

Single-Molecule Nanoscale Drug Carriers with Quantitative Supramolecular Loading

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A- Experimental Methods:

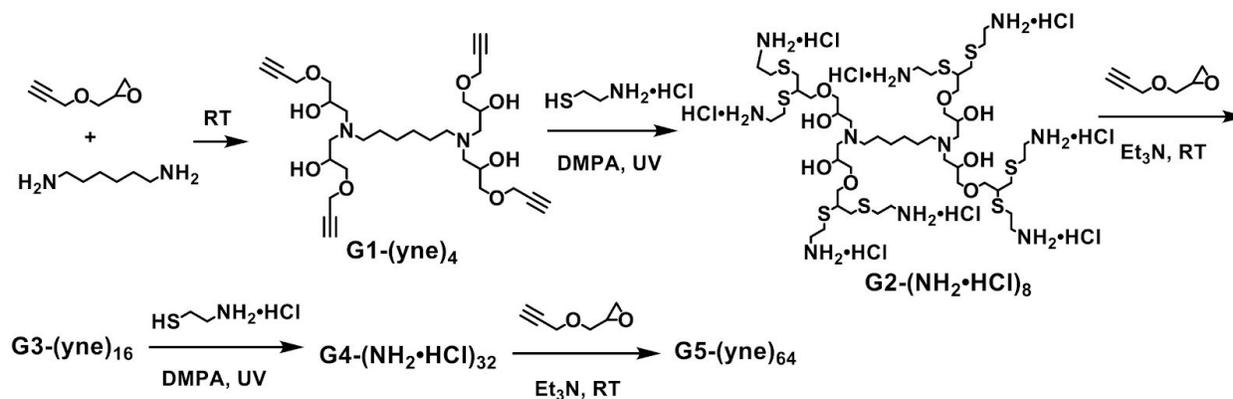
A.1- Materials

Copper(II) sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) (ACS grade, BDH), ascorbic sodium (99+%, Alfa Aesar), sodium azide (VWR), methanesulfonyl chloride (98%, BeanTown Chemical), chloroacetyl chloride (98%, Alfa Aesar), tert-butyl carbazate (95+%, Matrix Scientific), adamantan-1-amine (98%, Oxchem Corp), hexamethylenediamine (98%, Sigma-Aldrich), *p*-xylylenediamine (97%, Bide Pharmatech), 2,2-dimethoxy-2-phenylacetophenone (DMPA, 99%, Chem Impex) cysteamine hydrochloride (99%, Chem Impex), polyethylene glycol monomethylether 2000 (Sigma-Aldrich), Resazurin sodium salt (Sigma-Aldrich), and doxorubicin hydrochloride (98%, Bide Pharmatech) were used as received. Glycidyl propargyl ether¹⁻³ and CB[7] azide^{4,5} were prepared according references. All other chemicals and solvents were analytical grade and purified through appropriate methods.

A.2- Instruments and Methods

UV irradiation reactions were carried out in a Rayonet RPR-100 photochemical reactor equipped with RMR-3500A lamps. ¹H nuclear magnetic resonance (NMR), ¹³C NMR, correlation spectroscopy (COSY), and heteronuclear single-quantum correlation spectroscopy (HSQC) were acquired on a Bruker 500 MHz spectrometer at 25°C. Size exclusion chromatography (SEC) was equipped with a Waters 515 HPLC pump, Polymer Standards Services (PSS) columns (GRAM, 10⁴, 10³ and 10² Å) at 55 °C with DMF flow rate = 1.00 mL/min and a Waters 2414 differential refractive index (RI) detector. The hydrodynamic size (Dh) of the samples were determined using dynamic light scattering (DLS) equipped with Zetasizer Nano-ZS (He-Ne laser wavelength at 633 nm, Malvern Instruments, Malvern, UK). The levels of Dox-Ad were determined by Gilson analytical RP-HPLC using a Gemini (Phenomenex) C18 column (250 mm x 4.6 mm) with 5 µm fused silica beads isocratically eluted with a 50:50 v/v acetonitrile/water with 0.1% NH₄OH. The system was run at a flow rate of 1 mL/min, and Dox-Ad was detected at 260 nm.

A.3- Dendrimer Synthesis



Synthesis of G1(-yne)₄. Hexamethylenediamine (0.725 g, 6.25 mmol) was dissolved in MeOH (3.0 ml) in a 20 mL vial and glycidyl propargyl ether (5.60 g, 50.0 mmol) was added. The reaction was stirred for 24 hours at room temperature. Solvent was evaporated under reduced pressure and DCM (5 mL) was added to dissolve the residue, from which the solution was precipitated into 200 mL of hexane. The precipitated oil was collected and dissolved in DCM (5 mL) and the solution was again precipitated into 200 mL of hexane. The procedure was repeated once more and the product was collected, and residual hexane remained was removed by adding DCM and concentrating under reduced pressure. The purified product [G1(-yne)₄, 3.50 g] was dried in a vacuum oven at room temperature to an oil. ¹H NMR (500 MHz, CDCl₃): δ (ppm) = 4.20 (d, *J* = 2.4 Hz, O-CH₂CCH, 8H), 3.86 (m, 4H), 3.57 (m, 4H), 3.49 (m, 4H), 2.56 (m, 12H), 2.45 (m, 4H), 1.45 (m, 4H), 1.29 (m, 4H).

Synthesis of G2-(NH₂·HCl)₈. G1(-yne)₄ (2.49 g, 4.41 mmol), cysteamine hydrochloride (11.97 g, 105.9 mmol), and DMPA (0.23 g, 0.9 mmol) were dissolved with MeOH (20 ml) in a 100 mL flask. The mixture was purged with nitrogen for 20 minutes and then UV-irradiated for 6 hour with stirring. The reaction mixture was precipitated into 2:1 mixture of acetone and DCM (v : v, 300 mL) and the precipitate was collected by centrifuge. The precipitate was redissolved in 20 mL of MeOH and precipitated into 2:1 (v:v) mixture of acetone and DCM (300 mL). This procedure was repeated three more times and the product was collected; residual acetone/DCM was removed by freeze-drying. The purified product [G2-(NH₂·HCl)₈, 4.68 g] was obtained as a solid. ¹H NMR (500 MHz, D₂O): δ (ppm) = 4.27 (m), 3.84-3.55 (m), 3.42-3.18 (m), 3.02-2.87 (m), 1.77 (m), 1.42 (m).

Synthesis of G3(-yne)₁₆. G2-(NH₂·HCl)₈ (0.68 g, 0.46 mmol) and glycidyl propargyl ether (1.71 g, 15.3 mmol) were dissolved in MeOH (3.0 ml) in a 20 mL vial and triethylamine (0.64 mL) was then

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added to free the amine. The reaction was stirred for 24 hours at room temperature. Solvent was removed under reduced pressure and the residue was dissolved in 50 mL of DCM. The DCM layer was washed successively with 5% NaOH (50 mL×2) and water (50 mL×1) and then dried over Na₂SO₄. The total volume was reduced to 5 mL and precipitated into hexane (50 mL). The precipitated oil was collected and dissolved with DCM (5 mL) and the solution was precipitated into 200 mL of hexane. The procedure was repeated once more before the product was collected, and residual hexane was removed by adding DCM and concentrating under reduced pressure. The purified product [G3-(yne)₁₆, 0.40 g] was dried in a vacuum oven at room temperature to an oil. ¹H NMR (500 MHz, CDCl₃): δ (ppm) = 4.20 (m), 3.89 (m), 3.74-3.35 (m), 3.08-2.40 (m), 1.44 (m), 1.27 (m).

Synthesis of G4-(NH₃Cl)₃₂. G3-(yne)₁₆ (0.40 g, 0.134 mmol), cysteamine hydrochloride (1.60 g, 14.1 mmol) and DMPA (0.02 g, 0.078 mmol) were dissolved in MeOH (5 ml) in a 20 mL vial. The reaction and purification procedures were exactly the same as those of the synthesis of G2-(NH₂·HCl)₈. The purified product [G4-(NH₂·HCl)₃₂, 0.55 g] was obtained as a solid. ¹H NMR (500 MHz, D₂O): δ (ppm) = 4.31–3.97 (m), 3.93-2.70 (m), 1.74 (m), 1.39 (m).

Synthesis of G5-yne₆₄. G4-(NH₃Cl)₃₂ (0.9 g, 0.136 mmol) and glycidyl propargyl ether (1.1 g, 9.82 mmol) were dissolved in MeOH (3.0 ml) in a 20 mL vial. Then triethylamine (0.53 mL) was added and the reaction was stirred for 24 hours at room temperature. The purification procedures were exactly the same as for the synthesis of G3-(yne)₁₆. The purified product [G5-(yne)₆₄, 1.23 g] was dried in a vacuum oven at room temperature to an oil. ¹H NMR (500 MHz, CDCl₃): δ (ppm) = 4.20 (m), 4.00-3.35 (m), 3.08-2.40 (m), 1.44 (m), 1.28 (m).

Synthesis of G5-PEG₅₆CB[7]₈. G5-(yne)₆₄ (57.0 mg), CB[7] azide (45.6 mg), CuSO₄·5H₂O (1.6 mg), PMDETA (N,N,N',N'',N''-pentamethyldiethylenetriamine) (1.33 uL), and DMF (7 mL) were added to a 25 mL Schlenk flask. The flask was degassed by three freeze-pump-thaw cycles. At the last cycle, the flask was opened while frozen to quickly add sodium ascorbate (25.0 mg, 0.125 mmol) into the flask before re-capping the flask. The flask was vacuumed and backfilled with N₂ for 5 cycles before being immersed in a thermostatic oil bath at 50 °C to thaw the mixture and initiate the reaction. After 48 hours stirring at 50 °C, the reaction mixture was frozen by liquid nitrogen and PEG azide (PEG-N₃) (620 mg) and sodium ascorbate (25.0 mg, 0.125 mmol) were added to the frozen mixture before re-capping the flask. The flask was vacuumed and backfilled with N₂ for 5 cycles before being immersed in a thermostatic oil bath at 50 °C to thaw the mixture solution and initiate the reaction.

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After 24 hours more stirring at 50 °C, the organic solvent, copper catalyst, and excess of PEG azide were removed by washing the reaction mixture with >40 mL of water using a Corning Spin-XUF Concentrator with molecular weight cutoff of 5k Da. The product G5-CB[7]₈-PEG (0.7 g) was obtained as a fine powder by freeze-drying.

Synthesis of G5- PEG₆₀CB₄. G5-yne₆₄ (17.5 mg), CB[7] azide (7.0 mg), CuSO₄·5H₂O (0.25 mg), PMDETA (0.21 uL), DMF (2 mL), and PEG azide (PEG-N₃) (181 mg) were used. The reaction and purification procedures were exactly the same as those in the synthesis of G5-CB[7]₈-PEG. The product G5-CB[7]₄-PEG was obtained as a fine powder by freeze-drying.

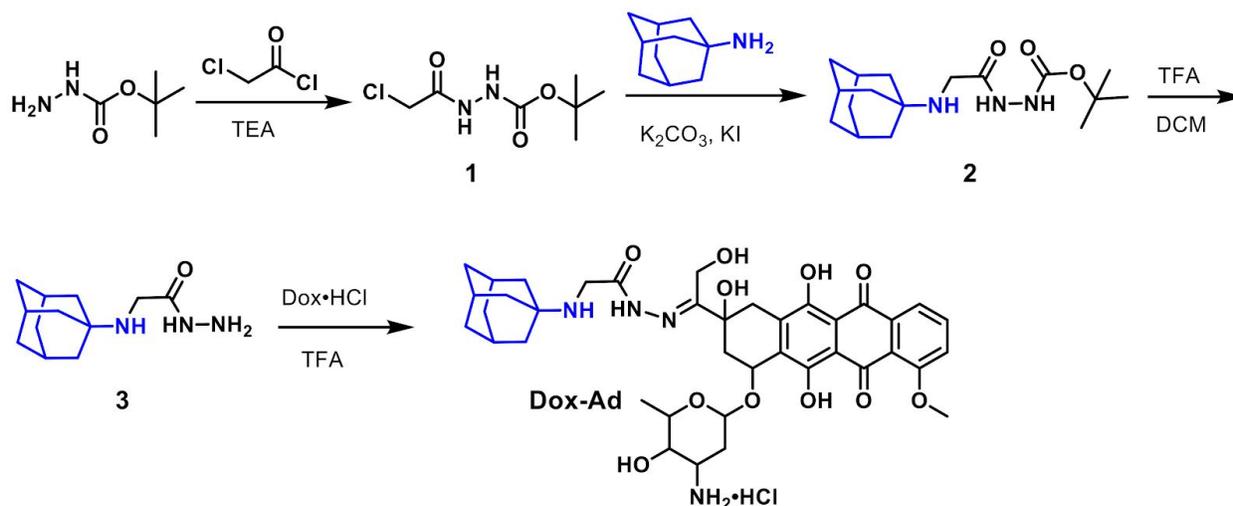
Synthesis of G5- PEG₅₂CB₁₂. G5-yne₆₄ (8.0 mg), CB[7] azide (9.7 mg), CuSO₄·5H₂O (0.18 mg), PMDETA (0.15 uL), DMF (2 mL), and PEG azide (PEG-N₃) (105 mg) were used. The reaction and purification procedures were exactly the same as those in the synthesis of G5-CB[7]₈-PEG. The product G5-CB[7]₁₂-PEG (60 mg) was obtained as fine powder by freeze-drying.

Estimation of CB7 content. A stock solution of G5-(CB7)₈-PEG (146 mg) in 2 mL of D₂O was prepared. A sample of the stock solution (0.2 mL) was mixed with 1,4-phenylenedimethanamine dihydrochloride (probe) (0.08 mg). The ¹H-NMR spectrum of this mixture was recorded in D₂O. In the obtained spectrum, the integrals of signals at δ = 6.56 ppm (A_{threaded}) and δ = 7.46 ppm (A_{free}) were taken carefully.

The content of CB7 from this 0.2 mL stock solution in micromole was calculated as:

$$C_{CB[7]} = \frac{0.08}{MW_{probe}} \times \frac{A_{threaded}}{A_{threaded} + A_{free}} \text{ (mmol)}$$

For the determination of CB7 content, two samples were taken and ¹H-NMR spectra of each sample with probe was recorded. The average of two independent experiments were used to verify CB[7] content in each sample.

A.4- Doxorubicin Prodrug Synthesis:

Synthesis of compound 1. Chloroacetyl chloride (7.9 mL, 0.1 mol) was slowly added to the solution of triethylamine (14.0 mL, 0.1 mol) and tert-Butyl carbazate (13.2 g, 0.1 mol) in 20 mL of DCM that was placed into a chilled ice bath. After the addition of chloroacetyl chloride, the cooling bath was removed and the reaction mixture was stirred for additional 3 hours. Then, the reaction mixture was diluted into 100 mL of DCM and washed with H₂O (100mL) three times. The DCM layer was dried over Na₂SO₄ and concentrated under reduced pressure. The obtained crystal-like solid (Compound 1, 13.6 g) was used directly for the next step. ¹H NMR (**Figure S4**, 500 MHz, CDCl₃): δ (ppm) = 8.28 (s, 1H), 6.60 (s, 1H), 4.13 (s, 2H), 1.49 (s, 9H).

Synthesis of compound 2. The reaction mixture of adamantly amine (3.02 g, 0.02 mol), compound **1** (4.16 g, 0.02 mol), K₂CO₃ (8.28 g, 0.06 mol), KI (2.32 g, 0.02 mol), and THF (100 mL) was stirred at 65°C for 3 hours. Solvent was then removed under reduced pressure. The residue was suspended in 100 mL of DCM and washed with H₂O (100mL) three times. The DCM phase was dried over Na₂SO₄ and concentrated under reduced pressure. The obtained crystal-like solid (Compound **2**, 5.7 g) was used directly for the next step. ¹H NMR (**Figure S4**, 500 MHz, CDCl₃): δ (ppm) = 3.38 (s, 2H), 2.07 (s, 3H), 1.71-1.55 (m, 12H), 1.48 (s, 9H).

Synthesis of compound 3. Compound **2** (3.35 g, 10.3 mmol) was dissolved in 20 mL DCM and TFA (10 mL) was added. The reaction mixture was stirred at RT for 4 hours. The volume was reduced under vacuum and the residue was precipitated into hexane. The sticky precipitate was washed with hexane (30 mL) three times and dried under reduced pressure. The final product

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(Compound 3, 5.9 g) was obtained as viscous oil. ^1H NMR (**Figure S4**, 500 MHz, $\text{DMSO-}d_6$): δ (ppm) = 4.00 (s, 2H), 2.25 (s, 3H), 1.89 (s, 6H), 1.80-1.60 (m, 6H).

Synthesis of Dox-Ad. Compound 3 (717 mg, 3.2 mmol), doxorubicin hydrochloride (136 mg, 0.234 mmol), and TFA (50 μL) were dissolved in MeOH (10 mL). The reaction mixture was stirred in the dark for 48 hours. Then, the volume of the mixture was reduced to about 1 mL and precipitated into 15 mL of ethyl acetate. The precipitate (Dox-Ad) was collected by centrifuge, washed by ethyl acetate (5 mL) three times and dried under vacuum overnight. ^1H NMR (500 MHz, $\text{DMSO-}d_6$): δ (ppm) = 13.31 (s, 1H), 11.01 (s, 1H), 8.72 (s, 2H), 7.91 (m, 3H), 7.85 (s, 2H), 7.66 (d, J = 3.2 Hz, 1H), 5.80 (t, J = 4.5 Hz, 1H), 5.67 (s, 1H), 5.49 (d, J = 5.8 Hz, 1H), 5.33 (d, J = 2.5 Hz, 1H), 4.96 (t, J = 7.2 Hz, 1H), 4.41 (m, 2H), 4.01 (t, J = 6.8 Hz, 1H), 3.97 (s, 3H), 3.80 (d, J = 16.0 Hz, 1H), 3.65 (d, J = 16.0 Hz, 1H), 3.56 (m, 1H), 3.36 (d, J = 17.0 Hz, 1H), 3.36 (m, 1H), 2.76 (d, J = 17.0 Hz, 1H), 2.60 (m, 1H), 2.10 (m, 1H), 2.07 (s, 3H), 1.92-1.68 (m, 2H), 1.75 (s, 6H), 1.62 (d, J = 12.0 Hz, 3H), 1.54 (d, J = 12.0 Hz, 3H), 1.16 (d, J = 6.5 Hz, 3H). ESI-MS (Advion Single-Quadripole CMS-L): 748.472 (observed) 748.339 (expected)

A.5- Determination of K_{eq} by ^1H -NMR competition experiments

^1H -NMR competition experiments were performed on a 500 MHz Bruker NMR spectrometer, according to the method described by Isaacs and colleagues.^{6,7} Briefly, NMR samples were prepared at three different ratios of a) Dox-Ad, b) a competitive guest with a previously reported binding constant, and c) free unmodified **CB[7]**. Samples were prepared in D_2O and allowed to reach equilibrium for times ranging from ~30 minutes for the weakest guest to 4 days for the strongest guests. Equilibrium was verified by tracking the measured ^1H -NMR resonances until the spectra became constant. ^1H -NMR spectra were then acquired for each sample. The resonances which could be clearly assigned to the bound (marked as *' in **Figure S8**) and the free (marked as * in **Figure S9**) guest and did not overlap with any other signals were integrated. The concentrations of the bound and free guest were obtained by this method, also yielding the concentration for the bound and the free form of the competing guest as well. In these equilibrium mixture, the K_{rel} values were calculated using the relationships below.^{6,7} K_{rel} values for 3 independent experiments with varying and known ratios of each of the three components were averaged to obtain the final estimated K_{eq} values reported in the paper.

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$$K_{rel, test/known} = \frac{[test]_{bound}[known]_{free}}{[test]_{free}[known]_{bound}}$$

K_{rel} can alternatively be expressed as following:

$$K_{rel, test/known} = \frac{K_{eq, test}}{K_{eq, known}}$$

Rearranging yields the following expression for K_{eq} :

$$K_{eq, test} = K_{rel, test/known} \times K_{eq, known}$$

A.5- Drug Loading and Release Studies:

Drug Loading. Stock solution of prodrug at a concentration of 1.11 mM (1 mg/mL) were prepared. The loading of prodrug was realized by mixing this prodrug stock solution with G5-CB[7]₈-PEG stock solution. Then, the mixed solution was injected into the HPLC for testing, measuring absorbance at 260 nm.

Sample 1: 40 uL G5-(CB[7])₈-PEG stock + 20 uL prodrug stock (1 eq of prodrug to CB[7] content)

Sample 2: 40 uL G5-(CB[7])₈-PEG stock + 40 uL prodrug stock (2 eq of prodrug to CB[7] content)

Sample 3: 0 uL G5-(CB[7])₈-PEG stock + 20 uL prodrug stock

Doxorubicin release. G5-(CB[7])₈-PEG loaded with 1 equivalent of was added to a dialysis tube (3.5K MWCO; Slide-A-Lyzer, Thermo Scientific) and dialysed against 14 ml of phosphate buffer at pH 7.4, 6.5, 5.5, and 4.0. The tubes were capped and gently agitated at room temperature with a shaker. At serial time intervals, 1 mL of the buffer solution was taken and 1 mL of fresh buffer solution of the same pH was replaced. The fluorescence intensity of Dox released was measured at 596 nm with an excitation wavelength of 480 nm using a microplate reader (Infinite M200 PRO, Tecan). The absolute amount of DOX released was calculated based on a calibration curve from known concentration of doxorubicin.

A.6- Cell Assays:

Cytotoxicity Assay. MDA-MB-231 cells were seeded in 96 well plates at a density of 1×10^4 cells per well with 200 μ L complete media (DMEM + 10% FBS) and cultured for 24 hrs. The different concentrations of G5-CB[7]₈-PEG + Dox-Ad and free doxorubicin in fresh complete media were

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prepared and added to each well. The cells were incubated in testing media for 72 hrs. To each well, 20 μ L of alamarBlue test solution was added. After incubation at 37 °C for 2 hrs, the fluorescence intensities were measured with a microplate reader (ex: 560 nm, em: 590 nm). The results were expressed as percentage viability relative to controls. Each concentration was done in triplicate. Samples were fit to a standard dose-response function (GraphPad Prism v7) to determine the IC₅₀.

Intracellular Localization. MDA-MB-231 cells were seeded on 10 mm² glass coverslips placed in 6-well plates and incubated for 24 hrs. Then, the cells were incubated with nanomedicine and free dox for 1 hour. After being stained with LysoTracker Green according to the manufacturer's protocol, the cells were fixed with 4% paraformaldehyde immediately. Nuclei were stained with DAPI. Intracellular localization was visualized using EVOS FL Auto cell imaging system.

B. Supplemental Data:

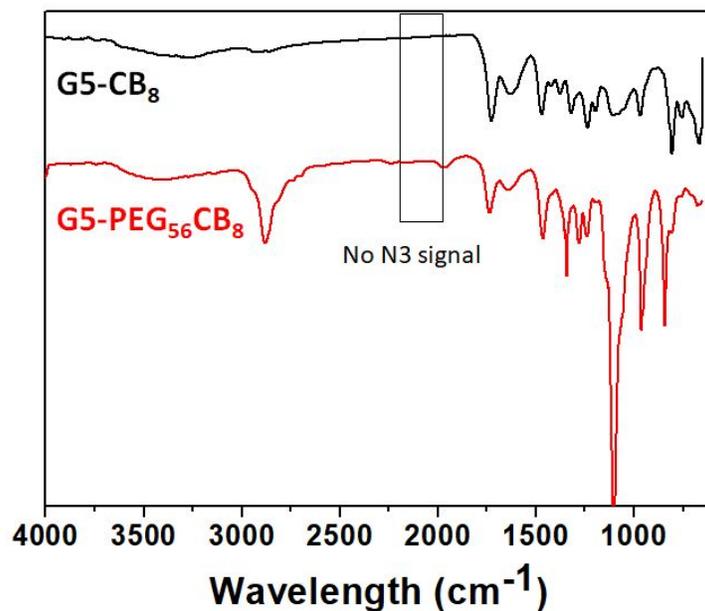


Figure S1. FT-IR spectra of the products after \ alkyne-azide 'click' coupling.

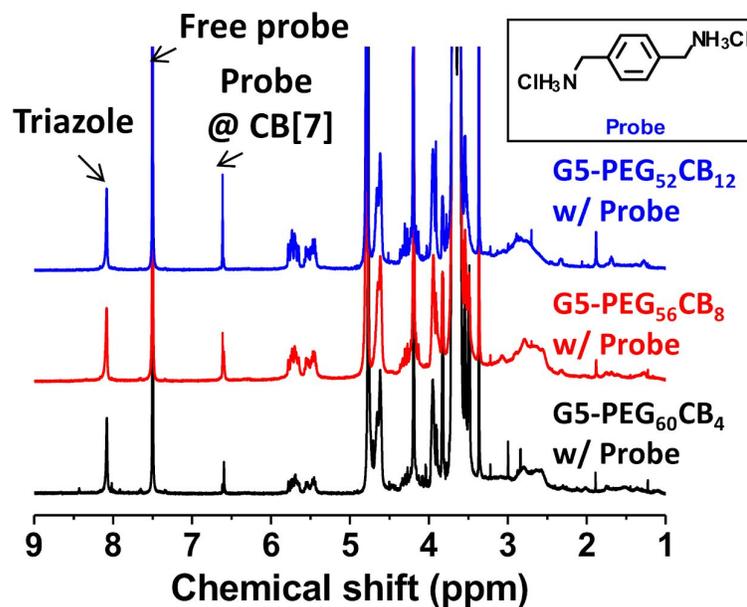


Figure S2. ¹H-NMR of dendrimers functionalized with four (black line), eight (red line) and twelve (blue line) CB[7] with *p*-xylylenediamine guest used as a probe in D₂O.

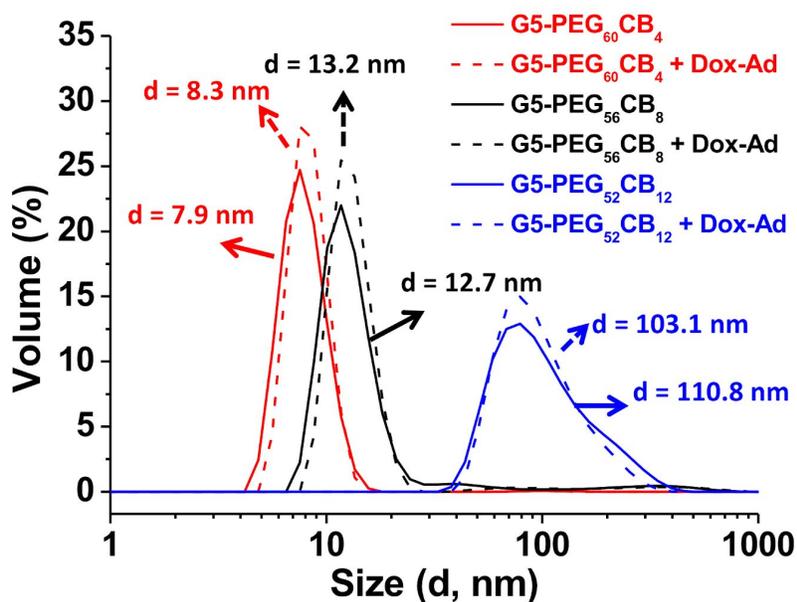


Figure S3. DLS size measurements of the dendrimers functionalized with four (red line), eight (black line) and twelve (blue line) CB[7] before (solid line) and after (dashed line) prodrug loading in PBS.

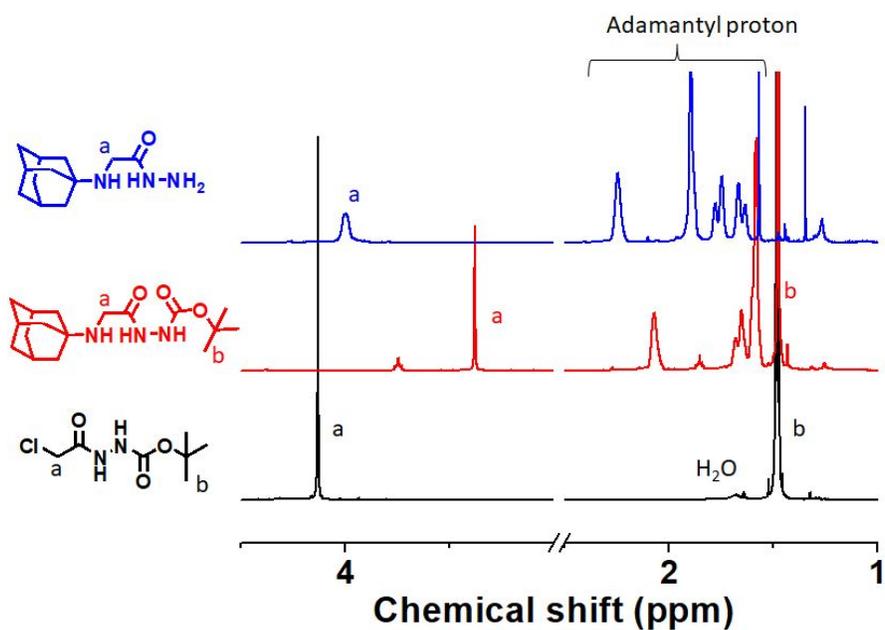


Figure S4. ¹H-NMR spectra of compound 1 (black line), compound 2 (red line) in CDCl₃ and compound 3 (blue line) in DMSO-*d*₆.

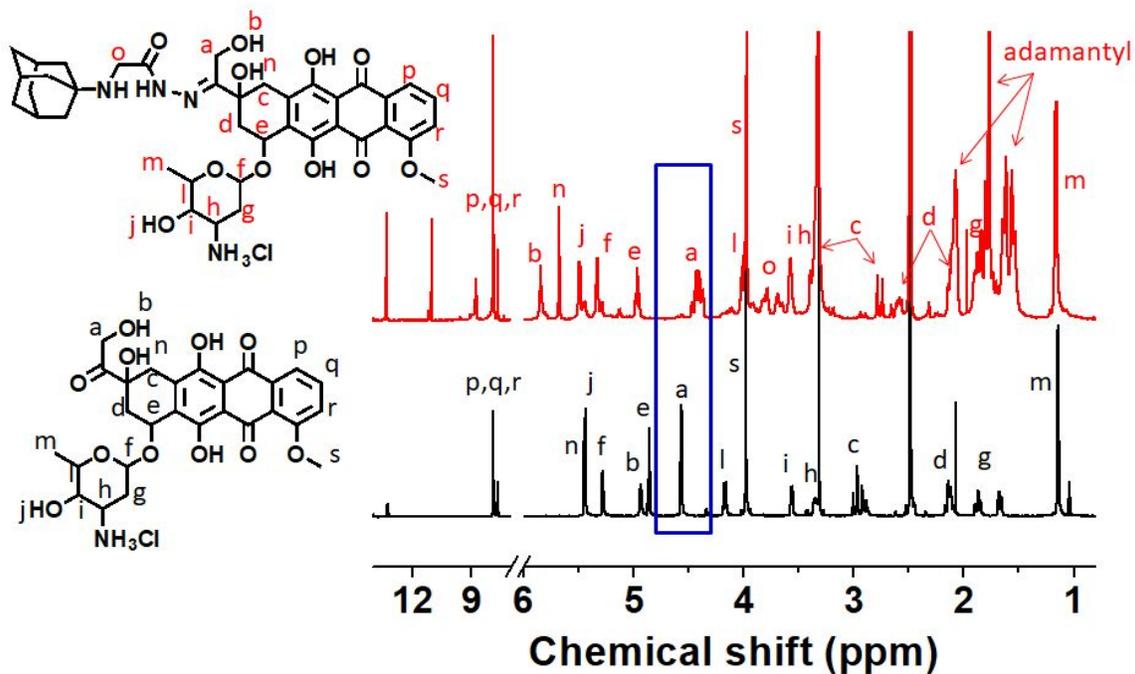


Figure S5. ^1H -NMR spectra of free doxorubicin hydrochloride (black line) and Dox-Ad (red line) in $\text{DMSO-}d_6$.

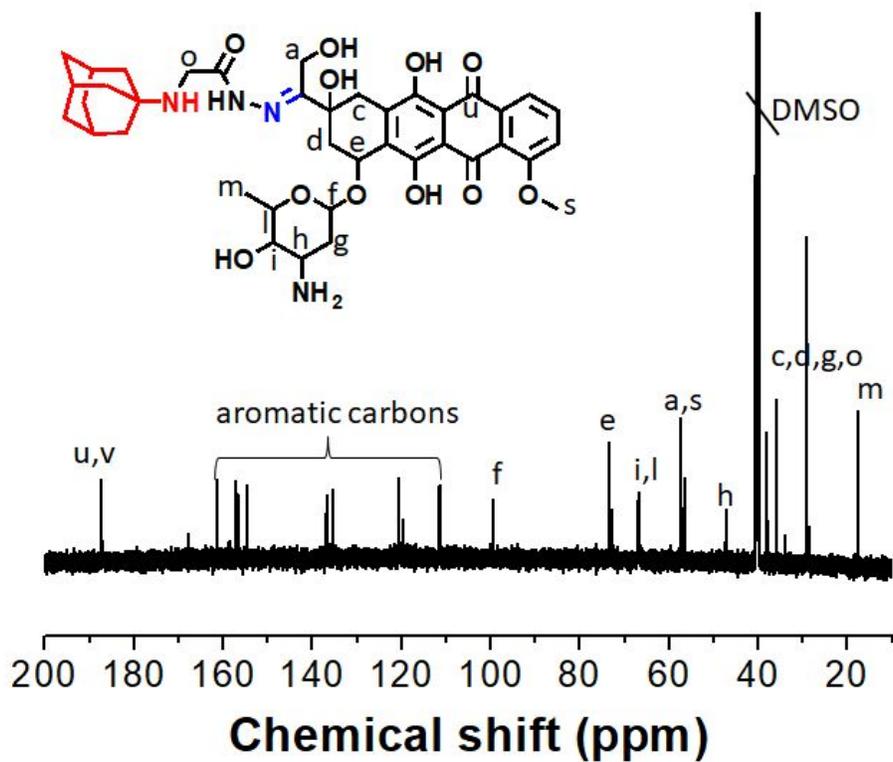


Figure S6. ^{13}C NMR spectrum of Dox-Ad in $\text{DMSO-}d_6$.

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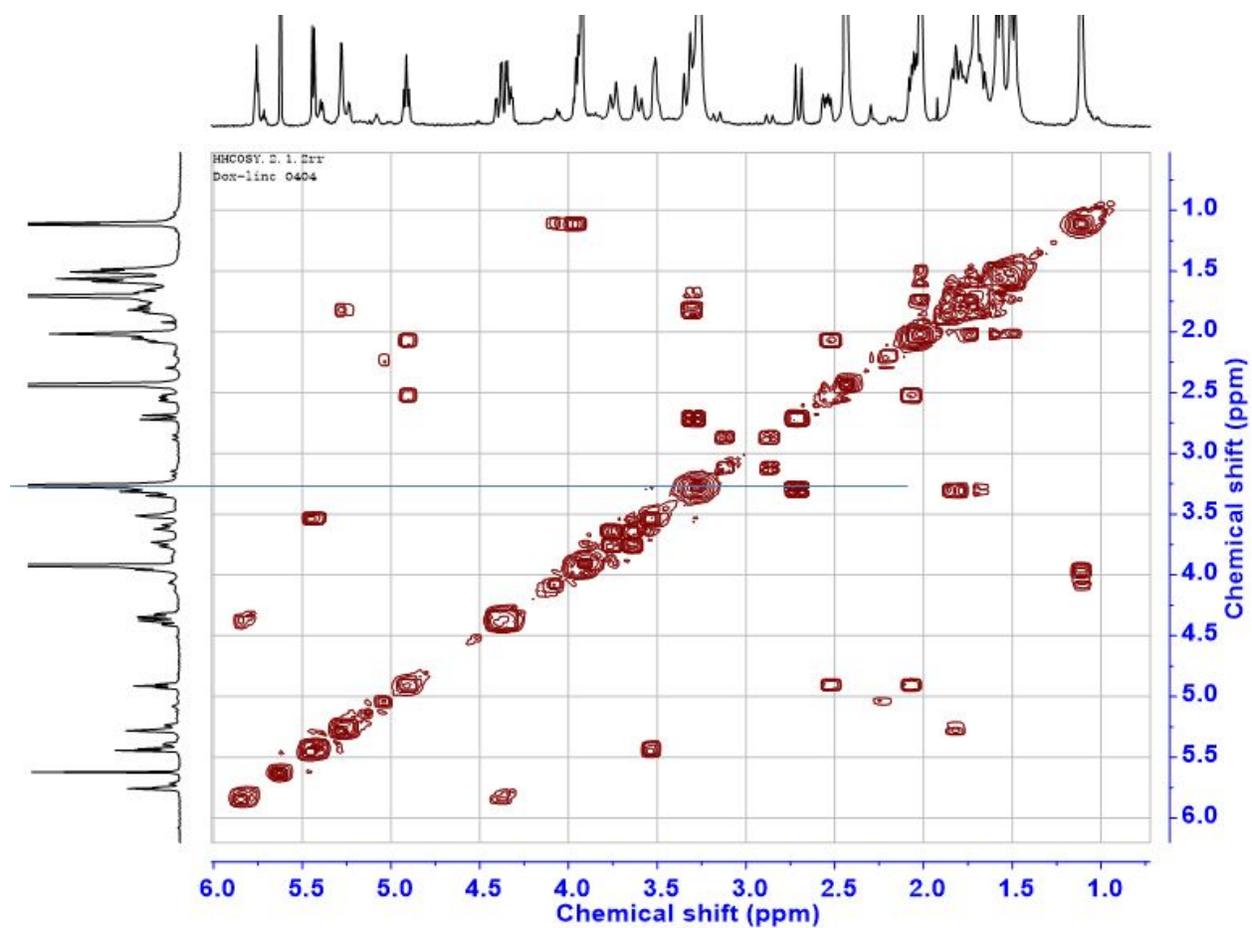


Figure S7. H-H COSY spectrum of Dox-Ad in DMSO- d_6 .

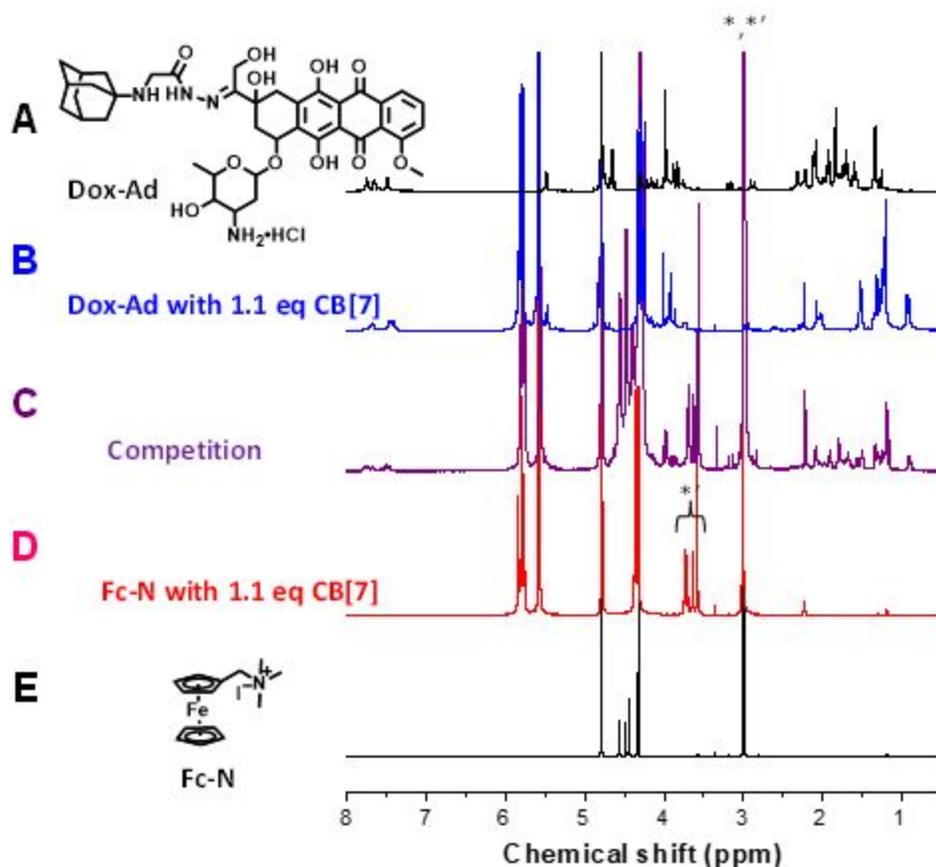


Figure S8. Example ¹H-NMR spectra from guest competition studies to determine K_{eq} for Dox-Ad. (A) Dox-Ad, (B) model guest with CB[7], (C) Dox-Ad (1.67 mM) and competitive guest (14.21 mM) with CB[7] (3.93 mM), (D) competitive guest Fc-N with CB[7] and (E) competitive guest Fc-N in D₂O. K_{rel} value of 9.89 ± 3.29 was obtained from averaging three different competition experiments with different ratios of guest, competitor, and CB[7]. Comparing to the literature-reported value of K_{eq} of $3.31 \times 10^{11} \text{ M}^{-1}$ for the competitor Fc-N,⁶ the K_{eq} for this guest was determined at $(3.26 \pm 1.07) \times 10^{12} \text{ M}^{-1}$. (*'-bound signal, *-free signal)

C. Supporting References:

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