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High-density doxorubicin-conjugated polymeric nanoparticles via ring-opening metathesis polymerization

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General Considerations. All synthetic manipulations were performed under a dry nitrogen atmosphere using either standard Schlenk techniques or an inert-atmosphere glovebox, unless otherwise noted. HPLC-grade tetrahydrofuran (THF) and methylene chloride were dried over neutral alumina via the Dow-Grubbs solvent system[1] installed by Glass Contours. Solvents were collected under argon, degassed under vacuum, and stored under nitrogen in a Strauss flask prior to use. ^{1}H and ^{13}C NMR spectra were recorded on a Varian INOVA 500 FT-NMR spectrometer (500 MHz for ^{1}H NMR, 125 MHz for ^{13}C NMR). ^{1}H NMR data are reported as follows: chemical shift {multiplicity (b = broad, s = singlet, d = doublet, t = triplet, q = quartet, qn = quintet, and m = multiplet), integration, and peak assignments}. ^{1}H and ^{13}C chemical shifts are reported in ppm downfield from tetramethylsilane (TMS). Peak assignments were made with the assistance of ACD Labs software.

High resolution electron impact mass spectrometry (HREIMS) and atmospheric pressure chemical ionization mass spectrometry (APCIMS) data were obtained on a VG70-250SE high resolution mass spectrometer. Electrospray ionization mass spectrometry (ESIMS) data was obtained on a Micromass Quattro II Triple Quadrupole mass spectrometer. Elemental analyses were provided by Atlantic Microlab, Inc. (Norcoss, GA). Molecular weights relative to polystyrene standards were measured on a Waters gel-permeation chromatograph (GPC) equipped with Breeze software, a 717 autosampler, Shodex KF-G guard column, KF-803L and KF-806L columns in series, a Waters 2440 UV detector, and a 410 RI detector. HPLC-grade THF was used as the eluent at a flow rate of 1.0 mL/min and the instrument was calibrated using polystyrene standards (Aldrich, 15 standards, 760-1,800,000 Daltons). All flash chromatography was carried out using a 56-mm inner diameter column containing 200-mm of silica gel under a positive pressure of lab air. Spectra/Por RC (MWCO = 3500) dialysis membranes were purchased from Spectrum Laboratories. Formvar/Carbon, 400 mesh copper TEM grids were purchased from Ted Pella. Absorption spectra were recorded on a Varian Cary 5000 UV-Vis-NIR spectrophotometer using a Starna quartz cell (path length = 10mm).

Materials. Catalyst $Ru(PCy_3)_2$=CHPh (**5**) was purchased from Strem Chemicals and used as received. Deuterated solvents were purchased from Cambridge Isotope Laboratories and used without further purification, except for $CDCl_3$, which was distilled over calcium hydride and vacuum transferred into an air-tight solvent bulb followed by transfer to an inert-atmosphere glovebox. 5-*Exo*-norbornene-2-ol (**1**) was prepared according to literature procedure.[2,3] 5-(4-{2-Exo-[2-(2-{-2[2-(2-Methoxy-ethoxy)-ethoxy]-ethoxy}-ethoxy)-ethoxy]-ethoxymethyl}-benzyloxy)-bicyclo[2.2.1]hept-2-ene (**4**) was prepared according to literature procedure.[4] Doxorubicin was purchased from Hande Tech Development Company. All other reagents were purchased from Aldrich and used without further purification unless otherwise noted.

***Exo*-2-norbornenyl *p*-nitrophenyl carbonate (2)**. Into a 50-mL Schlenk flask was added 5-*exo*-norbornene-2-ol (200 mg, 1.8 mmol) and 4-nitrophenyl chloroformate (1.28 g, 6.3 mmol). The flask was placed under nitrogen and dry CH_2Cl_2 (10 mL) was added. Pyridine (440 µL, 5.4 mmol) was added via syringe and the solution was stirred at 0 ^oC for 2 h and overnight at room temperature. The solution was transferred to a 100-mL separatory funnel with additional CH_2Cl_2 (20 mL) and washed with saturated brine (3 x 50 mL). The organic layer was dried over Na_2SO_4 and filtered over a Buchner funnel. The solvent was removed on the rotary evaporator and the residue was purified by flash chromatography (30% hexanes/CH_2Cl_2) to give a white solid (500 mg, 1.8 mmol, 99%). ^1H NMR ($CDCl_3$): δ 1.58-1.85 (m, 4H, 3- and 7-norbornenyl-*H*$_2$), 2.94 (b, 1H, 1-norbornenyl-*H*), 3.08 (b, 1H, 4-norbornenyl-*H*), 4.75 (m, 1H, 2-norbornenyl-*H*), 6.01 (m, 1H, 6-norbornenyl-*H*), 6.32 (m, 1H, 5-norbornenyl-*H*), 7.40 (d, 2H, *J* = 8.5 Hz, aromatic-*H*), 8.29 (d, 2H, *J* = 9.0 Hz, aromatic-*H*). ^{13}C NMR ($CDCl_3$): δ 34.7 (3-norbornenyl-*C*), 40.8 (4-norbornenyl-*C*), 46.5 (7-norbornenyl-*C*), 47.5 (1-norbornenyl-*C*), 81.1 (2-norbornenyl-*C*), 122.0 (2 Ar*C*), 125.5 (2 Ar*C*), 132.2 (6-norbornenyl-*C*), 142.0 (5-norbornenyl-*C*), 145.5 (O-*C*O-O), 152.5 (Ar*C*), 155.8 (Ar*C*). APCIMS: *m/z* 276.1 $(M+H)^+$. Anal.: Calcd. for $C_{14}H_{13}NO_5$: C, 61.09; H, 4.76; N, 5.09. Found: C, 60.99; H, 4.79; N, 4.93.

3'-carbamate norbornene-conjugated doxorubicin (3). Into a foil-wrapped 50-mL Schlenk flask were added **2** (142 mg, 0.5 mmol) and doxorubicin•HCl (200 mg, 0.34 mmol). The flask was placed under nitrogen and dry DMF (10 mL) was added via cannula, followed by the addition of triethylamine (96 µl, 1.0 mmol) via syringe. The solution was stirred for 18 h at room temperature and concentrated on a rotary evaporator. The residue was redissolved in methylene chloride (100 mL), transferred to a 250-mL separatory funnel, and washed with deionized water (3 x 100 mL). The organic layer was collected and filtered to remove an insoluble dark red precipitate. The solvent was removed on the rotary evaporator and the dark red residue was purified by flash chromatography (EtOAc-CH_2Cl_2-MeOH; 8.5:1:0.5) to yield a bright red solid (230 mg, 0.3 mmol, 98%). ^1H NMR ($CDCl_3$): δ 1.30 (bd, 3H), 1.53-1.87 (bm, 4H), 2.17 (d, 2H), 2.32 (d, 2H), 2.81 (b, 1H), 3.01 (d, 1H), 3.27 (d, 1H), 3.68 (b, 1H), 3.87 (b, 1H) 4.10 (b, 3H), 4.13 (b, 1H), 4.54 (bs, 1H), 4.77 (m, 3H), 5.01 (bd, 1H), 5.31 (bs, 1H), 5.52 (bs, 1H), 5.92 (bs, 1H), 6.19 (bs, 1H), 7.41 (d, 1H), 7.80 (t, 1H), 8.05 (d, 1H). ^{13}C NMR ($CDCl_3$): δ 17.1, 30.4, 34.2, 34.8, 35.9, 40.7, 46.3, 47.0, 47.6, 56.9, 65.8, 67.5, 70.0, 76.0, 96.3, 101.0, 111.6, 111.8, 118.6, 120.1, 121.1, 132.8-136.0 (m, 7 Ar*C*), 141.2, 155.9, 156.4, 161.3, 186.9, 187.4, 214.1. ESIMS: *m/z* 678.2 $(M-H)^-$, 713.9 $(M+Cl)^-$. Anal.: Calcd. for $C_{35}H_{37}NO_{13}$(•CH_3OH): C, 60.75; H, 5.81; N, 1.97. Found: C, 60.97; H, 5.57; N, 2.00.

Block Copolymer (4_{15}-b-3_{15}). In an inert-atmosphere glovebox, monomer **4** (23.3 mg, 0.046 mmol) was weighed into a 20-mL scintillation vial equipped with a magnetic stirring bar. A 9:1 mixture of dry $CDCl_3$/CD_3OD (2 mL) was added, followed by a solution of catalyst **5** (2.5 mg, 0.003 mmol) in 9:1 dry $CDCl_3$/CD_3OD (1 mL). The mixture was stirred for 45 min at room temperature. An aliquot (100 µL) was removed, quenched with ethyl vinyl ether, and analyzed by GPC (M_n = 6300, M_w/M_n = 1.19). Subsequently, a solution of monomer **3** (30 mg, 0.044 mmol) in 9:1 dry $CDCl_3$/CD_3OD (1.5 mL) was added and stirred for an additional 60 min. The polymerization was terminated with the addition of ethyl vinyl ether (1 mL). The copolymer **4**$_{15}$-*b*-**3**$_{15}$ (39.8 mg, 76%) was isolated as a dark red solid by precipitation in cold pentanes (200 mL), filtering, and washing with fresh pentanes (3x 50 mL). ^1H-NMR ($CDCl_3$): δ 0.89 (m), 1.26 (m), 1.57-2.06 (b), 2.11 (s), 2.18 (m), 2.33 (d), 2.59 (b), 2.71 (b), 3.05 (m), 3.38 (s), 3.54 (m), 3.65 (b), 4.10 (bs), 4.54 (bm), 4.77 (bm), 5.31-5.52 (bm), 7.05 (s), 7.47 (s), 7.80 (m), 8.06 (m). GPC: M_n = 12,550; PDI = 1.31. The theoretical M_n was calculated to be 17,901. The observed difference between the experimental and calculated M_n values can be attributed to a decreased hydrodynamic radius with respect to linear GPC polystyrene standards. ^1H-NMR integration of the peaks at 3.38 and 4.10 ppm (corresponding to the terminal methoxy protons in

monomer **4** and the CH_3O-aromatic protons in monomer **3**, respectively) showed a 3:2.4 ratio, which matches closely with the 1:1 stoichiometry used in the reaction. Comparing the absorbance (0.755 a.u.) of $\mathbf{4}_{15}$-*b*-$\mathbf{3}_{15}$ (1 mg in 10 mL THF) against a calibration curve of the doxorubicin monomer (ε = 8884 cm^{-1} M^{-1}) in THF (Figure S1) also confirmed the complete incorporation of doxorubicin with an abosorbance corresponding to 15 units.

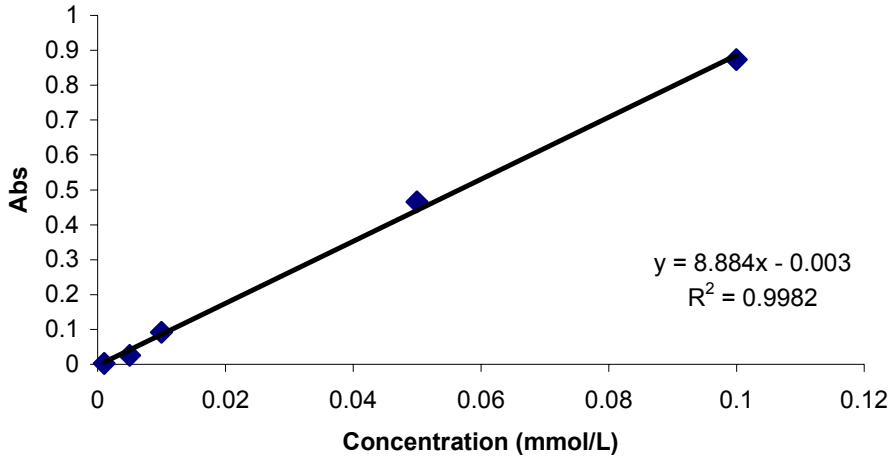

Figure S1. The absorbance calibration curve for 3'-carbamate norbornene-conjugated doxorubicin **(3)** in THF.

General Procedure for the Preparation of Nanoparticle Solutions. Aqueous solutions of the block copolymer were prepared by dialysis. A stock solution of the copolymer (0.01 wt%) in DMSO was stirred for 4 h at room temperature to ensure complete polymer solubilization. An aliquot (2.5 mL) was transferred to a 4-mL scintillation vial and set to stir vigorously. Ultrapure water (Millipore 18.2 MΩ•cm resistivity) was added to the stirring copolymer solution at a rate of 1 drop (10 μL, 0.35 wt%) per every 10 s using a micro-pipette until the solution contained 18 wt% water. The resulting aggregate solution was placed in a dialysis tube (Spectra/Por RC, 3-mL Float-a-lyzer, MWCO = 3500) and dialyzed against ultrapure water in a 500-mL Erlenmeyer flask with the dialysis solution changed every 30 min. Complete removal of DMSO from the filtrate after 48 h was verified by UV-Vis spectroscopy as indicated by the disappearance of the UV cut-off for DMSO at 268 nm.

Transmission Electron Microscopy. Transmission electron microscopy (TEM) was performed on a Hitachi H8100 microscope operating at an accelerating voltage of 200 kV. For the observation of the size and distribution of the copolymer nanoparticles, samples (5 μL) were deposited from aqueous solutions of the copolymer nanoparticles onto copper EM grids (400 mesh, Formvar/carbon-coated). Water was allowed to evaporate from the grids at atmospheric pressure and room temperature. Negative staining was performed by exposing the grids to a solution of 2 wt% uranyl acetate (5 μL) for 2 min. The grids were tapped dry with a filter paper to remove the excess stain. The samples were air-dried before TEM measurement.

Light-Scattering Measurements. Dynamic light-scattering (DLS) measurements were performed on a Brookhaven Instruments Corp. photon correlation spectrometer (BI-200 SM goniometer) fitted with a Brookhaven Instruments BI-9000AT digital correlator and a 300-mW argon ion laser at 514 nm. The scattering angle used was 90°. A refractive index-matching bath of filtered decalin (0.2 m) surrounded

the scattering cell, and the temperature was fixed at 25 ˚C. Correlation data were fitted–using the method of cumulants[5]–to the logarithm of the correlation function, yielding the diffusion coefficient, *D*. The hydrodynamic diameters (D_H) of the nanoparticles were calculated using *D* and the Stokes-Einstein equation ($D = k_B T/3\pi\eta D_H$, where k_B is the Boltzmann constant, *T* is the absolute temperature, and η is the solvent viscosity). The polydispersity factor of the nanoparticles, represented as μ_2/Γ^2, where μ_2 is the second cumulant of the decay function and Γ is the average characteristic line width, was calculated by the cumulant method. CONTIN algorithms were used in the Laplace inversion of the autocorrelation functions to confirm particle size distributions.[6] All analyses were performed with the supplied instrument software (BIC Dynamic Light Scattering Software).

***In Vitro* Doxorubicin Release Experiments**. Nanoparticle solutions (3.0 mL, 18 wt% H_2O in DMSO) containing 0.01 wt% of the copolymer were prepared as stated above. The solution was transferred (300 μL) to a foil-wrapped 1.5-mL safe lock eppendorf tube. The tube was centrifuged for 30 min at 10K rpm. An aliquot of the supernatant was removed and the absorbance at 480 nm was compared in triplicate versus that of ultrapure water as the initial point (Table S1). Although the nanoparticle solution does absorb light at 480 nm, rigorous centrifugation reduces this absorbance to zero, within experimental limits. In contrast, a solution of doxorubicin•HCl retains its 480-nm absorbance even after being subjected to the same centrifugation procedure.

The supernatant was then replaced with pH 4 HCl-adjusted ultrapure water and the tube was vortexed for several minutes until the polymer was redispersed. The tube was shaken for three hours and 30 min on an NMR-tube shaker and then centrifuged for 30 min at 10K rpm. An aliquot of the supernatant was removed and its absorbance at 480 nm was measured as an indication of the release of doxorubicin. The aliquot was then added back to the eppendorf tube. The tube was then resubjected to vortexing and shaken as described above with occasional sampling and monitoring of the solution absorbance at 480 nm. The release of doxorubicin was monitored over a 24-h period. The theoretical molecular weight of the copolymer was used to determine the concentration of doxorubicin at 100% release from the copolymer. The theoretical absorbance at this concentration was calculated from a calibration curve (Figure S2) for doxorubicin•HCl.

Table S1. Control experiments for doxorubicin release

Measurement number	Absorbance of 0.01 wt% nanoparticles[a]	Absorbance of Supernatant after centrifugation[b]	Absorbance of 0.1-mM doxorubicin•HCl[a]	Absorbance of Supernatant after centrifugation[b]
1	0.1044	0.0026	0.9316	0.8926
2	0.1052	0.0048	0.8841	0.8508
3	0.1083	0.0008	0.9197	0.9039

[a] The absorbance of the nanoparticle solution before centrifugation was taken to ensure that the nanoparticles did absorb at 480 nm as a comparative measurement. The absorbance of the 0.1-mM standard doxorubicin•HCl was taken before centrifugation as a comparative measurement.

[b] After centrifuging the nanoparticle solution for 30-min at 10K rpm, the absorbance of an aliquot of the supernatant was taken to show that the absorbance is reduced to nearly zero. After centrifuging the 0.1-mM doxorubicin•HCl solution for the same amount of time, the absorbance of the solution does not change significantly and is assumed to be negligible for practical considerations.

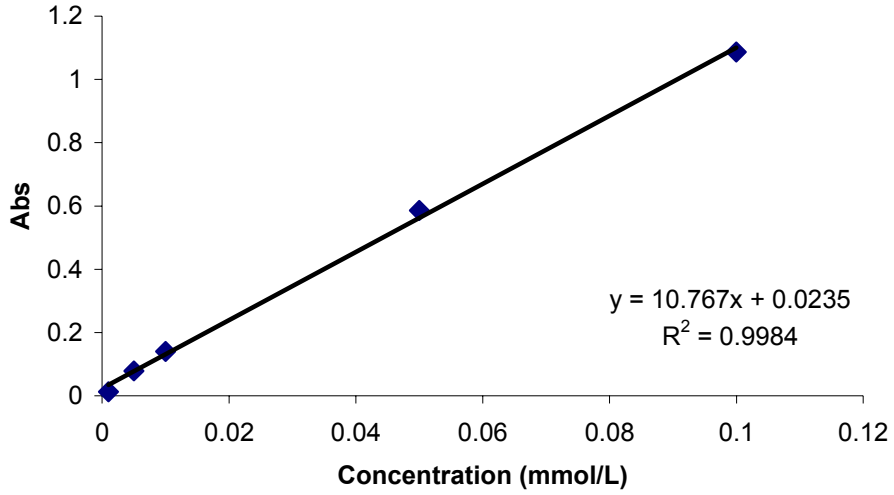

Figure S2. The absorbance calibration curve for doxorubicin•HCl in pH 4 water.

Table S2. Doxorubicin release data from nanopaticles of copolymer $\mathbf{4}_{15}$-*b*-$\mathbf{3}_{15}$.

Time (h)	Absorbance (at 480 nm)[a]	Average Absorbance	Release (%)	Standard Deviation
	0.0663			
0.5	0.0834	0.0722	19.52	0.968
	0.0670			
	0.1238			
1	0.1030	0.106	28.65	1.65
	0.0912			
	0.1661			
4	0.1168	0.1292	34.93	3.25
	0.1048			
	0.1666			
8	0.1451	0.1652	44.64	1.94
	0.1838			
	0.1667			
12	0.1694	0.1683	45.49	0.142
	0.1688			
	0.1815			
24	0.1667	0.1748	47.25	0.751
	0.1763			

[a] Each sample was measured three times.

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