

1 **Chitosan/carrageenan nanoparticles: Effect of cross-linking with tripolyphosphate**
2 **and charge ratios**

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20 **ABSTRACT**

21 Chitosan/carrageenan/tripolyphosphate nanoparticles were prepared by polyelectrolyte
22 complexation/ ionic gelation, the latter compound acting as cross-linker. The
23 incorporation of the three components in the nanoparticle matrix was assessed by
24 analytical techniques (FTIR, XPS and TOF-SIMS).

25 Using chitosan/carrageenan nanoparticles as control, the effect of the cross-linker in the
26 particles properties was studied. A decrease in size (from 450-500 nm to 150-300 nm)
27 and in zeta potential (from +75 - +85 mV to +50 - +60 mV), and an increase in
28 production yield (from 15-20% to 25-35%), and in stability (from one week to up to 9
29 months) were observed. Also, a correlation between positive to negative charge ratios in
30 the formulations and the above characteristics was established.

31 The small size and high positive surface charge make the developed
32 chitosan/carrageenan/tripolyphosphate nanoparticles potential tools for an application in
33 mucosal delivery of macromolecules.

34

35 **Keywords:** chitosan, cross-linking, *k*-carrageenan, nanoparticles, tripolyphosphate

36

37 **1. Introduction**

38 Polymeric nanoparticles have been used increasingly in various fields, such as drug
39 delivery, imaging and tissue engineering, the first being, by far, the most reported
40 application. The main reason justifying the widespread use of polymeric nanoparticles
41 relies on the displayed high surface-to-volume ratio which improves the loading
42 capacity of the selected molecule, while providing its protection. In addition, increased
43 drug absorption might be attained by the capacity of nanoparticles to reduce epithelial
44 resistance to transport (de la Fuente, Csaba, Garcia-Fuentes & Alonso, 2008; Rawat,
45 Singh & Saraf, 2006; Reis & Ribeiro, 2006).

46 Many polymers have been used to prepare these vehicles, but those of natural origin are
47 often preferred because, as compared to synthetic counterparts, they comply more easily
48 with the requisites of biocompatibility, biodegradability and absence of toxicity that are
49 mandatory in any biomedical application (Liu, Jiao, Wang, Zhou & Zhang, 2008;
50 Malafaya, Silva & Reis, 2007). Chitosan (CS) and carrageenan (CRG) are two marine-
51 derived polymers which belong to the above mentioned class, and have demonstrated in
52 a previous study the ability to assemble into nanoparticles of 400-600 nm (Grenha et al.,
53 2010). CS is a cationic polysaccharide composed of repeating units of *N*-
54 acetylglucosamine and *D*-glucosamine that are β -(1-4)-linked (Figure 1), and presents
55 well-documented favorable properties for drug delivery such as biocompatibility,
56 biodegradability, low toxicity (Dornish, Hagen, Hansson, Peucheur, Vedier &
57 Skaugrud, 1997; Hirano, Seino, Akiyama & Nonaka, 1988) and mucoadhesiveness
58 (Lehr, Bouwstra, Schacht & Junginger, 1992). CRG is another polysaccharide, extracted
59 from red seaweed (van de Velde, Knutsen, Usov, Rollemay & Cerezo, 2002) and
60 composed of galactose and anhydrogalactose units, linked by glycosidic bonds (Figure
61 1) (Lim, Gwon, Choi, Shin & Nho, 2010). Due to its half-ester sulfate moieties,

62 carrageenan displays a strong ionic nature and exhibits a high capacity to react with
63 proteins (Malafaya, Silva & Reis, 2007; Mohamadnia, Zohuriaan-Mehr, Kabiri,
64 Jamshidi & Mobedi, 2007). There are two types of carrageenan that evidence gel-
65 forming ability, *k*- and *i*-, *k*-carrageenan gels being more firm than those obtained with *i*-
66 carrageenan, which are more elastic and soft (Bixler, 1993). The assembly of the
67 referred CS/CRG nanoparticles was mediated by polyelectrolyte complexation (Grenha
68 et al., 2010), a method that uses very mild conditions, avoiding harmful organic solvents
69 or high shear forces. Therefore, it has the general capability of protecting the
70 encapsulated molecules and retaining their activity during the encapsulation, which are
71 its principal advantages (Mohanraj & Chen, 2006; Saboktakin, Tabatabaie, Maharramov
72 & Ramazanov, 2010; Grenha, 2012). This methodology involves the interaction
73 between a chitosan with high degree of protonation and a polyanion, permitting the
74 rapid formation of nanoparticles. Their size, as well as other characteristics, might be
75 modulated by adjusting formulation parameters like the type of materials composing the
76 particles matrix, their concentration and mass ratios, amongst others (Calvo et al. 1997a;
77 Grenha, 2012).

78 In many cases, for instance if the nanoparticles are to be applied in mucosal delivery, it
79 is important to ensure that their size will permit the contact with the epithelial surface,
80 an effect that is maximised for particles between 50 and 500 nm (Desai, Labhasetwar,
81 Amidon & Levy, 1996; Jani, Halbert, Langridge & Florence, 1990). Preparing
82 nanoparticles in this size range is facilitated by the use of adequate cross-linking agents.
83 Tripolyphosphate (TPP) is a non-toxic polyanion (Figure 1) known for its capacity to
84 cross-link chitosan, a reaction mediated by electrostatic forces, resulting in the
85 formation of ionic cross-linked networks (Janes, Calvo & Alonso, 2001; Mi, Sung,
86 Shyu, Su & Peng, 2003).

87 The objective of this work was to produce CS/CRG nanoparticles, including in the
88 formulation TPP as cross-linking agent, and to evaluate the effect of the presence of this
89 polyanion on the properties of nanoparticles, namely concerning size, surface charge
90 and stability. To do so, different amounts of cross-linker were used and formulations
91 with different polymeric mass ratios were tested. Reduced size and strong positive
92 surface charge would improve the nanoparticles contact with mucosal epithelial
93 surfaces, which is very positive when considering an application in mucosal drug
94 delivery.

95

96 **2. Experimental**

97 **2.1. Materials**

98 Chitosan (low molecular weight, deacetylation degree = 75-85%), pentasodium
99 tripolyphosphate, glycerol and glacial acetic acid were supplied by Sigma Chemicals
100 (Germany). *k*-carrageenan and potassium bromide (KBr) were obtained from FMC
101 Biopolymer (Norway) and Riedel-del-Haën (Germany), respectively. Ultrapure water
102 (Milli-Q Plus, Millipore Iberica, Spain) was used throughout.

103

104 **2.2. Nanoparticles preparation**

105 CS/CRG/TPP nanoparticles were prepared by a modification of a previously
106 described methodology (Grenha et al., 2010), based on the polyelectrolyte complexation
107 of CS with CRG and additional ionic gelation of chitosan with TPP anions. Briefly, CS
108 was dissolved in 1% (w/w) acetic acid to obtain a solution of 1 mg/mL and CRG and
109 TPP were dissolved in purified water to obtain stock solutions of 2.5 and 1.0 mg/mL,
110 respectively. Different volumes of the latter solutions were mixed in order to obtain
111 volumes of 0.8 mL of solutions with the required concentrations of both components.

112 The spontaneous formation of nanoparticles occurs upon incorporation, under gentle
113 magnetic stirring at room temperature, of the aforementioned solutions into 2 mL of the
114 CS solution, corresponding to final theoretical CS/CRG/TPP ratios varying from 4/1/0
115 to 7/1/1 (w/w).

116 Nanoparticles were concentrated by centrifugation at 16 000 x g on a 10 µL glycerol
117 layer for 30 min at 15 °C (centrifuge 5804R, Eppendorf, Germany). The supernatants
118 were discarded and nanoparticles were resuspended in 200 µL of purified water.

119

120 **2.3. Nanoparticles physicochemical characterization**

121 The production yield of nanoparticles was calculated by gravimetry. Fixed volumes
122 of nanoparticle suspensions were centrifuged (16 000 x g, 30 min, 15 °C), and
123 sediments were freeze-dried over 24 h at -34 °C, followed by a gradual increase in
124 temperature until 20 °C, using a Labconco freeze dryer (Labconco, USA) ($n = 3$).

125 The process yield (P.Y.) was calculated as follows: P.Y. (%) = (nanoparticle
126 sediment weight/total solid weight) x 100.

127 The morphological examination of CS/CRG/TPP nanoparticles was conducted by
128 transmission electron microscopy (TEM) (JEM-1011, JEOL, Japan). The samples were
129 stained with 2% (w/v) phosphotungstic acid and placed on copper grids with Formvar[®]
130 films for TEM observation.

131 Measurements of nanoparticle size and zeta potential were performed on freshly
132 prepared samples by photon correlation spectroscopy and laser Doppler anemometry,
133 respectively, using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). For the
134 analysis of particle size and determination of the electrophoretic mobility, each sample
135 was diluted to the appropriate concentration with ultrapure water and placed in the

136 electrophoretic cell. Each analysis was performed at 25 °C. Three batches of each
137 formulation were analyzed ($n = 3$).

138

139 **2.4. Nanoparticle stability study**

140 Aliquots of nanoparticle formulations with and without TPP (formulations 5/1/1 and
141 5/1/0, respectively) were stored at 4 °C. Nanoparticle sizes and zeta potentials were
142 monitored as a function of time for 250 days, using the technique described above ($n =$
143 3).

144

145 **2.5. Nanoparticles chemical analysis**

146 *2.5.1. Fourier transform infrared (FTIR) spectroscopy*

147 The interactions between the different components of the nanoparticulate systems
148 were analyzed by FTIR. Infrared spectra of the specimen powders, namely CS, CRG
149 and TPP, and CS/CRG/TPP nanoparticles (formulation 5/1/1), were recorded using a
150 FTIR spectrophotometer (Tensor 27, Bruker, Germany). Prior to the assay, the samples
151 were gently triturated with KBr and compressed into discs.

152 For each spectrum a 32-scan interferogram was collected in transmittance mode with
153 a 4 cm^{-1} resolution in the $4000\text{--}400\text{ cm}^{-1}$ region at room temperature.

154

155 *2.5.2. Surface analysis by X-Ray photoelectron spectroscopy (XPS) and time-of-* 156 *flight secondary ion mass spectrometry (TOF-SIMS)*

157 The surface of CS/CRG/TPP nanoparticles was analyzed to determine their chemical
158 composition. To do so, a droplet of nanoparticles (formulations 4/1/1 and 5/1/1) was
159 placed directly on a polished monocrystalline silicon wafer, used as a sample holder.
160 The droplet was then allowed to dry in a desiccator, prior to the analyses. The surface of

161 the samples was analyzed by XPS (K-Alpha ESCA, Thermo Scientific, UK) and TOF-
162 SIMS (TOF-SIMS IV, Ion-TOF GmbH, Germany). Solutions of the different
163 compounds (CS, CRG and TPP) were analysed separately as controls.

164 The XPS measurements were carried out using monochromatic Al-K α radiation ($h\nu =$
165 1486.6 eV), and photoelectrons were collected from a take-off angle of 90° relative to
166 the sample surface. The X-Ray monochromatic spots were 400 μm in diameter and the
167 correspondingly sampling area was 0.1256 mm^2 . Measurements were performed in
168 constant analyzer energy (CAE) mode with 100 eV pass energy for survey spectra and
169 20 eV pass energy for high-resolution spectra. Charge referencing was done by setting
170 the lower binding energy C 1s photopeak at 285.0 eV, the C 1s hydrocarbon peak
171 (Briggs & Seah, 1983). Surface elemental composition was determined using the
172 standard Scofield photoemission cross section. Residual vacuum in the analysis
173 chamber was maintained at around 3×10^{-9} mbar.

174 For TOF-SIMS analyses, samples were bombarded with a pulsed bismuth ion beam
175 (Bi^{3+}) generated with a liquid metal ion gun operated at 25 keV and a 45° incidence
176 with respect to the sample surface. The secondary ions generated were extracted with a
177 10 kV voltage, and their time-of-flight from the sample to the detector was measured in
178 a reflectron mass spectrometer. Electron flood gun charge compensation was necessary
179 during measurements. A raster size of 500 $\mu\text{m} \times 500 \mu\text{m}$ was used, and at least three
180 different spots were analyzed under the “static” condition with ion doses of 2×10^{12}
181 ions/ cm^2 . The calibration of the mass spectra in the positive mode was based on
182 hydrocarbon peaks such as CH_3^+ , C_2H_3^+ , C_3H_5^+ and C_7H_7^+ . Negative spectra were
183 calibrated to the C^- , C_2^- , C_3^- , C_4^- , C_2H^- , C_3H^- and C_4H^- peaks before further analysis. The
184 experimental conditions (ion type, beam voltage, and primary ion dose) were
185 maintained constant for each experiment.

186

187 **2.6. Statistical analysis**

188 The t-test and the one-way analysis of variance (ANOVA) with the pair wise
189 multiple comparison procedures (Student-Newman-Kleus Method) were performed to
190 compare two or multiple groups, respectively. All analyses were run using the
191 SigmaStat statistical program (Version 3.5, SyStat, USA) and differences were
192 considered to be significant at a level of $p < 0.05$.

193

194 **3. Results and discussion**

195 **3.1. CS/CRG/TPP nanoparticles characterization**

196 CS/CRG/TPP nanoparticles were produced by a very mild polyelectrolyte
197 complexation/ionic gelation method, as described in the Experimental Section. Briefly,
198 when the three components are mixed, an electrostatic interaction is established between
199 the positively charged amino groups of CS and the negatively charged sulphate and
200 phosphate groups of CRG and TPP, respectively, leading to the nanoparticle formation
201 in a process derived from inter- and intramolecular linkages mediated by the anionic
202 molecules (Janes, Calvo & Alonso, 2001). TPP affords a further intense interaction, as it
203 provides a cross-linking effect. Figure 2 displays a TEM microphotograph of
204 representative CS/CRG/TPP nanoparticles, showing a spherical morphology and
205 compact structure.

206 An estimation of the positive to negative (+/-) charge ratios for the different
207 formulations was made, based on the following assumptions: 1) Chitosan was
208 considered to have one positive charge per deacetylated monomer and, since it has a
209 deacetylation degree of 75-85%, a mean value of 0.8 positive charges per monomer was
210 used. According to a reported method (Ma et al, 2008) an average monomeric molecular

211 weight of 169 g/mol for this deacetylation degree was obtained; 2) Carrageenan was
212 assumed to be in the sodium salt form, to which corresponds a mass of 408 g/mol and a
213 negative charge per disaccharide monomer unit; 3) Pentasodium tripolyphosphate has a
214 molar mass of 368 g/mol and five negative charges per anion. The mass of each
215 compound in every formulation was then converted to moles of charge and the +/-
216 molar ratio was calculated.

217 As can be seen in Figure 3, formulations with mass ratios below 4/1/1 (as 2/1/0.5 and
218 3/1/1), which evidence a +/- charge ratio around 1 (Figure 3A), resulted in precipitation
219 (Figure 3B). The observed precipitation for lower ratios is due to the presence of an
220 excess of anionic charges, which neutralize chitosan positive charges and, thus, reduce
221 or eliminate electrostatic repulsion, leading to precipitation. In fact, a 1:1 +/- charge
222 stoichiometry does not mean that complete charge neutralization will occur, due to
223 different charge spacings in the intervenient species and to steric constraints. However,
224 one may assume a preferential interaction between the sulphate and the ammonium
225 groups, both weakly hydrated, instead of with the strongly hydrated counterions
226 (Crouzier & Picart, 2009). The same assumption should apply to TPP polyanion. This
227 would lead to mainly an intrinsic charge matching in detriment of an extrinsic charge
228 compensation and, thus, to a small deviation from neutrality.

229 On the contrary, the formulation corresponding to a mass ratio of 7/1/0, which
230 displays a +/- charge ratio of 13.5, did not lead to nanoparticle formation. As observed
231 by other authors and, according to the described formation mechanism of nanoparticles,
232 which is mediated by electrostatic interactions, the absence of nanoparticle formation is
233 attributed to an insufficient number of anionic charges to neutralise CS amino groups
234 (Calvo, Remuñán-López, Vila-Jato & Alonso, 1997a; Fernández-Urrusuno, Calvo,
235 Remuñán-López, Vila-Jato & José Alonso, 1999). For this reason, Figure 4 only depicts

236 the physicochemical characteristics of CS/CRG/TPP nanoparticles of mass ratios
237 between 4/1/0 and 6/1/1.

238 Observing in Figure 4A the data corresponding to formulations containing only
239 CS/CRG (4/1/0 to 6/1/0), no significant differences are observed for both size and zeta
240 potential, which lay around 450-500 nm and +80 mV, respectively. However, bearing in
241 mind the observations of Figure 3A, concerning charge ratios, there is a tendency to
242 decrease the size from the formulation 4/1/0 (charge ratio of 7.7) to 6/1/0 (charge ratio
243 of 11.6), which is attributed to the increase in the charge ratio, as reported elsewhere
244 (Chen, Mohanraj, Wang & Benson, 2007; Nizri, Magdassi, Schmidt, Cohen & Talmon,
245 2004; Nizri, Makarsky, Magdassi & Talmon, 2009). Increasing CRG content in the
246 formulation (from 6/1/0 to 4/1/0) leads to a lower charge ratio due to the negative
247 charge of CRG sulphate groups. This resulted, as expected, in a zeta potential decrease.
248 Comparing these data with some reported previously for CS/CRG nanoparticles
249 (Grenha et al., 2010), we observe similar tendencies but smaller nanoparticle sizes in the
250 present work for comparable formulations, which might be explained by the use of
251 chitosan and carrageenan from different suppliers (van de Velde, Knutsen, Usov,
252 Rollemay & Cerezo, 2002). In Figure 4B it is seen that the production yields are
253 relatively low (13-19%) and comparable in all cases. In the present study, CS/CRG
254 nanoparticles were used as control.

255 The incorporation of TPP in the matrix of nanoparticles succeeded in providing an
256 effective cross-linking effect. More specifically, the presence of TPP resulted in
257 alterations on all the typical characterization parameters, namely the nanoparticle size
258 and zeta potential (Figure 4A), as well as production yield (Figure 4B). For the highest
259 amount of TPP incorporated (mass ratio of 1 compared to chitosan), particle size
260 decreased to 176-208 nm ($p < 0.05$) and zeta potential also decreased from +80 mV to

261 approximately +50 mV ($p < 0.05$). Size decrease is attributed to the cross-linking effect,
262 which induces the condensation of polymeric chains, resulting in smaller particles. The
263 simultaneous decrease in zeta potential is a consequence, not only of the inclusion of a
264 negatively charged material in the matrix of nanoparticles, but also of the general size
265 decrease, which possibly exposes a lower number of charged groups because of the
266 diminished surface. Interestingly, it can be observed that the incorporation of a mass
267 ratio of only 0.5 of TPP is enough to induce a clear effect on size and zeta potential, but
268 a higher amount of cross-linking agent (mass ratio of 1 compared to chitosan) is
269 necessary to produce a significant effect on production yield ($p < 0.05$). In fact, if the
270 formulation 4/1 is considered as example, it is seen by observation of Figure 4 that the
271 addition of 0.5 TPP does not modify the production yield, but it significantly decreases
272 both the nanoparticles size, by 45% (from 491 nm to 269 nm), and the zeta potential, by
273 25% (from +78 to +54 mV). A further increase of 0.5 TPP, resulting in a final
274 CS/CRG/TPP = 4/1/1, results in a significant increase of the production yield to 36%,
275 while the changes in the size and zeta potential, although significant ($p < 0.05$), are not
276 very pronounced, as size decreases 61 nm and zeta potential 4 mV. This trend was
277 similarly observed for the remaining formulations and, in all cases, the polydispersity
278 index was lower than 0.3. The inclusion of TPP in the nanoparticles was also observed
279 to result in unimodal size distribution.

280 The production yield practically doubled in all formulations with the highest amount
281 of TPP ($p < 0.05$), reaching a maximum of 36% for CS/CRG/TPP = 4/1/1. This
282 behaviour could be explained by the specific mechanism of nanoparticle formation,
283 according to which a determined amount of negative charges is necessary to provide a
284 certain degree of neutralisation of chitosan amino groups, which leads to the formation
285 of nanoparticles. When adding 0.5 TPP the cross-linking occurs, decreasing the size of

286 nanoparticles, but the amount of phosphate groups is not enough to increase the number
287 of formed nanoparticles, an effect that is observed when TPP amount is doubled. The
288 obtained results are in accordance with the ability of TPP to cross-link chitosan,
289 demonstrating in this case the capacity to decrease the size of chitosan-based
290 nanoparticles obtained by polyelectrolyte complexation with another polymer.

291 These results demonstrate, as a whole, that the charge ratio plays a critical role in the
292 production of nanoparticles by electrostatic interactions. In fact, it is shown that the
293 final properties of CS/CRG/TPP nanoparticles can be adjusted by modulating charge
294 ratios.

295

296 **3.2. Nanoparticles stability study**

297 The data corresponding to nanoparticles size evolution over time is depicted in
298 Figure 5, for formulation CS/CRG/TPP = 5/1/1 and for the corresponding CS/CRG
299 formulation, which was used as control. The rationale of conducting this assay in water
300 was the interest in obtaining information on the nanoparticles stability in the
301 resuspension medium. In the cases where the nanoparticles are an intermediate product
302 of the final drug delivery system, as reported in some works of our group (Grenha,
303 Seijo, Serra & Remuñán-López, 2007; Al-Qadi, Grenha & Remuñán-López, 2011), this
304 could avoid the need of extra procedures to stabilize the nanoparticles, as a
305 lyophilization step for instance.

306 The formulation without TPP tends to demonstrate some degree of size variation,
307 although not statistically significant, accompanied by an increase in the polydispersity
308 after 7 days of storage. On the contrary, the formulation containing TPP evidenced
309 improved stability, without signals of significant size variation during the experimental
310 period of 250 days. Zeta potential was also monitored in this study, but no alterations

311 were found over time for either of the assayed formulations (data not shown). Other
312 authors reported the stability of CS/TPP nanoparticles when stored at 5 °C for 15 days
313 in non-buffered medium, further observing an increase in the polydispersity after that
314 period, as the standard deviation of mean size of the nanoparticles registered a
315 significant increase (López-León, Carvalho, Seijo, Ortega-Vinuesa & Bastos-González,
316 2005). The results found in the present study for the CS/CRG/TPP formulation indicate
317 that TPP acts as a stabiliser, possibly because of its cross-linking effect, which causes
318 polymeric molecules to establish stronger interactions with each other to form a more
319 stable structure, less prone to aggregation. Actually, cross-linking reactions have been
320 described to improve the properties of particulates (Mi, Sung, Shyu, Su & Peng, 2003),
321 hydrogels (Sung, Huang, Chang, Huang & Hsu, 1999) and scaffolds (Adekogbe &
322 Ghanem, 2005), amongst other structures.

323

324 **3.3. Chemical analysis of nanoparticles**

325 As commented above, formulations containing TPP showed different
326 physicochemical characteristic as compared to those without TPP, which suggests that
327 TPP is in fact incorporated in the matrix of nanoparticles. Nevertheless, an indubitable
328 demonstration of TPP presence is possible only by chemical analysis of the
329 formulations. In this manner, specific techniques of chemical analysis, such as FTIR,
330 XPS and TOF-SIMS, were used to characterize the chemical composition of
331 nanoparticles, the last two techniques referring to surface analysis.

332

333 *3.3.1. FTIR analysis*

334 The FTIR spectrum of CS/CRG/TPP nanoparticles is depicted in Figure 6, along with
335 the spectra of all the materials separately (CS, CRG and TPP), which were used as

336 controls in this assay. TPP spectrum presents two intense absorption bands at 1147 and
337 906 cm^{-1} , attributed, respectively, to P=O and P-O along with P-O-P. The overlapping
338 of the former with the sulphate band of *k*-carrageenan (van de Velde, Knutsen, Usov,
339 Rollemay & Cerezo, 2002) and of the latter with the carbohydrate bands, renders the
340 detection of their presence in the nanoparticles ambiguous. Moreover, those bands are
341 expected to shift upon protonation and hydrogen bonding (Jiang, Saxena, Song, Ward,
342 Beveridge & Myneni, 2004), which may occur during particle formation. However, the
343 collapse of the sulphate and polysaccharide bands observed in the nanoparticles may be
344 accounted for if TPP is present, especially if shifting of the 1147 cm^{-1} band occurred.
345 Also, the band at 894 cm^{-1} in the nanoparticles may be attributed to a shift in the 906
346 cm^{-1} band of TPP. The amide bands are masked by the 1644 cm^{-1} bending band
347 of adsorbed water (Wilson, Smith, Kacurakova, Saunders, Wellner & Waldron, 2000)
348 and the new 1539 cm^{-1} absorption of the amino groups in protonated CS.

349

350 3.3.2. Surface analysis by XPS and TOF-SIMS

351 XPS is one of the most commonly used techniques of surface analysis. Upon
352 exposure of the sample to an X-ray beam, the binding energies of characteristically
353 emitted photoelectrons are measured, providing information on the elements from
354 which they originate, as well as their chemical bonding. Table 1 displays the percentage
355 of each chemical element present in the sample of either controls (CS, CRG and TPP)
356 or nanoparticles. The final chemical composition of a sample can be obtained from core
357 photoemission intensity peak areas using the Shirley background subtraction technique
358 from the survey spectra. The element composition can be quantified by using X-ray
359 photoelectron intensity values and the Scofield theoretically derived set of atomic
360 sensitivity factors. Some of the samples showed an intensive silicon signal (data not

361 shown), which is attributed to the substrate, as a consequence of an incomplete coating
362 of the substrate surface with the sample. These Si signals do not compromise the
363 obtained results and are not included in Table 1. The survey of controls detected the
364 expected elements, such as carbon (C), oxygen (O), nitrogen (N), phosphorus (P) and
365 sulfur (S). The obtained CS composition (64.1% C, 29.6% O and 6.3% N) is similar to
366 that observed by Silva *et al.*, who used the same CS type to produce membranes and
367 obtained 66.4% C, 28.0% O and 5.6% N. In addition, these authors report an O/N ratio
368 of 4.98, which is close to that of the present work (4.69) (Silva *et al.*, 2008).

369 In this work, the prepared nanoparticles were found to contain approximately 57% C,
370 35% O, 4% N, 2% P and 1% S, the content of P being necessarily attributed to the
371 cross-linking agent TPP, and that of S having origin in CRG. The obtained atomic
372 percentages of C, O, N and P were comparable to those reported in other works for the
373 analysis of CS/TPP nanoparticles (53.8% C, 33.8% O, 4.5% N and 2.7% P) (Calvo,
374 Remuñan-López, Vila-Jato & Alonso, 1997b; Grenha, Seijo, Serra & Remuñan-López,
375 2007). The slight variations can be explained by the use of CS of different
376 characteristics and the analysis of nanoparticles with different compositions and mass
377 ratios. The C/N ratio of nanoparticles assayed in the present study (14.3) is slightly
378 higher than those reported by Grenha *et al.* (11.9)(Grenha, Seijo, Serra & Remuñan-
379 López, 2007) and Calvo *et al.* (10.9)(Calvo, Remuñan-López, Vila-Jato & Alonso,
380 1997b). This difference is attributed to the presence of an extra compound in the
381 nanoparticles formulation (CRG), which increased the amount of C, thereby increasing
382 the C/N ratio.

383 Unexpectedly, a certain amount of C and N were detected in the TPP and CRG
384 samples, respectively. This effect was reported by other authors as corresponding to the
385 atmospheric exposure of the samples, which led to the adsorption of some adventitious

386 carbon (Barr & Seal, 1995; Swift, 1982) and nitrogen (Allott, Curtis, Hall, Harriman &
387 Battarbee, 1995; Baltrusaitis, Jayaweera & Grassian, 2009; Edwards, Zak, Kellner,
388 Eisenlord & Pregitzer, 2011; Rao, Rao & Ppabhakaran, 1987) on the samples surface.

389 In contrast to photoelectron spectroscopy techniques such as XPS, TOF-SIMS not
390 only provides information on the elements present on analysed surfaces, but also offers
391 detailed molecular information with high sensitivity. This technique has been useful in
392 the characterization of surface chemistry of pharmaceutical systems (Barnes, Kempson
393 & Prestidge, 2011). Figure 7 displays the negative mass spectra, between 50 and 110
394 mass/u, obtained by TOF-SIMS for each of the analysed samples.

395 The spectrum of CS (Figure 7A) evidences a peak at m/z 58 corresponding to
396 C_2H_4NO (Al-Qadi, Grenha & Remuñán-López, 2011), as well as peaks at m/z 59
397 ($C_2H_3O_2$), 69 (C_3HO_2), and 71 ($C_3H_3O_2$). Typical fragments of chitosan were also
398 detected in the sample of control chitosan, such as $C_{14}H_9NO$, $C_4H_{21}N_{14}O$ and $C_8H_{15}NO_6$
399 (data not shown), the latter representing one of the typical units of chitosan molecule,
400 *N*-acetyl-D-glucosamine (Grenha, Remunan-Lopez, Carvalho & Seijo, 2008; Grenha,
401 Seijo, Serra & Remuñán-López, 2007). The CRG spectrum (Figure 7B) is dominated by
402 SO_2 , SO_3 and SO_4H peaks, at m/z 64, 80 and 97, respectively. Smaller peaks, attributed
403 to saccharide species, as well as to SO_4 , are also present. TPP spectrum (Figure 7C)
404 consists of two peaks, corresponding to PO_2 and PO_3 , at m/z 63 and 79 (Al-Qadi,
405 Grenha & Remuñán-López, 2011).

406 As it is demonstrated in Figure 7 C and D, the mass spectra of both nanoparticle
407 formulations evidence peaks that are characteristic of all the previous spectra, which
408 correspond to controls. The obtained results are in agreement with those previously
409 observed in the XPS analysis, indicating the presence of all the components (CS, CRG
410 and TPP) in the nanoparticles and suggesting a homogeneous distribution of the various

411 constituents through their matrix. However, given the novelty of this technique, few
412 references were found reporting results of the application of TOF-SIMS on similar
413 materials and those found report fragment peaks with higher masses (mass/u), thus, the
414 establishment of comparisons with previously developed works was very scarce.

415

416 **4. Conclusions**

417 In this work, nanoparticles comprising CS, CRG and TPP were produced and
418 characterized using several techniques such as photon correlation spectroscopy and laser
419 Doppler anemometry, TEM, FTIR, XPS, and TOF-SIMS. The three components were
420 identified in the FTIR, XPS and TOF-SIMS spectra of the nanoparticles, thus indicating
421 an effective association of all the materials. In particular, their detection by the surface
422 analysis techniques suggests a homogeneous distribution through the nanoparticles'
423 matrix.

424 TPP acted as a cross-linker agent and, therefore, enabled the production of
425 nanoparticles with smaller size, apart from increasing their production yield. In
426 addition, the presence of TPP in the nanoparticle matrix increased their stability,
427 providing a shelf-life of at least 9 months. Charge ratios were demonstrated to play a
428 critical role in the nanoparticles formation, since a ratio around 1 leads to precipitation,
429 owing to charge neutralization, while very high charge ratios do not provide enough
430 charges to permit an interaction that induces nanoparticle formation. Overall, it was
431 demonstrated that by modulating charge ratios, the final properties of CS/CRG/TPP
432 nanoparticles can be adjusted to specific applications.

433 Taking into account the small size and high positive charge displayed by the
434 developed nanosystems, they are considered to hold potential for an application in
435 mucosal delivery of macromolecules.

436

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442

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575

Table 1 - Surface composition (atomic percentage) determined by XPS of CS, CRG, TPP and CS/CRG/TPP nanoparticles of different ratios.

Element	CS (%)	CRG (%)	TPP (%)	4/1/1 NP (%)	5/1/1 NP (%)
C	64.1	57.4	16.0	57.5	57.9
O	29.6	38.2	63.6	35.2	35.1
N	6.3	1.1	0	4.0	4.0
P	0	0	20.4	2.0	1.7
S	0	3.3	0	1.3	1.3
Ratio O/N	4.69	35.7	0	8.75	8.73
Ratio C/N	10.2	53.6	0	14.3	14.4

CS: Chitosan, CRG: Carrageenan; NP: Nanoparticles; TPP: Tripolyphosphate

576 **Figure captions**

577 **Fig. 1.** Chemical structures of materials composing the matrix of nanoparticles:

578 chitosan, *k*-carrageenan and sodium tripolyphosphate.

579 **Fig. 2.** TEM microphotograph of representative CS/CRG/TPP (4/1/1) nanoparticles.

580 **Fig. 3.** Representative scheme of A) positive/negative charge ratio of each formulation

581 (white fill: formation of nanoparticles; dark grey: precipitation; light grey: inability to

582 form nanoparticles); B) the ability to form nanoparticles according to formulation

583 composition: precipitation (\square); nanoparticles (\bullet); solution (\diamond).

584 **Fig. 4.** Effect of CS/CRG mass ratio and TPP amount on **A)** nanoparticle size (round

585 marks) and zeta potential (triangular marks) and **B)** production yield of nanoparticle

586 (square marks). White: 0 TPP; grey: 0.5 TPP; black: 1 TPP (mean \pm SD, $n = 3$).

587 **Fig. 5.** Evolution of CS/CRG (\circ) and CS/CRG/TPP (\bullet) nanoparticle size as a function

588 of time, upon storage at 4 °C (mean \pm SD, $n = 3$).

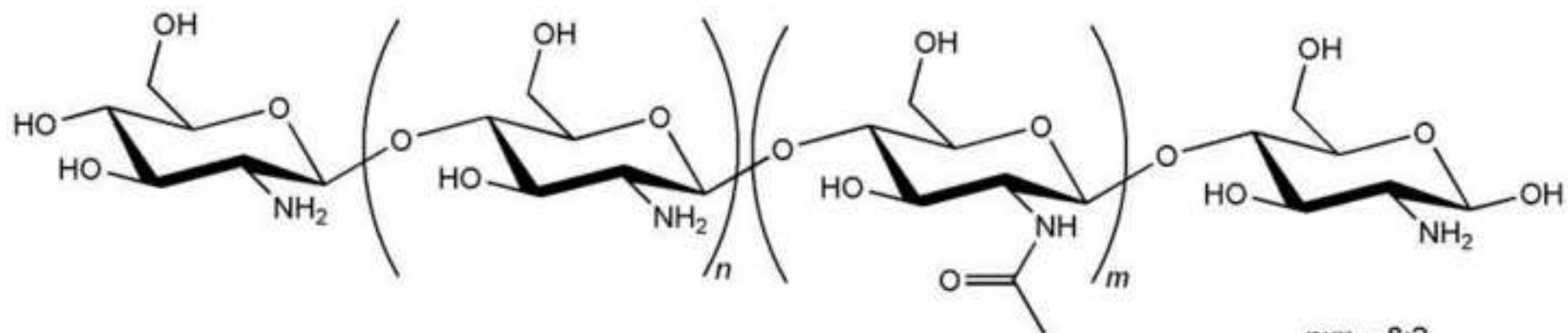
589 **Fig. 6.** FTIR spectra of CS, CRG, TPP and CS/CRG/TPP (5/1/1) nanoparticles.

590 **Fig. 7.** Negative ion mass spectra obtained by TOF-SIMS analysis of (A) CS, (B) CRG,

591 (C) TPP, (D) CS/CRG/TPP = 4/1/1 nanoparticles and (E) CS/CRG/TPP = 5/1/1

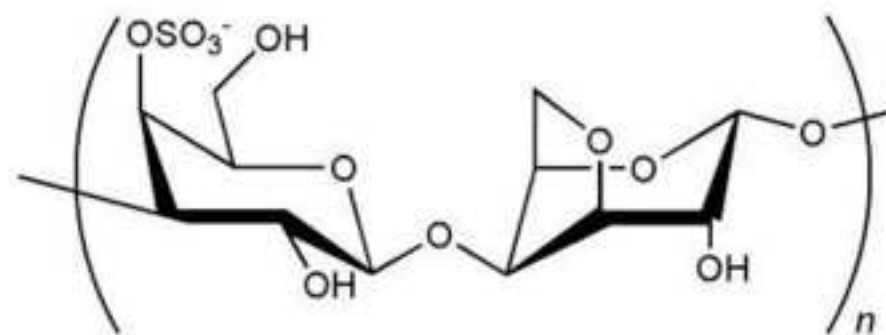
592 nanoparticles.

Figure 1
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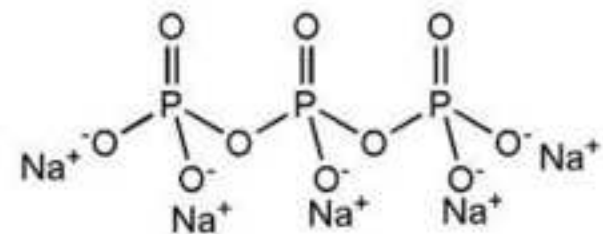


Chitosan (CS)

$n:m \approx 8:2$



k-Carrageenan (CRG)



Sodium triphosphate (TPP)

Figure 2
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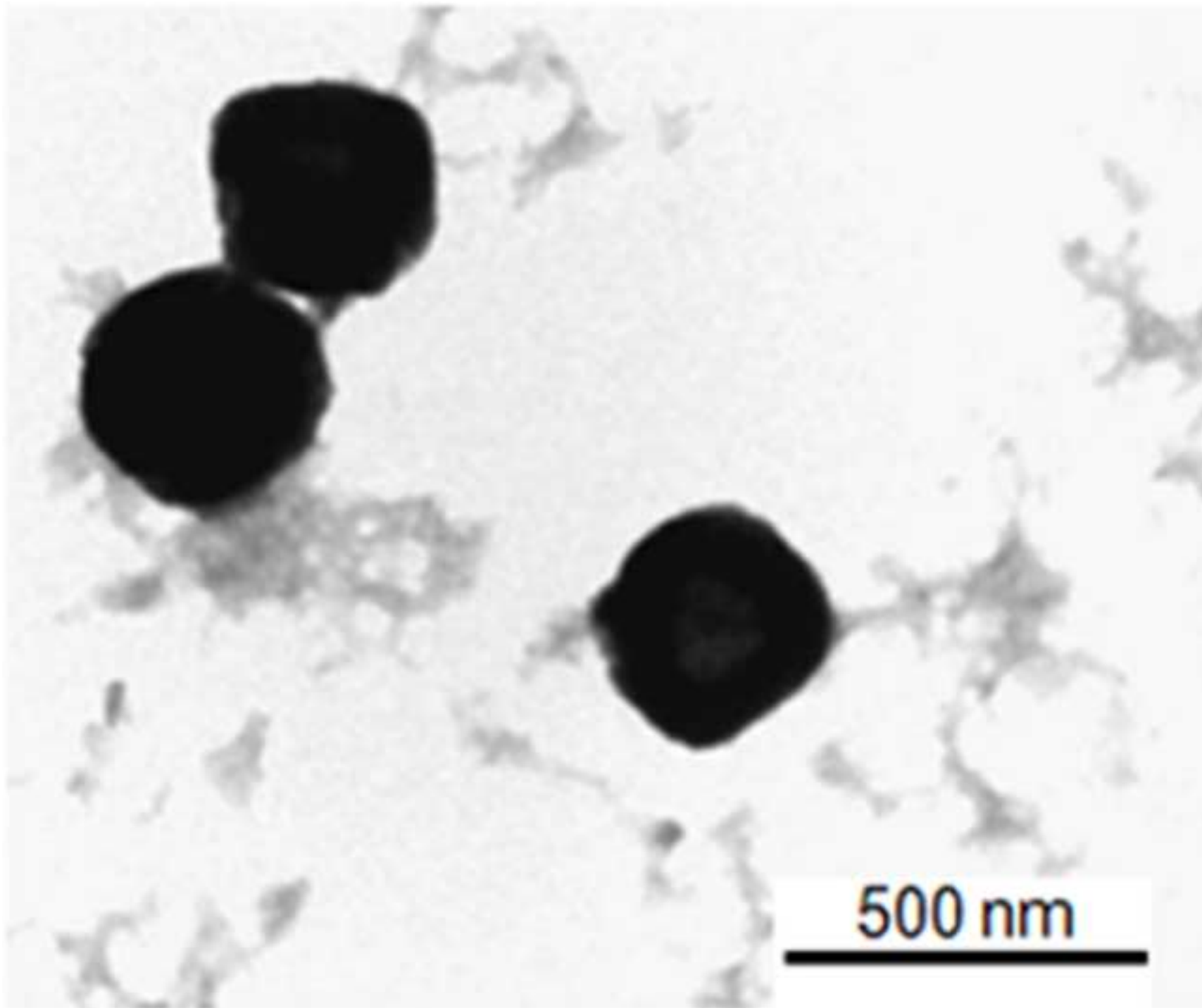


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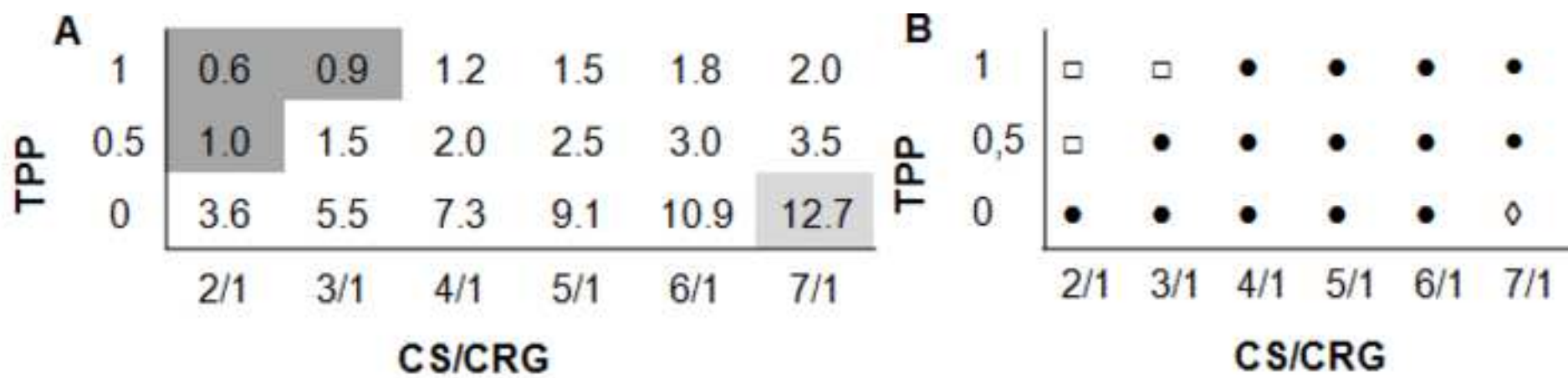


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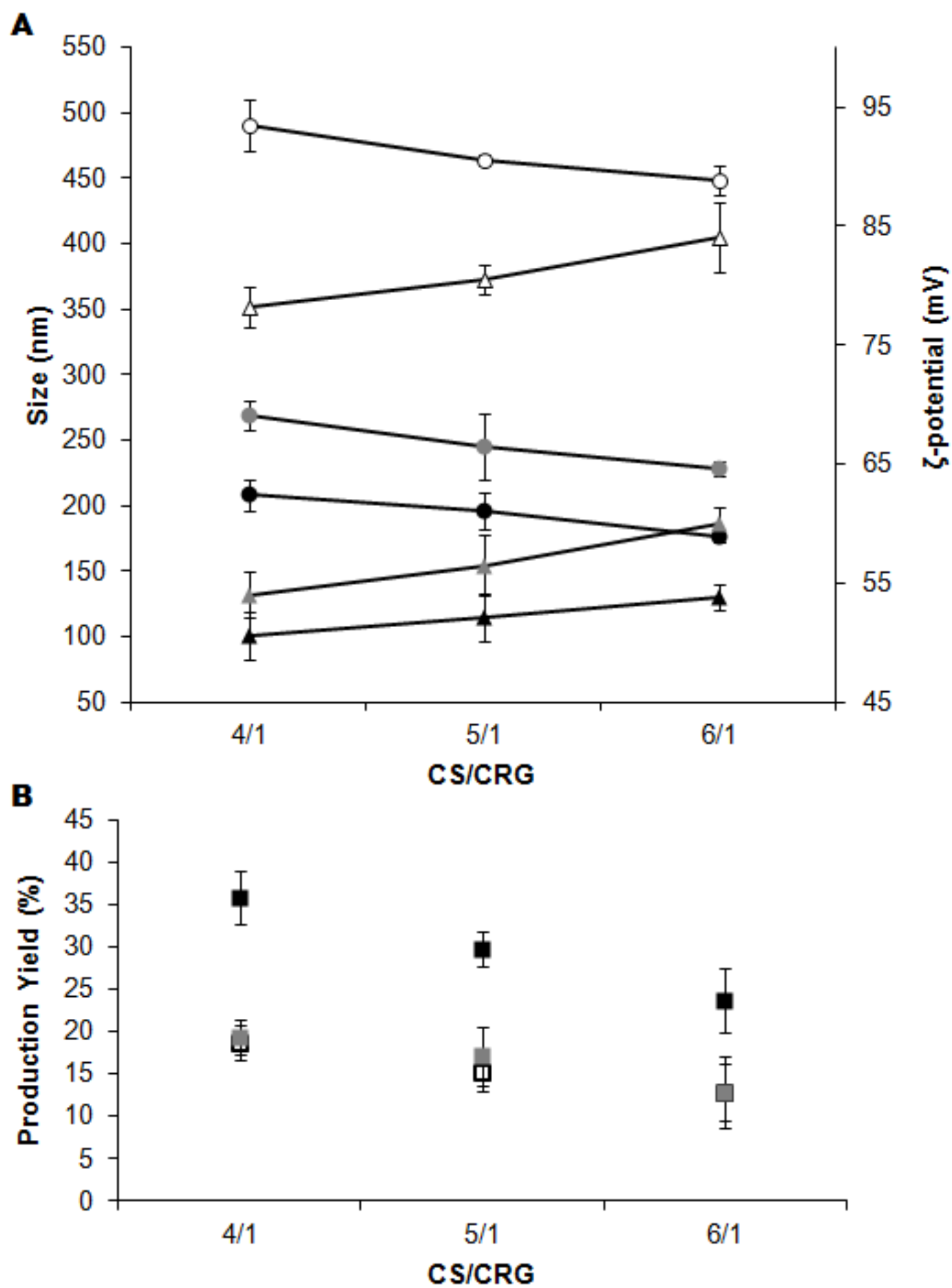


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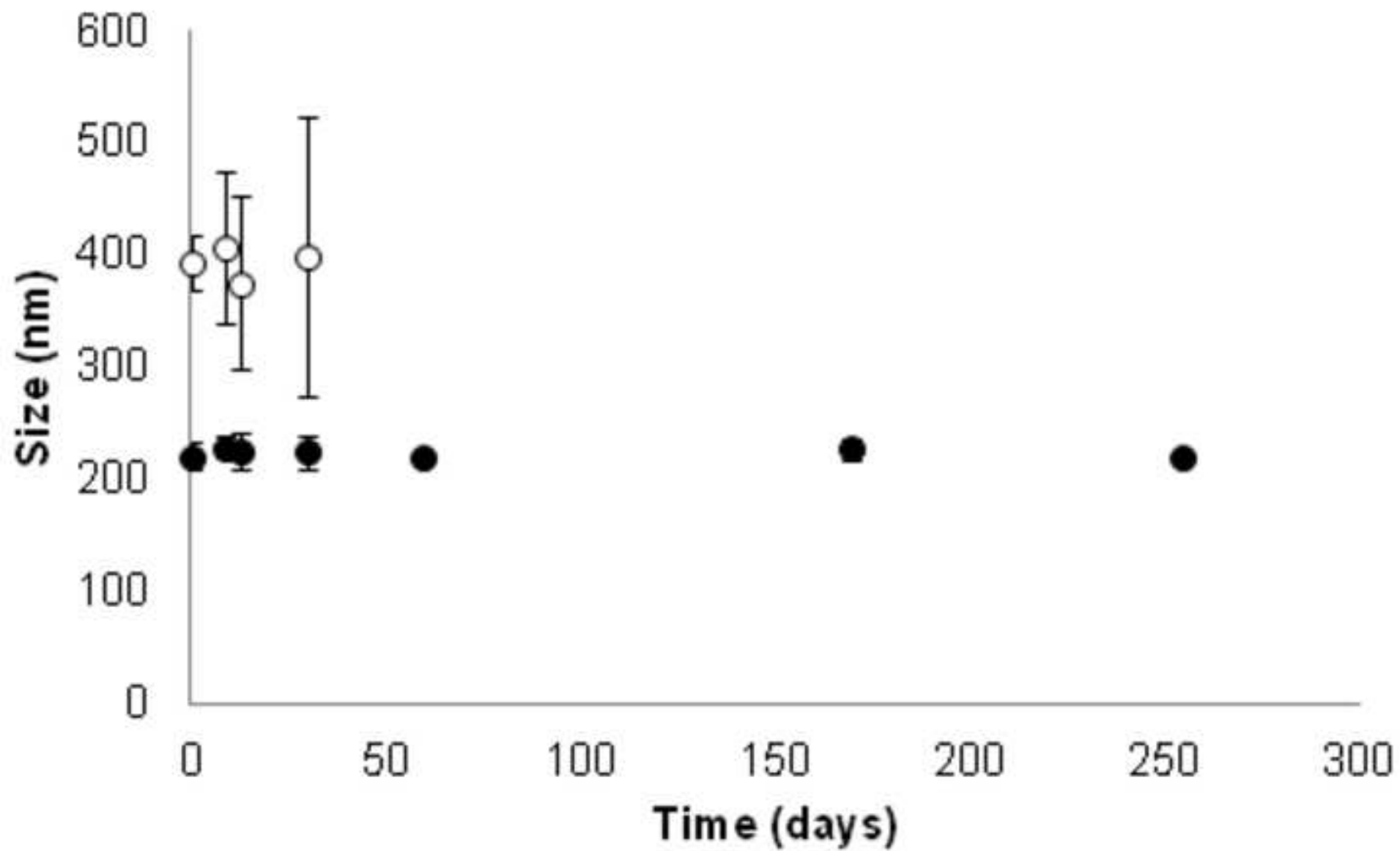


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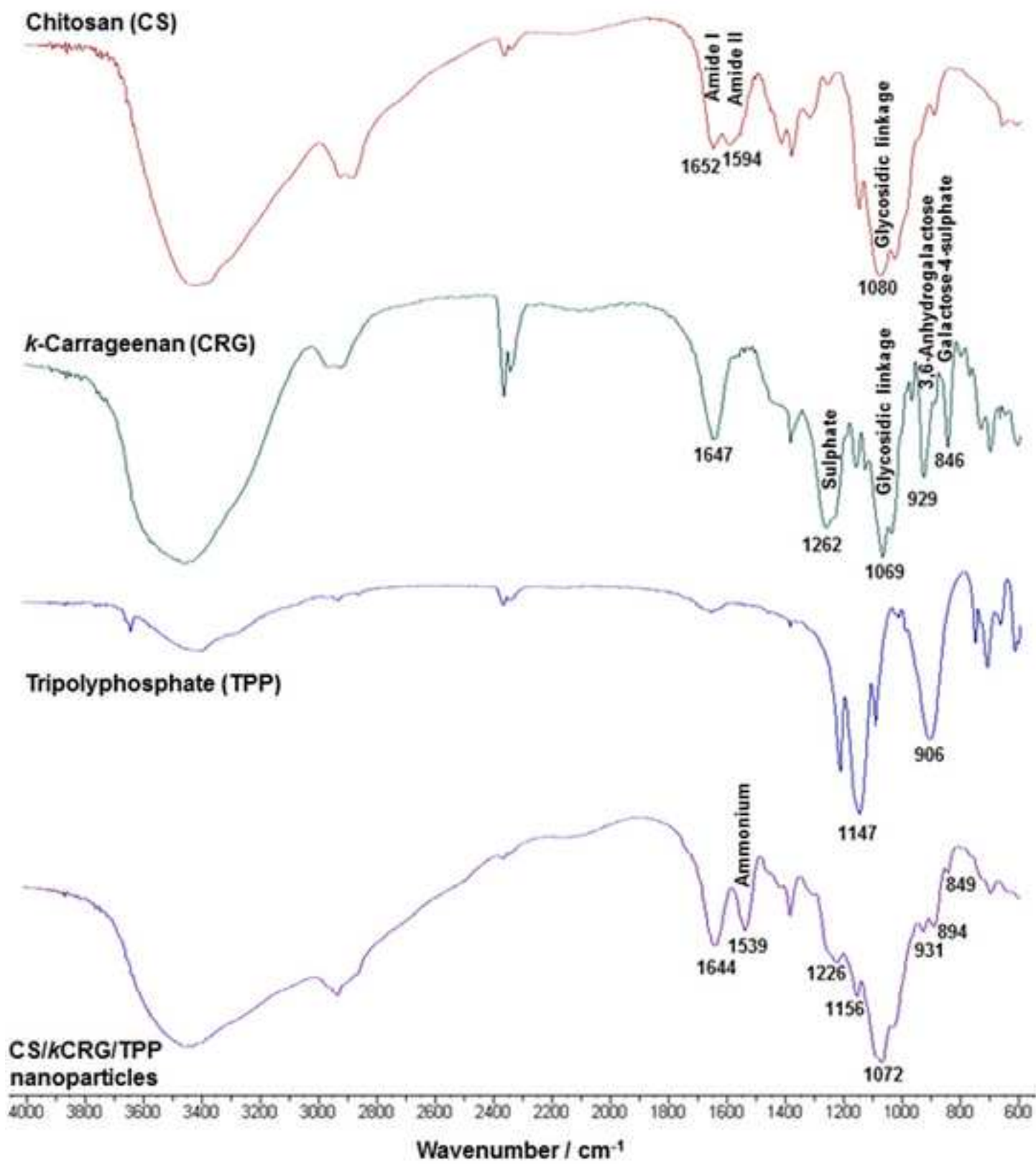


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