PROTEIN-POLYMER CONJUGATES VIA GRAFT-\textit{FROM} RING-OPENING METATHESIS POLYMERIZATION

by

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DEDICATION

To my family; to my friends.
# TABLE OF CONTENTS

List of Figures ................................................................................................................................. 5

Acknowledgements .......................................................................................................................... 7

Abstract ........................................................................................................................................ 8

Chapter I: Introduction .................................................................................................................... 9

Chapter II: Design, Results, and Discussion .................................................................................. 20
  2.1 Hydrophilic Ligands .................................................................................................................. 20
  2.2 Water-Soluble Grubbs’ Catalyst ............................................................................................... 23
  2.3 Activity and Stability of Water-Soluble Grubbs’ Catalyst ....................................................... 26
  2.4 Protein Surface Macroinitiator ................................................................................................. 33
  2.5 ROMP From Protein Surface .................................................................................................. 39

Chapter III: Materials and Synthesis ............................................................................................. 49
  3.1 Materials .................................................................................................................................. 49
  3.2 Instrumentation ......................................................................................................................... 49
  3.3 Synthesis .................................................................................................................................. 51

Chapter IV: Future Direction .......................................................................................................... 59
  4.1 High Aspect Ratio Nanoparticles ............................................................................................. 59
  4.2 ROMP from TMV Surface ......................................................................................................... 62
  4.3 Pharmacokinetics of TMV-Polymer Conjugates ...................................................................... 63
  4.4 TMV-Polymer Conjugates as MRI Contrast Agents ................................................................. 64

Appendix I: Spectra and Chromatograms ...................................................................................... 67

References ....................................................................................................................................... 75
# LIST OF FIGURES

| Table 1. | Classification of protein-based therapeutics | ................................................................. | 9 |
| Figure 1. | General mechanisms for ATRP and RAFT | ................................................................. | 13 |
| Figure 2. | General schemes showing olefin metathesis | ................................................................. | 14 |
| Figure 3. | Synthesis of G3 | .................................................................. | 15 |
| Figure 4. | Functionalized pyridyl ligands | .................................................................. | 16 |
| Figure 5. | Effects of pH and copper on Grubbs’ catalyst | ................................................................. | 17 |
| Figure 6. | Pyridyl sulfobetaine ligand synthesis | .................................................................. | 21 |
| Figure 7. | Alternative zwitterionic ligand synthesis | .................................................................. | 22 |
| Figure 8. | PEG-pyridyl ligand synthesis | .................................................................. | 22 |
| Figure 9. | Ligand exchange mechanisms | .................................................................. | 24 |
| Figure 10. | Structure and appearance of water-soluble Grubbs’ catalyst | ................................................................. | 25 |
| Figure 11. | $^1$H-NMR of water-soluble Grubbs’ catalyst | ................................................................. | 26 |
| Figure 12. | NMR decomposition study of water-soluble Grubbs’ catalyst | ................................................................. | 27 |
| Figure 13. | Deuterium exchange mechanisms in D$_2$O | .................................................................. | 28 |
| Figure 14. | UV decomposition study of water-soluble Grubbs’ catalyst | ................................................................. | 29 |
| Figure 15. | ROMP in organic conditions | .................................................................. | 31 |
| Figure 16. | ROMP kinetics in aqueous conditions | .................................................................. | 33 |
| Figure 17. | Surface modification of lysozyme via EDC coupling | ................................................................. | 34 |
| Figure 18. | Surface modification of lysozyme via norbornene anhydrides | ................................................................. | 35 |
| Figure 19. | Assembly of ROMP macroinitiator | .................................................................. | 36 |
| Figure 20. | General scheme for graft-*from* ROMP | .................................................................. | 39 |
Figure 21. Synthetic scheme for graft-from ROMP .............................................40
Figure 22. PAGE and SEC results from graft-from ROMP ..............................42
Figure 23. Graft-from block copolymerization ...............................................43
Figure 24. PAGE and SEC results, living graft-from ROMP, trial 1 .................45
Figure 25. PAGE and SEC results, living graft-from ROMP, trial 2 ...............46
Figure 26. Preparation of PEG-substituted pyridyl ligand ..............................51
Figure 27. Preparation of PEG monoester norbornene monomer ..................52
Figure 28. Preparation of azido-PEG-norbornene monomer ........................53
Figure 29. Preparation of norbornene-functionalized lysozyme ......................54
Figure 30. Preparation of Grubbs’ 3rd generation catalyst .............................55
Figure 31. Preparation of water-soluble Grubbs’ catalyst .............................56
Figure 32. Structure of tobacco mosaic virus .................................................60
Figure 33. General scheme of ROMP from TMV surface .............................63
Figure 34. Fluorescent copolymers from TMV surface ................................64
Figure 35. Gd(DOTA)-functionalized polymers from TMV surface ..............65
Figure A1. NMR spectrum of PEG-substituted pyridyl ligand ......................67
Figure A2. NMR spectrum of PEG-monoester norbornene monomer .............68
Figure A3. NMR spectrum of azido-PEG-norbornene ................................69
Figure A4. NALDI spectrum of PEG-substituted pyridyl ligand ....................70
Figure A5. NALDI spectrum of PEG-monoester norbornene monomer ..........71
Figure A6. ESI mass spectrum of azido-PEG-norbornene ............................72
Figure A7. SEC of crude polymer conjugate samples – trial 1 ......................73
Figure A8. SEC of crude polymer conjugate samples – trial 2 ......................74
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Protein-Polymer Conjugates via Graft-From Ring-Opening Metathesis Polymerization

Abstract

by

SERGEY A. ISAROV

Protein therapeutics and biopharmaceuticals are used in a wide variety of clinical applications such as drug delivery and diagnostics. Unfortunately, protein-based therapies possess significant limitations as a result of degradative processes that restrict the lifetime of proteins in vivo. This project explores the growth of conformationally rigid polymers derived via graft-from ring-opening metathesis polymerization (ROMP) directly from the surface of proteins. We predict that these polymers can protect proteins from degradation, while bearing useful functionalities to improve their utility and behavior within biological systems. We report a novel aqueous ROMP technique for the growth of polymers from the surface of lysozyme. This technique represents the first example of the use of ROMP for the growth of polymers directly from the surface of a biological substrate under physiological conditions. Via extensive proof-of-concept studies, we report the successful formation of a protein ROMP macroinitiator that subsequently facilitated graft-from ROMP to afford protein-polymer conjugates of high molecular weights. Further optimization of reaction conditions will be performed in order to more precisely control polymer length, composition, and polymerization kinetics. Future studies will investigate ROMP from the surface of tobacco mosaic virus (TMV) to form high aspect ratio nanoparticle conjugates.
CHAPTER I: INTRODUCTION

Protein therapeutics represent a significant area of biomedical research and have, in recent years, found application in almost every field of medicine. They possess far-reaching applications such as antibody- and enzyme-based therapies, drug-, gene-, and contrast agent-delivery vehicles, as well as diagnostics and vaccines. Hundreds of such applications are at the forefront of current biomedical research and many have already reached or surpassed clinical trials. Since the initial discovery of the therapeutic uses of recombinant insulin in the early 1980s, over 100 protein therapies are now approved for clinical use by the FDA. In fact, these therapies have enjoyed such a wide variety of clinical applications that the FDA now employs a separate system for the functional classification of the various groups of protein-based pharmaceuticals (Table 1).

<table>
<thead>
<tr>
<th>Group I – Protein therapeutics with enzymatic or regulatory activity</th>
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<tbody>
<tr>
<td>• Ia: Replacing a protein that is deficient or abnormal</td>
</tr>
<tr>
<td>• Ib: Augmenting an existing pathway</td>
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<td>• Ic: Providing a novel function or activity</td>
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<th>Group II – Protein therapeutics with special targeting activity</th>
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<tr>
<td>• IIa: Interfering with a molecule or organism</td>
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<td>• IIb: Delivering other compounds or proteins</td>
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<th>Group III – Protein vaccines</th>
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<tr>
<td>• IIIa: Protecting against a deleterious foreign agent</td>
</tr>
<tr>
<td>• IIIb: Treating an autoimmune disease</td>
</tr>
<tr>
<td>• IIIc: Treating cancer</td>
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| Group IV – Protein diagnostics                              |

The clinical success of these therapies has given rise to a significant interest in protein engineering in an effort to develop and otherwise improve them further.\textsuperscript{4} Biological therapeutics of this type have gained interest in part due to their known mechanism of action, biological specificity, and reduced toxicity. These benefits stem in part from the fact that many biological macromolecules used therapeutically are either already produced in the body, or are modified versions thereof. However, significant challenges in administering these therapies still exist. Proteins typically have a short circulation lifetime due to a variety of degradative processes such as immune-mediated clearance, hydrolysis by serum proteases, and receptor-mediated endocytosis.\textsuperscript{5}

As a result, protein-based therapies are short-lived and typically require repeated intravascular administration throughout the course of a given disease or treatment. In order to mitigate these limitations, classical methods to protect therapeutic proteins from degradation \textit{in vivo} have involved the conjugation of chemically and biologically inert polymers to the protein surface. Among the most widely-used of such protective materials is \textit{poly}(ethylene glycol) (PEG).\textsuperscript{6} PEG polymer chains are hydrophilic, non-toxic and weakly immunogenic. As such, they can serve as excellent protective groups for biological macromolecules.\textsuperscript{7}

The bioconjugation of proteins with PEG (PEGylation) improves protein pharmacokinetics by increasing circulation lifetime and reducing enzymatic degradation with minimal effect on macromolecular structure and activity.\textsuperscript{8} First, PEG chains increase the water solubility and physicochemical stability of proteins both \textit{in vivo} as well as throughout shelf storage.\textsuperscript{7,8} Second, PEGylation increases the hydrodynamic radius of proteins thereby minimizing renal clearance.\textsuperscript{4} Third, their low immunogenicity can serve
to reduce the potential of immunogenic cargo to which they are attached from provoking an immune response.\textsuperscript{4,6,9} Finally, PEG chains form a relatively inert physical barrier around proteins that prevent degradative enzymes or antibodies from detecting and otherwise interacting with the protein surface.\textsuperscript{7} By increasing circulation time, PEG bioconjugation can serve to assist in targeted delivery and bioaccumulation of PEG-bearing proteins within specific sites of disease such as tumor tissue or atherosclerotic plaques.\textsuperscript{6}

The first clinically-approved PEGylated protein was a derivative of adenosine deaminase, accepted into service by the FDA in 1990.\textsuperscript{6} As of 2011, a total of nine FDA-approved PEGylated protein therapies were in clinical use and as of 2012, eleven additional PEGylated proteins were at the stage of clinical trials.\textsuperscript{1,7–9} A notable example is PEGylated interferon α2a which is currently among the most widely used injectable treatments for hepatitis C.\textsuperscript{10}

Through the process of PEGylation the circulation lifetime of protein-polymer conjugates can be increased, but the production of these protein-polymer conjugates has proven to be technically challenging. Typically, conjugates are formed via “graft-to” methods where a pre-formed polymer is attached directly to a protein surface.\textsuperscript{11,12} This approach is both kinetically and thermodynamically unfavorable as multiple macromolecules with a low concentration of reactive sites are being brought together. Additionally, separating the protein-polymer conjugate from unreacted starting materials is difficult since the two components are usually of similar size. In order to alleviate these problems, we propose a graft-from approach wherein polymerization is initiated in a multivalent fashion directly from the surface of the protein. Polymers are subsequently
grown from that surface, consuming soluble monomer in the reaction solution. This graft-
*from* approach is favorable over the graft-*to* approach for two reasons. First, it allows for a significant kinetic advantage due to the increased concentration of reactive sites available for polymer growth. In this case, the concentration of reactive sites is at least equal to the concentration of monomer, and the concentration of monomer is much higher than the concentration of protein. Therefore, the reactive site concentration-protein concentration ratio is much higher than that of the graft-*to* method, where a much lower molar amount of polymer is usually required relative to that of protein. Second, the graft-
*from* approach allows for a much simpler purification process due to the great difference in size between the protein-polymer conjugate and monomer units. A simple filtration or dialysis may be sufficient to adequately isolate or purify protein-polymer conjugate product.

Polymerization from the protein surface using PEGylated monomers provides the ability to more directly control both the length of the polymer chain as well as a variety of other characteristics such as the architecture of the polymer and the rigidity of its backbone. Specifically, in the case of living polymerizations such as atom transfer radical polymerization (ATRP), reversible addition fragmentation chain transfer polymerization (RAFT) or ring-opening metathesis polymerization (ROMP), simply controlling the concentration of monomer in the reaction solution at the beginning of polymerization will yield proportionally higher or lower polymer lengths. Furthermore, the subsequent addition of a variety of reactive monomers either in sequence or simultaneously can yield block or random copolymers in a “one-pot” fashion. In addition, if several types of orthogonal polymerization chemistries are used at the same time, conjugating different
types of polymers on the same protein can be achieved. With a graft-from approach, polymer-reinforced biological macromolecules can be more easily produced and purified, providing an improved route for the development of high-performance protein therapies.$^{13,14}$

Due to recent advances in controlled radical polymerization (CRP) chemistry, especially those associated with the execution of radical polymerizations in aqueous solution, a large number of methods have been reported for the graft-from preparation of protein-polymer conjugates using CRP chemistries. Most notable are those of RAFT and ATRP (Figure 1)$^{15,16}$ The ATRP mechanisms involves halide exchange between a growing polymer chain radical and an uncharged monomer species by way of electron exchange with a transition metal complex intermediate.$^{17,18}$ In contrast, the RAFT mechanism generally involves the decomposition of a radical initiator and its propagating reaction with monomers bearing radical leaving groups capable of initiating subsequent polymerization cycles.$^{19}$ Such polymerization methods are quickly becoming ubiquitous in graft-from polymer bioconjugation due to their speed, robustness, and tolerance of mild experimental conditions.$^{16}$

Figure 1. General mechanisms for A: ATRP and B: RAFT.$^{16}$
The methodology proposed in this project represents the first time ROMP has been used to grow polymers directly from the surface of proteins. ROMP chemistry is extremely powerful due to its ability to quickly form low-dispersity polymers of targeted molecular weights and architectures, while being exceptionally tolerant of a variety of functional groups.\textsuperscript{20} The origins of ROMP arose from the initial discovery of the polymerization of cyclic olefins by Herbert Sousa Eleuterio in 1957.\textsuperscript{21,22} Over the next 50 years, significant research interest in olefin metathesis via transition metal catalysis gave rise to the most notable contributions in the field by Robert H. Grubbs and Richard R. Schrock in the 1980s and 90s, earning them the 2005 Nobel Prize in Chemistry.

\textbf{Figure 2.} General scheme showing \textbf{A}: ring-opening metathesis (ROM), \textbf{B}: ring-closing metathesis (RCM), and \textbf{C}: cross metathesis (CM) mechanisms. ROMP shown with norbornene monomer. $M$=metal center of ROMP catalyst; $A,B$=almost any organic or inorganic group.
Although many transition metal complexes in high oxidation states have been investigated, those of molybdenum, tungsten, and ruthenium enjoyed the greatest attention due to their ability to catalyze ring-opening, ring-closing, and cross-metathesis reactions (Figure 2) with highest fidelity.\textsuperscript{20,23,24} Among these various types of reactions, ring-opening metathesis is unique in that it allows for a polymer chain to grow in length with every catalytic cycle and therefore is designated as a polymerization technique known as ROMP.

In 2002 Jennifer Love \textit{et al.} developed a ruthenium-based heterocyclic carbene complex that was able to catalyze ROMP reactions in organic media at astonishingly fast rates. This catalyst, known as Grubbs’ third generation catalyst (G3), initiated polymerization reactions almost six orders of magnitude faster than its predecessor, Grubbs’ second generation catalyst (G2). The principle behind this drastic increase in catalytic activity involves the lability of exchangeable ligands around the ruthenium metal center. G2-type catalysts typically possess five ligands: an \(N\)-heterocyclic carbene (NHC), two halogens, one benzylidene, and an “L-type” tricyclohexylphosphine group.

\[
\text{Mes} \backslash \begin{array}{c} N \backslash \text{Mes} \end{array} \quad \text{Mes} \backslash \begin{array}{c} N \backslash \text{Mes} \end{array}
\]

\[
\begin{array}{c} \text{Cl} \end{array} \quad \begin{array}{c} \text{Cl} \end{array} \quad \begin{array}{c} \text{P(Cy)}_3 \end{array}
\]

\[
\begin{array}{c} \text{Ru} \quad \text{Cl} \quad \text{Cl} \quad \begin{array}{c} \text{P(Cy)}_3 \end{array} \quad \begin{array}{c} \text{R} \end{array} \quad \begin{array}{c} \text{Mes} \end{array}
\]

Figure 3. G2: Grubbs’ second generation catalyst, being converted into G3: Grubbs’ third generation catalyst bearing two pyridyl ligands with optional functionalization (\(R\)).
(P(Cy)$_3$, Figure 3, G2). In this configuration, the catalyst maintains relatively high stability. However, the P(Cy)$_3$ group must dissociate from the complex in order to become catalytically active. In the case of G3, the P(Cy)$_3$ group is replaced with two “L-type” pyridyl ligands, most commonly pyridine or bromopyridine (Figure 3, G3). The pyridyl ligands are significantly more labile than P(Cy)$_3$, dissociate much more easily, and therefore allow much faster initiation of the catalytic cycle.$^{25}$

Up until very recently, ROMP chemistry has been limited almost exclusively to organic media because the ruthenium carbene (Grubbs’) catalysts required for ROMP are only soluble and active in organic solvents with relatively low polarity such as dichloromethane (DCM). Early attempts at simple olefin metathesis in water using transition metal salts such as Ru(Cl$_3$)(H$_2$O)$_n$ showed significant promise, yielding higher achievable molecular weights and lower polymer dispersities than analogous ROMP reactions in inert organic solvents.$^{26-28}$ As a result, the ability to perform ROMP in aqueous media represents an exciting new area of research. Although a number of

![Figure 4](image-url)

**Figure 4.** Functionalized pyridyl water-soluble ligands used by Emrick et al. to modify Grubbs’ catalyst. A: Pyridyl phosphorylcholine; B: Pyridyl-triazole PEG; C: Pyridyl PEG ether.
ruthenium-based catalysts have been described for cross-metathesis, very few have been reported for ROMP, especially those that show comparable polymerization activity to ROMP in organic media. Typically, the most successful modifications of Grubbs’ catalyst to form a water-soluble complex involve the use of functionalized pyridyl ligands bearing hydrophilic groups. Some of the earliest work on this type of modification was done by Todd Emrick et al. using either PEGylated or zwitterionic phosphorylcholine ligands as means by which to bring Grubbs’ catalyst into aqueous solution (Figure 4). Emrick showed that in spite of the solubility challenges, successful ROMP chemistry in water could be performed using this type of water-soluble Grubbs’ catalyst, together with water-soluble norbornene monomers. These seminal results proved that although challenging, free neutral polymerizations typically of a single block could take place in water, albeit only under certain conditions.

![Figure 5](image-url). Effects of (A) low pH and (B) copper on the activation of Grubbs’ catalyst.
Emrick discovered that the “L-type” functionalized pyridyl ligands around the water-soluble Grubbs’ catalyst are much less labile in water than in organic media and therefore required either highly acidic conditions or a copper additive in order to dissociate quickly enough to facilitate polymerization (Figure 5). In an acidic environment, the pyridyl ligands are equilibrium-protonated, making it more difficult for them to attach to the catalyst complex. In the presence of copper, the competitive coordination of the pyridyl ligands to copper ions allows them to more easily dissociate for the catalyst complex. Emrick concluded that ROMP in water at a pH of around 1.5 proceeds much more efficiently than at neutral pH in the presence of copper. Under acidic conditions, quantitative monomer conversions and low dispersities were achieved whereas in the presence of copper, monomer conversions of only up to 70% were achieved with appreciable increase in dispersity as shown by gel-permeation chromatography.32

Applied ROMP chemistry in water, such as the proposed growth of polymers from the surface of a biological substrate is a previously unexplored area of research. Developing a novel ROMP technique specifically for the purpose of grafting from proteins has a number of important advantages as well as some unique challenges. When designing a multifunctional protein-polymer conjugate, ROMP chemistry has the potential to be instrumental in allowing for the fine-tuning of polymer size and composition, and hence particle shape and surface chemistry. ROMP is an extremely rapid living polymerization, which is significant particularly because it allows for the formation of polymers of low dispersity while, under ideal conditions, consuming most if not all monomer starting material, often requiring little to no purification. In addition,
unlike many radical polymerization techniques, ROMP catalysts have extremely high turnover capacities making it easier to synthesize polymers of more complex architectures. Furthermore, ROMP proceeds strictly by way of an olefin metathesis mechanism, and as such only consumes monomers bearing accessible olefin groups at established rates. These rates vary significantly depending on the chemical, electronic, and steric environment associated with the given double bond. For example, ring-strained cyclic and bicyclic olefins such as cyclooctenes and norbornenes are incorporated into polyolefins via ROMP very rapidly, while terminal olefins and internal olefins slowly form homo or heterodimers via the process of single-cycle cross metathesis. As a result of its monomer specificity and differential kinetics, ROMP provides an orthogonal source of monomers to existing graft-from methods using controlled radical polymerizations (CRP).

There are a number of important benefits to the use of ROMP for protein bioconjugation. Given its specificity for monomers bearing cyclic olefins allows ROMP to be uniquely bioorthogonal to existing CRP polymerization chemistries currently used to modify proteins using graft-from techniques. The ability to utilize multiple polymerization methods on a single protein opens the door to the incorporation of a variety of polymer functionalities impossible with either ROMP or CRP methods alone. In addition, the unsaturated polyolefin backbone afforded by ROMP such as those of poly(norbornene) is much more rigid than either the PEG backbone or the acrylate and acrylamide backbones of polymers derived via CRP. Conjugating a rigid polymer to a protein surface may serve to better protect the protein from degradation by more effectively restricting access to it by serum proteases and antibodies.
CHAPTER II: DESIGN, RESULTS, AND DISCUSSION

2.1 Hydrophilic Ligands

The first critical objective of this project was to design and synthesize a proprietary Grubbs’ catalyst variant that would possess ample solubility in aqueous media while retaining the majority of its catalytic activity. Our initial strategy involved the synthesis of a zwitterionic pyridyl sulfobetaine ether ligand (PSL, Figure 6) that would be subsequently associated with Grubbs’ catalyst thereby solubilizing it in water. The principal rationale behind the use of a zwitterionic ligand is predicated on the assumption that due to its relatively small molecular weight and high water solubility, this type of ligand would bring the catalyst into aqueous solution most efficiently. The synthesis of this ligand involved two steps. First, ring-opening amination of sultone with dimethylaminopropanol forms a sulfobetaine. Second, Williamson ether linkage between the sulfobetaine and 4-bromomethyl pyridine hydrobromide forms the zwitterionic ligand. Unfortunately, although the first step of the synthesis proceeded with very high yield, no appreciable product could be purified from the second step. The primary issue was identified to be the difficulty in selection of a solvent that would properly solubilize both starting materials while increasing the availability of a free nucleophile. For example, although both starting materials dissolved readily in water, the reaction did not proceed due to the low aqueous availability of the nucleophile. Conversely, polar aprotic solvents such as dimethyl sulfoxide (DMSO) and dimethylformamide (DMF) would be expected to increase the availability of the nucleophile, but were unable to properly solubilize the starting materials at low enough temperatures.
Three alternative strategies were employed to troubleshoot this problem. First, the two steps of the ligand synthesis were attempted in reverse, by initially coupling the dimethylaminopropanol to the pyridine prior to performing the ring-opening reaction with sultone. The reasoning behind this approach was that the solubility of the resulting dimethylaminopropane-pyridyl ether would be higher than that of the sulfobetaine in aprotic solvents. Although better product conversion was achieved, adequate purification of the final product in high enough yield was not possible. Second, the reaction between the sulfobetaine and pyridine was conducted under biphasic conditions with water and DMF in equal volumes using tetra-n-butylammonium bromide (TBAB) as a phase transfer catalyst. Unfortunately, throughout a 24-hour period, no desired product was observed in either organic or aqueous layers via TLC or $^1$H-NMR. Finally, an unconventional approach involved reacting sultone with dimethylaminopyridine (DMAP) in an effort to open the sultone ring with the dimethylamine group of DMAP. However, this reaction most likely results in an alternative unusable major product that can form as a result of the opening of the sultone ring with the reactive pyridyl nitrogen on DMAP (Figure 7). Although this reaction proceeded very efficiently and pure product was isolated in very high yield, $^1$H-NMR and mass spectral characterization of the product could not discriminate between the desired minor product and the possible alternative

![Figure 6. Synthetic scheme of pyridyl sulfobetaine ether ligand (PSL) with sulfobetaine intermediate.](image-url)
unusable major product, both of which possess identical molecular weight, proton distribution, and zwitterionic properties.

![Chemical structure](image)

**Figure 7.** Alternative synthesis of zwitterionic pyridyl sulfobetaine ligand using sultone and DMAP reagents. Two possible products may form: only one contains a free pyridyl nitrogen available for association with Grubbs’ catalyst.

As a result of the difficulty in obtaining pure zwitterionic ligands in adequate yield, an alternative strategy was selected. This approach involved the synthesis of a pyridyl PEG ether ligand (PPL, **Figure 8**) similar to that described earlier by Emrick (**Figure 4C**). The synthesis of this ligand was more straightforward, involving the Williamson ether linkage of poly(ethylene glycol) monomethyl ether (average molecular weight of 500 Da.) to bromomethyl pyridine hydrobromide in anhydrous tetrahydrofuran (THF). Although the reaction time of this synthesis is considerably longer than that of the zwitterionic ligand, it was possible to purify the PPL in high enough yield to use for subsequent modification of Grubbs’ catalyst.

![Synthetic scheme](image)

**Figure 8.** Synthetic scheme of PEG pyridyl ligand (PPL).
2.2  Water-Soluble Grubbs’ Catalyst

Once enough PPL was purified, the next step involved a ligand exchange reaction with Grubbs’ catalyst. Based on previous work by Emrick, two ligand exchange reactions for this purpose are described (Figure 9). In the first method, Grubbs’ second-generation catalyst (G2) is directly modified by equilibrium exchange of the P(Cy)3 group with the desired functionalized pyridyl ligand. After the reaction is complete, the new catalyst complex can be dissolved in water, causing the released P(Cy)3 to precipitate out of solution. This P(Cy)3 precipitate can then be easily removed by filtration or centrifugation. In the second method, G2 is first converted to Grubbs’ third-generation catalyst (G3) using pyridine as its new “L-type” ligand. The G3 is purified, and then subsequently used to perform the equilibrium ligand exchange reaction. Purification of catalyst in this method is considerably longer, as it involves the step-wise removal of volatile pyridine under reduced pressure. Typically, when the desired new ligand is below a critical molecular weight, it can complex directly with G2 in one step according to the first method. Above that molecular weight, the direct attachment to G2 is not efficient and potentially harmful to the catalyst. In this case, attachment to G3 via the second method is safer, likely due to the higher lability of the pyridine groups around the catalyst.33,34

The hydrophilic PPL groups synthesized for the purposes of this project are of an average molecular weight of 500 Daltons. Since this ligand is slightly different that any previously used, both methods for modification of Grubbs’ catalyst were attempted. When the PPL was added to a solution of G2 in anhydrous dichloromethane (DCM), an instant color change from dark maroon to dark yellow was observed, with a subsequent
formation of disperse precipitate that was difficult to remove via centrifugation. The expected characteristic forest green color of G3-type Grubbs’ catalyst attached to pyridine was not observed and therefore it was concluded that the PPL was too large to directly complex with G2.

Fortunately, the alternative exchange reaction between the PPL and G3 proceeded with great success (Figures 9B, 10A). PPL was added directly to a solution of G3 in anhydrous DCM and the solution maintained a dark green color throughout the ligand exchange process, with no formation of precipitate. The purification of modified G3 was significantly more time consuming. After the initial addition of PPL, the solution was allowed to stir for 30 minutes under nitrogen atmosphere to allow for equilibrium to be


**Figure 9.** Ligand exchange mechanisms for the modification of Grubbs’ catalyst. A: Low molecular weight modification. Direct ligand exchange with G2. B: High molecular weight modification. G2 conversion to G3 using volatile pyridine, with subsequent ligand exchange.
reached between the functionalized and non-functionalized pyridines. Next, all solvent and free pyridine were removed via reduced pressure for another 30 minutes. Following the evacuation of the reaction flask, the remaining residue was resuspended in more anhydrous DCM and the stir-pump cycle was repeated six more times. After the final pump cycle, the green oily residue was resuspended in water or aqueous buffer by sonicating the solution for five minutes. It is important to note that the water-soluble Grubbs’ catalyst complex is more weakly soluble in water than the PPL alone, and therefore takes several minutes to fully dissolve and form a clear dark green aqueous solution (Figure 10B). After resuspension in aqueous solvent, the solution was centrifuged for several minutes to remove any insoluble particulates, and the supernatant was ready for use directly for aqueous ROMP. A method for quickly and accurately quantifying catalyst concentration in aqueous solution has not yet been established. Further studies in the use of inductively-coupled plasma-mass spectrometry (ICP-MS) in order to determine the molar concentration of ruthenium in aqueous solution as a function of its absorbance at various wavelengths is one potential future research area which may lead to a quick and accurate method for the determination of free catalyst concentration.

**Figure 10.** A: Structure of water-soluble Grubbs’ catalyst bearing two PEGylated pyridyl ligands. B: Dilute aqueous solution of water-soluble Grubbs’ catalyst with characteristic forest green color that is slightly more yellow than that of catalyst solutions in organic solvents such as DCM.
2.3 Activity and Stability of Water-Soluble Grubbs’ Catalyst

The catalytic mechanism of ROMP is highly sensitive to the chemical and electronic configuration of the ruthenium complex. However, the specific effects that dissolution in water may have on its structure and function are poorly understood. Therefore, following the development of a modified water-soluble Grubbs’ catalyst, its activity and stability under aqueous conditions had to be thoroughly investigated.

The “gold standard” for the determination of the health of Grubbs’ catalyst is the observation of the presence, intensity, and chemical shift of the catalyst’s alkylidene proton by $^1$H-NMR. This proton is unique for a number of reasons. First, its location on the carbon that resides adjacent to the double bond between the ruthenium central atom and the benzyl group causes it to be so electron deficient that it can be easily observed with a chemical shift far downfield on an NMR spectrum (Figure 11). Second, its proximity to the metal center makes its electronic environment very sensitive to the presence or absence of other surrounding ligands and therefore its chemical shift will reflect changes in the configuration of ligands around the metal center.

![Figure 11. $^1$H-NMR spectra of regular Grubbs’ third-generation catalyst (G3 regular) and its water-soluble modified species (G3 modified) in deuterated chloroform. Strong alkylidene singlet is visible far downfield at ~19 ppm.](image-url)
By simply observing the intensity and chemical shift of the alkylidene proton over time, changes in the structure and stability of the water-soluble Grubbs’ catalyst in water can be easily determined. A time-course $^1$H-NMR study was designed to monitor the alkylidene signal of a fresh batch of aqueous Grubbs’ catalyst solution in D$_2$O. The alkylidene signal was monitored over a period of 12 hours to determine changes in its shift and intensity. Three important observations were made based on the results of the time-course NMR experiment. First, the intensity of the alkylidene peak in D$_2$O was significantly lower than that of the water-soluble Grubbs’ catalyst in deuterated chloroform (CDCl$_3$, Figure 12A). Second, there were three alkylidene-type signals observed on the NMR of the catalyst in D$_2$O, instead of the one signal that is seen in the NMR of the same compound in CDCl$_3$ (Figure 12B). Third, the three alkylidene-type signals observed on the NMR spectrum of the modified catalyst in D$_2$O did not change intensity or position throughout the course of the 12-hour experiment (Figure 12C).

Figure 12. A: $^1$H NMR spectrum of water-soluble Grubbs’ catalyst in D$_2$O, showing multiple low-intensity peaks in the alkylidene region. B: 12-hour time course study of alkylidene peaks. C: Relative integration over time of signals in the alkylidene region.
All of these observations can be explained by a series of studies performed by Lynn et al. on the behavior and reactivity of ruthenium alkylidenes in protic solvents. Lynn reported that in the presence of deuterium, an exchange process takes place wherein the deuterium atom replaces both the alkylidene proton as well as one of the chlorine atoms and pyridyl ligands in the ruthenium complex. This mechanism explains the significant decrease in signal intensity of the alkylidene proton on the NMR, as the deuterium-exchanged alkylidene cannot be detected. Furthermore, this mechanism also explains the presence of numerous alkylidene peaks, as the chemical shift of the peaks will be different depending on the number of chlorine atoms and pyridyl ligands associated with the catalyst complex (Figure 13). Finally, the observation that the alkylidene peaks did not change position or intensity over a 12-hour period supports the conclusion that once the exchange of deuterium atoms reaches equilibrium, the catalyst does not degrade in appreciable amounts as would have been indicated by either the disappearance or shifting of alkylidene signals over time.

Figure 13. Various species of water-soluble Grubbs’ catalyst in D₂O, showing D₂O exchange for chlorine as well as deuterium exchange for alkylidene proton. Species that generate NMR signals are enclosed in boxes.
Additional stability experiments were conducted by monitoring the UV absorbance of water-soluble Grubbs’ catalyst complex solutions at 330 nm and at 680 nm. The 330 nm absorption band corresponds to metal-to-ligand charge transfer (MLCT) between the ruthenium and NHC as well as the benzyl ligands. The results of the UV experiment revealed that within a relatively short time frame, the absorption at 330 nm decreases at an average rate of 0.06 Au/hour, corresponding to a total signal loss of approximately 25% after the first 10 hours (Figure 14). The maximum absorbance at this wavelength was only visible at very low catalyst concentrations. Conversely, the absorbance residing at 680 nm was only visible at very high catalyst concentrations, and increased over time at approximately the same rate (Figure 14). Although the exact mechanism for this process is currently unknown, it is likely that these observations are a direct result of catalyst decomposition in response to exposure to UV light.

Figure 14. UV time course studies of aqueous solutions of water-soluble Grubbs’ catalyst in D₂O. Spectra were taken at 15-minute intervals over the course of 12 hours. A: Dilute solution of catalyst, monitoring at 330 nm, showing a gradual decrease in maximum absorbance. B: Concentrated solution of catalyst, monitoring at 680 nm, showing a gradual increase in maximum absorbance.
There exists a discrepancy between the lack of appreciable decomposition of the catalyst as seen by $^1$H-NMR and the changes in maximum absorbance shown by UV. It is possible that due to the high rate of exchange between the alkylidene proton and deuterium atom that any gradual catalyst decomposition, however minimal it may be, is not visible via $^1$H-NMR. However, it can be concluded that even in the event that the catalyst decomposes at a rate proportional to what is shown by UV experiments, this rate is nonetheless relatively slow, and allows for ample time to perform both free polymerizations as well as graft-from polymerizations before significant amount of catalyst decomposition has taken place.

Catalyst activity studies were conducted by growing free poly(norbornene) in both organic and aqueous media in order to determine the activity of the catalyst as well as its rate of initiation. To study the viability of the water-soluble Grubbs’ catalyst, three ROMP experiments were conducted where polymerizations with $M_n$ targets of 20, 30, and 40 kDa were synthesized in organic media (Figure 15A). These polymers were then purified by precipitation into cold pentane, and subjected to gel-permeation chromatography (GPC) to determine degree of polymerization and polymer dispersity (Figure 15B). Unfortunately, due to technical issues with GPC equipment, accurate measurements of either parameter were not possible. However, GPC tracings showed peaks for each polymer at approximately 20 kDa, supporting the conclusion that the modified catalyst was, in fact catalytically active. Furthermore, a second peak was also seen in each case at a slightly lower retention time, corresponding to a higher molecular weight. It is not immediately evident what may be causing this peak to appear however, it is possible that incomplete conversion of G3 to water-soluble Grubbs’ catalyst may have
resulted in two species of catalyst to be present, both of which retained activity in organic media, and catalyzed polymerizations at different rates. Alternatively, the monomer solution may have contained a mixture of both PEGylated and non-PEGylated norbornene, both of which would be soluble in organic media but could be incorporated into polymers at different rates.

Figure 15. A: Synthetic scheme of ROMP in organic conditions using water-soluble Grubbs’ catalyst. B: GPC traces of 20, 30, and 40 kDa purified polymer samples.
Finally, polymerization kinetics experiments were performed in order to determine the rate of catalytic initiation in aqueous conditions. For this experiment, an aqueous solution of water-soluble Grubbs’ catalyst was added to an NMR tube containing a solution of PEGylated norbornene monomer at a known concentration (Figure 16A). After addition, the NMR tube was quickly agitated and $^1$H-NMR spectra were immediately collected at one-minute intervals for 60 minutes (Figure 16B). The relative integration of monomer and polymer signals over time afforded rates of monomer consumption and polymer formation. This experiment showed that although the polymerization initiates very quickly, a monomer conversion of only 40% was achieved (Figures 16C, 16D). However, these experiments were conducted prior to later studies that revealed the optimization of reaction conditions and therefore a number of factors, such as the concentration of copper sulfate, may have contributed to poor monomer conversion. Aside from the degree of monomer conversion, the first order catalytic initiation constant could be calculated by observing the rate of monomer consumption over the first several minutes (Figure 16E). Based on the observed rate of initiation, it was shown that the first order initiation constant for the water-soluble Grubbs’ catalyst is $2.0 \times 10^{-3}$ s$^{-1}$. This rate resides in between the previously-reported initiation constants for G2 ($0.003 \times 10^{-3}$ s$^{-1}$) and G3 ($200.0 \times 10^{-3}$ s$^{-1}$).$^{25}$

Further extensive studies in catalyst stability both free in solution as well as bound to protein surfaces is required to more accurately determine the half-life of the modified catalyst species as well as it’s reactivity in aqueous environments. Additionally, the exact mechanism of catalyst decomposition in water, as well as how this mechanism compares to that of Grubbs’ catalyst in organic conditions must also be elucidated.
2.4 Protein Surface Macroinitiator

The general strategy for the assembly of a macroinitiator for ROMP on the surface of a protein involves the attachment of norbornene groups to reactive residues on the protein surface, and subsequent docking of a water-soluble Grubbs’ catalyst to the norbornene via single-cycle cross metathesis across the cyclic olefin. Initial attempts at

Figure 16. A: Synthetic scheme of ROMP in aqueous conditions using water-soluble Grubbs’ catalyst. B: $^1$H-NMR time-course study of aqueous ROMP. Spectra were collected every 30 minutes for 12 hours. Hourly spectra are shown with monomer and polymer signals selected. C: Relative integration of monomer and polymer signals over time. D: Expanded view of ROMP initiation from 0 to 6 minutes. E: Initiation kinetics of aqueous ROMP.
the modification of the protein surface were conducted using bovine serum albumin (BSA). Norbornene monocarboxylic acid, activated with EDC, was reacted with BSA in a 1:9 DMSO-phosphate buffer mixture, to form amide linkages between the norbornene acid groups and primary amines of lysine residues on the protein surface. Despite several attempts at the EDC coupling reaction, adequate conversion of lysine residues to be visible via MALDI-MS could not be achieved. It is possible that due to the large molecular weight of BSA, together with its propensity to form homodimers and homotrimers, quantitative conversion and characterization of this reaction may not be possible.

As an alternative strategy, lysozyme was used to alleviate the aforementioned problems. Lysozyme is an excellent model protein due to its established structure, relatively small size, and very low propensity to dimerize. Initial attempts at the surface modification of lysozyme using the EDC coupling reaction described above showed unremarkable conversion of lysine residues, with an average of two modifications per protein, out of a possible total of seven primary amines at the protein surface: six lysine residues and the protein amino terminus. (Figure 17) In order to improve conversion, an alternative modification method was used. In this method, a reaction of a large excess of norbornene dicarboxylic anhydride (NDA) formed amide linkages with primary amines at surface of lysozyme (Figure 18A).

![Figure 17. Synthetic scheme of DMAP-catalyzed EDC coupling of norbornene carboxylic acid with lysine residues on the surface of lysozyme.](image)
Early attempts at this reaction yielded significantly higher residue conversion, averaging 4 modifications per protein with little to no unreacted lysozyme remaining as shown by MALDI-MS. The reaction was completed within 90 minutes and purification was achieved with simple spin filtration against several aliquots of clean buffer (Figure 18B). Further tuning of reaction conditions to 75 equivalents of NDA and a pH adjustment to approximately 8 during the course of the reaction significantly improved conversion, with an average of 5 modifications per protein, including the amino terminus, with no unreacted lysozyme starting material. (Figure 18C).

Figure 18. A: Synthetic scheme of functionalization of lysine residues on the surface of lysozyme with norbornene dicarboxylic anhydride. B, C: MALDI-TOF spectra of purified lysozyme-norbornene products after amide formation at pH 6 and 8, respectively.
Once isolated, the lysozyme-norbornene conjugate was allowed to react with a large molar excess of water-soluble Grubbs’ catalyst in the presence of copper sulfate in order to quickly form the macroinitiator complex at the protein surface (Figure 19). A very large excess of catalyst is used in order to expedite the formation of the macroinitiator as well as to prevent cross-metathesis between norbornene groups at lysine residues on different proteins. The copper sulfate additive is required to facilitate metathesis by assisting the abstraction of one pyridyl ligand from the ruthenium center, thereby converting the complex into its catalytically active form (Figure 5). Following a brief reaction time of approximately 30 minutes, the protein surface macroinitiator must be thoroughly purified prior to initiating any graft-from ROMP. This purification step is essential as any remaining free catalyst can itself initiate free polymerizations in solution and would render it impossible to control Mₙ targets.

![Synthetic scheme of the assembly of ROMP macroinitiator.](image)

**Figure 19.** Synthetic scheme of the assembly of ROMP macroinitiator. **NDA** – norbornene dicarboxylic anhydride. **Ru=CHPh** – water-soluble Grubbs’ catalyst.

Initial aqueous reactions involving the water-soluble Grubbs’ catalyst contained copper sulfate at very low concentrations, typically in a 1:1 molar ratio with the catalyst itself. However, significant amount of precipitate was observed when copper sulfate is added to the reaction solution. Further investigation into the formation of this precipitate
revealed that above a pH of approximately 4.9, dilute copper sulfate solutions gave rise to the formation of insoluble copper hydroxide in water, and insoluble copper phosphate in phosphate buffers. For this reason, all later aqueous metathesis chemistry was performed in the presence of copper sulfate at concentrations of approximately 150 mM to maintain a soluble copper sulfate concentration at high enough levels to facilitate ROMP.

Purification of the lysozyme-macroinitiator complex has proven to be very challenging. Due to the large excess of water-soluble Grubbs’ catalyst used to assemble the macroinitiator complex, and given the strict requirement that no free catalyst can be present prior to initiating ROMP, the macroinitiator must be put through a very thorough purification process. Furthermore, despite extensive efforts to characterize the degree of stability and rate of degradation of the water-soluble Grubbs’ catalyst in aqueous solutions, it is still not definitively known whether the catalyst maintains its optimal activity over long periods of time nor whether it can withstand extensive chemical or mechanical purification. Finally, the reactivity of the catalyst when attached to the protein surface relative to its activity while free in solution is also not known. For these reasons, the purification of the macroinitiator complex must not only be extremely thorough, but must also be done as quickly as possible in order to minimize any potential decomposition of the catalyst over time.

Early attempts at macroinitiator purification have been through the use of centrifugal spin filtration with a molecular weight cut-off of approximately 3 kDa. Spin filtration using these filters is very effective when purifying the lysozyme-norbornene conjugate, but is much slower and less efficient when purifying the macroinitiator. One possible explanation for this is that although the water-soluble Grubbs’ catalyst is water-
soluble, its solubility in water is not very high and therefore may have a propensity to aggregate in the spin filter membrane at higher concentrations. These aggregates significantly slow down filtration and as a result, several hours are required to remove what is likely only a fraction of free catalyst from the crude macroinitiator solution. Two alternative strategies for the isolation of macroinitiator were also attempted. The first involved precipitation of the protein complex in a 20% PEG solution (average molecular weight 8 kDa). Concentrated solutions of high-molecular weight PEG have been shown to effectively precipitate proteins at around room temperature. However, no precipitate was observed upon the addition of PEG, even at concentration of up to 30% w/v. The second alternative approach was the use of ammonium sulfate to precipitate the protein complex. In this case, a significant amount of dark green precipitate was immediately observed upon the addition of 20% (NH₄)₂SO₄. The heterogeneous solution was then centrifuged to form a dark green oily pellet, and the pellet was resuspended in buffer. Unfortunately, it is unlikely that the resuspended pellet contained purified macroinitiator. Immediately after addition of the ammonium sulfate and formation of the precipitate, the supernatant above the solid precipitate appears almost completely colorless. If the macroinitiator complex were the only substance precipitating out of solution, the supernatant would be expected to contain a large excess of dissolved excess Grubbs’ catalyst, which is either dark green when it is healthy or yellow-brown when decomposed. Given that the supernatant of the ammonium sulfate solution was mostly colorless, it is likely that little free Grubbs’ catalyst remained in solution.

Further investigation into the quick and thorough purification of this compound is required. Additional studies will explore the possibility of using an organic extraction
with chloroform to isolate protein macroinitiator in the aqueous phase, exploiting the Grubbs’ catalyst’s propensity to preferentially dissolve in the organic phase. Finally, another future alternative method for the assembly of the macroinitiator complex involves the addition of a molar excess of lysozyme protein to a solution of Grubbs’ catalyst in order to saturate the catalyst in surface reactive sites. This approach is risky as it could result in possible cross-metathesis between lysine residues, but given the limited success of other purification methods, as well as the known decrease in ROMP polymerization kinetics in water, it is possible that no cross-metathesis will take place.

2.5 ROMP from Protein Surface

In spite of the difficulties inherent in the purification of the protein surface macroinitiator complex, the most successful method employed thus far is extensive spin filtration to remove the majority of excess free catalyst. The purified macroinitiator was then subsequently used for ROMP from the surface of lysozyme by addition of macroinitiator to a solution of water-soluble norbornene monomer (Figure 20). The norbornene monomer used for all ROMP experiments was a PEG monoester norbornene synthesized in large scale via DMAP-catalyzed EDC-coupling with monocarboxylic acid norbornene (Figure 27).

Figure 20. Synthetic scheme of the assembly of the ROMP macroinitiator, and subsequent graft-from ROMP to form protein-polymer conjugate. NDA – norbornene dicarboxylic acid anhydride. Ru=CHPh – water-soluble Grubbs’ catalyst.
The ROMP reaction is also performed in the presence of copper sulfate, which must be added again as the copper sulfate used in the assembly of the macroinitiator is in large part removed during its purification. Initial graft-*from* ROMP reactions were proof-of-concept studies designed to show that a polymer can be grown via ROMP from the protein surface and the protein-polymer conjugate can be purified and characterized. Once macroinitiator complex and copper sulfate was added to a solution of PEG-norbornene monomer, the reaction was allowed to stir for approximately 60 minutes prior to the addition of diethylene glycol monovinyl ether (DGME) to terminate the polymerization (Figure 21). Throughout these 60 minutes, aliquots of crude polymerization solution were removed at intervals of 1, 10, and 60 minutes and added to solutions of DGME to terminate the reaction at different times to monitor polymerization kinetics. The terminal olefin on DGME undergoes cross-metathesis across the double bond adjacent to the catalyst on the end of each growing polymer chain, effectively cleaving the catalyst and terminating further chain growth.

**Figure 21.** Synthetic scheme of ROMP from protein surface using PEG monoester norbornene monomer, and DGME terminator.
After 1 minute, a high molecular weight protein-polymer conjugate was seen, centered at approximately 100 kDa (Figure 22A). This reaction was complete in 30 minutes and led to nearly full conversion of lysozyme to the lysozyme-polymer conjugate. After 60 minutes of polymerization, reactions were quenched and purified by a centrifugal molecular weight cut off filter.

To evaluate the conjugation results, both PAGE gels and size exclusion chromatography (SEC) were performed to determine approximate molecular weight and conversion percentage. Gel results indicated a high molecular weight smear near the top of the gel, at approximately 170 kDa (Figure 22A) as it has been seen in other protein-polymer conjugates synthesized via graft-from polymerizations.\textsuperscript{38,39} Encouraged by this result, the gel was stained using a barium iodide staining technique, known to stain for PEG.\textsuperscript{40} The barium iodide stain matched the Coomassie stain, indicating successful protein-polymer conjugate formation (Figures 22B, 22C). Finally, SEC was performed to determine percent conversion and approximate polymer dispersity. The elution of the protein-polymer conjugate shifted to from ~14 mL to ~7 mL indicating a substantial increase in molecular weight (Figure 22D). The chromatogram revealed two high-molecular weight peaks, which can be attributed to a distribution of catalyst-modified sites on the protein, as can be seen in the MALDI spectrum of norbornyl-modified lysozyme. The polymer chains were targeted to be of high molecular weight, thus the addition or subtraction of a single polymer chain on the protein would result in a change of conjugate molecular weight of up to approximately 30 kDa. In addition, no free lysozyme remained after polymerization, indicating complete initiation.
Finally, we took advantage of ROMP as a living polymerization to grow block copolymers from the lysozyme macroinitiator using a prepared azido-PEG norbornene as the second monomer (Figure 28). After one hour of polymerization using PEG norbornene, azido-PEG norbornene monomer was added to form a second block (Figure 23A). Block copolymer formation was monitored using size exclusion chromatography (SEC). The two peaks associated with the initial single block PEG-norbornene conjugate
eluted at a lower volume and coalesced into a single peak at the void volume of the column (Figure 23C), indicating increased molecular weight. To confirm reactivity of the second block, a post-polymerization reaction via copper-catalyzed azide-alkyne cycloaddition (CuAAC) was employed to determine reactivity of the azido group. An alkyne dye (OregonGreen488, Figure 23B) was clicked onto the conjugate to create a fluorescent label whose maximum absorbance resides at 498 nm. By monitoring the SEC trace at this wavelength, a significant peak corresponding to the absorbance of the dye at the elution volume of the protein-polymer conjugate is observed (Figure 23C). This result indicates both successful block formation and the ability to functionalize the conjugate following polymerization.

![Figure 23](image)

Figure 23. A. Synthetic scheme of graft-from ROMP block copolymerization. B. OregonGreen488 alkyne dye (OG). C. FPLC chromatogram of purified block copolymer fluorescent conjugate.
Two subsequent graft-from trials involved multiple simultaneous polymerizations, each with different reaction conditions. These trials were designed to show more specifically, a change in the degree of polymerization (DP) as a function of monomer concentration as well as to further optimize the polymerization conditions in order to achieve better conversion and initiation. In these experiments, five aliquots of water-soluble Grubbs’ catalyst were added to separate reaction solutions, each containing increasing equivalents of monomer.

In the first trial, 100 equivalents of water-soluble catalyst were used to modify the lysozyme-norbornene conjugate with an average of 3 modifications per protein. The macroinitiator complex was purified with extensive spin filtration, and all metathesis reactions took place in the presence of 3 mM copper sulfate. Five polymerizations were initiated simultaneously by introducing equimolar amounts of macroinitiator to solutions of 0, 5, 50, 100, and 200 molar equivalents of monomer per moles of protein conjugate and allowed to react for 45 minutes.

PAGE results showed streaking in the lanes of each sample with an observable increase in molecular weight proportional to an increase in monomer equivalents (Figure 24A). Unreacted macroinitiator complex was also visible as a hazy band in each lane, indicating an incomplete polymer initiation. SEC results verified that polymer initiation was very poor, with each tracing showing a predominant macroinitiator peak with unremarkable increase in shouldering at a lower elution volume corresponding to polymer chain growth (Figures 24B, 24C).
In the second trial, 300 equivalents of catalyst were used to modify the lysozyme-norbornene conjugate with an average of 5 norbornene modifications per protein. The macroinitiator complex was purified via precipitation with ammonium sulfate as described previously, and all metathesis reactions took place in the presence of 200 mM copper sulfate. Five polymerizations were initiated simultaneously by introducing equimolar amounts of macroinitiator to solutions of 0, 10, 50, 100, and 500 equivalents of monomer per moles of protein conjugate and allowed to react for 2 hours. PAGE results showed little to no streaking in the lanes that corresponded to 0, 5, and 50 equivalents (Figure 25A). A low-molecular weight streak was seen in the 100 equivalents lane, and a strong high-molecular weight streak was seen in the 500 equivalents lane. No unreacted macroinitiator was visible on the gel in any lane.

Figure 24. A. PAGE gel of unpurified graft-from reaction mixture demonstrating polymer size dependence on monomer concentration. L = ladder, Lane 1 = lysozyme wild type, Lane 2 = lysozyme carboxynorbornene, Lane 3-7 = ROMP reaction in the presence of 0, 5, 50, 100, and 200 equivalents monomer. B. FPLC chromatogram of crude polymer conjugate samples. Full chromatogram is shown in Figure A7 (Appendix I). C. Expanded view of protein-polymer conjugate peaks.
In spite of the inconclusive PAGE results, SEC results showed a significantly improved degree of initiation, with new high molecular weight peaks appearing to increase in size proportional to the equivalents of monomer used (Figures 25B, 25C). Multiple peaks were visible at retention times corresponding to higher molecular weights, indicating the formation of multiple protein-polymer conjugate species with varying degrees of polymer initiation. Although an unreacted macroinitiator peak was seen in every sample, a much larger protein-polymer conjugate peak was also present. In the sample corresponding to 500 equivalents of monomer, the macroinitiator peak was significantly smaller than the conjugate peak, indicating a high degree of initiation.

The PAGE data in Figures 24A and 25A indicate a significant loss of protein content relative to lysozyme wild-type and lysozyme-norbornene standards, in spite of

Figure 25. A. PAGE gel of unpurified graft-from reaction mixture demonstrating polymer size dependence on monomer concentration. L = ladder, Lane 1 = lysozyme wild type, Lane 2 = lysozyme carboxynorbornene, Lane 3-7 = ROMP reaction in the presence of 0, 10, 50, 100, and 500 equivalents monomer. B. FPLC chromatogram of crude polymer conjugate samples. Full chromatogram is shown in Figure A8. C. Expanded view of protein-polymer conjugate peaks.
the fact that conjugate lanes were loaded with a much higher concentration of protein. At the same time, the SEC results of the corresponding samples, shown in Figures 25B and 25C, indicate that protein is in fact present at relatively high concentration. One possible explanation for this discrepancy involves the preparation of the samples for PAGE analysis. Each protein-polymer conjugate sample is prepared for PAGE, in part, by addition of 2 µL of dithiothreitol (DTT) as a strong reducing agent for disulfide bonds, of which lysozyme has four.\textsuperscript{41} Upon addition of DTT to the sample, an immediate precipitate is observed. This precipitate is not observed, however, when DTT is added to wild-type protein samples as well as those of lysozyme-norbornene. It can therefore be concluded that the DTT is interacting with the water-soluble Grubbs’ catalyst or its associated ligands via either reductive or competitive mechanisms thereby causing it to lose its hydrophilic properties and ultimately bringing it out of aqueous solution. In addition, protein macroinitiator as well as protein conjugated with very low molecular weight polymer maintains a high enough catalyst-protein content ratio that this interaction with the catalyst may have the ability to bring such conjugate species out of solution as well. In future studies, SDS-PAGE will be conducted on these samples without the use of DTT in order to further investigate these effects.

Nevertheless, the most likely cause for incomplete surface initiation in each case is the incomplete purification of macroinitiator complex. If purification is incomplete and free Grubbs’ catalyst is still present when monomer is added, the free catalyst likely initiates polymerization at a much faster rate than that of catalyst docked on the protein surface. Therefore, competitive monomer consumption into free polymer by unattached catalyst in solution may play a role in slowing down or otherwise inhibiting
polymerizations from initiating effectively by catalyst at the protein surface. Additional polymerization experiments are required to determine which experimental parameters most strongly affected the significant increase in initiation in trial 2, as well as to determine a method for complete initiation.

In conclusion, an aqueous ROMP technique has been developed for the growth of polymers from the surface of lysozyme. This technique represents the first example of the use of ROMP for the growth of polymers directly from the surface of a biological substrate under physiological conditions. Proof-of-concept studies have determined successful formation of protein ROMP macrorinitiator species as well as protein-polymer conjugates of high molecular weights. Further optimization of reaction conditions must be performed in order to more precisely control polymer length, composition, and polymerization kinetics.
CHAPTER III: MATERIALS AND SYNTHESIS

3.1 Materials

Dichloromethane (DCM, reagent grade), DCM (extra dry), and deuterated chloroform were purchased from Acros Organics. Methanol, tetrahydrofuran (THF) (dried over molecular sieves), ethyl acetate, pentane, and copper (II) sulfate pentahydrate (CuSO₄) were purchased from Fisher Scientific. Dimethyl sulfoxide was purchased from AMRESCO. 4-bromomethyl pyridine hydrobromide, cis-5-Norbornene-exo-2,3-dicarboxilic anhydride, O-(2-aminoethyl)-O′-(2-azidoethyl) pentaethylene glycol, and Grubbs’ 2nd generation catalyst were purchased from Sigma-Aldrich. 5-Norbornene-endo-2-carboxilic acid, poly(ethylene glycol) monomethyl ether (Mₙ = 350), and sodium hydride (60% oil dispersion) were purchased from Alfa-Aesar. Diethylene glycol monovinyl ether (DGME) was purchased from TCI. 4-dimethylaminopyridine (DMAP) was purchased from AnaSpec, Inc. N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) was purchased from Chem-Impex International. Egg white lysozyme was purchased from VWR. All reagents were used directly, without further purification.

3.2 Instrumentation

¹H- and ¹³C-NMR spectra were obtained using either a 300 MHz Varian Gemini spectrometer or a 600 MHz Varian Inova NMR spectrometer. All NMR spectra were analyzed against residual solvent peaks. Matrix-assisted laser desorption-ionization
(MALDI) and nanostructure-assisted laser desorption-ionization (NALDI) spectra were obtained with a Bruker Autoflex III MALDI-TOF-TOF mass spectrometer equipped with a 200 Hz Smartbeam II laser system. For all MALDI experiments, samples were analyzed in reflectron positive ion mode as a 1:1 solution with dihydroxybenzoic acid (DHB) matrix using a Bruker ground steel target plate in the range of $m/z = 10 – 20$ kDa. For all NALDI experiments, neat samples were analyzed in linear positive ion mode using a Bruker NALDI nanostructured target plate accessory in the range of $m/z = 100 – 1000$ Da. Electrospray ionization mass spectrometry (ESI-MS) was performed using a Thermo Scientific LCQ DECA ion trap mass spectrometer equipped with a 4 kV electrospray source. UV-Vis measurements were acquired using a Shimadzu BioSpec-nano UV-Vis spectrophotometer. Gel permeation chromatography (GPC) was performed on a Shimadzu Prominance GPC instrument equipped with a Shimadzu RID10A differential refractometer detector. Stationary phase was two Phenomenex 10E3A size exclusion columns in sequence maintained at 40 °C. GPC mobile phase was anhydrous THF at a flow rate of 0.7 mL/min. Size exclusion chromatography (SEC) was performed using a GE Healthcare AKTA-FPLC 900 chromatograph equipped with a Superdex 75 10/300 GL size exclusion column. For all SEC experiments, the mobile phase was 50 mM phosphate buffer (pH 7.4) at a flow rate of 0.4 mL/min. SDS polyacrylamide gel electrophoresis (PAGE) was performed on Novex NuPAGE 4-12% bis-tris protein gels (1.0mm x 12 well) (35 minutes, 200 V, 10X MES SDS-PAGE running buffer, pH 8.3). Gels were stained with Coomassie SimplyBlue SafeStain (Life Technologies). Barium iodide staining was performed according to literature by incubation with a 5% solution of barium chloride (Fisher) followed by a 0.1 M aqueous solution of iodine (Fluka).
3.3 Synthesis

**Para-poly(ethylene glycol)-substituted pyridine.** The ligand was prepared based on a previously-reported Williamson ether synthesis protocol. Poly(ethylene glycol) monomethyl ether ($M_n = 350$, 2.1 g, 6.0 mmol) was added dropwise to a slurry of sodium hydride (432 mg, 6.0 mmol) in 15 mL dry THF and stirred for 30 minutes under nitrogen atmosphere at 25 °C. 4-bromomethyl pyridine hydrobromide (2.28 g, 9.0 mmol) was then added, followed immediately by another portion of sodium hydride (432 mg, 6.0 mmol) and the reaction was allowed to stir vigorously under nitrogen atmosphere for 12 hours at 25 °C. The heterogeneous mixture was filtered via fine fritted funnel, and the filtrate concentrated by reduced pressure. The clear orange oil was then subjected to silica gel chromatography using a 5% MeOH:CH$_2$Cl$_2$ mobile phase which afforded pure product as a clear colorless oil (399 mg, 15%). $^1$H-NMR (300.1 MHz, CDCl$_3$, 17 °C, ppm): $\delta = 8.56$ (2H, d, $J = 6.0$ Hz) 7.26 (2H, d, $J = 6.0$ Hz) 4.58 (2H, s) 3.64 (PEG methylene, m, $J = 27$ Hz) 2.98 (3H, s). MS (NALDI-TOF) $m/z$: [M + Na$^+$] Calculated for C$_{19}$H$_{33}$NO$_7$Na ($n = 6$) 410.47; Found 409.705 (18%), Calculated for C$_{21}$H$_{37}$NO$_8$Na ($n = 7$) 454.53; Found 453.756 (63%), Calculated for C$_{23}$H$_{41}$NO$_9$Na ($n = 8$) 498.58; Found 497.797 (100%), Calculated for C$_{25}$H$_{45}$NO$_{10}$Na ($n = 9$) 542.63; Found 541.844 (60%), Calculated for C$_{27}$H$_{49}$NO$_{11}$Na ($n = 10$) 586.68; Found 585.857 (19%). For all subsequent stoichiometric

![Figure 26. Preparation of PEG-substituted pyridyl ligand.](image-url)
calculations using this product, an $M_n$ of 475 Da was assumed as given by the highest-abundance peak shown by NALDI-MS (Figure A1).

![Figure 27. Preparation of PEG monoester norbornene monomer (4).](image)

**Poly(ethylene glycol) monoester norbornene.** The monomer was prepared based on a previously-reported procedure.$^{43}$ 5-Norbornene-endo-2-carboxylic acid (1.38 g, 10 mmol), poly(ethylene glycol) monomethyl ether (3.50 g, 10 mmol), and DMAP (122.17 mg, 1.0 mmol) were dissolved in 25 mL dry CH$_2$Cl$_2$. EDC (1.92 g, 10 mmol) was then added and the mixture was refluxed at 30 °C for 12 hours. The mixture was then concentrated under reduced pressure to a white sticky oil which was taken up into ethyl acetate (100 mL) and water (50 mL). The organic layer was washed with two aliquots of saturated sodium bicarbonate solution (100 mL) and two aliquots of brine (100 mL). The organic layers were combined, concentrated under reduced pressure, and the clear oily residue was subjected to silica gel chromatography using a gradient of 1% - 6% MeOH:CH$_2$Cl$_2$ in increments of 100 mL to yield pure product as a clear pale-yellow oil (2.4 g, 58%). $^1$H-NMR (300.1 MHz, CDCl$_3$, 17 °C, ppm): $\delta$ = 6.15 (1H, m, $J$ = 6.0 Hz) 5.92 (1H, m, $J$ = 9.0 Hz) 4.15 (2H, PEG methylene, m, $J$ = 18 Hz) 3.65 (PEG methylene, m, $J$ = 18 Hz) 3.53 (PEG methylene, m, $J$ = 9.0 Hz) 3.36 (3H, s) 3.20 (1H, br s) 2.94 (1H, s) 2.88 (1H br s) 1.87 (1H, m, $J$ = 24 Hz) 1.42 (1H, d, $J$ = 15 Hz) 1.34 (1H, m) 1.26 (1H,
d, $J = 9.0 \text{ Hz}$). MS (NALDI-TOF) $m/z$: [M] Calculated for C$_{15}$H$_{24}$O$_5$ ($n = 3$) 284.35; Found 284.762 (10%), Calculated for C$_{28}$H$_{24}$O$_6$ ($n = 4$) 328.40; Found 328.848 (37%), Calculated for C$_{19}$H$_{32}$O$_7$ ($n = 5$) 372.46; Found 372.914 (76%), Calculated for C$_{21}$H$_{36}$O$_8$ ($n = 6$) 416.51; Found 416.968 (100%), Calculated for C$_{25}$H$_{40}$O$_9$ ($n = 7$) 460.56; Found 461.023 (95%), Calculated for C$_{25}$H$_{44}$O$_{10}$ ($n = 8$) 504.62; Found 505.093 (73%), Calculated for C$_{27}$H$_{48}$O$_{11}$ ($n = 9$) 548.67; Found 549.128 (44%), Calculated for C$_{29}$H$_{52}$O$_{12}$ ($n = 10$) 592.72; Found 593.175 (20%). For all subsequent stoichiometric calculations using this product, an $M_n$ of 417 Da was assumed as given by the highest-intensity peak shown by NALDI-MS (Figure A2).

![Figure 28. Preparation of Azido-PEG-Norbornene monomer.](image)

**Preparation of azido-PEG-norbornene.** To a 20 mL scintillation vial equipped with a magnetic stir bar was added O-(2-Aminoethyl)-O'-(2-azidoethyl) pentaethylene glycol, (160 mg, 0.46 mmol), dissolved in 2.0 mL CH$_2$Cl$_2$. The vial was then capped with a rubber septum, and cis-5-Norbornene-exo-2,3-dicarboxilic anhydride (50 mg, 0.3 mmol), dissolved in 1.0 mL in CH$_2$Cl$_2$, was then slowly added dropwise over 30 minutes. After an additional 30 minutes of stirring at 25 °C, the solution was washed with 10 mL 1.0 M HCl solution (2X) and then with 10 mL brine (2X). The organic layer was then collected and concentrated under reduced pressure to yield product as a clear colorless oil.
(89 mg, 59%). $^1$H-NMR (300.1 MHz, CDCl$_3$, 17 °C, ppm): $\delta = 6.18$ (2H, s) 3.66 (PEG methylene, m, $J = 33$ Hz) 3.57 (2H, s) 3.45 (2H, m, $J = 12$ Hz) 3.38 (2H, t, $J = 9$ Hz) 3.07 (2H br d, $J = 21$ Hz) 2.57 (2H, q, $J = 30$ Hz) 2.24 (1H, d, $J = 9$ Hz) 1.51 (1H, d, $J = 9$ Hz). MS (ESI-TOF) $m/z$: [M + Na$^+$] Calculated for C$_{23}$H$_{36}$N$_4$O$_8$Na 519.24; Found 519.27 (Figure A3).

Norbornene-functionalized lysozyme. To a 20 mL scintillation vial equipped with a magnetic stir bar was added egg white lysozyme (10 mg, 0.7 µmol), dissolved in 9.0 mL phosphate buffer (pH 7.4), and stirred at 25 °C for 10 minutes until no solid particulates were visible. cis-5-Norbornene-exo-2,3-dicarboxilic anhydride (5.7 mg, 35 µmol) was dissolved in 1.0 mL DMSO, and slowly added to the lysozyme solution dropwise over 60 minutes. After an additional 30 minutes of stirring, free norbornene was removed using a centrifugal molecular cut-off filter (3.5 kDa, 3X, 10k rpm, 90 minutes). The resulting supernatant was diluted to 10 mL of lysozyme-carboxynorbornene solution that was stored at a concentration of 1.0 mg/mL as verified by UV-VIS spectroscopy at a wavelength of 280 nm ($\varepsilon_{280} = 38,940$ cm$^{-1}$ M$^{-1}$). Complete conversion and number of lysine modifications was verified via MALDI-MS (Figures 18B, 18C).
Grubbs’ 3rd generation catalyst. Grubbs’ 2nd generation catalyst was converted to the 3rd generation catalyst according to a previously reported procedure.25 To a 20 mL scintillation vial equipped with a magnetic stir bar was added Grubbs’ 2nd generation catalyst, [(H2IMes)(P(Cy)3)(Cl)2Ru=CHPh] (100 mg, 0.12 mmol). The red powder was dissolved directly in 1.0 mL pyridine, and allowed to stir under nitrogen atmosphere for 5 minutes at 25 °C until all red color disappeared to yield a clear, dark green solution. 10 mL of cold pentane was then added and the solution was allowed to stir for 5 more minutes. The green precipitate that formed was isolated via fine fritted funnel, and washed with 20 mL of cold pentane. The green powder was then collected, dried under reduced pressure, and stored as pure Grubbs’ 3rd generation catalyst of the form [(H2IMes)(py)2(Cl)2Ru=CHPh], (66 mg, 76%).

Figure 30. Preparation of Grubbs’ 3rd generation catalyst.
PEG-substituted Grubbs’ catalyst. A 10 mL round-bottom flask equipped with a magnetic stir bar was charged with Grubbs’ 3rd generation catalyst (40 mg, 55 µmol), dissolved in 0.5 mL dry CH₂Cl₂. Para-poly(ethylene glycol)-substituted pyridine (100 mg, 200 µmol), dissolved in 0.5 mL dry CH₂Cl₂, was then added and the solution was allowed to stir under nitrogen atmosphere at 25 °C for 30 minutes. The solution was then thoroughly concentrated under reduced pressure (without heating) for 10 minutes to yield a dark green oily residue. The residue was re-dissolved in 1.0 mL dry CH₂Cl₂ and allowed to stir under nitrogen atmosphere at 25 °C for 30 minutes again. This stir-pump cycle was repeated six more times. After the last cycle, the dark green oily residue was re-suspended in 2.0 mL degassed phosphate buffer (pH 7.4), agitated, and subjected to centrifugation at 12k rpm for 10 minutes to remove insoluble particulates. The dark green supernatant was removed via pipette and used directly for aqueous metathesis chemistry. Since a small red pellet of insoluble ruthenium was observed after centrifugation, a 90% conversion was assumed between Grubbs’ 3rd generation catalyst and its water-soluble species for future stoichiometric calculations.
Preparation of protein-polymer conjugate. Norbornene-functionalized lysozyme (3.0 mg, 0.21 µmol) in 1.0 mL degassed phosphate buffer (pH 7.4) was added to a 20 mL scintillation vial equipped with a magnetic stir bar. 2.0 mL of the prepared aqueous solution of PEG-substituted Grubb’s catalyst is quickly added (36 mg, 50 mmol) followed immediately by CuSO₄ (10 mg, 40 µmol) added as 100 µL of 100 mg/mL solution in phosphate buffer (pH 7.4) and the mixture was allowed to stir under nitrogen atmosphere at 25 °C for 45 minutes. The dark green crude macroinitiator complex solution was separated from free catalyst using a centrifugal molecular weight cutoff filter (3.5 kDa, 3x, 10k rpm, 90 minutes). In the meantime, a solution of poly(ethylene glycol) monoester norbornene monomer (31.4 mg, 63 µmol), dissolved in 1.0 mL degassed phosphate buffer (pH 7.4), was prepared in a 20 mL scintillation vial equipped with a magnetic stir bar and allowed to stir at 25 °C for 30 minutes to ensure adequate dissolution. Following the filtration of the macroinitiator complex, the green supernatant (concentrated to 1.0 mL) was added to the norbornene monomer solution, followed immediately by another portion of CuSO₄ (10 mg, 40 µmol) added as 100 µL of 100 mg/mL solution in phosphate buffer (pH 7.4) and the mixture was allowed to stir under nitrogen atmosphere at 25 °C for 60 minutes. For block copolymerization, azido-PEG norbornene (10.8 mg, 21 µmol) was then added and the reaction was allowed to stir under nitrogen atmosphere at 25 °C for 60 more minutes. In both cases, the polymerization was terminated by quenching the reaction mixture with an excess of diethylene glycol monovinyl ether (2 mL) and allowing the solution to stir for 30 minutes. The crude protein:polymer conjugate solution was separated from small molecules using a centrifugal molecular weight cutoff filter (30 kDa, 5x, 10k rpm).
Copper-catalyzed azide-alkyne cycloaddition (CuAAC). The click reaction was performed according to a previously reported procedure.\(^4\) 500 µL of 0.5 mg/mL protein:polymer diblock conjugate solution in phosphate buffer was placed in a 2.0 mL eppendorf tube. A pre-mixed solution of 2.5 µL 50 mM CuSO\(_4\) and 12.5 µL 50 mM 3-tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) was then added. 50 µL Oregon Green 488 alkyne dye (Invitrogen) was also added, followed immediately by 25 µL of a freshly-prepared 50 mM solution of ascorbic acid (Acros). The mixture was vortexed to ensure adequate mixing, and allowed to stand in the dark for 60 minutes. Afterwards, the diblock conjugate was isolated using a centrifugal molecular cut off filter (3.5 kDa, 2x, 10k rpm) to yield a pale green supernatant containing dye-activated protein:polymer conjugate.
CHAPTER IV: FUTURE DIRECTION

4.1 High Aspect Ratio Nanoparticles

Elongated nanoparticles of high aspect ratio possess improved pharmacokinetics, tumor homing, and tissue penetration over analogous spherical and globular particles. As a result, high aspect ratio nanomaterials have shown very strong applicability in drug, gene, and contrast agent delivery, separation and filtration of analytes within complex mixtures (i.e. plasma), glucose and protein sensing, and selective interaction with tissues and macromolecules.\textsuperscript{3,45,46} Additionally, it has been extensively reported that high aspect ratio nanoparticles are less susceptible to phagocytosis by alveolar macrophages – a property which, under the right circumstances, is therapeutically useful due to its enhancement of particle circulation time.\textsuperscript{47} Generally, the bioavailability of the nanoparticle heavily relies on it’s aspect ratio, chemical composition, and surface chemistry. As a result of improved pharmacokinetics, deposition of the therapeutic particle at the target site can be significantly improved. Furthermore, reduced macrophage activation prevents collateral pathogenesis such as liver toxicity and other tissue injury.\textsuperscript{48}

Unfortunately, existing methods for the fabrication of these particles are graft-to approaches that have similar limitations as those discussed earlier, and it is therefore extremely difficult to tune many of these important properties, especially when dealing with multivalent conjugates where many polymer attachments are involved. To further enhance the affectivity of nanoparticle-based therapeutics, the graft-from methodology can also be applied to nanoparticles, providing the same benefits as would be expected...
for protein therapies. The graft-from approach utilizes a biological polymer scaffold as template for chemical synthesis. This technique not only allows for the tuning of nanoparticle aspect ratio but also for very strict synthetic control over polymer composition and architecture. The ability to control polymer length and polymer distribution along the nanoparticle surface is particularly important in order to create monodisperse nanoparticles whose size fits a narrow clinically useful profile. For example, particles larger than 1 µm, may accumulate unnecessarily in certain highly perfused tissues such as the liver and kidneys, or aggregate to cause capillary occlusion. On the other hand, particles smaller than 10 nm are more likely to be subjected to rapid renal clearance.⁹

The biological nanoparticle of choice for this project is tobacco mosaic virus (TMV), a single-stranded RNA virus that self-assembles naturally into a hollow rod-shaped structure that has a length of 300 nm and a diameter of 18 nm (Figure 32). The

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**Figure 32.** Structure of TMV. A: Entire TMV assembly, B: Section of TMV showing internal RNA structure surrounded by coat proteins, with reactive lysine residue shown in red. C: Single coat protein with reactive lysine residue shown in red.
structure of TMV consists of a single-stranded RNA core surrounded by 2,130 coat protein subunits on its exterior surface, making it an excellent biological template for the synthesis of polymeric nanoparticles as its interior and exterior surfaces can be independently functionalized. The TMV used in this project is a lysine variant, engineered with a T158K mutation to introduce a reactive lysine residue at the C-terminus of the protein where chemical modification can be easily performed. This C-terminus is particularly useful because it protrudes away from the protein directly into the solvent space, making it especially well-positioned for chemical modification. Furthermore, the regularly spaced lysine residues throughout the surface of TMV allow for a very uniform distribution of polymer initiation sites, and therefore polymer conjugations. The elongated shape of TMV has been shown to be highly beneficial to biodistribution due to enhanced vessel margination and faster diffusion across biological membranes. As an example, in the case of solid tumor tissue undergoing extensive angiogenesis, poorly–supported vasculature lacks consistent smooth muscle support and is highly fenestrated, allowing narrow rod-shaped structures to easily penetrate the spaces between endothelial cells. These properties further improve the particle’s passive diffusion into tumor tissue by way of enhanced permeability and retention (EPR). It is clear that a protective polymer coating conjugated to a high aspect ratio nanoparticle such as TMV sets the stage for the development of extremely high-performance nanomaterials. These viral nanoparticle conjugates can possess extended half-lives in vivo and their shape and surface chemistry can be tuned via graft-from polymer conjugation to target specific tissues and cells. Furthermore, the hollow shape of TMV allows the independent functionalization of both interior and exterior surfaces, providing ample surface area for
multifunctional modifications. One such possibility is the loading of hydrophilic or immunogenic cargo on the inside of the virus that would otherwise be unable to travel effectively though the circulation.

4.2 ROMP from TMV Surface

A recombinant variant of TMV bearing lysine residues on its exterior surface will be used as a substrate for conjugate nanoparticle synthesis (Figures 32C, 33). These lysine residues will first be functionalized with exo-oxanorbornene dicarboxilic anhydride (ODA), and degree of conversion will be monitored via MALDI-MS. Similar to the functionalization of lysozyme, we predict that given the high reactivity of the free amine with the anhydride group on the norbornene, that a large proportion of solvent-accessible lysine residues can be quickly and easily functionalized in this way. Following activation of the lysine residues, an excess of water-soluble Grubbs’ catalyst will be introduced, immobilizing it onto the exterior surface of TMV to form a TMV macroinitiator. PEG-functionalized exo-monoester oxanorbornenes as monomers will be used because the additional oxygen atom at the bridgehead of the oxanorbornene as well as the free carboxylic acid group remaining after the amide linkage of the norbornene will both assist in the solubility of these groups in an aqueous solution. Polymers of a relatively small molecular weight (~10 kDa) will then be grown from the surface of TMV to form a TMV-poly(norbornene) nanoparticle conjugate whose size and surface composition will be characterized via MALDI-MS, electrophoresis, size exclusion chromatography, DLS, and TEM. These nanoparticle conjugates will be fluorescently tagged by adding on a second block bearing fluorescent markers. (Figure 34).
Pharmacokinetics of TMV-Polymer Conjugates.

TMV conjugated with PEG has been previously shown to possess favorable biocompatibility in vivo, with elongated circulation time and slower tissue clearance versus analogous spherical nanoparticles. To determine the biodistribution of our conjugates, a 10 mg/kg prepared and purified solution of TMV-poly(oxanorbornene) will be introduced via tail vein injection into healthy 10-14 week old BALB/c mice. In vivo fluorescence imaging of the whole animal will be performed at a number of time points between 4 and 96 hours following injection. At these time points, mice will be sacrificed and organs such as heart, liver, spleen, and kidney will be collected and imaged via fluorescent molecular tomography (FMT) to determine local biodistribution, reported as percent injected dose per gram of tissue. Mice injected with directly-PEGylated TMV as well as wild type TMV will be used as controls to study how the pharmacokinetics of wild-type TMV and TMV-PEG compare to that of TMV-polymer conjugate. All fluorescence measurements will be normalized to a standard curve as given by the fluorescence of in vitro solutions of our conjugate in plasma at a number of known concentrations.
Immune shielding afforded by the polymer conjugation will also be investigated by performing sandwich ELISA experiments using anti-TMV antibodies, also against TMV-PEG controls. It is hypothesized that antibody detection of TMV will be markedly reduced as a result of bioconjugation with PEGylated poly(norbornene) due to its chemical and structural properties discussed earlier.

### 4.4 TMV:Polymer Conjugates as MRI Contrast Agents

Functionalized poly(oxanorbornene) bearing chelated gadolinium (III) ions will be grown from the surface of TMV and studied in vivo as potential tumor-targeted contrast delivery agents. Oxanorbornene dicarboxylic acid anhydride monomers will be functionalized with a DOTA (1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid) groups charged with chelated paramagnetic gadolinium (III) ions. (Figure 35) Although slightly more expensive and synthetically challenging, DOTA groups are used preferentially to other chelator agents such as diethylene triamine pentaacetic acid.
(DPTA) due to the improved propensity of DOTA to protect the Gd ions from competitive chelation or co-chelation with zinc and copper ions *in vivo*. A PEG group will be attached to the remaining arm of the oxanorbornene, to increase aqueous stability. Low molecular weight polymers of these diester oxanorbornenes will be grown from the surface of TMV by the same methods as previously described. Conjugates will be characterized using PAGE, MALDI-MS, SEC, DLS, and TEM to ensure that TMV retains its self-assembled structure following bioconjugation. The resulting TMV-polymer conjugate will possess a surface chemistry that is densely functionalized with gadolinium (III) ions per unit surface area. We believe that the ability of TMV to effectively carry a dense payload of contrast material will significantly improve its effectiveness as an MRI imaging agent by providing enhanced tissue contrast at sites of accumulation.

![Figure 35](image)

**Figure 35.** Scheme of Gd(DOTA)-functionalized with *poly*(oxanorbornene) grown from TMV surface.

The T1 ionic relaxivity of gadolinium (III) conjugated to the TMV surface ions was previously shown to be improved as compared to that of free Gd(DOTA). The rationale behind this is believed to be that mounting the contrast agent on a rigid substrate...
increases ionic relaxivity by reducing its tumbling rate.\textsuperscript{54} In other words, the more rigidly the gadolinium behaves in solution, the better the MRI contrast is expected to be. In previous studies, Gd(DOTA) was mounted directly to the surface of TMV by reaction with tyrosine residues on its coat protein. The method we propose will not only allow the attachment of a much higher density of gadolinium ions per unit surface area of TMV, but also doing so using a much more rigid molecular framework of the \textit{poly}(norbornene) backbone. After TMV-polymer conjugates bearing Gd(DOTA) groups will be created, their ionic relaxivity will be studied \textit{in vitro} using a pre-clinical MRI instrument and compared to that of Gd(DOTA) directly attached to TMV (TMV-Gd(DOTA)) and free Gd(DOTA) in solution.

TMV-Gd(DOTA) conjugate nanoparticles have been previously studied in their ability to target and effectively resolve atherosclerotic plaques in MRI imaging studies. These studies demonstrated that TMV serves as an effective means by which to increase tissue contrast by delivering a large cargo of gadolinium ions and increasing their ionic relaxivity at concentrations that are significantly lower than existing diagnostic standards.\textsuperscript{55} We propose using Gd(DOTA)-functionalized \textit{poly}(oxanorbornene) to deliver even higher loading levels of gadolinium mounted in an even more rigid fashion to further build on these seminal observations. A prepared and purified solution of TMV-\textit{poly}(oxanorbornene) bearing Gd(DOTA) functionalities will be injected via tail vein catheter at a concentration of 0.2 mg/kg into 8-week-old female BALB/c mice bearing orthotopic 4T1 mammary tumor xenograft cells. Following injection, MRI scans will be taken at multiple time intervals between 30 and 120 minutes to determine tumor localization.
Figure A1. $^1$H-NMR spectrum of PEG-substituted pyridyl ligand.
Figure A2. $^1$H-NMR spectrum of PEG-monoester norbornene monomer (4).
Figure A3. $^1$H-NMR spectrum of azido-PEG-norbornene (5).
Figure A4. NALDI spectrum of PEG-substituted pyridyl ligand.
Figure A5. NALDI spectrum of PEG-monoester norbornene monomer.
Figure A6. ESI Mass spectrum of azido-PEG-norbornene.
Figure A7. FPLC (SEC) chromatogram of crude polymer conjugate samples – trial 1.
Figure A8. FPLC (SEC) chromatogram of crude polymer conjugate samples – trial 2.
REFERENCES


