Amphiphilic Dendritic Supramolecular Assemblies for Drug Delivery

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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 °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 °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.

Synthesis

The synthesis of the poly(glycerol-succinic acid) – PEG dendritic macromolecule 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)$_2$-PEG:

(**[G3]-PGLSA**-OH)$_2$-PEG (1.82 g, 0.212 mmol), and 2-(*cis*-1,3-*O*-benzylidene glycerol)succinic acid mono ester anhydride (15.93 g, 29.36 mmol) were dissolved in THF (50 mL) and stirred under nitrogen. DMAP (0.537 g, 4.40 mmol) was added and stirring was continued for 14 hours. Any remaining anhydride was quenched by the addition of n-propanol (2.5 mL, 28 mmol), which was allowed to stir for another 5 hours. The reaction was diluted with DCM (50 mL) and washed with 0.1 N HCl (100 mL), saturated sodium bicarbonate (100 mL 3x), and brine (100 mL). The organic phase was dried with Na_2SO_4, filtered, and concentrated before the PEG-based dendrimer was precipitated in ethyl ether (400 mL) and collected to yield 3.11 g of a white solid (87 % yield). ^{1}H NMR ($CDCl_3$): δ 2.61 (broad m, 180, -C$\underline{H}_2$-C$\underline{H}_2$-), 2.70 (broad m, 64, -C$\underline{H}_2$-C$\underline{H}_2$-), 3.43 (t, 2, -C$\underline{H}_2$-C$\underline{H}_2$-), 3.56-3.65 (broad m, 286, -C$\underline{H}_2$-C$\underline{H}_2$-), 3.78 (t, 2, -C$\underline{H}_2$-C$\underline{H}_2$-), 4.11 (broad m, 125, -C$\underline{H}_2$-CH-C$\underline{H}_2$-), 4.23 (broad m, 125, -C$\underline{H}_2$-CH-C$\underline{H}_2$-), 4.68 (m, 32, -CH$_2$-C$\underline{H}$-CH$_2$-), 5.20 (m, 30, -CH$_2$-C$\underline{H}$-CH$_2$-), 5.49 (s, 32, C$\underline{H}$), 7.32 (m, 93, arom. C$\underline{H}$), 7.46 (m, 62, arom. C$\underline{H}$). ^{13}C NMR ($CDCl_3$): δ 172.28 (C$\underline{O}$OR), 171.90 (C$\underline{O}$OR), 171.60 (C$\underline{O}$OR), 138.04 (C$\underline{H}$), 129.26 (C$\underline{H}$), 128.48 (C$\underline{H}$), 126.21 (C$\underline{H}$), 101.29 (C$\underline{H}$), 70.76 (C$\underline{H}_2$), 69.46 (C$\underline{H}$), 69.15 (C$\underline{H}_2$), 66.53 (C$\underline{H}$), 62.57 (C$\underline{H}_2$), 29.34 (C$\underline{H}_2$), 29.18 (C$\underline{H}_2$), 29.02 (C$\underline{H}_2$), 28.83 (C$\underline{H}_2$). FTIR: ν (cm^{-1}) 2865 (aliph. C-H stretch), 1734 (C=O). MALDI MS M_w: 17289, M_n: 16968, PDI: 1.02. SEC M_w: 8110, M_n: 7950, PDI: 1.02. T_g = 5.3.

Synthesis of (**[G4]-PGLSA**-OH)$_2$-PEG:

Pd(OH)$_2$/C (10 % w/w) was added to a solution of (**[G4]-PGLSA**-bzld)$_2$-PEG (2.88 g, 0.170 mmol) in 30 mL of 2:1 DCM/methanol. The apparatus for catalytic hydrogenolysis was evacuated and filled with 60 psi of H_2 before shaking for 8 hours.

The catalyst was filtered off and washed with DCM (20 mL). The PEG-based dendrimer was isolated after evaporation of solvents to give 2.86 g of a white solid (98 % yield). ^{1}H NMR ($(CD_3)_2CO$): δ 2.64 (broad m, 248, -C<u>H</u>$_2$-C<u>H</u>$_2$-), 3.49-3.60 (broad m, 296, -C<u>H</u>$_2$-C<u>H</u>$_2$-), 3.66 (broad m, 50, -C<u>H</u>$_2$-CH-C<u>H</u>$_2$-), 3.82 (broad m, 42, -C<u>H</u>$_2$-CH-C<u>H</u>$_2$-), 4.04-4.16 (broad m, 66, -C<u>H</u>$_2$-C<u>H</u>-C<u>H</u>$_2$-), 4.28 (broad m, 124, -C<u>H</u>$_2$-C<u>H</u>-C<u>H</u>$_2$-), 4.86 (m, 10, -CH$_2$-C<u>H</u>-CH$_2$-), 5.27 (m, 30, -CH$_2$-C<u>H</u>-CH$_2$-). ^{13}C NMR ($(CD_3)_2CO$): δ 172.20 (<u>C</u>OOR), 70.45 (<u>C</u>H$_2$), 70.10 (<u>C</u>H), 69.92 (<u>C</u>H$_2$), 65.96 (<u>C</u>H), 62.31 (<u>C</u>H$_2$). FTIR: ν (cm^{-1}) 3445 (OH), 2931 (aliph. C-H stretch), 1713 (C=O). MALDI MS M_w: 14402, M_n: 14146, PDI: 1.02. SEC M_w: 9130, M_n: 8980, PDI: 1.02. T_g = -18.0.

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)$_2$-PEG$_{3400}$:

For molar calculations we assumed a molecular weight of 14265, corresponding to a PEG core with an exact MW of 3400. 25 mg (1.75 x 10^{-6} mol) of the (**[G4]-PGLSA**-OH)$_2$-PEG$_{3400}$ dendrimer was dissolved in 1.0 mL of CH_3OH. 1.0 mg (1.75 x 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. Samples for NMR were prepared with deuterated solvents.

10-Hydroxycamptothecin (HCPT) encapsulated (**[G4]-PGLSA**-OH)$_2$-PEG$_{3400}$:

For molar calculations we assumed a molecular weight of 14265, corresponding to a PEG core with an exact MW of 3400. 25 mg (1.5 x 10^{-6} mol) of the (**[G4]-PGLSA**-OH)$_2$-PEG$_{3400}$ dendrimer was dissolved in 2.0 mL of CH_3OH. A solution of 0.5 mg (1.5 x 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 HCPT was measured via UV-Vis ($\varepsilon_{382} = 28{,}000$) and found to be 120 μM. The encapsulated drug-dendrimer solution was then stored in the dark, at room temperature, until further use.

NMR Experiments

NMR data were recorded at 25 ^{o}C in 5 mm NMR tubes using Varian Inova 500 MHz NMR spectrometer with 5 mm Varian probes. 500 MHz ^{1}H 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.

(**[G4]-PGLSA**-OH)$_2$-PEG$_{3400}$ Encapsulated Reichardt's Dye:

^{1}H NMR spin lattice relaxation time constants (T_1) were measured using an inversion recovery (180^{o} - τ - 90^{o}) sequence with a repeat period > ($5T_1 + \tau$). Twenty spectra with 32 scans/spectrum were acquired for the dendrimer encapsulated Reichardt's dye complex in D_2O with a recovery delay of 13.8 s and τ values ranging from 0.025 to 25 s. ^{1}H NMR spin-spin relaxation time constants (T_2) were measured using a Carr-Purcell-Meiboom-Gill (CPMG) experiment with a repeat period > ($5T_1 + \tau$). 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. Thirty spectra with 16 scans/spectrum were acquired for the dendrimer encapsulated Reichardt's dye complex in D_2O with a recovery delay of 25.8 s and τ values ranging from 0.008 to 0.24 s. Phase sensitive 2D NOESY spectra were recorded on the 500 MHz spectrometer with a 5.5 kHz SW, 2000 points, 1 s RD, a mixing time of 450 ms, and 32 scans per increment. 300 Time increments were collected and zero filled to 2000 points with Gaussian weighting in both dimensions.

Cell Culture

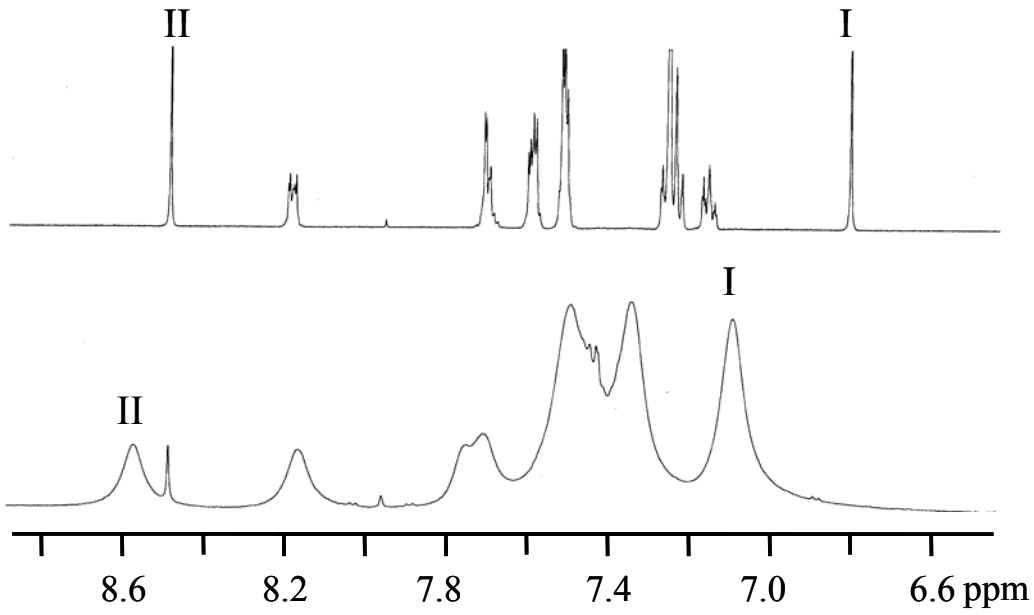
The human colon cancer cell line HT-29 was obtained from ATCC. The cells were cultured in medium (MEM medium, 10% FBS, 1% nonessential amino acids, 0.01 mg/μL bovine insulin) at 37 °C in a humidified atmosphere composed of 5% CO_2. Cells were harvested from subconfluent cultures using trypsin/EDTA (0.05% / 0.02%) and were suspended in medium. Cell viability was determined using an Erythrosin B exclusion method.

SRB Cytotoxicity Assays

The cytotoxicity of the (**[G4]-PGLSA**-OH)$_2$-PEG$_{3400}$ dendrimer, free HCPT and encapsulated HCPT was determined using the HT-29 cell line and the sulforhodamine B (SRB) assay.[2] Cells were plated into a 96-well plate at a density of 2 x 10^4 cells/mL, 100 μL per well, and incubated for 22 hours (37 °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 HCPT, or dendrimer encapsulated HCPT. The tests were conducted in replicates of 8 for each concentration. The cells were incubated at 37 °C/ 5% CO_2. Following the incubation period of 0.5 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 °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.; Middleton, C.; Kim, J.; Kim, T.; Grinstaff, M. W. *J. Am. Chem. Soc.* 2002, **124**, 5291.
(2) Padilla De Jesus, O.; Ihre, H.; Gange, L.; Fréchet, J. M. J.; Szoka, F. *Bioconjugate Chem.* 2002, **13**, 453.



SI Figure 1 ^{1}H NMR for (top) Reichardt's dye in CD_3OD and (bottom) (**[G4]-PGLSA**-$OH)_2$-PEG_{3400} encapsulated Reichardt's dye in D_2O.