Polymer Brushes

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Highly Efficient "Grafting onto" a Polypeptide Backbone Using Click Chemistry**

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A cell's extracellular matrix consists of macromolecules, such as glycoproteins, proteoglycans, and collagen, which control both the cell's mechanical structure and their microenvironment.[1] These features provide physical cues that are necessary to induce various cell functions and morphologies. An important goal of tissue engineering is to mimic the environment of the extracellular matrix on several levels: mechanically, chemically, and architecturally.[2] To accomplish this goal, new synthetic methods are necessary to mimic the structure of these complex macromolecules. We have developed a synthetic method to form highly-functionalized, grafted polypeptides that can be made to mimic complex biomacromolecules, such as glycoproteins and proteoglycans. Although these new synthetic polypeptides are much simpler than natural peptides, they still adopt the α-helical conformation of natural polypeptides; various chemical moieties can be attached to mimic the microenvironment of the extracellular matrix. These polymers have several features that make them very attractive for biological applications, including low toxicity, biodegradability, tunable structures, and well-controlled dimensions.

The synthesis of polypeptide homopolymers has been reported using a well-studied *N*-carboxyanhydride (NCA) ring-opening polymerization (ROP), which can accommodate a wide range of monomers containing various functional groups.[3–6] In particular, the carboxylic acid group (e.g. glutamate and aspartate) and the amine moiety (e.g. lysine) of amino acids have been used to add chemical complexity, such as pharmaceutical drugs, that dictate hydrophobicity or pH responsiveness.[5,7,8] However, functionalization of polypeptides synthesized by NCA ROP has several limitations. Because of the nature of the polymerization, the type of monomer that can be used is limited to NCAs with alkyl end-groups or NCAs where the functional group is protected. When creating polypeptides with functional carboxylic acid or amino groups, a three-step process is often required: 1) polymerization with the protected functional group; 2) deprotection of the functional group; and 3) functionalization. If a high degree of functionalization is required, the chemical moieties that can be added are limited to small molecules and low-molecular-weight oligomers. The addition of polymeric side chains at a high grafting density using a "grafting onto" method, has not yet been acheived. Li et al. reported a grafting efficiency of 36% for poly(γ-benzyl-L-glutamate)-g-poly(ethylene glycol) with a poly(ethylene glycol) (PEG) molecular weight $M_w = 350\ g\,mol^{-1}$ [9], and Feuz et al. reported a grafting efficiency of 48% for poly(L-lysine-g-PEG) with PEG MW = 1000, 2000, and 5000 $g\,mol^{-1}$.[10]

To overcome the limitations of NCA ROP, we have synthesized a new NCA monomer by incorporating a terminal alkyne group that is available for click chemistry. Click reactions, which were first described by Sharpless et al., refer to a series of highly efficient reactions, that include the 1,3-dipolar cycloaddition reaction between an alkyne and an azide to form a triazole.[11] These reactions have received a significant amount of attention because of their high reaction efficiency, mild reaction conditions, functional group tolerance, and few byproducts.[11] In recent years, click chemistry has been used in a wide variety of polymer applications, including functionalization of polymers with small molecules, formation of diblock polymers, formation of new dendrimers, formation of macromonomers, cross-linking of micelles, and the "grafting onto" method for the formation of molecular brushes.[12–20] Herein, we report the synthesis of poly(γ-propargyl-L-glutamate) (PPLG) and the attachment of different lengths of azide-terminated poly(ethylene glycol) (PEG-N_3). This model system demonstrates the high efficiency of "grafting onto" polymer side chains while maintaining the α-helical conformation of the polypeptide backbone.

The synthetic strategy employed in our study is shown in Scheme 1. The alkyne-containing monomer, γ-propargyl-L-glutamate *N*-carboxyanhydride (**2**), was synthesized by a two-step process. γ-Propargyl-L-glutamate hydrochloride (**1**) was prepared by the reaction of a propargyl alcohol with glutamic acid, mediated by trimethylsilyl chloride.[21] **1** was then reacted with triphosgene in ethyl acetate to form the NCA monomer (**2**).[22]

PPLG (**3**) was prepared by ROP of **2** initiated by heptylamine in *N*,*N*-dimethylformamide (DMF). The polymerization was monitored by observing the disappearance of

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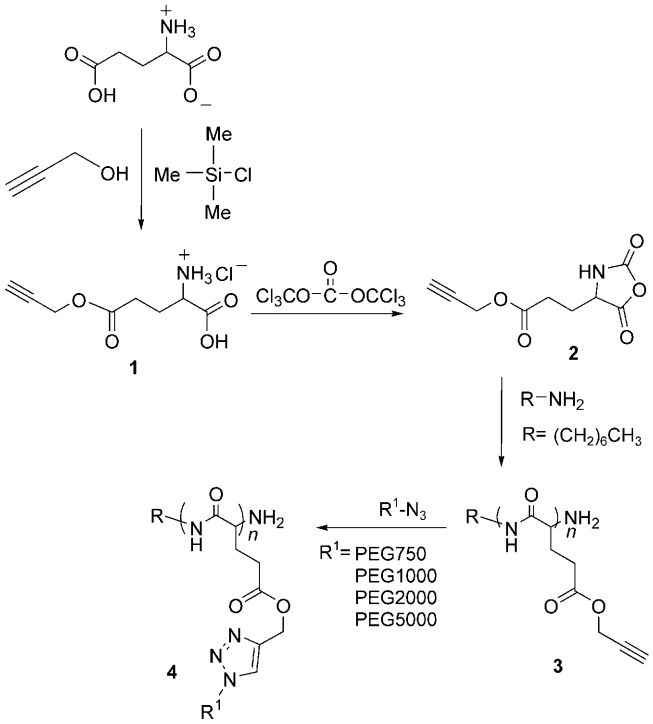

Scheme 1. Synthesis of PPLG, and PEG side chain coupling using click chemistry.

characteristic peaks from NCA(1790 and 1850 cm^{-1}) using an FTIR spectrometer.[7] After 2–3 days, the peaks disappeared, and the polymer was purified by precipitation out of solution using diethyl ether. The resulting PPLG had a degree of polymerization of $n = 40$ (by DMF gel permeation chromatography (GPC), $M_n = 8513$, polydispersity index PDI = 1.449; Figure 3A). The relatively broad molecular weight distribution is typical of a primary amine initiated NCA ROP. There are several strategies presented in the literature to minimize the side reactions associated with this type of polymerization;[3,23–28] a brief discussion can be found in the Supporting Information.

To synthesize the PPLG-g-PEG polymer, PPLG was coupled with PEG-N_3 using a CuBr/*N*,*N*,*N*,*N*′,*N*′-Pentamethyldiethylenetriamine (PMDETA) catalyst in DMF, with a molar ratio of alkyne/azide/CuBr/PMDETA of 1:2:0.33:0.33, for all molecular weights of PEG-N_3 used, and at various ratios for PEG1000-N_3 to further characterize the side chain grafting. After the reaction was complete, the reaction solution was passed through a short aluminum oxide column to remove the catalyst. The functionalized polymers were purified by dialysis and characterized by ^{1}H NMR spectroscopy, FTIR spectrometry, GPC, and circular dichroism (CD).

The kinetics of the PEG-N_3 coupling reaction were determined using a PEG1000-N_3 side chain, and a reaction molar ratio of alkyne/azide/CuBr/PMDETA equal to 1:1:0.1:0.1, using GPC. The molar ratios were lowered to slow down the kinetics such that they could be observed by GPC. Samples were taken from the reaction mixture (40 μL) at various time points; GPC samples were prepared from these by dilution with 750 μL DMF followed by addition of 5 μL of a toluene standard. Consumption of PEG-N_3 was determined by comparing the peak area of the PEG-N_3 curve to the toluene reference peak. Figure 1 shows the GPC traces

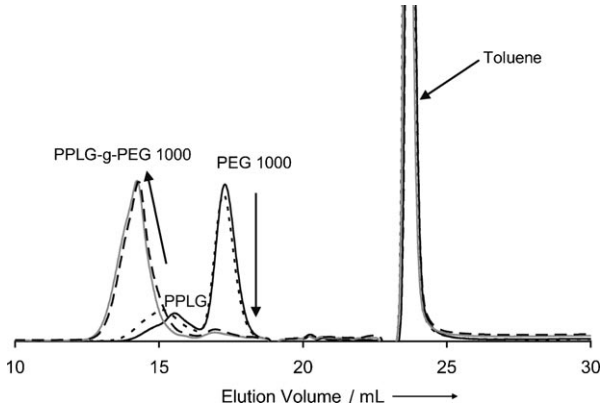

Figure 1. Evolution of DMF GPC traces as a function of reaction time. —— 0 min, ----- 5 min (8.9% conversion), —— 35 min (95.8%), – – – 125 min (95.8%).

(DMF) and conversion as a function of reaction time. As indicated by the overlap between the 125 min trace and the 35 min trace, the reaction had proceeded to completion after 35 min. The conversion of PEG-N_3 was determined by GPC at 35 min to be 95.8%.

We also used ^{1}H NMR spectroscopy to monitor side chain grafting. Figure 2 shows the ^{1}H NMR spectrum of pure PPLG, PPLG-g-PEG1000 at 50% functionalization, and PPLG-g-PEG at near-complete functionalization. It can be seen from a comparison of Figure 2A and 2B that the ester peak b has decreased, and a new ester peak k has appeared; furthermore, the peak m representing the methyl group next to the

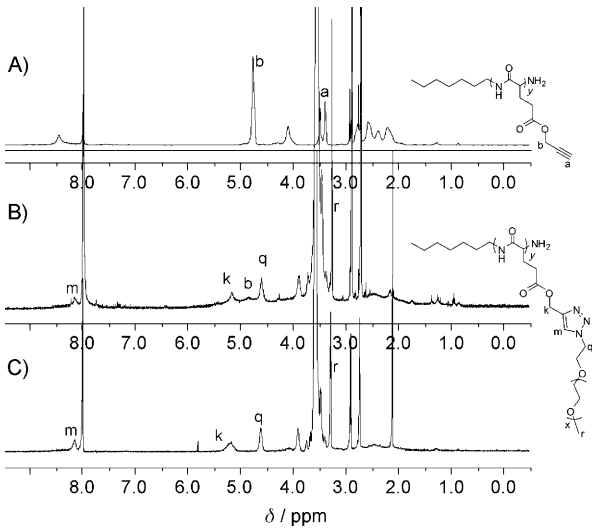

Figure 2. ^{1}H NMR spectrum of (A) PPLG in [D_7]DMF. (B) PPLG-g-PEG (MW = 1000 g mol^{-1}) with a feed ratio PPLG/PEG-N_3 of 1:0.5 in [D_7]DMF. (C) PPLG-g-PEG (MW = 1000 g mol^{-1}) with a feed ratio PPLG/PEG-N_3 of 1:2 in [D_7]DMF.

nitrogen atom of the triazole group appears. The alkyne peak a is not observed because it overlaps with the PEG-N_3 peaks. On comparing Figure 2B and 2C, ester peak b has completely disappeared, with a corresponding further increase in the intensity of ester peak k. In Figure 2B and 2C, no peaks from the original backbone are present that can be used to determine the grafting efficiency. Therefore, to determine the grafting efficiency, a small sample of the crude reaction solution was concentrated down to a solid, dissolved in [D_7]DMF, and a ^{1}H NMR spectrum was acquired. The conversion of the PEG-N_3 into the triazole was calculated by comparing the area under peaks m and f.[29] Based on this conversion (49.6% for PPLG-g-PEG1000), and the initial feed ratio of PEG-N_3 to PPLG (1:2.01), the grafting efficiency could then be determined (99.6%). These results are consistent with those observed by GPC (DMF) for the PPLG-g-PEG1000 system. Similar ^{1}H NMR spectra were observed for PPLG-g-PEG with PEG polymer chains of MW 1000, 2000, and 5000 g mol^{-1}. As shown in Table 1, the grafting efficiency (for a feed ratio of PPLG/PEG-N_3 of 1:2) was close to 100% in each case.

Table 1: Summary of DMF GPC results, and grafting efficiency determined by NMR.

	M_n [g mol^{-1}]	M_p [g mol^{-1}]	PDI[a]	Y_{graft}
PPLG	7043	6870	1.38	–
PPLG-g-PEG 750	14134	18080	1.42	98.9 ± 1.3%
PPLG-g-PEG 1000	14999	22223	1.40	96.3 ± 2.2%
PPLG-g-PEG 2000	34443	41884	1.22	–[b]
PPLG-g-PEG 5000	97082	99058	1.19	97.4 ± 2.8%

[a] Polydispersity index. [b] Not tested.

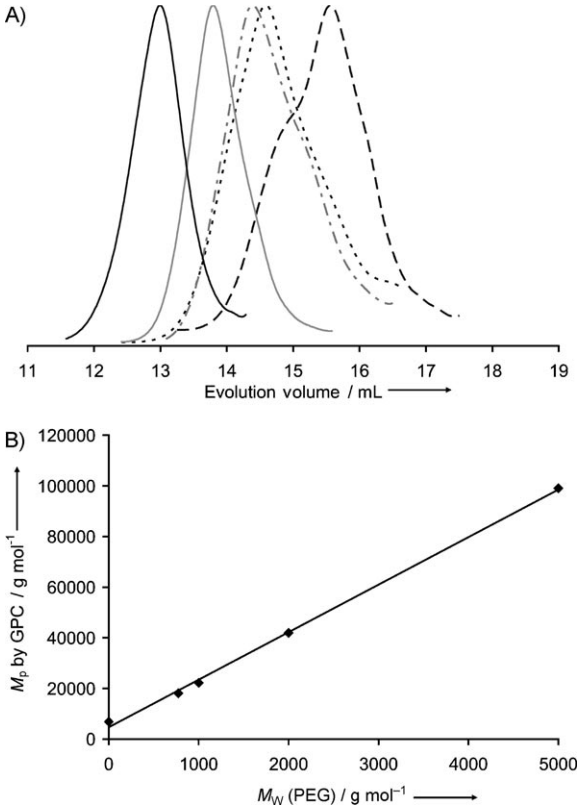

Figure 3. A) DMF GPC traces for PPLG-g-PEG. ––– PPLG, ----- PPLG-g-PEG 750, – – – PPLG-g-PEG 1000, ——PPLG-g-PEG 2000, —— PPLG-g-PEG 5000. B) PPLG-g-PEG molecular weight as a function of grafted PEG-N_3 molecular weight.

Figure 3A shows the GPC traces (DMF) of different molecular weight PPLG-g-PEG polymers prepared with a PPLG/PEG ratio of 1:2. All of the grafted copolymers show an increase in molecular weight while maintaining a narrow molecular weight distribution. This molecular weight increase also indicates that the grafting method does not degrade the peptide backbone. In Figure 3B, the PPLG-g-PEG molecular weight increases linearly with increasing side chain length, which indicates that the grafting efficiency remains consistent for different molecular weights of side chain.

The observed grafting efficiencies are higher than those of similar systems that utilize grafting-onto approaches found in the literature, including those involving click chemistry. Gao and Matyjaszewski synthesized a similar system of PHEMA-g-PEG; the highest PEG-N_3 (MW = 750) grafting efficiency of 88.4% was obtained at an alkyne/azide ratio of 1:8.5.[29] They suggested that the grafting efficiency was lower than 100% owing to steric congestion. Parrish and Emrick reported PEG-grafted aliphatic polyester systems with PEG molecular weights of up to 1100 g mol^{-1}, and grafting efficiencies between 70–80%.[30] Parrish, Breitenkamp, and Emrick reported a poly(α-propargyl-δ-valerolactone)-g-PEG system and obtained a PEG-N_3 (MW = 1100) grafting efficiency of 43%.[31] We hypothesize that the high grafting efficiency achieved with PPLG (nearly 100%) is a result of the rigid α-helical structure of the polymer backbone. Synthetic peptides, and in particular substituted poly(L-glutamates), are known to form stable α-helices when in various organic solvents, or when solvent cast from volatile organic solvents.[32,33] This stable α-helical structure causes the alkyne-terminated side chains to protrude outward from each repeat unit, thereby increasing the availability of the side chains for coupling. The α-helical structure is present throughout the reaction, initially from the PPLG backbone. Once the reaction reaches a high grafting density, the steric repulsion between the grafted PEG chains causes the graft polymer to maintain the shape of a symmetrical brush polymer, with the most favorable backbone conformation being an α-helix.[10]

To confirm the hypothesis that PPLG adopts an α-helical structure, liquid phase FTIR spectrometry was performed on a sample of PPLG dissolved in DMF. The α-helical conformation was identified by the strong C=O amide I absorption at 1658 cm^{-1} and the N–H amide II absorption at 1549 cm^{-1} (see Supporting Information, Figure S1).[34,35] Furthermore, CD spectroscopy was performed in water (DMF is not a suitable solvent for CD) to confirm the presence of an α-helical structure in PPLG and PPLG-g-PEG at different grafting densities and with different molecular weight side chains. To obtain a CD spectra of PPLG, a block copolymer was synthesized (PEG_{114}-b-$PPLG_{26.6}$; for synthesis and characterization, see Supporting Information). In all cases, the

characteristic negative ellipticity of an α-helix was observed at 208 nm and 222 nm.[36,37] At 50% substitution, and at near 100% substitution, the backbone adopts an α-helical conformation (Figure 4A). The less pronounced minima at 209 nm and 222 nm are a result of an increased presence of

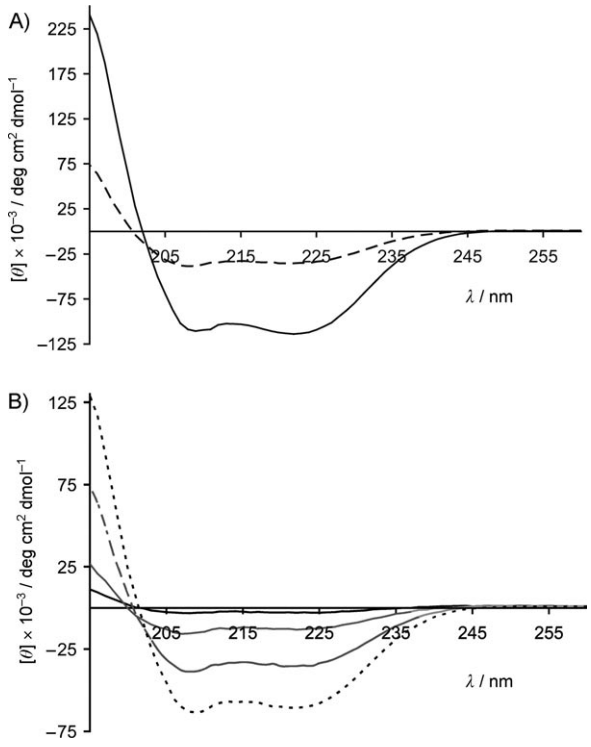

Figure 4. A) CD in water of PPLG-g-PEG 1000 (2.5 mg mL^{-1}) at ca. 50% and ca. 100% grafting. – – – PEG 1000 Y_{graft} 100%, —— PEG 1000 Y_{graft} 50%. B) CD of PPLG-g-PEG (2.5 mg mL^{-1}) at different molecular weights, all with close to 100% grafting. —— PEG 5000, —— PEG 2000, – – –1000, ----- PEG 775.

PEG side chains, which decrease the concentration of the α-helix backbone. In Figure 4B, the characteristic α-helix minimum were observed for all molecular weights of the PEG side chain. Thus, the characteristic α-helix peaks observed by FTIR spectrometry and CD spectroscopy indicate that the polymer backbone does have an α-helix structure; the rotating helical arrangement of these groups increases their availability along the backbone for coupling with the PEG-N_3 side chains.

In summary, we have described a new synthetic method to form highly functionalized grafted polypeptides. A new NCA monomer of γ-propargyl-L-glutamate and a new polymer, PPLG, have been synthesized. This new polymer provides a means of attaching a wide variety of molecules, which vary in both size and hydrophobicity, to a polypeptide using a single-step click reaction. The combination of NCA ROP methodology and click chemistry provides a versatile synthetic approach to develop molecules that mimic the complex architectures of natural peptides. We have shown that PEG chains with molecular weights that vary from 750 g mol^{-1} to 5000 g mol^{-1} can be attached to the PPLG backbone with nearly perfect grafting densities. A grafting density of 95.8% was obtained at an alkyne/azide reaction ratio of 1:1 and grafting densities of 96.3–98.9% were obtained at reaction ratios of alkyne/azide of 1:2. These grafting efficiencies are higher than similar PEG-grafting systems reported in the literature.[29–31] The extremely high efficiency achieved with PPLG is a result of the rigid α-helical structure of the polymer backbone, which causes the alkyne-terminated side chains to protrude outward from each repeat unit, thus increasing their availability for coupling.

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