicit anti-IL10 antibodies at any timepoint. The previously employed BGG-IL10 conjugate vaccine (B10) was used as positive control and elicited anti-IL10 IgY titers both as primary and booster vaccine (week 4 mean egg yolk anti-IL10 ~1.5mg/mL).

When used for the primary immunization the polymers did not yield anti-IL10 IgY, however, several were able to act as boosters when B10 was used to prime the hens (Figure 3.5). The most striking trend we observed was a marked decline in antibody

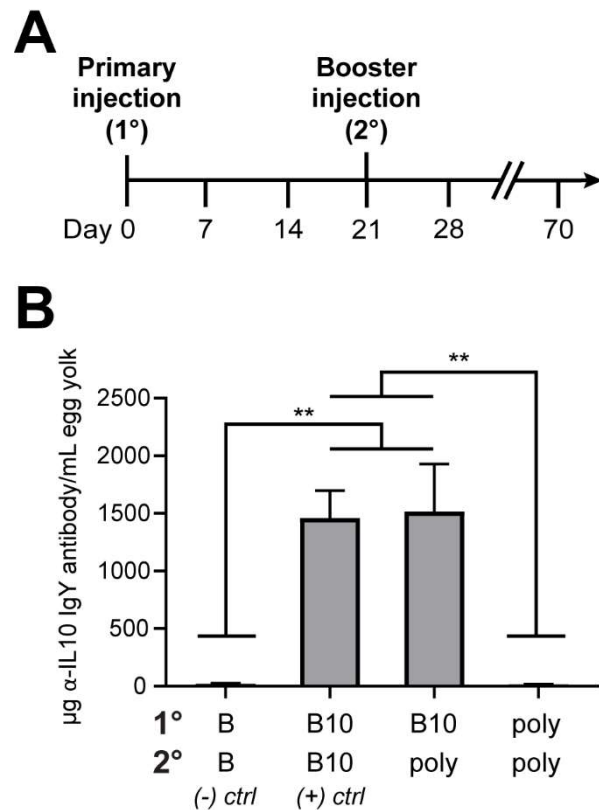


Figure 3.5. A) 3 hens per group were vaccinated on day 0 followed by boosters on day 21. B) Average yolk concentration of anti-IL10 IgY on week 4 following one of three vaccination protocols. Hens were vaccinated with either BGG carrier negative control, BGG-IL10 conjugate (B10), or polymer followed by boosting with B10 or polymer. Only B10 was successful at eliciting initial titers but some polymers proved effective as boosters.

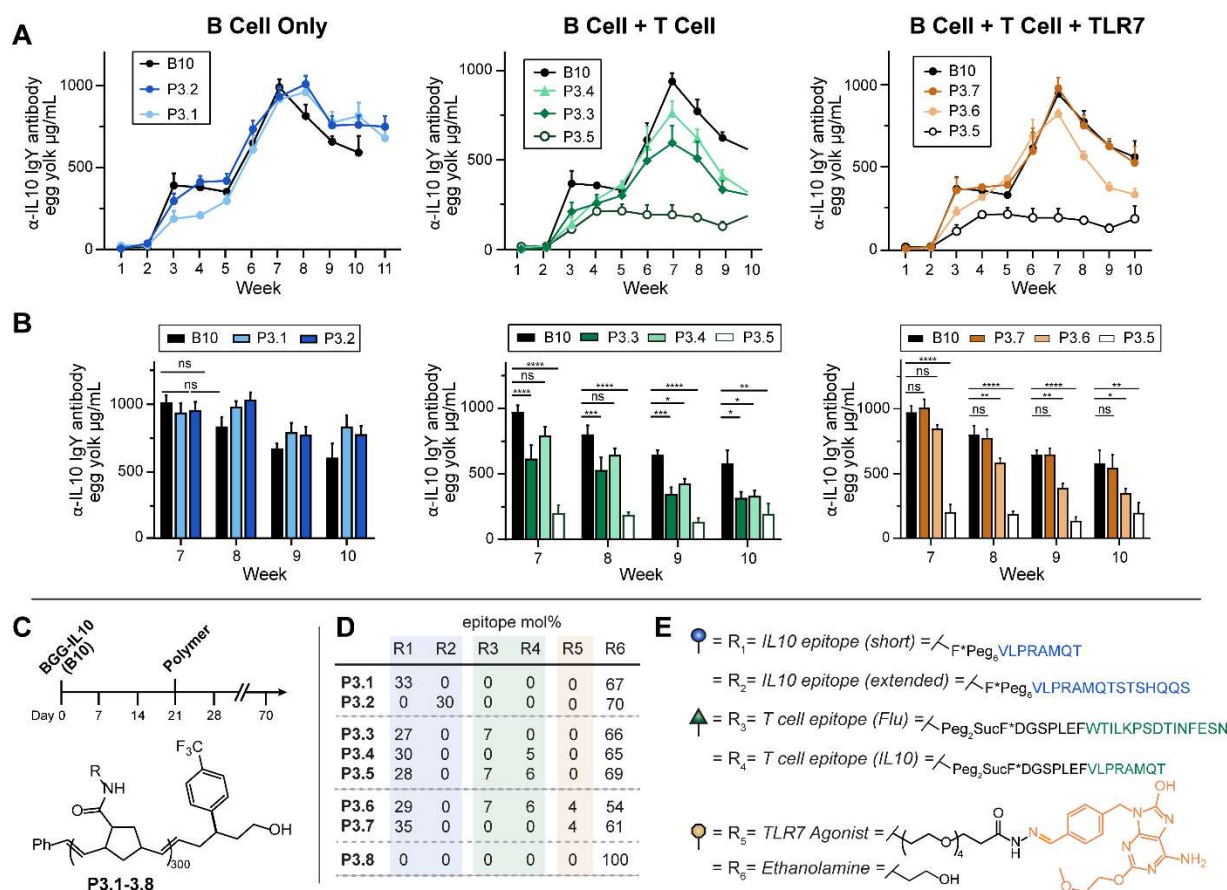


Figure 3.6. A) Weekly mean anti-L10 IgY titers presented as $\mu\text{g/mL}$ egg yolk for each set of polymer boosters grouped by immune epitopes. All titers are compared to those elicited by B10 in black.

B) Mean antibody titers during peak production weeks 7-10 for all three polymer groups compared to B10.

C) Vaccination schedule and polymer vaccine backbone structure.

D) polymer panel composition and epitope loading characterized by ^1H and ^{19}F NMR. E) Amino acid sequences and structures of the immune epitopes included on the polymer vaccines in this trial.

titers for birds boosted with polymers bearing T cell epitopes (Figure 3.6). Whereas **P3.1** and **P3.2**, functionalized with short and long IL10 sequences respectively, match and then exceed B10 titers at and past week 7 ($0.75\text{-}1.0\text{mg/mL}$ egg yolk IgY), **P3.5** functionalized with influenza and IL10 T cell epitopes yielded a plateau of IgY production at $\sim 0.25\text{mg/mL}$ after week 3. Inclusion of the TLR7 agonist rescued these

titers to a great extent in the case of **P3.6**, nearly restoring them to those observed with B10 booster. However, optimum results were achieved when the T cell epitopes and TLR7 agonist were abandoned altogether. We were gratified to find that the extended IL10 epitope possessed greater immunogenicity than the shorter sequence as we predicted since **P3.2** consistently outperformed **P3.1** at all time points. We were particularly excited to see that both of these polymers prolonged peak antibody secretion relative to the standard B10 vaccination protocol since this is the principle barrier to cost-effective production of anti-IL10 antibodies from chickens.

3.5 *Discussion*

Due to the highly defined structures of our polymer antigens we are able to provide insight into the design of more effective vaccine constructs. While our polymers did not prime immune responses in chickens, several proved efficient as vaccine boosters. This may have occurred for a few reasons. First, even BGG can be made to induce tolerogenic responses when differentially dosed.^{213,214} It is possible that our polymers are more efficient at presenting IL10 than anticipated resulting in attenuation of the inflammatory response by protein overload.^{213,214} Secondly, emulsive adjuvants such as Freund's and RIBI appear particularly effective at boosting immune responses to BGG vaccines^{214,215} but have been exchanged for small molecule adjuvants with our polymer system in mammals.^{196,212} This implies that the emulsive conditions in Freund's adjuvant lead to changes in B10 structure or assembly, possibly aggregation, which may increase its immunogenicity.²¹⁶ Native BGG is poorly immunogenic,^{213–215} and exists as monomers and dimers in aqueous solution even at extremely high concentrations.²¹⁷ It would be interesting, therefore, to test larger polymer aggregates for their ability to

prime immune responses in hens, however, this did not fall within the scope of our original trial parameters and so is planned for future studies.

We were surprised to find that including immune epitopes additional to the target antigen yielded less effective polymer vaccines. It is possible that the T cell epitopes recruit regulatory T cells (T_{reg}) suppressing the immune response,²¹⁸ consistent with our antigen's identity as a self-epitope.^{219,220} While the avian immune system is distinct from that in mammals, TLR7 agonism has been shown to enhance the immunosuppressive abilities of murine T_{reg} s.²²¹ A similar phenomenon may have occurred here and our results support a nuanced role for TLR7 in the avian immune system wherein pro-^{202–204} and anti-inflammatory²²¹ induction through TLR7 is antigen dependent. Additionally, T cells can induce antibody class switching through an array of cytokines.²²² In our study, it is possible that T cell recruitment skewed antibody production towards non-secreted isotypes thus reducing IgY titers.

We were highly encouraged that our simplest polymers proved to be the most effective vaccine boosters since this suggests that the cost of anti-IL10 IgY production can be decreased by using less complex vaccine constructs. We believe that further optimization of our scaffold is possible through modifying polymer size and shape. By mimicking either the smaller nanometer scale monomers and dimers of BGG²¹⁷ or the higher order structures which may form in Freund's adjuvant, it could be possible for IL10 functionalized materials to prime chicken immune responses and further exceed IgY production relative to the standard B10 construct. This approach is particularly attractive since it can be performed without synthesis of expensive immune epitopes and might yield increased antibody titers with low cost vaccines. Thus, we believe this

research will guide further development of chemical tools for systematic investigation of immunology and production of therapeutic antibodies.

3.6 Experimental Details

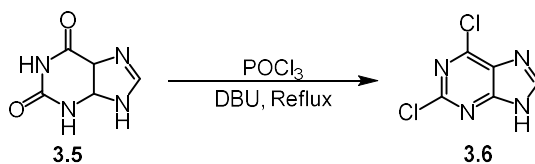
Materials. All chemicals were purchased from Sigma-Aldrich (MilliporeSigma). The ruthenium carbene catalyst **3.2**,¹⁵⁵ (*R, Z, E*)-6-methoxy-3-(4-(trifluoromethyl)phenyl))hex-5-en-1-ol capping agent **3.3**, and succinimidyl ester-substituted norbornene polymer **3.4** were prepared as described previously.¹²¹ Dry organic solvents were obtained from a solvent purification system (Pure Process Technology) or by standing over a quarter volume of 4 angstrom molecular sieves for 3 days prior to use. Flash chromatography (FC) was performed using SiliaFlash Irregular Silica Gel P60 from Silicycle and analytical thin layer chromatography (TLC) was performed using Siliaplate TLG-R10014B-323 with F254 fluorescence indicator. GM-1 ganglioside was purchased from Carbosynth LLC (Compton, Berkshire, United Kingdom). Anhydrous DMSO obtained from Sigma-Aldrich (MilliporeSigma) was used for polymer conjugation reactions. Water (H₂O) was purified with a MilliQ purification system (Millipore). Sterile, USP grade DMSO was purchased from Sigma-Aldrich (MilliporeSigma) for *in vivo* polymer formulations.

Instrumentation. ¹H and ¹⁹F nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance Neo-600 spectrometer equipped with a 5mm helium-cooled QCI-F cryoprobe. Chemical shifts were reported relative to trimethylsilane or residual solvent peaks in parts per million (DMSO-*d*₆: ¹H NMR δ 2.50). Polymer NMR spectra were acquired using samples in DMSO-matched NMR Microtubes (Shigemi, Inc) or 3mm SampleJet NMR tubes (Bruker). Spectra were analyzed using MestreNova processing software. Polymer spectra were recorded with a relaxation delay equivalent to four times the longest T₁ value (generally D₁ = 10). The degree of polymerization (DP)

was determined based upon integration of the terminal phenyl protons relative to polymer backbone alkene protons. The mole fraction of conjugated TLR7 ligand was determined based upon integration of the benzyl protons relative to polymer alkene protons. The mole fraction of conjugated peptide was determined based upon integration of peptide relative to polymer ^{19}F NMR spectroscopy signals.

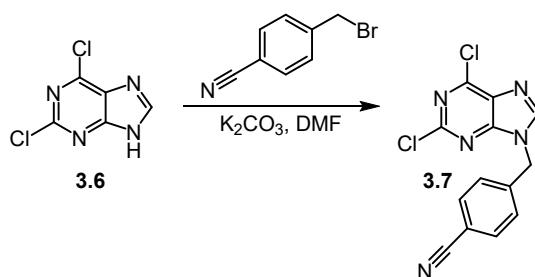
Peptide Synthesis General Procedure. Peptides were generated using standard solid-phase peptide synthesis (SPPS) with Fmoc-protected amino acids and pre-loaded Wang-Thr resin (short IL10 peptide), pre-loaded Wang-Ser resin (extended IL10 epitope), or unloaded NovaPEG Rink Amide resin (T cell epitopes). SPPS was performed using a Liberty Blue Automated Microwave Peptide Synthesizer. Peptides were cleaved from the resin with 2-3 h incubation at rt in 82.5% trifluoroacetic acid (TFA), 5% H_2O , 5% phenol, 5% thioanisole, and 2.5% 1,2-ethanedithiol and precipitated into cold ether. The precipitate was collected by centrifugation then washed with cold ether and dried under vacuum. HPLC (Waters Prep) purification on a C3 column (Agilent Zorbax 300SB) yielded pure peptide. Peptide purity and mass were confirmed using liquid chromatography-mass spectrometry (LC-MS) (Agilent/Advion) and MALDI (Bruker).

TLR7 Agonist Synthesis

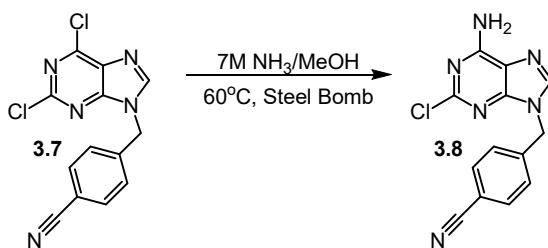


Compound 3.6. Compound **3.6** was produced according to a modified procedure in the cited reference.²²³ To a flame dried, N_2 flushed 1L 3-neck flask equipped with a dry reflux condenser add 47.8g xanthine (314mmol, 1 equiv.), 294mL phosphorous (V) oxychloride (3.14mol, 10 equiv.), and cool to 0°C . To a flame dried, N_2 flushed 500mL round bottom flask add 376mL 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) (2.52mol, 8 equiv.). Add the DBU dropwise to the reactant solution via cannula expecting vigorous reaction on DBU addition. Note that a thick yellow-green putty forms on DBU contact with POCl_3 necessitating occasional cleaning of the cannula during DBU addition as POCl_3 vapors contact the cannula. Once DBU addition is complete, heat the reaction to reflux (approximately 110°C) and allow the reaction to proceed for 6 hours before cooling to room temperature. At this stage the reactant solution is a dark-brown to black viscous syrup which is poured into 2.5L of ice containing approximately 500mL water. A substantial quantity of the reactant solution will be left behind in the reaction vessel and is recovered momentarily. As the reactant solution contacts aqueous solution a thick black tar forms which will rapidly congeal on the bottom of the Erlenmeyer. This tar is brought into solution by cautious addition of 50wt% aq. NaOH aiming to bring the final pH of the solution to 4. **CAUTION:** Extreme care must be taken during this stage of the workup since rapid and violent reaction between 50wt% aq. NaOH and the remaining POCl_3 can occur if the tar becomes dislodged from the bottom of the Erlenmeyer flask at

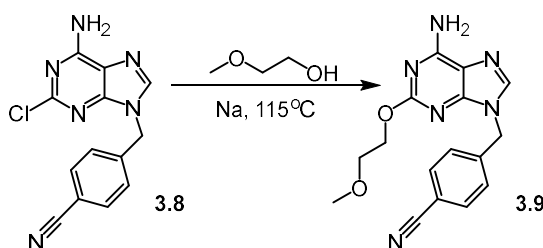
any stage. In one instance a golf-ball sized globule of tar arose from the parent syrup and the solution immediately erupted to the top of the fume hood scattering extremely acidic tar across the work area. 50wt% NaOH is employed due to the large quantity of acid present and the need to minimize the volume of aqueous solution introduced which will complicate extraction. Once enough NaOH has been added to reach homogeneity, use the aqueous solution to recover the reactant solution remaining in the 3-neck flask adding 50wt% NaOH as necessary to effect dissolution of the tar as before. After all of the syrup has dissolved bring the pH of the aqueous solution to 4. Note that if pH 6 is exceeded vigorous exothermic reaction will ensue arising presumably from hydrolysis of **3.6** to starting material. Filter the solution over a pad of celite and extract the aqueous phase with 2x 700mL portions EtOAc, dry over MgSO₄, and concentrate. The crude should concentrate to a neon yellow solid. Recrystallize the material from approximately 2.4L boiling MeOH using a combination of the following to induce crystallization. If crystals do not form at 0°C cool the solution to -78.5°C in a dry ice acetone bath. If crystals still do not form allow the reactant solution to warm to room temperature and place the solution on a rotovap without a water bath allowing enough solvent to be removed to form a thick layer of ice on the outside of the rotovap flask before attempting crystallization again. The initial crop of crystals is filtered off and a second crop is obtained by concentrating the mother liquor to approximately half of its original volume and crystallizing again. This will result in introduction of a bright yellow contaminant, however, this has not proven consequential in the following transformation. Product was obtained as fluffy, light yellow crystalline needles. Yield: 24% ¹H NMR (500 MHz, Methylene Chloride-*d*₂) δ 13.69 (brs, 1H dissociable N-H), 8.27 (s, 1H).



Compound 3.7. Compound **3.7** was synthesized according to a modified procedure for compound **2** in the cited reference.²⁰⁰ To a 250mL round bottom flask add 8g **3.6** (42.3mmol, 1 equiv.), 11.62g 4-(bromomethyl)benzonitrile (59.9mmol, 1.4 equiv.), 18.14g K₂CO₃ (131mmol, 3.1 equiv.), dissolve in 135mL DMF, and allow the reaction to proceed overnight. Pour the reactant solution into 4L H₂O and extract with 2x 800mL portions EtOAc, dry over MgSO₄, and concentrate. Note that the product precipitates on pouring into H₂O and will rapidly form a thick yellow resin if allowed to stand which complicates its handling. Therefore, it is advisable to quickly perform the first extraction after pouring into H₂O. Purify the material by FC using 400mL silica and 4L 8%:17%:75% EtOAc:Acetone:Hexanes. The crude and product are only sparingly soluble in EtOAc, CH₂Cl₂, CHCl₃, acetone, MeOH or combinations thereof and no suitable solvent was found for loading the material on the column. Therefore, the material is dry loaded by dissolution in a large volume of EtOAc and concentrated on 50mL of silica. Product is a white solid with R_f = 0.08 in the running solvent. Yield: 42%. ¹H NMR (500 MHz, Methylene Chloride-*d*₂) δ 8.15 (s, 1H), 7.68 (d, *J* = 8.4 Hz, 2H), 7.40 (d, *J* = 8.4 Hz, 2H), 5.49 (s, 2H).

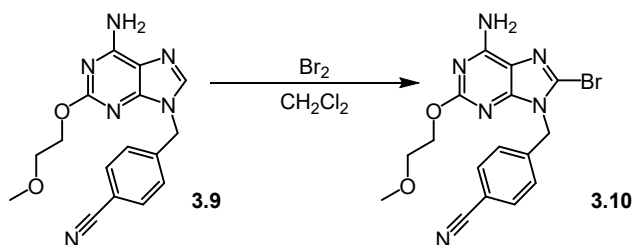


Compound 3.8. Compound **3.8** was synthesized according to a modified procedure for compound **3** in the cited reference.²⁰⁰ To a flame dried, N₂ flushed steel bomb equipped with a stir bar add 2.43g **3.7** (8mmol, 1 equiv.) and 100mL 7M NH₃/MeOH before sealing (all screws must be secured with Teflon tape). Heat the vessel to 50°C and allow the reaction to proceed overnight. After heating overnight, cool the vessel to 0°C and cautiously open the reaction vessel positioning a hood sash between oneself and the reaction. Collect the product by filtration, wash the filter cake with 2x 20mL portions MeOH, and air-dry the product by pulling air over the filter pad for 2 hours. Product is a chalk-like white solid with R_f = 0.14 on 5% MeOH:CH₂Cl₂. Yield: 70%. ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.07 (s, 1H), 7.67 (d, *J* = 8.3 Hz, 2H), 7.38 (d, *J* = 8.3 Hz, 2H), 5.40 (s, 2H).



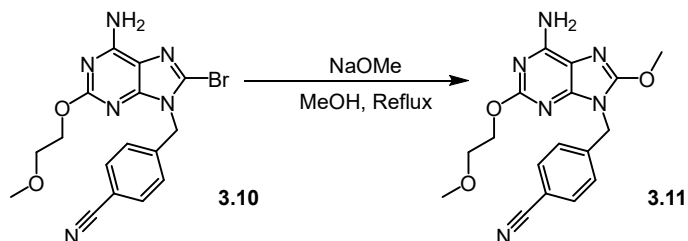
Compound 3.9. Compound **3.9** was synthesized according to a modified procedure for compound **4** in the cited reference.²⁰⁰ To a flame-dried, N₂ flushed 2L round bottom flask add 2.97g **3.8** (10.4mmol, 1 equiv.), and dissolve in 1L dry 2-(methoxy)ethanol. To a flame-dried, N₂ flushed 250mL round bottom flask add 90mL dry 2-(methoxy)ethanol

and 360mg sodium metal (15.7mmol, 1.5 equiv.) and allow the reaction to continue until effervescence ceases. Add the 2-(methoxy)ethoxide solution to the reactant solution, heat to 115°C, and allow the reaction to proceed for 8 hours before concentrating. Partition the concentrate between 400L each EtOAc and H₂O, isolate the organic phase, dry over MgSO₄, and concentrate. Purify the material by FC using 450mL silica and 4L 5% MeOH:CH₂Cl₂. Product is a white solid with R_f = 0.14 in the running solvent. Yield: 37%. ¹H NMR (500 MHz, DMSO-*d*₆) δ 8.07 (s, 1H), 7.82 (d, *J* = 8.3 Hz, 2H), 7.44 (d, *J* = 8.3 Hz, 2H), 7.27 (brs, 2H), 5.36 (s, 2H), 4.34 – 4.24 (m, 2H), 3.62 – 3.53 (m, 2H), 3.26 (s, 3H).



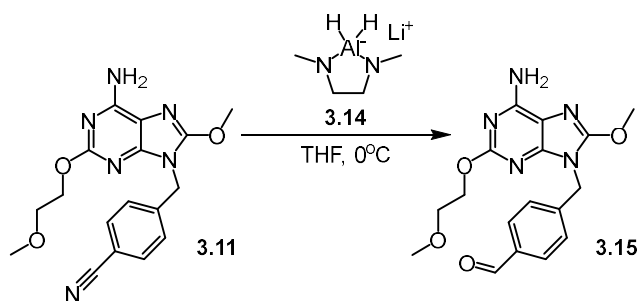
Compound 3.10. Compound **3.10** was synthesized according to a modified procedure for compound 5 in the cited reference.²⁰⁰ To a flame-dried, N₂ flushed 1L round bottom flask equipped with an oversized stir-bar add 500mg **3.9** (15.4mmol, 1 equiv.), dissolve in 385mL dry CH₂Cl₂, add 5mL Br₂ (97.6mmol, 6.3 equiv.) and bring the reaction to a vigorous stir such that the stir bar tumbles about the vessel rather than remaining in a single path. This will prevent conglomeration of a yellow-insoluble material which will accrue in a ring around a rotating stir bar. Despite extensive attempts, no combination of solvents was found to even partially dissolve this insoluble yellow material and failure to prevent its appearance is associated with greatly diminished yields. This material will also form if the reactant solution is fully concentrated at any stage prior to

chromatographic purification. After stirring overnight, wash the reactant solution with 2x 750mL portions 100mM sodium thiosulfate (11.6g Na₂S₂O₃ in 750mL H₂O), 1x 750mL portion saturated NaHCO₃, dry over MgSO₄, and reduce the organic layer's volume to that suitable for loading onto the column without fully concentrating by rotary evaporation to avoid formation of insoluble yellow material and product loss. Purify the material by FC using 200mL silica and a solvent gradient of 600mL each 0.6%, 1.2%, 1.8%, 2.4%, and 3% MeOH:CH₂Cl₂. Product is a white solid with R_f = 0.12 on 3% MeOH CH₂Cl₂. Yield: on average 47% yield was obtained, however, when measures to prevent formation of insoluble material were determined yields of ~65% were routinely obtained. ¹H NMR (500 MHz, Chloroform-*d*) δ 7.83 (d, *J* = 8.3 Hz, 2H), 7.49 (brs, 2H), 7.39 (d, *J* = 8.3 Hz, 2H), 5.35 (s, 2H), 4.30 (m, 2H), 3.59 (m, 2H), 3.27 (s, 3H).



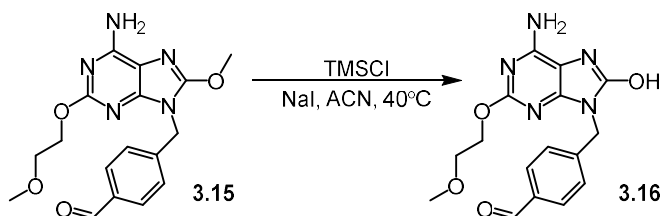
Compound 3.11. Compound **3.11** was synthesized according to a modified procedure for compound 6 in the cited reference.²⁰⁰ To a flame dried, N₂ flushed 500mL round bottom flask add 150mL dry MeOH, cool to 0°C, and add 2.93g sodium metal (127mmol, 70 equiv.). When effervescence has ceased add 750mg **3.10**, heat to reflux, and allow the reaction to proceed for 5 hours before concentrating. Partition the concentrate between 150mL each CH₂Cl₂ and H₂O, wash with a further 2x 150mL portions H₂O, dry over MgSO₄, and concentrate. Purify the material by FC using 125mL silica and a solvent

gradient of 500mL each 1%, 1.5%, and 2% MeOH:CH₂Cl₂ and 1.5L 2.5% MeOH:CH₂Cl₂. Product is a white solid with R_f = 0.13 on 5% MeOH:CH₂Cl₂. Yield: 49% noting that some product is lost to co-elution with low R_f impurity. The specified column conditions represent what was deemed an appropriate balance between time input and yield given that exhaustive conditions were required to achieve full resolution. ¹H NMR (500 MHz, Chloroform-*d*) δ 7.60 (d, *J* = 8.4 Hz, 2H), 7.40 (d, *J* = 8.3 Hz, 2H), 5.14 (s, 2H), 4.44 (m, 2H), 4.09 (s, 3H), 3.74 (m, 2H), 3.42 (s, 3H).



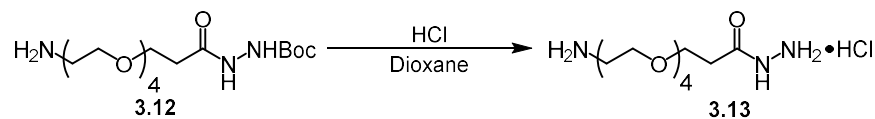
Compound 3.11. Compound **3.15** was synthesized according to a modified procedure for compound **7**²⁰⁰ using the custom reductant **3.14**²²⁴ prepared directly prior to reaction according modified procedures in the cited references. To a flame dried, N₂ flushed 5mL round bottom flask add 1mL 1.0M lithium aluminum hydride (LAH) (1.0mmol, 1 equiv. with respect to N,N'-Dimethylethylenediamine) in THF and cool to -78°C. To a flame dried, N₂ flushed 6mL vial add 108μL N,N'-Dimethylethylenediamine (1mmol, 1 equiv. with respect to LAH) and dissolve in 1mL dry THF. Add the amine solution to the reductant solution and allow 15 minutes for effervescence to cease at which stage the stock solution is a heterogeneous chalky white-blue. To a flame-dried, N₂ flushed 5mL round bottom flask add 50mg **3.11** (141μmol, 1 equiv.), dissolve in 780μL dry THF, and cool to 0°C. To this solution add 500μL of the reductant solution and allow the reaction to congeal to a spongy yellow solid over the course of

approximately 5-10 minutes and allow to stand under N₂ for 2 hours. After standing for 2 hours, hydrolyse the aluminum-imine complex by addition of 20mL 1.2M HCl, extract with 2x 20mL portions EtOAc, 2x 20mL portions CH₂Cl₂, combine the organic phases, dry over MgSO₄, and concentrate. Product is an oily yellow-white solid with R_f = 0.23 on 5% MeOH:CH₂Cl₂. Yield: on average 62% was obtained, however, at the end of the optimization sequence upwards of 85% was regularly achieved. The product is isolated as a mixture of aldehyde and hydrate tautomers which is reflected in the NMR assignments. If the experimenter is concerned about contamination with starting material an NMR sample can be acidified to shift equilibrium towards aldehyde confirming or denying assignments to each tautomer. ¹H NMR (500 MHz, DMSO-*d*₆) δ 9.98 (s, 0.88H, aldehyde), 8.32 (s, 0.29H, hydrate) 7.89 (d, *J* = 8.2 Hz, 1.45H, aldehyde aromatic), 7.43 (d, *J* = 8.2 Hz, 1.39H, aldehyde aromatic), 7.35 (d, *J* = 8.2 Hz, 0.41H, hydrate aromatic), 7.25 (d, *J* = 8.2 Hz, 0.47H, hydrate aromatic) 5.16 (s, 1.66H, aldehyde benzyl), 5.05 (s, 0.36H, hydrate benzyl) 4.28 (m, 2H, aldehyde and hydrate ethoxy methylene), 4.05 (s, 3H, aldehyde and hydrate aromatic ether), 3.59 (m, 2H, aldehyde and hydrate ethoxy methylene), 3.28 (s, 2.30H, aldehyde methoxy ether), 3.22 (s, 0.65H, hydrate methoxy ether).

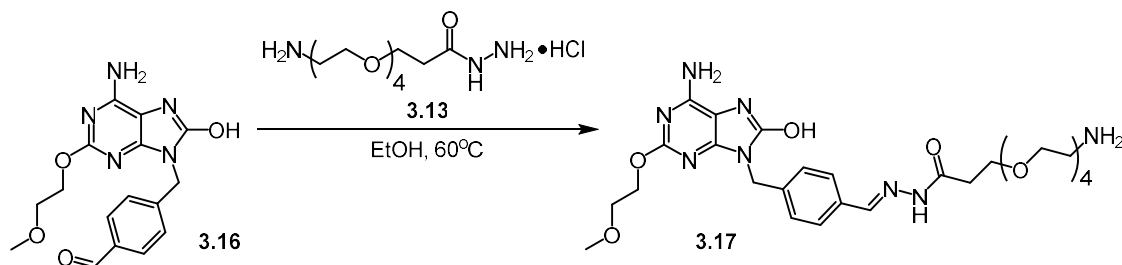


Compound 3.16. Compound **3.16** was synthesized according to a modified procedure for compound **8** in the cited reference.²⁰⁰ In a 25mL round bottom flask concentrate a solution of 23.2mg **3.15** (64.9μmol, 1 equiv.) in a few mL CH₂Cl₂, add a stir bar,

exchange the atmosphere for N₂, add 33.4mg NaI (223μmol, 3.4 equiv.), dissolve in 6.5mL dry CAN, and heat to 40°C. To this solution add dropwise 28.3μL chlorotrimethylsilane TMSCl (223μmol, 3.4 equiv.) and allow the reaction to proceed for 4 hours. Allow the reactant solution to cool to room temperature and centrifuge the reactant solution in a 12mL Falcon tube keeping the precipitate though expecting product in the supernatant (during one iteration the product precipitated at this stage and, since it is known to be highly insoluble in ACN once concentrated from the reactant solution it is best to exercise caution before discarding any supernatant or precipitate from this workup). After centrifuging concentrate the supernatant at which stage the product should be highly reluctant to enter ACN and is triturated by addition of 1mL boiling ACN followed by cooling to 0°C, centrifugation, and decanting expecting product as the precipitate in this iteration. Rinse the centrifuge with 2x 1mL portions ACN to recover as much product as possible from the walls of the tube and concentrate. Product is obtained as a brown solid which is insoluble in essentially all common laboratory solvents tested except, to some extent, DMSO. Yield: 35%. Product is obtained as a mixture of aldehyde and hydrate as reflected in the NMR assignments. ¹H NMR (500 MHz, Methanol-*d*₄) δ '9.87 (s, 0.09H, aldehyde), 7.80 (d, *J* = 8.1 Hz, 0.19H, aldehyde aromatic), 7.50 (d, *J* = 8.1 Hz, 0.20H, aldehyde aromatic) 7.33 (d, *J* = 8.2 Hz, 1.81H, hydrate aromatic), 7.30 (d, *J* = 8.2 Hz, 1.80H), 7.23 (s, 1H, hydrate), 5.03 (s, 0.19H, aldehyde benzyl) 4.95 (s, 1.78H, hydrate benzyl), 4.48 (m, 1.73H, hydrate ethoxy methylene), 4.45 (m, 0.25H, aldehyde ethoxy methylene) 3.64 (m, 2H, aldehyde and hydrate ethoxy methylene), 3.28 (s, 3H, aldehyde and hydrate methyl ether).



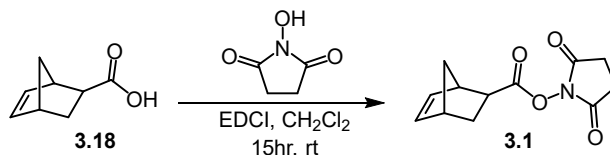
Compound 3.13. To a 2.5mL vial equipped with a stir bar add 20mg **3.12** (52.7 μ mol, 1 equiv.), dissolve in 200 μ L 4M HCl-Dioxane, and allow the reaction to proceed for 4 hours before concentrating. Remove the excess HCl by azeotrope with 3x 1mL portions CHCl_3 . Yield: $\geq 95\%$. Product is a greenish tinted clear oily solid. ^1H NMR (500 MHz, Methanol- d_4) δ 3.72 – 3.47 (m, 20H).



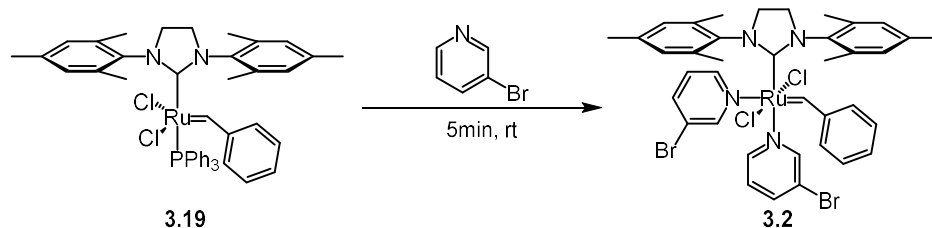
Compound 3.17. To a 6mL vial equipped with a stir bar add 7.6 mg (22.1 μ mol, 1 equiv.) aldehyde **5.11**. Prepare a solution of 100mg/mL Amino-PEG₄-Hydrazide Dihydrochloride **5.13** in 50mM pH 6 acetic acid (AcOH)/ sodium acetate (NaOAc) buffered ethanol (EtOH), add 221 μ L (22.1 μ mol Hydrazide, 1 equiv.) of the stock solution to the reaction vial, heat to 60 $^\circ\text{C}$, and allow the reaction to proceed for 3 h before concentrating. Purify the material by pipette column using C-18 functionalized silica gel and 12mM pH 5 AcOH/NaOAc buffered 60 vol.% $\text{H}_2\text{O}:\text{CH}_3\text{CN}$ expecting product **5.9** to elute in fractions 2 and 3 (0.5mL fractions). Product **5.9** is a white powder with $R_f = 0.22$ on C-18 TLC plates using the column mobile phase. Yield: 39%. ^1H NMR (400 MHz, Methanol- d_4) δ 8.13 (s, 0.57H, trans-hydrazide), 7.78 (d, $J = 8.2$ Hz, 0.3H, cis-hydrazide), 7.68 (d, $J = 8.1$ Hz, 1.16H, trans-hydrazide), 7.48 (d, $J = 8.0$

Hz, 0.65H, cis-hydrazide), 5.08 (s, 2H), 4.64 – 4.52 (m, 2H), 3.86 (dt, $J = 9.7, 6.0$ Hz, 2H), 3.80 – 3.55 (m, 22H), 3.40 (s, 3H), 3.15 (q, $J = 5.6$ Hz, 2H). M/Z $[M+H]^+$
Calculated: 605.3042, Measured: 605.3043.

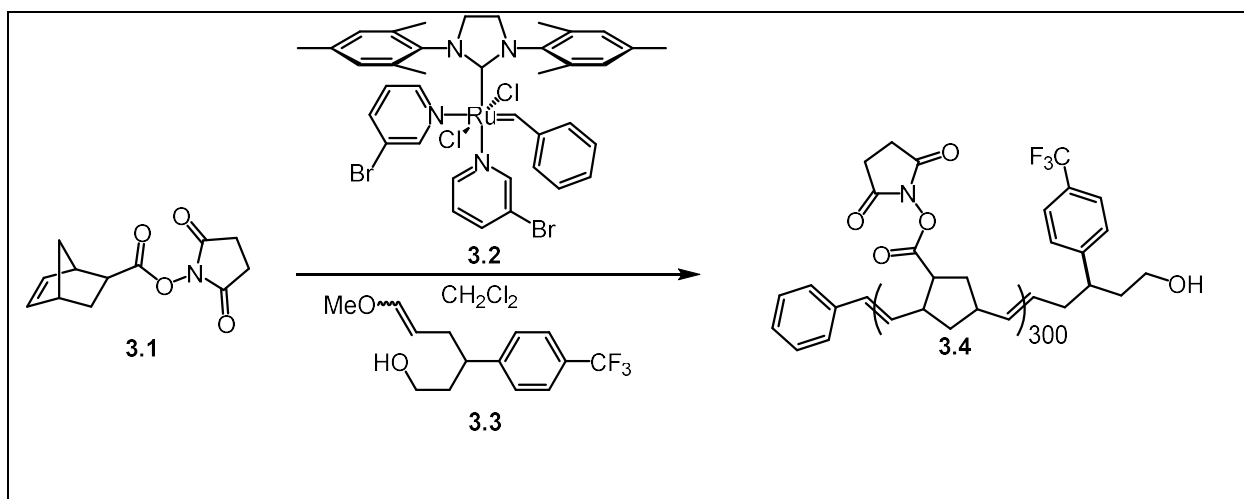
Monomer, Catalyst and Polymer Synthesis



Compound 3.1: Compound **3.1** was synthesized according to a modified procedure for compound 15-NHS in the cited reference.¹⁵⁴ To a flame dried N_2 flushed 100mL round bottom flask add 2g Exo-Norbornene-5-Carboxylic Acid **3.18** (14.5mmol, 1 equiv.), 2.33g N-Hydroxysuccinimide (20.2mmol, 1.4 equiv.), 3.05g 1-Ethyl-3-(3-Dimethylaminopropyl)carbodiimide•HCl (15.9mmol, 1.1 equiv.), dissolve in 24.1mL dry CH_2Cl_2 , and allow the reaction to proceed overnight before concentrating. The reactant solution remains clear and colorless throughout. Partition the concentrate between 90mL each EtOAc and H_2O , extract the aqueous phase with a further 2x 90mL portions EtOAc, combine the organic phases, wash with 2x 45mL portions saturated NH_4Cl , 2x 90mL portions saturated $NaHCO_3$, 1x 90mL portion brine, dry over $MgSO_4$, and concentrate. Product is an off-white solid. Average Yield: 80%. Minimum Yield: 73%. Maximum Yield: 88%. ^1H NMR (600 MHz, Chloroform- d) δ 6.23 (dd, $J = 5.7, 3.0$ Hz, 1H), 6.17 (dd, $J = 5.7, 3.1$ Hz, 1H), 3.32 – 3.24 (m, 1H), 3.02 (m, 1H), 2.86 (d, $J = 6.6$ Hz, 5H), 2.53 (ddd, $J = 9.0, 4.5, 1.4$ Hz, 1H), 2.07 (dt, $J = 12.0, 3.8$ Hz, 1H).



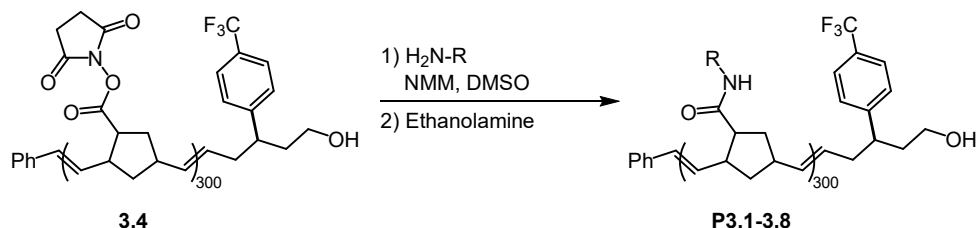
Compound 3.2: Catalyst **3.2** was synthesized according to a modified literature procedure for compound 6 in the cited reference.¹⁵⁵ To a 24mL vial add 200mg Grubbs' II catalyst **3.19** (236 μ mol, 1 equiv.), 223 μ L 3-Bromopyridine (2.33mmol, 10 equiv.), and stir to a bright green paste over 5 minutes with a glass rod. To this paste add 4mL Hexanes and cool to 4°C in a refrigerator overnight after which the supernatant has taken on a faint yellow-green color. After cooling overnight, collect the catalyst by filtration over a Büchner funnel and rinse the filtrate with 3x 4mL portions Hexanes. Allow the catalyst to air dry overnight before storage. Product is a bright green powder which is best stored under N₂ for periods of more than a few weeks. Average Yield: 73%. Minimum Yield: 54%. Maximum Yield: 88%.



Polymer 3.4. Polymer **3.4** was synthesized according to a modified procedure for polymer 2a in the cited reference. To a flame dried N_2 -flushed 5mL round bottom flask add 100mg monomer **3.1** (425 μmol , 300 equiv.), dissolve in 1.32mL dry CH_2Cl_2 , and cool to -15°C in a brine slush. In a flame dried N_2 -flushed 6mL vial prepare a solution of 12.5mg/mL Grubbs' III catalyst **3.2** in dry CH_2Cl_2 , add 100 μL of the catalyst stock (1.41 μmol , 1 equiv.) to the reactant solution and allow the reaction to proceed at -15°C for 15 minutes. After stirring for 15 minutes, quench by addition of 100 μL of a 310mg/mL solution of vinyl ether **3.3** (113 μmol , 80 equiv., synthesized as described in the cited reference) and allow the solution to warm to room temperature as the reaction proceeds for 1 hour. After stirring for 1 hour, purify the material by precipitation by adding the reactant solution dropwise to 80mL ether, centrifuging and decanting. Allow the precipitate to air-dry for at least 1 hour before taking up in 2mL CH_2Cl_2 and repeating the precipitation process once more. Product is a white solid as obtained from precipitation which concentrates to a translucent pink-brown film if rotovapped. Capping efficiency $\sim 80\%$. PDI = 1.10. ^1H NMR (600 MHz, Methylene Chloride- d_2) δ 7.59 (m, 1.76H ortho $-\text{CF}_3$ phenyl signal), 7.35 (m, 6.1H, phenyl cap and meta $-\text{CF}_3$

phenyl signal), 5.78 – 5.12 (m, 852H, solvent overlap on alkenes), 3.61 – 2.99 (m, 300H), 2.81 (d, $J = 18.7$ Hz, 1352H), 2.27 (d, $J = 16.7$ Hz, 243H), 2.04 (d, $J = 23.1$ Hz, 229H), 1.60 (s, 227H), 1.46 – 1.07 (m, 262H).

Polymer Functionalization General Procedure



To an Eppendorf tube was added a 0.1 M solution of **3.4** in DMSO (2.18 mg, 9.267 μmol monomer units, 1 eq), a 20.0 mg mL^{-1} (w/v) solution of CatD-sensitive Influenza peptide in DMSO (1.37 mg, 0.463 μmol , 0.05 eq), and a 20.0 mg mL^{-1} (w/v) solution of CatD-sensitive IL10 peptide in DMSO (0.961 mg, 0.463 μL , 0.05 eq). *N*-Methylmorpholine (20.4 μL , 0.185 mmol, 20 eq) was added. The tube was capped and placed on a mechanical rotator to mix for 6-7 h at room temperature. A 20.0 mg mL^{-1} (w/v) solution of IL10 peptide **5.5** in DMSO (5.25 mg, 3.71 μmol , 0.40 eq) was added. The tube was allowed to mix for 15-20 h. A 0.1 M solution of TLR7 ligand **3.17** (0.280 mg, 0.463 μg , 0.05 eq) was added and mixed for 4 h. Ethanolamine was added (22.4 μL , 0.37 mmol, 40 eq) and allowed to mix for 2-3 h. The polymer solution was diluted 5-10 fold with water and loaded onto a 3 mL 20K MWCO dialysis cassette (Pierce). The polymer solution was dialyzed into 1L water for 24 hours with the water reservoir being changed 4-5 times. The sample was concentrated by lyophilization to yield a white flocculent solid. ^1H NMR spectroscopy in DMSO- d_6 was used to determine the percent TLR7 ligand substitution and ^{19}F NMR spectroscopy was used to determine the percent IL10,

CatD-Influenza, and CatD-IL10 substitution on **P3.4**. The same procedure was used to prepare all functionalized polymers (**P3.1 – P3.8**), but with modification of the specific ligand combinations. Polymer stock solutions were prepared in USP grade DMSO (3 mg/mL IL10 peptide, 20 – 30 μ M polymer) and used for subsequent experiments.

Cathepsin D Cleavage Assay

To ensure that the CatD-sensitive IL10 and Influenza epitopes were substrates for CatD they were subjected to an *in vitro* cleavage assay. Peptide substrates were dissolved in DMSO to generate a 25 mM stock solution. These peptides were diluted into assay buffer (0.1 mM NH_4OAc , 0.2 mM NaCl , pH 3.5) to yield a 25 μ M solution. Recombinant murine cathepsin D (R&D Systems) was activated in assay buffer at rt at 20 μ g/mL for 10 minutes before dilution to 2.5 μ g/mL in assay buffer. Activated cathepsin D and peptide substrate solutions were combined 1:1 to begin the digestion assay (final volume 100 μ L). The reaction was quenched via the addition of 1% (v/v) TFA. Samples were lyophilized, resuspended in 1:1 (v/v) $\text{CH}_3\text{CN}:\text{H}_2\text{O}$, and desalted with MALDI beads. Desalted samples were spotted with α -cyano-4-hydroxycinnamic acid matrix solution prior to MALDI-TOF analysis (Bruker Microflex).

Hen Immunizations

Animals were maintained in accordance with the University of Wisconsin-Madison Research Animal Resources Center and College of Agricultural and Life Sciences Animal Care Committee, both of which adhere to both national and local guidelines. Hyline W36 broiler hens in lay were individually caged in battery cages on raised wire flooring and fed a standard commercial diet. Protein conjugate or polymer (300 μ g IL10) in 1.5

mL sterile PBS was formulated with equivalent volume of Freund's Complete Adjuvant (primary, day 0) or Incomplete Adjuvant (booster, day 21). Each bird was administered 1 mL of vaccine formulation intramuscularly in the breast or thigh muscle. Eggs were collected daily, recorded, and stored in a temperature-controlled egg cooler until sampled.

Measurement of Egg Yolk Anti-IL10 IgY

Enzyme-linked immunosorbent assay (ELISA) was used to quantify IL10 peptide specific IgY in egg yolk. IL10 peptide-conjugated ovalbumin was prepared with the previously described glutaraldehyde procedure.^{225,226} IL10-Ova protein conjugate was diluted 1:1000 in sodium carbonate buffer, pH 9.6, and incubated overnight at 4°C (100 µL/well) in a Costar EIA/RIA flat bottom polystyrene 96 well plate. The plate was washed with PBS tween 20 and blocked with protein-free blocking buffer for 1-2 h at rt. Affinity purified egg yolk IgY specific to the IL10 VLPRAMQT peptide sequence (GeneTel Lab, Madison, WI) with a known concentration was used as a standard curve starting at 425 ng/mL and diluted two-fold in 1% milk seven times. Egg yolk samples were prepared by diluting egg yolk (1:10) in acidified PBS pH 4.5, vortexing, and incubating at 4 °C overnight. Samples were centrifuged at 3000 x *g* for 10 minutes, and supernatants were diluted in 1% milk at 1:100-1:200 and added to the blocked plate (100 µL/well). The samples and standards were incubated for 1 h and then discarded. The plate was washed five times with PBS tween. Goat anti-chicken IgY-HRP (Bethyl) was diluted in protein free blocking buffer at 1:10,000, added to the plate, and incubated for 30 minutes at rt. After washing the plate six times, 100 µL 1-step TMB-ELISA was added to the plate and allowed to develop until the standard was adequately developed

when 0.5 M H₂SO₄ was then added to the plate. An EL800 plate reader was used to read absorbance at 506 nm. Samples were compared to the standard curve on each plate to determine IL10 VLPRAMQT peptide-specific antibody titers (expressed at IgY/egg yolk [mg/mL]).

Statistical Analysis

Comparisons of egg yolk anti-IL10 IgY were made using two-way analysis of variance (ANOVA), followed by Dunnett's test for multiple comparison with Prism 8 (GraphPad software). *P* values less than 0.05 were considered statistically significant and marked with a single asterisk. *P* values less than 0.002 were marked with two asterisks. *P* values less than 0.0002 were denoted with three asterisks. *P* values less than 0.0001 were marked with four asterisks. All values are reported as the mean ± SEM.

Acknowledgements

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Chapter 4: Chemoselective, Post-Polymerization Modification of Bioactive, Degradable Polymers

Reproduced with permission from Fishman, Joshua M.; Zwick, Daniel B.; Kruger, Austin K.; Kiessling, Laura L. Chemoselective, Post-Polymerization Modification of Bioactive, Degradable Polymers. *Biomacromolecules*. **2019**, *20* (2), 1018-1027.

Contributions: Polymer synthesis and characterization were performed by Joshua Fishman and Austin Kruger. Cellular assays and microscopy were performed by Daniel Zwick.

4.1 *Abstract*

Degradable polymers are a necessary advancement for promoting sustainability and addressing unmet medical needs. Tailoring degradable polymers is challenging because installing functional group-rich side chains is difficult when the backbone itself is susceptible to degradation. A convenient means of side chain installation is through post-polymerization modification (PPM). In functionalizing polyoxazinones, a class of degradable polymers generated by the ring-opening metathesis polymerization (ROMP), we predictably found PPM challenging. Even the versatile azide–alkyne cycloaddition click reaction was ineffective. To solve this problem, we screened PPM reactions whose efficiencies could be assessed using photochemistry (excimer formation). The mildest, pH-neutral process was functionalization of a ketone-containing polymer to yield either oxime (acid labile)- or alkyoxylamine (stable)-substituted polymers. Using this approach, we equipped polymers with fluorophores, reporter groups, and bioactive epitopes. These modifications imbued the polymers with distinctive spectral properties and biological activities. Thus, polyoxazinones are now tunable through a modular method to diversify these macromolecule's function.

4.2 *Introduction*

Synthetic polymers have proven invaluable tools for investigation of otherwise recalcitrant biological systems.¹²² While the degree of control now possible over polymer structure is immense²²⁷ and has demonstrated ability to profoundly influence biology,^{58,67,196,228} most synthetic polymers possess non-degradable backbones severely limiting their applications in medicine.^{229,230} The identification of new degradable materials is therefore valuable;²³¹ yet the challenge is to ensure that degradable polymers possess properties consummate to their extant non-degradable counterparts including control over structure and function.

A handful of polymerization reactions can yield degradable polymers with control over molecular weight and dispersity. For example, the anionic ring-opening polymerization (ROP) of cyclic monomers with hydrolysable linkages such as esters, carbamates, and phosphoesters can introduce degradable functionality into a linear polymer backbone.²³² Still, ROP must be conducted under stringently dry conditions, the monomers must be of ultrahigh purity, and the monomers cannot contain functionality susceptible to transesterification or termination reactions during polymerization. Chemoselective “click” reactions²³³ can enable polymers generated by ROP to be modified via postpolymerization modification (PPM). Although multiple click reactions have been used in PPM to generate specialty non-degradable polymers, many of these functionalization reactions can lead to backbone degradation.^{234,235} To mitigate side reactions, click PPM reactions on ROP backbones must be optimized to favor efficient conjugation over degradation.

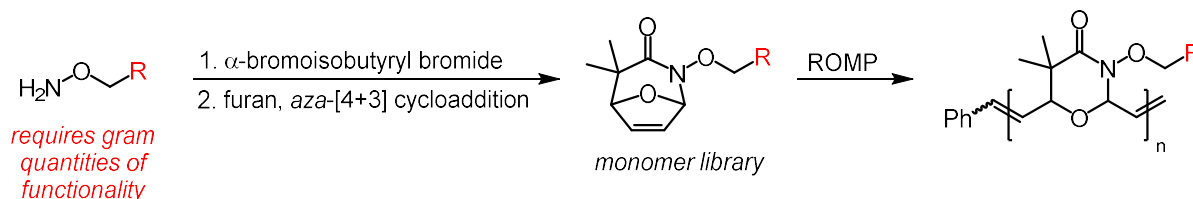
An alternative approach is to employ a living radical copolymerization (LRP) of functionalized monomers. For example, reversible addition–fragmentation chain

transfer (RAFT) polymerization can be used to intersperse ester linkages within a poly(vinyl acetate) backbone.²³⁶ While RAFT polymerization is more air and moisture tolerant than anionic ROP and can proceed in aqueous media,^{237–239} the cyclic ketene acetal comonomers needed to introduce degradable linkages require specialty handling.²³² Additionally, these cyclic monomers suffer from low incorporation into the polymer chain,^{240–242} with the exception of specific monomer pairs.^{243,244} We therefore examined alternative methods to generate degradable, functionalized polymers.

Like ROP and RAFT, the ring-opening metathesis polymerization (ROMP) can be a living polymerization that affords defined polymers that can be tailored for multiple desired applications.^{245,246} Metathesis has multiple benefits:^{155,247} catalysts can polymerize monomers that possess polar functional groups, the reaction can occur at room temperature, and the polymerization kinetics are fast. These features of ROMP have yielded scaffolds that can be precisely tailored for myriad purposes including materials,^{248–251} electronic,^{252,253} and biological applications.^{212,254–260} Indeed, ROMP can accommodate functionality incompatible with other polymerization methods. Additionally, the molecular weight, dispersity, and backbone architecture of ROMP products are all controllable through judicious choice of polymerization conditions.^{261,262} Pragmatically, ruthenium catalysts are air and moisture compatible; however, with few exceptions,^{263–268} they afford polymers consisting of backbones that are not degradable. While recent reports of degradable materials from ROMP are promising, many rely on introduction of degradable linkages by copolymerization which yields only partially degradable materials.^{265,266,269} We sought to use ROMP to generate fully degradable polymers with diverse functionality.

To this end, we reported that bicyclic oxazinones undergo ROMP to afford polyoxazinones,²⁶³ which are stable yet degradable under acidic or basic conditions. We diversified the polyoxazinone backbone by generating a monomer library with various hydroxamic ester substituents (Figure 4.1A).

A) Previous Work - polymer diversification achieved using *grafting-through* approach



B) This Work - polymer diversification achieved using post-polymerization modification

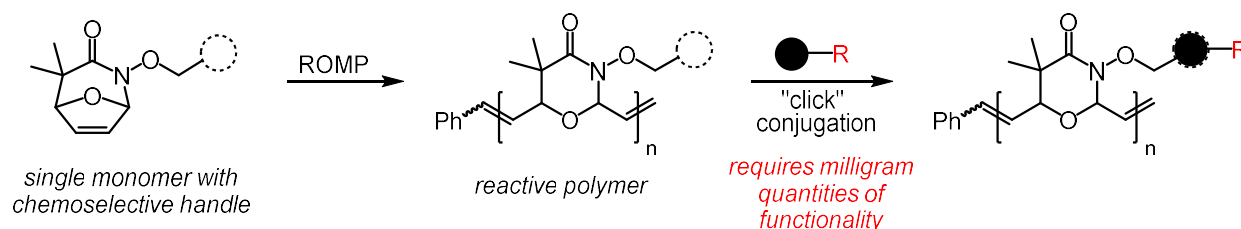


Figure 4.1. ROMP can afford degradable polyoxazinones for tailoring through two strategies: A) *grafting-through* using pre-functionalized monomers or B) post-polymerization modification (PPM) of a reactive polymer precursor.

The monomers were synthesized in a two-step sequence starting from a range of *O*-functionalized hydroxylamine precursors.²⁷⁰ This approach requires that each unique monomer be accessed separately. The efficiency of producing tailored polyoxazinones via the grafting-through approach previously described could be improved using PPM. A grafting-from PPM strategy is advantageous for generating bioactive polymer probes because fluorophores and biological ligands are often costly to produce and only available in milligram quantities. Furthermore, PPM enables the rapid synthesis of polymers decorated with multiple functional moieties with control and standardization

of polymer chain length between samples. Such features are important when testing differences in bioactivity due to ligand type or density.^{64,122,146,271–280}

We envisioned an effective PPM strategy would streamline the synthesis of bioactive polyoxazinones (Figure 4.1B). Since we previously demonstrated that polyoxazinones degrade when the pH is below 4.5 or above 10,²⁶³ what was needed was an efficient chemoselective reaction occurring at neutral pH values. Several transformations failed, but oxime formation was effective. Reaction of ketone-bearing polymers with alkoxyamines afforded high degrees of conjugation without decomposition. The efficiency of conjugation was assessed by quantifying excimer fluorescence, a rapid measure of the extent of modification. The synthetic strategy was used to generate cell-permeable polymers and synthetic antigens, highlighting the utility and biocompatibility of functionalized polyoxazinones.

4.3 Results

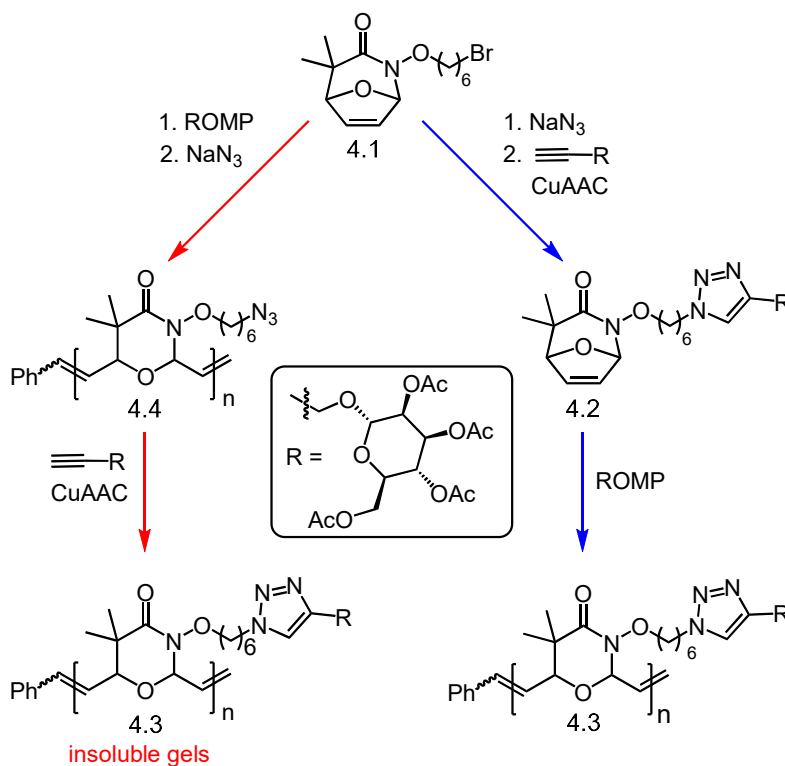
The click azide-alkyne cycloaddition (CuAAC) has been useful in generating tailored materials²⁸¹ and was an attractive avenue to explore for PPM of polyoxazinones. given its high chemoselectivity and occurrence at neutral pH. We could not access azide-substituted polymers by direct polymerization of azido-monomers, as azide groups can react with ruthenium catalysts, but we could access azido-polymers through halide displacement.²⁸² We previously reported the synthesis of bicyclic oxazinone monomer **4.1** bearing an alkyl halide (Scheme 4.1).²⁶³ Monomer **4.1** was subjected to ROMP and the resulting polymer was exposed to sodium azide to yield polyoxazinone **4.4**, a polymer poised for PPM via CuAAC. We anticipated this reaction would be successful, as similar processes had been used to modify nondegradable ROMP

polymers.^{146,276,278,280,283}

However, polymer **4.4** was not transformed to the desired polymer but rather to an insoluble gel (Scheme 4.1). Gel formation was unexpected as the polymer product generated by a different procedure was soluble. Specifically, we had previously converted monomer **4.1** to the corresponding azide and appended a peracetylated

propargyl mannoside using the click reaction to afford monomer **4.2**. Monomer **4.2** was successfully polymerized to afford well behaved, protected glycopolymer **4.3**. Thus, the polymer expected from CuAAC on **4.4** should be soluble, but it was not. The insolubility of **4.4** appeared to arise from its ability to chelate to intermediate organometallic complexes formed during copper-mediated azide–alkyne cycloaddition. Specifically, when polymer **4.4** was stirred under conditions amenable to CuAAC, but without the alkyne present, no gelation or side reactions were observed. Thus, while CuAAC can be used for monomer functionalization, it was ineffective for PPM (Scheme 4.1). Copper-free azide–alkyne cycloaddition occurs with the use of strained alkynes.^{284,285} Indeed, exposure of polymer **4.4** to commercially available azadibenzocyclooctyne-acid

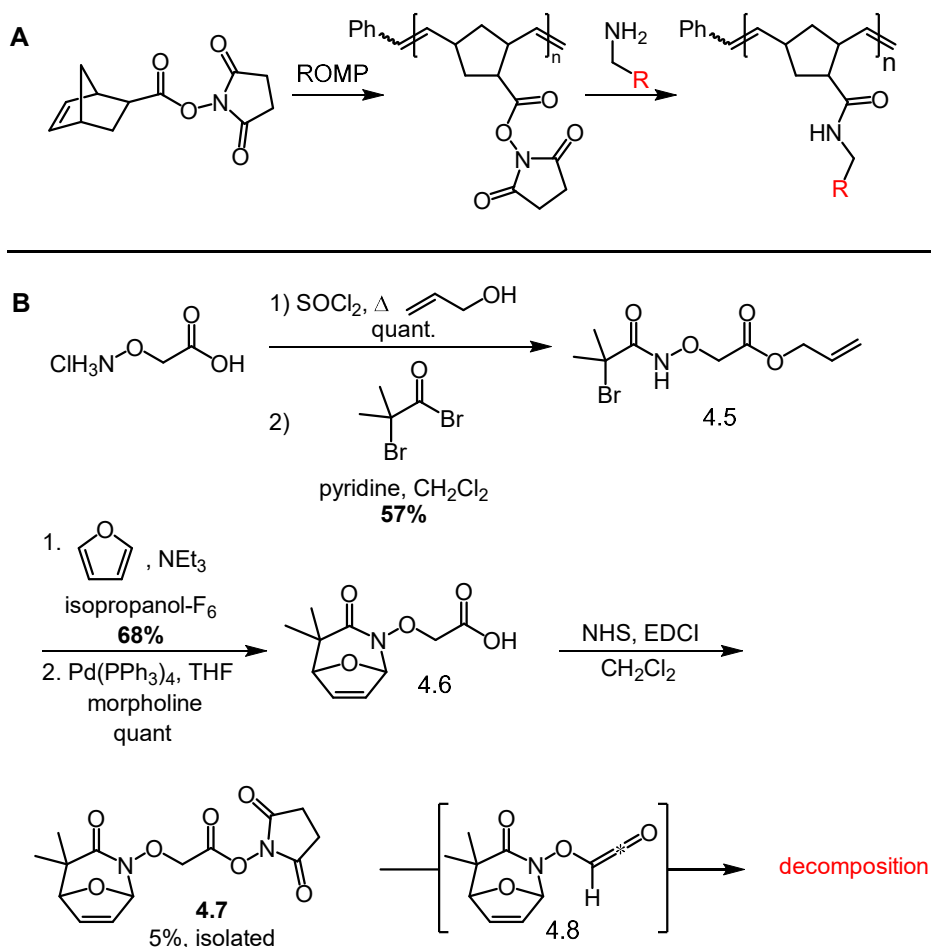
Scheme 4.1. CuAAC Affords Monomer 4.2 Variants but Its Application to Polymer 4.4 Afforded an Insoluble Gel



(ADBCOA) led to a non-cross-linked polymer with >90% of the azide moieties converted to the expected triazole product (see Experimental Details). However, the use of ADBCOA affords a polymer product with large, hydrophobic groups; even with free acids present the product was insoluble in aqueous solutions. Additionally, the polymer product from the strain-promoted click reaction increased in dispersity from 1.4 to 2.1, which is undesirable when precisely defined multivalent biological probes are needed. Therefore, we searched for a useful alternative chemoselective handle.

Polynorbornene substrates bearing N-hydroxysuccinimidyl esters can be elaborated to generate compositionally complex macromolecules (Scheme 4.2A),^{256,274,286}

Scheme 2. Unexpected Instability of Monomer Bearing an Activated Ester

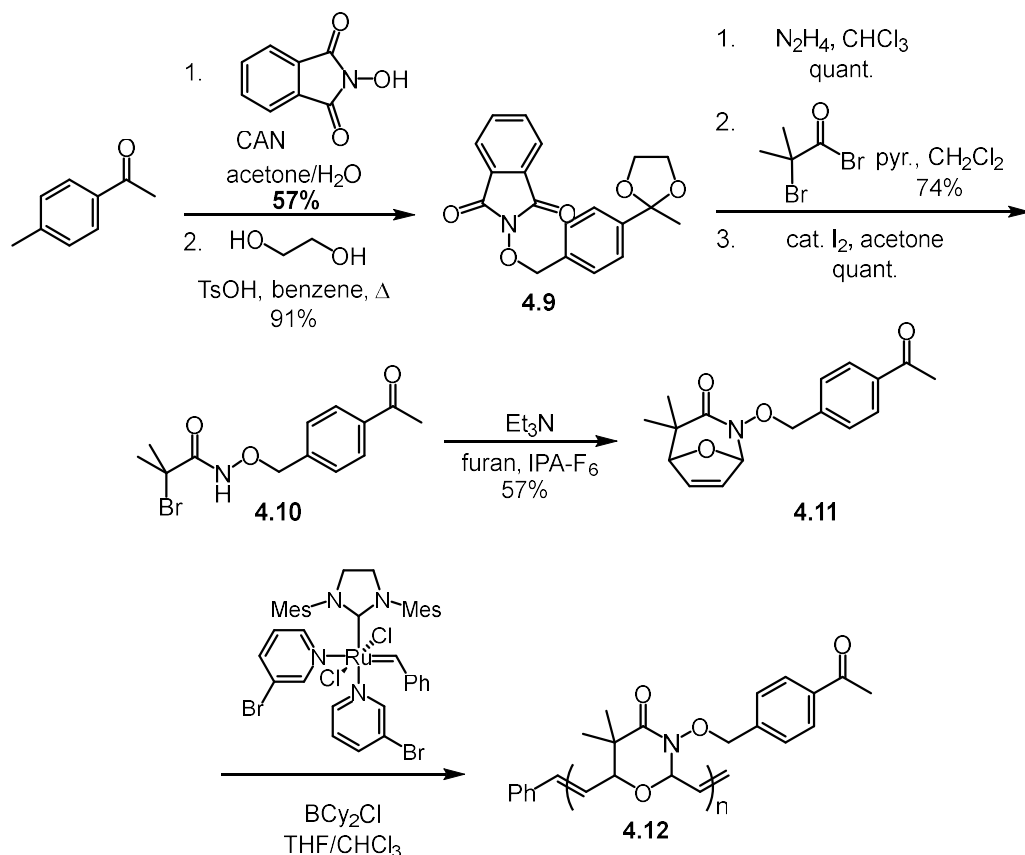


Polymers bearing succinimidyl esters can undergo facile functionalization by PPM, as amine-containing ligands can be appended via amide bond formation. To capitalize on this strategy, we targeted the synthesis of a bicyclic oxazinone monomer containing a succinimidyl ester (Scheme 4.2B). Starting with *O*-(carboxymethyl) hydroxylamine, the carboxylic acid was esterified with an allyl group and then acylated with bromoisobutyryl bromide (BIBB) to afford hydroxamic ester **4.5**. Bicyclic oxazinone **4.6** was subsequently generated using an aza-[4+3] cycloaddition with furan followed by allyl ester removal. The latter process could be conducted in the presence of the bicyclic olefin using tetrakis(triphenylphosphine) palladium and morpholine to yield carboxylic acid **4.6**. Attempted esterification with N-hydroxysuccinimide afforded the desired monomer **4.7** in low yield. We surmise that the modest yield of **4.7** results from the production of ketene **4.8**. This ketene intermediate could participate in side reactions, such as a cycloaddition with the bicyclic olefin, to erode yields. This mode of ketene formation has been observed in other systems in which a carboxylic acid with an electron withdrawing heteroatom at the α carbon was activated for esterification.²⁸⁷ We therefore pursued an alternative approach.

Macromolecules have been modified previously using chemoselective oxime formation. In one manifestation, proteins engineered to contain the unnatural amino acid *p*-acetyl phenylalanine react selectively with alkoxyamines.^{288–290} Inspired by these studies, we focused on synthesizing a polyoxazinone displaying an acetophenone derivative. Compound **4.9** is readily accessible in gram quantities (Scheme 4.3);²⁹¹ therefore, we were attracted to it as a building block to assemble acetophenone-containing bicyclic oxazinones and polyoxazinones.

The oxime PPM strategy was readily implemented. The required bicyclic oxazinone monomer was assembled via a 1,3 dipolar cycloaddition between compound **4.10** and furan. Compound **4.10** was accessed from *p*-methylacetophenone. After installation of the protected alkoxyamine, the ketone was converted to a 1,3-dioxane **4.9**. Carbonyl protection was used to avoid self-condensation upon liberation of the free hydroxylamine. The resulting compound was acylated to generate the α -bromo hydroxamic ester, and the dioxane group was removed using mild conditions (catalytic iodine) to afford ketone **4.10**. Mild conditions were critical as α -bromohydroxamic esters rapidly decompose to acrylamide N-oxides in acidic or basic solutions.

Scheme 4.3. Synthesis of Ketone Bearing Polymer



Finally, oxazinone monomer **4.11**, displaying an acetophenone moiety, was successfully generated from an aza-[4 + 3] cycloaddition with furan. Our initial attempts to

polymerize monomer **4.11** using a solvent system of tetrahydrofuran (THF) and chloroform afforded polyoxazinone **4.12**, but conversion was unexpectedly low (Table 4.1, entry 6). Substrates that contain hydroxamic esters can be problematic for such metathesis reactions because the precursors and their polymer products can chelate the metallocarbene catalyst to stymy turnover.^{263,292} One potential solution is to add a Lewis acid that binds to polar groups and thereby frees the carbene catalyst to mediate metathesis.²⁹² To this end, we added chloro-dicyclohexylborane (10 mol % BCy₂Cl) to the reaction mixture. The result was increased polymer conversion and good molecular weight control over a range of monomer to catalyst loading ratios (Table 4.1).

Table 4.1. ROMP of Monomer 4.11

[4.11] ₀ /[cat]	Conv (%) ^a	<i>M</i> _n ^{GPC} (g/mol) ^b	PDI ^b
15/1	83	7800	1.3
25/1	87	13700	1.5
50/1	87	16000	1.5
100/1	89	23400	1.4
200/1	84	40000	1.9
200/1	24	25800	2.0

^aBased on ¹H NMR integrations of monomer olefin signals to polymer olefin signals. ^bCalibrated with polystyrene standards, eluted 1.0mg/mL in THF. ^cWithout BCy₂Cl.

We next investigated methods to elaborate acetophenone-functionalized polymer **4.12**. Nondegradable polynorbornene-derived polymers containing ketone functionality have been decorated through formation of acyl hydrazones,^{276,293} but these linkages are acid labile, which would result in the release of the bioactive group from the polymer backbone. For bioactive polymers to function as probes, they must be stable. We turned to oxime linkages because they are more hydrolytically stable than acyl hydrazones at physiological pH.²¹¹ To generate these polymers, we envisioned exposing alkoxyamines to ketone-containing polymers.

The conjugation reactions of alkoxyamine ligands to ketone-bearing biomacromolecules, such as proteins, are typically conducted in acetate buffer.^{290,294}

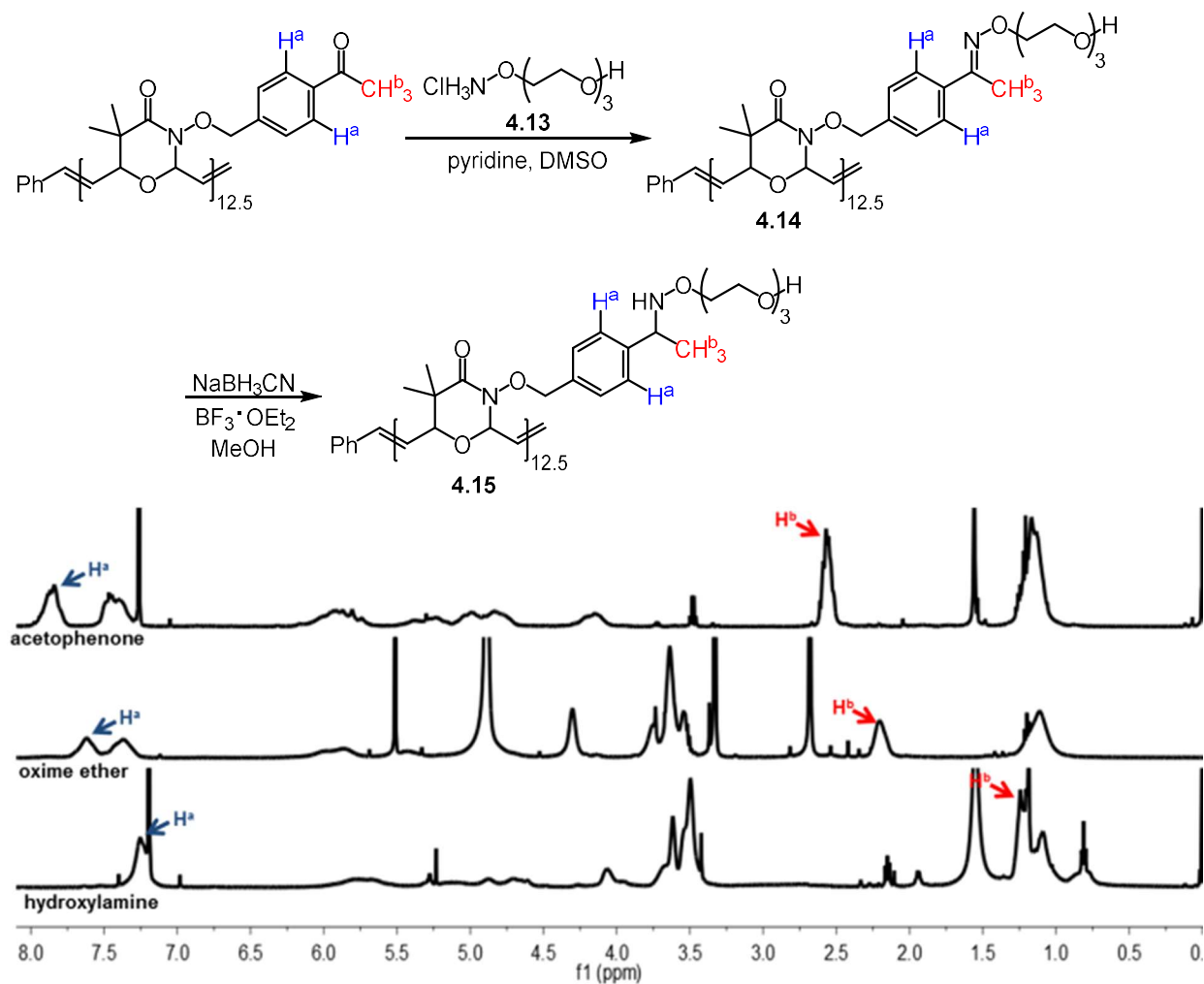


Figure 4.2. Postpolymerization modification of ketone-containing **4.12** with alkoxyamine **4.13** affords oxime-substituted polymer **4.14**. Subsequent chemoselective reduction afforded an alkylamine-displaying polymer **4.15**. The ^1H NMR data indicate that the reactions proceeded with high efficiencies. The low aqueous solubility of polyoxazinone **4.12** and the acid lability of the oxazinone linkages of the polymer backbone render these conditions incompatible. We therefore used an oligoethylene glycol ligand **4.13** terminated with a hydroxyamine to screen

alternative conditions (Figure 4.2). With a dimethyl sulfoxide and pyridine cosolvent, we used an excess of alkoxyamine **4.13** (1.5–2.0 equiv) to produce oxime conjugate

4.14.²⁹⁵ Proton NMR signals indicative of aryl ketones were present in the starting material ($H^a = \delta$ 7.8 ppm;

$H^b = \delta$ 2.6 ppm), and

within 24 h of

functionalization, signals

consistent with oxime

4.14 ($H^a = \delta$ 7.6 ppm; $H^b = \delta$ 2.2 ppm) were

detected (Figure 4.2).

Analysis of the resulting

sample with gel

permeation

chromatography (GPC)

revealed a shift in

retention time to a

shorter elution volume

(Figure S4.1) consistent

with the expected

increase in polymer mass upon modification. These data indicate that hydroxylamine ligands can be attached with negligible backbone decomposition and with remarkably high conjugation efficiency.

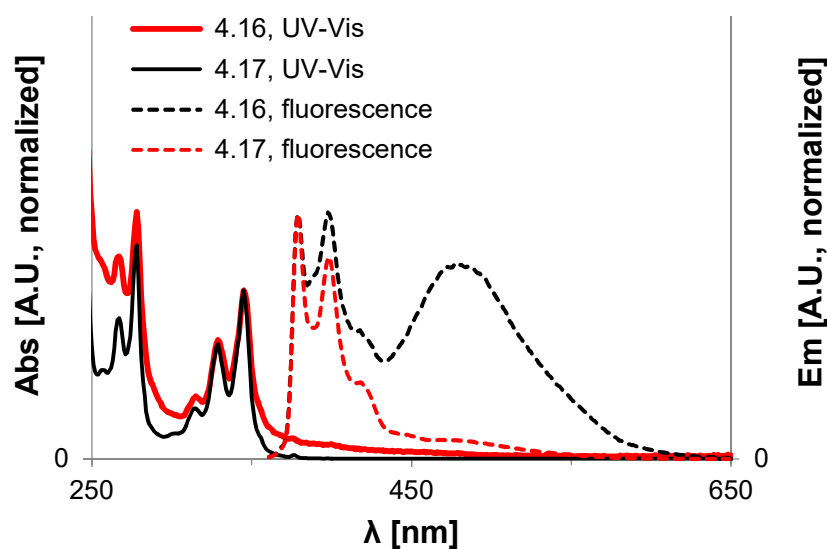
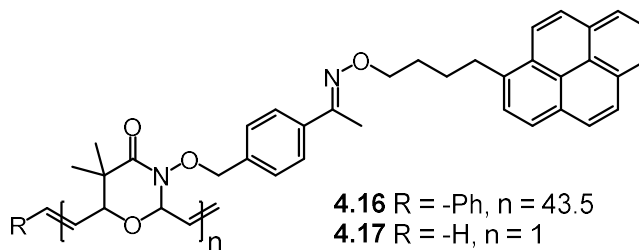


Figure 4.3. Polyoxazinone **4.16** was generated by exposing polymer **4.12** to *O*-(4-pyrenylbutyl)hydroxylamine. The resulting polymer **4.16** displays excimer fluorescence but monomer **4.17** does not. Representative UV-Vis and fluorescence spectra are shown at 50 μ M pyrene, a concentration that does not promote solvent-induced dimerization (see Experimental Details).

We also verified the post-polymerization modification of **4.12** by exploiting the differences in optical properties of proximal chromophores. These spectroscopic changes can be observed using a fluorimeter, instrumentation that is present in most biological laboratories. Specifically, we exposed the ketone bearing polymer to *O*-(4-pyrenylbutyl) hydroxylamine to afford pyrene-substituted polymer **4.16** (Figure 4.3). When two pyrene derivatives are proximal, they give rise to spectra indicative of excimer formation.²⁹⁶ Excimer formation manifests as a broad fluorescent emission band centered at 480 nm, and the ratio of excimer to monomer emission can be quantified [Ex(480)/Ex(378)]. When polymer **4.16** was exposed to 50 μ M pyrene, an emission band at 480 nm was observed leading to an exciplex ratio of 0.79, a value indicative of excimer formation (Figure 4.3). Pyrene concentrations up to 500 μ M gave similar Ex(480)/Ex(378) ratios. In contrast, in this concentration range, neither free hydroxylamine-pyrene ligand nor model “1-mer” **4.17** afforded an exciplex band. Thus, excimer formation from polymer **4.16** results from a high local concentration of the pendant residues on the polymer backbone. The consistent excimer ratios for **4.16** are indicative of efficient oxime formation. Therefore, exciplex formation can be used to qualitatively confirm PPM in a simple and rapid test.

An advantage of PPM is that it allows for the attachment of multiple, pendant groups in a single step. This feature enables study of polymer structure-activity relationships: differentially modified polymers can be readily compared as their backbones are identical. A wide range of different moieties, including ligands and fluorophores or other reporter groups, can be appended through PPM.²³⁵ Bioactive ROMP polymers have been used as probes to optimize and understand the mechanisms by which multivalent ligands affect cell surface receptor signaling and function.^{255,297}

Polyoxazinones, however, have not been assessed for biological activity. We therefore synthesized polymer **4.18**, which bears the immunogenic epitope 2,4-dinitrophenol (DNP) and the water-soluble fluorophore AlexaFluor488 (AF488) (Figure 4.4), and tested this agent as an antigen.

A DNP-responsive B cell line was used to assess the ability of polymer **4.18** to activate immune signaling.

Antigen activation and internalization by B lymphocytes are processes critical for immunity, as they can elicit antibody production. B cell responses are activated upon antigen engagement of a membrane-bound immunoglobulin receptor termed the B cell receptor (BCR). A B cell line (A20HL)^{298–300} with a DNP binding BCR can be activated by multivalent displays of DNP epitopes to mediate antigen endocytosis.^{146,212,256,286} Specifically, upon antigen engagement with the BCR, both the ligand and receptor are internalized and rapidly

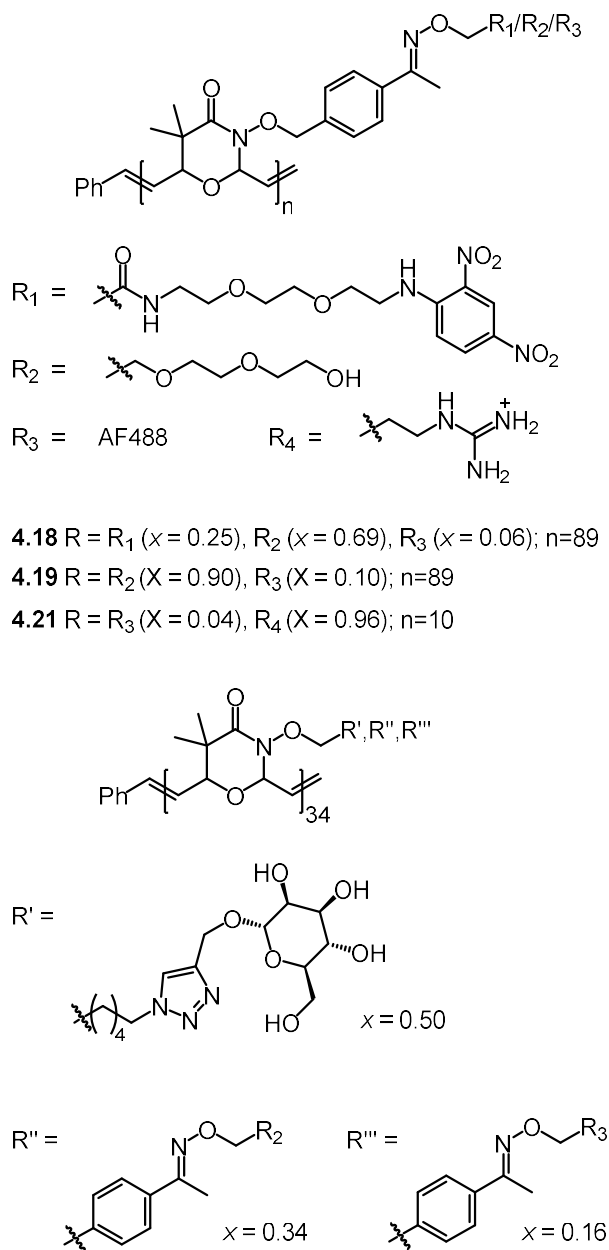


Figure 4.4. Compositions of polyoxazinones modified via PPM with a fluorophore (AAF) and biological recognition elements. x = mole fraction of ligand.

Polymer fluorescence was coincident with that due to the BCR, an observation consistent with BCR-mediated internalization (Figure 4.5B). The internalized polymer and BCR also colocalized with the early endosomal marker transferrin, indicating that the polymeric antigen undergoes trafficking to relevant sites within the immune cells (Figure 4.5C). Finally, internalization was ablated at 4 °C, a finding consistent with uptake via BCR-mediated endocytosis (Figure 4.5A). Taken together, the uptake and trafficking of polymer **4.18** is consistent with it activating physiological processes involved in B cell-mediated immunity.

We carried out additional experiments to assess whether the oxazinone backbone could engage in off-target biological interactions. When DNP-specific B cells were exposed to fluorophore-labeled polymer **4.19**, which bears nonbinding oligoethylene glycol groups, no internalization was observed (Experimental Details Figure 4.2a). Similarly, mannose-substituted polymer **4.20** was not taken up at either temperature (Experimental Details Figure 4.2b). These results indicate the groups displayed on the polyoxazinone backbone, not the backbone itself, are essential for recognition and therefore polymer endocytosis.

We also have been interested in synthesizing polymers that penetrate cell membranes. One solution to this problem is to conjugate a binding epitope to the polymer that can trigger receptor-mediated endocytosis, as described above for the BCR. This strategy can enable cell type-specific uptake; however, compounds that exploit generic uptake mechanisms are also being sought. One such strategy is to incorporate a transporter domain or a protein transduction domain mimic.^{303–306} Short arginine-rich amino acid sequences that can complex with negatively charged sulfated glycosaminoglycans (GAGs) found on the cell surface can promote uptake of compounds

to which they are attached.^{307–309} While the mechanisms underlying internalization are still being elucidated, the process is typically energy-dependent and relies on deformations in the curvature of the membrane.³¹⁰ We previously showed ROMP products functionalized with an oligoguanidinium artificial transduction domain (ATD) are internalized by cells.^{282,310,311} Because uptake and trafficking can be sensitive to molecular features, we tested whether the polyoxazinone backbone can be taken up by cells. Ketone containing polymer **4.12** was decorated with guanidinium groups to afford polymer **4.21** (Figure 4.4). Polymer **4.21** is relatively short because longer oligoguanidinium species are not readily internalized.^{282,305}

Polyoxazinone **4.21** was generated with an ATD using a guanidinium-functionalized hydroxylamine (Figure 4.4). The fluorophore AF488 also was conjugated to visualize internalization. The cell-permeability of the polymer was tested in both A20HL B cells, which express a DNP-specific BCR, and in A20 cells, which do not. Internalization was observed in both cell lines. While uptake occurred readily after 30 min at 37 °C, none was observed at 4 °C (Figure 4.6). The temperature dependence of polymer internalization is consistent with an active mode of cell penetration.³¹¹ These data indicate that polyoxazinones can be functionalized to promote either receptor-mediated endocytosis or ATD-facilitated uptake highlighting the utility of this backbone and the advantages of PPM.

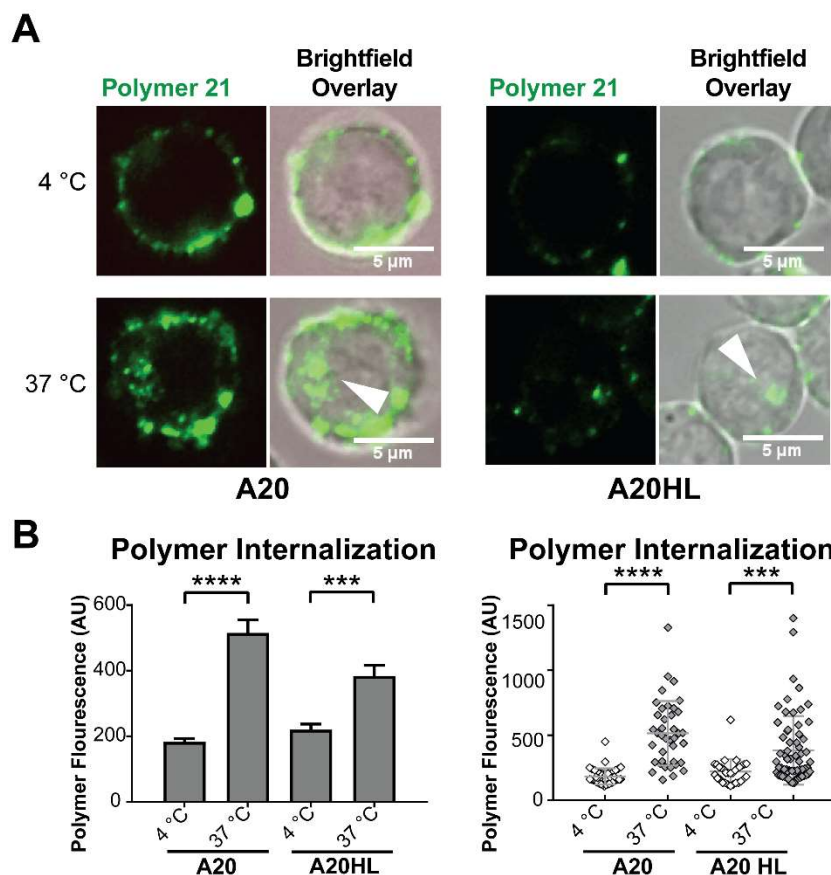


Figure 4.6. Polyguanidinium polymer **4.21** was internalized by A20 and A20HL B cells at 37 °C but not at 4 °C. Arrows indicate intracellular fluorescence. Total internalized and surface polymer fluorescence was measured using at least 30 cells for each condition and shown as bar and scatter plots. *** $P < 0.001$, **** $P < 0.0001$ (Mann–Whitney test). Error bars represent the standard error of the mean for one of 3 independent experiments.

4.4 Discussion

ROMP combines chain length control, fast polymerization kinetics, and tolerance to air and moisture during polymerization. This polymerization method also provides access to synthetic scaffolds that can be elaborated to afford biologically active polymer probes. The use of ROMP to generate polyoxazinones demonstrated that ROMP can afford degradable polymers with tailored functionality from a single monomer. Still, our previously reported grafting-through synthesis of polyoxazinones required quantities of

highly functionalized monomers that can be difficult to obtain and that limit comparisons between polymers bearing different epitopes.

PPM facilitates the synthesis of functionalized backbones from ROMP. Nondegradable ROMP scaffolds have been elaborated using click handles, such as activated esters,^{212,256,274,278,286} azides,^{146,276,280,283} α -chloroacetamides,²⁸² maleimides,²⁷⁸ and ketones.^{276,293} We found that we could decorate polyoxazinones from the polymerization of ketone-containing monomer **4.11**, which is readily accessible through a dipolar cycloaddition. The resulting clickable polyoxazinone scaffold represents a new reactive and degradable scaffold.

Despite the existing collection of chemoselective reactions, the problems we encountered equipping the polyoxazinone backbone with an orthogonally reactive group highlight the intrinsic challenges in modifying degradable backbones for biological studies. We avoided bio-orthogonal reactions that yield a hydrophobic linker, as their application dramatically and unpredictably influenced the properties of the polymers.²³⁵ The CuAAC click reaction has the advantage that the resulting linker is small. Though the CuAAC has been used on some degradable scaffolds, the reaction can be problematic for substrates with a high density of heteroatoms. In our backbone, these heteroatoms are intrinsic to the polymer's ability to undergo degradation, but they apparently suppress the desired click reaction. Attempts to install a succinimidyl ester resulted in monomer decomposition, presumably through ketene formation. Ultimately, we utilized an oxime linkage. The neutral conditions used for its introduction promoted desired transformation over backbone degradation and facilitated the transformation of polymers into biologically active multivalent probes.

We verified the successful PPM of polyoxazinones through both NMR and fluorescence spectroscopy. The latter could detect the conjugation of a pyrene fluorophore, as pyrene exciplex formation was induced.³¹² We anticipate that this approach can be used generally to monitor the efficiency of PPM modification.³¹²

An obstacle to polymer-based therapeutics and polymer probes is accessing the cell's interior.^{313,314} We used oxime formation to generate polymers with biologically relevant functionality and reporter groups. This synthetic strategy afforded compounds that undergo cellular uptake either through receptor-mediated endocytosis or using an artificial transduction domain. To this end, polyoxazinones that can engage the BCR undergo endocytosis by B lymphocytes and thereby function as synthetic antigens. Furthermore, the polymer was routed to endosomal compartments that can lead to antigen processing. Alternatively, the conjugation of guanidinium groups to the polyoxazinone backbone enabled uptake into cells, presumably through a process similar to that employed by protein-transduction domains. Thus, the heteroatom-rich polyoxazinone scaffold is compatible with cellular uptake. The ability to functionalize polyoxazinones opens up avenues to create a new class of polymer probes that can traverse membranes.

4.5 *Conclusions*

The covalent functionalization of macromolecules using PPM is a powerful means of synthesizing functional materials. The identification of new degradable polymer scaffolds amenable to PPM facilitates the production of polymer probes. Our studies show that polyoxazinones can be used as biological probes; their activities are similar to existing polynorbornene or polycyclooctene ROMP scaffolds, but they can be degraded.

Our conditions for polyoxazinone elaboration do not require large quantities of the modifying agent. Additionally, the acid labile oxime bond that results could be converted to a non-hydrolyzable alkoxyamine through reduction. Because PPM is exceptionally valuable when tailoring a scaffold for biological applications, we generated polymers decorated with biologically relevant moieties, including fluorophores, reporter groups, and bioactive epitopes. These modifications imbued the polymers with distinctive spectral properties and biological activities. In this way, the properties of polyoxazinones are now tunable through covalent PPM, an economical and modular method to diversify a macromolecule's function. We anticipate these findings will facilitate the generation of degradable polymers for diverse applications in materials science and biology.

4.6 Experimental Details

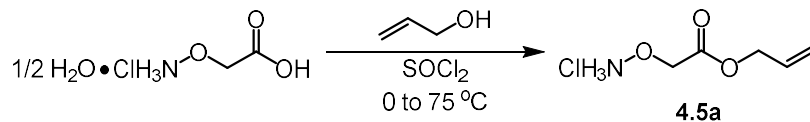
Materials and General Information. All commercially available reagents were purchased from Sigma-Aldrich (St. Louis, MO). The fluorescent dye, Alexa Fluor 488 C5-aminooxyacetamide bis(triethylammonium) salt, was purchased from Invitrogen (Grand Island, NY) as a hydroxylamine conjugate. Monomers **4.1** and **4.2**, polymers **4.3** and **4.4**,²⁶³ the phtahlimidoacetophenone precursor to **4.11**,²⁹¹ and catalyst **4.18**¹⁵⁵ were synthesized as described previously. Dry tetrahydrofuran (THF) was distilled over sodium/benzophenone or obtained from a solvent purification system (Pure Process Technology). Dichloromethane was distilled over calcium hydride or obtained from a solvent purification system (Pure Process Technology). Triethylamine was distilled over calcium hydride. Dimethylsulfoxide (DMSO), *N,N*-dimethyl formamide (DMF), diethyl ether, hexanes, ethyl acetate (EtOAc), and methanol were used as received. All reactions were run under an inert atmosphere of N₂ unless otherwise specified. Reactions were stirred using Teflon coated magnetic stir bars. All glassware and stir bars were stored in oven or flame-dried and N₂ flushed before use.

Analytical thin layer chromatography (TLC) was carried out on E. Merck (Darmstadt) TLC plates pre-coated with silica gel 60 F254 (250 µm layer thickness). Analyte visualization was accomplished using a UV lamp and by charring with potassium permanganate stain (Fischer, 1.5 g in 300 mL water with 6.5% K₂CO₃ (w/v) and 5 mL 5% NaOH (w/v)). Flash column chromatography was performed on SiliaFlash® P60 (Silicycle; Quebec City, Canada; 40-63 µm particle size).

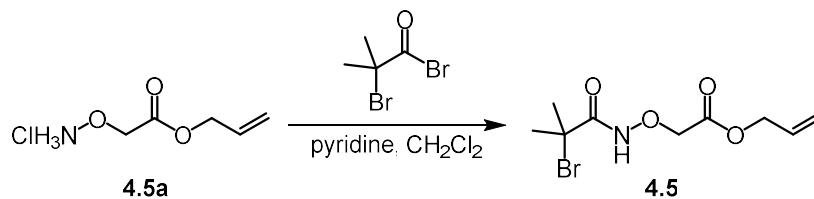
¹H and ¹³C nuclear magnetic resonance (NMR) spectra were obtained using an Avance-400 MHz spectrometer or Avance-500 MHz spectrometer. Chemical shifts are reported relative to tetramethylsilane or residual solvent peaks in parts per million (CHCl₃: ¹H: δ

7.26, ^{13}C : δ 77.23; MeOH- d_4 : ^1H : δ 3.31, ^{13}C : δ 49.00). Peak multiplicity is reported as singlet (s), doublet (d), doublet of doublets (dd), doublet of doublet of doublets (ddd), doublet of doublet of triplets (ddt), triplet (t), doublet of triplets (dt), quartet (quart), pentet (pent), multiplet (m), AB quartet (ABX_2). Monomer conversion was determined by taking an aliquot of the polymerization milieu after vinyl ether quench and taking the ratio of olefin protons assigned to the polymer backbone and residual monomer double bonds as observed using ^1H -NMR. High resolution electrospray ionization mass spectra (HRESI-MS) were obtained on a Micromass LCT mass spectrometer. Room temperature GPC-size exclusion chromatography (SEC) analysis (Viscotek GPC max) was performed on 300 x 7.5 mm PolyPor 5 μm mixed columns from Polymer Laboratories with a mobile phase of THF (1.0mL/min, 40 $^\circ\text{C}$ or an Agilent 1260 Infinity high performance liquid chromatography (HPLC) equipped with a dual Agilent PL1110-6500 columns using a mobile phase of 25mM LiBr in DMF with a flow rate of 1mL/min at 60 $^\circ\text{C}$. Data was analyzed using OmniSEC software (Viscotek Inc.) to determine M_n , M_w , and polydispersity index (M_n/M_w). Columns were calibrated with 10 narrow polystyrene standards (Polymer Laboratories S-M2-10 kit). UV-Vis absorption spectra were obtained on a Varian Cary 50-Scan UV-Visible Spectrophotometer and fluorescence emission spectra were obtained on a Hitachi F-4500 Fluorescence Spectrophotometer. All optical measurements were taken in a quartz cuvette with chloroform as the solvent.

Experimental Procedures and Analytical Data

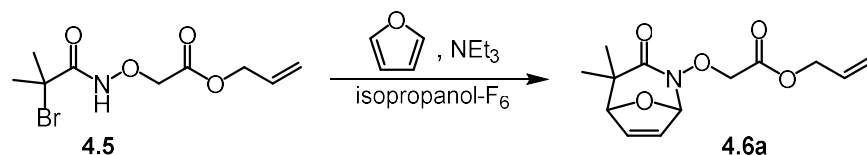


Compound 4.5a. To a suspension of *O*-(carboxymethyl) hydroxylamine hemichloride (2.00 g, 18.3 mmol) in allyl alcohol (36 mL) at 0°C was added thionyl chloride (1.6 mL, 22.0 mmol), dropwise, over 15 min. The solution was moved to a 75°C oil bath and stirred for 2 h. The solution was cooled to room temperature (rt) and the solvent was removed *in vacuo* to afford an orange-brown residue. The residue was taken up in methanol and treated with activated charcoal. After 15 min, the charcoal was removed by filtration and the solvent was removed *in vacuo* to afford the allyl ester **5a** (3.18 g, quant), which was taken on without further purification. ^1H -NMR (MeOH- d_4 , 400 MHz): δ 5.98 (ddt, $J = 17, 11, 5$ Hz, 1H), 5.39 (d, $J = 17$ Hz, 1H), 5.29 (d, $J = 11$ Hz, 1H), 4.90 (broad s, 4H), 4.75 (s, 2 H, under solvent). ^{13}C -NMR (MeOH- d_4 , 100 MHz): δ 169.0, 132.8, 119.5, 71.5, 67.3. HRESI-MS calcd for $\text{C}_5\text{H}_9\text{NO}_3$ $[2\text{M}+\text{H}]^+$ 263.1238; found 263.1246.

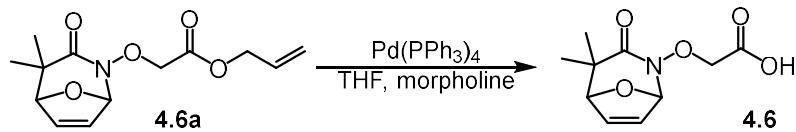


Compound 4.5. To a stirring solution of **4.5a** (2.94 g, 17.5 mmol) and pyridine (2.82 mL, 35.0 mmol) in dichloromethane (70 mL) at 0°C was added α -bromoisobutyryl bromide (2.14 mL, 17.3 mmol), dropwise. The solution was stirred for 15 h, then diluted with dichloromethane (35 mL) and then washed with water (2 x 70 mL). The organic

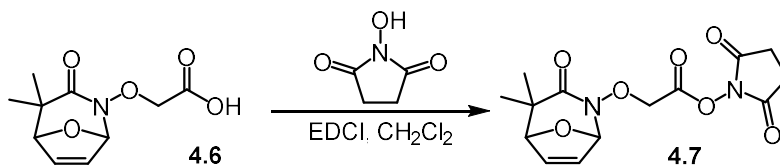
phase was dried and concentrated and the crude residue was purified by silica column chromatography (25% EtOAc/hex) to afford compound **4.5** (2.81 g, 58%). $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ 9.91 (s, 1H), 5.93 (ddt, J = 17, 11, 5 Hz, 1H), 5.43-5.29 (m, 2H), 4.70 (dt, J = 6, 1 Hz, 2H), 4.53 (d, 2H), 1.94 (s, 6H). $^{13}\text{C-NMR}$ (CDCl_3 , 100 MHz): δ 169.7, 169.3, 131.2, 119.7, 72.1, 66.2, 58.7, 32.3. HRESI-MS calcd for $\text{C}_9\text{H}_{14}\text{BrNO}_4$ $[\text{M}+\text{H}]^+$ 280.01; found 280.0179.



Compound 4.6a. To a stirring solution of **4.5** (1.00 g, 3.53 mmol) in 1,1,1,3,3,3-hexafluoroisopropanol and furan [1:1 (v/v), 0.25 M wrt **5**] at 0 °C was added triethylamine (1.00 mL, 7.06 mmol) dropwise over 5 min. Stirring continued at 0 °C for 5 min, then the reaction mixture was allowed to warm to rt over 30 min. The volatile compounds were removed under reduced pressure and the residue was purified by flash column chromatography (25% EtOAc/hexanes) to afford allyl ester **4.6a** (648 mg, 68%). $^1\text{H-NMR}$ (CDCl_3 , 300 MHz): δ 6.76 (dd, J = 5.9, 1.0 Hz, 1H), 6.51 (dd, J = 5.9, 2.9 Hz, 1H), 6.00 (d, J = 1.2 Hz, 1H), 5.94 (ddt, J = 17.4, 10.6, 5.6 Hz, 1H), 5.36 (dq, J = 17.4, 1.4 Hz, 1H), 5.29 (dq, J = 10.8, 1.4 Hz, 1H), 4.70 (m, 2H), 4.55 (s, 2H), 4.50 (d, 1.8 Hz, 1H), 1.48 (s, 3H), 1.06 (s, 2H). $^{13}\text{C-NMR}$ (CDCl_3 , 100 MHz): δ 177.8, 169.4, 135.5, 135.0, 131.5, 119.2, 92.4, 87.2, 72.8, 65.8, 49.7, 26.8, 20.0. HRESI-MS calcd for $\text{C}_{13}\text{H}_{17}\text{NO}_5$ $[\text{M}+\text{H}]^+$ 268.1180; found 268.1178.

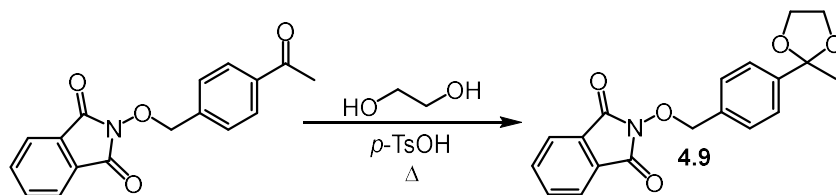


Compound 4.6. To a solution of the precursor allyl ester **4.6a** (200 mg, 0.75 mmol) and morpholine (0.32 mL, 3.77 mmol) in THF (20 mL) was added Pd(PPh₃)₄ (83 mg, 0.075 mmol). The solution was stirred at rt for 1 h and then the reaction was concentrated *in vacuo*. The residue was dissolved in sat. NaHCO₃ (25 mL) and washed with dichloromethane (3 x 20 mL). The aqueous solution was acidified to pH = 2 with 1 M HCl and the solution was extracted with EtOAc (4 x 50 mL). The combined EtOAc phase was dried and concentrated to afford compound **4.6** (174 mg, quant), which was taken on without further purification. ¹H-NMR (CDCl₃, 400 MHz): δ 10.36 (s, 1H, -OH), 6.76 (dd, *J* = 5.9, 0.8 Hz, 1H), 6.60 (dd, *J* = 6.1, 2.9 Hz, 1H), 5.64 (d, *J* = 1.0 Hz, 1H), 4.59 (AB quartet, *J* = 17.3 Hz, 2H), 4.59 (d, *J* = 2.8 Hz, 1H), 1.52 (s, 3H), 1.11 (s, 3H). ¹³C-NMR (CDCl₃, 100 MHz): δ 179.6, 170.8, 135.6, 135.2, 91.7, 87.0, 73.4, 49.1, 26.8, 19.6. HRESI-MS calcd for C₁₀H₁₃NO₅ [M+H]⁺ 228.0867; found 228.0863.

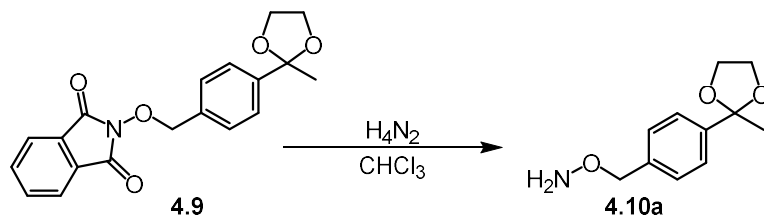


Compound 4.7. To a stirring solution of **4.6** (471 mg, 2.07 mmol) in dichloromethane (2.5 mL) was added *N*-hydroxysuccinimide (337 mg, 2.94 mmol) and EDCI (442 mg, 2.86 mmol). The solution was stirred at rt for 15 h. The reaction was diluted with dichloromethane (10 mL) and washed with 5% citric acid solution (2 x 20 mL), sat. NaHCO₃ (3 x 20 mL), and brine (1 x 20 mL). The organic phase was dried and

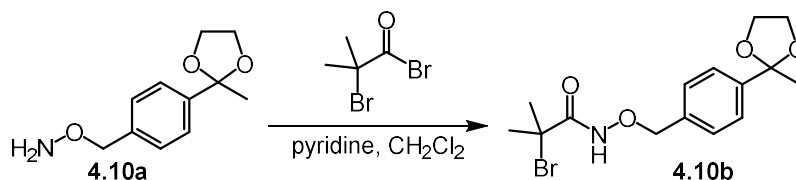
concentrated. The residue was purified by silica column chromatography (75% EtOAc/hex) to afford compound **4.7** (35 mg, 5%). $^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 6.70 (dd, $J = 6.5, 1.0$ Hz, 1H), 6.51 (dd, $J = 6.5, 2.0$ Hz, 1H), 5.90 (d, $J = 1.0$ Hz, 1H), 4.87 (s, 2H), 4.50 (d, 2.0 Hz, 1H), 2.88 (s, 4H), 1.50 (s, 3H), 1.06 (s, 3H). $^{13}\text{C-NMR}$ (CDCl_3 , 125 MHz): δ 178.3, 168.8, 165.4, 135.4, 135.2, 92.5, 87.2, 70.61, 50.0, 26.8, 25.7, 19.9. HRESI-MS calcd for $\text{C}_{14}\text{H}_{16}\text{N}_2\text{O}_7$ $[\text{M}+\text{H}]^+$ 325.1030; found 325.1025.



Compound 4.9. To a solution of *N*-(4-acetylbenzyloxy)phthalimide (Terent'ev, 2012 #54)²⁹¹ (1.95 g, 6.53 mmol) in benzene (25 mL) was added ethylene glycol (1.35 mL, 24.2 mmol) and *p*-toluene sulfonic acid monohydrate (12 mg, 0.063 mmol). The solution was then refluxed under Dean-Stark conditions for 15 h. The solution was diluted with dichloromethane (80 mL) and washed with brine (2 x 60 mL) followed by water (2 x 60 mL). The organic layer was dried over Na_2SO_4 , filtered, and concentrated to afford **4.9** as a white solid (2.13 g, 95%). $^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 7.85-7.80 (m, 2H), 7.77-7.72 (m, 2H), 7.56-7.48 (m, 4H), 5.21 (s, 2H), 4.06-4.00 (m, 2H), 3.78-3.71 (m, 2H), 1.64 (s, 3H). $^{13}\text{C-NMR}$ (CDCl_3 , 125 MHz): δ 163.5, 144.5, 134.5, 133.2, 129.7, 128.9, 125.6, 123.5, 108.7, 79.7, 64.4, 27.5. HRESI-MS calcd for $\text{C}_{19}\text{H}_{17}\text{NO}_5$ $[\text{M}+\text{H}]^+$ 340.1180; found 340.1176.

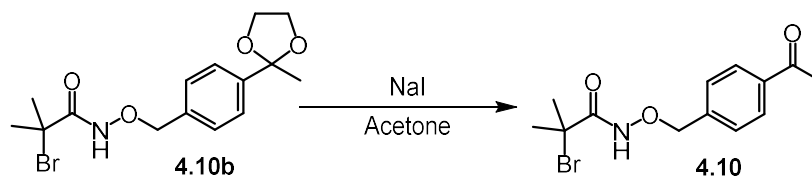


Compound 4.10a. To a stirring solution of **4.9** (517 mg, 1.52 mmol) in CHCl_3 (4.7 mL) was added hydrazine (0.11 mL, 3.35 mmol, anhydrous). The solution was stirred for 1.5 h at rt, during which time a white precipitate appeared. The solution was diluted with CHCl_3 (2 mL) and the insoluble solids were filtered off. The filtrate was concentrated and the resultant yellow oil, **4.10a**, was taken on without further purification (322 mg, quant). $^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 7.52-7.45 (m, 2H), 7.37-7.31 (m, 2H), 5.41 (broad s, 2H, $-\text{NH}_2$), 4.69 (s, 2H), 4.07-3.97 (m, 2H), 3.81-3.73 (m, 2H), 1.65 (s, 3H). $^{13}\text{C-NMR}$ (CDCl_3 , 125 MHz): δ 143.1, 137.0, 18.2, 125.5, 108.8, 77.7, 64.4, 27.6. HRESI-MS calcd for $\text{C}_{11}\text{H}_{15}\text{NO}_3$ $[\text{M}+\text{H}]^+$ 210.1125; found 210.1118.

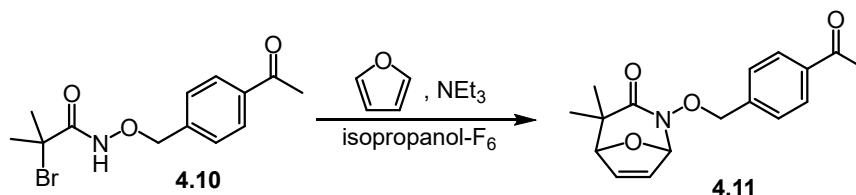


Compound 4.10b. To a stirring solution of **4.10a** (315 mg, 1.51 mmol) at 0 °C was added pyridine (0.26 mL, 3.24 mmol) followed by α -bromoisobutyryl bromide (0.19 mL, 1.51 mmol), which was added dropwise. The solution was stirred for 1 h at rt. The solution was diluted with dichloromethane (10 mL) and washed with water (2 x 9 mL). The organic layer was dried over Na_2SO_4 , filtered, and concentrated. The resultant residue was purified by flash column chromatography (35% EtOAc/hexanes). Compound **4.10b** was obtained as an off-white solid (400 mg, 74%). $^1\text{H-NMR}$ (CDCl_3 ,

500 MHz): δ 9.09 (s, 1H, NH), 7.55-7.48 (m, 2H), 7.43-7.36 (m, 2H), 4.93 (s, 2H), 4.09-3.99 (m, 2H), 3.81-3.71 (m, 2H), 1.94 (s, 6H), 1.65 (s, 3H). ^{13}C -NMR (CDCl_3 , 125 MHz): δ 169.6, 144.2, 134.3, 129.4, 125.7, 108.7, 78.0, 64.5, 59.4, 32.4, 27.6. HRESI-MS calcd for $\text{C}_{15}\text{H}_{20}\text{BrNO}_4$ $[\text{M}+\text{H}]^+$ 358.0649; found 358.0643.

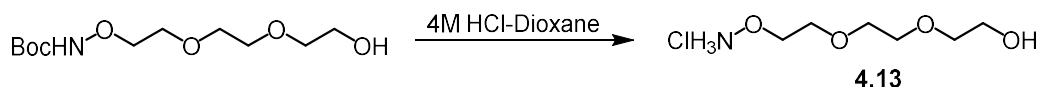


Compound 4.10. To a stirring solution of **4.10b** (937 mg, 2.61 mmol) in acetone (10.8 mL) was added iodine (66 mg, 0.26 mmol). The solution was stirred for 10 min at rt, then concentrated to $\sim 1/4$ total volume. The remaining solution was diluted with dichloromethane (26 mL) and washed with 5% $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ (1 x 26 mL) and water (2 x 26 mL). The organic layer was dried over Na_2SO_4 , filtered, and concentrated to afford **4.10** as a white solid (823 g, quant). ^1H -NMR (CDCl_3 , 500 MHz): δ 9.32 (s, 1H, NH), 7.92-7.86 (m, 2H), 7.47-7.41 (m, 2H), 4.92 (s, 2H), 2.53 (s, 3H), 1.86 (s, 6H). ^{13}C -NMR (CDCl_3 , 125 MHz): δ 197.8, 169.9, 140.0, 137.2, 129.2, 128.6, 77.4, 58.9, 32.2, 26.7. HRESI-MS calcd for $\text{C}_{13}\text{H}_{16}\text{BrNO}_3$ $[\text{M}+\text{H}]^+$ 314.0387; found 314.0377.

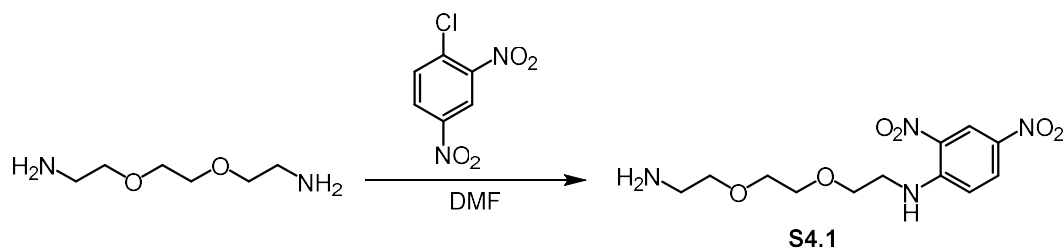


Compound 4.11. To a stirring solution of **4.10b** (89.5 mg, 0.28 mmol) in furan (0.57 mL) and 1,1,1,3,3,3-hexafluoroisopropanol (0.57 mL) at 0 °C was added triethylamine (79 μL , 0.57 mmol) dropwise. The solution was then stirred at rt for 30 min and then concentrated. The resultant residue was purified by flash column chromatography (40%

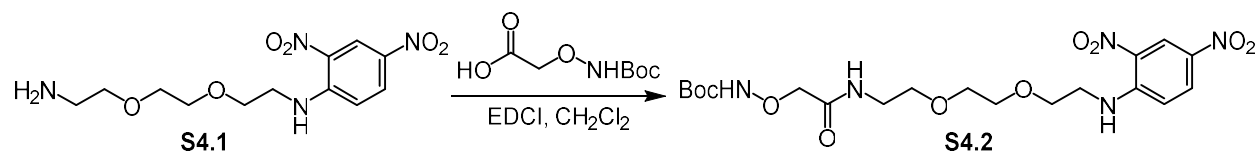
EtOAc/hexanes). Monomer **4.11** was obtained as a clear oil (50 mg, 58%). $^1\text{H-NMR}$ (CDCl_3 , 500 MHz): δ 8.02-7.90 (m, 2H), 7.58-7.49 (m, 2H), 6.63 (dd, $J = 6.0, 1.0$ Hz, 1H), 6.48 (dd, $J = 6.0, 1.8$ Hz, 1H), 5.28 (d, $J = 1.1$ Hz, 1H), 4.98 (AB quartet, $J = 11.3, 2$ Hz), 4.49 (d, $J = 1.8$ Hz, 1H), 2.61 (s, 3H), 1.48 (s, 3H), 1.06 (s, 3H). $^{13}\text{C-NMR}$ (CDCl_3 , 125 MHz): δ 197.7 (C), 176.1 (C), 140.7 (C), 137.2 (C), 135.2 (CH), 134.8 (CH), 129.5 (CH), 128.5 (CH), 91.3 (CH), 87.3 (CH), 77.0 (CH_2 , under solvent), 49.3 (C), 26.9 (CH_3), 26.7 (CH_3), 19.7 (CH_3). HRESI-MS calcd for $\text{C}_{17}\text{H}_{19}\text{NO}_4$ $[\text{M}+\text{H}]^+$ 302.1387; found 302.1391.



Compound 4.13. 2-(2-(2-(N-Boc aminoxy)ethoxy)ethoxy)ethanol⁴ (24 mg, 0.09 mmol) was dissolved in a 4 M solution of hydrochloric acid in 1,4-dioxane (0.79 mL). The solution was stirred for 1 h at rt and then concentrated. Excess acid was azeotroped with dichloromethane (3 x 0.5 mL). Compound **4.13** was isolated as a clear oil (20 mg, quant). $^1\text{H-NMR}$ (MeOH-d_4 , 500 MHz): δ 4.24-4.16 (m, 2H), 3.90-3.80 (m, 2H), 3.73-3.65 (m, 6H), 3.61-3.56 (m, 2H). $^{13}\text{C-NMR}$ (CDCl_3 , 125 MHz): δ 72.8, 72.2, 70.6, 70.1, 69.2, 60.8. HRESI-MS calcd for $\text{C}_6\text{H}_{16}\text{NO}_4$ $[\text{M}-\text{Cl}]^+$ 166.1074; found 166.1076.

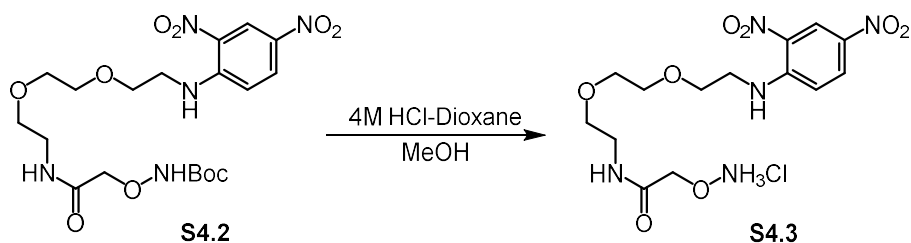


Compound S4.1. To a solution of 2,2'-(ethylenedioxy) bis(ethylenediamine) (1.0 mL, 6.80 mmol) in DMF (1.5 mL) was added 1-chloro-2,4-dinitrobenzene (138 mg, 0.68 mmol) in DMF (6 mL), dropwise. The solution was then stirred for 30 min at rt. The solution was diluted with dichloromethane (20 mL) and washed with water 2 x 26 mL). The organic layer was dried over Na₂SO₄, filtered, and concentrated to afford **S4.1** as a yellow oil (197 mg, 92%). ¹H-NMR (CDCl₃, 400 MHz): δ 9.13 (d, *J* = 2.7 Hz, 1H), 8.82 (s, 1H, NH), 8.27 (dd, *J* = 9.5, 2.6 Hz, 1H), 6.96 (d, *J* = 9.5 Hz, 1H), 3.85 (t, *J* = 5.3 Hz, 2H), 3.76-3.65 (m, 4H), 3.61 (q, *J* = 5.1 Hz, 2H), 3.53 (t, *J* = 5.3 Hz, 2H), 2.88 (t, *J* = 5.2 Hz, 2H), 1.44 (s, 2H, NH₂). ¹³C-NMR (CDCl₃, 100 MHz): δ 148.4, 136.1, 130.5, 130.3, 124.3 114.0, 73.6, 70.8, 70.3, 68.6, 43.3, 41.8. HRESI-MS calcd for C₁₂H₁₈N₄O₆ [M+H]⁺ 315.1300; found 315.1290.

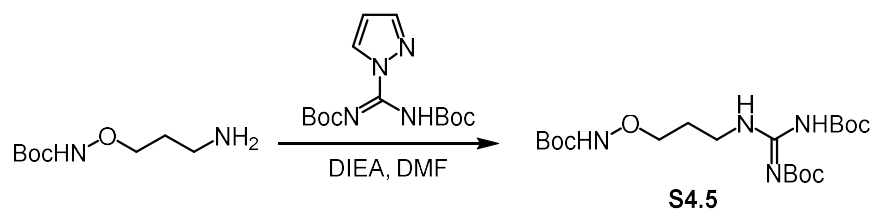


Compound S4.2. To a stirring solution of **S4.1** (25 mg, 0.08 mmol) and (N-Boc-aminoxy) acetic acid (19 mg, 0.10 mmol) in dichloromethane (5 mL) was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (48 mg, 0.25 mmol). The solution was stirred for 16 h at rt. The solution was diluted with dichloromethane (4 mL)

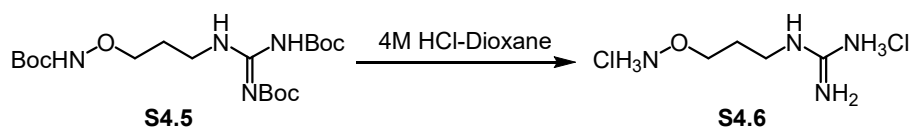
and washed with water (1 x 6 mL) and brine (1 x 6 mL). The organic layer was dried over Na₂SO₄, filtered, and concentrated. The resultant residue was purified by flash column chromatography (48% acetone/hexanes) to afford compound **S4.2** (29 mg, 75%). ¹H-NMR (CDCl₃, 400 MHz): δ 9.15 (d, *J* = 2.6 Hz, 1H), 8.82 (broad s, 1H, NH), 8.28 (dd, *J* = 9.5, 2.3 Hz, 1H), 7.95 (broad s, 1H, NH), 7.57 (s, 1H, NH), 6.95 (d, *J* = 9.5 Hz, 1H), 4.33 (s, 2H), 3.86 (t, *J* = 5.3 Hz, 2H), 3.76-3.65 (m, 4H), 3.60 (q, *J* = 5.3 Hz, 4H), 3.53 (q, *J* = 5.4 Hz, 2H), 1.47 (s, 9H). ¹³C-NMR (CDCl₃, 100 MHz): δ 168.8, 157.4, 148.4, 136.1, 130.5, 130.3, 124.5, 114.0, 82.9, 76.1, 70.7, 70.4, 69.7, 68.5, 43.3, 38.8, 28.1. HRESI-MS calcd for C₁₉H₂₉N₅O₁₀ [M+H]⁺ 488.1988; found 488.1987.



Compound S4.3. To a stirring solution of **S4.2** (29 mg, 0.06 mmol) in methanol (0.2 mL) was added a 4 M hydrochloric acid solution in 1,4-dioxane (0.5 mL). The solution was stirred for 1 h at rt and then concentrated. Excess acid was azeotroped off with chloroform (3 x 0.5 mL). Compound **S4.3** was isolated as a yellow oil (25 mg, quant). ¹H-NMR (MeOH-d₄, 500 MHz): δ 9.06 (d, *J* = 2.7 Hz, 1H), 8.32 (dd, *J* = 9.5, 2.7 Hz, 1H), 7.24 (d, *J* = 9.6 Hz, 1H), 4.57 (s, 2H), 3.83 (t, *J* = 5.2 Hz, 2H), 3.73-3.63 (m, 6H), 3.61 (t, *J* = 5.5 Hz, 2H), 3.45 (t, *J* = 5.4 Hz, 2H). ¹³C-NMR (MeOH-d₄, 125 MHz): δ 168.2, 148.4, 135.7, 130.2, 129.7, 123.3, 114.7, 70.7, 70.2, 69.9, 69.0, 68.4, 42.6, 38.7. HRESI-MS calcd for C₁₄H₂₂N₅O₈ [M-Cl]⁺ 388.1465; found 388.1463.

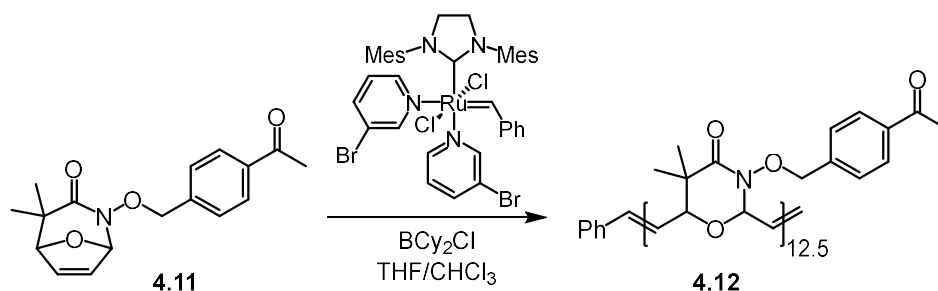


Compound S4.5. To a stirring solution of *N*-Boc, *O*-(3-aminopropyl) hydroxylamine⁵ (310 mg, 1.0 mmol) and *N*, *N'*-di-Boc-1*H*-pyrazole-1 carboxamidine (475 mg, 2.50 mmol) in DMF (8.7 mL) was added DIEA (0.35 mL, 2.0 mmol). The solution was stirred at rt for 18 h and then concentrated. The residue was suspended in water (20 mL) and extracted successively with dichloromethane (3 x 15 mL) and ethyl acetate (4 x 15 mL). The combined organic phase was dried and concentrated. The residue was purified by silica column chromatography (50% EtOAc/hex). Compound **S5** was isolated as a white solid (370 mg, 85%). ¹H-NMR (CDCl₃, 500 MHz): δ 11.47 (s, 1H), 8.50 (s, 1H), 7.66 (s, 1H), 3.94 (t, *J* = 5.7 Hz, 2H), 3.57 (q, *J* = 6.3 Hz, 2H), 1.87 (pent, *J* = 6.2 Hz, 2H), 1.51 (s, 9H), 1.49 (s, 9H), 1.48 (s, 9H). ¹³C-NMR (CDCl₃, 125 MHz): δ 163.4, 156.9, 156.3, 153.2, 83.2, 81.5, 79.4, 74.0, 37.8, 28.3, 28.3, 28.1, 27.9.



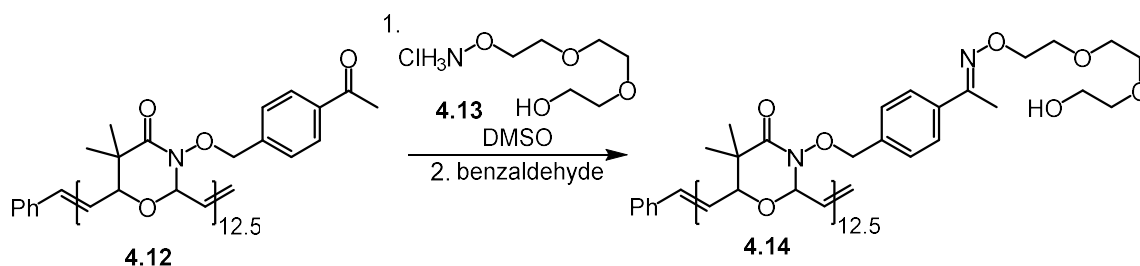
Compound S4.6. Compound **S5** (365 mg, 0.84 mmol) was dissolved in a 4 M solution of HCl in 1,4-dioxane (6.8 mL) at 0 °C. The solution was stirred and allowed to warm to rt over 20 h. The solution was concentrated and excess acid was azeotroped with toluene (3 x 6 mL) to afford compound **S6** as a white solid (140 mg, 81%). ¹H-NMR (MeOH-d₄, 500 MHz): δ 4.16 (T, *J* = 6.1 Hz, 2H), 3.34 (t, *J* = 7.1 Hz, 2H, under solvent), 2.01 (pent,

$J = 6.5$ Hz, 2H). ^{13}C -NMR (MeOH- d_4 , 125 MHz): δ 157.3, 72.0, 37.6, 26.9. HRESI-MS calcd for $\text{C}_4\text{H}_{13}\text{N}_4\text{O}$ $[\text{M}]^+$ 133.1084; found 133.1079.



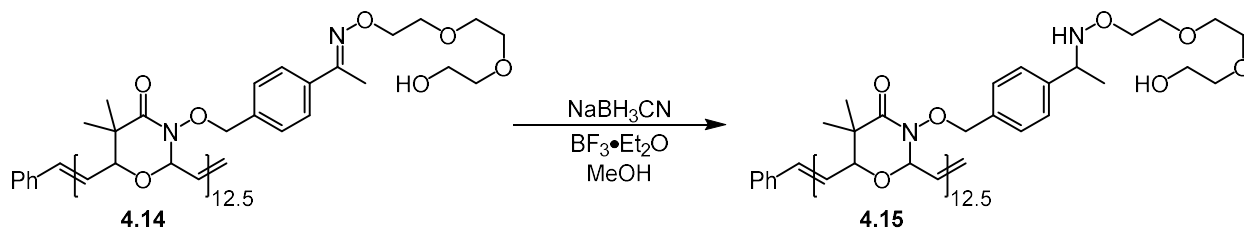
General Procedure for Polymer Synthesis. To a stirring solution of **4.11** (60 mg, 0.20 mmol) in tetrahydrofuran (0.1 mL) under a blanket of argon was added a 1 M solution of chlorodicyclohexyl borane in hexanes (20 μL) and then ruthenium carbene catalyst in chloroform (0.1 mL) (Note: polymer chain length is dictated by the ratio of monomer to catalyst). The solution was stirred for 30 min at rt. The reaction was quenched with ethyl vinyl ether (0.1 mL) and 4 drops of methanol and the resulting mixture was stirred overnight (in cases where needed 0.1–0.2 mL chloroform was also added to resolubilize any precipitated polymers). The solution was triturated into diethyl ether (30 mL) and the solids were collected by centrifugation. The solids were then dissolved in minimal chloroform and retrituated until all of the unreacted monomer was removed as determined by NMR (usually achieved within 3 rounds). Polymer **4.12** was isolated as a white solid. ^1H -NMR (CDCl_3 , 500 MHz): δ 8.03–7.70 (m, 2H), 7.60–7.30 (m, 2H + end group Ph Hs), 6.30–5.60 (m, 2H), 5.55–5.12 (m, 1H), 5.12–4.60 (m, 2H), 4.40–3.85 (m, 1H), 2.67–2.40 (m, 3H), 1.35–0.92 (m, 6H). Note: Monomers were purified before each polymerization.

General Procedure for Oxime Conjugation. Polymer **4.12** (n eq wrt acetophenone) was taken up in a solution of hydroxylamine ligand **4.13** (1.5n eq) in DMSO so the final concentration of acetophenone was 40 mM. Pyridine (8n eq) was added and the solution was stirred at rt for 24 h. Benzaldehyde (1.5n eq) was added and the solution was stirred for 1 h (benzaldehyde caps any unreacted hydroxylamine and aids in polymer purification in next step). The solution was triturated into 1.25 mL of diethyl ether, and the solids were collected by centrifugation. Trituration was repeated until all small molecules were removed as determined by NMR.

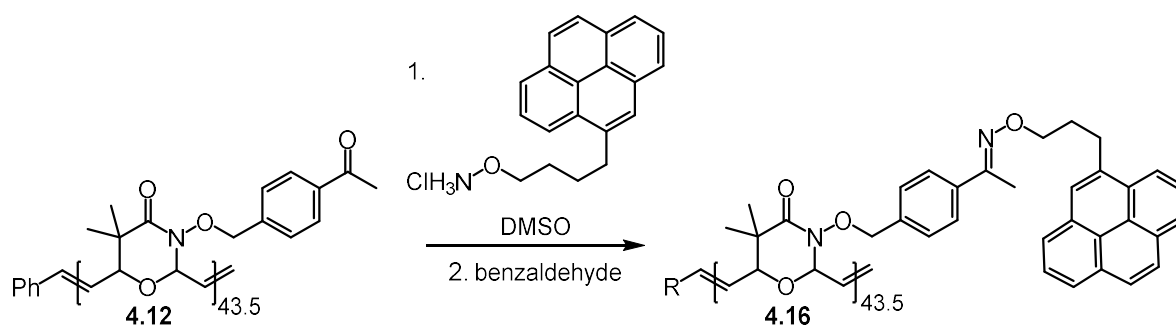


General Procedure for Oxime Conjugation. Polymer **4.12** (n eq wrt acetophenone) was taken up in a solution of hydroxylamine ligand **4.13** (1.5n eq) in DMSO so the final concentration of acetophenone was 40 mM. Pyridine (8n eq) was added and the solution was stirred at rt for 24 h. Benzaldehyde (1.5n eq) was added and the solution was stirred for 1 h (benzaldehyde caps any unreacted hydroxylamine and aids in polymer purification in next step). The solution was triturated into 1.25 mL of diethyl ether, and the solids were collected by centrifugation. Trituration was repeated until all small molecules were removed as determined by NMR. ¹H-NMR **4.14** (MeOH-d₄, 500 MHz): δ 7.75-7.20 (m, 4H + end group Ph protons), 6.35-5.65 (m, 2H), 5.55-

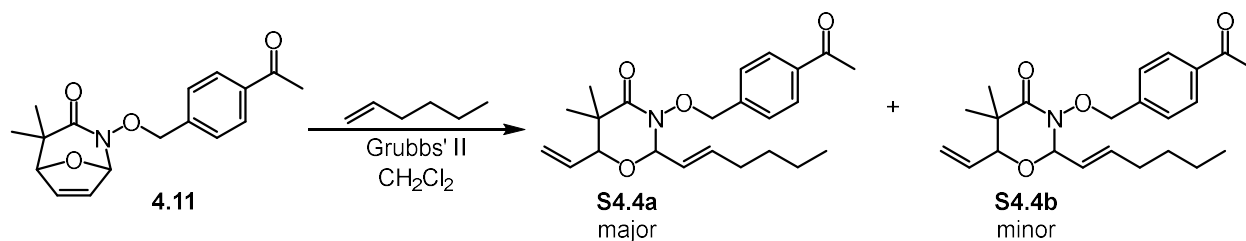
5.25 (m, 1H), 5.15-4.60 (m, 2H, under solvent), 4.45-4.10 (m, 3H), 3.85-3.45 (m, 10H), 2.30-2.05 (m, 3H, 1.30-0.85 (m 6H).



Polymer 4.15. To a solution of **4.14** (1.6 mg, 3.47 μmol wrt oxime) and sodium cyanoborohydride (16.4 mg, 0.26 mmol, 75 eq) in methanol (0.25 mL) at 0 °C was added boron trifluoride diethyl etherate (21 μL , 0.17 mmol, 50 eq). The solution was stirred for 30 mins at 0 °C and at rt for an 1h. The reaction was quenched with saturated sodium bicarbonate solution (0.5 mL). The solution was diluted with chloroform (0.5 mL) and the biphasic mixture was separated. The aqueous phase was extracted with chloroform (2 x 0.5 mL) and the combined organic layer was dried over Na_2SO_4 , filtered, and concentrated to afford **4.15** as a tacky solid (1.4 mg, 87%). ^1H -NMR (CDCl_3 , 500 MHz): 7.45-7.05 (m, 4H, overlap with solvent), 6.15-4.40 (m, 6H), 4.30-3.82 (m, 3H), 3.78-3.15 (m, 10H), 1.30- 1.00 (m, 9H).

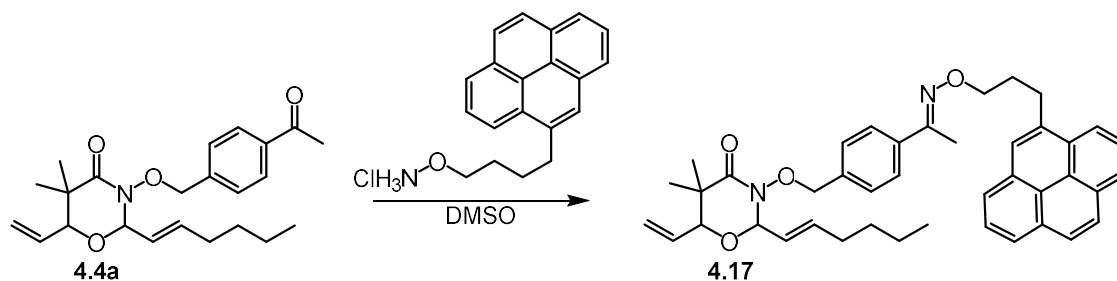


Polymer 4.16. Polymer **16** was synthesized following the general procedure using *O*-(4-pyrenyl butyl) hydroxylamine hydrochloride¹ as the ligand. ¹H-NMR (CDCl₃, 500 MHz): δ 8.30-7.45 (m, 11H), 7.45-7.15 (m, 2H, under solvent peak), 6.10-4.50 (m, 6H), 4.30-3.80 (m, 3H), 3.45-3.10 (m, 2H), 2.60-2.35 (m, 1H, OH), 2.25-2.05 (m, 3H), 2.0-1.75 (m, 4H), 1.15-1.00 (m, 6H).



Compound S4.4a and S4.4b. To a stirring solution of **4.11** (75 mg, 0.25 mmol) and 1-hexene (0.15 mL, 1.24 mmol) in dichloromethane (0.63 mL) was added Grubbs' 2nd Generation catalyst (**GII**, 21 mg, 0.025 mmol) in dichloromethane (0.27 mL). The solution was stirred for 2 h at rt and then the reaction was quenched by the addition of ethyl vinyl ether (0.4 mL). After an additional hour of stirring, the solution was concentrated *in vacuo* and the crude residue was purified by flash column chromatography (20% ethyl acetate/hexanes). Products **S4.4a** (66 mg, 69%) and **S4.4b**

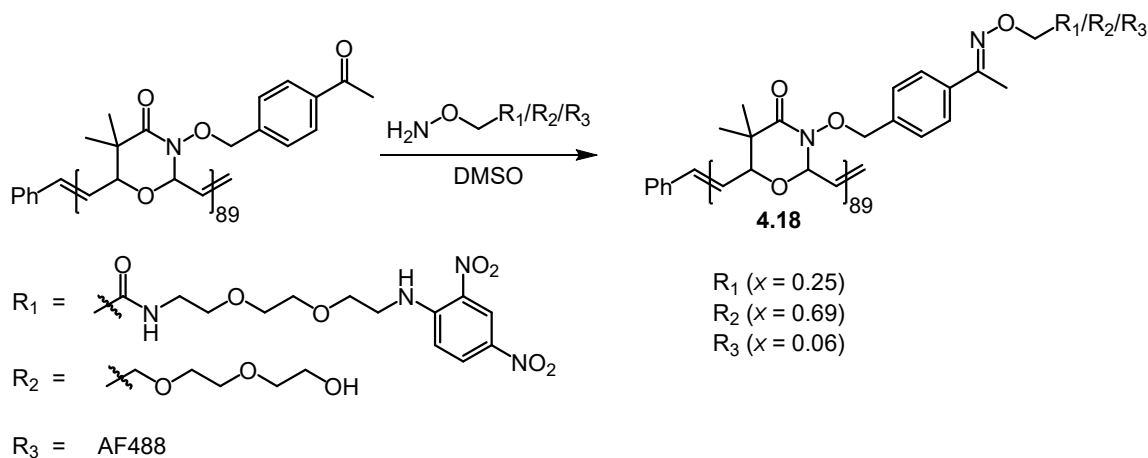
(20 mg, 18%) eluted at distinct retention times and were isolated as brown oils. **S4.4a**: $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ 7.93 (d, J = 8.3 Hz, 2H), 7.48 (d, J = 8.3 Hz, 2H), 5.99 (dt, J = 15.4, 6.8 Hz, 1H), 5.79 (ddd, J = 17.2, 10.6, 6.5 Hz, 1H), 5.51 (ddt, J = 15.4, 7.7, 1.4 Hz, 1H), 5.38-5.26 (m, 2H), 5.16 (d, J = 7.7 Hz, 1H), 5.07 (d, J = 10.2 Hz, 1H), 4.82 (d, J = 10.2 Hz, 1H), 4.00 (d, J = 6.4 Hz, 1H), 2.59 (s, 3H), 2.15-2.05 (m, 2H), 1.42-1.27 (m, 4H), 1.23 (s, 3H), 1.14 (s, 3H), 0.88 (t, J = 7.1 Hz, 3H). $^{13}\text{C-NMR}$ (CDCl_3 , 100 MHz): δ 197.8, 173.9, 140.4, 139.9, 137.3, 132.0, 129.3, 128.5, 125.4, 119.5, 91.6, 82.8, 76.7, 45.1, 32.0, 30.9, 26.8, 22.4, 21.0, 20.6, 14.0. HRESI-MS calcd for $\text{C}_{23}\text{H}_{31}\text{NO}_4$ $[\text{M}+\text{H}]^+$ 386.2326; found 386.2328. **S4.4b**: $^1\text{H-NMR}$ (CDCl_3 , 400 MHz): δ 7.92 (d, J = 8.3 Hz, 2H), 7.48 (d, J = 8.3 Hz, 2H), 5.97 (dt, J = 15.4, 6.7 Hz, 1H), 5.73 (dt, J = 15.4, 6.7 Hz, 1H), 5.50 (ddt, J = 15.4, 7.8, 1.3 Hz, 1H), 5.42 (ddt, J = 15.5, 7.4, 1.3 Hz, 1H), 5.15 (d, J = 7.8 Hz, 1H), 5.07 (d, J = 10.2 Hz, 1H), 4.82 (d, J = 10.2 Hz, 1H), 2.59 (s, 3H), 2.15-2.01 (m, 4H), 1.44-1.21 (m, 11H), 1.09 (s, 3H), 0.93-0.84 (m, 6H). $^{13}\text{C-NMR}$ (CDCl_3 , 100 MHz): δ 197.8, 174.2, 140.5, 139.8, 137.2, 137.1, 129.3, 128.3, 125.8, 124.7, 91.6, 83.0, 76.6, 45.3, 32.3, 31.9, 31.2, 30.8, 26.8, 22.4, 22.3, 21.1, 20.8, 14.0, 14.0. HRESI-MS calcd for $\text{C}_{27}\text{H}_{39}\text{NO}_4$ $[\text{M}+\text{H}]^+$ 442.2952; found 442.2962.



Compound 4.17. Compound **S4.4a** (4.8 mg, 12.5 μmol) was taken up in a 19.0 mg/mL solution of *O*-pyrenyl hydroxylamine hydrochloride in DMSO (252 μL). The solution was stirred at rt for 24 h and then diluted with dichloromethane (1.5 mL). The

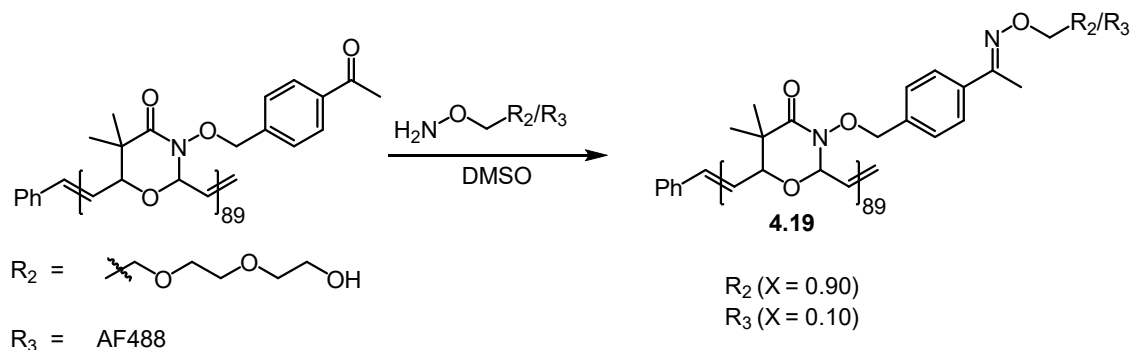
solution was washed with brine (3 x 1 mL), dried, and concentrated. The crude residue was purified by flash column chromatography (15 → 25% ethyl acetate/hexanes).

Compound **4.17** was isolated as a brown oil (4 mg, 49%). ¹H-NMR (CDCl₃, 500 MHz): δ 8.29 (d, *J* = 9.3 Hz, 1H), 8.15-7.95 (m, 7H), 7.89 (d, *J* = 7.8 Hz, 1H), 7.61 (d, *J* = 8.2 Hz, 2H), 7.36 (d, *J* = 7.8 Hz, 2H), 5.95 (dt, *J* = 15.4, 6.7 Hz, 1H), 5.79 (ddd, *J* = 17.2, 10.7, 6.5 Hz, 1H), 5.49 (ddt, *J* = 15.4, 7.7, 1.5 Hz, 1H), 5.36-5.28 (m, 2H), 5.11 (d, *J* = 7.4 Hz, 1H), 5.04 (d, *J* = 9.8 Hz, 1H), 4.79 (d, *J* = 9.8, 1H), 4.29 (t, *J* = 6.4 Hz, 2H), 3.98 (d, *J* = 6.5 Hz, 1H), 3.41 (t, *J* = 7.4 Hz, 2H), 2.21 (s, 3H), 2.15-2.07 (m, 2H), 2.03-1.89 (m, 4H), 1.42-1.32 (m, 4H), 1.23 (s, 3H), 1.15 (s, 3H), 0.90 (t, *J* = 7.2 Hz, 3H). ¹³C-NMR (CDCl₃, 125 MHz): δ 154.0, 139.7, 137.3, 137.0, 135.9, 132.1, 131.6, 131.1, 130.5, 129.9, 129.7, 128.0, 127.7, 127.6, 127.4, 127.3, 126.7, 126.1, 125.9, 125.5, 125.0, 124.9, 124.8, 123.6, 119.4, 91.5, 82.7, 74.1, 45.1, 34.8, 33.4, 32.0, 30.9, 29.4, 29.2, 28.4, 25.4, 21.0, 20.7, 12.8, 11.6. HRESI-MS calcd for C₄₃H₄₈N₂O₄ [M+H]⁺ 657.3687; found 657.3690.

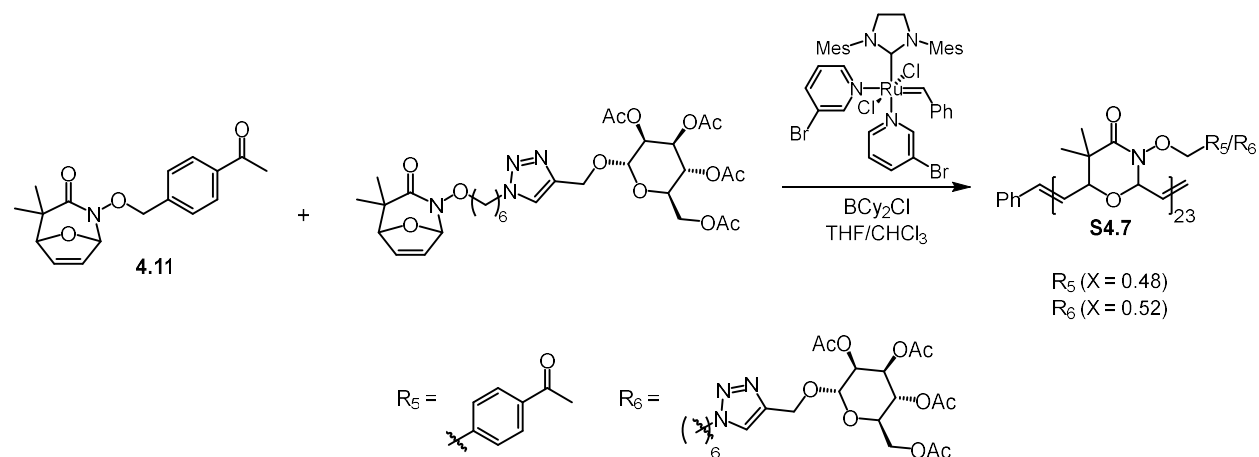


Polymer 4.18. Polymer **18** was synthesized following the general procedure using oligoethylene glycol derivative **13**, dinitrophenyl derivative **S3**, and AF488 in a 0.5:0.42:

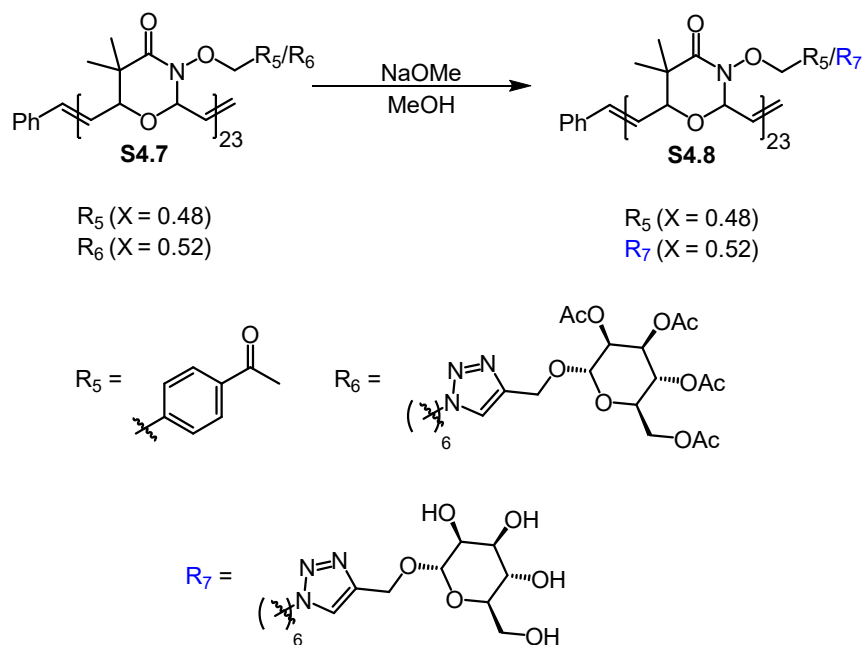
0.08 ratio (mol/mol/mol). $^1\text{H-NMR}$ (500 MHz, DMSO-d_4): Set backbone signal at δ 6.30-5.60 ppm to 2H. Signal at 8.34 (s, 1xH) was used to determine level of AF488 substitution. Signal at 8.13 (m, 1xH) was used to determine level of DNP substitution. Found 95% conversion to oxime; 25% DNP, 6% AF488, 69% $\text{PEG}_3\text{-OH}$ conjugation.



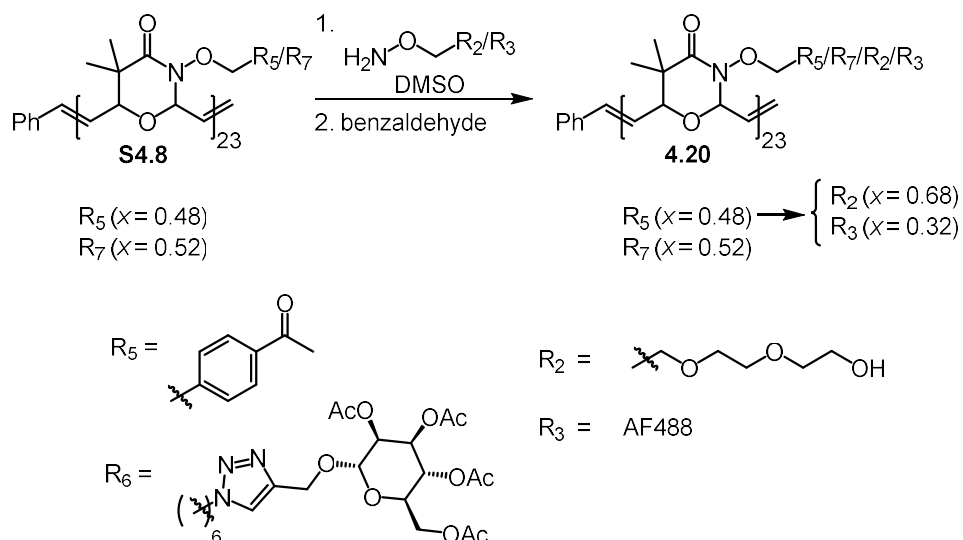
Polymer 4.19. Polymer **4.19** was synthesized following the general procedure using oligoethylene glycol derivative **4.13** and AF488 in a 0.9 : 0.1 ratio (mol/mol). $^1\text{H-NMR}$ (500 MHz, DMSO-d_4): Set backbone signal at δ 6.10-5.55 ppm to 2H. Used signal at 8.30 (s, 1xH) to determine AF488 content. Used signal at 3.70-3.30 (m, 10xH) to determine PEG content. Found quantitative conversion to oxime; 90% $\text{PEG}_3\text{-OH}$, 10% AF488 conjugation.



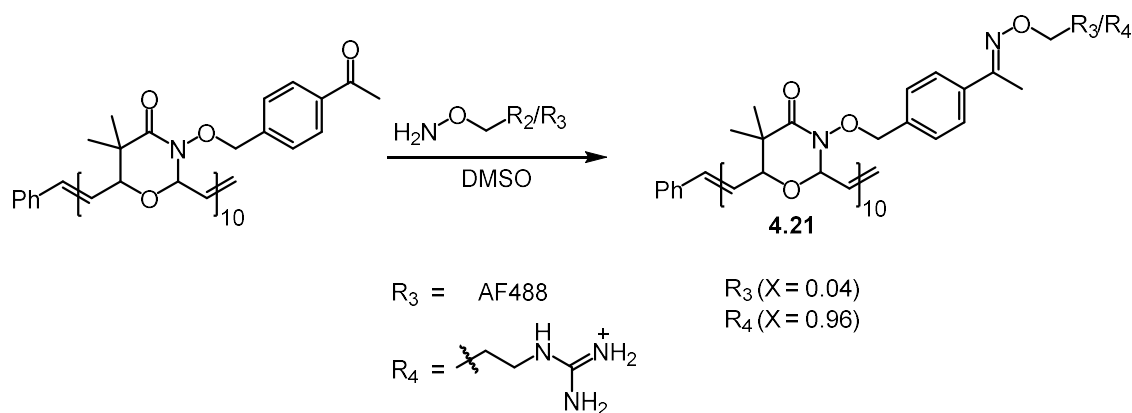
Polymer S4.7. To a solution of monomer **4.11** (30 mg, 0.10 mmol) and monomer **4.2** (68 mg, 0.10 mmol) in THF (0.1 mL) under atmosphere of Ar was added BCy_2Cl from a 1 M solution in hexanes (20 μ L) followed by a solution of catalyst 3-bromopyridine-substituted ruthenium carbene catalyst (4.43 mg, 5 μ mol) in chloroform (0.08 mL). The solution was stirred for 50 min at rt and then the polymerization was quenched by the addition of ethyl vinyl ether (0.1 mL). The precipitated solids were re-dissolved in minimal chloroform and the solution was triturated into diethyl ether (30 mL). Intermediate polymer **S4.7** was collected by centrifuge. ^1H -NMR (500 MHz, $CDCl_3$). Set backbone signal at δ 6.30-5.60 ppm to 2H. Used signals at 7.59 (broad s, 1xH, triazole-H) and 2.03 (s, 3xH, acetate) to determine $Ac_4Mannose$ content. Signals at 7.89 (m, 2xH) and 2.59 (broad s, 3xH, acetophenone methyl) were used to determine acetophenone content. Found 50% acetophenone, 50% $Ac_4Mannose$ incorporation.



Polymer S4.8. To a stirring solution of **S4.7** (5.1 mg, 4.4 μmol (wrt mannose)) in DMSO (0.2 mL) was added a 0.5 M solution of sodium methoxide in methanol (17.6 μL). The solution was stirred for 20 min at rt and then acidified with a 0.23 M solution of anhydrous HCl in methanol (45 μL). The neutralized solution was triturated into 1.25 mL diethyl ether and the solids were isolated via centrifugation. Intermediate polymer **S4.8** was dissolved in DMSO- d_6 over 16 h before ^1H -NMR analysis. ^1H -NMR (500 MHz, DMSO- d_6). Set backbone signal at δ 6.25-5.35 ppm to 3H. Signals at 8.09 (broad s, 1xH, triazole-H) and 1.77 (s, 2xH) were used to determine mannose content. Signal at 7.90 (m, 2xH) was used to determine acetophenone content. Found 50% acetophenone, 50% mannose incorporation.

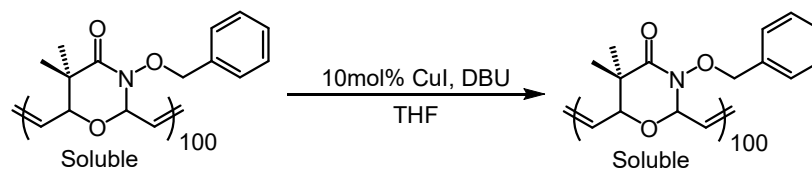


Polymer 4.20. To a solution of polymer **S4.8** (0.96 mg, 1.17 μmol (wrt ketone)) in DMSO (100 μL) was added alkoxyamine **4.13** (0.24 mg, 0.88 μmol) from a 16.4 mg/mL stock solution in DMSO and Alexa Fluor 488 C5-aminooxyacetamide bis(triethylammonium) salt (AF488) (0.73 mg, 0.88 μmol) from a 68.2 mg/mL stock solution in DMSO. Pyridine (1.131 μL , 10.4 μmol) was added and the solution was stirred at room temperature in the dark for 24 h. Benzaldehyde (1.79 μL , 1.76 μmol) was added and the solution was stirred for 1 h. The solution was triturated into diethyl ether (1.25 mL) and the solids were collected by centrifugation. Trituration was repeated until all small molecules were removed as determined by NMR. ^1H -NMR (500 MHz, DMSO- d_6). Set backbone signal at δ 6.30-5.15 ppm to 3H. Used signals at 8.06 (broad s, 1xH, triazole-H) and 1.74 (broad s, 2xH) to determine mannose content. Used signal at 8.34 (s, 1xH) to determine AF488 content. Found quantitative conversion to oxime; 50% mannose, 34% PEG₃OH, 16% AF488 conjugation.



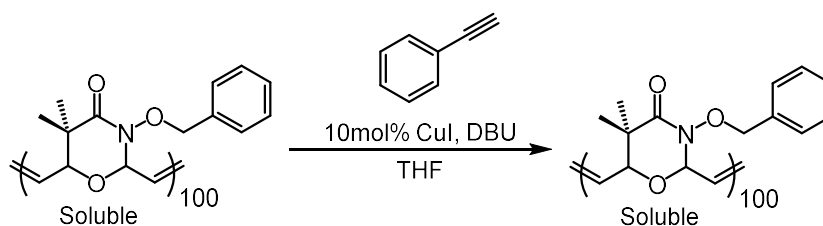
Polymer 4.21. Polymer **21** was synthesized following the general procedure using **S4.6** and AF488 as ligands in a 97:3 ratio (mol/mol). Triturating the solution into diethyl ether did not remove all excess ligand. The polymer was purified using a PD-10 desalting column. $^1\text{H-NMR}$ (500 MHz, MeOH-d_4): δ 7.65-7.05 (m, 4H), 6.10-5.60 (m, 2H), 5.55-5.30 (m, 1H), 5.00-4.55 (m, 2H, under solvent), 4.40-3.85 (m, 3H), 2.10-2.00 (m, 3H), 1.95-1.80 (m, 2H), 1.15-0.85 (m, 6H). Found 90% conversion to oxime; 86% guanidinium, 4% AF488.

Investigation of copper-mediated gelation of polyoxazinones



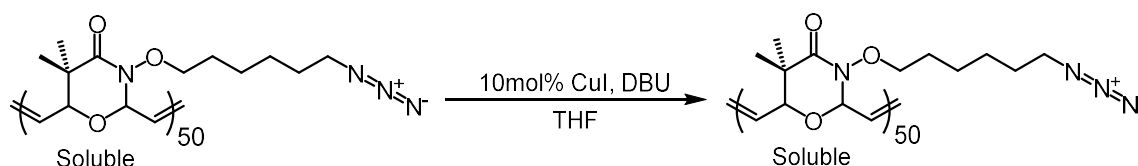
Experiment 1. In a 2.5mL vial was concentrated 4.3mg *O*-Benzyl-Polyoxazinone 100mer (151nmol, 1 equiv.) in a few hundred microliters CH_2Cl_2 and a stir bar added. In a 6mL vial was prepared a stock solution of 1.35 $\mu\text{L/mL}$ 1,8-Diazoabicyclo[5.4.0]undec-7-ene (DBU) in THF. This stock solution was used to prepare a final stock solution of

1.35μL/mL DBU and 1.78mg/mL Copper (I) Iodide. To the polymer concentrate was added 156μL of the DBU/CuI (DBU: 1.41μmol, 0.093 equiv. with respect to monomer, CuI: 1.46μmol, 0.097 equiv. with respect to monomer) solution and the reaction was allowed to proceed for 19 hours with occasional monitoring for gelation during the first 5 hours of reaction. After stirring for 19 hours the solution was concentrated and taken up in CDCl₃ for ¹H NMR analysis which indicated the presence of substantial quantities of *O*-Benzyl-Polyoxazinone 100mer. No precipitate or gel was observed.



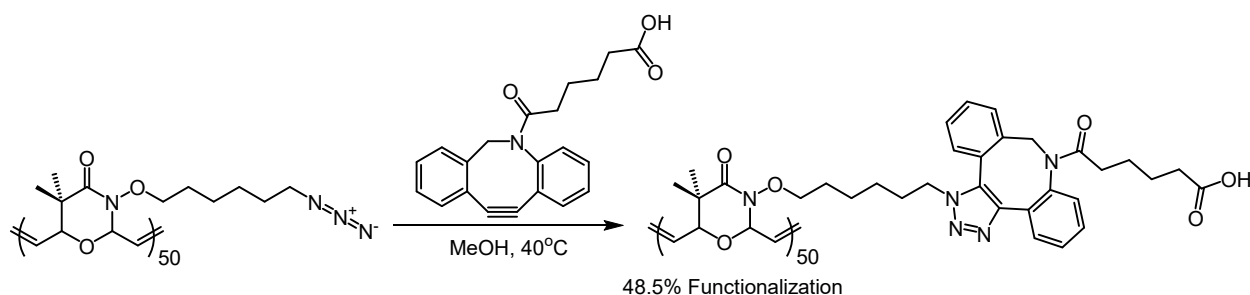
Experiment 2. To a flame-dried Nitrogen-flushed 6mL vial equipped with a stir bar was added 20mg (70.4nmol, 1 equiv.) *O*-Benzyl-Polyoxazinone 100mer. In a separate flame-dried Nitrogen-flushed 6mL vial was prepared a stock solution of 1.27μL/mL DBU and 11.3μL/mL Phenylacetylene in dry THF. Using this solution, a final stock solution of 1.27μL/mL DBU, 11.3μL/mL Phenylacetylene, and 1.63mg/mL Copper (I) Iodide was prepared in a flame-dried Nitrogen-flushed vial in dry THF. 824μL of this solution (DBU: 7.01μmol, 0.1 equiv. with respect to monomer, Phenylacetylene: 84.8μmol, 1.2 equiv. with respect to monomer, CuI: 7.05μmol, 0.1 equiv. with respect to monomer) was added to the polymer concentrate and the reaction allowed to proceed under a Nitrogen atmosphere for 17 hours with occasional monitoring for gelation during the first 2 hours of reaction. After stirring for 17 hours the solution was concentrated, suspended in CDCl₃, passed over a 0.22μM syringe filter, and analyzed by ¹H NMR which indicated significant quantities of *O*-Benzyl-Polyoxazinone 100mer.

Note that the precipitate is thought to be CuI which was found to be insoluble in 0.5-1mL CDCl₃ in the amounts used in this experiment even on heating. It is hypothesized that in experiment 1 the quantity of CuI was small enough to allow for dissolution in 0.5-1mL CDCl₃.

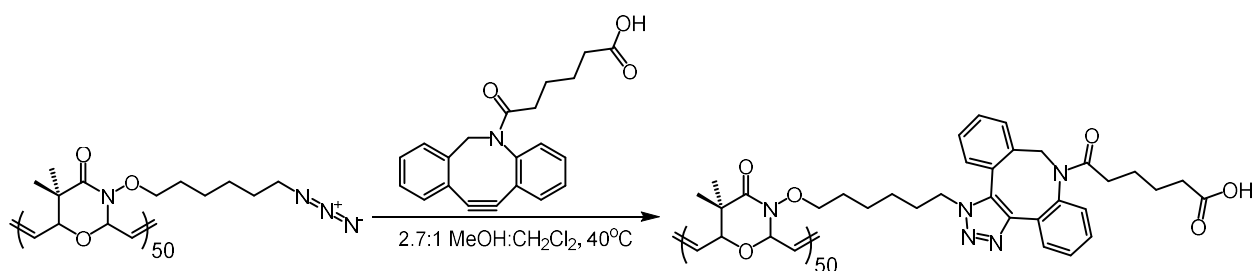


Experiment 3. In a 6mL vial was concentrated 39.9mg *O*-6-Azidoethyl Polyoxazinone 50mer²⁶³ and high vacuum pulled on the material for 2 hours before flushing with Nitrogen. The concentrate was taken up in 1.26mL dry THF and 630μL (*O*-6-Azidoethyl Polyoxazinone 50mer: 20.0mg, 1.36μmol, 1 equiv.) of the solution was transferred to a flame-dried Nitrogen-flushed 5mL round bottom flask equipped with a stir bar. In a flame-dried Nitrogen-flushed 6mL vial was prepared a stock solution of 2.69μL/mL DBU in dry THF. Using this solution, a final stock solution of 2.69μL/mL DBU and 3.43mg/mL Copper (I) Iodide was prepared in a flame-dried Nitrogen flushed 6mL vial and 824μL (DBU: 14.9μmol, 0.22 equiv. with respect to monomer, CuI: 14.8μmol, 0.22 equiv. with respect to monomer) of this stock solution was added to the polymer solution and the reaction maintained under a Nitrogen atmosphere for 16 hours with occasional monitoring for the first 2 hours of reaction for gelation. After stirring for 16 hours the reactant solution was concentrated and taken up in CDCl₃ and passed over a 0.22μM syringe filter to remove insoluble CuI for ¹H NMR analysis which indicated a

substantial quantity of *O*-6-Azidoethyl Polyoxazinone 50mer. No gelation was detected during the experiment.

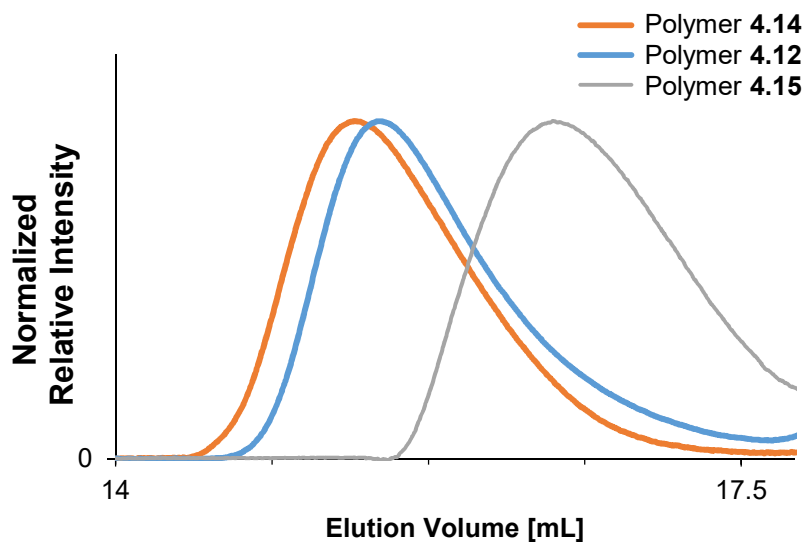


Polymer S4.9. In a 2.5mL was concentrated a solution of 3.8mg *O*-6-Azidoethyl Polyoxazinone 50mer²⁶³ (258nmol, 1 equiv.) in a few hundred microliters CH₂Cl₂ and a stir bar added. The concentrate was dissolved in 100μL MeOH and 5.2mg Azadibenzocyclooctyne Acid (ADIBO-A) (15.6μmol, 1.21 equiv. with respect to monomer). The reaction was heated to 40°C and allowed to proceed for 1 hour at which point the ADIBO-A appeared nearly fully consumed by TLC (100% EtOAc) and the reactant solution was concentrated. The concentrate was taken up in 200μL CH₂Cl₂ and added dropwise to 1.2mL Diethyl Ether in a 1.5mL Eppendorf tube, centrifuged at 12,000G for 2 minutes, and the supernatant decanted. The precipitate was taken up in 200μL CH₂Cl₂ and re-precipitated by repeating the above procedure to afford 1.5mg of orange solid which was found to be roughly 48.5% functionalized by NMR. ¹H-NMR (CD₂Cl₂, 600 MHz): 7.72-7.05 (m, 3.9H, partial functionalization), 6.29-5.73 (m, 2H), 5.37ppm (s, 1H, overlap with solvent), 4.61-4.18 (m, 2H), 4.07-3.72 (m, 1H), 2.58-0.63 (br m, 22H). PDI = 4.19

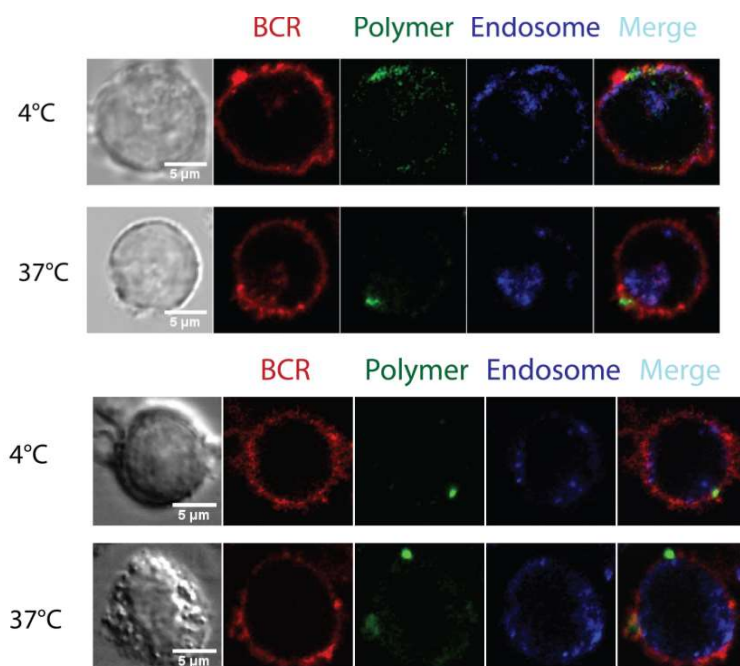


Polymer S4.10. In a 6mL vial was concentrated a solution of 14mg *O*-6-Azidohexyl Polyoxazinone 50mer (951nmol, 1 equiv.) in a few hundred μL CH_2Cl_2 and a stir bar was added. The concentrate was dissolved in 100 μL CH_2Cl_2 followed by addition of 268 μL MeOH which induced brief formation of a white precipitate, though, this material re-dissolved on swirling for about 5 seconds. To this solution was added 19mg Azadibenzocyclooctyne Acid (ADIBO-A) (57.0 μmol , 1.2 equiv. with respect to monomer) and the reaction was heated to 40°C and monitored by TLC (100% EtOAc). The starting material was mostly consumed over a period of 6 hours and the baseline polymer spot became fluorescent under UV irradiation as the reaction proceeded. On removing from the oil bath a fine off-white precipitate formed which concentrated to a yellow-brown solid. The concentrate was taken up in 200 μL CH_2Cl_2 and added dropwise to 1.2mL Diethyl Ether in a 1.5mL Eppendorf tube, centrifuged at 12,000G for 2 minutes, and the supernatant decanted. The precipitate was taken up in 200 μL CH_2Cl_2 and re-precipitated by repeating the above procedure to afford 12.5mg (yield = 42%) of 91.5% functionalized material. Product was an orange-tinted white solid as obtained from precipitation which concentrated from CH_2Cl_2 to afford a beige solid. ^1H -NMR ($\text{CD}_2\text{Cl}_2/\text{MeOD}$, 400MHz): 7.63-6.96 (m, 7.32H, partial functionalization), 6.21-5.6 (m,

2H), 5.60-5.33 (m, 1H, overlap with solvent), 4.52-4.15 (m, 6H) 4.02-3.65 (m, 1H), 3.31-3.22 (m, 1H), 2.49-0.61 (m, 21H, partial functionalization). PDI = 2.13



Experimental Details Figure 4.1. GPC characterization of polymer **4.12** ($RI_{\max} = 15.47$ mL, PDI = 1.32), **4.14** ($RI_{\max} = 15.34$ mL, PDI = 1.31) and **4.15** ($RI_{\max} = 16.45$ mL, PDI = 1.28). A shift in retention time to a lower elution volume upon oxime formation indicates successful attachment of **4.13**. Due to the lower solubility of polymer **4.15** in THF as compared to **4.12** or **4.14**, there is a shift to a higher elution volume upon oxime reduction. The constant PDI measurements indicate the backbone is stable throughout these two transformations.



Experimental Details Figure 4.2. A20HL B-cells, which express a DNP-binding BCR, were exposed to polymers A) **4.19**, conjugated to PEG₃OH, and B) **4.20**, conjugated to mannose. Neither substrate was internalized by the A20HL cells or co-localized with either the BCR or transferrin positive endosomes.

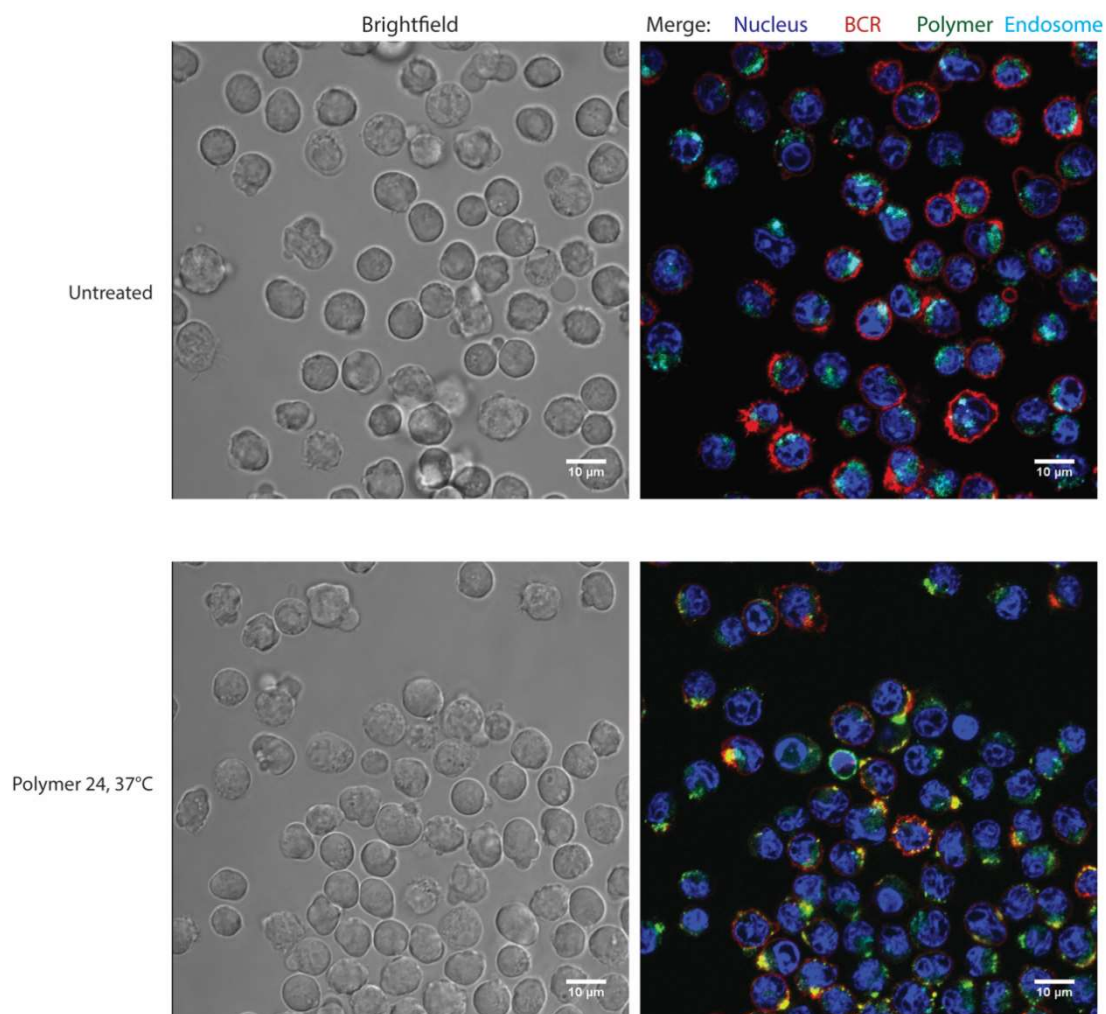


Figure S4.3. Field of view of B lymphocytes (A20HL cells) treated with polymer **4.18** (5 μg/mL) at 37 °C as described above.

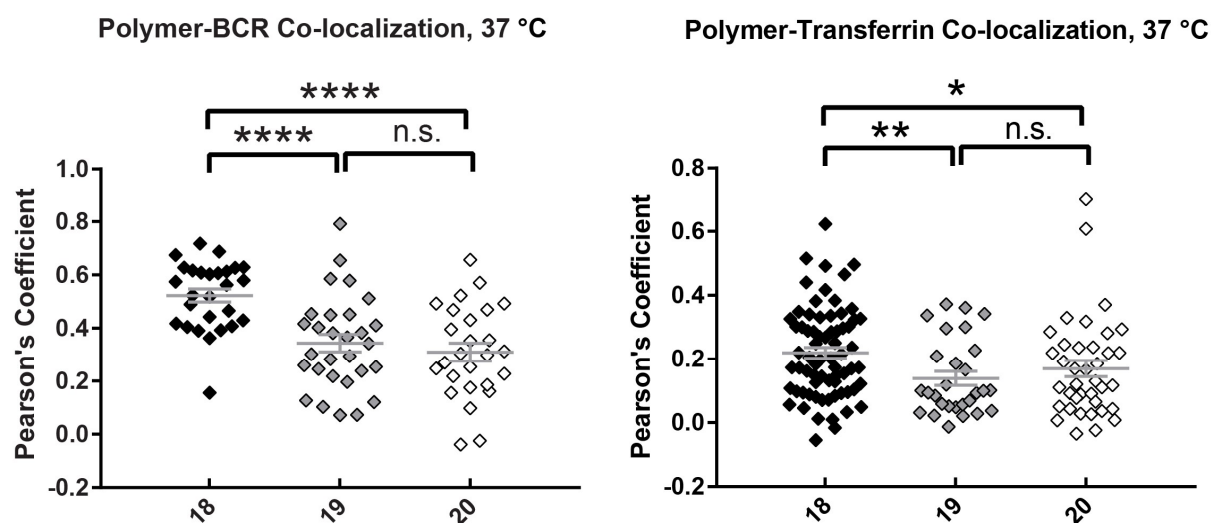
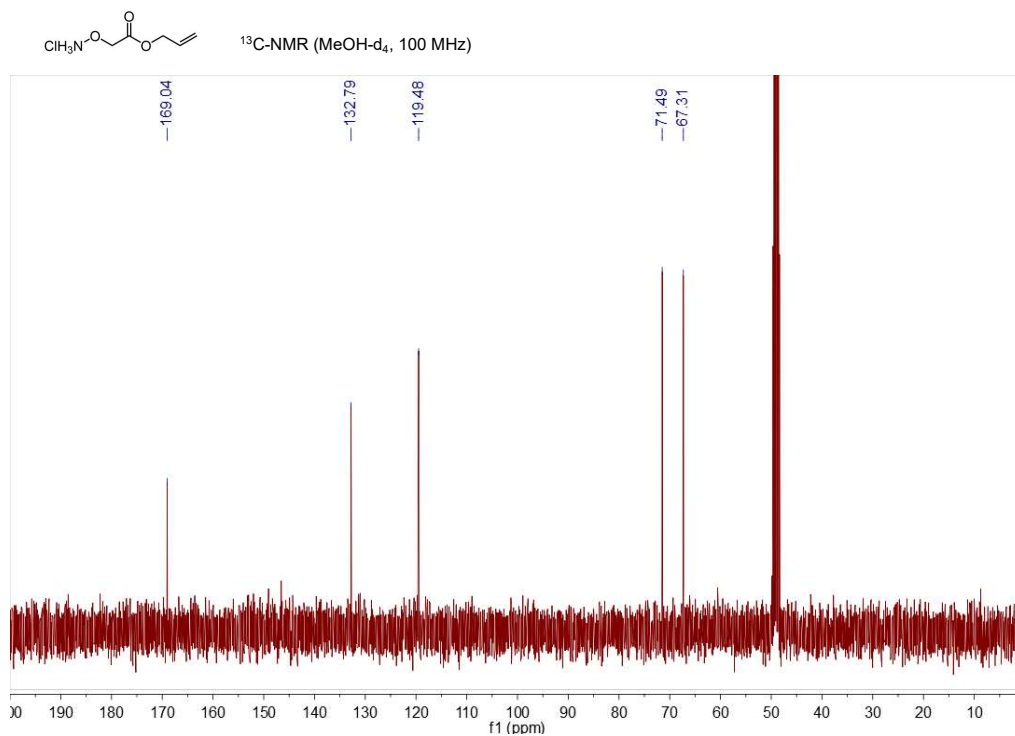
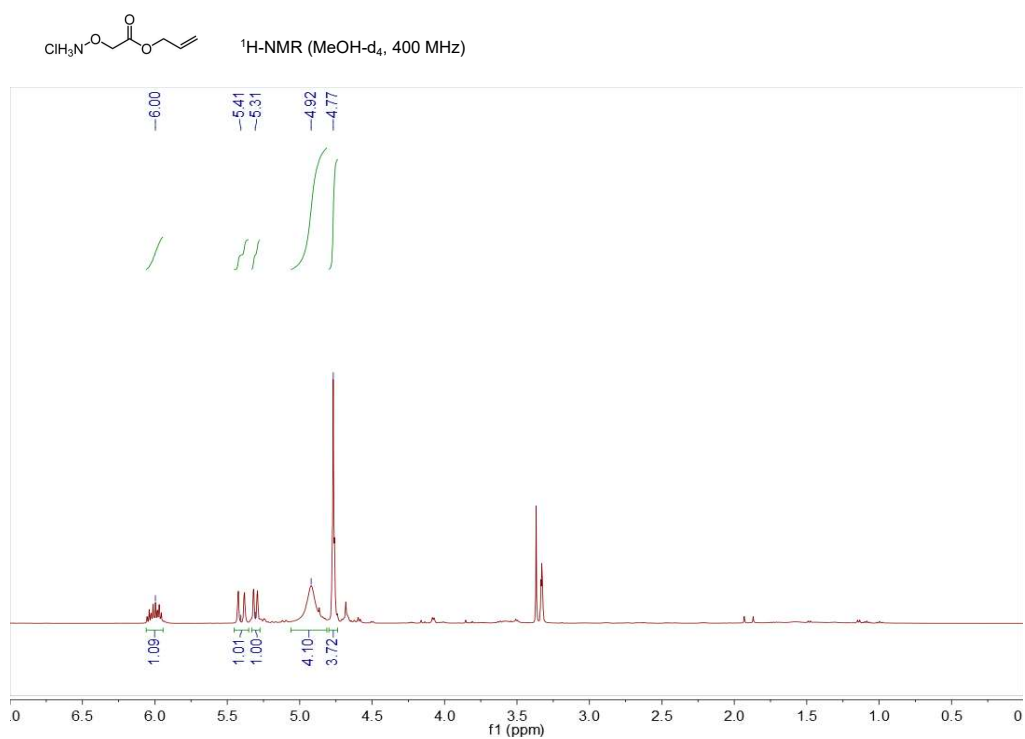


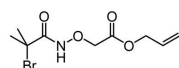
Figure S4. A20HL B-cells, which express a DNP-binding BCR, were exposed to polymers **18**, **19**, and **20** (Figure 5). Quantification of uptake data. Left: Polymer co-localization with BCR at 37 °C. Right: Polymer co-localization with early endosomal marker transferrin at 37 °C. The Pearson's coefficient was calculated using at least 25 cells for each condition. * $P=0.0295$, ** $P<0.01$, **** $P<0.0001$ (Mann-Whitney test). Arrow indicates intracellular fluorescence. Data are representative of 3 independent experiments. Error bars represent the standard error of the mean for one of the 3 independent experiments. Bar graphs derived from these scatter plots are shown in Figure 5B.

^1H and ^{13}C NMR Spectra

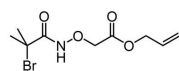
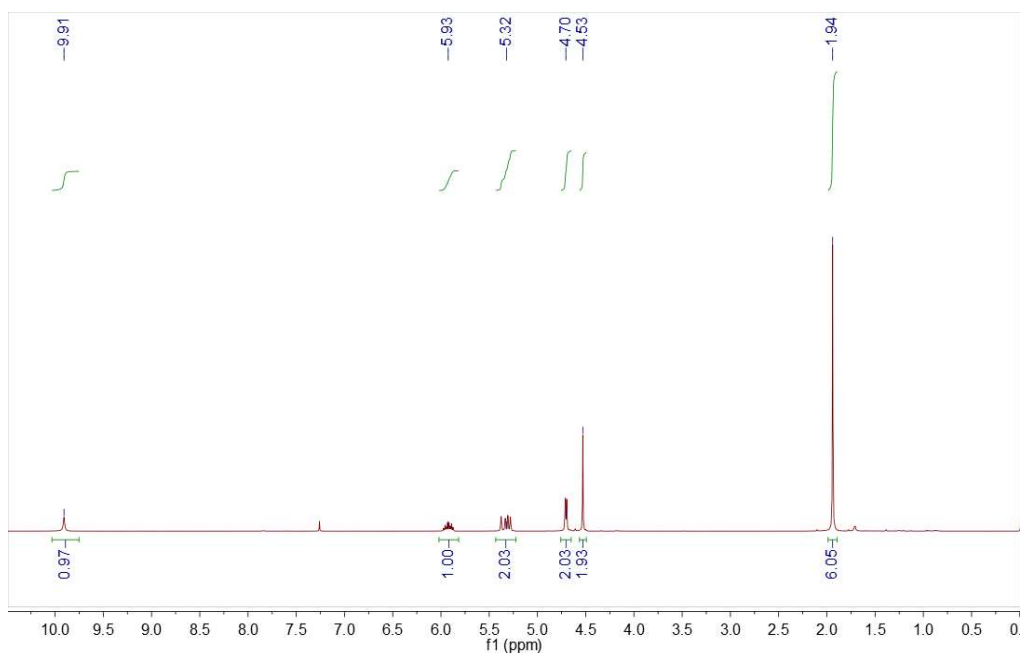
Compound 4.5a



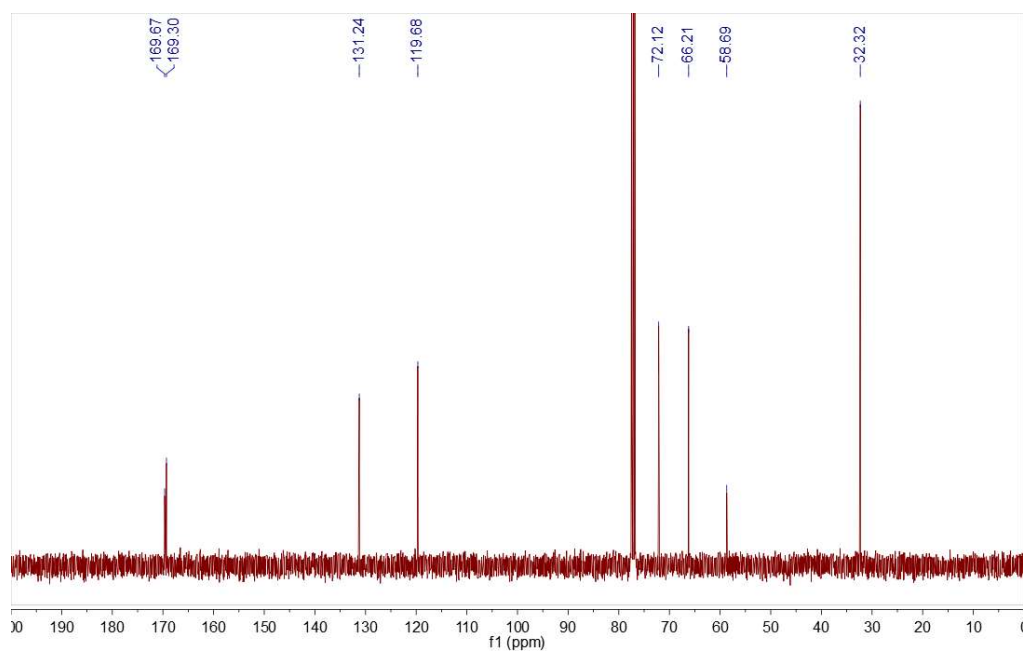
Compound 4.5



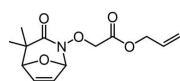
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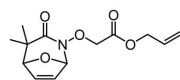
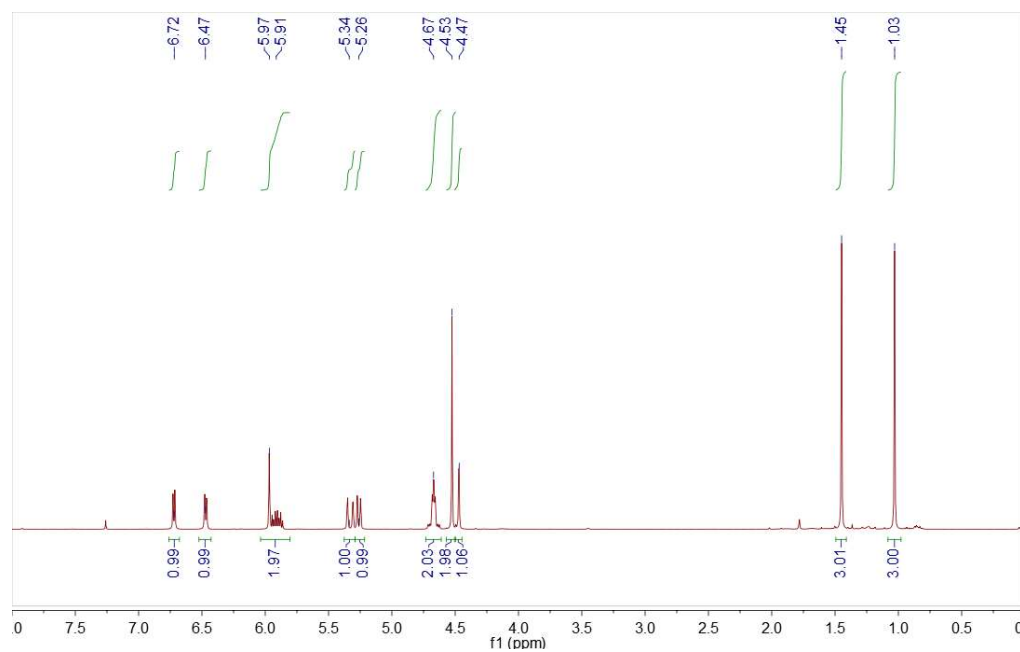
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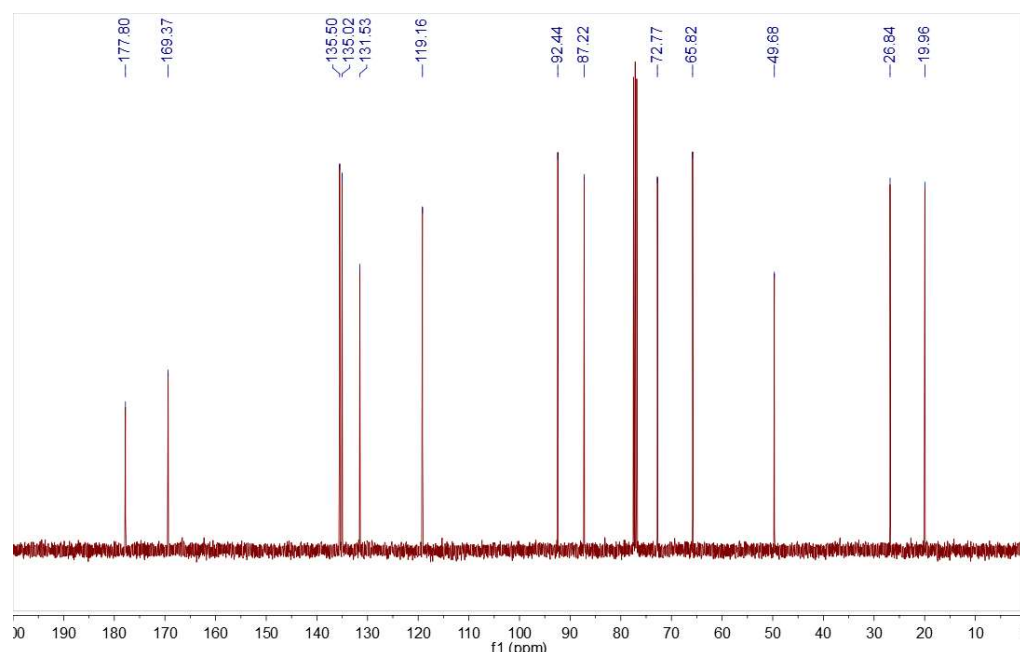
Compound 4.6a



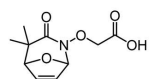
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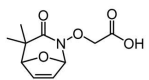
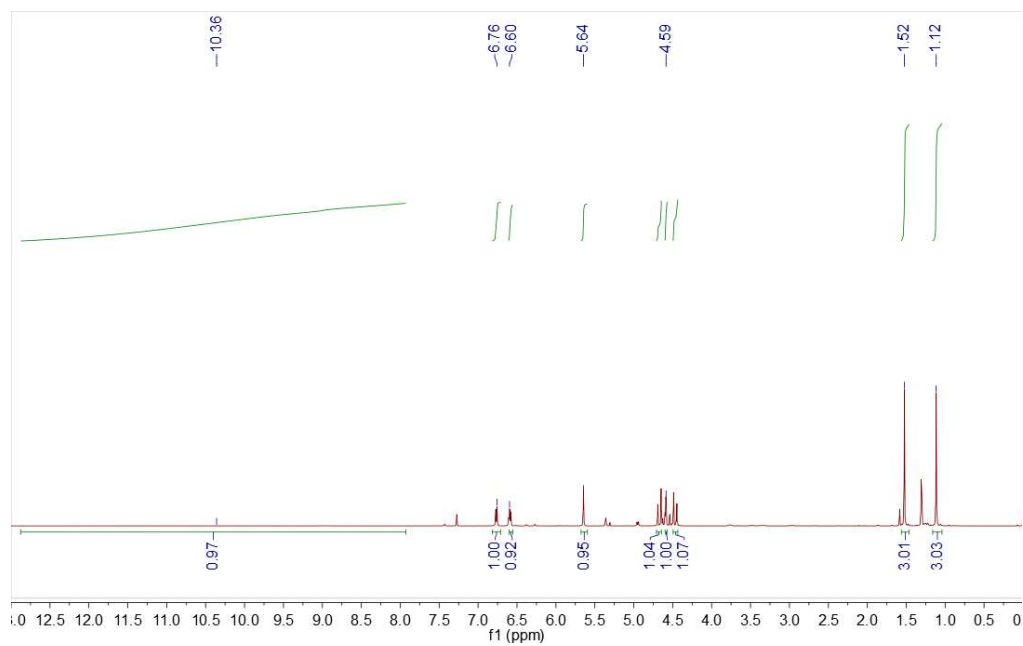
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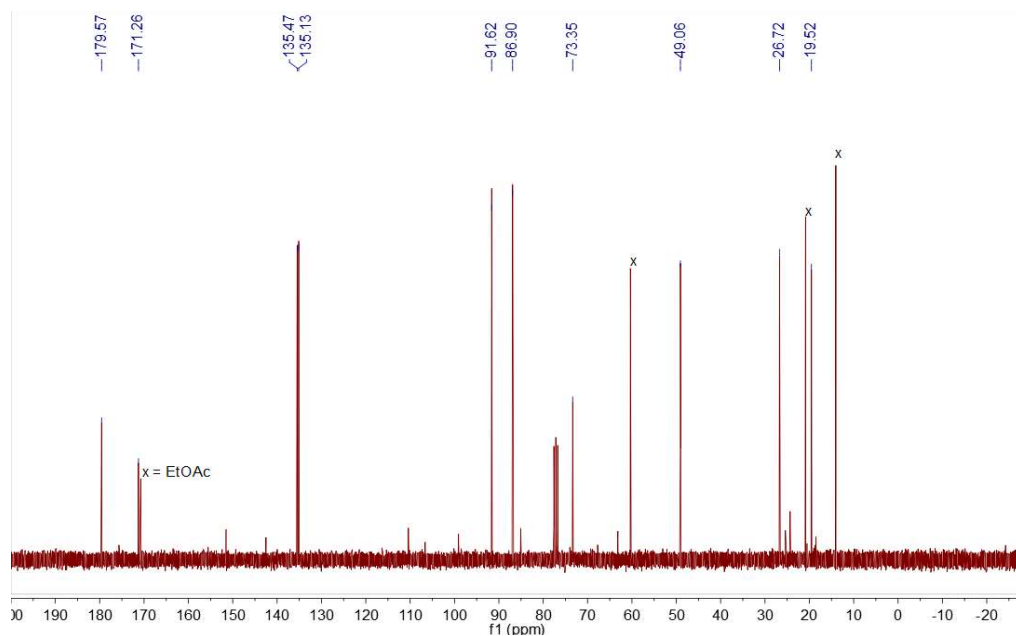
Compound 4.6



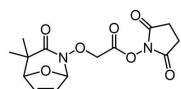
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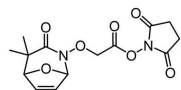
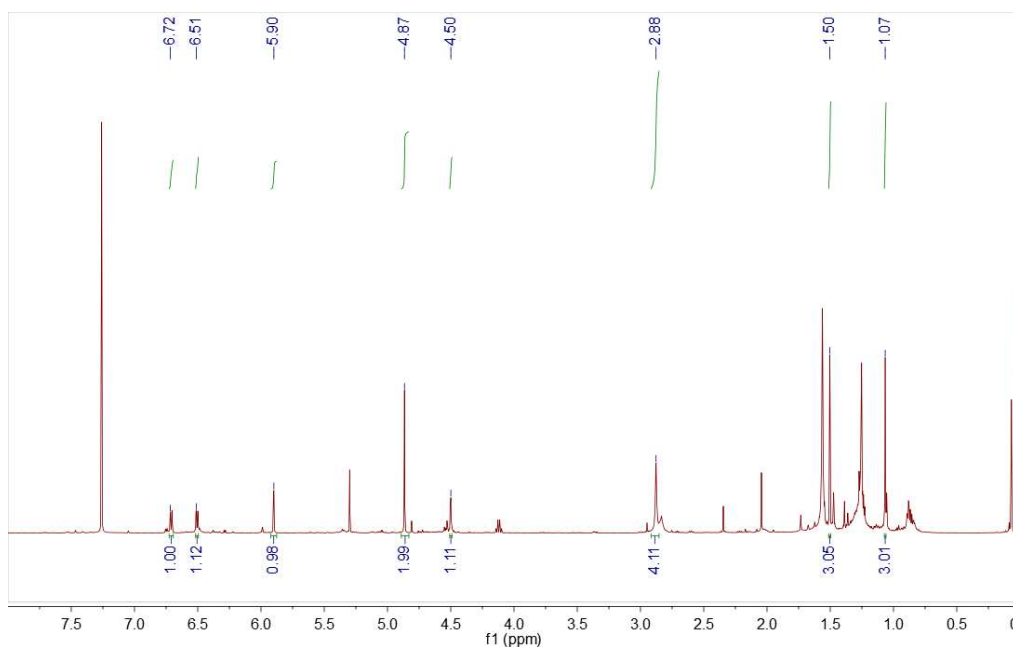
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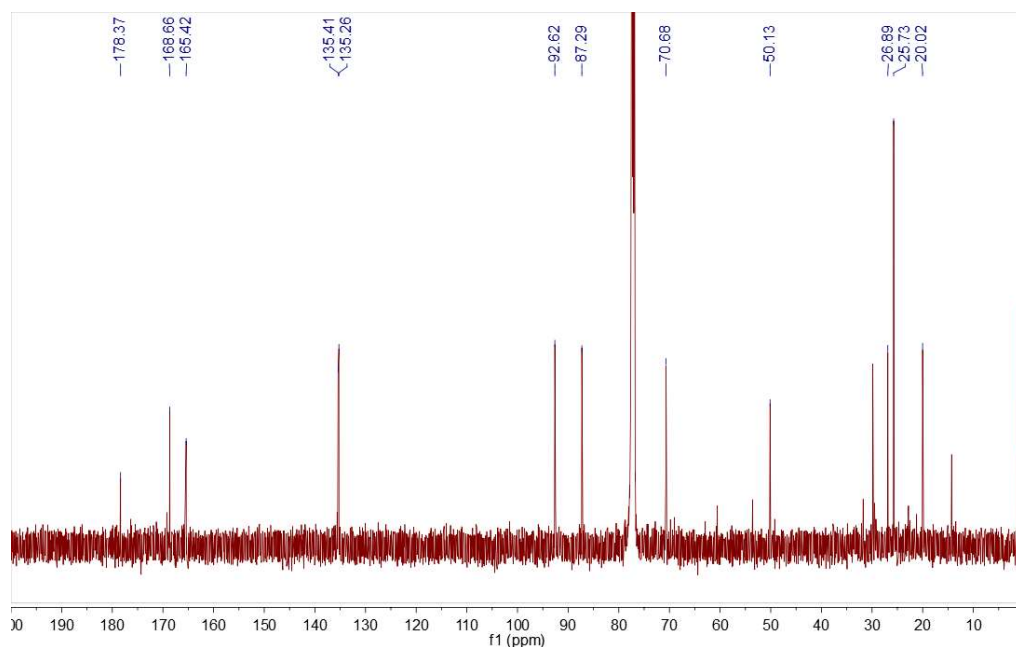
Compound 4.7



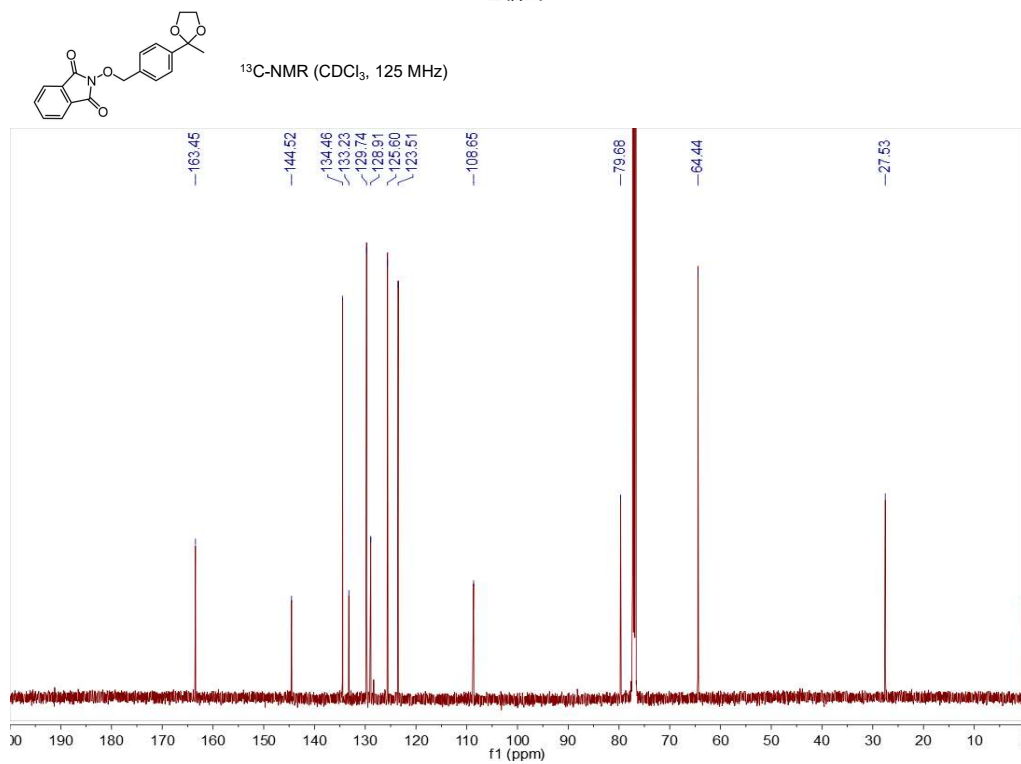
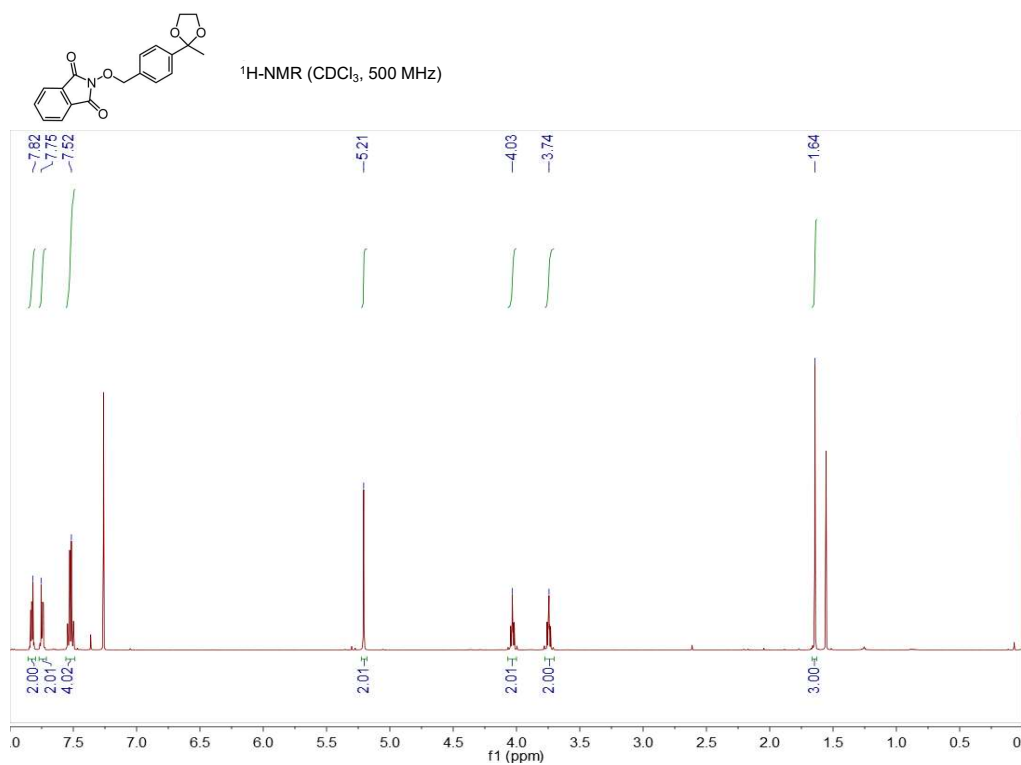
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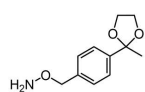
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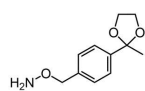
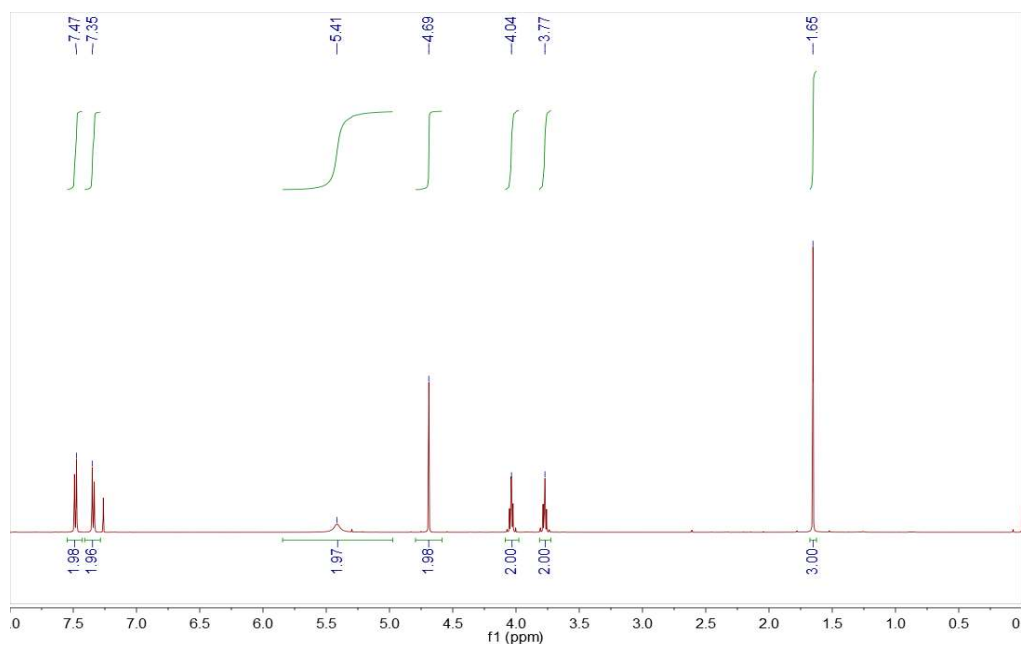
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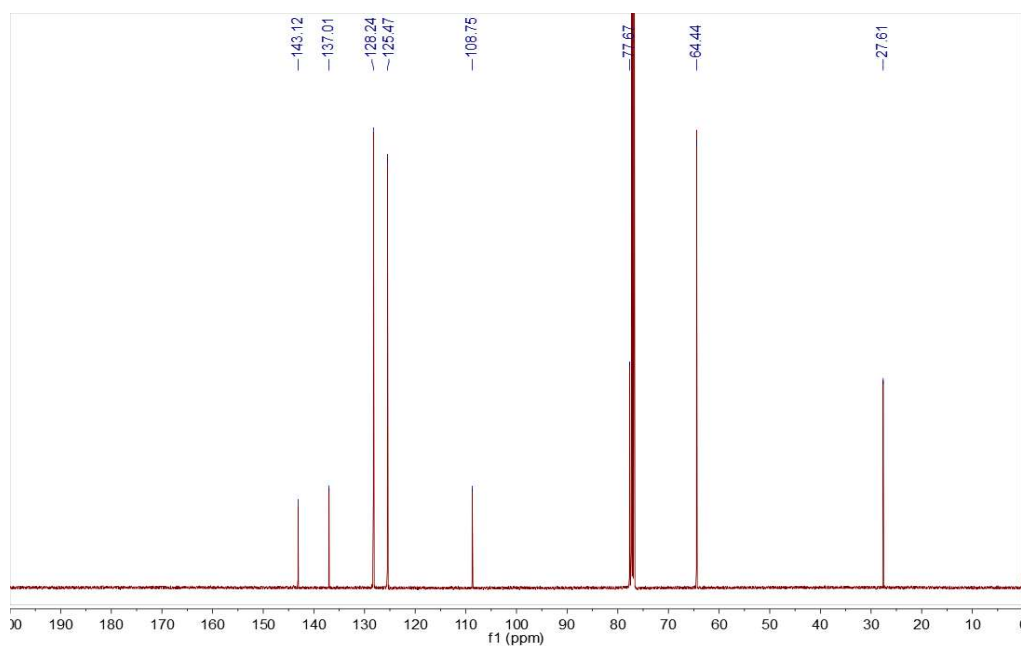
Compound 4.10a



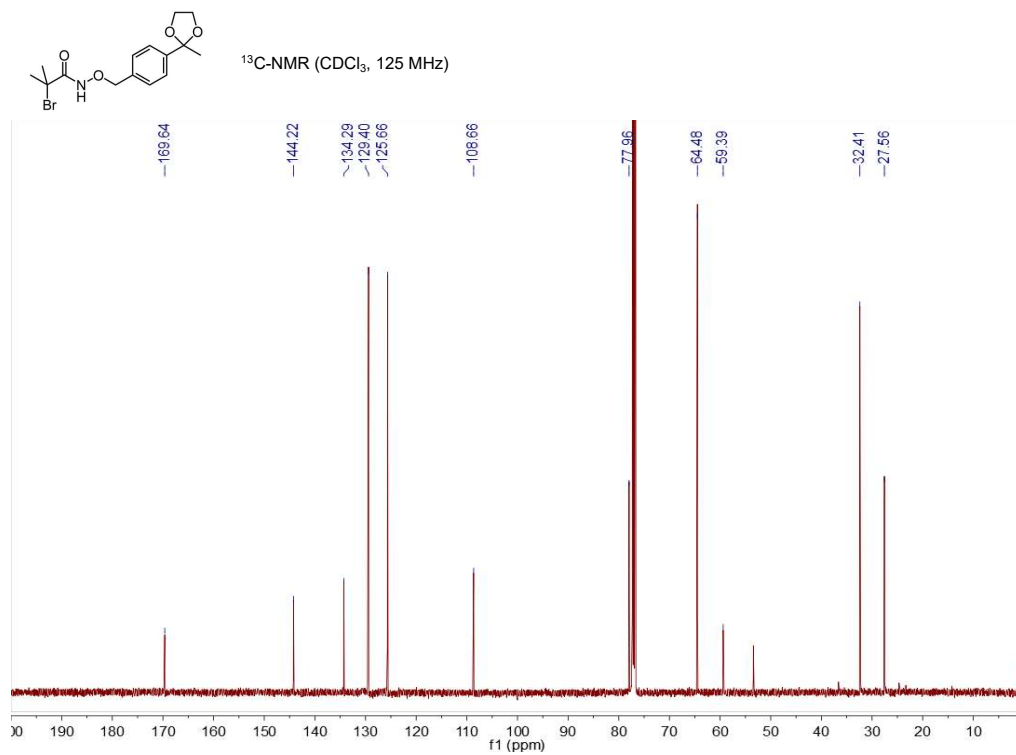
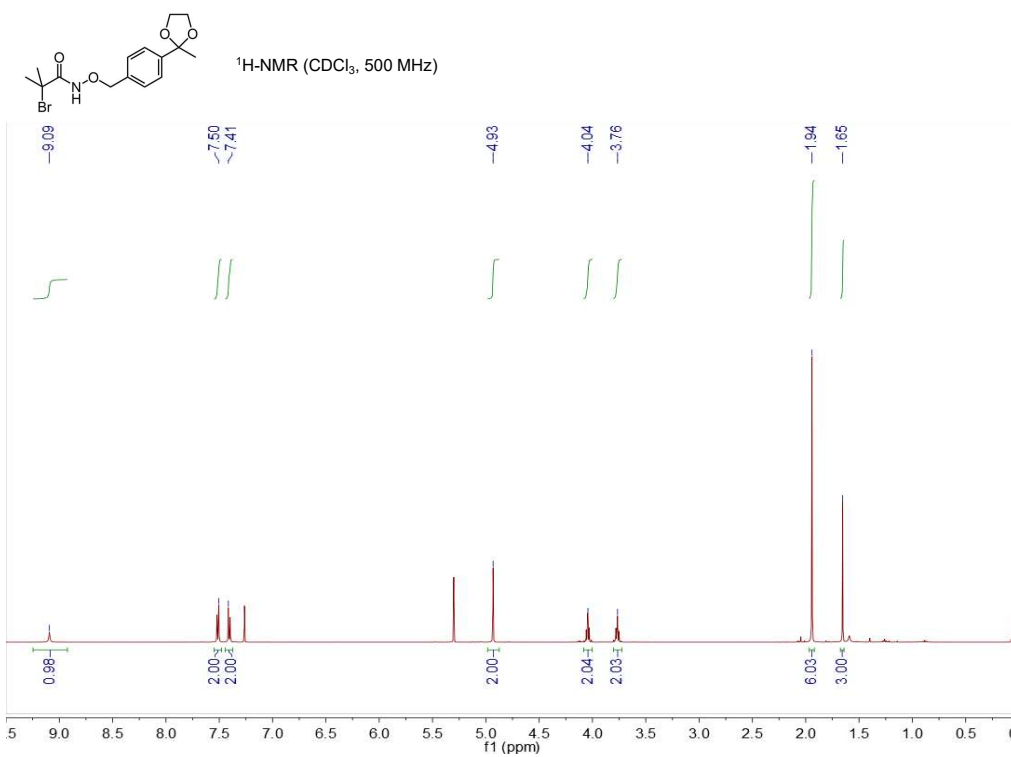
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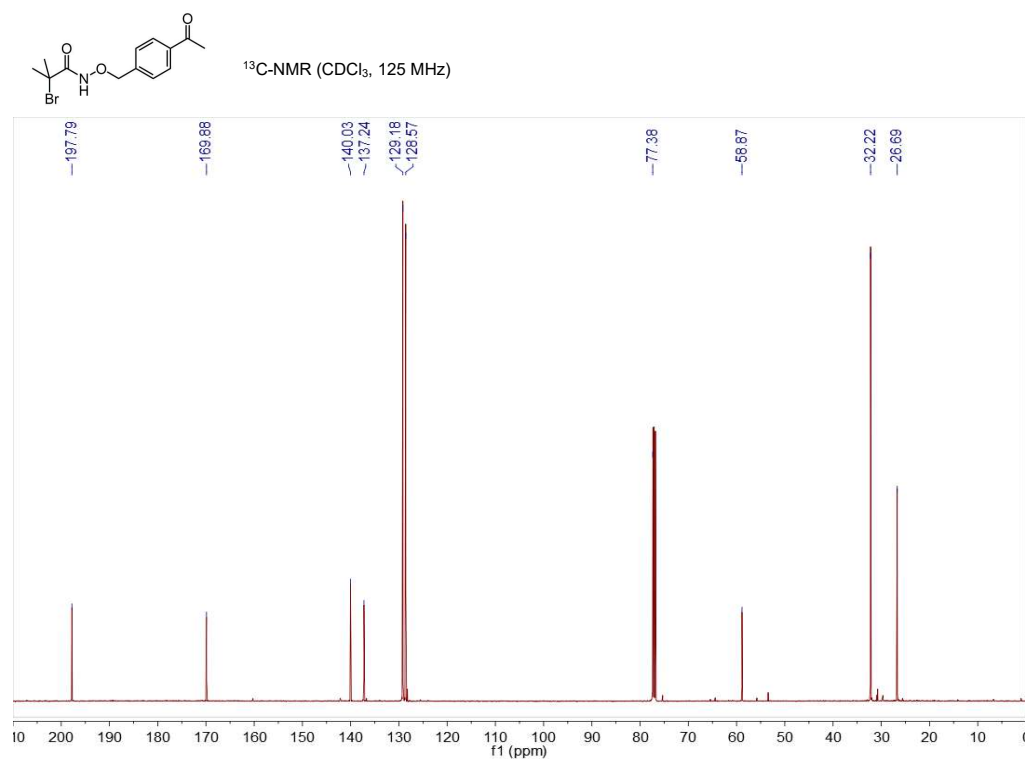
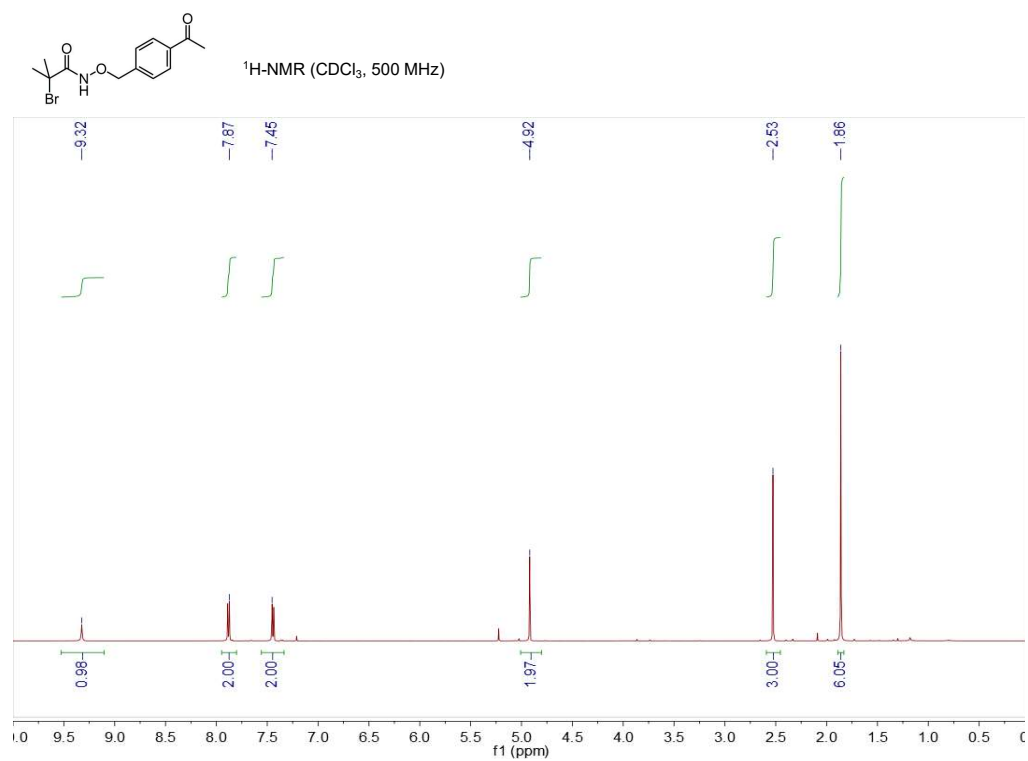
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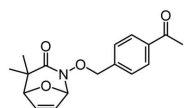
Compound 4.10b



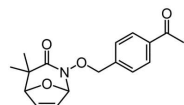
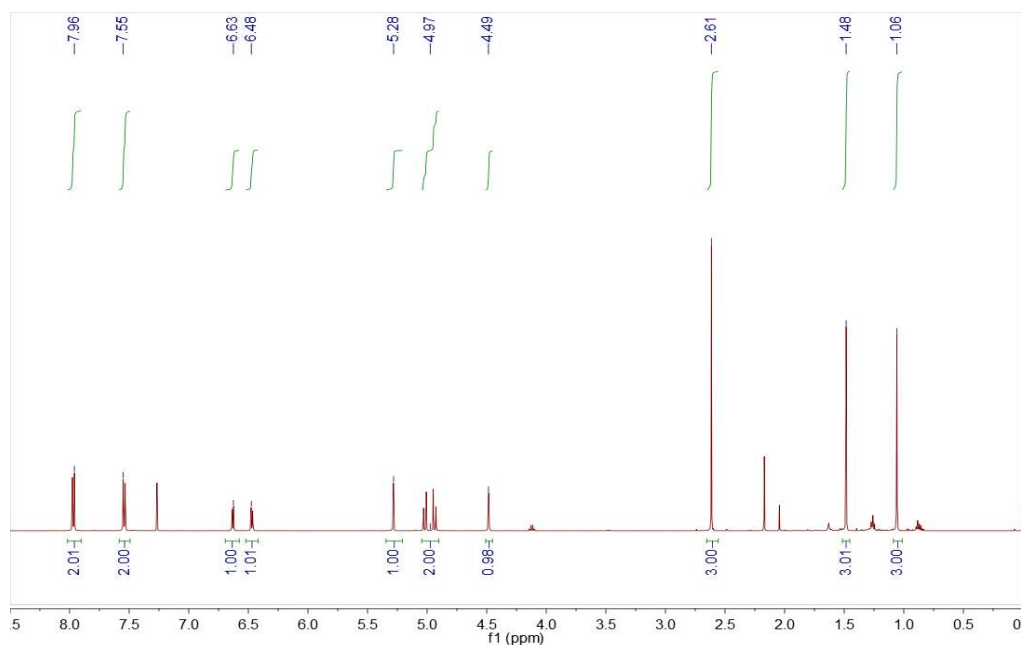
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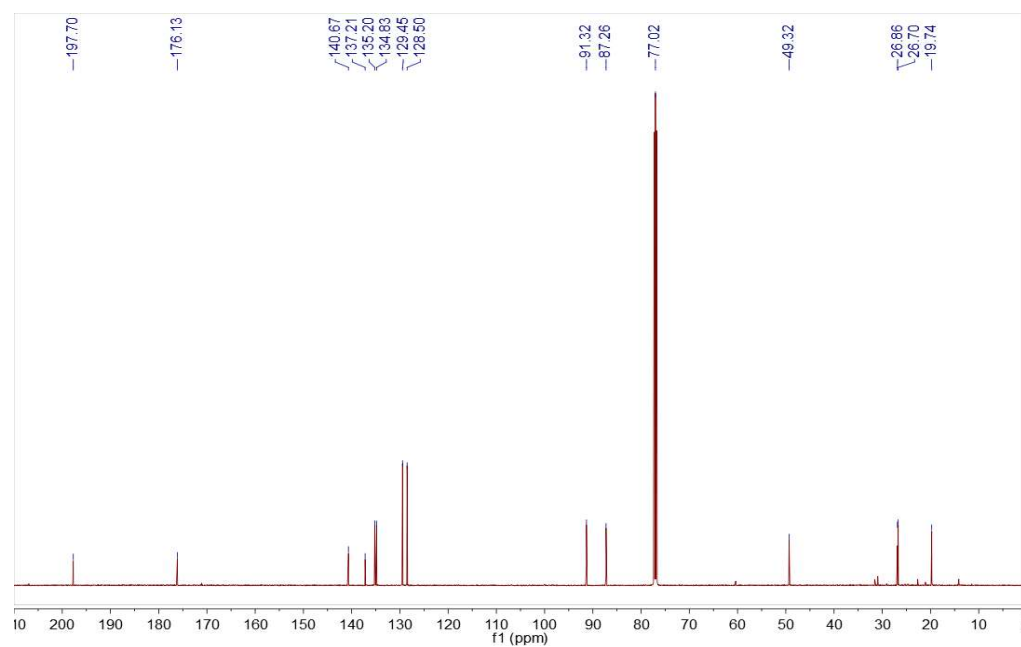
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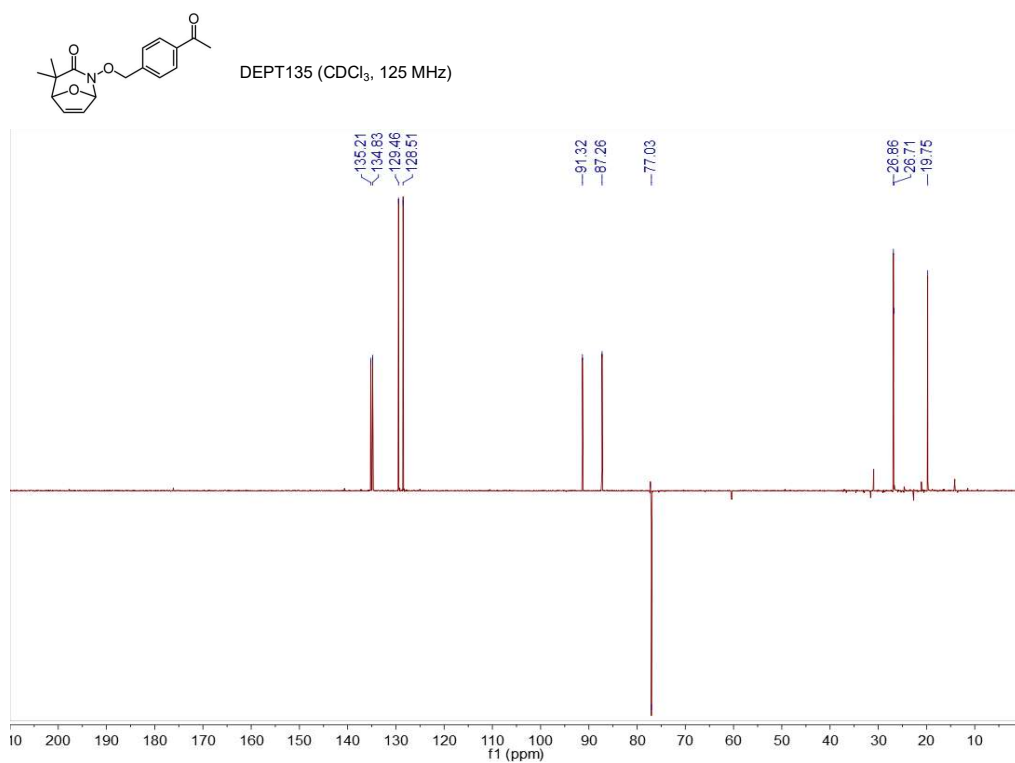
11
¹H-NMR (CDCl₃, 500 MHz)



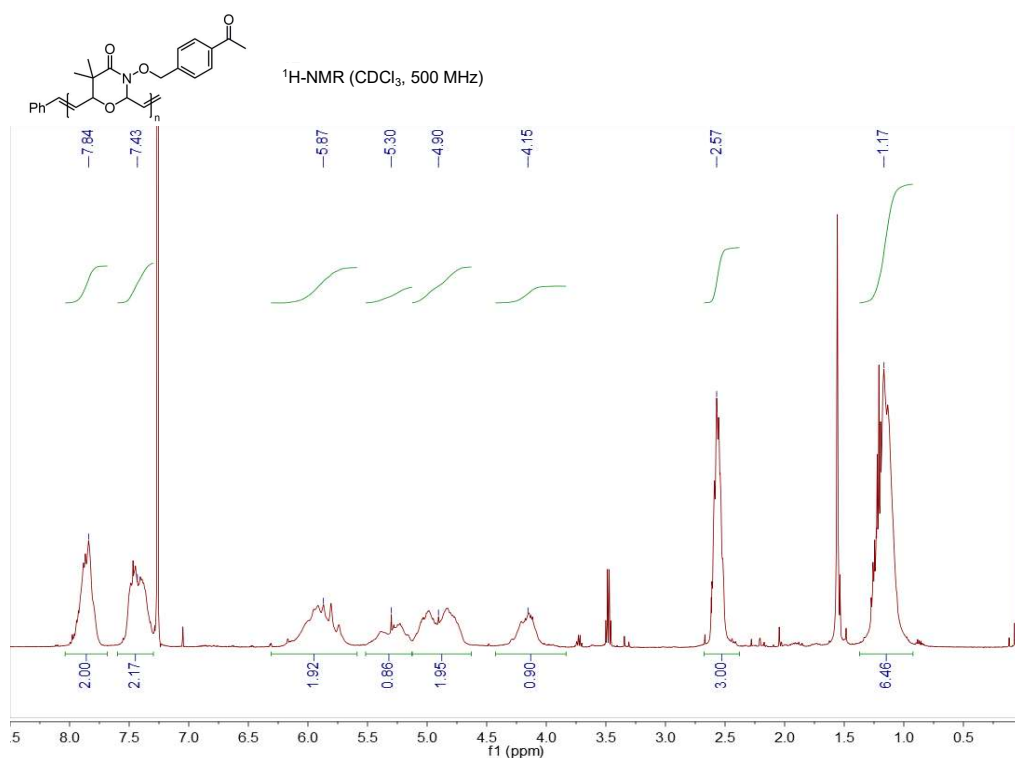
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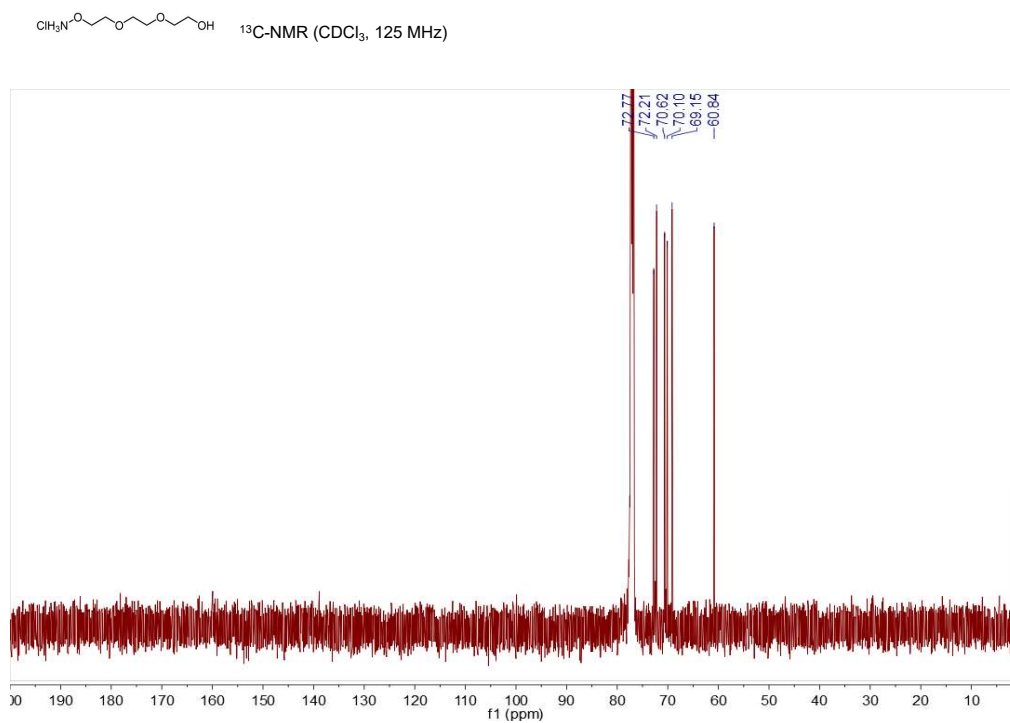
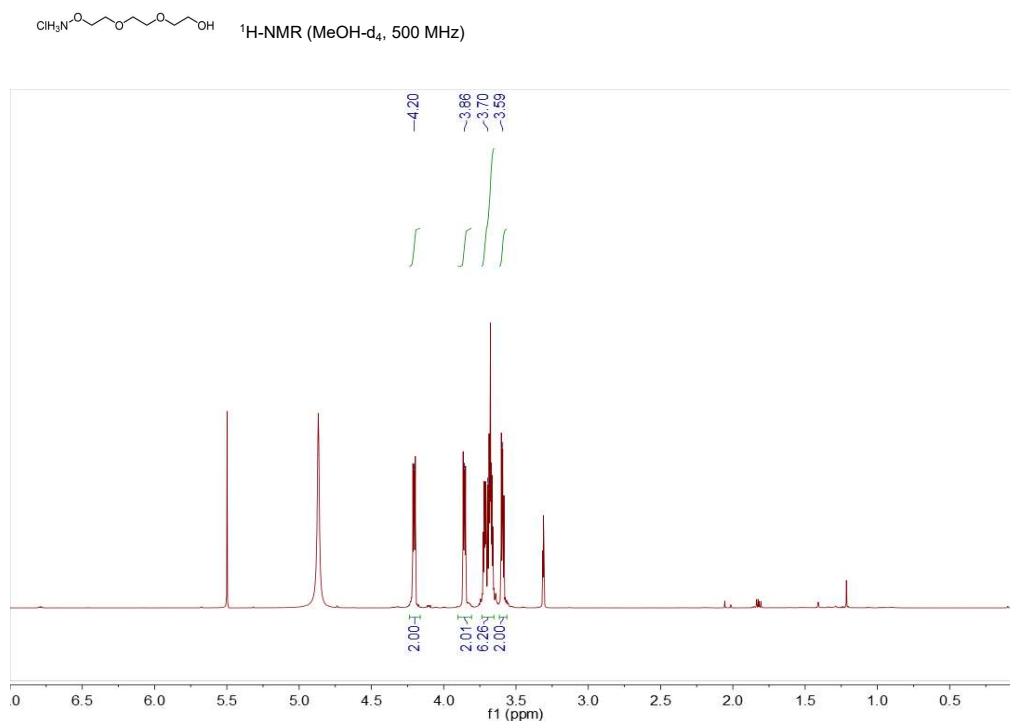
Compound 4.11 DEPT



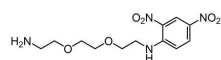
Polymer 12



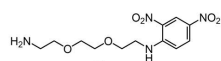
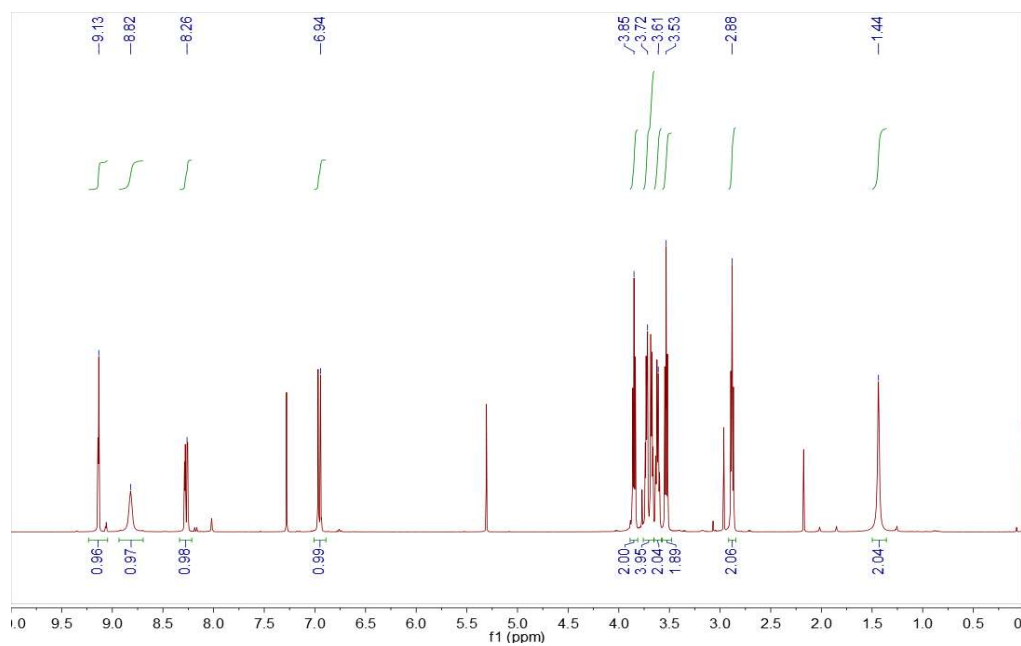
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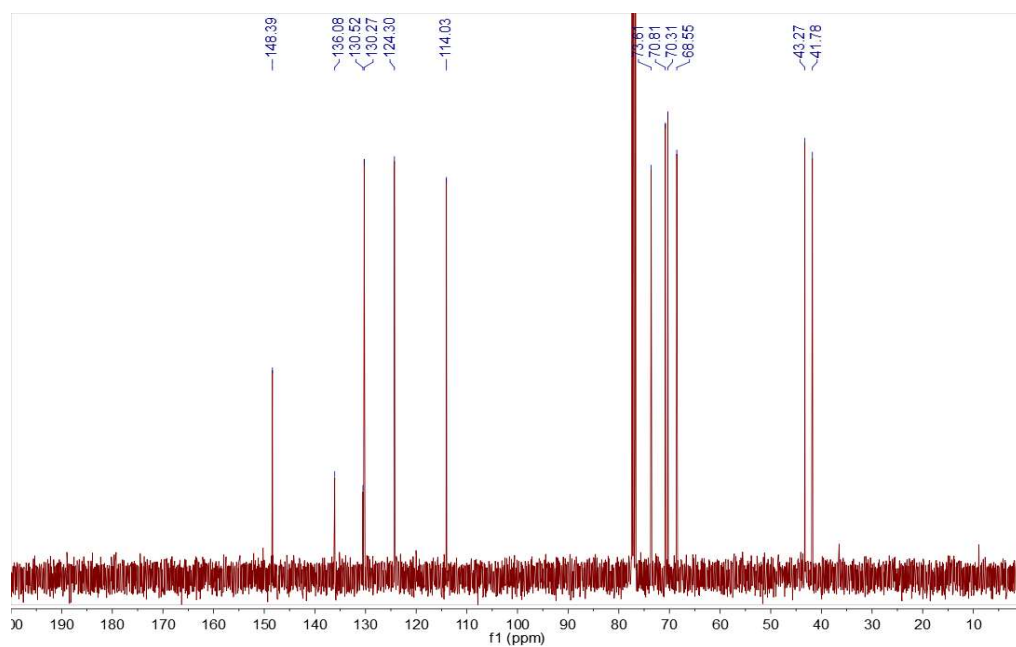
Compound S4.1



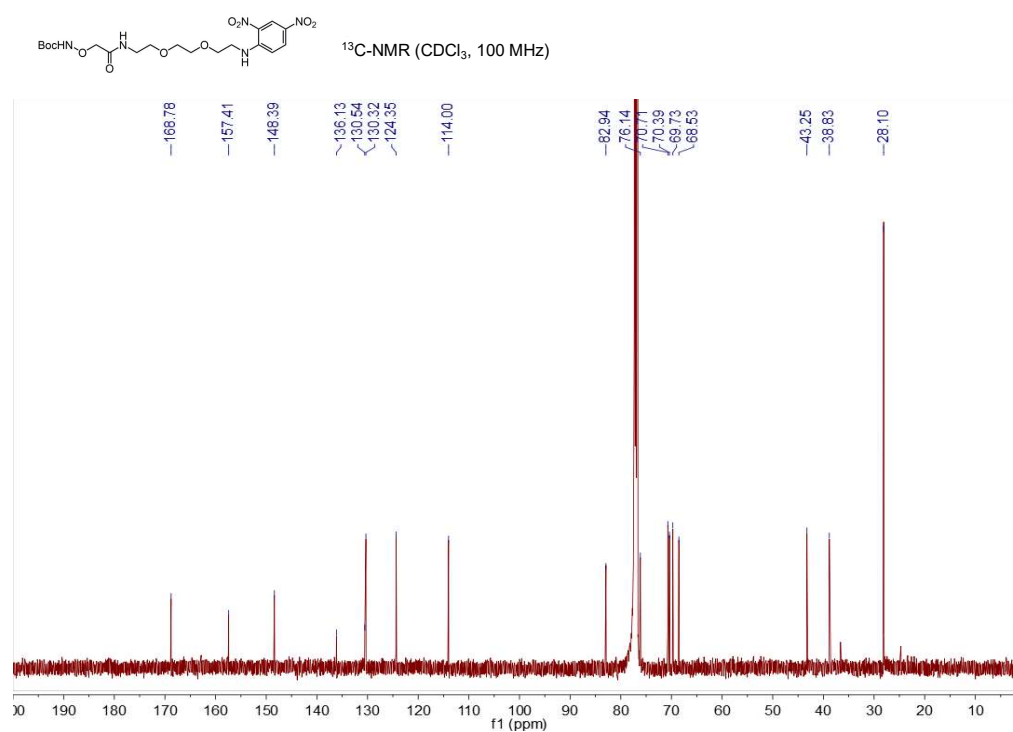
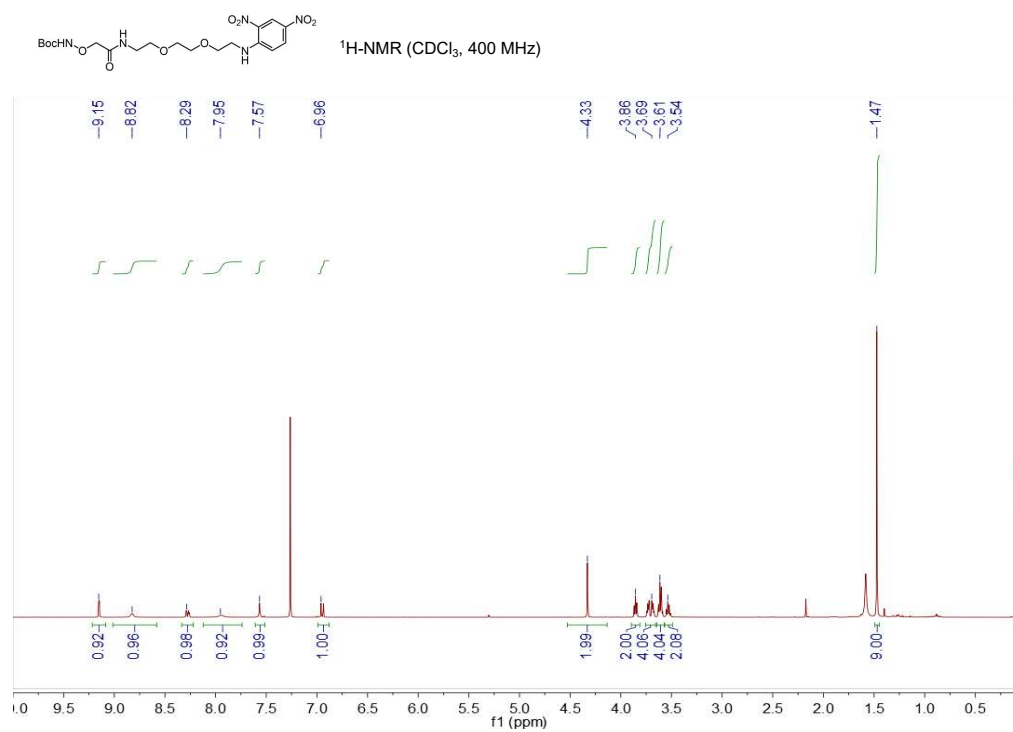
$^1\text{H-NMR}$ (CDCl_3 , 400 MHz)



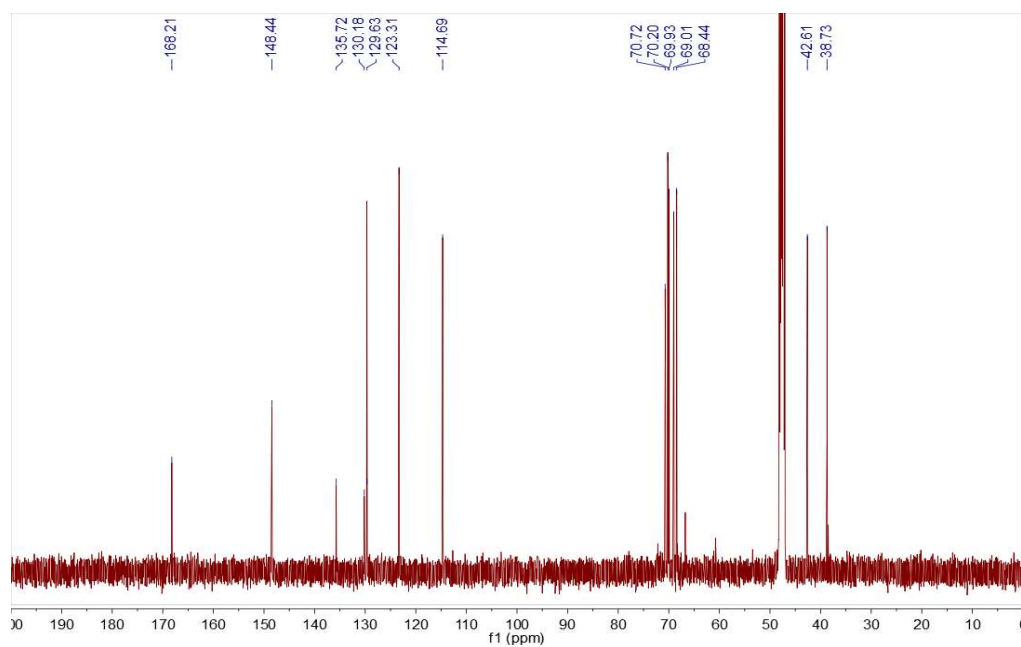
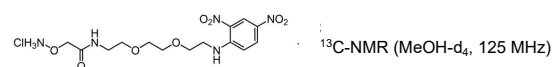
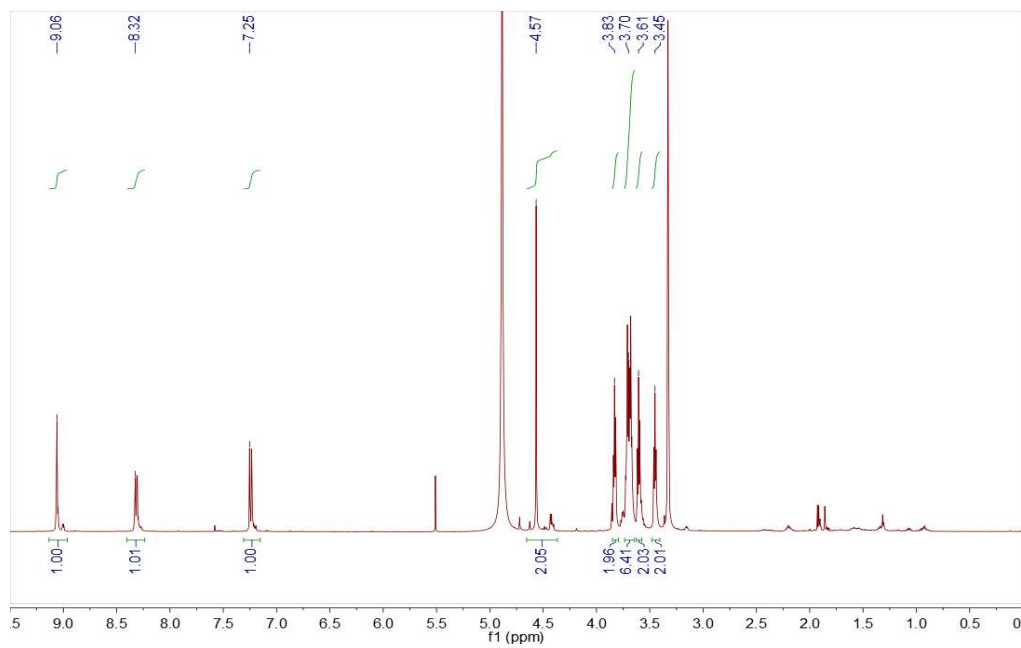
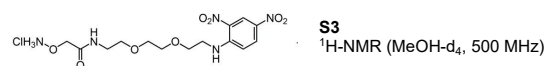
$^{13}\text{C-NMR}$ (CDCl_3 , 100 MHz)



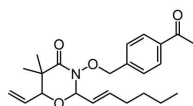
Compound S4.2



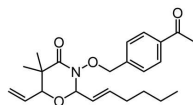
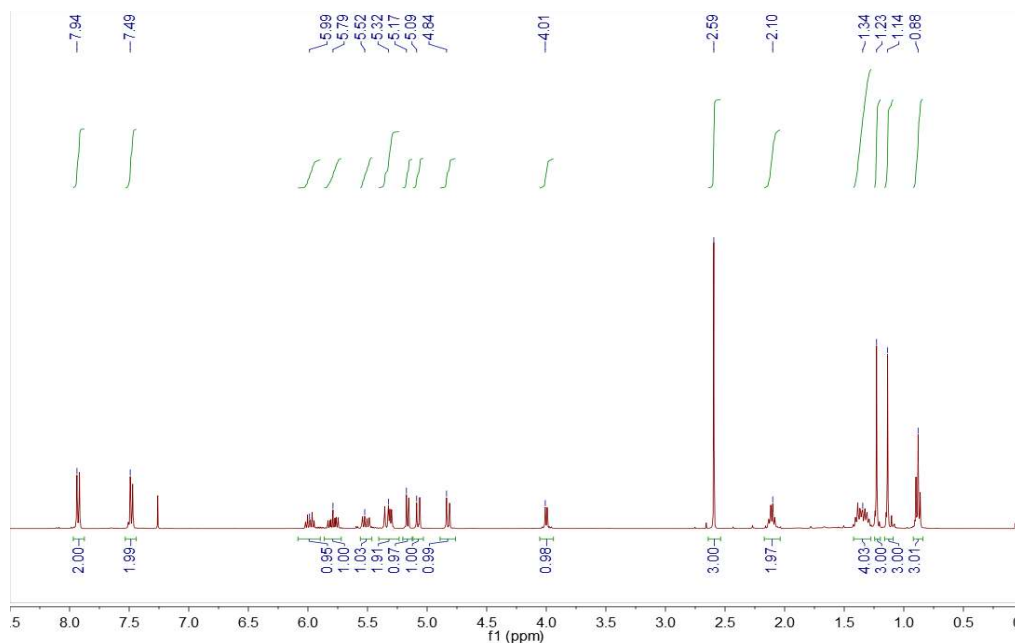
Compound S4.3



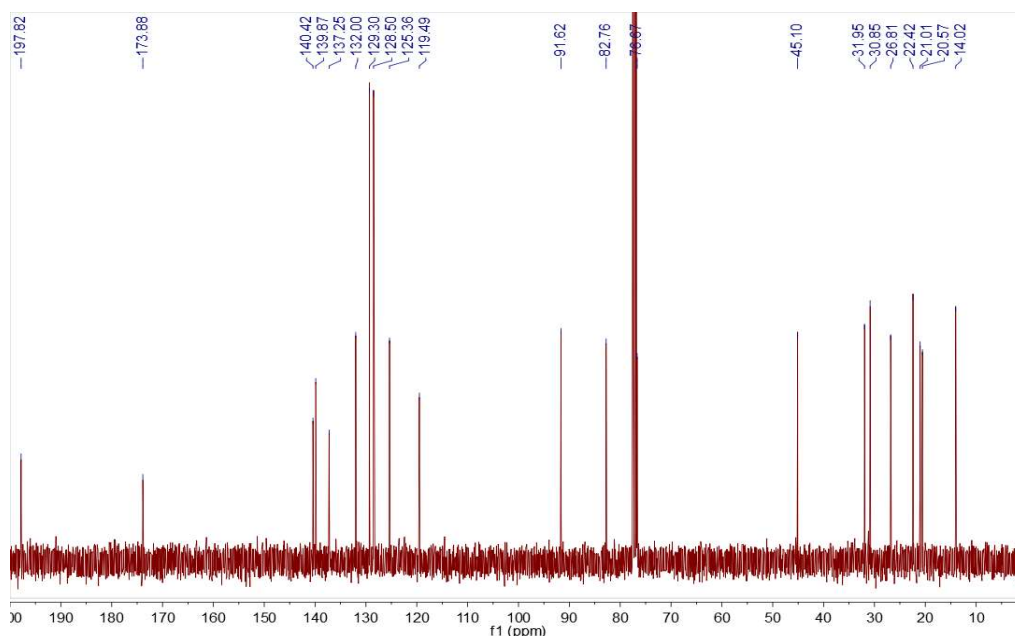
Compound S4.4a



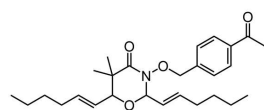
¹H-NMR (CDCl₃, 400 MHz)



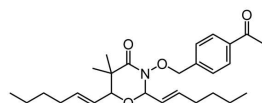
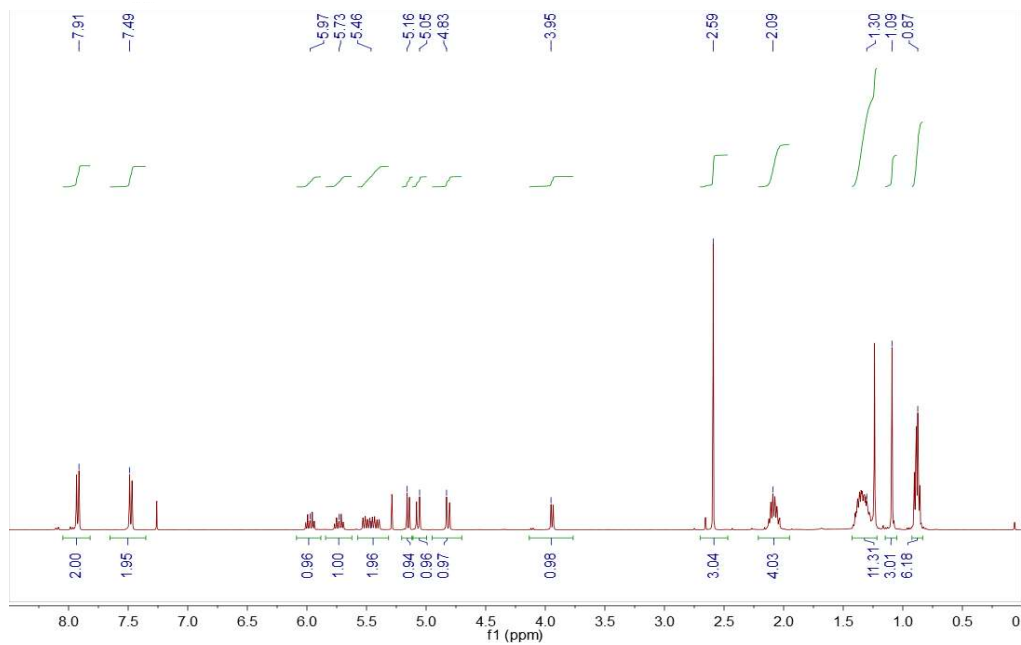
¹³C-NMR (CDCl₃, 100 MHz)



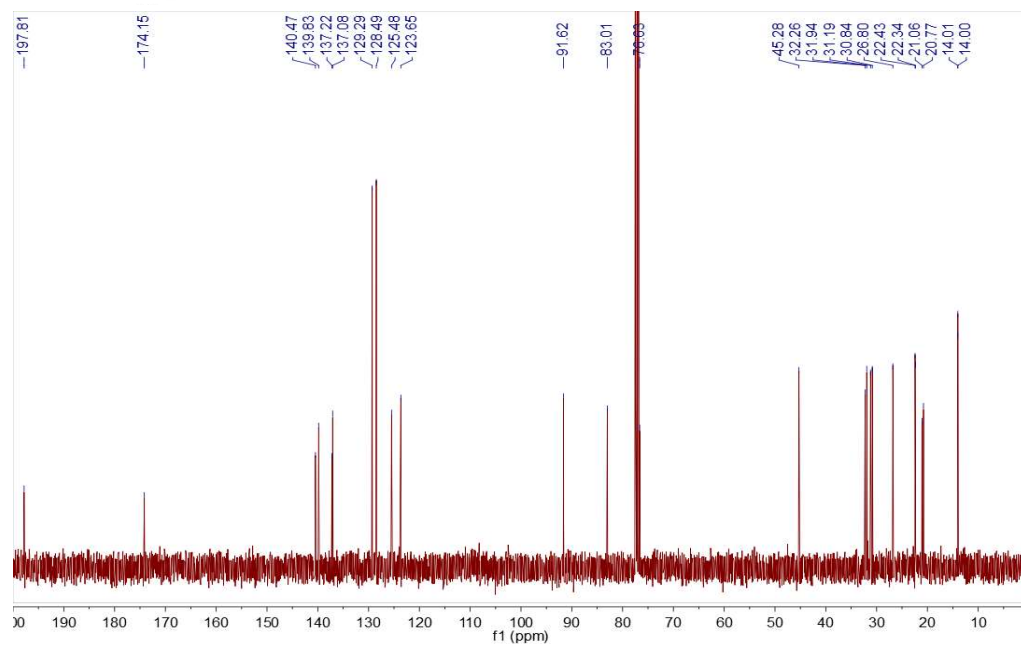
Compound S4.4b



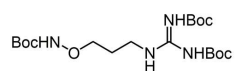
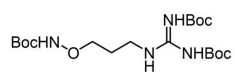
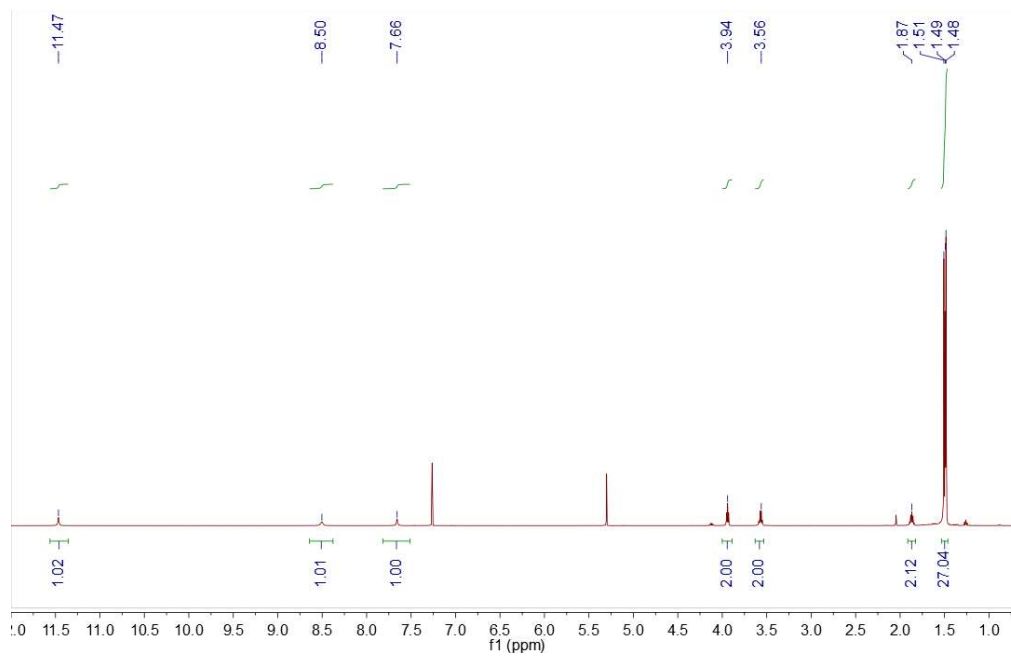
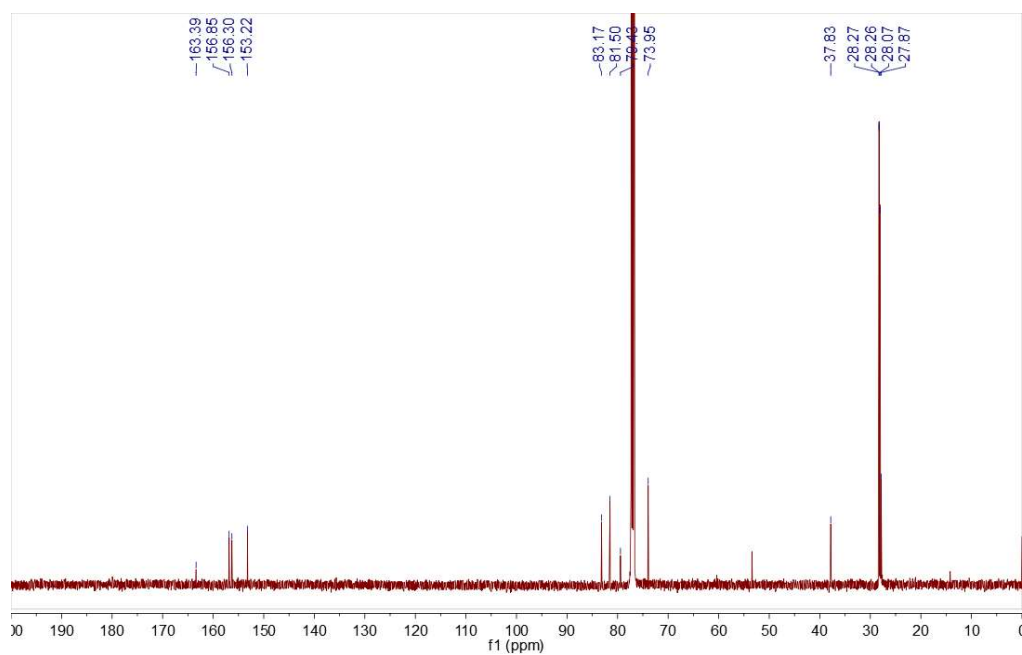
¹H-NMR (CDCl₃, 400 MHz)



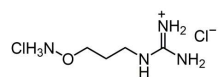
¹³C-NMR (CDCl₃, 100 MHz)



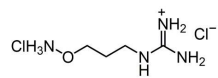
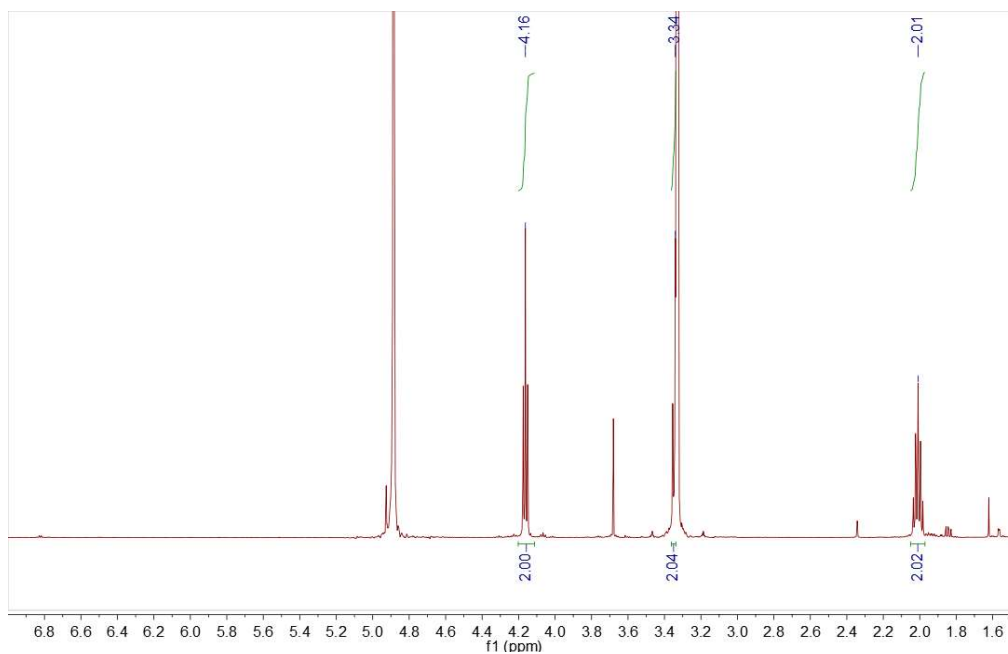
Compound S4.5

¹H-NMR (CDCl₃, 500 MHz) ^{13}C -NMR (CDCl_3 , 125 MHz)

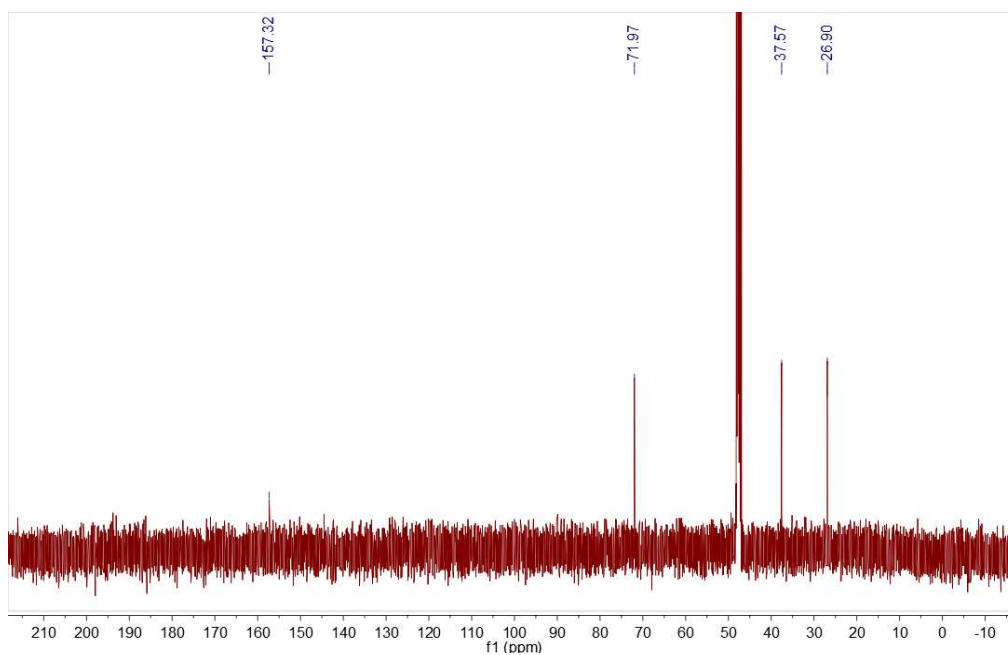
Compound S4.6



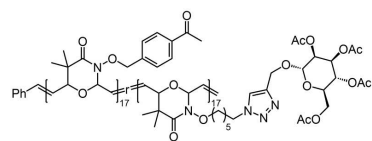
$^1\text{H-NMR}$ (MeOH- d_4 , 500 MHz)



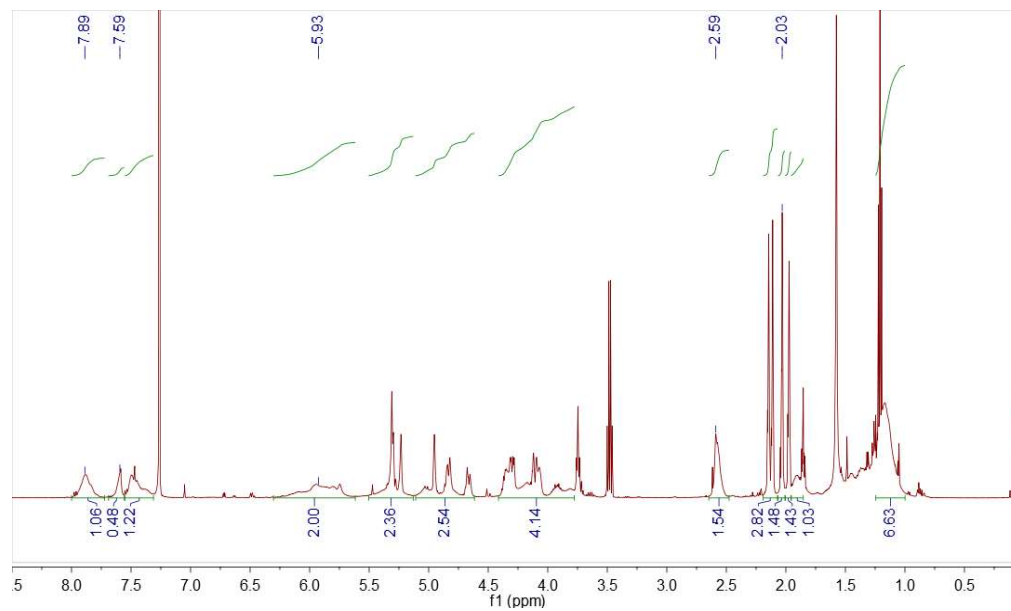
$^{13}\text{C-NMR}$ (MeOH- d_4 , 125 MHz)



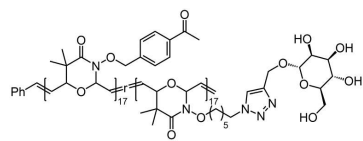
Polymer S4.7



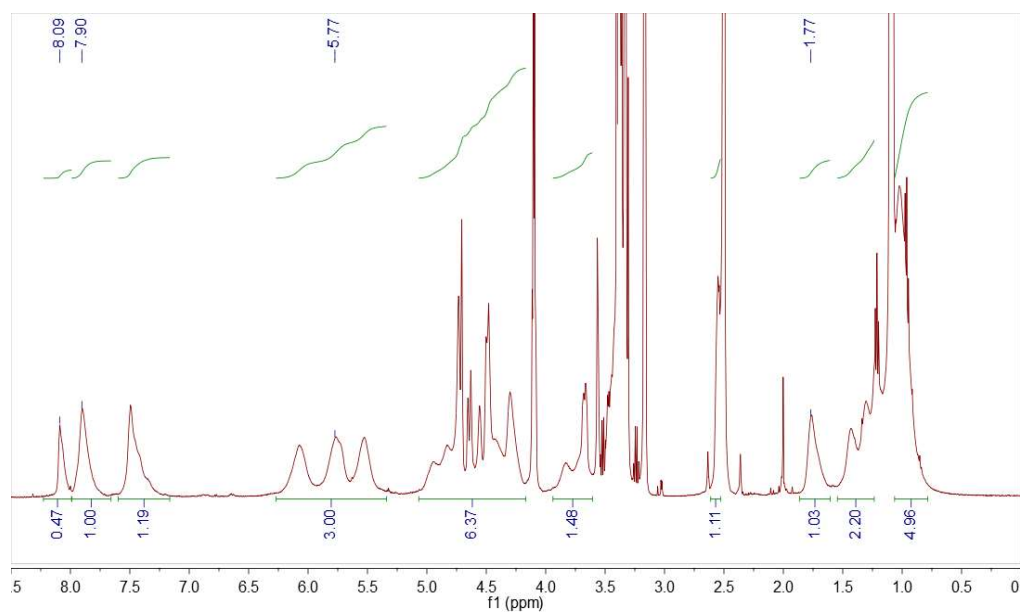
¹H-NMR (CDCl₃, 500 MHz)



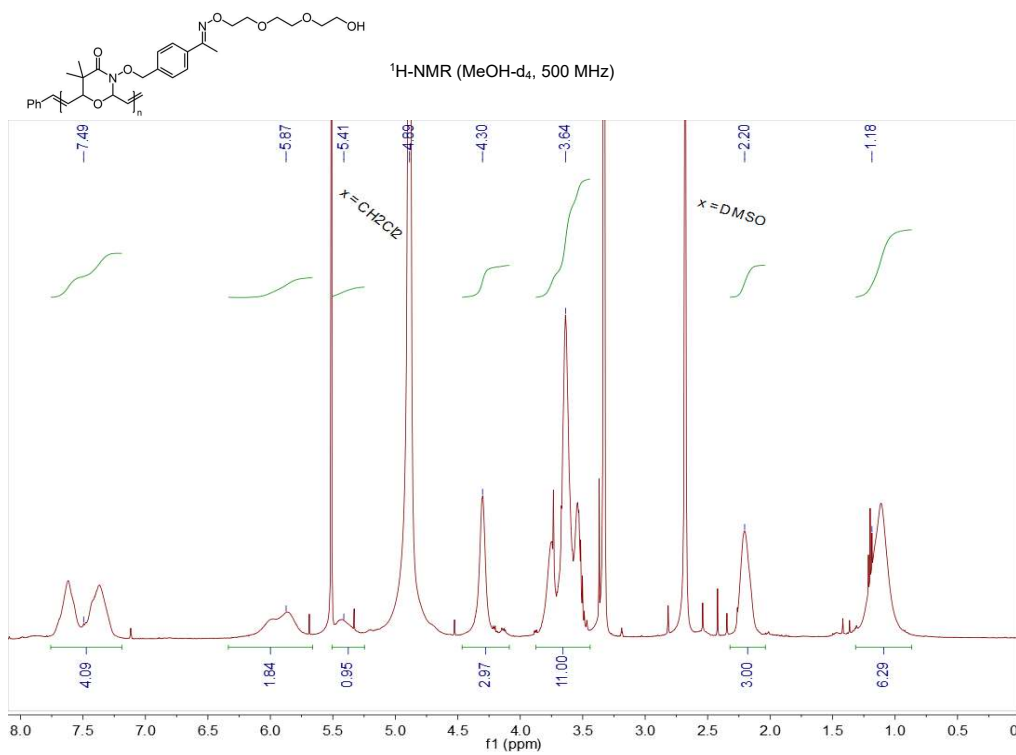
Polymer S4.8



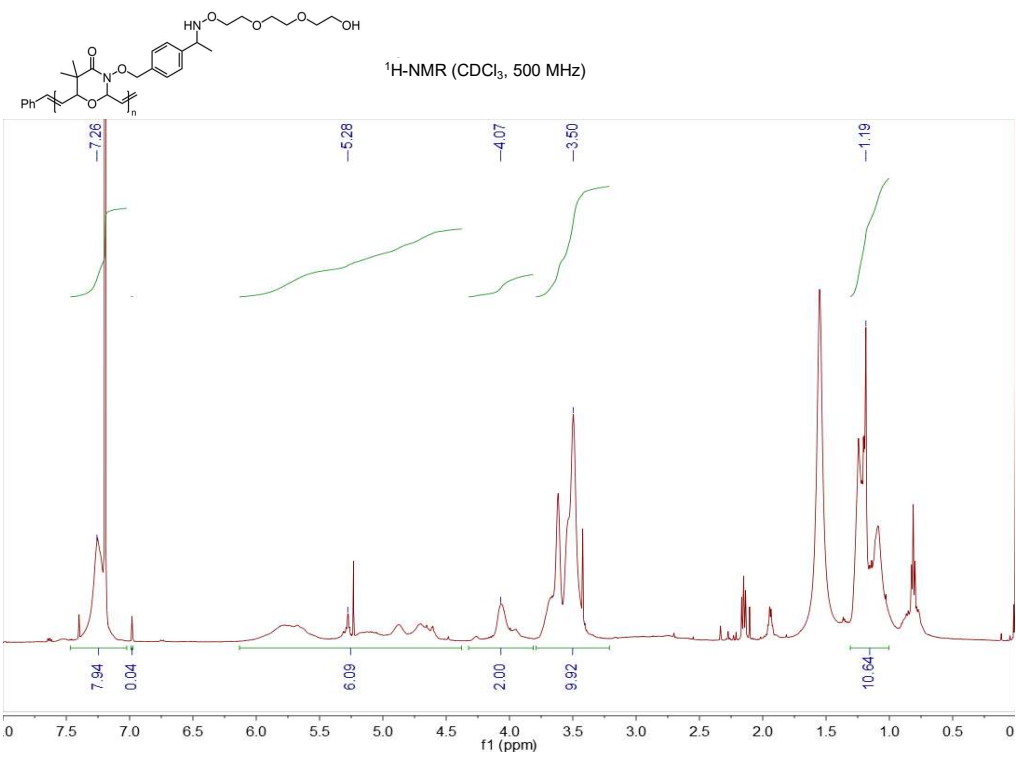
¹H-NMR (DMSO-d₆, 500 MHz)



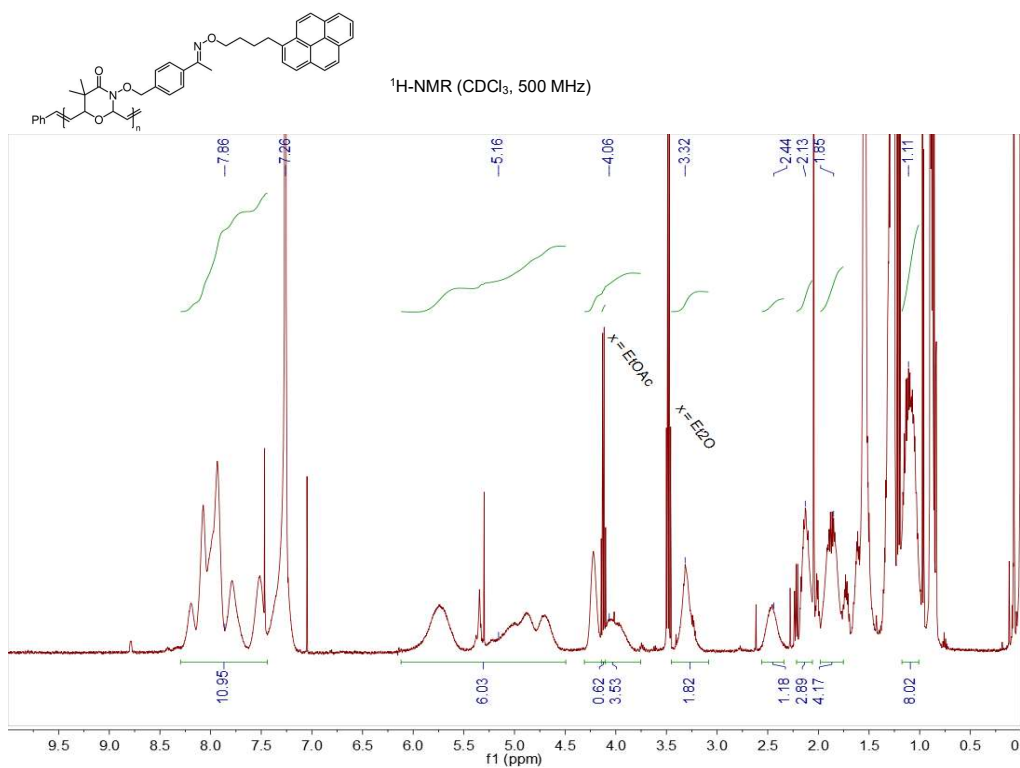
Polymer S4.14



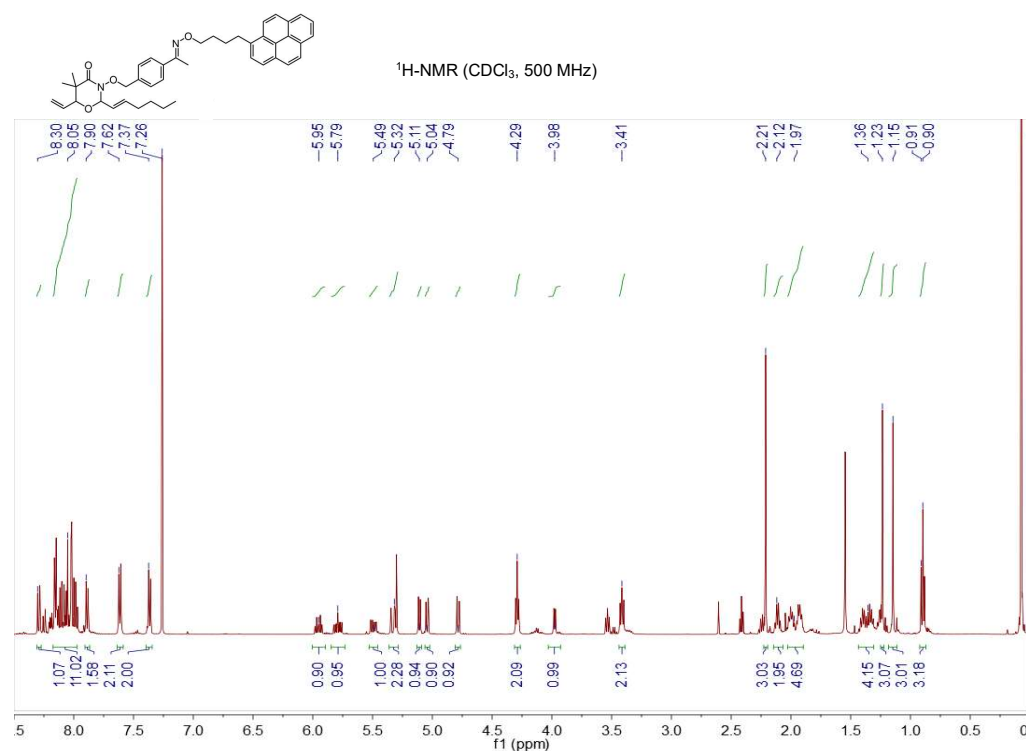
Polymer S4.15



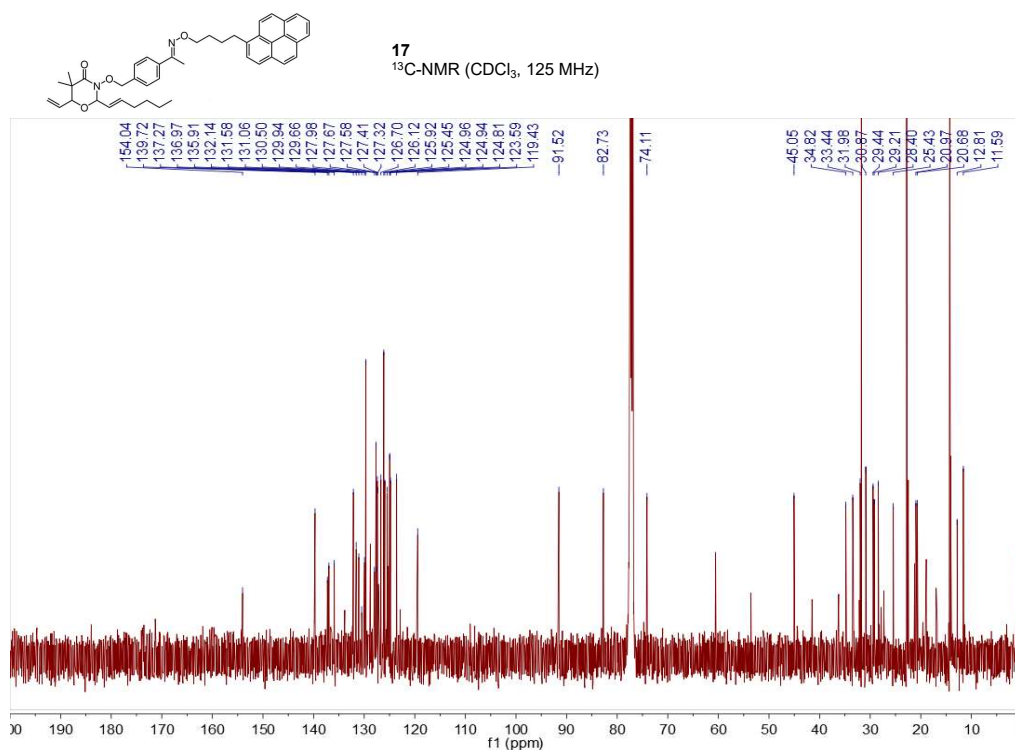
Polymer 4.16



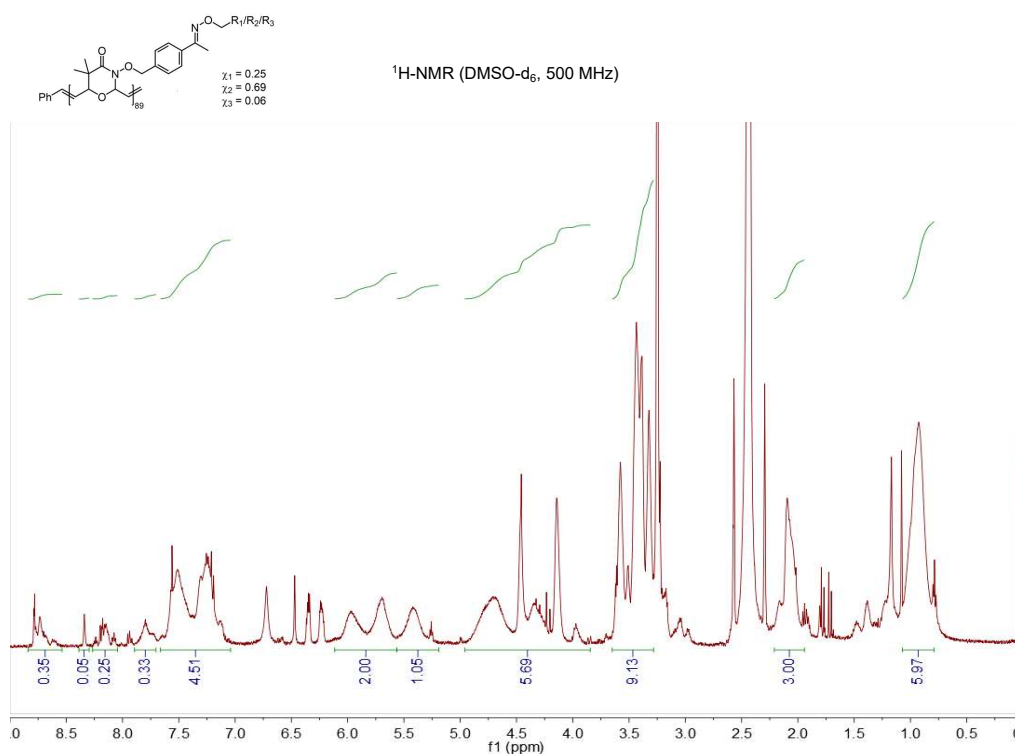
Polymer 4.17



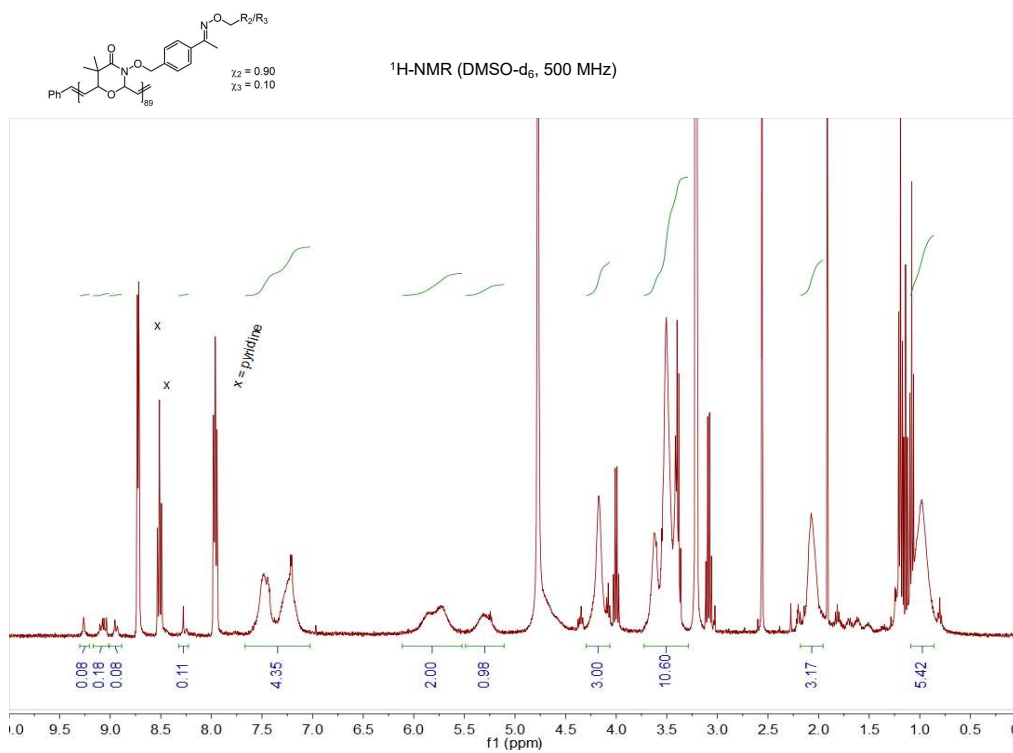
Polymer 4.17



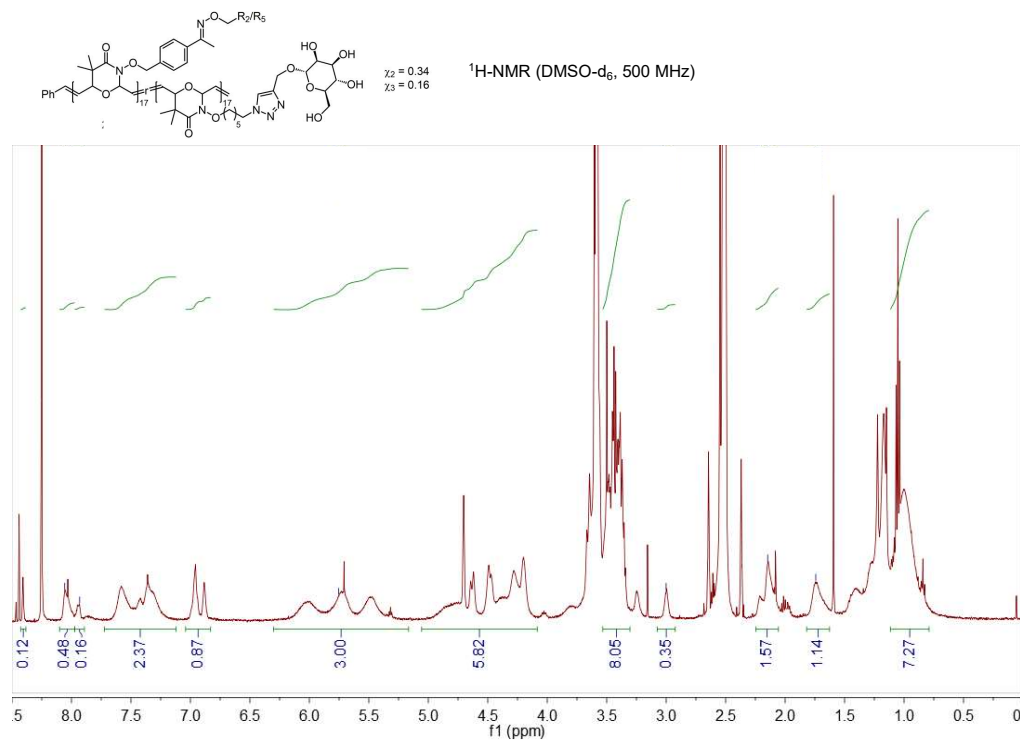
Polymer 4.18



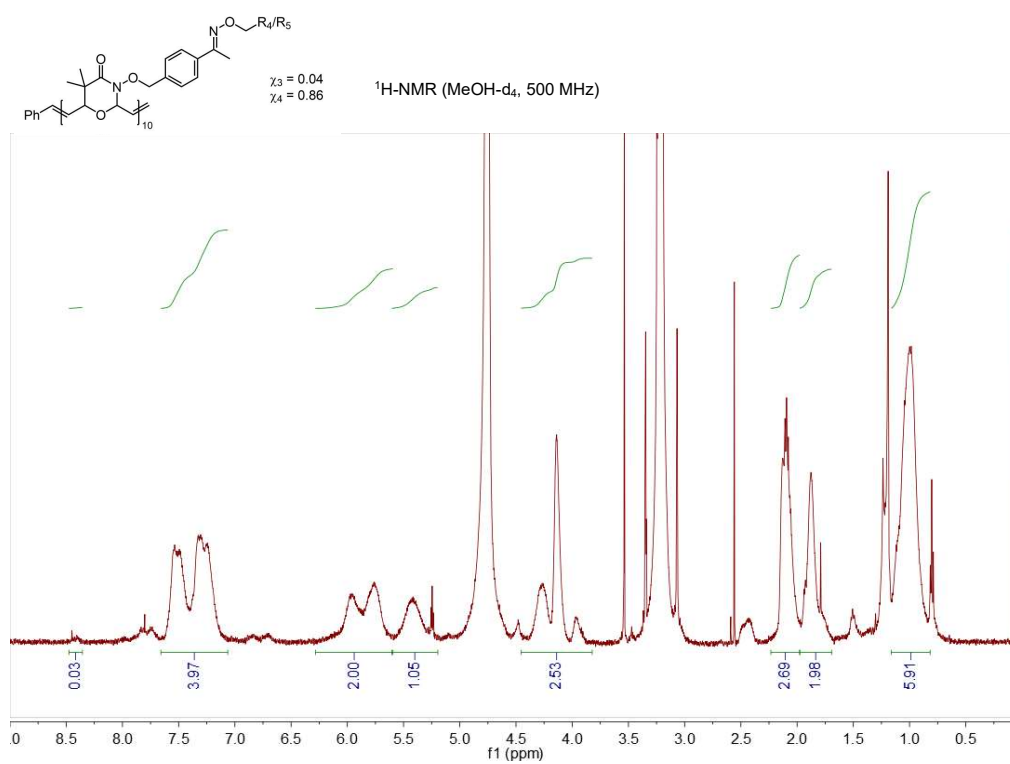
Polymer 4.19



Polymer 4.20



Polymer 4.21



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