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Best practices for purification and characterization of PAMAM dendrimer

Douglas G. Mullen[†,‡], **Ankur Desai**[‡], **Mallory A. van Dongen**[‡,§], **Mark Barash**[‡,§], **James R. Baker Jr.**[‡], and **Mark M. Banaszak Holl**[†,‡,§,*]

[†]Program in Macromolecular Science and Engineering, University of Michigan, Ann Arbor, MI 48109

[‡]Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan, Ann Arbor, MI 48109

[§]Department of Chemistry, University of Michigan, Ann Arbor, MI 48109

Abstract

Poly(amidoamine) (PAMAM) dendrimer are branched polymers with low degrees of heterogeneity. Current synthesis methods, however, result in substantial batch variability. We present our optimized procedure for post-synthesis (and post-market) purification of a generation 5 PAMAM dendrimer by membrane dialysis and demonstrate its effectiveness and limitations using a representative lot of biomedical grade dendrimer. This method successfully removes trailing generation defect structures, thereby reducing the heterogeneity of the material (PDI reduced from 1.04 to 1.02). Optimized analytical techniques to characterize the unpurified and purified dendrimer are also detailed. The efficiency of the purification method is successfully monitored by these analytics and dendrimer parameters that are critical for subsequent modification reactions and biological evaluation (M_n, M_w, PDI, average number of end groups) obtained. To provide better definition of the variability that should be expected between lots of synthesized material, HPLC traces for three additional commercial lots of dendrimer are also presented.

Introduction

First developed in the 1970's, dendrimers are a unique class of branched polymers with characteristically low polydispersity making these materials attractive for a number of different applications. In particular, poly(amido amine) dendrimers have received intense attention as candidates for a variety of biomedical applications including targeted drug delivery,[1–4] diagnostic imaging,[5] and gene delivery.[6] In addition to being relatively monodisperse (PDI = 1.01 for some lots of generation 5 (G5) dendrimer), PAMAM dendrimers are an ideal size to interact with biological processes (1 – 10nm),[7] are structurally well-defined, possess a hydrophilic back-bone, and have numerous modification sites to which different functional ligands can be attached for synergistic effects.[8] Although PAMAM dendrimers are very uniform as compared to typical polymers or nanoparticles,[9] the material is not a single molecular species and perhaps more importantly, substantial variations can exist between synthetic lots. It is important for researchers working with these materials to appreciate these aspects of this polymer class. Furthermore, in order to

*Corresponding author: Mark M. Banaszak Holl, mbanasza@umich.edu, 930 N. University Avenue, Ann Arbor, MI 48109-1055. TEL: 734-763-2283, FAX: 734-763-2283.

effectively prepare PAMAM dendrimer for use in many of the referenced applications, it is necessary to carefully purify and characterize each individual lot of material.

The structural heterogeneity in PAMAM dendrimers results from undesired side-reactions that occur during synthesis. These structural defects have been described by several groups and include missing arms, dimers, and trailing generations. [10–13] Important parameters including the mean number of terminal arms, the number average molecular weight and PDI of the polymer are directly affected by these defect structures. The relative amount of each defect structure is also highly sensitive to the reaction conditions, resulting in significant variations in the material composition of different batches. Consequently, accurate characterization of these parameters for each batch of dendrimer is essential to achieve reproducibility in subsequent ligand conjugations, as well as to understand the biological activity of the material. Finally, by removing trailing generation defect structures through purification, the variations between batches of dendrimer for these parameters can be reduced.

This manuscript describes our ‘best practices’ for purifying and characterizing G5 PAMAM dendrimer, and provides our most current understanding of the material composition and batch variations for commercially sourced material. This report focuses on G5 PAMAM dendrimer because it provides an excellent combination of sufficiently-sized hydrophilic polymer backbone to support a mean of about ten hydrophobic dyes, drugs, or targeting agents, while still being small enough to effective transport through biological tissues. Biomedical Grade Generation 5 PAMAM dendrimer, a top commercial grade of dendrimer, was purchased from Dendritech Inc. and then purified by membrane dialysis to remove a portion of the trailing generation defect structures. The purified and un-purified material was then characterized by GPC, potentiometric titration, and reverse-phase HPLC. This analysis provided the number average and weight average molecular weight, PDI, and the mean number of primary amines per dendrimer. Purification by dialysis was found to reduce the heterogeneity of the material and increase the number average molecular weight of the polymer. HPLC and GPC characterization of two additional lots of Biomedical Grade G5 PAMAM dendrimer purchased from Dendritech Inc. and one lot of G5 PAMAM dendrimer synthesized by Dendritech Inc. but purchased from Sigma-Aldrich is also provided. This second analysis shows the degree of heterogeneity and lot variability that should be expected from commercially sourced G5 PAMAM dendrimer.

Experimental Methods

Materials

Biomedical Grade Generation 5 (G5) PAMAM dendrimer Lot # 0905-18-E5.0-PB (**Lot A**), 0205-06-E5.0-LD (**Lot C**), and 1006-13-E5.0-PB (**Lot D**) was purchased from Dendritech Inc. Generation 5 PAMAM dendrimer #536709-5G (**Lot B**) was also manufactured by Dendritech Inc. but was purchased through Sigma-Aldrich Co. Water was purified using a Barnstead Nanopure Infinity system. 10,000 molecular weight cut-off dialysis tubing (Spectra/Por 7 Dialysis membrane, nominal flat width: 45 mm, diameter: 29 mm) was purchased from Spectrum Laboratories Inc. and washed with Nanopure water before use. Deuterium oxide for NMR characterization 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.

Dendrimer Purification

Methanol was removed from a solution of **Lot A** Biomedical Grade G5 Dendrimer under reduced pressure to produce a viscous oil. The dendrimer was redissolved in water and then

lyophilized to obtain a dry weight (5.1310 g). The dry dendrimer was dissolved with 80 mL of DI water and divided into 4 aliquots (**Aliquots 1, 2, 3**, and **4**). Four 10,000 MWCO dialysis membranes were prepared with a length of approximately 14 inches. Weighted clips were attached to one end of each dialysis tube and 20 mL of the dendrimer/water solution were added to each dialysis membrane tube. Residual air was removed from each tube and the tubes were sealed with floating clips, attached so that the length of each dialysis tube was approximately 7 inches. A foam float was affixed to each of the floating clips and the dialysis tubes and placed into glass jars filled with one gallon of Nanopure water each. Slow stirring was utilized. Nanopure water was exchanged 9 times over 3 days. The minimum time between exchanges was 3 hrs. After purification, the dendrimer was removed from the dialysis tubes and placed in glass scintillation vials. The purified dendrimer (**Purified Aliquots 1, 2, 3** and **4**) was lyophilized for 3 days to yield 5.0238 g (98%) of a glassy material. The dried product was stored under nitrogen at −25 °C.

GPC

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 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 counter ion to make the dendrimer surfaces hydrophobic. Dendrimer samples were prepared at approximately 1mg/mL in water with 0.14 wt% TFA and an injection volume of 35 μL was used. A 35 μL injection of water with 0.14 wt% TFA was also run to obtain baseline signals. Dendrimer traces at 210 nm were processed by subtracting the baseline trace at 210nm and normalizing to the peak maximum.

Potentiometric Titration

Potentiometric titration was carried out using a Mettler Toledo MP220 pH meter and a Mettler Toledo InLab 430 pH electrode at room temperature, 23 °C. A 20 mL solution of 0.1

N NaCl was added to Purified Aliquot 1 Dendrimer (80.7 mg) to shield amine group interactions.[2] The pH of the dendrimer solution was lowered to pH = 2.01 using 0.1034 N HCl. A 25 mL Brand Digital Buret III was used for the titration with 0.1024 N NaOH. NaOH was titrated into the dendrimer solution in 0.06 mL increments (2 drops). A high rate of stirring was maintained throughout the titration. The numbers of primary and tertiary amines were determined by from the titration curve with NaOH as previously described.[2] This process was also conducted for the Unpurified Dendrimer Lot A and the Upurified Dendrimer Lot B.

Results and Discussion

The purification of PAMAM dendrimer has been an important step in our laboratory to prepare material for non-viral gene delivery mechanistic studies,[14,15] polycation-cellular membrane interaction studies[15–19] and for modification with functional ligands such as a targeting moieties, therapeutic molecules and/or imaging agents.[3,20–22] By removing many of the trailing generation defect structures, the purification process improves the uniformity of the material. This is important because each batch of dendrimer has a varying amount of trailing generation defect structures, which can affect the observed material properties. In particular, the particle pharmacodynamics and biodistribution are expected to vary as a function of size for the defect populations.[23,24] The different water solubilizing capabilities of the defect structures are also likely to influence biodistribution.[25] Reducing the variability in the relative amount of each defect population will improve the consistency of the bulk pharmacodynamics and biodistribution properties. It is important to note that this method does not remove the highest molecular weight trailing generation (Mn estimated to be 14,200 g/mol) or the dimer defect structure with a Mn estimated to be 58,000 g/mol. After each purification has been completed it is critical to conduct a full characterization of the material.

Due to the scale of material purified in this manuscript, purification was conducted using four dialysis membranes. Approximately 1.28g of dendrimer was placed in each dialysis membrane. HPLC and GPC characterization of each aliquot was conducted independently so as to detect any variations that might have from the use of multiple membranes.

GPC molecular weight analysis

GPC was used to characterize the dendrimer both before and after purification. Figure 1 shows the GPC data from the Light Scattering and Differential Refractive Index detectors for the Unpurified Lot A dendrimer sample as well as one of the four purified samples. A comparison of **panels a** and **b** reveals that the dialysis process reduced the amount of smaller sized dendrimer particles that elute at 22.5 minutes. This reduction is consistent with the increase in the weight and number average molecular weight for the purified dendrimer samples. Table 1 contains the number average molecular weight, the weight average molecular weight and the PDI for the different dendrimer samples. The dialysis process increased M_n of the material by approximately 3,000 g/mol and reduced the PDI from 1.043 to an average of 1.018. Previous studies have found that the error of the M_n measurement to be ± 1,000 g/mol.[22] Consequently, by this analytical technique, the purification process had the same efficiency for all four dendrimer aliquots.

HPLC resolves major dendrimer defect structures

Reverse phase HPLC has been previously developed for analysis of PAMAM dendrimer.[26,27] HPLC traces at 210 nm for the four purified dendrimer aliquots and the Unpurified Lot A dendrimer can be seen in Figure 2. In addition to the major peak in each trace at 19.1 min, several smaller peaks can be seen. These peaks have been previously

shown to be composed of trailing generation defect structures and the dimer defect structure.[26] In the trace for the Unpurified Lot A dendrimer, two additional peaks can be found with retention times at 16.9 min and 17.8 min. It is these dendrimer defect structures that are successfully removed by dialysis and result in the recorded increase in Mn from 23,100 ± 1,000 to 26,200 ± 1,000 (the average of the four purified aliquots). Presumably these defects are the trailing generation with same size and molecular weight of a generation 2 (Mn ~ 3,300 g/mol) and 3 (Mn ~ 6,900 g/mol) dendrimer, respectively. Purification by membrane dialysis, however, does not remove the dendrimer defect strctures at 18.5 min (G4-sized defect structures) and the dimer structures at 19.3 min. Finally, by this HPLC analysis, excellent consistency is found between the four aliquots of purified material indicating a consistent performance of the dialysis membranes.

Mean number of end groups per dendrimer

Accurate determination of the mean number of end groups (primary amines) per dendrimer is critical in order to calculate reaction stoichiometries as well as determine the mean number of functional ligands that become conjugated to the dendrimer. Theoretically, a G5 dendrimer has 128 primary amines. Due to the numerous defect structures, in practice this value is significantly lower. The use of potentiometric titration to calculate the mean number of primary amines per dendrimer has been described earlier (Figure 3).[2] Although we continue to use the same calculation (# end groups = mole NaOH consumed / mole dendrimer), the titration procedure has been modified. Instead of using a glass burette, we have increased the precision of the titration by using a Digital Burette to add the 0.1024 N NaOH in 0.06 mL increments. The mean number of end groups for the Purified Aliquot 1 grade dendrimer was found to be 109 ± 5, which is an increase in the mean number of end groups per dendrimer from the Unpurified Lot A dendrimer (96 ± 5). Interestingly the mean number of end groups for the Unpurified Lot B (Sigma-Aldrich) dendrimer was 116 ± 5. Although this material has the highest mean number of end groups and closest to the theoretical maximum of 128, it is incorrect to conclude that the Lot B material is the most uniform material. Rather, the high mean number of end groups for the Sigma-Aldrich dendrimer is a consequence of the large amount of dimer that was observed in both the GPC and HPLC characterization.

Dendrimer Variability in Commercial Lots

In an effort to provide better definition of the variability that exists between lots of commercially sourced dendrimer, HPLC and GPC characterization was performed on Lots B–D. This analysis is important because the true range of material characteristics for commercially-sourced dendrimer has received little attention. GPC results can be found in Table 1 and HPLC results are shown in Figure 4 along with Unpurified Lot A and Aliquot 4. As reflected in the PDI, the degree of heterogeneity for Lots B–C (PDI = 1.062, 1.052 and 1.082, respectively) is greater compared to both Lot A (PDI = 1.043) and the purified Aliquots 1–4 (PDI = 1.018). In addition to the differences in the PDI values, the HPLC traces in Figure 4 show clear differences in the relative amount of the defect structures in each lot. A peak fitting analysis was used to quantify the amount of each defect class (G2, G3, G4 and G5-dimer) (Table 2). In general, the lots ranged from 80 – 90% G5 dendrimer topology with G5 dimer as the largest impurity present (5 – 13%) followed by decreasing amounts of G4, G3, and G2 trailing generations.

Conclusion

PAMAM dendrimers have excellent uniformity compared to most polymers and other nanoparticles as purchased or typically synthesized. Additional post-market purification and characterization of generation 5 poly(amidoamine) dendrimer, however, remains important

for those laboratories interested in applying these materials for biomedical applications and achieving consistent material properties; particularly pharmacodynamics and biodistribution properties . In this instance, minimization and understanding of the variations that exist between commercial batches, and removal of the trailing generations, is important. This report has described our current 'best practices' for purification of G5 PAMAM dendrimer by dialysis followed by characterization by GPC, potentiometric titration, and HPLC. This analysis serves to clarify that although dendrimers are often described as a polymer with excellent PDI (as low as 1.01), this is only attained after extensive purification and commercial material can have substantial variability in defect concentration.

Acknowledgments

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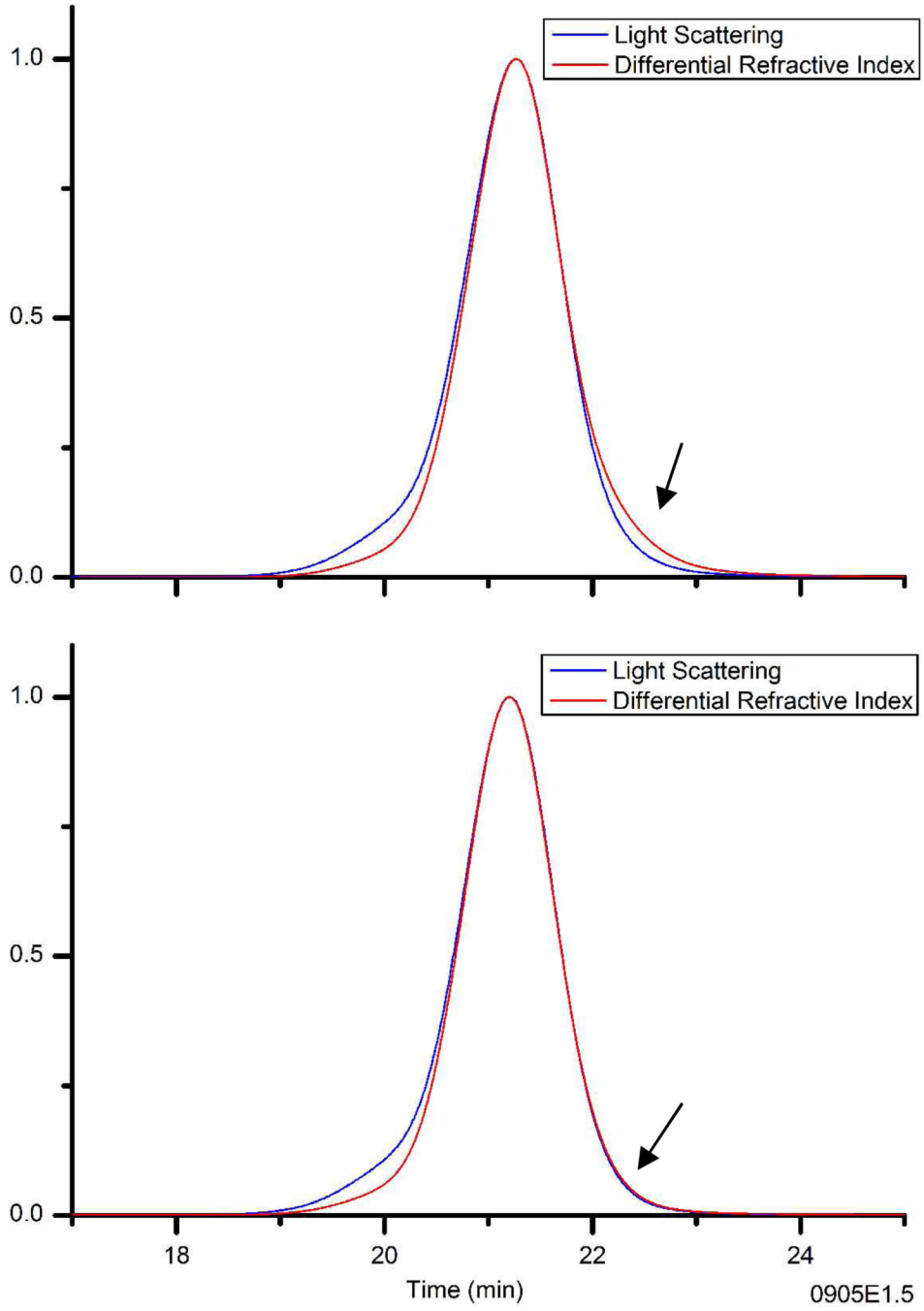

Figure 1.

Characterization of PAMAM dendrimer using a GPC based Light Scattering Detector and a Differential Refractive Index Detector. **a)** Unpurified Lot A Dendrimer. **b**) Purified Dendrimer (Aliquot 1). The purification method reduces the amount of smaller dendrimer particles (trailing generation defects) that elute at 22.5 minutes.

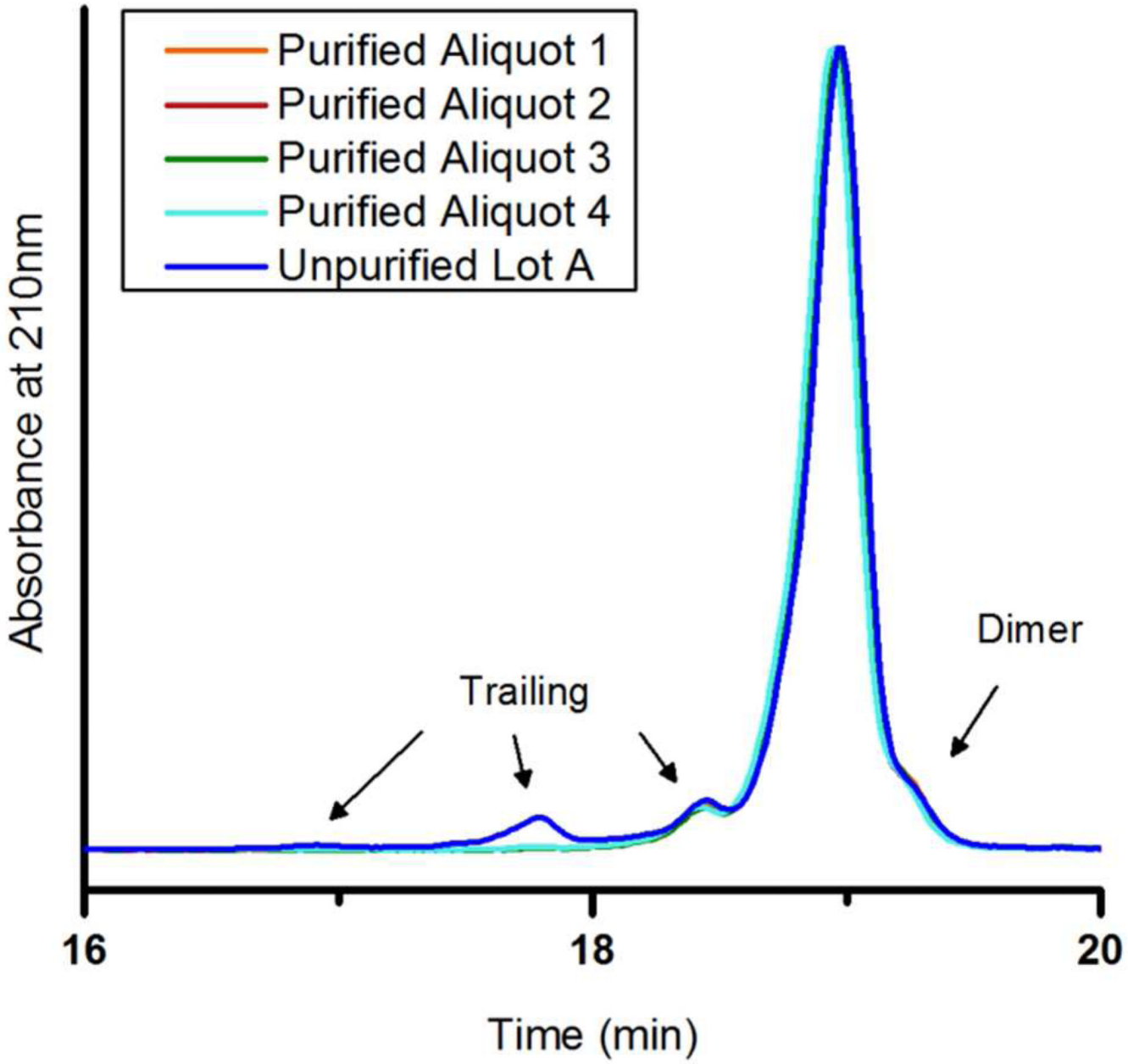

Figure 2.
Normalized HPLC traces at 210nm for Unpurified Lot A dendrimer and Purified Dendrimer Aliquots 1, 2, 3 and 4. Note that Purified Aliquots 1–4 have overlapping traces. Purification by membrane dialysis successfully removes the trailing generation defect structures with elution times of 16.9 min and 17.8 min but does not remove the trailing generation defect structures at 18.5 min or the dimer defects at 19.3min.

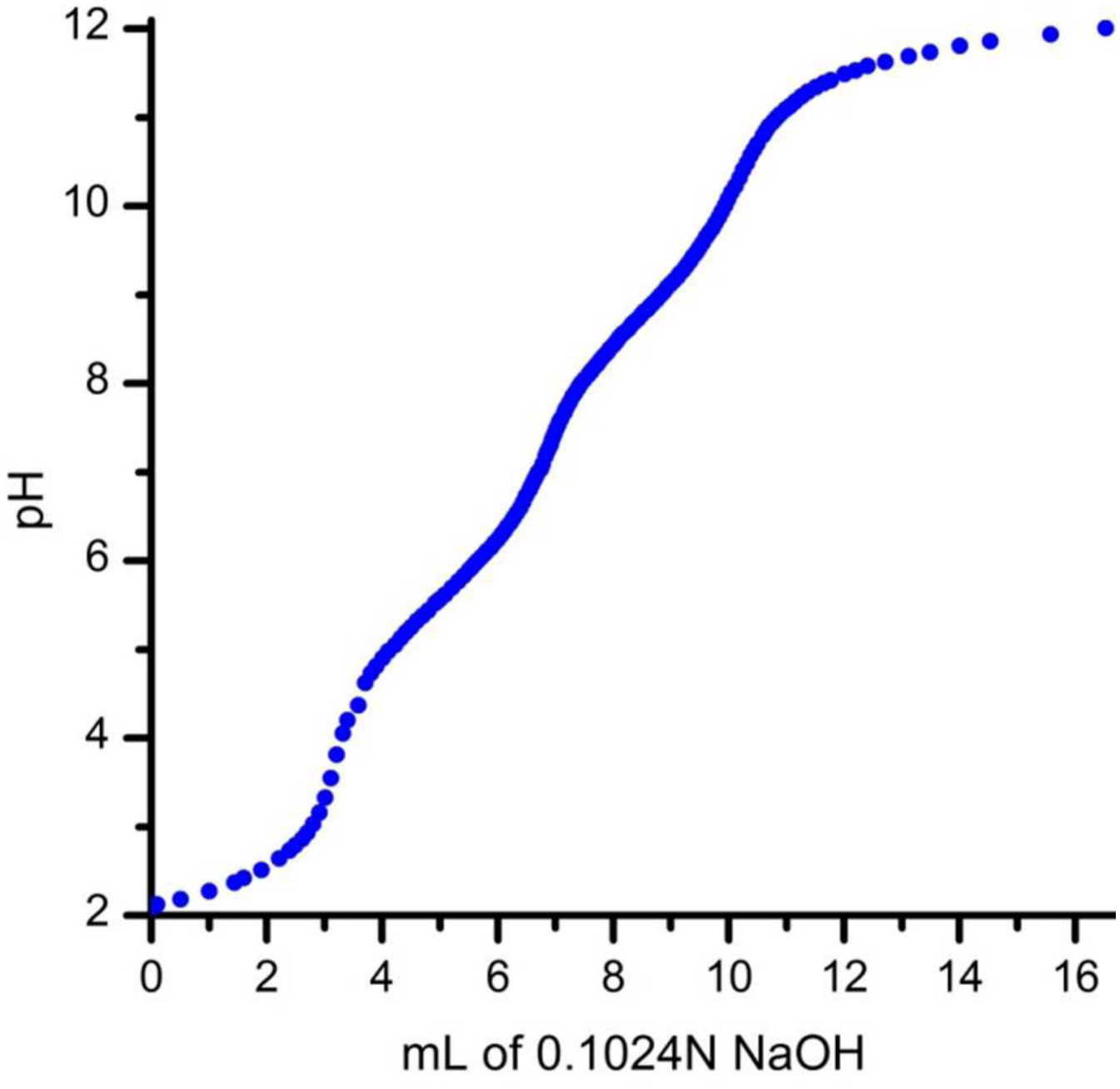

Figure 3.
Potentiometric titration of the Purified Dendrimer (Aliquot 1).

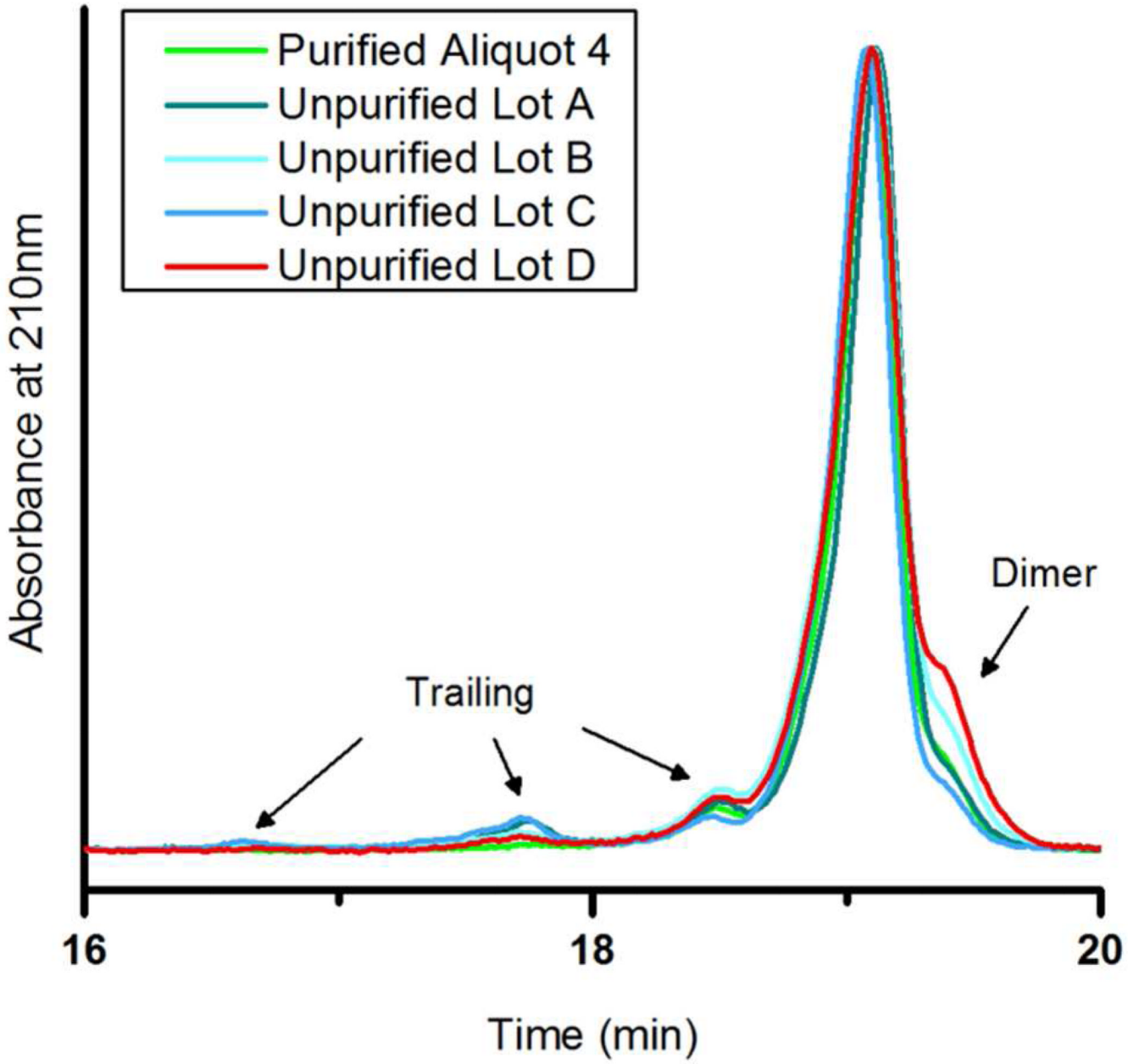

Figure 4.
Normalized HPLC traces at 210nm for Unpurified Dendrimer Lots A, B, C and D as well as Purified Dendrimer Aliquot 4.

Table 1

Molecular weight and end group analysis of Unpurified Dendrimer Lots A–D and Purified Dendrimer Aliquots 1–4

Dendrimer	Mn	Mw	PDI	# of End Groups
Unpurified Lot A	23,100 ± 1,000	24,100 ± 1,000	1.043	96 ± 5
Purified Aliquot 1	27,000 ± 1,000	27,500 ± 1,000	1.017	109 ± 5
Purified Aliquot 2	25,500 ± 1,000	25,900 ± 1,000	1.017	
Purified Aliquot 3	26,700 ± 1,000	27,200 ± 1,000	1.018	
Purified Aliquot 4	25,600 ± 1,000	26,100 ± 1,000	1.019	
Unpurified Lot B	24,900 ± 1,000	26,500 ± 1,000	1.062	116 ± 5
Unpurified Lot C	24,500 ± 1,000	25,800 ± 1,000	1.052	
Unpurified Lot D	27,900 ± 1,000	30,300 ± 1,000	1.085	

Table 2

Percentage of Generational Defect Structures Present in Purified and As-Received G5 PAMAM Dendrimer.

	Purified Aliquot 4	Unpurified Lot A	Unpurified Lot B	Unpurified Lot C	Unpurified Lot D
G2	0	0	<1	<1	0
G3	0	3	1	3	1
G4	4	4	5	3	5
G5	89	88	85	90	81
G5-dimer	7	5	9	5	13