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GRGDS-Functionalized Poly(lactide)-graft-poly(ethylene glycol) Copolymers: Combining Thiol-Ene Chemistry with Staudinger Ligation

Dorothee E. Borchmann, Niels ten Brummelhuis, and Marcus Weck*

Molecular Design Institute and Department of Chemistry, New York University, New York, NY 10003, United States

Abstract

A tri(ethylene glycol)-containing lactide analogue was synthesized via thiol-ene chemistry between a bi-functional triethylene glycol and allyl lactide. Subsequent tin-octoate-catalyzed ring-opening polymerization yielded well-defined poly(lactide)-*graft*-poly(ethylene glycol) copolymers with molecular weights of 6000 g/mol and polydispersity indices of 1.6. The tri(ethylene glycol) chains along the copolymers contain azide termini that are capable of 'click'-type postpolymerization functionalization. The utility of this strategy was demonstrated via successful Staudinger ligation to install the Gly-Arg-Gly-Asp-Ser (GRGDS) peptide.

Introduction

Biodegradable polyesters including poly(lactide) (PLA), poly(ϵ -caprolactone), and poly(glycolide) have become increasingly popular in biomedical applications, including for the creation of tissue engineering scaffolds.¹ The facile hydrolysis of these materials *in vivo* allows for renal clearance of small molecule fragments over time.² One major drawback of polyesters, however, is their hydrophobicity, which makes them prone to non-specific biomacromolecule adsorption.³ Consequently, copolymers of PLA with poly(ethylene glycol) (PEG), which is known to reduce non-specific protein adsorption,⁴ have been reported.^{5,6} The Baker group synthesized PEG-containing lactide monomers in several steps, the final being a self-condensation of two hydroxyl-acids containing PEG chains.⁷ Our group reported a convergent synthesis of PEG-containing lactides via 1,3-dipolar cycloaddition of PEG_n-azides ($n = 3, 7$, and 40) via a norbornenyl lactide analogue.⁸ Polymerization yielded poly(lactide)-*graft*-poly(ethylene glycol) (PLA-*g*-PEG) brush copolymers. Steric crowding at the monomer reactive site due to the bulky norbornene group, however, led to prolonged polymerization times and broad polydispersity indices (PDIs). In this contribution, we report a new tri(ethylene glycol) (TEG)-containing lactide monomer synthesized by thiol-ene radical chemistry between an allyl lactide⁹ and a bifunctional 2-(2-(2-azidoethoxy)ethoxy)ethanethiol (N₃-TEG-SH). We suggest that this approach can be used to create other functional lactide monomers in an efficient way. Our PEG-lactides contain terminal azide groups that allow for post-polymerization functionalization. Thus, our strategy combines two orthogonal high-yielding coupling reactions to give functional and biodegradable polymers.

*Corresponding Author: marcus.weck@nyu.edu.

Supporting Information. Mechanistic scheme of the Staudinger Ligation, hydrolysis data of PLA-*g*-PEG, procedure for tap-GRGDS synthesis, spectroscopic data, and detailed MALDI-ToF assignments. This material is available free of charge via the Internet at <http://pubs.acs.org>.

A plethora of conjugation reactions which use azides have been reported,¹⁰ the most popular being the copper(I)-catalyzed 1,3-dipolar cycloaddition of azides with alkynes, which can exhibit all the characteristics of a 'click'-reaction.¹¹ Studies of the Bertozzi group¹² and our group¹³ have shown that copper cannot be removed completely from systems containing PEG-chains. Since Cu(I) is toxic and should be avoided in biomaterials synthesis, we explored the Staudinger ligation^{14,15} that links an azide with a triarylphosphine (tap) containing an electrophilic trap as an alternative (a mechanistic scheme can be found in the Supporting Information). The Staudinger ligation has been used extensively in chemical biology for applications including cell labeling, protein immobilization, and glycan labeling.¹⁶ In addition, the Staudinger ligation has been used in polymer chemistry, with seminal work being accomplished by Wu¹⁷ and van Hest¹⁸ in 2009. Since then, the scope has expanded to include the synthesis of RAFT agents containing tap functionalities.¹⁹ Staudinger ligation has also been used for the synthesis of biomaterials combining proteins or peptides with PEG²⁰ and poly(amidoimine).²¹ The resultant frameworks, however, are only capable of attaching a single unit on the polymer end, and, more importantly, lack a biodegradable scaffold. In contrast, the PLA-*g*-PEG copolymers described in this contribution represent completely biodegradable architectures and allow for several copies of tap-modified molecules to be attached to a single backbone, owing to the azides being present on the PEG-chain ends of each repeating unit. As a proof of principle, we report the functionalization of the PLA-*g*-PEG copolymers with the Gly-Arg-Gly-Asp-Ser (GRGDS) pentapeptide. RGD-containing peptides are derived from the extracellular matrix protein fibronectin²² and are known to promote cell adhesion through integrin receptors.²³ The new material is designed to bind to cells containing integrin receptors while repelling other biomacromolecules through the PEG-chains of the copolymer and could ultimately be used as tissue engineering scaffold.

Experimental Section

Materials

All chemicals were purchased from Sigma-Aldrich and used as received unless otherwise noted. Toluene used for polymerizations was dried over sodium/benzophenone and distilled under reduced pressure. Tetrahydrofuran was refluxed with sodium/benzophenone and distilled to remove the radical inhibitor. Deuterated solvents for NMR spectroscopy were purchased from Cambridge Isotope Labs (Andover, MA, USA) and used as received. Spectra/Por® 6 membranes (Spectrum Labs; Rancho Dominguez, CA, USA) were used for dialysis. Ultrafiltration discs (Millipore; Billerica, MA, USA) were used as received.

Characterization

¹H NMR and ¹³C NMR spectra were recorded either on a Bruker AC 400MHz or Bruker AVIII 600 MHz spectrometers at room temperature unless stated otherwise. All chemical shifts are reported in ppm. Mass spectra were obtained on an Agilent 1100 Series Capillary LCMSD Trap XCT Spectrometer using methanol as solvent unless otherwise indicated. High resolution mass spectra were acquired on an Agilent 6224 Accurate-Mass TOFLC/MS with acetonitrile as solvent. IR spectra were obtained on a Nicolet Magna-IR 760 spectrometer. Size-exclusion chromatography (SEC) was carried out using a Shimadzu pump coupled to a Shimadzu RI detector. A 0.03 M LiCl solution in *N,N*-dimethylformamide was used as eluent at a flow rate of 1 mL/min with a temperature of 60°C. A set of Polymer Standards columns (AM GPC gel, 10 µm, pre-column, 500 Å and linear mixed bed) was used. M_w^{app} , M_n^{app} , and PDI represent the apparent weight-average molecular weight, apparent number-average molecular weight, and polydispersity index, respectively. MALDI-ToF spectra were acquired on a BrukerUltrafleXtreme MALDI tandem mass spectrometer in positive mode. α -Cyano-4-hydroxycinnamic acid in THF was

used as matrix. A saturated sodium acetate solution was spotted on top of the matrix layer as dopant salt and the compound of interest was applied in its respective NMR solvent.

Synthesis of 2-(2-(2-azidoethoxy)ethoxy)ethanethiol (2)—Azido-triethyleneglycol-tosylate²⁴ (517 mg; 1.57 mmol; 1 eq.) and potassium thioacetate (269 mg; 2.35 mmol; 1.5 eq.) were dissolved in 10 mL ethanol. The mixture was refluxed for one hour. Then the solids were filtered off and the solvent was evaporated under reduced pressure. The residue was dissolved in water and extracted three times with diethyl ether. The organic layer was dried over sodium sulfate and filtered. The solvent was removed *in vacuo* and the crude product was dissolved in ten times its weight of 1.25 N HCl in methanol. The mixture was degassed with a stream of Argon for ten minutes and then heated to reflux for four hours. After cooling, the mixture was diluted with water and methanol was removed under reduced pressure. The remainder was extracted four times with diethyl ether. The organic phase was dried over sodium sulfate, filtered, and the solvents were evaporated *in vacuo*. The crude residue was purified by column chromatography on silica (eluent: dichloromethane:methanol 98:2), yielding 234 mg of 2-(2-(2-azidoethoxy)ethoxy)ethanethiol as a clear oil (1.22 mmol; 78%). The product was stored under argon at -20°C to minimize disulfide formation. ¹H NMR (400 MHz, CDCl₃) δ = 3.70-3.61 (m, 8H, O-CH₂-CH₂-O); 3.40 (t, *J* = 5.19 Hz, 2H, N₃-CH₂); 2.71 (dt, *J* = 6.39 Hz, 8.11 Hz, 2H, HS-CH₂); 1.60 (t, *J* = 8.11 Hz, 1H, S-H). ¹³C NMR (100 MHz, CDCl₃) δ = 73.0, 70.7, 70.3, 70.1, 50.7, 24.3. IR (poly(ethylene) film cards) ν (cm⁻¹) = 2912, 2849, 2108, 1734, 1461, 1346, 1293, 1118, 818. MS-ESI (M+Na)⁺ *m/z* calcd for C₆H₁₃N₃O₂SNa 214.25, found 214.06.

Synthesis of N₃-TEG-Lactide (10) [3-(3-((2-(2-azidoethoxy)ethoxy)ethyl)thio)propyl]-6-methyl-1,4-dioxane-2,5-dione] (3)—Allyl lactide (500 mg; 2.94 mmol; 1 eq) and N₃-PEG₃-SH **2** (843 mg; 4.41 mmol; 1.5 eq) were dissolved in 1.5 mL distilled THF. DMPA (150 mg; 0.2 eq) was added and the mixture was degassed for 15 min. The mixture was irradiated with UV light (15W UVP Black Ray UV Bench Lamp XX-15L) while stirring for one hour at 4°C. The reaction mixture was concentrated under reduced pressure and purified by column chromatography on silica gel (eluent: hexanes:ethyl acetate 3:2). 720 mg of product (1.99 mmol; 68%) was obtained as a yellow oil. The product (as well as its precursor allyl lactide) is enriched in one diastereomer; for analysis, the major diastereomer is described. ¹H NMR (400 MHz, CDCl₃) δ = 5.01 (q, *J* = 6.74 Hz, 1H, quarternary ring-proton 1); 4.95 (q, *J* = 3.24 Hz, 1H, quaternary ring-proton 2); 3.68-3.61 (m, 8H, O-CH₂-CH₂-O); 3.38 (t, *J* = 5.38 Hz, 2H, N₃-CH₂); 2.71 (t, *J* = 6.69 Hz, 2H, CH₂-S); 2.64 (t, *J* = 7.07 Hz, 2H, CH₂-S); 2.26-2.19 (m, 1H, CH₂-CH₂-CH₂-S); 2.10-2.03 (m, 1H, CH₂-CH₂-CH₂-S); 1.91-1.76 (m, 2H, CH₂-CH₂-CH₂-S); 1.66 (d, *J* = 6.74 Hz, 3H, CH₃). ¹³C NMR (100 MHz, CDCl₃) δ = 167.4, 166.7, 75.5, 72.4, 71.2, 70.8, 70.5, 70.2, 50.8, 32.0, 31.5, 29.1, 24.3, 15.9. IR (poly(ethylene) film cards) ν (cm⁻¹) = 2913, 2850, 2105, 1767, 1461, 1348, 1244, 1121, 1048, 992, 773. MS-ESI (M+Na)⁺ *m/z* calcd for C₁₄H₂₃N₃O₆S 361.13, found 361.13.

Synthesis of PLA-g-PEG (4)—408 mg (1.13 mmol, 30 eq.) of N₃-TEG-lactide **3** were transferred to a Schlenk flask and dried under vacuum for one day. The Schlenk tube was transferred to a nitrogen-filled glove box. 900 μ L of dry toluene were added. A stock solution of 460 μ L tin(II) octoate (1.13 mmol) and 410 μ L benzyl alcohol (3.8 mmol) in 10 mL dry toluene was prepared and 100 μ L of this solution were added to the monomers in the Schlenk reactor (*i.e.* tin octoate 1.5%, benzyl alcohol 1 eq.). The closed Schlenk flask was taken out of the glove box and heated at 110°C for 24h. Then, the solvent was evaporated *in vacuo*. The polymer was dialyzed for three days against acetone with solvent changes after 12h, 24h and 48h. 306 mg of polymer were obtained (75% yield). ¹H NMR

(600 MHz, acetone- d_6) δ = 7.44-7.34 (m, 0.3H; benzyl alcohol initiator); 5.33-5.08 (m, 2H); 3.75-3.56 (m, 8H); 3.44-3.34 (m, 2H); 2.75-2.62 (m, 4H); 2.20-1.98 (m, 2H); 1.87-1.70 (m, 2H); 1.64-1.50 (m, 3H). ^{13}C NMR (600 MHz, DMSO- d_6) δ = 209.0; 195.0; 71.2; 70.8; 70.3; 70.1; 69.9; 50.6; 31.3; 31.1; 25.4; 20.8; 17.2. IR (poly(ethylene) film cards) $\nu(\text{cm}^{-1})$ = 2922, 2866, 2101, 1720, 1712, 1451, 1358, 1280, 1185, 1090, 647.

Synthesis of GRGDS-polymer conjugate (6)—55 mg of polymer **4** were dissolved in 1 mL of DMF. Phosphine-GRGDS **5** (50 mg; 0.06 mmol; 0.4 eq. with regard to the azides) was dissolved in 1 mL DMF and added to the polymer. After stirring for 30 minutes, 350 μL of water were added. The reaction mixture was then stirred for 36h. The crude product was concentrated under reduced pressure and re-dissolved in sodium acetate buffer (pH 5): acetonitrile 4:6. Ultrafiltration was performed by placing this solution in the ultrafiltration chamber, applying nitrogen pressure to the chamber to reduce the volume to 5 mL and adding new solvent. The chamber was flushed in this fashion three times. Subsequently, two wash runs with water:acetonitrile (1:1) were performed. Pure product **6** was obtained after freeze-drying and analyzed by ^1H NMR spectroscopy, ^{31}P NMR spectroscopy, and MALDI-TOF spectrometry (see Figures and Supporting Information).

Results and Discussion

Our monomer synthesis utilizes allyl lactide⁹ functionalized with tri(ethylene glycol) (N_3 -TEG-SH). We reasoned that an alkyl linker to the TEG-chain compared to the previously reported norbornenyl linker⁸ should alleviate steric crowding at the monomer reactive site and lead to improved molecular weights and polydispersity indices. Scheme 1 illustrates the synthesis employed to yield PLA-*g*-PEG **4**.

Thiol-ene chemistry based functionalization of allyl lactide **1**⁹ with bifunctional N_3 -TEG-SH **2**, synthesized by two consecutive substitution reactions from commercially available 2-(2-(2-chloroethoxy)ethoxy)ethanol, yielded the TEG-containing lactide monomer with an azide-functionalized TEG-chain-terminus. For the thiol-ene reaction, we explored both thermal (using azobisisobutyronitrile as the radical initiator) and UV (using 2,2-dimethoxy-2-phenylacetophenone (DMPA) as the radical initiator) reaction conditions. Only 26% product was isolated under thermal reaction conditions whereas the DMPA-catalyzed reaction afforded 68% isolated yield. This is in close analogy to the literature which has reported that photocouplings proceed with higher efficiency and functional group tolerance compared to their thermal analogs.²⁵ The highest yields were obtained when the reaction was performed at 4°C. The disappearance of the alkene resonances at 5.8 ppm and the appearance of new signals around 3.5 ppm and 2 ppm for the TEG chain in the ^1H NMR spectrum confirmed successful product formation of monomer **3** (Figure 1). The successful thiol-ene reaction is evidenced by the signals of the methylene groups next to the thioether functionality at 2.7 ppm.

Monomer **3** was readily polymerized using tin(II) octoate-catalyzed ring-opening polymerization (ROP) with benzyl alcohol as the initiator in dry toluene affording polymer **4** as highly viscous oil, which is soluble in dichloromethane, acetone, dimethyl sulfoxide, *N,N*-dimethylformamide, and acetonitrile:H₂O 1:1. The catalyst loading for the polymerization was 1.5 mol% with regard to the monomer and we aimed to obtain a 30mer. ^1H -NMR end-group analysis indicated that the polymer had a number average molecular weight (M_n) of $6.0 \cdot 10^3$ g/mol, which corresponds to a degree of polymerization (DP) of 17. Size-exclusion chromatography (SEC), gave an apparent M_n (M_n^{app}) of $11.0 \cdot 10^3$ g/mol with a polydispersity index (PDI) of 1.6 (Figure 2), which corresponds to a narrower molecular weight distribution than observed for the previously published system (PDI = 2.1).⁸ The discrepancy of molecular weights obtained by NMR spectroscopy versus

SEC is not surprising, since the SEC results are reported versus linear poly(styrene) standards. We also analyzed **4** by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-ToF MS). Molecular weight species ranging from 5 to 18 repeat units were observed with the most intense peaks around 7–9 repeat units. Most likely the molecular weight is underestimated due to the fact that lower molecular weight species are detected more easily, because they undergo desorption-ionization more easily.²⁶

In order to assess whether polymer **4** is degradable we exposed polymer-coated glass slides to 1N hydrochloric acid at elevated temperatures. By liquid chromatography mass spectrometry (LC-MS) the degradation products lactoyl lactate and sodium 2-hydroxy-5-mercaptopentanoate were observed (Supporting Information).

The functionalization of the azide-containing polymer **4** with a peptide was achieved through a Staudinger ligation reaction (Scheme 2). We chose to avoid the copper(I)-catalyzed 1,3-dipolar cycloaddition between azides and alkynes¹¹ due to the presence of the sulfur-containing TEG-chain in our system. It has been shown that copper(I) can be complexed by PEG-chains^{12,13} and sulfur is known to be a good ligand for copper(I). Reaction conditions for the peptide-polymer conjugation were screened with the small molecule analogue 1-methyl-2-diphenylphosphino-terephthalate,²⁷ which was synthesized according to the literature. We found that the reaction proceeded readily at room temperature in DMF. One equivalent of water was added as a proton source after 30 minutes. Two equivalents of phosphines per azide were necessary to obtain complete functionalization. We used the ¹H NMR resonances at δ = 8.20 ppm and 8.40 ppm to verify complete functionalization (Supporting Information).

After determining the optimized Staudinger ligation conditions using the model reaction, triarylphosphine (tap) modified GRGDS peptide **5** was synthesized by solid phase peptide synthesis (SPPS) using the Fmoc/^tBu protecting group methodology. Before cleavage from the resin and deprotection of the side-chain functional groups, 1-methyl-2-diphenylphosphinoterephthalate was attached to the peptide employing an *O*-(7-azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU)-catalyzed coupling reaction. In a previously reported procedure to synthesize tap-RGD derivatives, resin-bound carbodiimide has been used to create phosphine-modified peptides, however, racemization of the C-terminal amino acid was observed.²¹ We used a modified literature procedure by Kiick *et al.*²⁷ and employed HATU as coupling agent with the peptide still on the resin. After simultaneous cleavage and deprotection followed by purification (three-fold precipitation into cold diethyl ether) peptide tap-GRGDS **5** was obtained.

We aimed for 20% reaction of the azide groups with peptide. A previous report by Anderson and Hawker²⁸ demonstrated that nanoparticle uptake and RGD-loading of the latter are proportional up to a functionalization of 20% with integrin-binding peptides. A higher RGD-loading did not lead to increased cell uptake, presumably due to receptor saturation. Thus, only 0.4 equivalents of **5** were used based on the previously performed model reaction with 1-methyl-2-diphenylphosphino-terephthalate. After coupling, the reaction mixture could not be purified solely by dialysis against aqueous solutions of acetonitrile. Unbound peptide was observed in the ¹H NMR spectrum. We hypothesize that bound and unbound peptides form salt bridges with each other and thus did not separate simply by diffusion. After ultrafiltration and freeze-drying, however, the GRGDS-peptide-polymer conjugate **6** was obtained in high purity. The material was analyzed by ¹H NMR and ³¹P NMR spectroscopies and MALDI-ToF mass spectrometry. ¹H NMR spectroscopy was used to determine the degree of functionalization. The integration of the PLA-backbone protons at δ = 5.16 ppm was compared to the ring protons of the substituted phenyl ring of the triaryl

phosphine oxide moiety at $\delta = 8.19$ ppm and 8.41 ppm (Figure 3). For the tap-GRGDS-PLA-*g*-PEG **6**, a functionalization of 14 % was obtained (average of around two peptides per polymer chain). The functionalization is slightly lower than the targeted 20%, which might be due to the steric crowding of the tap-peptides compared to the small molecule analogue. ^{31}P NMR spectroscopy showed a clear shift from -5 ppm for the phosphine in **5** to 29 ppm for the phosphine oxide of **6**, indicating successful oxidation of the phosphine group and thus conjugation.

MALDI-ToF MS disclosed a significant shift in molecular weight from **4** to the tap-peptide-functionalized conjugate **6** (Figure 4 and Supporting Information). The molecular weight of **4** has its highest molecular weight peaks at $5.5 \cdot 10^3$ g/mol, while the peptide-polymer conjugate **6** shows maximum molecular weights around $7.5 \cdot 10^3$ g/mol. With a molecular weight gain of 792 g/mol per peptide after successful Staudinger ligation, this corresponds to two or three peptides per polymer chain, and, with a degree of polymerization of 17 for the mother polymer **4**, a functionalization of 11–17 %, which corroborates the ^1H NMR integration result.

Conclusion

In this contribution we present a convergent synthesis to a tri(ethylene glycol)-containing lactide derivative with an azide moiety on the TEG-chain terminus using thiol-ene chemistry. Subsequent ROP yielded well-defined PLA-*g*-PEG copolymers with azides available for post-polymerization modifications. As a proof of principle, we explored the Staudinger ligation to synthesize materials based on PLA-*g*-PEG and the GRGDS peptide sequence. This is the first report of a biodegradable, comb-shaped peptide-polymer conjugate of this type synthesized by Staudinger ligation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

^1H -NMR spectra of allyl lactide **1** and N_3 -TEG-lactide **3** in comparison. Disappearance of the allylic protons shows the successful conversion to N_3 -TEG-lactide **3**.

Figure 2.
SEC-trace of polymer **4** (poly(styrene) standards, refractive index detection, 0.3 M LiCl in *N,N*-dimethylformamide as eluent)

Figure 3. Characterization of tap-GRGDS-conjugate **6** by ^1H -NMR in DMSO-d_6 (400MHz).

Figure 4. MALDI-ToF of N₃-PEG-PLA **4** (A) in comparison to tap-GRGDS-PLA **6** (B) with an average of two peptides per polymer.

Scheme 1.

Formation of N₃-TEG-lactide **3** by thiol-ene reaction between allyl lactide **1** and bifunctional tri(ethylene glycol) **2** and subsequent polymerization of **3** to give polymer **4**.

Scheme 2.

Post-polymerization functionalization of PLA-*g*-PEG with tap-GRGDS **5** under Staudinger conditions in DMF/water at room temperature.