Aging process of Polyamidoamine dendrimers: effect of pH and shaking in the fluorescence emission and aggregation-state

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Declaration of interest

None.

Abbreviations

DG4.0: PAMAM dendrimers of generation 4.0; DG4.5: PAMAM dendrimers of generation 4.5; DLS: dynamic light scattering; dpr: days post-reconstitution; NTIF: non-traditional intrinsic fluorescence; PAMAM: polyamidoamine; TEM: Transmission Electron Microscopy.

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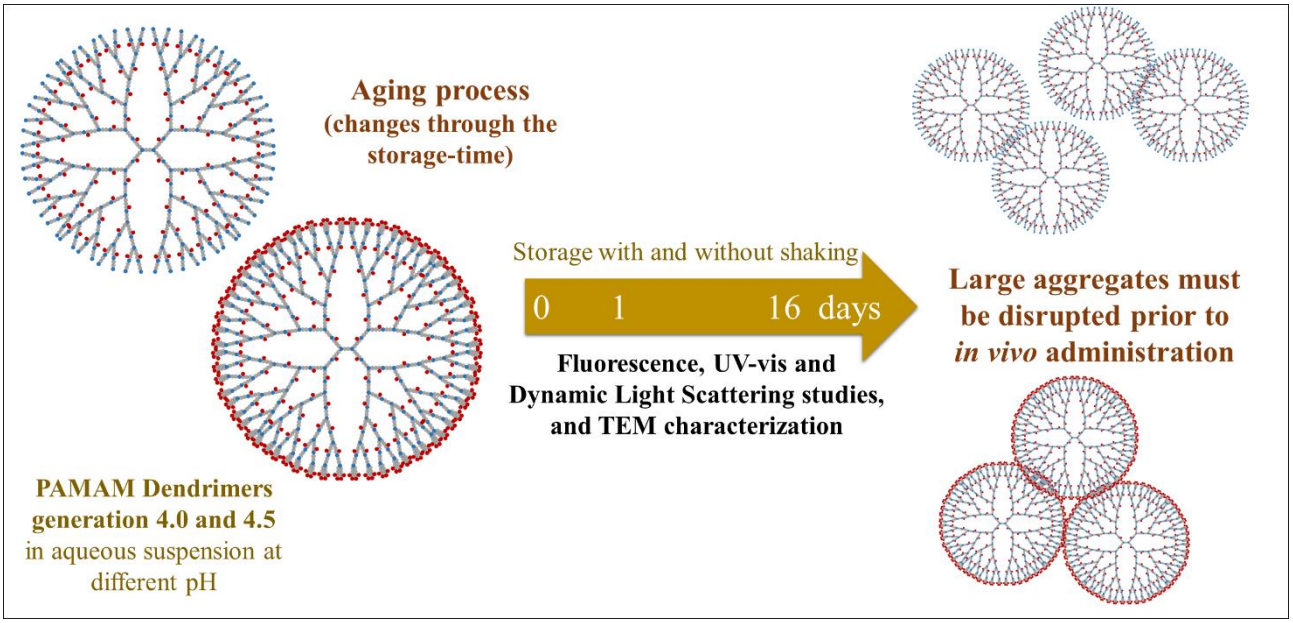
Abstract

In the last years, it has been discovered and intensely studied the non-traditional intrinsic fluorescence of PAMAM dendrimers. Nevertheless, their aging process in aqueous suspension is scarcely studied, being unknown the causes of the observed changes in their fluorescence properties. Hence, this work aims to characterize the PAMAM dendrimers of generation 4.0 (DG4.0) and 4.5 (DG4.5) through the aging process at three different pH conditions, stored with or without shaking. We studied, up to 16 days, the UV-Vis absorption, the fluorescence emission, and the size of dendrimers/aggregates. In a different way than the already published work, we demonstrated that there is no chemical change in dendrimers through the aging process, even though changes in fluorescence emission were observed. Besides, we have put in evidence that changes in the agglomeration patterns of dendrimers would not be related to change in the fluorescence emission thought aging. Moreover, we demonstrated that DG4.5 formed large aggregates in water that need to be disrupted by shaking previous to an *in vivo* administration.

Keywords: PAMAM dendrimers, Aging Process, Aggregation Patterns, Spectroscopy, Non-Traditional Fluorescence.

Graphical abstract



INTRODUCTION

Dendrimers, also known as cascade molecules, are three-dimensional polymers obtained by organic synthesis, consisting of a core and branches that grow radially forming a surface with multiple terminal groups. In the 80s, Tomalia first synthesized dendrimers from an ethylenediamine core with polyamidoamine (PAMAM) branches, which converted in the most used up to date. Since that moment, PAMAM dendrimers emerged in the multiple fields of application of nanotechnology, especially as promising drug delivery systems [1–6], but also as a nanodrugs *per se* for their anti-bacterial, anti-prion, anti-viral, anti-coagulant, anti-oxidant, anti-inflammatory, and anti-aggregation proteins properties [7–10].

In the last 20 years, it has been discovered and intensely studied the non-traditional intrinsic fluorescence (NTIF) of PAMAM dendrimers, which arises from the confinement of non-traditional heteroatomic fluorophores [11]. So far, it has been postulated that the internal tertiary amines and amide bonds (which are transformed to imidic acid in acid pH) are the fluorophores responsible for the NTIF of PAMAM dendrimers [12,13]. Also, it has been postulated that the NTIF of PAMAM dendrimers depends on the concentration [14,15], the generation [16,17], the temperature [16], the aggregation state [18,19], the conformation state [20], the polarity of the solvent [18,19] and the pH [15]. Particularly, Wang et al. (2004) showed an increase in the fluorescence emission of dendrimers in aqueous suspension as the pH decreases, which could be due to (i) the protonation of tertiary amines, (ii) the increment of hydrogen bonds, or (iii) chemical reactions that instead of new fluorescent motifs[15]. Also, Wang et al. (2007) showed an increase in the fluorescence emission of PAMAM dendrimers n aqueous suspensions as the sample aging. Specifically, they founded that the fluorescence emission increased with time and was stabilized after 75 or 450 minutes at pH= 2 or pH= 7, respectively [16]. These authors propose that the increase in fluorescence may be due to (i) changes in the state of aggregation of dendrimers or (ii) chemical reactions dependent on oxygen presence that produce new fluorescent motifs [16]. However, Wang et al. (2010) showed that the changes in fluorescence emission of dendrimers are reversible when the pH is modified, and thus demonstrated that there is no chemical reaction involved [17]. Likewise, it has been shown a dependence between the NTIF and the aggregation-state and confinement of the non-traditional fluorophores motifs of other macromolecules. In this sense, Jasmine et al. (2009) concluded that (i) the fluorescence emission of PAMAM dendrimers is maximized when they are forming aggregates of approximately 70 nm and (ii) the free dendrimers would emit less fluorescence because they would have greater degrees of freedom for de-excite by non-radiative ways[18].

As far as we know, the aging process of PAMAM dendrimers in aqueous suspension was hardly studied, so the causes of the changes in their properties throughout aging still unknown. Therefore, it is important to study the effect of the aging process on the properties of dendrimers, since they are used as drug delivery systems or as nanodrugs. Above all, the aggregation-state should be determined before *in vivo* intravenous administration, due to the possible toxic effects of the agglomerates. Also, the fluorescence should be determined, since it would allow analyzing their biodistribution in biological systems.

In this context, we aimed to characterize the PAMAM dendrimers through the aging process at different storage conditions. For this, we selected PAMAM dendrimers of generation 4.0 (DG4.0) and 4.5 (DG4.5), both with ethylenediamine core, since they are the most used as drug delivery systems [21–27]. The DG4.0 has 64 amine terminal groups, while the DG4.5 has 128 carboxylic terminal groups, so these dendrimers have opposite charges under physiological conditions that could modify their behavior through the aging process. We analyzed the UV-Vis absorption, the fluorescence emission, and the size of dendrimers/aggregates for 16 days in samples at different pH with or without shaking storage. In this sense, the characterization of dendrimers is essential for predicting and elucidating its properties, and no single technique could completely describe them.

MATERIALS AND METHODS

PAMAM dendrimer of generation 4.0 (DG4.0) (CAS N° 412449) and generation 4.5 (DG4.5) (CAS N° 470457), both with ethylenediamine core, were purchased from Sigma-Aldrich (Merck, Argentina). For the preparation of the dendrimer samples to be characterized, methanol from the commercial suspensions was evaporated at 25 °C in a SpeedVac concentrator SAVANT® AES1010 (Thermo Fisher Scientific, EE. UU.). The solid residues were reconstituted at room temperature in deionized water at different pH, maintaining a constant dendrimer concentration of 24 µM. In our work, distilled deionized water (Millipore filtration system) at pH acid (pH 2-3), neutral (pH 6,5-7,5), or basic (pH 11-12) were used to study the effect of the protonation state of dendrimers in their characteristics. The pH of the solutions was adjusted with dilute hydrochloric acid or sodium hydroxide solutions. Also, to study the effect of the storage conditions, a set of samples of each dendrimer was fixed through the storage, whereas the other set was shaken at 100 rpm in a linear shaker DLab SK-L180-E (Dragon Lab, Argentina). All the samples, with or without shaking, were stored in the dark at 28 °C through the 16 days of analyzes.

The characterization of DG4.0 and DG4.5 suspension at different pH, with or without shaking, was carried out by UV-vis spectroscopy, Fluorescence emission spectroscopy, Dynamic Light Scattering (DLS), and Transmission Electron Microscopy (TEM) (**Figure 1**).

UV-Vis experiments were performed in a Jasco V-550 spectrophotometer, with a resolution of 0.5 nm and the absorption detection range between 190 and 450 nm. The UV-Vis absorption of both DG4.0 and DG4.5 in the three pH conditions, with and without shaking, were studied at 0, 1, 2, 9, and 16 days post-reconstitution (dpr).

Fluorescence experiments were performed in a Scinco FS-2 spectrofluorometer, with a resolution of 0.5 nm. The excitation wavelength was 390 nm, and the emission detection range was between 400 and 600 nm. The fluorescence emission of both DG4.0 and DG4.5 in the three pH conditions, with and without shaking, were studied at 0, 1, 2, 9, and 16 dpr. Also, the emission every 10 min up to 250 min post-reconstitution were analyzed with this equipment. The area under the fluorescence emission curve was integrated for each sample at each analysis time. For comparison, the result obtained in each time was relativized to the area under the curve of each sample at time 0 post-reconstitution. DLS assays were done in a Nano Zetasizer ZEN3600 (Malvern Instrument, UK) at 25 °C. The laser employed was a He-Ne emitting at 633 nm. The refractive index used for DG4.0 was 1.35, for DG4.5 was 1.34, and for the dispersant was 1.33. Mie Scattering Theory was used to get the diameter of the samples and the size distribution in number, volume, and intensity percentages. The DLS characterization of both DG4.0 and DG4.5 in three pH conditions, with and without shaking, were studied at 0, 1, 2, 9, and 16 dpr.

TEM images were obtained in a Turbo Transmission Electron Microscope EM 301 (Philips). The suspensions of DG4.0 and DG4.5 at neutral pH, with and without agitation, were deposited on a copper grid (300 mesh) and covered with a formvar/carbon film. The excess liquid was removed with a filter paper. The samples were stained with 1-3% uranyl acetate and the images were obtained using an acceleration voltage of 60 kV.

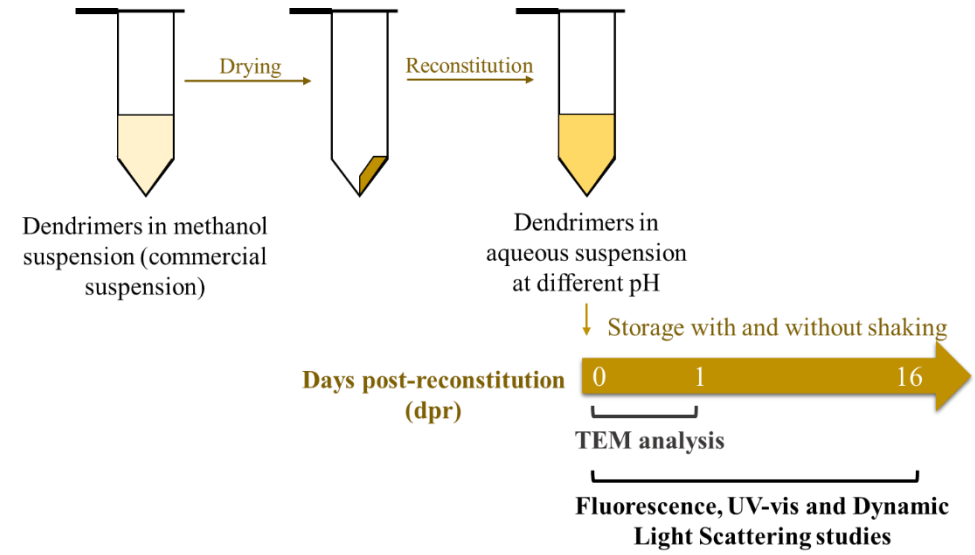


Figure 1 – Scheme of dendrimers-suspension obtaining, storage, and characterization through the days post-reconstitution (dpr).

RESULTS AND DISCUSSION

The PAMAM dendrimers of generation 4.0 (DG4.0) and 4.5 (DG4.5) are widely used as drug delivery systems since they have a particular structure with internal pockets, capable of holding drug molecules inside, and a hyperbranched surface, where the drugs can be covalently anchored [2]. Furthermore, these two specific types of dendrimers are often used together and compared given that they have a similar internal chemical structure and size, but opposite external electrostatic charges at physiological pH [28,29]. Both DG4.0 and DG4.5 have amide bonds and tertiary amines on their internal branches. The DG4.0 has primary amines on their terminations, which give it a positive charge at physiological pH, while the DG4.5 has carboxylic acids on their terminations, which give it a negative charge at physiological pH [20,30].

Although dendrimers have been used for more than a decade, the effect of aging on their UV-Vis absorption, fluorescence emission, and aggregation-state properties is still unknown. In this work, to deeply characterize the aging process of DG4.0 and DG4.5, UV-Vis spectroscopy, fluorescence emission spectroscopy, DLS, and TEM were carried out through the post-reconstitution days in aqueous suspension at different pH, with or without shaking storage condition.

In **Figure 2,** the UV-Vis absorption spectra of both types of dendrimers (DG4.0 and DG4.5) at neutral pH after storage with or without shaking are presented. In addition, in **Figures S1 and S2,** the UV-Vis absorption spectra under the three pH conditions (acid, neutral and basic pH) are shown. No changes in the UV-Vis absorption spectra of DG4.0 or DG4.5 were observed over 16 days post reconstitution (dpr) at acidic, neutral or basic pH, with or without shaking storage condition. Therefore, with these

results, we ensured that no chemical changes in the dendrimers in any condition during the aging process of the samples have occurred. These results are consistent with the hypothesis that the aging process is not mediated by an irreversible chemical change in dendrimers, but is mediated by a change in their protonation- or aggregation-state [18].

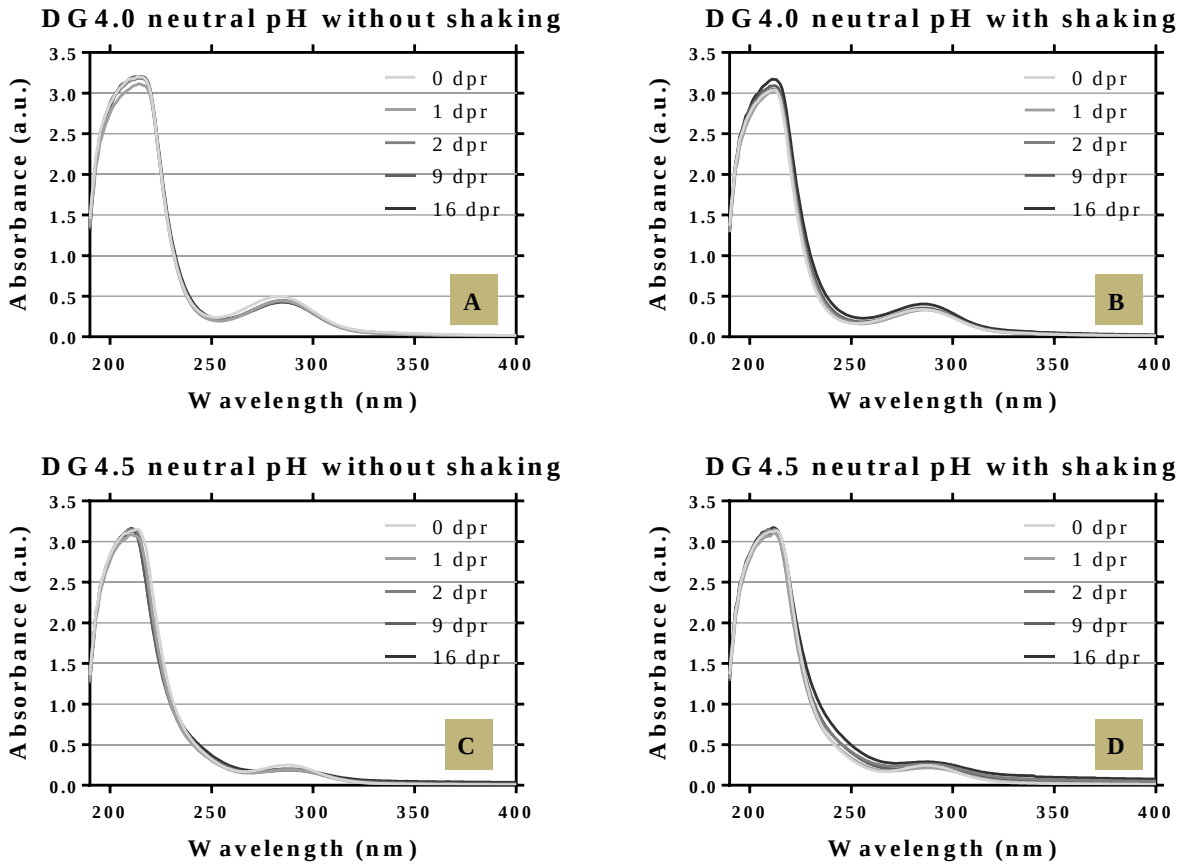


Figure 2 – UV-Vis absorption at neutral pH of PAMAM DG4.0 (A and B) and DG4.5 (C and D) stored without (A and C) or with (B and D) shaking.

In Figure 3 the UV-Vis absorption spectra of DG4.0 (Figures 3 A and B) and DG4.5 (Figures 3 C and D) under the three pH conditions (acid, neutral, and basic pH) at 16 dpr are compared. It can be observed that, both in storage without (Figures 3 A and C) or (Figures 3 B and D) shaking, the UV-Vis absorption spectra of both dendrimers showed changes as a function of pH. These changes in function of pH were observed in every day of analyzes (Figures S1 and S2). In all cases, it could be seen that, from acidic to basic pH, there was a shift in the maximum absorbance of the internal amides towards higher wavelengths (from 210 to 216 nm) and an increase in the absorbance intensity of the internal tertiary amines (at 280 nm). The mentioned changes in the absorption of internal amides and tertiary amines could due to changes in the physicochemical environment in which they are found, specifically in the pH of the medium. The changes in the physicochemical environment

could mediated changes in the state of protonation of the internal (amides and tertiary amine) and terminal (primary amine and carboxylic acid) groups of the dendrimers [30,31]. At acid pH, the internal tertiary amines are protonated (-NH_3^+-) since the pH is lower to its pKa, and the amide bonds are tautomerized to imidic acid (-C-O^-=NH^+-) [13,31]. On the contrary, at basic pH, the internal tertiary amines are deprotonated (-NH_2-) and the amide bonds are not tautomerized (-C=O-NH-). The change in the protonation-state of tertiary amine could lead to an increment in the absorption intensity at 280 nm, while the tautomerization of amide bonds could lead to a shift in the maximum of absorbance.

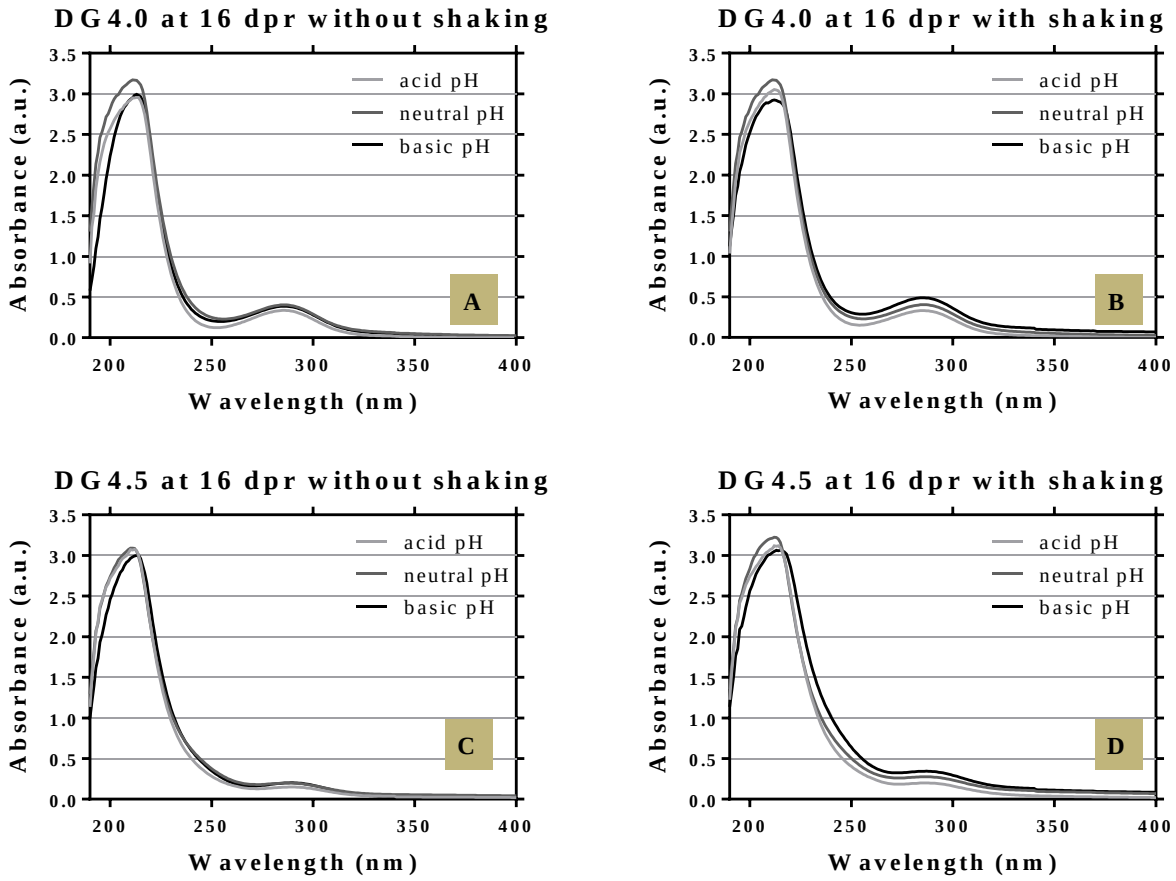


Figure 3 – UV-Vis absorption spectra at different pH of PAMAM DG4.0 (A and B) and DG4.5 (C and D) stored without (A and C) or with (B and D) shaking for 16 days.

The fluorescence emission spectra of DG4.0 and DG4.5 are showed in Figures S3 and S4, respectively, as a function of aging-time and the different pH and shaking studied conditions. The fluorescence of DG4.5 was higher than that of DG4.0 for the three pH conditions (acid, neutral, and basic pH). The higher fluorescence intensity of DG4.5 compared to DG4.0 could be because they have more non-traditional chromophore groups (amides and tertiary amines). The results are in accordance with those previously shown by Wang et al. (2007), where the fluorescence emission depended on the dendrimer

generation [16]. On the other hand, the fluorescence of both dendrimers at acid pH was higher than that at basic pH. These changes could be due to the change in the physicochemical environment of dendrimers and the change in the protonation-state, as we previously discussed for the UV-Vis absorbance results.

From the obtained fluorescence emission spectra (Figures S3 and Figure S4) we determined the relative fluorescence emission for each sample compared to its fluorescence emission at the initial time of the aging process (0 dpr). The relative fluorescence emission, at different pH, of both dendrimers stored with or without agitation, are presented in **Figures 4 and 5**.As can be seen in the Figure 4 A, C, and E and in Figures 5 A, C, and E, there were no changes in the relative fluorescence emission of any of the dendrimers at short analysis times (less than 250 min). These results are contradicted with those previously reported by Wang et al. (2007)[16] , who observed an increment in the fluorescence emission even at lowers aging time. This difference could be since a lower concentration of dendrimers was used in our work (24 µM instead of 700 µM), which modifies the emission patterns [14,15]. However, changes in the relative emission were observed as the dendrimer samples aged for 16 days, as can be seen in Figures 4 B, D, and F and Figures 5 B, D, and F. Accurately, DG4.0 presented a significant increase in relative fluorescence emission as a function of time at the three pH studied, showing an increase of up to five times (Figures 4 B and D). Only in the condition of basic pH (Figure 4 F), a difference was observed between the samples with shaking, compared to that without shaking. Instead, it was observed that the fluorescence increased with the aging for DG4.5, but it only did up to two times (Figures 5 B, D, and F). In DG4.5, no significant changes were observed when the samples were shaking. In this respect, the fluorescence emission of the DG4.0 significantly increases with the aging time, while that of the DG4.5 only slightly increases. Since Wang et al. (2007) [16] had only studied the aging process of cationic dendrimers (DG4.0), we do not have a counterpart with which to compare our results obtained for DG4.5.

Concerning this analysis, Tomalia et al. (2019) described in their recent review that the fluorescence of PAMAM dendrimers could be increased when some external force makes more rigid their branches since its removes mechanisms of relaxation (vibrational mode are restricted) so the probability of photon emission (fluorescence) increases [11]. In consequence, to study the effect of different conditions in the aging process of the dendrimers, we shake one of the samples and fix the other, because we expected that the samples with shaking have differentiated interaction between dendrimers and more dissolved oxygen than the samples without shaking. However, no significant differences were observed in the fluorescence emission of dendrimers under different shaking conditions, except for DG4.0 at basic pH.

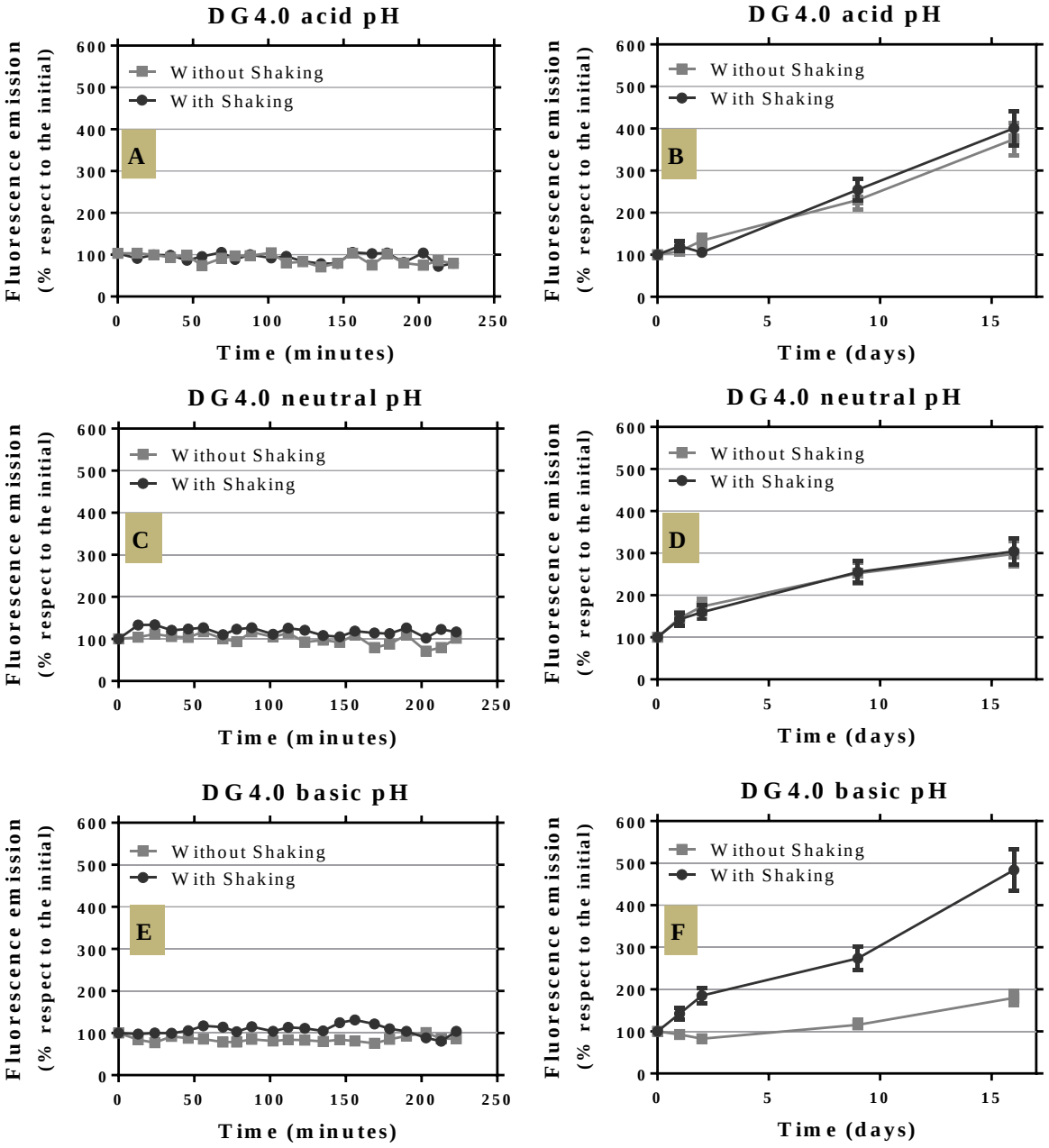


Figure 4 – Relative fluorescence emission at acid (A and B), neutral (C and D) or basic (E and F) pH of PAMAM DG4.0 stored without (A, C and E) or with (B, D and F) shaking. The A, C and E graphs show the results obtained during 250 min after resuspension, while the B, D and F graphs show the results obtained at 1, 2, 9, and 16 days post-reconstitution.

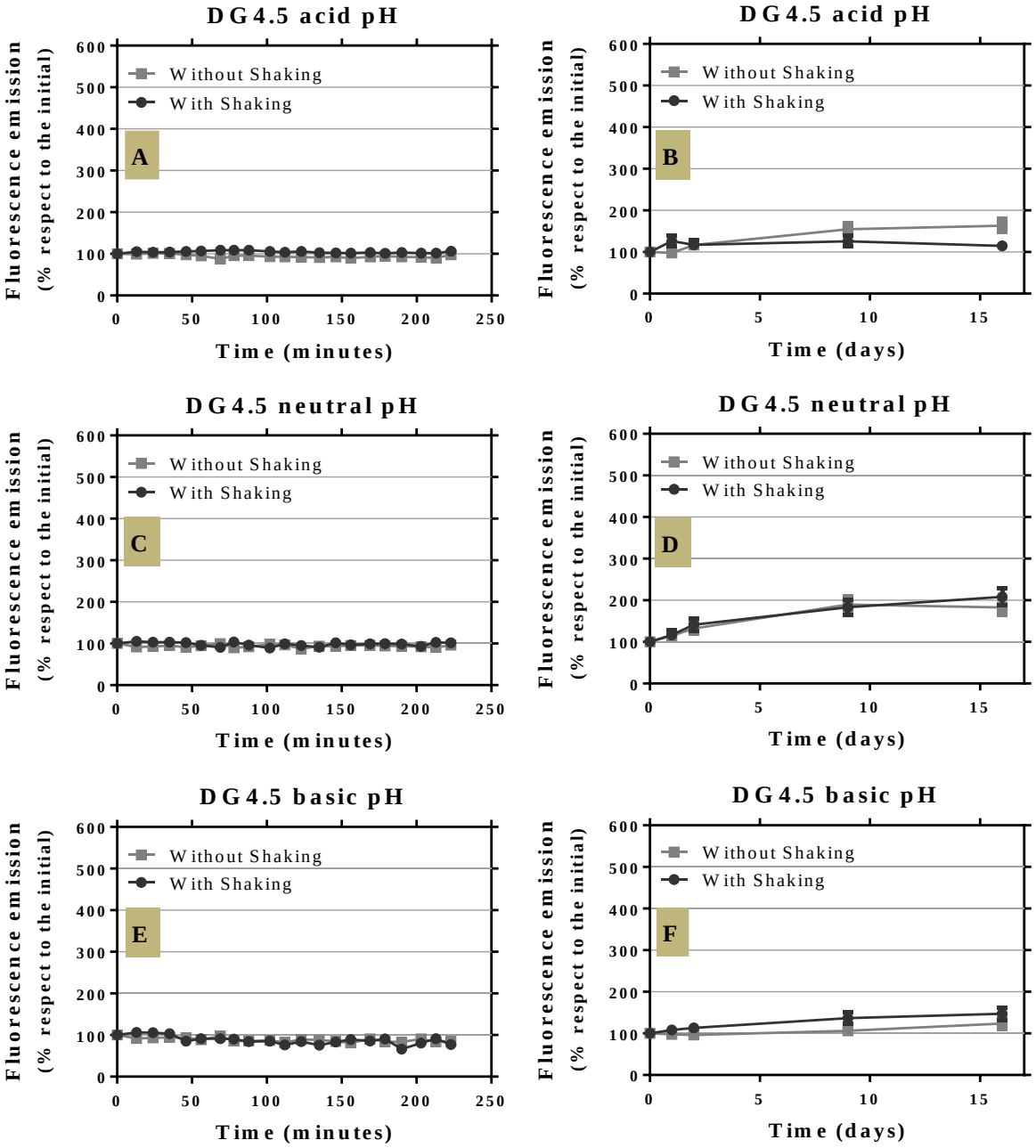


Figure 5 – Relative fluorescence emission at acid (A and B), neutral (C and D) or basic (E and F) pH of PAMAM DG4.5 stored without (A, C and E) or with (B, D and F) shaking. The A, C and E graphs show the results obtained during 250 min after resuspension, while the B, D and F graphs show the results obtained at 1, 2, 9, and 16 days post-reconstitution..

In order to analyze whether the aggregation-state of the dendrimers plays an important role in the change of the fluorescence emission throughout the aging time, we studied the particle size distribution of DG4.0 and DG4.5 in the three different pH, with or without shaking condition. The diameters of

free dendrimers or their aggregates as a function of pH and the aging time, with or without shaking, are presented in **Figure 6**. These diameters correspond to the means of the populations with the highest amount of particles (%) in the particle size distributions measured as the number percentage, exemplified in **Figure 7.** In the samples of DG4.0 reconstituted at neutral and basic pH (Figures 6 C and E), it was observed that dendrimers are free dispersed (less than 5 nm) from 0 to 16 dpr. On the other hand, for DG4.0 at acid pH (Figure 6 A), it was observed that small aggregates of up to 12 nm were formed immediately after reconstitution. These small aggregates were disassembled to give rise to free dendrimers after 1 dpr. These results (Figure 6 A, C, and E) demonstrate that the dispersed state of the DG4.0 in the three pH studied remains constant over time, without presenting significant aggregation phenomena.

Otherwise, the formation of large aggregates (between 350 and 600 nm) was observed in the DG4.5 samples immediately after reconstitution in the three pH-conditions (Figure 6 B, D, and F). In the samples with shaking, the aggregates disarmed after 1 dpr, giving rise to free dispersed dendrimers (less than 5 nm). In the samples without shaking, the disaggregation was observed after 2 dpr only in neutral-pH condition (Figure 6 D). On the contrary, in the samples at acid and basic pH without shaking, the aggregates remained relatively constant for the 16 days of study (Figures 6 B and F).

About these results, it has been described that dendrimers could adopt different conformations that would produce changes in the packing of their internal and terminal groups, depending on the conditions in which they are found. For example, full-generation PAMAM dendrimers (such as DG4.0) exhibit open conformations at low pH due to electrostatic repulsion between internal tertiary amines and external primary amines, both protonated, which stiffens the dendrimer's branches and force away from the inside. On the other hand, at basic pH, the branches retract because of the hydrogen bridges between the inner tertiary amines and the terminal primary amines, both deprotonated, a process known as back-folding and results in a compact structure [20,30]. Therefore, intermediate-generations PAMAM dendrimers (such as DG4.5), show open conformations at both acidic and basic pH, but a more compact structure at physiological pH [30]. Likewise, the polarity and purity of the solvent affect the conformation of the dendrimers due to the back-folding process of the terminal groups [30]. Particularly, to a lower solvation-capacity of the medium, greater back-folding of the dendrimers. In NMR studies, it was observed that polar dendrimers in non-polar solvents have more considerable intra- and inter-molecular interactions, resulting in a compact interior and the aggregation of dendrimers, while in polar solvents they have an open structure [20]. These observations would explain our results and why after the process of methanol-evaporation and reconstitution in water, the dendrimers remain physically aggregated, although their surface groups

have charges that would induce an electrostatic repulsion process. Also, it would explain the need to provide energy to the system through agitation to disarm these aggregates.

It is important to highlight that the observed aggregation/disaggregation patterns do not explain the changes in the fluorescence emission of both DG4.0 and DG4.5, so the change of the aggregation state of dendritic ramifications would not be responsible for the modification of the NTIF emission. These results contradict those previously reported by Jasmine et al. (2009) who assured that the change in the aggregation-state of dendrimers, mediated by the type of solvent, modified the fluorescence intensity [18]. Given this contradiction, we believe that the change in the type of solvent modifies the physicochemical environment in which the dendrimers are located, which modifies their excitation/relaxation pattern regardless of the state of aggregation.

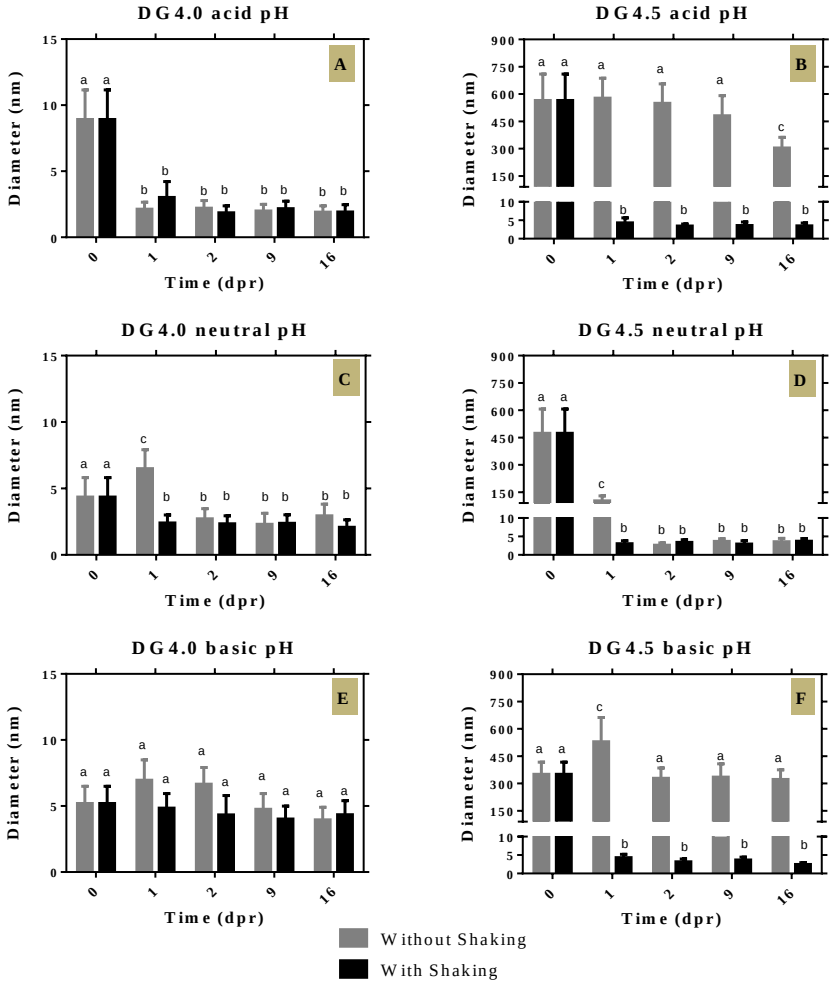


Figure 6 – Diameter (in nm) measured at acid (A and B), neutral (C and D) or basic (E and F) pH for PAMAM DG4.0 (A, C and E) and DG4.5 (B, D and F) after storage without or with shaking. The results were analyzed statistically by two-way ANOVA. The samples with different letters (a,b,c) are statistically different ($p<0.05$) between them.

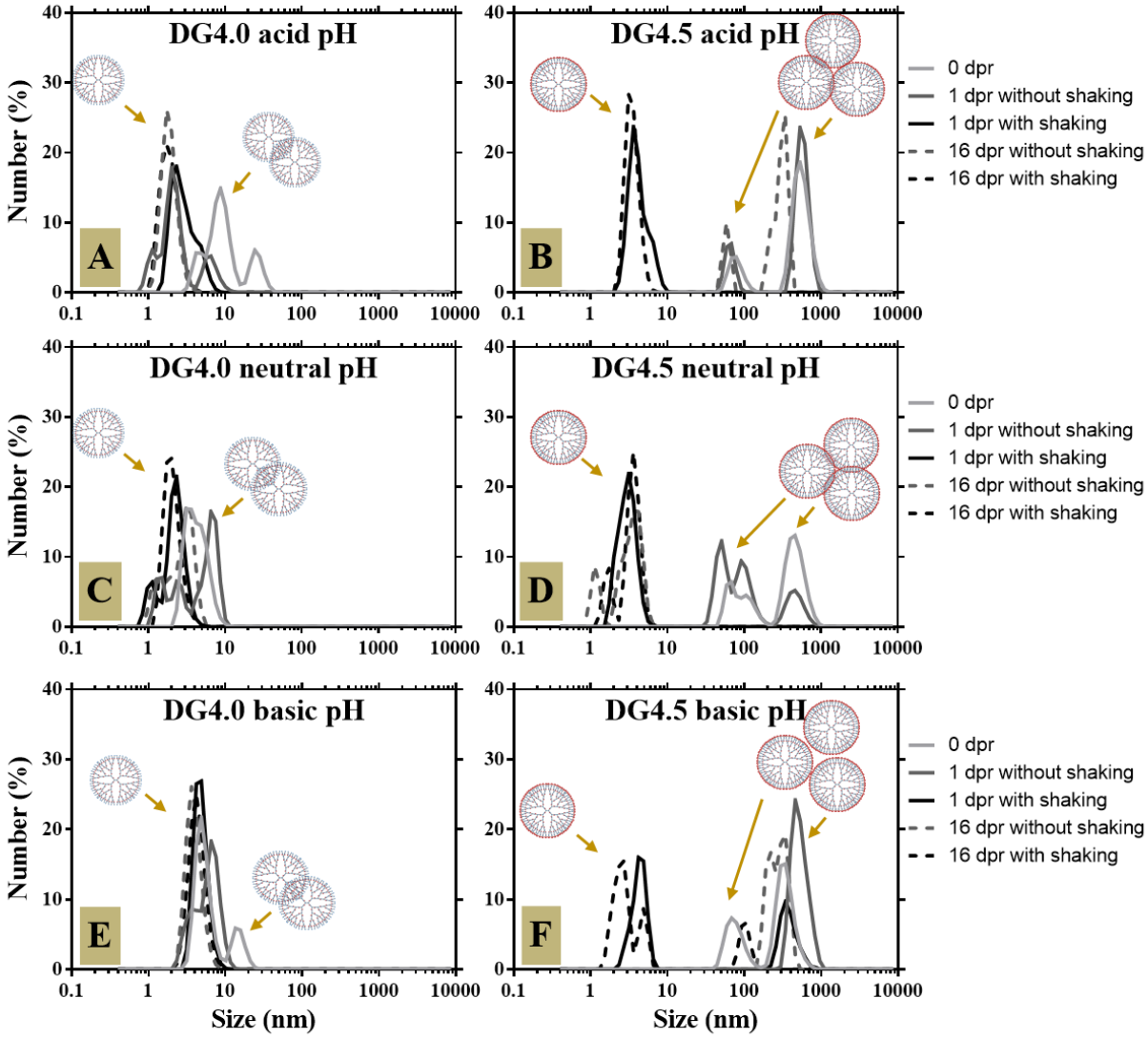


Figure 7 – Particle size distribution at acid (A and B), neutral (C and D) or basic (E and F) pH, determined as number percentage, of PAMAM DG4.0 (A, C and E) and DG4.5 (B, D and F) stored without or with shaking at 0, 1, and 16 days post reconstitution.

As dendrimers are usually applied as drug delivery systems in physiological media (neutral pH), we decided to confirm the results obtained by DLS using TEM. The TEM micrographs of DG4.0 and DG4.5 at neutral pH, with or without shaking for 24 h (1 dpr), are presented in **Figure 8**. It can be seen that while DG4.0 is mostly free and dispersed in both conditions, DG4.5 has a large number of aggregates in the condition without shaking. Hence, the results determined by DLS in the samples at

neutral pH are coincident with those obtained by TEM.

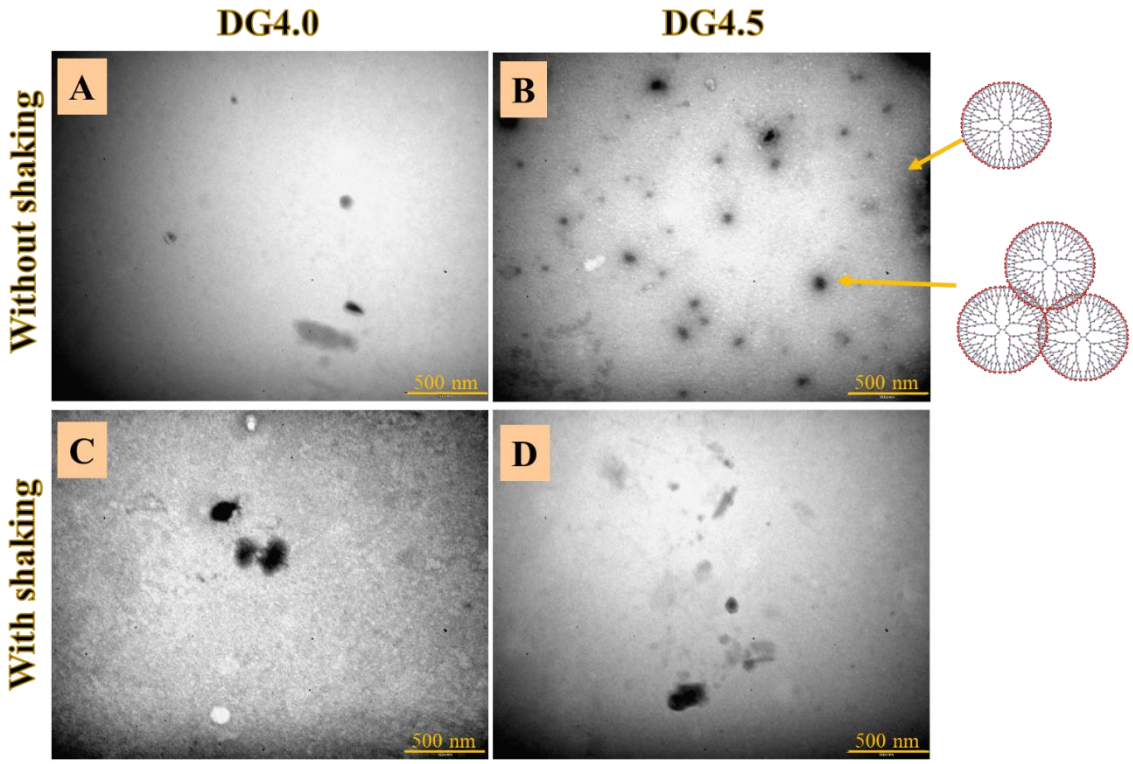


Figure 8 – TEM images of PAMAM DG4.0 (A and C) and DG4.5 (B and D) at neutral pH stored without (A and B) or with (C and D) shaking for 1 day post-reconstitution.

It is essential to highlight the biomedical implications of dendrimer aggregation and the need for a shaking process for at least 24 h to induce the disaggregation before an intravenous or intranasal administration. For example, Win-Shwe et al. (2014) performed DLS measurements of DG4.0 in methanol where they have a size of 3.4 ± 0.9 nm; after methanol evaporation and reconstitution in water, where they showed two populations, one of 5.7 ± 1.4 nm and other of 976 ± 391 nm (aggregates of dendrimers); and after 24 h storage at 4 °C, where aggregates disappear leaving a single population of 5.6 ± 2.3 nm [32]. Our work highlights the need to also shake the DG4.5 samples during storage.

CONCLUSION

We have studied the aging process of PAMAM dendrimers by analyzing their UV-Vis absorption, their fluorescence emission, and their aggregation-state over time. In a different way than the already published work, we have put in evidence that changes in the agglomeration patterns of dendrimers would not be related to change in the fluorescence emission throughout aging. Also, we demonstrated that DG4.5 formed large aggregates in water that need to be shaken previous an *in vivo* administration.

This fact is essential to consider when the dendrimers are going to be used in biomedical applications as drug delivery systems or as a nanodrug.

Acknowledgments

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