Structural and Physicochemical Characteristics of One-Step PAMAM Dendrimeric Nanoparticles

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Abstract

The recently developed One-Step Poly(amidoamine) (OS-PAMAM) dendrimers stand out for their characteristics as the high drug-load capacity and cell-delivery improvement of therapeutic agents. The OS-PAMAM dendrimers have proven to be useful in the biomedical field as nanocarrier or nanosystems for therapy. In the present research it was encouraging to determine their physicochemical characteristics which were compared with commercial PAMAM generation-6 (G6). The spectroscopic measurement of amides, nanoparticle size (10-30 nm), polydispersity index and zeta potential measurements correlates with the commercial product. The OS-PAMAM has cavities detected by AFM, and the force analysis showed the same adhesion force and different elasticity than PAMAM-G6. The molecular weight (MW) was 10 times lower than the commercial one for both techniques employed, resembling the MW of a PAMAM generation-3 (G3). OS-PAMAM/PAMAM-G6 MS-MS mirror plots demonstrate chemical equivalency amongst herein analyzed dendrimers, with the advantage that OS-PAMAM is produced with a faster and low-cost synthetic protocol, which allows them to be applied for both research and industry in the biomedical field.

Keywords: Poly(amidoamine), Nanotechnology, Molecular Weight, PFG-DOSY NMR, orthogonal polydispersity index.

1. Introduction

Polyamidoamine (PAMAM) dendrimers are organic nanoparticles (NPs) that have occupied a prominent position in research and industry because of their well-described characteristics, high molecular loading ability, and their wide range of applicability in fields like biomedicine for controlled drug delivery, gene therapy, theragnostic and vaccines [1,2].

PAMAM dendrimers have an arborescent construction and present a generational growth form, which is based on a nucleus initiator called zero generation (G0), from which are added functional groups in the periphery resulting in homo-structural plates between focal points (branching points). Thus, a dendrimer with six branches from the centre to the periphery is called a dendrimer of generation 6 (G6) [3,4].

Since the pioneer method of synthesis reported by Tomalia et al. 1986 [3], various strategies have been proposed for improving the PAMAM dendrimers synthesis, including divergent and/or convergent synthesis alongside click chemistry and Microwave-Assisted Reactions [5–7]. The divergent synthesis consists of a multifunctional initiator core that grows to the periphery through two reactions where dendrimers can reach generation 10. However, structural defects are a critical concern for high generation dendrimers. They inevitably arise from incomplete reactions or side reactions that occur because of the increasing number of reactions and steric hindrance as the dendrimer grows. Additionally, it is challenging to remove defective structural molecules because of their similar chemical composition [8].

On the other hand, in the convergent synthesis, dendrimers are built from small molecules starting from the surface of the dendrimer to end up attached to a central core through several inward-directed reactions. Otherwise, it can be so much controlled, and the impurities can be separated more easily, but the reactivity to reach the interior core is often considerably reduced because of the increasing steric congestion as the dendrimer generation increases. Therefore, only low-generation dendrimers can be successfully prepared using the convergent method [4,9]. A combined divergent/convergent method has been further developed and applied for dendrimer synthesis. This approach includes the synthesis of building blocks using

a divergent approach followed by the convergent dendrimer assembly [8], also optimized by click chemistry and Microwave-Assisted to deliver simplified and accelerated dendrimer synthesis [6,10]. Nevertheless, the obtention of high generation PAMAM dendrimers in the desired quantity, with consistent quality and low cost and energy expenditure, remains challenging [5].

The recently developed OS-PAMAM dendrimers obtained by a novel synthesis method remain the beneficial characteristics such as high load capacity and the increased efficiency in drug delivery —like the commercial one— previously reported [11]. Moreover, the time, cost, and energy of manufacturing are reduced, enabling their massive production and application as platforms for several molecules in the biomedical field and others [12]. In this work, the novel synthesis method was employed, and the physicochemical characteristics of OS-PAMAM were compared with a commercial PAMAM G6. Hence, the characteristic functional groups expected for PAMAM dendrimers were assigned from ultraviolet-visible spectrophotometry (UV-Vis) and Fourier-Transform Infrared spectroscopy (FT-IR). Particle size distribution as a function of polydispersity indexes (PDI) as well as zeta potential (ZP) were measured by dynamic light scattering (DLS). Polydispersity in solution of both OS-PAMAM and PAMAM G6 was monitored with pulsed-field gradient saturation transfer echo water-presaturation diffusion ordered spectroscopy {PFG-STE-$H_2O_{(presat)}$-DOSY} [13] to obtain diffusion coefficients related to weighted average molecular weights (Ṁw) and PDIs of both PAMAM moieties in the solution state. Furthermore, molecular weights (MW) and PDIs obtained with Size-exclusion chromatography (SEC) were orthogonally compared with data obtained with PFG-STE-$H_2O_{(presat)}$-DOSY as well as with PDIs from DLS. Also, morphology, distribution pattern, and mechanical characteristics in flat surfaces by atomic force microscopy (AFM) were analyzed. Finally, an analysis of MS-ESI-QToF was employed to obtain the experimental high-energy (10-45 eV) *m/z* fragmentation profiles and compare in a mirror plot the m/z shared between OS-PAMAM and PAMAM-G6.

2. Materials and methods

2.1 Materials

The main reactants, methyl acrylate and ethylenediamine (Sigma-Aldrich) were used for the optimized method. The commercial PAMAM dendrimer G6 (PAMAM G6), 5 wt. % in methanol from Merck, USA was used as control. The methanol used for all the synthesis was purchased in J.T. Baker. For PFG-STE-$H_2O_{(presat)}$-DOSY NMR analysis, deuterium oxide (99.9% deuteration, CAS No. 7789-20-0) from Merck Millipore was employed. The water used in all the experiments was purified using a Simplicity® UV Water Purification System (Merck Millipore). All chemicals used in this study were analytically pure.

2.2 Synthesis method

The synthesis of OS-PAMAM dendrimers is explained in patent application No. MXA2018008247 and was carried by modifying different variables such as the proportion of ethylenediamine and methyl acrylate reactants, stirring speed, and vacuum pressure, which allows the generation of temperature gradients throughout the synthesis. In this way, a polymerization is achieved over time and the Michael addition reactions and amidations are modulated in the same reaction. The reaction was carried out in methanol, under vacuum pressure which generated a decreasing temperature gradient throughout the synthesis from 15 °C to 5 °C. The vacuum also promotes solvent elimination as well as low molecular weight molecules, that are byproducts of parallel reactions. Another set of impurities was removed by a dialysis membrane of 2000 kDa. As a result, a yellow viscous solution was obtained, the reaction yield was 45%, and after the purification step by dialysis, it was 23% [14].

2.3 UV-Vis and FT-IR spectroscopy

The UV-Vis was performed in a spectrophotometer (NanoDrop 2000, Thermo Scientific), 20 mg/mL of the OS-PAMAM dialyzed and commercial standard PAMAM-G6 were measured in a range from 200 to 800 nm in type 1 water and

compared through the two main absorption bands at 210-216 nm and 280-285 nm, corresponding to the backbone structure of secondary and tertiary amides, characteristics of PAMAM structures. Further, both synthesized OS-PAMAM and commercial PAMAM-G6 were dried at 65°C, and 5 mg of the samples were characterized by Fourier-transform infrared spectra (FT-IR) to obtain the primary and secondary amides, also characteristic functional groups through ATR (Frontier Optica, PerkinElmer, USA).

2.4 Dynamic Light Scattering

The particle size distribution, PDI, and ZP were measured by dynamic light scattering (DLS). The three batches of OS-PAMAM dendrimers were filtered by an Acrodisc® Syringe Filter of 0.2 µm, suspended in methanol (99.9%) at 20 mg/mL and stored at 2- 8°C. All suspensions (2 mL) were measured at 25°C, and the average value was obtained after two repetitions with 25 accumulations, taking into count the PDI. Also, the average ZP was obtained after one repetition and five accumulates. These characteristics were monitored through the time (0-4 months) and compared with PAMAM G6 stored at the same temperature, concentration, and analytic method in a Particulate Systems (NanoPlus, GA, USA) equipment.

2.5 Size Exclusion Chromatography

The polydispersity of the molecular weight from both OS-PAMAM and PAMAM-G6 dendrimers was determined by size-exclusion chromatography (SEC). A 5 µL-volume of both dendrimer samples (30 mg/mL) was injected into an Acquity UPLC system (Waters®; M.A., USA) equipped with an Acquity UPLC BEH SEC 200 Å column (4.6 mm x 300 mm, 1.7 µm) (Waters®), which was maintained at 30°C during the analysis. The separation was achieved using a 50 mM phosphate/150 mM NaCl buffer solution (pH 6.0) as a mobile phase at 0.4 mL/min (JT. Baker; N.J., USA). Data were acquired at 215 nm and processed using the software Empower. The molecular weight was determined by linear regression of the maximum value in absorbance units (AU) based on the MW standard from 1.5 to 670 kDa, Cat 151-1901 (Biorad, CA, USA).

Three batches of OS-PAMAM and G6 were compared using their PDI values. For this purpose, the SEC profile of each sample was exported from Empower® to Excel (Microsoft, WA, USA) and the PDI values were determined using the method described using formulas 1 to 3 [15]:

$$(1)\ \overline{M}_n = \frac{\sum_i^n (W_i) PW_i}{\sum_i^n (W_i / M_i) PW_i} \qquad (2)\ \overline{M}_w = \frac{\sum_i^n (W_i M_i) PW_i}{\sum_i^n (W_i) PW_i} \qquad (3)\ PDI = \frac{\overline{\mathrm{M}}_\mathrm{w}}{\overline{\mathrm{M}}_\mathrm{N}}$$

Where W_i is the intensity (AU) of the observed size (kDa), PW_i is the percentual intensity (weighted intensity) and M_i is the observed size in the SEC analysis, which was determined using a logarithmic equation determined by the retention time of the components of the MW standard. Structural heterogeneity of dendrimer samples was qualitatively computed in terms of a second derivative analysis of the SEC profile (Figure 4B), whereas homogeneous samples will produce a single antiphase chromatogram pattern, whilst a set of several antiphase patterns are expected within second derivative analysis of heterogeneous samples.

2.6 Atomic Force Microscopy

The dendrimers OS-PAMAM and PAMAM-G6 were analyzed in a Bioscope Catalyst AFM (Bruker, Germany) to know the shape, size, and mechanical properties (adhesion force and elasticity). In the experimental tests, 30 µL of suspension with dendrimers were deposited and dried on a piece of glass slice of dimension 5 mm × 5 mm. The glass slide was first cleaned by immersion in ethanol absolute for 20 min, followed by rinsing with distilled water. It was employed a pyramidal tip SNL Bruker of 2 nm in a 300 nm ramp size with 0.4 of a trig threshold. Before using the Thermal Tune Method, the deflection sensitivity of the cantilever was determined in a liquid fluid by using the value of the inverse of the slope of the force curve while the cantilever was in contact with a hard glass surface to obtain the force measurements. The averages of the deflection sensitivity (s) and elasticity constant (k) determined at five different points were 16.57 nm/V and 0.57 N/m, respectively, and the peak force setpoint was performed at 7Nn. The images were analyzed by using the NanoScope Analysis 1.5 Software.

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2.7 NMR – Pulsed Field Gradient Diffusion Ordered Spectroscopy

For each batch, 540 µL of liquid buffered PAMAM-G6 and OS-PAMAM solution were dissolved in 60 uL of D_2O (99.9% deuteration, CAS No. 7789-20-0), for having a 9:1 ratio of H_2O:D_2O solvent system. All NMR spectra were recorded on a Bruker 600 AVANCE III HD equipped with a 5mm 1H / D TXI probe head with a z-gradient. The following set of NMR experiments were conducted at a controlled temperature of 303 K: a) Standard ^{1}H-one dimensional direct-polarization NMR experiments (Figure 8 and F2-projections in Figure 9A) were carried out with 16 transients of 32768 complex points, having recycling delays of 8 s and with acquisition times of 1700 ms, produced experimental times of 2 minutes 35 seconds per PAMAM dendrimer batch. No apodization function was applied during Fourier Transform for proton spectra depicted in Figures 8A and 8C. In contrast a Resolution Enhancement post-processing was applied for spectra depicted in Figures 8B and 8B with a Lorentz-to-Gauss line-sharpening apodization; b) acquisition and processing details of PFG-STE-H_2O(presat)-DOSY have been recently published [13,16], whereas, for the present study, the following key NMR parameters were used: 64 gradient levels with a linear increase from 2 to 98%, a gradient strength up to 54G/cm and 8 transients per gradient increase comprising 65536 complex points were used. The diffusion delay ($\boldsymbol{\Delta}$) was 100 ms, and the length of the square diffusion encoding gradient pulse (δ) was 4.2 ms to assure accurate gradient encoding-decoding signal attenuation. No apodization function within the direct F2 dimension was needed for semi-Fourier transformation prior to Inverse-Laplace indirect F1 transformation.

In this present work the comparative analysis between OS-PAMAM and PAMAM G6 was performed by DOSY-NMR to obtain the relative MW. Hence, it was measured the diffusion coefficient (D) of each chemical-shift assigned resonance and related with the hydrodynamic radius by the Stokes−Einstein equation (with stick boundary conditions):

$$R_H = \frac{k_B T}{6\pi\eta D}$$

where kB is the Boltzmann constant, T is the temperature (in K), and η is the viscosity of the solution (~9.7 cP for D_2O at 30 °C) [17].

The obtained diffusion coefficients were internally referenced with respect to the water signal at a value of $-7.629 \times 10^{-10} m^2 s^{-1}$ (see figure 8A, diffusion signal correlating with the water ^1H chemical shift at 4.7 ppm). Experimental polydispersity indexes in the solution state (**Đ** in figure 8A) in turn related to weight average molecular weights (**$\dot{M}_w$** in figure 8A) correlated with the experimental diffusion coefficient distributions at the low concentration regime [13] of both PAMAM-G6 and OS-PAMAM dendrimers are reported as figure legends.

2.8 Mass Spectrometry coupled to Liquid Chromatography

The PAMAM-G6 and OS-PAMAM samples were prepared at a concentration of 100 uM with a 50:50 methanol: acetonitrile mixture. Subsequently, 10 ul of each sample was injected into an Acquity® UPLC Class I system (Waters; MA, USA) equipped with a 1.7 µm (2.1 mm x 100 mm) BEH300 C4 column 45°C (Waters®; MA, USA). The samples were eluted using water+formic acid (0.1%) (phase A) and acetonitrile+formic acid (0.1%) (phase B) as follows: 100% of phase A from 0 to 1 min followed by a linear gradient of 100 - 0% from 1 to 11 min of phase A; all reagents used in this analysis were MS grade (Sigma-Aldrich, MO, USA). Then, the mass analysis was performed on a Vion® hybrid Quadrupole Time of Flight Spectrometer equipped with an Electro Spray Ionization (ESI) source. The mass spectrometer was operated in positive polarity and MSE sensitivity mode from 50 to 4000 m/z; the collision energies were 5.00 eV (low) and 10.00 eV to 45.00 eV (high collision energy ramp) with 2 seconds of scanning time. ESI parameters were set at 140°C (source temperature), 600°C (desolvation temperature), 30 eV (cone voltage), 100 L/h (cone gas), 1,200 L/h (desolvation gas), and 2.75 kV (capillary voltage). A 10-pg/µL [Glu1]-Fibrinopeptide B solution (Waters®) was infused during the MS analysis at 20 µL/min for mass correction. Data were acquired and processed using UNIFI® software (version 1.9.4.053) (Waters®), on which the chemical formula the PAMAM and their reported fragments [16] were loaded. The m/z signals of PAMAM and their fragments

were searched in the experimental data acquired from the high energy channel considering a 25 ppm error.

2.9 Statistical analysis

All the obtained results were determined at least in triplicate and analyzed using the SigmaPlot® software (Systat Software Inc., USA). The averages and Pearson linear regressions were determined using Microsoft Excel®, (Microsoft, USA) and differences between groups were analyzed using one-way analysis of variance (ANOVA) and the Student-Newman-Keuls as a post-hoc test. A value of $p \leq 0.05$ was considered statistically significant.

3. Results and discussion

3.1 UV-Vis and FT-IR spectroscopy of OS-PAMAM and PAMAM-G6

The OS-PAMAM dendrimers have presented promising applications within the biomedicine field [9]. Therefore, the characteristics and reference values of OS-PAMAM dendrimers were determined in comparison to the commercial PAMAM-G6. For that, three OS-PAMAM batches were randomly selected and measured by UV-Vis. The maximum absorbance at 210-216 nm and 280-285 nm, corresponding to secondary and tertiary amides, match the wavelengths found in PAMAM-G6 and previous reports, were the height of the peak centered at ~280 nm changes upon protonation of the intradendrimer tertiary amines [18,19] (Figure 1A). Further, this signal of tertiary amines was consistent through the time up to 16 weeks of observation without significant changes, indicating a complete reaction and stability at 5 ± 3 °C (Figure 1B) of a full generation PAMAM dendrimer (Figure 1C).

The functional groups of OS-PAMAM were also compared with PAMAM-G6 by FT-IR. The stretching frequencies of OS-PAMAM at 3270 cm^{-1} of N-H, 1637 cm^{-1} of C=O, 3089 cm^{-1} of C-H, and the N-H bend at 1546 cm^{-1}, match with peaks at 3275 cm^{-1}, 1637 cm^{-1}, 3087 cm^{-1}, and 1547 cm^{-1} corresponding to secondary amides,

respectively, of PAMAM-G6 dendrimers (Figure 2). Those peaks have been widely reported for PAMAM dendrimers across different generations [20,21]

3.2 Size, Polydispersity Index and Zeta potential

To know the OS-PAMAM properties in colloidal solutions and determine its stability at 5 ± 3 °C, a DLS analysis was performed using the PAMAM-G6 as a reference. The average size was obtained through the hydrodynamic size of three batches of OS-PAMAM and the commercial one; all measurements were performed after the same storage conditions at 0, 2, and 4 months (Table 1).

The size was closely related to the PDI, which indicates the heterogeneity of a sample based on size. OS-PAMAM and PAMAM-G6 sized ~ 10 nm with PDI of 0.2 - 0.5; approximately 20 nm when PDI ranges 0.5 - 1.0, and ~ 30 nm indicates PDI values above the unity (Figure 3 A-B). Therefore, a directly proportional relationship between the size and the PDI for OS-PAMAM and PAMAM-G6 with R^2 =0.9127 (Figure 3A) and R^2 =0.8278 (Figure 3B), respectively, across independent batches and measurements was determined. Although there was no significant increase in the average size or PDI, as a function of storage time in most batches of OS-PAMAM and PAMAM G6, batch 3 presented an increase in polydispersity and size. Polydispersity can occur due to size distribution or agglomeration of the OS-PAMAM during analysis. Other studies have demonstrated that a higher PDI number indicates that NPs are less monodisperse, with a larger size, and will be more likely to form agglomerates over time [22,23]. Furthermore, the ZP of these two NPs was compared, whereas values of ~ 50 mV were found for PAMAM-G6 and ~ 14 mV for OS-PAMAM (Figure 3-C and D). The ZP differences between dendrimers could be attributed to the generation since an increase in zeta potential has been observed as a function of generation [24]. However, it should be considered that ZP values are not an absolute measurement of NPs stability. For example, solid lipid nanoparticles with positive ZP have a long-circulating half-life due to the absorption of protein components in the blood, while NPs with negative ZP can be cleared by the reticuloendothelial system. Furthermore, this parameter is very important for biomedical applications because toxicity has been linearly correlated with high

positive values of ZP [25,26]. Indeed, controlling and validating these parameters from the synthesis is crucial for the useful applications of dendrimeric NPs in different fields [27,28].

3.3 Molecular weight comparisons of OS-PAMAM and PAMAM-G6

The molecular weight (MW), and reproducibility between batches were measured by size exclusion chromatography (SEC). All batches of OS-PAMAM (green, blue and orange chromatograms, figure 4) and PAMAM-G6 (black, Figure 4) respectively present a maximum in their chromatograms at 4.55 min and 3.2 minutes. Both MW were calculated by linear regression of the MW marker curve (red in figure 4A), resulting in 8.5 kDa and 86 kDa for the three OS-PAMAM batches and PAMAM-G6 correspondingly (Figure 4A). The MW of PAMAM-G6 was higher in comparison to the theoretical MW reported (58,048 kDa). Differences between theoretical and experimental MW values of the dendrimers may be associated with various phenomena, such as nonspecific interactions between PAMAM-G6 and SEC column [29]. According to other reports, the intrinsic viscosity of the dendrimers decreases as generation increases, which could affect the measurement of low and high generations with the same analytical method, i.e., G3 vs G6 [30,31].

The average PDI obtained in the SEC analysis for OS-PAMAM batches was 1.05 ± 0.039, whilst a polydispersity index of 3.16 was computed for PAMAM-G6 (Table 2), in clear agreement with the overestimated 86 kDa SEC molecular weight of said dendrimer. Interestingly, the SEC distribution pattern is wider in OS-PAMAM, suggesting a larger structural heterogeneity. In contrast, the chromatographic peak of the PAMAM-G6 -with a sharper chromatogram linewidth- indicates structural homogeneity of the more dispersed dendrimer. This physicochemical difference amongst products is unambiguously revealed when their SEC profiles are subjected to a Second Derivative Analysis (**ΔΔ**Absorbance, Figure 4B): A single antiphase signal is revealed for the structurally homogeneous PAMAM-G6 dendrimer, as a result of deconvoluting the SEC chromatogram with the **ΔΔ**Absorbance procedure For the contrary, the **ΔΔ**Absorbance deconvolution of broad OS-PAMAM chromatographic SEC peaks produces a more complex pattern, as the result of a

weighted sum of several antiphase peaks, as a consequence of the structural heterogeneity that One-Step PAMAM moieties are presenting. To the best of our knowledge, this post-processing SEC treatment has not been presented so far as a qualitative method for revealing structural heterogeneity of polyamidoamide dendrimers. In addition, when OS-PAMAM and PAMAM-G6 are analyzed by SEC at pH 2.5 both products reduce their hydrodynamic ratio as both polydispersions elute at shorter retention times [15] but the peak of OS-PAMAM widens whereas PAMAM-G6 gets sharper. This suggests that OS-PAMAM polydispersity includes several structures with different pKa whereas PAMAM-G6 has a reduced number of structures with a closer pKa among them. Besides, OS-PAMAM may include structures different to PAMAM. A previous MW report of commercial PAMAM G5 by SEC has indicated the presence of trailing generation impurities and oligomerized defects, like for instance, non-ideal amidation, that can represent approximately 30% of the sample by weight (Figure S1) [32]. For instance, in comparisons, the higher peak should be taken as the full-body PAMAM structure [33,34].

Despite the structural heterogeneity of OS-PAMAM, the three tested batches evinced the same behavior, which may facilitate the reproducibility of its biologic application (Figure S2) [35,36]. Several studies have demonstrated the encapsulation efficiency as well as biocompatibility of highly dendronized polymers, indicating its potential biomedical application [37–39].

3.4 Comparative analysis of size, shape, and force measurements by AFM

The shape of OS-PAMAM and PAMAM-G6 was compared by AFM. The images showed the distribution over the slide for both NPs. The average size was measured through a maximum vertical radius (Rz) of cross-sections in particles analyzed in all images from different fields over the slide. A similar Rz (height) of 14.21 ± 2.81 nm and 13.30 ± 2.56 nm for OS-PAMAM and PAMAM-G6, respectively, were obtained (Figure 5A-B). Further, a mostly spherical shape for both NPs was observed, as reported in other studies [40,41]. However, superficial irregularities in OS-PAMAM dendrimers were observed, suggesting the presence of cavities formed between

393 ramifications which explain the minor MW of OS-PAMAM, and the collapsed
394 structure on the slide, which can form agglomerates (Figure 4, 5-C).

395 The force measurements of OS-PAMAM and PAMAM G6 dendrimer was also
396 obtained by AFM, comparing the differences in adhesion and elasticity (DMT
397 Modulus) between the surface of the glass and each nanomaterial. The analysis was
398 performed by pushing the AFM tip onto the surface of the samples and monitoring
399 force-versus-distance curves. Automatic analysis of curves using NanoScope
400 Analysis Software generates maps of mechanical properties distribution with
401 topographical imaging [42] (Figure 6A-7A). The highest value of several cross-
402 sections of adhesion (nN) and elasticity (MPa) from 50 NPs was average. For
403 adhesion, the average Rz of OS-PAMAM and PAMAM-G6 were 21.878 ± 3.67 nN
404 and 18.18 ± 3.55 nN, respectively, and they did not present significant differences
405 and the interaction between the dendrimers and the AFM tip employed were similar
406 (Figure 6B). The OS-PAMAM presented a larger adhesion force than reported for
407 PAMAM-G4-NH_2 with different tip substrate [43]. Adhesion between the polymer
408 surface and AFM-tip is controlled by the chemical groups at or near the surface.
409 Despite the low MW of OS-PAMAM on comparison to PAMAM G6, the similar
410 adhesion force could be due to related surface functional groups between both
411 dendrimers [44,45]. Dendrimers in biomedical applications interact with cargo
412 molecules and cells, and the force of adhesion provides the fundamental basis for
413 the hypothetical notion that high binding avidity [46].

414 Regarding the elasticity in Derjaguin-Muller-Toporov (DMT) modulus maps, the
415 samples themselves are softer and thickness than the glass. Differential Rz average
416 values were 27.69 ± 3.11 MPa and 37.80 ± 4.42 MPa for OS-PAMAM and PAMAM-
417 G6, respectively, with significant differences between dendrimers (Figure 7A). In
418 general, the OS-PAMAM DMT modulus values decreased significantly compared
419 with PAMAM-G6 (Figure 7B). An increase in elasticity concerning the molecular
420 weight and density of the NPs has been observed [42,47]. Besides, a much higher
421 elastic modulus has been observed for individual G4 than G3 dendrimers molecules
422 due to their more shape persistent conformation [48]. Hence some biophysical

characteristics of OS-PAMAM aggregates surfaces given by the adhesion were comparable to PAMAM-G6. Otherwise, the DMT modulus differences suggest that OS-PAMAM has a more flexible conformation than PAMAM-G6.

3.5 Structural analysis, diffusion coefficient distributions, weighted average molecular weight, polydispersity indexes and structural homogeneity of OS-PAMAM and PAMAM-G6 by Pulsed-Field Gradient Diffusion Ordered (PFG-DOSY) NMR spectroscopy

OS-PAMAM dendrimers were synthesized with the use of a modified Michael addition method between ethylenediamine and acrylic acid methyl ester; commonly, PAMAM has polar pockets comprised mainly of alkyl chains, tertiary amines, and amido scaffolds [14]. Proton nuclear magnetic resonance (^{1}H NMR) has provided a robust analysis for structural studies and identity evaluation of PAMAM dendrimers. Also, PAMAM-drug hosts interactions were previously analyzed by ^{1}H NMR titrations [49] through dipolar NOESY correlation-exchanges [50] and PFG NMR [51] ^{1}H NMR Chemical shift assignment of PAMAM dendrimer has been described [52]: 2.39 ppm (120H, br, −NCH_2 CH_2 CONH−, Ha); 2.60 ppm (56H, br, −CONH CH_2 CH_2 N−, Hb); 2.69 ppm (120H, br, −NCH_2 CH_2 CONH−, Hc); 2.79 ppm (64H, br, −$CONHCH_2$ CH_2 NH_2 , Hb′); 3.21 ppm (56H, br, −$CONHCH_2$ CH_2 N−, Hd); 3.26 ppm (64H, br, −$CONHCH_2$ CH_2 NH_2, Hd′).

First, standard ^{1}H-one dimensional direct-polarization NMR spectra of commercial PAMAM-G6 (Figure 8A and B) and OS-PAMAM (Figure 8C and D) with identical post-processing treatment, reveal subtilities comprising structural heterogeneity and polydispersity of both samples. Spectra from figures 8A and 8C were only Fourier Transformed with no post-processing treatment, whilst Figures 8B and 8D were apodized with a Lorentz-to-Gauss line sharpening of 6 Hz and a Gaussian center position at 20% of the Free Induction Decay (FID) for a resolution enhancement, despite a dramatic loose of sensitivity. In a first glance, ^{1}H-one dimensional direct-polarization NMR spectra of PAMAM-G6 with no application of any weighting

function (Figure 8A), reveal linewidths at half height (LWHH) of 15.71 Hz for −CONH **CH_2** CH_2 N− resonances at δ= 3.39 ppm and 3.025 ppm, and even broader LWHHs of 63.72 Hz (δ= 2.78 ppm, −CONH CH_2 **CH_2** N- spin system), 56.74 Hz (δ= 2.59 ppm, −N**CH_2** CH_2 CONH− spin system), 20.95 Hz (δ= 2.42 ppm, −CONH CH_2 **CH_2** N− spin system) and 21.82 Hz (δ= 2.34 ppm, −NCH_2 **CH_2** CONH− spin system). Broader ^{1}H LWHH in OS-PAMAM spectrum with no post-processing (Figure 8C) does not have an origin from higher polydispersity but rather due to higher structural heterogeneity [35] as unambiguously revealed in Figure 8D, in comparison to Figure 8B. Resolution enhancement achieved with identical Lorentz-to-Gauss weighting functions in both dendrimers ^{1}H NMR spectra produces different results. Line sharpening apodization applied to PAMAM-G6 proton spectrum produces equivalent LWWHs (Figure 8B) as when no apodization function was applied (Figure 8A). In contrast, broad resonances of OS-PAMAM ^{1}H NMR spectrum (Figure 8C) are deconvoluted into narrower frequencies of c.a. 1 to 1.5 Hz of LWHHs (Figure 8D) when resolution enhancement is achieved with a Lorentz-to-Gauss apodization function identically applied as in PAMAM-G6 ^{1}H spectrum (Figure 8B). The set of narrow resonances revealed in Figure 8D strongly suggest that OS-PAMAM presents an important structural heterogeneity due to defects in the dendrimer structure during the synthesis (Section 2.2) and a lack of dendrimer purity such as with the commercial PAMAM-G6.

Analysis of two-dimensional PFG-STE-H2O(presat)-DOSY spectra of both dendrimers demonstrate at first glance, differences in diffusion coefficient distributions between both dendrimers: the OS-PAMAM showed higher diffusion coefficient distributions around a lower logarithmic diffusion coefficient average of **Log DC_{av}** = -8.8 m^2s^{-1} ± 0.05 than the PAMAM-G6 counterpart (**Log DC_{av}** = -9.6 ± 0.05 m^2s^{-1}, Figure 9A). Furthermore, experimental solution-state DOSY-MW distributions obtained from diffusion coefficient distributions [13] are represented as histograms (Figure 9B) and expressed in Da for PAMAM dendrimers ranging from G1 to G6. PAMAM-G6 weighted average molecular weight of MW= 55711.86 ± 0.7 corresponds to the theoretical value elsewhere reported [53], whilst OS-PAMAM weighted average molecular weight of MW= 5056.47 ± 0.7 strongly suggest the

presence of a PAMAM-G3 dendrimer, in full agreement with SEC data, predicting an average MW of 8.5kDa for OS-PAMAM (Figure 4). Finally, solution-state polydispersity indexes (PDI, **Đ** in figure 9A) are experimentally obtained from the molecular weight distributions at the low concentration regime from the experimental average diffusion coefficient distributions (**Log DC_{av}** in Figure 9A) and the width of each distribution, represented by the experimental diffusion coefficient distributions [13]. Table 2 resumes the solution-state MW and PDIs of OS-PAMAM and PAMAM-G6 obtained with non-invasive DOSY-NMR spectroscopy for DLS (Table 1) and SEC (Figure 4), finding orthogonality amongst the three techniques. Present results are in agreement with other studies reporting that PAMAM G3 is around 7 kDa [29,54]. Moreover, lower logarithmic diffusion coefficient average of OS-PAMAM (**Log DC_{av}** = -8.8 m^2s^{-1} ± 0.05) that in turn weights higher diffusion coefficient distributions responsible to produce lower PDI and average MW values, with respect PAMAM-G6, as revealed with DLS, SEC and PFG DOSY NMR (Table 2), in combination with the observed set of narrow resonances in ^{1}H NMR spectrum apodized with a line sharpening weighting function (Figure 8D), allows to conclude that herein produced OS-PAMAM presents lower polydispersity but higher structural heterogeneity, with respect its more polydisperse but more homogeneous PAMAM-G6 counterpart.

In complement, the molecular weight of G4 dendrimer is 14195 Da measured by MALDI-TOF mass spectrometry, whereas the associated polydispersity index (PDI) of the said dendrimer is 1.01, obtained by gel permeation chromatography [51]. All these data suggest that the molecular weight of OS-PAMAM dendrimer equates to a PAMAM-G3.

3.6 Structural characterization by MS-MS

As part of the physicochemical characterization of OS-PAMAM and PAMAM-G6, and taking advantage of the sensitivity of MS-ESI-QToF, we obtained their experimental high-energy (10-45 eV) *m/z* fragmentation profiles and compared in a mirror plot, as shown in Figure 10. It was found that in the range of 50 - 600 *m/z* both samples share, among other masses, the values of *m/z* 571.4013, 457.3241, 367.2433,

355.2460, 343.2431, 325.2336, 241.1646, 229.1643, 199.1542, 127.0851, 98.0609 and 85.0761. These *m/z* values belong to the decomposition pathway by retro-Michael reactions for the species [M + 5H] 5+, [M + 7H] 7+ and [M + 9H] 9+ of the PAMAMs of G1 G2 and G3 respectively. This correspondence with the values calculated for the ionic precursors and the path of the fragments for G0 (Table of Figure 10) demonstrate the identity of OS-PAMAM. On the other hand, in the enlarged section of Figure 10, from 471 to 482 *m/z*, the species of 479.0647 *m/z* reported for the intramolecular cyclization of the ester group was not found, which is one of the typical side-products of the Michael addition. This indicates that during the synthesis of OS-PAMAM cyclic side-product are not formed [55].

In addition, to determine the reproducibility between OS-PAMAM batches the high-energy *m/z* profiles of batch 1 and 2 were compared. It was found that both batches are highly similar containing charge mass values of the characteristic fragments of the G0, G1, G2 and G3 dendrimers at the same relative intensity (Figure S3). The identification of the most abundant fragments of OS-PAMAM will be useful for biomedical applications such as for determining the elimination pathways in pharmacokinetic surveys using urine as the main elimination via ([26,56,57].

4. Conclusion

In summary, we performed a detailed physicochemical characterization of OS-PAMAM compared with commercial PAMAM-G6. Our finding reveals that OS-PAMAM is a dendrimer with lower polydispersity but higher structural heterogeneity, with respect its G6 commercial counterpart. Size exclusion chromatography and nuclear magnetic resonance spectroscopy have found excellent orthogonality to demonstrate the polydispersity and structural homogeneity of herein analyzed dendrimers. For the first time, two postprocessing treatments - **ΔΔ**Absorbance in SEC and Lorentz-to-Gauss resolution enhancements in NMR spectra have revealed by their principles, the structural heterogeneity of herein produced OS-PAMAM, that could in turn be used to reveal defects within the dendrimer structure as well as the

purity of novel or traditional PAMAM synthetic approaches. Interestingly, OS-PAMAM structural heterogeneity and polydispersity is conserved amongst batches. That relation not only remains between repetitions but also over time up to 4 months at 5 ± 3 °C. In general, the ZP of OS-PAMAM were less positive than PAMAM-G6. However, ZP was consistent between batches and time for both dendrimers. The AFM morphological analysis showed a circular shape in all dendrimers, and cavities were detected in OS-PAMAM. Further, the adhesion force did not present significant differences, while the elasticity (DMT Modulus) was higher in PAMAM-G6, which represents a similar chemical surface but a different internal structure. The MW of OS-PAMAM is around 10 times smaller than commercial G6, as orthogonally detected by SEC and PFG-STE-H2O(presat)-DOSY, whereas lower OS-PAMAM masses are explained due to their cavities. OS-PAMAM / PAMAM-G6 MS-MS mirror plots demonstrate chemical equivalency amongst herein analyzed dendrimers, with a slight advantage that OS-PAMAM is produced with a faster and low-cost synthetic protocol. These novel NPs has been previously studied as a drug delivery system [11]; however, more biological studies will be carried out to compare the biomedical applicability with commercial PAMAM, taking into account the structural heterogeneity, polydispersity and synthetic reproducibility.

5. Glossary

OS-PAMAM: PAMAM dendrimers obtained by a One-Step method of synthesis

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7. Disclosure

The author reports no conflicts of interest in this work.

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Tables

Table 1. Average particle size, polydispersity index, and zeta potential of OS-PAMAM and PAMAM-G6 through 4 months at 5 ± 3 °C.

		Month	Average size ± SD (nm)	PDI	Average ZP ± SD (mV)
OS-PAMAM	Batch 1	0	20.4 ± 0.7 20.4 ± 0.7	0.700 0.747	14.39 ± 0.46
		2	10.2 ± 0.4 30.6 ± 1.1	0.478 0.99	11.2 ± 1.39
		4	20.5 ± 0.8 10.3 ± 0.4	0.765 0.47	9.94 ± 2.15
	Batch 2	0	10.2 ± 0.3 20.4 ± 0.7	0.255 0.761	16.64 ± 1.04
		2	20.4 ± 0.7 10.2 ± 0.3	0.860 0.276	16.11 ± 1.02
		4	11.6 ± 1.4 10.2 ± 0.3	0.302 0.246	11.83 ± 1.64
	Batch 3	0	20.4 ± 0.7 10.2 ± 0.4	0.584 0.372	12.22 ± 3.17
		2	20.5 ± 0.8 30.6 ± 1.1*	0.848 1.194	9.62 ± 1.14
		4	30.6 ± 1.1* 30.6 ± 1.1*	1.060 1.172	8.88 ± 1.13
PAMAM G6		0	20.5 ± 0.8 20.6 ± 0.9 30.9 ± 1.4	0.814 0.753 1.019	49.22 ± 1.09
		2	10.3 ± 0.4 20.6 ± 0.9 24.8 ± 5	0.451 0.912 0.934	57.02 ± 2.34
		4	20.8 ± 1 10.7 ± 0.5 30.7 ± 2.8	0.567 0.487 1.231	46.99 ± 2.05

Note. Data represent the mean ± SD (n=25) of 2 and 3 independent measurements of OS-PAMAM and PAMAM G6 respectively. Significative differences between months * $P < 0.01$ with respect to time zero.

Table 2. Orthogonality between polydispersity indexes (PDI, **Đ** in figure 8A) and average molecular weights (Da) of OS-PAMAM and PAMAM-G6 obtained in the present study with Dynamic Light Scattering (DLS), Size Exclusion Chromatography (SEC) and Diffusion Ordered (DOSY) NMR spectroscopy. Zeta Potential (ZP) is highlighted for both dendrimers. PDIs with DLS herein reported were constructed by the arithmetical average and standard deviations of the full set of both OS-PAMAM batches and PAMAM-G6 dendrimers through four months reported in Table 1. The SEC-PDI of OS-PAMAM here reported comes from the analysis of three batches.

	DLS		**SEC**		**DOSY**	
	OS-PAMAM	PAMAM-G6	OS-PAMAM	PAMAM-G6	OS-PAMAM	PAMAM-G6
PDI	0.671 ± 0.31 ZP~14 mV	0.796 ± 0.24 ZP~ 20 mV	1.05 ± 0.039	3.16	1.005 ± 0.05	1.02 ± 0.05
MW			8500	83000	5056.47	55711.86

Captions

Figure 1. UV-Vis spectra of OS-PAMAM and PAMAM-G6. (A) Spectrum with characteristic signals of three independent production batches of OS-PAMAM vs PAMAM G6. (B) The signals corresponding to tertiary amides from 280 to 285 nm were taken as a reference value to monitor batches' integrity and control PAMAM-G6 through time until four months at 5 ± 3 °C.(C) Structure of generation 3 (G3) PAMAM dendrimer with amino groups at the periphery. The ethylenediamine core is circumvented by a dashed line, reproduced from Abbasi et al., 2014 [4].

Figure 2. FT-IR spectra of compared dendrimers. The signals of OS-PAMAM (black) at 3270 cm^{-1} / 1637 cm^{-1} and 3089 cm^{-1} / 1546 cm^{-1} correspond with the signals at 3275 cm^{-1} / 1637 cm^{-1} of primary and 3087 cm^{-1} / 1547 cm^{-1} of secondary amides, respectively, from PAMAM-G6 dendrimers (blue).

Figure 3. Size and polydispersity index (PDI) relation and potential zeta profile. The average hydrodynamic sizes as a function of the polydispersity index (PDI) of (A) OS-PAMAM (n = 18) and (B) PAMAM G6 (n = 9) were determined. Representative zeta potential profile of (C) OS-PAMAM and (D) PAMAM G6. All data were obtained under the same analytic method conditions by dynamic light scattering (DLS).

Figure 4. Molecular weight (MW) between OS-PAMAM batches and PAMAM G6 by SEC. (A) Chromatogram of PAMAM-G6 dendrimer at 215 nm (black line) was compared to three independent batches of OS-PAMAM (green, blue, and orange line) at pH 6.8. MW was determined by linear regression of the maximum value in absorbance units (A.U.) based on the MW standard curve (data not showed). (B) Second derivative analysis of OS-PAMAM batches and PAMAM G6.

Figure 5. Size and morphological analysis of dendrimers by AFM. Heightmap of (A) PAMAM-G6 and (B) OS-PAMAM, both average sizes were analyzed through the maximum vertical radius (Rz) of cross-sections (n=50). (C) The dotted line represents a close-up of the 3D image of the typical shape of OS-PAMAM and PAMAM-G6 dendrimers.

Figure 6. AFM adhesion force profiles between dendrimers. (A) 2D (left) and 3D (right) topographical imaging of OS-PAMAM and PAMAM-G6 was performed through automatic

analysis of curves using NanoScope Analysis Software monitoring force-versus-distance curves. (B) Average adhesion force values (nN) of cross-sections from OS-PAMAM and PAMAM-G6. Data represent mean ± S.D. (n=50).

Figure 7. AFM elasticity modulus differences between OS-PAMAM and PAMAM G6. (A) The elasticity in DMT modulus maps of OS-PAMAM and PAMAM-G6 was performed through automatic analysis of curves using NanoScope Analysis Software comparing hard (slide) and elastic (dendrimers) surfaces. (B) Average elasticity values (MPa) of cross-sections from OS-PAMAM and PAMAM-G6. Data represent mean ± SD (n=50), * P< 0.01 vs OS-PAMAM.

Figure 8. Standard ^{1}H-one dimensional direct-polarization NMR spectra. Spectra of PAMAM-G6 (blue) and OS-PAMAM (red) with any apodization prior to Fourier Transform (A and C) and proton spectra weighted with a Lorentz to Gauss line sharpening apodization function (B and D) used to reveal sample heterogeneity.

Figure 9. Solution-state PFG-STE-H2O(presat)-DOSY of PAMAM-G6 and OS-PAMAM. For obtaining average diffusion coefficient distributions (DCD), weighted average molecular weights and polydispersity indexes. (A) PFG-STE-H2O (presat)-DOSY–F2 assigned- NMR spectra of PAMAM batches: Commercial G6 PAMAM (Black) and synthetized OS-PAMAM (gray). Below each logarithmic diffusion coefficient average (Ln DCav, highlighted with a horizontal dotted line) are reported the weight-average molecular weights ($\dot{M}w$) and the polydispersity indexes (PDI) per case. (B) Relative molecular weights [Mw = f(DCD, DCav)] of each chemical-shift assigned resonance obtained from DCD and DCav relationships represented as histograms and expressed in Daltons, are compared with reported molecular weights of PAMAM dendrimers by generation (G1: 1430 Da; G2: 3256 Da; G3: 6909 Da; G4: 14215 Da; G5: 28826 Da and G6: 58048 Da, from http://www.dendritech.com/pamam.html).

Figure 10. LC/ESI-QTOF-MS in high energy spectrum of precursor and fragmentation ions of OS-PAMAM compared with commercial PAMAM-G6. The precursor ions shared between OS-PAMAM and PAMAM-G6 were indicated according to the theoretical values reported for PAMAM G0-G3 and were colored in black. The precursor ions (m/z) of OS-PAMAM dendrimers dominated by a common fragmentation pattern based on the retro-Michael

mechanism were colored in blue. The table below summarizes the theoretical m/z values of the OS-PAMAM and the PAMAM-G6 compared. All OS-PAMAM values present an error of less than 10 ppm concerning the theoretical one. *The theoretical values were obtained from Ulaszewska et al., 2013 [55].

Figure S1. Ideal (A) and non-ideal (B) amidation of PAMAM G0 dendrimers.

Figure S2. Molecular weight (MW) of OS-PAMAM and PAMAM G6 by SEC. Chromatogram at 215 nm of PAMAM-G6 dendrimer (blue line) compared to 3 independent batches of OS-PAMAM (green, red, and purple line) at pH 2.5 condition before five months stored at commercial conditions (methanol at 5 ± 3 °C). MW of OS-PAMAM batches was calculated considering PAMAM-G6 as reference.

Figure S3. LC/ESI-QTOF-MS in high energy spectrum of precursor and fragmentation ions of OS-PAMAM batches. The precursor ions shared between OS-PAMAM batch 1 (L1) and batch 2 (L2) indicate the reproducibility of the synthesis method between batches.