Published in final edited form as:

Macromol Chem Phys. 2009 May 22; 210(10): 823–. doi:10.1002/macp.200900025.

Maleimide Functionalized Poly(ϵ -caprolactone)-*b*-poly(ethylene glycol) (PCL-PEG-MAL): Synthesis, Nanoparticle Formation, and Thiol Conjugation

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Summary

Carboxylic acid terminated poly(ϵ -caprolactone)s (PCL-COOHs) with narrow polydispersity were synthesized and coupled with poly(ethylene glycol) (HO-PEG-OH) to afford PCL-PEG-OH copolymers. The hydroxyl groups in the PCL-PEG-OHs were then converted to maleimide groups to afford maleimide terminated PCL-PEG-MALs that contained 70–90% maleimide functionality. Nanoparticles with maleimide functionality on their surfaces were prepared by impingement mixing. Particle sizes and size distributions were determined by dynamic light scattering. Conjugation of reduced glutathione with model maleimides and two MAL-functional nanoparticles was also demonstrated. The amount of accessible maleimide on the particle surface was measured using Ellman's reagent to range between ~51–67%.

Keywords

block copolymer nanoparticles; biocompatible; glutathione conjugation; NMR spectroscopy

Introduction

Low bioavailability limits the effectiveness of hydrophobic drugs. One approach for addressing this problem has been to develop various drug delivery systems. For example, liposomes,[1] polymeric micelles,[2] hydrogels,[3] dendrimers,[4] and microparticles[5] have been developed with the additional aims of controlling the drug release process and targeting specific sites in the body. Among these systems, polymeric micelles formed by self-assembly of amphiphilic block copolymers in aqueous solution have been widely investigated as potential drug delivery carriers.[6] An attractive feature of using amphiphilic block copolymers is the versatility and ease with which one can control the size and properties of the resultant micelles by changing chemical composition, molecular weights, or block ratios.[7] The delivery vehicles can also be stabilized through core[8] or shell[9] crosslinking. Additionally, it is feasible to functionalize block copolymer micelles or nanoparticles with specific ligands or marker molecules to achieve targeted delivery of bioactive and/or imaging agents to specific sites.[10–12]

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Supporting information for this article is available at the bottom of the article's abstract page, which can be accessed from the journal's homepage at <http://www.mcp-journal.de>, or from the author.

Poly(ϵ -caprolactone)-*b*-poly(ethylene glycol) block copolymer (PCL-PEG) is of special interest in biomedical applications. Gao and coworkers[12a] reported the use of maleimide terminated poly(ϵ -caprolactone)-*b*-poly(ethylene glycol) (PCL-PEG-MAL) micelles to deliver the antitumor agent doxorubicin. A cyclic pentapeptide, cRGD, was attached to the micelle's surface through a maleimide-thiol conjugation reaction to enhance the uptake of micelles in tumor endothelial cells. In their work, PCL-PEG-MAL was synthesized by ring-opening polymerization of ϵ -caprolactone using maleimide terminated poly(ethylene glycol) (HO-PEG-MAL) as the initiator, a strategy that is dependent on the availability of different molecular weight, hetero-functional HO-PEG-MAL initiators. We have developed and describe here i) an alternate route for preparing PCL-PEG-MALs; ii) the use of these functionalized block copolymers to form maleimide-containing PCL-PEG nanoparticles; and iii) the facile conjugation of *L*-glutathione[13] with these maleimide functionalized nanoparticles. The use of a carboxylic acid to initiate the polymerization has advantages: 1) without the need of converting the end functional group; 2) combined with the fractionation technique described, this approach could provide several different molecular weight PCL-COOH in one synthesis.

Experimental

Carboxylic Acid Terminated Poly(ϵ -caprolactone) (3, PCL-COOH)

Octanoic acid (**2**, 0.91 g, 6.3 mmol), ϵ -caprolactone (**1**, 14.4 g, 126 mmol), and camphor sulfonic acid (CSA, 5.0 mg) were added to a 50 mL culture tube. The mixture was purged with nitrogen for 10–15 min. The tube was placed in a pre-heated sand bath at 230 °C for 24 h. (Caution: The sand bath and reaction vessel should be placed behind a safety shield.) The reaction mixture was quenched by cooling the system to ambient temperature. ^1H NMR spectroscopy of the crude mixture indicated that the conversion of ϵ -caprolactone was >95%. The crude product was dissolved in 80 mL of THF, and ~150 mL of methanol was slowly added to the magnetically stirred solution of PCL. The solution was allowed to stand overnight without stirring, and the precipitate was filtered to give the 1st fraction [M_n = 6900 g/mol (6.9K), 4.7 g, Table 1 & Figure 1]. The filtrate was processed by two additional sequential addition/filtration cycles, each using 150 mL of methanol. This provided 2nd and 3rd fractions [4600 g/mol (4.6K), 3.6 g; 3200 g/mol (3.2K), 1.8 g, respectively]. Evaporation of all solvent from the solution and addition of 50 mL of methanol to the solid afforded the 4th fraction [2600 g/mol (2.6K), 1.5 g]. ^1H NMR (500 MHz, CDCl_3): δ 4.06 (t, $-\text{COOCH}_2-$, J = 6.5 Hz), 2.37 (t, $-\text{CH}_2\text{COOH}$, J = 7.5 Hz), 2.31 (t, $-\text{CH}_2\text{COO}-$, J = 7.5 Hz), 1.65 (bm, $-\text{CH}_2-$), 1.38 (bm, $-\text{CH}_2-$), 1.29 (bm, $-\text{CH}_2-$), and 0.88 (t, $-\text{CH}_3$, J = 7.0 Hz). ^{13}C NMR (125 MHz, CDCl_3): δ 173.8, 64.4, 34.3, 28.6, 25.7, and 24.8.

Acid Chloride Terminated Poly(ϵ -caprolactone) (4, PCL-COCl)

PCL-COOH (**3**, M_n = 6.9K, 1.2 g, 0.17 mmol) was dissolved in 10 mL of dry CH_2Cl_2 in a 25 mL flask. About 10 μL of dimethylformamide (DMF) was added via a wiretrol to the flask. Oxalyl chloride (0.1 mL, 1.2 mmol) was added via a syringe. The reaction mixture was stirred at ambient temperature for 12 h. CH_2Cl_2 and excess oxalyl chloride were removed under vacuum to afford PCL-COCl (**4**). The disappearance of the ^1H NMR resonance at 2.4 ppm and appearance of a new resonance at 2.9 ppm indicated essentially complete conversion of $-\text{CH}_2\text{COOH}$ to $-\text{CH}_2\text{COCl}$.

Hydroxyl Terminated Poly(ϵ -caprolactone)-*b*-poly(ethylene glycol) (5, PCL-PEG-OH, M_n = 6.9K-4.6K)

Excess PEG-diol (M_n = 4.6 K, 10.0 g, 2.2 mmol) in 30 mL of CH_2Cl_2 was dried overnight over ~1 g of 3Å molecular sieves. This PEG solution, pyridine (0.1 mL), and DMAP (5 mg) were added to the flask containing the above PCL-COCl (**4**, M_n = 6.9K, ~1.2 g, 0.17 mmol).

The reaction mixture was stirred at ambient temperature for 12 h. The solvent was evaporated under vacuum. CH₃OH (50 mL) was added to the solid and the suspension was stirred for 1 h. The solid **5** was separated by centrifugation (5000 rpm, 15 min) and decantation of the methanol soluble fraction. This purification step was repeated 5 times to provide 70–80% of the theoretical mass of **5**, in which unreacted PEG-diol could not be detected (see Figure S2 in the Supporting Information).

3-Chloro-2,5-dioxo-1-pyrrolidinepropanoyl Chloride (**6**)

β-Alanine (8.9 g, 100 mmol) was first dissolved in 15 mL of water. Maleic anhydride (9.8 g, 100 mmol) was added to the solution in one portion, and the mixture was vigorously stirred for 3 h to produce a slurry. The solid was filtered and washed with 3×50 mL of water, 50 mL of ethanol, and 50 mL of ether to give the amic acid **7** (yield: 9.3 g, 50%).^[14] Dry amic acid **7** was refluxed in excess thionyl chloride for 30 min (or was treated with 3 equivalents of oxalyl chloride in the presence of a catalytic amount of DMF in methylene chloride at ambient temperature for 12 h). Excess thionyl chloride (or oxalyl chloride) was removed under vacuum to give a viscous yellow liquid **6** (yield > 95%). ¹H NMR (500 MHz, CDCl₃): δ 4.66 (dd, 1H, -CHCl-, *J* = 4.0, 9.0 Hz), 3.90 (t, 2H, NCH₂CH₂-, *J* = 7.0 Hz), 3.35 (dd, 1H, -CHClCH_aH_b-, *J* = 9.0, 19.0 Hz), 3.28 (t, 2H, NCH₂CH₂-, *J* = 7.0 Hz), and 2.94 (dd, 1H, -CHClCH_aH_b-, *J* = 4.0, 18.5 Hz). ¹³C NMR (125 MHz, CDCl₃): δ 172.6, 172.5, 171.5, 48.7, 43.8, 39.4, and 34.8.

Maleimide Terminated PCL-PEG (**7**, PCL-PEG-MAL)

PCL-PEG-OH (**5**, 6.9K–4.6K, 1.0 g, ~0.083 mmol) was dissolved in 10 mL of CH₂Cl₂. Pyridine (0.1 mL) and DMAP (5 mg) were added. Neat acid chloride **6** (132 mg, 0.6 mmol) was added at ambient temperature. The reaction mixture was stirred overnight and the CH₂Cl₂ was removed under vacuum. The solid was suspended in methanol and stirred for 1 h. This mixture was centrifuged, and the supernatant layer of solution was decanted. The residual solid was dissolved in CH₂Cl₂ and triethylamine (TEA, 2.0 mL) was added. The mixture was stirred for 4 h at ambient temperature and the CH₂Cl₂ was evaporated under vacuum. Dialysis in methanol (Supporting Information) afforded a slightly yellow solid **7** (yield: 880 mg, 87%). The ¹H NMR spectrum (Figure 2) indicates 70–90% maleimide functionality.

2-Methoxyethyl 3-(2,5-Dioxo-2,5-dihydro-1*H*-1-pyrrolepropanoate (**11a**)

Acid chloride **6** (3.56 g, 16.0 mmol) and 2-methoxyethanol (**10a**, 1.07 g, 14.0 mmol) were dissolved in 50 mL of CH₂Cl₂. Pyridine (2.62 mL, 32 mmol) and DMAP (10 mg) were added to the CH₂Cl₂ solution at 0 °C and it became dark red immediately. The solution was stirred at room temperature for 12 h and the CH₂Cl₂ was removed under vacuum. A co-solvent of ethyl acetate and hexanes (1:1 v/v) was added, and a black solid precipitated. The remaining brown solution was concentrated and purified by MPLC (1:1 hexanes: ethyl acetate) to give a colorless liquid in 80% yield. The ¹H NMR spectrum indicated that the sample contained ca. 10% of maleimide **11a** and 90% of a 3-chlorosuccinimide [2-methoxyethyl-3-(3-chloro-2,5-dioxopyrrolidin-1-yl)propanoate]: ¹H NMR (300 MHz, CDCl₃): δ 4.66 (dd, 1H, -CHCl-, *J* = 3.9, 8.7 Hz), 4.22 (t, 2H, -COOCH₂CH₂-, *J* = 4.5 Hz), 3.87 (t, 2H, -NCH₂CH₂-, *J* = 6.9 Hz), 3.58 (t, 2H, -COOCH₂CH₂-, *J* = 4.5 Hz), 3.33 (dd, 1H, -CHClCH_aH_b-, *J* = 8.7, 18.9 Hz), 2.92 (dd, 1H, -CHClCH_aH_b-, *J* = 3.9, 18.6 Hz), and 2.70 (t, 2H, -NCH₂CH₂-, *J* = 6.9 Hz). ¹³C NMR (75 MHz, CDCl₃): δ 172.9, 172.8, 170.6, 70.4, 64.1, 59.0, 48.9, 39.4, 35.3, and 31.8.

Triethylamine (TEA, 50 μL) was added to a solution of the above mixture (20.0 mg, 76 μmol) in 1 mL of CH₂Cl₂. The solution was stirred for 4 h and concentrated under vacuum, and the residue was purified by MPLC (1:1 hexanes: ethyl acetate) to give 14.3 mg of

maleimide **11a** (83.5%). ^1H NMR (500 MHz, CDCl_3): 6.71 (s, 2H, $-\text{CH}=\text{}$), 4.23 (t, 2H, $-\text{COOCH}_2\text{CH}_2-$, $J = 5.0$ Hz), 3.84 (t, 2H, $-\text{NCH}_2\text{CH}_2-$, $J = 7.0$ Hz), 3.58 (t, 2H, $-\text{CH}_2\text{OCH}_3$, $J = 5.0$ Hz), 3.38 (s, 3H, CH_3), and 2.68 (t, 2H, $-\text{NCH}_2\text{CH}_2-$, $J = 7.0$ Hz). ^1H NMR (500 MHz, D_2O): 6.89 (s, 2H, $-\text{CH}=\text{}$), 4.25 (t, 2H, $-\text{COOCH}_2\text{CH}_2-$, $J = 4.5$ Hz), 3.85 (t, 2H, $-\text{NCH}_2\text{CH}_2-$, $J = 6.5$ Hz), 3.69 (t, 2H, $-\text{CH}_2\text{OCH}_3$, $J = 4.5$ Hz), 3.39 (s, 3H, CH_3), and 2.75 (t, 2H, $-\text{NCH}_2\text{CH}_2-$, $J = 6.5$ Hz). ^{13}C NMR (125 MHz, CDCl_3): 170.9, 170.5, 134.4, 70.4, 64.0, 59.1, 33.8, and 33.0. HR-ESI-MS ($\text{M}+\text{H}^+$): found (calcd) 228.0903 (228.0872).

Maleimide Terminated Poly(ethylene glycol) Monomethyl Ether (**11b**, PEG-MAL)

Acid chloride **6** (0.44 g, 2.0 mmol) and MeO-PEG-OH (**10b**, $M_n = 5.0\text{K}$, 5.0 g, 1.0 mmol) were dissolved in 20 mL of CH_2Cl_2 . Pyridine (0.25 mL, 3 mmol) and DMAP (5 mg) were added to the CH_2Cl_2 solution at 0 °C. The mixture was stirred at room temperature for 12 h. TEA (0.2 mL) was added via syringe. After 4 h CH_2Cl_2 was removed under vacuum to give a brown solid. The solid was dissolved in THF and precipitated by addition of ether twice. Because of the difficulty in removing the TEA $\cdot$ HCl salt from the system, membrane dialysis in methanol was used to purify the maleimide terminated PEG. The crude mixture was dissolved in ~25 mL of methanol, and the solution was added to a dialysis membrane tube (see Supporting Information for additional details). The membrane was sealed and suspended in 2000 mL of methanol and the solution was stirred gently. After 2 h the methanol was replaced by a fresh batch of methanol and the process was repeated four times. The PEG solution was then concentrated under vacuum to afford maleimide terminated PEG **11b**.

L-Glutathione (**12**) Model Conjugation with Maleimide **11a** to Give **13a**

L-Glutathione (**12**, 23.8 mg, 78 μmol) and maleimide **11a** (17.7 mg, 78 μmol) were added to 2 mL of deionized water. Maleimide **11a** gradually dissolved during the course of the reaction. After 4 h water was evaporated under vacuum to give the maleimide-thiol conjugate **13a**. ^1H NMR spectroscopy in D_2O showed essentially full conversion of both reactants. ^1H NMR (500 MHz, D_2O): δ 4.72 (dd, 1/2H, $-\text{SCH}_2\text{CH}-$, $J = 5.5, 7.5$ Hz), 4.70 (dd, 1/2H, $-\text{SCH}_2\text{CH}-$, $J = 5.0, 8.5$ Hz), 4.25 (br t, 2H, $\text{CH}_3\text{OCH}_2\text{CH}_2-$, $J = 4.5$ Hz), 4.10 (dd, 1/2H, $-\text{SCH}-$, $J = 4.0, 9.0$ Hz), 4.07 (dd, 1/2H, $-\text{SCH}-$, $J = 4.0, 9.0$ Hz), 4.00 (s, 2H, $-\text{NHCH}_2\text{COOH}$), 3.85 (t, 1H, $-\text{CH}(\text{NH}_2)-$, $J = 6.5$ Hz), 3.84 (t, 2H, $-\text{NCH}_2-$, $J = 6.5$ Hz), 3.70 (br t, 2H, CH_3OCH_2- , $J = 4.5$ Hz), 3.41 (s, 3H, CH_3-), 3.38 (dd, 1/2H, $-\text{SCHH}-$, $J = 5.5, 14.5$ Hz), 3.34 (dd, 1H, $-\text{SCH}(\text{C}=\text{O})\text{CHH}-$, $J = 9.0, 9.0$ Hz), 3.31 (dd, 1H, $-\text{SCH}(\text{C}=\text{O})\text{CHH}-$, $J = 9.0, 9.0$ Hz), 3.26 (dd, 1/2H, $-\text{SCHH}-$, $J = 5.5, 14.0$ Hz), 3.20 (dd, 1/2H, $-\text{SCHH}-$, $J = 8.0, 14.0$ Hz), 3.04 (dd, 1/2H, $-\text{SCHH}-$, $J = 9.0, 14.0$ Hz), 2.74 (t, 2H, $-\text{CH}_2\text{COO}-$, $J = 6.5$ Hz), 2.73 (dd, 1/2H, $-\text{SCH}(\text{C}=\text{O})\text{CHH}-$, $J = 4.5, 14.5$ Hz), 2.69 (dd, 1/2H, $-\text{SCH}(\text{C}=\text{O})\text{CH}_2-$, $J = 4.0, 14.0$ Hz), 2.56 (m, 2H, $-\text{CH}_2\text{CONH}-$), and 2.20 (q, 2H, $-\text{CH}_2\text{CH}_2\text{CONH}-$, $J = 7.5$ Hz). HR-ESI-MS ($\text{M}+\text{H}^+$): found (calcd) 535.1746 (535.1710). HR-ESI-MS ($\text{M}+\text{Na}^+$): found (calcd) 557.1499 (557.1529).

L-Glutathione (**12**) Model Conjugation with PEG-MAL **11b** to Give **13b**

PEG-MAL **11b** ($M_n = 5.0\text{K}$, 50% maleimide functionality based on ^1H NMR analysis; 20 mg; ~2 μmol of MAL) and L-glutathione (**12**, 0.6 mg, 2 μmol) were dissolved in 2 mL of H_2O . The reaction mixture was stirred at ambient temperature overnight. Water was removed under vacuum. ^1H NMR analysis of the residue (D_2O) showed essentially complete conversion to **13b** for both glutathione and maleimide (Figure 3).

“Flash NanoPrecipitation” Experiment

In a typical run PCL-PEG-MAL (6.9K-4.6K, 30 mg, 70–90% MAL functionality) and PCL-PEG-OH (6.9K-4.6K, 330 mg) were dissolved in 10 mL of THF. The mixed polymer

solution was loaded into a 100-mL gas tight syringe, and deionized water (10 mL) was loaded in a separate 100-mL syringe. The syringes were inserted into the same syringe pump and the outlets connected via teflon tubing to the inlet ports of a two-jet CIJ mixer.[15] Mixing occurred upon impinging the two streams into the mixer head at a flow rate of 72 mL/min or a jet velocity of 6.1 m/s. The outlet stream was directed into a beaker containing 80 mL of deionized water. The total injection time was approximately 10 seconds. The final solution was used directly for glutathione conjugation and DLS measurement without filtration or dialysis.

Results and Discussion

Synthesis of PCL-PEG-MAL (7)

The synthesis of PCL-PEG-MAL **7** is summarized in Scheme 1. PCL-COOH (**3**), having one each of a non-functional and a carboxylic acid end group, was prepared, fractionated, and converted to its acid chloride derivative, PCL-COCl (**4**). This was coupled with (excess) PEG-diol to provide PCL-PEG-OH **5**. Esterification of the terminal, primary alcohol in **5** with the acid chloride **6**, which carries a masked maleimide subunit, followed by elimination of HCl gave the target **7** from **5** in a one-pot procedure. A major advantage of this strategy is that many combinations of different molecular weights of PCL-COOH and HO-PEG-OH are readily accessible, which allows synthesis of a series of different molecular weight PCL-PEG-MALs (**7**).

PCL-COOH (**3**) was prepared by ring-opening polymerization (ROP) of ϵ -caprolactone (**1**) at 230 °C using octanoic acid (**2**) as the initiator and camphor sulfonic acid (CSA) as an acid catalyst.[16] The choice of **2** as the initiator was guided by the need to have one non-functional end group as well as by its relatively low volatility. When $M_n = 2.2$ K was targeted, PCL-COOH (**3**) with M_n of 7.1 K and PDI = 1.75, as measured by GPC (PS standard), was obtained. This PCL-COOH was fractionated by dissolving PCL in THF followed by sequential addition of methanol to afford four fractions with relatively narrow molecular weight distributions (Figure 1, Table 1). Following fractionation, each of the four fractions has a relatively small PDI [1.14–1.24 from GPC (Figure 1, panel A) and 1.04–1.09 by MALDI-TOF MS (Figure 1, panel B)].

PCL-COOH (**3**) was treated with oxalyl chloride in the presence of a catalytic amount of DMF to afford the acid chloride terminated PCL **4**. No evidence for chain cleavage or other degradation chemistry during this reaction was detected by GPC or ^1H NMR analysis. The quality of PCL-COCl (**4**) is critical for the next coupling reaction; it could be evaluated either by direct ^1H NMR analysis or by coupling with PEG-NH₂ followed by GPC analysis of the product mixture (see Supporting information). The ^1H resonance at 2.4 ppm in PCL-COOH (**3**) corresponds to the methylene group alpha to the terminal carboxyl group ($-\text{CH}_2\text{COOH}$). This was replaced by a new resonance at 2.9 ppm ($-\text{CH}_2\text{COCl}$) in **4**, indicative of formation of the acid chloride; typical samples of PCL-COCl showed greater than 90% of acid chloride functionality by comparison of the intensity of the ^1H resonance of $-\text{CH}_2\text{COCl}$ with that of the PCL backbone protons. PCL-COCl (**4**) with $M_n = 2.6$ K and PEG-NH₂ with $M_n = 6.0$ K were chosen to study the coupling efficiency. This amide bond coupling was performed in the presence of triethylamine so that all of the primary amine in PEG-NH₂ was free to react. As expected, greater than 90% conversion was observed based on GPC analysis (see Supporting Information), which also indicated high quality of PCL-COCl (**4**).

In the coupling of PCL-COCl (**4**) with PEG-diol (HO-PEG-OH, 4.6K) a ten-fold excess of HO-PEG-OH was used to minimize formation of PCL-PEG-PCL triblock copolymer. PEG-diol was dried over 3 Å molecular sieves in CH_2Cl_2 solution or under high vacuum for 48 h at 40 °C prior to use. Moisture present during the coupling reaction can compete for

consumption of PCL-COCl. As a precaution, HO-PEG-OH was pretreated with ~1 equivalent (relative to PCL-COCl) of acid chloride **6** before the addition of PCL-COCl (**4**). In one instance where the efficiency of the coupling of HO-PEG-OH with **4** to give **5** was directly compared without vs. with pretreatment by **6**, the coupling yield improved from ~50% to ~90%. remove the unreacted, excess PEG-diol from the product **5**, the crude material was washed repeatedly with MeOH. The progressive increase in purity of **5** could be monitored by GPC analysis (see Supporting Information). After the fifth wash cycle, no remaining PEG-diol was detected. This purification protocol was efficient; 75~85% yield was achieved over three runs. Since the PCL-PEG-OH copolymer **5** coeluted with starting PCL-COOH (**3**) by GPC, **5** was further analyzed by ^1H NMR spectroscopy. Analysis of the PEG (3.32 ppm) vs. PCL (4.08 ppm) backbone ^1H NMR resonances indicated high conversion of **4** to **5**. The block copolymer **5** was further characterized by MALDI-TOF MS ($M_n \sim 11,000 \text{ g mol}^{-1}$), but the spectrum was of marginal signal-noise quality (see Supporting Information) because of the known difficulties[17] in ionization of block copolymers under MALDI analysis.

Coupling of two other molecular weight PCL-COCl (**4**, $M_n = 8.6\text{K}$, and 4.6K) with the same molecular weight HO-PEG-OH ($M_n = 4.6\text{K}$) was successful. The resulting copolymers were purified using the same purification procedure and the results are summarized in Table 2 (entry 1). However, when lower MW PCL-COCl (3.2K or 2.6K) was coupled with PEG-diol, the efficiency of mass recovery was low because of the higher solubility in methanol of these copolymers having smaller PCL blocks.

The last synthetic operation to produce a PCL-PEG-MAL (**7**) was conversion of the terminal hydroxyl group in **5** to a maleimide functionality. 2-Methoxyethanol and poly(ethylene glycol) (MeO-PEG-OH, 5.0 K) were chosen as a model compound upon which to develop this end group transformation. We first attempted a Mitsunobu reaction to convert MeO-PEG-OH to maleimide terminated poly(ethylene glycol) (PEG-MAL) by direct displacement of the (activated) primary alcohol with maleimide.[18] Although this reaction proceeded well (~70%) in our hands for the low molecular weight alcohol 2-methoxyethanol, when we attempted to apply it to the $5,000 \text{ g/mol}$ PEG-OH, the maximum maleimide functionality we achieved was only around 10% (^1H NMR analysis), so we devised a different strategy.

We considered use of the known acid chloride **9**. Acylation of **5** with **9** should give the PCL-PEG-MAL **7**. We attempted to prepare **9** (Scheme 2) by the reported procedure:[14] amic acid **8** (from the reaction of maleimide with β -alanine) was refluxed in thionyl chloride. However, we observed nearly complete (>95% by ^1H NMR analysis) formation of the succinimide derivative **6**, in which HCl had added in conjugate fashion to the alkene; **9** was only a trace component. Gentler treatment of **8** with oxalyl chloride at room temperature in the presence of catalytic amount of DMF also resulted in nearly quantitative formation of acid chloride **6**.

Assuming we could later reveal the maleimido group by mild base promoted elimination of HCl from a 3-chlorosuccinimide like **6**, we studied the acylation of 2-methoxyethanol (**10a**) and 5.0K MeO-PEG-OH (**10b**) with **6**. Pyridine was used as the stoichiometric base (in methylene chloride) to promote ester formation. In the case of **10a**, an aliquot of the reaction mixture was examined by ^1H NMR spectroscopy, which indicated the intermediacy of the expected 3-chlorosuccinimidoyl ester. Subsequent addition of a stronger amine base, triethylamine, promoted facile elimination to provide the maleimido-ester conjugates **11a** (65% isolated yield) and **11b** (~85% yield) from the corresponding model alcohols **10a** and **10b**.

This two-stage, one-flask operation was then used to convert the PCL-PEG-OH (**5**; 4.6K-4.6K, 6.9K-4.6K and 8.6K-4.6K) to PCL-PEG-MALs (**7**) (Scheme 1). An approximate ten-fold excess of acid chloride **6** was used. After treatment with excess triethylamine, CH₂Cl₂ was removed under vacuum. The contaminating low molecular weight species (e.g., TEA•HCl) were dialyzed away from **7** into methanol. The resulting PCL-PEG-MALs (**7**) were judged to have 70–90% maleimide functionality (¹H NMR analysis, Figure 2). The results are summarized in Table 2 (entry 2).

Model Maleimide-Thiol Conjugation using *L*-glutathione (**12**)

The reaction between maleimide and thiol groups in water (or buffer) is widely used for conjugation of, e.g., cysteine-containing peptides and proteins.[19] The low molecular weight maleimide **11a** and *L*-glutathione (**12**) were used as models for a maleimide-thiol conjugation reaction. Since both of these molecules are small, the reaction progress could be easily monitored directly by ¹H NMR spectroscopy (Figure 3). A 1:1 molar ratio of these two compounds was mixed in D₂O at ambient temperature. The solubility of the maleimide derivative **11a** in water is limited, but over the course of several hours it gradually dissolved as the reaction proceeded to completion. The resonances in the ¹H NMR spectrum of the conjugate **13a** were assigned based on analysis by homonuclear gradient correlation spectroscopy (GCOSY).

PEG-MAL **13b** was also conjugated with *L*-glutathione (**12**) in D₂O using the same protocol. After 4 h resonances at δ 4.59 and 2.98 ppm in the starting *L*-glutathione and at 6.88 ppm in the PEG-MAL **13b** had disappeared. As with **13a**, eight new resonances, corresponding to the PEG-MAL/glutathione conjugate, were observed in the ¹H NMR spectrum (Figure 3). The conjugation reaction was also studied in pH = 6.5 phosphate buffer and similar results were obtained.

PCL-PEG-MAL Nanoparticle Formation by Impingement Mixing

Nanoparticles, having advantageous and controllable size distributions, have been generated from PCL-PEG block copolymers using “Flash NanoPrecipitation” (FNP).[15] In this process two (or more) solvent streams are simultaneously and rapidly introduced into a confined impingement jet (CIJ) mixer. When one is a solution of PCL-PEG block copolymer in, e.g., THF, and the other is a THF-miscible but poor solvent for one of the blocks (e.g., water), then rapid precipitation of particles occurs upon mixing. The hydrophilic PEG blocks preferentially cover the particle surfaces.[20,21] With a goal of eventually derivatizing such particles with biomacromolecules, we explored the *coprecipitation* by FNP of PCL-PEG-MALs **7** with unfunctionalized PCL-PEG (6.9K-4.6K).

A mixture of PCL-PEG-MAL and PCL-PEG in THF was mixed with H₂O in a two-stream CIJ mixer (~0.02 mL chamber volume) at a total flow velocity of 72 mL min⁻¹ (see Experimental Section). After mixing, particle sizes were determined by dynamic light scattering (DLS) at a 90° angle, and the data were analyzed using the Regularized Positive Exponential Sum (REPES) model (Figure 4).[22] The mass average particle size, $\overline{d_w}$, was estimated by DLS using Equation (1),

$$\overline{d_w} = \frac{\sum n_i m_i d_i}{\sum n_i m_i} = \frac{\sum n_i d_i^4}{\sum n_i d_i^3} \quad (1)$$

where n_i and m_i are the number and the mass of particles with the diameter of d_i , respectively. The *span* to describe polydispersity is defined by Equation (2),[23]

$$span=(d_{90}-d_{10})/d_{50} \quad (2)$$

where, d_{10} , d_{50} , and d_{90} are diameters at which the cumulative sample mass is under 10%, 50%, and 90%, respectively.

Representative particles size distribution histograms (percentage of mass vs. particle diameter) were plotted in Figure 4 (see Supporting information for Intensity and Number average particle sizes, Table S1). Compared to the results in panels A and B, the sample shown in panel C, with a larger *span* (1.95 vs. 1.30 and 1.41) and mass average diameter (35 nm vs. 27 and 20 nm), had a relatively larger portion of bigger particles. The presence of uncoupled PCL homopolymer in PCL-PEG-MAL (8.6K-4.6K) might lead to such a difference.

L-Glutathione[13] Conjugation Directly to MAL-functional Nanoparticles

It is not trivial to determine the amount of maleimide on a particle surface directly. Therefore, we used an indirect measurement. Thiol conjugation with maleimides is highly efficient—essentially quantitative in our hands for the case of **11a** reacting with L-glutathione to give **13a** (Scheme 2). Free thiol can be quantified reliably even at low concentration by an ultraviolet spectroscopic assay that uses Ellman's reagent. [24] (see Supporting Information for details).

To determine the efficiency of thiol-maleimide conjugation on the nanoparticles' surfaces, each of two samples was incubated for 4 h with ~2 equivalents (relative to the theoretical amount of maleimide) of glutathione (50 μ M) in pH 8 buffer solution. The amount of unreacted glutathione was then quantified against a standard curve by the Ellman determination, from which the amount of initial maleimide could then be deduced. Ca. 51% and 67% conjugation was determined by this method for PCL-PEG (6.9K-4.6K)/PCL-PEG-MAL (8.6K-4.6K) and PCL-PEG (6.9K-4.6K)/PCL-PEG-MAL (4.6K-4.6K), respectively. The fact that neither particle is completely conjugated suggests that some of the maleimide end groups either are buried within the nanoparticles, presumably to a somewhat greater extent for the higher molecular weight PCL-PEG, and/or sufficiently sterically hindered by other surface or interfacial phenomena to render them less reactive. Conjugation of bovine serum albumin (BSA) on the surface of the maleimide-functionalized particles was also successfully demonstrated. [25]

Conclusion

PCL-COOH was successfully synthesized by ring-opening polymerization of ϵ -caprolactone in high yield (>95%) using octanoic acid as initiator in the presence of a catalytic amount of CSA. Narrow distribution PCL-COOHs (PDI~1.14–1.24) were achieved by fractionation of crude PCL-COOH (PDI~1.75) in THF/methanol cosolvent. Three different molecular weight PCL-PEG-OHs (8.6K-4.6K, 6.9K-4.6K and 4.6K-4.6K) were successfully synthesized by reactive coupling of excess HO-PEG-OH with three different molecular weight PCL-COCl. Pretreatment of HO-PEG-OH with acid chloride **6** before the addition of PCL-COCl improved the coupling efficiency. The hydroxyl group in each of these PCL-PEG-OHs was then converted to maleimide functionality by end-capping with **6** followed by TEA treatment to promote elimination and reveal the maleimide.

Model thiol-maleimide conjugation between L-glutathione and maleimide **11a** or PEG-MAL **11b** ($M_n = 5.0K$), analogs of PCL-PEG-MAL copolymer, preceded well. Complete conversion of both glutathione and maleimide was obtained. Impingement mixing of PCL-

PEG-MAL (8.6K-4.6K, 6.9K-4.6K or 4.6K-4.6K) and PCL-PEG (6.9K-4.6K) resulted in formation of maleimide surface-functionalized nanoparticles. The mass average sizes were ca. 20–40 nm. The amount of accessible maleimide functionality on the nanoparticle surfaces was estimated by Ellman analysis to be 51% and 67% conjugation for 8.6K-4.6K and 4.6K-4.6K PCL-PEG-MAL nanoparticles, respectively. We are pursuing this strategy as a means for conjugating other biologically relevant molecules (e.g., proteins, vaccines, or imaging agents) to these biocompatible particles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported in part by the IPRIME (Industrial Partnership for Research in Interfacial and Materials Engineering) program at the University of Minnesota, in part by a grant awarded by the National Institutes of Health (CA-76497), and in part by a grant awarded by the NSF-NIRT (CTS-0506966).

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Figure 1. Analysis of size distributions of crude and fractionated PCL-COOHs (3). Fractions 1–4 range from high to low molecular weight, respectively. Panel A) GPC traces (peak intensities are normalized). Panel B) MALDI-TOF MS.

Figure 2.
 ^1H NMR spectrum of PCL-PEG-MAL 7 (6.9K-4.6K) in CDCl_3 .

Figure 3.

^1H NMR spectra (in D_2O) of maleimide **11a**, the conjugation product **13a** (from **11a** and **12**), *L*-glutathione (**12**), and the conjugation product **13b** (from **12** and **11b**). In PEG polymer **13b**, the proton resonances from each end group (methoxy and glutathione-maleimide adduct) are evident by inspection vis-à-vis those from the analogous protons in the simpler progenitors **11a**, **13a**, and **12**, even in the presence of the dominant PEG methylene backbone resonance at ~ 3.5 ppm. For the detailed assignment of the resonances between δ 2 and 5 ppm for these **11–13**, see Supporting Information.

Figure 4.

Particle sizes and size distribution determined by DLS using the REPES model at 90° and plotted as mass% vs. diameter. Each measurement was made on the unfiltered effluent solution. Panel A: PCL-PEG-MAL (4.6K-4.6K)/PCL-PEG (6.9K-4.6K), $\overline{d}_w = 27$ nm, $span = 1.30$. Panel B: PCL-PEG-MAL (6.9K-4.6K)/PCL-PEG (6.9K-4.6K), $\overline{d}_w = 20$ nm, $span = 1.41$. Panel C: PCL-PEG-MAL (8.6K-4.6K)/PCL-PEG (6.9K-4.6K), $\overline{d}_w = 35$ nm, $span = 1.95$.

Scheme 1.

Scheme 2a.

^a a) i) SOCl_2 , Δ , neat or ii) $(\text{COCl})_2$, DMF (cat.), rt, CH_2Cl_2 ; b) i) **10**, pyridine, 4-dimethylaminopyridine (DMAP, cat.), rt, CH_2Cl_2 ; ii) Et_3N , rt; c) glutathione (**12**, 1 equiv), H_2O or D_2O , rt.

Table 1

Molecular weights and molecular weight distributions (polydispersity index, PDI) of fractionated PCL-COOHs (**3**) by GPC and MALDI-TOF MS.

samples	mass (g)	M_n (PDI) ^a [kg/mol]	M_n (PDI) ^b [kg/mol]
crude	14.4	7.1 (1.75)	2.1 (1.20)
fraction 1	4.7	15.8 (1.24)	6.9 (1.05)
fraction 2	3.6	11.3 (1.21)	4.6 (1.09)
fraction 3	1.8	8.0 (1.21)	3.2 (1.08)
fraction 4	1.5	5.3 (1.14)	2.6 (1.04)

^a from GPC at room temperature using THF as eluent, relative to polystyrene standard;

^b from MALDI-TOF MS using dithranol as matrix.

%Coupling efficiency (**4** with 4.6K PEG-diol) and final %maleimide functionality for three different molecular weight PCL-PEG-MALs (**7**).

Table 2

entry	analyte	Block copolymer molecular weights		
		4.6K-4.6K	6.9K-4.6K	8.6K-4.6K
1	%coupling of PCL-COCl 4 ^a	>90	>90	~70 ^b
2	%maleimide functionality ^c	70–90%	70–90%	70–90%

^a calculated by integrating the ratios of PCL to PEG backbone resonances by ¹H NMR analyses of PCL-PEG-OHs (**5**), assumes all HO-PEG-OH was removed by methanol washing;

^b product of reaction performed without pretreatment of HO-PEG-OH with acid chloride **6**;

^c estimated by integrating the ratio of various pairs of maleimide resonances (*i.e.*, p, q, and r in Figure 2) vs. those from the octanoic acid initiator (*i.e.*, a in Figure 2) and ¹³C satellite resonances of the PEG or PCL backbone protons (*i.e.*, k–n or i and e, respectively, in Figure 2).