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Hydrolysis resistant functional polypeptide scaffold for biomaterials

Wade Wang^{a,c} and Paula T. Hammond^{b,c*}

Abstract

For biomaterials, rich functionality often comes at a cost of precise control over structure. In this paper, we have developed a hydrolysis resistant polypeptide that marries the potential for expansive modification with synthetic control. This polymer, poly(γ -propargyl-L-glutamine) may be prepared from commercially available or previously synthesized poly(L-glutamate) by routine amide bond coupling reactions on a multi gram scale. Afterwards, the polymer may be altered to suit many applications through modification of the side chains or end group by orthogonal and quantitative reactions. The ease of synthesis and versatile nature of poly(γ -propargyl-L-glutamine) makes it an unique and ideal scaffold for biomaterial applications.

Introduction

There is a clear dichotomy between synthetic polymers created in a laboratory and natural polymers isolated from biological sources. Natural polymers have found many applications as biomaterials given their complex functionality and innate biological activity. However, these polymers are commonly ill defined, difficult to isolate, difficult to modify, and susceptible to batch to batch variation. On the other hand, synthetic polymers allow for precise control and modification of chemical structure and functionality through advances in living polymerization and click chemistry. While many simple polymers, such as poly(ethylene glycol), have been used to much success in biomaterial applications, it is difficult to endow biological activity to these polymers through chemical modification. Other synthetic polymers that are created by controlled polymerization but more easily modified are excellent candidates to replace simple synthetic polymers when complex functionality or biological activity are required.

Polypeptides produced by N-carboxyanhydride ring opening polymerization are an appealing material for the replacement of natural polymers in biological applications. The polymer backbone is inherently biocompatible and polymers with varying side chains can be synthesized to suit specific applications.¹ In particular, poly(γ -propargyl-L-glutamate) (PPLG) is an α -helical polypeptide that may be modified through copper-catalyzed azide-alkyne cycloaddition (CuAAC). The controlled polymerization and modification methods enable precise control of structure, making it a promising material for the replacement of natural polymers in biological applications.^{2–9} However, the limited lifetime of ester bonds on the side chain precludes PPLG from being used in long term applications or under harsh conditions where polymer degradation is undesirable such as polymeric platforms for organoids, hydrogels for long term 3D cultures, surface functionalization for implant

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Supplementary information is available

coatings, and scaffolds for tissue engineering. In addition, the increased lability of the ester bond under basic conditions or high temperatures limits the employment of otherwise useful post polymerization reactions or processing conditions.

One approach to synthesis of hydrolysis resistant functional polypeptides involves coupling of amines to poly(L-glutamate). This may be accomplished by displacement of a glutamic acid ester with an amine through nucleophilic acyl substitution. Despite being the most direct route to poly(L-glutamate) derivatives, this approach requires the use of excess amine and organic solvents. Commercially available poly(γ -benzyl-L-glutamate) (PBLG) reacts with amines, though achieving high conversion requires the use of catalysts or aggressive reaction conditions that may result in side reactions.¹⁰ Poly(γ -trichloroethyl-L-glutamate) features a better leaving group as the ester, resulting in greater reactivity, but requires additional monomer synthesis and polymerization of the amino acid monomer. In addition, complete conversion without excess amine is still not guaranteed.^{11,12} Alternatively, amines may be coupled to poly(L-glutamate) by reagents such as EDC or DMT-MM.^{13,14} While this method has generated interesting materials, grafting density is limited. Literature examples of these couplings routinely report incomplete grafting, ranging from 50% to 90%, despite stoichiometric or excess amounts of amine. Synthesis of complex biomaterials often involves conjugation of high molecular weight components in low concentrations and aqueous medium, which would further reduce the grafting efficiency.

Thus, replacing amide bond coupling with a more robust chemistry, such as CuAAC, would be beneficial in the synthesis of these materials. CuAAC, commonly referred to as click chemistry, has recently generated great attention due to its ease of execution, effectiveness, and tolerance of diverse functional groups. Previous examples of glutamic acid derivatives capable of post polymerization modification with CuAAC have not demonstrated hydrolytic stability⁵ or quantitative modification of every repeat unit with azides or alkynes.¹³ To improve upon other approaches in synthesizing functional polypeptides, we have designed a hydrolysis resistant variant of PPLG through post polymerization modification of PBLG. Deprotection followed by amide bond coupling yields a polymer that retains α -helical structure, modular nature, and low dispersity of ester PPLG, but greatly improves hydrolytic stability by replacing the ester bond with an amide. Furthermore, we have found the synthetic route to be highly robust and scaleable. The direct transformation of the poly(L-glutamate) backbone allows access to a broad molecular weight range.

Experimental

Triphosgene, 3-(3-Dimethylaminopropyl)-1-ethyl-carbodiimide hydrochloride (EDC), 1-Hydroxybenzotriazole hydrate (HOBt), and γ -Benzyl-L-glutamate were obtained from Chem-Impex. 5-Norbornene-2-methylamine was obtained from TCI America. *N,N*-Dimethylformamide (DMF) was dried and stored over 3Å molecular sieves under an argon atmosphere prior to use. Lithium phenyl-2,4,6- trimethylbenzoylphosphinate (LAP)¹⁵ and 2-(2-azidoethoxy)ethanol¹⁶ were synthesized according to literature procedure. Regenerated cellulose 1 kDa molecular weight cutoff dialysis membrane was obtained

from Spectrum Labs. All other reagents and materials were purchased from Sigma-Aldrich and used as received.

^1H and ^{13}C NMR spectra were obtained in CDCl_3 , dimethyl sulfoxide- d_6 or deuterium oxide (Cambridge Isotope Laboratories) using a Bruker Avance 400 MHz NMR spectrometer at 25 °C. Circular dichroism (CD) spectroscopy was done on an Aviv model 202 CD spectrometer at 25 ± 0.1 °C, sampling every 1 nm with a 3 s averaging time over a range of 190–260 nm (bandwidth = 1.0 nm). Measurements were taken in a quartz cell of 1 mm path length at a concentration between 0.1 – 0.5 mg/mL. Gel permeation chromatography (GPC) measurements in DMF with 10 mM LiBr were carried out using a Waters 1525 binary pump system equipped with two Polypore columns operated at 75 °C, series 2414 refractive index detector, and 717plus autosampler. Waters Breeze Chromatography Software Version 3.30 was used for data collection as well as data processing. The instrument was calibrated against narrow molecular weight poly(methyl methacrylate) standards.

γ -Benzyl-L-glutamate N-carboxyanhydride

To a dispersion of γ -benzyl-L-glutamate (25.0 g, 112 mmol) in THF (200 mL) in a 1 L two neck round bottom flask equipped with a magnetic stir bar and rubber septum was added triphosgene (30.0 g, 100 mmol) in one portion. The mixture was stirred and sparged with a steady stream of argon under reflux for 2 h then cooled to RT. Hexanes (700 mL) were added and the mixture was allowed to sit for 7 h. The resulting precipitate was collected by vacuum filtration and washed 4 times with hexanes under a blanket of argon. The resulting solid was added to a flame dried 1 L flask, dissolved in anhydrous THF (300 mL), and recrystallized via solvent diffusion under a layer of hexanes (700 mL) in an argon atmosphere at RT. The solids were collected by vacuum filtration under a blanket of argon, washed 4 times with hexanes, and dried *in vacuo* to give white crystals (25.7 g, 103 mmol, 92%). ^1H NMR (400 MHz, CDCl_3) δ : 7.46 – 7.31 (m, 5H), 6.49 (s, 1H), 5.37 – 5.01 (m, 2H), 4.40 (s, 1H), 2.63 (t, J = 6.3 Hz, 2H), 2.42 – 2.23 (m, 1H), 2.23 – 2.01 (m, 1H). ^{13}C NMR (100 MHz, CDCl_3) δ : 172.57, 169.46, 151.76, 135.28, 128.86, 128.77, 128.55, 67.30, 57.16, 30.11, 27.08.

poly(γ -benzyl-L-glutamate) (PBLG)

A flame dried 100 mL Schlenk flask equipped with a magnetic stir bar and rubber septum was charged with NCA (3.28 g, 13.2 mmol) and DMF (30 mL). 5-Norbornene-2-methylamine (16.9 μL , 0.132 mmol) was added via micropipette with stirring and the reaction was sparged with a steady stream of argon at RT for 48 h. The reaction was added to water and the resulting precipitate was collected by centrifugation, washed 3 times with water, and dried *in vacuo* to give a white powder (2.44 g, 11.9 mmol repeat units, 90%). ^1H NMR (400 MHz, DMSO) δ : 8.37 (s, 1H), 7.48 – 7.03 (m, 5H), 6.17 – 5.94 (m, 0.03H), 5.24 – 4.81 (m, 2H), 4.11 (s, 1H), 2.80 – 1.55 (m, 4H).

poly(γ -propargyl-L-glutamine) (PPLQ)

A round bottom flask equipped with a stir bar was charged with PBLG (6.00 g, 27.4 mmol repeat units) and THF (72 mL). The solution was cooled to 0 °C and a chilled solution of lithium hydroxide (1.70 g, 41.1 mmol) in water (24 mL) was added. The reaction was vigorously stirred at 4 °C for 12 h, diluted with water, and extracted 3 times with diethyl

ether (3 x 100 mL). The organic layer was discarded and the pH of the aqueous layer was adjusted to 1-2 with 6M HCl. Residual ether was evaporated by sparging with compressed air and the solution was frozen and lyophilized to give a white solid. The solid was added to a round bottom flask equipped with a stir bar and DMF (25 mL), HOBt hydrate (ca. 20% water) (6.95 g, 41.1 mmol), and propargylamine (2.46 mL, 38.4 mmol) were added. EDC (6.83 g, 35.6 mmol) was added portionwise with stirring. After stirring for 24 h methanol (250 mL) was added. The precipitate was collected by centrifugation, washed with water, and dried *in vacuo* to give a white powder (2.63 g, 15.8 mmol repeat units, 58%). ¹H NMR (400 MHz, DMSO) δ: 8.22 (s, 1H), 8.09 (s, 1H), 6.16 – 5.93 (m, 0.02H), 4.21 (s, 1H), 3.85 (s, 2H), 3.07 (s, 1H), 2.15 (s, 2H), 1.82 (d, *J* = 48.3 Hz, 2H).

Poly(γ-((1-(2-(hydroxymethoxy)ethyl)-1H-1,2,3-triazol-4-yl)methyl)-L-glutamine) (PPLQ-g-EO2)

PPLQ (0.40 g, 2.4 mmol repeat units), DMF (10 mL), 2-(2-azidoethoxy)ethanol (0.34 mL, 2.9 mmol), and PMDETA (50 μL, 0.24 mmol) were added to a centrifuge tube. The tube was capped and vortexed to form a homogenous solution. The solution was sparged with a steady stream of argon for 20 minutes. Afterwards, copper (I) bromide (34 mg, 0.24 mmol) was added in one portion. The tube was quickly capped, vortexed for 3 minutes, and allowed to sit for 12 h. Diethyl ether (40 mL) was added to the reaction and the resulting emulsion was separated by centrifugation. The diethyl ether was discarded and the remaining liquid was resuspended in DI water (5 mL). Dowex M4195 resin was added until the solution no longer appeared blue. The resin was removed by gravity filtration, the solution was dialyzed against water (MWCO 1000, RC), frozen, and lyophilized to give a white powder (0.65 g, 2.2 mmol repeat units, 91%). ¹H NMR (400 MHz, D₂O) δ: 8.02 (s, 1H), 6.19 – 5.81 (m, 1H), 4.56 (s, 2H), 4.41 (s, 1H), 4.26 (s, 1H), 3.91 (s, 2H), 3.61 (s, 2H), 3.53 (s, 2H), 2.36 (s, 2H), 2.16 – 1.80 (m, 2H).

Photochemical reaction

PPLQ-g-EO2 (35 mg), β-mercaptoethanol (0.28 μL), and LAP (0.44 mg) were added to 0.70 mL of deuterium oxide in a quartz cuvette. The solution was illuminated for 10 minutes by a 100 W long wave mercury UV lamp placed 7 cm above the sample.

Hydrolysis study

Hydrolysis of amide linked PPLQ and ester linked PPLG was determined by NMR. Polymer samples were rendered water soluble by grafting with an oligomeric ethylene glycol, dissolved in deuterium oxide (20 mg/mL), and the pH was adjusted by the addition of monosodium dihydrogen phosphate, sodium bicarbonate, or potassium carbonate to a final concentration of 10 mM. Sodium dutoxide was added to a final concentration of 150 mM. These additives gave solutions of pH 4.5, 8.0, 12, and 14, respectively, as determined by pH paper with pH increments of 0.5. A 23:77 ratio of monosodium dihydrogen phosphate to disodium monohydrogen phosphate was added to a final concentration of 250 mM for a solution of pH 7.4 at 37 °C. For PPLG and PPLQ, the peak corresponding to the aromatic proton on the triazole ring was used to determine extent of hydrolysis. Prior to hydrolysis, the peak is located at δ8.08 ppm. After hydrolysis, the peak is located at δ8.04 ppm. For determining PPLQ hydrolysis at pH 14, the triazole peak diminished over time due to proton exchange with the deuterated solvent. Instead, the

peaks at δ 3.90 ppm and δ 3.85 ppm corresponding to protons on the oligomeric ethylene glycol were used to determine hydrolysis (see Fig. S4).

Results/Discussion

A polypeptide with a functional group connected by an amide linkage on the side chain may be synthesized through either direct polymerization of a monomer containing an amide group or post polymerization modification of a polymer to install the functional group through amide bond coupling. A few examples of ring opening polymerization of NCA monomers containing an amide group on the side chain¹ encouraged attempts to synthesize the polymer through polymerization of a monomer bearing an amide linked alkyne group on the side chain. Propargyl groups were selectively installed on the ϵ -amine of lysine and γ -carboxylate of L-glutamate by blocking the α -amine and α -carboxylic acid with protecting groups or chelation with copper.¹⁷ However, attempted synthesis of the NCA monomer by the Fuchs-Farthing method with triphosgene¹⁸ did not yield the expected product. Alternative methods of NCA synthesis by protection of the α -carboxylic acid with *tert*-butyldimethylsilyl chloride followed by oxalyl chloride and DMF were also unsuccessful (see Fig. S1).¹⁹

Ultimately, a post polymerization modification strategy succeeded in the synthesis of amide linked PPLQ. Synthesis of PPLQ begins with the synthesis and subsequent polymerization of γ -Benzyl-L-glutamate N-carboxyanhydride to form poly(γ -benzyl-L-glutamate). The ester is saponified under basic conditions to produce the free carboxylic acid which is then coupled with propargylamine (see Fig. 1).

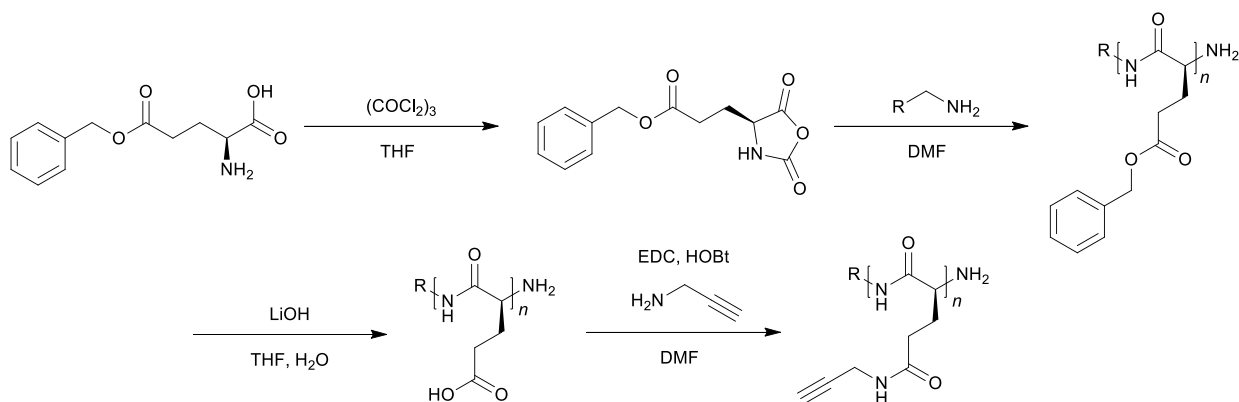


Figure 1. Synthesis of amide linked PPLQ by post polymerization modification

The end of the PPLQ polymer chain may also be tuned by choice of amine initiator to give greater control over the final structure. The synthesis of end-functional polymers is prerequisite to forming end linked networks or attachment of targeting ligands. A norbornene group on the end of the polymer enables end functionalization after grafting by reactions with either a thiol or tetrazine. The norbornene group was installed on the end of PPLQ by initiating the polymerization of PBLG with a norbornene amine, and the

subsequent post polymerization modifications are designed to preserve the norbornene group. Various conditions were screened for the deprotection of PBLG including hydrobromic acid in acetic acid, anisole in methanesulfonic acid²⁰, and trimethylsilyl iodide.^{21,22} Only basic deprotection in lithium hydroxide preserved the norbornene end group while maintaining the α -helical structure of the polypeptide (see Fig. S2). Likewise, a few conditions were screened for the coupling of propargylamine to poly(L-glutamate). Uronium/aminium type coupling reagents as well as EDC alone or EDC with NHS were not successful. However, EDC with HOBt yielded the desired polymer (see Fig. S3).

The deprotection of benzyl esters on a polypeptide under basic conditions is not standard and does result in some racemization of the polypeptide when compared to known acidic deprotection methods that do not induce racemization. However, poly(L-glutamate) obtained by this deprotection method is still predominantly α -helical. Furthermore, the effect of racemization on secondary structure is diminished in both PPLQ and PPLQ-g-EO2 as determined by circular dichroism and FTIR (see Fig. S5-S7). Because the secondary structure of PPLQ and PPLQ-EO2 is similar despite deprotection method, basic deprotection as described is suitable for when end group functionality is a priority. Otherwise, PPLQ may still be synthesized by acidic deprotection of PBLG with no subsequent changes to the procedure.

The synthesis of amide linked PPLQ has some notable advantages compared to the synthesis of ester linked PPLG. First, γ -benzyl glutamic acid is commercially available in both the L and D isomer. The convenient source of starting material, purification of the NCA by recrystallization, and quantitative post polymerization modification enables easy scale up of the synthesis to ten gram scale and larger. Moreover, the polymerization of the NCA monomer, γ -benzyl-L-glutamate NCA, is one of the most studied and characterized of all NCA polymerizations.²³ If desired, the NCA monomer as well as PBLG or poly(L-glutamate) are widely commercially available, enabling the synthesis of amide linked PPLQ in labs that may not be well equipped for organic chemistry.

Amide linked PPLQ retains all the positive characteristics of ester linked PPLG while bolstering the hydrolytic stability. NMR shows quantitative conversion of glutamic acid repeat units to γ -propargyl-L-glutamate (see Fig. 2) and GPC shows that the low dispersity characteristic of NCA polymerization is retained (see Fig. 3). In this regard, amide linked PPLQ synthesized by post polymerization modification is comparable to the hypothetical properties of amide linked PPLQ synthesized by polymerization of a NCA monomer bearing an alkyne. Furthermore all alkyne groups on the polymer are accessible to modification by CuAAC with various azides. This suggests that the polymer forms a stable α -helix in solution, effectively displaying the alkynes on the side chains to enhance their reactivity⁵. The α -helical structure is further confirmed by CD spectroscopy (see Fig. 4). However, upon grafting, the secondary structure shifts to a random coil with some elements of a PPII helix (see Fig. S6). This structure may be favored by the possibility of side chain to backbone hydrogen bonding enabled by the amide bonds on the side chain.²⁴

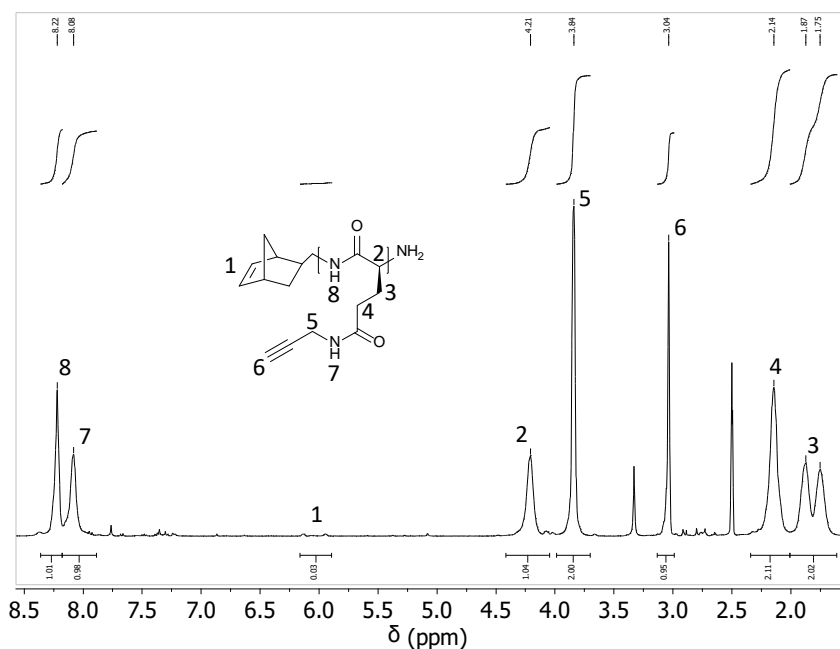


Figure 2. NMR of amide linked PPLG in DMSO-d₆. Additional peaks at δ 2.50 and 3.33 correspond to DMSO and water, respectively. Residual signals from HOBT, EDC, and DMF are from δ 7.0 – 8.0 and δ 2.5 – 3.0.

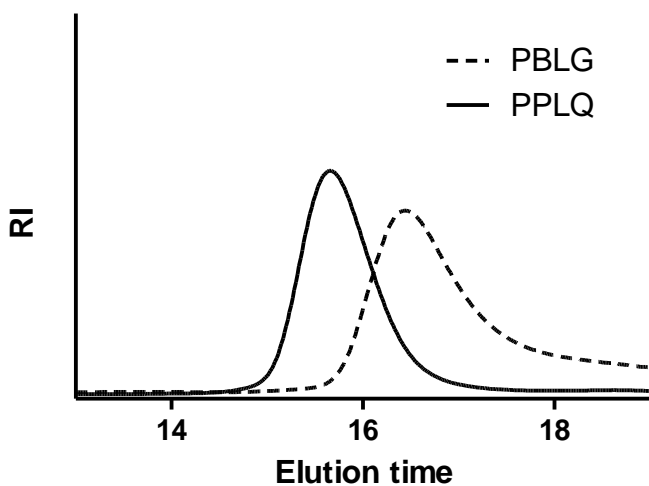


Figure 3. GPC trace of initial PBLG and derived PPLQ. For PPLQ, the calculated M_n is 11.6 kDa and PDI 1.11. For PBLG, the calculated M_n is 5.1 kDa and PDI 1.17. M_n and PDI were calculated based off PMMA standards.

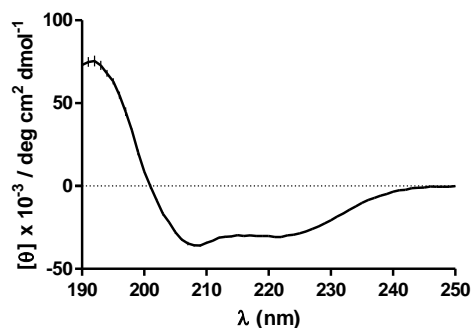


Figure 4. Circular dichroism spectrum of PPLQ in hexafluoroisopropanol (0.17 mg/mL).

To test the accessibility of the norbornene end group, PPLQ was first grafted with an azide modified diethylene glycol to render the polymer water soluble. The grafted product, PPLQ-g-EO2, was reacted with β -mercaptoethanol as a model thiol using LAP as a photoinitiator and illumination by a UV lamp. NMR confirmed the consumption of norbornene groups (see Fig. 5).

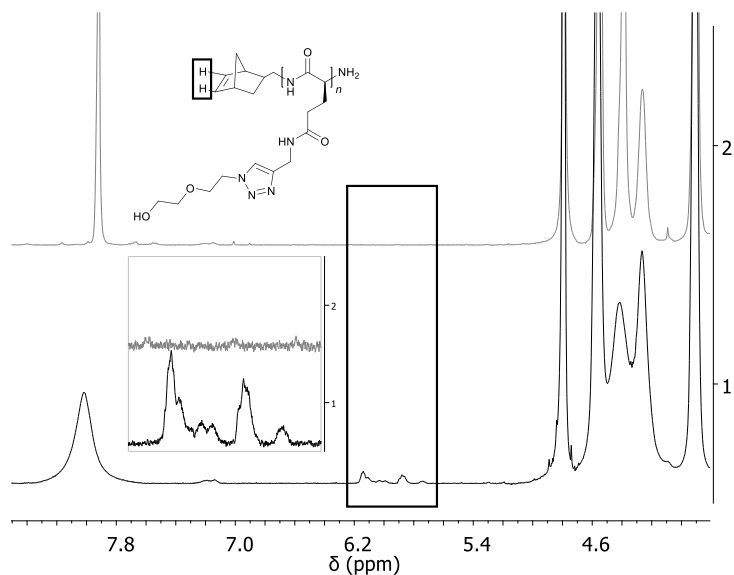


Figure 5. NMR analysis in D_2O detailing consumption of the norbornene end group of PPLQ in a model reaction with β -mercaptoethanol. The bottom spectra is before illumination; the top spectra is following illumination. The insert depicts the highlighted vinyl protons of norbornene on the end of PPLQ-g-EO2 prior to and after the photoreaction.

The hydrolytic stability of amide PPLQ was evaluated over time by NMR spectroscopy. Various aqueous environments of pH 4.5, 8, 12, and 14 at room temperature were tested as well as a physiologically relevant environment of pH 7.4 at 37°C. Ester PPLG was quickly hydrolyzed in pH 12 and nearly instantly hydrolyzed at pH 14. The hydrolysis of ester PPLG at either slightly basic pH and physiological conditions was slower, but

apparent. Amide PPLQ showed no detectable hydrolysis other than at pH 14. Even at extremely basic pH, the hydrolysis of amide PPLQ is slow. As hypothesized, converting the side chain linkage from ester to amide greatly improved hydrolytic stability (see Fig. 6).

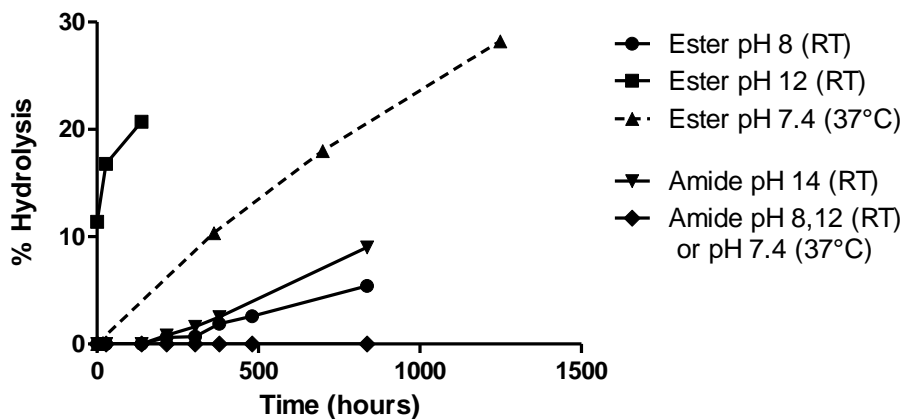


Figure 6. Hydrolysis over time for various functional groups of both ester and amide PPLG at different pH. Hydrolysis was undetectable for amide PPLQ at pH < 14 and both ester PPLG and amide PPLQ at acidic pH.

Conclusion

By converting the side chain linkage from an ester to an amide, the stability of PPLG in neutral and basic aqueous environments, especially at elevated temperatures, has been greatly improved. The improved stability makes amide PPLQ suitable for use in long term cell culture or other biological settings in addition to harsher pH environments. Although the propargyl group is installed after polymerization, the polymer shows complete modification comparable to polymerization of a propargyl containing monomer. In addition, other unique characteristics of ester PPLG such as the potential for quantitative grafting by CuAAC are retained. Amide PPLQ exhibits many desirable properties for biomaterial applications such as facile functionalization and controlled synthesis. There is potential for modification to endow a diverse set of properties and mimic the structure and function of natural polymers. This new polymer, PPLQ, expands the already versatile nature of PPLG.

Conflicts of Interest

There are no conflicts to declare.

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