Polymersome-Forming Amphiphilic Glycosylated Polymers: Synthesis and Characterization

Ahmed M. Eissa,[1,2] Michael J.P. Smith,[1] Artur Kubilis,[1] Jackie A. Mosely,[1] Neil R. Cameron[1]

[1]Department of Chemistry and Biophysical Sciences Institute (BSI), Durham University, South Road, Durham DH1 3LE, United Kingdom

[2]Department of Polymers, Chemical Industries Research Division, National Research Centre (NRC), Dokki, Cairo, Egypt

Correspondence to: N. R. Cameron (E-mail: n.r.cameron@durham.ac.uk)

Received 30 July 2013; accepted 18 September 2013; published online 16 October 2013

DOI: 10.1002/pola.26959

ABSTRACT: Copper-catalyzed azide-alkyne cycloaddition (CuAAC) was used to prepare glycosylated polyethylene (PE)–poly(ethylene glycol) (PEG) amphiphilic block copolymers. The synthetic approach involves preparation of alkyne-terminated PE-*b*-PEG followed by CuAAC reaction with different azide functionalized sugars. The alkyne-terminated PE-*b*-PEG was prepared by etherification reaction between hydroxyl-terminated PE-*b*-PEG ($M_n \sim 875$ g mol^{-1}) and propargyl bromide and azidoethyl glycosides were prepared by glycosylation of 2-azidoethanol. Atmospheric pressure solids analysis probe-mass spectrometry was used as a novel solid state characterization tool to determine the outcome of the CuAAC click reaction and end-capping of PE-*b*-PEG by the azidoethyl glycoside group. The aqueous solution self-assembly behavior of these amphiphilic glycosylated polymers was explored by TEM and dye solubilization studies. Carbohydrate-bearing spherical aggregates with the ability to solubilize a hydrophobic dye were observed. The potential of these amphiphilic glycosylated polymers to self-assemble via electro-formation into giant carbohydrate-bearing polymersomes was also investigated using confocal fluorescence microscopy. An initial bioactivity study of the carbohydrate-bearing aggregates is furthermore presented. © 2013 Wiley Periodicals, Inc. J. Polym. Sci., Part A: Polym. Chem. **2013**, *51*, 5184–5193

KEYWORDS: amphiphiles; amphiphilic copolymers; ASAP mass spectrometry; block copolymers; click glycosylation reaction; electro-formation; functionalization of polymers; giant vesicles; polymersomes; self-assembly; vesicles

INTRODUCTION Polymers that can self-assemble into various nanostructures in a selective solvent have found many potential applications in the areas of biomaterials,[1] sensors,[2] emulsion stabilization,[3] and electronics.[4,5] In particular, amphiphilic block copolymers are capable of forming a range of nanoscale species including micelles, nanoparticles, and vesicles.[6] Anchoring a bioactive functional group to the backbone provides the possibility of active targeting of the polymer to a particular site *in vivo*. In particular, carbohydrate-bearing polymers are of considerable interest due to their importance and potential applicability in materials science and biomedicine.[7] Amphiphilic glycopolymers, synthetic polymers with pendent carbohydrate moieties, have been prepared previously and demonstrated to be able to form a variety of nanostructures including spherical micelles,[8,9] worm-like micelles,[10] nanocapsules,[11] and vesicles.[12–15] These complex supramolecular structures display bioactivity, which originates from the ability of the pendent carbohydrate moieties on the surface of the self-assembled structures to interact with carbohydrate binding proteins (lectins).[16] Many recognition events are thought to proceed by specific carbohydrate–lectin interactions.[17] From the molecular recognition sense, finding suitable functional polymeric carriers which are noncytotoxic, nonimmunogenic, and stable at a wide range of pH is highly desirable.

The synthesis of bioactive amphiphilic glycopolymers is challenging, involving the construction of specific block copolymers by controlled polymerization and the synthesis of either polymerizable carbohydrate derivatives or functionalized glycans capable of attachment to a reactive polymer precursor. A simpler approach to amphiphilic glycosylated polymers would involve the end-functionalization of commercially available amphiphilic block copolymers. However, these are quite rare; notable examples include poly(ethylene oxide)-*b*-(propylene oxide)s (PEO-PPOs). One interesting example is poly(ethylene-*b*-(ethylene glycol)) (PE-*b*-PEG) which is readily available at a low average molecular weight ($M_n \sim 875$ g mol^{-1}). It combines the biocompatibility and physicochemical properties of the water soluble PEG block with the hydrophobic PE chains that can associate leading to the formation of different nanoscale morphologies. This has

been demonstrated by several studies that report the creation of a wide array of nanostructures including large crystal platelets,[18,19] nanodisks,[20] wormlike micelles,[21] and spherical multicore micelles.[22] At present, however, only a limited number of examples of PE-based materials capable of forming vesicles exist.[23,24] We therefore chose to explore the glycosylation of PE-*b*-PEG block copolymers as a facile route to the construction of polymersome-forming amphiphilic glycosylated polymers.

Click chemistry, in particular the Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC) reaction, has received significant attention from polymer chemists in the field of glycopolymer synthesis due to the high efficiency and technical simplicity of the reaction.[25] In this contribution, a click glycosylation reaction between alkyne end-capped PE-*b*-PEG and different azidoethyl functionalized sugars was used to afford amphiphilic block copolymers. Characterization of the glycosylated PE-*b*-PEGs was challenging due to their limited solubility behavior and low molecular weight; hence, atmospheric pressure solids analysis probe (ASAP) mass spectrometry,[26] a technique that has been used to analyze polymers in the solid state, was used for direct analysis of the glycosylated polymer products. Interestingly, despite poor solubility, the glycosylated PE-*b*-PEGs were found to be able to self-assemble in aqueous solution to form spherical aggregates that can solubilize a hydrophobic fluorescent molecule, Nile Red. The bioactivity of these glycosylated macromolecular aggregates was investigated using a turbidimetric assay using the lectin Concanavalin A (Con A). The assembly of glycosylated PE-*b*-PEGs into polymersomes was also attempted using the electroformation method. Preliminary evidence presented here suggests these polymers are promising precursors for the formation of glycosylated giant unilamellar vesicles (GUVs) which have potential application in medicine as model systems to study biological processes mediated by carbohydrates.

EXPERIMENTAL

Materials

Polyethylene-*block*-poly(ethylene glycol) H-$(PE)_n$-*b*-$(PEG)_m$-OH ($M_n \sim 875$, ethylene oxide ~20 wt %) was purchased from Sigma–Aldrich. 2-Azidoethanol was prepared following a reported procedure.[27,28] All other chemicals and reagents used in the synthesis were also purchased from Sigma–Aldrich and used without further purification. All dry solvents were obtained from the Solvent Purification System, Department of Chemistry, Durham University.

Instrumentation and Measurements

^{1}H-NMR spectra were recorded using the deuterated solvent lock on a Varian Mercury spectrometer at 400 MHz. Chemical shifts are quoted in ppm, relative to tetramethylsilane (TMS), as the internal reference. ^{13}C-NMR spectra were recorded at 101 MHz (2000 scans) using continuous broadband proton decoupling and a 3 s recycle delay and are therefore not quantitative; chemical shifts are quoted in ppm, relative to $CDCl_3$ (77.55 ppm).

Solid-state NMR spectra were obtained using either a Varian VNMRS spectrometer operating at 100.56 MHz for ^{13}C (399.88 MHz for ^{1}H) or a Varian Unity Inova instrument operating at 75.40 MHz for ^{13}C (299.82 MHz for ^{1}H). They were obtained under magic-angle spinning conditions with spin-rates in the range 10,000–14,000 Hz. Carbon NMR spectra were recorded with cross-polarization (typically with a recycle delay of 1.0 s and a contact time of 1.00 ms) or direct excitation (typically with a recycle delay of 1.0 s) and with ^{1}H decoupling. ^{1}H spectra were obtained with direct excitation. Samples were run as prepared and spectral referencing is with respect to external, neat TMS.

The design of ASAP and discussion of its modes of operation have been previously discussed.[29] ASAP MS measurements were performed on a Xevo QToF mass spectrometer (Waters Corp., Manchester, United Kingdom). Positive ions were recorded between 50 and 2000 Da. The majority of ion source parameters remained constant throughout the experiments including capillary voltage (3.00 kV), corona current (2.5 μA), extraction cone voltage (2 V), source temperature (150 °C), nitrogen cone gas flow (0 L h^{-1}), and nitrogen desolvation gas flow (600 L h^{-1}). The remaining ion source parameters desolvation gas temperature (400–450 °C) and sample cone voltage (10–50 V) were optimized during the experiments to maximize the intensity of the observed ions.[30]

FTIR spectra were recorded on a Perkin Elmer 1600 series FTIR spectrometer fitted with Diamond ATR crystal unit.

Confocal fluorescence microscopy observations were performed on a Nikon Diaphot microscope coupled with Bio-Rad μRadiance confocal scanning system equipped with a Nikon Plain 50× NA 0.85 oil-immersion objective using He–Ne laser with excitation at 543 nm in combination with a PMT and emission filter at 570LP for detection.

TEM images were obtained from a Hitachi H-7600 instrument operating at 100 kV. Aqueous solutions of glycosylated polymer (PE-*b*-PEG β-D-glucoside **9**) were prepared by the solvent-switch method (see below, "Fluorescent Dye Entrapment" section). The sample (10 μL) was absorbed onto a freshly glow-discharged grid (400 mesh copper grid coated with Formvar) for 1 min before the excess was removed by blotting. The grid was then dipped in a drop of 1% aqueous uranyl acetate solution (as a staining agent) for 30 s and the excess was removed by blotting. The TEM (camera system) is operated using AMT 600 software.

Synthesis of Alkyne End-Capped PE-*b*-PEG 1

A solution of PE-*b*-PEG (5.0 g, 5.7 mmol) in dry THF (50 mL) was added dropwise to a slurry of NaH (165 mg, 6.9 mmol) in dry THF (10 mL) at 0 °C in a three-necked round-bottom flask, equipped with an addition funnel and a rubber seal septum, under N_2 atmosphere. The mixture was

stirred for 30 min and propargyl bromide (80% in toluene, 800 μL, 6.9 mol) was added via a syringe. The mixture was kept at ambient temperature for a further 2 h and then stirred at 70 °C for 6 h. DCM (50 mL) was added and the solution was washed with HCl (1 N aqueous solution) followed by distilled water (3×). The solvent was removed under reduced pressure to give the alkyne end-capped PE-*b*-PEG **1** (4.6 g, 88%).

^{1}H-NMR (400 MHz, $CDCl_3$) δ 4.14 (s, 2H, —O—CH_2—C≡CH), 3.58 (m, 12H, —CH_2—CH_2—O—), 3.37 (t, $J = 7.08$ Hz, 2H), 2.81 (s, 1H), 2.36 (s, 1H, —CH_2—C≡CH), 1.20 (bs, 49H, CH_3—CH_2—CH_2—), (t, $J = 6.59$ Hz, 3H, CH_3—CH_2—).

Synthesis of 2-Azidoethanol 2

In a round-bottom flask, sodium azide (7 g, 108 mmol) was dissolved in water (20 mL). 2-Chloroethanol (5.6 mL, 83 mmol) was added and the mixture was then stirred at 80 °C for 24 h. The reaction mixture was cooled down to room temperature, saturated with sodium chloride, and the product was extracted with DCM (3× 20 mL). The organic layer was dried over sodium sulfate and then the solvent was removed under reduced pressure to obtain 2-azidoethanol **2** as a colorless liquid (7.5 g, 80%).

^{1}H-NMR (400 MHz, $CDCl_3$) δ 3.71 (q, $J = 5.7$ Hz, 2H, —CH_2—OH), 3.37 (t, $J = 5.0$ Hz, 2H, —CH_2—N_3), 2.60 (t, $J = 5.8$ Hz, 1H, —CH_2—OH). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 61.40 (—CH_2—OH), 53.49 (—CH_2—N_3).

Synthesis of 2′-Azidoethyl 2,3,4,6-Tetra-*O*-acetyl-*β*-D-glucoside 3

In a two-necked 100-mL round-bottom flask, *β*-D-glucose penta-acetate (5.0 g, 12.8 mmol) was dissolved in dry DCM (25 mL) under N_2 atmosphere. 2-Azidoethanol **2** (1.9 g, 22.3 mmol) was added to this solution by a syringe. The resulting solution was stirred under N_2 and cooled to 0 °C. $BF_3{\cdot}Et_2O$ (2.1 mL, 16.7 mmol) was then added dropwise at 0 °C. The mixture was stirred for 1 h at 0 °C and then overnight at ambient temperature. The mixture was diluted with DCM (50 mL) and washed with cold water and with saturated aqueous $NaHCO_3$, dried over anhydrous sodium sulfate, and concentrated in rotary evaporator to obtain a viscous liquid crude residue. The product was purified by recrystallization from the solution of methanol in order to obtain the 2′-azidoethyl-2,3,4,6-tetra-*O*-acetyl-*β*-D-glucoside **3** (2.5 g, 45%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.15 (t, $J = 9.47$ Hz, 1H), 5.03 (dd, $J = 10.0$, 9.3 Hz, 1H), 4.95 (dd, $J = 9.6$, 7.9 Hz, 1H), 4.53 (d, $J = 7.9$ Hz, 1H), 4.19 (dd, $J = 12.3$, 4.7 Hz, 1H), 4.10 (dd, $J = 12.3$, 2.5 Hz, 1H), 3.96 (ddd, $J = 10.7$, 4.9, 3.5 Hz, 1H), 3.71–3.56 (m, 2H), 3.47–3.38 (m, 1H), 3.22 (ddd, $J = 13.4$, 4.9, 3.3 Hz, 1H), 2.02 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.94 (s, 3H). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 170.53, 170.16, 169.30, 100.66, 72.81, 71.99, 71.11, 68.42, 68.41, 61.87, 50.54, 20.65, 20.60, 20.52. HR ESI MS: *m/z* 440.1267 corresponds to $[C_{16}H_{23}N_3O_{10}]^+$ within 3 ppm. FTIR: ∼2100 cm^{-1} (C—N_3 stretching vibration), ∼1750 cm^{-1} (C═O stretching vibration).

Synthesis of 2′-Chloroethyl α-L-Fucoside 4

In a two-necked 100-mL round-bottom flask, a mixture of α- and *β*-L-fucose (1.64 g, 10.00 mmol), 2-chloroethanol (10 mL), and Dowex 50W-100 (H^+) (1.00 g) was stirred under N_2 atmosphere at 80 °C for 2 h. The mixture was cooled to room temperature. The resin was removed by filtration and washed with ethanol (3× 2 mL). The solvent was removed from the filtrate under reduced pressure. The crude product was recrystallized with ethanol to give the 2′-chloroethyl α-L-fucoside **4** as crystals (1.2 g, 55%).

^{1}H-NMR (400 MHz, D_2O): δ 4.84 (d, $J = 3.9$ Hz, 1H), 4.07 (q, $J = 7.1$, 6.4 Hz, 1H), 3.88–3.61 (m, 7H), 1.11 (d, $J = 6.6$ Hz, 3H). ^{13}C-NMR (101 MHz, D_2O): δ 98.60, 71.81, 69.54, 68.47, 67.99, 66.89, 43.41, 15.25.

Synthesis of 2′-Chloroethyl 2,3,4-Tri-*O*-acetyl-α-L-fucoside 5

In a one-necked round-bottom flask, 2-chloroethyl α-L-fucoside **4** (400 mg, 1.76 mmol) was dissolved in a mixture of pyridine/acetic anhydride (1:1, v/v, 5 mL) at ambient temperature for 2 h. The reaction was quenched by stirring with ice (6 g) for 1 h. The product was extracted with DCM (60 mL) and washed with 1 M HCl (3× 40 mL) and brine (3× 40 mL). The organic layer was dried over sodium sulfate and then the solvent was removed under reduced pressure. The crude product was purified by flash column chromatography on silica gel using dichloromethane/EtOAc (9:1, v/v) as an eluent to give the 2′-chloroethyl 2,3,4-tri-*O*-acetyl-α-L-fucoside **5**, $R_f = 0.55$ (373 mg, 60%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.31 (dd, $J = 10.5$, 3.4 Hz, 1H), 5.24 (dd, $J = 3.4$, 1.3 Hz, 1H), 5.09–5.00 (m, 2H), 4.18 (q, $J = 7.0$, 6.5 Hz, 1H), 3.86 (dt, $J = 11.0$, 5.4 Hz, 1H), 3.68 (ddd, $J = 11.1$, 6.0, 5.0 Hz, 1H), 3.59 (t, $J = 5.6$ Hz, 2H), 2.10 (s, 3H), 2.02 (s, 3H), 1.93 (s, 3H), 1.08 (d, $J = 6.6$ Hz, 3H). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 170.55, 170.54, 169.99, 96.47, 71.09, 68.60, 68.12, 67.91, 64.74, 42.71, 20.80, 20.68, 20.63, 15.85. HR ESI MS: *m/z* 337.20 corresponds to $[M(^{37}Cl) + Na]^+$, *m/z* 375.00 corresponds to $[M(^{35}Cl) + Na]^+$ and *m/z* 273.20 corresponds to $[M\text{-}(OCH_2CH_2Cl)]^+$.

Synthesis of 2′-Azidoethyl 2,3,4-Tri-*O*-acetyl-α-L-fucoside 6

In a two-necked 100-mL round-bottom flask, a suspension of 2′-chloroethyl 2,3,4-tri-*O*-acetyl-α-L-fucoside **5** (440 mg, 1.25 mmol), NaI (188 mg, 1.25 mmol), and sodium azide (813 mg, 12.5 mmol) in DMF (6 mL) was stirred at 80 °C for 18 h. The solvent was evaporated under reduced pressure. DCM (150 mL) was added and washed with water (30 mL) and with brine (3× 30 mL). The organic layer was dried over sodium sulfate and the solvent was removed under reduced pressure to give the 2′-azidoethyl 2,3,4-tri-*O*-acetyl-α-L-fucoside **6** (400 mg, 90%).

^{1}H-NMR (400 MHz, $CDCl_3$): δ 5.31 (dd, $J = 10.2$, 3.4 Hz, 1H), 5.25 (dd, $J = 3.4$, 1.3 Hz, 1H), 5.08 (d, $J = 3.7$ Hz, 1H), 5.05 (d, $J = 1.2$ Hz, 1H), 4.12 (q, $J = 6.5$ Hz, 1H), 3.80 (ddd, $J = 10.8$, 6.1, 3.3 Hz, 1H), 3.55 (ddd, $J = 10.6$, 7.0, 3.3 Hz,

1H), 3.40 (ddd, $J = 13.4$, 7.0, 3.3 Hz, 1H), 3.31 (ddd, $J = 13.4$, 6.1, 3.3 Hz, 1H), 2.10 (s, 3H), 2.02 (s, 3H), 1.92 (s, 3H), 1.09 (d, $J = 6.5$ Hz, 3H). ^{13}C-NMR (101 MHz, $CDCl_3$): δ 170.60, 170.53, 169.97, 96.49, 71.10, 67.97, 67.89, 67.18, 64.70, 50.47, 20.78, 20.67, 20.62, 15.88. HR ESI MS: *m/z* 382.18 corresponds to $[M + Na]^+$. FTIR: ~2100 cm^{-1} (C—N_3 stretching vibration), ~1750 cm^{-1} (C=O stretching vibration).

General Procedure for Synthesis of Glycosylated PE-*b*-PEG Block Copolymers via Click Chemistry

In a two-necked 100-mL round-bottom flask, equipped with a condenser, a mixture of alkyne end-capped PE-*b*-PEG **1** (500 mg, 0.55 mmol), the respective 2′-azidoethyl 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucoside **3** or 2′-azidoethyl 2,3,4-tri-*O*-acetyl-α-L-fucoside **6** (0.55 mmol), copper (II) sulfate pentahydrate (14 mg, 0.055 mmol, in 0.5 mL of water), and sodium ascorbate (22 mg, 0.11 mmol, in 0.5 mL of water) in THF (50 mL) was stirred at reflux (70 °C) for 24 h. The mixture was concentrated using a rotary evaporator and the residue was poured into water. The solid material was collected by filtration and freeze-dried to give the corresponding acetylated glycosylated PE-*b*-PEG **7** and **8**, respectively.

PE-b-PEG Per-O-acetylated β-D-Glucoside 7

Yield: ~95%. ^{1}H-NMR (400 MHz, $CDCl_3$); cloudy solution: δ 7.54 (s, 1H), 5.19–4.88 (m, 4H), 4.69–4.31 (m, 6H), 4.27–3.98 (m, 4H), 3.84 (t, $J = 9.6$ Hz, 1H), 3.79–3.45 (m, 33H), 3.37 (t, $J = 6.8$ Hz, 2H), 2.03 (s, 3H), 1.96 (s, 3H), 1.93 (s, 3H), 1.90 (s, 3H), 1.62 (s, 7H), 1.50 (t, $J = 7.1$ Hz, 3H), 1.36 (s, 2H), 1.18 (m, 114H), 0.81 (t, $J = 6.0$ Hz, 3H). FTIR: 1749 cm^{-1} (C=O stretching vibration), complete disappearance of the azide peak at ~2098 cm^{-1}.

PE-b-PEG Per-O-acetylated α-L-Fucoside 8

Yield: ~91%. ^{1}H-NMR (400 MHz, $CDCl_3$); cloudy solution: δ 7.73 (s, 1H), 5.30–5.25 (m, 2H), 5.12 (m, 1H), 5.02 (bs, 1H), 4.68 (s, 2H), 4.58 (m, 2H), 4.09 (m, 1H), 3.87–3.79 (m, 2H), 3.70–3.56 (m, 33H), 3.44 (t, $J = 6.7$ Hz, 2H), 2.16 (s, 3H), 2.04 (s, 3H), 1.99 (s, 3H), 1.65–156 (m, 9H), 1.25 (m, 114H), 1.10 (d, $J = 6.5$ Hz, 3H), 0.87 (bs, 3H). FTIR: 1752 cm^{-1} (C=O stretching vibration), complete disappearance of the azide peak at 2097 cm^{-1}.

In a one-necked 100-mL round-bottom flask, the respective peracetylated glucose end-capped PE-*b*-PEG **7** or peracetylated fucose end-capped PE-*b*-PEG **8** (0.3 mmol) was stirred with K_2CO_3 (0.2 g) in a mixture of MeOH/H_2O (50 mL, 20:1) at 40 °C for 2 h. The mixture was first neutralized and then poured into THF. The solid material was dried under reduced pressure to give the corresponding glyco end-capped PE-*b*-PEG **9** and **10** (0.3 g, 85%).

PE-b-PEG β-D-Glucoside 9

Solid state ^{13}C-NMR (101 MHz, direct polarization): δ 144.76, 124.52, 103.09, 70.59, 50.38, 32.80, 26.22, 24.46, 14.98. FTIR: 3369 (broad, —O—H stretching vibration), complete disappearance of the carbonyl peak at 1749 cm^{-1}.

PE-b-PEG α-L-Fucoside 10

Solid state ^{13}C-NMR (101 MHz, direct polarization): δ 144.91, 126.03, 99.30, 70.53, 50.70, 32.78, 27.70, 24.37, 16.75, 14.93. FTIR: 3364 (broad, —O—H stretching vibration), complete disappearance of the carbonyl peak at 1752 cm^{-1}.

Fluorescent Dye Entrapment

A mixture of PE-*b*-PEG *β*-D-glucoside **9** (2 mg) and Nile Red (1 mg) was stirred in 1 mL of methanol. While stirring, 1 mL of water was added dropwise and the mixture was stirred for 15 min. The organic solvent was removed using a rotary evaporator at 40 °C under reduced pressure. The evaporation process was continued for 1 h to ensure the complete removal of the organic solvent. A photograph of the resultant solution was taken.

Electro-Formation Process

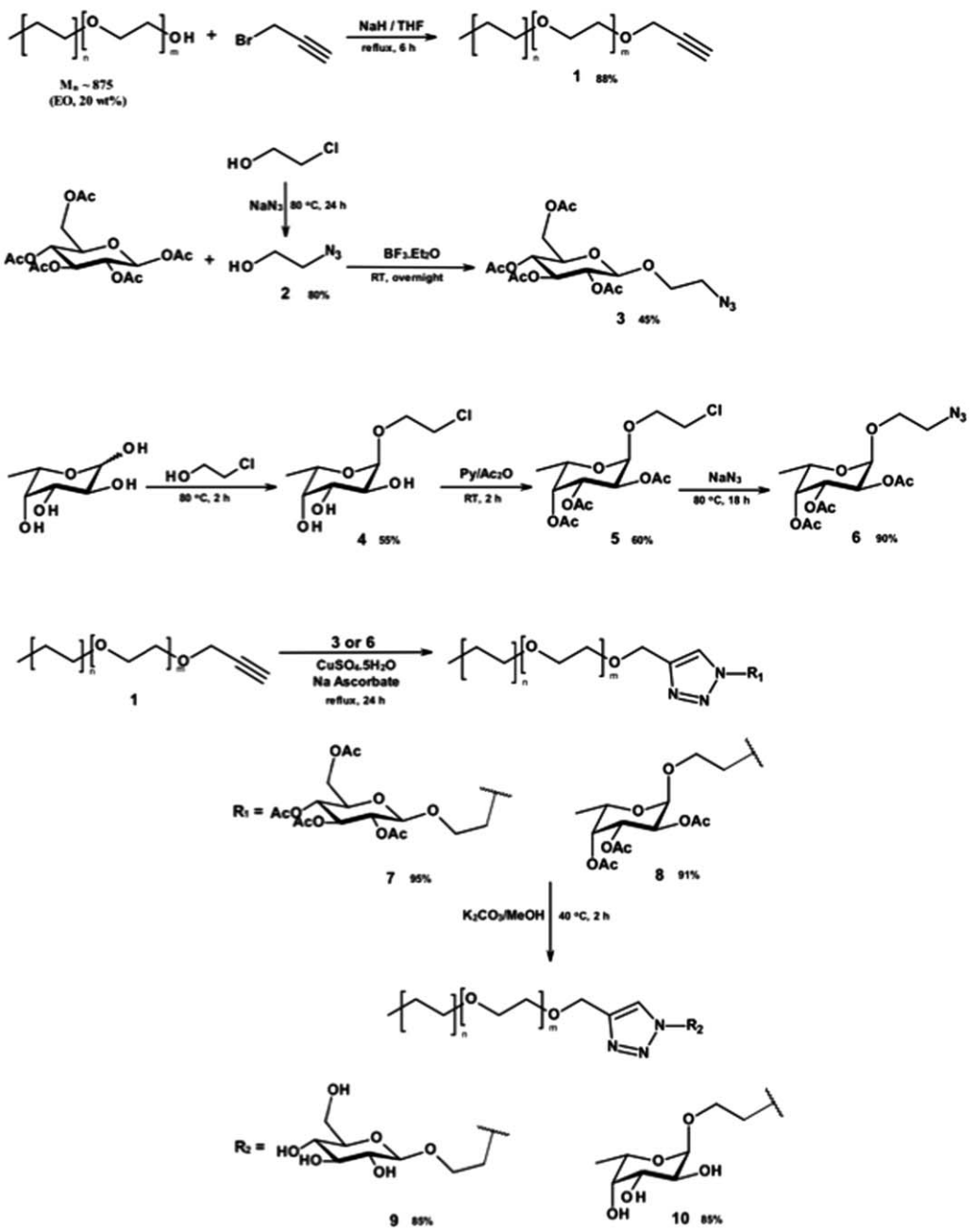
GUVs were formed using a protocol similar to a modification[31] of the original method designed by Discher et al.,[32] for polymersome electro-formation from highly hydrophobic copolymers. A 20 μL drop of polymer solution in chloroform/methanol (4:1) containing Nile Red (0.05 mol %) was first deposited on each of two indium tin oxide (ITO) coated glass slide electrodes. The solvent was then evaporated under vacuum to form thin films on the ITO slides. Before applying an electric field of ~1.2 V with frequency of ~10 Hz, a rubber spacer was sandwiched between the two slide electrodes and filled with sucrose solution (200 mM). The temperature was kept at 70 °C for 3 h. The sucrose solution containing the polymersomes was carefully transferred to a glucose solution (220 mM) in a glass slip bottomed container to be ready for phase contrast observation under the bright field mode and confocal fluorescence microscopy.

Turbidimetric Assay

Concanavalin A (Con A) was dissolved (~1 mg/mL) in HBS buffer (10 mM HEPES, 90 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH = 7.4), and the resulting solution was gently mixed. Turbidity measurements were performed by adding the Con A solution (400 μL) to a dry quartz cuvette (500 μL volume, 1 cm path length). A filtered solution of the ligand aggregate of interest in HBS buffer (prepared by solvent-switch method from methanol as described in the "Fluorescent Dye Entrapment" section without incorporating the dye and filtered using Cronus 25mm Glass Microfibre Syringe Filters 1.2 μm) was then added (100 μL at 0.2 mg/mL). Upon addition, the solution was mixed vigorously using a micropipette before placement in a Varian Cary-100 UV–Vis spectrophotometer attached with temperature controller. Absorbance data were recorded at 420 nm for 10 min.

RESULTS AND DISCUSSION

The CuAAC click reaction was used to synthesize different amphiphilic polymers end-functionalized with one carbohydrate unit. The synthetic approach involves preparation of alkyne-terminated PE-*b*-PEG followed by CuAAC reaction



SCHEME 1 Synthesis of glycosylated PE-*b*-PEG block copolymers via CuAAC click reaction.

with different azidoethyl functionalized sugars, Scheme 1. The use of azidoethyl glycosides ensures the polymers possess the native *O*-glycosyl linkage and are, therefore, capable of binding to lectins.

The alkyne-terminated block copolymer was prepared by etherification reaction between commercially available hydroxyl-terminated PE-*b*-PEG ($M_n \sim 875$ g mol^{-1}) and propargyl bromide. The hydroxyl groups of the PE-*b*-PEG were quantitatively converted into alkyne functionalities to produce the alkyne-terminated analogue, according to ^{1}H-NMR spectroscopy. The ^{1}H-NMR spectrum of the starting material (hydroxyl-terminated PE-*b*-PEG) shows all the peaks corresponding to the PE and the PEG segments as well as peaks at 3.3 ppm, 3.7 ppm, 1.5 ppm, and 1.7 ppm, which can be assigned to the methylene groups in the PE at the junction of the PE-PEG blocks and the methylene groups near the hydroxyl end group, Figure 1(A). The successful alkyne-end capping of PE-*b*-PEG was indicated by the appearance of peaks due to the alkyne proton (—CH_2—C≡*CH*) and the CH_2 protons adjacent to the alkyne group (—O—*CH_2*—C≡CH) at 2.36 and 4.14 ppm, respectively, Figure 1(B). In addition, disappearance of the peaks due to the hydroxyl groups and the CH_2 protons attached to the hydroxyl groups at the chain end in PE-*b*-PEG at 2.81 ppm and 3.7 ppm, respectively, were observed [Fig. 1(A)].

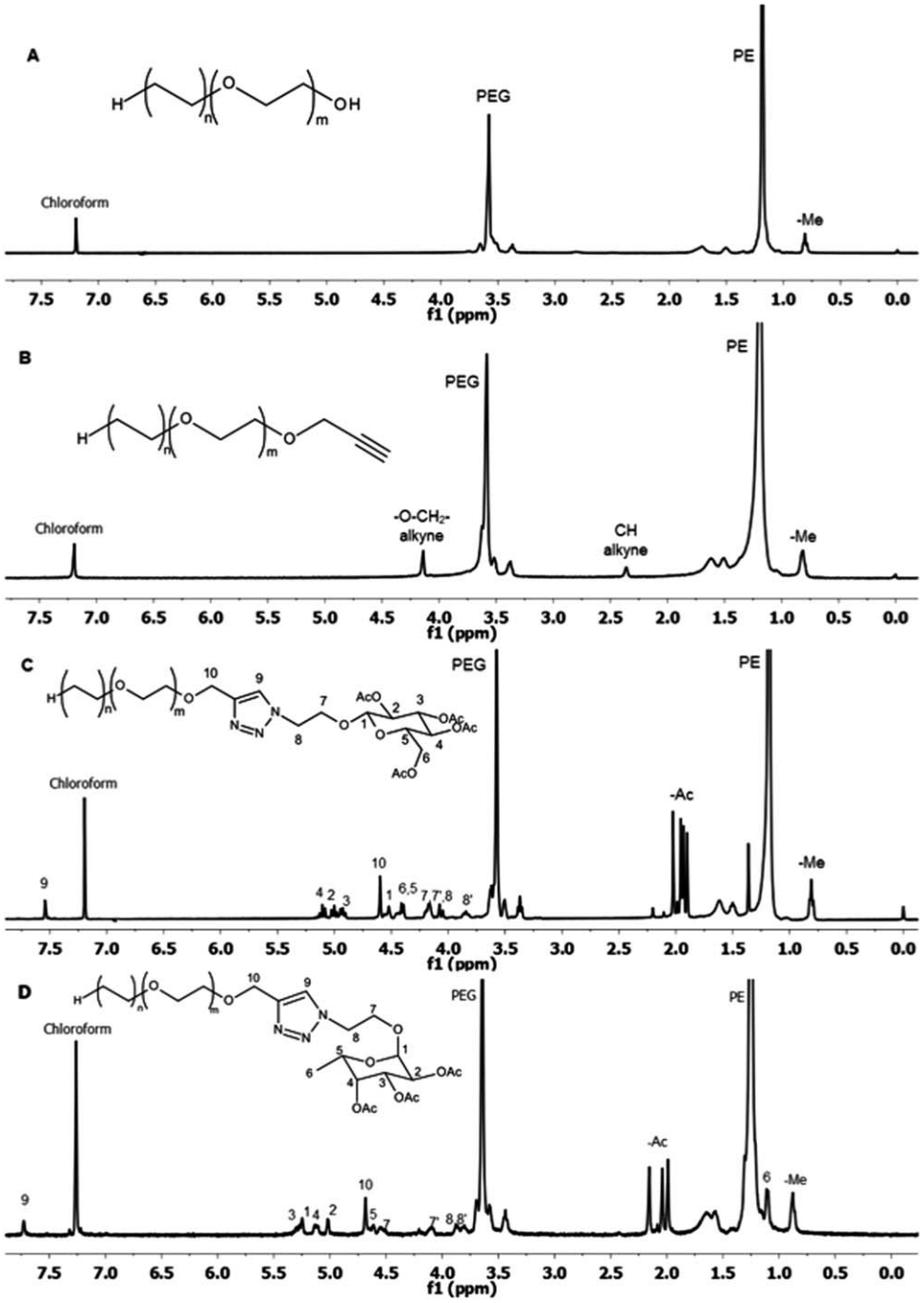


FIGURE 1 Comparison of ^{1}H-NMR spectra of (A) PE-*b*-PEG, (B) alkyne terminated PE-*b*-PEG, (C) protected-glucose functionalized PE-*b*-PEG **7**, and (D) protected-fucose functionalized PE-*b*-PEG **8** in $CDCl_3$.

2′-Azidoethyl 2,3,4,6-tetra-*O*-acetyl-*β*-D-glucoside **3** was prepared from *β*-D-glucose penta-acetate using a similar procedure to that reported previously.[33] However, to access 2′-azidoethyl 2,3,4-tri-*O*-acetyl-α-L-fucoside **6**, another synthetic approach was implemented.[34] The synthesis involved acid-catalyzed glycosylation of L-fucose to give 2′-chloroethyl α-L-fucoside **4** which was then *O*-acetylated and treated with NaN_3/NaI in DMF to give 2′-azidoethyl 2,3,4-tri-*O*-acetyl-α-L-fucoside **6** in good yield.

CuAAC click reactions were performed on the alkyne-terminated PE-*b*-PEG **1** and the 2′-azidoethyl per-*O*-acetylated glycosides **3** and **6** using $CuSO_4{\cdot}5H_2O$/sodium ascorbate to produce PE-*b*-PEG per-*O*-acetylated glycosides **7** and **8**, respectively. The successful end-capping of PE-*b*-PEG with the carbohydrate groups via click reaction was demonstrated by the appearance of a peak at ~7.6 ppm corresponding to the aromatic triazole proton as well as all peaks due to carbohydrate moiety and their protecting acetyl groups and

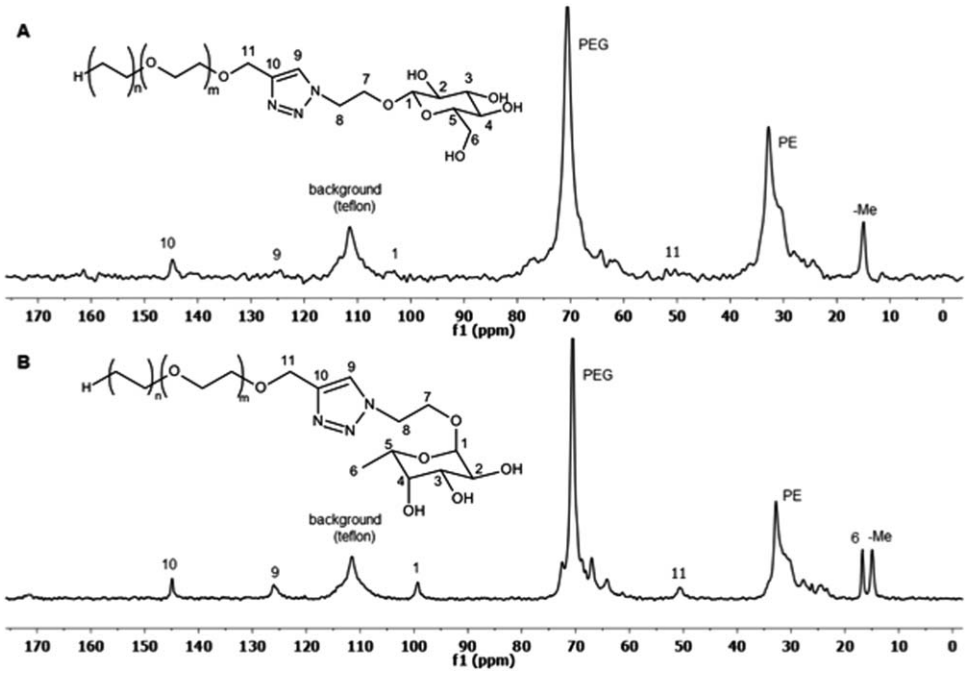


FIGURE 2 Solid state ^{13}C-NMR (direct polarization) spectra of (A) PE-*b*-PEG *β*-D-glucoside **9** and (B) PE-*b*-PEG α-L-fucoside **10**.

PE-*b*-PEG segment, Figure 1(C,D). De-*O*-acetylation of **7** and **8** afforded PE-*b*-PEG *β*-D-glucoside **9** and PE-*b*-PEG α-L-fucoside **10**, respectively. The glycosylated PE-*b*-PEG showed poor solubility, therefore, the choice of characterization tools to be used for these materials was limited. The solid state ^{13}C-NMR spectra of the glycosylated PE-*b*-PEG compared with those of the *O*-acetylated ones showed complete disappearance of the carbonyl peak at ~171 ppm due to the acetyl protecting groups, Figure 2. FTIR spectra also showed disappearance of the carbonyl band at 1750 cm^{-1} as well as appearance of a broad absorption band at ~3370 cm^{-1} due to —OH stretching vibrations.

Atmospheric pressure solids analysis probe-mass spectrometry (ASAP-MS) was used as a characterization tool for the amphiphilic glycosylated block copolymers. The technique, which was introduced in [11,26] permits direct analysis of samples thus minimizing sample preparation.[35] It has also been shown to be an effective technique to ionize compounds regardless of polarity or volatility[36] and has previously been applied to the analysis of low molecular weight synthetic polymers.[30] The advantage to this study was the ability to analyze poorly soluble low molecular weight polymers directly from synthesis products, thus avoiding interference from matrix ions that would otherwise confuse MALDI-TOF-MS experiments. ASAP-MS analysis of solid PE-*b*-PEG per-*O*-acetylated α-L-fucoside **8** did not show peaks corresponding to the intact copolymer **8**. Nevertheless, there are a number of peaks that provide structurally useful information that permit characterization of the sample. A polymeric molecular weight distribution (▼) is observed in the *m/z* 450–900 region of the spectrum, Figure 3. A mass difference between the peaks of 28 Da (C_2H_4) suggests that this polymeric distribution is the result of successful ionization of the PE block of the copolymer. Indeed, accurate mass analysis of *m/z* 477.5232, a peak in the (▼) series, gave an empirical formula of $C_{34}H_{69}$, confirming the presence of a straight chain poly(ethylene) molecule.

In addition to the distribution of peaks that correspond to the hydrophobic portion of the molecule, there are a number of peaks denoting structurally significant ions that allow for characterization of the hydrophilic part. The spectrum base

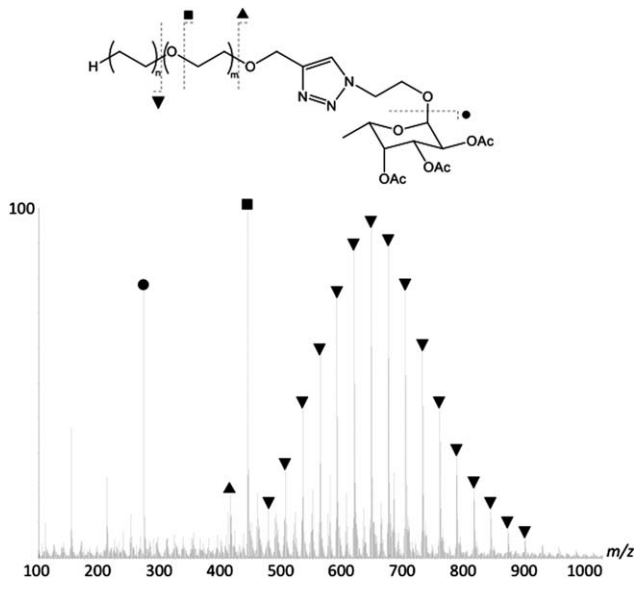


FIGURE 3 Bond cleavage diagram and ASAP-MS of PE-*b*-PEG per-*O*-acetylated α-L-fucoside **8**.

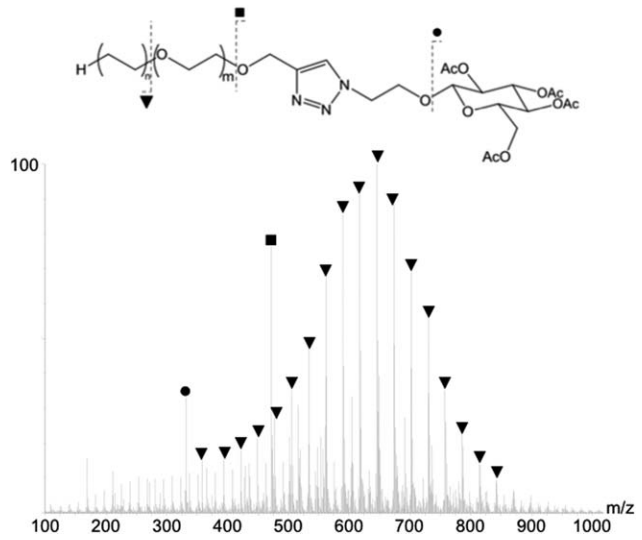


FIGURE 4 Bond cleavage diagram and ASAP-MS of PE-*b*-PEG per-*O*-acetylated *β*-D-glucoside **7**.

peak at *m/z* 443.1624, labeled (■) in Figure 3, was found to have an empirical formula of $C_{19}H_{29}N_3O_9$, which corresponds to cleavage at the bond labeled to (■) in the structure and correlates with the expected structure formed during the click reaction. Additional structural evidence for this hydrophilic part of the molecule is found at *m/z* 414.5187 (▲), Figure 3, which is a second ion containing the fucose ring and the ring formed by the click reaction. The protected fucose moiety is also detected at *m/z* 273.1059 (•) providing a third piece of information regarding this part of the molecule.

ASAP-MS of PE-*b*-PEG per-*O*-acetylated *β*-D-glucoside **7** showed no presence of intact copolymer ions **7**, Figure 4. However, this spectrum does provide significant ions that allude to the structure of the intact species. The series of peaks labelled (▼) is identical to that previously observed in the mass spectra of copolymers **8** and **10**, where accurate and precise mass analysis indicate the presence of a purely hydrocarbon based species as shown in Figure 4. Moreover, the ions in the lower mass region of the spectrum provide complementary information to that gained from the PE molecular weight distribution. At *m/z* 472.1, the peak labeled (■) represents an ion with an empirical formula of $C_{19}H_{26}N_3O_{11}$ that corresponds to the click-derived functional group as shown in Figure 4. Similar to the PE-*b*-PEG per-*O*-acetylated *α*-L-fucoside, ASAP MS of PE-*b*-PEG per-*O*-acetylated *β*-D-glucoside contains a peak that corresponds to the protected sugar functionality (•).

To investigate the self-assembly of these amphiphilic glycosylated PE-*b*-PEGs, aggregates were prepared by the commonly used "solvent-switch" method, in which methanol was used to solubilize both the copolymer and the dye Nile Red. Water was added gradually with stirring until self-assembly took place. The organic solvent was then removed by evaporation. The resultant colloidal solution was found to be fluorescent under illumination with long-wave (365 nm) UV light, Figure 5. This demonstrates the entrapment of Nile Red in the hydrophobic interior of the self-assembled morphologies.

TEM was attempted to explore the morphology of PE-*b*-PEG *β*-D-glucoside **9** aggregates formed by solvent-switch method, Figure 6. A solution of the polymer was dried onto a TEM grid and stained with uranyl acetate, then imaged by TEM. The resulting micrographs show that this glycosylated polymer forms a phase characterized by a majority of spherical aggregates with a highly hydrophobic core. It is believed that carbohydrate moieties represent the corona of these aggregates, hence holding the stain much less than the PE hydrophobic core. It also appears that these aggregates may be able to interact through their coronas (carbohydrate moieties). At this stage, no further investigations were carried out to identify the exact morphology and/or intermolecular binding interactions of these aggregates.

These positive results prompted us to continue and investigate the self-assembly of the amphiphilic glycosylated PE-*b*-PEGs into polymersomes. The electro-formation method, which was pioneered by Angelova and Dimitrov in 1986,[37] was used to produce homogeneous GUVs.[38] This method is similar to film rehydration; however, an electric field is applied to facilitate hydration. The applied electric field has been found to enhance vesicle formation by decreasing membrane tension, inducing periodic motions (mechanical stress), and increasing interlayer repulsion through electroviscous/electrostatic effects.[39]

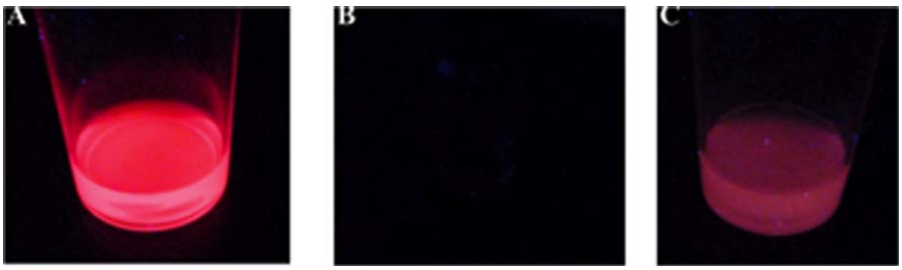


FIGURE 5 Photographs of (A) Nile Red solution in methanol, (B) Nile red in water, and (C) colloidal solution of PE-*b*-PEG *β*-D-glucoside and Nile Red in water under illumination with long-wave UV light ($\lambda = 365$ nm). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

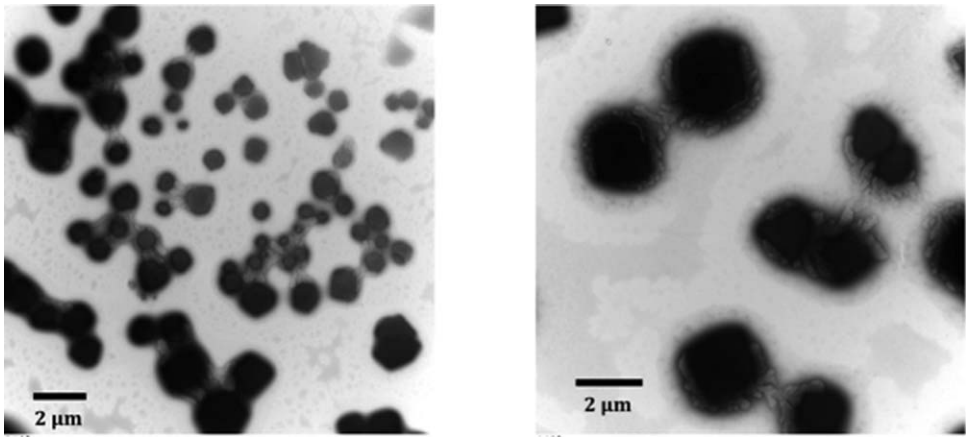


FIGURE 6 TEM images of assemblies formed by PE-*b*-PEG β-D-glucoside **9**.

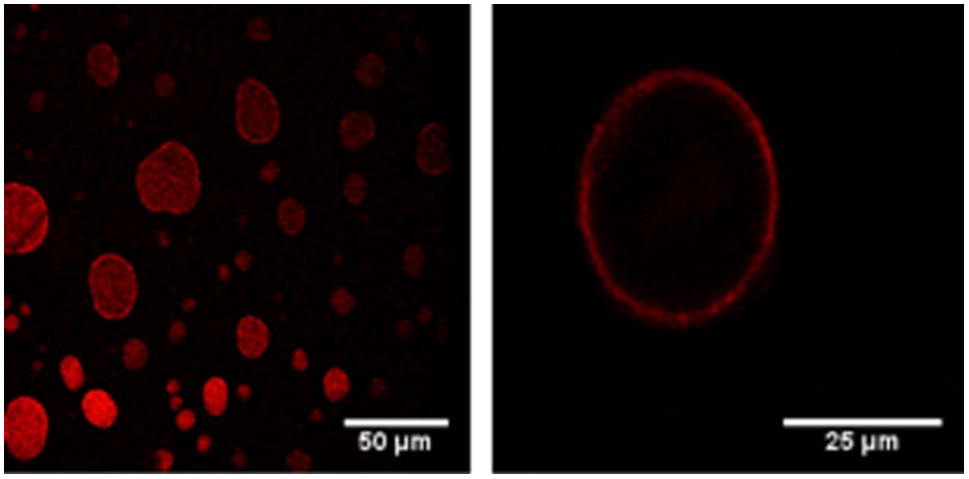


FIGURE 7 Confocal images of giant unilameller vesicles formed from PE-*b*-PEG β-D-glucoside **9** (visualized with Nile Red). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

A custom-made electro-formation kit was used in this study. A glycosylated PE-*b*-PEG film was solution-cast in the presence of Nile Red on the surfaces of both electrodes of ITO-coated glass plates. Application of AC voltage between the two electrodes during the swelling of the polymer in water resulted in the formation of GUVs of 25–50 μm in diameter as revealed by confocal fluorescence microscopy, Figure 7. Electro-formation is reported to allow faster and better-controlled formation of GUVs compared with spontaneous processes.[40] With the standard electro-formation method, the temperature is usually kept above the phase-transition temperature of the material. However, in this study, the electro-formation was carried out at 80 °C which is slightly lower than the T_m of the glycosylated PE-*b*-PEG which was found to be 109 °C based on differential scanning calorimetry analysis. Despite this, GUVs were observed.

The ability of these glycosylated macromolecular assemblies to bind with their relevant lectins (carbohydrate binding proteins) was evaluated using a well-established turbidimetric assay,[41] Figure 8. In this turbidity assay, the Con A tetramer is mixed with an excess of the multivalent ligand under investigation, inducing a rapid precipitation. The change in turbidity is related directly to the formation of Con A clusters in solution mediated by the appropriate multivalent ligand. As expected,

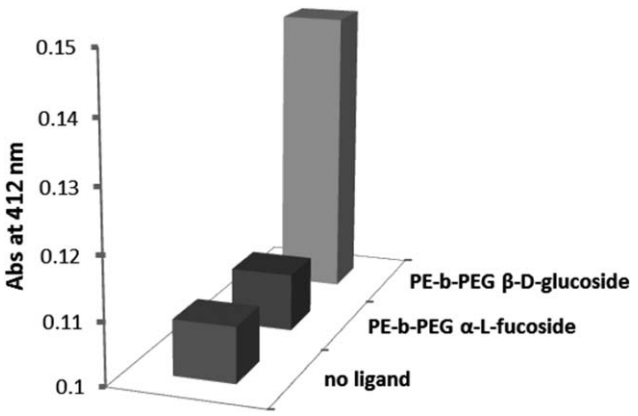


FIGURE 8 Assessment of the binding of nanoscale aggregates of glycosylated polymers to Con A by turbidimetry.

PE-*b*-PEG β-D-glucoside **9** aggregates can bind with Con A. In contrast, PE-*b*-PEG α-L-fucoside **10** aggregates did not show any binding affinity toward Con A. These results indicate that D-glucose moieties are presented on the surface of PE-*b*-PEG β-D-glucoside **9** aggregates in a densely multivalent manner and are available for binding to the relevant lectin. These results also show the potential of these glyco-aggregates to bring about targeted drug delivery to specific cells.

CONCLUSIONS

A facile and efficient synthetic approach based on a CuAAC click reaction of alkyne-terminated PE-*b*-PEG with different azidoethyl glycosides was successfully developed to prepare glycosylated amphiphilic block copolymers with different carbohydrate functionalities (glucose or fucose). The prepared glycosylated PE-*b*-PEGs were found to have poor solubility behavior. ASAP mass spectrometry was shown to provide unique structural information that allowed full characterization of these glycosylated block copolymers. The capability of these glycosylated PE-*b*-PEG to form spherical aggregates in aqueous solution with the ability to solubilize a hydrophobic dye (Nile Red) was also demonstrated by TEM. Pleasingly, the functional amphiphilic block copolymers were found to self-assemble using the electro-formation method into giant polymersomes with diameter of 25–50 μm in aqueous solution as revealed by confocal fluorescence microscopy. Preliminary bioactivity study (turbidimetric assay) of the obtained glycosylated polymer aggregates indicated the accessibility and selectivity of carbohydrate moieties to their relevant lectin. These functional polymersomes could prove to be interesting structures as a result of the carbohydrate groups present on their surface.

REFERENCES AND NOTES

1 L. Pinchuk, G. J. Wilson, J. J. Barry, R. T. Schoephoerster, J.-M. Parel, J. P. Kennedy, *Biomaterials* **2008**, *29*, 448–460.

2 S. Nayak, L. A. Lyon, *Angew Chem Int Ed Engl* **2005**, *44*, 7686–7708.

3 I. W. Hamley, Block Copolymers in Solution Fundamentals and Applications **2005**, Wiley & Sons, Chichester.

4 A. J. Ryan, *Nature* **2008**, *456*, 334–336.

5 C. Tang, E. M. Lennon, G. H. Fredrickson, E. J. Kramer, C. J. Hawker, *Science* **2008**, *322*, 429–432.

6 A. Blanazs, S. P. Armes, A. J. Ryan, *Macromol. Rapid Commun.* **2009**, *30*, 267–277.

7 A. M. Eissa, N. R. Cameron, In Bio-Synthetic Polymer Conjugates; H. Schlaad, Ed.; Springer: Berlin, Heidelberg, **2013**; Vol. 253, pp 71–114.

8 M. Hetzer, G. Chen, C. Barner-Kowollik, M. H. Stenzel, *Macromol. Biosci.* **2010**, *10*, 119–126.

9 G. Chen, S. Amajjahe, M. H. Stenzel, *Chem. Commun.* **2009**, 1198–1200.

10 N. R. Cameron, S. G. Spain, J. A. Kingham, S. Weck, L. Albertin, C. A. Barker, G. Battaglia, T. Smart, A. Blanazs, *Faraday Discuss* **2008**, *139*, 359–368.

11 B. S. Kim, W. Y. Yang, J. H. Ryu, Y. S. Yoo, M. Lee, *Chem. Commun.* **2005**, 2035–2037.

12 G. Pasparakis, C. Alexander, *Angew Chem Int. Ed. Engl.* **2008**, *47*, 4847–4850.

13 L. You, H. Schlaad, *J. Am. Chem. Soc* **2006**, *128*, 13336–13337.

14 Z. Hordyjewicz-Baran, L. You, B. Smarsly, R. Sigel, H. Schlaad, *Macromolecules* **2007**, *40*, 3901–3903.

15 H. Schlaad, L. You, R. Sigel, B. Smarsly, M. Heydenreich, A. Mantion, A. Masic, *Chem. Commun.* **2009**, 1478–1480.

16 S. Hehir, N. M. Plourde, L. Gu, D. E. Poree, W. J. Welsh, P. V. Moghe, K. E. Uhrich, *Acta Biomater.* **2012**, *8*, 3956–3962.

17 J. Holgersson, A. Gustafsson, M. E. Breimer, *Immunol. Cell Biol.* **2005**, *83*, 694–708.

18 A. Ramzi, M. Prager, D. Richter, V. Efstratiadis, N. Hadjichristidis, R. N. Young, J. B. Allgaier, *Macromolecules* **1997**, *30*, 7171–7182.

19 J. Wang, J. H. Horton, G. Liu, S.-Y. Lee, K. J. Shea, *Polymer* **2007**, *48*, 4123–4129.

20 L. Yin, M. A. Hillmyer, *Macromolecules* **2011**, *44*, 3021–3028.

21 H. Schmalz, J. Schmelz, M. Drechsler, J. Yuan, A. Walther, K. Schweimer, A. M. Mihut, *Macromolecules* **2008**, *41*, 3235–3242.

22 T. Li, W. J. Wang, R. Liu, W. H. Liang, G. F. Zhao, Z. Li, Q. Wu, F. M. Zhu, *Macromolecules* **2009**, *42*, 3804–3810.

23 H. Gao, Y. Tang, Z. Hu, Q. Guan, X. Shi, F. Zhu, Q. Wu, *Polym. Chem.* **2013**, *4*, 1107–1114.

24 X. Shi, Y. Zhao, H. Gao, L. Zhang, F. Zhu, Q. Wu, *Macromol. Rapid Commun.* **2012**, *33*, 374–379.

25 S. Slavin, J. Burns, D. M. Haddleton, C. R. Becer, *Eur. Polym. J* **2011**, *47*, 435–446.

26 C. N. McEwen, R. G. McKay, B. S. Larsen, *Anal. Chem.* **2005**, *77*, 7826–7831.

27 K. D. Park, P. Morieux, C. Salome, S. W. Cotten, O. Reamtong, C. Eyers, S. J. Gaskell, J. P. Stables, R. Liu, H. Kohn, *J. Med. Chem.* **2009**, *52*, 6897–6911.

28 H. R. Pfaendler, V. Weimar, *Synthesis* **1996**, 1345–1349. https://www.thieme-connect.de/ejournals/abstract/10.1055/s-1996-4390.

29 A. D. Ray, J. Hammond, H. Major, *Eur. J. Mass Spectrom* **2010**, *16*, 169–174.

30 M. J. P. Smith, N. R. Cameron, J. A. Mosely, *Analyst* **2012**, *137*, 4524–4530.

31 R. Dimova, U. Seifert, B. Pouligny, S. Forster, H. G. Dobereiner, *Eur. Phys J. E* **2002**, *7*, 241–250.

32 B. M. Discher, Y. Y. Won, D. S. Ege, J. C. M. Lee, F. S. Bates, D. E. Discher, D. A. Hammer, *Science* **1999**, *284*, 1143–1146.

33 X.-H. Yang, Y.-H. Zhou, H.-J. Liu, X.-X. Guo, *Acta Crystallogr. Sect. E* **2009**, *65*, O2651–U2770.

34 J. H. Ni, S. Singh, L. X. Wang, *Bioconjugate Chem* **2003**, *14*, 232–238.

35 C. McEwen, S. Gutteridge, *J. Am. Soc. Mass Spectrom.* **2007**, *18*, 1274–1278.

36 S. Trimpin, K. Wijerathne, C. N. McEwen, *Anal. Chim. Acta* **2009**, *654*, 20–25.

37 M. I. Angelova, D. S. Dimitrov, *Faraday Discuss* **1986**, *81*, 303–311.

38 G. Battaglia, A. J. Ryan, *J. Phys. Chem. B* **2006**, *110*, 10272–10279.

39 V. Malinova, S. Belegrinou, D. Bruyn Ouboter, W. Meier, In Organic Electronics; T. Grasser, G. Meller, L. Li, Eds.; Springer: Berlin, Heidelberg, **2010**; Vol. 223, pp 213–258.

40 P. Walde, K. Cosentino, H. Engel, P. Stano, *ChemBioChem* **2010**, *11*, 848–865.

41 C. W. Cairo, J. E. Gestwicki, M. Kanai, L. L. Kiessling, *J. Am. Chem. Soc.* **2002**, *124*, 1615–1619.