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Isolation and Characterization of Dendrimer with Precise Numbers of Functional Groups

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Experimental Methods:

Reagents and Materials:

Biomedical grade Generation 5 PAMAM (poly(amidoamine)) dendrimer was purchased from Dendritech Inc. and purified as described in the synthesis section. MeOH (99.8%), acetic anhydride (99.5%), triethylamine (99.5%), dimethylformamide (99.8%), acetone (ACS reagent grade = 99.5%), methyl 3-(4-hydroxyphenyl)propanoate (97%), sodium azide (99.99%), 1-bromo-2-chloroethane (98%), ethyl acetate (EtOAc) 99.5%, 18-crown-6, K₂CO₃, NaCl, 1N HCl, 2 M KOH, *N*-(3-dimethylaminopropyl)*N*'-ethylcarbodiimide (= 97.0%) (EDC), *N*-hydroxysuccinimide (98%) (NHS), D₂O, and volumetric solutions (0.1 M HCl and 0.1 M NaOH) for potentiometric titration were purchased from Sigma Aldrich Co. and used as received. 10,000 molecular weight cut-off centrifugal filters (Amicon Ultra) and Hexanes (HPLC grade) were obtained from Fisher Scientific. 1x and 10x phosphate buffer saline (PBS) (Ph = 7.4) without calcium or magnesium was purchased from Invitrogen.

Nuclear Magnetic Resonance Spectroscopy

All ¹H NMR experiments were conducted using a Varian Inova 400 MHz instrument. 10s delay time and 64 scans were set for each dendrimer sample. Temperature was controlled at 25 °C. For experiments conducted in D₂O, the internal reference peak was set to 4.717 ppm. Based upon measuring T₂* values and empirical studies to ensure that the chosen delay was long enough to avoid any decreased peak intensity associated with spin saturation, the delay for all integration studies was set to 10 s.

Gel Permeation Chromatography

GPC experiments were performed on an Alliance Waters 2695 separation module equipped with a 2487 dual wavelength UV absorbance detector (Waters Corporation), a Wyatt HELEOS Multi Angle Laser Light Scattering (MALLS) detector, and an Optilab rEX differential refractometer (Wyatt Technology Corporation). Columns employed were TosoHaas TSK-Gel Guard PHW 06762 (75 mm × 7.5 mm, 12 μm), G 2000 PW 05761 (300 mm × 7.5 mm, 10 μm), G 3000 PW 05762 (300 mm × 7.5 mm, 10 μm), and G 4000 PW (300 mm × 7.5 mm, 17 μm). Column temperature was maintained at 25 ± 0.1 °C with a Waters temperature control module. The isocratic mobile phase was 0.1 M citric acid and 0.025 wt % sodium azide, pH 2.74, at a flow rate of 1 mL/min. The sample concentration was 10 mg/5 mL with an injection volume of 100 μL. The weight average molecular weight, M_w, has been determined by GPC, and the number average molecular weight, M_n, was calculated with Astra 5.3.14 software (Wyatt Technology Corporation) based on the molecular weight distribution.

Potentiometric Titration

Potentiometric titration was carried out using a Mettler Toledo MP220 pH meter and a Mettler Toledo InLab 430 pH electrode at room temperature, 23 °C. A 10 mL solution of 0.1 N NaCl was added to purified G5 PAMAM dendrimer **1** (127.5 mg) to shield amine group interactions. The pH of the dendrimer solution was lowered to pH = 2.01 using 0.1034 N HCl. A 25 mL Brand Digital BuretteTM III was used for the titration with 0.0987 N NaOH. The numbers of primary and tertiary amines were determined by from the titration curve with NaOH as previously described.^[1]

Analytical Reverse Phase High Performance Liquid Chromatography

HPLC analysis was carried out on a Waters Delta 600 HPLC system equipped with a Waters 2996 photodiode array detector, a Waters 717 Plus auto sampler, and Waters Fraction collector III. The instrument was controlled by Empower 2 software. For analysis of the conjugates, a C5 silica-based RP-HPLC column (250 x 4.6 mm, 300 Å) connected to a C5 guard column (4 x 3 mm) was used. The mobile phase for elution of the conjugates was a linear gradient beginning with 100:0 (v/v) water/acetonitrile and ending with 20:80 (v/v) water/acetonitrile over 30 min at a flow rate of 1 mL/min. Trifluoroacetic acid (TFA) at 0.14 wt % concentration in water as well as in acetonitrile was used as a counter ion to make the dendrimer surfaces hydrophobic. Elution traces of the dendrimer-ligand conjugate were obtained at 210 nm. We have previously shown that 210 nm is a convenient wavelength to monitor PAMAM dendrimers because absorbance is not significantly affected by varying amounts of conjugated ligand and Beer's Law is followed.^[2] Run-to-run reproducibility of retention time was 0.016 min which is ~4% of the magnitude of the peak-to-peak separation noted in this analysis.

Semi-preparative Reverse Phase High Performance Liquid Chromatography

HPLC isolation was carried out on a Waters Delta 600 HPLC system equipped with a Waters 2996 photodiode array detector, a Waters 2707 auto sampler, and Waters Fraction collector III. The instrument was controlled by Empower 2 software. For analysis of the conjugates, a C5 silica-based RP-HPLC column (250 x 21.20 mm, 10µ 300 Å) connected to a C5 guard column (50 x 21.20 mm) was used. The mobile phase for elution of the conjugates was a linear gradient beginning with 100:0 (v/v) water/isopropanol and ending with 60:40 (v/v) water/isopropanol over 25 min at a flow rate of 10 mL/min. Trifluoroacetic acid (TFA) at 0.14 wt % concentration in water as well as in isopropanol was used as a counter ion to make the dendrimer surfaces hydrophobic. Elution traces of the dendrimer-ligand conjugate were obtained at 210 nm.

Synthesis

The G5-(NH₂)₁₁₂ dendrimer was conjugated to Azide and Ac groups. Ac refers to the acetyl termination, and Azide to the Azide Ligand.

Compound 1: Azide Ligand (3-(4-(2-azidoethoxy)phenyl)propanoic acid)

1a. To a solution of methyl 3-(4-hydroxyphenyl)propanoate (1.699 g, 9.43 mmole) in dry acetone (47.5 mL) was added anhydrous K₂CO₃ (3.909 g, 0.0283 mole) followed by 1-bromo-2-chloroethane (1.563 mL, 0.01886 mole). The resulting suspension was refluxed for 43 h with vigorous stirring. The reaction mixture was cooled to room temperature and the salt was removed by filtration followed by washing with portions of EtOAc (3 x 70 mL). The crude material was purified by silica chromatography (25:75 EtOAc:Hexane) and the solvent was removed under vacuum to give the desired product, methyl 3-(4-(2-chloroethoxy)phenyl)propanoate **1a**, as an oil (0.75 g, 33%). ¹H NMR (500 MHz, CDCl₃, 25 °C) *d* = 7.121 (d, *J* = 8.7, 2H), 6.843 (d, *J* = 8.7, 2H), 4.206 (t, *J* = 5.9, 2H), 3.798 (t, *J* = 5.9, 2H), 3.664 (s, 3H), 2.895 (t, *J* = 7.8, 2H), 2.598 (t, *J* = 7.8, 2H).

1b. To a solution of methyl 3-(4-(2-chloroethoxy)phenyl)propanoate **1a** (0.75 g 3.1 mmole) in anhydrous DMF (6.1 mL) was added 18-crown-6 (3.4 mg, 0.013 mmole) and sodium azide (0.44 g, 6.8 mmole). The resulting solution was heated at 78 °C for 11 h. The reaction mixture was cooled to room temperature, diluted with ethyl acetate (50 mL), washed with a saturated

NaHCO₃ solution (4 x 70 mL), and then dried over MgSO₄. The solvent was removed under vacuum to give methyl 3-(4-(2-azidoethoxy)phenyl)propanoate **1b** as a yellow oil (0.58 g, 75%) ¹H NMR (500 MHz, CDCl₃, 25 °C) *d* = 7.125 (d, *J* = 8.6, 2H), 6.849 (d, *J* = 8.6, 2H), 4.129 (t, *J* = 5.0 2H), 3.666 (s, 3H), 3.581 (t, *J* = 5.0, 2H), 2.899 (t, *J* = 7.8, 2H), 2.600 (t, *J* = 7.8, 2H).

1c. To a solution of methyl 3-(4-(2-azidoethoxy)phenyl)propanoate **1b** (3.88 g, 0.0156 mole) in methanol (102 mL) was added potassium hydroxide (2 M, 28.3 mL, 0.0566 mole). The resulting solution was refluxed at 70 °C for 3 h. The solution was cooled to room temperature and condensed under reduced pressure. The residue was dissolved in water (30 mL) and was acidified by addition of 1N HCl to pH 1. The white cloudy solution was diluted with EtOAc. Layers were separated and the aqueous layer was extracted with EtOAc (2 x 70 mL). The combined organic extracts were washed with a saturated NaCl solution and dried over MgSO₄. Solvent was evaporated under vacuum to give the (3-(4-(2-azidoethoxy)phenyl)propanoic acid) **1c** as a white solid (3.44 g, 93.9%). ¹H NMR (500 MHz, CDCl₃, 25 °C) *d* = 7.139 (d, *J* = 8.5, 2H), 6.859 (d, *J* = 8.5, 2H), 4.132 (t, *J* = 5.0 2H), 3.584 (t, *J* = 5.0, 2H), 2.909 (t, *J* = 7.7, 2H), 2.653 (t, *J* = 7.7, 2H).

Dendrimer 1: Purification of Generation 5 PAMAM Dendrimer G5-(NH₂)₁₁₂

The purchased G5 PAMAM dendrimer was purified by dialysis, as previously described,^[2] to remove lower molecular weight impurities including trailing generation dendrimer defect structures. The number average molecular weight (27,100 g/mol ± 1,000) and PDI (1.018 ± 0.014) was determined by GPC. Potentiometric titration was conducted to determine the mean number of primary amines (112 ± 5).

Dendrimer 2: G5-(NH₂)₁₀₈-Azide_{4,3}

The Azide Ligand (19.4 mg, 82.7 μmole), EDC (31.7 mg, 0.165 mmole), and NHS (21.9 mg, 0.190 mmole) were dissolved in anhydrous acetonitrile (4.861 mL). The resulting solution was stirred under nitrogen for 1 hr. The resulting solution was added by syringe pump to a solution of G5 PAMAM dendrimer **1** (451.9 mg, 16.53 μmole) in DI water (100 mL). The resulting reaction mixture was stirred for 12 hrs under nitrogen at room temperature. The product was purified using 10,000 MWCO centrifugal filtration devices. Purification consisted of 2 cycles to concentrate the solution, 1 cycle using 1x PBS (without magnesium and calcium) and four cycles using DI water. Each cycle was 10 minutes at 5,000 rpm. The purified dendrimer was lyophilized for three days to yield a white solid (368.9 mg, 79%). ¹H NMR integration determined the mean number of Azide Ligands per dendrimer to be 4.3.

Dendrimer 3: G5-Ac₁₀₈-Azide_{4,3}

Dendrimer **2** (365.2 mg, 12.89 μmole) was dissolved in anhydrous methanol (30.0 mL). Triethylamine (297 μL, 2.12 mmole) was added to this mixture and stirred for 30 minutes. Acetic anhydride (174 μL, 1.8 mmole) was added in a dropwise manner to the dendrimer solution. The reaction was carried out in a glass flask, under nitrogen, at room temperature for 24 hours. Methanol was evaporated from the resulting solution and the product was purified using 10,000 MWCO centrifugal filtration devices. Purification consisted of 1 cycles to concentrate the

solution, 2 cycle using 1x PBS (without magnesium and calcium) and four cycles using DI water. The purified dendrimer was lyophilized for three days to yield a white solid (258.4 mg, 61%). ¹H NMR integration determined that the dendrimer was fully acetylated.

Dendrimer 4: G5-Ac₁₁₂

Dendrimer **1** (126.2 mg, 4.620 μmole) was dissolved in anhydrous methanol (24.0 mL). Triethylamine (144 μL, 1.03 mmole) was added to this mixture and stirred for 30 minutes. Acetic anhydride (78.1 μL, 0.827 mmole) was added in a dropwise manner to the dendrimer solution. The reaction was carried out in a glass flask, under nitrogen, at room temperature for 24 hours. Methanol was evaporated from the resulting solution and the product was purified using 10,000 MWCO centrifugal filtration devices. Purification consisted of 1 cycle to concentrate the solution, 2 cycle using 1x PBS (without magnesium and calcium) and four cycles using DI water. The purified dendrimer was lyophilized for three days to yield a white solid (105.5 mg, 71%). ¹H NMR integration determined that the dendrimer was fully acetylated. Integral values for the interior dendrimer protons f, g, and e (see Figure 3 for assignments) were found to be 487, 260 and 487, respectively.

Isolation Procedure

Dendrimer 3 was injected into the HPLC system for 12 consecutive runs. Each injection used 18.2 mg of material in 910 μL of water w/0.14% TFA. A 30 minute run time and a 10 minute delay between runs were used. Beginning at 20 min 0 s in each run, 120 fractions were collected using the Waters Fraction Collector at 4 s intervals. Fractions for all 12 runs were collected in the same set of test tubes.

Fractions were combined based on the peak fitting analysis to obtain the 9 isolated dendrimer-ligand samples (Samples 0-8). 50 μL from each sample was removed and used for analytical HPLC characterization. Purification of each sample was necessary due to the low pH (~1.9) of the HPLC solvent system. This process is described in detail for Sample 0 and was repeated for Samples 1-8.

Sample 0: Fractions 11-18 (20m40s-21m12s) were combined, diluted with an equal volume of 1x PBS (w/o Mg or Ca) and aspirated with nitrogen to evaporate isopropanol. The concentrated sample was lyophilized for 1 day to yield a white powder. The dried sample was then re-dissolved in 2.5 mL of 10x PBS (w/o Mg or Ca) and purified using PD-10 desalting columns. DI water was used as the mobile phase for the column purification step. The sample was then lyophilized for 2 days to yield a white solid (10.4 mg).

Sample 1: Fractions 34-38 (22m12s-22m32s) 10.1 mg. The purification process was repeated twice for Sample 1.

Sample 2: Fractions 52-55 (23m24s-23m40s) 8.5 mg.

Sample 3: Fractions 66-69 (24m20s-24m36s) 9.5 mg.

Sample 4: Fractions 78-81 (25m8s-25m24s) 8.7 mg.

Sample 5: Fractions 87-89 (25m44s-25m56s) 5.4 mg.

Sample 6: Fractions 96-98 (26m20s-26m32s) 6.7 mg.

Sample 7: Fractions 105-107 (26m56s-27m8s) 1.5 mg.

Sample 8: Fractions 115-117 (27m36s-27m48s) 1.8 mg.

References

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