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## **Design of blue crab chitosan responsive nanoparticles as controlled-release nanocarrier: Physicochemical features, thermal stability and in vitro pH-dependent delivery properties**

Marwa Hamdi, Rim Nasri, S.M. Li, Moncef Nasri, Murat Kaya, Hela Kchaou, Chentir Imene, Ola Abdelhedi, Mourad Jridi

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## Manuscript Details

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<b>Title</b>	Design of blue crab chitosan responsive nanoparticles as controlled-release nanocarrier: Physicochemical features, thermal stability and in vitro pH-dependent delivery properties.
<b>Article type</b>	Research Paper

### Abstract

In this study, carotenoproteins (CPs) were encapsulated in blue crab chitosan (CS)-tripolyphosphate (TPP) nanoparticles by ionotropic gelation and CS-protein isolate (PI) complex coacervation. The success of CPs encapsulation was confirmed by FT-IR spectroscopy, TGA and XRD techniques. Particle size and thermal stability of nanoparticles was dependent to the encapsulation methods. Indeed, a regular distribution and spherical shape, with size range of about 300 (ionotropic gelation) – 600 nm (complex coacervation), were observed by SEM analysis. The encapsulation efficiency and loading capacity of CPs were about 74% and 31% for the complex coacervation and 89% and 47% for the ionotropic gelation approaches, respectively. In vitro release studies showed an initial swift effect, followed by a slow CPs release. The highest amount of released CPs and the lowest release time were detected with the ionotropic gelation method. Further, in vitro release kinetics of CPs were found to be medium dependent, where ethanol displayed higher released CPs amount with longer release time, compared to PBS (pH ~ 6.8). These findings suggest that the encapsulation technique obviously affected the particles structure, and the glass transition temperature, and the mass loss of encapsulated materials. The better CPs stabilization was obtained for the ionotropic gelation nanoparticles.

<b>Keywords</b>	Blue crab chitosan; nanoparticles engineering; In vitro release.
<b>Manuscript category</b>	Carbohydrates, Natural Polyacids and Lignins
<b>Corresponding Author</b>	Marwa Hamdi
<b>Corresponding Author's Institution</b>	ENIS
<b>Order of Authors</b>	Marwa Hamdi, Rim Nasri, Suming Li, Moncef Nasri
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## Submission Files Included in this PDF

### File Name [File Type]

Cover Letter R.docx [Cover Letter]

Response Letter IJBM.docx [Response to Reviewers]

Highlights R.docx [Highlights]

Abstract R.docx [Abstract]

Manuscript R.docx [Manuscript File]

Figure captions R.docx [Figure]

Figures R.docx [Figure]

Graphical Abstract R.pdf [Figure]

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Sfax, Tunisia, 26<sup>th</sup> of September 2019

Dear Editor,

Please find enclosed our revised paper entitled « **Design of blue crab chitosan responsive nanoparticles as controlled-release nanocarrier: Physicochemical features, thermal stability and *in vitro* pH-dependent delivery properties** » to be considered for publication in your journal « **International Journal of Biological Macromolecules** ».

We are in a full awareness of the fact that chitosan use as nanocarrier system is not an innovation in itself, and you may consider our results as have been discussed in the literature or as an expected tenet, but the purpose of our work is, first, the valorization of a species of blue crab that constitutes a real threat to the aquatic Tunisian ecosystem.

Above all of that and concretely, the manuscript discussed the difference between nanoencapsulation techniques, in terms of particles physicochemical features, thermal stability and *in vitro* release kinetics of carotenoproteins, chosen as model.

In this aspect, two approaches were chosen to be investigated, ionotropic gelation and complex coacervation. Regarding the complex coacervation, a new polymer was used, blue crab protein isolate, which was extracted, characterized and applied in our laboratory and it is the subject of another manuscript being submitted.

To this end, several techniques were used to deeply characterize the blue crab chitosan-based nanoparticle, especially, the Scanning Electron and Atomic Force Microscopies, the thermogravimetric and differential scanning calorimetry analyses, etc.

In our opinion, this study would open up a new avenue to understand well the nanoparticles characteristics dependence to the applied technique, helping readers to choose the better nanoencapsulation technique, considering the desired and triggered application.

All authors agree to submit the work to « **International Journal of Biological Macromolecules** ».

This work has not been published before, and is not under consideration for publication anywhere else.

I hope it satisfy the required needs and your approval.

King regard and respect.

Thanking you.

**Dr. Marwa HAMDI**

Laboratory of Enzymatic Engineering and Microbiology, National School of Engineers of Sfax,  
Sfax. B.P.1173, 3038 Sfax, Tunisia.

E-mail: [marwahamdi50@yahoo.fr](mailto:marwahamdi50@yahoo.fr)

Sfax, Tunisia, September 2019 the 26<sup>th</sup>

Dear Editor,

We found the reviewers' feedback very helpful. We revised our manuscript and included all their recommendations. These are listed below with our accompanying justifications. We are confident that the clarifications provided here will address all the referees' remarks and we hope that our manuscript will be finally accepted for publication in "Food Hydrocolloids".

Thanking you,

### **Response to reviewers' comments**

#### **Reviewer #1:**

First, we thank the reviewer for his constructive and helpful suggestions. All corrections suggested were incorporated in the text and highlighted in red.

This paper untitled «Design of blue crab chitosan responsive nanoparticles as controlled-release nanocarrier: Physicochemical features, thermal stability and *in vitro* pH dependent delivery properties» is well written and structured.

In my opinion the paper presents results which could be published in IJBIOMAC after minor revisions.

In order to improve the quality of the manuscript, the following points should be corrected.

#### **Remarks**

1. L 28: replace drug by bioactive molecules;

Yes, we agree with the reviewer comment and mistake corrected as recommended. Please see line 29.

2. L31-34: Please rewrite and be more accurate in the possible avenue of the present work;

Yes, we agree with the reviewer comment and the conclusion in the abstract section was corrected as recommended. Please see lines 32-35.

3. L 49- 50: Such a repetition, please rewrite;

Yes, we agree with the reviewer comment and corrected as recommended.

4. L 49- 50: L 52: Replace “Inactivation” by a more adequate word;

Yes, we agree with the reviewer comment and corrected as recommended.

5. L83 - L83: Please justify the encapsulation of carotenoproteins from blue crab shell with Chitosan and protein isolate from the same blue crab shell;

Yes, we agree with the reviewer and more details were given in the introduction section.

Please see lines 77-85:

*«The aim of this study was to encapsulate carotenoproteins from blue crab shells by complex coacervation, using chitosan and protein isolate from the same species, and ionotropic gelation with chitosan and tripolyphosphate. The physicochemical properties, morphology, the pigment storage stability and in vitro release kinetics, were comparatively evaluated, which would open up a new avenue to better understand the nanoparticles characteristics dependence to the applied technique. In fact, blue crab species is, nowadays, extensively found in the Mediterranean, especially, in Tunisian coasts causing various damages regarding fishing. Thus, above all of that and concretely, the purpose of our work is additionally the valorization blue crabs used for chitosan, carotenoproteins and protein isolate extraction».*

6. In general, the introduction is unbalanced; there are more details on the encapsulation definitions. it would be interesting to mention some research about chitosane as a nanocarrier, carotenoids encapsulation and its limitation to more highlight the importance of the present study;

Yes, we agree with the reviewer comments and overall the introduction section was improved as recommended.

7. L 91: Why do author mention the phenolic compounds content in CPs?

Authors mention the phenolic compounds content in CPs just to give more details about the chemical composition of CPs, since no further purification steps were conducted. Mistake was corrected. Please see line 91.

8. L 93: Replace recuperation by recovery;

Yes, we agree with the reviewer and the mistake was corrected as recommended. Please see line 91.

9. L113-L114: Repetition;

Yes, we agree with the reviewer and the mistake was corrected as recommended. Please see the Section 2.2.2.

10. L 191 – L193: Justify the chosen temperature and time used in thermal stability;

Yes, we agree with the reviewer and more details were given. Please see lines 203-207:

*«Thermal stability of freeze-dried CS-based nanoparticles based on CS was studied at 80, 100 and 120 °C (sterilization temperatures) for 5, 15 and 30 min, since commercialized products are frequently exposed to heat treatment, the most used processing method in food industry, during their preparation. Particles size and zeta potential were evaluated at each desired time interval».*

11. L213-L225: Too long, please sum up.

In general, the discussion contains too long sentences. It is preferable to shorten the sentences for a better arguments and explanations comprehension.;

Yes, we agree with the overall discussion was revised as recommended.

12. L 327: Delete “in the present work”.

Yes, we agree with the reviewer and the mistake was corrected as recommended. Please see lines 333-334.

13. According the obtained results, the ionotropic gelation was more effective to encapsulate CPs and the obtained nanoparticules could be used in drug delivery. According to the characteristic of CC nanoparticules, which application would author recommend? please add it to the conclusion.;

Yes, we agree with the reviewer and the conclusion was improves as recommended.

Please see lines 570-580.



## **Highlights**

- Blue crab chitosan as nanocarrier for carotenoids (CPs) stabilization was used;
- Ionotropic gelation and complex coacervation approaches were adopted;
- Higher entrapment efficiency and loading charge were reached via the ionotropic gelation;
- Microencapsulation enhanced thermal properties and stability of carotenoproteins;
- Ionotropic gelation allowed the highest amount and the lowest release time of CPs.

## **Abstract**

In this study, carotenoproteins (CPs) were encapsulated in blue crab chitosan (CS)-tripolyphosphate (TPP) nanoparticles by ionotropic gelation and CS-protein isolate (PI) complex coacervation. The success of CPs encapsulation was confirmed by FT-IR spectroscopy, TGA and XRD techniques. Particle size and thermal stability of nanoparticles was dependent to the encapsulation methods. Indeed, a regular distribution and spherical shape, with size range of about 300 (ionotropic gelation) – 600 nm (complex coacervation), were observed by SEM analysis. The encapsulation efficiency and loading capacity of CPs were about 74% and 31% for the complex coacervation and 89% and 47% for the ionotropic gelation approaches, respectively. *In vitro* release studies showed an initial swift effect, followed by a slow CPs release. The highest amount of released CPs and the lowest release time were detected with the ionotropic gelation method. Further, *in vitro* release kinetics of CPs were found to be medium dependent, where ethanol displayed higher released CPs amount with longer release time, compared to PBS (pH ~ 6.8). These findings suggest that the encapsulation technique obviously affected the particles structure, and the glass transition temperature, and the mass loss of encapsulated materials. The better CPs stabilization was obtained for the ionotropic gelation nanoparticles.

**Keywords:** Blue crab chitosan; nanoparticles engineering; *In vitro* release.

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4 1           **Design of blue crab chitosan responsive nanoparticles as**  
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6 2           **controlled-release nanocarrier: Physicochemical features, thermal**  
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9 3           **stability and *in vitro* pH-dependent delivery properties.**  
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13 4   Marwa Hamdi <sup>a\*</sup>, Rim Nasri <sup>a,b</sup>, Suming Li <sup>c</sup>, Moncef Nasri <sup>a</sup>  
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15           <sup>a</sup> Laboratory of Enzyme Engineering and Microbiology, University of Sfax, National Engineering School of Sfax,  
16           B.P. 1173, 3038 Sfax, Tunisia.  
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20 7   <sup>b</sup> Higher Institute of Biotechnology of Monastir, University of Monastir, Monastir, Tunisia.  
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23           <sup>c</sup> European Institute of Membranes, UMR CNRS 5635, University of Montpellier, Place Eugene Bataillon, 34095  
24           Montpellier Cedex 5, France.  
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31 11   \* **Corresponding author:** Marwa Hamdi, Laboratory of Enzyme Engineering and  
32           Microbiology, University of Sfax, National Engineering School of Sfax, B.P. 1173, 3038 Sfax,  
33           Tunisia. **Tel:** 216 25740373 / 216 54186612; **E-mail:** marwahamdi50@yahoo.fr.  
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62 **19 Abstract**  
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65 20 In this study, carotenoproteins (CPs) were encapsulated in blue crab chitosan (CS)-  
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67 21 tripolyphosphate (TPP) nanoparticles by ionotropic gelation and CS-protein isolate (PI)  
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69 22 complex coacervation. The success of CPs encapsulation was confirmed by FT-IR  
70  
71 23 spectroscopy, TGA and XRD techniques. Particle size and thermal stability of nanoparticles  
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73 24 was dependent to the encapsulation methods. Indeed, a regular distribution and spherical shape,  
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75 25 with size range of about 300 (ionotropic gelation) – 600 nm (complex coacervation), were  
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77 26 observed by SEM analysis. The encapsulation efficiency and loading capacity of CPs were  
78  
79 27 about 74% and 31% for the complex coacervation and 89% and 47% for the ionotropic gelation  
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81 28 approaches, respectively. *In vitro* release studies showed an initial swift effect, followed by a  
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85 30 with the ionotropic gelation method. Further, *in vitro* release kinetics of CPs were found to be  
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87 31 medium dependent, where ethanol displayed higher released CPs amount with longer release  
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89 32 time, compared to PBS (pH ~ 6.8). These findings suggest that the encapsulation technique  
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91 33 obviously affected the particles structure, and the glass transition temperature, and the mass  
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93 34 loss of encapsulated materials. The better CPs stabilization was obtained for the ionotropic  
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95 35 gelation nanoparticles.  
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38 **Keywords:** Blue crab chitosan; nanoparticles engineering; *In vitro* release.  
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121 **43 1. Introduction**  
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123 44 Recently, encapsulation has been widely considered as a viable and effective approach  
124  
125 45 for active protection of biomolecules with interesting functional properties. Encapsulation is a  
126  
127 46 process consisting in the incorporation of an active substance into a coating matrix in order to  
128  
129 47 protect it from the external environment (against oxidation and degradation during storage) and  
130  
131 48 control its release in a chosen environment [1,2]. The controlled release process allows  
132  
133 49 encapsulated substances to reach their target in active form [3,4]. Encapsulation has its  
134  
135 50 applications in many areas, including pharmacies for the controlled release of the active  
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137 51 ingredient, in cosmetics for the protection of the active ingredient, in food for taste masking,  
138  
139 52 flavor protection, and detergency for encapsulated fragrance for softener or laundry [5].  
140  
141

142 53 Various techniques have been developed and used for nanoencapsulation purposes, such  
143  
144 54 as emulsification, coacervation, emulsification–solvent evaporation, nanoprecipitation,  
145  
146 55 supercritical fluid, and inclusion complexation technique. These methods can produce capsules  
147  
148 56 in the nanometer range varying from 10 to 1000 nm [6]. Drug or biomolecules release from  
149  
150 57 nanoparticles takes place by several mechanisms including surface erosion, disintegration,  
151  
152 58 desorption and diffusion [7,8].  
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155 59 A variety of nanoparticles, based on several biocompatible and degradable natural  
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157 60 polymers (mainly polysaccharides), has been used. Due to its unique physicochemical and  
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159 61 biopharmaceutical properties, in addition to its biodegradable character [9], chitosan is  
160  
161 62 considered effective in drug delivery systems. In fact, in such applications, water solubility and  
162  
163 63 the presence of a positive charge are recommended properties, in order to react with negatively  
164  
165 64 charged polymers, macromolecules and polyanions in an aqueous environment [10]. Thus,  
166  
167 65 chitosan-based delivery systems show great potential for delivering anticancer, antibacterial,  
168  
169 66 antifungal, anti-inflammatory drugs, vaccines, nucleic acids, peptides and therapeutic proteins,  
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171 67 DNAs and genes, etc. [11].  
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179  
180 68            Additionally, carotenoids and carotenoproteins are widely applied in food, cosmetics and  
181  
182 69            pharmaceutical industries, because of their various bioactivities, mainly antioxidant,  
183  
184 70            antimicrobial, anticancer, immunomodulatory, antidiabetic and anti-inflammatory effects [12].  
185  
186 71            Nevertheless, the major difficulties to value their application is their limited  
187  
188 72            solubility/dispersibility in aqueous media and their sensitivity to oxidation and/or degradation,  
189  
190 73            during food processing, drug formulation, etc. [13]. Encapsulation is the emerging technology  
191  
192 74            that offers a mean to overcome these problems, mainly the protection of carotenoids against  
193  
194 75            harsh environmental and the improvement of their stability during processing and storage  
195  
196 76            circumstances [14].  
197  
198

199 77            The aim of this study was to encapsulate carotenoproteins from blue crab shells by  
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201 78            complex coacervation, using chitosan and protein isolate from the same species, and ionotropic  
202  
203 79            gelation with chitosan and tripolyphosphate. The physicochemical properties, morphology, the  
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205 80            pigment storage stability and *in vitro* release kinetics, were comparatively evaluated, which  
206  
207 81            would open up a new avenue to better understand the nanoparticles characteristics dependence  
208  
209 82            to the applied technique. In fact, blue crab species is, nowadays, extensively found in the  
210  
211 83            Mediterranean, especially, in Tunisian coasts causing various damages regarding fishing. Thus,  
212  
213 84            above all of that and concretely, the purpose of our work is additionally the valorization blue  
214  
215 85            crabs used for chitosan, carotenoproteins and protein isolate extraction.  
216  
217  
218

## 219 86            2. Materials and methods

### 220 87            2.1. Materials

221  
222 88            CS with medium molecular weight of ~125 kDa, acetylation degree of 11% and intrinsic  
223  
224 89            viscosity [ $\eta$ ] of 3203 ml/g (0.1% (w/v) in 1% acetic acid) was extracted from blue crab *Portunus*  
225  
226 90            *segnis* shells, as described in a previous study [15]. CPs, from blue crab shells, contained  
227  
228 91             $4242.86 \pm 60.61 \mu\text{g g}^{-1}$  extract of total carotenoids, with a recovery yield of 84% [16]. Blue crab  
229  
230 92            muscle protein isolate (PI) was prepared based on the pH-shifting method [17], allowing the  
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239 93 recuperation of more than 85% (dry weight) proteins, with an extraction yield of 16% (dry  
240  
241 94 weight).

242  
243 95 Tween 80, glacial acetic acid, sodium tripolyphosphate (TPP) and all other chemicals of  
244  
245 96 analytical grade were purchased from Sigma-Aldrich Chemical Co. (St. Louis, USA). Milli-Q  
246  
247  
248 97 water was used throughout this study.

## 249 98 **2.2. Conception of blue crab chitosan nanoparticles loaded with carotenoproteins**

### 250 99 **2.2.1. Encapsulation of CPs into CS-PI nanoparticles by complex coacervation**

251  
252  
253 100 CS-PI nanoparticles loaded or not with CPs were prepared based on the complex  
254  
255 101 coacervation method by using CS and PI as wall materials, as described by Chang *et al.* [18].  
256  
257 102 PI at a concentration of 0.5% (w/v) was dissolved in Milli-Q water and solution's pH was  
258  
259 103 adjusted to 10.0 using NaOH 1M, while CS solution (0.5 %, w/v) was prepared in acetic acid  
260  
261 104 0.15 M (pH 4.0). Both solutions were stirred for 1 h, and then, incubated for 24 h at 4 °C to  
262  
263 105 ensure complete hydration. Afterwards, PI and CS stock solutions were centrifuged at 4 °C,  
264  
265 106 4000 g for 15 min and supernatants were mixed, to reach the desired PI to CS mass ratios of  
266  
267 107 1/4, 1/1, 4/1, 8/1, 16/1 and 20/1 (w/w). The mixture was stirred vigorously and stirring was  
268  
269 108 continued for additional 2 h to ensure the cross-linking between CS and PI. In another aspect,  
270  
271 109 complex coacervation pH was optimized considering the zeta potential of aqueous solutions of  
272  
273 110 PI and CS, the turbidity of the PI-CS mixtures and the yield of the dried coacervates  
274  
275 111 measurements [13].

276  
277  
278 112 To encapsulate CPs into the nanoparticles, a stock solution was gradually dropped, under  
279  
280 113 vigorously stirring, into the CS stock solution at a CS/CPs mass ratio of 1/0.8. The CPs-loaded  
281  
282 114 nanoparticles were stored at 4 °C overnight to uphold decantation, then, filtered and washed  
283  
284 115 twice with Milli-Q water. To obtain powdered nanoparticles, CS-PI coacervates were  
285  
286 116 lyophilized and stored at 4 °C for subsequent analysis.

296  
297  
298 117 The coacervate yield was determined by measuring the coacervate dried mass to the total  
299  
300 118 biopolymer mass in triplicate and expressed as follows [19]:  
301

$$302 \quad \text{Coacervate yield (\%)} = \frac{M_i}{M_0} \times 100 \quad \text{Eq (1)}$$

303  
304  
305  
306 120 where  $M_0$  is the total biopolymers weight used to make the biopolymers solutions and  $M_i$  is the  
307  
308 121 weight of the dried coacervate phase.  
309

310 122 The PI and CS strength of electrostatic interaction (SEI) at each required pH value was  
311  
312 123 thereby determined. The optimum pH range, which allows complexation to occur between PI  
313  
314 124 and CS, was selected based on the maximum SEI range, and subsequently used to optimize the  
315  
316 125 PI-CS weight ratio.  
317

### 318 319 126 **2.2.2. Encapsulation of CPs in CS-TPP nanoparticles by ionotropic gelation**

320  
321 127 For the ionotropic gelation, as reported by Haider *et al.* [20], CS solution was prepared,  
322  
323 128 at a concentration of 3.2 mg/ml, in acetic acid 0.15 M. To remove any undissolved particles,  
324  
325 129 CS solution was centrifuged at 3000 g, 4 °C for 15 min. The pH of the solution (50 ml) was  
326  
327 130 adjusted to 4.0. Afterwards, Tween-80 (80 mg) was added as emulsifier and the mixture was  
328  
329 131 stirred at 60 °C for 30 min. Simultaneously, CPs solution was prepared by dissolving 128 mg  
330  
331 132 in 4 ml ethanol, and subsequently gradually dropped, into the CS solution, under vigorously  
332  
333 133 stirring, to reach the desired CS/CPs weight ratio of 1/0.8. Agitation was sustained for 20 min,  
334  
335 134 and then, 30 ml of TPP solution (1.87 mg/ml; pH 4.0) were added drop wise under continuous  
336  
337 135 stirring to the mixture solution for 30 min, to reach a CS-TPP weight ratio of 1/0.35. After  
338  
339 136 complete addition of TPP, mixing was continued for 30 min, to guarantee the complete and the  
340  
341 137 perfect gelation between CS and TPP.  
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344  
345 138 The mixture was, thereafter, centrifuged at 4000 g for 30 min at 4 °C, and the obtained  
346  
347 139 pellet, containing nanoparticles, was rinsed with Milli-Q water (25 ml), and subsequently,  
348  
349 140 freeze-dried.  
350



## 141 **2.3. Synthetized CPs-loaded CS-based nanoparticles characterizations**

### 142 **2.3.1. Zeta potential and particles size measurement**

143 The zeta potential, as well as the particles size were determined using a laser scattering  
144 particles size distribution analyzer Litesizer 500 (Anton Paar, GmbH, France). A minimum of  
145 three replicate tests was carried out for each formulation at 25 °C and the mean zeta potential  
146 and particles size values, besides standard deviations were obtained with the software included  
147 with the instrument.

### 148 **2.3.2. Turbidimetric analysis**

149 The turbidity was measured with a spectrophotometer (T70, UV/vis spectrometer, PG  
150 Instruments Ltd., China) at 600 nm, and the optimum pH for complex coacervation is regarded  
151 as the pH value at which the highest light absorption occurred.

### 152 **2.3.3. CPs encapsulation efficiency and loading charge measurement**

153 The amount of CPs loaded in CS nanoparticles was calculated based on TGA/DTG  
154 (derivative thermal gravimetric) analysis [21]. The loading capacity (LC) is defined as the  
155 quantity of loaded CPs per 100 g of nanoparticles, while the amount of loaded CPs based on  
156 the initial CPs (in feed) corresponds to the encapsulation efficiency (EE). Therefore,  
157 considering the DTG thermograms, LC and EE were determined from the equations (2) and (3),  
158 respectively, given below:

$$159 \quad \% LC = \frac{\text{Content of loaded CPs}}{\text{Mass of nanoparticles}} \times 100 \quad \text{Eq (2)}$$

$$160 \quad \% EE = \frac{\text{Content of loaded CPs}}{\text{Content of initial CPs}} \times 100 \quad \text{Eq (3)}$$

### 161 **2.3.4. Spectroscopic characterization of CS nanoparticles**

162 Nanoparticles were applied to the Fourier transform infrared spectroscopy (FTIR,  
163 NEXUS of ThermoFisher) equipped with an attenuated reflection accessory (ATR) containing

414  
415  
416 164 a diamond/ZnSe crystal, at room temperature (25 °C) in the spectral range frequencies of 650-  
417  
418 165 4000 cm<sup>-1</sup>. Prior to analysis, calibration was performed via background spectrum recorded from  
419  
420 166 the clean and empty diamond and for each spectrum, 32 scans of interferograms were averaged  
421  
422  
423 167 and the spectral resolution was 4 cm<sup>-1</sup>. Data analysis and treatment were carried out by using  
424  
425 168 the OMNIC Spectra software (ThermoFisher Scientific).

426  
427 169 To further investigate the structural characteristics of the prepared nanoparticles, XRD  
428  
429 170 patterns were recorded using an X-ray diffractometer (D8, Advance Bruker XRD  
430  
431 171 diffractometer, Germany). Ni-filtered Cu K $\alpha$  radiation ( $k = 1.5406 \text{ \AA}$ ) was used to record the  
432  
433 172 X-ray powder patterns. The relative intensity was recorded in the scattering range  $2\theta$  of 5–50°  
434  
435 173 with a step size of 0.02° and a counting time of 5 s/step, with an error of  $\pm 1^\circ$ .

### 437 438 174 **2.3.5. Thermal properties of CS-based nanoparticles**

439  
440 175 Differential scanning calorimeter (Modulated DSC Q20, TA Instruments), equipped with  
441  
442 176 a liquid nitrogen cooling system, was used to investigate the thermal properties of nanoparticles.  
443  
444 177 The DSC analysis allowed the estimation of melting and crystallization points, as well as the  
445  
446 178 glass transition of the macromolecular materials. Thermal profiles of nanoparticles were  
447  
448 179 analyzed in a temperature range of 0-225 °C, at a heating rate scan of 10 °C/min, under nitrogen  
449  
450 180 flow rate of 50 ml/min. Thermograms were analyzed by using the TA Universal V4.5A  
451  
452 181 software.

453  
454 182 Thermal stability of nanoparticles was further studied using Thermogravimetric analyzer  
455  
456 183 (TGA Q500 High Resolution, TA Instruments), operating under nitrogen flow (60 ml/min).  
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458 184 TGA analysis is based on the assessment of the progressive change in mass, expressed in  
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460 185 percentage (%) as a function of temperature. Samples were heated from 25 to 700 °C at a  
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462 186 heating rate of 20 °C/min and their weight, initially about 4 mg, was constantly measured with  
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464 187 an accuracy of 0.01 mg. Thermograms were analyzed by using the TA Universal V4.5A  
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466 188 software.

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475 189 **2.3.6. Nanoparticles microstructure analysis**  
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477 190 The microstructure of nanoparticles was determined using scanning electron microscopy  
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479 191 (SEM, Hitachi S4800), at an angle of 90° to the surface, using different magnifications. Prior  
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482 192 to imaging, nanoparticles were fixed on the SEM support using double side adhesive tape, and  
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484 193 observed up to a 2000 x magnification, under an accelerating voltage of 2.0 kV and an absolute  
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486 194 pressure of 60 Pa, after sputter coating with a 5 nm thick gold.

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488 195 Atomic force microscopy (AFM, Agilent Pico Plus) analysis was performed to further  
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490 196 characterize the morphology of unloaded and CPs-loaded CS nanoparticles. Imaging was  
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492 197 carried out in tap mode in air with standard Si cantilever, a spring constant of 0.6 N/m and an  
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494 198 estimated tip radius of 10 nm. The scan area was 2 μm × 2 μm, 256 lines and taken at a rate of  
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496 199 0.8 s/lines. The study was realized at room temperature with 54% level of humidity. The scan  
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498 200 speed was adjusted at 1.01 lines/s, the resonance frequency and the distance between tip and  
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500 201 nanoparticles were applied at 161.02 kHz and 9–11 nm, respectively.

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503 202 **2.3.7. Nanoparticles thermal stability**  
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505 203 Thermal stability of freeze-dried CS-based nanoparticles based on CS was studied at 80,  
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507 204 100 and 120 °C (sterilization temperatures) for 5, 15 and 30 min, since commercialized products  
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509 205 are frequently exposed to heat treatment, the most used processing method in food industry,  
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511 206 during their preparation. Particles size and zeta potential were evaluated at each desired time  
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513 207 interval.  
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517 208 **2.4. Study of CPs *in vitro* release from nanoparticles**  
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519 209 Ethanol and PBS (pH ~6.8) were used as media models for an *in vitro* CPs release study.  
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521 210 In brief, wet samples (10 mg) and media (1.2 ml) were placed in a microtube and incubated at  
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523 211 ambient temperature. At sampling time, the incubated mixture was centrifuged and 100 μl of  
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525 212 supernatant was collected. Evaluation of the amount of CPs released was determined using a  
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527 213 spectrophotometer at a wavelength of 480 nm. An equal volume of fresh media was then  
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534 214 replaced in the mixture, and the same procedure was repeated for the subsequent sampling.  
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536 215 Cumulative percent of released CPs was obtained by dividing the cumulative amount of  
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538 216 released CPs at each sampling time point ( $M_t$ ) to the initial weight of the CPs-loaded in the  
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540 217 sample ( $M_0$ ), and schemed as a function of release time. All *in vitro* release experiments were  
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542 218 performed in triplicate.

$$\% \text{ Cumulative release} = \sum_{t=0}^t M_t / M_0 \times 100 \quad \text{Eq (4)}$$

## 2.5. Statistical analysis

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552 221 All experiments were carried out in duplicate and average values with standard deviation  
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554 222 errors are reported. Mean separation and significance were analyzed using the SPSS software  
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556 223 package ver. 17.0 professional edition (SPSS, Inc., Chicago, IL, USA) using ANOVA analysis.  
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558 224 Differences were considered significant at  $p < 0.05$ .

## 3. Results and Discussion

### 3.1. Optimization of the complex coacervation conditions between CS and PI

#### 3.1.1. Influence of CS and PI solutions' pH

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Complex coacervation, the combination of two oppositely-charged polymers causing interaction and precipitation of complex polymers, is a liquid-liquid phase separation mechanism resulting from the formation of intramolecular electrostatic complexes between the macromolecules [22]. In this context, PI and CS complexation was subsequently investigated and optimized.

The evolution of the zeta potential values of the PI and CS solutions in the selected pH range (2.0-10.0) is revealed in **Fig. 1A**. Indeed, the zeta potential of the PI solution was positive below pH 4.5, where negatively charged groups ( $\text{COO}^-$ ) are less abundant than positively charged groups ( $\text{NH}_3^+$ ). For all pH values above pH 4.5, the negativity of the PI increased

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592  
593 237 gradually with the increase of the pH, where the number of carboxyl groups ( $\text{COO}^-$ ) exceeded  
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595 238 the number of amine groups ( $\text{NH}_3^+$ ). The amphoteric character of the proteins containing both  
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597 239 amine and carboxyl functional groups resulted in the PI zeta potential pH dependence.

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600 240 Regarding CS, it was positively charged at pH values below 8.0 and the zeta potential  
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602 241 level began to increase with the decrease of pH (**Fig. 1A**). The positive charge of the CS is due  
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604 242 to the acid protonation of the amine groups ( $\text{NH}_3^+$ ) of the glucosamine units. The CS zeta  
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606 243 potential reduction subsequently to the pH rising could be assigned to the deprotonation of the  
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608 244 CS amine groups, by interaction of the proton of the amino group of the CS with strong  
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610 245 hydroxyl groups from the added NaOH [18]. This deprotonation led thereby to CS loss of its  
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612 246 charge, becoming insoluble at pH values exceeding 8.0.

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614 247 Since physico-mechanical and thermal characteristics of the complex coacervates are  
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616 248 controlled by the extent of the interaction between the contributing matrix-wall polymers, the  
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618 249 strength of the electrostatic interaction (SEI), defined as the product of the absolute values of  
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620 250 the zeta potential of PI and CS, otherwise, their binding force during complex coacervation, is  
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622 251 a critical parameter affecting the complex coacervation process [23]. The SEI values for the PI  
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624 252 and CS solutions reached the highest value of approximately  $600 \text{ mV}^2$  at pH 6.0, as displayed  
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626 253 in **Fig. 1A**, indicating that the strongest attraction between both PI and CS was around this pH  
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628 254 value, further supported with an almost neutral zeta potential of PI-CS complex coacervates at  
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630 255 pH 6.0.

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633 256 Additionally, the yield and turbidity of complex coacervates are used as key parameters  
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635 257 in the optimization process and are recognized as the most reliable parameters [23]. **Fig. 1B**  
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637 258 displays the yield of the complex coacervates at a selected pH range, chosen based on the  
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639 259 highest value of SEI ( $600 \text{ mV}^2$ ). As it can be seen, the yield of complex coacervates increased  
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641 260 significantly ( $p < 0.05$ ) with pH increase from pH 4.0 to pH 6.0, suggesting that this range could  
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643 261 be the point at which the formation of insoluble complexes was initiated [25]. The highest yield  
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652 262 of 90% ( $p<0.05$ ) was observed at pH 6.0, above which, the yield of complex coacervates started  
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654 263 to decrease significantly ( $p<0.05$ ).

656 264 This finding was due to an increase of negative charge of PI that was unable to complex  
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659 265 with CS (decrease of its positive charge), triggering thereby a disturbance of the charge balance  
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661 266 between PI and CS. This optimal pH correlated well with the SEI values that were found to be  
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663 267 higher around pH 6.0. Moreover, the decrease in the coacervates yield due to a decrease in the  
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665 268 SEI (Fig. 1A), and thereby a drop-in complexation between PI and CS, could be due to  
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667 269 alkalization of the mixed dispersion above pH of 6.0 leading to dissociation of the complex  
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669 270 coacervates.

671 271 Turbidity analysis data show similar trend, with a significant ( $p<0.05$ ) decrease in  
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673 272 turbidity between pH 6.5 and 8.0, suggesting that the strongest complexation between PI and  
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675 273 CS occurred in the pH range of 5.0-6.5, with an optimum at pH 6.0.

### 678 274 3.1.2. PI-to-CS weight balance effect

680 275 In another aspect of the complex coacervation optimization, it is recognized that the  
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683 276 protein-polysaccharide mixing ratio sensibly influenced the charge balance of their mixture  
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685 277 [26]. The turbidity and the coacervate yield of mixtures equilibrium phase, considering different  
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687 278 PI to CS mass ratios at an optimal pH of 6.0, are illustrated in Fig. 1C. Data reveal that when  
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689 279 PI-CS mass ratio increased, the coacervate yield and turbidity values improved significantly  
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691 280 ( $p<0.05$ ), reaching the highest levels at the PI-CS ratio of 8/1. An additional increase in PI-CS  
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693 281 mass ratios to 20/1 (w/w) resulted in a significant diminution in the coacervates yield and  
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695 282 turbidity values ( $p<0.05$ ). In fact, the maximum of coacervate production yield requires the  
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697 283 highest quantity of PI and CS moieties in the mixture to be able to interact with each other.  
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699 284 However, minor complex coacervate yield could be a consequence of redundant PI-to-CS  
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701 285 weight ratios, implying that an outnumber of un-complexed either PI or CS molecules exists  
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704 286 [7]. Therefore, the optimal PI-CS mixing ratio of 8/1 was recorded.

### 3.2. Size and Zeta potential of CPs-loaded nanoparticles

The diameters of CS nanoparticles with and without CPs (ionotropic gelation and complex coacervation) were determined. **Table 1** shows that unloaded nanoparticles obtained by ionotropic gelation and complex coacervation possessed an average diameter of  $\sim 7.26 \mu\text{m}$  and  $9.76 \mu\text{m}$ , and polydispersity indexes of 42.9% and 62.37%, respectively. The mean diameter of CS nanoparticles decreased with the incorporation CPs, with an average diameter of  $4.72 \mu\text{m}$  (ionotropic gelation) and  $4.87 \mu\text{m}$  (complex coacervation) and polydispersity indexes of 37.94% (ionotropic gelation) and 47.04% (complex coacervation), indicating to variable extend the nanoparticles narrow size distribution [3]. The light scattering technique might measure the particle size as reflected by the hydrodynamic diameters of aggregate or hydrated individual particles.

The results reflected that the aqueous aggregation and swelling of CPs-loaded CS nanoparticles were lower than those of unloaded CS nanoparticles, regardless the applied microencapsulation process, i.e. ionotropic gelation or complex coacervation [6]. In fact, the CS nanoparticles are developed based on the electrostatic interaction of CS with TPP (ionotropic gelation) and CS with PI (complex coacervation), which will modulate their size. In another side, the adsorption of CS onto the CPs droplets is the elementary process of CPs-loaded CS nanoparticles formation. The lower agglomeration in CPs-loaded CS nanoparticles might be due to hydrophobic CPs molecules that forced it to entrap inside.

Considering the encapsulation method, different particle sizes distributions were observed for the nanoparticles, being smaller ( $7.26$  to  $4.72 \mu\text{m}$ ) for ionotropic gelation than those of the complex coacervation ( $9.76$  to  $4.87 \mu\text{m}$ ). This is interesting because the particle size of nanoparticles could sensitively influence their chemical and physical properties [27]. In fact, the smaller particles are more easily solubilized, compared to larger particles with higher surface area. Further, CS molecular weight drop led to the decrease of its viscosity, influencing

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769  
770 312 the particle size, and the simultaneously increased capability of cross-linking to shape smaller  
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772 313 structures [28].  
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774 314 In addition, unloaded nanoparticles exhibited zeta potential values of +51.43 mV and  
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776 315 +49.42 mV, for ionotropic gelation and complex coacervation, respectively (**Table 1**). The  
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778 316 occurrence of positive zeta potential values, reflecting the specific positively charged surface  
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781 317 of CS that could be due to the contribution of protonated amino groups present on the surface  
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783 318 of particles [29]. However, the zeta potential decreased to values of +45.3 mV (ionotropic  
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785 319 gelation) and +46.8 mV (complex coacervation) for CPs-loaded nanoparticles. This finding  
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787 320 reflected that the surface positive charge was reduced due to the loading of CPs and the  
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789 321 shielding effect of protonated NH<sub>2</sub> group by CPs on CPs-loaded nanoparticles, mitigating  
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791 322 therefore the dispersion stability of nanoparticles in water [30,31]. **Although the slight decrease**  
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793 323 **in the zeta potential values after the encapsulation of CPs, the nanoparticules still significantly**  
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795 324 **stable, with zeta potential values exceeding +30 mV [32].**  
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798 325 Bagre *et al.* [33] described in a previous work an inversion of positive potential to  
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800 326 negative value when alginate was coated over chitosan nanoparticles, with an increase in the  
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802 327 size and an improved release profile of enoxaparin facilitating the oral delivery of this bioactive  
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804 328 substance. In line with results from the present work, several studies have reported that zeta  
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806 329 potential values of CS nanoparticles were reduced when drugs, such as ascorbic acid [34] and  
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808 330 eugenol [35] were incorporated.  
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### 811 331 **3.3. Loading charge and entrapment efficiency of CPs**

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813 332 **Although spectrophotometry and high-performance liquid chromatography (HPLC) are**  
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815 333 **effective techniques to determine the content of molecules loaded in the particles, these latter**  
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817 334 **were not useful, in the present work. Indeed, CPs did not dissolve in an aqueous HCl solution,**  
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819 335 **besides the complete destruction of particles and dissolution of molecules in the medium that**  
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821 336 **should be considered.** Thereby, TGA/DTG thermograms were applied to determine the content  
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829 337 of CPs loaded in nanoparticles [20,36], where CPs-loaded nanoparticles were successfully  
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831 338 degraded as described by Wang *et al.* [13].  
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833 339 **Fig. 2A** shows that CS nanoparticles exhibited three step weight loss at temperatures  
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835 340 below 150 °C (moisture loss), 180-310 °C (degradation of CS) and above 310 °C (loss of  
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837 341 crosslinked CS with TPP or PI). Compared with the derivative thermogravimetry (DTG)  
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839 342 thermograms of the bare nanoparticles, CPs-loaded CS nanoparticles revealed a weight loss in  
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841 343 four stages. The new weight loss range, at temperatures ranging from 250 to 310 °C, is ascribed  
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843 344 to the degradation of CPs, with a drastic weight mass loss up to 92% in the case of free CPs.  
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845 345 The rate of maximum weight loss corresponding to temperature was considered as the  
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847 346 decomposition temperature (Td), clearly observed as a peak in the DTG thermogram, as shown  
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849 347 in **Fig. 2b**. These results confirmed the successful loading of CPs into CS nanoparticles and the  
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851 348 improvement of CPs heat stability by nanoencapsulation, using both systems ionotropic  
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853 349 gelation and complex coacervation.  
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856 350 The LC and EE are calculated and presented in **Table 2**, based on the percentage of weight  
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858 351 loss at temperatures ranging from 250 to 310 °C, reflecting the amount of loaded CPs, as above  
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860 352 described in the section 2.3.3, based on Eq (2) and (3). The LC of CPs was found to be 31.87%  
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862 353 and 46.43% for nanoparticles performed based on the complex coacervation and ionotropic  
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864 354 gelation approaches, respectively. Similarly, nanoparticles prepared based on the complex  
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866 355 coacervation methodology revealed lower EE level of 73.93%, compared to nanoparticles  
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868 356 performed using the ionotropic gelation approach, with EE of 88.54% (**Table 2**). In general,  
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870 357 chitosan displays the capability of rapid gelation process upon contact with polyanions, through  
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872 358 the creation of inter and intramolecular crosslinking interactions mediated by these polyanions.  
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874 359 Consequently, particles are easily shaped subsequently to mixing of TPP and chitosan's amino  
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876 360 groups [35].  
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888 361 The LC and EE values obtained in the current study are comparable or even greater when  
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890 362 compared against forward obtained data for chitosan-based nanoparticles, prepared by the  
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892 363 ionotropic gelation [30,37,38] and complex coacervation [22,39-40] methods, and applied as  
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894 364 nanocarriers for hydrophobic drugs/molecules.  
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### 897 365 **3.4. Effective loading of CPs into CS nanoparticles**

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899 366 In the present study, physicochemical characterization techniques involving FTIR and  
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901 367 XRD were further adopted to evaluate and confirm the success and the effective loading of CPs  
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903 368 into CS nanoparticles.  
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906 369 FTIR spectra of CPs, CS, PI, blank nanoparticles and CPs-loaded nanoparticles,  
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908 370 displaying physicochemical interactions are reported in **Fig. 3**. CPs profile showed  
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910 371 characteristic bands at  $1487\text{ cm}^{-1}$ ,  $1414\text{ cm}^{-1}$  and  $1269\text{ cm}^{-1}$  (aromatic compounds or carboxylic  
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912 372 acids, such as phenolic compounds),  $1770\text{ cm}^{-1}$  and  $1572\text{ cm}^{-1}$  (fatty acid esters and aromatic  
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914 373 compounds such as primary amines, respectively),  $3002\text{ cm}^{-1}$ ,  $2944\text{ cm}^{-1}$  and  $2864\text{ cm}^{-1}$  (alkyl  
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916 374 group from proteins) [42,43]. In fact, CPs are stable complexes, in which the carotenoids are  
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918 375 bound to a high-density lipoprotein [44]. For nanoparticles spectra, absorption bands assigned  
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920 376 to  $\text{NH}_2$  and OH groups stretching vibrations at  $3423\text{ cm}^{-1}$ , indicated hydrogen bonding between  
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922 377 CS with TPP (ionotropic gelation) and CS with PI (complex coacervation). In addition, bands  
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924 378 detected at  $1649\text{ cm}^{-1}$ ,  $1579\text{ cm}^{-1}$ ,  $1093\text{ cm}^{-1}$ ,  $894\text{ cm}^{-1}$  corresponded to amide I, amide II, C-  
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926 379 O-C and pyranose ring, respectively [28,45]. Upon comparison with unloaded nanoparticles, as  
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928 380 a results of hydrogen bonding between CPs and CS, TPP or PI, the band of  $\text{NH}_2$ -OH group  
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930 381 stretching in CPs-loaded nanoparticles has been broadened and shifted to  $3374\text{ cm}^{-1}$ , which  
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932 382 indicated changes in the CS and PI structure following the encapsulation procedure [5]. The  
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934 383 presence of bands at  $1566\text{ cm}^{-1}$  and  $1420\text{ cm}^{-1}$ , corresponding to C-C aromatic ring in both  
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936 384 native CPs as well as CPs-loaded nanoparticles, signposted the successful interaction between  
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938 385 CPs, CS and TPP or PI [20,29]. The occurrence of electrostatic forces that act between  
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947 386 positively charged CS nanoparticles and negatively charged CPs, besides the hydrogen bonding  
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949 387 and other non-covalent interaction, could be a possible mechanism of interaction between CS  
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951 388 and CPs, facilitating its loading. Data abovementioned infers the formation of nanoparticles  
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953 389 (ionotropic gelation and complex coacervation) and the effective encapsulation of CPs into  
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955 390 them.

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958 391 **Moreover, XRD is a useful tool to explore the nanostructure of matter. Therefore, XRD**  
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960 392 **patterns of CS, blank nanoparticles, and CPs-loaded nanoparticles are presented in Fig. 4.** CS  
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962 393 displayed typical fingerprints of semi-crystalline chitosan, with strong reflections at around  
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964 394  $2\theta=10.8^\circ$  and  $2\theta=20.1^\circ$ , showing high degree of crystallinity [15]. After electrostatic  
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966 395 interaction with TPP (ionotropic gelation) and with PI (complex coacervation), peak alterations  
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968 396 and shifts were observed with reduction of peak intensity (**Fig. 4**). Indeed, after cross-linking,  
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970 397 no peak was detected in the diffractogram of nanoparticles, reflecting the destruction of the  
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972 398 native CS packing structure [20,21]. It is well-known that the width of X-ray diffraction peak  
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974 399 is related to the size of crystallite, the broadened peak usually results from imperfect crystal  
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976 400 [46]. Consequently, the broad peak of nanoparticles may be caused by the cross-linking reaction  
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978 401 between CS and TPP or PI that could destroy the crystalline structure of CS, involving greater  
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980 402 disarray in chain alignment in the nanoparticles after complexation [21,31]. In addition, a new  
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982 403 peak was found in the diffractogram of nanoparticles at  $2\theta$  of around  $28^\circ$ . These distinct  
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984 404 differences reflected the modification in the arrangement of molecules in the crystalline matrix  
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986 405 induced by ionic complexation. Regarding the diffraction spectra of CPs-loaded nanoparticles,  
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988 406 compared with the unloaded nanoparticles, the characteristic peak at  $2\theta$  of  $19^\circ$  confirmed the  
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990 407 presence of CPs within nanoparticles. Thus, XRD analysis revealed the successful  
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992 408 encapsulation of CPs in nanoparticles, clearly showing the modification in their packing  
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994 409 structure.  
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1006 410 Accordingly, on behalf of FTIR and XRD data, it is possible to conclude that the two  
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1008 411 steps emulsion and ionic gelation between CS and TPP (ionotropic gelation), besides  
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1010 412 complexation between CS and PI (complex coacervation), are suitable for the encapsulation of  
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1012  
1013 413 CPs into nanoparticles [47].  
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### 1015 414 **3.5. Thermal characteristics of CPs-loaded nanoparticles**

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1017 415 Thermal transition profiles for CS, CPs and nanoparticles from the DSC thermograph  
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1019 416 allowed to draw further evidences towards the effectiveness of the encapsulation process. DSC  
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1021 417 thermograms of unloaded and CPs-loaded nanoparticles are shown in **Fig. 5**. The unloaded  
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1023 418 nanoparticles showed specific prominent peaks ascribed to CS. In fact, an endothermic peak at  
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1025 419 nearly 100 °C, labeled as dehydration temperature, and a glass transition temperature peak at  
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1027 420 202 °C, linked to polymer thermal decomposition [45], were detected. Furthermore, CPs  
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1029 421 showed a couple of specific melting endotherms at around 100-135 °C that disappeared in CPs-  
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1031 422 loaded nanoparticles thermograms, regardless the applied encapsulation technique. This could  
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1033 423 be explained by molecular dispersion of CPs in the polymeric nanoparticles losing its  
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1035 424 crystallinity, and suggesting thermal stabilization of CPs due to the interaction of CS with TPP  
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1037 425 (ionotropic gelation) and CS with PI (complex coacervation). This interaction is further  
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1039 426 substantiated by the absence of any characteristic peaks of PI and TPP.  
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1043 427 In addition, the slight reduction in CS dehydration temperature to 85 °C and the lower  
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1045 428 glass transition temperatures, reaching 198 °C and 177 °C, for the ionotropic gelation and  
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1047 429 complex coacervation, respectively (**Fig. 5**), reflected the changes involved in the polymer  
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1049 430 chains order of arrangement upon CPs loading polymeric nanoparticles [30,48].  
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1051 431 **All the data displayed by the DSC thermographs of the prepared nanoparticles could be**  
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1053 432 **correlated with their corresponding TGA curves above described in Fig. 2. Accordingly, it**  
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1055 433 **could be concluded that the initial melting temperature of CS nanoparticles was effectively**  
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1057 434 **enhanced upon CPs encapsulation.**  
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### 3.6. Morphological characterization of CPs-loaded nanoparticles

The morphology of the nanoparticles was further observed by SEM, since, as above described, the shape and size of particles have strong effect on the fluidity of nanoparticles and thereby their solubility [49]. The SEM images reveal that the size of the blank nanoparticles was higher compared to the CPs-loaded nanoparticles (**Fig. 6**), which is concurrent to the laser scattering particles size distribution findings (**Table 1**). Such characteristic was likewise reported in several studies [50-52]. The swelling of the CS layer adjoining the individual particles, and/or the aggregation of single particles while dispersed in water might be the basis of larger diameter of unloaded nanoparticles [21]. Moreover, more agglomerated particles were observed for unloaded CS nanoparticles (**Fig. 6a (A) and Fig. 6b (A)**), probably as a result of the water absorption behavior of this matrix, generally depending on the chemical properties of surface, and which can be reduced through drying [50]. Conversely, lower agglomerated particles with more uniform appearance were observed for CPs-loaded CS nanoparticles (**Fig. 6a (B) and Fig. 6b (B)**).

In another side, SEM micrographs clearly display that the morphology of the nanoparticles varied significantly depending on the applied formulation method. For both CPs-loaded nanoparticles, the aggregation of particles was further visible, and those aggregates seemed to be melting and combining with each other. As it is shown in **Fig. 6b**, the complex coacervation nanoparticles demonstrated spheroidal shapes and non-smooth surface, likely due to the contribution of PI content with high molecular weight that delay the surface contraction, avoiding the regular spheres formation [51]. Particle morphology of the ionotropic gelation-based nanoparticles (**Fig. 6a**) showed collapsed irregular particles with abundant roughness and cracks on the surface.

Different particle sizes were observed for the nanoparticles, being larger (~ 600 nm) for complex coacervation than those of the ionotropic gelation powder (~ 300 nm). This finding

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1123  
1124 460 was in the same line with data of the size measured by the light scattering technique, reflecting  
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1126 461 that the aggregation or swelling in water of CPs-loaded nanoparticles were lower than those of  
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1128 462 unloaded nanoparticles, conceivably due to the hydrophobicity of CPs molecules entrapped  
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1130  
1131 463 inside the nanoparticles.

1132  
1133 464 To better study the CPs-loaded CS nanoparticles' microstructure, 3D AFM imaging was  
1134  
1135 465 performed (**Fig. 7**). Data reveal that CPs-loaded CS nanoparticles prepared by using the  
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1137 466 ionotropic gelation technique were more uniform in size as compared to CPs-loaded CS  
1138  
1139 467 nanoparticles based on the complex coacervation approach (**Fig. 7a**). Maximum height of 3.1  
1140  
1141 468 nm was observed for ionotropic gelation-based CPs-loaded nanoparticles and 0.21  $\mu\text{m}$  for CS-  
1142  
1143 469 based nanoparticles according to the complex coacervation method (**Fig. 7b**).

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1145 470 Our findings from SEM and AFM studies demonstrated that the incorporation of CPs  
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1147 471 decreased significantly the size of CS-based nanoparticles. Further, CS-based nanoparticles  
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1149 472 unloaded or loaded with CPs prepared by using the ionotropic gelation procedure were smaller  
1150  
1151 473 and more homogenously dispersed as compared to the complex coacervation-achieved  
1152  
1153 474 nanoparticles, suggesting that CPs could behave like surfactant by additional increase of the  
1154  
1155 475 dispersity and thereby CS-based nanoparticles' surface area [49].

### 1156 476 **3.7. Stability study of CPs-loaded CS nanoparticles**

1157  
1158  
1159 477 Lyophilized CPs-loaded CS nanoparticles were subjected to stability studies at 80, 100  
1160  
1161 478 and 120 °C (sterilization temperatures) for 5, 15 and 30 min. Particle size (**Fig. 8a**) and zeta  
1162  
1163 479 potential (**Fig. 8b**) were determined at each desired time interval.

1164  
1165 480 Droplet size diameter is reported to be a key factor able of physical stability illustration  
1166  
1167 481 and physicochemical behavior modification of encapsulated compounds [53]. **As shown in Fig.**  
1168  
1169 482 **8a, whatever the encapsulation method used, the particle diameter of CPs loaded nanoparticles**  
1170  
1171 483 **increased significantly with the increase of heat time from 5 to 30 min and temperature from**  
1172  
1173 484 **80 to 120 °C ( $p < 0.05$ ). Indeed, size values increased from 5.4  $\mu\text{m}$  to 10.45  $\mu\text{m}$  after an**

1181  
1182  
1183 485 incubation time of 30 min at 80 °C, and to 10.73 after a 30 min incubation time at 120 °C, in  
1184  
1185 486 the case of the complex coacervation process (**Fig. 8a (A)**). This finding implies an increase in  
1186  
1187 487 the number of larger particles and a decrease in the narrow distribution of the suspension size,  
1188  
1189 488 and thereby, its meager thermal stability. In fact, there is an intensification in particles' collision  
1190  
1191 489 frequency, typically under heat treatment, and thereafter the unfold of proteins present within  
1192  
1193 490 the particles, which could uphold particles aggregation [54]. In addition, nanoparticles size  
1194  
1195 491 revealed a significant increase with temperature augmentation, which could be assigned to PI  
1196  
1197 492 denaturation, regarding the CS-PI based nanoparticles, promoting the aggregation of  
1198  
1199 493 nanoparticles due to modifications in their configuration, triggered by higher temperatures [55].

1200  
1201 494 Conversely, the particle size of CPs loaded nanoparticles prepared via the ionotropic  
1202  
1203 495 gelation approach was smaller after a 30 min heating reaction time at 120 °C (10.01  $\mu\text{m}$ )  
1204  
1205 496 ( $p < 0.05$ ). This finding interestingly demonstrated that CS crosslinked with TPP protected CPs  
1206  
1207 497 molecules from heat-induced aggregation (**Fig.8a (B)**). Moreover, CPs loaded nanoparticles  
1208  
1209 498 prepared by ionotropic gelation were found to exhibit a good heat-stability at 80 °C. In fact, it  
1210  
1211 499 has been previously reported that CS nanoparticles exhibited an increase in particle size during  
1212  
1213 500 storage, mainly due to the swelling, the aggregation of the particles and the interaction of the  
1214  
1215 501 chains of free polymers with the particles network [56].

1216  
1217 502 In another side, zeta potential data, often considered as particle suspensions and colloidal  
1218  
1219 503 samples stability sign, were recovered (**Fig. 8b**). Indeed, a high zeta potential (either positive  
1220  
1221 504 or negative) is required to maintain particle distance and protect their stability, allowing a  
1222  
1223 505 sufficient repulsive energy [49,54]. Indeed, a low zeta potential value (almost null) led to faster  
1224  
1225 506 particles coagulation and sedimentation. Nevertheless, at high zeta potential, electrostatic  
1226  
1227 507 repulsion forces between particles allow the improvement of their stability. Thus, micro-  
1228  
1229 508 systems moderate stability is observed with zeta potential around  $\pm 30$  mV, good stability at  
1230  
1231 509 around  $\pm 50$  mV and excellent stability at zeta potential value  $\geq \pm 60$  mV [57]. It is worthy to

1240  
1241  
1242 510 mention that zeta potential of heated CPs-loaded nanoparticles was decreasing with the rise of  
1243  
1244 511 heating temperature and incubation time (**Fig. 8b**). Despite this decrease, data from zeta  
1245  
1246 512 potential analysis suggest that the tested nano-carriers prepared by ionotropic gelation and  
1247  
1248  
1249 513 complex coacervation should be sufficiently stable against heat with average zeta potential  
1250  
1251 514 more than +30 mV in both cases.

1252  
1253 515 Findings regarding heat resistance of CPs-loaded nanoparticles corroborate well with  
1254  
1255 516 above mentioned results in the present study. In fact, lower encapsulation efficiency might  
1256  
1257 517 indicate that there is too much uncapsulated core on the nanoparticle's surface [22,34], as  
1258  
1259 518 perceived in complex coacervation-based nanoparticles, which can explain its lower stability.  
1260  
1261 519 Compared to ionotropic gelation, the achieved nanoparticles better stability can be ascribed to  
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1263 520 Tween-80, used as non-ionic surfactant that delays the establishment of aggregates, promoting  
1264  
1265  
1266 521 thereby the formation of CPs-loaded nanoparticles.

1267  
1268 522 Overall results in the present study suggest that, due its cross-linking effect, stimulating  
1269  
1270 523 polymeric molecules to create tougher interactions with each other, TPP acts as stabilizer,  
1271  
1272 524 improving nanoparticles structural stability. Subsequently, CS-TPP, as nanocarrier, could be  
1273  
1274 525 adopted as a tool to protect active and sensible molecules against oxidation.

### 1277 526 **3.8. *In vitro* release kinetics assessments of CPs from CS nanoparticles**

1278  
1279 527 It is well known that drug or biomolecules release from nanoparticles take place by  
1280  
1281 528 several mechanisms, including surface erosion, disintegration, diffusion and desorption [16,18].  
1282  
1283 529 The *in vitro* release study of CPs from prepared nanoparticles was subsequently monitored to  
1284  
1285 530 understand and to recognize the release mechanism and kinetics of CPs, which is decisive for  
1286  
1287 531 ulterior applications of CPs-loaded nanocarriers [8,58].

1288  
1289 532 The *in vitro* release profiles of CPs from the nanoparticles are reported in **Fig. 9**,  
1290  
1291 533 considering the amount of CPs released as function of different times, measured at 480 nm,  
1292  
1293  
1294 534 based on a calibration curve of CPs concentration (**Data not shown**). The *in vitro* release



1299  
1300  
1301 535 scheme of CPs from nanoparticles can be labelled as a biphasic process (**Fig. 9**), with an initial  
1302  
1303 536 rapid release followed by subsequent slower release. The initial swift release was attributed to  
1304  
1305 537 the CPs molecules entrapped near the surface, where the dissolution rate of the polymer near  
1306  
1307  
1308 538 the surface is high [21,37,59]. **In the second stage, the release rate was rather slow, attaining a**  
1309  
1310 539 **plateau**, probably due to the diffusion of the CPs dispersed into the CS matrix as the dominant  
1311  
1312 540 mechanism, and thus, nearly no further release of CPs (**Fig. 9**). Supplementary release of CPs  
1313  
1314 541 required the swelling and/or degradation of the compact CS-TPP and CS-PI nanoparticles.

1316 542 Similar results of encapsulated materials were reported with chitosan nanoparticles, with  
1317  
1318 543 swift initial release followed subsequently by a slow release [5,14,22,49]. It is important to  
1319  
1320 544 pointed out that the deliverance of a drug by diffusion **related the swelling and the**  
1321  
1322 545 **transformation from glassy into rubbery polymer matrix, as a result of the penetration of**  
1323  
1324 546 **medium into the particle system [2,60].**

1327 547 In the present study, two media were used, ethanol and PBS (pH~6.8), as CPs are  
1328  
1329 548 dissolved well in both solvents. The release shapes of CPs in ethanol and PBS were to variable  
1330  
1331 549 extend analogous, with the typical biphasic delivery process. Otherwise, the release of CPs in  
1332  
1333 550 ethanol reached a plateau within 90 min (ionotropic gelation) and 150 min (complex  
1334  
1335 551 coacervation). Nonetheless, reaching a plateau in PBS, required a shorter time, such as 30 min  
1336  
1337 552 (ionotropic gelation) and 90 min (complex coacervation). This might be an outcome of the  
1338  
1339 553 weakening of the electrostatic interaction between the cationic carrier (CS) and anionic TPP  
1340  
1341 554 (inotropic gelation) or PI (complex coacervation), resulting in faster and shorter release time.  
1342  
1343 555 Due to the higher solubility of CPs in ethanol, the quantity of CPs released in ethanol was higher  
1344  
1345 556 than that in PBS.

1348 557 In terms of the encapsulation methodology, the amount of CPs released from  
1349  
1350 558 nanoparticles prepared by ionotropic gelation (**Fig. 9a**) was found to be greater, with shorter  
1351  
1352 559 release interval than that of nanoparticles prepared using the complex coacervation approach

1358  
1359  
1360 560 **(Fig. 9b)**. In fact, mainly due to better surface-to-volume ratio, nanoparticles with reduced  
1361  
1362 561 particle size may result in an easy and fast deliverance of entrapped CPs. Furthermore, the  
1363  
1364 562 quantity of released CPs was found to be modulated by the amount of CPs entrapped. In fact,  
1365  
1366 563 the high LC, reaching more than 46% with the ionotropic gelation process vs. ~32% for the  
1367  
1368 564 complex coacervation **(Table 2)**, provided a fast release rate and a concomitantly high amount  
1370  
1371 565 of released CPs [28].  
1372

1373 566 Hereafter, data in the present research indicate that the CS based nano-systems,  
1374  
1375 567 engineered by the ionotropic gelation and complex coacervation, are suitable for controlling the  
1376  
1377 568 release of CPs.  
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#### 1380 569 **4. Conclusion**

1382 570 CPs-loaded nanoparticles were successfully prepared by complex coacervation and  
1383  
1384 571 ionotropic gelation approaches, as confirmed by FTIR, TGA/DTG and XRD analytical  
1385  
1386 572 techniques. The nanoparticles were spherical in shape with positively charged surfaces and size  
1387  
1388 573 depending on the formulation approach, where ionotropic gelation allowed the development of  
1389  
1390 574 smaller nanoparticles, with higher LC and EE. The *in vitro* release studies indicated that the  
1391  
1392 575 content of encapsulated CPs influenced its release rate from nanoparticles, with higher released  
1393  
1394 576 CPs and shorter release interval, adopting the ionotropic gelation approach. **Therefore, the**  
1395  
1396 577 **effectiveness of CS-TPP, as a nano-carrier biopolymer for encapsulating bioactive substances,**  
1397  
1398 578 **and its potential use in several fields, namely for drug delivery. Moreover, the good thermal**  
1400  
1401 579 **stability of CS-PI nanoparticules, obtained by complex coacervation, signposted that CS-PI**  
1402  
1403 580 **coacervate could be suitable to be used for encapsulation of thermally sensitive materials.**  
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## **Figure captions**

**Figure 1:** Zeta potential of PI, CS and CS-PI coacervates, and strength of electrostatic force (SEI) (A). Complex coacervates yield and turbidity vs. pH (B). Turbidity and the yield of complex coacervates at pH 6.0 with different PI-CS mass ratios (C).

**Figure 2:** (a) ATG thermograms of nanoparticles via ionotropic gelation (A) and complex coacervation (B). (b) DTG thermograms of CS-based microcapsules via ionotropic gelation (A) and complex coacervation (B).

**Figure 3:** ATR-FTIR spectra of nanoparticles via ionotropic gelation (A) and complex coacervation (B).

**Figure 4:** XRD patterns of nanoparticles prepared via ionotropic gelation (A) and complex coacervation (B).

**Figure 5:** DSC profiles of CS-based microcapsules via ionotropic gelation (A) and complex coacervation (B).

**Figure 6:** a) SEM imaging of unloaded (A) and CPs-loaded (B) nanoparticles via ionotropic gelation. b) SEM imaging of unloaded (A) and CPs-loaded (B) nanoparticles via complex coacervation.

**Figure 7:** a) AFM images of (A) surface topographic and phase, and (B) 3D image of unloaded CPs-loaded nanoparticles prepared by using the ionotropic gelation procedure. b) Surface topographic and phase (A), and (B) 3D image of CPs-loaded nanoparticles elaborated according to the complex coacervation method, at 2×2 μm area.

**Figure 8:** Particle size (a) and zeta potential (b) evolution vs. temperature and incubation time of CPs-loaded nanoparticles using ionotropic gelation (A) and complex coacervation (B).

**Figure 9:** *In vitro* release assessments of CPs from nanoparticles prepared using ionotropic gelation (A) and complex coacervation (B).

Fig. 1

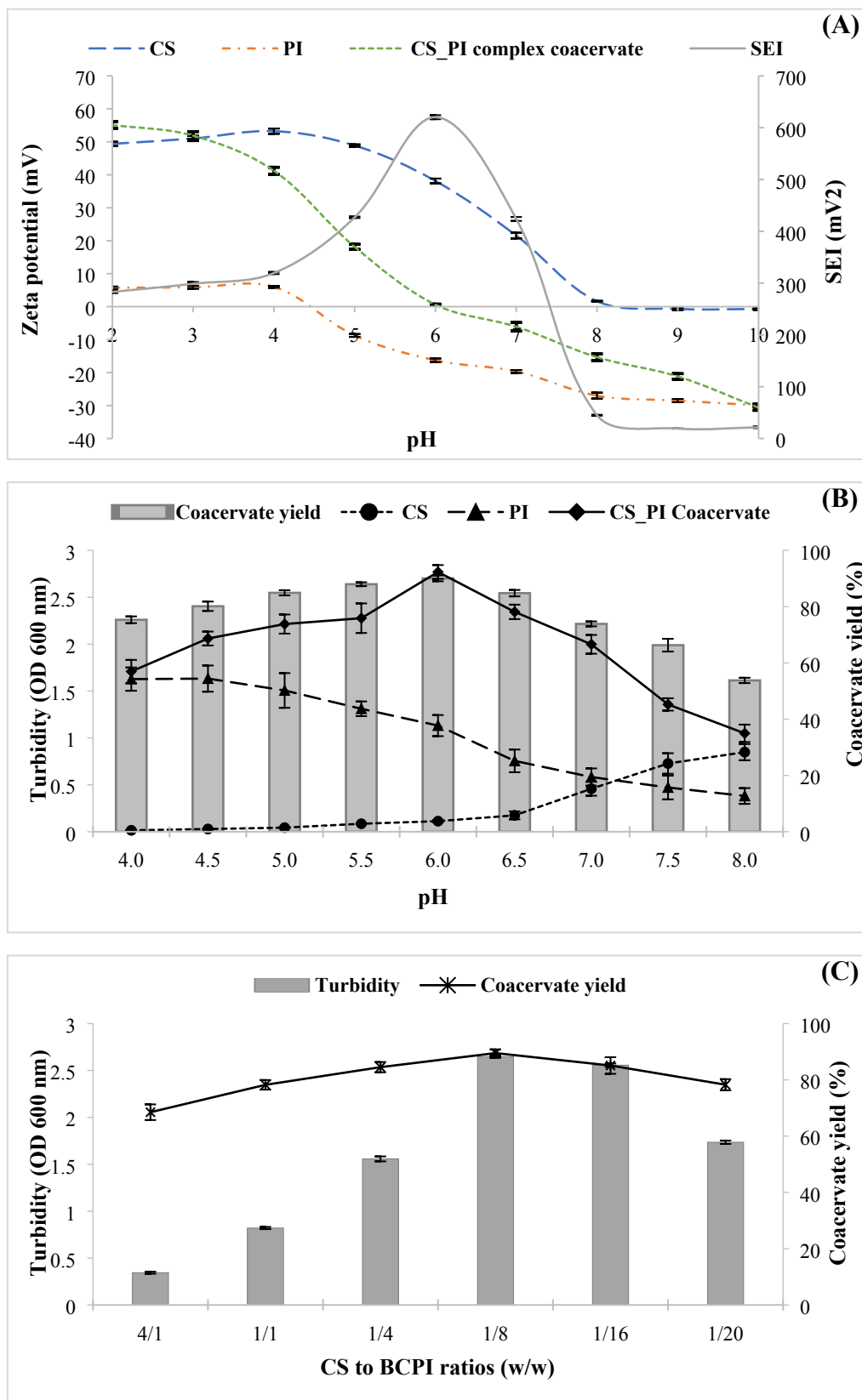
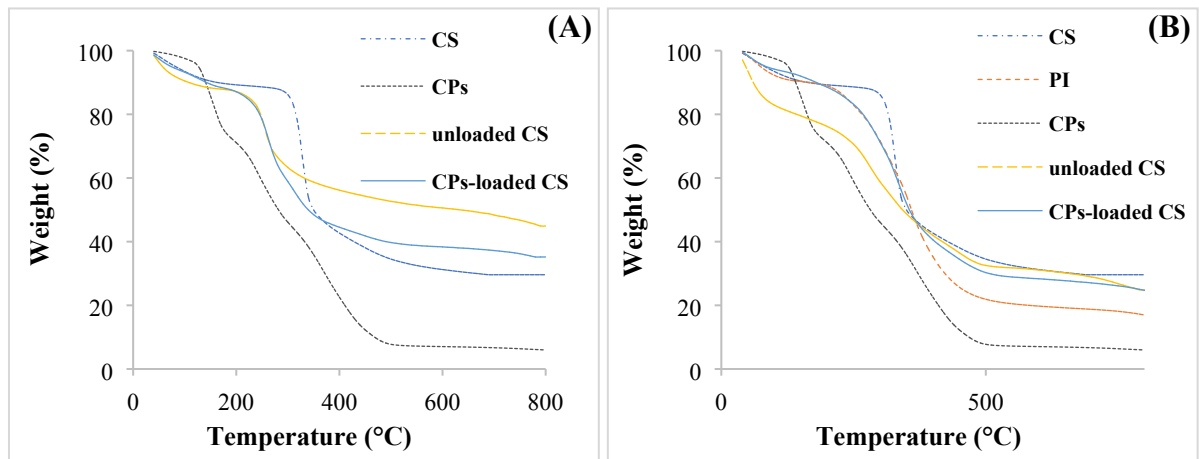
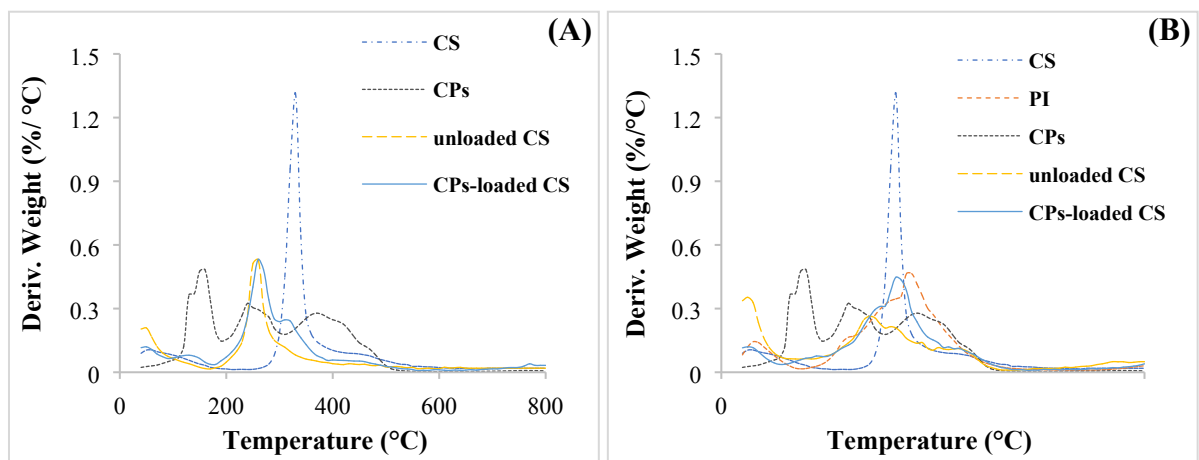


Fig. 2

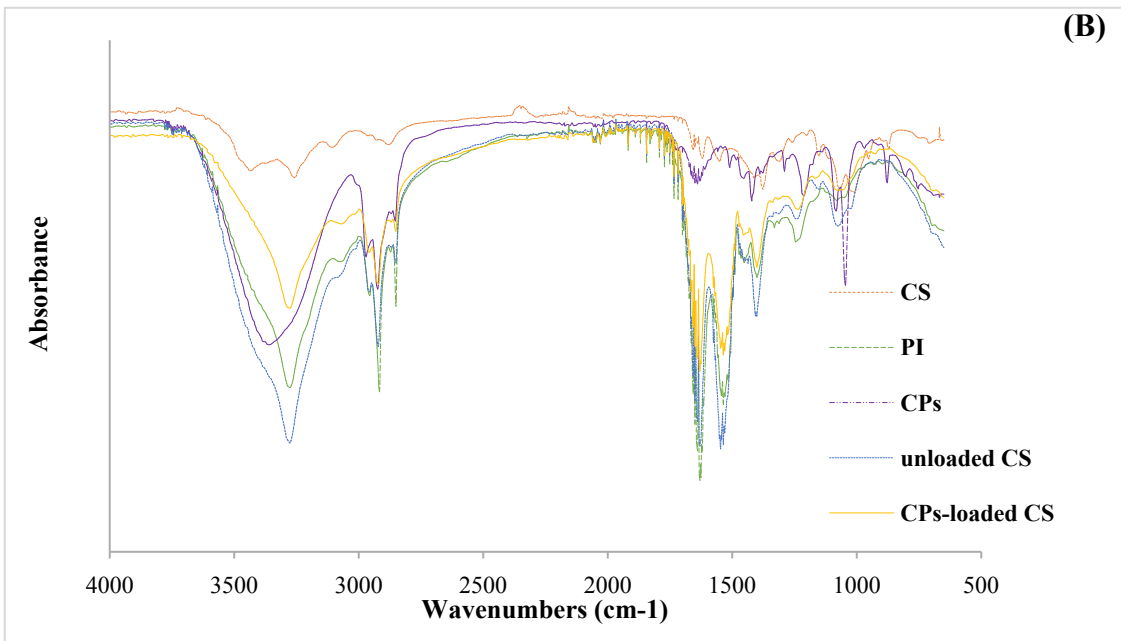
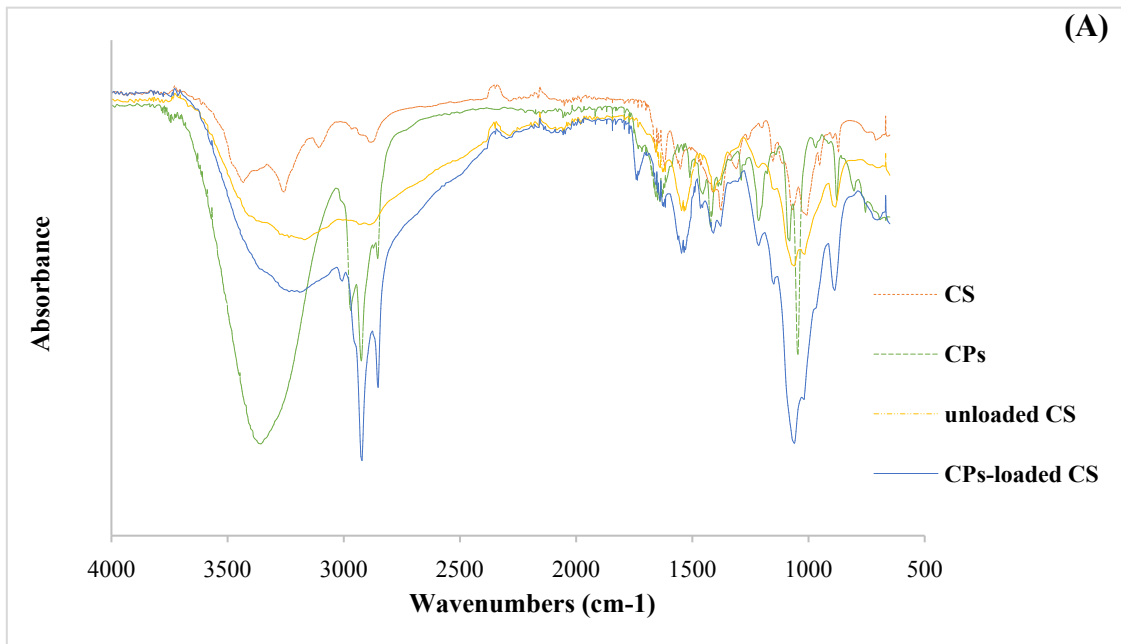
(a)



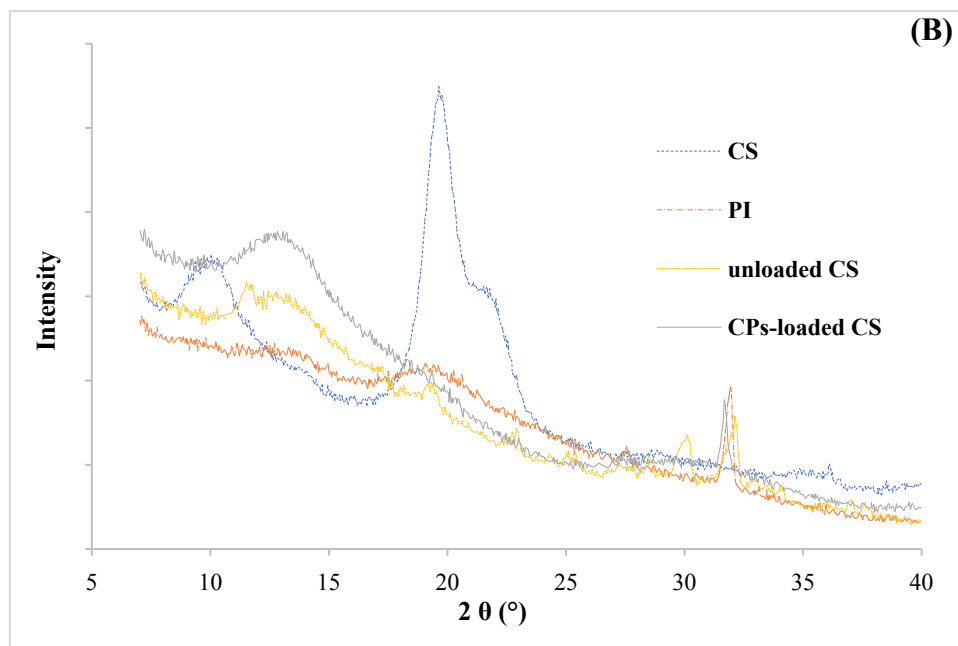
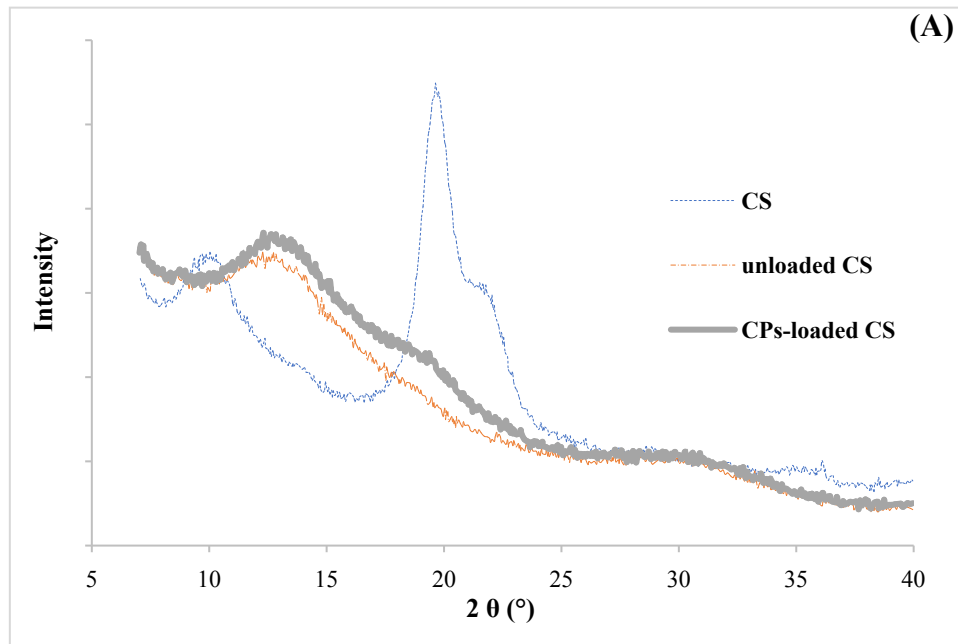
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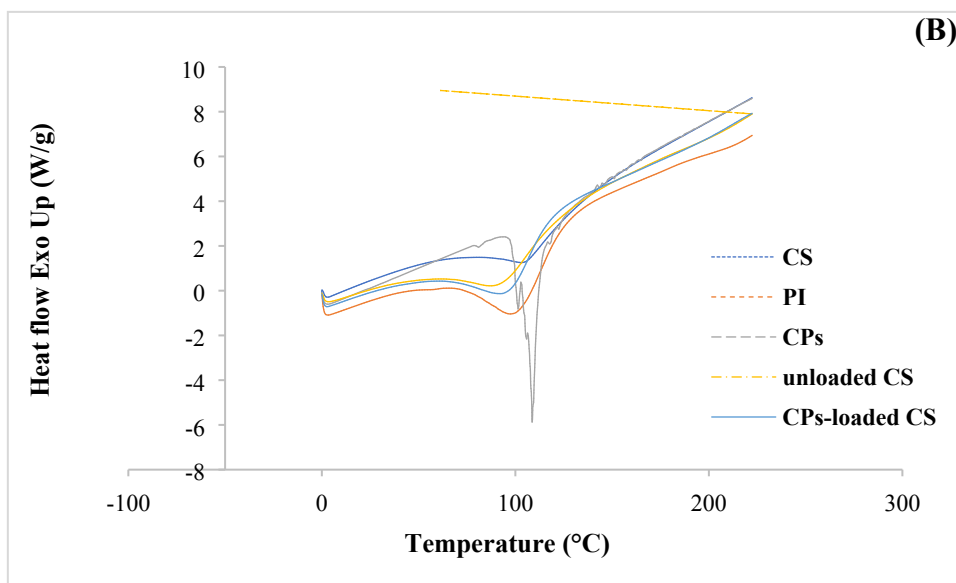
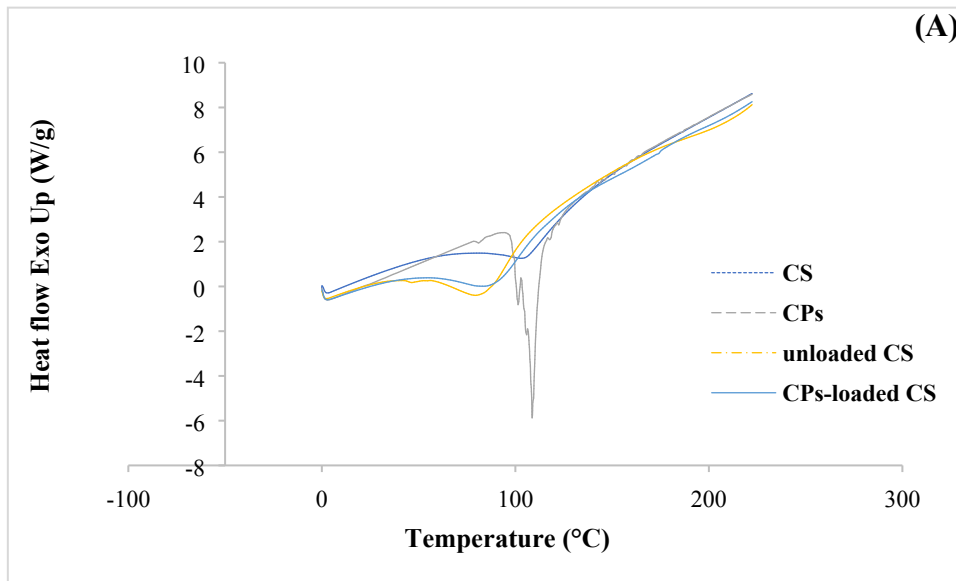
**Fig. 3**



**Fig. 4**



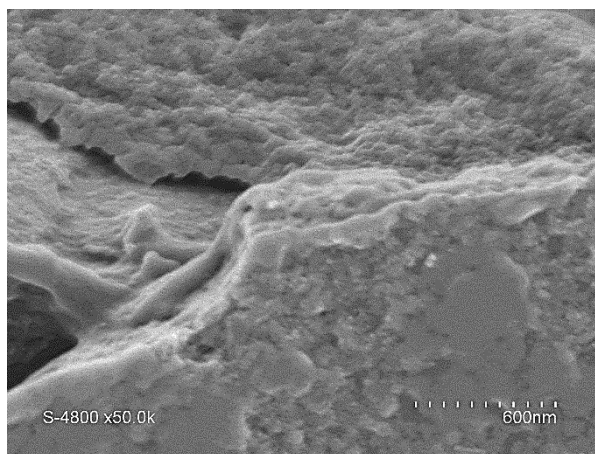
**Fig. 5**



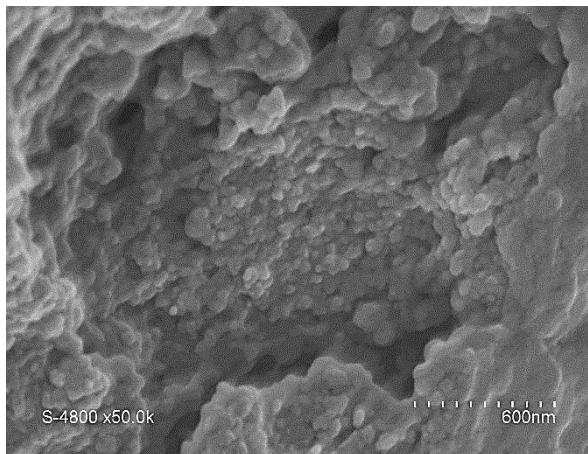
**Fig. 6**

**(a)**

**A**

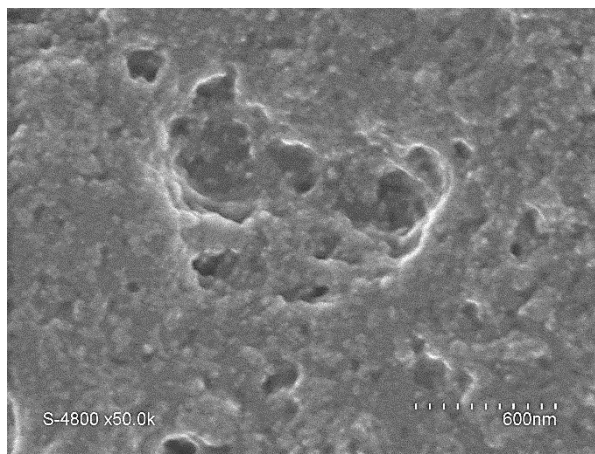


**B**



**(b)**

**A**



**B**

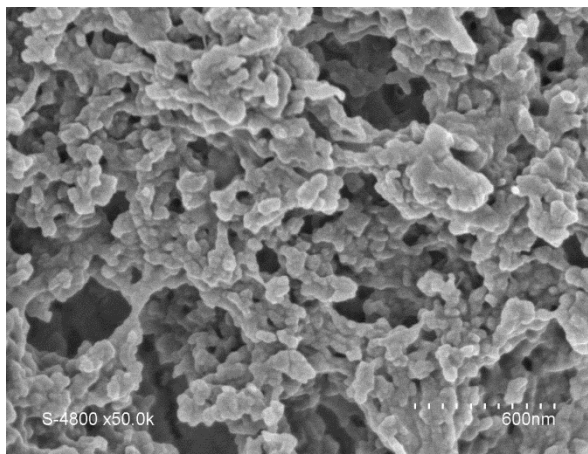
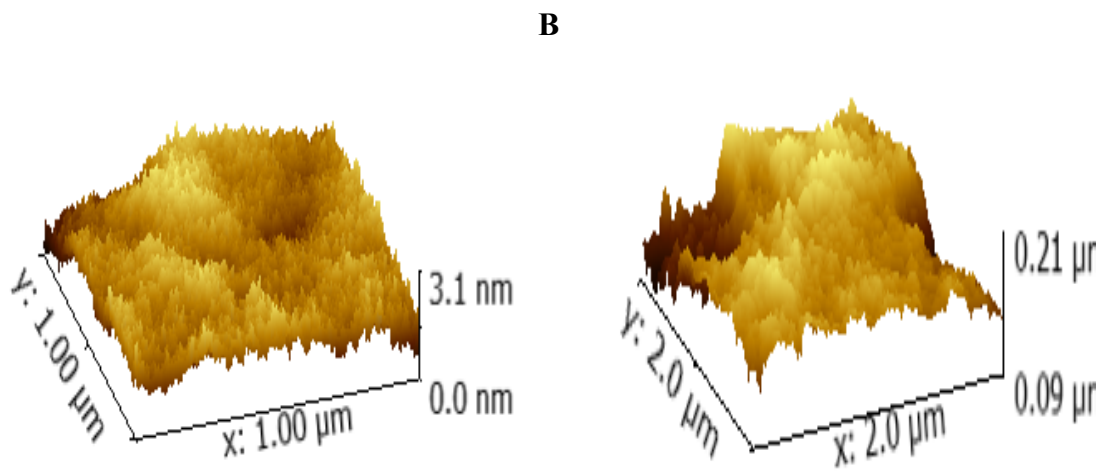
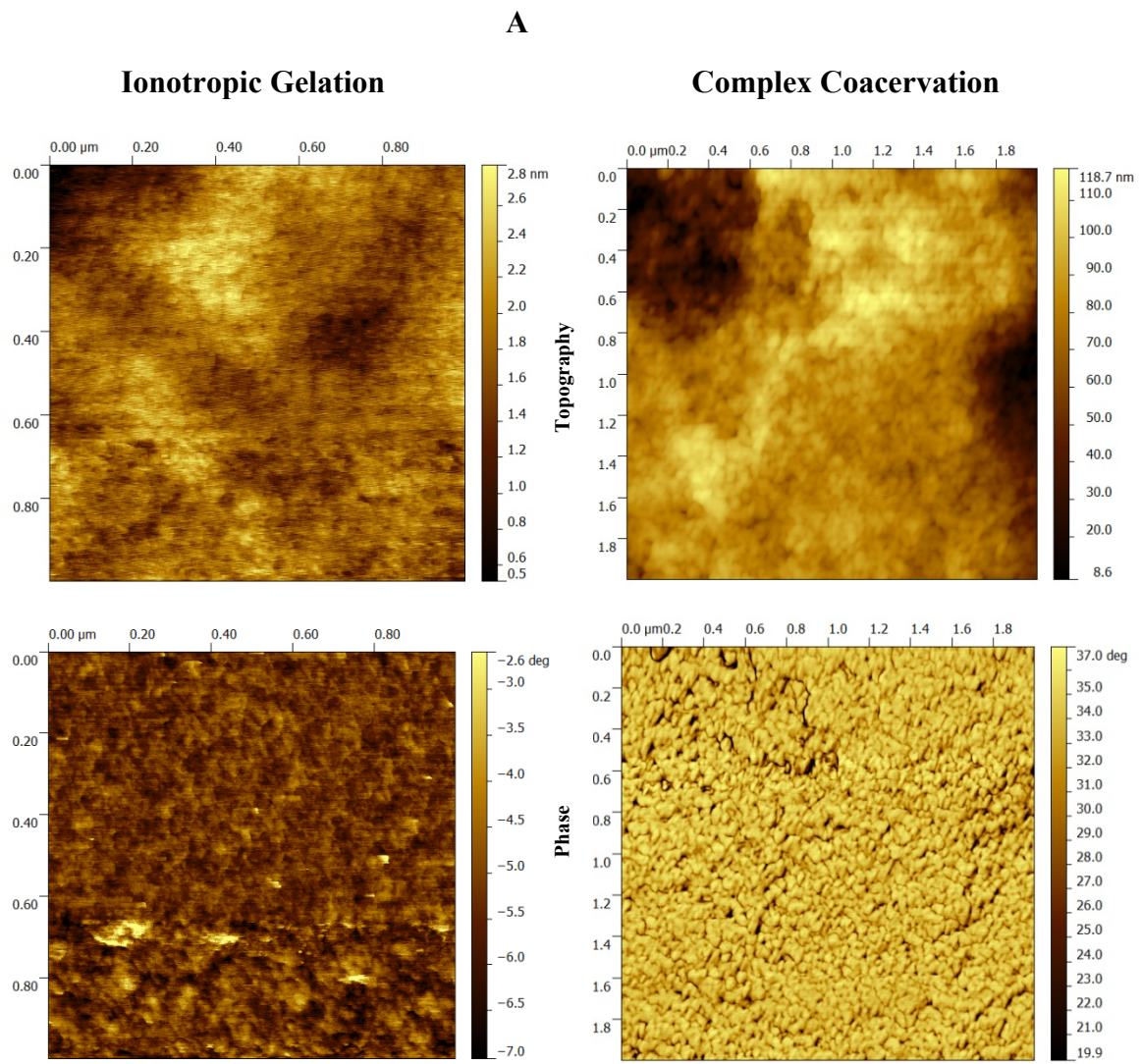


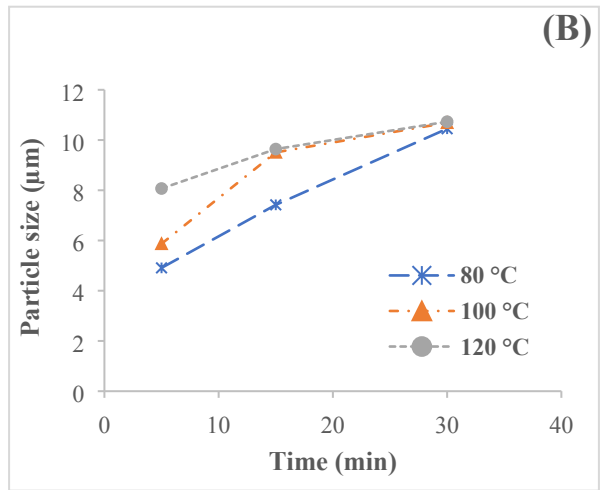
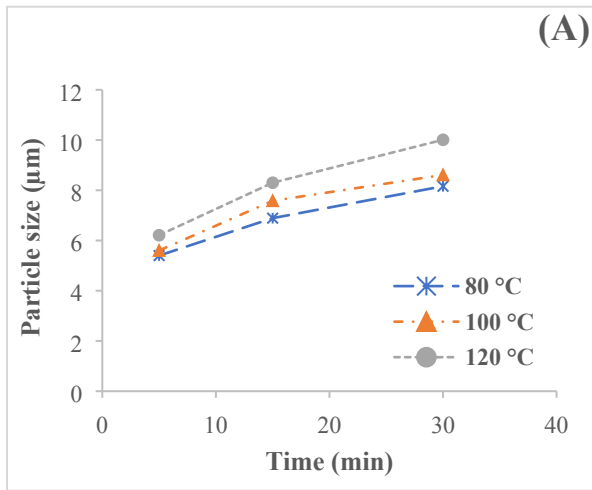


Fig. 7



**Fig. 8**

**(a)**



**(b)**

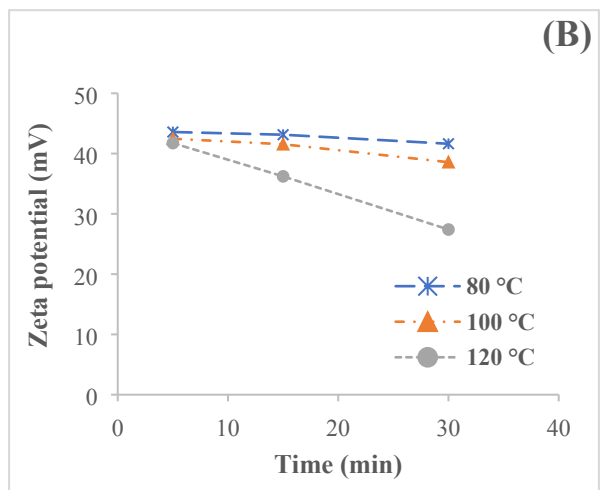
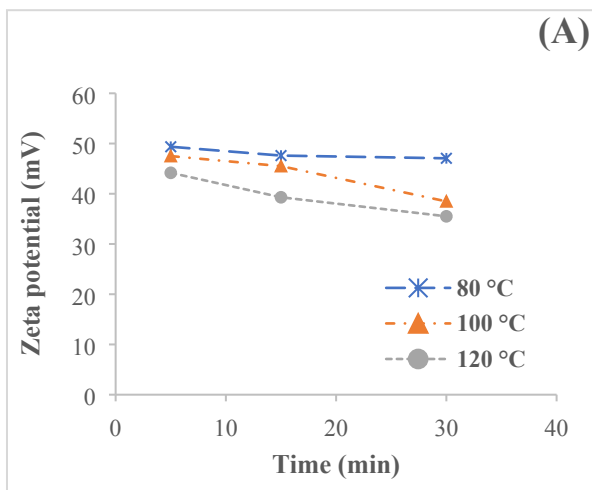
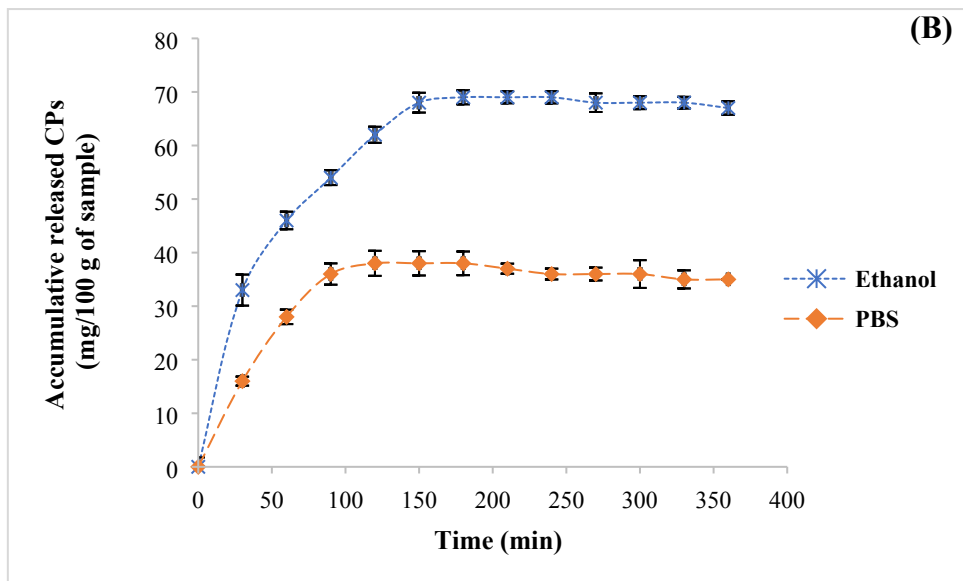
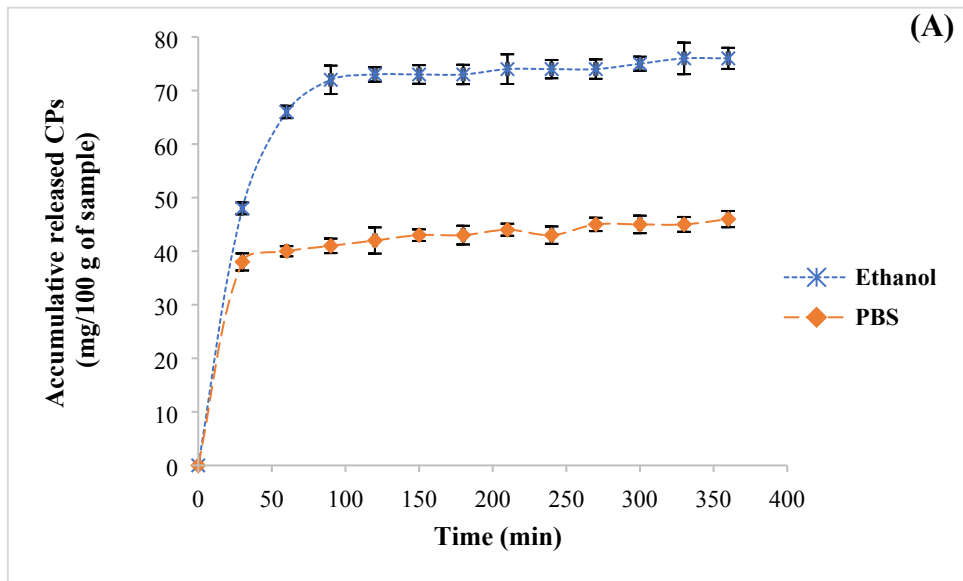
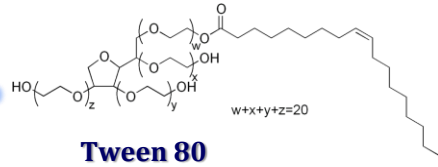


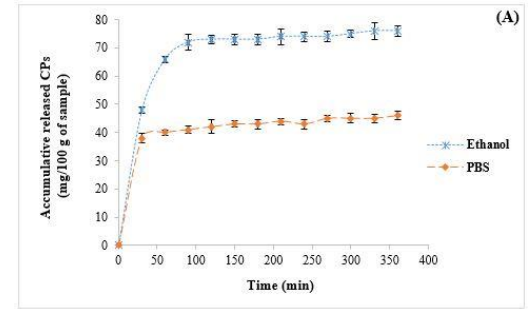
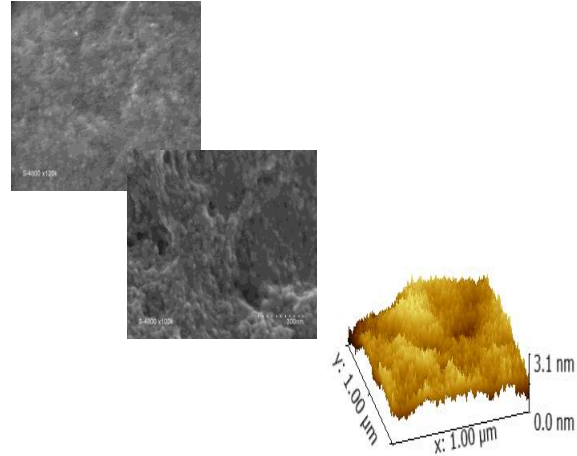
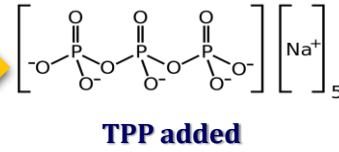
Fig. 9



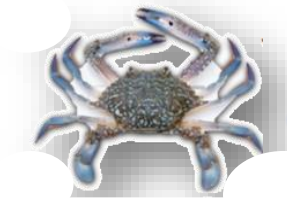
# Ionotropic Gelation



*Emulsification*



**In vitro release**



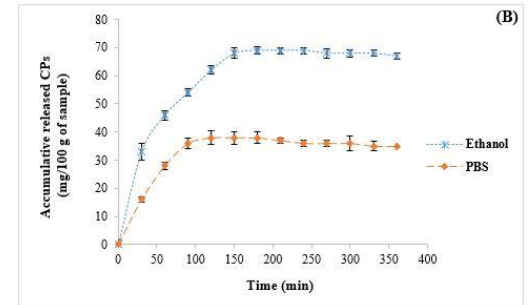
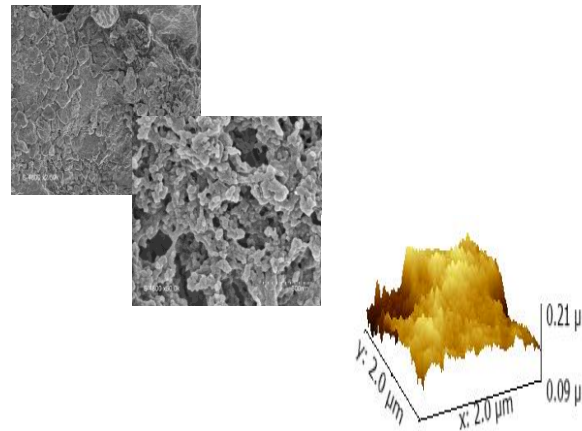
**Chitosan BCC**  
AD = 8%  
Mw = 115 000 g mol<sup>-1</sup>

**Carotenoproteins CPs**



**Proteins Isolate BCPI**

*pH 6.0*  
*BCC to BCPI of 1/8*



**In vitro release**

# Complex Coacervation

**Table 1:** Particle size ( $\mu\text{m}$ ), polydispersity index (%) and zeta potential (mV) of CS-based nanocapsules.

		Particle size ( $\mu\text{m}$ )	Polydispersity index (%)	Zeta potential (mV)
Iontropic gelation	unloaded CS	$7.26 \pm 0.01^{\text{aB}}$	42.90	$51.43 \pm 0.73^{\text{aA}}$
	CPs-loaded CS	$4.72 \pm 0.06^{\text{bB}}$	37.94	$45.30 \pm 1.38^{\text{bA}}$
Complex coacervation	unloaded CS	$9.76 \pm 0.09^{\text{aA}}$	62.37	$49.42 \pm 0.68^{\text{aB}}$
	CPs-loaded CS	$4.87 \pm 0.01^{\text{bA}}$	47.04	$46.80 \pm 0.24^{\text{bB}}$

**CPs:** carotenoproteins; **CS:** blue crab chitosan.

\* Results are the means of three determinations  $\pm$  standard deviation.

<sup>(a-b)</sup> Different letters in the same column are significantly different for the same process, as determined by ANOVA test ( $p < 0.05$ ).

<sup>(A-B)</sup> Different letters in the same column are significantly different between the ionotropic gelation and the coacervation complex approaches, as determined by ANOVA test ( $p < 0.05$ ).

**Table 2:** Encapsulation efficiency (%) and loading charge (%) of CPs into CS-based nanocapsules.

	EE (%) *	LC (%) **
Ionotropic gelation	88.54	46.43
Complex coacervation	73.93	31.87

**CPs:** carotenoproteins extract; **CS:** blue crab chitosan; **EE:** encapsulation efficiency; **LC:** loading charge.

\* The loading capacity (LC%) is defined as the quantity of loaded CPs per 100 g of nanoparticles.

\*\* The encapsulation efficiency (EE%) corresponded to the amount of loaded CPs based on the initial CPs (in feed).