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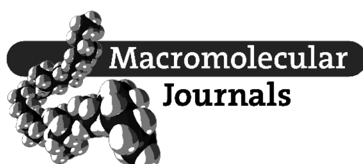

Supporting Information

for *Macromol. Rapid Commun.,* DOI: 10.1002/marc.201400650

Biodegradable Micelles Capable of Mannose-Mediated Targeted Drug Delivery to Cancer Cells

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Materials and Methods

Materials and cells. Boc-L-tyrosine was purchased from Chem-Impex International (Des Plaines, IL, USA) and used as received. L-lacOCA was prepared according to published procedures, purified by two recrystallisations in diethyl ether, and stored at -30 °C in a glove box.[1] Anhydrous dichloromethane (DCM), hexane, and tetrahydrofuran (THF) were dried by columns packed with alumina and stored in a glove box. Anhydrous dimethylformamide (DMF) was dried by passing the solvent through a column packed with 4Å molecular sieves. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used as received unless otherwise specified.

A549 cells (human lung adenocarcinoma) and HepG-2 cells (human hepatocellular carcinoma) were purchased from the American Type Culture Collection (Rockville, MD, USA), and cultured in DMEM supplemented with 10% fetal bovine serum (FBS).

Instrumentation. NMR spectra were recorded on a Varian U500 (500 MHz) or a VXR-500 (500 MHz) spectrometer. Gel permeation chromatography (GPC) experiments were performed on a system equipped with an isocratic pump (Model 1100, Agilent Technology, Santa Clara, CA, USA), a DAWN HELEOS multi-angle laser light scattering detector (MALLS detector, Wyatt Technology, Santa Barbara, CA, USA) and an Optilab rEX refractive index detector (Wyatt Technology, Santa Barbara, CA, USA). The detection wavelength of HELEOS was set at 658 nm. Separations were performed using serially connected size exclusion columns (100 Å, 500 Å, 10^3 Å and 10^4 Å Phenogel columns, 5 μm, 300 × 7.8 mm, Phenomenex, Torrance, CA, USA) at 60 °C using DMF containing 0.1 M LiBr as the mobile phase. The MALLS detector was calibrated using pure toluene with no need for calibration using polymer standards and was used for the determination of the absolute molecular weights (MWs). The molecular weight of polymer was determined from the *d*n/*d*c value calculated offline by means of the internal

calibration system processed by the ASTRA V software (Version 5.1.7.3, Wyatt Technology). Infrared spectra were recorded on a Perkin Elmer 100 serial FTIR spectrophotometer calibrated with polystyrene film. Lyophilization was conducted on a Labconco FreeZone lyophilizer (Kansas City, MO, USA). Particle size and dispersity were measured with a ZetaPlus dynamic light scattering detector (15 mW laser, incident beam at 676 nm, Brookhaven Instruments, Holtsville, NY, USA). Fluorescence spectrum was recorded on a PekinElmer LS 55 fluorescence spectrometer (Santa Clara, CA, USA). Transmission electron microscopy (TEM) studies were performed with a JEOL 2100 Cryo TEM at a voltage of 80 kV. Samples were prepared by drop-casting micelle solutions onto 200 mesh carbon film supported copper grid (Electron Microscopy Sciences, Hatfield, PA, USA) and then air-drying at room temperature before measurement.

Synthesis of 1,2,3,4,6-penta-*O*-acetyl- α/β-D-mannopyranose (M2). 1,2,3,4,6-penta-*O*-acetyl-α/β-D-mannopyranose was prepared according to the procedure reported by Jakeman.[2] D-Mannose (10.00 g, 55.6 mmol, **M1**), pyridine (45 mL), and acetic anhydride (52 mL, 556 mmol) were combined in a 250 ml flask and stirred at room temperature. The mannose was dissolved gradually. After stirring for 4 h, the reaction mixture was diluted with ice-water (100 mL) and extracted with DCM (100 mL) three times. The combined organic layers were washed with 1 M aqueous HCl (3 × 200 mL), H_2O (200 mL), saturated aqueous $NaHCO_3$ (150 mL), and H_2O (200 mL). The organic layer was dried over magnesium sulfate ($MgSO_4$) and the solvent was removed under reduced pressure. The product was obtained as colorless syrup (19.80 g, 50.7 mmol, 91 % yield) which was a mixture of α and β anomers. This product was used in the next synthetic step without any further purification. ^{1}H NMR ($CDCl_3$, 500 MHz): α diastereomer δ 5.84 (d, 1H, 1-*H*), 5.46 (m, 1H, 2-*H*), 5.26 (m, 1H, 4-*H*), 5.10 (m, 1H, 3-*H*), 4.08-4.27 (m, 2H, CH_2OAc), 3.77-3.80 (m, 1H, 5-*H*), 2.18 (s, 3H, C(O)CH_3), 2.07 (s,

3H, C(O)C*H*$_3$), 2.06 (s, 3H, C(O)C*H*$_3$), 2.02 (s, 3H, C(O)C*H*$_3$), 1.98 (s, 3H, C(O)C*H*$_3$). β diastereomer δ 6.05 (d, 1H, 1-*H*), 5.28-5.32 (m, 2 H, 3-*H* and 4-*H*), 5.23 (m, 1H, 2-*H*), 4.08-4.27 (m, 2H, C*H*$_2$OAc), 4.00-4.05 (m, 1H, 5-*H*), 2.15 (s, 3H, C(O)C*H*$_3$), 2.14 (s, 3H, C(O)C*H*$_3$), 2.07 (s, 3H, C(O)C*H*$_3$), 2.06 (s, 3H, C(O)C*H*$_3$), 2.02 (s, 3H, C(O)C*H*$_3$). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 168.12-170.68, 90.5, 70.7, 68.8, 68.4, 65.6, 62.2, 20.6-20.9. ESI-MS (m/z): Calcd $C_{16}H_{22}O_{11}$ 390.1 (M); found: 413.3 $[M + Na]^+$.

Synthesis of 2'-bromoethyl 2,3,4,6-tetra-*O*-acetyl-α-D-manno-pyranoside (M3). M2 (5.37 g, 13.8 mmol) and 2-bromoethanol (0.98 mL, 13.8 mmol) were dissolved in DCM (50 mL). Then, boron trifluoride etherate (5.8 mL, 47.2 mmol) was added to the solution and stirred in the dark under a nitrogen atmosphere for 3 h and monitored by TLC (ethyl acetate/hexane, 1:1) until complete disappearance of the starting material. After addition of DCM (100 mL), the reaction mixture was neutralized by adding saturated sodium bicarbonate solution (100 mL) and the resulting solution was washed with deionized (DI) water (2 × 200 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated to dryness under reduced pressure. The resulting oil was then purified using silica gel chromatography (ethyl acetate/hexane (1:1, v/v)). The relevant fractions were collected, combined and concentrated to dryness under reduced pressure to yield 2'-bromoethyl 2,3,4,6-tetra-*O*-acetyl-α-D-manno-pyranoside (**M3**) as a colorless powder (2.05 g, 32 % yield). ^{1}H NMR ($CDCl_3$, 500 MHz): δ 5.27-5.36 (m, 3H, 2-*H*, 3-*H* and 4-*H*), 4.88 (d, 1H, 1-*H*), 4.12-4.27 (m, 3H, 5-*H* and 6-*H*), 3.86-4.00 (m, 2H, OC*H*$_2$), 3.52 (t, 2H, C*H*$_2$Br), 2.16 (s, 3H, C(O)C*H*$_3$), 2.11 (s, 3H, C(O)C*H*$_3$), 2.05 (s, 3H, C(O)C*H*$_3$), 2.00 (s, 3H, C(O)C*H*$_3$). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 170.04, 170.21, 170.78, 170.81, 97.94, 69.91, 69.21, 69.12, 68.67, 66.19, 62.60, 29.97, 21.06, 20.94, 20.90, 20.86. ESI-MS (m/z): Calcd $C_{16}H_{23}BrO_{10}$ 456.1 (M); found: 477.3 $[M + Na]^+$.

Synthesis of 2'-azidoethyl 2,3,4,6-tetra-*O*-acetyl-α-D-manno-pyranoside (M4). **M3** (1.40 g, 3.0 mmol) and sodium azide (1.00 g, 15.4 mmol) were dissloved in anhydrous DMF (30 mL) and stirred at 60 ^{o}C for 6 h. The reaction mixture was filtered and concentrated to dryness under reduced pressure. The dry powder was dissolved in DCM (50 mL) and then washed with DI water (4 × 50 mL). The combined organic layers were dried over $MgSO_4$, filtered, and concentrated to dryness under reduced pressure to obtain **M4** (1.15 g, 92 % yield) as white powder. IR (neat); v = 2950, 2107, 1745, 1674, 1435, 1476, 1229, 1139, 1092, 1045, 979, 906 cm^{-1}. ^{1}H NMR ($CDCl_3$, 500 MHz): δ 5.27-5.36 (m, 3H, 2-*H*, 3-*H* and 4-*H*), 4.87 (d, 1H, 1-*H*), 4.11-4.31 (m, 2H, 6-*H*), 4.05 (m, 1H, 5-*H*), 3.65-3.89 (m, 2H, OCH_2), 3.41-3.52 (m, 2H, CH_2N_3), 2.16(s, 3H, C(O)CH_3), 2.10(s, 3H, C(O)CH_3), 2.05(s, 3H, C(O)CH_3), 1.99(s, 3H, C(O)CH_3). ^{13}C NMR ($CDCl_3$, 500 MHz): δ 170.77, 170.16, 169.95, 169.90, 97.87, 69.51, 68.97, 67.19, 66.10, 62.58, 50.48, 21.02, 20.89, 20.86, 20.81. ESI-MS (m/z): Calcd $C_{16}H_{23}N_3O_{10}$ 417.1 (M); found: 440.1 $[M + Na]^+$.

Synthesis of 2'-azidoethyl-*O*-α-D-mannopyranoside (M5). A small piece of sodium metal was added to **M4** (1.15 g, 2.8 mmol) in methanol (MeOH, 30 mL). After 45 min, the solution was acidified with Amberlite IR-120H^+ (Note: The Amberlite IR-120H^+ was washed with MeOH before using.) to pH 6. The mixture was filtered and evaporated under reduced pressure to afford **M5** as a white crystalline solid (0.61 g, 89 % yield). ^{1}H NMR (DMSO-d_6, 500 MHz): δ 4.68 (d, 1H, 1-*H*), 3.76-3.80 (m, 4H, 2-*H*, 3-*H*, 4-*H* and 5-*H*), 3.61-3.67 (m, 2H, 6-*H*), 3.53-3.57 (m, 2H, $CH_2CH_2N_3$), 3.34-3.49 (m, 2H, CH_2N_3). ^{13}C NMR (DMSO-d_6, 500 MHz): δ 100.66, 73.87, 71.47, 70.87, 67.58, 66.29, 61.94, 50.67. ESI-MS (m/z): Calcd $C_8H_{15}N_3O_6$ 249.1 (M); found: 272.3 $[M + Na]^+$.

Synthesis of 5-[4-(prop-2-yn-1-yloxy)benzyl]-1,3-dioxolane-2,4-dione (Tyr(alkynyl)-OCA). Tyr(alkynyl)-OCA was synthesized as described previously.[3, 4] ^{1}H NMR ($CDCl_3$, 500 MHz): δ

7.17 (d, 2H, Ar*H*), 6.96 (d, 2H, Ar*H*), 5.27 (t, 1H, alpha-*H*), 4.68 (d, 2H, -PhOCH_2C≡CH), 3.19-3.35 (m, 2H, -CH_2PhOCH_2C≡CH), 2.52 (t, 1H, -PhOCH_2C≡C*H*), ^{13}C NMR ($CDCl_3$, 500 MHz): δ 166.5, 157.8, 147.9, 131.0, 124.5, 115.7, 80.1, 78.4, 75.9, 56.0, 35.8.

Synthesis of poly(Lac-OCA)-*b*-poly(Tyr(alkynyl)-OCA) (PLA-*b*-PTA) copolymer. Polymerization was proceeded in the glove box. L-lacOCA (69.8 mg, 0.6 mmol, 30 equiv.) was dissolved in DCM (2 mL) followed by addition of pyrenebutanol (200 μL, 0.1 M, 1 equiv.) and 4-(dimethylamino)pyridine (DMAP, 200 μL, 0.1 M, 1 equiv). The polymerization was allowed to proceed for 16 h at room temperature. Tyr(alkynyl)-OCA (49.2 mg, 0.2 mmol, 10 equiv.) was added and the reaction lasted for another 16 h. After the polymerization was complete, the poly(Lac-OCA)$_{30}$-*b*-poly(Tyr(alkynyl)-OCA)$_{10}$ (PLA$_{30}$-*b*-PTA$_{10}$) was precipitated with diether ether and dried under vacuum (82.3 mg, 92.5 % yield).

Synthesis of poly(Lac-OCA)-*b*-(poly(Tyr(alkynyl)-OCA)-*g*-mannose) (PLA-*b*-(PTA-*g*-mannose)). PLA$_{30}$-*b*-PTA$_{10}$ (45 mg, 0.1 mmol of alkyne group), **M5** (49.8 mg, 0.2 mmol) and *N*,*N*,*N'*,*N''*,*N''*-pentamethyldiethylenetriamine (PMDETA, 50 μL, 0.2 mmol) were dissolved in DMF (2 mL). Into the solution, copper(I) bromide (CuBr, 14 mg, 0.1 mmol) was added and stirred for 24 h in glove box. The solvent was removed under vacuum and the residue was dissolved in DI water (5 mL). The crude product was purified by ultrafiltration using Amicon Ultra-4 centrifugal filter unit (MWCO 3 kDa, Millipore, Billerica, MA, US) and lyophilized (65.0 mg, 93.6 % yield).

Preparation of micelles and determination of critical micelle concentration (CMC). PLA$_{30}$-*b*-(PTA$_{10}$-*g*-mannose) (10.0 mg) was dissolved in DMF (2 mL) and DI water (20 mL) was slowly added under vigorous stirring. After vigorous stirring for another 2 h at room temperature, the micelles were obtained and further purified by ultrafiltration using Amicon Ultra-4 centrifugal filter unit (MWCO 3 kDa, 3500 rpm) to remove DMF and lyophilized. The CMC of

the micelle was determined using Nile Red (NR) as a fluorescence probe.[5] NR in THF (0.1 mg/mL, 10 μL) was added to a glass vial via a microsyringe. After THF was evaporated, a micellar solution (2 mL) was added. The concentration of the micellar solution varied from 5×10^{-4} to 0.25 mg/mL. Then the solution was racked for more than 1 h. Finally, fluorescence spectra were recorded with the excitation wavelength at 557 nm.

Preparation of DOX-Loaded Micelles. DOX-loaded micelles were readily prepared using the cosolvent method.[6] Briefly, DOX·HCl (5.3 mg, 9.2 mmol) and 1.5-fold molar amount of triethylamine (TEA, 2 μL, 13.8 mmol) were dissolved in DMSO (5 mL) and stirred at room temperature for 2 h.[7] Subsequently, PLA_{30}-*b*-(PTA_{10}-*g*-mannose) (10.0 mg) were completely dissolved in DMF (1.5 mL) and then mixed with DOX solution (1 mL, 1 mg). DI water (20 mL) was added slowly by using a microsyringe under vigorous stirring. After being stirred for another 2 h, the solution was further purified by ultrafiltration using Amicon Ultra-4 centrifugal filter unit (MWCO 3 kDa, 3500 rpm) to remove DMF and free drug and the micelles were then lyophilized. For determination of drug loading content (DLC) and drug loading efficiency (DLE), the DOX-loaded micelles were dissolved in DMSO and analyzed with the DOX amount by fluorescence spectroscopy, wherein calibration curve was obtained with DOX solution in DMSO at different DOX concentrations. DLC and DLE were calculated according to the following equations:

DLC (wt %) = (weight of loaded drug / weight of polymer) × 100 %

DLE (wt %) = (weight of loaded drug / weight of drug in feed) × 100 %

***In vitro* release of DOX from PLA_{30}-*b*-(PTA_{10}-*g*-mannose) micelles.** The release profiles of DOX from PLA_{30}-*b*-(PTA_{10}-*g*-mannose) micelles were studied using a dialysis bag (MWCO 3500 Da) at 37 ^{o}C. To acquire sink conditions, *in vitro* drug release test was performed at low drug concentration.[8] Briefly, DOX-loaded freeze-dried micelles (5 mg) was dispersed in 3 mL

of the respective PBS buffer and transferred into dialysis bag (MWCO 3500 Da). It was immersed in 47 mL of PBS solution (pH 5.4 or 7.2) in a beaker. The beaker was then placed in a 37 ^{o}C water bath and stirred at 120 rpm. At desired time intervals, 2 mL release medium was taken out and replenished with an equal volume of fresh media. The amount of DOX released was determined by spectrofluorimetry (Ex=485 nm, Em=590 nm), and the cumulative release (CR) was calculated as described previously.[9]

Cell uptake. HepG-2 and A549 cells were seeded on 96-well plates at 1×10^4 cells/well and cultured for 24 h before replacement of fresh media (100 μL/well). DOX-micelles or free DOX were added at the final DOX concentrations of 2, 5, 10, 20, and 50 μM. After incubation at 37 °C for different periods of time (0.5, 1, 2, 4, 8 h), cells were washed three times with PBS and lysed with the RIPA lysis buffer. DOX content in the cell lysate was measurement by spectrofluorimetry (E_x = 480 nm, E_m = 590 nm) and total protein content was quantified using the BCA kit (Promega, Madison, WI, USA). The uptake level was expressed as nmol DOX per mg protein. In order to elucidate the mannose receptor-mediated targeting effect of micelles, the cell uptake study was performed in the media supplementary with 100, 300, 600, and 1000 μmol/L of mannose.

MTT assay. The cytotoxicity of blank micelles, DOX-loaded micelles, and free DOX against HepG-2 and A549 cells was evaluated using the MTT assay. Cells were seeded on 96-well plates at 1×10^4 cells/well and cultured for 24 h before replacement of fresh media (100 μL/well). DOX-loaded micelles and free DOX were added at the final concentration ranges of 0-100 μM DOX-equiv for free DOX and 0-250 μM DOX-equiv for DOX-micelles. Blank micelles were added at the same amount as their DOX-loaded analogues. Cells were further incubated for 72 h before viability assessment. The IC_{50} values were expressed as concentration (μM) of DOX-equiv.

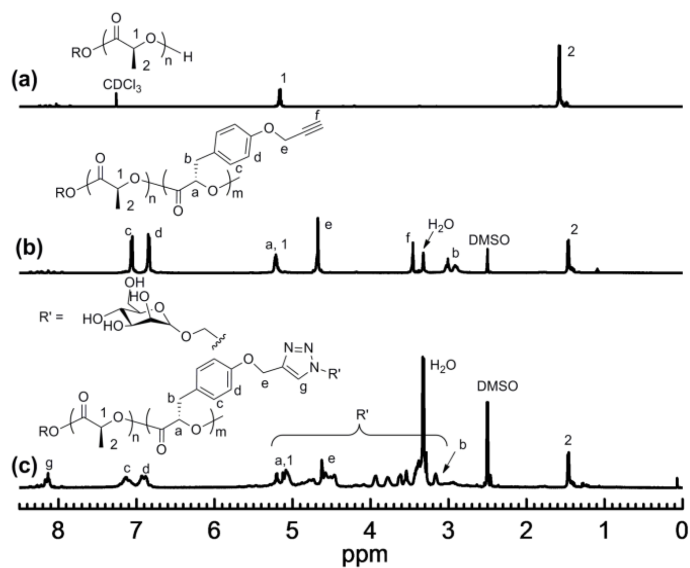

Figure S1. ^{1}H NMR spectra of PLA (a), PLA-*b*-PTA (b), and PLA-*b*-(PTA-*g*-mannose) (c).

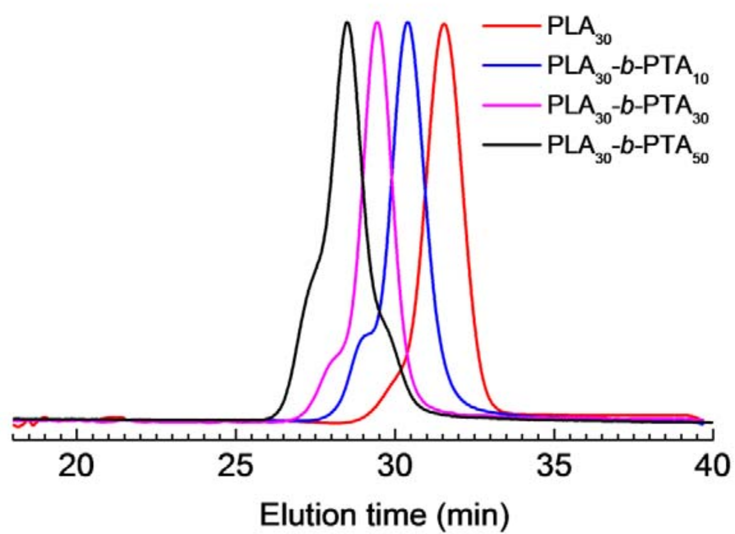

Figure S2. Overlay of GPC curves of PLA_{30}, PLA_{30}-*b*-PTA_{10}, PLA_{30}-*b*-PTA_{10} and PLA_{30}-*b*-PTA_{50}.

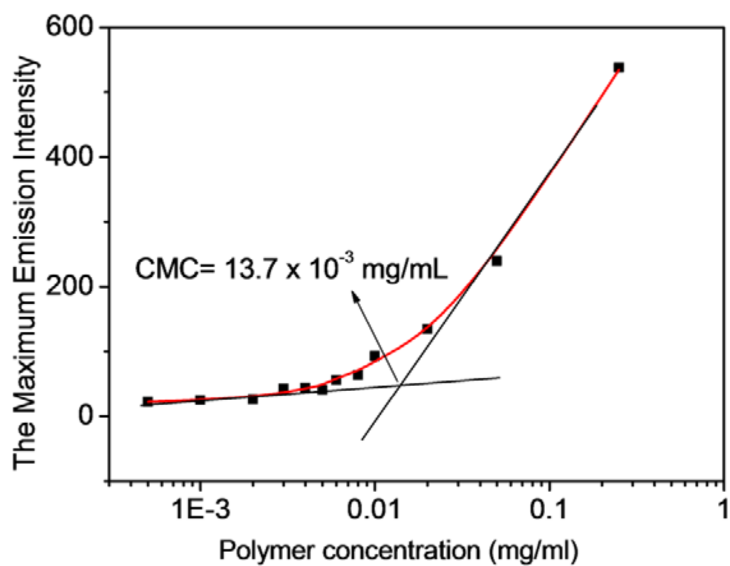

Figure S3. The fluorescence intensity at the maximum emission wavelength as a function of PLA_{30}-*b*-(PTA_{10}-*g*-mannose) concentration when using NR as the probe.

Table S1. Properties of PLA-*b*-(PTA-*g*-mannose) micelles in water.

Sample	CMC (mg/L)	Diameter (nm)	PDI
PLA_{30}-*b*-PTA_{10}	13.7	80.8	0.20
PLA_{30}-*b*-PTA_{30}	31.5	106.6	0.26
PLA_{30}-*b*-PTA_{50}	40.5	130.8	0.23

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