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Structural design of zein-cellulose nanocrystals core-shell microparticles for delivery of curcumin

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Abstract: The novel core-shell microparticles were fabricated to deliver curcumin using hydrophobic zein microparticles as the core and hydrophilic cellulose nanocrystals (CNCs) as the shell. CNCs were utilized to regulate the physicochemical properties, structure, stability, and *in vitro* digestion of the microparticles. FTIR analysis showed that electrostatic attraction and hydrophobic interactions were responsible for the assembly of zein-CNCs core-shell microparticles. Transmission electron microscopy (TEM) confirmed that the microstructure of the microparticles was dependent on the level of CNCs adsorbed. Furthermore, the rise of CNCs level delayed the release of curcumin from the core-shell microparticles in the gastrointestinal tract and reduced its bioaccessibility. In this study, the potential of utilizing hydrophilic nanoparticles was explored to stabilize hydrophobic microparticles through interparticle interactions, which was useful to develop the novel food grade microparticles with desirable properties using natural materials with different hydrophilicities and hydrophobicities for the application in functional foods and dietary supplements.

Key words: Zein, Cellulose nanocrystals, Core-shell microparticle, Curcumin, Microstructure, *In vitro* digestion

1. Introduction

Food core−shell microparticles have been widely developed through the layer-by-layer deposition method. Generally, based on the molecular characteristics (molecular weight, charge, stiffness and digestibility) of the biopolymer used, the mass raito and sequence of biopolymers can be designed to precisely control the thickness, charge, permeability and environmental response of the shell (Johnston, Cortez, Angelatos, & Caruso, 2006). The layer-by-layer self-assembly between biopolymers is mainly driven by electrostatic interaction, hydrophobic force and hydrogen bonding (Chen et al., 2020; Wei, Zhan, et al., 2020). Core-shell microparticles can be used to enhance the physicochemical stability and bioaccessibility of nutraceuticals entrapped (Liu, Wu, Selomulya, & Chen, 2011), which show a great potential through combination of the advantages of both the core and shell materials (Hendrickson, Smith, South, & Lyon, 2010). Materials of different properties can be combined to fabricate core−shell microparticles (Hu et al., 2019), which allows the encapsulation and delivery of nutraceuticals. Due to their unique structural properties, the core-shell microparticles showed a variety of promising applications such as in foods (Augustin & Hemar, 2009), pharmaceutics (Elzoghby, Samy, & Elgindy, 2012), and biomedicines (Yu et al., 2018).

Proteins and polysaccharides are the most common biopolymers as the host materials for the layer-by-layer assembly in the fabrication of food microparticles due

35 to their sustainability and nutritional values. For example, carboxymethyl chitosan
36 (CMCS) has been utilized to coat the zein nanoparticles for delivery of vitamin D3 (Luo,
37 Teng, & Wang, 2012). The encapsulation effiency and stability of vitamin D3 were
38 improved in zein nanoparticles with CMCS coating. Thymol-loaded zein nanoparticles
39 were stabilized with sodium caseinate and hydrochloride double layers to improve their
40 redispersibility and antimicrobial activity (Zhang et al., 2014). Recently, in our group,
41 zein-carrageenan core-shell nanoparticles were developed to co-encapsulate curcumin
42 and piperine to enhance their photothermal stability and modulate the release of
43 nutrients during *in vitro* digestion (Chen et al., 2020).

44 Nevertheless, different from the deposition of polyelectrolyte on the surface of
45 particles, different particles can be mutually stabilized and formed distinct hierarchical
46 structures through various interactions (Li et al., 2020; Madivala, Fransaer, & Vermant,
47 2009). In a real food system, there are a large number of particle-particle interactions
48 in both bulk and interfacial phases. Most previous studies focused on the particle-
49 particle interaction at the interface, exploring the influence of different colloidal
50 particles on the stability of the interface (Katepalli, John, Tripathi, & Bose, 2017; Sarkar,
51 Ademuyiwa, et al., 2018). There are very limited reports on the interaction between
52 particles in the bulk phase, especially for applications in the food or pharmaceutical
53 industries. Interparticle interactions often affect their motions in the system

(Vesaratchanon, Nikolov, & Wasan, 2009). In a concentrated suspension, particles can interact with each other and form particle clusters and particle networks, where these interactions control the motion of particles (Kourki & Famili, 2012). Moreover, steric repulsion, short- and long-range hydrodynamic interactions, and particle shape affect the structure and the diffusion of different particles (Li et al., 2020). Hu et al. (2019) designed the core-shell microparticles based on gel-network-restricted antisolvent method, in which zein nanoparticles were used to cover microgel beads from polysaccharides. The resulting microparticles demonstrated the restricted swelling properties and sustained release of encapsulants. These studies showed that interparticle interactions and nanoparticle-stabilized microparticles have a great potential in functional foods.

Zein is an important plant protein for the application in food delivery systems and biomedical treatments (Patel & Velikov, 2014). Due to its unique amphiphilicity, zein could be self-assembled into the particle-based vehicles through multiple techniques (such as anti-solvent, solvent-evaporation, and pH shifting). However, zein alone tends to agglomerate strongly during the rise in solvent polarity due to its strong hydrophobicity, which causes the particle size to grow and form larger aggregates (Wei, Sun, Dai, Zhan, & Gao, 2018). Cellulose nanocrystals (CNCs) are rod-shaped nanoparticles with high crystallinity, which are usually extracted from wood, cotton,

73 bacteria, algae and other bioresources (Hu, Ballinger, Pelton, & Cranston, 2015). The
74 superiority of CNCs in applications of food and pharmaceutical industries stems from
75 their advantages such as high aspect-ratio, high surface-area-to-volume ratio, and
76 excellent physicochemical stability (Klemm et al., 2011; Moon, Martini, Nairn,
77 Simonsen, & Youngblood, 2011). Owing to their environmental sustainability, CNCs
78 show the promising applications in commercial formulations such as medicine,
79 household detergents and personal care supplies. Despite the CNCs are generally
80 recognized as hydrophilic (due to extensive hydroxylation), the highly ordered polymer
81 chains allow for a "hydrophobic edge" to the nanocrystals with amphiphilic properties
82 (Capron & Cathala, 2013; Kalashnikova, Bizot, Bertoncini, Cathala, & Capron, 2013).
83 Up to now, there exists no available information on the particle-particle interaction and
84 the effect of nanoparticle coatings on the stability of microparticles and the sustained
85 release of loaded nutrients.

86 In this study, curcumin was selected as a model polyphenol to be loaded into the
87 core-shell microparticles. We aimed to introduce CNCs to improve the functional
88 performance of curcumin-loaded zein-CNCs core-shell microparticles. The influence
89 of the level of CNCs on the interparticle interactions and microstructure of zein-CNCs
90 core-shell microparticles was investigated. Furthermore, the *in vitro* digestion behavior
91 of the core-shell microparticles in the gastrointestinal tract (GIT) was characterized.

92 This study revealed the effect of particle-particle interactions on the physicochemical
93 properties and *in vitro* digestion of the zein-CNCs core-shell microparticles for the
94 protection of curcumin and provided the theoretical basis in the design of the
95 nanoparticle-stabilized microparticles as delivery systems.
96
97 **2. Materials and methods**
98 *2.1. Materials*
99 Zein (protein content: 91.3%), pepsin from porcine gastric mucosa (P7000),
100 porcine pancreatic lipase type 2 (L3126) and bile salts (1:1 mixture of cholic acid and
101 deoxycholic acid, 48305) were purchased from Sigma-Aldrich (USA). The pepsin has
102 a reported activity of 474 units/mg and the lipase had a reported activity of 100–500
103 units/mg (using olive oil). The CNCs with a diameter of 5–20 nm and length of 100–
104 200 nm were obtained from Shanghai ScienceK Nanotechnology Ltd. Cellulose
105 nanocrystals (CNCs) were isolated by sulfuric acid hydrolysis of wood fibers.
106 Curcumin (greater than 98%) (CAS: 458-37-7) was obtained from Adamas-Beta
107 (Shanghai, China). Other chemical agents and materials were acquired from
108 Eshowbokoo Biological Technology (China).
109
110 *2.2. Fabrication of curcumin loaded zein microparticles*

111 Curcumin loaded zein microparticles were fabricated by the solvent-evaporation
112 method according to our previous report (Wei, Yu, et al., 2019). Briefly, 3.0 g zein was
113 dissolved in 300 mL 70% (v/v) aqueous ethanol solution with magnetic stirring at 500
114 rpm for 3 h. Thereafter, 0.30 g curcumin was dissolved in the solution and continuously
115 stirring overnight. The solution was then evaporated at 45 °C for 30 min and the
116 remaining volume was set to 100 mL, which was then diluted to 200 mL. The pH of
117 particle suspensions was adjusted to 4.0 using 1M HCl. The sample was centrifuged
118 (Sigma 3k15, Germany) at 725 × g for 10 min and the supernatants were obtained. Part
119 of samples was stored at 4 °C and the other part was freeze-dried for 48 h to obtain
120 powder samples. The curcumin loaded zein microparticle was named as Z-cur.
121
122 *2.3. Fabrication of curcumin loaded zein-CNCs core-shell microparticles*
123 The CNC suspensions with the desired concentrations were obtained by dispersing
124 1.5 g CNC powder into 100 mL deionized water and followed by ultrasonic treatment
125 (10 min, 400 W) using probe-type sonicator. Then the CNC suspensions were diluted
126 to different concentrations (0.10%, 0.25%, 0.50%, 0.75%, 1.00%, 1.25%, and 1.50%,
127 w/v) with deionized water. The pH of the CNC suspensions was adjusted to 4.0 by
128 adding 0.1 M HCl or NaOH. In all samples, 40 mM NaCl was maintained in the aqueous
129 phase to partially screen the surface charge of CNCs, thereby promoting their interfacial

130 packing. The zein-CNCs core-shell microparticles were fabricated by mixing 15 mL of
131 different concentrations of CNC suspensions with 15 mL of zein microparticle
132 suspensions prepared at 12000 rpm (Ultra Turrax, model T25, IKA Labortechnic,
133 Staufen, Germany). After the addition of CNC suspensions, the microparticle
134 suspensions were further homogenized for another 5 min. The different core-shell
135 microparticles were termed as Z/0.10C-cur, Z/0.25C-cur, Z/0.50C-cur, Z/0.75C-cur,
136 Z/1.00C-cur, Z/1.25C-cur, and Z/1.50C-cur according to different concentrations of
137 CNCs.

138

139 *2.3. Particle characteristics*

140 Particle size and zeta-potential of the microparticles were determined by a Nano-
141 ZS90 (Zetasizer, Malvern Instruments Ltd., Worcestershire, UK). Stokes-Einstein
142 equation and Smoluchowski model were applied in the measurement of particle size
143 and zeta potential, respectively. The samples were diluted to avoid multiple light-
144 scattering effect. The type of cuvette used is DTS1060 and the scattering angle is 90°.
145 The refractive index (RI) of aqueous phase was set as 1.45 and the RI of the
146 microparticles was set as 1.52. All measurements were carried out at 25 °C in triplicate.

147

148 *2.4. Physicochemical stability under different environmental stresses*

149 *2.4.1. Physical stability*

150 The physical stability of the microparticles was analyzed with the LUMiSizer

151 (L.U.M. 290 GmbH, Germany) based on accelerating the destabilization by
152 centrifugation. Specifically, 1.8 mL of sample was centrifugated at 3000 rpm for 1 h at
153 25 °C with the fixed interval of 20 s (Wei et al., 2018).

154 *2.4.2. Photostability*

155 The stability of curcumin in the microparticles against UV light was evaluated.
156 Briefly, 20 mL of fresh microparticle suspensions in a transparent glass vial was
157 incubated in a controlled light cabinet (Q-Sun, Q-Lab Corporation, Ohio, USA) for 120
158 min under the exposing light condition (35 °C, 0.35 W/m^2) (Wei et al., 2018). The
159 retention rate of curcumin was plotted against time. The determination of curcumin in
160 the microparticles was conducted according to our previous method (Wei, Wang, et al.,
161 2020). Briefly, 1 mL of sample was mixed with 4 mL ethanol through vortex oscillation
162 for 120 s. The mixture was then centrifugated at 10,000 g for 30 min. The supernatant
163 was collected and diluted with 80% aqueous ethanol solution (v/v). A UV-1800
164 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) was applied to measure the
165 absorbance at 426 nm. The curcumin content was calculated according to a standard
166 curve (R^2=0.999).

167 *2.5.3. Thermal stability*

168 Briefly, 5 mL of fresh microparticle suspensions in a transparent glass vial was
169 heated at 55, 70 and 85 °C for 30 min and then cooled down to 25 °C (Wei, Zhang, et

al., 2019). The particle size, zeta-potential, and content of curcumin were then analyzed as described above.

2.5.4. pH stability

Fresh microparticle suspensions were adjusted to pH 2.0, 6.0 and 9.0 with 1 M NaOH or HCl (Joye, Davidov-Pardo, & McClements, 2015). After stored for 24 h at 25 ℃, the particle size and zeta-potential of the microparticles were measured as described above.

2.5.5. Ionic stability

Fresh microparticle supensions were mixed with different amounts of NaCl for 1 h to ensure complete dissolution. The NaCl concentration of the particle suspensions was adjusted to 10, 50, and 100 mM (Joye et al., 2015). After stored for 24 h, the particle size and zeta-potential of the microparticles were determined.

2.6. Fluorescence spectroscopy

The intrinsic fluorescence of protein in the microparticles was determined with a fluorescence spectrophotometer (F-7000, Hitachi, Japan). The emission spectra were collected between 290 and 450 nm at a scanning speed of 100 nm/min after being excited at 280 nm at 25 ℃. The concentration of the protein was set as 0.20 mg/mL.

189 *2.7. Fourier transform infrared spectroscopy (FTIR)*

190 Infrared spectra of microparticles were analyzed with a Spectrum 100 Fourier
191 transform spectrophotometer (PerkinElmer, UK). The mixture of 2.0 mg sample and
192 198.0 mg of pure KBr was ground into fine powder and pressed into a pellet at 20 MPa
193 for 60 s. The spectra were acquired after 64 scans at a wavenumber range from 4000 to
194 400 cm^{-1} with a 4 cm^{-1} resolution. Pure KBr powder was used as a baseline.

195

196 *2.8. XRD*

197 The molecular arrangement of particle powders was measured by an X-ray
198 diffractometer (Brucker D8, Odelzhausen, Germany) with a Cu anode, 40 kV voltage
199 and current of 40 mA. The scan was ranged from 4 ° to 40 ° (2θ) with a step size of
200 0.02 °and step time of 5 s (Huang et al., 2017).

201

202 *2.9. Transmission electron microscopy (TEM)*

203 The morphology of samples was analyzed using Tecnai 200 transmission electron
204 microscope (FEI Company, Philips, NL-5600 MD, Eindhoven, Netherlands), operating
205 at an accelerating voltage of 60 kV. The dispersion at pH 4.0 was diluted into 0.2 mg/mL
206 with distilled water (pH 4.0), and one drop of the dispersion was placed on a 200-mesh
207 carbon coated copper grid. Images with various magnifications were acquired at 25 kV.

208

209 *2.10. In vitro gastrointestinal digestion*

210 An *in vitro* gastrointestinal model was applied in this study with some
211 modifications (Wei, Yang, et al., 2020):

212 *Stomach phase*: 30 mL of the particle dispersions was mixed with 60 mL of
213 simulated gastric fluid (SGF) containing 0.0032 g/mL pepsin to mimic gastric digestion.
214 The pH was adjusted to 2.0 and the sample was then swirled at 150 rpm in a shaking
215 incubator at 37 °C for 1 h.

216 *Small intestine phase*: 40 mL of gastric digesta was transferred into a 100 mL glass
217 beaker and then adjusted to pH 7.0. Thereafter, 40 mL of simulated intestinal fluid (SIF)
218 containing 5 mg/mL bile salt and 0.4 mg/mL pancreatin was mixed with digesta in
219 reaction vessel. The pH was adjusted to 7.0 and the samples were held under continuous
220 vibration at 150 rpm in a shaking incubator at 37 °C for 1 h to mimic small intestine
221 digestion.

222 The size, release percentage of curcumin and structure of the microparticles were
223 determined after being digested periodically (every 30 min). An aliquot of raw digesta
224 was centrifugated at 10000 g for 30 min at 4 °C, and the supernatant was filtered with
225 a 0.45 μm filter. The amount of curcumin released was calculated by following the
226 equation below:

227 $$\text{Release percentage } (\%) = \frac{\text{released curcumin (mg)}}{\text{entrapped curcumin (mg)}} \times 100\% \quad (1)$$

228 The bioaccessibility of curcumin was determined after the intestinal digestion. Part
229 of the digesta was processed using a high-speed centrifuge at 15,000 g for 60 min at 4 °C
230 and the micelle phase containing the solubilized curcumin was collected. The
231 bioaccessibility of curcumin was defined as the fraction of curcumin released from the
232 food matrix and solubilized within mixed micelles present in the small intestine before
233 it could be absorbed (Yao, Xiao, & McClements, 2014). The curcumin contents
234 extracted from the initial microparticles and micelle fraction were determined
235 according to the method described in section 2.4. The bioaccessibility (%) of curcumin
236 was calculated by following the equation below:

237 $$Bioaccessibility\ (\%) = \frac{C_{micelle}}{C_{initial\ microparticle}} \times 100\% \quad (2)$$

238 where $C_{micelle}$ represented the concentration of curcumin in the micelle fraction and
239 $C_{initial\ microparticle}$ represented the total concentration of curcumin encapsulated into the
240 microparticles.

241

242 *2.11. Statistical analysis*

243 All experiments were performed at least three times and the means and standard
244 deviations were calculated. ANOVA analysis (Duncan's multiple range tests) was used
245 to determine statistical differences ($p < 0.05$) between samples.

246

247 **3. Results and discussion**

248 *3.1. Particle properties*

249 The size of curcumin loaded zein microparticles was 1017.3 ± 17.6 nm, which was
250 the lowest among all microparticles (Fig. 1A). With the adsorption of CNCs at a low
251 level (Z/0.10C-cur), the size of zein-CNCs core-shell microparticles was markedly
252 (p<0.05) increased to 3663.7 ± 226.3 nm. It was assumed that CNCs could not
253 completely cover the surface of the zein microparticles, and therefore the negatively
254 charged CNCs formed the bridges between the positively charged microparticles
255 through the electrostatic attraction, promoting the particle aggregation. Interestingly,
256 the size of zein-CNCs core-shell microparticles continued to decrease as the level of
257 CNCs increased until it reached 1.00% (w/v). The phenomenon indicated that as the
258 concentration of CNCs increased, the surface of the core-shell microparticles was
259 gradually covered by CNCs until they were saturated, which provided sufficient
260 interparticle repulsion to prevent the particle bridging.

261 With the adsorption of CNCs onto zein microparticles, the zeta-potential of the
262 microparticles was changed from a positive (Z-cur: 45.40 ± 1.06 mV) to a negative
263 value near zero (Z/0.10C-cur: - 2.73 ± 2.14 mV) (Fig. 1B), revealing that zein
264 microparticles and CNCs formed the zein-CNCs core-shell microparticles mainly

265 through the electrostatic attraction. The addition of CNCs at a low level neutralized the
266 positive charge on the zein microparticles and weakened the electrostatic repulsion
267 between the particles. As the proportion of CNCs continued to increase, the zeta-
268 potential of the core-shell microparticles gradually increased and reached a plateau at
269 Z/1.00C-cur, which provided sufficient the electrostatic repulsion to prevent the particle
270 aggregation. This result was consistent with the size of zein-CNCs core-shell
271 microparticles.
272
273 *3.2. Physicochemical stability under different environmental stresses*
274 *3.2.1. Physical stability*
275 The curcumin loaded zein microparticles were unstable and the particle
276 aggregation occurred due to the hydrophobic interaction (Fig. 1C). With the adsorption
277 of CNCs at a low level, the physical stability of zein-CNCs core-shell microparticles
278 was reduced after CNCs was adsorbed. Among all the microparticles, Z/0.10C-cur was
279 the most unstable due to the largest particle size. As aforementioned, the addition of
280 CNCs reduced the electrostatic repulsion between the particles and induced the bridging
281 between the particles, thereby decreasing the stability of the particles. With the rise of
282 CNCs level, the physical stability of zein-CNCs core-shell microparticles was gradually
283 improved. When the concentration of CNCs exceeded above 0.50% (w/v), the physical
284 stability of zein-CNCs core-shell microparticles became better than that of zein

285 microparticles. When the concentration of CNCs was between 0.75%-1.00% (w/v), the
286 physical stability of zein-CNCs core-shell microparticles was greatly enhanced, which
287 showed that the CNCs have completely covered the surface of curcumin loaded zein
288 microparticles, providing the sufficient steric and electrostatic repulsion between the
289 particles.

290 *3.2.2. Photo stability*

291 Among all the microparticles, the curcumin entrapped in the zein microparticles
292 degraded most quickly under the exposure to UV light (Fig. 2A). Although the aromatic
293 side groups and double bonds in zein molecules could absorb part of UV light, the
294 curcumin content in the zein microparticles was greatly reduced to 17.42 ± 2.53% after
295 2 hours of light exposure (Luo et al., 2013). With the rise of CNCs level, the photo
296 stability of curcumin in the core-shell microparticles was progressively improved. The
297 retention rate of curcumin reached the maximum (30.73 ± 2.76%) in Z/0.75C-cur after
298 120 min of UV radiation. The low quantity of CNCs could not fully cover the surface
299 of zein microparticles, so that more light could enter into the interior of the
300 microparticles through the interfacial gaps between CNCs, leading to the degradation
301 of curcumin. The enhanced adsorption of CNCs could protect more surface area of
302 curcumin loaded zein microparticles, retarding the penetration of light and the
303 degradation of curcumin. It was reported that the introduction of the CNCs into

304 polyvinyl alcohol films decreased the transparency with the strong anti-ultraviolet
305 ability (Dai, Huang, & Huang, 2018). Nevertheless, as the concentration of CNCs
306 reached over 1.00% (w/v), the photo stability of curcumin entrapped in the core-shell
307 microparticles was slightly diminished. This result might be explained that the smaller
308 microparticles had a larger surface area, which allowed more light to penetrate into the
309 inside of the microparticles, resulting in more degradation of curcumin.

310 *3.2.3. Thermal stability*

311 The size of zein microparticles remained basically stable after heating (Fig. 2B).
312 However, there was an obvious increase in the particle sizes of Z/0.25C-cur and
313 Z/0.50C-cur during thermal treatment. At the low proportion of CNCs, the surface
314 charge of the zein microparticles was neutralized and the zeta-potential dropped greatly
315 (Fig. 2C). Meanwhile, thermal treatment promoted the diffusion and collision of the
316 particles, and therefore CNCs tended to form the bridges between the microparticles
317 through hydrophobic and electrostatic interactions. With the increase in CNCs level,
318 the thermal stability of zein-CNCs core-shell microparticles was continuously
319 improved due to enhanced steric and electrostatic repulsion, revealing that the
320 introduction of CNCs endowed the interface with a great thermal resistance owing to
321 its stable structure and strong intermolecular hydrogen bonding (Dai et al., 2018).

322 Thermal treatment caused the most severe degradation of curcumin in zein
323 microparticles, which only remained 72.93 ± 1.63% after heating (Fig. 2D). The

thermal stability of curcumin entrapped in zein-CNCs core-shell microparticles was greatly enhanced compared to zein microparticles with the adsorption of CNCs. Although adding a small amount of CNCs caused the aggregation of the core-shell microparticles (Fig. 2B), CNCs improved the thermal stability of curcumin entrapped. With the rise in CNCs level, the retention rate of curcumin loaded in Z/0.50C-cur was markedly (p<0.05) elevated to 92.96 ± 0.04%. This result might be ascribed to the increase in the thickness of the "protective" particle layer formed by the adsorption of CNCs on the surface of zein microparticles, which reduced the transmit of heat from the external environment to the interior of the particles, thereby inhibiting the degradation of curcumin. When the level of CNCs continued to rise, the retention rate of curcumin in the core-shell microparticles still kept constant.

3.2.4. pH stability

At pH 2, the size of zein microparticles remained basically stable (Fig. 3A). However, with the addition of CNCs, zein-CNCs core-shell microparticles appeared to be agglomerated, especially at a low level of CNCs. This phenomenon was mainly attributed to the fact that the pI of zein is far away from the pH of the environment, which made the surface of zein microparticles carried a large amount of positive charge (Fig. 3B). Strong electrostatic repulsion effectively inhibited the particle aggregation. In an acidic environment, the negative charge carried by CNCs was greatly reduced,

343 which decreased the zeta-potential of the zein-CNCs core-shell microparticles. The
344 reduced electrostatic repulsion promoted the formation of bridges between the particles.
345 With the rise of CNCs level, the stability of core-shell microparticles was obviously
346 improved at pH 2.

347 In the neutral environment, the greatest increase was observed in the size of zein
348 microparticles (Fig. 3A). Since the zeta-potential was close to 0 at pH 6, there existed
349 obvious aggregation between zein microparticles due to no electrostatic repulsion (Fig.
350 3B) (Wei et al., 2018). With the adsorption of CNCs, zein-CNCs core-shell
351 microparticles showed the constant sizes at pH 6. CNCs with a large magnitude of
352 negative charges were adsorbed on the surface of zein microparticles to provide
353 sufficient steric and electrostatic repulsion (Pandey et al., 2018). When pH was elevated
354 to 9.0, the size of zein microparticles transformed to the initial size at pH 4. It is worth
355 noting that the size of all zein-CNCs core-shell microparticles was significantly (p<0.05)
356 reduced, even lower than their initial sizes at pH 4. Additionally, the zeta-potential of
357 all the microparticles was increased. Both zein microparticles and CNCs carried a large
358 amount of negative charges and generated strong electrostatic repulsion, which made it
359 difficult for CNCs to adsorb to the surface of zein microparticles and form stable "core-
360 shell" structured microparticles.

361 *3.2.5. Ionic stability*

362 The size of zein microparticles and zein-CNCs core-shell microparticles

363 remained stable at 10 mM (Fig. 3C). Nevertheless, the size of the core-shell
364 microparticles was increased greatly with the rise in ionic strength, which was
365 consistent with their zeta-potential (Fig. 3D). The presence of salt reduced the zeta-
366 potential of the core-shell microparticles due to the electrostatic screening. The surface
367 charge carried by the core-shell microparticles was screened by counter-ions, and
368 therefore the electrostatic repulsion between the particles was greatly reduced (Wei, Li,
369 et al., 2020). The insufficient repulsion between the microparticles was
370 disadvantageous to keep their stability against aggregation. It is worth noting that when
371 the ionic strength was increased from 50 mM to 100 mM, the zeta-potential of all
372 microparticles was further reduced, but the most obvious increase of particle size
373 occurred in Z/0.50C-cur and Z/0.75C-cur. This phenomenon was reasonably explained
374 by that the electrostatic shielding promoted the bridging of CNCs on the surface of
375 different microparticles, leading to further aggregation between the microparticles.
376
377 *3.3. Fluorescence spectrum*
378 A characteristic fluorescence emission peak was observed at 304 nm after being
379 excited at 280 nm as a indicative of a high proportion of tyrosine residues in zein (Fig.
380 4A) (Shukla & Cheryan, 2001). There was no significant change in the wavelength of
381 emission maximum but the fluorescence intensity of zein varied with different

382 formulations. The interparticle interaction between zein microparticles and CNCs
383 enhanced the fluorescence intensity of zein, revealing that the micro-environment of
384 tyrosine residues became apolar (Sun, Wei, Li, Dai, & Gao, 2017b). With the rise of
385 CNCs level, the fluorescence intensity of zein was continuously increased. These
386 results suggested that although CNCs were only adsorbed on the surface of zein
387 microparticles instead of traditional complexation of protein and polysaccharides, the
388 presence of CNCs could still change the internal microenvironment of zein
389 microparticles and alter the conformation of protein through molecular interactions.
390
391 *3.4. FTIR*
392 As shown in Fig. 4B, the frequencies of the bands assigned to the amide I and II
393 regions of Z-cur remained at 1657.03 and 1515.77 cm^{-1}. An absorption band at 3324.68
394 cm^{-1} was attributed to O-H stretching. Another absorption band observed at 2934.65
395 cm^{-1} was assigned to C-H stretching (Sun et al., 2017a). With the addition of CNCs,
396 the absorption peak of hydrogen bonds in zein-CNCs core-shell microparticles was
397 shifted from 3324.68 to 3338.66 cm^{-1} because of the formation of hydrogen bonds (Fig.
398 4B). Upon increasing the CNCs level, the absorbance at 3338.66 cm^{-1} of Z/0.10C-cur
399 was gradually shifted to 3351.19 cm^{-1} of Z/1.25C-cur. The phenomenon manifested that
400 CNCs were adsorbed onto the zein microparticles mainly through hydrogen bonding,

401 which could also alter the microenvironment of zein. Meanwhile, the incorporation of
402 CNCs into zein microparticles caused the shift corresponding to C-H stretching. With
403 the rise of CNCs content, the absorption peak associated with C-H stretching was
404 shifted from 2929.34 (Z/0.10C-cur) to 2907.16 cm^{-1} (Z/1.25C-cur). These shifts were
405 associated with interparticle interactions between zein microparticles and CNCs, which
406 involved electrostatic interactions, hydrophobic forces, and hydrogen bonds (Davidov-
407 Pardo, Joye, & McClements, 2015). The absence of new peaks formed suggested that
408 microparticles and nanoparticles were physically combined without the formation of
409 covalent bonds. The band corresponding to amide I depended on the secondary
410 structure of the protein backbone at around 1658 cm^{-1} remained unchanged, which
411 indicated that the secondary structure of zein was not affected by the incorporation of
412 CNCs.
413
414 *3.5. XRD*
415 As depicted in Fig. 4C, pure curcumin showed the crystalline state with sharp
416 characteristic diffraction peaks. After encapsulation into zein microparticles, Z-cur
417 showed a relatively flat pattern and no obvious diffraction peaks of curcumin occurred
418 in zein microparticles. The phenomenon suggested that curcumin was completely
419 entrapped into zein microparticles with its amorphous nature. When CNCs were

adsorbed onto the surface of zein microparticles, the XRD intensity of zein-CNCs core-shell microparticles was slightly increased. The crystallinity of biopolymers represented the order of molecular structure in their complexes (Shaikh, Ankola, Beniwal, Singh, & Kumar, 2009). The incorporation of CNCs might induce the aggregation of the microparticles and increase the crystallinity of zein-CNCs core-shell microparticles because of its highly ordered structure.

3.6. Morphological observation

Through the observation of TEM, zein microparticles showed a spherical shape with around 1 μm in diameters (Fig. 5), which was consistent with the results by dynamic light scattering. It is worth noting that zein microparticles seriously aggregated due to the strong hydrophobicity. The TEM image suggested that the CNCs were stiff, needle-like particles of a nearly perfect crystalline structure, forming a compact network-type architecture, which was ascribed to the high aspect ratio of CNCs, i.e., the ratio of length to diameter (L/D). The microstructure of zein-CNCs core-shell microparticles was dependent on the concentration of CNCs. At the low level of CNCs (Z/0.10C-cur), part of the CNCs were adsorbed on the surface of zein microparticles and part of the CNCs would extend into the aqueous phase to enhance the stability of the microparticles. Nevertheless, part of the surface of zein microparticles was still not

covered by CNCs, hence the microparticles was still aggregated through the hydrophobic interaction. As the CNCs level was elevated, the particles were still aggregated because of the partial cross-linking between the particles. The low quantity of CNCs was insufficient to cover the whole particle surface and therefore formed bridging between neighboring particles (Z/0.25C-cur and Z/0.50C-cur). When the level of CNCs was further increased, more CNCs were completely adsorbed on the surface of zein microparticles, and excessive CNCs extended into the aqueous phase. The morphology indicated that CNCs were successfully interacted with zein microparticles to generate zein-CNCs core-shell microparticles, and the presence of CNCs provided sufficient steric and electrostatic repulsion for core-shell microparticles to avoid particle aggregation.

As a supplement, scanning electron microscopy (SEM) was also used to observe the microstructure of the core-shell microparticles (Fig. S1). The individual spherical zein microparticles exhibited a smooth surface. The CNCs were adsorbed to the surface of adjacent particles and formed the bridges between the particles. When the concentration of CNCs continued to increase, they were gradually adsorbed to the surface of zein microparticles, showing a compact hydrophobic-hydrophilic core-shell structure. At a higher level of CNCs, they formed a thicker shell to protect the curcumin loaded zein microparticle core, and the excessive CNCs entered into the aqueous phase.

458

459 *3.7. In vitro gastrointestinal digestion*

460 *3.7.1 Particle size*

461 The *in vitro* digestion fate of curcumin loaded zein-CNCs core-shell microparticles
462 was investigated in the gastrointestinal tract (GIT). The size of all the microparticles
463 was increased within the gastric phase (Fig. 6A), indicating that the proteolysis of zein
464 occurred in all the microparticles due to the presence of pepsin, leading to particle
465 aggregation. It is worth noting that when the lower content of CNCs adsorbed to zein
466 microparticles, the core-shell microparticles showed serious aggregation in the gastric
467 phase. In an acidic environment, CNCs lost a large amount of negative charges, which
468 reduced the electrostatic repulsion between the microparticles. When the content of
469 CNCs was not enough to cover the surface of zein microparticles, CNCs tended to form
470 the bridges between the microparticles to promote particle aggregation. When the
471 content of CNCs increased, the degree of particle aggregation was gradually diminished.
472 In spite of additional steric repulsion for the core-shell microparticles provided by the
473 increase in CNCs, but it was still insufficient to keep them stable at pH 2, which was
474 confirmed by the morphology of different microparticles during *in vitro* digestion (Fig.
475 S2).

476 The particle size of gastric digesta was decreased greatly after they were

477 transferred to the intestinal phase. The neutral environment of the intestinal phase made
478 both zein microparticles and CNCs negatively charged, which transformed the
479 interaction between zein microparticles and CNCs and the interaction between
480 microparticles from electrostatic attraction to electrostatic repulsion. This phenomenon
481 promoted the separation between the microparticles and made the structure of zein-
482 CNCs core-shell microparticles collapse. Nevertheless, with the extension of the
483 digestion time in the small intestine, the size of the microparticles was gradually
484 increased due to the proteolysis catalyzed by trypsin, resulting in the deconstruction
485 and aggregation of the core-shell microparticles.

486 *3.7.2. Release of curcumin*

487 Fig. 6B showed the release of curcumin from zein microparticles and zein-CNCs
488 core-shell microparticles at different *in vitro* digestion times. Among all the samples,
489 zein microparticles showed the highest release percentage of curcumin during the whole
490 digestion, which released 22.71% of curcumin in the gastric phase and 61.46% of
491 curcumin in total. The release of curcumin from the core-shell microparticles was
492 dependent on the level of CNCs added. The incorporation of CNCs greatly restricted
493 the release of curcumin from zein-CNCs core-shell microparticles. With the increase in
494 CNCs level, the total release percentage of curcumin of the core-shell microparticles
495 was markedly reduced from 50.40% (Z/0.25C-cur) to 30.88% (Z/1.00C-cur) during the

496 gastrointestinal tract. On one hand, CNCs formed a dense coating around the curcumin
497 loaded zein microparticles (Fig. S1), despite the fluctuation of pH during the digestion
498 could affect the adsorption of CNCs on the surface of the microparticles. Meanwhile,
499 CNCs should be resistant to hydrolysis by digestive enzymes as a dietary fibre (Mackie
500 et al., 2019; Nsor-Atindana et al., 2017). The formation of an indigestible physical
501 barrier around zein microparticles made CNCs suitable for controlling digestion of
502 zein-CNCs core-shell microparticles. On the other hand, at the higher content of CNCs,
503 the excessive CNCs could extend from the surface of the core-shell microparticles into
504 the aqueous phase and form a network structure between the microparticles (Fig. 5),
505 which retarded the diffusion and contact of protease and microparticles and reduced the
506 release of curcumin.

507 *3.7.3. Curcumin bioaccessibility*

508 The bioaccessibility of curcumin in zein-CNCs core-shell microparticles was
509 consistent with the release of curcumin in the GIT. Zein microparticles showed the
510 highest bioaccessibility of curcumin (16.66 ± 2.36%) among all the samples. With
511 the addition of CNCs, the curcumin was less bioaccessible in the core-shell
512 microparticles compared to zein microparticles. As aforementioned, indigestible CNCs
513 formed a densely packed interface that restricted the access of proteases to the core-
514 shell microparticles, which effectively reduced the curcumin release and subsequently

its bioaccessibility. Besides, CNCs adsorbed could limit the proximity of the negatively charged bile salts through charge repulsion. The excessive CNCs could extend into the aqueous phase and form a network structure (Fig. 5), which delayed the formation and diffusion of bile salts and mixed micelles. This finding is consistent with the results that the addition of CNCs delayed the lipid digestion of emulsions during intestinal digestion (Bai et al., 2019; Sarkar, Li, Cray, & Boxall, 2018).

4. Conclusion

In this study, CNCs were introduced to improve the chemical stability of curcumin and modulated the *in vitro* digestion of zein-CNCs core-shell microparticles. CNCs adsorbed to the surface of zein microparticles to form the core-shell microparticles through electrostatic attraction, hydrophobic interaction, and hydrogen bonding. At the low level of CNCs, the microparticles were aggregated because of the partial cross-linking. When the content of CNCs was increased, CNCs covered completely the surface of zein microparticles, and the presence of CNCs provided sufficient steric and electrostatic repulsion for the core-shell microparticles to avoid particle aggregation. Meanwhile, indigestible CNCs could form a compact shell that restricted the access of proteases and bile salts to the core of the microparticles, which effectively reduced the curcumin release and bioaccessibility. The zein-CNCs core-shell microparticles developed in this work have potential for application in functional foods. In the future, their functionality still needs to be evaluated comprehensively through *in vivo* experiments.

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Notes

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