Determination of Molecular Weight, Particle Size, and Density of High Number Generation PAMAM Dendrimers Using MALDI−TOF−MS and nES−GEMMA

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ABSTRACT: In this work we present the characterization of PAMAM dendrimers from generation two (G2) up to ten (G10) with a focus on the G5−G10 dendrimers with matrix-assisted laser desorption/ionization linear mass spectrometry (MALDI−MS) and nanoelectrospray gas-phase electrophoretic mobility molecular analysis (nES−GEMMA). For the first time the molecular masses of high-mass dendrimers G8−G10 were determined by MALDI−MS and nES−GEMMA, techniques which are based on different physicochemical principles. Obtained experimental data allows the determination of the molecular mass (up to 580 kDa with a precision below ±0.9%), of the spherical size (from 3.3 to 14.0 nm with a precision of ±0.2 nm) and the calculation of their densities. Amounts in the nanogramm range were sufficient for an analysis that could be performed within several minutes. The results based on these methods for high-generation dendrimers exhibited an excellent correlation and were compared with published data using techniques based on different principles.

Introduction

Dendritic (branchlike) architecture has been recognized as complete new building scheme for polymers as early as 1926 by Herman Staudinger within his macromolecular hypothesis.[1] This kind of architecture lead to polymeric molecules,[2,3] called dendrimers, which faced a rapidly increasing importance in the recent decade,[4−8] since they are recognized as a whole new type of well-defined functionalized building blocs for a bottom-up synthesis of nanoscale objects with designable properties.[9−12] Unlike other polymers, dendrimers possess a well-defined globular shape with sizes in the low nm range and structure, with a clear distinction between core and surface, which makes them suitable for applications like catalysis and as delivery vehicles[7] for small drug molecules.[13] Various interesting molecular architectures using dendrimers have already been realized, such as self-assembly macromolecule structures between different types of dendrimers,[14,15] dendrimers coupled with various polymers in different arrangements,[16−19] dendrimers with hydrophobic core and hydrophilic surface, which may be used as artificial liposomes, and dendrimers coupled to peptides, antibodies, DNA, and carbohydrates, for example. The goal of these architectures is to create a whole set of new macromolecular properties which could not have been realized otherwise.

To be able to create desired dendrimer products and structures, dendrimer properties (e.g., molecular weight, hydrophobic vs hydrophilic character, number and type of free reaction groups, and average diameter and density) have to be sufficiently characterized during macromolecular synthesis steps, and this must be done before their use in the above-mentioned applications. Despite this fact, analysis seems to be a step behind this in importance, as was already stated by Caminade et al.[20] The main reason for this fact is that dendrimers pose a new challenge to all analytical techniques as they exhibit exponentional growth of surface and mass during their stepwise synthesis (producing the next higher generation) as well as covering a mass range from a few kDa in the case of generation one (G1) to one MDa for generation ten (G10).

A special, very important subclass of the dendrimers, namely poly(amido-)amine (PAMAM) dendrimers, will be the focus of this work. PAMAM dendrimers resemble proteins in their size and particular chemical structure (a huge number of peptide bonds). But while proteins can fold oddly or change their shape, dendrimers are more or less cemented structures with strong and rigid bonds. Because of these attributes they are already used to replace proteins in immunodiagnostics[21,22] and in vitro gene expression applications,[23] for example. Therefore, it is not surprising that PAMAM dendrimers are the first class of dendrimers which have been successfully commercialized,[3] which makes their detailed characterization even more important.

Until now, size exclusion chromatography (SEC),[24−28] slab gel electrophoresis (SDS−PAGE),[29] capillary electrophoresis (CE),[29,30] small-angle X-ray scattering (SAXS),[31,32] and small-angle neutron scattering (SANS),[33,34] atomic force microscopy (AFM),[35−38] transmission electron microscopy (TEM),[39] and mass spectrometry[40,41] have been applied to determine the molecular mass, diameter, and shape of PAMAM dendrimers. Other techniques that focus on a chemical characterization such as the characterization of surface groups or the abundance of certain structure elements will not be discussed in this work, since the presented experimental data are solely focused on molecular mass, size, and density determination. In the following section, we give a brief overview of the afore-mentioned techniques as well as their advantages and disadvantages. A detailed description of other analytical techniques applied to dendrimers can be found in the following reviews.[20,42−44]

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SEC is a widely and routinely used separation technique which allows the separation of analytes according to their size in solution. The main advantages of SEC are easy handling and a high dynamic range concerning molecular weight determination. When this method was applied to determine the molecular mass of dendrimers, a systematic error between expected and with SEC determined molecular mass values was found.[24] This error could be linked to the different shape of the commonly used calibrants, which are linear polystyrol polymers, and the spherically shaped dendrimers. This shape dependency of the molecular weight determination is a general phenomenon of SEC and thus makes this method somehow problematic in the use of molecular weight determination of dendrimers particularly for higher generations.

CE and SDS−PAGE are analytical techniques widely used for the analysis of biopolymers. Both techniques separate analytes according to their electrophoretic migration behavior, which strongly depends on the number of charged surface groups, the pH of the buffer solution, and the cross section of the molecule. For molecular weight determination, as it is carried out with proteins, denaturation and the attachment of sodium dodecyl sulfate (SDS) molecules to the polypeptide backbone is inevitable as it levels the charging differences between different types of proteins and leads to a direct relationship between migration behavior and molecular weight. Applying these techniques to analyze dendrimers, it is possible to differentiate between different generations of one particular dendrimer species and between dendrimer particles of one generation with different surface modification. Both techniques lack the ability to determine the size or molecular mass of the dendrimers.[30] However, a qualitative analysis of the change of dendrimer populations during synthesis and modification steps has been possible with these methods.[20]

SAXS and SANS are both scattering techniques which allow the determination of the radius of gyration of the dendrimer molecules as well as the electron density distribution inside the dendrimer particles in solution. With these techniques, it was possible to prove that PAMAM dendrimers change their characteristic shape from starlike (varying electron density to radius function) to rigid-sphere-like (constant electron density along the outer radius of the particle). The advantage of both techniques is that the determined value is an average of a vast amount of scattering events on many dendrimer particles, and that measurements are made with dendrimers in solution. However, both techniques also have their disadvantages, as considerable experimental expense as well as high expertise in interpreting the experimental data is required.[31−34]

AFM is a microscopic technique that allows one to investigate the shape as well as mechanical properties of single dendrimer particles. The strongest limitation of AFM in the determination of dendrimers is the softness of these organic molecules, which limits the resolution and hampers the analysis of dendrimers smaller than G5.[35−38] Another observed effect is the deformation and loss of the spherical shape of dendrimers during adsorption on substrate surfaces, which poses problems in the size determination. With scanning of the whole dendrimer particle, it was possible to circumvent this problem and determine the volume and calculate a spherical-equivalent diameter.[35,36,38]

TEM is an additional microscopic method whose resolution depends on the energy of the primary electron beam. For samples that provide enough contrast and regularity, even atomic resolution is possible. This method was used to determine the diameter of G5 up to G10 PAMAM dendrimers in vacuum.[39] However, as dendrimers are organic molecules with a weak contrast, a staining step must be carried out before analysis, which may lead to a systematic error in the determined particle dimensions, but as TEM can be carried out at low temperature, the losing-shape effect as described for AFM can be avoided.[39]

Mass spectrometry is an analytical technique preliminary used for the exact molecular weight determination of various high-mass biopolymers. The MALDI−TOF−MS technique was a major break-through for the mass spectrometric analysis of molecules with molecular weights higher than 2 kDa and was first presented in 1988 by Hillenkamp, Karas, and Tanaka.[45,46] Whereas today most MALDI−MS experiments are performed with proteins, carbohydrates, and oligonucleotides,[47] the analysis of dendrimers with this technique is also already described in the literature up to G4.[40,41,47−50] Observed imperfections and deviations from the calculated molecular weights of the dendrimer samples were attributed to chemical defects. Additionally, fragmentation of dendrimers was observed for dendrimers absorbing at a similar wavelength of the applied laser wavelength used for desorption/ionization.[48,49] As in the case of proteins or DNA fragments, post-source decay (PSD) fragmentation of PAMAM dendrimers of small size (up to G4) was also observed with MALDI−TOF−MS.[50] Beside MALDI−MS, electrospray ionization (ESI)−MS is another mass spectrometric technique for the exact molecular weight determination already applied to dendrimers.[51,52] Advantages of MALDI−TOF−MS (in the linear mode) are its high molecular weight range, sensitivity, and mass accuracy.[53] The main disadvantages of this technique are the rather high instrumentation cost and the search for an optimal matrix and a proper sample preparation method for a given analyte. ESI−MS has the advantage of generating highly charged molecular ions, which reduces the mass-to-charge ratio and makes molecules with higher mass accessible to mass analyzers other than TOF systems. However, for the determination of the actual molecular mass, the exact charge states of the multiply charged ions are required. For this purpose, peaks representing adjacent charge states have to be resolved, which is difficult for many high mass molecules[54] and was not possible for, e.g., a PAMAM G6 dendrimer (for a higher generation dendrimer, it is even more difficult) despite combining ESI with a Fourier-transform ion cyclotron analyzer.[40]

The nanoES−GEMMA (electrospray gas-phase electrophoretic mobility molecular analyzer), which is used in this study, is a relatively new technique that combines the benefits of the low charge levels (one or two charges per molecule) of the MALDI with the process of desorption/ionization from the bulk solution by the nano-ES process. It uses a differential mobility analyzer (DMA) for the separation of the singly charged particle by their electrophoretic mobility diameter (EMD) at atmospheric pressure. Such devices have been in use since a long time in aerosol science for the analysis of submicrometer to micrometer aerosol particles;[55] their design was recently adapted to extend the measurable size range into the low single digit nanometer region, or in terms of molecular mass, into the low kDa mass range. A condensation particle counter (CPC) is used for the detection of the separated singly charged particles (ions), offering single particle detection allowing high sensitivity (10^3 particles or ions/cm^3). Furthermore, a direct relationship between molecular mass and diameter has already been demonstrated for globular proteins[56] and small DNA fragments,[57] allowing the use of the nES−GEMMA to determine the molecular mass of high molecular weight proteins and noncovalent protein complexes. Also the size analysis of bacteriphages,[58] viruses and virus fragments,[59] high mass protein complexes[56,60] and polymer (poly(ethylene glycol), polysytyrene

and poly(methyl methacrylate)) mixtures[61] as well as small (≤G4) PAMAM dendrimers[62] was demonstrated with this method.

In this work, we present now the characterization (molecular mass, size, and average density) of PAMAM dendrimers up to G10 with the two independent techniques MALDI−TOF−MS and nES−GEMMA as well as a comparison with other techniques such as SAXS, SANS, AFM, TEM, and data provided by the manufacturer as well as theoretical values based on molecular dynamics calculations.[63]

Materials and Methods

Chemicals. All amine-terminated PAMAM dendrimers with an ethylenediamine core were obtained as 5−20% solutions in methanol (G2−G7 from Sigma-Aldrich, G9 and G10 from Dendritech). Ammonium acetate and acetonitrile (ACN) of analytical grade were acquired from Merck. Trifluoroacetic acid (TFA, 99%) was purchased from Riedel-deHaen and 2,4,6-trihydroxyacetophenon monohydrate (THAP) from Fluka, both analytical grade. Ammonium acetate and TFA solutions were made by means of analytical grade water from Merck. For MALDI−TOF−MS calibration bovine insulin (Sigma-Aldrich) and the Invitromass HMW Calibrant Kit (Invitrogen) were applied.

MALDI—TOF—MS Instrument. MALDI mass spectrometry was carried out on an Axima TOF^2 tandem instrument from Shimadzu Biotech Kratos Analytical. The device is a high resolution, floor-standing instrument equipped with a pulsed nitrogen laser (wavelength, 337 nm; pulse width, 4 ns), an integrated 2 GHz transient recorder, a curved field reflector, and a differential pumped collision gas cell. The instrument was operated in the positive ion linear (flight path 1.2 m) mode applying an accelerating voltage of 20 kV without delayed extraction.

Calibration for PAMAM dendrimers from G4 to G10 was performed externally by using the Invitromass HMW Calibrant Kit, which contained various recombinant standard proteins with given molecular weights in the mass range from 30 kDa to 160 kDa. The calibration kit was applied according to the manufacturer's instructions, but in contrast to the instruction manual, THAP as MALDI matrix solution was applied for external calibration. Calibration for PAMAM dendrimer G2 was performed externally using the singly and doubly protonated molecule of bovine insulin. All mass spectra were acquired by averaging 50−200 unselected and consecutive laser shots on the same preparation by automatic rastering. In order to calculate relative standard deviations (RSDs) for molecular weight determination, each PAMAM dendrimer sample was analyzed ten times using different sample spots on the same MALDI target. All mass spectra were smoothed using the company-supplied Savitzky-Golay algorithm.[64] The applied peak (*m*/*z* determination) detection method was threshold-centroid at 50% height of the peak maximum.

Sample Preparation for MALDI—MS Analysis. Prior to analysis, THAP was dissolved in 1/1 (v/v) 0.1% TFA/ACN mixture to give a final MALDI matrix concentration of 10 mg/mL. Prior to use, the all day freshly prepared matrix solution was vortexed thoroughly. For MALDI−TOF−MS analysis, 1 uL of PAMAM dendrimer stock solution was vacuum-dried at room temperature in order to remove all solvents. Afterward, the residue was dissolved completely in a 9/1 (v/v) 0.1% TFA/ACN solvent mixture to give a final PAMAM dendrimer concentration of 10 nmol (assuming an ideal molecular weight)/mL which was equivalent to the following mass concentrations: 0.03 mg/mL (G2), 0.14 mg/mL (G4), 0.29 mg/mL (G5), 0.58 mg/mL (G6), 1.16 mg/mL (G7), 4.67 mg/mL (G9), and 9.34 mg/mL (G10). One part of the resulting PAMAM dendrimer solution was mixed with two parts of THAP matrix solution in an Eppendorf tube. Then 0.5 uL aliquots of this matrix/sample mixture were deposited on several different spots on a stainless steel microtiter format MALDI target and dried under a gentle stream of air at room temperature forming smooth crystalline layers.

nES—GEMMA Instrument. The GEMMA system consists of a nanoelectrospray (nES) unit, a nanodifferential mobility analyzer (nDMA), and an ultrafine condensation particle counter (uCPC) as detector (all parts from TSI Inc). The nanoES generated multiply charged particles are charge-reduced in a bipolar air environment produced by α-radiation from a Po-210 radioactive source (NRD). This charge-reduction process results in neutral and only singly charged molecules/nanoparticles. The operating particle size range of the instrument combination is between 3 (limited by the particle detection with the uCPC) and 65 nm (the upper scan limit of the nDMA when using maximum sheath flow for maximum nDMA resolution). The nDMA was operated with negative voltage polarity on the central electrode, thus separating and detecting the positively charged fraction of the generated molecules/nanoparticles. The detection limit of the complete GEMMA system in terms of particle concentration is 1 singly charged nanoparticle/cm^3. However, due to the limited efficiency of charging process concentration of airborne dendrimer particles of at least 10000 particles (equivalent to approximately 4 pg for G5 molecules)/cm^3 are necessary to obtain appropriate particle count statistics across the whole sizing (3−30 nm)/scanning range within a reasonable time frame (120 s per scan). Ten scans were averaged for each final size spectrum presented in this paper.

Nano Electrospray Conditions. For all measurements, the settings of the nanoES source, buffer concentrations, and solution conductivity were identical. Samples in 20 mM ammonium acetate buffer (pH 7.4) were electrosprayed. For this solvent system and spray-chamber design we found that a stable cone-jet mode operation was obtained at 2 kV and 0.3 L/min CO_2 (99.995%, Air Liquide)/1L/min compressed air (99.999% synthetic air, Air Liquide). A pressure of 4 psi was applied onto the sample solution for sample introduction, which resulted in a sample solution flow of 67 nL/min through the fused silica capillary (25 μm inner diameter and 160 μm outer diameter, length 25 cm uncoated; supplied by TSI Inc). The primary generated droplet size, which is determined by the conductivity of the solvent system and the sample flow through the capillary as well as the inner diameter of the capillary was 150 nm, corresponding to a flow of 2×10^{11} droplets/minute or about 1.7×10^8 droplets/cm^3. The electrospray process was operated in the positive ion mode, which means that droplets with a high initial number of positive charges were produced.

Sample Preparation for nES—GEMMA Analysis. For the nES−GEMMA, all of the above-mentioned commercially available PAMAM dendrimer stock solutions were diluted directly with a 20 mM aqueous ammonium acetate buffer (pH 7.4) to a concentration avoiding the formation of PAMAM cluster ions ($[nM]^+$, $n \geq 2$) consisting of 2 and more dendrimer molecules through the ES process. This concentration was 20 μg/mL for G5, G6, G7, G9, and G10 and 2 μg/mL for G2 and G4.

Results and Discussion

Exact Molecular Weight Determination with MALDI Mass Spectrometry. The MALDI−TOF mass spectra of PAMAM dendrimers G2−G10 are shown in Figure 1, parts a-g. Most mass spectra exhibit mainly singly charged molecular ions and to a lesser degree doubly charged molecular ions (see Figure 1, parts a−e). Sometimes the mass spectra show additionally (e.g., in the case of G5 and G6, Figure 1, parts c and d) singly charged dimeric molecular ions. Beside the singly charged molecular ion peak the doubly charged molecular ion peak was found as well. The mass spectrum of PAMAM dendrimer G9 showed beneath singly and doubly charged molecular ions also an abundant signal at *m*/*z* ∼94000 (Figure 1 f). The peak observed at this *m*/*z* ratio was not a triply charged molecular ion peak of PAMAM G9 but either a fragment ion of the analyte molecule or with a much higher probability an impurity. Since no comparable molecular ion peaks (or fragment ions) were observed in the mass spectra of PAMAMs with generations lower than 9, it is most likely that this peak derived from an

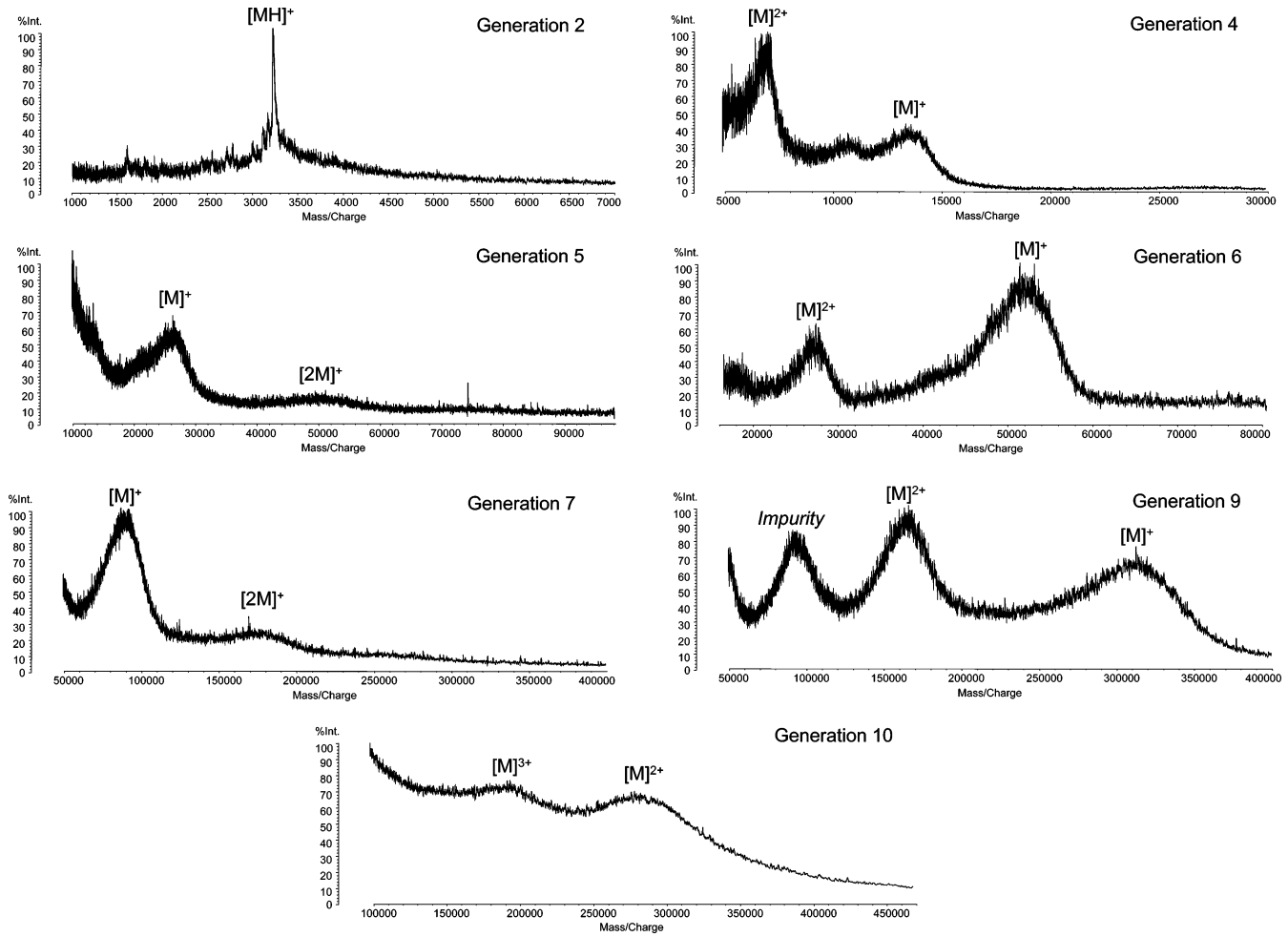

Figure 1. Positive ion MALDI mass spectra in the linear mode of PAMAM dendrimers of generations (a) G2, (b) G4, (c) G5, (d) G6, (e) G7, (f) G9, and (g) G10.

Table 1. Summary of the Average Molecular Weights Determined with MALDI−TOF Mass Spectrometry and the Calculated Molecular Weights of Various Generations of PAMAM Dendrimers[a]

PAMAM generation	theoretical no. of NH_2 groups based on an ideal structure	theoretical mol wt based on an ideal structure [kDa]	MALDI−TOF--MS derived mol wt [kDa]	RSD [%]	mol wt difference between theoretical and measured values [%]
2	16	3.256	3.233	0.0	−0.6
4	64	14.21	13.32	0.2	−6.3
5	128	28.82	25.92	0.4	−10.1
6	256	58.05	50.16	0.3	−13.6
7	512	116.49	90.97	0.9	−21.9
9	2048	467.14	323.30[b]	1.2	−30.8
10	4096	934.69	579[c]	1.4	−38.1

[a]The differences (given in percent) shown in the right column refer to the measured molecular weights compared with the calculated molecular weights in case of ideal structure/synthesis (unless noted otherwise, the given molecular weights are based on the singly charged molecular ion peak). [b] Molecular weight determination based on the doubly charged molecular ion peak. [c] Molecular weight determination based on the triply charged molecular ion peak.

impurity within the original sample. Possible impurities are precursors of earlier synthesis steps, like G7 or G8, which were not completely removed or converted. Given a similar chemical structure of analytes, molecules with lower molecular weight tend to show more intense peaks in MALDI mass spectrometry due to better desorption/ionization yield. Thus, the impurity can actually be present in the sample solution in a much lower concentration than the PAMAM dendrimer G9. In the case of PAMAM G10 only the doubly and triply charged molecular ions, but no singly charged molecular ion was observable (Figure 1g) despite the fact that the instrument can handle a *m*/*z* range up to 600 000. Concerning the full peak widths at half-maximum (fwhm) of the singly charged molecular ion analyte peaks, it was interesting to notice that well-defined proteins of similar molecular weight had substantially lower FWHMs than dendrimers. The high fwhm values of the PAMAM dendrimer analyte peaks could be explained by incomplete synthesis and nonoptimized purification after synthesis. In the case of PAMAM dendrimers G2−G7, the singly charged molecular ion peaks were used for molecular weight determination (Table 1). Because instrument calibration was limited to 160 kDa, for PAMAM G9 the doubly charged and for PAMAM G10 the triply charged molecular ion peak was applied for molecular weight determination. Nevertheless, the molecular weight of the triply charged molecular ion peak of PAMAM G10 was slightly out of the external calibration range, which was limited with

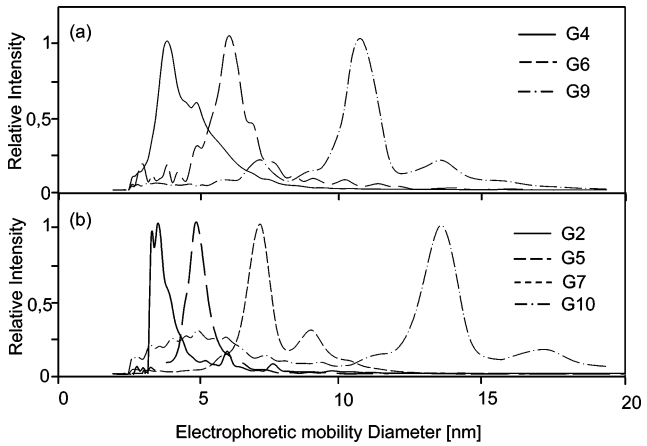

Figure 2. nES−GEMMA spectra of PAMAM dendrimers (a) G4, G6, and G9 as well as (b) G2, G5, G7, and G10.

m/*z* 159081. The molecular weights of all PAMAM dendrimer samples determined with MALDI−TOF−MS were below the calculated molecular weight for ideal synthesis (Table 1). This difference between measured and calculated molecular weight increased continuously with increasing generation of the PAMAM dendrimers and was already surprisingly high for lower PAMAM dendrimer generations (G4, minus 6.3%, G5: minus 10.1%). This observation was corroborated by nES−GEMMA data shown and discussed below. Interestingly, in the case of G4 and G5, the singly- charged analyte peaks included molecular weights above the calculated molecular weights for ideal PAMAM dendrimer synthesis, indicating impurity attachments or difficulties associated with the MALDI desorption/ionization. In the case of G6 and G7 the upper limit of the singly charged analyte peaks coincide with the molecular weights calculated for ideal PAMAM dendrimer synthesis. Most likely, the synthesis of PAMAM dendrimers was not complete, and as a consequence, the difference between the calculated and actual molecular weight increased with every synthesis step. It has to be stated in this context that the MALDI mass spectrometric derived molecular weight in the linear mode is independent of the 3-dimensional structure of the dendrimer molecule and that under our MALDI conditions no considerable metastable decay was observed.[49,50] The obtained precision of molecular weight determination with MALDI−TOF−MS in the linear mode across all analytes was ±0.4%.

Size Determination with nES—GEMMA. Figure 2 shows the GEMMA size spectra of PAMAM dendrimers G4, G6, and G9 (Figure 2a) and G2, G5, G7, and G10 (Figure 2b). The typical amount of particles being characterized within one spectrum is between 10^4 and 10^7, thus characterizing an ensemble and not single particle properties as is done in TEM, AFM, SAXS, and SANS. As can be seen, the location (size) of the $[M]^+$ peak maximum increases steadily with the number of the generation. For G7, G9, and G10, cluster dimers ($[2M]^+$ ion) could be observed (Figure 2). Furthermore, in the GEMMA spectra of PAMAM dendrimers G9 and G10, some impurities (particles of smaller size) could be detected (Figure 2). As these impurities are also high molecular weight components, which is also confirmed by MALDI−MS, it can be assumed that they are either incomplete products of the G9 and G10 dendrimer synthesis or dendrimers of smaller generations, which were not successfully removed after the synthesis. As can be seen in Table 2, the diameters determined by nES−GEMMA measurements agree well with values derived from AFM measurements of different origin,[35,36,38] from vitrified solutions by TEM,[39] and from SAXS[31,32] and SANS[33,34] (diameter of gyration) studies with an average difference of about 10% within one dendrimer generation. This difference in size between the different studies is well within the described standard deviations resulting from the experimental method uncertainties and the polydispersity of the dendrimers themselves. The only exceptions are the two SAXS studies, whose results for generations smaller than 7 agree with TEM, AFM, and GEMMA measurements but which found significantly smaller diameters than the other methods for PAMAM dendrimers G7 and higher. This phenomenon is discussed in the literature[31,32] and can be attributed to higher order scattering events inside the particle, which distort the scatter angle and results subsequently in an underestimation of the particle diameter.

Molecular Weight and Density Determination Based on nES-GEMMA. As mentioned before, the nES−GEMMA data can not only be used for the determination of the average particle diameter, but also, with appropriate calibration, for the determination of the molecular weight. For this reason, the GEMMA system was calibrated with common globular proteins, which are essentially all of spherical shape according to Bacher et al.[56] Another approach to estimate the molecular mass of a dendrimer particle is the calculation of the average particle volume and the calculation of its mass with an assumed density. As was found by molecular dynamics calculations of dendrimers,[63] a density of about 50 atoms/nm^3 or, when multiplied with the average atom mass of the PAMAM dendrimers, 0.52 kg/L, can be assumed for the bulk volume of the dendrimer particle. Both approaches were used for the calculation and determination of the molecular mass from GEMMA derived particle diameters (Table 3).

As can be seen in Table 3, both approaches clearly overestimate the molecular mass of the PAMAM dendrimer G2 particle compared to the MALDI−MS data. This result can be explained by the fact that such small dendrimers cannot be viewed as rigid spheres, but more as starlike branched molecules (disklike), with a significant distance between its branches, thus possessing neither spherical shape, which is known to bias the determined electrophoretic mobility diameter, nor the assumed bulk density. For higher generation PAMAM dendrimers, where spherical shape, rigidity and bulk properties of the dendrimer particle can be assumed, both approaches do not over estimate but instead underestimate the molecular weight of the dendrimers by ∼18% but agree very well with each other.

The reason for this difference can either be rooted in the determined particle diameter, or in the explicitly (derived from molecular dynamics) or implicitly (as in the case of the protein calibration curve) set particle density. As particle diameters between different analytical methods showed good agreement (as discussed in the paragraph before), particle densities were calculated from molecular dynamics,[63] taken from the manufacturer[65] and from MALDI mass spectrometric and nES−GEMMA data (Figure 3).

As can be seen in Figure 3, the by the means of nES−GEMMA determined average protein density (∼0.54 kg/L) is nearly identical to the average dendrimer density found in molecular dynamics calculations, thus explaining the good agreement between the molecular mass derived from protein calibrations and calculated from the determined particle volume and the theoretical density. The series representing the density values derived from MALDI−TOF−MS and nES−GEMMA data shows an increase of the particle density from 0.31 (G2) to 0.65 (G5). PAMAM generations above G5 show constant density, thus confirming the transition from starlike branching

Table 2. Average Particle Diameter Obtained in This Work with nES−GEMMA and in Literature, from SAXS, SANS, TEM and AFM, Where AFM Diameters Were Calculated from Determined Particle Volumes, Assuming a Spherelike Shape

	average particle diameter [nm]									
	GEMMA	AFM[35]	AFM[36]	AFM[38]	TEM[39]	SAXS[31]	SAXS[32]	SANS[33]	SANS[34]	manufacterer[a,68]
G2	3.3									2.9
G4	4.3					3.4	3.6	3.9		4.5
G5	5.1	4.5		4.9	4.3	4.8	4.4	4.9	4.4	5.4
G6	6.4	5.6	5.9	5.9	6.9	5.3	5.7	6.1		6.7
G7	7.6	7.2	7.1	7.1	8.0	6.4	6.5	7.2		8.1
G9	11.2	12.5	10.5	10.5	12.4	9.8	9.2			11.4
G10	14.0	15.6			14.7	11.5	10.8		-	13.5

[a] According to manufacturer medium diameter determined with dilution viscosimetry, SAXS, and SANS.

Table 3. Molecular Mass Determination Based on MALDI−TOF−MS and nES−GEMMA and Molecular Dynamics[63]

	molecular mass (kDa)		
PAMAM generation	MALDI−MS[a]	nESI−GEMMA[b]	molecular dynamics[c]
G2	3.24	5.17	5.68
G4	13.32	12.3	13.1
G5	25.92	20.5	21.5
G6	50.16	41.6	42.6
G7	90.97	71.0	71.4
G9	323.3	239	231
G10	579	482	454

[a] External protein calibration. [b] Calibration function molecular mass (Da) = $131.77 \times x^{3.106}$; $r^2 = 0.9943$ derived from measurements of well-defined proteins as standards. [c] Assuming a particle density of 0.52 kg/L, which was obtained by molecular dynamics calculations.[63]

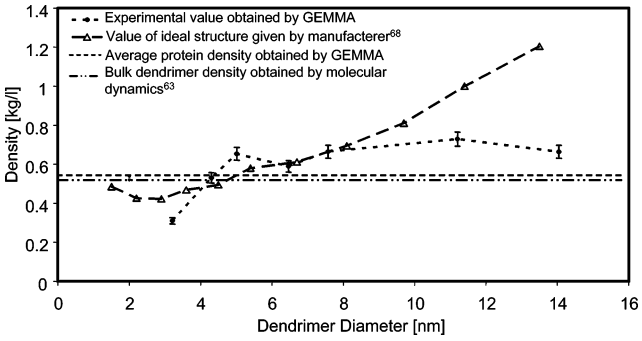

Figure 3. Plot of theoretical and experimental PAMAM dendrimer particle densities vs particle diameter.

to rather rigid spheres with bulk properties as was found by SAXS,[31,32] SANS,[33,34] and AFM[35,36,38] studies.

However, the difference in density between proteins and dendrimers, for which the values were calculated based on molecular dynamics,[63] to experimental data leads directly to the observed differences between the molecular masses derived from MALDI−MS and the protein calibration and via density calculated values from the nES−GEMMA experiments. One reason for this difference to theory might be that the dendrimer particles are not surrounded by solvent environment as in the molecular dynamics simulation. This amplifies intramolecular forces and might lead to a more dense packing inside the particle and a higher average bulk density. Proteins, as used in the calibration, are not branchlike but are linear in their primary structure, which also might lead to differences in particle density, even when the elemental distribution is roughly the same.

The theory of ideal dendrimer growth behavior postulates a nearly linear, constant increase of the diameter of dendrimers, especially for generations above 4, where globular particle shape can be assumed, but an exponentional molecular mass growth (values derived from manufacturer[61]). This leads to a steady increase of the postulated dendrimer density with every generation above 4; for smaller generations a slight decrease of density is postulated, since there the starlike branching leads to an increase of the distance between the different branches (dashed line with triangles in Figure 3). Theory and determined densities agree very well until G7. However, according to our MALDI−MS and nES−GEMMA data, the density of G9 and G10 PAMAM dendrimers lies significantly below theoretical values. The reason for this deviation between our experimental values and values calculated for ideal dendrimer structures is probably a congestion-induced *De Gennes dense packing*,[9,66,67] as was postulated for high generation dendrimers in the literature, which leads to an incomplete saturation of free branching spots during the synthesis of the next generation.

Conclusions

In this work, we have shown that molecular mass determination of PAMAM dendrimers up to G10 was generally possible with MALDI−TOF−MS analysis. The observed average precision was ±0.4%. An increasing difference between the theoretical molecular masses for ideal dendrimer growth/synthesis and molecular masses determined with MALDI−TOF−MS with increasing number of generation was found. Analysis with MALDI−TOF−MS provided molecular weights up to 40% below the calculated theoretical molecular weight. nESI-GEMMA analysis of the electrophoretic mobility diameter of dendrimer particles of all generations was possible with a precision of ±0.2 nm. All diameter values were in excellent agreement with values found by various other analytical techniques such as SAXS, SANS, TEM, and AFM. The polydispersity of the molecules and the distribution of diameters of the analyzed dendrimer generations were found to be significantly higher than for proteins in the same molecular mass and size range. This effect is probably the result of statistically incomplete attachment of the next dendrimer shell, an error that propagates in dendrimer synthesis as every dendrimer generation is the starting material of the synthesis of the succeeding generation. An attempt to calculate the molecular mass of PAMAM dendrimers via their particle diameter with different approaches resulted in a considerable discrepancy to the MALDI−MS data. The calculated density of the dendrimer particles based on the MALDI−MS and nES−GEMMA data showed a significant difference in protein densities (0.54 kg/L) and densities calculated from molecular dynamic results (0.52 kg/L) as well as densities derived from dendrimer growth theory (up to 1.2 kg/L). Our results indicate that PAMAM dendrimer particles of G4 or higher possess a constant average density of 0.65 kg/L, which is caused by tight packing of the branches inside the particle.

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