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Fabrication of zein/quaternized chitosan nanoparticles for encapsulation and protection of curcumin

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Abstract:

In this article, nanoparticles (NPs) from a water-soluble chitosan (CS) derivative (N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride, HTCC) and zein had successfully been assembled via a low-energy phase separation method. The fabricated NPs were investigated for the first time to encapsulate and protect curcumin (Cur). The particle size and zeta potential of zein-HTCC NPs varied from 66 to 170 nm and +36.3 to +62.5mV, respectively. The encapsulation efficiency (EE) was greatly improved to 94.9% after HTCC coating, compared with 85.2% that using zein as a single encapsulant. The microstructure of the NPs was revealed by transmission electron microscopy (TEM). The physicochemical and structural analysis showed that the electrostatic interactions and hydrogen bonds were major forces responsible for NPs formation. The encapsulation forms were evaluated for their efficiency in overcoming Cur's heat and UV sensitivity, which improve the stability about 2.7 fold, 3.5 fold and 2.5 fold when disposed with 60 °C treatment for 30 min, 80 °C treatment for 1 min and ultraviolet radiation for 2 h at zein: $HTCC_l$=1:1. The results of stability and DPPH assays indicated the protection of bioactivity as encapsulated. Zein-HTCC NPs were believed to be a promising delivery system for supplementation or treatment of hydrophobic nutrients or drugs.

Keywords: zein HTCC curcumin stability protection

1. Introduction

Bioactive compounds have been intensively investigated in recent years for these health-beneficial properties and for potential applications in the fields of pharmaceutic, nutraceuticals, and functional foods[1, 2]. Among them, polyphenols have attracted many researchers' attention because of their anti-oxidant, anti-inflammatory, and anti-cancer properties[3]. Together with some other plant-derived polyphenols, Cur is among the best characterised polyphenols since it is primarily used as a food colourant and more important, it has antioxidant, antibacterial, antifungal, antiviral, anti-inflammatory, antiproliferative, and pro-apoptotic effects[4-6]. Unfortunately, the application of Cur has been limited by its poor water solubility and unstability what is limiting its bioavailability, thus impending its conversion from cooking to clinical applications[7, 8]. Therefore, exploitation of Cur as a functional food and nutraceutical ingredient or pharmaceutical compound is feasible only when encapsulated in a delivery system, which is capable of stabilizing and protecting it from degradation while preserving its biological activities and enhancing its bioavailability[9].

Many encapsulation approaches have been applied to increase the water solubility and/or bioavailability of Cur such as liposome[10], polymeric micelles[7], emulsion[11], complex[12], NPs[13], etc. Among these approaches, biodegradable polymer NPs offer promising enhanced functional properties of bioactive compounds which are unstable against processing and severe storage condition, such as heating, ultraviolet radiation[14]. Over the past decades, NPs systems based on proteins including gelatin, collagen, casein, albumin and whey protein have been studied for delivering drugs,

nutrients, bioactive peptides and probiotic organisms[15]. In most cases, due to the isoelectric point (pI) of protein, the formulations of NPs greatly influenced by the pH condition, making the formulations unstable. Therefore, considerable endeavors have been taken in investigating the associative interactions between natural proteins and polysaccharides in order to improve the stability of NPs[16]. As an alcohol-soluble protein obtained from corn, zein has attracted widespread interest in delivery systems due to its inherent excellent biocompatibility and biodegradability[17]. Zein has been extensively investigated in the encapsulation of bioactive compounds because of its capability to form self-assembled NPs[18, 19]. It has thus been utilized in food and pharmaceutical applications, such as heparin, gitoxin, fish oil, and the like[20-22]. CS, a natural polyaminosaccharide obtained from N-deacetylation of chitin, with distinctive biological properties such as non-toxicity, biocompatibility, biodegradability and antimicrobial activity, has been widely used in biomaterial applications[14]. However, in neutral and basic environments, the CS molecules lose their charge and precipitation will occur due to the pKa of CS (6.5)[23]. To overcome this drawback and expand its use, functional groups have been introduced into CS to make it water-soluble[24-27]. Among the derivatives of CS, quaternized CS has attracted much attention due to the properties of retaining cationic charges at neutral pH, good water solubility, antibacterial activity, mucoadhesivity, enhanced antioxidant activity, and enhanced cellular penetration[28]. N-(2-hydroxyl) propyl-3-trimethyl ammonium chitosan chloride (HTCC) can be prepared by a relatively easy chemical reaction of CS and 2,3-Epoxypropyltrimethyl ammonium chloride (EPTMAC)[29]. The presence of

positive charge is expected to increase the mucoadhesive nature of CS, which leads to an increased residence time and enhanced bioavailability[30].

In the present work, HTCC with different molecule, which showed higher aqueous solubility than CS in a much broader pH range, was synthesized and used to prepare zein-HTCC NPs. The Cur/zein-HTCC NPs delivery system was developed using a liquid-liquid phase separation approach. The characteristics of Cur encapsulation and protection system have been studied using transmission electron microscopy (TEM), Fourier transform infrared spectroscopy (FT-IR), X-ray diffraction (XRD), fluorescence spectra and DPPH measurements. Additionally, the Cur encapsulation and protection for zein-HTCC NPs were evaluated. Furthermore, the light stability studies of free Cur and Cur/zein-$HTCC_1$ NPs using the UV absorbance were investigated. The results illustrated zein-HTCC NPs greatly increased the water solubility of Cur and had great potential application for bioactive compounds in food and medicinal fields.

2. Materials and methods

2.1. Materials

Zein (Mw=19k and 22k Da) was purchased from Tokyo Chemical Industry, Co., Ltd. (Tokyo, Japan). CS with different molecular weight and all with 91% deacetylation were supplied by Zhejiang Yuhuan Ocean Biochemistry Co., Ltd. (China). Curcumin (95.0% purity) was purchased from National Medicine Group Chemical Reagent Co., Ltd. 2,3-Epoxypropyltrimethyl ammonium chloride (EPTMAC) was purchased from Shandong Dongying Chemical Co., Ltd. with a purity of 96%. Other chemicals

used were of analytical grade. All the solutions used in the experiments were prepared using ultrapure water through a Millipore (Millipore, Milford, MA, USA) Milli-Q water purification system.

2.2. Fourier Transform Infrared Spectroscopy (FT-IR) and ^{1}H-nuclear Magnetic Resonance (^{1}H NMR) spectroscopy

FT-IR spectra were obtained with a Jasco 4100 series with an attenuated total reflection cell (Jasco Inc., Easton, MO). All samples were prepared as KBr pellets and were scanned against a blank KBr pellet background.

^{1}H NMR spectra were obtained on a Mercury 400 spectrometer (400 MHz for ^{1}H) in D_2O containing a small amount of CD_3COOD at 25 ℃.

For pH dependence of the samples water solubility, the test samples (0.5g) were dissolved in 1% HAc (50 ml). With stepwise addition of NaOH solution (1 M), the transmittance of the solutions was recorded with a UV-vis spectrophotometer (UV-1100, MAPADA) at 600 nm.

2.3. Synthesis of HTCC

The HTCC was prepared in a similar manner to the method reported by Lim and Hudson[31]. Briefly, CS (2.0 g, 12.3mmol) was dispersed in isopropyl alcohol (20.0mL) and the solution was adjusted to pH 9, stirring the mixture until the CS was evenly dispersed, heating the solution to 80°C. EPTMAC (11.22 g, 73.8mmol) was dissolved in water and added to CS suspension at 1 h intervals. After reaction for 6 h, the reaction mixture was precipitated by acetone, washed repeatedly until the solution become neutral, dissolved in distilled water. The end-product was obtained by freeze

drying after 5-day dialysis.

2.4. Degree of quaternization (DQ)

The DQ of the HTCC was measured by titrating theamount of Cl^{-1} ions on the HTCC with aq $AgNO_3$ solution. DQ is defined as the mol ratio of bonded EPTMAC per mol of glucosamine calculated from the original mass of CS and its degree of deacetylation (DD)[31, 32]. Thoroughly dried HTCC (0.1000 g) was dissolved in deionized water (100mL) and conductometrically titrated with 0.017 M $AgNO_3$ aq solution. Solution conductivities were monitored with conductometer 644 Metrohm, Swiss. During the titration, the temperature of the solution was kept constant (20.4-20.5 °C) using a water bath.

2.5. Molecular weight determination

The average molecular weight (Mw) of quaternized CS was determined by using the gel permeation chromatography (GPC) in conjunction with multi-angle static light scattering detector (DAWN HELEOS II, WYATT, USA) and refractive index detector (Optilab T-rEX, WYATT, USA). All samples were dissolved in acetate buffer (pH 4.5) and then filtered through nylon syringes filters (450nm) (Vertical chromatography Co., Ltd. Thailand). The mobile phases, 0.5 M AcOH and 0.5 M AcONa (acetate buffer pH 4.5), were used at a flow rate of 0.6mL/min at 30℃. Then the injection volume of 20μL was used.

2.6. Preparation of zein-HTCC NPs and Cur loading

Zein was dissolved in aqueous ethanol solutions (75% v/v) to obtain a stock solution with final concentration of 5mg/mL. HTCC solution was prepared by dissolving

155 weighed HTCC powder into water. Then, 1mL of zein solution was rapidly mixed
156 with 7mL of HTCC solution with different concentration. The solution was under
157 vigorous stirring until a single phase was formed, consisted of different weight ratios
158 of zein: HTCC at 3:1, 2:1, 1:1, 1:2 and 1:3, respectively.
159 For Cur loading, the stock of 20 mg/mL Cur prepared with ethanol was firstly mixed
160 with zein solution for 60 min. The formulation containing Cur was prepared by
161 adding the above solution dropwise to $HTCC_1$ solution with magnetic stirring,
162 resulting in different weight ratios of zein: $HTCC_1$ at 3:1, 2:1, 1:1, 1:2 and 1:3,
163 respectively. The finally obtained Cur concentration was 50μg/mL.

164 **2.7. Characterizations of NPs**

165 2.7.1. Particle size and zeta potential
166 Dynamic laser scattering (DLS) and zeta potential measurements of all blank and
167 Cur/zein-HTCC NPs were performed on a commercial laser light scattering
168 instrument (Malvern ZEN3690, Malvern Instruments) at 25°C and 90° scattering
169 angle.

170 2.7.2 Morphology observation
171 Transmission electron microscope (TEM) images were taken on a JEM-2100F (JEOL,
172 Japan). The samples were prepared by dropping solution onto copper grids coated
173 with carbon and then dried naturally.

174 2.7.3. X-ray diffraction (XRD) and fluorescence spectrum
175 Molecular arrangement of Cur, zein and $HTCC_1$ in powder, as well as the NPs and
176 Cur/zein-$HTCC_1$ NPs was compared by powder X-ray diffraction patterns acquired at

room temperature on a Bruker D8-Advance Diffractometer (Bruker AXS Inc., Madison,WI, USA) with backgroundless sample holders. The data were collected over an angular range from 5° to 50° 2θ in continuous mode using a step size of 0.02° 2θ and step time of 5 seconds.

The fluorescence emission spectra of Cur were determined using a Cary Eclipse fluorescence spectrophotometer (Varian Instruments, Walnut Creek, CA) as the excitation wavelength. Cur (10μg/mL) was dissolved in ethanol and 10%v/v ethanol. The excitation wavelength was set at 420 nm, and the emission spectra were ranged from 450 to 700 nm. The slit openings were set at 5 nm for both excitation and emission.

2.8. Encapsulation of Cur

Cur in the percolated solutions was determined by a UV-vis spectrophotometer (UV-1100, MAPADA) at 428 nm. The free Cur was obtained by calculating the Cur content that were ultracentrifuged at 4000 × g for 30 min in a refrigerated centrifuge (TGL-20000cR) with angle rotor. The encapsulation efficiency (EE) and loading capacity (LC) were defined as the drug content that was entrapped into zein-HTCC NPs and calculated as follows:

$$EE(\%) = \frac{\text{Total Cur - Free Cur}}{\text{Total Cur}} \times 100$$

$$LC(\%) = \frac{\text{Total Cur - Free Cur}}{\text{Weight of zein - HTCC NPs}} \times 100$$

2.9. Cur protection

To elucidate the effect of encapsulation on the stability of Cur against external severe processing, we compared with remnant content of free Cur with that of entrapped Cur

199 in zein-HTCC NPs after thermal treatment and ultraviolet radiation. Free and
200 encapsulated Cur with concentration of 10μg/mL shared pasteurization treatment
201 (60°C, 30 min or 80 °C, 1 min) and ultraviolet radiation (30 W, 254 nm) with 40 cm
202 distance was taken into account for the protective effect. For the Cur encapsulated
203 NPs, ethyl alcohol was added and extracted with the same volume for 4 h, and then
204 evaporated overnight at 40 °C under vacuum[33]. The existent Cur was calculated
205 through the absorption value at 428 nm.
206 For light stability studies, the UV absorbance of free Cur (10μg/mL in 10% v/v
207 ethanol) and Cur/zein-$HTCC_1$ were recorded at 428 nm for 24 h under ambient
208 conditions.

209 **2.10. Radical-scavenging activity by DPPH method**

210 To guarantee the bioactivity of encapsulated Cur, the antioxidant activity of Cur was
211 measured according to the DPPH method with minor modification[34, 35]. Briefly, the
212 scavenging activity assay was carried out by monitoring the absorbance of an
213 ethanolic solution of DPPH (100μM) at 517 nm in the presence and absence of the
214 test compounds at room temperature with a UV-vis spectrophotometer. The
215 antioxidant activity of Cur was expressed as the percentage of DPPH that was
216 decreased in comparison with that of the control condition (i.e., the testing solution
217 without the presence of Cur) after 30 min preservation in the dark.

218 **3. Results and discussion**

219 **3.1. Synthesis and characterization of HTCC**

220 In basic aqueous solution, CS with different molecular weight (Mw) was coupled with

EPTMAC to give the water-soluble HTCC, in which the amino group of CS was suffciently nucleophilic to induce ring-opening of EPTMAC (Scheme 1) [23]. The Mw and DQ of HTCC were shown in Table 1. The DQ of the HTCC was measured by conductometric titration of Cl^{-1} with 0.017 M aq $AgNO_3$ solution. The amount of $AgNO_3$ used at the end point equaled the amount of Cl^{-1} ions presented on the HTCC[31].

The FT-IR spectra of CS and HTCC were measured with KBr pellets in the range of 500-3750 cm^{-1}. In the spectrum of HTCC, the characteristic peak (1568 cm^{-1}) representing NH_2 deformation was weakened and two new peaks positioned at 1483 and 2916 cm^{-1} were appeared (Fig. 1a), which were attributed to the bending mode and flex mode of $-CH_3$ in quaternized ammonium, indicating that the introduction of the quaternary ammonium salt group on CS backbone[24, 36]. To further confirm the success of the reaction, ^1H NMR analysis of CS and HTCC was performed in CD_3COOD-D_2O. The NMR spectra of the samples were shown in Fig. 1b and Fig. 1c. As evidence of the reaction, methyl groups in the quaternary ammonium salt group were observed as a very strong peak at 3.2 ppm.

Fig. 1d showed the pH dependence of CS and HTCC solutions. At low pH (pH < 6.0), the transmittance was close to 100% not only for the HTCC solution but also for the CS solutions. When the pH increased from 6.0 to 7.0, the transmittance of the CS solution rapidly dropped, and the solution became opaque. In contrast, the transmittance of HTCC solution did not changed. These results illustrated that HTCC has a better solubility in neutral and basic conditions than CS[32].

3.2. Optimization of the Formulation

Due to cationic properties of HTCC, zein can be investigated to form NPs with HTCC to develop novel drug delivery systems. The effects of preparation parameters on particle size and zeta potential in different formulations were summarized in Table 2 and Table 3.

The particle size of Cur-encapsulated zein NPs without $HTCC_1$ coating was 134 nm. After $HTCC_1$ coating was applied on zein NPs, the particle size varied with the weight ratios of zein and $HTCC_1$. With the increase of $HTCC_1$ ratios, particle size of Cur/zein-$HTCC_1$ NPs increased from around 66 to 156 nm. At zein-$HTCC_1$ ratio of 3:1, 2:1 and 1:1, the particle size was even lower than of the zein NPs which might be due to the reason that opposite surface charge of zein and $HTCC_1$ could let these two kinds of compounds more close to each other[37]. Besides, the NPs had a small PDI less than 0.16 except for Cur/zein NPs which had a greater PDI of 0.25. The zeta potential of Cur/zein NPs was −17.3 mV. After coated by $HTCC_1$, the zeta potential of NPs became highly positive in the range of +36.3 to +42.8 mV which slightly augmented with the increase of $HTCC_1$ concentrations. These observations confirmed that $HTCC_1$ was successfully coated on the surface of Cur/zein NPs by electrostatic interactions. The encapsulation efficiency (EE) of different formulations was also demonstrated in Table 2. The EE of zein NPs without $HTCC_1$ was around 85.2% and increased to 92.7% at a zein-$HTCC_1$ ratio of 1:1 (Table 2). This could be ascribed to the $HTCC_1$ through electrostatic interactions, resulting in the thick and dense Cur/zein- $HTCC_1$ NPs and therefore an increase of EE.

The effect of HTCC Mw on the NPs size, zeta potential, PDI, EE and LC was also investigated (Table 3). The size increased as Mw of HTCC increased (the ratio of zein: HTCC was kept at 1:1). This phenomenon might be due to longer molecular chains of HTCC with larger Mw entangled with negatively charged zein NPs through ionic interactions would give rise to bigger complex. Moreover, zeta potential increased with the HTCC Mw. This observation can be easily explained by the stronger electrostatic interaction between HTCC and zein. Higher Mw of HTCC with higher degree of quaternization was expected to provide more positive charge and compact NPs because a greater number of trimethylammonium groups of HTCC interacted with zein NPs. Moreover, the wide distribution led to an increase in PDI. Seen from Table 3, higher Mw of HTCC led to higher EE because the longer chain of HTCC molecule could entrap more Cur[27].

3.3. Influence of pH on zein NPs and zein-HTCC$_1$ NPs

To study the effect of pH on the precipitation kinetics of zein NPs and zein-HTCC$_1$ NPs, all samples were studied by dispersing the pre-formed formations in deionized water adjusted to pH from 2 to 11 using 0.1 N HCl or 0.1 N NaOH. Zein has an isoelectric pH of 6.2 and hence the particles have tendency to aggregate at neutral-basic pH. At pH 6 of the zein NPs solution, the precipitation appeared (Fig. 2a). However, it kept small particle size and PDI for zein-HTCC$_1$ NPs at this pH (Fig. 2b). The zeta potential of zein NPs was positive at pH <6, while the zeta potential was negative at pH >7 (Fig. 2a). At acid condition, the decrease in the zeta potential of zein-HTCC$_1$ might be due to the stronger ion strength, leading to the charge screening

287 effect. Moreover, at highly alkaline pH value, the decline of the zeta potential of
288 zein-HTCC$_1$ NPs was attributed to the augment of zeta potential (negative charge) of
289 zein NPs. The similar observation was also revealed in other studies[37,38]. The particle
290 size of zein-HTCC$_1$ NPs kept almost constant from pH 3 to 10 (Fig. 2b). The PDI in
291 the whole pH range (2-11), was less than 0.2. Our finding was that the zein-HTCC$_1$
292 NPs were stable at a broad range of pH, which was an appealing advantage for further
293 application.

294 **3.4. Morphological Observation**

295 The morphological observation of zein NPs, zein-HTCC$_1$ NPs and Cur/zein-HTCC
296 NPs were performed by TEM. Figure 3a showed a typical size distribution profile of
297 zein NPs. TEM micrograph displayed that zein NPs without HTCC coating shared
298 features of a spherical shape (Fig. 3b). After zein NPs were coated by HTCC$_1$, the
299 complex formed sphere NPs with smooth surface and much smaller and more
300 homogeneous diameter (Fig. 3c and 3d). The reduced particle size might be
301 contributed by the electrostatic interactions between zein NPs and HTCC$_1$ molecules.
302 As shown in Fig. 3e-3i, the incorporation of Cur did not cause morphological changes.
303 With the Mw of HTCC increased, the size of NPs increased. The TEM diameter of the
304 NPs was smaller than that obtained from DLS. This phenomenon may be due to the
305 inherent difference in detection of the particle size between DLS and TEM. DLS
306 provides the data of the NPs swollen in solution, whereas that obtained by TEM
307 showed the images of the NPs spread and dried on copper grids coated with carbon
308 film[39, 40].

3.5. FT-IR, XRD and fluorescence spectrum

Figure 4a showed spectra of zein, zein-$HTCC_1$ NPs, Cur/zein NPs and Cur/zein-$HTCC_1$ NPs. In the original spectra of zein (Fig. 4a) and HTCC (Fig. 1a), the bands of hydrogen bonds were at 3441, and 3477 cm^{-1}, respectively. However, after formation of NPs, shift of hydrogen bonds occurred, and the peaks were at 3422, 3432, 3438 cm^{-1} in the spectra of zein-$HTCC_1$ NPs, Cur/zein NPs and Cur/zein-$HTCC_1$ NPs respectively, suggesting the hydrogen bonding was formed between Cur and zein, zein and $HTCC_1$. Therefore, the hydrogen bonding among zein, HTCC, and Cur was considered to be one of the major forces facilitating NPs formation. The amide I band of zein at 1646 cm^{-1} showed the prominent C=O stretching and the amide II band at 1549cm^{-1} involving C-N stretching. It shifted significantly to 1639 and 1541 cm^{-1}, respectively in Cur/zein NPs, suggesting that the electrostatic interactions were another intermolecular force between Cur and zein. Compared with zein, the bands of amide I and amide II groups shifted to 1655 and 1542 cm cm^{-1}, respectively, in zein-$HTCC_1$ NPs, indicating the electrostatic interactions between zein and $HTCC_1$. A further shift of these bands occurred after Cur was encapsulated into NPs. Besides, because both zein and Cur are highly hydrophobic molecules, the hydrophobic interactions could also contribute to the formation of NPs.

The XRD patterns of the NPs and pure Cur were shown in Figure 4b. The major characteristic peaks of Cur were at appeared at 8.90, 12.26, 14.54, 17.24, 23.33, 24.60 and 25.52 degree, indicative of their highly crystalline nature[41]. In contrast, zein showed two flatter humps instead of sharp peaks, indicating the amorphous nature of

the protein[42]. Cur specific peaks disappeared in all the formations of suggesting that Cur in the NPs or the complex did not exist as a crystal form which provided additional evidence of encapsulation.

Considering that Cur itself is a fluorescent compound, and the fact that the fluorescence spectrum of a compound is usually affected by its microenvironment, we compared the emission spectrum of Cur in zein-HTCC NPs with that of Cur in ethanol and 10%v/v ethanol. Cur has very poor water solubility (11ng/mL) and is a major factor that limits its in vivo bioavailability. Zein-$HTCC_1$ NPs significantly increased the water solubility of Cur, as evidenced from the evident increase in Cur fluorescence compared to the free Cur in 10%v/v ethanol (Figure 4c). The fluorescence of Cur in zein-$HTCC_1$ NPs was lower than the fluorescence of Cur in 100% ethanol which also was anther evidence for encapsulation. The emission peak of Cur in ethanol was at 542 nm, while the peak shifted to 531 nm when Cur was encapsulated in zein-$HTCC_1$ Cur. This result further confirmed that the microenvironment of Cur was changed upon Cur-encapsulation[4].

3.6. Cur protection

As we know, Cur's applications are limited due to its low water solubility and sensitivity to alkaline conditions, thermal treatment, ultraviolet radiation, metallic ions, enzymes, oxygen and ascorbic acid, thus restricting its bioavailability[43]. However, pasteurization and radiation sterilization were common technologies in food processing industry. The thermal and ultraviolet and light stability of encapsulated Cur was estimated in comparison to non-encapsulated Cur (Fig. 5a). After 60 °C treatment

for 30 min, 80 ℃ treatment for 1 min and ultraviolet radiation for 2 h, the unchanged Cur was reduced to 21.9%, 29.2% and 24.5%, respectively for free Cur (Fig. 5a). When Cur was loaded into zein NPs, the retention of Cur was 74.5%, 73.5% and 60.4%, respectively (Fig. 5a). Interestingly, the zein/$HTCC_1$ NPs protective effect was more obvious. When the weight ratio of zein/$HTCC_1$ was 1:1，the Cur preserved could reach to 79.2%, 77.3% and 60.5%, respectively, when subjected 30 min 60 ℃ treatment, 1 min 80 ℃ treatment and 2 h ultraviolet radiation (Fig. 5a). Compared with free Cur at the same treatments, the zein/$HTCC_1$ protected Cur content was enhances about 2.7 fold, 3.5 fold and 2.5 fold. However, in the same proportion, protective effect had no further increased as the Mw increased (Fig. 5a). The results clearly showed that the stability of encapsulated Cur at the weight ratio of zein: $HTCC_1$ = 1:1, could be protected and improved, which was helpful to broaden Cur potential application in the food field. As can be seen from Figure 5b, the absorbance of free Cur decreased rapidly to 50% within 30 min, while the absorbance of Cur in zein-$HTCC_1$ NPs decreased gradually and the absorbance was seen up to 12h(Fig. 5c). The light stability of Cur was enhanced dramatically after encapsulation in zein-$HTCC_1$ NPs.

3.7. Antioxidant activity

Antioxidants can scavenge free radicals and maintain its safety, nutritional quality, functionality and palatability by retarding the process of lipid peroxidation, which is one of the major reasons for deterioration of food and pharmaceutical products during processing and storage[44]. Cur is a natural compound reported to possess strong

antioxidant activity. In our study, we used the DPPH scavenging method to verify the radical-scavenging ability of zein-HTCC NPs loaded Cur. Without any treatment in control group, the antioxidative effectiveness of disposed Cur was lower than that of free Cur (Fig. 6), indicating its instability in food process, which has been found in previous studies and consistent with above results[45]. The antioxidant activity of free Cur was about 72.2%, revealing the higher antioxidant activity, while the scavenging rate decreased to 39.9%, 41.6% and 30.6% with 60 ℃ treatment for 30 min, 80 ℃ treatment for 1 min and ultraviolet radiation for 2 h (Fig. 6). When encapsulated into zein NPs, under the same treatments，the antioxidant activity increased as compared with the free Cur except for free Cur in control group without any treatment (Fig. 6). Comparatively, the Cur/zein-HTCC NPs was given a higher antioxidant activity in the same conditions. Moreover, in the weight ratio of zein: $HTCC_1$ = 1:1, the DPPH radical-scavenging ability of protected Cur were improved 13.3%, 26.9% and 29.0% compared with free Cur, when the solutions were pasteurized at low and high temperature and ultraviolet radiation respectively (Fig. 6). However, with the Mw increased，the antioxidant activity did not further enhanced，which also co-responded with the protection experiments (Fig. 6). The obtained results confirmed that the loaded Cur still retained its free radical scavenging ability, even after it had been subjected to an encapsulation.

4. Conclusion

In this work, zein-HTCC NPs was fabricated successfully as a novel delivery system for Cur, using a low-energy liquid-liquid dispersion method. By coating Cur/zein NPs

with HTCC, particle size was dramatically changed and zeta potential was increased to be highly positive, depending on Mw of HTCC. Hydrogen bonding and electrostatic interaction as well as hydrophobic interaction were considered to be the major forces facilitating the formation of NPs. Cur was encapsulated in zein-HTCC NPs, improving its solubility and stability and preserving its bioactivity in pasteurization and ultraviolet radiation, which broadened its application in functional foods and medical field. The worthwhile endeavor elucidated proteins/polysaccharides complex was feasible to solubilize and protect sensitive amphiphilic bioactive compounds and has extensive potential in food and medicinal application with various purposes.

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507 **Figure captions:**

508 Fig.1 FT-IR spectra of CS and HTCC (a). ^{1}H NMR spectra data of CS (b) and HTCC (c). pH
509 dependence of water solubility of CS and HTCC (d); the concentrations of CS and HTCC are 1%
510 (w/v). CS represented the one made for HTCC. HTCC represented the Mw of 8.708×103. Data
511 were displayed as mean±SD (n=3).

512

513 Fig.2 Effect of pH on particle size, zeta potential and PDI from 2 to 11.0 on zein NPs and zein-
514 $HTCC_1$ NPs prepared at zein: $HTCC_1$ ratio of 1: 1 w/w. Digital photographs were shown inset.
515 Data were displayed as mean±SD (n=3).

516

517 Fig.3 Size distribution of zein NPs (a) and zein-$HTCC_1$ NPs (c) prepared at zein: $HTCC_1$ ratio of
518 1:1 w/w. TEM photos of zein NPs (b) and zein-$HTCC_1$ NPs (d). TEM images of zein- $HTCC_1$ NPs
519 (e), zein-$HTCC_2$ NPs (f), zein-$HTCC_3$ NPs (g), zein-$HTCC_4$ NPs (h), zein-$HTCC_5$ NPs (i) at
520 zein-HTCC ratio of 1:1 w/w with the concentration of Cur at 50μg/mL.

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522 Fig.4 FT-IR (a) and XRD (b) of different samples. Cur, curcumin powder; zein, zein powder;
523 zein-$HTCC_1$, zein-$HTCC_1$ NPs prepared using zein: $HTCC_1$ ratio of 1: 1 w/w; Cur/zein,
524 curcumin-loaded zein NPs at curcumin concentration of 50μg/mL; Cur/zein-$HTCC_1$,
525 curcumin-loaded zein-$HTCC_1$ NPs prepared using zein: $HTCC_1$ ratio of 1: 1 w/w at curcumin
526 concentration of 50μg/mL. Fluorescence spectra of curcumin (10μg/mL) in ethanol, 10% ethanol
527 solution and Cur-loaded zein-$HTCC_1$ NPs solution (Cur equivalent of 10 μg/mL) (c).

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529 Fig.5 Cur protection by zein-HTCC with different Mw of HTCC at the zein-HTCC ratio of 1:1
530 w/w in the heat and ultraviolet treatment (a). Absorbance of Cur (10μg/mL) in 10% ethanol
531 solution (b) and Cur-loaded zein-$HTCC_1$ NPs (Cur equivalent of 10 μg/mL) solution (c) as a
532 function of time. Data were displayed as mean±SD (n=3).
533
534 Fig.6 Radical scavenging activity improvement by Cur entrapment with different Mw of HTCC at
535 the zein-HTCC ratio of 1:1 w/w. Control group indicated samples without any treatments. Data
536 were displayed as mean±SD (n=3).
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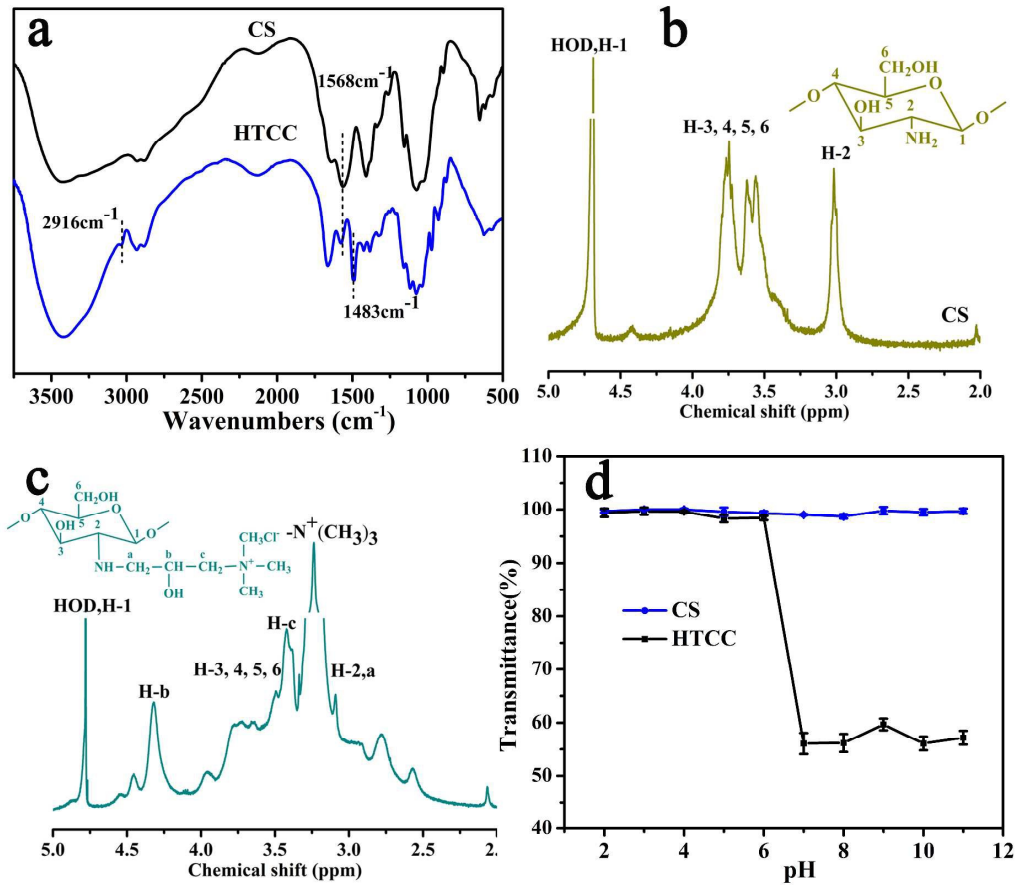
551 Fig.1



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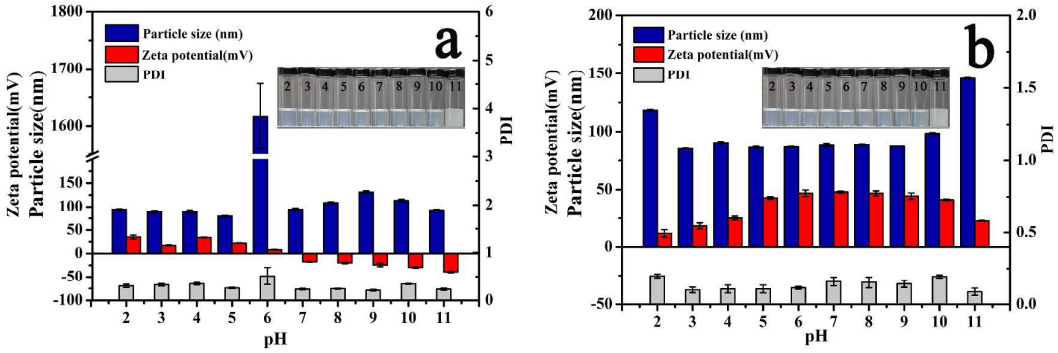
563 Fig.2



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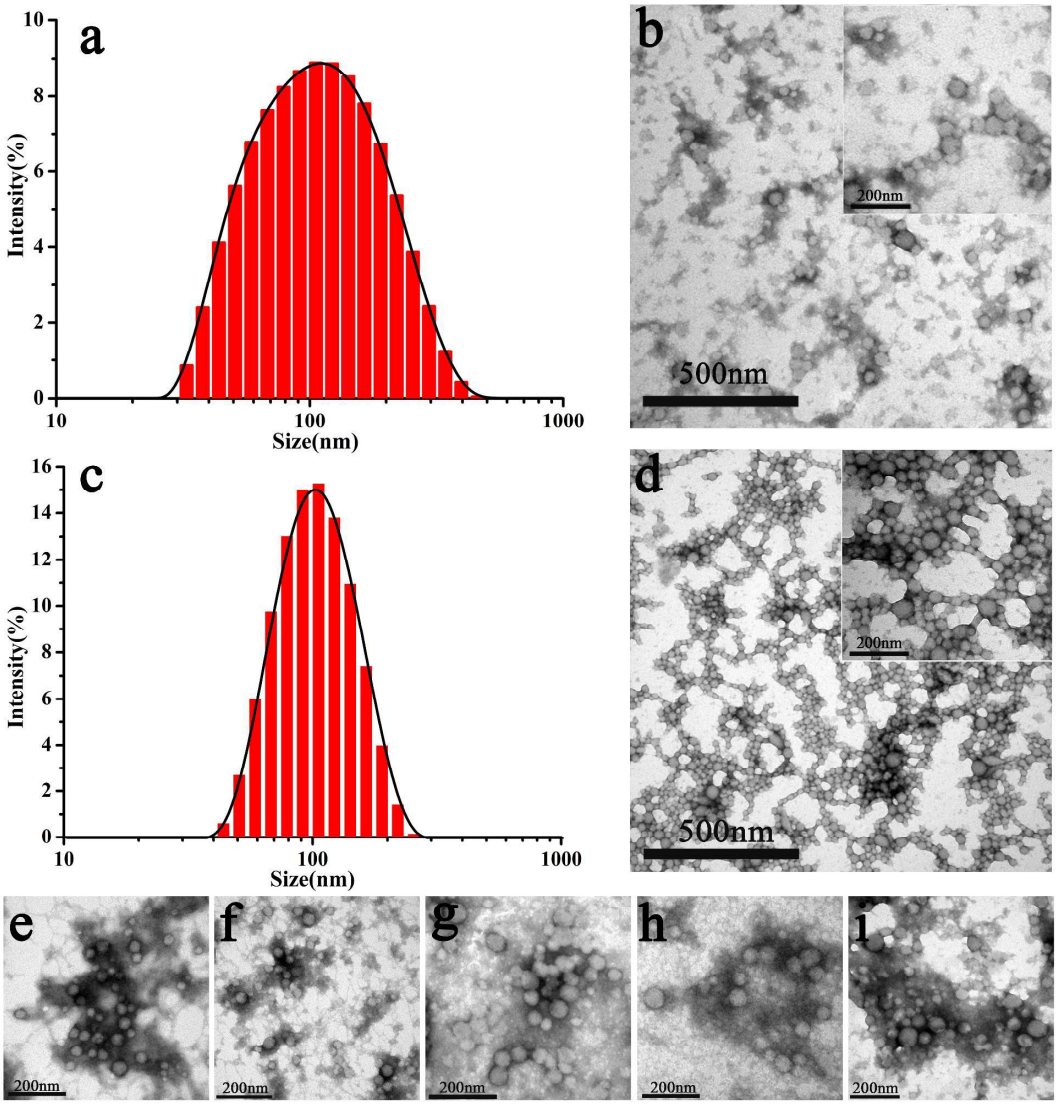
582 Fig.3



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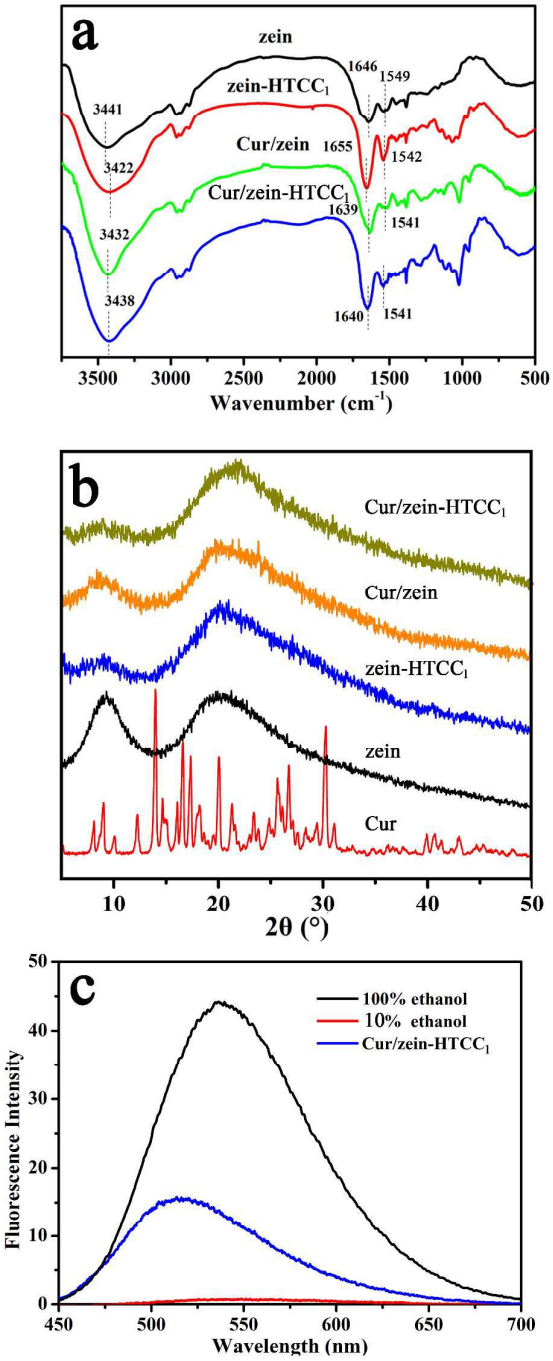
591 Fig.4



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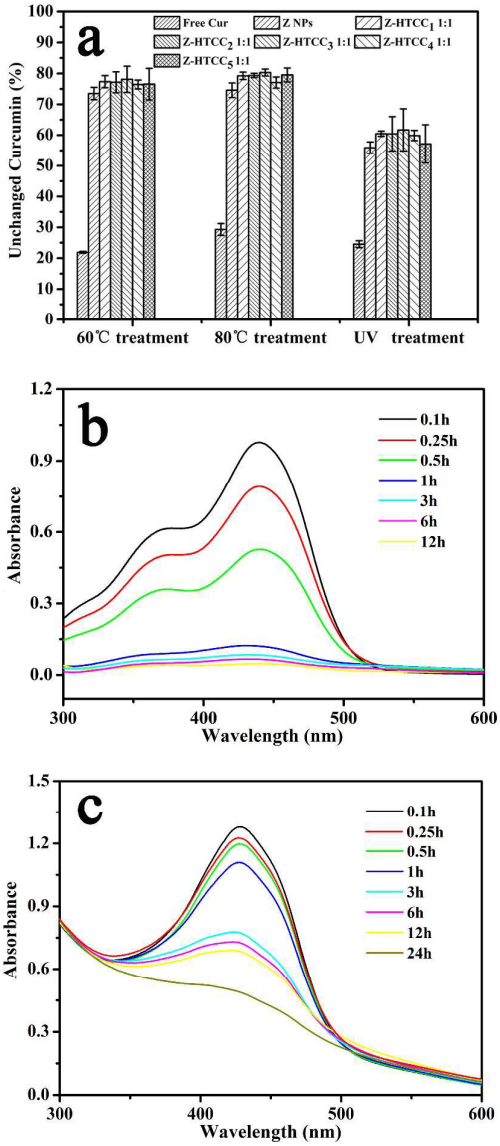
596 Fig.5



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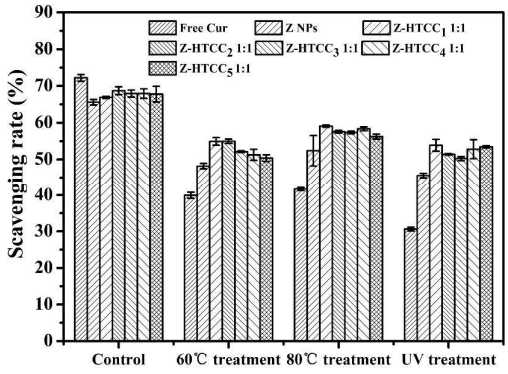
604 Fig.6



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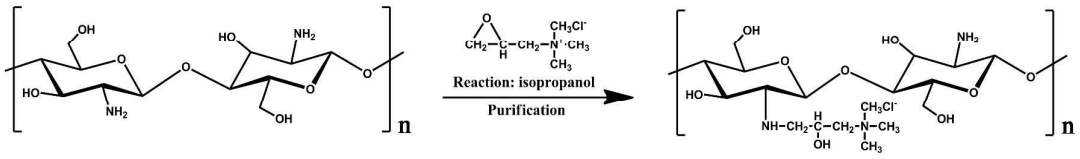
622 Scheme 1



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624 Scheme 1. Synthesis of the HTCC

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643 Table 1

644 Table 1 Properties of HTCC

Sample	Degree of quaternization	M_W
$HTCC_1$	0.623±0.02	8.708×10^3
$HTCC_2$	0.869±0.03	1.688×10^5
$HTCC_3$	0.879±0.04	3.322×10^5
$HTCC_4$	0.907±0.03	7.051×10^5
$HTCC_5$	0.921±0.05	1.832×10^6

645 $HTCC_1$-$HTCC_5$ represented HTCC with different quaternization degree and different molecular

646 weight. Data were displayed as mean±SD (n=3).

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662 Table 2

663 Table 2 Effect of zein: $HTCC_1$ weight ratios on the size, PDI, zeta potential, EE and

664 LC of NPs

Samples	Size(nm)	PDI	Zeta potential(mV)	EE(%)	LC(%)
Z	134.8±2.9	0.25±0.02	-17.3±1.5	85.2±1.2	8.5±0.12
Z-$HTCC_1$ 3:1	66.4±0.30	0.15±0.01	36.3±0.6	87.7±1.2	6.5±0.11
Z-$HTCC_1$ 2:1	79.1±0.30	0.14±0.02	37.9±0.6	89.4±2.4	5.7±0.16
Z-$HTCC_1$ 1:1	121.0±1.6	0.13±0.01	38.3±3.8	92.7±1.7	4.4±0.08
Z-$HTCC_1$ 1:2	154.0±2.0	0.13±0.03	39.0±0.6	89.7±2.4	2.8±0.08
Z-$HTCC_1$ 1:3	156.3±1.8	0.16±0.05	42.8±1.3	86.8±4.1	2.0±0.10

665 Z represents zein NPs without HTCC coating with the concentration of Cur at 50μg/mL. Other

666 samples represent formulations with different mass ratios of zein-$HTCC_1$ with the concentration of

667 Cur at 50μg/mL. PDI, polydispersity. EE (%), encapsulation efficiency. LC (%), loading capacity.

668 Data were displayed as mean±SD (n=3).

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680 Table 3

681 Table 3 Effect of HTCC Mw on the size, PDI, zeta potential, EE and LC of NPs

Samples	Size(nm)	PDI	Zeta potential(mV)	EE(%)	LC(%)
Z-HTCC$_2$ 1:1	105.1±2.1	0.29±0.04	48.6±3.1	92.9±3.9	4.4±0.10
Z-HTCC$_3$ 1:1	141.9±7.0	0.36±0.06	49.1±2.6	93.5±2.4	4.4±0.06
Z-HTCC$_4$ 1:1	158.3±3.1	0.48±0.03	54.2±4.7	93.9±1.2	4.4±0.08
Z-HTCC$_5$ 1:1	177.0±4.3	0.52±0.03	62.5±1.2	94.9±1.9	4.5±0.08

682 Samples represent formulations with different Mw of HTCC at the zein-HTCC ratio of 1:1 with
683 the concentration of Cur at 50μg/mL. PDI, polydispersity. EE (%), encapsulation efficiency.
684 LC(%), loading capacity. Data were displayed as mean±SD (n=3).

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