

Synthetic Polypeptide-Based Hydrogel Systems for Biomaterials

by

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B.A. Chemistry, Mathematics

Washington & Jefferson College, 2012

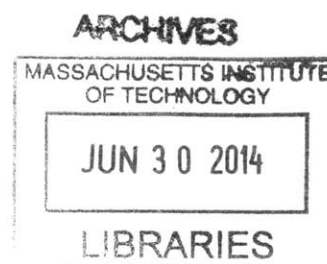
Submitted to the Department of Chemistry in Partial Fulfillment of the  
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## **Abstract**

Hydrogels formed from synthetic polypeptides generated by ring opening polymerization (ROP) of  $\alpha$ -amino acid *N*-carboxyanhydrides (NCAs) present a robust material for modeling the interaction between extracellular matrix (ECM) properties and cellular phenomena. The unique properties of the polypeptide backbone allow it to fold into secondary structures and the ability to modify the side chain presents the opportunity to display chemical functionalities that dictate cellular signaling. The ability to induce cells to form tissue is a chemical and engineering challenge due to the fact that cells need physical support in the form of a 3D scaffold with both chemical and mechanical signals. The Hammond group previously reported the combination of synthetic polypeptides with modified side chains available for click chemistry at quantitative grafting efficiencies. Herein, new schemes for hydrolytically stable versions of the polymer system with click functionality are introduced. Additionally, a new random copolymer, poly( $\gamma$ -propargyl-L-glutamate-co- $\gamma$ -allyl-L-glutamate) (PPALG) is presented that exploits both the azide-alkyne and thiol-ene click reactions to allow orthogonal side chain modification to increase chemical complexity and ultimately allow a library of “designer” gel systems to be generated.

Thesis supervisor: Paula T. Hammond

Title: David H. Koch Professor in Engineering

*For my loving parents,  
John and Suzanne Martin*

## Acknowledgements

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Finally, I am forever grateful and blessed to have my amazing parents, John and Suzanne Martin, who instilled in me the importance of education, kindness, and respect. They have provided me with a lifetime of unconditional love, support, and laughter. Thank you for being there at all times – I am honored to dedicate this work to you.

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## Introduction

### Motivation

Tissue engineering, a major branch of biomedical research, is a multidisciplinary area that thrives on the designs and research of cell biologists, chemical engineers, materials scientists, chemists, and clinical researchers all aiming to attain the ultimate goal of tissue and organ regeneration. Although each day in the United States an average of 79 individuals receive traditional organ transplants, another 18 individuals pass away each day due to a shortage of donated healthy organs. Currently, over 120,000 individuals in the United States are on the waiting list for a possibly life-saving organ transplant.<sup>1</sup> This number has steadily increased over the last ten or so years; whereas, the number of traditional transplants has remained relatively constant (Figure 1).

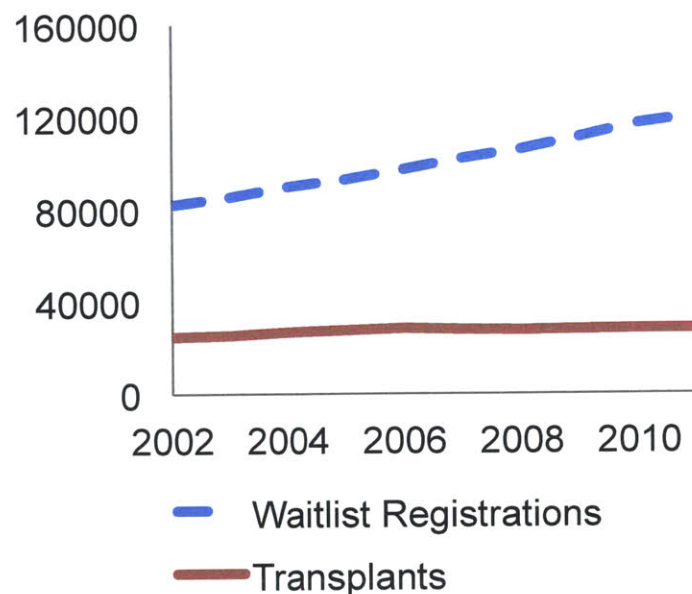


Figure 1. Number of waitlist registrations and traditional organ transplants in the United States between 2002 and 2011.

Although the ability to regenerate or even create tissues or organs for this application is very appealing, the complexity both chemically and mechanically

has presented many challenges to researchers since the late 1980s and early 1990s.<sup>2</sup> These early papers broadly described strategies for the alternative to organ transplantation that utilized isolated cells (derived from the patient or from a donor), tissue-inducing substances (e.g., growth factors or cytokines), and finally, placing the cells on or within matrices.<sup>2</sup> The ability to induce cells to form tissue is a chemical and engineering challenge due to the fact that the cells need physical support in the form of a 3D scaffold that possesses both chemical and mechanical signals. The scaffold present in the natural environment is referred to as the extracellular matrix (ECM) and serves as a “channel for cell-cell communication, which is effected through interactions with a number of cell surface receptors and ECM proteins, which, in turn, facilitate events like adhesion, migration, proliferation, and metastasis.”<sup>3</sup> The extracellular matrix is a dynamic environment that is constantly being secreted and remodeled by its resident cells and, therefore, this presents the challenge in designing biomaterials for use in tissue engineering as the synthetic scaffold.

## Hydrogels

Among the materials used for this application, hydrogels are receiving increased attention due to their ability to retain a high amount of water, high biocompatibility (both biodegradability and bioresorbability), and ability to assist in the exchange of metabolites (oxygen and nutrients).<sup>4,5</sup>

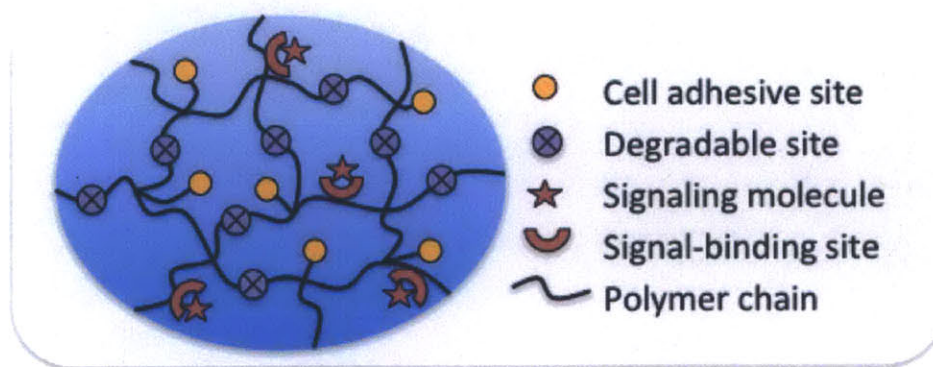


Figure 2. Schematic of a cross-linked polymer gel network with biologically relevant chemical functionalities.

Hydrogels, defined as polymer networks that are insoluble in water but swell to an equilibrium volume while maintaining their shape, can also be easily and predictably modified with cell adhesion ligands necessary for cell growth (Figure 2). The fate of the cells will depend highly on cell-substrate interactions and these will be mediated by the properties of the chosen hydrogel. Therefore, in choosing a material for the hydrogel composition, one must consider whether the polymer forming the gel is natural or synthetic, degradable or non-degradable, homopolymer, copolymer, block-copolymer, etc.<sup>6</sup> The hydrogel can also be either physically (reversibly) cross-linked via molecular entanglement or some secondary interaction such as ionic bonding, hydrogen bonding, or hydrophobic interactions or chemically (permanently) cross-linked through covalent bonding. Natural gels are composed of various protein fibrils and fibers interwoven within a hydrated network of glycosaminoglycan chains.<sup>5</sup> Common natural hydrogel systems such as hyaluronic acid or gelatin are thus abundant in functionality but difficult to predictably modify and present a batch-to-batch inconsistency. Some synthetic hydrogel systems such as polyethylene glycol or poly(lactic-co-glycolic) acid are readily prepared with controlled chain length; however, their simplicity hinders the ability to add necessary functionality.

## **Synthetic Polypeptides**

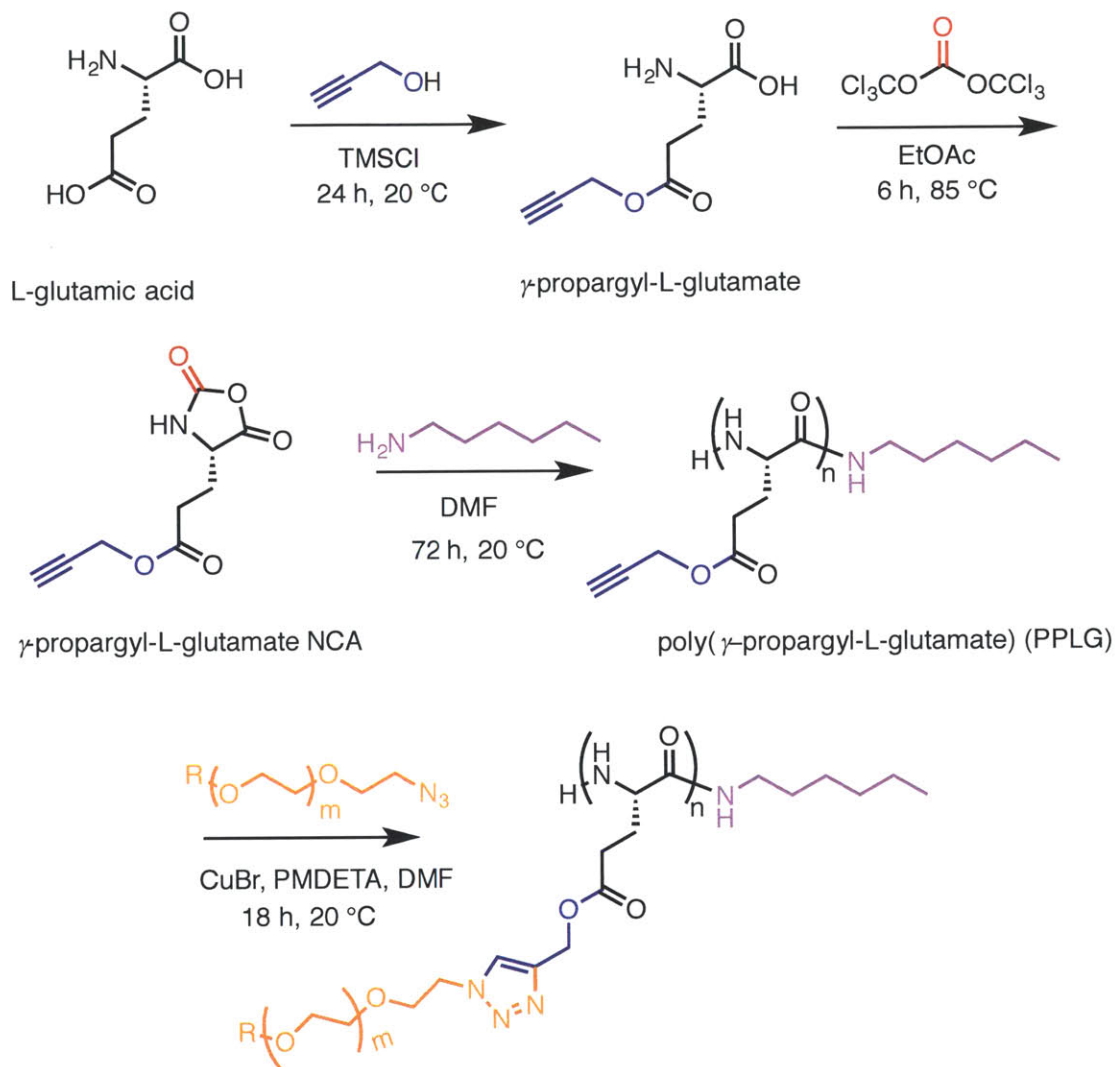
Natural biological systems produce polypeptides of precisely controlled sequences derived from the 20 naturally occurring amino acids. These peptide polymers have advantages over other synthetic polymers due to their ability to possess stable secondary structures ( $\alpha$ -helices,  $\beta$ -sheets, etc.) in solution due to cooperative hydrogen bonding. Synthetic polypeptides prepared by the ring-opening polymerization (ROP) of amino acid *N*-carboxyanhydrides (NCAs) have existed for decades and can “allow the optimal display of surface moieties that dictate cell signaling and molecular docking and provide unique opportunities to design supramolecular shape and function.”<sup>7</sup> The use of NCA monomers derived from the naturally occurring amino acids is, however, limited based on the



monomer side groups possible. Many procedures require protection and deprotection steps due to the presence of additional nucleophiles in the amino acid that may interfere with the mechanism of NCA formation. Similarly, the use of charged amino acids leads to side chain charge repulsion that affects the final chain length and relative composition of the polymer.<sup>7</sup> The synthesis of highly grafted or modified polymeric peptides is thus very promising. However, the difficulty of post-modification of the pendant groups along the polymer backbone can lead to low degrees of functionalization and steric effects can also limit incorporation of amino acids modified with large macromolecular side chains during polymerization.<sup>7</sup> Thus, highly quantitative functionalization chemistries such as “click” reactions have been developed and paired with the synthetic polypeptide schemes to generate designer libraries of polymers that can be used in hydrogels for a wide variety of tissue engineering applications.

### **Combining Click Chemistry and Polypeptides**

Click reactions are “inherently simple, efficient, and selective, with facile protocols, tolerance to a wide variety of functional groups, and quantitative yield.”<sup>7</sup> Popularized by Sharpless, these reactions typically proceed at ambient temperature and pressure, are easily purified, and produce byproducts that are not highly toxic. The incorporation of click reactions into schemes aimed at producing synthetic polypeptides with diverse pendant functionalities is very powerful. The first example of the use of click chemistry paired with an NCA polymerized species was presented by Engler of the Hammond group.<sup>8</sup> In this work a new NCA monomer,  $\gamma$ -propargyl-L-glutamate, was synthesized that incorporates a pendant alkyne group available for the Sharpless alkyne-azide click reaction. Through this scheme a triazole linkage attaches the desired functionality to the polymer (poly( $\gamma$ -propargyl-L-glutamate) or PPLG) backbone through a “grafting onto” method while maintaining the  $\alpha$ -helical conformation of the polypeptide backbone. The synthetic strategy employed in the study is shown in Scheme 1.



Scheme 1. Synthesis of PPLG through a ring-opening polymerization and subsequent side chain coupling using an azide-alkyne click reaction.

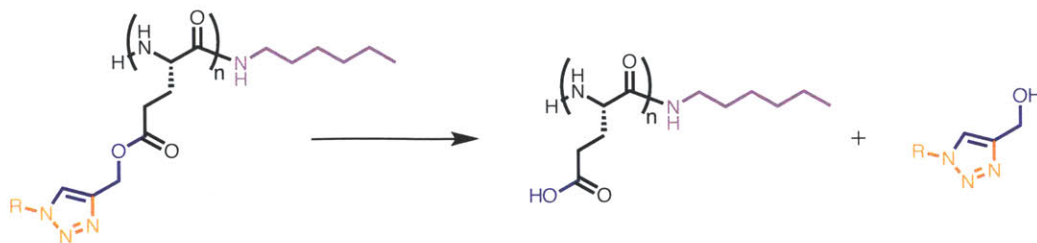
The alkyne-bearing monomer,  $\gamma$ -propargyl-L-glutamate *N*-carboxyanhydride was synthesized by reacting the carboxylic acid moiety of L-glutamic acid with propargyl alcohol to form the ester linkage followed by phosgenation in ethyl acetate to form the NCA ring. PPLG was then prepared by ring-opening polymerization initiated by a primary amine in *N,N*-dimethylformamide (DMF). Engler was able to graft a variety of functionalities to the pendant alkyne group including polyethylene glycol (PEG) chains, thiols, and amines with grafting efficiencies upwards of 95%. This is remarkable due to the fact that grafting

efficiencies for macromolecular species onto a polymer backbone are typically between 60-80% or less.<sup>9,10</sup> This capability provides an opportunity to create new types of graft and functional copolymers that are inaccessible with other methods generating biomimetic and bioinspired polymers.<sup>7</sup> Using this and other polypeptide systems, the Hammond group is addressing some major challenges in hydrogel systems. This includes independently tuning the stiffness and permeability of hydrogels that control cell differentiation. For example, if a 50:50 mixture of L- and D-glutamic acid is used to synthesize PPDLG, it will form a more flexible random coil instead of the rigid-rod-like  $\alpha$ -helical structure of PPLG. The hydrogels formed with chemical cross-linking from these polypeptides, PPLG and PPDLG, with the same degree of polymerization and cross-linking density will have similar swelling and permeability; however, they will have large differences in stiffness.<sup>11</sup> These systems can be used to create a designer library of hydrogels through variation of the chain length, chirality, grafting density, and cross-linking density. It is the goal of the work herein to extend the aforementioned studies by designing and synthesizing new polymer backbones that enable functionality to be readily accessible through facile click reactions. These new polymeric systems can be used to form hydrogels for modeling the interaction between extracellular matrix properties and cellular phenomena.

## **Results and Discussion**

### **Amide-Linked Hydrolytically Stable Polypeptides**

Although PPLG has many applications (drug delivery, antimicrobials, gene delivery, and hydrogels), the functionality grafted onto the polymer backbone is ultimately attached by an ester linkage. These ester side chains may be hydrolyzed which releases the clicked on functionality and exposes a carboxylic acid moiety (Scheme 2).



Scheme 2. The modified pendant group of PPLG may be hydrolyzed revealing a carboxylic acid moiety.

Therefore, in some biological environments, enzymatic ester cleavage could cause reduced activity of the attached functionality such as with sugar units.<sup>7</sup> Engler quantified this hydrolysis with respect to time for PPLG functionalized with, for example, a secondary amine (Figure 3).<sup>12</sup>

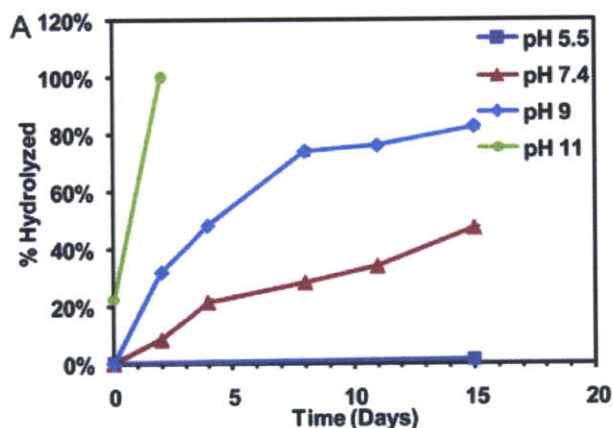
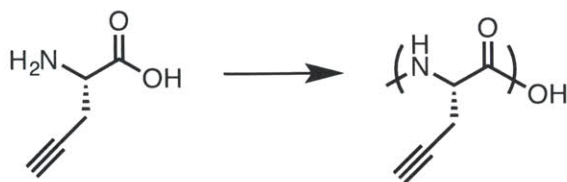


Figure 3. Percentage of ester side chains hydrolyzed as a function of time for PPLG (DP = 75) functionalized with a secondary amine.<sup>12</sup>

Although one can track the percentage of hydrolyzed side chains, it is difficult to know precisely where along the backbone this has occurred. Additionally, for longer-term cell studies, it is desirable to have a hydrolytically stable polymer platform. Thus, one goal of the work herein was to design and synthesize an alternative to PPLG that could be used in hydrogels for an extended period of time.

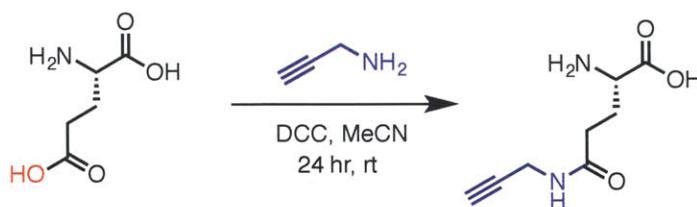
The process of designing a hydrolytically stable PPLG alternative began by investigating the use of poly( $\alpha$ -propargyl-L-glycine) shown in Scheme 3.



Scheme 3. Use of  $\alpha$ -propargyl-L-glycine to form a polymer with an accessible and hydrolytically stable pendant alkyne functionality for click reactions.

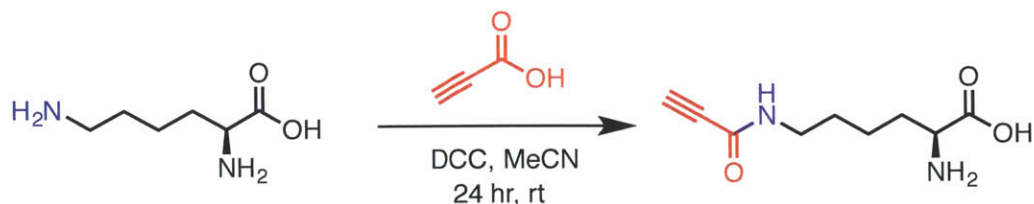
The starting material,  $\alpha$ -propargyl-L-glycine is commercially available although expensive. The  $\alpha$ -propargyl-L-glycine NCA was prepared using similar conditions as the NCA formed in Scheme 1 but in low yield. The resulting NCA was polymerized (again using similar conditions as Scheme 1) but precipitated out of solution before the reaction had gone to completion. Although numerous solvents, mixtures, and additives were investigated, the polymer could not reach beyond a degree of polymerization of ten as calculated by DMF gel permeation chromatography (GPC) with poly(methyl methacrylate) (PMMA) standards.<sup>13</sup> Therefore, alternative polymer schemes were subsequently investigated.

The next logical synthetic scheme was to make use of an amide-linked polymer, starting with either glutamic acid or lysine. Due to the increased nucleophilicity of nitrogen over oxygen, an amide bond is far more hydrolytically stable than an ester bond. Therefore, the ester linkage in PPLG could be replaced with this longer lasting amide linkage. Although these bonds are readily formed, a peptide-coupling agent is required due to a lack of electrophilicity at the carbonyl carbon of the carboxylate. Before purchasing expensive protected amino acids, the coupling of an unprotected amino acid was attempted with the corresponding functionality possessing the terminal alkyne group. This was attempted with both L-glutamic acid and L-lysine (Schemes 4 and 5).<sup>13</sup>



Scheme 4. Formation of an propargyl amide linkage via addition of propargyl amine to L-glutamic acid through a DCC coupling.

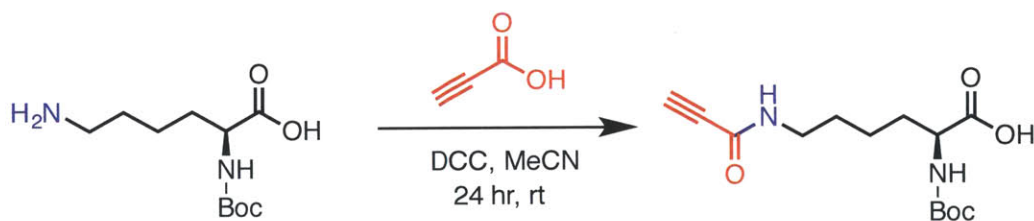




Scheme 5. Synthesis of an amide-linkage via addition of propynoic acid to L-lysine through a DCC coupling.

*N,N'*-dicyclohexylcarbodiimide (DCC) was the chosen peptide-coupling reagent used to enhance the electrophilicity of the carboxylate group by activating the charged oxygen into a better leaving group. These reactions were performed under argon atmosphere at room temperature for 24 hours; however, the purification proved extremely difficult due to non-selective amide coupling.<sup>13</sup>

Due to the challenges occurring with the unprotected amino acids, *N*<sub>α</sub>-(*tert*-butoxycarbonyl)-L-lysine was purchased (Scheme 6) and reacted with the same electrophile under the same conditions as above (Scheme 5).

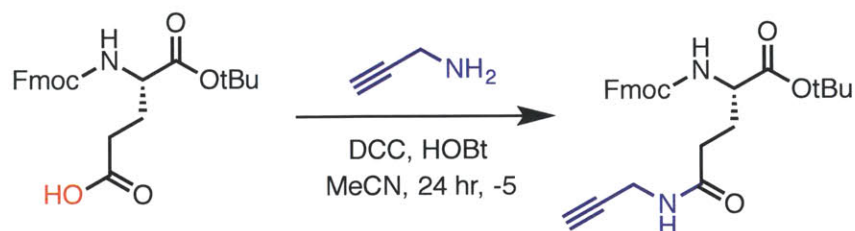


Scheme 6. Synthesis of an amide-linkage via addition of propynoic acid to Boc-Lys-OH through a DCC coupling.

In this synthetic scheme with the partially protected amino acid, unfortunately, the unprotected nucleophilic amine of the Boc-Lys-OH reacted with the unprotected carboxylate moiety of another molecule of lysine instead of the desired electrophile bearing the alkyne moiety.

This non-selective amide formation caused me to devise a scheme with an amino acid protected at both the  $\alpha$ -amine and the carboxylate that would subsequently need to be deprotected for the formation of the *N*-carboxyanhydride ring. Therefore, *N*- $\alpha$ -Fmoc-L-glutamic acid  $\alpha$ -*tert*-butyl ester

was purchased and used under the same reaction conditions as Scheme 4 with the addition of 1-hydroxybenzotriazole (HOBt) to increase reaction yields (Scheme 7) and at reduced temperature.



Scheme 7. Formation of an amide linkage bearing a terminal alkyne through the addition of propargyl amine to Fmoc-Glu(OtBu)-OH via DCC coupling.

I successfully performed this reaction as supported by  $^1\text{H}$  nuclear magnetic resonance spectroscopy (NMR) (Figure 4).

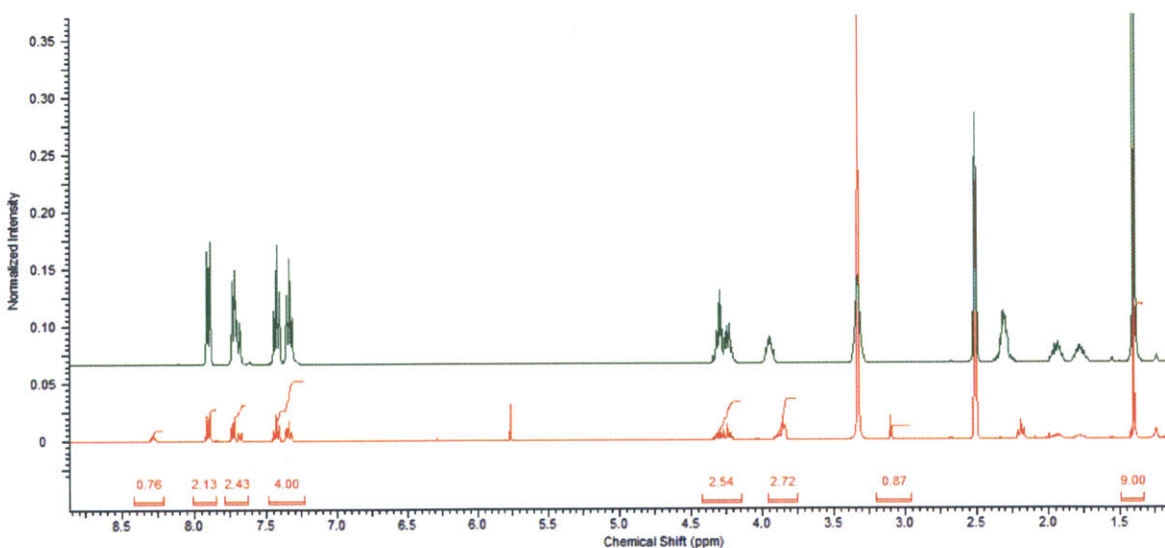


Figure 4.  $^1\text{H}$  NMR spectra of unreacted Fmoc-Glu(OtBu)-OH (top) and the propargyl amine coupled product shown in Scheme 7 (bottom).

The above spectra (Figure 4) show the formation of the propargyl amide linkage through the appearance of the peak at a chemical shift of 3.09 ppm corresponding to the alkyne proton (bottom spectrum). Additionally, the

appearance of the peak at a chemical shift value of 8.28 ppm corresponds to the amide proton of the product. Finally, the total integration of the two peaks at 3.85 and 4.29 ppm was four in the starting material and increased to six to account for the two methylene protons of the propargyl group. Although this first step in the monomer synthesis was successful and could subsequently be reacted under the same conditions as used to form PPLG to polymerize, this synthetic scheme was not continued due to cost and more promising alternatives.

Next, the PyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate) peptide coupling reagent (Figure 5) was considered to react the Boc-protected lysine (Scheme 6) with 5-hexynoic acid in excess (Scheme 8). The use of an excess of our desired carboxylate group would ensure that the Boc-protected lysine would not couple with another molecule of itself.

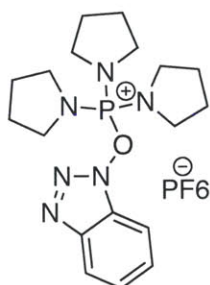
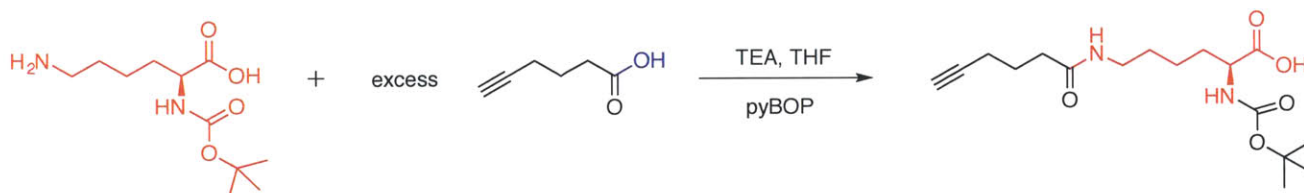


Figure 5. Chemical structure of the PyBOP peptide coupling reagent.



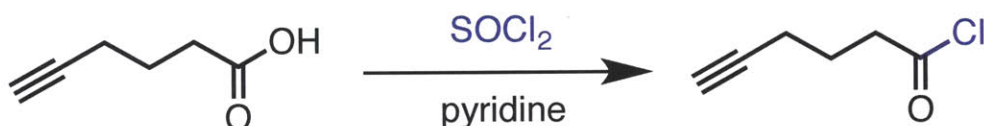
Scheme 8. Coupling of Boc-Lys-OH with 5-hexynoic acid with PyBOP to form an amide linkage possessing a terminal alkyne pendant group for click chemistry.

This reaction was performed in tetrahydrofuran (THF) in the presence of triethylamine (TEA). Although this reaction was also successfully performed, the



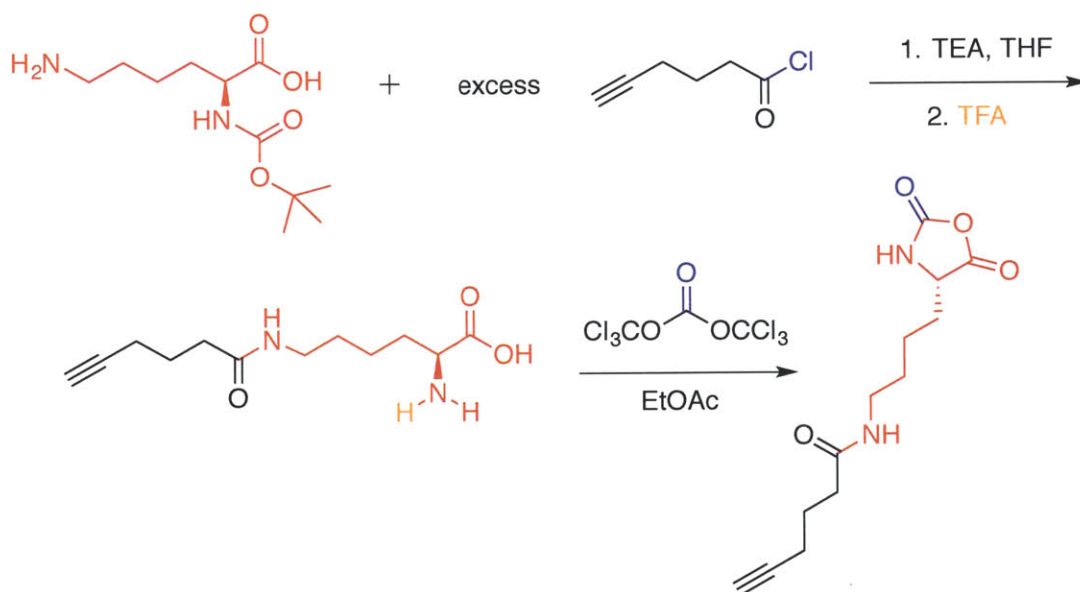
purification proved extremely difficult and I was unable to remove byproducts via column chromatography despite use of various eluents.

Finally, I devised a scheme that would not require the use of a peptide coupling reagent by using an electrophile much more potent than a carboxylate group. This synthetic method exploits the reactivity of acid chlorides by coupling the Boc-protected lysine with 5-hexynoyl chloride. The latter compound can readily be prepared from 5-hexynoic acid (Scheme 9).



Scheme 9. One step conversion of 5-hexynoic acid to 5-hexynoyl chloride through use of thionyl chloride.

This Boc-protected lysine can then be coupled with this species in THF in the presence of TEA and subsequently deprotected to form the NCA ring (Scheme 10).



Scheme 10. Synthesis of an amide-linked NCA monomer with a terminal alkyne group for click chemistry via an acid chloride coupled with Boc-Lys-OH.

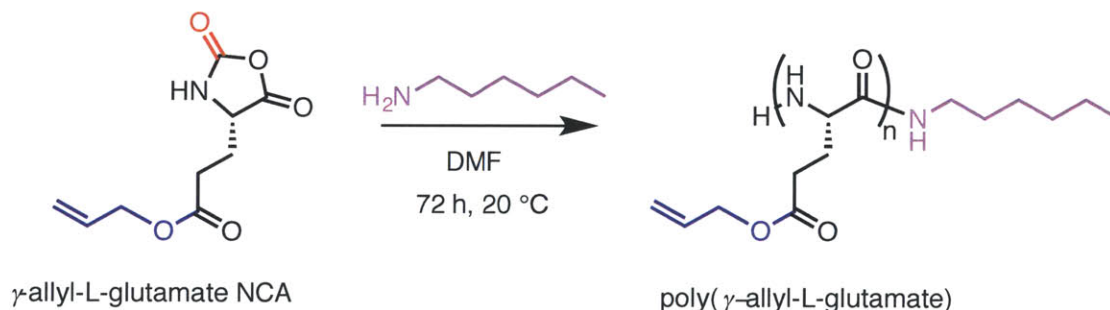
This scheme is still currently under investigation in the Hammond group and will produce hydrolytically stable synthetic polypeptides with click functionality that can undergo azide-alkyne click reactions to easily and efficiently attach the desired functionality. These polymers can then be cross-linked to form designer gels with tunable chain length, stiffness, permeability, and chemical displays that provide cells with a dynamic structural environment.

### **Introducing an Orthogonal Click Chemistry**

The Cu(I)-catalyzed variation of the Huisgen cycloaddition described previously is a 1,3-dipolar cycloaddition reaction that enables the efficient grafting of chemical moieties onto the PPLG backbone. As mentioned, this combination of click chemistry and synthetic polypeptide NCAs is a powerful tool for generation of synthetic mimics of biological molecules. Although the azide-alkyne click reaction has received the greatest attention, a number of high-yielding and orthogonal reactions qualify under the acknowledged criteria of click chemistry in terms of chemoselectivity and equimolarity.<sup>7</sup> One example of another readily accessible click chemistry is the thiol-ene type reaction. Light-mediated thiol-ene radical reactions effectively combine the classical benefits of click reactions with the advantages of a photoinitiated process. Thus, this click reaction can proceed without the need for a metal catalyst as required in the Sharpless azide-alkyne cycloaddition. Thiol-ene reactions proceed rapidly and quantitatively under ambient atmospheric conditions. The ability to graft thiols onto ene-modified polymer substrates can provide materials with specific properties not achievable by other methods.<sup>14</sup> Therefore, aligned with the approach used to design PPLG, a second goal of this work was to combine the alkyne side group with an additional “clickable group” also incorporated directly along the backbone during the polymerization of the NCA. Although in choosing an orthogonal pendant group several functionalities were considered, the alkene was chosen due to its robustness not only in the thiol-ene reaction but also due to the accessibility of other functionalities in one step by traditional organic synthesis methods.

### Synthesis of Poly( $\gamma$ -Allyl-L-Glutamate)

L-glutamic acid

 $\gamma$ -allyl-L-glutamate

The polymer product was confirmed by  $^1\text{H}$  NMR and a representative spectrum is shown in Figure 6. This batch of PALG has a degree of polymerization of 25 by  $^1\text{H}$  NMR as evidenced by the peak at a chemical shift of 5.88 ppm corresponding to the alkene proton labeled “c” and the peak at a chemical shift of 5.22 ppm corresponding to the terminal alkene protons labeled “d” (Figure 6). Note that the

integrations are referenced to the peak at a chemical shift of 0.86 ppm corresponding to the methyl group protons of the initiator labeled “a”.

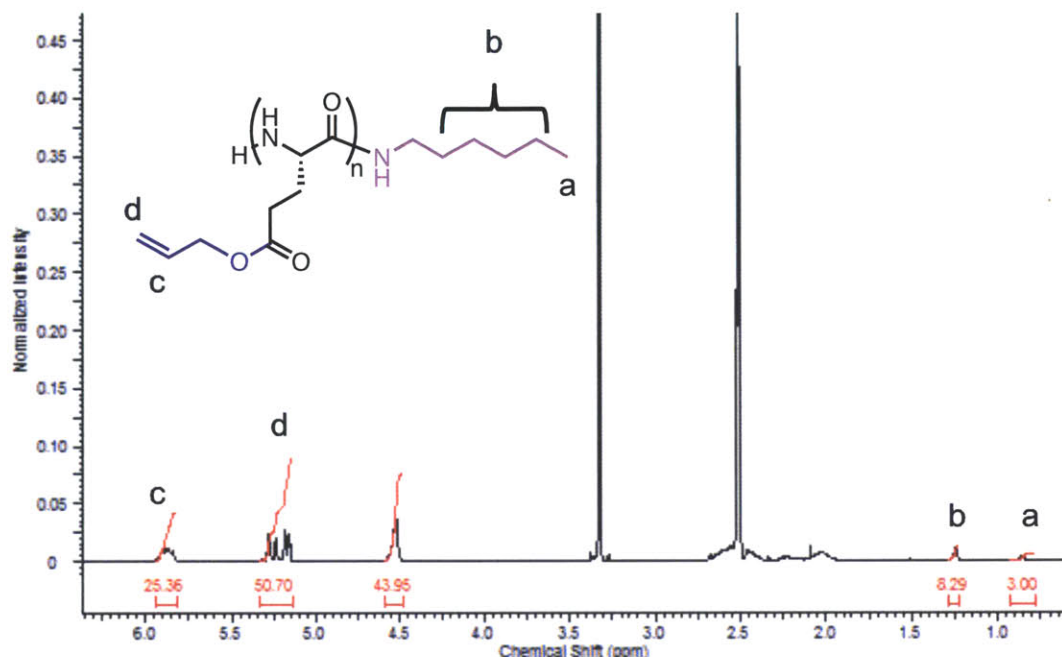


Figure 6. Representative <sup>1</sup>H NMR spectrum of poly(γ-allyl-L-glutamate) (PALG).

The poly(γ-allyl-L-glutamate) (PALG) system has the benefit of adopting an  $\alpha$ -helical conformation in solution similar to PPLG that allows the pendant alkene groups to be free from steric hindrance and accessible for functionalization.<sup>15</sup> This ability to achieve diverse structures by side-chain conjugation allows control over polymer bioactivity and solubility and the ability to exploit chirality allows control over stiffness and permeability of the resultant gel.

### Co-polymers with Orthogonal Click Groups

Although the poly(γ-allyl-L-glutamate) system provides a robust platform for side chain functionalization through highly efficient grafting with the thiol-ene click reaction, the goal was to design copolymers to increase the complexity and availability with orthogonal click reactions to create a library of designer hydrogels. Therefore, I began attempting the synthesis of the random copolymer, poly(γ-propargyl-L-glutamate-co-γ-allyl-L-glutamate) (PPALG). The syntheses of

the monomers,  $\gamma$ -propargyl-L-glutamic acid NCA and  $\gamma$ -allyl-L-glutamic acid NCA, were conducted separately according to Schemes 1 and 11, respectively. These reactive monomers were dried under high vacuum overnight and combined together in DMF with a heptylamine initiator under argon atmosphere. After 24-72 hours (depending on the degree of polymerization), the resultant PPALG was precipitated in diethyl ether and underwent additional aqueous purification. The product was then lyophilized overnight. The resulting random copolymer PPALG is shown in Figure 7.

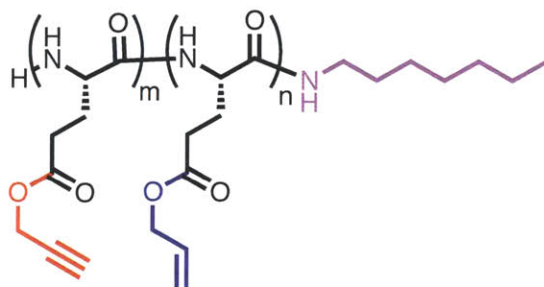


Figure 7. Chemical structure of the random copolymer PPALG with pendant alkyne and alkene groups available for click chemistry.

The polymer product was confirmed by  $^1\text{H}$  NMR and a representative spectrum is shown in Figure 8.

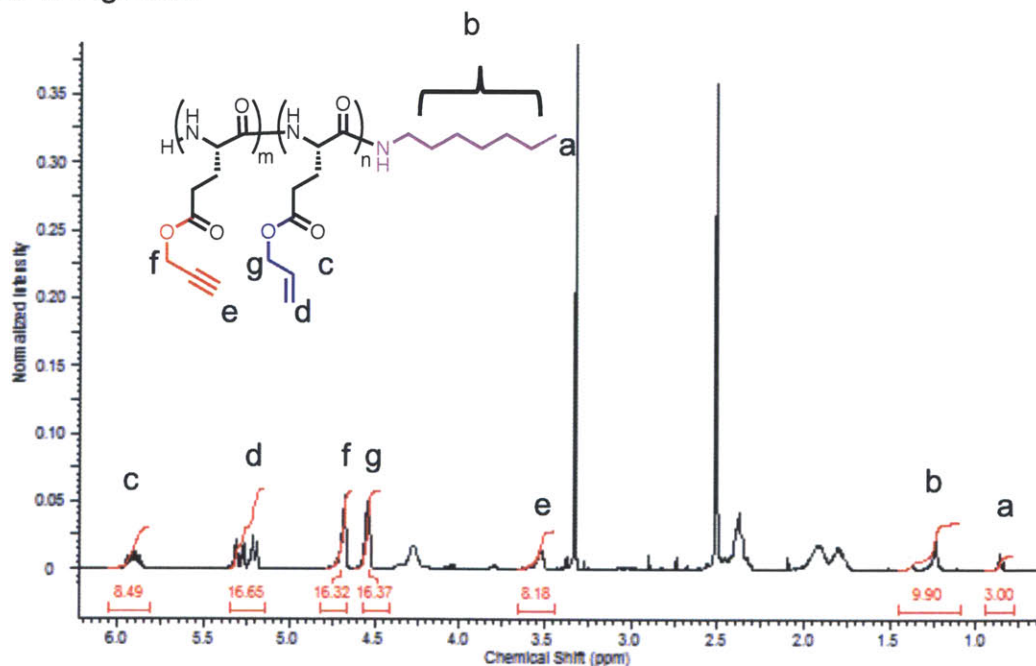


Figure 8. Representative  $^1\text{H}$  NMR spectrum of PPALG.



This batch of PPALG has a degree of polymerization of 16 by  $^1\text{H}$  NMR as evidenced by the peak at a chemical shift of 5.91 ppm corresponding to the alkene proton labeled “c” and the peak at a chemical shift of 5.26 ppm corresponding to the terminal alkene protons labeled “d” (Figure 8). Note that the integrations are referenced to the peak at a chemical shift of 0.86 ppm corresponding to the methyl group protons of the initiator labeled “a”. The synthesis of this polymer allows access to a variety of previously unavailable functionalities and combinations of functionalities to be grafted onto the polymer backbone in a nearly quantitative fashion. The two clickable groups, a terminal alkyne and a terminal alkene, can provide the opportunity to perform orthogonal click reactions at specific units along the polymer backbone. Since the efficiency of the Sharpless azide-alkyne click reaction had previously been quantified<sup>8</sup>, I decided to perform a thiol-ene click reaction with my PPALG products.

### Thiol-ene Click Chemistry with PPALG

As previously mentioned, the thiol-ene click reaction can be photochemically induced and performed at ambient temperature and pressure. This radical process proceeds according to the mechanism depicted in Figure 9.

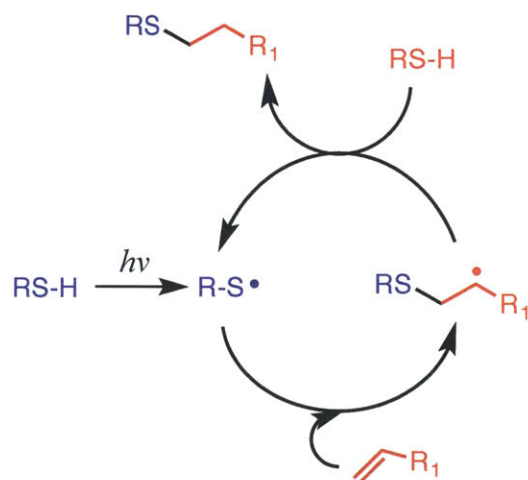


Figure 9. General thiol-ene coupling reaction mechanism.

The alkene functionality present at the pendant side chains of the polymer can couple with the sulfur radical generated from the thiol reagent. Thiols are

available commercially and can also be readily prepared on a typical laboratory scale by several methods. The thiol purchased for this study was poly(ethylene glycol) methyl ether thiol (Figure 10).

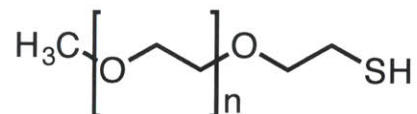


Figure 10. Poly(ethylene glycol) methyl ether thiol with  $M_n = 800$ .

A macromolecular species was chosen for the ability to follow the reaction by GPC and to compare the grafting efficiency to previous work. The reaction of PPALG and PEG methyl ether thiol was performed at ambient and temperature and pressure under UV light for 24 hours in DMF. No chemical initiator was used for this process. The expected product structure is shown in Figure 11.

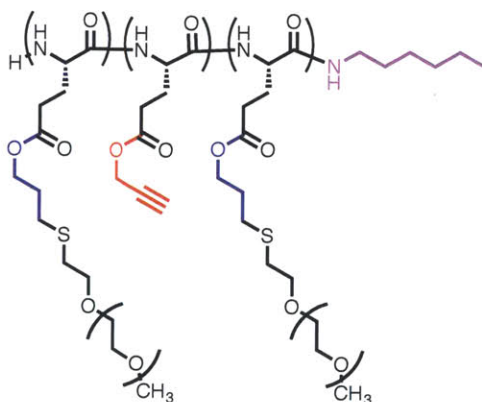


Figure 11. Generalized chemical structure for PPALG after grafting onto with PEG methyl ether thiol.

The number average molecular weight of several batches of PPALG was determined before and after conducting the thiol-ene click reaction and are shown in Table 1.

Polymer	Before thiolene click		After thiolene click	
	Mn	PDI	Mn	PDI
PPALG-1	6105	1.24	11769	1.09
PPALG-2	8794	1.26	11877	1.11
PPALG-9	13466	2.14	19520	1.5

Table 1. Number average molecular weight ( $M_n$ ) and polydispersity index (PDI) data for three different batches of PPALG before and after performing the thiolene click reaction with poly(ethylene glycol) methyl ether thiol ( $M_n = 800$ ).

The molecular weight of the three tested batches (1, 2, and 9) of PPALG increased in all cases after 24 hours as determined by DMF GPC with PMMA standards. A representative GPC trace is shown below for PPALG-1 (Figure 12).

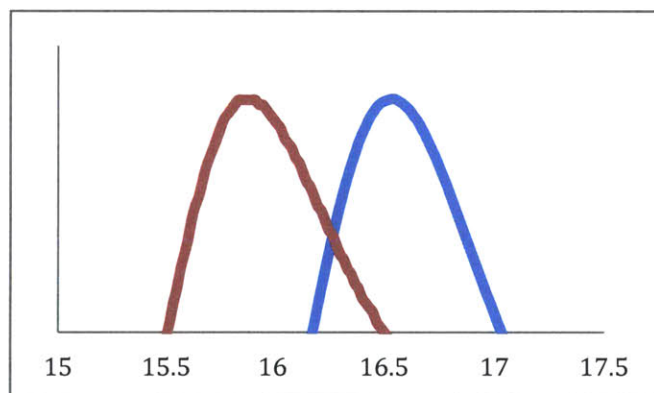


Figure 12. Representative DMF GPC trace for PPALG-1 before (left peak, red) and after (right peak, blue) click reaction with PEG methyl ether thiol.

To calculate the grafting efficiency, the ratio of propargyl and allyl side chains was determined by  $^1\text{H}$  NMR for each polymer batch. These fractions were then used to determine the average number of propargyl units and allyl units in a certain polymer. The number of allyl units in a batch of PPALG was then compared to the number of thiols that had been clicked on as determined by the increase in number average molecular weight by DMF GPC. Representative data is shown below for PPALG-2 (Tables 2, 3, and 4). Note that the molecular weight and degree of polymerization as determined by  $^1\text{H}$  NMR are typically less than as determined by DMF GPC with PMMA standards for these polymers.



Before thiolene click			After thiolene click			
Mn	PDI	DP	Mn	PDI	Mn Increase	# Thiols
8794	1.26	52.556	11877	1.11	3083	3.854

Table 2. DMF GPC data for PPALG-2 with PMMA standards before and after the thiol-ene click reaction with PEG methyl ether thiol.

DP	% Propargyl	% Allyl
33.913	92.627	7.373

Table 3.  $^1\text{H}$  NMR data for PPALG-2 before the thiol-ene click reaction.

DP from GPC	Propargyl Units	Allyl Units	Grafting Efficiency
52.56	48.68	3.88	<b>99.45</b>

Table 4. Calculation of the average number of propargyl and allyl units in PPALG-2 using data from  $^1\text{H}$  NMR and determination of grafting efficiency.

As one can see for this particular batch (PPALG-2), the average number of allyl units present was 3.88 and the number of thiols clicked onto the backbone as determined by DMF GPC was 3.854 using the stated manufacturer number average molecular weight. This yields a nearly quantitative grafting efficiency. Therefore, this PPALG system has great potential as a tool in designing and synthesizing polymers for hydrogels used for biomimetic or bioinspired materials. One can exploit the orthogonality of this thiol-ene click reaction and the azide-alkyne click reaction to build up complexity and tailor systems to a specific biological application.

## Materials

L-glutamic acid ( $\geq 99\%$ ) was purchased from both EMD Chemicals, Inc. and Sigma-Aldrich Co. and used without further purification. Propargyl alcohol, allyl alcohol ( $\geq 98.5\%$ ), chlorotrimethylsilane ( $\geq 99\%$ ), poly(ethylene glycol) methyl ether thiol, diethyl ether ( $\geq 98\%$ ), *N,N*-dimethylformamide (anhydrous, 99.8%), hexylamine (99%), *N* $_{\alpha}$ -Boc-L-lysine (99%), 5-hexynoic acid (97%), and propargyl amine (98%) were purchased from Sigma-Aldrich, Co. and also used without further purification. Fmoc-Glu-OtBu was purchased from Novabiochem

by EMD Chemicals, Inc. and used as received. Ethyl acetate was purchased from EMD Chemicals, Inc. and further dried over anhydrous magnesium sulfate ( $\text{MgSO}_4$ ).

All  $^1\text{H}$  nuclear magnetic resonance (NMR) spectra were obtained on a Bruker AVANCE III 400 MHz spectrometer with a SpectroSpin superconducting magnet and TopSpin 3.1 software at the Department of Chemistry Instrumentation Facility. Gel permeation chromatography (GPC) was conducted using a Waters 1525 with refractive index detection and an XBridge BEH C18 column at the Institute for Soldier Nanotechnologies. Polymer solutions were prepared at a concentration of 5 mg/mL in HPLC-grade DMF and passed through a 13mm syringe filter with 0.45  $\mu\text{m}$  PTFE membrane. The polymer solution was run through the GPC columns with DMF as eluent with 0.01 M LiBr.

## Conclusions

The ability to mimic aspects of the native extracellular matrix in terms of both mechanical and molecular signaling is highly desirable for a variety of tissue engineering applications such as organ regeneration. Hydrogels, with their ability to provide an aqueous environment and good biocompatibility, are at the forefront of research aimed at modeling extracellular matrix effects on cellular phenomena. The use of synthetic polypeptides (from ring-opening polymerization of  $\alpha$ -amino acid-*N*-carboxyanhydrides) in forming these gels has allowed systems with predictable structure and function. Additionally, the recent combination of NCA polymerization and click chemistry along the polymer backbone using modified amino acid monomers has increased the capabilities even further with nearly quantitative grafting efficiencies and access to a variety of functionalities. In continuing and expanding the PPLG work developed in the Hammond group, I was able to synthesize a hydrolytically stable version of the monomer using *N*- $\alpha$ -Fmoc-L-glutamic acid  $\alpha$ -*tert*-butyl ester coupled with propargyl amine to form an amide bond with a terminal alkyne available for click chemistry. I also developed several synthetic schemes to continue the design of a hydrolytically stable PPLG

alternative that are currently under investigation in the Hammond group. In addition to developing NCA polymers with clickable groups for longer-term cell studies, I have also successfully synthesized a random copolymer containing orthogonal click groups along the backbone. This system, poly( $\gamma$ -propargyl-L-glutamate-co- $\gamma$ -allyl-L-glutamate) (PPALG), provides the capability of combining the copper mediated azide-alkyne click reaction and the photoinitiated thiol-ene click reaction as orthogonal coupling methods for deriving higher-order structures and increased complexity from synthetic polypeptides. Additionally, I was able to successfully demonstrate the effectiveness and efficiency of the thiol-ene click reaction onto the PPALG backbone with a macromolecular species. Currently, the Hammond group is continuing investigation of the hydrolytically stable clickable polypeptide systems as well as the synthesis of random and block copolymers with orthogonal click chemistry capabilities and end-linked synthetic polypeptide systems.

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