Feasibility of cationic carbosilane dendrimers for sustainable protein sample preparation

Estefanía González-García[1,2], Javier Sánchez-Nieves[2,3,4,5], Francisco Javier de la Mata[2,3,4,5], María Luisa Marina[1,2], María Concepción García[1,2,*]

[1]Departamento de Química Analítica, Química Física e Ingeniería Química, Universidad de Alcalá, Ctra. Madrid-Barcelona, Km. 33.600, 28871 Alcalá de Henares, Madrid, Spain

[2]Instituto de Investigación Química "Andrés M. del Río", Universidad de Alcalá, Ctra. Madrid-Barcelona, Km. 33.600, 28871 Alcalá de Henares, Madrid, Spain

[3]Departamento de Química Orgánica y Química Inorgánica, Universidad de Alcalá, Ctra. Madrid-Barcelona, Km. 33.600, 28871 Alcalá de Henares, Madrid, Spain

[4]Networking Research Center for Bioengineering, Biomaterials and Nanomedicine (CIBER-BBN), Spain

[5]Instituto Ramón y Cajal de Investigación Sanitaria, IRYCIS, Spain.

*Corresponding author: concepcion.garcia@uah.es

Abstract

Protein sample preparation is the bottleneck in the analysis of proteins. The aim of this work is to evaluate the feasibility of carbosilane dendrimers functionalized with cationic groups to make easier this step. Anionic carbosilane dendrimers (sulphonate- and carboxylate-terminated) have already demonstrated their interaction with proteins and their potential in protein sample preparation. In this work, interactions between positively charged carbosilane dendrimers and different model proteins were studied when working under different pH conditions, dendrimer concentrations, and dendrimer generations. Amino- and trimethylammonium-terminated carbosilane dendrimers presented, in some cases, weak interactions with proteins. Unlike them, carbosilane dendrimers with terminal dimethylamino groups could interact, in many cases, with proteins and these interactions were affected by the pH, the dendrimer concentration, and the dendrimer generation. Moreover, dendrimer precipitation was observed at all pHs, although just second and fourth generation (2G and 4G) dendrimers resulted in the formation of complexes with proteins. Under experimental conditions promoting dendrimer-protein interactions, 2G dimethylamino-terminated dendrimers were proposed as an alternative to other methods used in analytical chemistry or analysis in which an organic solvent or a resin are required to enrich/purify proteins in a complex sample.

Keywords: carbosilane dendrimer; cationic dendrimer; interaction; protein enrichment; protein purification.

1. Introduction

Protein sample preparation involves all steps from the separation/isolation of proteins within a matrix to their purification/enrichment. All these operations imply the disruption of the interactions established between proteins and other molecules (lipids, nucleic acids, carbohydrate, pigments, phenols, etc.) in the sample [1]. Taking into account these interactions and the heterogeneity, complexity, and instability of proteins, protein sample preparation constitutes a great challenge. Protein extraction has been carried out based on the different solubility, hydrophobicity, isoelectric point, or molecular weight of proteins [2, 3]. It is common to extract proteins using an alkali buffer containing reducing, denaturing, or chaotropic agents. The coextraction of other molecules is usual during the extraction of proteins, which forces the introduction of a final purification step. This step currently involves the precipitation of proteins with an organic solvent (trichloroacetic acid, acetone, methanol, or phenol) [1, 4, 5]. These facts make these methodologies to be tedious, time-consuming and non-suitable for the extraction of small amounts of proteins. Moreover, they do not guarantee the complete removal of interfering compounds and, overall, they are non-sustainable [6, 7].

Nanomaterials, as gold nanoparticles, magnetic nanoparticles or carbon nanotubes among many others, have demonstrated to be a suitable alternative to conventional methodologies used in protein sample preparation. Indeed, different nanomaterials have been employed in the extraction, enrichment, and purification of proteins [8]. Moreover, they can be easily synthesized with a specific size and functionality, which enables the control of the selectivity in the extraction and purification of proteins [8, 9]. A kind of nanomaterials, scarcely exploited in protein sample preparation, are dendrimers. Dendrimers are hyperbranched macromolecules

constituted by a central core surrounded by branches called "dendrons" [10]. According to their ramification, they can be 1st generation dendrimers (1G), 2nd generation dendrimers (2G), 3rd generation dendrimers (3G), etc. The higher the dendrimer generation, the higher the number of functional groups. Their multivalent surface along with the microenvironment within their flexible branches, make them suitable to host guest molecules [11]. These characteristics have made possible their use in biomedical applications [10]. Most commonly used dendrimers are polyamidoamine (PAMAM) ones. They were the first kind of dendrimers to be synthesized and are commercially available. Interactions between hydrophilic PAMAM dendrimers and proteins have been studied in different works [12-15]. Moreover, PAMAM dendrimers have been functionalized on magnetic nanoparticles surface to be used for the immobilization of bovine serum albumin (BSA) [16]. Other known dendrimers are poly(propylene imine), polyester, or silicon-based dendrimers, among many others [17].

Carbosilane dendrimers are silicon-based dendrimer with silicon atoms as branching points between generations which results in a high kinetic and thermodynamic stability derived from strong C-Si bonds [18-20]. They are easily functionalized, present high flexibility, and are biocompatible. Moreover, they have a hydrophobic skeleton and, at the same time, they are water-soluble after functionalization with polar groups, which promotes both hydrophobic and hydrophilic interactions [18, 21]. Different works have demonstrated the interaction of carbosilane dendrimers (amino-, ammonium-, and carboxylate-terminated) with proteins [22-25]. These features make carbosilane dendrimers to be interesting in protein sample preparation. In fact, selectivity of carbosilane dendrimers can be monitored since they are easily functionalized with different groups. Moreover, they do not require the use

of high amounts of organic solvents or polluting reagents, as conventional methods, and they enable the significant reduction of extraction/purification times.

Our research group observed that anionic (sulphonate- and carboxylate-terminated) carbosilane dendrimers/dendrons could be applied to the extraction and purification of proteins [26-28]. Interactions between these dendrimers/dendrons and proteins greatly depended on the protein itself, pH, and dendrimer generation and concentration. In general, interactions were favored when using high dendrimer generations and acidic pHs [26, 27]. Especially interesting was the application of carboxylate-terminated carbosilane dendrimers to protein purification since they resulted in the precipitation of the dendrimer-protein complexes that could be easily separated from the remaining solution [27]. On the other hand, single-walled carbon nanotubes functionalized with sulphonate-terminated carbosilane dendrons were also successfully applied to the extraction of proteins [28]. More recently, carbosilane dendron-coated gold nanoparticles have also been applied in the extraction of proteins although the high strength of stablished interactions required the use of very harsh conditions to release extracted proteins [29]. Despite these encouraging results, further research is still required to make more readily the application of these nanosystems and even to reuse them.

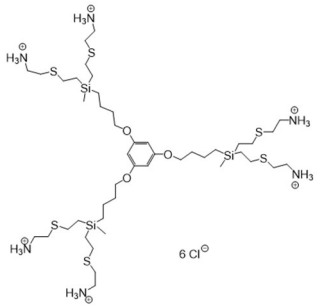
Cationic dendrimers have never been applied in protein sample preparation despite their great potential. Previous researches showed that cationic carbosilane dendrimers could interact with RNA forming RNA-dendrimer nanoparticle complexes that were useful for delivering antiHIV nucleic acids [30]. Moreover, cationic carbosilane dendrimers have also demonstrated a great potential to interact with HIV-derived

peptides [31]. These results support the interest of studying the feasibility of cationic dendrimers in protein sample preparation, which constitutes the main aim of this work.

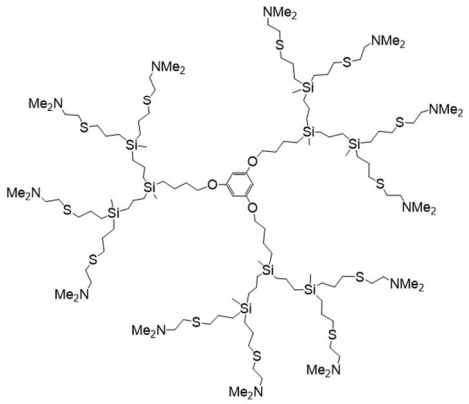
2. Materials and Methods

2.1. Reagents and materials

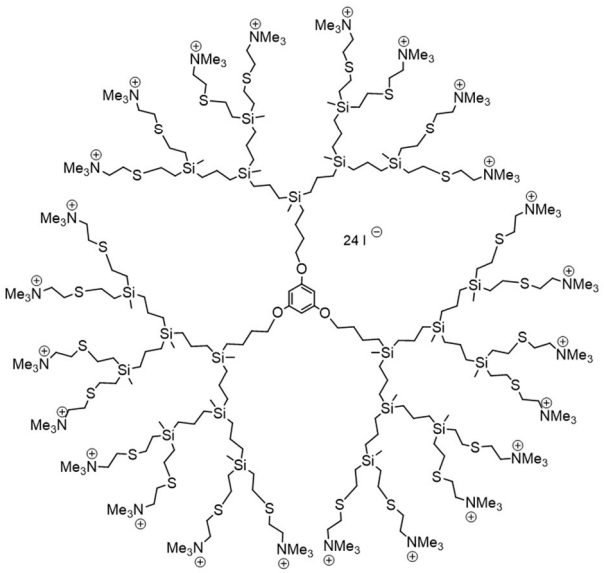
All chemicals and reagents were of analytical grade. Water was purified through a Milli-Q system from Millipore (Bedford, MA, USA). Trifluoroacetic acid (TFA), sodium hydroxide, β-mercaptoethanol, dithiothreitol (DTT), bovine serum albumin (BSA), lysozyme (Lyz) from chicken egg white, and myoglobin (Myo) from equine heart were from Sigma-Aldrich (Saint Louis, MO, USA). Tris(hydroxymethyl)aminomethane (Tris), hydrochloric acid (HCl), sodium dodecyl sulfate (SDS), and sodium chloride (NaCl) were from Merck (Darmstadt, Germany) and acetic acid, methanol (MeOH), tetrahydrofuran, ethanol, and acetone were from Scharlau Chemie (Barcelona, Spain). Laemmli buffer, Mini-Protean precast gels, Tris/glycine/SDS running buffer, Precision Plus Protein Standards (recombinant proteins expressed by *E. coli* with molecular weights of 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa), Bio-Safe Coomassie stain, silver stain kit, and Bradford reagent (Coomassie Blue G-250) were acquired at Bio-Rad (Hercules, CA, USA). Amino-terminated carbosilane dendrimers (A) ($G_1O_3(S\text{-}NH_2)_6$ (1G-A), $G_2O_3(S\text{-}NH_2)_{12}$ (2G-A), $G_3O_3(S\text{-}NH_2)_{24}$ (3G-A)), dimethylamino-terminated carbosilane dendrimers (D) ($G_2O_3(S\text{-}N(CH_3)_2)_{12}$ (2G-D), G_3O_3 $(S\text{-}N(CH_3)_2)_{24}$ (3G-D), and G_4O_3 $(S\text{-}N(CH_3)_2)_{48}$ (4G-D)), and trimethylammonium-terminated carbosilane dendrimers (T) ($G_1O_3(S\text{-}NMe_3^+)_6$ (1G-T), $G_2O_3(S\text{-}NMe_3^+)_{12}$ (2G-T), $G_3O_3(S\text{-}NMe_3^+)_{24}$ (3G-T), and $G_4O_3(S\text{-}NMe_3^+)_{48}$ (4G-T)) were prepared following methods previously described [32]. **Figure 1** shows the structures corresponding to different carbosilane dendrimer studied in this work.



1G amino-terminated



2G dimethylamino-terminated



3G trimethylammonium-terminated

Figure 1. Structures corresponding to the 1G, 2G, and 3G of the amino-, dimethylamino-, and trimethylammonium-terminated carbosilane dendrimers, respectively.

144 **2.2. Fluorescence measurements**

145 Intrinsic fluorescence of proteins was monitored with a RF-1501
146 spectrofluorometer (Shimadzu, Kyoto, Japan). The λ_{EXC} was fixed at 279–281 nm and the
147 spectra were recorded from 290 to 400 nm. Dimethylamino dendrimers were solubilized
148 in tetrahydrofuran while amino and trimethylammonium dendrimers were dissolved in
149 water. Dendrimer solutions at different pHs (1.8 (in 0.1% TFA), 7.5 (in Milli-Q water), 9.0
150 (in 5 mM Tris-HCl), and 12 (in 2 M NaOH)) containing standard proteins (BSA, Lyz, and
151 Myo) at different protein:dendrimer molar ratios (1:0, 1:1, 1:4, 1:8, 1:12, and 1:20) were
152 prepared by duplicate. Moreover, blank solutions of dendrimers were also prepared at
153 the different pHs. Mixtures were incubated for 30 min at room temperature. In those
154 cases where a precipitate was formed, solutions were centrifuged at 4000 g for 10 min
155 and fluorescence intensity of supernatants was registered immediately after. All
156 measurements were carried out by triplicate. Final fluorescence intensity was obtained
157 by subtracting the fluorescence of blanks.

158 **2.3. Gel electrophoretic separation**

159 Separation of proteins by sodium dodecyl sulfate-polyacrylamide gel
160 electrophoresis (SDS-PAGE) was carried out in a Mini-Protean system from Bio-Rad.
161 Protein solutions were mixed at a 1:1 (v/v) ratio with Laemmli buffer containing 5 % (v/v)
162 β-mercaptoethanol, while protein precipitates were directly dissolved in this buffer.
163 Resulting mixtures were heated at 100 ℃ for 5 min and introduced into the gel wells.
164 Next, a potential of 80 V was applied for 5 min for proteins loading into the gel, and,
165 then, a potential of 200 V for 30 min for protein separation. Finally, gels were treated
166 with a fixing solution containing water/MeOH/acetic acid (50/40/10 % (v/v/v)) during 30

167 min. For low protein concentration samples, silver staining was employed while
168 Coomassie Blue staining was used for high protein concentration samples.

169 Silver staining requires the use of a second fixing solution consisting of 10 %
170 EtOH/5 % acetic acid that was added twice for 15 min each. Afterwards, gels were
171 treated with an oxidizer for 5 min, washed with water, and stained with silver reagent
172 by shaking for 20 min. After washing for 1 min with water, the developing solution was
173 added. The reaction was stopped when bands were visible by adding water/acetic acid
174 (95/5 % (v/v)). Coomassie staining was carried out by treating the gel with Coomassie
175 Bio-Safe reagent for 1 h and, then, washing with water for 2 h.

176 **2.4. Protein extraction from plum seeds**

177 Protein extraction was carried out following the procedure of González-García et
178 al. [33] with some modifications. 30 mg of defatted plum seeds were mixed with 5 mL
179 of 100 mM Tris-HCl pH 7.5 containing 0.25 % (m/v) DTT, sonicated with a high intensity
180 focused ultrasound (30 % amplitude, 5 min), and centrifuged (10 min, 4000 g). The
181 supernatant was recovered and submitted to a purification step either by acetone
182 precipitation (4 ℃, 30 min) or by precipitation with dendrimers. Precipitates were
183 recovered by centrifugation (10 min, 4000 g). The concentration of proteins was
184 estimated according to the Bradford method [34] using a calibration curve prepared
185 with BSA (0.025 – 0.3 mg mL^{-1}).

186 **3. Results and discussion**

187 Interactions between cationic carbosilane dendrimers and proteins were
188 monitored by measuring the intrinsic fluorescence intensity and the maximum emission

189 wavelength of proteins in presence of increasing dendrimers concentrations and under
190 different pH conditions. For that purpose, three standard proteins (BSA, Lyz, and Myo)
191 covering a wide range of molecular weights (from 14.3 to 66.5 kDa) and isoelectric points
192 (from 4.7 to 11.5) were chosen. **Table 1** shows the size, isoelectric point, and net charge
193 of proteins depending on the pH. Intrinsic fluorescence of proteins is mainly due to
194 tryptophan (Trp) residues and it depends on the number of these residues and their
195 accessibility [35]: BSA (Trp134 and Trp213), Lyz (Trp28, Trp62, Trp63, Trp108, Trp111,
196 and Trp123), and Myo (Trp7 and Trp14). Any change in the Trp environment, due to e.g.
197 an interaction, could result in a modification of the fluorescence intensity. Three
198 different families of cationic carbosilane dendrimers were studied: amino-terminated
199 dendrimers (1G, 2G, and 3G), dimethylamino-terminated dendrimers (2G, 3G, and 4G),
200 and trimethylammonium-terminated dendrimers (1G, 2G, 3G, and 4G).

201 **Table 1.** Studied proteins molecular weight, isoelectric point, and theoretical estimation
202 of net charge at pHs 1.8, 7.5, and 9.0.

	Molecular weight (kDa)	Isoelectric point (pI)	Net charge*		
			pH 1.8	pH 7.5	pH 9.0
BSA	66.5	4.7	+ 97	- 17	- 22
Lysozyme	14.3	11.5	+ 17	+ 8	+ 8
Myoglobin	17.8	6.8	+ 31 (heme group, - 2)	0 (heme group, - 2)	0 (heme group, - 2)

203 * Values obtained from reference [27].

204 **3.1. Study of interactions between amino-terminated carbosilane dendrimers**
205 **and proteins**

206 Amino-terminated dendrimers present a pKa of near 9.4 and just after
207 deprotonation they become insoluble. At acidic pH, fluorescence studies revealed no

208 variation of fluorescence intensity and maximum emission wavelength of the three
209 standard proteins in presence of increasing concentrations (0 – 40 μM) of 1G, 2G, and
210 3G amino-terminated dendrimers. These results were expected since, at this pH, both
211 proteins and dendrimer are positively charged, and electrostatic interactions are
212 unlikely.

213 A similar behavior was observed at pH 7.5 and 9.0 for all three proteins.
214 Nevertheless, BSA and Myo, at these pHs, have net negative charge (-17 charges at pH
215 7.5 and -22 charges at pH 9.0 for BSA and -2 charges for Myo at both pHs [27]) and could
216 interact with positively charged amino-terminated dendrimers. These results could be
217 explained considering that the interaction between proteins and dendrimers, if any, was
218 far away from the Trp residues or that it did not significantly affect to the protein
219 conformation. In the case of Lyz, a constant fluorescence intensity was observed when
220 it was in contact with the dendrimers at pHs 7.5 and 9.0. These results would suggest a
221 lack of interaction which would agree with the fact that both Lyz and dendrimer are
222 positively charged.

223 At the highest pH studied, pH = 12, amino-terminated dendrimers are uncharged
224 and they form a precipitate. Under these conditions, BSA and Lyz showed a slight
225 variation of their intrinsic fluorescence, that could be due to the establishment of
226 interactions of non-electrostatic nature. However, Myo yielded a huge increase in the
227 emitted fluorescence intensity when the dendrimer was added (regardless the
228 generation). This increase was attributed to the *heme* group that is attached to Myo at
229 pHs higher than pH 2. When the *heme* group is close to a Trp residue, it can withdraw
230 its energy observing a low emitted fluorescence intensity [36]. Any change in the Myo

structure, e. g. due to an interaction with the dendrimer, can disturb this energy transfer from the Trp residue to the *heme* group triggering an increase in the emitted fluorescence intensity. This singular behavior of Myo in the interaction with dendrimers at neutral and basic pHs have been previously documented with anionic carbosilane dendrimers [26, 27].

Therefore, despite slight interactions between amino-terminated carbosilane dendrimers and proteins could be happening at neutral and basic pHs, these dendrimers do not seem to be suitable for protein sample preparation.

3.2. Study of interactions between trimethylammonium-terminated carbosilane dendrimers and proteins

Trimethylammonium-terminated dendrimers are positively charged at all studied pHs (1.8, 7.5, and 9.0). As can be observed in **Figure 2**, at acidic pH, all three proteins showed an almost constant fluorescence signal with 1G-T, 2G-T, and 3G-T dendrimers. A simple explanation could be the positive charge of both dendrimers and proteins at this low pH. Nevertheless, surprisingly, 4G-T dendrimers produced a decrease in the fluorescence intensity of the three proteins at acidic pH. This behavior could be attributed to the higher size of dendrimer branches that could trap proteins. Moreover, although the net charge of proteins was positive, they have sites with local negative charge that could interact with positive functional groups.

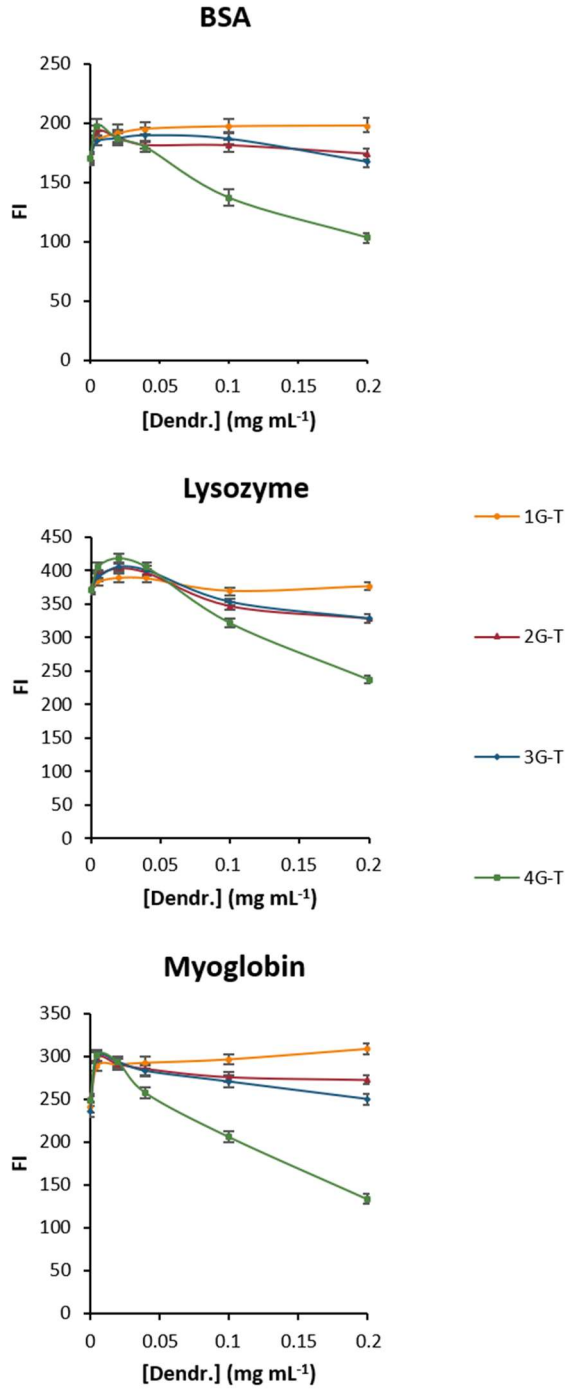


Figure 2. Variation of the fluorescence intensity (λ_{EXC} = 279–281 nm and λ_{EM} = 290–400 nm) of BSA, lysozyme, and myoglobin at 3 µM with increasing concentrations of the 1G, 2G, 3G, and 4G trimethylammonium-terminated carbosilane dendrimers at acidic pH after 30 min incubation at room temperature. Error bars show the standard deviation of two replicates measured by triplicate.

A deeper decrease was observed at higher pHs for all generations with BSA. In this case, the negative charge of BSA could promote electrostatic interactions of this protein with the dendrimer. The same reason can explain the fluorescence variation in the case of Myo at pH 9.0 (-2 charges). However, unlike BSA, Myo showed a drastic increase of fluorescence intensity at pH 9.0. This behavior can be explained considering the possible disruption of the energy transfer from Trp residues to the *heme* group, previously commented. On the other hand, Lyz showed the slighter reduction of fluorescence intensity when adding the 4G-T dendrimer at all pHs. Since both protein and dendrimer are always positively charged, interactions, if any, would have a non-electrostatic nature.

Although trimethylammonium-terminated dendrimers have demonstrated to interact with proteins, especially when using the 4G-T dendrimer, there is a limitation when trying to apply them to protein sample preparation, since interacting and non-interacting proteins remain in solution.

3.3. Study of interactions between dimethylamino-terminated carbosilane dendrimers and proteins

The pKa of dimethylamino-terminated dendrimers is about 9.5. However, these dendrimers are insoluble in water but soluble in tetrahydrofuran. For that reason, they do not reach the protonated state. The addition of water to dendrimers in tetrahydrofuran results in turbid solutions and their mixing with proteins increased, even more, the turbidity of the solutions. Dendrimers were mixed with proteins for 30 min and, after centrifugation, supernatants were recovered and analyzed. Results showed that the fluorescence intensity and the maximum emission wavelength of the

three standard proteins varied when increasing the dendrimer concentration at all studied pHs. At acidic pH, the addition of the dendrimer produced a reduction in the fluorescence intensity of BSA and Lyz that came along with a reduction in their maximum emission wavelength (blue-shift). This shift could be attributed to a structural change in the vicinity of Trp residues that may be promoted by the interaction of these proteins with the dendrimer [37]. This reduction can also be due to the precipitation of the protein along with the dendrimer. A similar situation was observed for BSA and Lyz at pH 7.5 and 9.0. Since dimethylamino- terminated dendrimers are uncharged, the observed fluorescence quenching could be attributed to the establishment of non-electrostatic interactions. Unlike them, Myo fluorescence intensity remained constant when adding the dendrimer at acidic pH but it showed a huge increase in the emitted signal when adding the dendrimer at pHs 7.5 and 9.0. This behavior was, again, attributed to the interaction of Myo with dendrimer near the *heme* group, interfering in the energy transfer from Trp residues to the *heme* group.

Despite these results could demonstrate the interaction of proteins with dendrimers, the observed fluorescence deactivation when Lyz and BSA were mixed with dendrimer could also be attributed to a collision (dynamic quenching) and not to the interaction between the protein and the dendrimer (static quenching). In order to find out whether this quenching was due to an interaction or a collision, further studies using SDS-PAGE were carried out. For that purpose, the supernatants obtained after mixing 2G-D, 3G-D, and 4G-D dendrimers and proteins were analyzed by SDS-PAGE. If fluorescence deactivation is due to a collision, the electrophoretic signal due to proteins will remain constant in the supernatant regardless the dendrimer concentration. If fluorescence deactivation is due to the interaction between proteins and the

303 precipitated dendrimer, the electrophoretic signal of proteins in the supernatant will
304 decrease when increasing the dendrimer concentration. **Figure 3.a.** shows the
305 electrophoretic band corresponding to BSA at acidic pH at two protein:dendrimer ratios
306 (1:8 and 1:20) and for the three studied generations. The same results were obtained at
307 all pHs and for all proteins. As observed, BSA band disappeared at a protein:dendrimer
308 ratio 1:20 for 2G-D and 4G-D dendrimers. This result suggested that proteins were
309 interacting with dendrimers and that protein-dendrimer complexes were formed and
310 precipitated. Nevertheless, this behavior was not observed for the 3G-D dendrimer since
311 the band intensity remained constant. Thus, in the case of 3G-D dendrimer, the collision
312 did not result in the formation of a protein-dendrimer complex but just in the
313 deactivation of protein fluorescence. The different behavior between even and odd
314 dendrimer generations could be related to the different assembly of dendrons in every
315 generation and to the different hydrophilic-hydrophobic balance.

316 To confirm that proteins were precipitating with 2G-D and 4G-D carbosilane
317 dendrimers at the protein:dendrimer ratio 1:20, they were applied to the simultaneous
318 capture of BSA, Lyz, and Myo from a mixture containing the three standard proteins. In
319 the case of BSA and Lyz, interactions were promoted at acidic pHs, followed by neutral
320 and basic pHs. Nevertheless, in the case of Myo, it was not observed any variation in the
321 intrinsic fluorescence intensity of the protein when adding the dendrimer at acidic pH
322 while a great increase in the fluorescence intensity was observed at pHs 7.5 and 9.0.
323 Taking into account these results, the simultaneous capture of the three proteins was
324 carried out at pH 7.5. **Figure 3.b.** shows the electrophoretic separation of proteins in the
325 supernatant and in the pellet at a protein:dendrimer ratio 1:20. As expected, protein
326 bands in the supernatants disappeared when adding both 2G-D and 4G-D dendrimers,

while they did appear in the precipitate, which demonstrated their interaction with proteins. These results showed the potential of these dendrimers in proteins sample preparation.

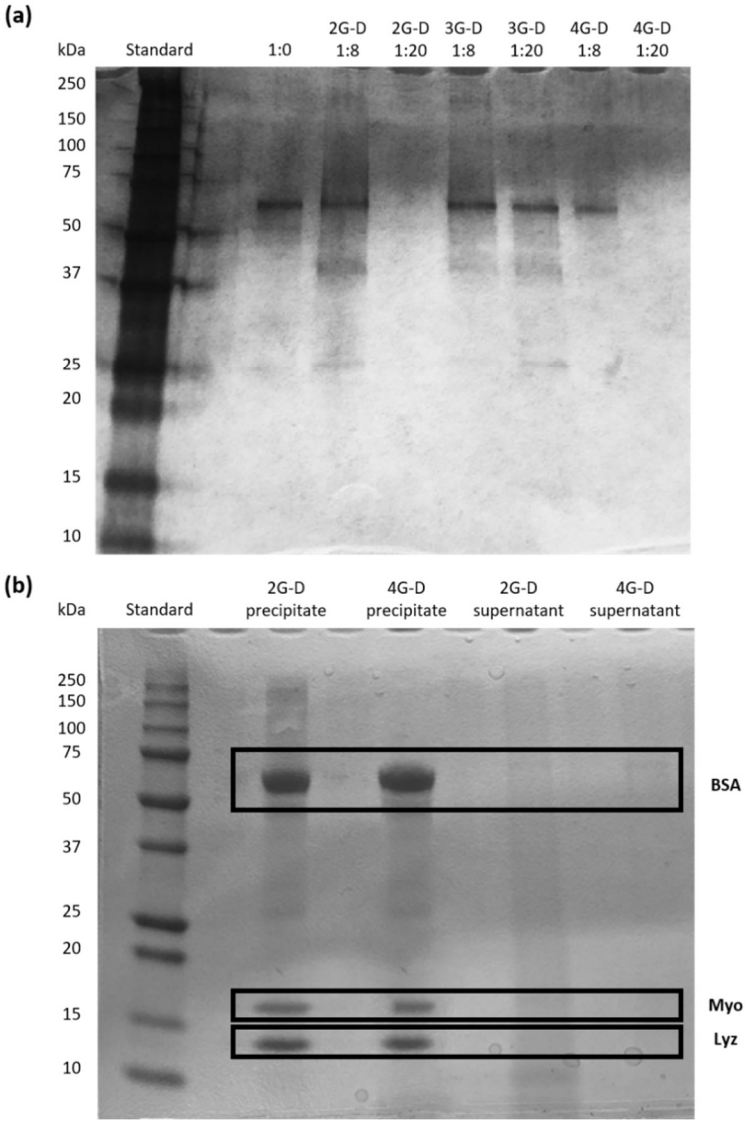


Figure 3. Gel electrophoretic profiles obtained by SDS-PAGE corresponding to **(a)** supernatants obtained after treating a BSA solution with different ratios (1:0, 1:8, and 1:20) of 2G, 3G, and 4G dimethylamino-terminated carbosilane dendrimers at acidic pH and **(b)** precipitates and supernatants obtained after the treatment of a protein mixture (BSA, Lyz, and Myo) with 1:20 ratio of 2G and 4G dimethylamino-terminated carbosilane dendrimers at neutral pH.

3.4. Application of dimethylamino-terminated carbosilane dendrimers to the enrichment/purification of proteins from a complex sample

To evaluate the feasibility of dimethylamino-terminated carbosilane dendrimers in protein sample preparation, they were applied to the enrichment/purification of proteins from a complex sample, plum seeds. Despite both 2G-D and 4G-D dendrimers enabled the precipitation of proteins, 2G-D dendrimers were selected for their easier synthesis.

Proteins from plum seeds were extracted using a buffered solution and a high intensity focused ultrasound probe. Then, the extracted plum seed proteins were purified using the 2G dendrimer at different concentrations, ranging from 4 to 200 µmol dendrimer/mg protein. As control, a traditional and non-sustainable procedure for the purification of proteins based on their precipitation with acetone (sample/acetone ratio 1:2 (v/v)), was carried out. The precipitated proteins were recovered by centrifugation, dissolved in the Laemmli buffer, and analyzed by SDS-PAGE (see **Figure 4.a.**). As observed in the gel image, a significant recovery of proteins was obtained when using dendrimer concentrations equal or higher than 50 µmol dendrimer/mg protein. Indeed, bands intensities were similar to the obtained when using the traditional precipitation with acetone. Taking into account the heterogeneity of plant proteins and that the results obtained with dendrimers were similar to the observed with the conventional method, it is possible to affirm that dendrimers present a high specificity for proteins, in general. Additionally, Bradford assay was employed to estimate the protein recovery obtained with the different dendrimer concentrations (see **Figure 4.b.**). This graphic displays the increase in the concentration of recovered proteins when increasing the

concentration of dendrimer used to precipitate them. Results showed that concentrations of 150-200 µmol dendrimer/mg protein yielded protein recoveries higher than 90 %. In fact, a concentration of 200 µmol dendrimer/mg protein enabled the precipitation of a 96.8 ± 1.1 %, which did not present significant differences ($p > 0.05$) with the recovery obtained when using the traditional acetone precipitation (100 %). Thus, these dendrimers constitute a greener alternative to the traditional organic solvent precipitation.

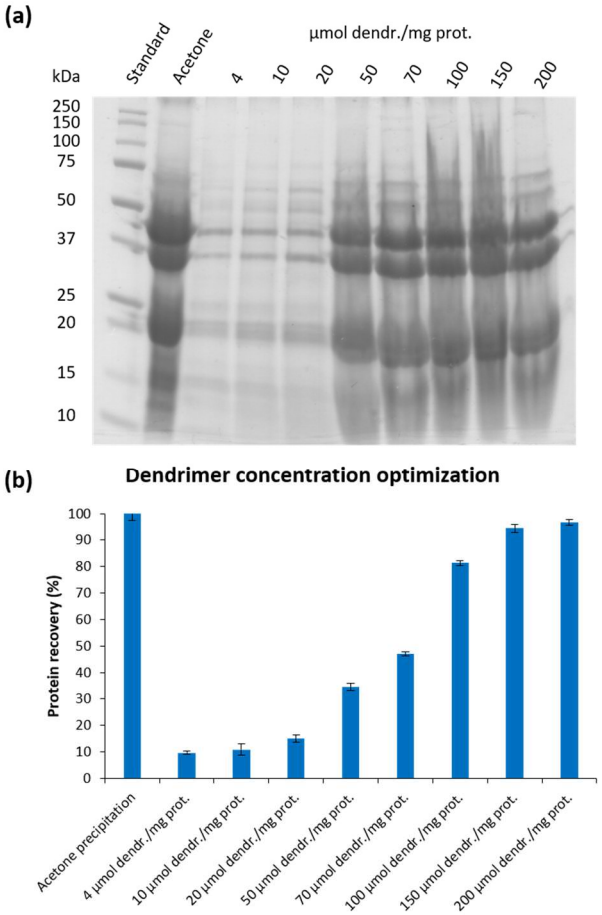


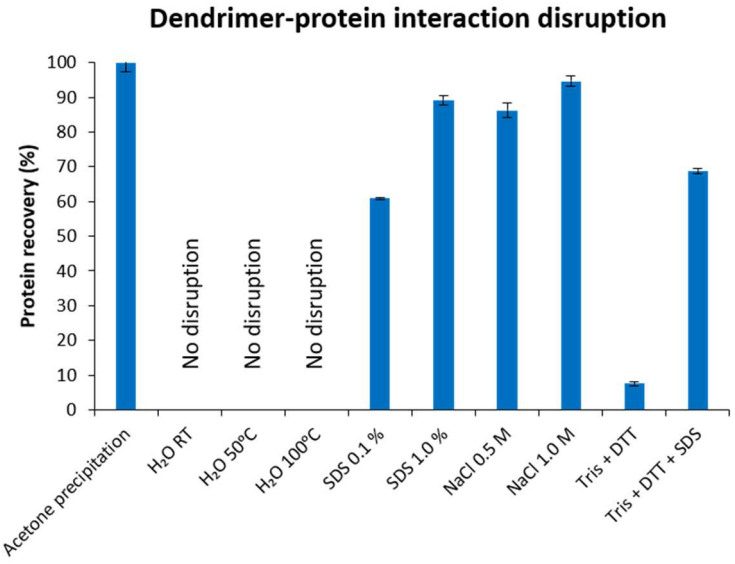
Figure 4. (a) Gel electrophoretic profile obtained by SDS-PAGE of the plum seed proteins precipitated with traditional acetone method and with increasing concentrations of 2G dimethylamino-terminated carbosilane dendrimers at neutral pH during 30 min and **(b)**

graphic representation of the corresponding protein recoveries estimated by Bradford assay. Error bars show the standard deviation of two replicates measured by triplicate.

Although protein-dendrimer complexes are perfectly disrupted in the Laemmli buffer used in SDS-PAGE, that contains SDS (2 % (m/v)) and β-mercaptoethanol (5 % (v/v)), these strong conditions cannot be used when proteins are extracted with purposes different from the electrophoretic separation (e.g. protein digestion). For that reason, different disruption conditions, based on bibliography [8, 27], were tested: water (room temperature, 50℃, and 100℃), SDS (0.1 and 1.0 % (m/v)), sodium chloride (0.5 and 1.0 M), and Tris-HCl buffer (containing DTT (0.5 % (m/v)) and containing SDS (0.5 % (m/v)) and DTT (0.5 % (m/v))). SDS and NaCl are common reagents used to disrupt the interaction between proteins and nanomaterials [8]. On the other hand, the use of SDS or DTT to disrupt protein-dendrimer interactions was also tested since they promote the denaturation of proteins. The recovered supernatants were analyzed by Bradford assay and results were displayed in **Figure 5**. As observed in this graphic, the use of just water at any temperature did not enable the disruption of the interaction. The use of Tris with DTT resulted in a very low recovery and the use of SDS (0.1 % (m/v)) or Tris-HCl buffer containing both DTT and SDS yielded moderate recoveries. The highest recoveries were obtained when using 1.0 M NaCl, followed by SDS (1.0 % (m/v)), and 0.5 M NaCl, which resulted in protein recoveries of 94.7, 89.3, and 86.3 %, respectively. However, the use of SDS in some applications, as protein hydrolysis or analysis, is limited, since SDS produces the enzymes denaturing or is incompatible with mass spectrometry. For that reason, the use of NaCl to disrupt the interaction between proteins and dendrimers is more suitable and suggested.

These results were compared with the obtained in a previous work using carboxylate-terminated carbosilane dendrimers, observing similar protein recoveries [27]. However, in that work, 3G dendrimers at acidic pH were required, while this work demonstrates that 2G cationic dendrimers, easier to synthesize than 3G dendrimers, at neutral pH, smoother conditions than acidic pH, can interact with proteins in a similar ratio to carboxylate ones.

Although the synthesis of carbosilane dendrimers require the use of some organic solvents, the proportional amount of solvent utilized to obtain the dendrimers needed for precipitating a certain amount of proteins is lower than the required to precipitate the same amount of proteins by the traditional strategy. Additionally, even though the synthesis of these dendrimers requires few steps, it enables the obtaining of an elevated amount of them, indicating that, overall, this methodology is less tedious than the conventional one. On the other hand, despite the reutilization of these dendrimers is limited, they have demonstrated a great potential in the enrichment/purification of proteins, which encourages the development of platforms based on these dendrimers that will allow its reuse [38].



410

411 **Figure 5.** Protein recoveries obtained after protein precipitation with 200 mmol 2G
412 dimethylamino-terminated carbosilane dendrimers/mg protein during 30 min and
413 subsequent interaction disruption using different media. Error bars show the standard
414 deviation of two replicates measured by triplicate.

415 4. Conclusions

416 This work demonstrates the feasibility of three different cationic carbosilane
417 dendrimers (amino-, trimethylammonium-, and dimethylamino-terminated) in protein
418 sample preparation. The study of the intrinsic fluorescence of proteins in presence of
419 amino-terminated dendrimers revealed the establishment of interactions between
420 myoglobin and dendrimers at a very high pH. The high size of 4G trimethylammonium-
421 terminated dendrimers made possible its interaction with proteins unlike same
422 dendrimers with lower generation. Since formed complexes remained in solution along
423 with non-interacting proteins, an additional step for their separation would be necessary
424 for their application in protein sample preparation. Deactivation of intrinsic proteins
425 fluorescence when they are mixed with 2G and 4G dimethylamino-terminated

426 dendrimers demonstrated the formation of protein-dendrimer complexes. Unlike even
427 generation dendrimers, a dynamic quenching was observed with 3G dimethylamino-
428 terminated dendrimer. Since protein-dendrimer interactions with 2G-D/4G-D
429 dendrimers came along with the formation of a precipitate, these dendrimers were
430 suitable for their use in protein sample preparation. 2G-D dendrimers were successfully
431 applied to the purification/enrichment of proteins extracted from plum seeds. In
432 comparison with a traditional purification method based on the precipitation of proteins
433 with acetone, dendrimers could precipitate 97 %. Most suitable solution for the
434 disruption of interactions, after purification, was 1 M NaCl that yielded a recovery of 95
435 %. These dendrimers suppose a more sustainable alternative than the conventional
436 precipitation of proteins using acetone. Future research in this line should address the
437 reusability of these platforms.

438

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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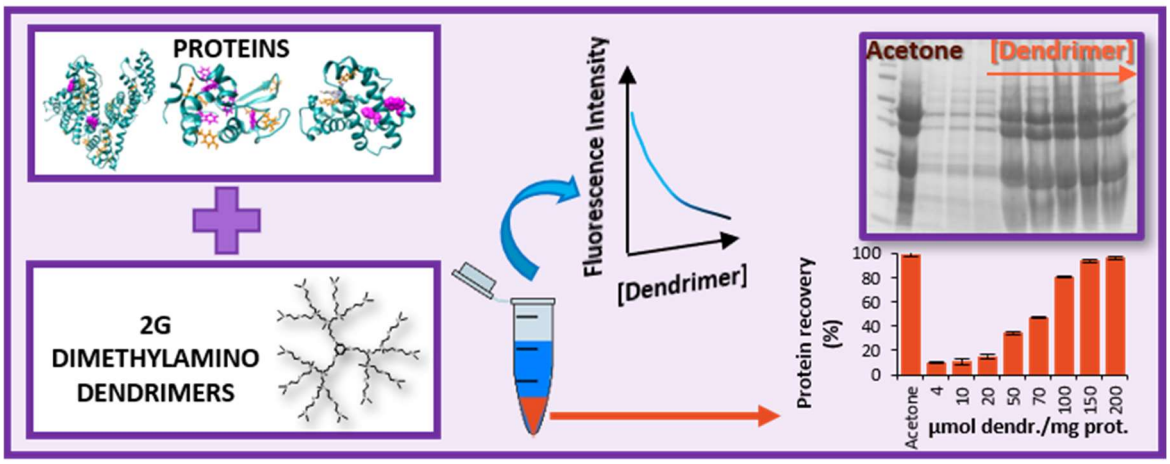

Graphical Abstract