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1 **Structural and physicochemical characteristics of One-** 2 **Step PAMAM dendrimeric nanoparticles**

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43 **Abstract**

44

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

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

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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

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

107 The recently developed OS-PAMAM dendrimers obtained by a novel synthesis
108 method remain the beneficial characteristics such as high load capacity and the
109 increased efficiency in drug delivery —like the commercial one— previously reported
110 [11]. Moreover, the time, cost, and energy of manufacturing are reduced, enabling
111 their massive production and application as platforms for several molecules in the
112 biomedical field and others [12]. In this work, the novel synthesis method was
113 employed, and the physicochemical characteristics of OS-PAMAM were compared
114 with a commercial PAMAM G6. Hence, the characteristic functional groups expected
115 for PAMAM dendrimers were assigned from ultraviolet-visible spectrophotometry
116 (UV-Vis) and Fourier-Transform Infrared spectroscopy (FT-IR). Particle size
117 distribution as a function of polydispersity indexes (PDI) as well as zeta potential
118 (ZP) were measured by dynamic light scattering (DLS). Polydispersity in solution of
119 both OS-PAMAM and PAMAM G6 was monitored with pulsed-field gradient
120 saturation transfer echo water-presaturation diffusion ordered spectroscopy {PFG-
121 STE-H₂O_(presat)-DOSY} [13] to obtain diffusion coefficients related to weighted
122 average molecular weights (\bar{M}_w) and PDIs of both PAMAM moieties in the solution
123 state. Furthermore, molecular weights (MW) and PDIs obtained with Size-exclusion
124 chromatography (SEC) were orthogonally compared with data obtained with PFG-
125 STE-H₂O_(presat)-DOSY as well as with PDIs from DLS. Also, morphology, distribution
126 pattern, and mechanical characteristics in flat surfaces by atomic force microscopy
127 (AFM) were analyzed. Finally, an analysis of MS-ESI-QToF was employed to obtain
128 the experimental high-energy (10-45 eV) m/z fragmentation profiles and compare in
129 a mirror plot the m/z shared between OS-PAMAM and PAMAM-G6.

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133 **2. Materials and methods**

134 **2.1 Materials**

135 The main reactants, methyl acrylate and ethylenediamine (Sigma-Aldrich) were used
136 for the optimized method. The commercial PAMAM dendrimer G6 (PAMAM G6), 5
137 wt. % in methanol from Merck, USA was used as control. The methanol used for all
138 the synthesis was purchased in J.T. Baker. For PFG-STE-H₂O_(presat)-DOSY NMR
139 analysis, deuterium oxide (99.9% deuteration, CAS No. 7789-20-0) from Merck
140 Millipore was employed. The water used in all the experiments was purified using a
141 Simplicity® UV Water Purification System (Merck Millipore). All chemicals used in
142 this study were analytically pure.

143 **2.2 Synthesis method**

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

156 **2.3 UV-Vis and FT-IR spectroscopy**

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

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

167 **2.4 Dynamic Light Scattering**

168 The particle size distribution, PDI, and ZP were measured by dynamic light scattering
169 (DLS). The three batches of OS-PAMAM dendrimers were filtered by an Acrodisc®
170 Syringe Filter of 0.2 µm, suspended in methanol (99.9%) at 20 mg/mL and stored at
171 2- 8°C. All suspensions (2 mL) were measured at 25°C, and the average value was
172 obtained after two repetitions with 25 accumulations, taking into count the PDI. Also,
173 the average ZP was obtained after one repetition and five accumulates. These
174 characteristics were monitored through the time (0-4 months) and compared with
175 PAMAM G6 stored at the same temperature, concentration, and analytic method in
176 a Particulate Systems (NanoPlus, GA, USA) equipment.

177 **2.5 Size Exclusion Chromatography**

178 The polydispersity of the molecular weight from both OS-PAMAM and PAMAM-G6
179 dendrimers was determined by size-exclusion chromatography (SEC). A 5 µL-
180 volume of both dendrimer samples (30 mg/mL) was injected into an Acquity UPLC
181 system (Waters®; M.A., USA) equipped with an Acquity UPLC BEH SEC 200 Å
182 column (4.6 mm x 300 mm, 1.7 µm) (Waters®), which was maintained at 30°C during
183 the analysis. The separation was achieved using a 50 mM phosphate/150 mM NaCl
184 buffer solution (pH 6.0) as a mobile phase at 0.4 mL/min (JT. Baker; N.J., USA).
185 Data were acquired at 215 nm and processed using the software Empower. The
186 molecular weight was determined by linear regression of the maximum value in
187 absorbance units (AU) based on the MW standard from 1.5 to 670 kDa, Cat 151-
188 1901 (Biorad, CA, USA).

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

$$193 \quad (1) \bar{M}_n = \frac{\sum_i^n (W_i)PW_i}{\sum_i^n (W_i/M_i)PW_i} \quad (2) \bar{M}_w = \frac{\sum_i^n (W_i M_i)PW_i}{\sum_i^n (W_i)PW_i} \quad (3) PDI = \frac{\bar{M}_w}{\bar{M}_n}$$

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

202 **2.6 Atomic Force Microscopy**

203 The dendrimers OS-PAMAM and PAMAM-G6 were analyzed in a Bioscope Catalyst
204 AFM (Bruker, Germany) to know the shape, size, and mechanical properties
205 (adhesion force and elasticity). In the experimental tests, 30 μ L of suspension with
206 dendrimers were deposited and dried on a piece of glass slice of dimension 5 mm \times
207 5 mm. The glass slide was first cleaned by immersion in ethanol absolute for 20 min,
208 followed by rinsing with distilled water. It was employed a pyramidal tip SNL Bruker
209 of 2 nm in a 300 nm ramp size with 0.4 of a trig threshold. Before using the Thermal
210 Tune Method, the deflection sensitivity of the cantilever was determined in a liquid
211 fluid by using the value of the inverse of the slope of the force curve while the
212 cantilever was in contact with a hard glass surface to obtain the force measurements.
213 The averages of the deflection sensitivity (s) and elasticity constant (k) determined
214 at five different points were 16.57 nm/V and 0.57 N/m, respectively, and the peak
215 force setpoint was performed at 7Nn. The images were analyzed by using the
216 NanoScope Analysis 1.5 Software.

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219 2.7 NMR – Pulsed Field Gradient Diffusion Ordered Spectroscopy

220 For each batch, 540 μL of liquid buffered PAMAM-G6 and OS-PAMAM solution were
221 dissolved in 60 μL of D_2O (99.9% deuteration, CAS No. 7789-20-0), for having a 9:1
222 ratio of $\text{H}_2\text{O}:\text{D}_2\text{O}$ solvent system. All NMR spectra were recorded on a Bruker 600
223 AVANCE III HD equipped with a 5mm $^1\text{H} / \text{D}$ TXI probe head with a z-gradient. The
224 following set of NMR experiments were conducted at a controlled temperature of
225 303 K: a) Standard ^1H -one dimensional direct-polarization NMR experiments (Figure
226 8 and F2-projections in Figure 9A) were carried out with 16 transients of 32768
227 complex points, having recycling delays of 8 s and with acquisition times of 1700 ms,
228 produced experimental times of 2 minutes 35 seconds per PAMAM dendrimer batch.
229 No apodization function was applied during Fourier Transform for proton spectra
230 depicted in Figures 8A and 8C. In contrast a Resolution Enhancement post-
231 processing was applied for spectra depicted in Figures 8B and 8B with a Lorentz-to-
232 Gauss line-sharpening apodization; b) acquisition and processing details of PFG-
233 STE- H_2O (presat)-DOSY have been recently published [13,16], whereas, for the
234 present study, the following key NMR parameters were used: 64 gradient levels with
235 a linear increase from 2 to 98%, a gradient strength up to 54G/cm and 8 transients
236 per gradient increase comprising 65536 complex points were used. The diffusion
237 delay (Δ) was 100 ms, and the length of the square diffusion encoding gradient pulse
238 (δ) was 4.2 ms to assure accurate gradient encoding-decoding signal attenuation.
239 No apodization function within the direct F2 dimension was needed for semi-Fourier
240 transformation prior to Inverse-Laplace indirect F1 transformation.

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

$$246 R_H = \frac{k_B T}{6\pi\eta D}$$

247 where k_B is the Boltzmann constant, T is the temperature (in K), and η is the viscosity
248 of the solution (~ 9.7 cP for D_2O at $30^\circ C$) [17].

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

256 **2.8 Mass Spectrometry coupled to Liquid Chromatography**

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

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

278 2.9 Statistical analysis

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

285

286 3. Results and discussion

287

288 3.1 UV-Vis and FT-IR spectroscopy of OS-PAMAM and PAMAM-G6

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

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

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

306 3.2 Size, Polydispersity Index and Zeta potential

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

312 The size was closely related to the PDI, which indicates the heterogeneity of a
313 sample based on size. OS-PAMAM and PAMAM-G6 sized ~ 10 nm with PDI of 0.2
314 - 0.5; approximately 20 nm when PDI ranges 0.5 - 1.0, and ~ 30 nm indicates PDI
315 values above the unity (Figure 3 A-B). Therefore, a directly proportional relationship
316 between the size and the PDI for OS-PAMAM and PAMAM-G6 with $R^2 = 0.9127$
317 (Figure 3A) and $R^2 = 0.8278$ (Figure 3B), respectively, across independent batches
318 and measurements was determined. Although there was no significant increase in
319 the average size or PDI, as a function of storage time in most batches of OS-PAMAM
320 and PAMAM G6, batch 3 presented an increase in polydispersity and size.
321 Polydispersity can occur due to size distribution or agglomeration of the OS-PAMAM
322 during analysis. Other studies have demonstrated that a higher PDI number
323 indicates that NPs are less monodisperse, with a larger size, and will be more likely
324 to form agglomerates over time [22,23]. Furthermore, the ZP of these two NPs was
325 compared, whereas values of ~ 50 mV were found for PAMAM-G6 and ~ 14 mV for
326 OS-PAMAM (Figure 3-C and D). The ZP differences between dendrimers could be
327 attributed to the generation since an increase in zeta potential has been observed
328 as a function of generation [24]. However, it should be considered that ZP values
329 are not an absolute measurement of NPs stability. For example, solid lipid
330 nanoparticles with positive ZP have a long-circulating half-life due to the absorption
331 of protein components in the blood, while NPs with negative ZP can be cleared by
332 the reticuloendothelial system. Furthermore, this parameter is very important for
333 biomedical applications because toxicity has been linearly correlated with high

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

337 3.3 Molecular weight comparisons of OS-PAMAM and PAMAM-G6

338 The molecular weight (MW), and reproducibility between batches were measured by
339 size exclusion chromatography (SEC). All batches of OS-PAMAM (green, blue and
340 orange chromatograms, figure 4) and PAMAM-G6 (black, Figure 4) respectively
341 present a maximum in their chromatograms at 4.55 min and 3.2 minutes. Both MW
342 were calculated by linear regression of the MW marker curve (red in figure 4A),
343 resulting in 8.5 kDa and 86 kDa for the three OS-PAMAM batches and PAMAM-G6
344 correspondingly (Figure 4A). The MW of PAMAM-G6 was higher in comparison to
345 the theoretical MW reported (58,048 kDa). Differences between theoretical and
346 experimental MW values of the dendrimers may be associated with various
347 phenomena, such as nonspecific interactions between PAMAM-G6 and SEC column
348 [29]. According to other reports, the intrinsic viscosity of the dendrimers decreases
349 as generation increases, which could affect the measurement of low and high
350 generations with the same analytical method, i.e., G3 vs G6 [30,31].

351 The average PDI obtained in the SEC analysis for OS-PAMAM batches was $1.05 \pm$
352 0.039 , whilst a polydispersity index of 3.16 was computed for PAMAM-G6 (Table 2), in
353 clear agreement with the overestimated 86 kDa SEC molecular weight of said
354 dendrimer. Interestingly, the SEC distribution pattern is wider in OS-PAMAM,
355 suggesting a larger structural heterogeneity. In contrast, the chromatographic peak
356 of the PAMAM-G6 -with a sharper chromatogram linewidth- indicates structural
357 homogeneity of the more dispersed dendrimer. This physicochemical difference
358 amongst products is unambiguously revealed when their SEC profiles are subjected
359 to a Second Derivative Analysis ($\Delta\Delta$ Absorbance, Figure 4B): A single antiphase
360 signal is revealed for the structurally homogeneous PAMAM-G6 dendrimer, as a
361 result of deconvoluting the SEC chromatogram with the $\Delta\Delta$ Absorbance procedure
362 For the contrary, the $\Delta\Delta$ Absorbance deconvolution of broad OS-PAMAM
363 chromatographic SEC peaks produces a more complex pattern, as the result of a

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

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

384 **3.4 Comparative analysis of size, shape, and force measurements by AFM**

385 The shape of OS-PAMAM and PAMAM-G6 was compared by AFM. The images
386 showed the distribution over the slide for both NPs. The average size was measured
387 through a maximum vertical radius (Rz) of cross-sections in particles analyzed in all
388 images from different fields over the slide. A similar Rz (height) of 14.21 ± 2.81 nm
389 and 13.30 ± 2.56 nm for OS-PAMAM and PAMAM-G6, respectively, were obtained
390 (Figure 5A-B). Further, a mostly spherical shape for both NPs was observed, as
391 reported in other studies [40,41]. However, superficial irregularities in OS-PAMAM
392 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-NH₂ 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

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

426

427 **3.5 Structural analysis, diffusion coefficient distributions, weighted average** 428 **molecular weight, polydispersity indexes and structural homogeneity of OS-** 429 **PAMAM and PAMAM-G6 by Pulsed-Field Gradient Diffusion Ordered (PFG-** 430 **DOSY) NMR spectroscopy**

431 OS-PAMAM dendrimers were synthesized with the use of a modified Michael
432 addition method between ethylenediamine and acrylic acid methyl ester; commonly,
433 PAMAM has polar pockets comprised mainly of alkyl chains, tertiary amines, and
434 amido scaffolds [14]. Proton nuclear magnetic resonance (^1H NMR) has provided a
435 robust analysis for structural studies and identity evaluation of PAMAM dendrimers.
436 Also, PAMAM-drug hosts interactions were previously analyzed by ^1H NMR titrations
437 [49] through dipolar NOESY correlation-exchanges [50] and PFG NMR [51] ^1H NMR
438 Chemical shift assignment of PAMAM dendrimer has been described [52]: 2.39 ppm
439 (120H, br, $-\text{NCH}_2\text{CH}_2\text{CONH}-$, Ha); 2.60 ppm (56H, br, $-\text{CONHCH}_2\text{CH}_2\text{N}-$, Hb);
440 2.69 ppm (120H, br, $-\text{NCH}_2\text{CH}_2\text{CONH}-$, Hc); 2.79 ppm (64H, br, $-\text{CONHCH}_2\text{CH}_2$
441 NH_2 , Hb'); 3.21 ppm (56H, br, $-\text{CONHCH}_2\text{CH}_2\text{N}-$, Hd); 3.26 ppm (64H, br,
442 $-\text{CONHCH}_2\text{CH}_2\text{NH}_2$, Hd').

443

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

453 function (Figure 8A), reveal linewidths at half height (LWHH) of 15.71 Hz for $-\text{CONH}$
454 $\text{CH}_2 \text{CH}_2 \text{N}-$ resonances at $\delta = 3.39$ ppm and 3.025 ppm, and even broader LWHHs of
455 63.72 Hz ($\delta = 2.78$ ppm, $-\text{CONH CH}_2 \text{CH}_2 \text{N}-$ spin system), 56.74 Hz ($\delta = 2.59$ ppm, $-\text{NCH}_2$
456 $\text{CH}_2 \text{CONH}-$ spin system), 20.95 Hz ($\delta = 2.42$ ppm, $-\text{CONH CH}_2 \text{CH}_2 \text{N}-$ spin system) and
457 21.82 Hz ($\delta = 2.34$ ppm, $-\text{NCH}_2 \text{CH}_2 \text{CONH}-$ spin system). Broader ^1H LWHH in OS-
458 PAMAM spectrum with no post-processing (Figure 8C) does not have an origin from
459 higher polydispersity but rather due to higher structural heterogeneity [35] as
460 unambiguously revealed in Figure 8D, in comparison to Figure 8B. Resolution
461 enhancement achieved with identical Lorentz-to-Gauss weighting functions in both
462 dendrimers ^1H NMR spectra produces different results. Line sharpening apodization
463 applied to PAMAM-G6 proton spectrum produces equivalent LWHHs (Figure 8B) as
464 when no apodization function was applied (Figure 8A). In contrast, broad resonances
465 of OS-PAMAM ^1H NMR spectrum (Figure 8C) are deconvoluted into narrower
466 frequencies of c.a. 1 to 1.5 Hz of LWHHs (Figure 8D) when resolution enhancement
467 is achieved with a Lorentz-to-Gauss apodization function identically applied as in
468 PAMAM-G6 ^1H spectrum (Figure 8B). The set of narrow resonances revealed in
469 Figure 8D strongly suggest that OS-PAMAM presents an important structural
470 heterogeneity due to defects in the dendrimer structure during the synthesis (Section
471 2.2) and a lack of dendrimer purity such as with the commercial PAMAM-G6.

472

473 Analysis of two-dimensional PFG-STE-H₂O(presat)-DOSY spectra of both
474 dendrimers demonstrate at first glance, differences in diffusion coefficient
475 distributions between both dendrimers: the OS-PAMAM showed higher diffusion
476 coefficient distributions around a lower logarithmic diffusion coefficient average of
477 $\text{Log DC}_{\text{av}} = -8.8 \text{ m}^2\text{s}^{-1} \pm 0.05$ than the PAMAM-G6 counterpart ($\text{Log DC}_{\text{av}} = -9.6 \pm$
478 $0.05 \text{ m}^2\text{s}^{-1}$, Figure 9A). Furthermore, experimental solution-state DOSY-MW
479 distributions obtained from diffusion coefficient distributions [13] are represented as
480 histograms (Figure 9B) and expressed in Da for PAMAM dendrimers ranging from
481 G1 to G6. PAMAM-G6 weighted average molecular weight of $\text{MW} = 55711.86 \pm 0.7$
482 corresponds to the theoretical value elsewhere reported [53], whilst OS-PAMAM
483 weighted average molecular weight of $\text{MW} = 5056.47 \pm 0.7$ strongly suggest the

484 presence of a PAMAM-G3 dendrimer, in full agreement with SEC data, predicting an
485 average MW of 8.5kDa for OS-PAMAM (Figure 4). Finally, solution-state
486 polydispersity indexes (PDI, \mathfrak{D} in figure 9A) are experimentally obtained from the
487 molecular weight distributions at the low concentration regime from the experimental
488 average diffusion coefficient distributions ($\text{Log DC}_{\text{av}}$ in Figure 9A) and the width of
489 each distribution, represented by the experimental diffusion coefficient distributions
490 [13]. Table 2 resumes the solution-state MW and PDIs of OS-PAMAM and PAMAM-
491 G6 obtained with non-invasive DOSY-NMR spectroscopy for DLS (Table 1) and SEC
492 (Figure 4), finding orthogonality amongst the three techniques. Present results are
493 in agreement with other studies reporting that PAMAM G3 is around 7 kDa [29,54].
494 Moreover, lower logarithmic diffusion coefficient average of OS-PAMAM ($\text{Log DC}_{\text{av}}$
495 $= -8.8 \text{ m}^2\text{s}^{-1} \pm 0.05$) that in turn weights higher diffusion coefficient distributions
496 responsible to produce lower PDI and average MW values, with respect PAMAM-
497 G6, as revealed with DLS, SEC and PFG DOSY NMR (Table 2), in combination with
498 the observed set of narrow resonances in ^1H NMR spectrum apodized with a line
499 sharpening weighting function (Figure 8D), allows to conclude that herein produced
500 OS-PAMAM presents lower polydispersity but higher structural heterogeneity, with
501 respect its more polydisperse but more homogeneous PAMAM-G6 counterpart.

502

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

508

509 **3.6 Structural characterization by MS-MS**

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

515 355.2460, 343.2431, 325.2336, 241.1646, 229.1643, 199.1542, 127.0851, 98.0609
516 and 85.0761. These m/z values belong to the decomposition pathway by retro-
517 Michael reactions for the species $[M + 5H]^{5+}$, $[M + 7H]^{7+}$ and $[M + 9H]^{9+}$ of the
518 PAMAMs of G1 G2 and G3 respectively. This correspondence with the values
519 calculated for the ionic precursors and the path of the fragments for G0 (Table of
520 Figure 10) demonstrate the identity of OS-PAMAM. On the other hand, in the
521 enlarged section of Figure 10, from 471 to 482 m/z , the species of 479.0647 m/z
522 reported for the intramolecular cyclization of the ester group was not found, which is
523 one of the typical side-products of the Michael addition. This indicates that during
524 the synthesis of OS-PAMAM cyclic side-product are not formed [55].

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

532

533 4. Conclusion

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

544 purity of novel or traditional PAMAM synthetic approaches. Interestingly, OS-
545 PAMAM structural heterogeneity and polydispersity is conserved amongst batches.
546 That relation not only remains between repetitions but also over time up to 4 months
547 at 5 ± 3 °C. In general, the ZP of OS-PAMAM were less positive than PAMAM-G6.
548 However, ZP was consistent between batches and time for both dendrimers. The
549 AFM morphological analysis showed a circular shape in all dendrimers, and cavities
550 were detected in OS-PAMAM. Further, the adhesion force did not present significant
551 differences, while the elasticity (DMT Modulus) was higher in PAMAM-G6, which
552 represents a similar chemical surface but a different internal structure. The MW of
553 OS-PAMAM is around 10 times smaller than commercial G6, as orthogonally
554 detected by SEC and PFG-STE-H₂O(presat)-DOSY, whereas lower OS-PAMAM
555 masses are explained due to their cavities. OS-PAMAM / PAMAM-G6 MS-MS mirror
556 plots demonstrate chemical equivalency amongst herein analyzed dendrimers, with
557 a slight advantage that OS-PAMAM is produced with a faster and low-cost synthetic
558 protocol. These novel NPs has been previously studied as a drug delivery system
559 [11]; however, more biological studies will be carried out to compare the biomedical
560 applicability with commercial PAMAM, taking into account the structural
561 heterogeneity, polydispersity and synthetic reproducibility.

562 **5. Glossary**

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

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579

580 **7. Disclosure**

581 The author reports no conflicts of interest in this work.

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785

786 **Tables**

787

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

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

790

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

794

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

803

	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

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814 Captions

815 **Figure 1. UV-Vis spectra of OS-PAMAM and PAMAM-G6.** (A) Spectrum with characteristic
816 signals of three independent production batches of OS-PAMAM vs PAMAM G6. (B) The signals
817 corresponding to tertiary amides from 280 to 285 nm were taken as a reference value to monitor
818 batches' integrity and control PAMAM-G6 through time until four months at 5 ± 3 °C. (C)
819 Structure of generation 3 (G3) PAMAM dendrimer with amino groups at the periphery. The
820 ethylenediamine core is circumvented by a dashed line, reproduced from Abbasi et al., 2014
821 [4].

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

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

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

837 **Figure 5. Size and morphological analysis of dendrimers by AFM.** Heightmap of (A)
838 PAMAM-G6 and (B) OS-PAMAM, both average sizes were analyzed through the maximum
839 vertical radius (R_z) of cross-sections ($n=50$). (C) The dotted line represents a close-up of the
840 3D image of the typical shape of OS-PAMAM and PAMAM-G6 dendrimers.

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

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

846

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

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

856

857 **Figure 9. Solution-state PFG-STE-H₂O(presat)-DOSY of PAMAM-G6 and OS-PAMAM.** For
858 obtaining average diffusion coefficient distributions (DCD), weighted average molecular
859 weights and polydispersity indexes. (A) PFG-STE-H₂O (presat)-DOSY-F2 assigned- NMR
860 spectra of PAMAM batches: Commercial G6 PAMAM (Black) and synthesized OS-PAMAM
861 (gray). Below each logarithmic diffusion coefficient average ($\ln \text{DC}_{\text{av}}$, highlighted with a
862 horizontal dotted line) are reported the weight-average molecular weights (\bar{M}_w) and the
863 polydispersity indexes (PDI) per case. (B) Relative molecular weights [$\bar{M}_w = f(\text{DCD}, \text{DC}_{\text{av}})$] of
864 each chemical-shift assigned resonance obtained from DCD and DC_{av} relationships
865 represented as histograms and expressed in Daltons, are compared with reported molecular
866 weights of PAMAM dendrimers by generation (G1: 1430 Da; G2: 3256 Da; G3: 6909 Da; G4:
867 14215 Da; G5: 28826 Da and G6: 58048 Da, from <http://www.dendritech.com/pamam.html>).

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

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

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

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

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