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Effect of Mass Transport in the Synthesis of Partially Acetylated Dendrimer: Implications for Functional Ligand−Nanoparticle Distributions

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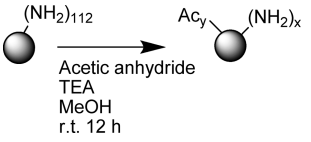
ABSTRACT: Partial acetylation of the amine-terminated poly(amidoamine) dendrimer has been used in the preparation of dendrimer particles conjugated with a wide variety of functional ligands including targeting moieties, therapeutic agents, and dye molecules. The effectiveness of mass transport during the partial acetylation reaction was found to have a major effect on subsequent distributions of dendrimer−ligand components and to be a major source of inconsistency between batches. This study has broad implications for a wide range of nanoparticle−ligand systems because it demonstrates that conjugates with the same mean ligand−particle ratios can have completely different distribution profiles.

Introduction

Nanoparticle-based platforms have emerged in the past decade as a truly disruptive technology with applications that include nanoassemblies,[1,2] sensing and detection,[3−6] targeted delivery,[7−11] imaging and diagnostics,[12−14] and probes of biological structure.[15] For many systems, an important design criterion is that the particles be charge neutral.[16−21] This requirement is often fulfilled with a surface modification step. In poly(amidoamine) (PAMAM) dendrimer-based systems, passivation of the surface primary amines has been frequently accomplished using a partial acetylation reaction (Scheme 1).[22] Because the partial acetylation results in a distribution of dendrimer particles with different numbers of modification sites, this passivation step is likely to have a major impact on the subsequent distribution of dendrimer−ligand components. At present, however, no studies have investigated the effect of the partial acetylation reaction on subsequent dendrimer−ligand distributions especially in the context of batch reproducibility. Specifically, no information is known about how the dendrimer−ligand distributions are affected by changes in the effectiveness of mass transport during the partial acetylation of the parent dendrimer.

The limitations of commonly used analytical techniques are arguably the main reason that the relationships between the passivation step and dendrimer−ligand distributions have not been characterized. Techniques such as nuclear magnetic resonance (NMR), ultraviolet/visible (UV−vis) spectroscopy, and elemental analysis only provide the mean number of ligands per particle. Although gel permeation chromatography (GPC), high-performance liquid chromatography (HPLC), and matrix-assisted laser desorption ionization time-of-flight (MALDI−TOF) have the potential to resolve the different nanoparticle-ligand components in a distribution, these techniques often fail to do so. PAMAM dendrimer have been conjugated with a wide variety of ligands including peptides,[23,24] T-antigens,[25,26] monoclonal antibodies,[27] folic acid,[28−31] and therapeutic agents,[28,32,33] yet only three studies exist where the distribution of dendrimer−ligand components was resolved.[34−36] Additionally, no examples exist where the distribution of dendrimer with different ratios of acetyl groups was resolved.

Scheme 1. General Scheme for Partial Acetylation of the G5-$(NH_2)_{112}$ Dendrimer



Our research group has become keenly interested in this subject because we found production of dendrimer-based platforms with consistent properties between batches to be challenging. In fact, one dendrimer platform that was approaching human trials is currently on hold due to insufficient control over batch consistency.[31,37,38]

Recently, we have noticed a dependence of the dendrimer−ligand distribution features on the batch of partially acetylated dendrimer. Our observations were made using two model ligands that enable us to resolve the distribution of dendrimer−ligand components with HPLC. This study presents three such sets of dendrimer−ligand conjugates($G5Ac_{76}$Alkyne, $G5Ac_{76}$Azide, and $G5Ac_{80}$Alkyne) produced with two batches of partially acetylated dendrimer and two small molecule ligands (Scheme 2 and Scheme 3). For each sample, the distribution of ligand/dendrimer ratios was quantified by HPLC and a peak fitting analysis. The distribution features were consistent for samples made with the same dendrimer, irrespective of the ligand. Major dendrimer−ligand distribution differences were found correlating with the batch of partially acetylated dendrimer. Our hypothesis was that the two dendrimer batches had different distributions of modification sites caused by differing acetyl group distributions. Furthermore, we hypothesized that the acetyl distribution differences resulted from the effectiveness of mass transport during the

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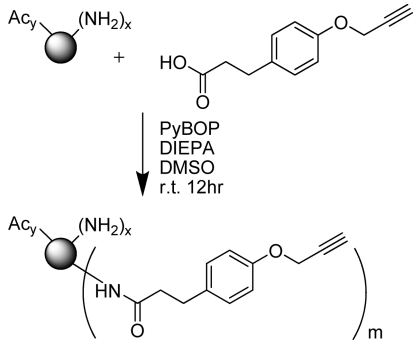
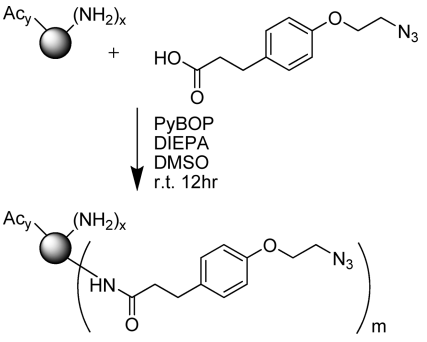
Scheme 2. General Scheme for Alkyne Ligand Conjugation to Partially Acetylated Dendrimer



Scheme 3. General Scheme for Azide Ligand Conjugation to Partially Acetylated Dendrimer



partial acetylation. This resulting distribution of modification sites then had a major impact on the subsequent dendrimer−ligand distributions.

To test our hypothesis, we conducted two partial acetylation reactions with effective and ineffective mass transport controlled by adjusting both dilution and stir rate parameters. No differences were detected between the two batches using NMR or HPLC characterization. The ligand was then conjugated to both batches producing material with the same mean number of ligands. The two batches had distinctly different distribution profiles consistent with our earlier observations. Our results indicate that mass transport has a major impact on the acetyl group distribution which in turn effects the distributions of all subsequent conjugated ligands. This mass transport-induced difference in acetyl group distribution then limits, and for ligands that are not sterically constrained, controls the attainable distribution of the functional ligands that are subsequently attached. The sensitivity of the acetylation reaction to mass transport quality makes reproducible distributions very difficult to achieve which is a substantial problem for batch-to-batch reproducibility. Broadly, this is a cautionary tale for many nanoparticle−ligand conjugates because the final two dendrimer−ligand conjugates appeared to be identical by common characterization techniques and yet had dramatically different distribution profiles.

Experimental Section

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 (TEA) (99.5%), dimethyl sulfoxide (99.9%), dimethylformamide (99.8%), acetone (ACS reagent grade ≥99.5%), *N,N*-diisopropylethylamine, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (98%), D_2O, 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 cutoff centrifugal filters (Amicon Ultra) were obtained from Fisher Scientific. The 1× phosphate buffer saline (PBS) (Ph = 7.4) without calcium or magnesium was purchased from Invitrogen. The alkyne ligand used (3-(4-(prop-2-ynyloxy)phenyl)-propanoic acid) was synthesized as described previously.[34] Synthesis of the azide ligand used (3-(4-(2-azidoethoxy)phenyl)-propanoic acid) was also previously described.[39]

Nuclear Magnetic Resonance Spectroscopy. All ^{1}H NMR experiments were conducted using a Varian Inova 400 MHz instrument. A 10 s delay time and 64 scans were set for each dendrimer sample. Temperature was controlled at 25 °C. For experiments conducted in D_2O, the internal reference peak was set to 4.717 ppm.

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). The DAWN HELEOS II utilized a 120 mW solid-state laser operating at 658 nm and the Refractive Index detector was a Differential RI detector operating at 658 nm. For dendrimer applications, the dn/dc was measured at a fixed wavelength, as mentioned above, by considering a 100% mass recovery for the sample. 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, 125 Å), G 3000 PW 05762 (300 mm ×7.5 mm, 10 μm, 200 Å), and G 4000 PW (300 mm ×7.5 mm, 17 μm, 500 Å). 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 (aqueous) 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.

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 a 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 × 4.6 mm, 300 Å) connected to a C5 guard column (4 × 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 counterion to make the dendrimer surfaces hydrophobic.

Synthesis. The G5-$(NH_2)_{112}$ dendrimer was conjugated to Ac, alkyne, and azide groups. Ac refers to the acetyl termination, alkyne to the alkyne ligand and azide to the azide ligand.

Dendrimer 1: Purification of Generation 5 PAMAM Dendrimer G5-$(NH_2)_{112}$. The purchased G5 PAMAM dendrimer was purified by dialysis, as previously described,[34] to remove lower molecular weight impurities including trailing generation dendrimer defect structures. The number-average molecular weight (27 100 g/mol ± 1000) 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: Synthesis of Partially Acetylated Dendrimer G5-Ac_{76}-$(NH_2)_{36}$. Purified G5 PAMAM dendrimer **1** (149.0 mg, 5.451 μmol) was dissolved in anhydrous methanol (22.0 mL). Triethylamine (69.1 μL, 0.496 mmol) was added to this mixture and stirred for 30 min. Acetic anhydride (40.5 μL, 0.397 mmol) was added to anhydrous methanol (5.0 mL) and the resulting mixture was added in a dropwise manner to the dendrimer

solution using an addition funnel. The reaction was carried out in a glass flask, under nitrogen, at room temperature for 24 h. Methanol was evaporated from the resulting solution and the product was purified using 10 000 MWCO centrifugal filtration devices. Purification consisted of one cycle (10 min at 5,000 rpm) using 1× PBS (without magnesium and calcium) and five cycles using DI water. The purified dendrimer was lyophilized for 3 days to yield a white solid (85.1 mg, 51%). ^{1}H NMR integration determined the degree of acetylation to be 68%.

Dendrimer 3: Synthesis of Partially Acetylated Dendrimer G5-Ac_{80}-$(NH_2)_{32}$. Purified G5 PAMAM dendrimer **1** (180.1 mg, 6.588 μmol) was dissolved in anhydrous methanol (26.8 mL). Triethylamine (83.6 μL, 0.600 mmol) was added to this mixture and stirred for 30 min. Acetic anhydride (45.3 μL, 0.480 mmol) was added to anhydrous methanol (7.3 mL) and the resulting mixture was added in a dropwise manner to the dendrimer solution using an addition funnel. The reaction was carried out in a glass flask, under nitrogen, at room temperature for 24 h. Methanol was evaporated from the resulting solution and the product was purified using 10,000 MWCO centrifugal filtration devices. Purification consisted of one cycle (10 min at 5,000 rpm) using 1× PBS (without magnesium and calcium) and five cycles using DI water. The purified dendrimer was lyophilized for 3 days to yield a white solid (124.5 mg, 62%). ^{1}H NMR integration determined the degree of acetylation to be 71.5%.

Synthesis of Dendrimer−Ligand Samples. All reaction steps were carried out in glass scintillation vials at room temperature under nitrogen. All samples were purified using 10,000 MWCO centrifugal filtration devices. Purification consisted of one cycle (10 min at 5,000 rpm) using 1× PBS (without magnesium or calcium) and five cycles using DI water.

Samples A−E: G5-Ac_{76}−$Alkyne_{(0.5,2.1,3.1,3.8,10.4)}$. Three stock solutions were prepared to synthesize samples A−E. A solution of partially acetylated dendrimer **2** (52.3 mg, 1.71 μmol) was prepared with anhydrous DMSO (8.00 mL). The alkyne ligand (6.2 mg, 30. μmol) was dissolved in DMSO (3.1 mL). Last, PyBOP (10.2 mg, 19.6 μmol) was dissolved in DMSO (2.04 mL).

Sample A. The alkyne ligand (0.063 mg, 0.31 μmol) in anhydrous DMSO (31.5 μL), was added to a solution of partially acetylated dendrimer **2** (6.40 mg, 0.210 μmol) in anhydrous DMSO (0.979 mL). *N,N*-Diisopropylethylamine (0.2 mg, 0.3 μL, 2.0 μmol) was added to the reaction mixture together with 1.018 mL of additional DMSO, and the resulting solution was stirred for 30 min. A solution of PyBOP (0.20 mg, 0.38 μmol) in anhydrous DMSO (32.1 μL) was added in a dropwise manner (0.1 mL/min) to the dendrimer solution. The resulting reaction mixture was stirred for 24 h under nitrogen and then purified using 10,000 MWCO centrifugal filtration devices. Purification consisted of one cycle (10 min at 5,000 rpm) using 1× PBS (without magnesium or calcium) and five cycles using DI water. The purified product **A** was lyophilized for 3 days to yield a white solid (2.9 mg, 45%). ^{1}H NMR integration determined the average number of alkyne ligands per dendrimer to be 0.5.

Sample B. Sample B was synthesized in the same manner as sample A, using partially acetylated dendrimer **2** (6.40 mg, 0.210 μmol) in anhydrous DMSO (0.979 mL), the alkyne ligand (0.19 mg, 0.93 μmol) in DMSO (0.094 μL), *N,N*-diisopropylethylamine (0.7 mg, 1 μL, 5 μmol), 0.890 mL of additional DMSO, and PyBOP (0.481 mg, 0.925 μmol) in anhydrous DMSO (96.2 μL). Sample B was purified and lyophilized in the same manner as sample A. The purified product sample B was a white solid (4.7 mg, 72%). ^{1}H NMR integration determined the average number of alkyne ligands per dendrimer to be 2.1.

Sample C. Sample C was synthesized in the same manner as sample A, using partially acetylated dendrimer **2** (6.40 mg, 0.210 μmol) in anhydrous DMSO (0.979 mL), the alkyne ligand (0.31 mg, 1.5 μmol) in DMSO (157.4 μL), *N,N*-diisopropylethylamine (1.2 mg, 1.6 μL, 9.3 μmol), 0.763 mL of additional DMSO, and PyBOP (0.802 mg, 1.54 μmol) in anhydrous DMSO (160.4 μL). Sample C was purified and lyophilized in the same manner as sample A. The purified product sample C was a white solid (7.1 mg, 108%). The extra mass indicated the presence of residual solvent. This did not affect the characterization of the material. ^{1}H NMR integration determined the average number of alkyne ligands per dendrimer to be 3.1.

Sample D. Sample D was synthesized in the same manner as sample A, using partially acetylated dendrimer **2** (6.40 mg, 0.210 μmol) in anhydrous DMSO (0.979 mL), the alkyne ligand (0.63 mg, 3.1 μmol) in DMSO (314.7 μL), *N,N*-diisopropylethylamine (2.4 mg, 3.2 μL, 18.6 μmol), 0.443 mL of additional DMSO, and PyBOP (1.6 mg, 3.08 μmol) in anhydrous DMSO (320.8 μL). Sample D was purified and lyophilized in the same manner as sample A. The purified product sample D was a white solid (3.3 mg, 50%). ^{1}H NMR integration determined the average number of alkyne ligands per dendrimer to be 3.8.

Sample E. Sample E was synthesized in the same manner as sample A, using stock solutions prepared on a different day. To partially acetylated dendrimer **2** (6.40 mg, 0.210 μmol) in anhydrous DMSO (1.730 mL) were added the alkyne ligand (0.60 mg, 3.1 μmol) in DMSO (314.7 μL), *N,N*-diisopropylethylamine (2.4 mg, 3.2 μL, 19 μmol), and PyBOP (1.6 mg, 3.1 μmol) in anhydrous DMSO (320.8 μL). Sample E was purified and lyophilized in the same manner as sample A. The purified product sample E was a white solid (2.2 mg, 32%). ^{1}H NMR integration determined the average number of alkyne ligands per dendrimer to be 10.4.

Samples F−H: G5-Ac_{76}-$Azide_{(1.9,4.4,7.7)}$. Three stock solutions were prepared to synthesize samples F−H. A solution of partially acetylated dendrimer **2** (52.3 mg, 1.71 μmol) was prepared with anhydrous DMSO (8.00 mL). The azide ligand (9.2 mg, 39.1 μmol) was dissolved in DMSO (4.6 mL). Last, PyBOP (10.2 mg, 19.6 μmol) was dissolved in DMSO (2.04 mL).

Sample F. The azide ligand (0.073 mg, 0.31 μmol) solution in anhydrous DMSO (43.9 μL), was added to a solution of partially acetylated dendrimer **2** (6.40 mg, 0.210 μmol) in anhydrous DMSO (0.979 mL). *N,N*-Diisopropylethylamine (0.71 mg, 1.0 μL, 5.6 μmol) was added to the reaction mixture together with 0.924 mL of additional DMSO, and the resulting solution was stirred for 30 min. The solution of PyBOP (0.160 mg, 0.308 μmol) in anhydrous DMSO (96.2 μL) was added in a dropwise manner (0.1 mL/min) to the dendrimer solution. The resulting reaction mixture was stirred for 24 h under nitrogen and then purified as described earlier. The purified product, sample F, was lyophilized for 3 days to yield a white solid (4.1 mg, 63%). ^{1}H NMR integration determined the mean number of azide ligands per dendrimer to be 1.9.

Sample G. Sample G was synthesized in the same manner as sample F, using partially acetylated dendrimer **2** (6.40 mg, 0.210 μmol) in anhydrous DMSO (0.979 mL), the azide ligand (0.36 mg, 1.5 μmol) in DMSO (181.3 μL), *N,N*-diisopropylethylamine (1.2 mg, 1.6 μL, 9.3 μmol), 0.787 mL of additional DMSO, and PyBOP (0.802 mg, 1.54 μmol) in anhydrous DMSO (160.4 μL). Sample G was purified and lyophilized in the same manner as sample F. The purified product, sample G, was a white solid (4.2 mg, 63%). ^{1}H NMR integration determined the mean number of azide ligands per dendrimer to be 4.4.

Sample H. Sample H was synthesized in the same manner as sample F, using partially acetylated dendrimer **2** (6.40 mg, 0.210 μmol) in anhydrous DMSO (0.979 mL), the azide ligand (0.70 mg, 3.1 μmol) in DMSO (362.5 μL), *N,N*-diisopropylethylamine (2.4 mg, 3.2 μL, 19 μmol), 0.443 mL of additional DMSO, and PyBOP (1.60 mg, 3.08 μmol) in anhydrous DMSO (320.8 μL). Sample H was purified and lyophilized in the same manner as sample F. The purified product, sample H, was a white solid (3.5 mg, 52%). ^{1}H NMR integration determined the mean number of azide ligands per dendrimer to be 7.7.

Samples I−M: G5-Ac_{80}-$Alkyne_{(0.4,0.7,2.7,6.8,10.2)}$. Three stock solutions were generated to synthesize samples I−M. A solution of the partially acetylated dendrimer **3** (22.4 mg, 0.728 μmol) was prepared with anhydrous DMSO (4.978 mL). The alkyne

Table 1. Comparison of the Average Number of Ligands per Dendrimer Computed by Two Independent Techniques (NMR Spectroscopy and HPLC) for Samples A−M[a]

		G5-Ac_{76}-Alkyne				
		NMR	HPLC			
		arithmetic mean	arithmetic mean	median	mode	no. of dendrimer species
dendrimer **2**	sample A‡	0.5 ± 0.1	0.4 ± 0.02	0	0	5
	sample B‡	2.1 ± 0.2	2.3 ± 0.09	1	0	10
	sample C‡	3.1 ± 0.3	3.4 ± 0.1	2	0	13
	sample D‡	3.8 ± 0.4	4.1 ± 0.2	3	0	13
	sample E‡	10.4 ± 1.0	11.0 ± 0.4	12	0	30
		G5-Ac_{76}-Azide				
		NMR	HPLC			
		arithmetic mean	arithmetic mean	median	mode	no. of dendrimer species
dendrimer **2**	sample F‡	1.9 ± 0.2	1.9 ± 0.08	1	0	13
	sample G‡	4.4 ± 0.4	4.2 ± 0.2	3	0	16
	sample H‡	7.7 ± 0.8	9.4 ± 0.4	10	0	26
		G5-Ac_{80}-Alkyne				
		NMR	HPLC			
		arithmetic mean	arithmetic mean	median	mode	no. of dendrimer species
dendrimer **3**	sample I	0.4 ± 0.04	0.4 ± 0.01	0	0	3
	sample J	0.7 ± 0.07	0.6 ± 0.02	0	0	4
	sample K	2.7 ± 0.8	2.8 ± 0.1	2	1	11
	sample L	6.8 ± 0.7	7.2 ± 0.3	7	7	17
	sample M	10.2 ± 1.0	11.2 ± 0.5	11	11	24

[a] Samples A−H were produced using dendrimer **2** as the parent partially acetylated dendrimer. Samples I−M were produced with dendrimer **3**. Dendrimer samples containing a distribution of ligands that are assigned as resulting from ineffective mass transport in the acetylation step are identified with a ‡.

ligand (9.9 mg, 49 μmol) was dissolved in DMSO (4.950 mL). Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (5.4 mg, 10. μmol) was dissolved in DMSO (1.000 mL).

Sample I. The alkyne ligand (29 μg, 0.15 μmol) in anhydrous DMSO (14.6 μL), was added to a solution of partially acetylated dendrimer **3** (4.40 mg, 0.143 μmol) in anhydrous DMSO (0.978 mL). *N,N*-Diisopropylethylamine (1.1 mg, 1.5 μL, 8.6 μmol) was added to the reaction mixture, and the resulting solution was stirred for 30 min. A solution of PyBOP (74 μg, 0.14 μmol) in anhydrous DMSO (13.8 μL) was added in a dropwise manner (0.1 mL/min) to the dendrimer solution. The resulting reaction mixture was stirred for 24 h under nitrogen and then purified as described earlier. The purified product, sample I, was lyophilized for 3 days to yield a white solid (3.7 mg, 84%). ^{1}H NMR integration determined the mean number of alkyne ligands per dendrimer to be 0.4.

Sample J. Sample J was synthesized in the same manner as sample I, using partially acetylated dendrimer **3** (4.4 mg, 0.14 μmol) in anhydrous DMSO (0.978 mL), the alkyne ligand (58 μg, 0.29 μmol) in DMSO (29.2 μL), *N,N*-diisopropylethylamine (0.2 mg, 0.3 μL, 2 μmol), and PyBOP (0.15 mg, 0.29 μmol) in anhydrous DMSO (28 μL). Sample J was purified and lyophilized in the same manner as sample I. The purified product, sample J, was a white solid (3.1 mg, 70%). ^{1}H NMR integration determined the mean number of alkyne ligands per dendrimer to be 0.7.

Sample K. Sample K was synthesized in the same manner as sample I, using partially acetylated dendrimer **3** (4.40 mg, 0.143 μmol) in anhydrous DMSO (0.978 mL), the alkyne ligand (0.15 mg, 0.72 μmol) in DMSO (73.0 μL), *N,N*-diisopropylethylamine (0.6 mg, 0.7 μL, 4 μmol), and PyBOP (0.37 mg, 0.72 μmol) in anhydrous DMSO (69 μL). Sample K was purified and lyophilized in the same manner as sample I. The purified product, sample K, was a white solid (3.6 mg, 80%). ^{1}H NMR integration determined the mean number of alkyne ligands per dendrimer to be 2.7.

Sample L. Sample L was synthesized in the same manner as sample I, using partially acetylated dendrimer **3** (4.40 mg, 0.143 μmol) in anhydrous DMSO (0.978 mL), the alkyne ligand (0.29 mg, 1.4 μmol) in DMSO (146 μL), *N,N*-diisopropylethylamine (1.1 mg, 1.5 μL, 8.6 μmol), and PyBOP (0.74 mg, 1.4 μmol) in anhydrous DMSO (138 μL). Sample L was purified and lyophilized in the same manner as sample I. The purified product, sample L, was a white solid (3.5 mg, 76%). ^{1}H NMR integration determined the mean number of alkyne ligands per dendrimer to be 6.8.

Sample M. Sample M was synthesized in the same manner as sample I, using partially acetylated dendrimer **3** (4.40 mg, 0.143 μmol) in anhydrous DMSO (0.978 mL), the alkyne ligand (0.44 mg, 0.14 μmol) in DMSO (978 μL), *N,N*-diisopropylethylamine (1.7 mg, 2.2 μL, 13 μmol), and PyBOP (1.1 mg, 2.1 μmol) in anhydrous DMSO (207 μL). Sample M was purified and lyophilized in the same manner as sample I. The purified product, sample M, was a white solid (2.7 mg, 57%). ^{1}H NMR integration determined the mean number of alkyne ligands per dendrimer to be 10.2.

Dendrimer 4: Synthesis of Partially Acetylated Dendrimer G5-Ac_{71}-$(NH_2)_{41}$. Purified G5 PAMAM dendrimer **1** (53.1 mg, 1.94 μmol) was dissolved in anhydrous methanol (7.9 mL). Triethylamine (24.6 μL, 0.177 mmol) was added to this mixture which was then stirred for 30 min. Acetic anhydride (14.4 μL, 0.141 mmol) was added to anhydrous methanol (2.2 mL), and the resulting mixture was added with a syringe pump to the dendrimer solution. The dropwise addition rate was 11.9 mL/h, and the stir-rate in the dendrimer solution was ∼900 rpm. The reaction was carried out in a glass flask, under nitrogen, at room temperature for 12 h. Methanol was evaporated from the resulting solution and the product was purified using 10,000 MWCO centrifugal filtration devices. Purification consisted of one cycle (10 min at 5,000 rpm) using 1× PBS (without magnesium and calcium) and five cycles using DI water. The purified dendrimer was lyophilized for 3 days to yield a white solid (45.6 mg, 77%). ^{1}H NMR integration determined the degree of acetylation to be 63%.

Dendrimer 5: Synthesis of Partially Acetylated Dendrimer G5-Ac_{82}-$(NH_2)_{30}$. Purified G5 PAMAM dendrimer **1** (50.1 mg, 1.83 μmol) was dissolved in anhydrous methanol (9.5 mL). Triethylamine (23.2 μL, 0.167 mmol) was added to this mixture and stirred for 30 min. Acetic anhydride (12.6 μL, 0.133 mmol) was added in a dropwise manner to the dendrimer solution.

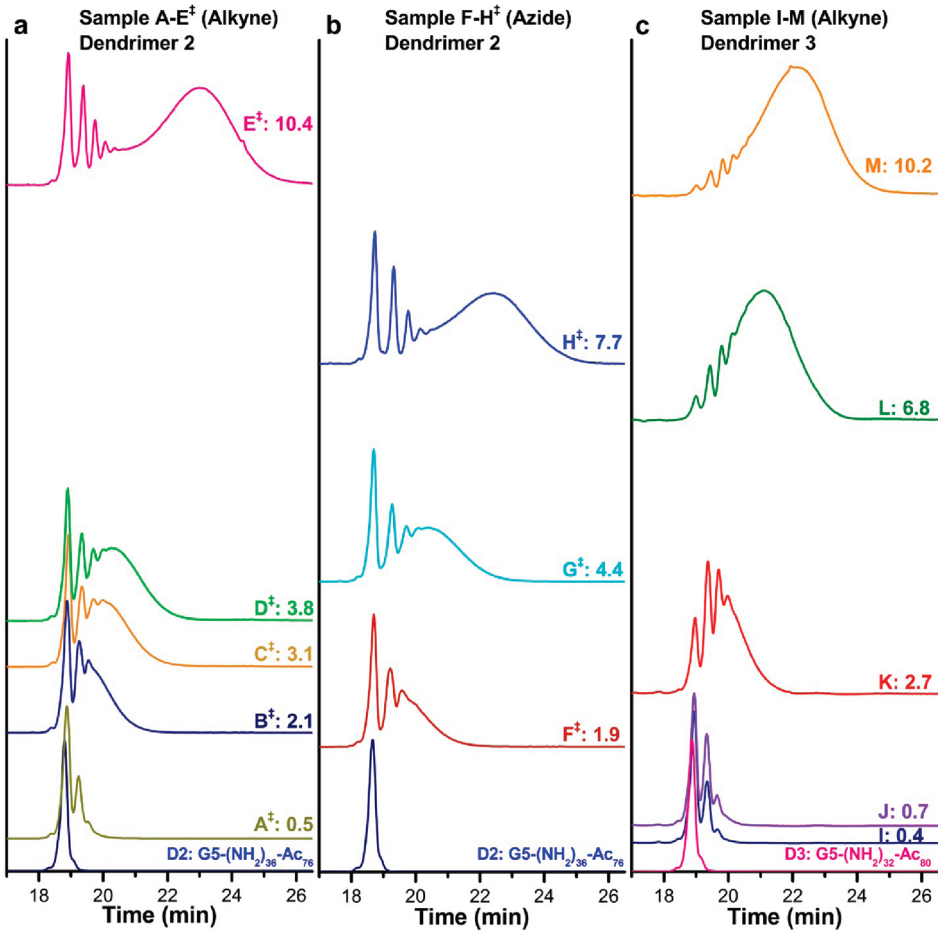


Figure 1. HPLC elution traces of dendrimer−ligand conjugates (samples A−M) at 210 nm. Traces were normalized to the largest peak and offset on the vertical axis based on the mean number of ligands per dendrimer. The mean number of ligands per dendrimer is listed by each trace. The HPLC trace of the unmodified parent dendrimer for each sample set is provided at the base of each panel (dendrimer **2** G5-Ac_{76}-$(NH_2)_{36}$ for panel a and b, and dndrimer **3** G5-Ac_{80}-$(NH_2)_{32}$ for panel c). (a) HPLC traces for the G5-Ac_{76}-Alkyne sample set (samples A−E). (b) HPLC traces for the G5-Ac_{76}-Azide sample set (samples F−H). (c) HPLC traces for the G5-Ac_{80}-Alkyne sample set (samples I−M). Dendrimer samples containing a distribution of ligands that are assigned as resulting from ineffective mass transport in the acetylation step are identified with a ‡.

The stir-rate in the dendrimer solution was low (600 rpm). The reaction was carried out in a glass flask, under nitrogen, at room temperature for 24 h. Methanol was evaporated from the resulting solution and the product was purified using 10,000 MWCO centrifugal filtration devices. Purification consisted of one cycle (10 min at 5,000 rpm) using 1× PBS (without magnesium and calcium) and five cycles using DI water. The purified dendrimer was lyophilized for 3 days to yield a white solid (46.6 mg, 62%). ^{1}H NMR integration determined the degree of acetylation to be 83%.

Samples N and O. Two stock solutions were generated to synthesize samples N and O. A solution of the Alkyne Ligand (7.2 mg, 35 μmol) was dissolved in DMSO (3.600 mL). Benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (PyBOP) (8.8 mg, 17 μmol) was dissolved in DMSO (1.760 mL).

Sample N. The alkyne ligand stock solution in anhydrous DMSO (1.1 mg, 5.1 μmol, 530 μL), was added to a solution of dendrimer **4** (12.8 mg, 0.422 μmol) in anhydrous DMSO (2.889 mL). *N,N*-Diisopropylethylamine (1.8 mg, 2.5 μL, 14 μmol) was added to the reaction mixture and the resulting solution was stirred for 30 min. The stock solution of PyBOP in anhydrous DMSO (2.7 mg, 5.2 μmol) 540 μL) was added in a dropwise manner (0.27 mL/min) to the dendrimer solution. The resulting reaction mixture was stirred for 24 h under nitrogen and then purified as described earlier. The purified product, sample N, was lyophilized for 3 days to yield a white solid (12.4 mg, 93%). ^{1}H NMR integration determined the mean number of alkyne ligands per dendrimer to be 6.6.

Sample O. The alkyne ligand (13.0 μg, 0.146 μmol), in anhydrous DMSO (14.6 μL), was added to a solution of dendrimer **5** (12.8 mg, 0.422 μmol) in anhydrous DMSO (2.889 mL). *N,N*-Diisopropylethylamine (1.8 mg, 2.5 μL, 14 μmol) was added to the reaction mixture, and the resulting solution was stirred for 30 min. The stock solution of PyBOP in anhydrous DMSO (2.7 mg, 5.2 μmol) 540 μL) was added in a dropwise manner (0.27 mL/min) to the dendrimer solution. The resulting reaction mixture was stirred for 24 h under nitrogen and then purified as described earlier. The purified product, sample N, was lyophilized for 3 days to yield a white solid (13.0 mg, 96%). ^{1}H NMR integration determined the mean number of alkyne ligands per dendrimer to be 6.8.

Results

Characterization of the Dendrimer−Ligand Samples A−M. The three conjugate sets were characterized by ^{1}H NMR spectroscopy and HPLC. We have previously shown that NMR integration combined with GPC and potentiometric titration provides the mean number of ligands per dendrimer.[34,35] Briefly, the mean number of end groups per G5-(NH_2) dendrimer **1** (112) was found by potentiometric tritration and the number-average molecular weight from GPC. The mean number of methyl protons per partially acetylated dendrimer was then calculated using the integral for the methyl protons, the integrals for the methylene protons on the amine-terminated arms and the mean number

of end groups per dendrimer. Finally, the mean number of methyl protons per dendrimer was used as a reference integral to quantify the mean number of ligands per dendrimer in the NMR spectra for samples A−M. The arithmetic mean number of ligands per dendrimer is reported in Table 1 for each sample. Similar to many nanoparticle-based systems, including a plethora of dendrimer−ligand conjugates,[30,33,37,40] the mean ligand-dendrimer ratio for samples A−M ranged from 0.4 to 10.4. In this table, and in all figures, data resulting from acetylated dendrimers that have been assigned as resulting from ineffective mass transport are identified with a ‡ to assist the reader in keeping track of the sample sets.

HPLC characterization of samples A−M was also performed using previously reported methods.[35] Elution traces recorded at 210 nm are plotted in Figure 1 by sample set. The trace of the parent dendrimer (dendrimer **2**, G5-Ac_{76}-$(NH_2)_{36}$; dendrimer **3**, G5-Ac_{80}-$(NH_2)_{32}$) is included at the base of each column. Discrete peaks, with the same peak shape as the parent dendrimer, can be seen within each HPLC trace. We have previously shown that each of these discrete peaks are composed of a dendrimer component with a different number of alkyne or azide ligands per particle.[34,35,39] The first discrete peak in each trace was found to be composed of dendrimer with 0 ligands. Dendrimer components with 1, 2, 3, ..., etc. ligands were found to have incrementally longer retention times. A previously developed peak fitting method deconstructed the HPLC trace with multiple copies of a fitting peak that had the same shape as the HPLC trace for the unmodified dendrimer (dendrimers **2** and **3**). This fitting method quantified the relative concentration of each dendrimer−ligand component in the distribution. With the relative concentration of each dendrimer−ligand component, the mean, median, and mode for each sample were calculated and are reported in Table 1. Quantified distributions for each sample are plotted by sample set in Figure 2 and in groups of similar ligand means in Figure 3.

Two major observations can be made from the quantified dendrimer−ligand distributions in Figures 2 and 3. First, the sample sets made using dendrimer **2** (G5-Ac_{76}−Alkyne and G5-Ac_{76}-Azide sample sets (samples A−H)) have the same distribution features. Comparing the distributions for samples B and F in Figure 3c found the number of components in each sample to be very similar (10 vs 13) and the difference between the concentration of each dendrimer−ligand component to be 3% or less. Remarkably, for the samples in these two sets (samples A−H) with means ranging from 0.5 to 10.4, the mode was 0. This highlights a very interesting distribution feature that is most apparent in samples E and H (Figure 2). Instead of a skewed-Poisson profile, the distributions are bimodal. High concentrations of dendrimer with low numbers of ligands (0, 1 and 2) are present with a second mode at a much higher dendrimer−ligand component (13 for sample H and 15 for sample E).

The second major observation from Figures 1−3 is that the G5-Ac_{80}-Alkyne set (samples I−M) which was produced using dendrimer **3**, does not have the same distribution features as the G5-Ac_{76}−Alkyne and G5-Ac_{76}-Azide sample sets (samples A−H). This difference can be best seen between sample E and M (Figure 3a). Whereas sample E is bimodal with a median of 12 and a mode of 0, sample M follows a skewed-Poisson distribution and has a median and mode of 11. The two samples have identical means by NMR (10.4 and 10.2), and yet sample M has 6 fewer dendrimer−ligand components (24 vs 30).

Characterization of Partially Acetylated Dendrimer Batches as a Function of Mass Transport Effectiveness. Characterization of the two partially acetylated batches of dendrimer (**4** and **5**) by NMR spectroscopy followed previously established protocols.[35] Peak integration provided a means to calculate the degree of acetylation. Dendrimer **4**, which was partially acetylated under effective stirring and dilution conditions, was found to be 63% acetylated. Because GPC and potentiometric titration had determined the starting dendrimer **1** to have a mean of 112 terminal arms per particle, the dendrimer in this batch had a mean of 71 Ac groups and 41 NH_2 groups. Dendrimer **5**, acetylated with ineffective stirring and undiluted acetic anhydride, was 73% acetylated with a mean of 82 Ac and 30 NH_2 groups.

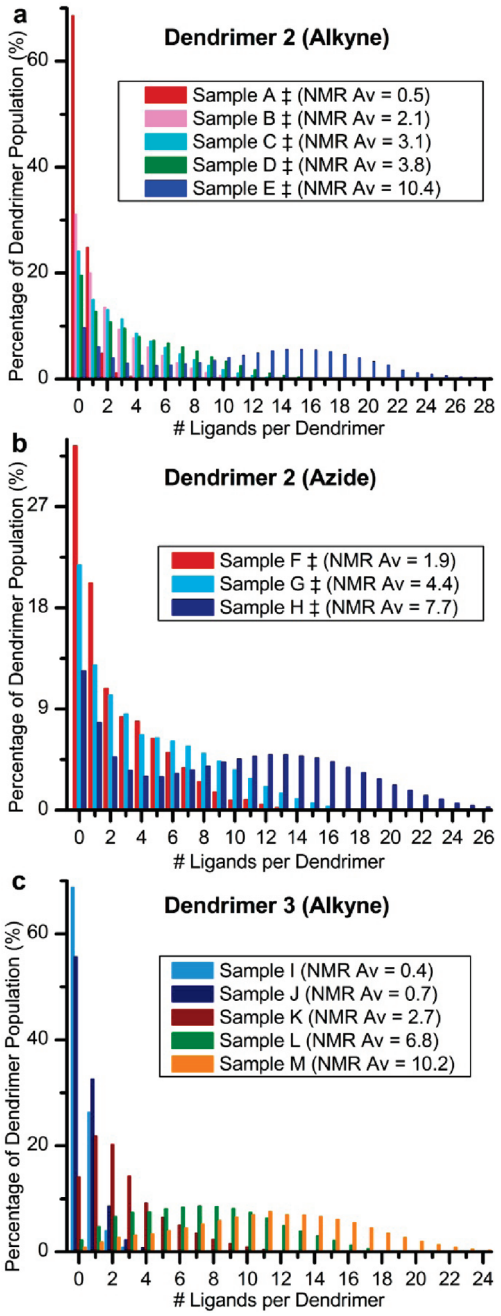


Figure 2. Quantified dendrimer−ligand distributions determined by the peak fitting enabled deconstruction of the HPLC traces. (a) Dendrimer−ligand distributions for the G5-Ac_{76}-Alkyne sample set (samples A−E). (b) Dendrimer−ligand distributions for the G5-Ac_{76}-Azide sample set (samples F−H). (c) Dendrimer−ligand distributions for the G5-Ac_{80}-Alkyne sample set (samples I−M). Dendrimer samples containing a distribution of ligands that are assigned as resulting from ineffective mass transport in the acetylation step are identified with a ‡.

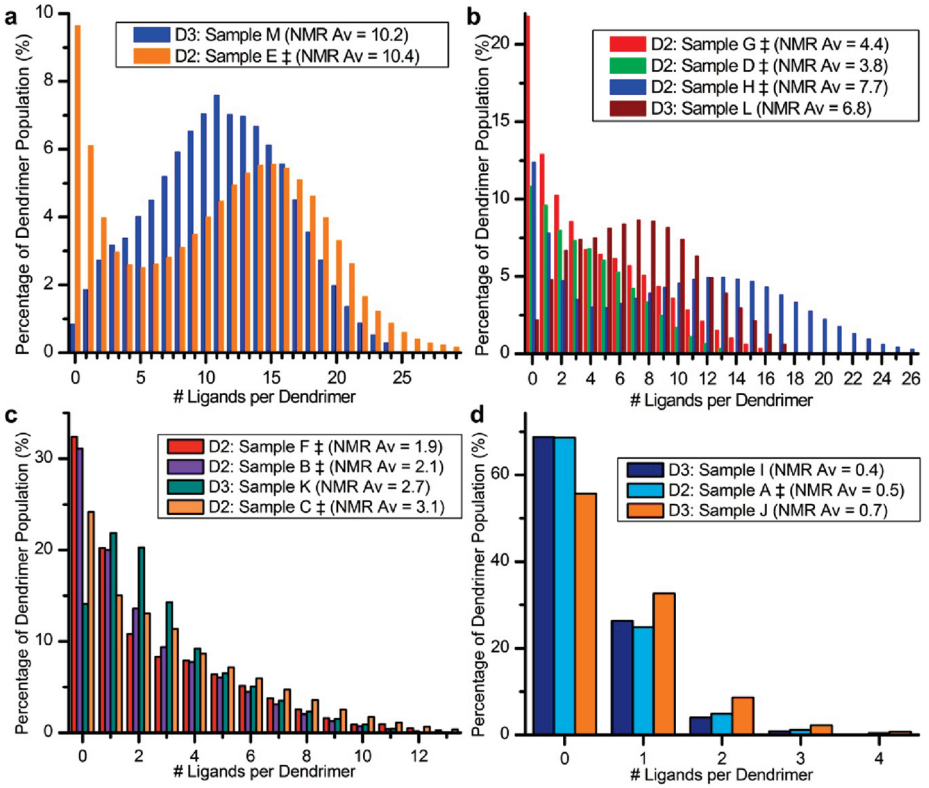


Figure 3. Comparison of the distribution of dendrimer−ligand species for samples with similar ligand means. The parent dendrimer is noted for each sample: D2 for dendrimer **2** and D3 for dendrimer **3**. (a) Distributions for samples M and E with ligand means of 10.2 and 10.4, respectively. (b) Distributions for samples with means between 4.4 and 6.8 (samples G, D, H, and L). (c) Distributions for samples with means between 1.9 and 3.1 (samples F, B, K, and C). (d) Distributions for samples with means between 0.4 and 0.7 (samples I, A, and J). Dendrimer samples containing a distribution of ligands that are assigned as resulting from ineffective mass transport in the acetylation step are identified with a ‡.

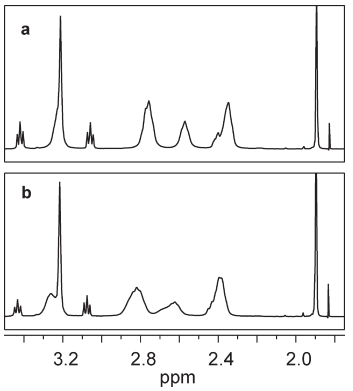


Figure 4. ^{1}H NMR spectra of partially acetylated dendrimer samples. (a) Spectrum of the partially acetylated dendrimer **4** produced under effective mass transport conditions (G5-Ac_{71}-$(NH_2)_{41}$). NMR peak integration determined the average degree of acetylation to be 63%. (b) Spectrum of the partially acetylated dendrimer **5** produced under ineffective mass transport conditions (G5-Ac_{82}-$(NH_2)_{30}$). NMR peak integration determined the average degree of acetylation to be 73%.

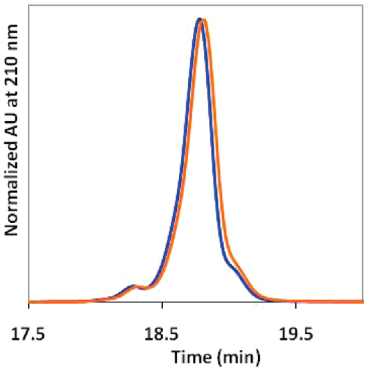


Figure 5. Normalized HPLC traces at 210 nm of partially acetylated dendrimer **4** (blue) and **5** (orange). Dendrimer **4** was synthesized under effective mass transport conditions while dendrimer **5** was produced with ineffective mass transport.

No direct information about the distribution of −Ac (and conversely $-NH_2$) groups can be obtained from the NMR spectra (Figure 4). To the level of detail at which the spectra have been analyzed in the past, the samples appear to be the same. A closer examination does find some interesting differences. First, there is a downfield chemical shift (a change of approximately 0.05 ppm) in Figure 4b for protons in the interior of the dendrimer structure. Second, the broad peak at 3.2 ppm in Figure 4a appears to be resolved into two different peaks in Figure 4b. These changes, however, cannot be interpreted to provide information about different distributions in the dendrimer structure.

Although close scrutiny of the NMR spectra in Figure 4 does find small differences, HPLC characterization of the two dendrimer batches (4 and 5) in Figure 5 reveal no differences between the two batches. The small peaks adjacent to the major peak have been previously determined to be different dendrimer defect structures (trailing generation and dimer defects).[41] The two major peaks do have slightly different retention times but this difference is within the run-to-run variability of the HPLC system (4%).

Characterization of Dendrimer−Ligand Samples N and O. NMR spectra for samples N and O are contained in Figure 6.

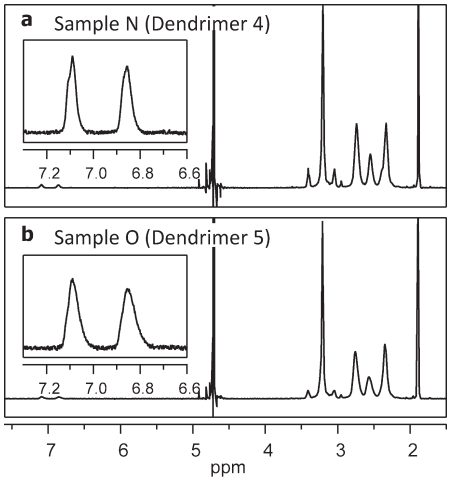


Figure 6. NMR spectra of sample N (a), produced using dendrimer **4** and sample O (b), produced using dendrimer **5**. An expanded view insert of the ligand aa′ bb′ protons at approximately 7.1 and 6.85 ppm is provided in both panels.

The mean number of ligands per dendrimer was found to be 6.7 and 6.6 respectively. In addition to having the same ligand mean, the NMR spectra reveals very few clues about any differences in the dendrimer−ligand distributions. Chemical shifts between the two spectra are very similar and the resolved semioverlapping peaks at 3.2 ppm in dendrimer 5 (Figure 4b) are no longer resolved in the ligand conjugated material. There is a difference in the FWMH for the ligand aa′ bb′ proton peaks at 7.1 and 6.9. fwhm is 7 hz wider for sample O.

HPLC traces for sample N and O are dramatically different (Figure 7). Sample N has a skewed-Poisson profile similar to the G5-Ac_{80}-Alkyne sample set (samples I−M). Sample O has the same, if not more pronounced bimodal distribution features as sample sets G5-Ac_{76}−Alkyne and G5-Ac_{76}-Azide (samples A−H). Differences between the two samples are even more apparent in the quantified distributions (Figure 8 and Table 2). The mode for sample O is zero with this component making up 27% of the sample. Conversely, the mode for sample N is 10 and this component makes up only about 7% of the sample. Furthermore, although the samples have the same means, sample O is significantly more heterogeneous than sample N (26 vs 20 dendrimer−ligand components).

Discussion

Consistent with previous publications from our group, the G5-NH_2 PAMAM dendrimer was purified and then fully characterized following purchase from Dendritech Inc. This process is necessary to both reduce the heterogeneity of the commercial polymer (dialysis can remove some of the trailing generation defect structures) and to secure an accurate understanding of the composition of the specific lot of material. We frequently observe differences between lots of the commercially synthesized dendrimer that are important to track for development purposes.

The distinct HPLC profile differences within samples A−M provided the motivation for this investigation. There is a clear dependence of the dendrimer−ligand distribution profile with the batch of partially acetylated dendrimer. In fact, the use of two different ligand molecules (alkyne vs azide) does not result in any differences in distribution features. Although the additional data is not included in this manuscript, we have observed variations in all of the material produced using this system correlating with the batch of partially acetylated dendrimer. The distribution profiles exhibited by samples

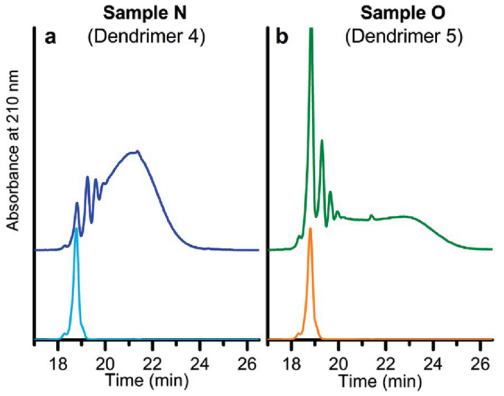


Figure 7. HPLC elution traces of samples N (a) and O (b) at 210 nm. The elution profile of the parent partially acetylated dendrimer (**4** and **5**, respectively) is included at the base of each column.

O and N are the most and least heterogeneous profiles that we have observed.

On the basis of our observations of samples A−M, our hypothesis was that dendrimers **2** and **3** had different distribution profiles of dendrimer-modification site ratios caused by a change in the quality of mass transport during the partial acetylation. Because the goal of the partial acetylation is to passivate an average of 60−80% of the dendrimer modification sites (primary amines), the reaction step occurs with an excess of modification sites per dendrimer relative to the molar amount of acetic anhydride. These conditions result in a distribution of dendrimer with different numbers of acetyl groups and consequently a distribution of dendrimer-modification site ratios. Assuming the partial acetylation reaction has perfect mass transport and is a purely stochastic process, the distribution profile would be expected to be similar to the Poisson distribution in Figure 9a. Such a distribution means that during the ligand conjugation reaction some dendrimer have a higher probability to react with ligand molecules. This causes an increase in the distribution heterogeneity.

We have observed such an increase in heterogeneity in our previous study that compared distributions between conjugates made with a G5-$(NH_2)_{112}$ dendrimer and with a partially acetylated dendrimer.[35] The dendrimer−ligand conjugates made with the G5-$(NH_2)_{112}$ had slightly skewed-Poisson distributions and the conjugate made with the partially acetylated dendrimer were more skewed. Given that ligand distributions do appear to be sensitive to a pre-existing acetyl group distribution, it is reasonable to hypothesize that the two dendrimer−ligand distribution profile types indicate that dendrimers **2** and **3** have significantly different acetyl group distributions. Under our hypothesis, in dendrimer **3**, used to make samples I−M, the acetyl distribution would be similar to the distribution in Figure 9a. For dendrimer **2**, the parent to samples A−H, the distribution of acetyl group-dendrimer ratios would be more heterogeneous and would include a large percentage of dendrimer components with zero or very few modification sites. This subpopulation would have a very low probability of subsequent ligand conjugation and would give rise to the high concentration of dendrimer with 0, 1, and 2 ligands in samples A−H.

The final part of our hypothesis is that this subpopulation of dendrimer−acetyl components is a consequence of ineffective mass transport. Because of the fast reaction kinetics of the acetylation reaction, locally high concentrations of acetic anhydride could easily generate the subpopulation of dendrimer with very few modification sites. The remaining dendrimer components would have a significantly increased probability of reacting with ligand molecules leading to an increase in heterogeneity.

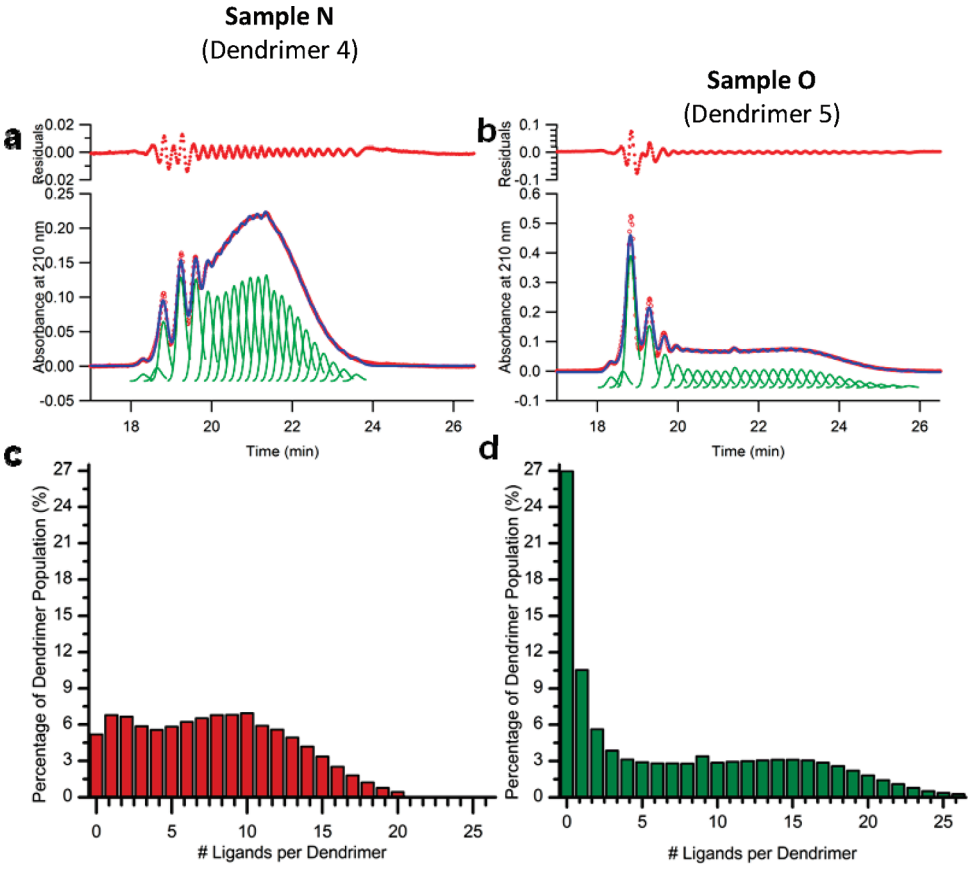


Figure 8. Distribution of dendrimer−ligand components for sample N and O quantified by the peak fitting method. (a) 210 nm HPLC elution data for sample N are shown in red dots, the multiple copies of the fitting peak is shown in green, and the summation of the fitting peaks are plotted in blue. Residual values in red have been multiplied by 10^6. (b) Fitted HPLC trace for sample O. The color scheme for panel b is the same as panel a. Residual values in red have been multiplied by 10^6. (c) Relative concentration of each dendrimer−ligand component in sample N. (d) Relative concentration of each dendrimer−ligand component in sample O.

Dendrimers **4** and **5** were synthesized to test our hypothesis. Dendrimer **4** was produced under effective mass transport conditions, defined by a high stir rate, dilution of acetic anhydride in methanol prior to addition and use of a syringe pump to achieve a slow, uniform addition of acetic anhydride. The conditions used for dendrimer **5** were characteristic of an ineffective mass transport regime. The stir rate was very low, acetic anhydride was not diluted with methanol before addition and a syringe pump was not used. As quantified in Figure 8, these two mass transport qualities ultimately result in dramatically different dendrimer−ligand distributions (samples N and O).

The dendrimer−ligand distributions in samples N and O are consistent with our hypothesis that the effectiveness of mass transport during the partial acetylation reaction has a major influence on subsequent dendrimer−ligand distributions. The ineffective mass transport dendrimer conjugate (sample O) has a bimodal dendrimer−ligand distribution with 27% of the dendrimer particles left unmodified by the ligand conjugation reaction. This profile is an extreme form of the bimodal profile originally observed in the dendrimer−ligand samples made with dendrimer **2** (samples A−H). Similar to the samples made with dendrimer **3** (samples I−M), the effective mass transport dendrimer (**4**) produces a dendrimer−ligand distribution that is a skewed-Poisson (sample N).

Even in this study, direct resolution of the distributions of dendrimer−actyl ratios is not achieved. It is clear, however, that the subsequent dendrimer−ligand distributions that are resolved provide indirect information about the distribution of dendrimer particles with different number of actyl groups. In the future, the alkyne and azide ligands may find a use as quality control tests to monitor the reproducibility of the partial acetylation as well as other ligand conjugations for which resolution of the distribution is not achieved.

Implications

The findings of this study should serve as a cautionary tale for nanoparticle−ligand systems. First, control of the passivation step is very important for achieving reproducible products. In particular, for dendrimer-based systems the introduction of a partial acetylation step may be the main obstacle to batch reproducibility. The sensitivity of this reaction to slight changes in mass transport as well as to changes in the starting G5-NH_2 dendrimer (variable amounts of dendrimer defect structures) makes this reaction hard to control. New platform designs should seek to eliminate the partial acetylation step and perform ligand conjugations to the G5-NH_2 dendrimer because of the variability on functional ligand distribution that is enforced by the variability in the acetylation distribution.

By performing the reactions in this sequence, a reproducible Poisson distribution of ligands is achievable which is the narrowest distribution that can be physically obtained for such solution phase reactions of small ligands conjugated to an excess of surface sites.[34,35] Distributions of this kind can show excellent targeted drug delivery *in vitro* and *in vivo*.[31,37,38] The remaining primary amines can then be passivated in a final acetylation step. In other nanoparticle systems, platform designs need to consider the fact that the passivation step typically results in a distribution of modification sites. This distribution then increases the heterogeneity of subsequent nanoparticle-ligand distributions.

Table 2. Comparison of the Average Number of Ligands per Dendrimer Computed by NMR Spectroscopy and HPLC for Sample N, Produced with Dendrimer 4, and Sample O, Produced with Dendrimer 5

	NMR	HPLC			
	arithmetic mean	arithmetic mean	median	mode	no. of dendrimer species
sample N (dendrimer **4**)	6.7 ± 0.7	7.8 ± 0.3	8	10	20
sample O (dendrimer **5**)	6.6 ± 0.7	7.0 ± 0.3	4	0	26

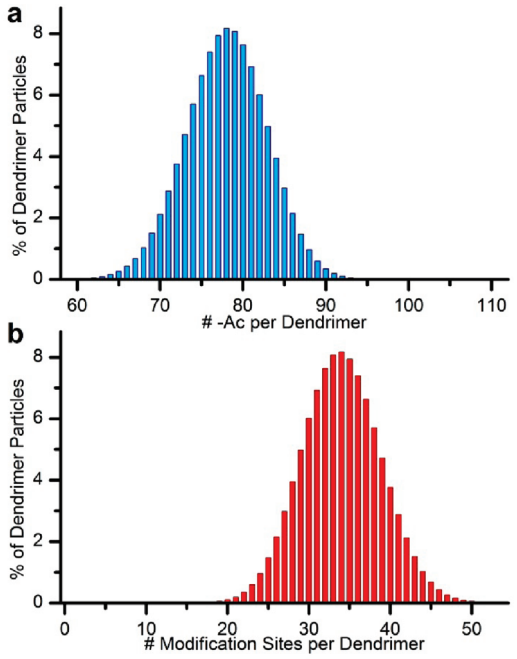


Figure 9. Theoretical distributions of dendrimer with different numbers of acetyl groups per particle (a) and the distribution of dendrimer with different numbers of modification sites that result from the partial acetylation (b).

Second, systems that achieve a reproducible mean number of ligands per particle may still not have a consistent distribution. It is very alarming that samples E and M had the same mean number of ligands per dendrimer and yet had completely different distributions. Only the HPLC analysis of the samples identified the differences. The batches of parent dendrimer for these samples (dendrimers **2** and **3**) were produced under what was thought to be the same synthetic conditions. Characterization of dendrimers **2** and **3** found that they were nominally the same material. Clearly this was not the case. The quality of mass transport during the partial acetylation reaction for dendrimer **2** was impaired relative to dendrimer **3**. The result of this impairment was a major difference in the dendrimer−ligand distribution that was only detected by HPLC. Similarly, characterization of dendrimer **4** and **5** with intentionally different mass transport conditions, failed to detect any differences in the material. Again, only the HPLC characterization of sample N and O revealed the even more extreme differences between the material. This is problematic because the capability to resolve the distribution of components does not exist for many nanoparticle-ligand systems in production today. Frequently, only the mean ligand-nanoparticle ratio is known for these systems. The results of this study indicate that it will be very important to go a step further and identify the distribution that gives rise to the mean. Failure to do so may make batch reproducibility a major challenge as these systems are moved to commercial scales. To this end, future studies need to focus on the relationships between the nanoparticle-ligand distributions and the material properties of the system. These studies will be very useful for defining the tolerable levels of variability in the distribution that will produce the same bulk properties.

Conclusion

In conclusion, the effectiveness of mass transport in the partial acetylation step has a major impact on subsequent dendrimer−ligand distributions. Our results indicate that mass transport effectiveness has a dramatic impact upon the dendrimer−acetyl distribution which in turn controls the distribution of ligands which can be achieved. Furthermore, this study demonstrates that using the mean ligand−nanoparticle ratio alone is insufficient to ensure that the batch reproducibility of the distribution is maintained.

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