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Generation 3 PAMAM dendrimer TAMRA conjugates containing precise dye/dendrimer ratios

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Abstract

The synthesis, isolation, and characterization of generation 3 poly(amidoamine) (G3 PAMAM) dendrimer containing precise ratios of 5-carboxytetramethylrhodamine succinimidyl ester (TAMRA) dye ($n = 1-3$) per polymer particle are reported. Stochastic conjugation of TAMRA dye to the dendrimer was followed by separation into precise dye-polymer ratios using rp-HPLC. The isolated materials were characterized by rp-UPLC, MALDI-TOF-MS, and ¹H NMR spectroscopy, UV-vis, and fluorescence spectroscopies.

Keywords

Poly(amidoamine) dendrimer; PAMAM; Fluorescent dye; rp-HPLC

1. Introduction

Cationic dendrimers have been extensively explored both as vectors for nonviral gene delivery and carriers in targeted drug delivery [1–3]. In order to image the location of the material in biological environments, a fluorescent dye is often conjugated to the dendrimer scaffold; however, the Poisson distribution of dye/dendrimer ratios resulting from typical stochastic synthesis conditions [4–6] gives rise to a range of photophysical [7] and biodistribution properties that limit interpretation of these experiments.

The experimental problems arising from the distribution of fluorophore to scaffold ratio is widely appreciated for polymers. Many groups have explored strategies to improve the control over fluorophore/dendrimer ratios since the relatively lower polydispersity index (PDI) of dendrimers and/or synthetic methodologies make this class of scaffold attractive for addressing the challenges [7–14]. Approaches include dye encapsulation [12–14],

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Author contributions

JM and KJ isolated G3 PAMAM monomer, conducted titration of G3 monomer, conducted acetylation and ¹H NMR integrations for G3 monomer, and conjugated/isolated G3-TAMRA_n-NH₂ conjugates. CAD performed UV-vis and ¹H NMR spectroscopy as well as fluorescence experiments on G3-TAMRA_n-NH₂ conjugates and performed MALDI-TOF-MS and UPLC on both G3-NH₂ material and G3-TAMRA_n-NH₂ conjugates. JD isolated G3-NH₂ material and performed UPLC on isolated material. All authors worked on designing the experiments, interpreting results, and drafting the manuscript.

employing the core of the dendrimer as the dye [11], and convergent synthesis starting with the dye [8,9]. All of these approaches have particular advantages and limitations. Dye encapsulation is readily achieved by simple mixing but does not give homogeneous ratios [7] and rapidly leaches dye under biological conditions [13]. Convergent syntheses can give material structural control approaching molecular levels but is limited in terms of molecular weight (MW) that can be achieved and also gives dye/dendrimer ratios controlled by number of arms. Use of the dye as the core avoids hydrophobic decoration of the dendrimer surface but can be synthetically arduous, increases dendrimer rigidity, and limits dye loading. In our approach, we have stochastically dye-modified the divergently synthesized, commercially available, poly(amidoamine) (PAMAM) dendrimer system and employed reverse-phase high performance liquid chromatography (rp-HPLC) to separate the resulting population into the desired precise ratio dye/dendrimer fractions [4,7,15]. In principle, this strategy has the advantages of being flexible across multiple generations of hydrophilic dendrimer and, in addition to providing the precise ratio materials for desired applications (i.e. dendrimer containing 1, 2, or 3 dyes per particle), also generates the component fractions of typical stochastic mixtures, thus providing better insight into the properties and biological behavior of these commonly prepared materials. In fact, in a recent review article by Sokolova and Epple on synthetic pathways to make nanoparticles fluorescent, conjugating fluorescent dyes to polymer surfaces was touted as being one of the most efficient ways to achieve fluorescence in a nanoparticle due to the ease of the synthetic process and high fluorescent yield [16]. However, the major disadvantage was the need to ensure excess dye is removed as well as the formation of a polydisperse particle population [7,16].

The desire for a divergent synthesis of a covalently attached fluorescent dye to a polymer platform can also be noted in the need for quality cellular delivery materials such as gene delivery agents. Recently it has been reported that even slight differences in nanoparticle size and surface charge can affect the efficiency and pathway of cellular uptake [17,18]. In addition, the transfection efficiencies of dendrimers have been shown to correlate inversely with hydrophobicity of their surface groups [19] while other studies have shown that the conjugation of a hydrophobic fluorophore to a dendrimer increased the ability to deliver oligonucleotides to the nucleus of a cell [20]. The mechanisms for the molecular size fractionation and dye-hydrophobicity effects remain unknown, however the ability to further analyze the cellular uptake and biodistribution of the nanoparticle would benefit immensely by the introduction of better probes, specifically ones that are precisely controlled with respect to size, molecular weight, and numbers of fluorescent markers.

Recently, we reported the isolation and characterization of precise dye/dendrimer ratios (fluorescein and TAMRA) for generation 5 (G5) G5 PAMAM dendrimer [7]. Lower generation dendrimers are also of great interest for biological applications because of their reduced cost and, for some applications, preferred size [21,22]. For controlled photophysical and biodistribution properties, it is important to control MW as well dye/dendrimer ratio [23]. Defects expected in G3 PAMAM include trailing generations (G1 and G2) as well as G3-G3 dimer, which give a molecular weight range from ~1400 to 14,000 Da for the nominally 6909 Da material [24]. We now report the semi-preparative scale purification of commercially available G3-PAMAM. The G3 monomer (G3m) material was isolated and employed in the synthesis, separation, and characterization of G3m containing precise ratios

of 5-carboxytetramethylrhodamine dye to dendrimer ($G3-NH_2-TAMRA_n$ where $n = 1-4+$). The isolated fractions were characterized by 1H nuclear magnetic resonance (NMR) spectroscopy, reversephase ultra high performance liquid chromatography (rp-UPLC), matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS), and emission and absorption spectroscopy.

2. Experimental

2.1. Chemicals and methods

Biomedical grade Generation 3 PAMAM was purchased from Dendritech Inc., and purified by dialysis. 5-carboxytetramethylrhodamine (5-TAMRA) was purchased from Life Technologies and used as received. HPLC grade water, HPLC grade acetonitrile, and deuterium oxide were purchased from Fisher-Scientific and used as received. A 500 MHz Varian NMR instrument and deuterium oxide solvent were used for all 1H NMR measurements. All MALDI-TOF-MS measurements were performed using a sinapinic acid/sodium trifluoroacetate matrix on a Bruker Autoflex Speed MALDI MS.

2.2. Isolation of G3 PAMAM monomer (G3m)

Semi-preparative rp-HPLC was performed on Waters Delta 600HPLC system equipped with a Waters 2998 photodiode array detector, a Waters 2707 autosampler, and Waters Fraction collector III. The instrument was controlled by Empower 2 software. For analysis of the conjugates, a C18 silica-based RP HPLC column (250×10.0 mm, $300 \text{ \AA}$) connected to a C18 guard (10 $\times$ 10 mm) was used. The mobile phase for elution of the conjugates was a linear gradient beginning with 95:5 (v/v) water/acetonitrile (ACN) and ending with 55:65 (v/v) water/ACN over 18 min at a flow rate of 12.00 mL/min. Trifluoroacetic acid (TFA) at 0.10 wt% concentration in water or ACN was used as a counter ion to make the dendrimer surfaces hydrophobic. The conjugates were dissolved in the mobile phase (water with 0.1% TFA). The injection volume was 910 μ L with a sample concentration of approximately 15 mg/mL. Detection of eluted samples was performed at 210 nm.

2.3. Conjugation of 5-TAMRA to G3 PAMAM

6.35 mL of 5-TAMRA (1 mg/mL DMSO) was added dropwise to a solution of G3 PAMAM monomer (20.40 mg, 3.0×10^{-6} mol) in 0.1 M $NaHCO_3$ (8.0 mL) over a period of 2 h by a syringe $\times$ pump. The reaction mixture was left stirring at 20 $^\circ$ C for 24 h. The product was purified by Sephadex G-25 column eluted with MilliQ water. The first band collected was dialysed with a 3500 MWCO membrane cassette against MilliQ water for three days, exchanging washes every two hours. The purified dendrimer conjugate was lyophilized for three days to yield a red powder material (20.3 mg, 80%).

2.4. Isolation of precise dendrimer/dye ratios

The rp-HPLC was identical to that described above. For isolation of the conjugates, a C5 silica-based rp-HPLC column (250×10.0 mm, $300 \text{ \AA}$) connected to a C5 guard (10 $\times$ 10 mm) was used. The mobile phase for elution of the conjugates was a linear gradient beginning with 100:0 (v/v) water/ACN and ending with 20:80 (v/v) water/ACN over 30 min at a flow rate of 2.75 mL/min. TFA at 0.14 wt% concentration in water as well as in ACN

was used as a counter ion to make the dendrimer surfaces hydrophobic. The conjugates were dissolved in the mobile phase (90:10 water/ACN). The injection volume was 500 L with a sample concentration of approximately 5 mg/mL, and the detection of eluted samples was performed at 210 and 547 nm.

2.5. Analytical rp-UPLC

An analytical Waters Acquity UPLC (C18 silica-based column) controlled by Empower 2 software was employed to analyze G3-TAMRA conjugates for purity. A linear gradient mobile phase was employed beginning with 95:5 (v/v) water/acetonitrile and ending with 55:45 (v/v) water/acetonitrile over 22 min at a flow rate of 2.0 mL/min. TFA at 0.14 wt% concentration in water as well as in ACN was used as a counter ion to make the dendrimer surfaces hydrophobic. The injection volume was 3 µL with a sample concentration of approximately 1 mg/mL, and the detection of eluted samples was performed at 210 nm.

2.6. Emission measurements

Fluorescence measurements were taken at a concentration of 1.0×10^{-5} M using a Fluoromax-4 spectrometer. Excitation of 520 nm and emission of 580 nm was used with a slit width of 2 nm for all measurements taken.

2.7 Absorption measurements

A Shimadzu UV-1601 UV/vis spectrometer was used for all absorption measurements. Studies used a concentration of 1.0×10^{-5} M solution and slit width of 2.0 nm.

3. Results

In order to prepare materials with uniform photophysical and hydrophobicity properties, one must control both dye/polymer ratio and polymer MW distribution. For the strategy using the divergently synthesized PAMAM described here, the approach must start with purification and characterization of the commercial G3 PAMAM scaffold. Semi-preparative rp-HPLC reveals 3 major components in the as-received commercial sample: trailing generation G2 (2%), G3m (86%), and G3–G3 dimer (12%) (Scheme 1a). G2 dimer is also likely present in small amounts and is expected to elute along with the G3m fraction (estimated to be <0.3% of G3m peak based on the G3m to G3–G3 dimer ratio). Purification via dialysis using a membrane with a 3500 kDa cutoff successfully removed the lower MW trailing generations; however, similar to the results previously obtained for G5 PAMAM [25], it does not change the G3m to G3–G3 dimer ratio. We therefore employed semi-preparative rp-HPLC to isolate each major component. Fractions indicated by the colored bars (trailing generation G2 (purple), G3m (red), G3–G3 dimer (blue)) were collected and analyzed by rp-UPLC and MALDI-TOF MS (Scheme 1b and Fig. 1). MALDI-TOF-MS of the isolated fractions shows a change in m/z ratio with MW values of 3550, 6850, and 13,100 Da for trailing G2, G3m, and G3–G3 dimer, respectively (Table S1). We confirmed using MALDI-TOF-MS that the tail on the right side (high MW) of the dimer peak consisted only of dimer and contained no detectable G3–G3–G3 trimer. Thus, the MW distribution is ~3500–13,000 for the as-received commercial sample. For the materials used in this report,

G3 PAMAM purified by dialysis (G3d) has a MW distribution of ~6000–14,000 Da and the G3m material has a MW distribution of approximately 6000–7300 Da.

G3-NH₂-TAMRA_n samples were prepared using both G3d and G3m materials as the starting scaffold (Scheme 2). In both cases, G3-NH₂-TAMRA_n ($n = 1, 2a, 2b, 3, 4+$) was prepared by first performing a stochastic conjugation in which the succinimidyl ester of the TAMRA was allowed to react with an amine group on the dendrimer surface to yield an amide bond. A minimum ratio of 4 TAMRA:1 PAMAM was used in order to minimize the unconjugated product while maximizing the product populations containing 1–3 dyes:dendrimer. The resulting mixtures (G3d-NH₂-TAMRA_{2.2(avg)} and G3m-NH₂-TAMRA_{3.8(avg)}) were separated using semi-preparative rp-HPLC to isolate fractions containing well-defined dye/dendrimer ratios. The composition and purity of the isolated fractions were determined by ¹H NMR spectroscopy, reinjection onto an analytical rp-UPLC column (Scheme 2c & e), and MALDI-TOF MS (Figs. 2 and Table S1).

In both the semi-preparative (Scheme 2b & d) and analytical rp-HPLC data (Scheme 2c and e) double peaks are observed for orange/green and blue fractions assigned as G3-TAMRA₂ and G3-TAMRA₃ ratios, respectively. Interestingly, double peaks were not observed for either scaffold for the material assigned as G3-TAMRA₁. For the material formed using the G3d scaffold, we hypothesize that the multiple peaks arise from the presence of regioisomers resulting from TAMRA conjugation to different arms on the G3 scaffold (different elution times may occur for two TAMRA conjugated to the same major branch as opposed two TAMRA being conjugated to different branches) or from the presence of dye conjugated to both G3 monomer and G3–G3 dimer (different elution times result from the difference in MW between the G3m and G3–G3 dimer scaffolds). We have not previously observed such effects when separating G5-Ac-TAMRA_n or G5-NH₂-TAMRA_n [4,7,15]; however, the peak width of G3-NH₂ is about 1.4 times narrower than G5-NH₂ due to the approximately four-fold difference in MW between the generations. The observations that (i) the double peaks were still observed for the G3m scaffold and (ii) no peak splitting was observed for either scaffold for G3-TAMRA₁ supports the hypothesis that the peak splitting arises from the presence of regioisomers. In order to further test the physical origin of the peak splitting, we isolated fractions $n = 2a$ and $n = 2b$ for the G3m scaffold (Scheme 2d) and characterized them using rp-UPLC, MALDI-TOF-MS, and ¹H NMR, absorption, and fluorescence spectroscopies. Although there was a difference in retention time in the UPLC (Scheme 2e), the average number of TAMRA as determined by ¹H NMR spectroscopy ($2a = 2.0$; $2b = 2.1$) and similar MW as measured by MALDI-TOF-MS ($2a = 7620$; $2b = 7650$) support the assignment of both peaks as resulting from G3m-TAMRA₂ material. As expected for regioisomers, the emission (Fig. 3a) and absorption spectra (Fig. 3b) for 2a and 2b are somewhat different; however, they are more similar to each other than they are to the spectra for G3m-TAMRA₁ or G3m-TAMRA₃. The ability to resolve dye regioisomers for G3d and G3m because of narrower scaffold MW distribution is consistent with ability to resolve the different TAMRA/dendrimer ratios for the G3-NH₂-TAMRA_{n(avg)} and G3-NH₂-TAMRA₄₊ materials by MALDI-TOF MS (Figs. 2 and S1). By way of contrast, the different ratios of G5m-TAMRA_n were not resolvable by mass spectroscopy and the regioisomers present were not resolved [7]. The resolution of the various dye-dendrimer

ratios by MALDI-TOF MS provides important new proof that the isolation protocols presented here, and previously published [7], is successful in providing materials with a single dye-dendrimer starting from commercially available, divergently synthesized dendrimer material.

^1H NMR spectroscopy measures mean dye/dendrimer ratios and is routinely used to confirm stochastic averages for conjugation reactions [4,5,7,15,26] as well as to confirm the mean number of ligand/dendrimer present for sample fractions isolated using rp-HPLC [4,7,15]. The accuracy of this approach is limited by the dispersion of dendrimer MW, which generates a variation in number of protons present on the dendrimer scaffold. In order to provide the NMR estimate of the number of TAMRA/dendrimer, the number of terminal protons had to be determined for G3d and G3m by titration of each scaffold. Titration indicated a mean of 30 and 25-NH₂ end groups for G3d and G3m, respectively, out of the 32 theoretically possible for G3 PAMAM. Using approaches previously developed to quantify the number of non-exchangeable internal protons present for G5 PAMAM [5], it was determined that the G3d and G3m material contained means of 466 and 382 such protons, respectively. These values were employed to determine the mean TAMRA-G3d and TAMRA-G3m ratios from ^1H NMR spectra. Integration of the TAMRA peaks present in the $\delta = 6.5\text{--}8.5$ ppm region (9 protons) and the G3 PAMAM peaks from $\delta = 2.0\text{--}3.7$ ppm (466 or 382 protons) was used to determine the mean values of G3d-TAMRA_{2.2(avg)} and G3m-TAMRA_{3.8(avg)} for the as-prepared stochastic mixtures.

Assuming the 25-NH₂ end groups for G3m arise from the presence of missing arm defects [23], and using the measured MW of 6850 Da for G3m from MALDI-TOF-MS and NMR integration value, a MW of 8423 Da is predicted for G3m-NH₂-TAMRA_{3.8(avg)}. The measured MALDI-TOF MS value is 9184 Da, which is within 8% error of the calculated estimate. A similar analysis for G3d-NH₂-TAMRA_{2.2(avg)} gives a predicted MW of 7780 Da, which is within 9% of the measured mass of 8520 Da. Analysis of NMR integrations also provided the mean number of TAMRA for each isolated fraction and gave ratios of 0.8, 1.9, 2.0, 2.9, and 6.0 for G3m-NH₂-TAMRA_n ($n = 1, 2a, 2b, 3$, and $4+$), respectively. (Fig. S3, Table S2). A similar analysis of the ^1H NMR integrations for G3d-NH₂-TAMRA_n ($n = 1\text{--}4+$) gave ratios of 0.8, 2.4, 3.3, 4.6, respectively. The accuracy of this analysis is limited because each fraction does not contain the full MW distribution of the G3m (Scheme 1b) or G3d material. Instead, a subset of the full distribution was obtained for each fraction as illustrated in Schemes 2b & 2d, and thus the assignment of a mean of 466 (G3d) or 382 (G3m) internal protons for the calculation of the ratio is approximate in each case.

The increased number of TAMRA per dendrimer will result in an increase of molecular weight of 414 Da per dye conjugated. The molecular weight of each G3m-NH₂-TAMRA_n isolated fraction (Scheme 2d) ($n = 1, 2a, 2b, 3$, and $4+$) was measured using MALDI-TOF-MS. The average masses were 7190, 7620, 7650, and 7980 Da for $n = 1, 2a, 2b$, and 3 , respectively (Fig. 2). The observed peak-to-peak mass shifts observed for $n = 4+$ and for G3m-NH₂-TAMRA_{3.8(avg)} are roughly 420 and 450 Da, respectively in reasonable agreement with varying amount of TAMRA present for these two materials. Similar good agreement of 434 and 420 Da was observed for the peak to peak-to-peak mass shifts for $n = 4+$ and for G3d-NH₂-TAMRA_{2.2(avg)} (Fig. S1). Using the MALDI-TOF-MS molecular

weight difference between the G3m-TAMRA_n ($n = 1-3$) samples and the G3m-PAMAM, it can be estimated that the TAMRA/dendrimer ratios corresponding to the $n = 1, 2a, 2b$, and 3 samples are 0.8, 1.9, 1.9, and 2.7, respectively. A similar analysis for the G3d-TAMRA_n ($n = 1-3$) samples gives TAMRA/dendrimer ratios corresponding to 0.7, 1.8, and 2.8, respectively. The use of MALDI-TOF-MS to assign these ratios suffers from the same fundamental problem as the NMR analysis. Assigning the difference of the G3m-TAMRA_n and G3m mass as resulting from a mass change due to TAMRA conjugation is approximate because only a fraction of the G3m molecular weight distribution is obtained during isolation G3m-TAMRA_n. This means that an indeterminate convolution of the change in G3m mass distribution is present in the MALDI-TOF determined ratios. UPLC does not have the weakness of sampling only a fraction of the peak width distribution when assigning the TAMRA/dendrimer ratio. In this case, the full MW distribution, albeit a different one than the G3m starting material, is included in the analysis. For these reasons, we believe UPLC is the most accurate method for determining the TAMRA/dendrimer ratio for these materials.

Studies were also carried out to examine the impact of the dye to polymer ratio on the absorption and emission spectra. The absorption spectra of G3m-NH₂-TAMRA_n ($n = 1, 2a, 2b, 3, 4+$) show an increase in intensity at 520 nm and 560 nm (Fig. 3b). Although these samples are dilute in terms of polymer concentration ($\sim 10^{-5}$ M), the extinction coefficient does not show a linear relationship between absorption value and concentration as the conjugated dyes are held in close proximity to each other and do not follow Beer's Law [7,27,28]. The absorption spectrum for G3-TAMRA₂₋₄₊ shows the classic pattern intensity observed for J-aggregate TAMRA dimers, [27,28] despite being at a total concentration of dye in solution that is expected to give a monomer absorption profile (1.4×10^{-5} M). The effective "local dye concentration" for the TAMRA dye held in close proximity on the dendrimer is about 0.3 M for G3-TAMRA₂. The observation of the J-aggregate absorption pattern provides independent confirmation that two or more dye molecules are present on the dendrimer. We also note that the absorption patterns observed for G3m-NH₂-TAMRA₃ and G3-NH₂-TAMRA₄₊ samples are not simple additions of monomer and dimer spectra and that more complicated absorption processes result when three or more TAMRA are held in close proximity (Fig. 3c and d). A similar set of data was obtained for G3d-NH₂-TAMRA_n ($n = 1, 2a, 2b, 3, 4+$) and is illustrated in Fig. S4.

The emission spectra centered at 580 nm decrease in intensity with concentration, exhibiting the largest degree of quenching as the ratio of dye/polymer increases from 1 to 2 (Fig. 3a). These data indicate that for a stochastic sample the optimal mean value of n for TAMRA dye on G3 PAMAM to obtain highest degree of average fluorescence is about 1.2 (Fig. S5). This value is obtained by considering the weighted average of emission for all G3 components of a stochastic distribution including $n = 0$.

4. Conclusions

In summary, commercially available G3 PAMAM dendrimer was purified by dialysis to remove trailing generations (G3d) and by semi-preparative rp-HPLC to remove trailing generations and oligomers (G3m). The direct conjugation of TAMRA to G3d and G3m

resulted in a stochastic mixture that was separated into precise ratios of dye to dendrimer yielding G3-NH₂-TAMRA_n conjugates ($n = 1-3$). With these materials, we were able to measure the relative absorption and emission properties and directly relate these properties to the number of dye per polymer particle. Absorption increased with increasing n and emission decreased with increasing n . The absorption spectra also showed distinctive TAMRA dimer behavior for dye/dendrimer ratios greater than one. ¹H NMR spectroscopy and MALDI-TOF-MS provided valuable information for characterization of TAMRA to dendrimer ratios but were noted to suffer from the need to approximate key values employed in the ratio determination. Therefore, rp-UPLC was determined to be the most reliable for determining dye/dendrimer ratio. Published work exploring the aqueous fluorescence lifetime and cellular uptake and fluorescence lifetime of related G5 PAMAM materials [29] suggests all of the individual G3 species isolated will have unique biological properties and are thus of use as markers to explore cell sorting processes and as models for understanding the biological properties of conjugated polymers.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.mtcomm.2015.06.006>

Abbreviations

PAMAM	polyamidoaminodendrimer
TAMRA	5-carboxytetra methylrhodamine

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Fig. 1.

a) a stacked plot of MALDI-TOF-MS spectra for G3 PAMAM material fractions: as-received (black), trailing generation G2 (purple), G3m (red), and G3–G3 dimer (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

a) a stacked plot of MALDI-TOF-MS spectra for G3 PAMAM material fractions: as-received (black), trailing generation G2 (purple), G3m (red), and G3–G3 dimer (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

a) Emission and b) absorption spectra of G3m-TAMRA_n-NH₂ ($n = 1, 2a, 2b, 3, 4+$) conjugates at 1×10^{-5} M; 1 TAMRA (red), 2a (orange), 2b (green), 3 (blue), 4+ (purple), and average (black). c) Absorbance of TAMRA $n = 1-3$ at a concentration of 3×10^{-5} M. d) Absorbance of $n = 3$ (blue) and an equimolar mixture of $n = 1$ and $n = 2$ (brown). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Scheme 1.

Isolation of as-received commercial G3-NH₂ PAMAM by semi-preparative rp-HPLC. a) Semi-preparative rp-HPLC trace with isolated fractions indicated by colored bars. b) Analytical UPLC traces for each isolated dendrimer fraction: as-received (black), trailing generation G2 (purple), G3 monomer (red), and G3-G3 dimer (blue). Each trace is baseline corrected and normalized). (For interpretation of the references to color in this Scheme legend, the reader is referred to the web version of this article.)

Scheme 2.

Isolation of dendrimer conjugates G3-TAMRA_n-NH₂ (n = 1, 2a, 2b, 3, 4+) by semi-preparative rp-HPLC. a) Stochastic conjugation to form G3-TAMRA_n-NH₂. b) Semi-preparative rp-HPLC trace for G3d-TAMRA_{2.2}(avg) material with isolated bands identified. The relative content of each population of G3-TAMRA_n-NH₂ (n = 0–4+) is 5.8, 10.0, 16.8, 16.1, 51.2 %, respectively. c) Analytical rp-UPLC traces for G3d isolated fractions. d) Semi-preparative rp-HPLC trace for G3m-TAMRA_{3.8}(avg) material with isolated bands identified. The relative content of each population of G3-TAMRA_n-NH₂ (n = 0–4+) is 1.6, 3.8, 8.5, 10.7, 75.4% respectively. e) Analytical rp-UPLC traces for G3m isolated fractions. Analytical rp-UPLC traces are baseline corrected and normalized.