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Highly Efficient “Grafting onto” a Polypeptide Backbone by “Click Chemistry”

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A cell's extracellular matrix consists of macromolecules, such as glycoproteins, proteoglycans, and collagen, that control both the mechanical structure and the microenvironment.^[1] These properties 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 in order 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. While, these new synthetic polypeptides are much simpler than natural peptides, they still possess the α -helical conformation of natural polypeptides and 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.

Synthetic homopolymers of polypeptides have been synthesized using a well-studied N-carboxyanhydride (NCA) ring-opening polymerization (ROP) which utilizes a wide variety of monomers containing various functional groups.^[3-6] In particular, carboxylic acid (e.g. glutamate and aspartate) and amino (e.g. lysine) groups of the amino acids have been used to add chemical moieties such as pharmaceutical drugs and molecules 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 alkane 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) the deprotection of the functional group, and (3) the functionalization. If a high degree of functionalization is required, the added chemical moieties are limited to small molecules and low molecular weight oligomers. The addition of polymeric side chains using a “grafting onto” method at a high grafting density has not been attainable. Li et al. reported a grafting efficiency of 36% for poly(γ -benzyl-L-glutamate)-g-poly(ethylene glycol) (PEG) with a PEG MW=350Da^[9] and

Feuz et al. reported a grafting efficiency of 48% for poly(L-lysine-g-PEG) with PEG MW=1, 2, and 5kDa.^[10]

To overcome the limitations of NCA ROP, we have developed a new NCA monomer that contains an alkyne group that is available for “click chemistry.” “Click reactions,” described by Sharpless et al., refer to a series of highly efficient reactions including, 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, crosslinking of micelles, and the “grafting onto” method of forming molecular brushes.^[12-20] Herein we report the synthesis of Poly(Propargyl-L-Glutamate) (PPLG) and the attachment of different lengths of azide-terminated polyethylene glycol (PEG). 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 illustrated 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 a propargyl alcohol with glutamic acid mediated by chlorotrimethylsilane.^[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 with heptylamine initiator in dimethylformamide (DMF). The polymerization was monitored by observing the disappearance of the NCA characteristic peaks (1790 and 1850 cm^{-1}) on an FTIR spectrum.^[7] The peaks disappeared after 2-3 days and the polymer was purified by precipitation into diethyl ether. The resulting PPLG had a degree of polymerization of $n = 40$ (by DMF GPC, $M_n = 8513$, $PDI = 1.449$, 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 PPLG-g-PEG, PPLG was coupled with PEG-N₃ using CuBr/PMDETA as catalyst in DMF with a molar ratio of alkyne/azide/CuBr/PMDETA equal to 1/2/0.33/0.33 for all molecular weight PEG-N₃ and at various ratios for PEG1000-N₃ to further characterize the side chain grafting. After the reaction was complete, the reaction solution was passed through a short alumina oxide column to remove the catalyst. The functionalized polymers were purified by dialysis and characterized by ¹H NMR, FTIR, GPC, and circular dichroism (CD).

The kinetics of the PEG-N₃ coupling reaction was determined using a PEG1000-N₃ 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 and GPC samples were prepared by dilution with 750 μL DMF and addition of 5 μL of a toluene standard. Conversion of the PEG-N₃ was determined by comparing the peak area of the PEG-N₃ curve to the toluene peak. Figure 1 shows the DMF GPC traces and conversion as a function of reaction time. As indicated by the overlap of the 125 min trace and the 35 minute trace, the reaction was complete after 35 minutes. The conversion of PEG-N₃ by GPC at 35 minutes was 95.8%.

We also used ^1H NMR to monitor side chain grafting. Figure 2 shows the ^1H NMR spectrum of PPLG, PPLG-g-PEG1000 at 50% functionalization, and PPLG-g-PEG at nearly complete functionalization. When comparing Figure 2A to Figure 2B, the ester peak, b, has decreased and a new ester peak, k, has appeared; furthermore, the m peak, representing the methyl group next to the nitrogen of the triazole group appears. The alkyne peak, a, appears to also decrease but is not observed because it overlaps with the PEG- N_3 peaks. When comparing Figure 2B to Figure 2C, the ester peak, b, completely disappears and the appearance new ester peak, k, has further increased. In Figure 2B and Figure 2C, there are no peaks from the original backbone present that can be used to determine a grafting efficiency. To determine the grafting efficiency, a small sample of the crude reaction solution was concentrated down to a solid, dissolved in DMF-d^{17} , and an ^1H NMR was taken. From this sample, the conversion of the PEG- N_3 was determined by comparing the area under peaks m and f.^[29] Based on the conversion of the azide to triazole (for PPLG-g-PEG1000, $y_{\text{conversion}} = 49.6\%$) and the initial feed ratio of PEG- N_3 to PPLG (1/2.01), the grafting efficiency was determined ($y_{\text{graft}} = 99.6\%$). These results are consistent with those observed by DMF GPC for the PPLG-g-PEG1000 system. Similar NMR spectrum were observed for PPLG-g-PEG with PEG MW 1000, 2000, and 5000 g/mol. As shown in Table 1, the grafting efficiency of each molecular weight sample reacted with a feed ratio of PPLG/PEG- N_3 of 1/2 is close to 100%.

Figure 3A shows the DMF GPC traces 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 scales linearly with increasing side chain length indicating that the grafting efficiency is consistent for each side chain molecular weight.

The observed grafting efficiencies are higher than those of similar systems utilizing graft-onto approaches found in the literature, including those involving click chemistry. Gao and Matyjaszewski synthesized a similar system of PHEMA-g-PEG and the highest PEG- N_3 (MW=750) grafting efficiency obtained was 88.4% at an alkyne/azide ratio of 1/8.5.^[29] They suggest that the grafting efficiency is lower than 100% as a result of steric congestion. Parrish and Emrick reported PEG-grafted aliphatic polyester systems with PEG- N_3 up to 1100 molecular weight and grafting efficiencies between 70-80%.^[30] Parrish, Breitenkamp, and Emrick reported a Poly(α -Propargyl- δ -valerolactone)-g-PEG system where the PEG- N_3 1100 grafting efficiency obtained was 43%.^[31] We hypothesize that the high grafting efficiency of nearly 100%, achieved with PPLG, is a result of the rigid α -helical structure of the polymer backbone. Synthetic peptides, in particular substituted poly(L-glutamates) form stable α -helix when in various organic solvents and 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 increasing their availability for coupling. This α -helical structure is present throughout the reaction. Initially, the α -helical structure is 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 develop the shape of a symmetrical brush polymer with the most favorable backbone conformation of an α -helix.^[10]

To confirm the hypothesis that PPLG has an α -helical structure, liquid phase FTIR was performed on PPLG 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} (Supporting Information, Figure S-1).^[34, 35] In addition, circular CD 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, PEG₁₁₄-b-PPLG_{26.6} was synthesized (synthesis and characterization in the Supporting Information). For all samples, the characteristic negative ellipticity of an α -helix was observed at 208 nm and 222 nm.^[36, 37] As shown in Figure 4A, at 50% substitution and near 100% substitution, the backbone has an α -helical conformation. The increased minimums at 209 nm and 222 nm are a result of an increased presence of PEG side chains decreasing the concentration of α -helix backbone. In Figure 4B, the characteristic α -helix minimum were observed for all molecular weight of the PEG side chains. The characteristic α -helix peaks observed in FTIR and CD 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-N₃ side chains.

In summary, we have described a new synthetic method to form highly functionalized, grafted polypeptides. A new NCA monomer of propargyl-L-glutamate and a new polymer, PPLG has been synthesized. This new polymer provides a means of attaching a wide variety of molecules, varying in both size and hydrophobicity, to a polypeptide using a single step click reaction. The combination of NCA ROP and “click chemistry” provides a versatile synthetic approach to develop molecules which mimic the complex architecture of natural peptides. We have shown that PEG chains with varying molecular weight from 750Da to 5000Da can be attached to the PPLG backbone at nearly perfect grafting densities. A grafting density of 95.8% was obtained at an alkyne/azide reaction ratio of 1/1 and a grafting density of 96.3-98.9% was 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Evolution of DMF GPC traces as a function of reaction time

Figure 2. ^1H NMR spectrum of (A) PPLG in DMF-d_7 (B) PPLG-g-PEG 1000 with a feed ratio PPLG/PEG-N3 of 1/0.5 in DMF-d_7 (C) PPLG-g-PEG 1000 with a feed ratio PPLG/PEG-N3 of 1/2 in DMF-d_7

Figure 3. (A) DMF GPC traces for PPLG-g-PEG (B) PPLG-g-PEG molecular weight as a function of grafted PEG-N₃ molecular weight

Figure 4.

A) CD of PPLG-g-PEG 1000 (2.5 mg/mL) at ~50% and ~100% grafting in water B) CD of PPLG-g-PEG (2.5 mg/mL) at different molecular weights all with close to 100% grafting.

Scheme 1. Synthesis of PPLG and side chain coupling via click chemistry

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

	Mn (g/mol)	Mp (g/mol)	PDI	η_{graft}
PPLG	7043	6870	1.38	--
PPLG-g-PEG 750	14134	18080	1.42	98.9 $\pm$ 1.3%
PPLG-g-PEG 1000	14999	22223	1.40	96.3 $\pm$ 2.2%
PPLG-g-PEG 2000	34443	41884	1.22	Not Tested
PPLG-g-PEG 5000	97082	99058	1.19	97.4 $\pm$ 2.8%