The Implications of Stochastic Synthesis for the Conjugation of Functional Groups to Nanoparticles

Supporting Information

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I. Experimental Methods:

Dendrimer Purification

Generation 5 PAMAM dendrimer was purchased from Dendritech Inc. To remove lower molecular weight impurities and trailing generations the dendrimer was dialysed with a 10,000 MWCO membrane against DI water for three days, exchanging washes every 4 hours. The number average molecular weight (27,336 g/mol) and PDI (1.018 +/- 0.014) was determined by GPC. Potentiometric titration was conducted to determine the average number of primary amines (112).

Partial Acetylation

Purified Generation 5 PAMAM dendrimer (133.7 mg, 4.89 μmole) was dissolved in anhydrous methanol (21 mL). Triethylamine (68.5 μL, 0.491 mmole) was added to this mixture and stirred for 30 minutes. Acetic anhydride (37.1 μL, 0.393 mmole) was added to anhydrous methanol (4 mL) and the resulting mixture 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 five cycles (30 minutes at 5,000 rpm) using 1x PBS and five cycles using DI water. The purified dendrimer was lyophilized for three days to yield a white solid (138.4 mg, 92%). Number average molecular weight (30,660 g/mol) and PDI (1.026 +/- 0.015) were determined by GPC. ^{1}H NMR integration determined the degree of acetylation to be 70%.

Ligand Synthesis (3-(4-(prop-2-ynyloxy)phenyl)propanoic acid)

To a solution of methyl 3-(4-hydroxyphenyl)propanoate (2.18 g, 0.0121 mol) in dry acetone (56 mL) was added anhydrous K_2CO_3 (4.60 g, 0.0333 mol) followed by propargyl bromide (80% solution in toluene, 1.88 mL, 0.0126 mol). The resulting suspension was refluxed for 24 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. The filtrate was evaporated under vacuum to give the desired product as an oil (2.43g, 92%).

The crude product from above was dissolved in MeOH (60 mL). KOH (8 M, 5.0 mL, 0.040 mol) was added and the resulting mixture was heated at 70 °C for 1.5 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 $MgSO_4$. Solvent was evaporated under reduced pressure to give the desired product as a yellowish solid. (2.21 g, 97%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.12, (d, 2H, J = 8.74 Hz), 6.89 (d, 2H, J = 8.71 Hz), 6.89 (d, 2H, J = 8.71 Hz), 4.65 (d, 2H, J = 2.40 Hz), 2.89 (t, 2H, J = 7.74 Hz), 2.64 (t, 2H, J = 7.75 Hz), 2.49 (t, 1H, J = 2.40 Hz)

Ligand Conjugation to Dendrimer

The ligand was conjugated to the partially acetylated dendrimer in two consecutive reactions. First, a stock solution of the ligand 3-(4-(prop-2-ynyloxy)phenyl)propanoic acid (9.4 mg, 0.046 mmole) was generated with a mixture of DMF (6.899 mL) and DMSO (2.300 mL). To this mixture was added 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (123.5 mg, 0.644 mmole). The resulting solution was stirred for 4 h at room temperature to create the active ester form of the ligand.

A stock solution of partially acetylated dendrimer (77.1 mg, 2.51 μmole) was made with DI water (17.190 mL). This solution was partitioned into four aliquots, A-D (15.0 mg, 0.489 μmole each). Additional DI water (2.520 mL, 2.016 mL, 1.512 mL) was added to the first three aliquots (A-C). The active ester form of the ligand (0.504 mL, 2.521 μmole) in DMF/DMSO was added in a dropwise manner (0.1 mL/min) to the first aliquot (A) of dendrimer-water solution. Similarly, the activated ester form of the ligand was added to the second, third, and fourth aliquots (5.043 μmole, 7.565 μmole, and 10.087 μmole respectively). The resulting mixtures were stirred for 2.5 days. All reaction steps were carried out in glass flasks at room temperature under nitrogen. The four reaction mixtures were purified using 10,000 MWCO centrifugal filtration devices. Purification consisted of five cycles using 1x PBS and six cycles using DI water. All cycles were 30 minutes at 5,000 rpm. The resulting products (A-D) were lyophilized for three days to yield a white solid (14.6 mg, 16.5 mg, 15.7 mg, and 15.5 mg respectively).

HPLC Characterization

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 90:10 (v/v) water/acetonitrile and ending with 10:90 (v/v) water/acetonitrile over 25 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.

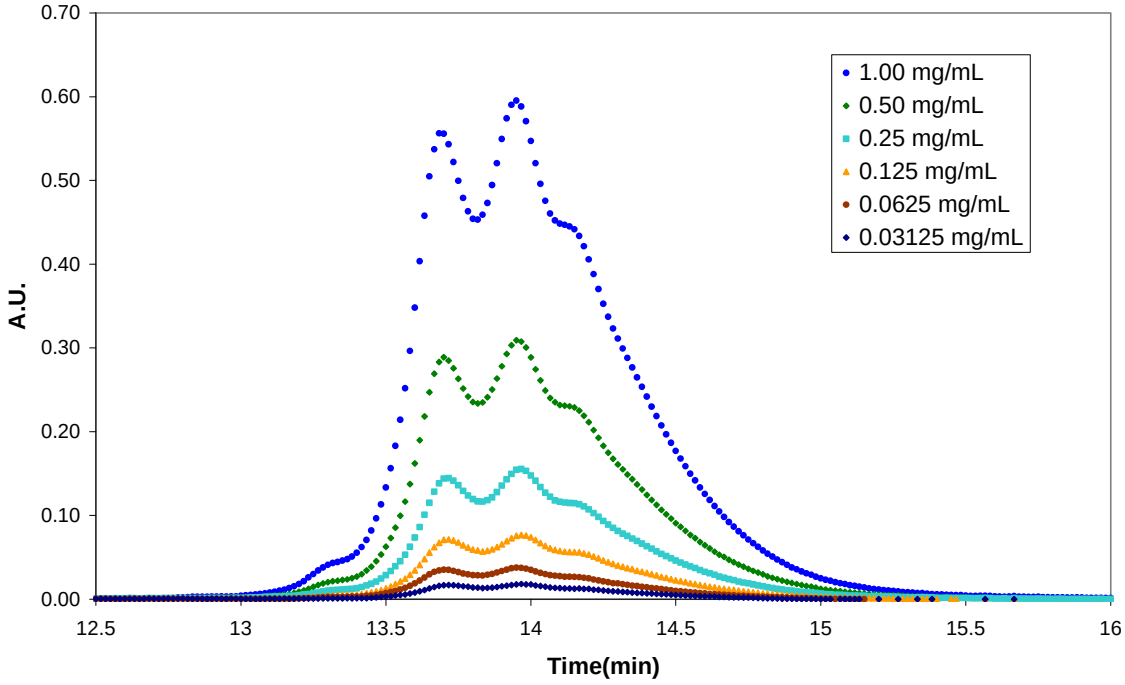
To determine the experimental error associated with the HPLC characterization, Sample D was injected five times over two days under identical conditions. The absorbance data was normalized against the major peak for each injection and standard deviations were computed at each time point. The standard deviations were normalized against absorbance and the average error in the range between 12.5 and 15.5 minutes was computed to be 4%.

II. Beer's Law: Dilution Study

A dilution study of Sample D was performed to demonstrate that the dendrimer conjugates follow Beer's Law at 210 nm. Solutions of Sample D with different concentrations were injected on the HPLC using the conditions detailed in the Methods

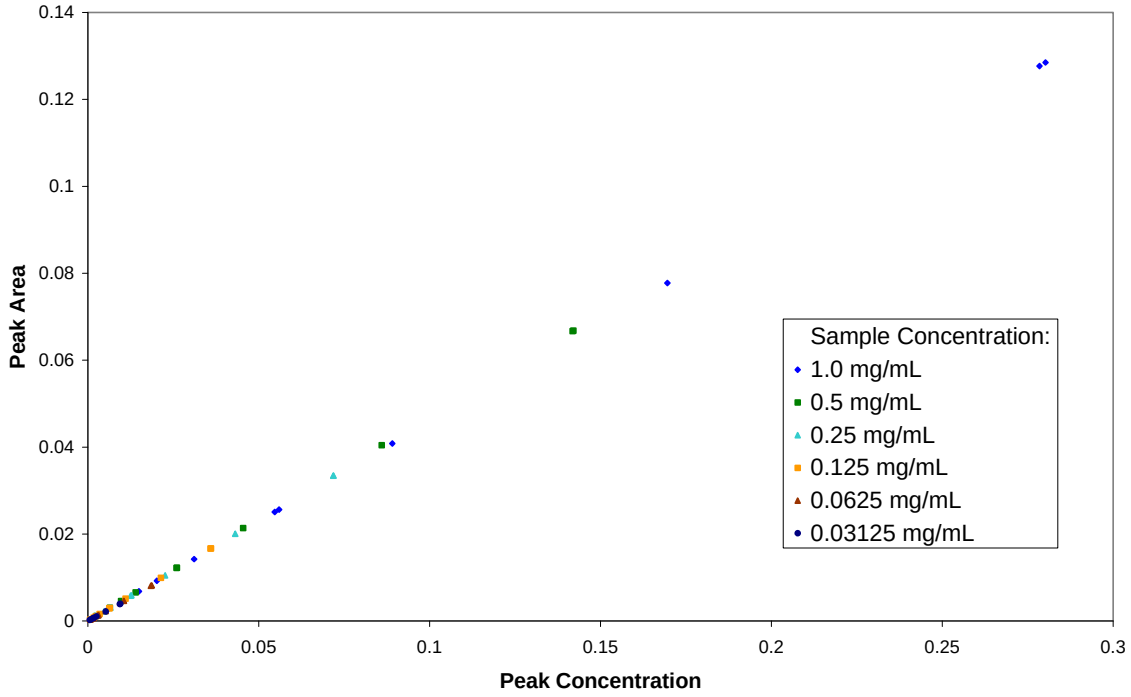
section of this publication. The elution profile at 210 nm of Sample D at varying concentrations can be found in Figure S1.

Figure S1: HPLC profile of Sample D injected at varying concentrations



Using the fitting procedure described earlier in this publication, peaks were fit to each of the elution profiles in Figure S1. Peak concentration was calculated as the product of the Peak Area Fraction and the Sample Concentration. The linear relationship found in Figure S2 between Peak Area and Peak concentration clearly demonstrates that Beer's Law is followed at 210 nm for the dendrimer conjugates in this study.

Figure S2: Peak Area vs. Peak Concentration



III. Characterization by Gel Permeation Chromatography (GPC)

GPC analysis was performed on the four dendrimer-ligand samples and the partially acetylated dendrimer using an Alliance Waters 2690/2695separations module (Waters Corp., Milford, MA) equipped with a Waters 2487 UV absorbance detector (Waters Corp.), a Wyatt Dawn DSP laser photometer (Wyatt Technology Corp., Santa Barbara, CA), an Optilab DSP interferometric refractometer (Wyatt Technology Corp.), and TosoHaas TSK-Gel Guard PHW 06762 (75 x 7.5 mm, 12 µm), G 2000 PW 05761 (300 x 7.5 mm, 10 µm), G 3000 PW 05762 (300 x 7.5 mm, 10 µm), and G 4000 PW (300 x7.5 mm, 17 µm) columns. Citric acid buffer (0.1 M concentration) with 0.025% sodium azide in water was used as a mobile phase, pH 2.74, using NaOH. Number Average Molecular Weight (Mn) and the Poly Dispersity Index (PDI) for each of the samples was calculated by using Astra software (version 4.9) (Wyatt Technology Corp.) and reported in Table S1. The general trend of increasing molecular weight is consistent with samples of increasingly higher averages of conjugated ligands.

Table S1: Number Average Molecular Weight and PDI calculated from GPC for the Dendrimer-Ligand conjugates

	Mn	PDI
G5Ac(70%)	30,660	1.026±0.015
Sample A	32,560	1.026±0.012
Sample B	33,200	1.025±0.012
Sample C	33,960	1.034±0.012
Sample D	35,080	1.024±0.012

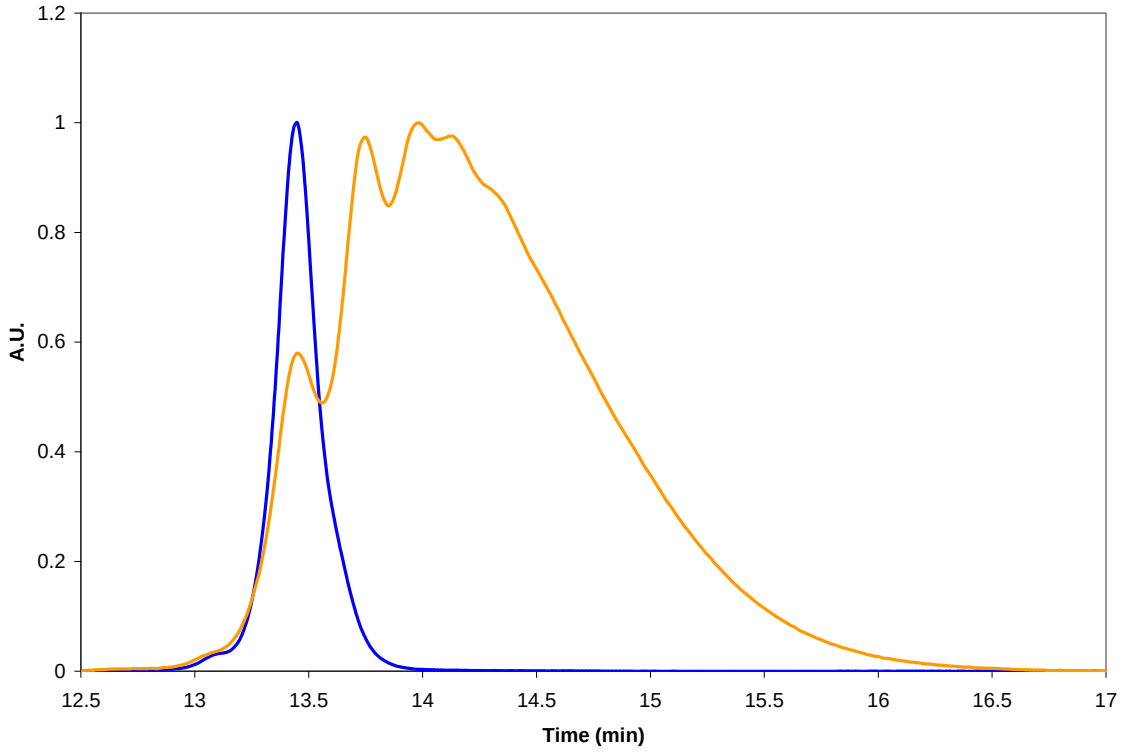
IV. Molecular Weight Characterization of the Dendrimer-Ligand Conjugates and Starting Material by MALDI-TOF

The four dendrimer-ligand samples and the partially acetylated dendrimer were characterized with a Micromass TofSpec-2E Matrix-Assisted, Laser-Desorption Time-of-Flight Mass Spectrometer. Spectra were acquired in Linear mode. The MALDI-TOF sample mixtures were prepared using 5 μL of the matrix trihydroxyacetophenone in equal amounts of water and acetonitrile (10 mg/mL), and 5 μL of the dendrimer in equal amounts of water and methanol (1 mg/mL). Each spot volume was 1 μL. Data summing and smoothing was applied post acquisition to each set of spectra. All processed spectra were normalized to the peak maximum.

V. Additional HPLC Profiles

The HPLC profiles for partially acetylated dendrimer and a dendrimer-ligand conjugate with an average of 3.1 can be found in Figure S3. Synthesis of the partially acetylated dendrimer is described in the Experimental Methods section of this paper. The dendrimer-ligand conjugate with an average of 3.1 ligands was synthesized following the procedure described for the four dendrimer-ligand samples investigated in this publication. Partially acetylated dendrimer for this dendrimer-ligand conjugate was 74% acetylated. A linear baseline subtraction was applied to both data sets and peak maxima were normalized.

Figure S3: HPLC profiles for partially acetylated dendrimer (blue) and partially acetylated dendrimer with an average of 3.1 ligands (orange).



VI. The Two Path Kinetic Model

Suppose a dendrimer has *n* ligands bound, but where *n* is small compared to the total number of dendrimer branches $M = 112$. Should another ligand encounter this dendrimer, there are two most probable avenues, that the ligand attaches to an available branch far from the influence of already-attached ligands, and that the ligand binds near a previously attached ligand. Given the low occupancy, we discount the possibility of a ligand binding near more than one other ligand.

Each avenue has an associated rate of attachment depending upon the probability of encountering such a site, the activation energy barrier, and an underlying collision frequency ω that is assumed to be the same for the two paths. The two parallel paths give two rate contributions, R_{n1} and R_{n2}, such that the total rate $R_n = R_{n1} + R_{n2}$.

The probability p_2 of a ligand binding at a site near a previously bound ligand is given by $p_2 = nze^{-E_{a2}/RT}$, where z is the effective coordination number, the number of sites

a bonded ligand influences, and E_{a2} is the activation energy barrier for this pathway, a value influenced by the proximity to the bound ligand. This leads to the rate $R_{n2} = nA_2 e^{-E_{a2}/RT}$ by multiplying with ω, giving the association of $A_2 = z\omega$.

The probability p_1 of a ligand binding to one of the other available sites is the probability of encountering an open site that doesn't belong to the other pathway. That is, $p_1 = [m - n(z+1)]e^{-E_{a1}/RT}$, where $m = 34$ is the number of non-acetylated sites, and E_{a1} is the activation energy barrier for this unassisted pathway. The term $n(z+1) = nz + n$ has a contribution from discounting sites under the influence of a bound ligard, nz, and the sites at which the previously bound ligands are bound, giving the n term. This leads to the rate $R_{n1} = A_1 e^{-E_{a1}/RT}$ with the association $A_1 = [m - n(z+1)]\omega$.

The effective coordination number z depends on the arrangement of the dendrimer branches and the number of those branches which are not acetylated. If we consider first nearest neighbor interactions the end of a given branch may be near a handful of others, say between four and six. We expect that $m/M = 30\%$ of these branches are ligated or open to ligation. Therefore, the effective coordination number is a number of order unity, say between one and two.

A numerical estimate of the effective coordination results in $z = 0.9$. This estimate was generated by randomly populating a 10x10 triangular (close-packed) lattice with 30 sites, then counting the number of neighbors each site had. This process was sampled 10 000 times, giving a low-skewed distribution of numbers of neighbors with the average being 0.9. This results in a range of values for A_2/A_1 from 0.0025 to 0.1 for n between 1 and 10, with an average value of 0.04.

Fitting the Model

The Master equation $\dot{c}_n = R_{n-1}c_{n-1} - R_n c_n$ was numerically integrated using an Euler method for 1000 steps. The time step is arbitrary, since the time derivative in the equation is only known to a constant of proportionality that is absorbed into A_1 and A_2 for each data set. Integrating for 10 000 steps instead of 1000 does not significantly change the result. A non-linear least-squares fit was conducted simultaneously for the four data sets, resulting in the following fitted values for the parameters.

	Fitted Value
$\frac{A_2}{A_1}e^{-(E_{a2}-E_{a1})/RT}$	0.58
L_1	2.0 E-5
L_2	4.7 E-5
L_3	7.4 E-5
L_4	9.8 E-5

A significant confirmation of the model is the recovery of the ligand concentration ratios. The constants L_1, L_2, L_3, L_4 are proportional to the ligand concentrations, though the constant of proportionality is not independently recoverable. Therefore, normalizing each by L_4, and normalizing the ligand concentrations by the amount used in the most concentrated data set allow comparable values.

	Fitted Values	Actual Values
L_1/L_4	0.20	0.25
L_2/L_4	0.47	0.5
L_3/L_4	0.75	0.75

The prediction for this method is a difference between the activation energy barriers of the two paths in the model. From the fit $\frac{A_2}{A_1}e^{-(E_{a2}-E_{a1})/RT} = 0.58$, we can solve for the energy difference $(E_{a1} - E_{a2}) = RT\log(0.58\frac{A_1}{A_2})$. After finding an estimate for $A_1/A_2 = 10$, we find the energy difference E_{a1}-E_{a2} = 6.2 ± 1.5 kJ/mol.