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Article

Modular Approach to the Functionalization of Polymersomes

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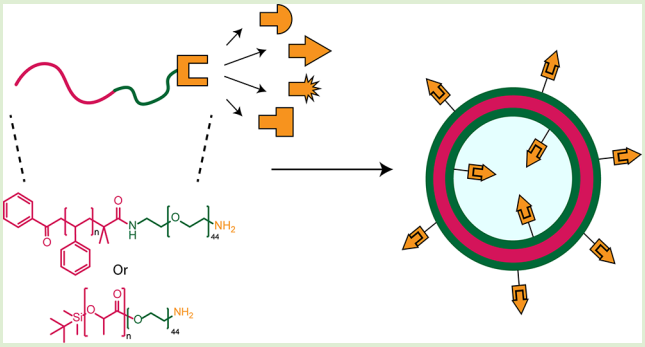
ABSTRACT: Functionalizing polymersomes remains a challenge due to the limitation in reaction conditions applicable to the chemistry on the surface, hindering their application for selective targeting. In order to overcome this limitation, functionalization can be introduced right before the self-assembly. Here, we have synthesized a library (32 examples) of PEG-*b*-PS and PEG-*b*-PDLLA with various functional groups derived from the amine-functionalized polymers, leading to functionally active polymersomes. We show that polymersome formation is possible via the general method with all functionalized groups and that these handles are present on the surface and are able to undergo reactions. Additionally, this methodology provides a general synthetic tool to tailor the functional group of the polymersome right before self-assembly, without limitation on the reaction conditions.



INTRODUCTION

In natural systems, amphipathic polymers are abundant building blocks that self-assemble to form ordered systems, such as vesicles and cell membranes. To emulate this behavior, polymeric systems that are biocompatible and lack toxicity have been developed and used for the construction of biomimetic materials that can be used for medical applications.[1−4] Polymersomes can be made using a wide variety of polymers[5,6] and have been used extensively as vesicles for drug delivery,[7−10] as nanoreactors,[11,12] and for motion.[13] In order to attain these functionalities, polymersomes require incorporation of specific materials or chemical groups. In the case of drug delivery and motion, this is often done by encapsulation.[14] However, encapsulation alone is quite limited in terms of chemical reactions and stability. Robust and efficient conjugation methods are essential to broadening the scope and accessibility of polymersomes in the medical field.[15,16] Common methods of conjugation used in the nanoparticle and polymersome field include click chemistry,[17−19] inverse-electron demand Diels−Alder,[20−22] oxime chemistry,[23−25] thiol−ene chemistry,[26−28] and Diels−Alder,[29−31] among others. However, this large toolbox of ligation techniques is not always easily accessible due to the limitations in functionalization of nanomaterials.

Many nanomaterials are made before the desired functional groups are introduced. After the self-assembly has taken place, new functional groups can be attached. Postmodification can however be very restrictive, due to low stability of membranes in various conditions or accessibility of the reactants.[32] Examples of postmodification are prevalent in literature, but are mostly made on a case-to-case basis. The group of Meier, for instance, has worked on functionalizing the surface of polymersomes to make them biocompatible, using hydrazones.[33] Similarly, azides have also been used to functionalize the surface,[34] and the group of Santos has looked into coating the surface with a long peptide chain for targeting.[35] Prefunctionalization of these materials would enable a faster and simpler formation of nanomaterials and also allow for dual-targeting upon postfunctionalization.[36] Although examples of this also exist within the literature, they are commonly made from scratch to be used for a single goal.[36,37] In all of these examples, a unifying strategy for prefunctionalization is lacking. A limitation is the need for the handles to be compatible with the synthetic preparation of the building block, as well as with the self-assembly process, as incompatibility may lead to loss of that functionality or morphology due to the lack of control in positioning of the handle.[38,39] In the case of poly(ethylene glycol)-*b*-polystyrene (PEG-*b*-PS), prefunctionalization of the polymer with different functional groups has proven to be successful, giving a window for further investigation into a prefunctional approach with various chemical handles.[40] For this reason, we decided to use the PEG-*b*-PS as well as the biodegradable poly(ethylene glycol)-*b*-poly(lactic acid) (PEG-

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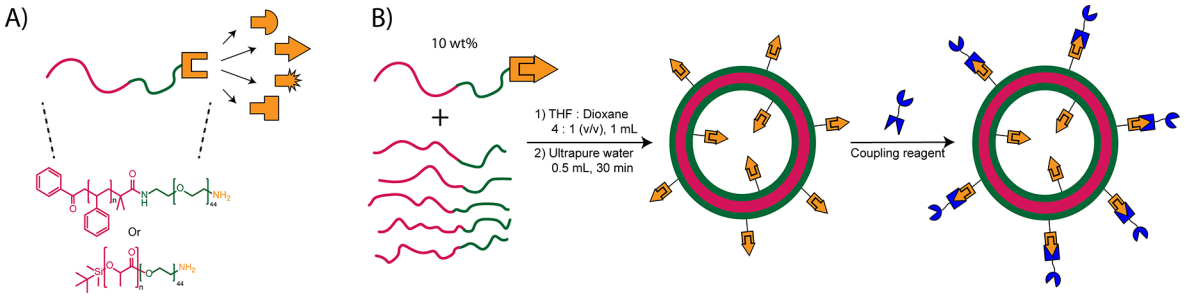

Figure 1. (A) Design of the modular PEG-*b*-PS and PEG-*b*-PDLLA polymers. The amine moiety functions as a base for attaching various functionalities. (B) Polymersomes are made via self-assembly upon slow addition of water (0.5 mL, 1 mL h^{-1}). 10% addition of the functionalized polymers during self-assembly provides functional handles, which can react for further decoration of the polymersome.

b-PDLLA) polymersome-based nanomotors reported by our group[13,41] as the basis for a library synthesis.

Designing a polymersome with the functionalization incorporated in the polymer before self-assembly could be achieved by direct polymerization of reactive handles to monomers. This is undesirable though, as this would increase the synthetic load immensely, because a new polymer has to be synthesized from scratch for every different ligation desired. Utilizing this approach also requires every synthetic step to tolerate the functional group of the polymer. Therefore, we have designed a method which combines the versatility of prefunctionalization with the high compatibility of post-functionalization. By first synthesizing a polymer with a functional handle that can, subsequently, form a stable bond with compounds that present the desired functional group, we make a polymer that can function as the base molecule for all other moieties that we want to attach. The reactivity on the polymer is hard to monitor via conventional methods such as NMR, IR and UV, due to the relatively small concentration of the handle compared to the size of the polymer. In order to overcome these problems, we have selected an amine functionality. The use of amine-functionalized polymers is already prevalent in the polymersome field, though mainly used for postmodifications. One example of premodification is shown by Zou et al., who have used amine-capped polymers to introduce new functionalities.[42] We aim to use their versatility for a simple to use prefunctionalization method. Amines can form incredibly stable amide bonds, for which reaction conditions have been researched immensely,[43−45] and completion of the reaction can be followed with 99.9% accuracy via the Kaiser test.[46,47] Amine-functionalized PEG-*b*-PS and PEG-*b*-PDLLA can bind various small molecules via an amide bond, which contains a different functionalization on the other end (Figure 1A). We aim to show the diversity possible via this modular approach by providing examples not only of moieties useful for chemical ligation, but also biotargeting and monitoring of the polymersome. When self-assembling the polymersome from the desired polymer, we then incorporate 10% of the functional polymer into the polymersomes (Figure 1B). To prove that the handles are available on the surface, we react DBCO-functionalized polymersomes with a 3-azidocoumarin derivative. This compound will become strongly fluorescent upon cycloaddition, allowing for the monitoring of its conjugation onto the polymersome, simply by measuring the fluorescence intensity.[48]

EXPERIMENTAL SECTION

Materials. All PEG polymers were obtained from AV Chemistry. All other reagents were obtained from commercial sources and were used without purification unless otherwise stated. Solvents were dried by passing over activated alumina columns in a MBraun MB SPS800 under a nitrogen atmosphere and stored under argon. Reactions were carried without the need for an inert atmosphere unless stated otherwise, in which case the reaction was performed under a dry atmosphere of argon. Standard syringe techniques were applied for the transfer of dry solvents and air- or moisture sensitive reagents. Styrene was passed over alumina to remove the inhibitor 4-*tert*-butylcatechol. Ultrapure Milli-Q water was obtained from QPOD Milli-Q system.

Instrumentation. Nuclear Magnetic resonance (NMR) characterization was carried out on a Bruker AVANCE HD nanobay console with a 9.4 T Ascend magnet (400 MHz) and a Bruker AVANCE III console with a 11.7 T UltraShield Plus magnet (500 MHz) equipped with a Bruker Prodigy cryoprobe, in chloroform ($CDCl_3$), DMSO-d_6, or THF-d_8. NMR spectra were recorded at 298 K unless otherwise specified. Chemical shifts are given in parts per million (ppm) with respect to tetramethylsilane (TMS, δ 0.00 ppm) as internal standard for ^{1}H NMR. Coupling constants are reported as *J* values in Hz. Peak assignment is based on 2D gDQCOSY, ^{1}H−^{13}C gHSQCED, and ^{1}H−^{13}C gHMBC spectra. Side group and end of chain signals separated from the bulk polymer ^{1}H signal are only reported when observed with clear s/n ratio and no overlap with polymer peaks, and may be (in)visible on other NMR spectrometers or with different concentrations. Gel permeation chromatography (GPC) equipped with PL gel 5 μm mixed D column calibrated for polystyrene (580−377400 g/mol) was carried out on a Shimadzu instrument with THF as eluent using differential refractive index and UV (254 nm) detectors. Transmission electron microscopy (TEM) was carried out on JEOL TEM 1400 equipped with a CCD camera at 60 kV. Samples were prepared by dropcasting 5 μL of appropriately diluted samples on a carbon-coated Cu grid (200 mesh) and dried overnight at room temperature. Cryogenic TEM was also carried out with JEOL TEM 2100. Malvern Zetasizer nano S was used for dynamic light scattering (DLS) measurements equipped with He−Ne laser of wavelength 633 nm. Fluorescence was measured on a Tecan Spark 200.

Synthesis of Polymers. *α-tert-Butyloxycarbonyl-polystyrene (**1**).* α-*tert*-Butyloxycarbonyl-polystyrene (**1**) was prepared as described by Tu et al.[49] CuBr (225 mg, 1.6 mmol, 2.3 equiv) was charged in a flame-dried Schlenk flask. PMDETA (330 μL, 1.6 mmol, 2.3 equiv) in anisole (5 mL) was added to the flask, after which it was vigorously stirred for 15 min. Styrene (28.7 mL, 250 mmol, 345 equiv) was added, and the mixture was degassed for 15 min. The mixture was cooled to 0 °C and *tert*-butyl-α-bromoisobutyrate (135 μL, 0.7 mmol, 1 equiv) was added, after which the mixture was degassed for another 15 min. The mixture was heated to 90 °C, and the progress of polymerization was followed by ^{1}H NMR. When the desired molecular length was obtained, 1-phenyl-1-trimethylsiloxyethene (9.55 mL, 46.4 mmol, 65.5 equiv) was added to terminate the reaction and left to stir for 2 h. Subsequently the reaction mixture was

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diluted with DCM, extracted with 50 mM EDTA solution twice and once with brine. The washed organic fraction was dried over $MgSO_4$ and concentrated by rotary evaporation. The concentrate was precipitated in ice cold MeOH and subsequently washed with cold MeOH (3×) and dried in vacuo to obtain **1** as a white solid (10.1 g, 64%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.98−7.95 (m, 2H, PS cap ortho), 7.59−7.54 (m, 1H, PS cap para), 7.49−7.45 (m, 2H, PS cap meta), 7.24−6.86 (m, PS arom. ortho and para), 6.58−6.28 (m, PS arom. meta), 2.30−1.70 (m, PS backbone C*H*), 1.70−1.17 (m, PS backbone CH_2), 1.30−1.21 (m, 9H, ^{t}Bu), 0.87−0.78 (m, 6H, *b*). ^{13}C NMR (125 MHz, $CDCl_3$) δ 198.3, 145.0, 137.1, 133.4, 128.6, 128.0, 127.7, 125.5, 79.4, 45.3, 44.2, 40.7, 29.0, 21.1. R_f 0.88 (AcOEt/Hept, 1:1 v/v). M_w/M_n 1.07.

*α-Carboxylic acid-polystyrene (**2**).* α-Carboxylic acid-polystyrene (**2**) was synthesized as described by Tu et al.[49] Polymer product **1** (10 g, 0.52 mmol, 1 equiv) was dissolved in 1,4-dioxane (100 mL), after which concentrated HCl (5 mL, 37%) was added. The mixture was left to reflux for 18 h at 110 °C. Progress of deprotection was followed by TLC. Once completed, the reaction mixture was evaporated in vacuo,and subsequently, the solid was dissolved in a minimal amount of DCM. The product was obtained by precipitation in cold MeOH. The precipitate was washed with cold MeOH (3×) and dried in vacuo to yield **2** as a white solid (8.84 g, 88%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.98−7.95 (m, 2H, PS cap ortho), 7.59−7.54 (m, 1H, PS cap para), 7.49−7.45 (m, 2H, PS cap meta), 7.24−6.86 (m, PS arom. ortho and para), 6.58−6.28 (m, PS arom. meta), 2.30−1.70 (m, PS backbone C*H*), 1.70−1.17 (m, PS backbone CH_2), 0.87−0.78 (m, 6H, *b*). ^{13}C NMR (125 MHz, $CDCl_3$) δ 198.6, 145.3, 137.1, 133.1, 128.7, 128.1, 128.3, 127.7, 125.7, 44.5, 41.8, 40.7, 26.1. R_f 0.81 (AcOEt/Hept, 1:1 v/v). M_w/M_n 1.06.

*α-Azide-poly(ethylene glycol)-b-polystyrene (**3**).* Polymer product **2** (8.7 g, 0.45 mmol, 1 equiv), α-azide-poly(ethylene glycol)-ω-amine (1.8 g, 0.90 mmol, 2 equiv), and DIPEA (0.134 g, 1.04 mmol, 2.3 equiv) were dissolved in DMF (100 mL). The solution was cooled to 0 °C, after which PyBOP (0.43 g, 0.83 mmol, 1.84 equiv) was added, and the mixture was allowed to warm to room temperature. The solution was left to stir for 168 h, in which time the reaction was followed by TLC. When finished, the reaction mixture was diluted with DCM and extracted with $NaHCO_3$ (4 wt % solution) and brine (2×) . The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The product was precipitated in ice cold MeOH and subsequently washed with ice cold MeOH (3×). The wet substance was dried in vacuo to yield **3** as a light yellow solid (6.61 g, 69%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.70−7.64 (m, 2H, PS cap ortho), 7.57−7.52 (m, 1H, PS cap para), 7.49−7.43 (m, 2H, PS cap meta), 7.24−6.86 (m, PS arom. ortho and para), 6.58−6.28 (m, PS arom. meta), 3.64 (br s, 176H, PEG), 2.30−1.70 (m, PS backbone C*H*), 1.70−1.17 (m, PS backbone CH_2), 1.00−0.93 (m, 6H, *b*). ^{13}C NMR (125 MHz, $CDCl_3$) δ 145.5, 139.2, 133.1, 128.2, 128.1, 128.0, 127.9, 125.5, 70.6, 45.3, 42.0, 40.7, 26.1. M_w/M_n 1.02.

*α-Amine-poly(ethylene glycol)-b-polystyrene (**4**).* Polymer product **3** (5.5 g, 0.263 mmol, 1 equiv) was dissolved in dry THF (80 mL). Triphenylphosphine (0.4 g, 1.52 mmol, 5 equiv) was added to the solution, and the reaction mixture was stirred for 48 h. A total of 100 mL of water was added, and the mixture was left to reflux at 100 °C for 16 h. Subsequently, the THF was evaporated in vacuo from the reaction mixture, and the remaining solution was alkalinized with NaOH to pH 10. The mixture was extracted with DCM, concentrated, and precipitated in ice cold MeOH. The precipitate was washed with ice cold MeOH (3×) to obtain **4** as a white solid (5.4 g, 97%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.70−7.64 (m, 2H, PS cap ortho), 7.57−7.52 (m, 1H, PS cap para), 7.49−7.43 (m, 2H, PS cap meta), 7.24−6.86 (m, PS arom. ortho and para), 6.58−6.28 (m, PS arom. meta), 3.64 (br s, 176H, PEG), 2.30−1.70 (m, PS backbone C*H*), 1.70−1.17 (m, PS backbone CH_2), 0.96−0.82 (m, 6H, *b*). ^{13}C NMR (125 MHz, $CDCl_3$) δ 145.3, 131.9, 128.2, 128.1, 127.9, 127.8, 70.6, 44.1, 42.1, 40.6, 26.4. M_w/M_n 1.02.

*α-Azide-poly(ethylene glycol)-b-polylactic acid (**5**).* α-Azide-poly(ethylene glycol)-*b*-polylactic acid (**5**) was prepared as described by Sherck et al.[50] N_3−PEG_{44}−OH (2.65 g, 1.2 mmol, 1 equiv) and D,L-Lactide (15.4 g, 107.1 mmol, 188 equiv) were dissolved in dry toluene (150 mL). The solution was concentrated, and the residual solvent was removed using a high vacuum. The solid was dissolved in dry DCM (130 mL) under argon. To this solution, DBU (188 μL, 0.6 mmol, 0.5 equiv) was added. The mixture was stirred and heated to 40 °C for 2 h. Afterward, the reaction mixture was concentrated in vacuo and subsequently precipitated in ice cold Et_2O (2×). The precipitate was dissolved in minimal 1,4-dioxane and lyophilized to yield **5** as a white solid (9.10 g, 91%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.33−4.49 (m, PDLLA C*H*), 3.57 (br s, 176H, PEG), 1.76−1.32 (m, PDLLA CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 206.9, 169.6, 70.6, 69.0, 16.6. M_w/M_n 1.09.

*α-Amine-poly(ethylene glycol)-b-polylactic acid (**6**).* Polymer product **5** (9.0 g, 0.6 mmol, 1 equiv) was dissolved in dry THF (70 mL). Triphenylphosphine (0.47 g, 1.8 mmol, 3 equiv) dissolved in dry THF (5 mL) was added to the solution and the reaction mixture was stirred for 48 h at 50 °C. A total of 50 mL of water was added, and the mixture was left to reflux at 100 °C for 16 h. Subsequently, the THF was evaporated in vacuo from the reaction mixture and the remaining solution was alkalinized with NaOH to pH 10. The mixture was extracted with DCM. The water layer was reduced in vacuo, concentrated, and precipitated in ice cold Et_2O. The precipitate was dissolved in minimal 1,4-dioxane and lyophilized to yield **6** as a white solid (7.9 g, 87%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.31−4.41 (m, PDLLA C*H*), 3.64 (br s, 176H, PEG), 1.63−1.38 (m, PDLLA CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 206.9, 169.6, 70.6, 69.0, 16.6. M_w/M_n 1.03.

*α-Amine-poly(ethylene glycol)-b-polylactic acid-TBS (**7**).* Polymer product **6** (7.5 g, 0.5 mmol, 1 equiv) was dissolved in dry DCM (20 mL) and degassed for 15 min. Et_3N (0.5 mL, 3.6 mmol, 7 equiv), TBDMSCl (492 mg, 3.26 mmol, 7 equiv), and DMAP (3.8 mg, 0.03 mmol; 0.06 equiv) were added to the solution. The reaction mixture was stirred for 16 h at 21 °C under an argon atmosphere. The mixture was concentrated in vacuo and precipitated in ice cold Et_2O. The precipitate was dissolved in minimal 1,4-dioxane and lyophilized to yield **7** as a white solid (5.5 g, 73%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.78−4.19 (m, PDLLA C*H*), 3.64 (br s, 176H, PEG), 1.63−1.38 (m, PDLLA CH_3), 0.92 (s, 9H, C(CH_3)$_3$), 0.15 (s, 6H, Si(CH_3)$_2$). ^{13}C NMR (101 MHz, $CDCl_3$) δ 206.9, 169.6, 70.6, 69.0, 16.7, 16.6, 8.6, 1.0. M_w/M_n 1.03.

*α-Acetylthiol-poly(ethylene glycol) ATRP initiator (**8**).* α-Acetylthiol-poly(ethylene glycol) ATRP initiator (**8**) was prepared as described by Tu et al.[49] α-Acetylthiol-poly(ethylene glycol) (250 mg, 125 μmol, 1 equiv) was dissolved in dry THF (25 mL) in a flamed-dried Schlenk flask. After adding Et_3N (52 μL, 375 μmol, 3 equiv), the mixture was cooled to 0 °C. α-bromoisobutyryl bromide (30.8 μL, 250 μmol, 2 equiv) was added dropwise. After addition, the resulting solution was stirred for 24 h while slowly warming to 21 °C. After the reaction, the white precipitate was filtered off and the solution was concentrated. The polymer was precipitated in ice-cold diethyl ether (2×) and dried in vacuo to obtain **8** as a white solid (184 mg, 73%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 4.37−4.28 (m, 2H, $CH_2CH_2OC(O)C(CH_3)_2Br$), 3.77−3.72 (m, 2H, $CH_2CH_2OC(O)C(CH_3)_2Br$), 3.64 (br s, 172H, PEG), 3.09 (t, J = 6.5 Hz, 6H, $AcSCH_2$), 2.34 (s, 3H, acetyl), 1.94 (s, 6H, C(CH_3)$_2$Br). ^{13}C NMR (125 MHz, $CDCl_3$) δ 195.5, 171.6, 68.8, 65.1, 70.5, 28.9, 30.8, 30.6.

Synthesis of Functionalized Polymers. *General Procedure for Coupling of Carboxylic Acid and NHS Derivatives to **4** (Method A).* Polymer product **4** (250 mg, 10.3 μmol, 1 equiv), DMAP (12.5 mg, 103 μmol, 10 equiv), and carboxylic acid or NHS derivative (103 μmol, 10 equiv) were dissolved in DCM (10 mL). EDC (19.6 mg, 103 μmol, 10 equiv) was added to the reaction mixture and was stirred at 21 °C. The progress of the reaction was followed by the Kaiser test.[46] Upon confirming a negative Kaiser test (>99% reacted), the reaction mixture was concentrated in vacuo and precipitated in ice cold MeOH. The solid was subsequently washed with ice cold MeOH (2×) and water (1×). The polymer product was dried overnight in vacuo to yield the functionalized PEG-*b*-PS polymer. NMR shifts are equal to **4**, with signals from the functional groups reported when visible.

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*General Procedure for Coupling of Carboxylic Acid and NHS Derivatives to **7** (Method B).* The carboxylic acid or NHS derivative (130 μmol, 10 equiv) and DIPEA (20 μL, 110 μmol, 9 equiv) were dissolved in DCM (1 mL), after which the solution was cooled to 0 °C. PyBOP (64.1 mg, 110 μmol, 9 equiv) was dissolved in DCM (0.5 mL), added dropwise to the cooled solution and subsequently stirred for 1 h. Then, polymer product **7** (200 mg, 13.0 μmol, 1 equiv) was dissolved in DCM (2 mL) and added to the stirring solution and allowed to slowly warm up to 21 °C. The progress of the reaction was followed by the Kaiser test.[46] Upon confirming a negative Kaiser test (>99% reacted), the reaction mixture was concentrated in vacuo and precipitated in ice cold Et_2O. The solid was subsequently dissolved in 1,4-dioxane and lyophilized to yield the functionalized PEG-*b*-PDLLA polymer. NMR shifts are equal to **7**, with signals from the functional groups reported when visible.

*General Procedure for Coupling of Halide Derivatives to **4** and **7** (Method C).* Polymer product **4** (250 mg, 10.3 μmol, 1 equiv) or **7** (200 mg, 13.0 μmol, 1 equiv) was dissolved in DCM (2 mL), after which Et_3N (20 equiv) was added to the solution. The acyl halide derivative (10 equiv) dissolved in DCM (1 mL) was added dropwise, and the mixture was stirred at 21 °C. The progress of the reaction was followed by the Kaiser test.[46] Upon confirming a negative Kaiser test (>99% reacted), the reaction mixture was concentrated in vacuo and precipitated in ice cold MeOH (PEG-*b*-PS) or Et_2O (PEG-*b*-PDLLA) and washed with the appropriate solvent (2×). The PEG-*b*-PS product was dried overnight in vacuo to yield the functionalized PEG-*b*-PS polymer. The PEG-*b*-PDLLA product was dissolved in 1,4-dioxane and lyophilized to yield the functionalized PEG-*b*-PDLLA polymer. NMR shifts are equal to **4** or **7**, with signals from the functional groups reported when visible.

*α-Alkyne-poly(ethylene glycol)-b-polystyrene (**4a**).* Method A: 4-pentynoic acid (6.67 mg); Yield: 66%. The alkyne peaks could not be seen on the NMR spectrum.

*α-Alkyne-poly(ethylene glycol)-b-polylactic acid (**7a**).* Method C: Propargyl chloroformate (12.7 μL); Yield: 66%. Handle ^{1}H NMR shifts: ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.77 (s, 1H).

*α-Dibenzocyclooctyne-poly(ethylene glycol)-b-polystyrene (**4b**).* Method A: Dibenzocyclooctyne-*N*-hydroxysuccinimidyl ester (41.2 mg); Yield: 62%. Handle ^{1}H NMR shifts: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.73−7.65 (m, 1H, aromatic), 5.23−5.12 (m, 2H, cyclooctane CH_2), 3.11−3.04 (m, 1H, $NHC(O)CH_2CH_2C(O)N$), 2.88−2.80 (m, 1H, $NHC(O)CH_2CH_2C(O)N$), 2.69−2.60 (m, 1H, $NHC(O)CH_2CH_2C(O)N$).

*α-Dibenzocyclooctyne-poly(ethylene glycol)-b-polylactic acid (**7b**).* Method B: Dibenzocyclooctyne-*N*-hydroxysuccinimidyl ester (52 mg); Yield: 70%. Handle ^{1}H NMR shifts: ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.86 (d, *J* = 7.9 Hz, 4H, aromatic), 7.68 (d, *J* = 8.1 Hz, 4H, aromatic), 2.79−2.70 (m, 4H, $NHC(O)CH_2CH_2C(O)N$).

*α-Aminooxy-poly(ethylene glycol)-b-polystyrene (**4c**).* Method A: (Boc-aminooxy) acetic acid (19.5 mg). The product was then dissolved in DCM (2 mL). Trifluoroacetic acid (11.9 mg, 103 μmol, 10 equiv) was added slowly in the solution. Subsequently, the reaction mixture was stirred for 16 h. Workup then proceeded according to Method A. Total yield: 57%. The aminooxy peaks could not be seen on the NMR spectrum.

*α-Aminooxy-poly(ethylene glycol)-b-polylactic acid (**7c**).* Method B: (Boc-aminooxy) acetic acid (24.6 mg). The product was then dissolved in DCM (2 mL). Trifluoroacetic acid (15.0 mg, 130 μmol, 10 equiv) was added slowly in the solution. Subsequently, the reaction mixture was stirred for 16 h. Workup then proceeded according to Method B. Total yield: 28%. Handle ^{1}H NMR shifts: ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.40 (s, 2H, NH_2).

*α-Bromine-poly(ethylene glycol)-b-polystyrene (**4d**).* Method C: α-bromoisobutyryl bromide (12.6 μL); Yield: 91%. Handle ^{1}H NMR shifts: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.94 (s, 6H).

*α-Bromine-poly(ethylene glycol)-b-polylactic acid (**7d**).* Method C: α-bromoisobutyryl bromide (16.5 μL); Yield: 71%. Handle ^{1}H NMR shifts: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.94 (s, 6H).

*α-Maleimide-poly(ethylene glycol)-b-polystyrene (**4e**).* Method A: 3-maleimidopropionic acid (17.2 mg); Yield: 84%. The alkene peaks could not be seen on the NMR spectrum.

*α-Maleimide-poly(ethylene glycol)-b-polylactic acid (**7e**).* Method B: 3-maleimidopropionic acid (21.7 mg); Yield: 63%. The alkene peaks could not be seen on the NMR spectrum.

*α-2-[4-(6-Methyl-1,2,4,5-tetrazine-3-yl)phenyl]-poly(ethylene glycol)-b-polystyrene (**4f**).* Method A: 2-[4-(6-methyl-1,2,4,5-tetrazine-3-yl)phenyl]acetic acid (23.7 mg); Yield: 88%. Handle ^{1}H NMR shifts: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.57−8.19 (m, 4H, aromatic), 3.10 (s, 3H, CH_3).

*α-2-[4-(6-Methyl-1,2,4,5-tetrazine-3-yl)phenyl]-poly(ethylene glycol)-b-polylactic acid (**7f**).* Method B: 2-[4-(6-methyl-1,2,4,5-tetrazine-3-yl)phenyl]acetic acid (29.9 mg); Yield: 86%. Handle ^{1}H NMR shifts: ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.55−7.35 (m, 4H, aromatic).

*α-Oxobutan-poly(ethylene glycol)-b-polystyrene (**4g**).* Method A: 3-(1,3-dioxolan-2-yl)propanoic acid (60 mg). After the coupling was determined to be successful, aq. HCl was added until the pH was <1. The reaction mixture was stirred for an additional 24 h. Workup then proceeded according to Method A. Yield: 97%. Handle ^{1}H NMR shifts: ^{1}H NMR (500 MHz, THF-d_8) δ 9.57 (s, 1H, aldehyde).

*α-Oxobutan-poly(ethylene glycol)-b-polylactic acid (**7g**).* Method B: 3-(1,3-dioxolan-2-yl)propanoic acid (75.7 mg). After the coupling was determined to be successful, aq. HCl was added until the pH was 4. The reaction mixture was stirred for an additional 24 h. Workup then proceeded according to Method B. Yield: 72%. Handle ^{1}H NMR shifts: ^{1}H NMR (500 MHz, $CDCl_3$) δ 9.82 (s, 1H, aldehyde).

*α-Hydroxyimino butanamide-poly(ethylene glycol)-b-polystyrene (**4h**).* Compound **4g** (500 mg, 20.5 μmol, 1 equiv) was dissolved in DCM (8 mL), after which hydroxalamine HCl (14.2 mg, 205 μmol, 10 equiv) and Et_3N (41.5 mg, 410 μmol, 20 equiv) were added to the mixture. The reaction mixture was left to stir for 16 h. Workup then proceeded according to Method A. Yield: 86%. The hydroximino peaks could not be seen on the NMR spectrum.

*α-Hydroxyimino butanamide -poly(ethylene glycol)-b-polylactic acid (**7h**).* Compound **7g** (50 mg, 3.25 μmol, 1 equiv), was dissolved in DCM (4 mL), after which hydroxalamine HCl (2.3 mg, 32.5 μmol, 10 equiv) and Et_3N (6.6 mg, 65 μmol, 20 equiv) were added. The solution was stirred for 16 h. Workup then proceeded according to Method B. Yield: 71%. The hydroximino peaks could not be seen on the NMR spectrum.

*α-Hydrazineylidene butanamide-poly(ethylene glycol)-b-polystyrene (**4i**).* Compound **4g** (200 mg, 8.2 μmol, 1 equiv) was dissolved in THF (4 mL), after which hydrazine hydrate (4.1 mg, 82 μmol, 10 equiv) was added. The solution was stirred for 16 h. Workup then proceeded according to Method A. Yield: 76%. The hydrazine peaks could not be seen on the NMR spectrum.

*α-Hydrazineylidene butanamide -poly(ethylene glycol)-b-polylactic acid (**7i**).* Compound **7g** (50 mg, 3.25 μmol, 1 equiv), was dissolved in THF (4 mL), after which hydrazine hydrate (1.6 mg, 32.5 μmol, 10 equiv) was added. The solution was stirred for 16 h. Workup then proceeded according to Method B. Yield: 54%. The hydrazine peaks could not be seen on the NMR spectrum.

*α-Norbornene-poly(ethylene glycol)-b-polystyrene (**4j**).* Method A: 5-norbornene-2-carboxylic acid (14.1 mg); Yield: 93%. Handle ^{1}H NMR shifts: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.13 (dd, *J* = 5.7, 3.1 Hz, 1H, alkene), 5.94 (dd, *J* = 5.7, 2.8 Hz, 1H, alkene), 3.20−3.10 (m, 1H, $HNC(O)CHCH$), 2.91 (dt, *J* = 8.6, 3.5 Hz, 1H, $HNC(O)CH$), 2.84 (s, 1H, $HNC(O)CHCH_2CH$), 1.84−1.33 (m, 2H, $HNC(O)CHCH_2$), 1.37−1.22 (m, 2H, bridge).

*α-Norbornene-poly(ethylene glycol)-b-polylactic acid (**7j**).* Method B: 5-norbornene-2-carboxylic acid (17.8 mg); Yield: 70%. Handle ^{1}H NMR shifts: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.28−5.88 (m, 2H, alkene).

*α-GlyGlyGly-poly(ethylene glycol)-b-polystyrene (**4k**).* Method A: *N-tert*-Butoxycarbonyl-glycyl-glycyl-glycine was prepared as described by Naumovich et al.[51] and subsequently used (37 mg). After the coupling was determined to be successful, aq. HCl was added until the

https://dx.doi.org/10.1021/acs.biomac.9b01734
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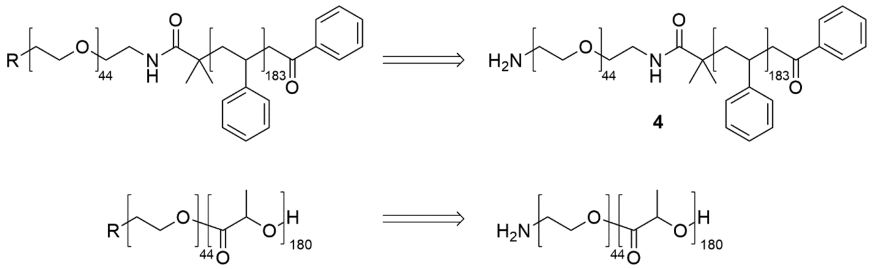
Scheme 1. Retrosynthesis of the Functionalized PEG-*b*-PS and PEG-*b*-PDLLA Polymers



pH was <1. The reaction mixture was stirred for an additional 24 h. Workup then proceeded according to Method A. Yield: 83%. Handle ^{1}H NMR shifts: ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.96−3.80 (m, 6H, Gly backbone).

*α-GlyGlyGly-poly(ethylene glycol)-b-polylactic acid (**7k**).* Method B: *N-tert*-Butoxycarbonyl-glycyl-glycyl-glycine was prepared as described by Naumovich et al.[51] and subsequently used (46.7 mg). After the coupling was determined to be successful, aq. HCl was added until the pH was 5. The reaction mixture was stirred for an additional 24 h. Workup then proceeded according to Method B. Yield: 85%. Handle ^{1}H NMR shifts: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.96−3.80 (m, 6H, Gly backbone).

*α-FITC-poly(ethylene glycol)-b-polystyrene (**4l**).* Fluorescein 5-isothiocyanate (8 mg, 20.6 μmol, 2 equiv) and **4** (250 mg, 10.3 μmol, 1 equiv) were dissolved in DMSO (0.5 mL). Et_3N (3 μL, 20.6 μmol, 2 equiv) was added, and the mixture was stirred in the dark for 4 h. The solvent was removed in vacuo. Workup then proceeded according to Method A. Yield: 81%. The FITC peaks could not be seen on the NMR spectrum. UV−vis λmax ($CHCl_3$)/nm 257, 461 and 486.

*α-FITC -poly(ethylene glycol)-b-polylactic acid (**7l**).* Fluorescein 5-isothiocyanate (19.5 mg, 50 μmol, 2 equiv) and **7** (200 mg, 25 μmol, 1 equiv) were dissolved in DMSO (0.5 mL). Et_3N (7.4 μL, 50 μmol, 2 equiv) was added, and the mixture was stirred in the dark for 4 h. The solvent was removed in vacuo. Workup then proceeded according to Method B. Yield: 50%. The FITC peaks could not be seen on the NMR spectrum. UV−vis λmax ($CHCl_3$)/nm 462 and 488.

*α-Acryolyl-poly(ethylene glycol)-b-polystyrene (**4m**).* Method C: Acryloyl chloride (7.7 μL); Yield: 69%. Handle ^{1}H NMR shifts: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.64 (d, J = 16.5 Hz, 1H, C*H*H═CHC(O)NH), 6.18 (d, J = 10.2 Hz, 1H, CH*H*═CHC(O)NH), 6.35 (dd, J = 16.3, 10.3 Hz, 1H, CHH═C*H*C(O)NH).

*α-Acryolyl-poly(ethylene glycol)-b-polylactic acid (**7m**).* Method C: Acryloyl chloride (9.8 μL); Yield: 99%. Handle ^{1}H NMR shifts: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.48 (d, J = 16.6 Hz, 1H, C*H*H═CHC(O)NH), 6.18 (d, J = 10.2 Hz, 1H, CH*H*═CHC(O)NH), 5.91 (dd, J = 16.5, 10.2 Hz, 1H, CHH═C*H*C(O)NH).

*α-Acetylthiol-poly(ethylene glycol)-polystyrene (**4n**).* CuBr (45 mg, 0.32 mmol, 3.2 equiv) was charged in a flame-dried Schlenk flask. PMDETA (66 μL, 0.32 mmol, 3.2 equiv) in anisole (0.5 mL) was added to the flask, after which it was vigorously stirred for 15 min. Styrene (5 mL, 43.6 mmol, 436 equiv) was added and the mixture was degassed for 15 min. The mixture was cooled to 0 °C and **8** (215 mg, 0.10 mmol, 1 equiv) was added after which the mixture was degassed for another 15 min. The mixture was heated to 90 °C, and the progress of polymerization was followed by ^{1}H NMR. When the desired molecular length was obtained, 1-phenyl-1-trimethylsiloxyethene (1.91 mL, 9.28 mmol, 92.8 equiv) was added to terminate the reaction and left to stir for 2 h. Subsequently, the reaction mixture was diluted with DCM, extracted with 50 mM EDTA solution twice and once with brine. The washed organic fraction was dried over $MgSO_4$ and concentrated by rotary evaporation. The concentrate was precipitated in ice cold MeOH and subsequently washed with cold MeOH (3×) and dried in vacuo to obtain **4n** as a white solid. Yield: 85%. Handle ^{1}H NMR shifts: ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.33 (s, 3H, acetyl). M_W/M_n 1.07.

*α-Thiol-poly(ethylene glycol)-polystyrene (**4o**).* Compound **4n** (300 mg, 14.3 μmol, 1 equiv) was dissolved in a MeOH (25 mL) and DCM (35 mL) mixture. K_2CO_3 (8 mg, 57.2 μmol, 4 equiv) was added to the solution. The mixture was stirred for 16 h at 21 °C. Subsequently the reaction mixture was diluted with AcOEt, extracted with 1 M HCl solution twice and once with brine. The washed organic fraction was dried over $MgSO_4$ and concentrated by rotary evaporation. The concentrate was precipitated in ice cold MeOH and subsequently washed with cold MeOH (3×) and dried in vacuo to obtain **4o** as a white solid. Yield: 83%. The sulfur peaks could not be seen on the NMR spectrum.

Synthesis of 3-Azido-7-hydroxycoumarin. 3-Azido-7-hydroxycoumarin was prepared as described by Yi et al.[52] with a yield of 15%.

Preparation of PEG-*b*-PS Polymersomes. Modified from a previous report,[53] a general procedure is described: MeO-PEG_{44}-*b*-PS_{170} (9 mg) and R-PEG_{44}-*b*-PS_{183} (1 mg) were dissolved in a mixture of THF and 1,4-dioxane (1 mL, 4:1 by volume) in a 15 mL capped vial with a magnetic stirrer. After dissolving the solution for 0.5 h at 21 °C, a syringe pump equipped with a syringe and a needle was used to deliver Milli-Q water with a rate of 1 mL/h for 0.5 h via a rubber septum, while vigorously stirring the mixture (900 rpm). When finishing the water addition, 10 mL of Milli-Q was added to the suspension, which ensured a rapid quenching of the PS domain within the bilayer of the polymersomes. The polymersomes were spun down via centrifuge and washed with Milli-Q a total of three times.

Preparation of PEG-*b*-PDLLA Polymersomes. MeO-PEG_{22}-*b*-$PDLLA_{94}$ (9 mg) and R-PEG_{44}-*b*-$PDLLA_{183}$ (1 mg) were dissolved in a mixture of THF and 1,4-dioxane (1 mL, 4:1 by volume) in a 15 mL capped vial with a magnetic stirrer. After mixing the solution for 0.5 h at 21 °C, an equivalent Milli-Q water was added via a syringe pump with a rate of 1 mL/h for 1 h via a rubber septum, while vigorously stirring the mixture (900 rpm). Afterward, the samples were dialyzed against 1 L of Milli-Q water for 24 h with a solution change after 1 h at 4 °C (membrane cut-off 12−14 kDa).

Click Reaction on DBCO-PEG-*b*-PS Polymersomes. A total of 20 μL of concentrated DBCO-PEG-*b*-PS polymersomes were diluted in 1 mL of Milli-Q water. 3-Azido-7-hydroxycoumarin (0.5 mg) was added to the solution and the mixture was stirred for 16 h in the dark before measuring fluorescence.

RESULTS AND DISCUSSION

Synthesis of the Base Polymer. From a retrosynthetic point of view, almost any functionalized group can be covalently attached to the polymers via an amide coupling to compounds **4** and **7** (Scheme 1). Amine chemistry is quite versatile, reacting highly effectively with carboxylic acids, esters, and acyl halides.[54] The amine-functionalized PEG-*b*-PS and PEG-*b*-PDLLA themselves can be made from the azide-functionalized polymers.[55,56] We envision this method being used generally for bottom-up built nanoparticles, since this

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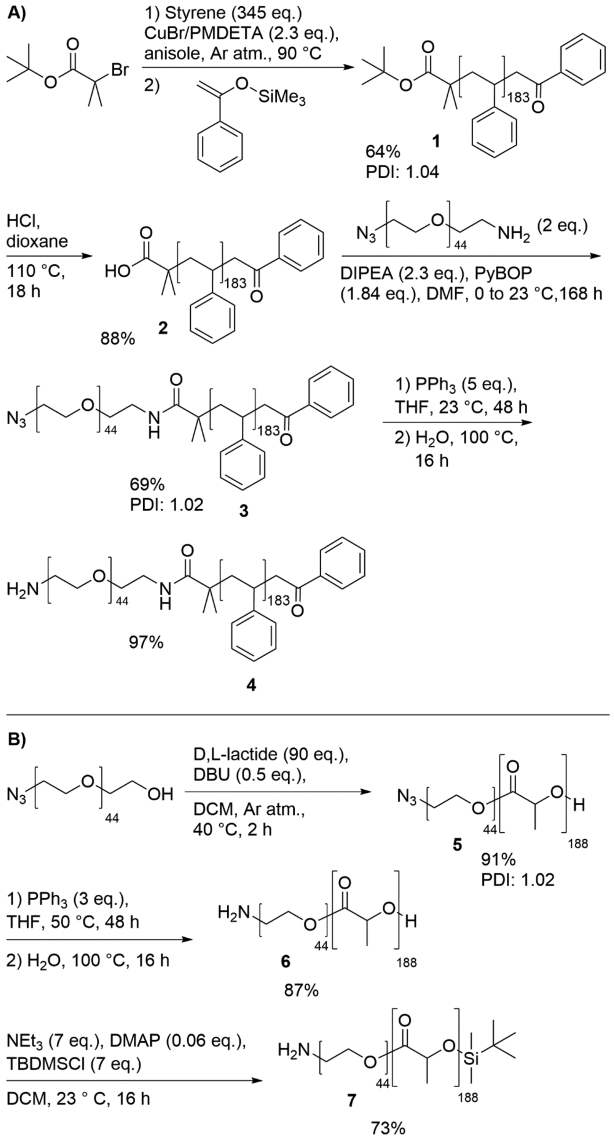
approach is possible for any polymer without reactive side groups.

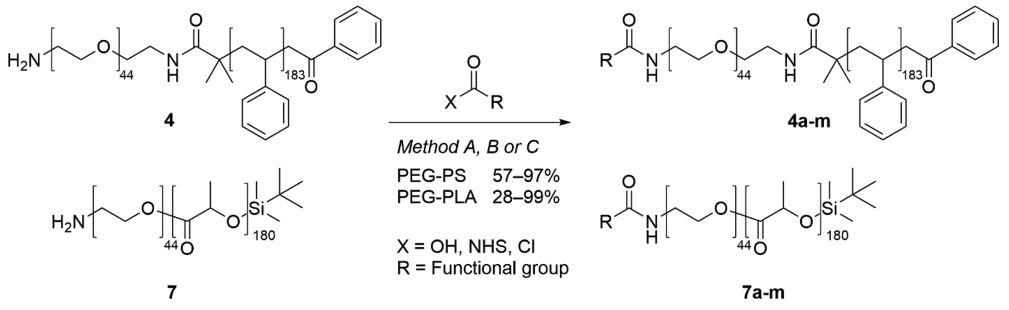
The forward synthesis of H_2N-PEG-*b*-PS **4** (Scheme 2A) commenced with the growth of polystyrene onto *tert*-butyl-*α*-bromoisobutyrate by ATRP, to obtain **1** with an average length of 183. This length was confirmed by ^1H NMR, and the GPC showed a PDI of 1.04. This was followed by deprotection to obtain **2**, as described in earlier work.[49] Next, we attached *α*-azide-PEG_{44}-*ω*-amine via an amide bond using benzotriazole-1-yloxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) as the coupling reagent, together with *N*,*N*-diisopropylamine (DiPEA) in DMF. This was done in contrast to previous work using amine functionalization of both ends of the polymer[40] in order to prevent triblock formation. Due to the large size of the polymers in comparison to the reactive groups, this reaction took 168 h to go to completion. Progress of the reaction was followed by TLC. Formation of **3** was corroborated by ^1H NMR and diffusion NMR, showing comparable diffusion speeds for both the PEG and PS signals, whereas uncoupled PEG has an approximately 10× faster diffusion speed compared to PS, indicating successful coupling (Figure S1). Finally, the azide was reduced by using a Staudinger reduction.[57] An excess of triphenylphosphine was added to the polymer in THF and allowed to stir for 48 h to ensure formation of the iminophosphorane. Afterward, water was used to reduce the iminophosphorane to the amine, which was shown on TLC. After precipitation, this afforded **4** in 97% yield.

Scheme 2. Strategy for the Synthesis of Amine-Functionalized PEG-*b*-PS and PEG-*b*-PDLLA Polymers



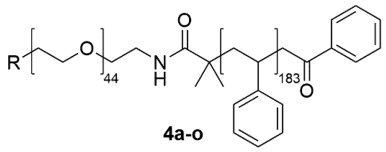
Library Synthesis. With the obtained amino-PEG-*b*-PS and amino-PEG-*b*-PDLLA block-copolymers, we started creating a library. This was done via three general methods, making it easy to couple carboxylic acids, NHS esters, and acyl halides (Scheme 3). Most of the functional handles on PEG-*b*-PS were introduced via method A, by means of amide coupling of carboxylic acids or NHS esters using EDC coupling reagent as well as DMAP.[58] Most of the functional handles on PEG-*b*-PDLLA were introduced via method B, using PyBOP and DIPEA as coupling reagents to form an amide bond from the respective carboxylic acids and NHS esters. For this method, it was discovered that activation of the acid should be performed before adding the polymer. When adding all reagents together, NMR showed that a portion of the polymer had decomposed as a result of the reaction conditions used. The PyBOP and DIPEA appear to have reacted with the polymer instead of activating the acid. This may have caused the ester bonds of

Scheme 3. Strategy for the Functional Handle Library Synthesis of PEG-*b*-PS and PEG-*b*-PDLLA Polymers

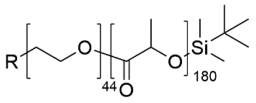


https://dx.doi.org/10.1021/acs.biomac.9b01734
Biomacromolecules 2020, 21, 1853−1864

Table 1. Scope of the Handle Functionalization Reaction



4a-o

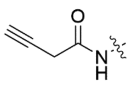


7a-m

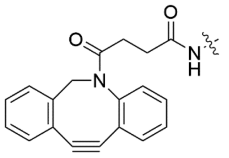
R =

3 69%
5 91%

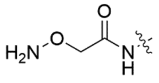
4 97%
7 73%



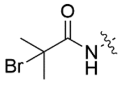
4a 66%
7a 66%



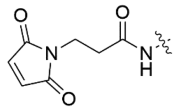
4b 62%
7b 70%



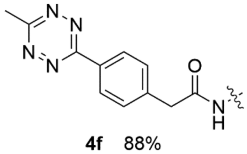
4c 57%
7c 28%



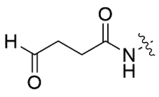
4d 91%
7d 71%



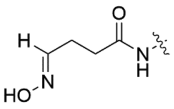
4e 84%
7e 63%



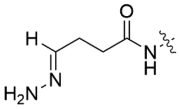
4f 88%
7f 86%



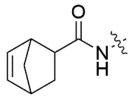
4g 97%
7g 72%



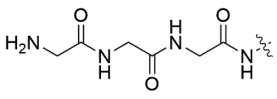
4h 86%
7h 71%



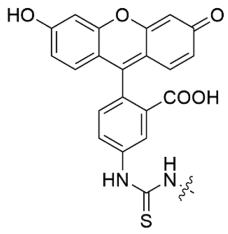
4i 76%
7i 54%



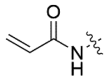
4j 93%
7j 70%



4k 83%
7k 85%



4l 81%
7l 50%



4m 69%
7m 99%

4n 70%

4o 83%

the PDLLA polymer to decompose. For this reason, the polymer is added 1 h after activation of the carboxylic acid. Finally, acyl halides were coupled via method C, using only Et_3N as a base. Due to the high reactivity of acyl halides,[43] no further coupling reagents are required.

Proving the attachment of the varying functional groups is difficult via the conventional methods used in organic chemistry. Due to the extremely small weight percent of the functional group compared to the bulk polymer, functional groups are virtually invisible on methods such as NMR and IR spectroscopy, not being able to overcome the noise of the spectra. For this reason as well, an amine functionality was chosen as the base module, as this provides another method of proving the successful functionalization. The amide couplings were all followed by means of a Kaiser test. This qualitative test shows the presence of free terminal amine in products by appearing either blue when free amine is present or yellow when there is not. The test is able to show 99.9% completion of the amide bond formation,[47] making it a very potent tool for ensuring full conversion of the polymer to the desired handle.

Using this method, virtually any functional group could be attached to the polymer, provided it does not react with the amine functionality. We aimed to make a library that shows the versatility of this approach, both from a synthetic side as well as a more biochemical side (Table 1). The azide (**3** and **5**) and amine (**4** and **7**) functional groups that were made during the synthesis of the modular polymers can already be used as handles, as has been demonstrated before.[13,19,59] First, we made the propargyl (**4a** and **7a**) and DBCO-functionalized polymers (**4b** and **7b**) in decent yields. These were chosen as examples for the click reaction, which is often used in biorthogonal chemistry. Commonly, the azide handle is placed on the nanoparticle,[18] but the reverse has also been shown to be successful in the biochemical field.[60] Next, an aminooxy group (**4c** and **7c**) was attached in moderate yields. Although full conversion was confirmed via the Kaiser test, the lower yield is most likely due to the precipitation not being optimal with this highly polar group. The aminooxy group allows the formation of extremely stable oxime linkages with aldehyde groups, making it easy to use for both chemical and biological purposes.[61] The addition of a halide linker led to the formation

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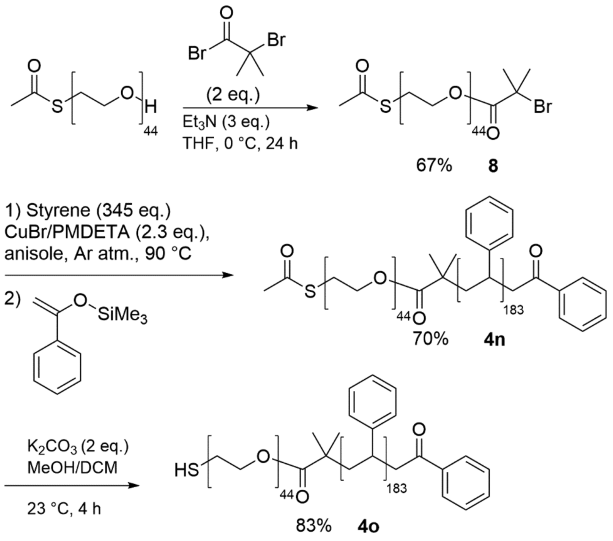
of the bromine-functionalized **4d** and **7d** in high yield. Having a bromine handle on the polymer allows for further polymerization after self-assembly via ATRP, as has been demonstrated.[49] Next, a maleimide group (**4e** and **7e**) was attached in good yield. These are commonly used in biorthogonal chemistry to perform Diels–Alder reactions[62] and couple to thiols.[63] Continuing with the biorthogonal linkage, a tetrazine group was attached as well with high yield (**4f** and **7f**). These versatile handles can perform inverse-demand Diels–Alder reactions with various functional groups, such as norbornenes and vinyl boronic acids.[64] An aldehyde functionality was made in two steps (**4g** and **7g**) with high yield. The aldehyde had a 1,3-dioxalane protecting group to ensure no imine was formed, which was removed after the amide coupling went to completion. The aldehyde functionality can be used to make oxime bonds, similar to the aminooxy functionality, as well as form imines. From the aldehyde, two other functional handles were made; an oxime (**4h** and **7h**) and a hydrazone (**4i** and **7i**). Oximes can undergo a Tiemann rearrangement, providing substituted ureas.[65] Hydrazones on the other hand are known for their ability to exchange functional groups with other hydrazones, making this an interesting linkage for exchanging functionalization on the nanoparticle.[66] A norbornene handle (**4j** and **7j**) was made as it can react with tetrazines,[21] as well as form nitrile oxide cycloadditions.[24]

In order to show the versatility of this modular approach, various examples other than biorthogonal linkage handles were made. First, a triglycine was attached to the polymer (**4k** and **7k**) in two steps, coupling and deprotection. Functionalization of nanomotors with peptides could make them recognizable by various receptors and enzymes. In this example, triglycine is recognized by the enzyme Sortase A, which allows for a biological approach for the functionalization of polymersomes.[67] Next, a commonly used dye, Fluorescein isothiocyanate (FITC), was attached to the polymers (**4l** and **7l**). This dye was chosen because it is one of the most popular fluorescent probes in the scientific field. FITC has been used mainly for fluorescence detection by labeling antibodies. This allows for detection of antigens, among others.[68] Due to its fluorescence, the presence of the FITC group was also determined by UV–vis (Figures S2 and S3).

Finally, an acryloyl handle was attached to the modular polymers (**4m** and **7m**). These handles can be used to form bonds with sulfur groups,[69] opening the possibility for peptide-conjugated vesicles using thiol chemistry. It is of course also possible to use sulfur handles on the nanovessicle, making it able to bind substrates that feature an acryloyl group. To our knowledge, however, there have been few reports of thiol-decorated vesicles being utilized in this context.[70] This drove us to also pursue a thiol handle as part of our library. Since no thiol suitable for our general coupling methods could be found commercially, we opted to synthesize the thiol-functionalized polymer from scratch (Scheme 4). An acetylated thiol-functionalized PEG was coupled to an ATRP initiator, forming **8** in moderate yield. The thiol is protected to ensure no thioesters could be formed. Afterward, polystyrene was grown onto the PEG similar to the formation of **1**, leading to an acetylated thiol-functionalized PEG-*b*-PS polymer **4n**. The protected thiol is also a useful handle when incorporating multiple functional groups onto a nanoparticle; it allows for selective reactivity on the other handle, after which the thiol can be deprotected to allow further reactivity. For this reason,

Scheme 4. Strategy for the Synthesis of Thiol-Functionalized PEG-*b*-PS Polymer



it is also incorporated in the library. Removal of the acetyl was performed by the use of a base in methanol, with addition of DCM to ensure solubility of the polymer. Upon removal of the acetyl moiety, **4o** was obtained in high yield.

Formation of Polymersomes. All polymersomes were made using 90% MeO-PEG$_{44}$-*b*-PS$_{170}$ and MeO-PEG$_{22}$-*b*-PDLLA$_{94}$, respectively, as the main component, since these materials are known to readily self-assemble into polymersomes,[71] adding 10% of the functionalized polymer. The decorated polymers are slightly longer or just as long as the unreactive polymers to ensure availability on the surface. After dissolving the polymer in organic solvent (4:1 THF/1,4-dioxane), 0.5 mL of ultrapure water was added slowly at 1 mL/h, inducing self-assembly into spherical polymersomes. Afterward, the PEG-*b*-PS polymersomes were quenched by adding 10 mL of ultrapure water, after which the suspension was transferred to a centrifuge tube. The suspension was purified 3× by centrifugation to remove organic solvent, and finally suspended in 1 mL of ultrapure water as a 10 mg/mL suspension. Polymersomes were made for all handles in the library. The integrity of the polymersomes was verified by transmission electron microscopy (TEM). All handles from the library were able to self-assemble into polymersomes without disturbance in the membrane morphology via the general method, despite size or polarity of the various handles (Figures S4–S20), comparable to polymersome formation without any functional polymers (Figure S21). DLS data also shows that all batches have similar size distributions (Figures S22–S39), averaging at 638 nm. The DBCO handle, the sterically largest and most hydrophobic of the functional handles, can be seen in Figure 2A. This sample was also visualized by cryo-TEM to show the undisturbed polymersome structure (Figures 2B and S40), as were the FITC and thiol handles (Figures S41–S43), due to their large potential impact on the structure. To ensure that the functionalized polymers are incorporated into the structure during the self-assembly, a 50× diluted FITC handle incorporated polymersome sample was examined by fluorescence spectrometry, showing an ∼440× increase in fluorescence compared to the blank (Figure S44). This high increase shows incorporation of the functionalized polymers in the structure. However, it is possible that large hydrophobic

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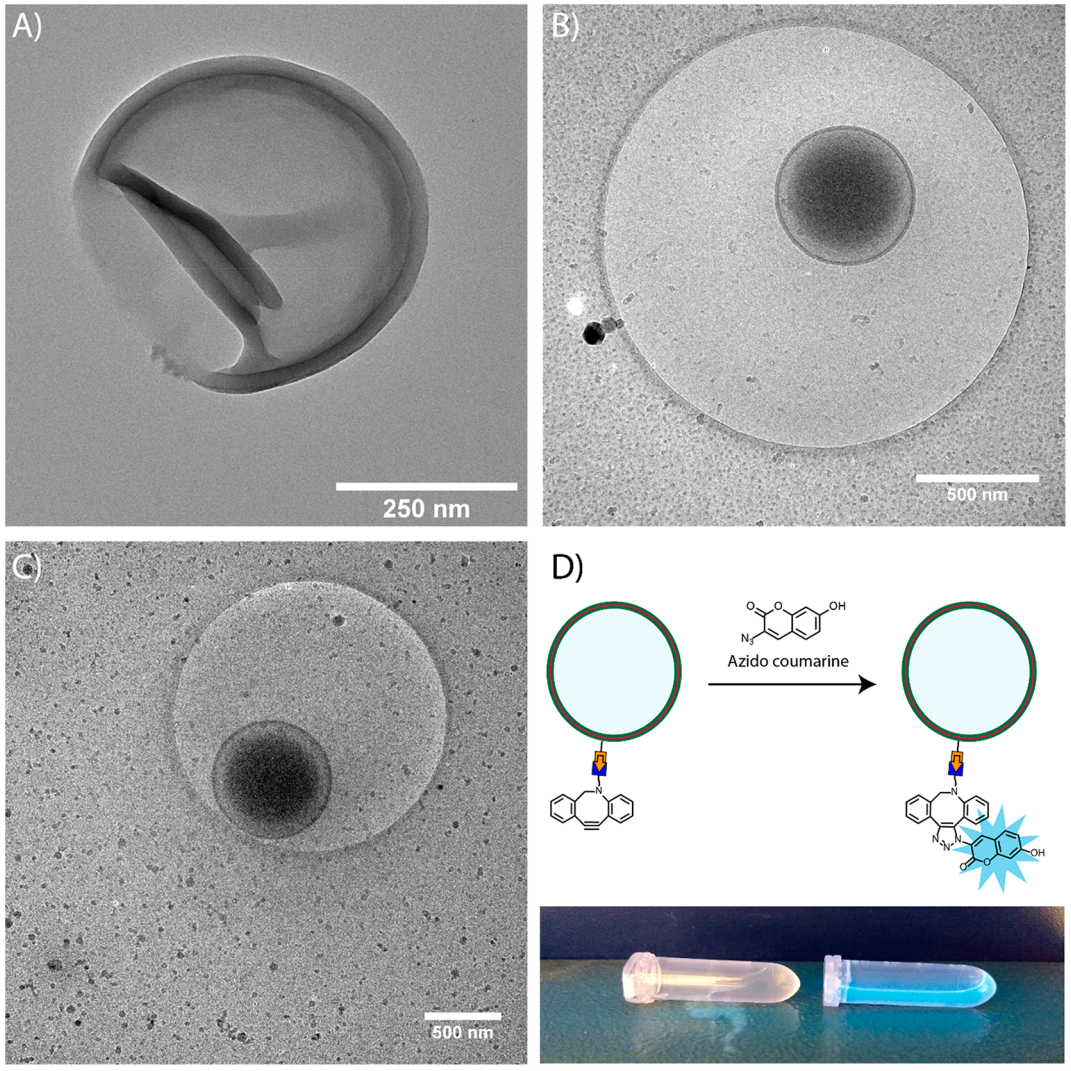

Figure 2. (A) TEM image and (B) cryo-TEM image of spherical 10% DBCO-PEG$_{44}$-*b*-PS$_{183}$ and 90% MeO-PEG$_{44}$-*b*-PS$_{170}$ polymersomes. (C) Cryo-TEM image of spherical 10% tetrazine-PEG$_{44}$-*b*-PDLLA$_{180}$ and 90% MeO-PEG$_{22}$-*b*-PDLLA$_{94}$ polymersomes. (D) Click reaction between DBCO-decorated PEG-*b*-PS polymersomes and 3-azido-7-hydroxycoumarin (right), leading to fluorescence. As a control, 3-azido-7-hydroxycoumarin was added to nonfunctionalized polymersomes, yielding no fluorescence (left).

probes fold into the polystyrene, making them unreachable for any reactivity to take place. This is mainly the case for the sterically larger and more hydrophobic handles. To verify the availability of the handles on the surface, we opted to test the reactivity of the largest and most hydrophobic entry in the library. For this reason, the DBCO linker was examined by reacting a 50× dilution of polymersomes with 3-azido-7-hydroxycoumarin in water overnight. The cycloaddition reaction was shown to have succeeded by confirming its fluorescence visually (Figure 2D), as well as via fluorescence spectroscopy, showing an ∼200× increase in fluorescence compared to the blank (Figure S44). Based on this evidence, we believe this indicates the accessibility of the wide range of functional handles presented in this library.

For PEG-*b*-PDLLA, the polymers were dissolved in 1 mL of organic solvent (4:1 THF/1,4-dioxane), after which an equivalent of ultrapure water was added slowly at 1 mL/h. All samples formed a cloudy solution, indicating the polymersome formation. Subsequently, the samples were dialyzed for 24 h against ultrapure water, with a solution change after 1 h to remove the organic solvent. The method of dialysis was chosen instead of quenching using ultrapure water, as was done for the PEG-*b*-PS polymersomes, because quenching the structures in a swift manner led to the formation of aggregates in some cases. From several samples, cryo-TEM images were taken to verify the morphology (Figures S45−S48). The tetrazine handle is highlighted in Figure 2C, as it is one of the more sterically larger and hydrophobic handles. The samples were analyzed by DLS to see their size distributions (Figures S49−S64) averaging at 354 nm. From these data it can be seen that the size and PDI of the polymersomes are influenced by the incorporation of different handles.

Polymersomes have been used for in vivo applications; however, sizes of ∼100 nm are optimal for biorelated applications.[72] The most common method for the fabrication of small-sized polymersomes is resizing through either extrusion or sonication.[73] Earlier work in our group has shown the possibility of producing small-sized polymersomes using the extrusion method.[74,75] These methods make it possible to reduce the size of the average size of the sample, making the polymersomes applicable for use in biomedical applications. Another challenge is the limitation in catalysts

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currently available to drive motion. We aim to broaden the fuel scope of block copolymer based nanomotors by using homogeneous catalysis, which has been absent from the nanomotor field, having only several examples in the literature.[76] This approach, however, requires options for chemical ligation to these polymer systems. We hope this general methodology to incorporate handles allows for an expansion in the variety of fuel sources and applications of nanomotors, allowing not only for the incorporation of catalysts and enzymes, but also drugs and imaging tools.

CONCLUSIONS

In conclusion, we have developed a general route for the synthesis of terminally functionalized PEG-*b*-PS and PEG-*b*-PDLLA via a modular approach. By placing a terminal amine on the polymers, a wide scope of functionalities can be achieved without the need for repeating the polymer synthesis for every different moiety. A library containing 32 different polymers has been synthesized. The examples used in the library reflect the wide scope of nanovesicle applications, introducing new chemical ligation techniques, as well as biochemical coupling options and fluorescence incorporation. Well-defined polymersome structures with 10% incorporation of the functional polymers were generated of every polymer in the library, of both the PEG-*b*-PS and the biodegradable PEG-*b*-PDLLA block copolymers. Self-assembly by using the fluorescent FITC-functionalized polymer showed the incorporation of these handles into the polymersome structure. Attachment of the fluorescent azidocoumarin to the DBCO-decorated polymersomes showed the availability of the functional groups, allowing for chemical ligation on the surface via click-reaction. Finally, we envision this methodology to be used for various polymer-based bottom-up systems, being applicable to a wide assortment of polymers, and leading to more covalent approaches in nanomotor and drug delivery design.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.biomac.9b01734.

TEM and cryo-TEM images of polymersomes, DLS data of polymersomes, fluorescence data, and NMR of all synthesized compounds (PDF)

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Notes

The authors declare no competing financial interest.

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