pH-driven preparation of caseinate-chitosan complex coacervation for co-encapsulation of hydrophobic curcumin and hydrophilic L-ascorbate: Stability and in vitro release propertiesrelease of curcumin in chitosan-stabilized caseinate particles

Xin Yin[a,b,c], Muhammad Aslam Khan[a,b], Weining Huang[a,b], Ning Li[d], Li Liang[a,b*], Paul Van der Meeren[c]

[a]State Key Lab of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu, China

[b]School of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu, China

[c]Particle and Interfacial Technology Group, Faculty of Bioscience Engineering, Ghent University, Coupure Links 653, B-9000 Gent, Belgium

[d]Guangzhou Puratos Food Co., Ltd, Guangzhou, Guangdong, China

[*]Corresponding author: Li Liang

Address: State Key Lab of Food Science and Technology, Jiangnan University, Wuxi, Jiangsu 214122, China

Telephone: +86(510)8519-7367

Email: liliang@jiangnan.edu.cn

Abstract

During the last years, the construction of binary particles has gained a growing attention for their use for the co-delivery of hydrophilic and hydrophobic bioactive compounds. The objective of this work was to assess the effectiveness of a L-ascorbate loaded chitosan coating on co-delivered curcumin in the caseinate core, by evaluating their interactions, protective effect as well as release behavior during *in vitro* simulated digestion. Caseinate-chitosan particles were fabricated by a combined facile pH conversion and ionic gelation method. Circular dichroism and infrared spectroscopy indicated that structural changes of caseinate were inhibited after coating with chitosan, and their interactions included hydrogen bonding and electrostatic interaction: electrostatic interaction occurred between L-ascorbate and chitosan, while hydrogen bonding primarily occurred between curcumin and caseinate. The particles could efficiently co-encapsulate around 47.6% L-ascorbate and 96.2% curcumin and exhibited a regulated release behavior *in vitro*, most of which were released in the intestine. The L-ascorbate-loaded chitosan coating greatly improved the storage and irradiation stability of curcumin. The $ABTS^{\cdot+}$ scavenging ability of the co-delivered system was significantly improved as compared to the two separate systems and basically unchanged during irradiation. These results can be used as helpful guide for future research to optimize bilayer particles for the co-delivery of multiple compounds with a beneficial health activity.

***Keywords*:** binary particle; L-ascorbate; curcumin; co-delivery; stability; regulated release

1. Introduction

Biopolymer-based edible carriers have been designed to overcome the challenge of bioactive compounds with chemical instability and low solubility by maximizing their function in commercial products. The pH-driven method is generally recognized as safe and utilizes the pH-dependent deprotonation-protonation of polyphenols and the unfolding-refolding properties of proteins [1]. As the major milk protein, caseinate has unique properties such as self-assembly properties and excellent gelation. It is thus an ideal candidate for food applications. However, caseinate is readily oxidized, which in turn accelerates the degradation of loaded polyphenols [2]. Furthermore, its instability in the gastrointestinal environment, as well as the low capture capacity for hydrophilic compounds pose significant challenges in fabricating stable delivery systems [3]. Accordingly, it is of major interest to fabricate biopolymer carriers with good colloid-chemical stability that can protect bioactive compounds from harsh conditions and control their release in a targeted manner.

Among the various modifications of caseinate-based carriers, the combination with polysaccharides has received considerable interest in polymer science. Advantageous structural features and better physicochemical properties can be attained in binary complexes than in either biopolymer [4]. Chitosan is a cationic polysaccharide composed of N-acetyl glucosamine and d-glucosamine units and can interact with other species owing to the presence of amino and hydroxyl groups along their chain. Chitosan-based carriers were reportedly used to capture bovine serum albumin and to improve its stability in a gastric environment [5]. In addition, tripolyphosphate (TPP)

as a cross-linking agent with pentavalent anions can ionically crosslink amine groups of chitosan to form nano-complexes, which have the capacity to entrap hydrophilic L-ascorbate [6]. With the promising findings on synergistic activity between hydrophilic and hydrophobic bioactive compounds, the focus on protein-polysaccharide particles has recently shifted to explore their potential in the application of co-delivery and the interactions between them [7].

Curcumin is the major polyphenol isolated from the rhizome of turmeric. Beyond being used as a spice and color-agent in the food industry, its consumption has been linked to the prevention and treatment of cancer, cardiovascular diseases, diabetes and other diseases thanks to its multiple bioactivities [8]. Meanwhile, antioxidants with lower hydrogen dissociation energy than curcumin appear to be effective co-factors to suppress the formation of phenolic radicals of curcumin [9, 10]. It was reported that the combination with ascorbic acid improved the antioxidant activity of curcumin as well as reduced the minimum inhibitory concentrations (MIC) against Candida strains [9]. Additionally, the co-intake of curcumin and ascorbic acid prevented ionizing radiation-induced cellular DNA damage in peripheral blood leukocytes [11]. These observations arouse the growing interest in the development of co-delivery systems of curcumin and ascorbic acid.

However, designing co-delivery carriers for curcumin and L-ascorbate remains quite challenging. Curcumin is hydrophobic with a LogP of about 4.16 [a], whereas L-ascorbate (L-SA) is the sodium salt of hydrophilic ascorbic acid, whose LogP is about -1.9 [b]. In this study, binary particles of caseinate and chitosan were constructed by

combining the pH conversion and ionic gelation methods. Curcumin was located in the caseinate and stabilized by a L-ascorbate-loaded polysaccharide coating. The binary particles were characterized by dynamic light scattering and atomic force microscopy, and the interactions between them were verified by infrared spectroscopy and circular dichroism. Moreover, the encapsulation, chemical stability and *in vitro* release behavior of curcumin and L-ascorbate were analyzed. The obtained results contribute to the design of co-delivery systems and to the exploration of their potential application in multi-functional foods.

2. Materials and methods

2.1. Materials

Sodium caseinate (SC), chitosan (with a deacetylation degree of about 75-85%), glacial acetic acid and sodium tripolyphosphate (TPP) were purchased from Sigma-Aldrich Co. (St. Louis, MO). Curcumin and 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) were provided by ChenGuang Biotech Group Co., Ltd. (Handan, China) and Aladdin Bio-Chem Technology Co. Ltd. (Shanghai, China), respectively. Sodium L-ascorbate (L-SA, purity≥98%) and other agents of analytical grade were purchased from SinoPharm CNCM Ltd. (Shanghai, China).

2.2. Preparation of L-ascorbate-loaded chitosan coating

L-ascorbate-loaded chitosan was induced by TPP according to a previously described method with slight modifications [12]. Firstly, the chitosan solution (0.6%, w/v) was hydrated in 1% (w/v) acetic acid solution, and then filtered through a 0.45 μm

syringe filter. L-ascorbate stock solution (600 μg/mL) and TPP solutions were sequentially added into the chitosan stock solution and stirred at 750 rpm for 2 h. The final weight ratio of chitosan to TPP was 4:1. Blank samples were prepared by replacing the L-ascorbate stock solution with the same volume of ultrapure water.

2.3. Preparation of chitosan-stabilized caseinate particles

Caseinate (0.2%, w/v) was dispersed in ultrapure water and kept overnight to ensure complete hydration. Curcumin power was added into the caseinate solution, adjusted to pH 12.0 and stirred for 1 h. Exactly 5 mL of chitosan stock solution without or with L-ascorbate was equally added into the caseinate solutions and held for 30 min under agitation. The pH of the mixed system was adjusted to 5.0 with either 0.1M HCl or NaOH. The final concentrations of chitosan and caseinate were both 0.1%, whereas that of L-ascorbate and curcumin were both 100 μg/mL.

2.4. Determination of particle size and ζ-potential

The particles were analysed at 25 °C on a NanoBrooker Omni Particle Size Analyzer (Brookhaven Instruments Ltd, New York, NY) with a He/Ne laser (λ=633 nm) and a scattering angle of 173°. The particle size was analyzed using a NNLS model, whereas the ζ -potential was calculated by the Smoluchowski equation based on phase analysis light-scattering (PALS) measurements.

2.5. Atomic force microscope (AFM)

Samples were diluted 20 times, dropped onto a freshly-cleaved mica surface and dried at room temperature for 12 h. The morphology was analyzed with a

Dimension fastscan AFM (Bruker Corporation, Billerica, MA) using the Tapping Mode.

2.6. Quantitation of L-ascorbate and curcumin

L-ascorbate was determined according to a previous method [13]. Briefly, 0.6 mL of sample was mixed with 1 mL of 4.5% metaphosphoric acid for 1 min, and then centrifuged at 3,500 g at 4 °C for 5 min. Exactly 1.0 mL of supernatant was collected and passed through a 0.22 μm syringe filter and injected into an HPLC system (Waters, Milford, MA) equipped with a 2695 separation module, a 2998 PDA detector and a T3 column (5 μm, 4.6 mm×250 mm, Waters, USA). All samples were protected from light, and kept in an ice bath to avoid loss of L-ascorbate during the process. The mobile phase consisted of a mixture of 0.3% (v/v) formic acid and 3% methanol. The flow rate was 1 mL/min and the column temperature was set at 35 °C. UV detection at 245 nm was used to analyze L-ascorbate.

For curcumin, 0.6 mL of sample was mixed with 2.4 mL ethanol for 1 min and then centrifuged at 10,000 g for 5 min at 4 °C. A mobile phase with acetonitrile, methanol and phosphoric acid (0.05%, v/v) in a volume ratio of 70:5:25 and UV detection at 425 nm were used to analyze curcumin[14].

2.7. Loading efficiency of L-ascorbate and curcumin

The loaded L-ascorbate and curcumin in the particles were separated from the aqueous suspension by ultracentrifugation at 100,000 g at 4 °C for 60 min using a himac CP100NX centrifuge (Hitachi Koki Co. Ltd., Tokyo, Japan) with a P100AT2-1014 rotor. The loading efficiency (LE) was calculated according to the following formula:

149 $$LE\ (\%) = \frac{C_0 - C_s}{C_0} \times 100 \quad (1)$$

150 Where, C_0 is the total concentration of bioactive compounds in the system and C_s is the
151 concentration of free compounds in the supernatant.

152 **2.8. Fourier transform infrared spectroscopy (FTIR)**

153 Samples before and after storage were freeze-dried in a Free Zone 2.5^{plus} freeze
154 dryer (Labconco Corp., Kansas City, MO). Freeze-dried samples were mixed with
155 potassium bromide at a mass ratio of 1:100 and then pressed into a transparent pellet.
156 FTIR spectra in the range of 4000-400 cm^{-1} were obtained on a Nicolet^{TM} iS^{TM} 10 FT-
157 IR Spectrometer (Thermo Fisher Scientific, Waltham, MA) by 64 scans with a
158 resolution of 4 cm^{-1}.

159 **2.9. Circular dichroism (CD)**

160 CD measurements were carried out in the far-UV region (190-250 nm) with n = 3
161 replications on a Chirascan V100 spectropolarimeter (Applied PhotoPhysics, Surrey,
162 United Kingdom). The optical path length was 0.1 cm. The background of chitosan/TPP
163 and bioactive compounds was subtracted from the raw spectra.

164 **2.10. Storage and irradiation stability**

165 Samples were stored at 45 °C in a JK-MGC-300A incubator (JKI, Shanghai, China)
166 or exposed to UVA light (λ~365 nm) by using an UVL-21 ultraviolet lamp (VWR
167 International Inc., West Chester, PA) with a fluence rate of 1 mW/cm^2. The contents of
168 L-ascorbate and curcumin were measured by HPLC as described above. Retention of

bioactive compounds during incubation was calculated by the equation as follows:

$$\text{Retention (\%)} = \frac{C_t}{C_0} \times 100 \quad (2)$$

Where, C_t is the concentration of L-ascorbate or curcumin at time t during incubation.

2.11. Antioxidant activity

The ABTS assay was performed according to a previous method with some modifications [15]. Briefly, a mixture of 7.4 mM ABTS and 2.6 mM $K_2S_2O_8$ in a volume ratio of 1:1 was placed in the dark for 12 h to produce an $ABTS^{\cdot+}$ solution. The radical cation solution was further diluted 25 times. After mixing 0.2 mL buffer or sample and diluted solution for 6 min, the absorbance of radical plus buffer (A_c) and sample (A_s) was recorded at 729 nm. The radical-scavenging activity was calculated as follows:

$$\text{Scavenging capacity (\%)} = \frac{A_c - A_s}{A_c} \times 100 \quad (3)$$

2.12. *In vitro* release assay

The release profile of curcumin and L-ascorbate was evaluated in simulated gastric and intestinal fluids [16, 17]. The simulated gastric fluid (SGF, pH 2) was composed of 0.32% (w/v) pepsin and 0.2% (w/v) sodium chloride. Exactly 5 mL of sample was distributed into 95 mL of SGF and incubated at 37 °C with continuous agitation for 2 h. Aliquots of digesta were taken at 0, 30, 60 and 120 min and replaced with fresh SGF. Then, exactly 20 mL of the above solution was mixed with 20 mL of simulated intestinal fluid (SIF) at pH 6.8, which contained 20 mM phosphate buffer, 0.5% (w/v) trypsin and 0.2% (w/v) sodium chloride. Four aliquots of 2 mL sample were withdrawn during the

intestinal digestion (at 180, 240, 300 and 360 min) and replaced with the same volume of fresh SIF. After centrifuging at 100,000 g for 60 min, the amount of bioactive compounds in the supernatant was measured. The cumulative release was calculated according to the following formula:

$$\text{Cumulative release (\%)} = \frac{W_t}{W_0} \times 100 \quad (4)$$

Where W_t and W_0 are the content of released bioactives at time t and the total content of bioactives in the system, respectively.

2.13. Statistical Analysis

All the experiments were carried out in triplicate. Data were presented as mean ± standard deviation and analyzed by a SPSS 20.0 statistical analysis system (IBM, Armonk, NY, USA) at 95% confidence.

3. Results and Discussion

3.1. Particle characterization

3.1.1. Particle charge, size and morphology

The pH-dependence of the electrical characteristics of the constituent polymers in the binary particles was analyzed to help understanding the formation and structure of the complexes. As shown in Fig. 1, the ζ-potential values of caseinate changed gradually from positive to negative as the pH increased from 2 to 10. There was a point of net charge around pH 4.7, which is close to the reported isoelectric point of caseinate (pH 4.6) [18]. The ζ-potential of chitosan was positive under acidic conditions due to

the protonation of amine groups. With the increase of pH to alkaline conditions, chitosan gradually lost most positive charges; the net charge of TPP-crosslinked chitosan was zero near pH 8 (Fig. 1). Some aggregation was visually observed with further increased pH due to its pH-responsive sol-gel transition properties [19]. The ζ-potential value of the binary particles was observed to be located in between the two individual components at any pH, indicating the occurrence of electrostatic interaction. According to the charge heterogeneity, still ample negative charges or patches are present in proteins at a pH close to and above the isoelectric point [20]. Additionally, similar to the chitosan system, the aggregation under neutral and alkaline conditions also occurred in binary particles (data not shown), which proved that the pH-dependence behavior of the binary particles was dominated by the chitosan coating. A similar phenomenon was observed in pectin-zein particles, where the core-shell structure of the binary particles was evidenced by the pH-responsive properties of the adsorbed anionic pectin [21].

The ζ-potential value of the binary particles measured at pH 5 was about +28 mV, which was not affected by curcumin (Table 1). Such observation was consistent with an earlier report of curcumin-fortified zein–pectin particles [21]. Concomitantly, the addition of curcumin increased the size of the binary particles, which was consistent with the behaviour of curcumin-loaded saponin particles [22]. Therefore, it was speculated that curcumin was incorporated in the hydrophobic core. However, addition of L-ascorbate slightly decreased the ζ-potential of the particles, which changed from +28 mV to +25 mV. This can be ascribed to the electrostatic attraction between the

carboxyl acid groups of L-ascorbate and amine groups of chitosan. As reported, the ζ-potential of TPP-crosslinked chitosan decreased with increased TPP concentration, whereby the optimized particles were obtained at a chitosan/TPP weight ratio of 2.5:1 [23]. In this study, the ratio of chitosan/TPP was 4:1. Hence, the amines of chitosan were not sufficiently ionic gelled by TPP, but partially bound to negatively-charged caseinate and L-ascorbate. This was also verified by the reduction in size of the binary particles upon addition of L-ascorbate (Table 1). Further co-encapsulated curcumin weakened the decreasing trend of the ζ-potential caused by L-ascorbate, which was probably due to the partial competitive substitution of L-ascorbate by curcumin. It is noteworthy that co-encapsulation of L-ascorbate and curcumin further increased the size of the particles to 777 nm.

The morphology of the binary particles was further evaluated by AFM. As observed in Fig. 2, the binary particles containing L-ascorbate showed compact, rather than lumpy edges as in the absence of L-ascorbate. In contrast, complexation with curcumin caused a greater contour size of the binary particles. This behavior was also in agreement with AFM images of curcumin complexation with soy protein isolate and soy soluble polysaccharide at pH 4 [24, 25]. The mean particle diameter determined by dynamic light scattering was larger than the particle size observed by AFM, which may be due to the swelling of protein/polysaccharide particles in aqueous medium [26].

3.1.2. Loading efficiency

The loading efficiency (LE) of curcumin in pH-driven prepared casein particles ranged from 70% to 100%, depending on the content of curcumin and carrier material

[1, 27]. As observed in Table 1, the LE of curcumin in binary particles was around 96%, and was not affected by L-ascorbate. In contrast, only 52% of L-ascorbate was encapsulated. According to previous reports, L-ascorbate hardly binds with negatively charged milk proteins (such as β-lactoglobulin) [13, 28]. Nevertheless, L-ascorbate readily interacted with the amino groups on the polymeric backbone of chitosan and could be entrapped within the chitosan layers [12]. Accordingly, the loading of L-ascorbate in binary particles was mainly ascribed to chitosan rather than caseinate. In the co-delivery system, curcumin decreased the LE of L-ascorbate to about 48% (Table 1), which can be explained in terms of the competitive substitution. This was also evident in thymoquinone/L-ascorbate fortified chitosan particles, whereby thymoquinone decreased the LE of L-ascorbate [29]. Additionally, the increased particle size of the co-delivery particles (Table 1), accompanied by a decrease of the surface area-to-volume ratio, might also result in a lower encapsulation of L-ascorbate in the surface coating.

3.1.3. Intermolecular interactions

The intermolecular interactions in the binary particles were analyzed by infrared (IR) spectrometry. As shown in Fig. 3A, the spectrum of chitosan revealed a broad and strong peak at 3428 cm^{-1} (O-H stretching vibrations), two main characteristic peaks of chitosan at 1675 and 1423 cm^{-1} (amide bands), as well as a peak at 1604 cm^{-1} (N-H vibration of the amino group). As for caseinate, it mainly exhibited a peak of hydrophilic O-H stretching at 3300 cm^{-1} and a strong vibration of hydrophobic C-H stretching at 3064 cm^{-1}, as well as the characteristic peaks for amide I (1670 cm^{-1}, C=O

276 stretching) and amide II (1542 cm^{-1}, C-N stretching) groups [30]. It was noted that the
277 peak at 3428 cm^{-1} in the spectra of chitosan shifted to 3339 cm^{-1} in binary particles,
278 verifying the occurrence of hydrogen bonding between caseinate and chitosan (Fig. 3A).
279 Similar results were also reported for quaternized chitosan-zein complexes, which
280 changed from 3477 to 3422 cm^{-1} [31]. Compared with chitosan and caseinate alone,
281 the shift of the amide bands (amide I at 1658 cm^{-1}, amide II at 1565 cm^{-1}) in the binary
282 particles represented the electrostatic interaction between the two [32].

283 In the case of curcumin, there were no characteristic peaks of carbonyl in the range
284 of 1800−1650 cm^{-1}, indicating that curcumin was present in the ketoenol tautomeric
285 form [33]. However, compared with native curcumin, numerous major peaks at 1634,
286 1604, 1509, 1430, 1280, 1032, and 966 cm^{-1} appeared in binary particles, revealing the
287 effective encapsulation of curcumin. Meanwhile, the characteristic peaks of curcumin
288 at 3509 cm^{-1} disappeared when it was encapsulated in binary particles, indicating the
289 presence of hydrogen bonding between the phenolic –OH of curcumin and biopolymers.
290 Such observations were consistent with an earlier report on the occurrence of hydrogen
291 bonds and hydrophobic interactions between curcumin and caseinate [34]. In addition,
292 upon addition of L-ascorbate, the peak of the amide I group shifted from 1658 to 1650
293 cm^{-1}, which was attributed to electrostatic interactions [32]. Compared to binary
294 particles without L-ascorbate, complexation with L-ascorbate increased the intensity of
295 the amide I contribution at 1650 cm^{-1} and the amide II peak at 1568 cm^{-1}. This may be
296 attributed to the hydrophilic interactions between polyphenols and protein groups such
297 as C=O, CN and NH, and a similar shift was also observed in tea polyphenol-casein

complex nanoparticles [35].

3.2. Evaluation of carrier storage stability

The storage stability of the delivery system was further verified by FTIR (Fig. 3B). Several variations were found in the spectra of chitosan, including the disappearance of the amide I at 1675 cm^{-1} and the enhanced intensity of the amino characteristic peak at 1600 cm^{-1}. This phenomenon may be explained by the degradation of chitosan during storage [36]. The new peaks in the caseinate spectrum at 2052 and 2144 cm^{-1} (Fig. 3B) were attributed to free $-NH_3^+$ groups and ascribed to the dissociation of bonds between $-NH_3^+$ and $-COO^-$ in oxidized proteins [37]. In contrast, this change did not occur in binary particles, indicating that these binary particles had an encouraging storage stability.

The structural changes of caseinate in binary particles during storage were further analyzed by CD measurements. The secondary structure of caseinate mainly presented a random coil structure [30] and was not affected by the addition of L-ascorbate before and after storage (Table 2). However, the addition of curcumin rearranged the secondary structure of caseinate, with a decrease in the random coil content and an increase in α-helices and β-sheets. This phenomenon may be explained by the disruption of internal hydrogen bonds of the peptide groups and the formation of intermolecular hydrogen bonds through competition between curcumin OH groups and peptide NH groups [38, 39]. Additionally, when compared to freshly prepared curcumin-fortified binary particles, the structural destabilization of caseinate was accelerated after storage. Such observation was consistent with an earlier study that

degraded resveratrol caused rigorous oxidation of protein-based carriers during storage at 45 °C [2]. Additionally, it was observed that curcumin-induced structural changes of caseinate in binary particles were suppressed by co-delivery of L-ascorbate. This behavior was in agreement with the improved storage stability of curcumin in the presence of L-ascorbate (Fig. 4), indicating a correlation between the stability of the carriers and the complexed curcumin.

3.3. Stability of co-loaded bioactive compounds

3.3.1. Storage stability

As shown in Figure 4, both free L-ascorbate and curcumin appreciably degraded during incubation. The storage stability of curcumin in binary particles was significantly improved. Compared to free curcumin, the retention of curcumin in binary particles was increased from 48% to 76% after storage for 30 days. Co-delivery of L-ascorbate further improved the retention of curcumin, reaching about 83%. EGCG as a hydrophilic polyphenol was also verified with a similar effect on the hydrophobic quercetin in W/O/W emulsion gels [40]. Combined with the above-mentioned results that co-delivered L-ascorbate inhibited the structural change of caseinate induced by curcumin (Table 2), it can be speculated that the degradation of curcumin was partially induced by oxidized caseinate. In the case of free L-ascorbate, only 23% remained after storage for 1 day and it degraded completely within 5 days (Fig. 4B). Loading L-ascorbate in binary particles also effectively inhibited the degradation. After 5 days of storage, still around 30% of L-ascorbate remained. However, co-delivered curcumin in binary particles slightly accelerated the degradation of L-ascorbate (Fig. 4B). This

342 phenomenon may be explained by the competitive substitution of curcumin for loaded
343 L-ascorbic, resulting in an increased content of L-ascorbate in free form (Table 1).
344 Meanwhile, it was also clear that the L-ascorbate was more readily oxidized than
345 curcumin (Fig. 4). As reported, the electrochemical potentials of curcumin and L-
346 ascorbate are 0.66 and 0.282 V, respectively [41, 42]. Compared to L-ascorbate
347 adsorbed on the chitosan layer, curcumin complexed within caseinate, with less contact
348 with the solution. Thus, L-ascorbate was the first to scavenge free radicals in the system
349 by donating electrons, providing a protective effect on curcumin in binary particles.

350 **3.3.2. Irradiation stability**

351 A gradual decrease in the retention of L-ascorbate and curcumin with the extension
352 of irradiation time was observed, while the irradiation stability of curcumin was lower
353 than that of L-ascorbate. The encapsulation in binary particles did not affect the
354 irradiation stability of L-ascorbate, but accelerated the degradation of curcumin. The
355 retention of curcumin in free form and in binary particles was about 80% and 59% after
356 irradiation for 4 h (Fig. 4C), respectively. Previous studies reported that α- and β-casein,
357 the major constituents of caseinate, exhibited a significant photo-oxidative sensitivity
358 [43]. Therefore, it was assumed that oxidized caseinate may act as a pro-oxidant, and
359 in turn accelerated the degradation of curcumin under irradiation. Additionally, co-
360 delivered L-ascorbate showed a significant inhibitory effect on the degradation of
361 curcumin, increasing the retention of curcumin from 59% to 73% after 4 h of irradiation
362 (Fig. 4C). On the other hand, co-encapsulated curcumin accelerated the degradation of
363 L-ascorbate, and the retention of L-ascorbate decreased from 87% to 66% after 4 h of

364 irradiation (Fig. 4D). These data suggest that L-ascorbate was sacrificed to protect
365 curcumin. A similar result was observed in nutritionally fortified whole and skimmed
366 milk, of which ascorbic acid significantly alleviated the light-induced degradation of
367 riboflavin [44].

368 The antioxidant activity was further characterized to verify the effectiveness of the
369 binary particles in delivering curcumin and/or L-ascorbate during irradiation. As shown
370 in Fig. 5, L-ascorbate possessed a higher $ABTS^{\cdot+}$ scavenging activity than curcumin.
371 The antioxidant activity of L-ascorbate in binary particles was lower than in free form,
372 which was possibly ascribable to the fact that complexation weakened the reactivity of
373 the hydroxyl and amino groups [45]. More specifically, the antioxidant activity of L-
374 ascorbate decreased with the irradiation time and was reduced by about 17% after 4 h
375 of irradiation, which was consistent with the decreased content (~15%, Fig. 4D). In
376 fact, L-ascorbate can be oxidized to dehydroascorbic acid and then form inactive
377 products under aerobic and acid conditions, such as 2-fluroic acid or 3-hydroxy-2-
378 pyrone [46]. For curcumin, encapsulation in binary particles obviously improved its
379 antioxidant activity. This may result from the improved solubility of curcumin in binary
380 particles, in line with an earlier report of encapsulated curcumin in zein/pectin particles
381 [47]. However, after 4 h of irradiation, the scavenging activities of pure and
382 encapsulated curcumin were decreased by 16% and 28%, respectively, which were both
383 lower than their corresponding degradation contents of 20% and 40%. In general, the
384 stability of dimethoxy curcumin, the auto-oxidation product of curcumin, is higher than
385 that of curcumin. This progress refers to the free radical initiated transfer of phenol (-

OH) to a methoxy group (-OCH_3), which is more electron donating than hydrogen [48]. Meanwhile, it was worthy to note that the scavenging activity of co-delivered binary particles was higher than either of curcumin and L-ascorbate in binary particles, and kept unchanged during the irradiation process. These results indicated that the co-delivery binary particles obtained an optimal stability and antioxidant activity.

3.4. *In vitro* simulated release

Figure 6 presents the cumulative release profiles of L-ascorbate and curcumin in binary particles during *in vitro* simulated digestion. There was a correlation between the initial release of L-ascorbate and curcumin from digestion (about 50% and 4%, respectively) and unloaded efficiency. The cumulative release of L-ascorbate during digestion mainly occurred in SIF, reaching to about 85% after 6 h of digestion. This phenomenon may be explained by the weakened electrostatic attraction of deprotonated chitosan with L-ascorbate in SIF (Fig. 1). The proportion of released curcumin in SGF was below 5%. After 6 h of digestion, the cumulative release of curcumin was above 90% (Fig. 6). Chitosan swollen in SIF had a looser structure, leading to burst release of the encapsulated compound [49]. Consequently, binary particles of caseinate/chitosan were an excellent co-delivery carrier to delay the release of curcumin and L-ascorbate in SGF.

4. Conclusions

Caseinate-chitosan particles were fabricated by a combined facile pH conversion and ionic gelation method. The binary particles showed excellent co-encapsulation properties and storage stability of hydrophobic curcumin and hydrophilic L-ascobate.

Co-delivered L-ascorbate could inhibit the secondary structural changes of caseinate during storage caused by the degradation of curcumin and was beneficial for maintaining the photostability of curcumin. More specifically, this co-delivery system revealed an excellent antioxidant activity and showed regulated release properties for bioactive compounds in SIF. These results could guide the design of biopolymer-based carriers with hydrophilic and hydrophobic target bioactives for the application in multi-functional foods.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgements

This work was supported by...

References

[a] Lu, Y, Lin, M, Zong, J, et al. Highly bioavailable curcumin preparation with a co-grinding and solvent-free process. *Food Sci Nutr.* 2020; 8: 6415– 6425. https://doi.org/10.1002/fsn3.1930

[b] R.C. Rose. Solubility properties of reduced and oxidized ascorbate as determinants of membrane permeation. Biochimica et Biophysica Acta (BBA) - General Subjects Volume 924, Issue 1, 16 April 1987, Pages 254-256

[1] K. Pan, Y.C. Luo, Y.D. Gan, S.J. Baek, Q.X. Zhong, pH-driven encapsulation of curcumin in self-assembled casein nanoparticles for enhanced dispersibility and bioactivity. Soft Matter, 10(35) (2014) 6820-6830.

[2] X. Yin, H. Cheng, Wusigale, H.H. Dong, W.N. Huang, L. Liang, Resveratrol stabilization and loss by sodium caseinate, whey and soy protein isolates: loading, antioxidant activity, oxidability. Antioxidants, 11(4) (2022) 647.

[3] G.Y. Liu, W.J. Huang, O. Babii, X.Y. Gong, Z.G. Tian, J.Q, Yang, Y.X. Wang, R.L. Jacobs, V. Donna, A. Lavasanifar, L.Y. Chen, Novel protein-lipid composite nanoparticles with an inner aqueous compartment as delivery systems of hydrophilic nutraceutical compounds. Nanoscale, 10(22) (2018) 10629-10640.

[4] Y.C. Luo, Food colloids binary and ternary nanocomplexes: Innovations and discoveries. Colloids and Surf. B Biointerfaces, 196 (2020) 111309.

[5] D.D. Yuan, J.C. Jacquier, E.D. O'Riordan, Entrapment of protein in chitosan-tripolyphosphate beads and its release in an in vitro digestive model. Food Chem., 229 (2017) 495-501.

[6] P. Mazancova, V. Nemethova, D. Trel'ova, L. Klescikova, I. Lacik, F. Razga, Dissociation of chitosan/tripolyphosphate complexes into separate components upon pH elevation. Carbohydr. Polym., 192 (2018) 104-110.

[7] E. Fleming, Y.C. Luo, Co-delivery of synergistic antioxidants from food sources for the prevention of oxidative stress. J. Agric. Food. Res. 3 (2021) ??.

[8] I. Muhammad, U. Azmat, S. Farhan, N. Muhammad, M. Umair Arshad, H.A. Rasul Suleria, Cucurmin, anticancer, & antitumor perspectives: a comprehensive review. Crit. Rev. Food Sci. Nutr., 58(8) (2018) 1271-1293.

[9] O.A.K. Khalil, O. Oliveira, J.C.R. Vellosa, A.U. de Quadros, L.M. Dalposso, T.K. Karam, R.M.

Mainardes, N.M. Khalil, Curcumin antifungal and antioxidant activities are increased in the presence of ascorbic acid. Food Chem., 133(3) (2012) 1001-1005.

[10] Y. Nimiya, W.C. Wang, Z.Y. Du, E. Sukamtoh, J.L. Zhu, E. Decker, G.D. Zhang, Redox modulation of curcumin stability: Redox active antioxidants increase chemical stability of curcumin. Mol. Nutr. Food Res., 60(3) (2016) 487-494.

[11] C.K.K. Nair, A. Menon, Consumption of antioxidant dietary agents, curcumin and vitamin C, protects cellular DNA from gamma-radiation. Int. J. Radiat Res. 11(1) (2013) 11-16.

[12] A. Alishahi, A. Mirvaghefi, M.R. Tehrani, H. Farahmand, S.A. Shojaosadati, F.A. Dorkoosh, M.Z. Elsabee. Shelf life and delivery enhancement of vitamin C using chitosan nanoparticles. Food Chem., 126(3) (2011) 935-940.

[13] L. Wang, Y.H. Gao, J. Li, M. Subirade, Y.D. Song, L. Liang. Effect of resveratrol or ascorbic acid on the stability of α-tocopherol in O/W emulsions stabilized by whey protein isolate: Simultaneous encapsulation of the vitamin and the protective antioxidant. Food Chem., 196 (2016) 466-474.

[14] F.G. Liu, D. Ma, X. Luo, Z.Y. Zhang, L.L. He, Y.X. Gao, D.J. McClements. Fabrication and characterization of protein-phenolic conjugate nanoparticles for co-delivery of curcumin and resveratrol. Food Hydrocoll., 79 (2018) 450-461.

[15] H. Li, D.F. Wang, C.Z. Liu, J.X. Zhu, M.H. Fan, X. Sun, T. Wang, Y. Xu, Y.P. Cao. Fabrication of stable zein nanoparticles coated with soluble soybean polysaccharide for encapsulation of quercetin. Food Hydrocoll., 87 (2019) 342-351.

[16] P. Wen, K. Feng, H. Yang, X. Huang, M.H. Zong, W.Y. Lou, N. Li, H. Wu. Electrospun core-shell structured nanofilm as a novel colon-specific delivery system for protein. Carbohydr. Polym., 169 (2017) 157-166.

[17] X. Yin, X.J. Fu, H. Cheng, Wusigale, L. Liang. α-Tocopherol and naringenin in whey protein isolate particles: Partition, antioxidant activity, stability and bioaccessibility. Food Hydrocoll., 106 (2020) 105895.

[18] C. Chang, T.R. Wang, Q.B. Hu, Y.C. Luo. Zein/caseinate/pectin complex nanoparticles: Formation and characterization. Int. J. Biol. Macromol., 104 (2017) 117-124.

[19] C. Qin, J. Zhou, Z. Zhang, W. Chen, Q. Hu, Y. Wang. Convenient one-step approach based on stimuli-responsive sol-gel transition properties to directly build chitosan-alginate core-shell beads.

Food Hydrocoll., 87 (2019) 253-259.

[20] C.G. de Kruif, F. Weinbreck, R. de Vries. Complex coacervation of proteins and anionic polysaccharides. Curr. Opin. Colloid Interface Sci., 9(5) (2004) 340-349.

[21] K. Hu, X.X. Huang, Y.Q. Gao, X.L. Huang, H. Xiao, D.J. McClements. Core-shell biopolymer nanoparticle delivery systems: Synthesis and characterization of curcumin fortified zein-pectin nanoparticles. Food Chem., 182 (2015) 275-281.

[22] S.F. Peng, Z.L. Li, L.Q. Zou, W. Liu, C.M. Liu, D.J. McClements. Improving curcumin solubility and bioavailability by encapsulation in saponin-coated curcumin nanoparticles prepared using a simple pH-driven loading method. Food Funct. 9(3) (2018) 1829-1839.

[23] K.I. Jang, H.G. Lee. Stability of chitosan nanoparticles for l-ascorbic acid during heat treatment in aqueous solution. J. Agric. Food. Chem., 56(6) (2008) 1936-1941.

[24] F.P. Chen, S.Y. Ou, Z. Chen, C.H. Tang, Soy soluble polysaccharide as a nanocarrier for curcumin, J. Agric. Food. Chem., 65(8) (2017) 1707-1714.

[25] F.P. Chen, B.S. Li, C.H. Tang, Nanocomplexation of soy protein isolate with curcumin: Influence of ultrasonic treatment, Food Res. Int., 75 (2015) 157-165.

[26] W.F. Xiong, C. Ren, J. Li, B. Li, Enhancing the photostability and bioaccessibility of resveratrol using ovalbumin-carboxymethylcellulose nanocomplexes and nanoparticles, Food Funct. 9(7) (2018) 3788-3797.

[27] B.J. Zheng, H.E. Lin, X.Y. Zhang, D.J. McClements, Fabrication of curcumin-loaded dairy milks using the pH-shift method: formation, stability, and bioaccessibility, J. Agric. Food. Chem., 67(44) (2019) 12245-12254.

[28] P. Puyol, M.D. Perez, L. Mata, M. Calvo, Study in interaction between beta-lactoglobulin and other bovin whey proteins with ascorbic-acid, Milchwissenschaft. 49(1) (1994) 25-27.

[29] N. Othman, M.J. Masarudin, C.Y. Kuen, N.A. Dasuan, L.C. Abdullah, S.N.A. Md Jamil, Synthesis and optimization of chitosan nanoparticles loaded with L-sscorbic acid and thymoquinone, Nanomaterials, 8(11) (2018) 19.

[30] H.M. Farrell, E.D. Wickham, J.J. Unruh, P.X. Qi, P.D. Hoagland, Secondary structural studies of bovine caseins: temperature dependence of beta-casein structure as analyzed by circular dichroism and FTIR spectroscopy and correlation with micellization, Food Hydrocoll., 15(4-6) (2001) 341-354.

[31] H.S. Liang, B. Zhou, L. He, Y.P. An, L.F. Lin, Y. Li, S.L. Liu, Y.J. Chen, B. Li, Fabrication of zein/quaternized chitosan nanoparticles for the encapsulation and protection of curcumin, Rsc Advances, 5(18) (2015) 13891-13900.

[32] Y.C. Luo, B.C. Zhang, M. Whent, L.L. Yu, Q. Wang, Preparation and characterization of zein/chitosan complex for encapsulation of alpha-tocopherol, and its in vitro controlled release study, Colloids Surf. B Biointerfaces 85(2) (2011) 145-152.

[33] C.S. Mangolim, C. Moriwaki, A.C. Nogueira, F. Sato, M.L. Baesso, A.M. Neto, G. Matioli, Curcumin-β-cyclodextrin inclusion complex: Stability, solubility, characterisation by FT-IR, FT-Raman, X-ray diffraction and photoacoustic spectroscopy, and food application, Food Chem., 153 (2014) 361-370.

[34] C.H. Tang, Nanocomplexation of proteins with curcumin: From interaction to nanoencapsulation (A review), Food Hydrocoll. 109 (2020) 106106.

[35] I. Hasni, P. Bourassa, S. Hamdani, G. Samson, R. Carpentier, H.A. Tajmir-Riahi, Interaction of milk α- and β-caseins with tea polyphenols, Food Chem., 126(2) (2011) 630-639.

[36] D.W. Ren, Y.L. Wang, D.C. Bao, W.Y. Xie, M.X. Jun, FTIR analysis of the enzymatic degradation of chitosan blend membranes, Spectrosc. Spect. Anal. 26(10) (2006) 1825.

[37] I. Nugrahani, R. Oktaviary, S. Ibrahim, T. Gusdinar, C. Apsari, FTIR method for peptide content estimation and degradation kinetic study of Canarium nut protein, Indones. J. Pharm. 31(2) (2020) 78-83.

[38] P. Barreto, J. Roeder, J. Crespo, G. Maciel, H. Terenzi, A. Pires, V. Soldi, Effect of concentration, temperature and plasticizer content on rheological properties of sodium caseinate and sodium caseinate/sorbitol solutions and glass transition of their films, Food Chem., 82(3) (2003) 425-431.

[39] T. Wang, P.C. Xu, Z.X. Chen, R. Wang, Mechanism of structural interplay between rice proteins and soy protein isolates to design novel protein hydrocolloids, Food Hydrocoll., 84 (2018) 361-367.

[40] X. Chen, D.J. McClements, J. Wang, L.Q. Zou, S.M. Deng, W. Liu, C. Yan, Y.Q. Zhu, C. Cheng, C.M. Liu, Coencapsulation of (-)-epigallocatechin-3-gallate and quercetin in particle-stabilized W/O/W emulsion gels: Controlled release and bioaccessibility, J. Agric. Food. Chem., 66(14) (2018) 3691-3699.

[41] A. Simic, D. Manojlovic, D. Segan, M. Todorovic, Electrochemical behavior and antioxidant and prooxidant activity of natural phenolics, Molecules, 12(10) (2007) 2327-2340.

[42] N.S. Jha, S. Mishra, S.K. Jha, A. Surolia, Antioxidant activity and electrochemical elucidation of the enigmatic redox behavior of curcumin and its structurally modified analogues, Electrochim. Acta, 151 (2015) 574-583.

[43] T.K. Dalsgaard, D. Otzen, J.H. Nielsen, L.B. Larsen, Changes in structures of milk proteins upon photo-oxidation, J. Agric. Food. Chem., 55(26) (2007) 10968-10976.

[44] K.H. Lee, M.Y. Jung, S.Y. Kim, Effects of ascorbic acid on the light-induced riboflavin degradation and color changes in milks, J. Agric. Food. Chem., 46(2) (1998) 407-410.

[45] S. Dubeau, G. Samson, H.A. Tajmir-Riahi, Dual effect of milk on the antioxidant capacity of green, Darjeeling, and English breakfast teas, Food Chem., 122(3) (2010) 539-545.

[46] J.P. Yuan, F. Chen, Degradation of ascorbic acid in aqueous solution, J. Agric. Food Chem, 46(12) (1998) 5078-5082.

[47] X.X. Huang, X.L. Huang, Y.S. Gong, H. Xiao, D.J. McClements, K. Hu, Enhancement of curcumin water dispersibility and antioxidant activity using core-shell protein-polysaccharide nanoparticles, Food Res. Int., 87 (2016) 1-9.

[48] J. Zhu, K.Z. Sanidad, E. Sukamtoh, G. Zhang, Potential roles of chemical degradation in the biological activities of curcumin, Food Funct, 8(3) (2017) 907-914.

[49] A.C. Carita, B. Fonseca-Santos, J.D. Shultz, B. Michniak-Kohn, M. Chorilli, G.R. Leonardi, Review article vitamin C: One compound, several uses. Advances for delivery, efficiency and stability, Nanomed-Nanotechnol. 24 (2020) 102117.

Web references

Drug Bank C. A. (2022). https://go.drugbank.com/drugs/DB11672/Accessed July 4, 2022

Drug Bank C. A. (2022). https://go.drugbank.com/drugs/DB00126/Accessed July 4, 2022