

Journal Article

**Formation of chitosan nanoparticles to encapsulate krill oil (Euphausia superba) for application as a dietary supplement**

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1           **Formation of chitosan nanoparticles to encapsulate krill oil (*Euphausia***  
2                                   ***superba*) for application as a dietary supplement**

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28           **Formation of chitosan nanoparticles to encapsulate krill oil (*Euphausia***  
29                                   ***superba*) for application as a dietary supplement**

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32  
33           **Abstract:**

34 Encapsulation of krill oil (KO), a rich source of eicosapentanoic (EPA) and  
35 docosahexanoic acid (DHA) was carried out in chitosan-TPP (tripolyphosphate)  
36 nanoparticles using a newly developed two-step process (i.e, formation of emulsion and later  
37 electrostatic interaction of chitosan with TPP). The encapsulation of KO in chitosan  
38 nanoparticles (CSNPs) was confirmed by using Fourier transform infrared spectroscopy  
39 (FTIR), X-ray diffraction (XRD) and Thermo gravimetric analysis (TGA) techniques.  
40 Loading capacity (LC) and encapsulation efficiency (EE) of the obtained particles were about  
41 9 – 25 and 33 – 59 % respectively, when the initial KO content was in the ratio of 0.25 – 1.25  
42 g/g of Chitosan. Bulk KO showed less protection to oxidation and showed more formation of  
43 hydroperoxides during first week as noted by FTIR. However, KO loaded CSNPs showed  
44 better prevention of KO towards oxidation with less hydroperoxide formation even after two  
45 weeks of storage at elevated temperature (45 °C). The obtained KO-loaded CSNPs were  
46 irregular in shape with an average particle diameter of < 130 nm as observed by SEM. The  
47 results obtained confirmed the suitability of the emulsion and later electrostatic interaction of  
48 CS with TPP for the formation of KO loaded CSNPs with greater EE & LC, which will  
49 enhance their usage in the Food and Pharmaceutical industries.

50

51 **Key Words:** Krill Oil, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction  
52 (XRD), Thermo gravimetric analysis (TGA), Oxidative stability

### 53 **1. Introduction**

54 Antarctic Krill (*Euphausia superba*) has recently emerged as a potential and rich alternative  
55 source of long chain omega-3 polyunsaturated fatty acids (LC ω-3 PUFAs) besides the algal and  
56 fish oils to be substituted as a dietary supplements. Krill oil (KO) contains long chain omega-3  
57 polyunsaturated fatty acids (LC ω-3 PUFAs), namely eicosapentaenoic acid (EPA, 20:5) and  
58 docosahexaenoic acid (DHA, 22:6) (Grandois, Marchioni, Minjie Zhao, Ennahar, & Bindler,  
59 2009). The fatty acids in fish oil are stored as triglyceride, whereas in KO approximately 30 – 65

60 % of the fatty acids are predominantly incorporated into phospholipids (Schuchardt et al., 2011;  
61 Tou, Jaczynski, & Chen, 2008). The particular and unique amphiphilic structural arrangement of  
62 phospholipids provides KO with a much better bioavailability (Schuchardt et al., 2011).  
63 Moreover, KO contains naturally occurring powerful antioxidants mainly astaxanthin (Deutsch,  
64 2007; Tou et al., 2008). Various researchers recommend use of KO to prevent chronic disorders  
65 like cardiovascular diseases, endocannabinoids, poor infant development, non-alcoholic fatty  
66 liver disease, premenstrual syndrome, inflammation and certain cancers. This preventive effect  
67 was credited to the synergistic action between KO constituents LC  $\omega$ -3 PUFAs, phospholipids  
68 and astaxanthin (Deutsch, 2007; Sampalis et al., 2003; Tur, Bibiloni, Sureda, & Pons, 2012).  
69 However, its limited solubility in water and rapid instability to oxidation had made it difficult to  
70 achieve these benefits (Bustos, Romo, Yáñez, Díaz, & Romo, 2003).

71 To avoid limited solubility (Dispersibility in aqueous media), and the oxidative instability of  
72 lipophilic compounds like KO, various researchers encapsulate them in protein and carbohydrate  
73 based matrices (Ilyasoglu & El, 2014; Zimet & Livney, 2009). In addition to the above  
74 mentioned benefits, nanoencapsulation of lipophilic compounds also increased their  
75 bioavailability (Fathi, Mozafari, & Mohebbi, 2012). However, protein and polysaccharides that  
76 have been used widely to encapsulate lipophilic compounds play a key role in attaining the  
77 benefits (Chen & Subirade, 2005; Wang et al., 2006). For example, Majeed et al, prepared clove  
78 oil loaded nanoemulsions using modified starch and Tween 80 based surfactants and attained  
79 controlled release of oil from starch based nanoemulsions. However, Tween 80 adsorbed onto  
80 the droplet and failed to provide the desired release of oil (Majeed et al., 2016).

81 Chitosan (CS), a cationic polysaccharide that has been used widely for encapsulation and  
82 delivery of lipophilic compounds due to its biodegradability (enzymatically degraded  
83 (Lysozyme) into fragments suitable for renal clearance), biocompatibility, non-antigenicity and  
84 low toxicity (Malafaya, Silva, & Reis, 2007). Recently, researchers used a two-step emulsion and  
85 ionic-gelation method to produce CS-TPP nanoparticles due to its simplicity and non-toxicity for  
86 targeted delivery of bioactives (Malafaya et al., 2007; Yang et al., 2011). In the two step,  
87 emulsion ionic-gelation procedure the latter involves electrostatic interaction between cationic  
88 groups of CS and anionic groups of TPP (Calvo, Remuñán-López, Vila-Jato, & Alonso, 1997;  
89 Kawashima et al., 1985; Yang et al., 2011). The electrostatic interaction between cationic groups  
90 of CS and anionic groups of TPP occurred by inter and intramolecular bonds (Calvo et al., 1997;  
91 Kawashima et al., 1985; Yang et al., 2011). Ionic-gelation based CSNPs have been used widely  
92 for the encapsulation, and targeted delivery of proteins (Kawashima et al., 1985; Xu & Du,  
93 2003), essential oils (Hosseini, Zandi, Rezaei, & Farahmandghavi, 2013; Keawchaon &  
94 Yoksan, 2011), drugs (Wang et al., 2006; Wu, Yang, Wang, Hu, & Fu, 2005), vitamins and  
95 nutrients (Chen & Subirade, 2005; Yoksan, Jirawutthiwongchai, & Arpo, 2010). Keawchaon  
96 and Yoksan revealed successful encapsulation of carvacrol in CS-TPP particles with extended  
97 shelf life and well retained functional properties (Keawchaon & Yoksan, 2011). Similarly,  
98 Hosseini et al, prepared oregano oil loaded CS-TPP nanoparticles by an additional step of oil-in-  
99 water emulsification prior to solidification of these droplets by CS & TPP (Hosseini et al., 2013).  
100 They confirmed regularly distributed, spherical shaped particles having size 40 – 80 nm with  
101 slow release characteristics. They reported more than 80 % release of oregano oil that was  
102 attributed to greater surface volume ratio due to smaller particle size. On the other hand, nano-  
103 capsules due to larger size reduced the surface volume ratio and ultimately influence the access  
104 of digestive enzyme, dispersibility of their products and finally influenced the efficacy of  
105 delivery system (Kim, Diab, Joubert, Canilho, & Pasc, 2016; Majeed et al., 2016). However,

106 loading of KO having a distinctive chemical structure of LC  $\omega$ -3 PUFAs into CSNPs at a nano-  
107 level size has not been elucidated. Therefore, the current study focuses on the fabrication,  
108 characterization and oxidative stability of KO loaded in CSNPs by two step process: oil-in-water  
109 emulsification, and ionic gelation (CS & TPP).

## 110 **2 Materials and methods**

### 111 **2.1 Materials**

112 Antarctic krill oil contained ~40 % total phospholipid, ~28 – 30 % total omega-3 fatty acids  
113 and  $\leq$  200 mg/kg astaxanthin as stated by the manufacturer (Hutai Biopharm Inc. (Sichuan,  
114 China). Partially deacetylated chitosan (CS; degree of deacetylation of 91.5 %), with average  
115 molecular weight of 100 kDa derived from crab shells was obtained from Golden-Shell  
116 Biochemical Co., Ltd. (Hangzhou, China). CS is a weak polyelectrolyte with a pKa value around  
117 6.5, which is positively charged in acidic conditions (Fan, Yan, Xu, & Ni, 2012). Tween 80,  
118 glacial acetic acid, Sodium tripolyphosphate (TPP) and all other chemicals used were of  
119 analytical grade, purchased from Sinopharm Chemical Reagent Co., Ltd., China. Double distilled  
120 water was used throughout this study.

### 121 **2.2 Preparation of KO-loaded CSNPs**

122 KO-loaded CSNPs were prepared using a modified version of the method described by  
123 (Calvo et al., 1997) and (Hosseini et al., 2013). A schematic illustration representing the CSNPs  
124 preparation procedure is shown in **Fig. 1**. Briefly, CS solution 1.5 % (w/v) was prepared by  
125 agitating CS in an aqueous acetic acid solution 1 % (v/v) at ambient temperature (25 – 28 °C) for  
126 24 h. The CS solution was centrifuged at 8000 rpm for 20 min, the supernatant was filtered  
127 through a 0.8  $\mu$ m pore size syringe filter. Tween 80 (0.5g, hydrophilic-lipophilic balance = 15)  
128 was added as a surfactant to the CS solution (40 mL) and the mixture was stirred at 45 °C for 2 h  
129 to obtain a homogeneous solution. KO was gradually dropped into the aqueous CS solution (40  
130 mL) and the system was homogenized using an Ultra-Turrax (T25, Ika-Werke, Staufen,  
131 Germany) at a speed of 13,000 rpm for 1 min and 16,500 for 2 min. The solution was positioned  
132 in an ice-bath to prevent heating. The content of KO was varied (0, 0.15, 0.30, 0.45, 0.60 and  
133 0.75 g) to obtain different weight ratios of CS to KO (1:0, 1:0.25, 1:0.50, 1:0.75, 1:1.00 and  
134 1:1.25 respectively). Subsequently, TPP solution (0.5 % v/v, 40mL) was then added drop wise  
135 into the o/w emulsion under continuous stirring and was agitated for 40 min. The particles  
136 formed were collected by centrifugation at 10,000  $\times$  g for 30 min at 20 °C and washed several  
137 times with water to remove or wash off excessive KO. Eventually, the wet particles were  
138 dispersed in 25 mL water by ultrasonication to produce a homogeneous suspension.  
139 Ultrasonication was performed using a (Jy98-IIIDN, 20 kHz, Ningbi Scientz Biotechnology Co.,  
140 Ltd., Ningbo, China) sonicator for 2 min in an ice bath. The suspensions were immediately  
141 freeze-dried at – 35 °C for 72 h and were stored in dry conditions at 25 °C.

### 142 **2.3 Characterization of KO-loaded CSNPs**

#### 143 **2.3.1 Z-average diameter and $\zeta$ -potential measurements**

144 The z-average diameter and the uniformity of particles in dispersion (particle size  
145 distribution) that is being measured as polydispersity index (PDI) for KO-loaded CSNPs were  
146 investigated by dynamic light scattering (DLS) using the Zetasizer Nano ZS<sup>®</sup> (Malvern

147 Instruments, Worcestershire, U.K.). To avoid multiple scattering effects, the nanoparticles were  
148 diluted 100-fold with purified water, placed in a cuvette and agitated well prior to measurements.  
149 Refractive indices of 1.45 for KO and 1.330 for water were used.  $\zeta$ -potential was determined by  
150 Laser Doppler Velocimetry using the Zetasizer Nano ZS<sup>®</sup> at a scattering angle of 173° at 25 °C.  
151 The diluted nanoparticles were placed in a folded capillary electrophoresis cell with count rate  
152 between 100 and 300 Kcps as described by Zainol et al. (Zainol et al., 2012). All the tests were  
153 performed in triplicate.

### 154 **2.3.2 Morphology of KO-loaded CSNPs**

155 The morphological characterization of the nanoparticles was done using SEM (Hitachi S-  
156 4800, Japan) at an accelerating voltage 2 kV. The powders were sprinkled onto double-backed  
157 cellophane tape attached to an aluminium stub before coating with gold-palladium in an argon  
158 atmosphere.

### 159 **2.3.3 Characterization using FTIR, TGA and XRD**

160 The infrared spectra of all samples were obtained using a Thermo Fisher Scientific Inc.,  
161 Nicolet iS10, FTIR spectrometer with KBr accessory. This instrument was operated with Nicolet  
162 OMNIC software (Version 8.2). For KO spectral acquisition, the liquid sample ( $\approx 2 \mu\text{L}$ ) was  
163 deposited on a KBr disk. The spectra were obtained using 16 scans at a resolution of  $4 \text{ cm}^{-1}$  over  
164 the frequency range of  $4000 - 400 \text{ cm}^{-1}$ . Before running each sample a background spectrum was  
165 obtained in air.

166 Contact angle was used to determine the interaction between KO and nanoparticles with sessile  
167 drop method. Briefly, KO ( $3 \mu\text{L}$ ) was carefully dropped with a dosing rate of  $0.5 \mu\text{L/s}$  onto the  
168 slides ( $20 \text{ mm} \times 50 \text{ mm} \times 1 \text{ mm}$ ) using  $2 \text{ mL}$  micrometer syringe (KDL Corp., Shanghai, China).  
169 The measurements were carried out in open air with relative humidity (30%) and at a room  
170 temperature of 25 °C. Both left and right contact angles expressed in degrees were automatically  
171 calculated from the digitalized image software belonging to the equipment (DataPhysics  
172 Instruments GmbH, OCA15EC, Germany). Measurements were taken in triplicate of each  
173 sample.

174 TGA was performed using a TGA/DSC 1 STARe (Mettler-Toledo, Switzerland) 25 – 600 °C  
175 with a heating rate of  $10 \text{ }^\circ\text{C/min}$  under nitrogen atmosphere. Each freeze-dried sample 6 – 10 mg  
176 was placed in the TGA furnace. The derivative thermogravimetric curves (DTG) and the first  
177 derivative of TG curves were calculated.

178 XRD patterns of packing materials were assessed by X-ray diffraction using a (Bruker AXS  
179 D8, Germany) diffractometer. The operation conditions were 40 kV and 40 mA with Cu K $\alpha$   
180 radiaton ( $\lambda = 1.5406 \text{ \AA}$ ). Samples were scanned in the  $2\theta$  range of  $5^\circ - 50^\circ$  at a speed of  $0.03^\circ$  per  
181 second.

### 182 **2.4 Determination of loading capacity (LC) and encapsulation efficiency (EE)**

183 The content of KO-loaded CSNPs was determined by TGA/DTG. Freeze dried CSNPs and  
184 KO-loaded CSNPs were placed in TGA furnace at 25 – 600 °C with a heating rate of  $10 \text{ }^\circ\text{C/min}$   
185 under nitrogen atmosphere and the weight loss percentage, obtained from TGA thermograms  
186 was used to determine the content of KO-loaded CSNPs. The loading capacity of KO (g/100g of  
187 sample) and encapsulation efficiency of KO (g/100g of sample) were thus calculated from eqs.  
188 (1) and (2) respectively.(Yoksan et al., 2010)

189 
$$LC (\%) = \frac{\text{weight of loaded KO}}{\text{Weight of sample}} \times 100 \quad (1)$$

190 
$$EE (\%) = \frac{\text{weight of loaded KO}}{\text{Weight of initial KO}} \times 100 \quad (2)$$

## 191 **2.5 Storage conditions**

192 For the lipid oxidation experiments, five grams of bulk oil and freeze-dried KO-loaded  
193 CSNPs were placed in 20 ml loosely capped amber glass bottles. Samples were stored at 45 °C  
194 for 4 weeks. The extent of lipid oxidation was investigated in terms of lipid hydroperoxide. All  
195 the experiments were carried out in duplicate.

### 196 **2.5.1 Determination of lipid oxidation**

197 In this study, lipid hydroperoxides, the primary oxidation products was monitored by FTIR  
198 (Guillén & Cabo, 1999, 2002). Each band frequency was obtained automatically from the  
199 instrument software command “find peaks” with an adequate threshold value near 85 %. The  
200 functional group vibration mode of each band was made by comparison with software spectral  
201 library as well as with literature data and similar experimental conditions of FTIR was applied  
202 for sample acquisition as utilised to confirm the loading of KO in CSNPs (See section 2.3.3).

## 203 **3 Results and discussion**

### 204 **3.1 Shape, size and surface charge of KO-loaded CSNPs**

205 KO-loaded CSNPs were prepared through the formation of oil droplets (including KO) and  
206 droplet solidification. The KO droplet formation in CS solution was achieved using the O/W  
207 emulsion technique. The solidification of each droplet was extended by ionic cross-linking of  
208 ammonium groups of CS molecules surrounding the KO droplet and phosphate groups of TPP.

209 The surface morphology of CSNPs and KO-loaded CSNPs were observed by SEM. **Fig. 2**  
210 **(a, b)** shows the CSNPs size varied between 100 – 300 nm that correlates with the findings of  
211 Yoskan (Yoksan et al., 2010). For KO-loaded CSNPs, the aggregations were also seemed that  
212 might be due to remaining KO around the particles with an average range of 80 – 130 nm (**Fig.**  
213 **2-c, d**).

214 The z-average diameter and PDI of CSNPs and KO-loaded CSNPs were examined by  
215 dynamic light scattering (DLS). **Fig. 3** shows that the z-average diameter and PDI of CS particles  
216 were about ~252 nm and 0.199, respectively. The z-average diameter of KO-loaded CSNPs were  
217 in the range of 229.5 – 182.4 nm. With increasing ratio of KO, the z-average diameter decreased  
218 (**Table 1**). The possible reason behind this reduction in particle size might be the coemulsifying  
219 properties of the oil constituents in the presence of surfactant that reduces the interfacial tension  
220 as various researchers reported this phenomenon for essential oil loaded nanoemulsions (Majeed,  
221 Antoniou, & Fang, 2014; Terjung, Löffler, Gibis, Hinrichs, & Weiss, 2012). However, the  
222 agglomeration and/or swelling of KO-loaded CSNPs in water were lower than those of CS  
223 particles. The obvious difference in the agglomeration of two nanoparticulate systems is the  
224 formation mechanism. The CS particles are formed by the electrostatic interaction of CS and  
225 TPP and their size will depend on how the molecules were mixed together. On the other hand,  
226 KO-loaded CSNPs are formed by the adsorption of CS onto the KO droplets. The lower  
227 agglomeration in KO-loaded CSNPs might be due to hydrophobic KO molecules that forced it to

228 entrap inside (Keawchaoon & Yoksan, 2011; Yoksan et al., 2010). The interesting fact about KO  
229 is that it possesses a large proportion of marine phospholipids (about 40 %) bonding with LC  $\omega$ -3  
230 PUFAs like EPA and DHA (Zhu, Zhuang, Luan, Sun, & Cao, 2015). Similarly, Shen and Lu et  
231 al. reported small z-average diameter of nanoparticles that can be credited to phospholipids in  
232 KO, having substantial inherent emulsifying power (Lu, Nielsen, Baron, Jensen, & Jacobsen,  
233 2012; Shen, Bhail, Sanguansri, & Augustin, 2014).

234 In addition,  $\zeta$ -potential of CSNPs gave a positive charge of + 37.7 mV as shown in **Fig. 3**.  
235 The positive surface charge arises due to ammonium groups of CS. With loading of KO, the  $\zeta$ -  
236 potential was decreased to + 26.6 mV. This reflects the CSNPs surface with increasing KO  
237 content. The reduction in  $\zeta$ -potential value was related to the number of TPP to CS charge groups  
238 as evident by the findings of (Antoniou et al., 2015). However, this reduction might be due to  
239 shielding effect of protonated  $-\text{NH}_2$  group by KO on CSNPs. Several studies have reported that  
240  $\zeta$ -potential values of CSNPs was reduced when drugs, i.e., ascorbic acid (Jang & Lee, 2008) and  
241 eugenol were (Woranuch & Yoksan, 2013) incorporated. This demonstrated that  $\zeta$ -potential  
242 value influenced reciprocally with increased drug content.

### 243 **3.2 Characterization of KO-loaded CSNPs**

244 CSNPs loaded with KO were characterized by Fourier Transform Infrared spectroscopy  
245 (FTIR). The results confirmed the presence of KO with characteristic peaks at  $3416\text{ cm}^{-1}$  (OH),  
246  $3012\text{ cm}^{-1}$  ( $=\text{C}-\text{H}$  stretching),  $3000 - 2800\text{ cm}^{-1}$  (C-H stretching),  $1740\text{ cm}^{-1}$  (C=O stretching  
247 band),  $1465\text{ cm}^{-1}$  ( $-\text{CH}_2-$  bending),  $1379\text{ cm}^{-1}$  ( $-\text{CH}_3$  bending),  $1091\text{ cm}^{-1}$  (CO stretching),  $971$   
248  $\text{cm}^{-1}$  (C=C stretching band) as shown in **Fig. 4a**.

249 However, CSNPs showed characteristics bands at  $3450\text{ cm}^{-1}$  (OH),  $2927\text{ cm}^{-1}$  (CH  
250 stretching),  $1640\text{ cm}^{-1}$  (amide I),  $1543\text{ cm}^{-1}$  (amide II),  $1155\text{ cm}^{-1}$  (P=O),  $1097\text{ cm}^{-1}$  (COC) and  
251  $891\text{ cm}^{-1}$  (pyranose ring) that suggests the complex formation between CS and TPP as a result of  
252 electrostatic interaction **Fig. 4b** (Bhumkar & Pokharkar, 2006; Xu & Du, 2003). Moreover, FTIR  
253 confirmed the incorporation of KO in CSNPS (**Fig. 4 c-g**) by comparing with characteristic  
254 peaks in the KO spectra. The occurrence of characteristic peak at same wave number in KO  
255 loaded CSNPs indicating no interaction with chitosan. Similarly, non-interaction behaviour of  
256 chitosan (hydrophilic) with oregano oil (hydrophobic) has earlier been reported by Hosseini et al  
257 when incorporated in CS-TTP nanoparticles (Hosseini et al., 2013). Further, this interaction was  
258 investigated using contact angle measurement and also showed no interaction between KO and  
259 CSNPs as shown in Figure **Fig. 5**. The contact angle of KO and CSNPs with air was 38.35 and  
260 25.84, respectively as shown in **Fig. 5a & b**. However, in case of increasing ratios of KO in  
261 loaded CSNPs the contact angle increased (26.28 – 36.46) that suggests increased  
262 hydrophobicity of KO (**Fig. 5 c-g**). On the other hand, with maximum KO loaded CSNPs (1:1 &  
263 1: 1.25) showed significant increase in contact angle (10 degree rise). Whereas, at lower ratios  
264 (1: 0.25 – 1: 0.75) of KO loaded CSNPs the contact angle was quite same (26.28 & 31.21) as  
265 appeared in KO with unloaded CSNPs (Fig. c,d). The possible reason behind this increase in  
266 contact angle at highest CS:KO mass ratios is the exposure of excessive oil to standard drop of  
267 KO (3 ul) used during this experimental procedure that resulted in increased hydrophobicity.  
268 These findings revealed that CS and CSNPs showed no interaction with KO. Contact angle  
269 measurement has already been used by variety of researchers to explain the interaction behaviour  
270 of hydrophobic and hydrophilic compounds (Liu et al., 2016; Shamsijazeyi et al., 2014).

271 On the other hand, the increase in CH stretching peak intensity at  $2869 - 2974\text{ cm}^{-1}$  reflects  
272 the location of KO in the CS matrix. These results were further strengthened as the increase in  
273 CH stretching peak intensity was observed with increasing KO content. Therefore, we can



274 consider CH stretching as a strong indicator of KO encapsulation in any matrix (Vongsvivut et  
275 al., 2012; Zhao, Wei, Liu, & Liu, 2014). Thus, emulsion and later electrostatic interaction of CS  
276 with TPP, a two-step process successfully encapsulated KO in CSNPs. (Section 3.3)

277 TGA has been used widely by a variety of researchers to confirm the weight change of  
278 material that is monitored as a function of temperature to evaluate its thermal stability (Yoksan et  
279 al., 2010). In our case, the degree of weight loss for CS alone and KO loaded CSNPs decreased  
280 with increasing temperature from 25 to 600 °C as shown in **Fig. 6A**. KO degradation showed one  
281 level of weight loss **Fig. 6A (a)**. Whereas, CS and KO loaded CSNPs showed two (**Fig. 6A-b**)  
282 and three levels of weight loss **Fig. 6A (c-g)**. Nam et al. reported the first and second level of  
283 weight loss for CS nanofibers that showed temperature ranges from 56 to 115 °C and 182 – 310  
284 °C, which corresponded to evaporation of moisture and decomposition of polymer, respectively  
285 (Nam, Park, Ihm, & Hudson, 2010). The rate of maximum weight loss corresponding to  
286 temperature was determined as the decomposition temperature ( $T_d$ ), which is clearly observed as  
287 a peak in the derivative thermogravimetry (DTG) thermogram, plotted in **Fig. 6B**. From the DTG  
288 thermogram, CSNPs exhibited one level  $T_d$  at 247 °C (**Fig. 6B-b**). By comparison between CS  
289 and KO-loaded CSNPs manifested new  $T_d$  range 327 – 331 °C (**Fig. 6B (c-g)**), which  
290 corresponded to the  $T_d$  of KO (**Fig. 6B-a**). The results confirmed the successful loading of KO  
291 into CSNPs. Similarly, Yoksan et al. reported increased thermal stability of successfully  
292 encapsulated ascorbyl palmitate in CSNPs (Yoksan et al., 2010). The weight loss percentage at  
293 this temperature range was thus used to compute the quantity of loaded KO (section 3.3)

294 XRD patterns of CS powder, CSNPs, and KO-loaded CSNPs are presented in **Fig. 7**.  
295 Generally, CS exhibits two peaks at  $2\theta$  of 10° and 20° (**Fig. 7a**), showing high degree of  
296 crystallinity. After electrostatic interaction with TPP, peak broadening and peak shifts were  
297 observed with reduction of peak intensity (**Fig. 7b**). In addition, a new peak is found in the  
298 diffractogram of CSNPs at  $2\theta$  of 23°. These distinct differences reflect the modification in the  
299 arrangement of molecules in the crystal lattice stimulated by ionic interaction (Bhumkar &  
300 Pokharkar, 2006; Yoksan et al., 2010). As compared with CSNPs, in the diffraction spectrum of  
301 KO-loaded CSNPs the characteristic peaks at  $2\theta$  of 18° confirmed the presence of KO within  
302 CSNPs. Thus, XRD analysis revealed the successful encapsulation of KO in CSNPs as it clearly  
303 showed change in the CS-TPP packing structure. So, on behalf of FTIR, TGA, and XRD we can  
304 conclude that two steps, emulsion and electrostatic interaction between CS and TPP is suitable  
305 for the encapsulation of KO in CSNPs.

### 306 **3.3 Encapsulation efficiency and loading capacity**

307 The TGA/DTG technique was applied for quantitative analysis of CSNPs in terms of weight  
308 loss at temperature ranging from 290 – 380 °C, corresponding to  $T_d$  of KO. The percentage of LC  
309 and EE of KO-loaded CSNPs were then calculated using **Eqs. (1)** and **(2)**, respectively, and are  
310 tabulated in **Table 1**. From TGA results, the percentage of LC was in the range of 8.8 to 24.7 %  
311 at 25 to 125 % (w/w) ratio of KO to CS (**Table 1**). LC percentage was dependent on initial KO  
312 content that was in agreement to the findings of other researchers who reported carvacrol or  
313 BSA loading in CSNPs was initial concentration dependent (Keawchaon & Yoksan, 2011; Xu  
314 & Du, 2003). EE of KO ranged from 33.3 to 58.9 %. Maximum EE value (58.9 %) was achieved  
315 at 1:0.25 (w/w) CS to KO ratio. However, with the increase of KO ratio, EE started to decrease  
316 as shown in **Table 1**. This might be due to saturation of CSNPs with KO (Hosseini et al., 2013;  
317 Yoksan et al., 2010), as it possesses a large proportion of marine phospholipids bonded with LC-  
318 PUFA and astaxanthin. No doubt, large proportion of phospholipids (about 50 %) in KO bounds  
319 with DHA, EPA and astaxanthin, which enhanced the solubility of these constituents in lipid

320 phase that consequently reduced its diffusion outside the nanoparticles (Zhu et al., 2015). The  
321 reduction in EE with increasing KO content suggests its loading in CSNPs is limited.

322 In addition to EE, LC was determined by FTIR using the CH stretching peak to determine  
323 the content of KO in CSNPs. The CH stretching peak at  $2925\text{ cm}^{-1}$  and the pyranose peak at  $891\text{ cm}^{-1}$   
324  $\text{cm}^{-1}$  were used as representative peaks of KO and CS, respectively. The CH stretching to  
325 pyranose peak ( $I_{2925}/I_{891}$ ) is shown in **Table 1**. CSNPs showed an  $I_{2925}/I_{891}$  value of 0.91 and for  
326 KO-loaded CSNPs, the value of  $I_{2925}/I_{891}$  was greater than 0.91 suggesting successful loading of  
327 KO in the nanoparticles. KO-loaded CSNPs with initial KO content (0.25 – 1.25 g/g) of CS  
328 showed  $I_{2925}/I_{891}$  values in the range of 1.14 – 1.81. The value of  $I_{2925}/I_{891}$  increased with  
329 increasing initial KO content. However, KO-loaded CSNPs with an initial KO content of 1 g/g of  
330 CS provided the maximum value of  $I_{2925}/I_{891}$  as shown in **Table 1**. These results confirmed the  
331 findings of TGA and we can conclude that the optimal weight ratio of CS to KO was 1:1.

### 332 **3.4 Oxidative stability**

333 The oxidative stability of bulk KO and KO containing CSNPs was evaluated using FTIR  
334 spectra that were determined after exposure with elevated oxidative stress ( $45\text{ }^{\circ}\text{C}$ ). FTIR  
335 spectroscopy has been used earlier to identify change in the functional groups of the sample that  
336 undergoes lipid oxidation (Voort, Ismail, Sedman, & Emo, 1994). **Fig. 8** illustrates obvious  
337 spectral changes in krill oil spectra as oxidation proceeds. However, peak shift in the ROOH  
338 region from  $\sim 3416\text{ cm}^{-1}$  to  $\sim 3377$  suggests the formation of hydroperoxides (**Fig. 8-A**). Whereas  
339 change in CO (initial absorption at  $\sim 1091$  &  $\sim 1077\text{ cm}^{-1}$  and gradual shifting to  $\sim 1093$  &  $\sim 1065$   
340  $\text{cm}^{-1}$  respectively) and *trans* region confirmed the formation of conjugated *trans* species ( $\sim 971$   
341  $\text{cm}^{-1}$ ) along with isolated *trans* absorptions ( $\sim 969\text{ cm}^{-1}$ ) as presented in (**Fig. 8-B**). In the case of  
342 KO containing CSNPs the ROOH peak shift varied with CS-KO weight ratios. For 1:1, it was  
343 moved to  $\sim 3431$  to  $\sim 3404\text{ cm}^{-1}$  and  $\sim 3424$  to  $\sim 3389\text{ cm}^{-1}$  at 1:1.25 ratio (**Fig. 8-C**). On the other  
344 hand, the triglyceride ester group peak shifts showed less dependency to CS-KO weight ratios  
345 and it was from  $\sim 1741$  to  $\sim 1739\text{ cm}^{-1}$  at 1:1 & 1:1.25 CS-KO weight ratios as shown in **Fig. 8-D**.

346 The occurrence of a larger shift  $\sim 39\text{ cm}^{-1}$  ( $\sim 3416 - \sim 3377\text{ cm}^{-1}$ ) in the ROOH band under  
347 oxidative stress as shown in **Fig. 8** has already been confirmed (Voort et al., 1994). Moreover,  
348 there was a shift back to higher wavenumbers ( $\sim 3425$ ) that might be due to breakdown of  
349 hydroperoxides to alcohols as evident by the findings of Gullién & Cabo and Voort et al.  
350 (Guillén & Cabo, 1999; Voort et al., 1994). In contrast a band shift of  $\sim 12\text{ cm}^{-1}$  of CO groups in  
351 the esters and only a slight ( $1 - 2\text{ cm}^{-1}$ ) shift in *cis*, conjugated *trans*, and isolated *trans* bands  
352 occurred. The KO showed an obvious decrease in the ROOH band and triglyceride ester groups  
353 during the first week of storage. KO-loaded CSNPs showed a modest decrease in band shifts  
354 even after two weeks of storage that suggests more oxidation prevention of KO in CSNPs.

355 The oxidation prevention of KO in KO-loaded CSNPs in terms of little change in band shift  
356 of ROOH and triglyceride ester groups under oxidative stress showed less availability of  
357 hydroperoxides to convert them back to aldehydes and ketones (Guillén & Cabo, 2002). Similarly  
358 Gullién and Cabo also reported a shift of the ROOH band towards lower wavenumbers as oils  
359 underwent oxidation, but to a somewhat lesser extent (Guillén & Cabo, 1999). Thus, it may be  
360 postulated that the ROOH band shift is due to extensive intermolecular hydrogen bonding of  
361 hydroperoxides (Russin, van de Voort, & Sedman, 2003).

### 362 **4 Conclusion**

363 The KO loaded CSNPs were prepared by a two-step, emulsion and later electrostatic  
364 interaction of CS with TPP showed average diameter of 80 – 130 nm as observed by SEM. The

365 loading capacity (LC) and encapsulation efficiency (EE) of KO in nanoparticles was about 8.8 to  
366 24.7 % and 33.3 to 58.9 %, respectively, when the ratio of KO to CS was 25 – 125 %. Moreover,  
367 the loading of KO into CSNPs was confirmed by the increment of CH stretching peak intensity  
368 at 2869 – 2974  $\text{cm}^{-1}$  (FTIR technique), a degradation temperature of 327 – 331 °C (TGA/DTG  
369 techniques), and the characteristic peaks at  $2\theta$  of 18° (XRD technique). Further, CSNPs were  
370 successful in preventing the oxidation of KO. The results confirmed the suitability of the  
371 emulsion and electrostatic interaction based method for the formation of KO loaded CSNPs with  
372 greater EE & LC that will enhance their usage in food and pharmaceutical industry. But, prior to  
373 their industrial usage further research is needed on the sensory perception, bioavailability and  
374 protection of encapsulate deterioration during product shelf life.

### 375 **Acknowledgement**

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506 **Highlights:**

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- KO loaded CSNPs were prepared using emulsion-electrostatic interaction method.

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- KO loaded CSNPs were irregular in shape with average diameter of < 130 nm.

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- CSNPs successfully entrap KO as evident by FTIR.

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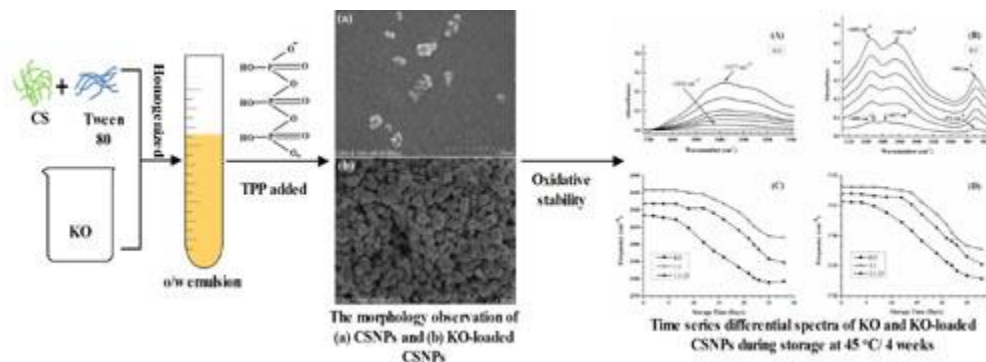
- KO loaded CSNPs prevented formation of hydroperoxides at elevated temperature.

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514 **Graphical Abstract**

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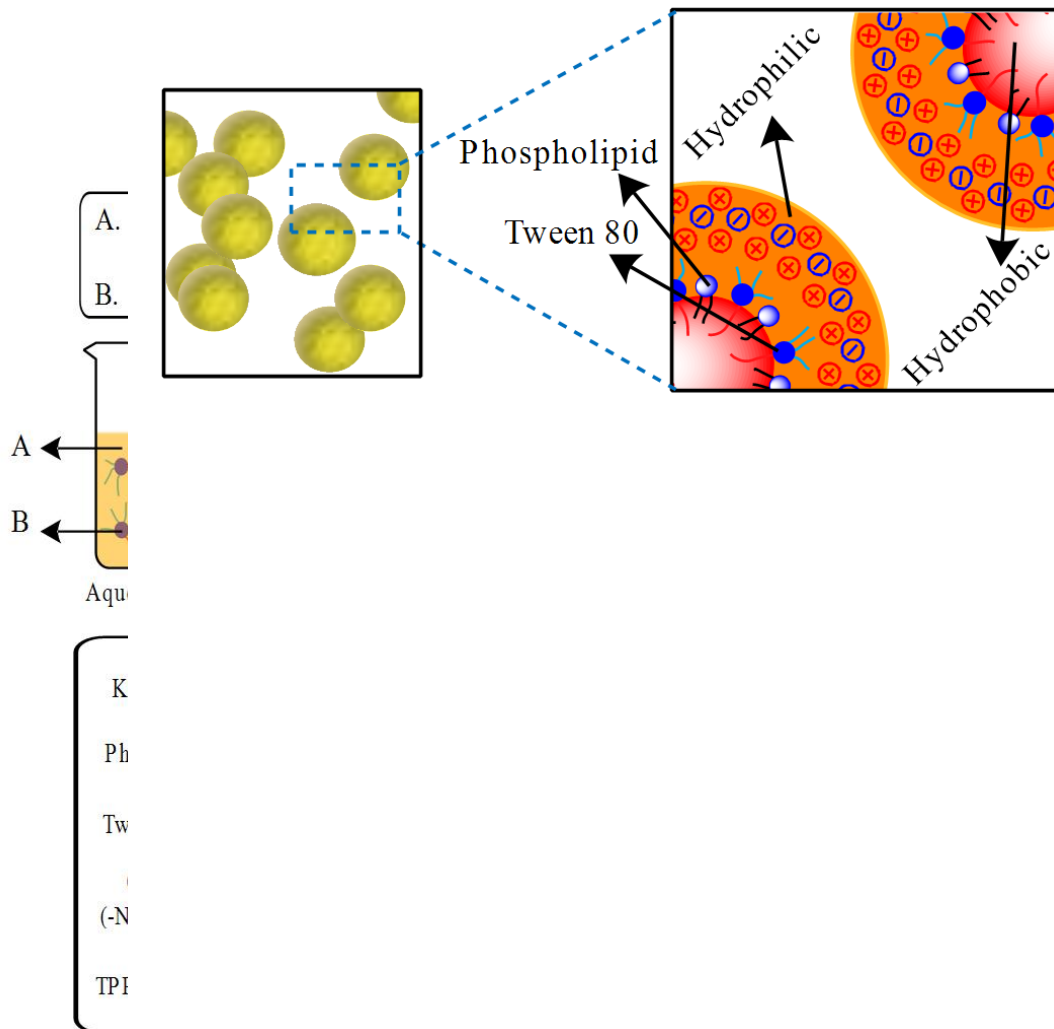
519 **Table 1.** Loading capacity (LC) and Encapsulation Efficiency (EE) of KO determined by TGA  
 520 technique, intensity ration of  $I_{2925/890}$  determined by FTIR technique, and z-average diameter and  
 521  $\zeta$ -potential value of CS and KO-loaded CSNPs.

| CS : KO<br>(w/w) | LC (%) | EE (%) | Z-average<br>diameter <sup>b</sup> | $\zeta$ -potential | FTIR <sup>a</sup>      |
|------------------|--------|--------|------------------------------------|--------------------|------------------------|
|                  |        |        | (nm)                               | (mV)               | ( $I_{2925}/I_{890}$ ) |
| <b>1:0.00</b>    | 0      | 0      | 252.0±4.9                          | 37.7±0.0           | 0.91                   |
| <b>1:0.25</b>    | 8.8    | 58.9   | 229.5±3.9                          | 35.2±0.2           | 1.14                   |
| <b>1:0.50</b>    | 13.3   | 47.1   | 218.6±0.3                          | 34.3±0.9           | 1.42                   |
| <b>1:0.75</b>    | 18.7   | 41.8   | 217.6±2.0                          | 31.0±0.5           | 1.60                   |
| <b>1:1.00</b>    | 21.8   | 37.0   | 191.3±0.2                          | 29.5±0.2           | 1.81                   |
| <b>1:1.25</b>    | 24.7   | 33.3   | 182.4±1.1                          | 26.6±0.4           | 1.80                   |

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 523 LC = (weight of loaded KO/weight of sample) × 100.  
 524 EE = (weight of loaded KO/weight of KO in feed) × 100.  
 525 <sup>a</sup>  $I_{2925}/I_{890}$  = Indicates the intensity ration of -CH stretching peak at 2925 cm<sup>-1</sup> to pyranose peak at  
 526 890 cm<sup>-1</sup>.  
 527 <sup>b</sup> Indicated values are reported as means ± standard deviation ( $n = 3$ )  
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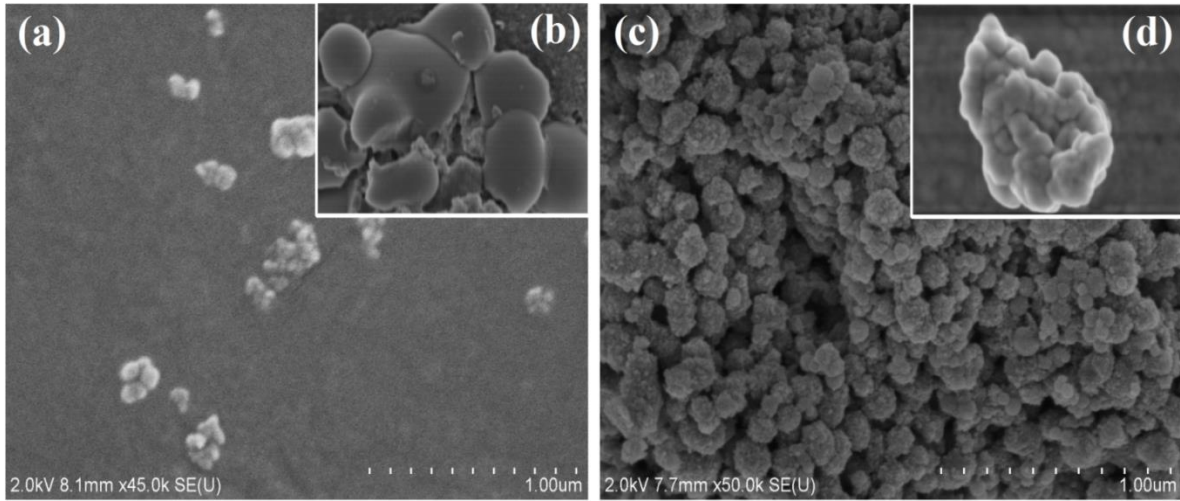


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Figure 1. Schematic illustration of KO-loaded CSNPs prepared by emulsion and electrostatic interaction of CS and TPP. O/W emulsion was stabilized by synergistic effect of two amphiphiles (i.e., tween 80 and phospholipids inherent in KO) in term of emulsification. A cartoon of formed KO-loaded CSNPs (inset) indicates the entrapment of oil droplet by absorption of surfactant molecules with their hydrophilic portions (light blue and dark blue of phospholipids and tween 80 respectively) oriented toward the aqueous phase and their hydrophobic portion (black and red of phospholipids and tween 80 respectively) anchored in the oil.



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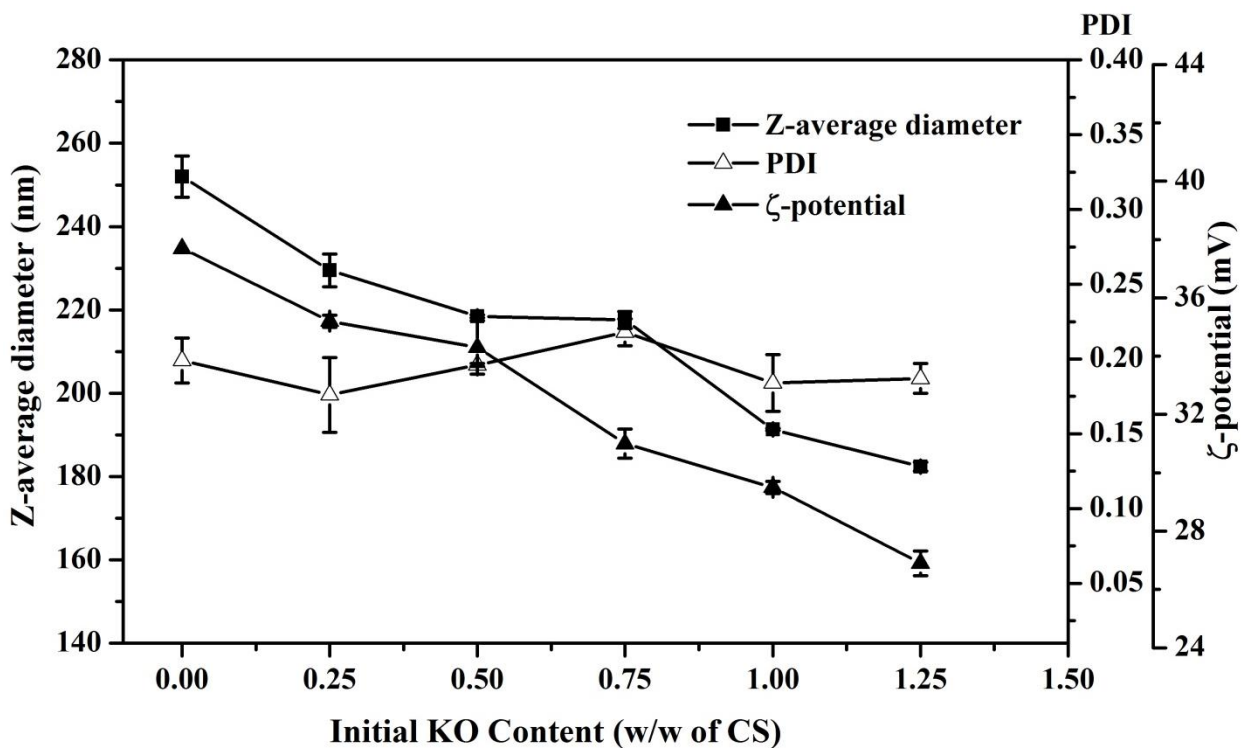
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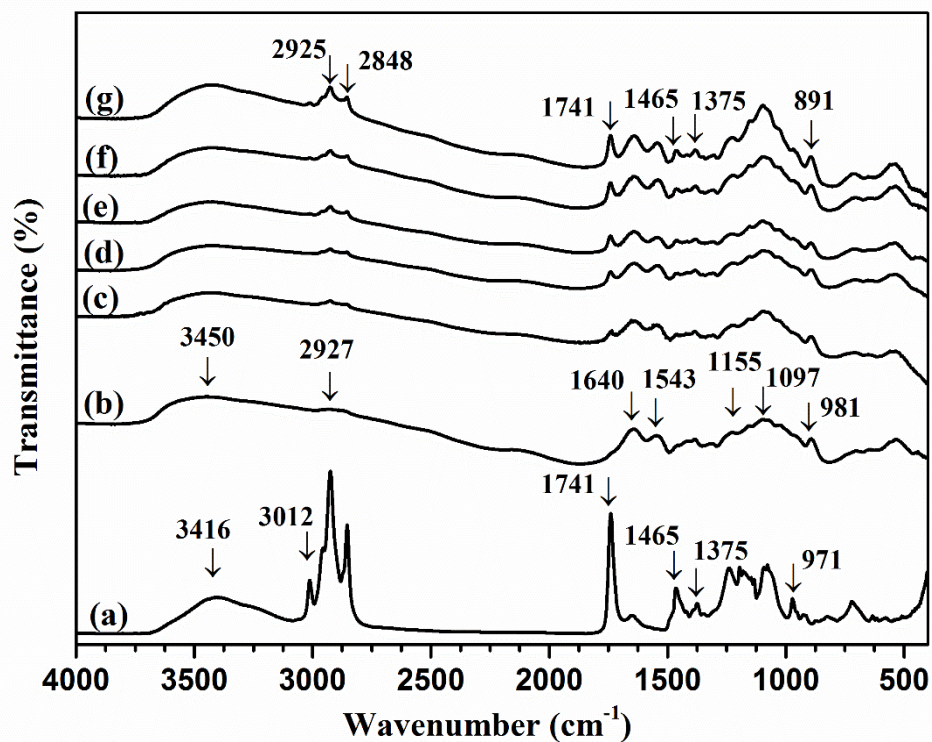
Figure 2. SEM micrographs at 2 kV of (a and b) CSNPs and (c and d) KO-loaded CSNPs prepared using an initial weight ratio of CS to KO of 1:1.00.

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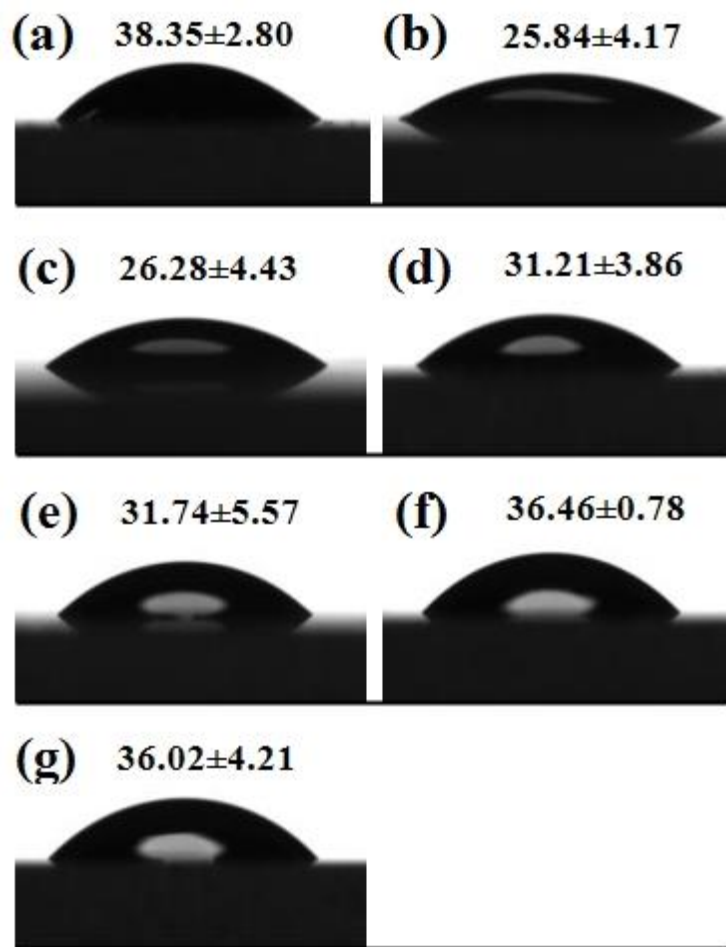
Figure 3. Z-average diameter, PDI and  $\zeta$ -potential of CSNPs and KO-loaded CSNPs with



554 different CS to KO weight ratios. Indicated values are the means  $\pm$  standard deviation ( $n = 3$ ).  
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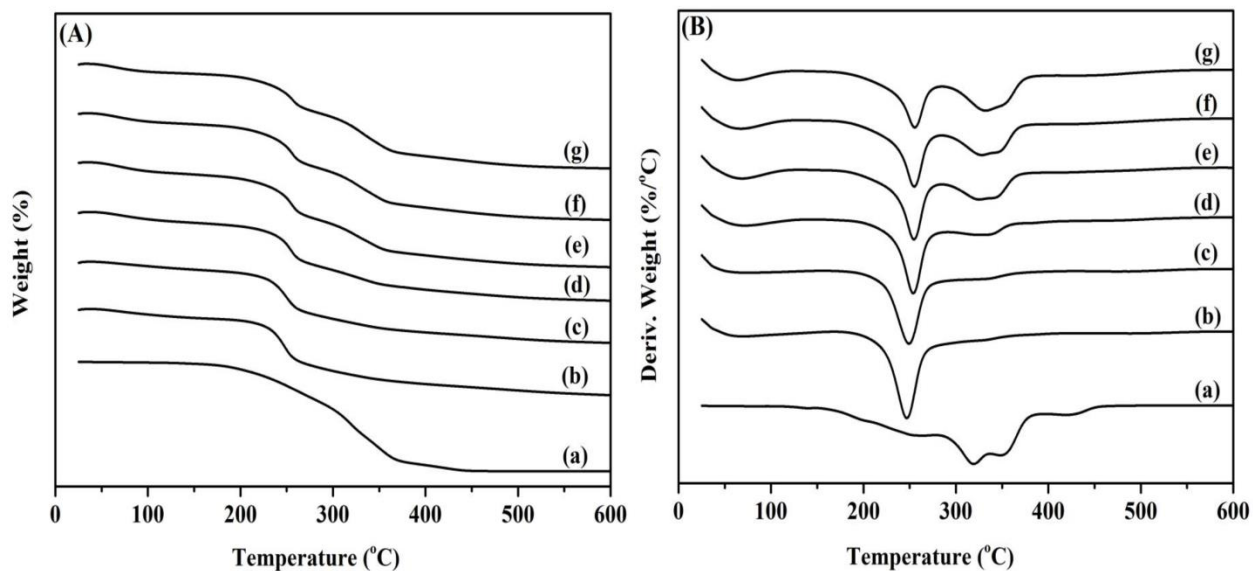


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 559 Figure 4. FTIR spectra of (a) KO, (b) CSNPs and (c)-(g) KO-loaded CSNPs prepared using  
 560 different CS to KO weight ratios: (c) 1:0.25, (d) 1:0.50, (e) 1:0.75, (f) 1:1.00, (g) 1:1.25.  
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 564 Figure 5: The surface contact angle values of (a) KO, (b) CSNPs and (c)-(g) KO-loaded CSNPs  
 565 prepared using different CS to KO weight ratios: (c) 1:0.25, (d) 1:0.5, (e) 1:0.75, (f) 1:1, (g)  
 566 1:1.25  
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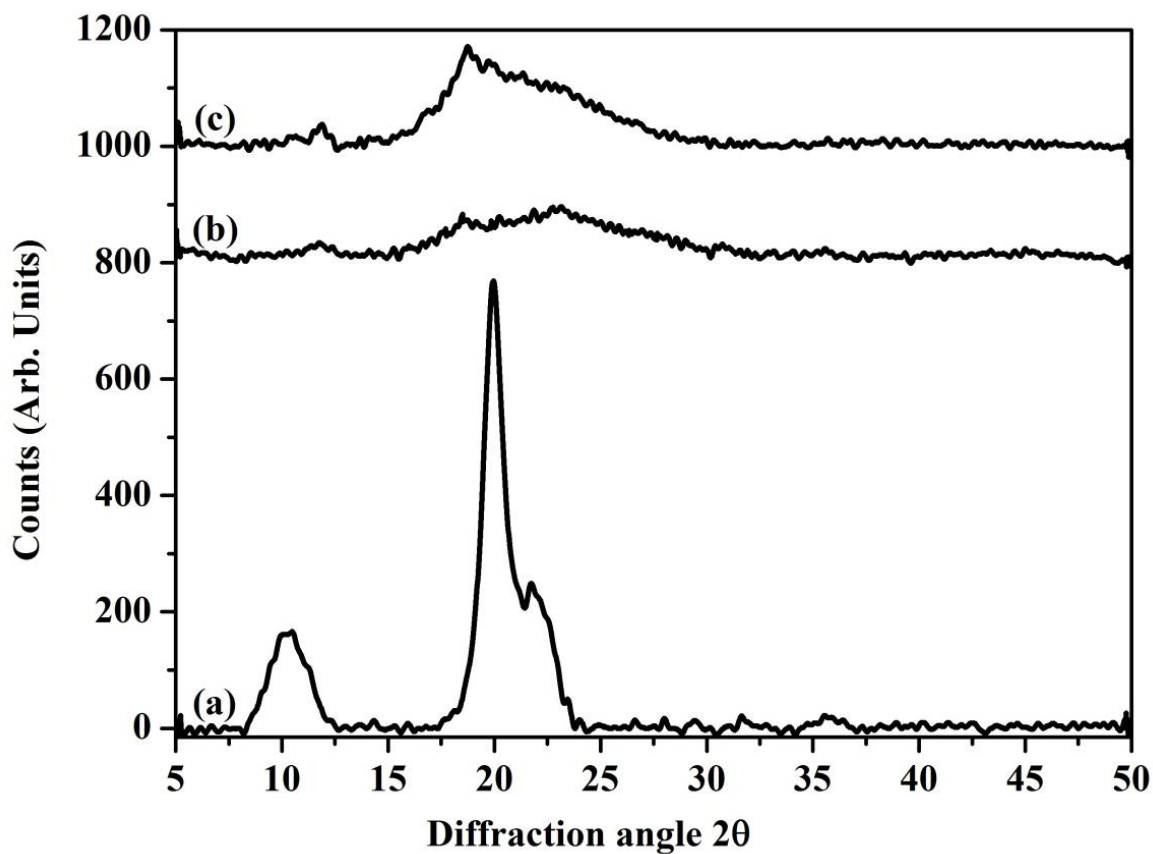


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 572 Figure 6. (A) TGA and (B) DTG thermograms of (a) KO, (b) CSNPs and (c)-(g) KO-loaded  
 573 CSNPs prepared using different CS to KO weight ratios: (c) 1:0.25, (d) 1:0.50, (e) 1:0.75, (f)  
 574 1:1.00, (g) 1:1.25.

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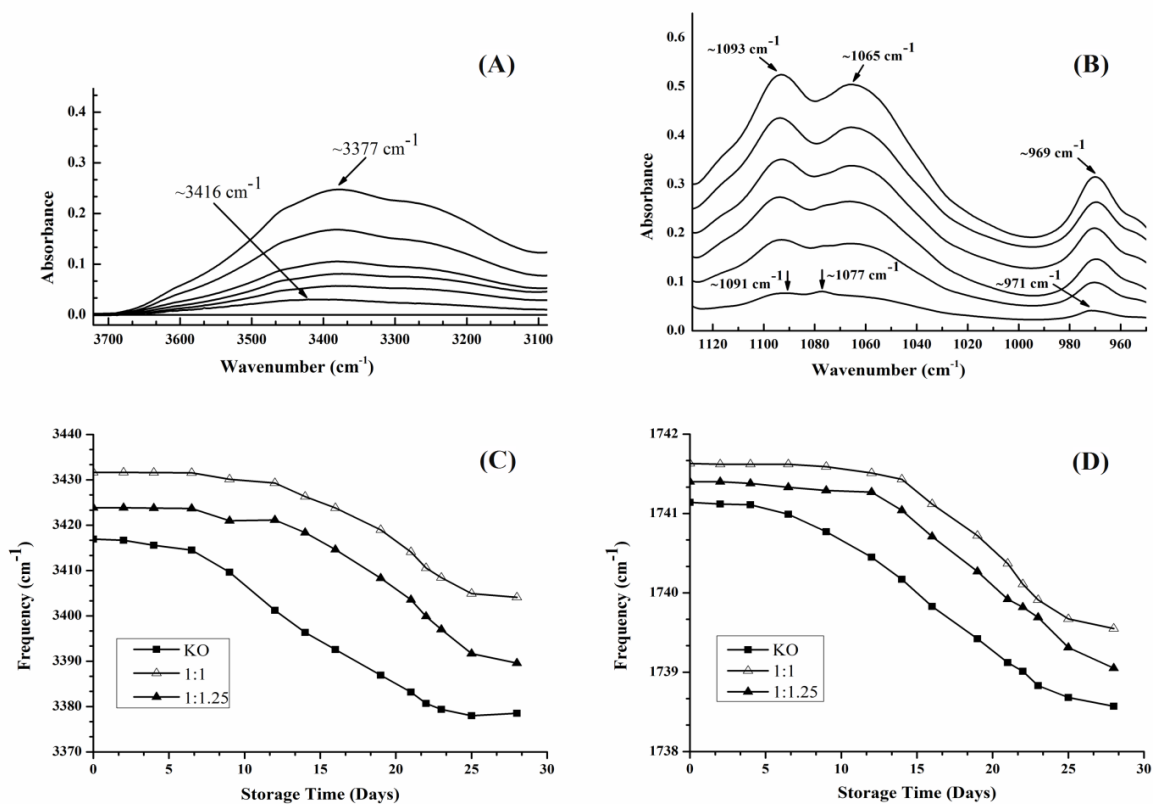
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579 Figure 7. XRD patterns of (a) CS powder, (b) CSNPs and (c) KO-loaded CSNPs.  
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 583 Figure 8. Time series differential spectra of KO and KO-loaded CSNPs during storage at 45 °C/  
 584 4 weeks: (A) ROOH region of KO (~3416 to ~3377 cm<sup>-1</sup>) absorptions, (B) CO region (~1091 to  
 585 ~1093 cm<sup>-1</sup> & ~1077 to ~1065 cm<sup>-1</sup>) and *trans* region of KO (~971 to ~969 cm<sup>-1</sup>) (C) Frequency  
 586 values of band near ~3416 cm<sup>-1</sup> (D) Frequency values of band near ~1741 cm<sup>-1</sup>.  
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