Dendritic Molecular Capsules for Hydrophobic Compounds

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Experimental Section

All solvents, except DMF, were dried and freshly distilled prior to use (pyridine with CaH and THF with Na). All chemicals were purchased from Aldrich or Acros as highest purity grade and used without further purification. All reactions were performed under nitrogen atmosphere at room temperature unless specified otherwise. NMR spectra were recorded on a Varian INOVA spectrometer (for ^{1}H and ^{13}C NMR, 400 MHz and 100.6 MHz respectively). FT-IR spectra were recorded on a Nicolet Smart MIRacle Avatar 360 using a zinc selenide crystal. MALDI-TOF mass spectra were obtained using a PerSpective Biosystems Voyager-DE Biospectrometry Workstation operating in the positive ion mode using 2-(4-hydroxyphenylazo)-benzoic acid (HABA). Elemental analysis was obtained from Atlantic Microlab, Inc. Size exclusion chromatography was performed using either THF as the eluent on a Polymer Laboratories PLgel 3 μm MIXED-E column (3 μm bead size) and a Rainin HPLC system (temp = 35 ^{o}C; flow rate = 1.0 mL/min), or a 0.25 M $NaNO_3$, 0.01 NaH_2PO_4 pH 7.0 solution as the eluent on a Polymer Laboratories PL Aquagel OH 30 column and a Rainin HPLC system (temp = 35 ^{o}C; flow rate = 1.0 mL/min). Polystyrene standards (1K, 4K, and 23K) were used for calibration. DMF = dimethylformamide, THF = tetrahydrofuran, DCC = dicyclohexylcarbodiimide, DMAP = 4-(dimethylamino)pyridine, DPTS = 4-dimethylaminopyridinium 4-toluenesulfate, DCU = 1,3-dicyclohexylurea, Pd/C = 10 % palladium on activated carbon. DSS = 2,2 dimethylsilapentane-5-sulfonic acid.

Synthesis. The synthesis of the poly(glycerol-succinic acid) dendrimers, PGLSA, were carried out as described in a previous publication.[1] A representative example of a dendrimer synthesis is described below.

Synthesis of **[G4]-PGLSA**-bzld: 2-(*cis*-1,3-*O*-Benzylidene glycerol)succinic acid mono ester (2.43 g, 8.67 mmol), [G3]-PGLSA (0.787 g, 0.153 mmol), and DPTS (1.30 g, 4.42 mmol) were dissolved in 10:1 THF/DMF (40 mL). The reaction flask was flushed with nitrogen and then DCC (2.63 g, 12.7 mmol) was added. The reaction was stirred at room temperature for 14 hours under nitrogen atmosphere. Upon completion, solvents were removed under vacuum and the remaining solids were redissolved CH_2Cl_2. The DCC-urea was filtered and washed with a small amount of CH_2Cl_2 (20 mL) and the solvent was

evaporated. The crude product was purified by silica gel chromatography, eluting with 3:97 to 5:95 methanol:CH_2Cl_2. The product was dissolved in CH_2Cl_2, filtered (to remove any DCU), and precipitated in ethyl ether at –20 ^oC to remove remaining DCC. The ethyl ether was decanted and the precipitate was exposed to reduced pressure to yield 1.50 g of a white powder (73 % yield). ^1H NMR ($CDCl_3$): δ 2.63 (m, 70, -C$\underline{H}_2$-C$\underline{H}_2$-), 2.72 (m, 146, -C$\underline{H}_2$-C$\underline{H}_2$-), 2.90 (m, 32, -C$\underline{H}_2$-C$\underline{H}_2$-), 4.14 (m, 100, -C$\underline{H}_2$-CH-C$\underline{H}_2$-), 4.25 (m, 100, -C$\underline{H}_2$-CH-C$\underline{H}_2$-), 4.70 (m, 32, -CH$_2$-C$\underline{H}$-CH$_2$-), 5.25 (m, 16, -CH$_2$-C$\underline{H}$-CH$_2$-), 5.52 (s, 32, C$\underline{H}$), 7.33 (m, 96, arom. C$\underline{H}$), 7.47 (m, 64, arom. C$\underline{H}$). ^{13}C NMR ($CDCl_3$): δ 172.27 (C$\underline{O}$OR), 171.90 (C$\underline{O}$OR), 171.57 (C$\underline{O}$OR), 138.08 (C$\underline{H}$), 129.25 (C$\underline{H}$), 128.47 (C$\underline{H}$), 126.23 (C$\underline{H}$), 101.27 (C$\underline{H}$), 69.49 (C$\underline{H}$), 69.13 (C$\underline{H}_2$), 66.54 (C$\underline{H}$), 62.45 (C$\underline{H}_2$), 29.34 (C$\underline{H}_2$), 29.02 (C$\underline{H}_2$), 28.83 (C$\underline{H}_2$). FTIR: ν̲ cm^{-1}) 2978 (aliph. C-H stretch), 1733 (C=O). MALDI MS 13536.8 m/z (MH^+) (Theory: 13534.7 m/z (M^+)). Elemental Analysis C: 58.20 %; H 5.56 % (Theory C: 58.04 %; H 5.56 %). SEC M_w: 9000, M_n: 8900, PDI: 1.01.

Synthesis of **[G4]-PGLSA**-OH (**1**)**:** Pd/C (10 % w/w) was added to a solution of benzylidine protected **[G4]-PGLSA**-bzld (0.477 g, 0.0352 mmol) in 9:1 THF/MeOH (20 mL). The flask for catalytic hydrogenolysis was evacuated and filled with 50 psi of H_2 before shaking for 10 hours. The catalyst was filtered and washed with 9:1 THF/MeOH (20 mL). The filtrate was evaporated to give 0.351 g of a colorless, viscous oil (93 % yield). ^1H NMR (CD_3OD): δ 2.65 (m, 244, -C$\underline{H}_2$-C$\underline{H}_2$-), 3.53 (m, 50, -C$\underline{H}_2$-CH-C$\underline{H}_2$), 3.65 (m, 22, -C$\underline{H}_2$-CH-C$\underline{H}_2$-), 3.81 (m, 28, -CH$_2$-C$\underline{H}$-CH$_2$-), 4.05 (m, 32, -C$\underline{H}_2$-CH-C$\underline{H}_2$-), 4.14 (m, 32, -C$\underline{H}_2$-CH-C$\underline{H}_2$-), 4.24 (m, 60, -C$\underline{H}_2$-CH-C$\underline{H}_2$-), 4.30 (m, 60, -C$\underline{H}_2$-CH-C$\underline{H}_2$-), 5.26 (m, 32, -CH$_2$-C$\underline{H}$-CH$_2$-). ^{13}C NMR (CD_3OD): δ 172.94 (C$\underline{O}$OR), 69.92 (C$\underline{H}$), 65.72 (C$\underline{H}_2$), 62.91 (C$\underline{H}_2$), 28.67 (C$\underline{H}_2$). FTIR: ν (cm^{-1}) 3444 (OH), 2931 (aliph. C-H stretch), 1729 (C=O). MALDI MS 10715.6 m/z (MH^+) (Theory: 10715.3 m/z (M^+)). Elemental Analysis C: 48.50 %; H 5.83 % (Theory C: 48.20 %; H 5.81 %). SEC M_w: 8800, M_n: 8720, PDI: 1.01.

Synthesis of **[G4]-PGLSA-**COONa (**2**)**:** [G4]-PGLSA-OH (0.140 g, 0.0131 mmol) was dissolved in pyridine (10 mL) and stirred while succinic anhydride (0.167 g, 1.68 mmol) was added. The reaction mixture was stirred for 16 hours before the pyridine was removed under reduced pressure. The contents were partially dissolved in DCM (15 mL), and 0.1 N HCl (15 mL) was then added and the mixture was stirred for an additional 15 minutes. After stirring, the organic and aqueous phases separated and a layer was formed between the two phases. While avoiding the interface, most of the aqueous and organic phases were removed. This washing procedure with 15 mL of DCM and 0.1 N HCl was repeated two more times. Any remaining organic or aqueous phase was removed first by rotoevaporation followed by lyopholization to yield 0.191 g of a highly viscous liquid (85 % yield). To dissolve the polymer in water, deionized water (10 mL) and brine (0.5 mL) were added to the solution and 0.05 N NaOH was added drop-wise to the stirring solution until the pH remained at 7.0. The dendrimer was purified via dialysis with 7,000 MW cutoff dialysis tubing for 24 hours in DI water. The water was then removed via lyopholization to obtain a white solid. ^1H NMR (D_2O): δ 2.32 (m, 130, -C$\underline{H}_2$-C$\underline{H}_2$-), 2.46 (m, 133, -C$\underline{H}_2$-C$\underline{H}_2$-), 2.58 (m, 228, -C$\underline{H}_2$-C$\underline{H}_2$-) 4.13-4.21 (m, 240, -C$\underline{H}_2$-CH-C$\underline{H}_2$-), 5.18 (m, 62, -CH$_2$-C$\underline{H}$-CH$_2$-). ^{13}C NMR (D_2O): δ 180.72 (C$\underline{O}$OH), 175.37 (C$\underline{O}$OH), 173.52 (C$\underline{O}$OR), 70.14 (C$\underline{H}$), 69.76 (C$\underline{H}$), 62.80 (C$\underline{H}_2$), 34.31 (C$\underline{H}_2$), 32.10

($\underline{C}H_2$), 30.72 ($\underline{C}H_2$), 29.01 ($\underline{C}H_2$). FTIR: ν (cm^{-1}) 3368 (OH), 2964 (aliph. C-H stretch), 1732 (C=O), 1567 (asym COO^- stretch), 1409 (sym COO^- stretch), 1149 (C-O stretch). MALDI MS 17168 *m/z* $[M + Na]^+$, 8602 *m/z* $[M + Na]^{2+}$, (Theory: 17120.0 *m/z* (M^+)). SEC M_w: 8330, M_n: 7780, PDI: 1.11.

Encapsulation Procedure. The encapsulation procedure requires both the dendrimer and hydrophobic compound to be soluble in a volatile organic solvent that is miscible with water.

Reichardt's dye encapsulated in **[G4]-PGLSA**-OH: 27 mg (2.52×10^{-6} mol) of the **[G4]-PGLSA**-OH dendrimer was dissolved in 2.0 mL of CH_3OH. Reichardt's dye (1.4 mg, 2.52×10^{-6} mol) was added to the CH_3OH solution and stirred for 10 minutes. H_2O (1.0 mL) was added to the solution and the solution was stirred for 1 hour. Next, the CH_3OH was removed via evaporation over several hours. The encapsulated dye-dendrimer solution was then stored at room temperature until further use. Samples for NMR analysis were prepared using deuterated solvents.

Reichardt's dye encapsulated in **[G3]-PGLSA**-OH: 25 mg (4.86×10^{-6} mol) of the **[G3]-PGLSA**-OH dendrimer was dissolved in 2.0 mL of CH_3OH. 2.7 mg (4.86×10^{-6} mol) of the Reichardt's dye was added to the CH_3OH solution and stirred for 10 minutes. H_2O (1.0 mL) was added to the solution and the solution was stirred for 1 hour. The CH_3OH was removed via evaporation over several hours. The encapsulated dye-dendrimer solution was then stored at room temperature until further use.

Reichardt's dye encapsulated in **[G2]-PGLSA**-OH: 25 mg (1.06×10^{-5} mol) of the **[G2]-PGLSA**-OH dendrimer was dissolved in 2.0 mL of CH_3OH. 5.9 mg (1.06×10^{-5} mol) of the Reichardt's dye was added to the CH_3OH solution and stirred for 10 minutes. H_2O (1.0 mL) was added to the solution and the solution was stirred for 1 hour. The CH_3OH was removed via evaporation over several hours. As the CH_3OH was removed from solution, Reichardt's dye began to precipitate from solution.

10-Hydroxycamptothecin (10HCPT) encapsulated **[G4]-PGLSA**-COONa: For molar calculations we assumed a molecular weight of 17823, corresponding to a half protonated/half sodium salt carboxylic acid terminated dendrimer. 25 mg (1.5×10^{-6} mol) of the **[G4]-PGLSA**-COONa dendrimer was dissolved in 2.0 mL of CH_3OH. A solution of (0.5 mg, 1.5×10^{-6} mol) 10-hydroxycamptothecin in 1.0 mL of CH_3OH was added to the dendrimer solution and stirred for 10 minutes. Next, 1.0 mL of H_2O was added to the CH_3OH solution and stirred for 1 hour. The uncovered solution was then stirred overnight in the dark to allow the CH_3OH to slowly evaporate. The remaining CH_3OH was removed via rotary evaporation over several hours. A small amount of drug precipitated from solution and was removed via centrifugation. The concentration of the encapsulated 10HCPT was measured via UV-vis ($\varepsilon_{382} = 28{,}000$) and found to be 200 μM. The encapsulated drug-dendrimer solution was then stored in the dark, at room temperature, until further use. Samples for NMR analysis were prepared using deuterated solvents.

UV-vis Experiments. Solutions of Reichardt's dye in solvents of varying polarities and concentrations were prepared. UV-vis spectra were recorded on a Hewlet Packard 8453 spectrophotometer. From the absorbance measurements, the $E_T(30)$ values were calculated for each solvent. Additionally, various equivalents of Reichardt's dye were encapsulated within **[G4]-PGLSA**-OH and the absorbances of the

resulting solutions were measured to determine the maximum encapsulant load within the dendrimer (SI Figure 4).

Light Scattering Experiments. Hydrodynamic radii (R_h) were measured for both the dendrimers and the dendrimer encapsulated Reichardt's dye at 25 ^oC using a Wyatt Mini-Dawn Quasi-elastic Light Scattering (QELS) Instrument. All solvents were filtered through 0.02 micron membrane filters. The R_h for the dendrimer in water (7 nm) or methanol (3 nm) was measured using a flow cell. The effect of solvent upon the R_h of the dendrimer (methanol (3 nm), water (16 nm), acetonitrile/water (30 nm), and THF (40 nm)) was measured using a microcuvette (mc). Hydrodynamic radius values acquired using the mc are overestimated when compared with values acquired with the flow cell due to small dust particulate. However, the mc sample size is an order of magnitude smaller then the flow cell, and the sample is easier to recover.

NMR Experiments. NMR data were recorded at 25 ^oC in 5 mm NMR tubes using Varian Inova 500 and 600 MHz NMR spectrometers with 5 mm Varian probes. 500 MHz ^1H NMR spectra of dye, dendrimer, and dye/dendrimer complex were obtained with a spectral width (SW) of 5.5 kHz, a 77^o pulse flip angle (5 μs), a 5.8 s acquisition time (AT), 1 sec relaxation delay (RD), and digitized using 64K points to obtain a digital resolution (DR) of 0.17 Hz/pt. 2D COSY spectra were recorded in absolute value mode with 5.5 kHz SW, 2000 data points, 1 s relaxation delay, and 16 scans/increment. 400 time increments were collected and zero-filled to 2000 points with sine-bell weighting in both dimensions before Fourier transformation and symmetrization of the 2D matrix. ^1H NMR spin lattice relaxation time constants (T_1) were measured using an inversion recovery ($180^o - \tau - 90^o$) sequence with a repeat period > ($5T_1 + \tau$). Thirteen spectra with 8 scans/spectrum were acquired for free Reichardt's dye in CD_3OD with a recovery delay of 16 s and τ values ranging from 0.12 to 16 s. Twenty spectra with 16 scans/spectrum were acquired for dendrimer alone and 48 scans/spectrum were acquired for dendrimer/encapsulated Reichardt's dye complex in D_2O with a recovery delay of 6 s and τ values ranging from 0.12 to 10 s. ^1H NMR spin-spin relaxation time constants (T_2) were measured using a Carr-Purcell-Meiboom-Gill (CPMG) experiment with a repeat period > ($5T_1 + \tau$). Thirty spectra with 16 scans/spectrum were acquired for unencapsulated Reichardt's dye in CD_3OD with a recovery delay of 16 s and τ values ranging from 0.05 to 1.5 s. The CPMG sequence from Varian was modified to include presaturation pulses for T_2 measurements of dye/dendrimer resonances with suppression of the water signal. Twenty spectra with 16 scans/spectrum were acquired for the dendrimer alone and dendrimer/encapsulated Reichardt's dye complex in D_2O with a recovery delay of 9 s and τ values ranging from 0.008 to 0.16 s. Phase sensitive 2D NOESY spectra were recorded on the 600 MHz spectrometer with a 6.3 kHz SW, 2000 points, 1 s RD, and 32 scans per increment. 400 Time increments were collected and zero filled to 2000 points with Gaussian weighting in both dimensions. Three experiments were carried out using mixing times of 300, 450 and 600 ms, respectively. For unencapsulated Reichardt's dye (in CD_3OD), NOE cross peaks were observed between the 8.10 and 7.64 ppm, 7.51 and 7.43 ppm, and 7.21 and 7.10 ppm multiplets, respectively (SI Figure 3). These NOE cross peaks arise from intra-ring NOE interactions within rings V, IV, and III, respectively.

The 8.39 ppm singlet from ring II shows off-diagonal cross peaks to the 8.10 ppm and 7.51 ppm multiplets, indicating distinct inter-ring NOE interactions between the meta protons of ring II and the ortho protons of the neighboring rings V and IV. In addition, the 6.73 ppm singlet from ring I reveals NOE cross peaks to the 7.51 and 7.18 ppm multiplets, consistent with inter-ring NOE interactions between the meta protons of ring I and the ortho protons of rings IV and III, respectively. 1D difference NOE spectra (NOEDS) for the dendrimer/10HCPT sample were generated from a spectrum recorded with a 6.5 s selective on-resonance irradiation of a succinic acid methylene signal at an estimated power level of 0.1 mw of power and a 5.8 s acquisition period to build up the steady state NOE and a control spectrum irradiated off-resonance of the methylene signal. Suppression of the water signal was achieved with a separate 1.5 s long presaturation pulse at the water frequency incorporated within the 6.5 s irradiation period. To improve free induction decay (FID) subtraction, the dendrimer/10HCPT sample was equilibrated in the magnet for 30 min before recording data. The NOEDS were obtained in an interleaved manner with 8 scans accumulated for the on- or off-resonance FID and looping around 128 times to achieve a good signal-to-noise ration with 1024 scans per FID. On a 600 MHz magnet, the shift difference between the TMS reference to water soluble DSS is 0.614 ppm upfield. The T_1 and T_2 relaxation behavior of the multiplet resonance from the dye may be influenced by scalar relaxation from spin-spin coupling in addition to the dipolar relaxation from adjacent protons. We thus focus on the proton singlets from rings I and II and consider their T_1 and T_2 relaxation as dominated by the intramolecular dipole-dipole relaxation mechanism which is described by equation 1 and 2, respectively, for spin ½ nuclei:

$$R_1 = \frac{1}{T_1} = \frac{3}{10}\cdot\frac{\gamma^2\cdot h^2}{r^6}\cdot\left(\frac{\tau}{1+\omega^2\tau^2}+\frac{4\tau}{1+4\omega^2\tau^2}\right) \quad (1)$$

$$R_2 = \frac{1}{T_2} = \frac{3}{10}\cdot\frac{\gamma^2\cdot h^2}{r^6}\cdot\left(1.5\tau+\frac{2.5\tau}{1+\omega^2\tau^2}+\frac{4\tau}{1+4\omega^2\tau^2}\right) \quad (2)$$

where r is the internuclear distance and γ and h are constants as usually defined. T_1 passes through a minimum at $\omega\tau \sim 0.6$. At 500 MHz, the T_1 minimum from dipolar relaxation is expected at $\sim \tau = 1.9 \times 10^{-10}$ s. Dividing equation 1 by 2 and rearranging terms gives:

$$\frac{T_2}{T_1} = \frac{5\tau+8\omega^2\tau^3}{5\tau+18.5\omega^2\tau^3+6\omega^4\tau^5} \quad (3)$$

In the fast motion limit (extreme narrowing condition), $\omega\tau << 1$, and the theoretical T_2/T_1 ratio is 1. Solving equation 2 explicitly using the measured relaxation times at 500 MHz for ring I of the dye in CD_3OD (T_1 = 1.81 s, T_2 = 1.44 s) gave $\tau = 4.60 \times 10^{-10}$ s. Using values for ring II (T_1 = 1.62 s, T_2 = 1.28 s) gave $\tau = 4.58 \times 10^{-10}$ s. In the slow motion limit, $\omega\tau >> 1$, the $\omega\tau$ squared terms dominate in equations 1 and 2, and equation 3 simplifies to:

$$\frac{T_2}{T_1} = \frac{8}{6\omega^2\tau^2+11} \quad (4)$$

The relaxation times for ring I of the encapsulated dye (T_1 = 0.715 s, T_2 = 0.022 s) gave $\tau = 2.05 \times 10^{-9}$ s. The values for ring II (T_1 = 0.738 s, T_2 = 0.021 s) gave $\tau = 2.14 \times 10^{-9}$ s.

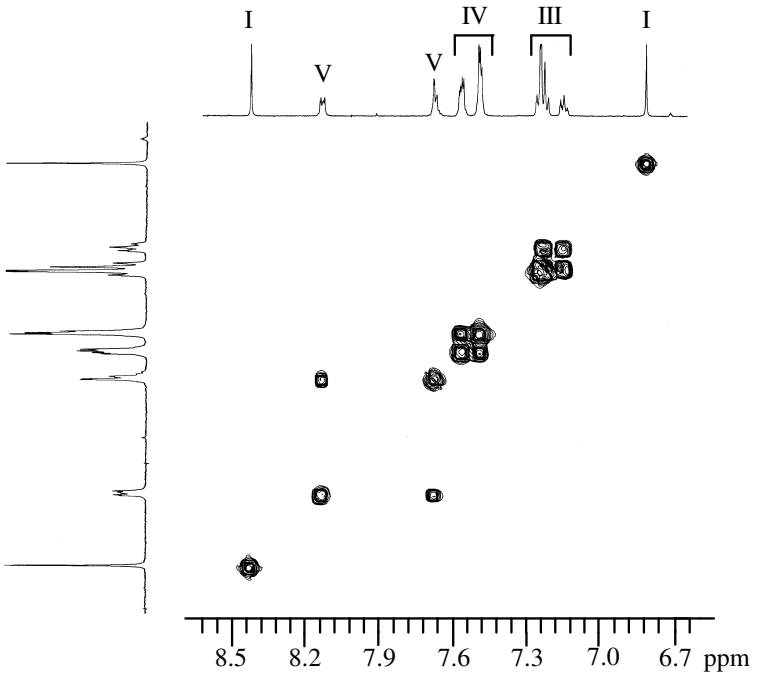
Cell Culture. The human breast cancer cell line MCF-7 was obtained from ATCC. The cells were cultured in medium (MEM medium, 10% FBS, 1% nonessential amino acids, 0.01 mg/μL bovine insulin) at 37 ^{o}C in a humidified atmosphere composed of 5% CO_2. Cells were harvested from subconfluent cultures using trypsin/EDTA (0.05% / 0.02%) and were suspended in medium. Cell viability was determined using an erythrosin B exclusion method.

Cytotoxicity Assay. The cytotoxicity of the dendrimer, free 10HCPT and encapsulated 10HCPT was determined using the MCF-7 cell line and the sulforhodamine B (SRB) assay.[2] Cells were plated into a 96-well plate at a density of 5 x 10^4 cells/mL, 100 μL per well, and incubated for 22 hours (37 ^{o}C and 5% CO_2) before the assay. The medium of each well was then replaced with 100 μL of antibiotic-free medium containing various concentrations of the dendrimer, free 10HCPT, or dendrimer encapsulated 10HCPT. The tests were conducted in replicates of 8 for each concentration. The cells were incubated at 37 ^{o}C/ 5% CO_2. Following the incubation period of 0.5, 1, or 2 hours the drug or dendrimer solutions were replaced with 100 μL of fresh medium. The media was changed every 3 days. After 5 days, the cells were fixed for 1 hour at 4 ^{o}C by adding 50 μL of ice-cold 10% trichloroacetic acid (TCA) after aspiration of the growth medium of each well. The wells were washed 5 times with cold water to remove the excess TCA and then air-dried at room temperature for several minutes. Once dry, each well received 100 μL of 0.4% SRB in 1% acetic acid followed with a 30-minute incubation at room temperature. The SRB solution was then aspirated off the cells and the wells were washed with 1% acetic acid. The wells were air-dried at room temperature for several minutes until moisture was no longer detected visually. The dye bound to the cells was dissolved by adding 100 μL of 10 mM Tris base, unbuffered, per well and agitated for 10-15 minutes at room temperature. The optical densities (OD) were obtained using a ThermoMax UV/vis microplate reader (Molecular Devices) at a wavelength of 562 nm. Optical density measured on wells containing cells that did not receive the drug represented 100% growth, and OD measured on wells containing no cells represented 0% growth.

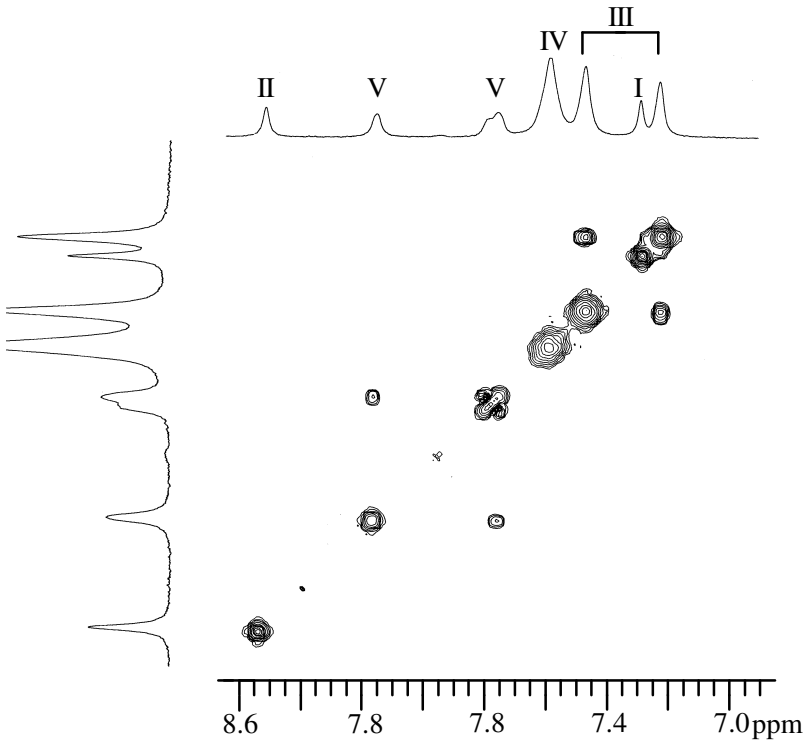
References:

(1) Carnahan, M. A.; Grinstaff, M. W. *Macromolecules* **2001**, *34*, 7648-7655.

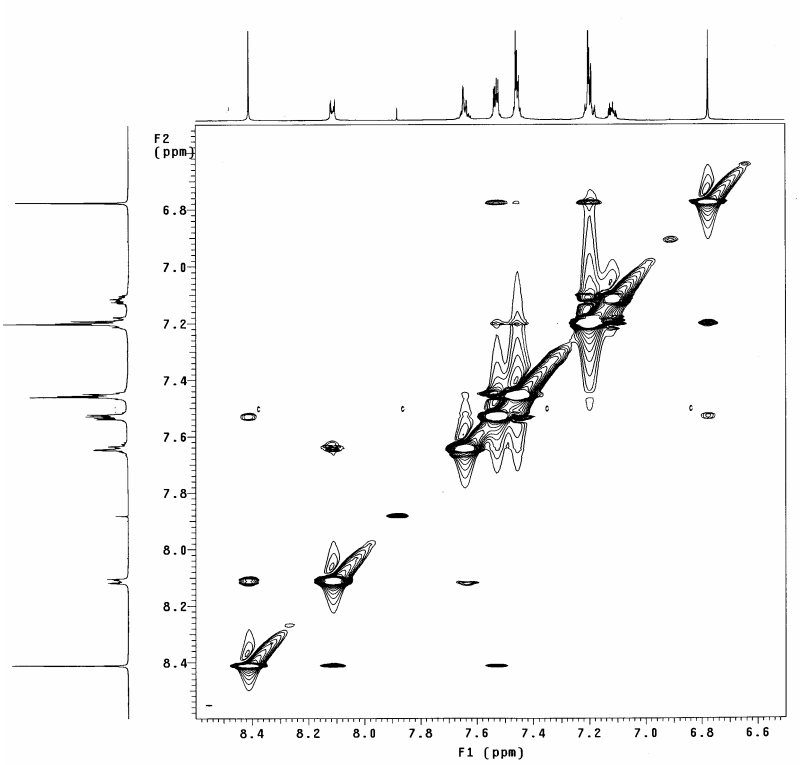
(2) De Jesus, O.; Ihre, H.; Gange, L.; Fréchet, J. M. J.; Szoka, F. *Bioconjugate Chem.* 2002, 13, 453-461.



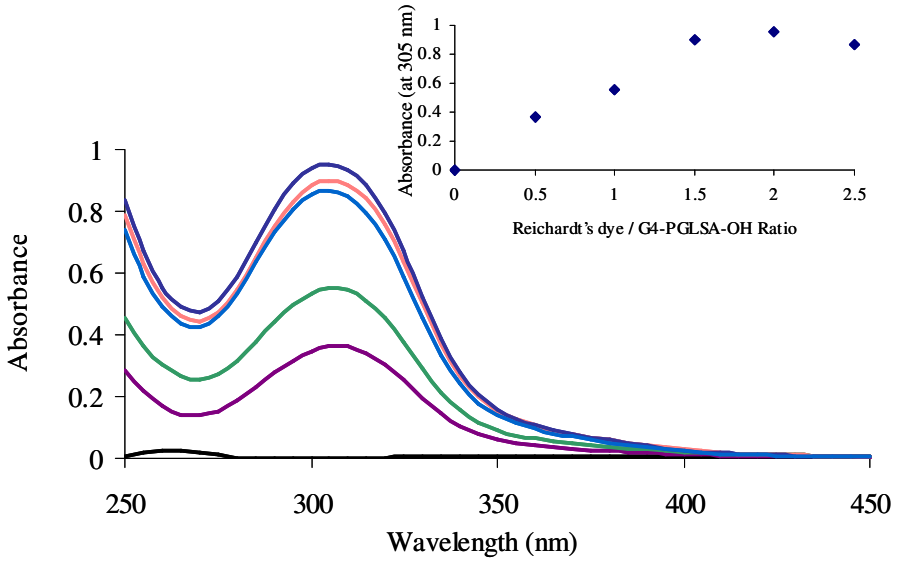
SI Figure 1. 2D ^{1}H NMR COSY of Reichardt's dye in CD_3OD (ppm from TMS).



SI Figure 2. 2D ^{1}H NMR COSY of Reichardt's dye encapsulated within **[G4]-PGLSA**-OH in D_2O (ppm from DMS).



SI Figure 3. Partial ^{1}H NOESY showing intramolecular NOE cross peaks among the aromatic protons of Reichardt's dye (in CD_3OD).



SI Figure 4. UV-vis spectrum for Reichardt's dye encapsulated within **[G4]-PGLSA**-OH dendrimers at various ratios. Insert: Comparison of absorbances (at 305 nm) for various ratios of dye:dendrimer, used to calculated the maximum number of dye molecules encapsulated within the dendrimer.