Supporting information for:

Paclitaxel-Conjugated PAMAM Dendrimers Adversely Affect Microtubule Structure through Two Independent Modes of Action

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Supporting Information

Supporting Methods

Synthesis and Characterization of the Paclitaxel Linker

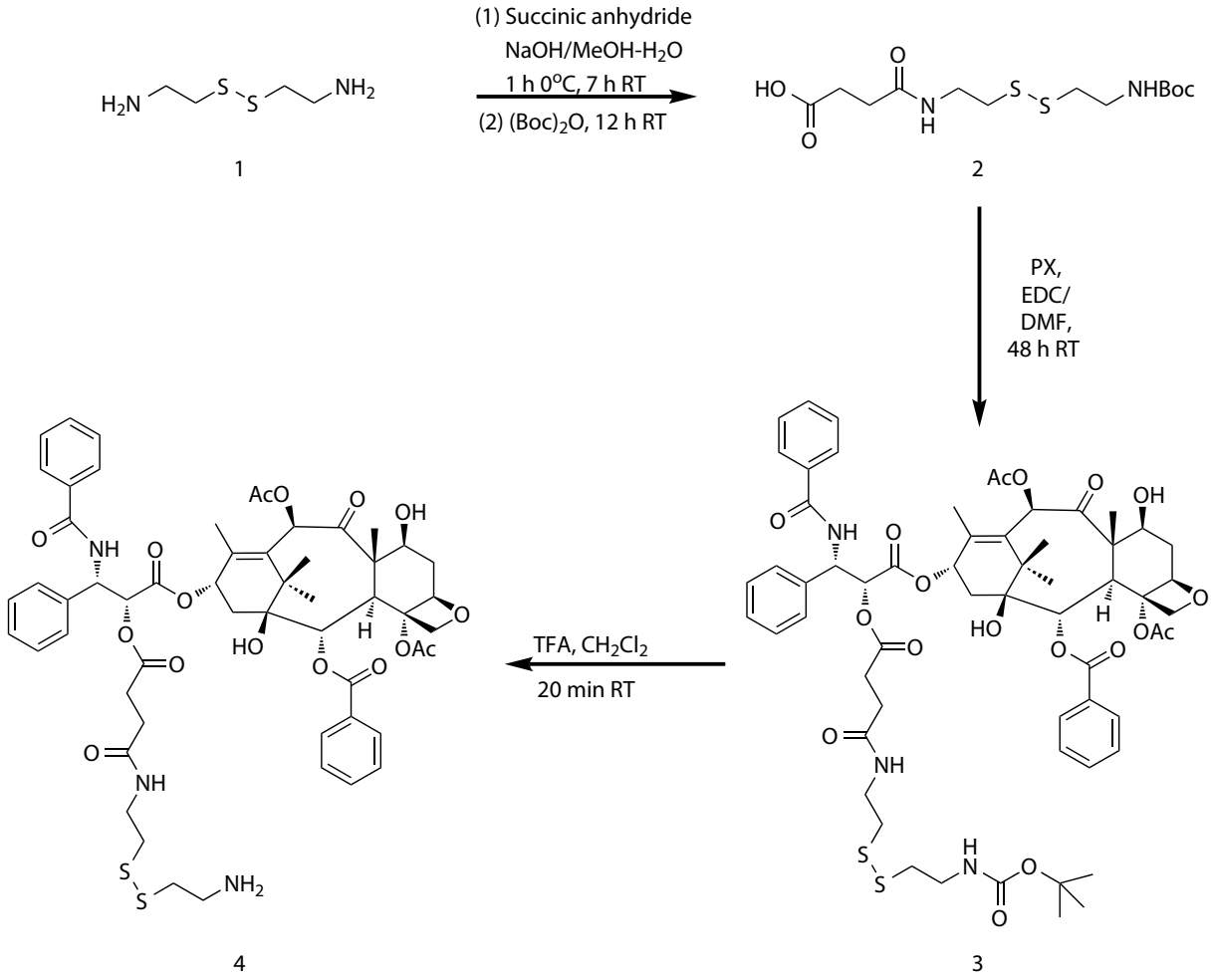

Figure S1: Synthesis of the paclitaxel linker

The synthetic scheme of the paclitaxel linker is shown in Figure S1.

Step 1. To a solution of cystamine dihydrochloride (**1**; 5.00 g, 22.2 mmol) in water (15 mL) was added NaOH (2.66 g, 66.6 mmol). The solution was diluted in MeOH (50 mL) and succinic anhydride was added (2.22 g, 22.2 mmol). The reaction was stirred at 0 °C for 1 h, then room temperature for 7 h. Boc anhydride ($(Boc)_2O$; 7.30 g, 33.3 mmol) was added to the mixture and the

resulting mixture was stirred at room temperature for 12 h. The mixture was concentrated *in vacuo*, and the aqueous residue was diluted with water (50 mL), basified with $NaHCO_3$ (5 %) to pH $\approx$ 9, extracted with ethyl acetate (150 mL), acidified with 1 M H_3PO_4 to pH $\approx$ 5, and extracted again with ethyl acetate (300 mL). The organic layer was concentrated *in vacuo* and purified by flash silica column chromatography (15:85 MeOH:CH_2Cl_2) to yield the N-Boc protected cystamine-succinic acid (**2**) as a white solid (1.64 g, 21 %). R_f (5 % MeOH:CH_2Cl_2) = 0.29. ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.46-3.44 (t, 2H), 3.33-3.3 (br m, 2H), 2.75-2.74 (t, 2H), 2.72 (t, 2H), 2.69 (t, 2H), 2.43 (br t, 2H), 1.36 (s, 9H) ppm.

Step 2. To a solution of paclitaxel (200 mg, 0.234 mmol) and the N-Boc protected cystamine-succinic acid (**2**; 87 mg, 0.246 mmol) in dimethylformamide (DMF; 10 mL) were added 4-dimethylaminopyridine (DMAP; 31 mg, 0.236 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; 31 mg, 0.254 mmol). The mixture was stirred at room temperature for 48 h. The mixture was concentrated *in vacuo* and then purified by flash silica column chromatography by eluting with 5-10 % MeOH/CH_2Cl_2 to yield the N-Boc protected paclitaxel linker derivative (**3**) as a pale yellow foam (209 mg, 75 %). R_f (5 % MeOH:CH_2Cl_2) = 0.46. ^{1}H NMR (400 MHz, CD_3OD): δ 8.08-8.05 (m), 7.93 (s), 7.82-7.76 (m), 7.66-7.61 (m), 7.58-7.48 (m), 7.44-7.35 (m), 7.26-7.20 (m), 6.8 (d), 6.41 (s), 6.11 (br t), 6.03 (m), 5.79-5.75 (m), 5.60 (m), 5.42-5.40 (t), 4.97 (d), 4.70 (d), 4.55 (br s), 4.36-4.22 (m), 4.14 (s), 3.79-3.71 (m), 3.41-3.38 (m), 3.31-3.26 (m), 3.12 (br m), 2.99-2.83 (m), 2.80 (s), 2.77-2.51 (m), 2.45-2.41 (m), 2.36-2.32 (m), 2.19-2.10 (m), 1.88 (d), 1.80-1.70 (m), 1.61 (s), 1.4 (br s), 1.10 (m) ppm. MS (ESI, positive ion mode): *m/z* (relative intensity, %) = 1210.4 (100) $[M+Na]^+$. HRMS (ESI) calculated for $C_{60}H_{73}N_3O_{18}S_2Na$ 1210.4228, found 1210.4271.

Step 3. To the N-Boc protected paclitaxel derivative (**3**; 50 mg, 0.042 mmol) was added a mixture of trifluoroacetic acid and CH_2Cl_2 (2 mL; 1:1). The mixture was stirred at room temperature for 20 min and then evaporated to dryness to yield the paclitaxel linker (**4**) as pale yellow oil. The ^{1}H NMR analysis indicated the complete deprotection of the N-Boc protecting group, and the

product was used without further treatment in the next step. R_f (0.5 % Et_3N/5 % MeOH:CH_2Cl_2) = 0.56. ^{1}H NMR (400 MHz, CD_3OD): δ 8.08-8.05 (m), 7.98-7.93 (m), 7.82-7.76 (m), 7.66-7.61 (m), 7.58-7.48 (m), 7.44-7.35 (m), 7.26-7.20 (m), 6.8 (d), 6.52 (d), 6.41 (s), 6.21 (br d) 6.11 (br t), 6.03 (m), 5.79-5.75 (m), 5.73-5.60 (m), 5.47-5.45 (m), 5.42-5.40(t), 5.29-5.24 (m), 4.97 (d), 4.70 (d), 4.6 (br s), 4.36-4.22 (m), 4.14 (s), 4.10-4.03 (m), 3.79-3.71 (m), 3.59-3.51 (m), 3.41-3.38 (m), 3.31-3.23(m), 3.12 (br m), 2.99-2.83 (m), 2.80 (s), 2.77-2.51 (m), 2.45-2.41 (m), 2.36-2.32 (m), 2.19-2.10 (m), 1.98 (d), 1.92-1.87 (m), 1.80-1.70 (m), 1.61 (s), 1.59-1.53 (m), 1.33 (br s), 1.10 (m), 1.01-0.94 (m), 0.89-0.83 (m) ppm. MS (ESI, positive ion mode): *m/z* (relative intensity, %) = 1088.4 (100) $[M+H]^+$. HRMS (ESI) calculated for $C_{55}H_{66}N_3O_6S_2$ 1088.3885, found 1088.3912.

Synthesis and Characterization of $PX_3Cy_{2-3}OH_{108}$-G5

The synthetic scheme of the $PX_3Cy_{2-3}OH_{108}$-G5 synthesis is shown in Figure S2.

To a solution of G5 PAMAM dendrimers ($(NH_2)_{114}$-G5; **5**; 100 mg; 3.7 μmol) in MeOH (10 mL) was added Cy5-NHS ester (14.2 mg; 18 μmol) and the mixture was stirred at room temperature for 16 h. To the resulting conjugate, $Cy_{2-3}(NH_2)_{108}$-G5 (**6**), was added glutaric anhydride (74 mg; 740 μmol) and the mixture was stirred at room temperature for 24 h. The mixture was then purified by MWCO 10 kD ultrafiltration to yield the carboxylated conjugate $Cy_{2-3}(COOH)_{108}$-G5 (**7**) as a sticky blue solid (83 mg; 47 %). To the carboxylated conjugate (**7**; 50 mg; 1.22 μmol) were added 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; 954 mg; 183 μmol) and NHS (23 mg; 200 μmol) in DMF (20 mL) and the mixture was stirred at room temperature for 8 h. To the resulting conjugate (**8**) was added the paclitaxel linker (**4**; 13.3 mg; 12.2 μmol) in triethylamine (TEA; 5 μL) and the mixture was stirred at room temperature for 12 h. To this mixture was added ethanolamine (ETA; 5 μL) and the mixture was stirred at room temperature for 16 h in order to quench the active NHS ester and neutralize the dendrimer surface. The resulting mixture was first purified by 10 kD dialysis against PBS and DI water for 3 runs each and then lyophilized to yield $PX_3Cy_{2-3}OH_{108}$-G5 (**9**) as a blue solid (48 mg; 82.0 %). MALDI-TOF-MASS analysis indicated that the $PX_3Cy_{2-3}OH_{108}$-G5 was the expected molecular weight,

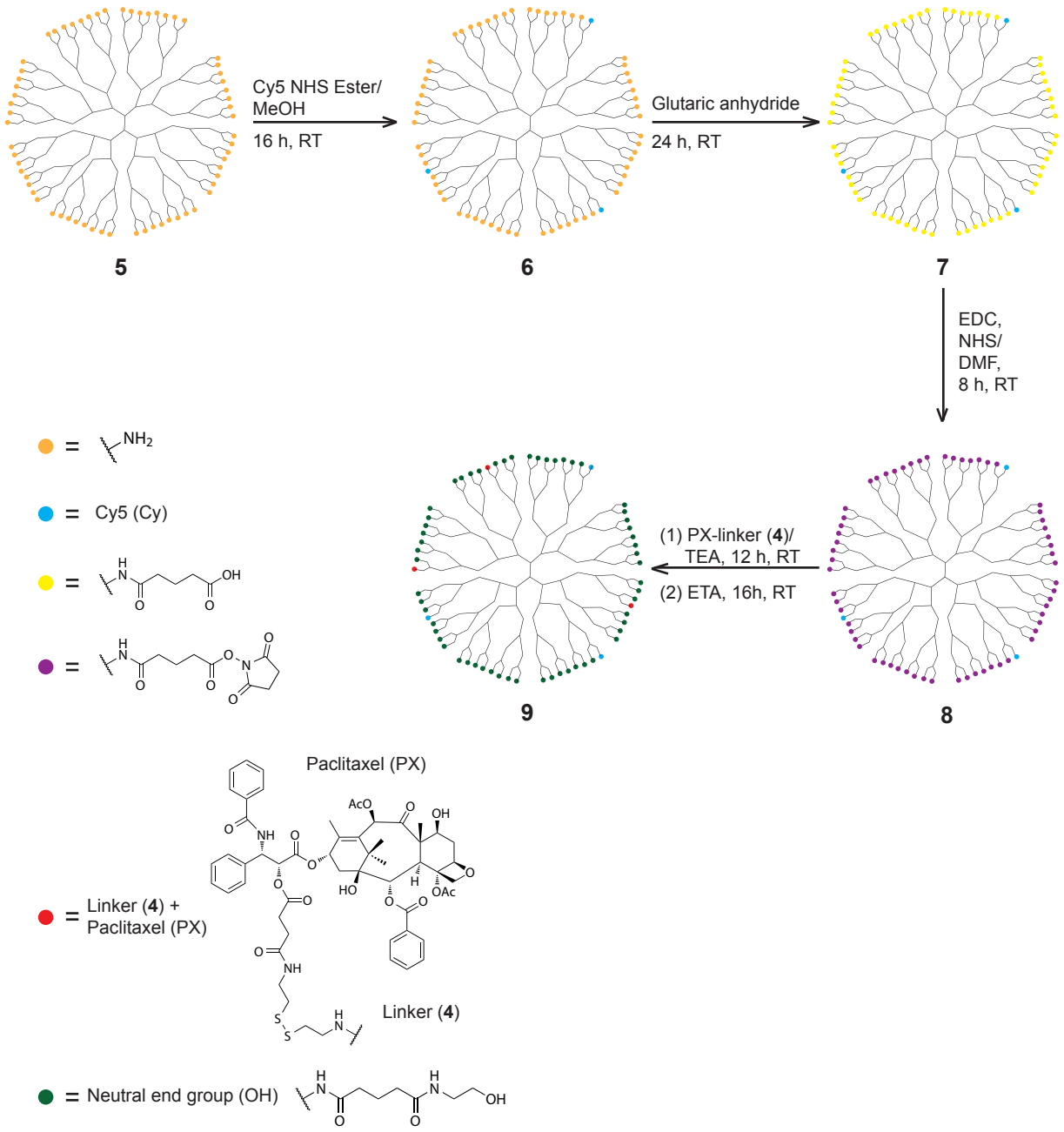

Figure S2: Synthesis of $PX_3Cy_{2-3}OH_{108}$-G5

48 kDa (Figure S3). The number of conjugated Cy5 dyes per dendrimer was determined to be 2.3, on average, using ultraviolet-visible spectroscopy (Figure S4), calibrated to free Cy5. The number of conjugated paclitaxel molecules per dendrimer was determined to be 3.2 using ^{1}H NMR spectroscopy (Figure S5). This was determined as follows. The peaks at δ (ppm) $\approx$ 7-8 belong

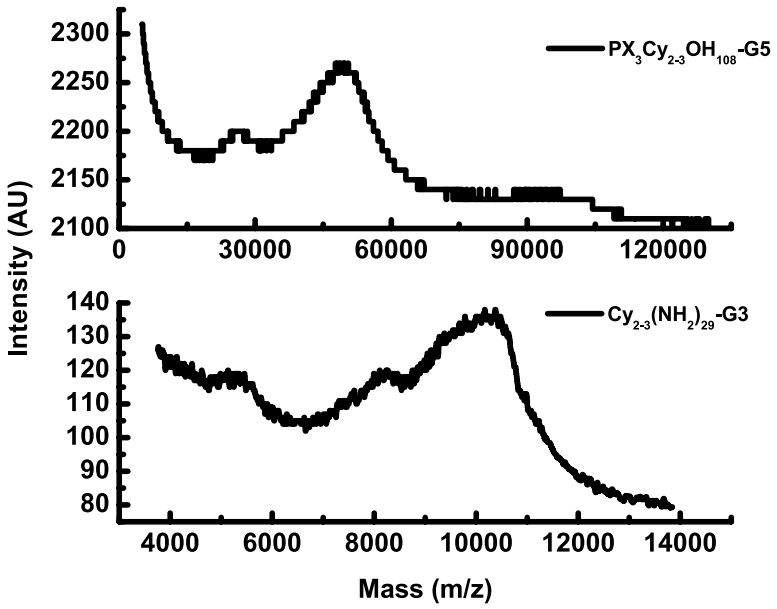

Figure S3: MALDI-TOF mass spectra of $PX_3Cy_{2-3}OH_{108}$-G5 (top panel) and $PX_3Cy_{2-3}OH_{26}$-G3 (bottom panel).

to the combination of three phenyl groups (15 H's) of paclitaxel and the aromatic groups (8 H's) of Cy5. The integration value for these combined signals was corrected to represent only paclitaxel by subtracting out the integration value calculated for the contribution of Cy5 molecule. For this correction, other signals at δ (ppm) $\approx$ 6–6.6, belonging to the protons in the triene conjugation system of Cy5 (3 H's), were used as a reference, and it was assumed that the mean number of Cy5 molecules per dendrimer is 2.3, as determined earlier by ultraviolet-visible spectroscopy (Figure S4).

Testing the Stability of Paclitaxel Conjugation to $PX_3Cy_{2-3}OH_{108}$-G5

$PX_3Cy_{2-3}OH_{108}$-G5 was tested for stability in water to confirm that the paclitaxel would not be spontaneously hydrolyzed from the dendrimer carrier at room temperature in the aqueous buffers used in this study over the maximum experimental time frame, $\leq$ 6 h. In order to test this, $PX_3Cy_{2-3}OH_{108}$-G5 was dissolved in DI water to a final concentration of 1 mg/mL. A 200 μL aliquot of this aqueous solution was tested for the presence of free paclitaxel after 0.17, 1, 2, 3, 6,

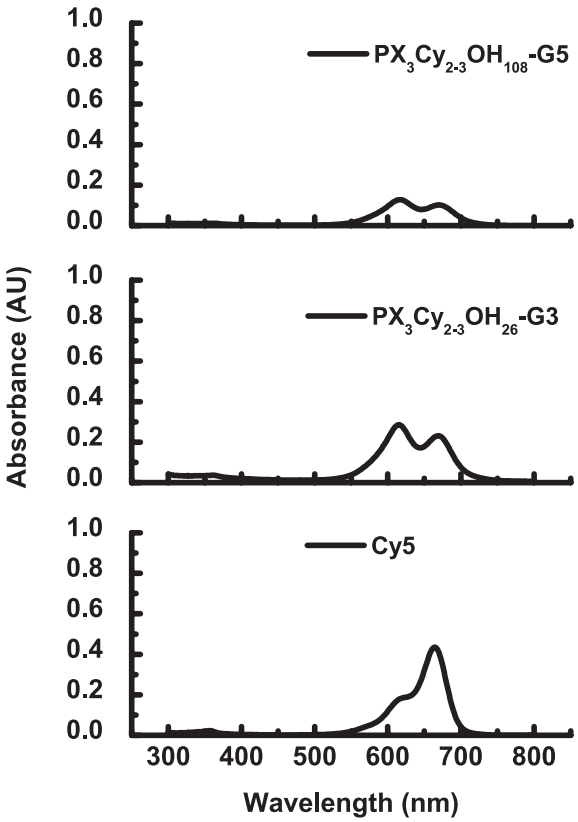

Figure S4: Ultraviolet-visible spectra of $PX_3Cy_{2-3}OH_{108}$-G5 (top panel), $PX_3Cy_{2-3}OH_{26}$-G3 (middle panel), and Cy5 (bottom panel).

and 20 h at room temperature. First, the dendrimers were removed from each aliquot by MWCO 10 kD ultrafiltration. Then the filtrate, containing any free paclitaxel that may have separated from the dendrimer during the incubation in water, was collected and the concentration of free paclitaxel was measured by ultra performance liquid chromatography (UPLC), using an Acquity Peptide Mapping System (Waters Corporation; Milford, MA) controlled by Empower 2 software and equipped with an Acquity BEH C4 column and a photodiode array detector. A gradient elution was used, using a mobile phase ranging from 99:1 - 20:80 (v/v) water:acetonitrile, containing 0.14 % trifluoroacetic acid. The UPLC spectra show that the paclitaxel linker of $PX_3Cy_{2-3}OH_{108}$-G5 is stable in water at room temperature for up to 20 h (Figure S6).

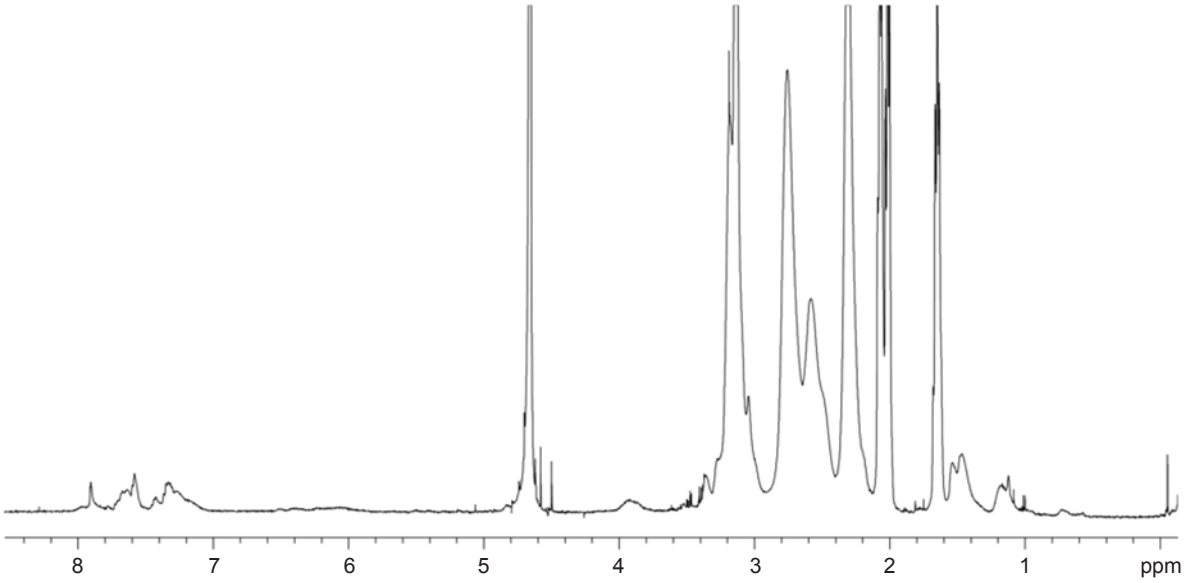

Figure S5: ^{1}H NMR spectrum of $PX_3Cy_{2-3}OH_{108}$-G5

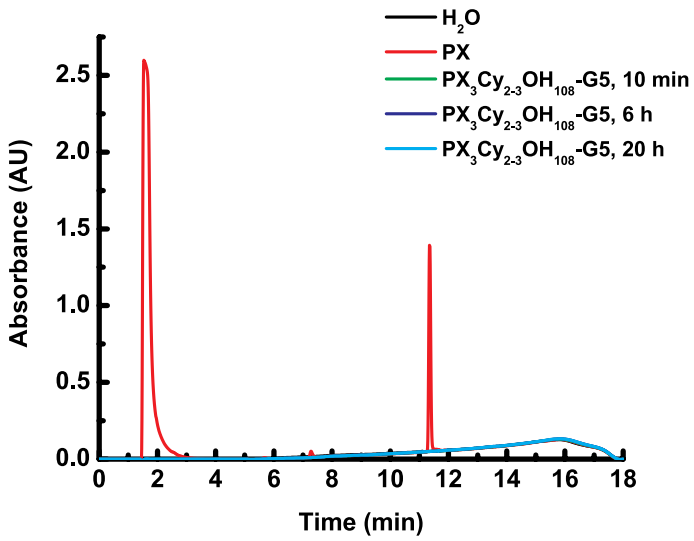

Figure S6: UPLC spectra of DI H_2O (black); (b) paclitaxel (PX) in DI water (red); or MWCO 10 kD filtrates from $PX_3Cy_{2-3}OH_{108}$-G5 incubated in DI water at room temperature for 10 min (green); 6 h (dark blue); or 20 h (light blue). Note that the first elution peak of the "PX" sample represents the solvent front while the second peak represents PX. All signals from the $PX_3Cy_{2-3}OH_{108}$-G5 filtrates are identical to that of water, and show no significant elution peaks at the time points that free PX elutes, indicating that the PX linker of $PX_3Cy_{2-3}OH_{108}$-G5 is stable in water at room temperature for up to 20 h.

Measuring the pKa of Tertiary Amines in the G5 PAMAM Dendrimer Core

For this measurement, a G5 PAMAM dendrimer with the same neutralizing surface modification as $PX_3Cy_{2-3}OH_{108}$-G5 (*i.e.* its terminal groups are hydroxyl groups), but with no conjugated Cy5 or paclitaxel, was used. This dendrimer, termed OH_{114}-G5, according to its surface modifications and stoichiometry, only contains titratable protons sites in the tertiary amines in the dendrimer core (compare with the structure of $PX_3Cy_{2-3}OH_{108}$-G5 shown in Figure 1). Potentiometric titration of OH_{114}-G5 was compared with that of $(NH_2)_{114}$-G5 (Figure S7). Prior to titration, 10 mg of

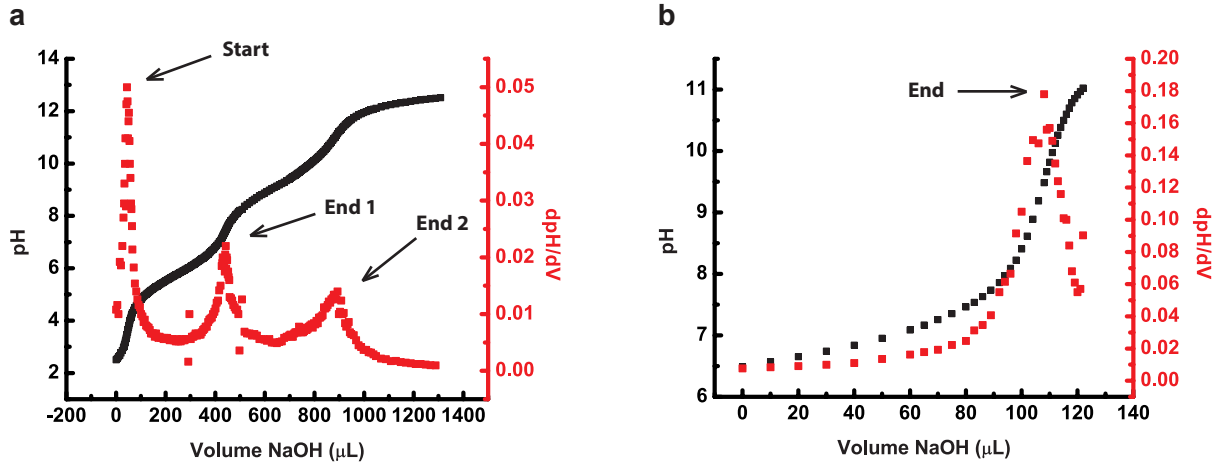

Figure S7: Potentiometric titration curves of (a) $(NH_2)_{114}$-G5, showing the start point of titration (start), endpoint of the tertiary amine titration (end 1) and endpoint of the primary amine titration (end 2); and (b) OH_{114}-G5, showing the endpoint of the tertiary amine titration (end).

dendrimer was dissolved in 1 mL of 0.1 N NaCl and the pH of the resulting solution was adjusted to 2.5. Potentiometric titration was conducted manually with a MP230 pH meter equipped with an InLab®Micro pH electrode (Mettler-Toledo; Columbus, OH). Assuming that both dendrimers have an equivalent titration start point, the pKa of the tertiary amines in OH_{114}-G5 was calculated as follows: pKa = 0.5 (Endpoint + Startpoint) = 0.5 (9.484 + 3.455) = 6.5 $\pm$ 0.2, where the error was determined from the raw data.

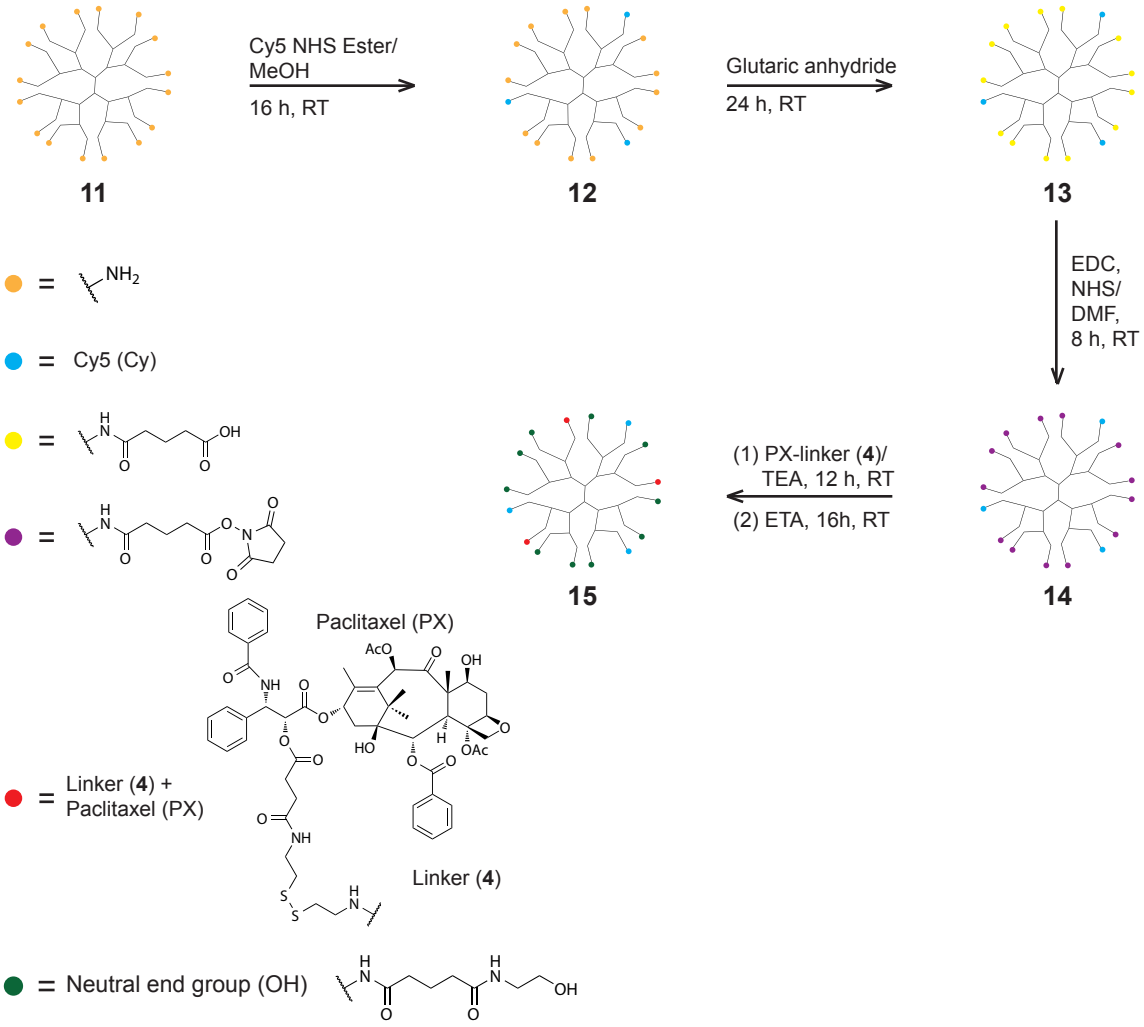

Figure S8: Synthesis of $PX_3Cy_{2-3}OH_{26}$-G3

Synthesis and Characterization of $PX_3Cy_{2-3}OH_{26}$-G3

The synthetic scheme of $PX_3Cy_{2-3}OH_{26}$-G3 is shown in Figure S8.

G3 PAMAM dendrimers were first purified using a 1 kDa MWCO dialysis membrane, achieving a relatively monodisperse population (polydispersity index (PDI) = 1.01–1.05, determined by gel permeation chromatography (GPC)).

To a solution of G3 PAMAM dendrimers ($(NH_2)_{32}$-G3; **11**; 20 mg; 2.87 μmol) in MeOH (5 mL) was added Cy5-NHS ester (11.3 mg; 14.2 μmol) and the mixture was stirred at room temperature for 16 h. To the resulting conjugate, $Cy_{2-3}(NH_2)_{29}$-G3 (**12**), was added gluataric anhydride (14.35

mg; 143.5 μmol) and the mixture was stirred at room temperature for 24 h. The mixture was then purified by MWCO 3 kD ultrafiltration to yield the carboxylated conjugate $Cy_{2-3}(COOH)_{29}$-G3 (**13**) as a sticky blue solid (16.3 mg; 54.7 %). To the carboxylated conjugate (**13**; 12.4 mg; 1.2 μmol) were added EDC and NHS (6.9 mg; 60 μmol) in DMF (5 mL) and the mixture was stirred at room temperature for 8 h. To the resulting conjugate (**14**) was added the paclitaxel linker (**4**; 10.9 mg; 10 μmol) in TEA (3 μL) and the mixture was stirred at room temperature for 12 h. To this mixture was added ETA (5 μL) and the mixture was stirred at room temperature for 16 h in order to quench the active NHS ester and neutralize the dendrimer surface. The resulting mixture was first purified by MWCO 3 kD dialysis against PBS and DI water for 3 runs each and then lyophilized to yield $PX_3Cy_{2-3}OH_{26}$-G3 (**15**) as a blue solid (12.3 mg 42 %). MALDI-TOF-MASS analysis indicated that the $Cy_{2-3}(NH_2)_{29}$-G3 was the expected molecular weight, 10.4 kDa (Figure S3). The number of conjugated Cy5 dyes per $PX_3Cy_{2-3}OH_{26}$-G3 was determined to be 2-3, on average, using ultraviolet-visible spectroscopy (Figure S4), calibrated to free Cy5. The number of conjugated paclitaxel molecules per $PX_3Cy_{2-3}OH_{26}$-G3 was determined to be 3.2 using ^{1}H NMR spectroscopy (Figure S9); by integrating the aromatic peaks located at δ 7-8 and

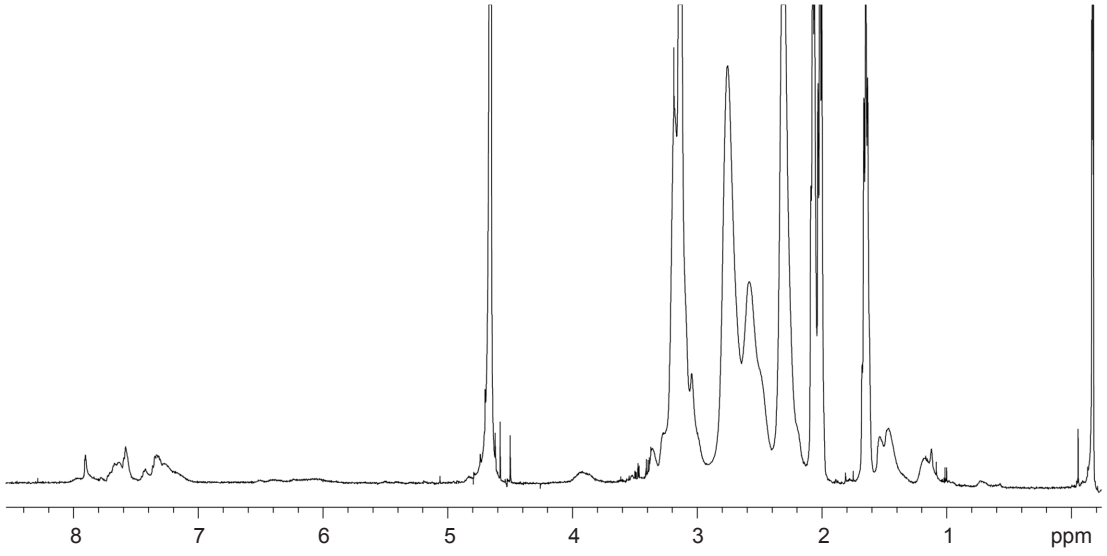

Figure S9: ^{1}H NMR spectrum of $PX_3Cy_{2-3}OH_{26}$-G3

subtracting out the aromatic protons due to the number of conjugated Cy5 molecules per dendrimer determined by ultraviolet-visible spectroscopy.