Analytical Methods

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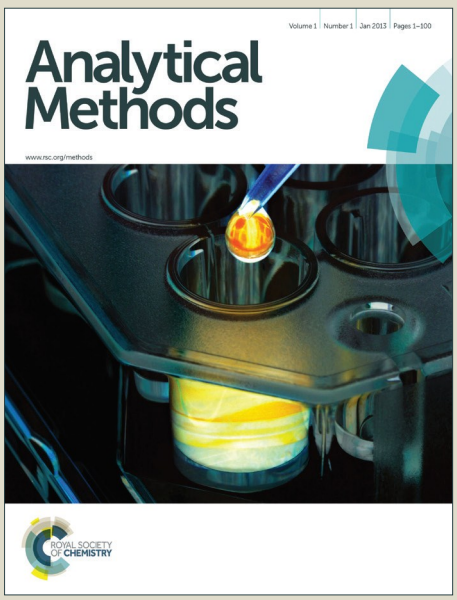

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Analytical Methods

Characterization of Polyamidoamino (PAMAM) Dendrimers Using In-Line Reversed Phase LC Electrospray Ionization Mass Spectrometry

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Generation 3 (G3) PAMAM dendrimers are symmetrical, highly branched polymers widely reported in the scientific literature as therapeutic agents themselves or as carrier scaffolds for various therapeutic agents. A large number of analytical techniques have been applied to study PAMAM dendrimers, but one that has been missing is in-line reversed phase LC electrospray ionization mass spectrometry (RP/LC/ESI/MS). To translate PAMAM dendrimers into therapeutic agents, a better understanding of their purity, stability and structure is required, and in-line RP/LC/ESI/MS is widely applied to all three of these analytical questions. In this study, we developed a robust in-line RP/LC/ESI/MS method for assessing stability, purity and structure of the G3 PAMAM dendrimers, and we also examined the reasons why previous attempts at method development failed. Using the RP/LC/ESI/MS method we uncovered several unique aspects of the chemistry of G3 PAMAM dendrimers. They are interconverted between two isomeric forms by dialysis, and under higher concentration levels there is an inter-molecular displacement reaction resulting, which degrades PAMAM dendrimers. Purification of G3 dendrimers by RP/LC was also previously unreported; so we slightly modified the LC/MS method for isolating individual components from a complex dendrimer mixture. Thus, we have developed a robust, comprehensive method for characterizing PAMAM dendrimers and their degradation.

Introduction

Dendrimers are symmetrical, highly branched polymers where the terminal positions can be functionalized with a wide variety of different chemical structures. Therapeutic and experimental drugs[1-4], dyes[5, 6], solubilizing chains[7] or a mixture of all three[8] can subsequently be added to these functional groups creating powerful multi-functional biological agents. There exist a large number of scientific reports regarding the therapeutic potential of dendrimers and especially polyamidoamino (PAMAM) dendrimers[9-12]. Also the dendrimers themselves are reported to possess therapeutic properties, but more often the PAMAM dendrimer has a therapeutic drug attached to the terminal group(s)[13].

Many analytical chemistry tools have been used to evaluate dendrimers, but by far the most common technique is 1H NMR[14-17]. Direct analysis of PAMAM dendrimers by MALDI[6, 18-22] and ESI[2, 23] has also been reported. While these techniques are useful they are not sufficient for evaluating the purity, stability and detecting individual components of complex dendrimer mixtures. One analytical technique missing from the dendrimer literature is in-line reversed phase/liquid chromatography/electrospray/mass spectrometry (RP/LC/ESI/MS), which is commonly used to evaluate the complex mixtures generated during studies of stability and purity. Here we report our development of a robust in-line RP/LC/ESI/MS method for the study of generation 3/generation 2.5 (G3/G2.5) PAMAM dendrimers. We employed this technique to examine the quality, stability and structures of commercially available dendrimers. In the course of this investigation, we were able to unravel some of the more arcane aspects of PAMAM dendrimer chemistry.

Experimental

Materials

All G3 and G2.5 PAMAM dendrimers (ethylenediamine core) were obtained from Dendritech (Midland, MI, USA) as solutions. All LC solvents and additives, water (H_2O), methanol (MeOH), acetonitrile (ACN), formic (FA), trifluoroacetic acid (TFA), 25% ammonium hydroxide were LC/MS quality and were purchased from Sigma-Aldrich (St. Louis, MO, USA). The glass-lined stainless steel PLRP-S HPLC column 2.1 mm ID 150 mm long and a pore size of 300Å with titanium frits was purchased from Michrom (Fremont, CA, USA). The monolithic fritless PLRP-S HPLC column 1 mm ID 250 mm long was purchased from Dionex (Sunnyvale, CA, USA). The semi-preparative PLRP-S HPLC column 7 mm ID 250 mm long with steel frits was purchased from Agilent Technologies (Santa Clara, CA, USA). The HPLC was a Waters (Milford, MA, USA) 1525u binary pump with an entirely PEEK/biocompatible injector and sample loop. The mass spectrometer was a Waters LCT Premier time of flight (ToF) unit equipped with the Z-spray ESI ion source. Data was acquired and analyzed using MassLynx 4.1 software and deconvolution was performed with MaxEnt 1.
Fraction collection was performed using an Agilent 1100 LC system with UV detection (214 nm) and a refrigerated fraction collector.
Amicon spin dialysis 3K MWCO regenerated cellulose tubes were purchased from EMD Millipore (Billerica, MA, USA

In-Line RP/LC/ESI/MS Analysis

All injections onto the analytical HPLC columns were diluted 1:1000 with solution A (1% ACN in water with 0.2% FA and 0.1% TFA), and 10 μL was injected. Solution B was 20% ACN in methanol with 0.2% FA and 0.1% TFA[24]. The LC analytical flow rate was 200 μL/min. The analytical LC gradient was initiated at 100% A and increased to 100% B in 12 min.
The MS was operated in positive ion V-mode (resolution = 5000). The deconvoluted data is displayed as molecular weights in daltons.
For the semi-preparative HPLC isolation of an impurity in G2.5 terminal carboxy PAMAM dendrimer 100 μL of undiluted sample was injected. The flow rate used was 1 mL/min. Solution A was 1% ACN in water and solution B was 20% ACN in methanol. Both solutions A and B contained 0.2% FA and 0.1% TFA. The gradient was monitored by off-line ESI/MS and the samples were collected using a cooled fraction collector. The pH of the solution in the collection vials was immediately increased from pH = 1.6 to 7 with approximately 50 μL of 25% ammonium hydroxide. The HPLC isolated materials were not stable over time at low pH. The stability of the commercial PAMAM dendrimers was evaluated by storing the 10% solutions in water at the vendor's suggested temperature of 4°C. The PAMAM dendrimers purchased as 10% solutions in methanol were also stored at 4°C.

The freeze-thaw experiments were conducted on the dendrimers (10% w/w in water) by freezing a 1 mL of sample at -20°C and waiting 30 min. The frozen sample was removed from the freezer and placed on the bench top for 20 min at which time the sample was a solution again. This was repeated for 2 more

cycles. It should be noted that after the second freeze-thaw the sample did not freeze again suggesting that the sample had dramatically degraded.

Dialysis of the 10% w/w in water G3/G2.5 PAMAM dendrimers was performed by first diluting the sample 1:1000 in LC/MS grade water. Then, 100 μL of this dilute solution was dialyzed according to the manufacturer's suggested procedure. 10 μL of the 10x concentrated dialyzed sample was injected into the analytical LC/MS system for analysis.

RP/LC Isolation of a Single Dendrimer Component by Fraction Collection

Semi-preparative isolation of a single component of a complex G2.5 terminal carboxy PAMAM dendrimer mixture was performed using a 7 mm ID PLRP-S column with a flow rate of 1 mL/min. The injection volume was 100 μL of a 10% solution in methanol. The solvents and gradient were otherwise identical to the analytical method described above. Fractions were collected at 1 min intervals. The elution profile was monitored with UV detection (214 nm). After collection of the appropriate fraction the pH of the isolated solution (pH = 1.6) was adjusted to pH =7 by adding approximately 50 μL of 25% ammonium hydroxide. Adjustment of the pH was necessary to prevent sample degradation.

RESULTS

The in-line RP/LC/ESI/MS method we employed was developed using an iterative process where as we conducted new experiments on different dendrimer samples we observed new LC/MS peaks appearing in the chromatogram. We then adjusted the operating parameters to optimize the detection of these new peaks while retaining the detection of previously observed LC/MS peaks. This stepwise refinement describes how the robust method reported here was developed.

The first dendrimer we examined using our in-line RP/LC/ESI/MS was the commercially available G3 terminal amino PAMAM (dendrimer A) in the version supplied as 10% in water. The analysis shown in Figure 1 was conducted immediately upon receipt of the sample so there was no storage time involved. The LC/MS trace showing a single peak at a retention time (RT) of 3.6 min is shown in Supplementary Figure S1 (Supporting Information), and the ESI MS of that LC peak is shown in Fig. 1. The retention time seems reasonable given that at pH = 1.6 the terminal amino dendrimer would be completely protonated, and hence very hydrophilic. The observed molecular weight (MW) agrees with the theoretical value of 6908.6 Da.

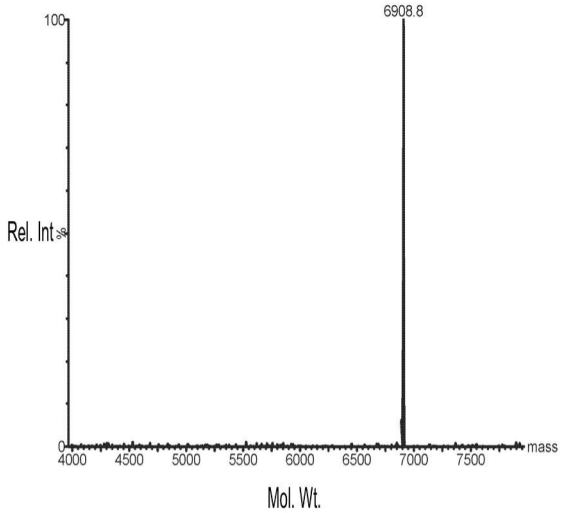

Figure 1. The positive ion deconvoluted ESI MS spectrum of the RP/LC peak at retention time 3.6 min. The expected mol. wt. was 6908.6 Da, which is within the expected accuracy of the deconvolution technique employed.

This same sample of G3 terminal amino PAMAM (dendrimer A) 10% w/w in water was then stored for 3 weeks at the manufacturer's suggested storage temperature of 4°C. The ESI/MS of that aged sample is shown in Fig. 2. There is quite a noticeable increase in the number of MS peaks, and many of them are separated by 114 Da. Clearly, the integrity of the sample has been compromised over a very short time period.

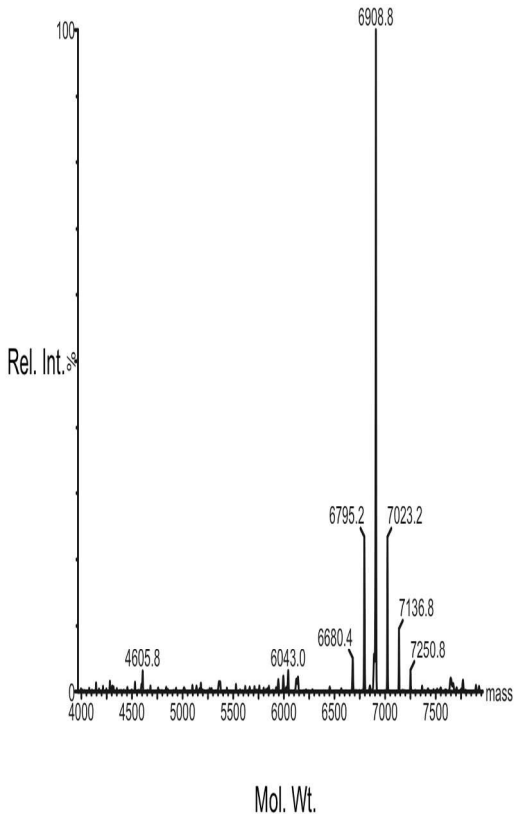

Figure 2. The RP/LC/ESI MS spectrum from a G3 terminal amino PAMAM sample stored for 3 weeks at 4°C, as recommended by the vendor. The retention time (3.6 min) is identical to the initial storage time zero sample, but there are new peaks observed both above and below the mol. wt. of 6908.8 Da. The dendrimer concentration was 10% w/w in water.

The MS spectra in Figures 1 and 2 were performed on the identical sample with Figure 1's analysis performed at storage time zero and Figure 2's analysis after 3 weeks of storage at 4°C. By comparing the 2 ESI/MS spectra, it is clear that the vendors suggested storage at 4°C was insufficient to preserve the samples structural integrity. Alternative storage conditions might be to freeze the sample at -20°C. However, the bulk sample solution would then be subjected freeze/thaw cycles of the aqueous solution. To test the stability of dendrimer A under freeze/thaw conditions, we froze an aliquot of the original sample (storage time = 0) by exposing it to minus 20°C for 30 min. and then slowly thawed the sample at room temperature over 20 min. The sample underwent a total of 3 freeze/thaw cycles. We observed on the second freeze attempt that after 30 min, the sample would not freeze solid. After the third freeze the sample would not freeze at all. The LC/MS trace of the sample after 3 freeze/thaw cycles is shown in Fig. 3, and the mass spectrum of the LC peak at RT 6.2 min is in Fig. 4. After only 3 freeze/thaw cycles, dendrimer A was completely degraded. The mass spectra of dendrimer A degradation products eluting at RT = 5.97 and 6.0 min are shown in Supplemental figures S2 and S3, respectively. The mass spectra show significant degradation of the dendrimer A and are quite complex with many MW peaks about 114 Da lower and higher than the starting molecular weight of 6909.

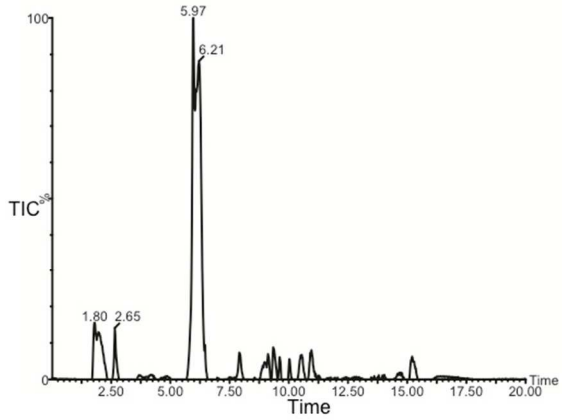

Figure 3. RP/LC/ESI/MS chromatogram of a sample of G3 terminal amino PAMAM dendrimer after three freeze-thaw cycles. No starting dendrimer was observed, and three new major LC peaks were observed at retention times 5.97, 6.0, and 6.21 min.

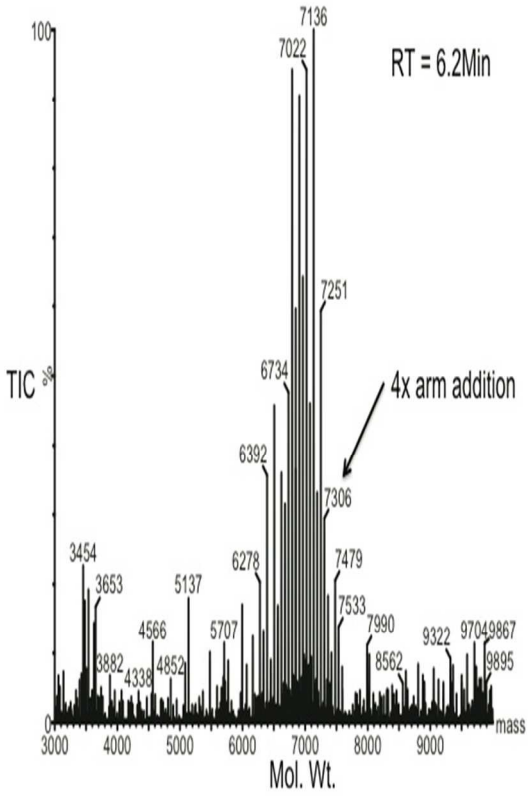

Figure 4. The RP/LC/ESI/MS spectrum of the LC peak at retention time 6.21 min after the sample was subjected to three successive freeze-thaw cycles.

In an attempt to increase the shelf life of dendrimer A, we tried to solvent exchange the water solution into methanol where we could store the solution at -20°C without freezing/concentrating the sample. We assumed that the problem observed with the water samples was the increased concentration of the dendrimer as it underwent the freezing process due to freezing point depression. To test whether dendrimer A was stable under dialysis conditions, we diluted the sample 1:1000 in water and dialyzed the diluted sample with a spin dialyzer equipped with a 3K MWCO regenerated cellulose membrane. This dialysis method does concentrate the sample by a factor of 5, but this still leaves the solution at a dilution factor of 200:1 relative to the starting concentration. The LC/MS trace of a 10 μL injection of dialyzed product is shown in Fig. 5. The mass spectrum of the peak at RT 12 min is shown in Fig. 6. The mass spectrum of the dendrimer (now referred to as dendrimer B) at RT = 12 min was very similar to the starting material, but the RT was completely different indicating some type of dramatic conformational change to dendrimer A.

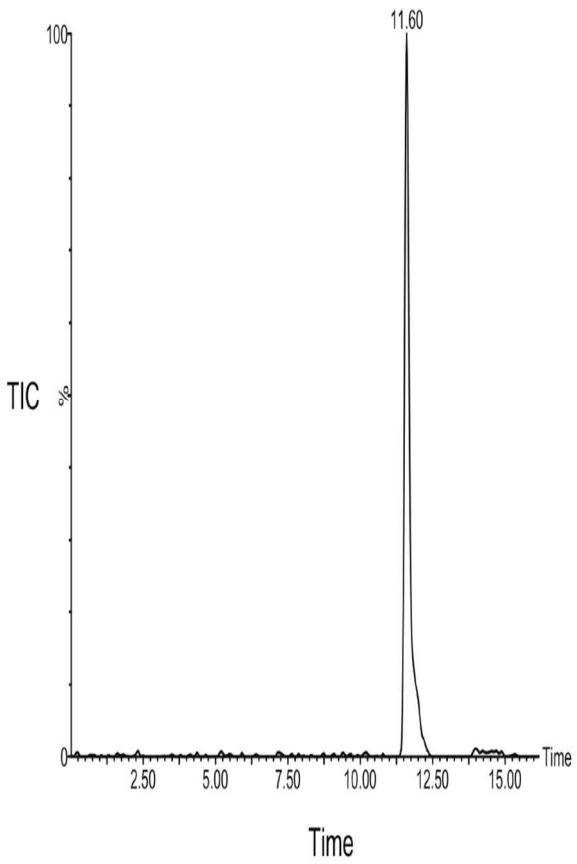

Figure. 5. RP/LC/ESI/MS chromatogram of G3 terminal amino PAMAM dendrimer after dialysis with a regenerated cellulose membrane. Dialysis resulted in the retention time for this sample being shifted from 3.6 to 11.6 min. This dendrimer conformer with the extended retention time observed using an HPLC column with a titanium frit is referred to as Dendrimer B.

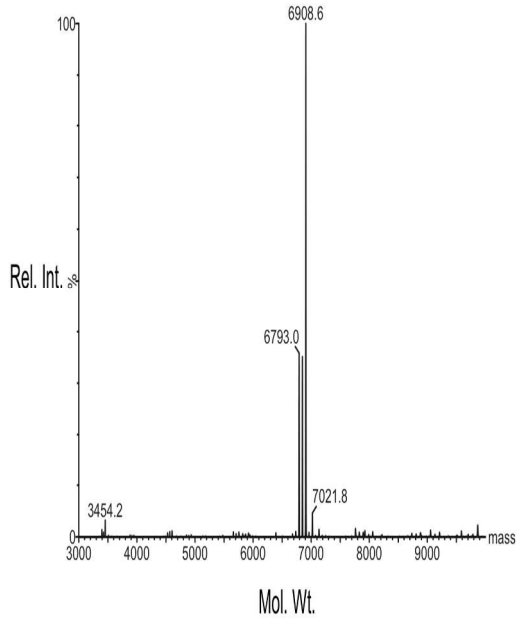

Figure 6. The RP/LC/ESI/MS spectrum of the LC peak at retention time 11.6 min of a G3 terminal amino PAMAM dendrimer after dialysis with a regenerated cellulose membrane and passed through an HPLC column containing a titanium frit.

Dendrimer A is also commercially available as a 10%w/w solution in methanol instead of water, so we opted to use this commercial material to test the hypothesis that there was a problem with our dialysis methodology. The LC/MS of the G3 terminal amino PAMAM dendrimer 10% w/w in methanol after a 1:1000 dilution with solvent A was identical to our post-dialysis dendrimer B. When we inquired about how the vendor generates the methanol version, they stated it was produced from the water solution by equilibrium dialysis using a regenerated cellulose membrane.

The terminal hydroxy G3 PAMAM dendrimer was purchased as a 20% solution in water. The mass spectrum is shown in Fig. 6 indicating a significant amount of degradation of the sample. Note that the degradation peaks are separated by 115 Da. Although our analytical method is quite effective in determining the purity of the all OH version of the G3 PAMAM dendrimer, this sample did not meet our criteria for chemical purity, and after repeated unsuccessful attempts to obtain a sample of suitable purity we did not pursue any further chemical modifications with the G3 terminal hydroxy dendrimer (Figure S4).

The G2.5 terminal carboxy PAMAM dendrimer (C) gave analogous LC/MS data to the G3 terminal amino dendrimer. The ESI/MS of dendrimer C is shown in Figure S5. The 10% solution in water of dendrimer C shows a single LC peak at RT 2 min, while the dendrimer in 10% w/w methanol gives a single peak at RT 4.1 min (S6). Dendrimer C in 10% water does not decompose after repeated freeze-thaw cycles. Dendrimer C also shows no sign of degradation when stored at 4°C for 6 weeks. After 6 months at 4°C in methanol dendrimer C does decompose, and the ESI/MS spectrum is shown in Supplemental figure S7.

Another analytical technique missing from conventional PAMAM dendrimer chemistry is the HPLC isolation of individual components from a mixture by semi-preparative HPLC. Our attempts to isolate components from a mixture containing compounds related to G3 terminal amino PAMAM dendrimer were completely unsuccessful in that the isolated component always decomposed prior to reinjection into the analytical column. However, we were successful with G2.5 terminal carboxy PAMAM dendrimers as long as we immediately adjusted the pH to 7 with ammonium hydroxide. After a 1000:1 dilution, the isolated component could then be re-injected into the analytical LC/MS system and an LC peak with the expected mol. wt. and retention time could be observed. We isolated a mono-methylated impurity from a 6 month-old G2.5 sample (Fig. 7) and were able to re-inject that sample and observe the expected mass spectrum (Fig. 8) at the expected retention time (Supplemental figure S8).

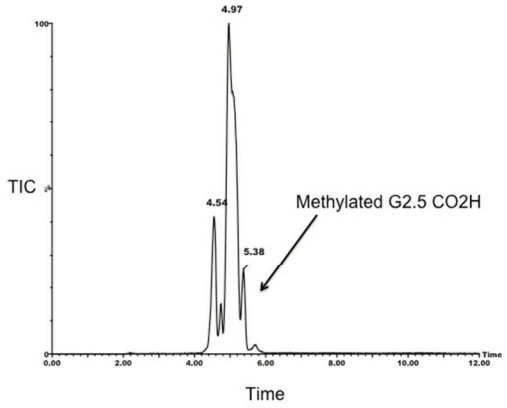

Figure 7. RP/LC/ESI/MS chromatogram of a G2.5 PAMAM dendrimer mixture stored in methanol for 6 months.

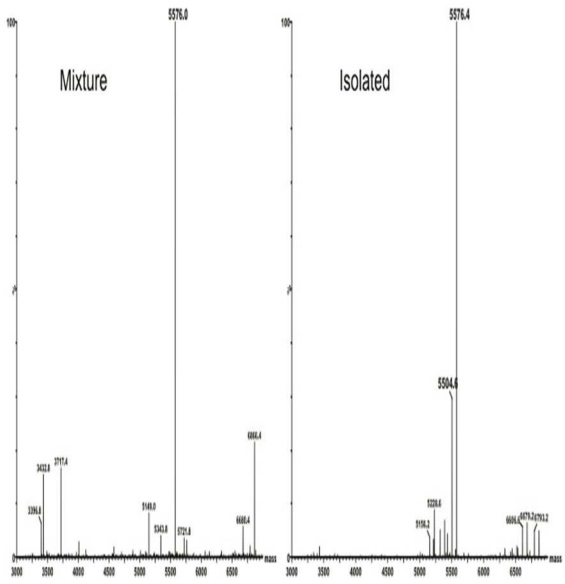

Figure 8. Comparison of the ESI MS data before and after HPLC isolation of the monomethylated G2.5 PAMAM dendrimer. There does appear to be some degradation of the isolated material, but overall the integrity of the isolated material is adequate.

DISCUSSION

Prior to this study, in-line RP/LC/ESI/MS was missing from the arsenal of analytical tools applied to the evaluation of dendrimer purity, stability and structure. Many of the results we observed are quite unexpected and rather complex. G3 PAMAM dendrimers are sold commercially as 10% w/w solutions in either water or methanol, and the suggested storage conditions specify refrigeration at 4°C. Freshly purchased G3 terminal amino PAMAM dendrimer as a 10% solution in water yields reasonable LC/MS data (Fig. 1). The molecular weight is correct, and there are only minor impurity peaks. Stability issues arise after only 3 weeks of storage at 4°C when the LC/MS data becomes much more complex with many new peaks visible in the mass spectrum. These new peaks are separated by 114 Da. A similar pattern of mass spec peaks is observed during freeze-thaw experiments, where freezing point depression increases the dendrimer concentration above the 10% level. The 114 Da difference could arise from a displacement reaction where the terminal amino group from another dendrimer molecule performs an SN2 type displacement at the methylene group alpha to the tertiary group, which when protonated is an excellent leaving group. The pH of the water solution was 6.8. The pKa range for the primary PAMAM amines is between 3 and 6, while the pKa range for the tertiary amines is between 7 and 9[25]. The proposed mechanism for G3 terminal amino PAMAM dendrimer degradation is shown in Fig. 9. This mechanism involves an inter-molecular displacement reaction, which would be highly sensitive to concentration effects. The pH would also be a factor in this mechanism, since at low pH the primary amines would be completely protonated and not a nucleophile. We adjusted the pH to <2 using TFA, and the resulting 10% dendrimer solution was able to undergo three freeze-thaw cycles without significant dendrimer degradation. This mechanism would also explain why the commercial product is never sold as a solid but exclusively as a 10% w/w solution.

Another possible source of 114 Da adduct ions is TFA (Mol. Wt. 114) and the possibility the observed series of mass peaks separated by 114 Da is the result of TFA adduct ions must be addressed. The ESI mass spectrum of all terminal amino G3 PAMAM dendrimer shown in Fig.1 does not display any adduct ions 114 Da higher in mass or any fragment ions 114 Da lower and the concentration of TFA (0.1%) is identical to that of the spectrum of degraded dendrimer showing a series 114 Da ions. The MS sample shown in Figure 2 was stored in the original water solution and not the HPLC solvent containing TFA therefore, adduct formation during storage was not a possibility. The observed series of 114 Da ions therefore cannot be the result of TFA interactions.

The mass spec peaks for degraded G3 terminal hydroxy PAMAM dendrimers are separated by 115 Da, which is also consistent with the proposed mechanism (Fig. 9). The substitution of a terminal amino group by hydroxyl results in a transferred group that is 1 Da higher in mass.

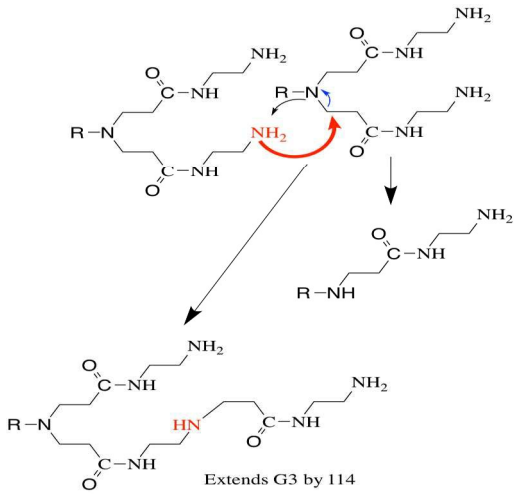

Figure 9. Proposed mechanism for the observed degradation of G3 terminal amino PAMAM dendrimer during storage, freeze-thaw and concentration.

For the synthetic chemist, this is an important observation since this dramatically limits the manner in which the starting dendrimer can be manipulated. Operations such as solvent exchange in vacuo are not permitted if the sample integrity of the starting dendrimer is to be maintained.

The G3 terminal hydroxy PAMAM dendrimer was purchased as a 20% solution in water, and significant amounts of degradation were observed upon receipt of the material. Again, this is consistent with a concentration-dependent degradation pathway.

The G2.5 terminal carboxy PAMAM dendrimer did not degrade as a function of concentration and was relatively stable at 4°C for at least 6 weeks. This observation is also consistent with the proposed mechanism, because the carboxyl group is a poor nucleophile.

The practical consequence of this finding is that the terminal carboxylate dendrimer is much more robust and is less likely to degrade during normal sample handling routines. G2.5 terminal carboxyl PAMAM dendrimer subjected to repeated freeze-thaw cycles showed little, if any, degradation. Solvent exchange from water to methanol by in vacuo removal of the water also produced little if any degradation. Consequently, we only use the carboxyl version as our starting material at this time, thus avoiding structural degradation of the starting material.

There are several scientific reports in the literature regarding PAMAM dendrimers existing in two forms[12]. In most cases there is no guidance given about how these were observed or under what circumstances they were encountered. There are, however, recent reports demonstrating the existence of two forms of dendrimer described as swollen and flat forms[16, 26, 27]. The mechanism of form conversion described in these papers involves a silica surface and a change in electrolyte concentration. In our present work, dialysis of the G3 terminal amino PAMAM dendrimer using a regenerated cellulose membrane converts the form having a short LC retention time (3.6 min) into a form displaying a long retention time (11.6 min). This conversion was observed under both low and neutral pH conditions. Intermediate conversion was also observed where both the short and long retention time versions were present after a shorter duration dialysis. The two forms displayed an identical observed molecular weight. This observation indicates that dialysis and the concomitant electrolyte reduction dramatically changed the dendrimer structure from one form to another. This type of structural change is also observed in the refolding of proteins by dialysis[28, 29]. Isolation of a recombinant protein often involves the use of inclusion bodies resulting in the unfolding of the target protein. The inclusion bodies are removed by dialysis, and the protein is subsequently refolded into the active form. In our case, dialysis would effectively remove low molecular weight electrolytes and affect a conversion of one dendrimer form into the other. Although more work is needed to elucidate the structural differences between these two forms, it is clear that in the present cases of G3 and G2.5 PAMAM dendrimers, RP/LC/ESI/MS, where the RP column contains a metal frit, is an effective tool in determining the presence of one or both of these structurally unique forms

The metal chelating properties of PAMAM dendrimers are also well-known[30-32]. Indeed, the terminal carboxylate dendrimer version is nearly equivalent in this respect to poly-EDTA, a well-known metal chelator. To determine whether metal interaction was playing a role in our observed LC/MS retention times, we substituted a monolithic PLRP-S fritless column for the titanium frit version we were typically using. We observed that the formerly long retention time peaks appeared at the same retention time as the pre-dialysis PAMAM dendrimers indicating that the long retention time was the result of some type of interaction with the titanium frits. The gradient we were running would eventually displace the dendrimer from the titanium frit. This would also explain why TFA was required in the mobile phase to observe these late-eluting dendrimers.

Another common tool missing from conventional dendrimer chemistry is isolation by HPLC of individual

components of complex dendrimer mixtures. In the case of G2.5 terminal carboxylate PAMAM dendrimers, we were successfully able to isolate a component from a dendrimer mixture and maintain the integrity of the isolated material. We isolated a methyl ester impurity from a degraded sample of G2.5 terminal carboxylate PAMAM dendrimer and were able to re-inject that material back onto an analytical LC and observe the expected mass spectrum. This technique worked only for the G2.5 dendrimer, since the isolated components of the terminal amino PAMAM dendrimer would degrade probably due to an increased concentration of the analyte in the fraction collector vial. We also found that ammonium hydroxide was required to neutralize the pH of the fractions collected during the HPLC isolation of the G2.5 methyl ester impurity.

CONCLUSIONS

We have developed a robust, comprehensive in-line RP/LC/ESI/MS method for evaluating the purity, stability and structures of G3/G2.5 PAMAM dendrimers. Using our method we encountered several previously unreported aspects of PAMAM dendrimer chemistry, such as the concentration-dependent degradation of the terminal amino and terminal hydroxy PAMAM dendrimers. This type of chemical analysis is critical if PAMAM dendrimers are to be utilized as therapeutic agents in the future. We also found that the G2.5 terminal carboxy version did not display this type of degradation and was consequently much more stable and easier to use. We also observed the formation of a second, metal-interacting form of the dendrimer, generated by dialysis using a regenerated cellulose membrane. Whether this metal-interacting form is related to previous reports regarding the existence of a second dendrimer form will require further study, but by using our RP/LC/ESI/MS method it is quite easy to detect the two different forms. Detection of this second form could be critical depending upon what type of chemical modification is being attempted. It also remains to be determined whether the two forms have different therapeutic profiles.

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