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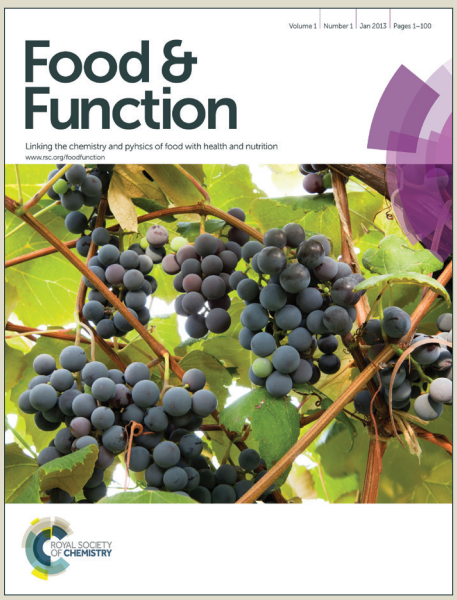

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Amphiphilic zein hydrolysate as a novel nano-delivery vehicle for curcumin

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Abstract: In this paper, we developed amphiphilic zein hydrolysate (ZH) as a novel delivery vehicle, which could be used for preparing curcumin (Cur) nanocomplexes. These ZH-Cur nanocomplexes exhibited spherical morphology with a monodisperse size distribution (<50 nm), and the dispersion was transparent, which could have a great application potential in nutraceutical-fortified food and clear beverages. The water solubility of curcumin was considerably enhanced by the nanocomplexation above 8200-fold (*vs* free curcumin in water). The good colloidal and storage stability of ZH-Cur nanocomplexes were greatly improved, and more than 60% of curcumin was retained in 72 h storage at ambient conditions. These phenomena appeared to be attributed to the fact that amphiphilic ZH displayed self-assembly properties in water solution and strong interfacial activity at oil-water interface, as confirmed by micelles formation and dynamic interfacial adsorption respectively. Fluorescence titration and FTIR results indicated the existence of strong hydrophobic interactions between ZH and Cur.

Key words: Zein Hydrolysate; Curcumin; Nanoparticles; Nanocomplexation

Introduction

Enriching staple foods and beverages with nutraceuticals is a promising approach for promoting health of broad populations. However, the enrichment with hydrophobic nutraceuticals is highly challenging due to their low water solubility, poor bioavailability and high sensitivity to degradation during production, shelf life and digestion.[1] Nanoparticle-based delivery systems, which can circumventing the pitfalls of poor water solubility and chemical instability, are very suitable for hydrophobic nutraceuticals.[2] Plant proteins (mainly soy proteins, zein and wheat gliadins) are widely available, low-cost and safer (less allergenic and low possibility to provoke zoonotic disease transmission). More importantly, they can be easily made into food colloidal delivery systems based on the occurrence of non-covalent interactions (including hydrophobic interactions, hydrogen bonding, electrostatic interactions, and aromatic stacking) with bio-active substances.[3]

Zein, a hydrophobic prolamine, contains a large number of hydrophobic amino acids. The inherent amphiphilic property of zein makes it a fine material in fabricating functional colloidal particles and controlling the release of drugs and nutritional supplements.[4, 5] By simple anti-solvent precipitation method,[6] many alcohol-soluble bioactive ingredients, such as polyphenols (thymol[7], curcumin[8], quercetin[9] and cranberry procyanidins[10]) and essential oils[11] have been encapsulated into zein particles because of their co-dissolution in water-alcohol solutions. This could hugely improve the chemical stability of such bioactive ingredients and the bioavailability through controlled-release *in vivo*. However, since zein colloidal particles are stabilized by surface repulsion (electrostatic stabilization), they tend to lose physical stability when approaching neutral pH and physiological pH in intestine, both leading to particles aggregation.[12] To overcome the drawbacks, stabilizers like sodium caseinate[6] or gum arabic[13] have been added to water solution. Unfortunately, zein colloidal

particles are usually in large scale (diameter >100 nm), and they are commonly turbid dispersions in aqueous phase, which greatly limit their applications in some food processing fields (e.g., clear drinks).

The self-assembly of amphiphilic polypeptides have attracted many attentions in recent years. Various nanostructures, such as micelles, nanotubes, nanofibers and nanoribbon, could be fabricated by the self-assembly of amphiphilic peptides.[14] Such nanostructures, as delivery vehicles, have great potential applications in transport and delivery of bioactive compounds, nutrients or additives.[3] Synthetic amphipathic proline-rich peptides based on N-terminal domain of γ-zein exhibited a prominent characteristic as a new carrier in nanotechnology.[15] Moreover, some amphiphilic protein hydrolysates, such as α-lactalbumin hydrolysate[16], were also found to have the self-assembling properties.

Proline-rich zein contains numerous amphipathic polypeptide fragments. However, there has been no report on the amphipathic characteristic of ZH and its applications as a nanocarrier. Studying these issues could pave the way for many important applications, such as the entrapment and protection of bioactive substance in food processing. In fact, we have recently found out that ZH exhibited excellent amphiphilic and self-assembly properties in water solution, showing an enormous potential in the application of food colloidal delivery systems. Curcumin (Cur), a hydrophobic polyphenol derived from the rhizome of herb, is endowed with a number of biological and pharmacological activities, such as antioxidant, anti-inflammatory, antimicrobial, and anticarcinogenic.[2] However, due to poor water solubility and chemical instability, bioavailability of Cur is hugely limited, impeding its conversion from cooking to clinical and functional foods applications.[17-19] The aim of this work was to study the amphiphilic properties of ZH, and a model

co-assembled nanocomplexes system based on ZH was established for the protected delivery of curcumin. In particularly, the interactions between ZH and Cur were also studied using fluorescence titration and FTIR methods.

Materials and Methods

Materials

Zein (>92%), Curcumin (~98% purity, from Curcuma longa) and pyrene (>99% purity) were purchased from Sigma-Aldrich (St. Louis, MO). *Alcalase* 2.4L (endoproteinase from Bacillus licheniformis, 2.4AU/g) was obtained from Novozymes North America Inc. (Franklinton, NC). Sodium caseinate (Na-Cas) (>90%) was obtained from Meryer chemical technology Co., Ltd (Shanghai, China). All other chemicals used were of analytical grade.

Preparation and characterization of ZH

ZH was prepared according to the method of *Kong, et al.*[20] with some modifications. Briefly, zein suspension in deionized water (3% w/v) was hydrolyzed with *Alcalase* at 50 °C in automatic potentiometric titrator (Metrohm). The enzyme to zein substrate ratio was 2:100 (mL/g). The pH of zein suspension was adjusted to 9.0 before hydrolysis was initiated, and maintained at 9.0 by continuously dropwise adding 1 M NaOH during hydrolysis. The process of enzymolysis was terminated when pH didn't drop in 5 min. After hydrolysis, the pH was brought to 7.0 using 1 M HCl, and the solution was then heated at 95 °C for 5 min to inactivate the enzyme. Then the hydrolysate was centrifuged at 10, 000 r/min, 25 °C for 20 min. The supernatant was dialyzed (100 Da cutoff) over night against deionized water and finally freeze-dried (Dura-Dry MP freeze-dryer, FTS Systems, Inc., Ridge, NY). The prepared ZH was placed in the ziplock bags and stored at 4 °C before use. To eliminate possible color interferences with the following chemical analyses, ZH solutions were

decolored by mixing with an equal volume of chloroform. The organic phase containing the extracted pigments was removed.

Protein solubility in specified hydrolysis time points was measured according to the biuret method[21]. ZH solutions were centrifuged at 1800 g for 10 min. Protein solubility (percent) was defined as the protein amount in the supernatant divided by the original protein amount and then multiplied by 100.

The degree of hydrolysis (DH) was monitored by the pH-Stat method.[22] The amount of alkali consumed in enzymatic reaction was recorded and used to calculate the DH. DH was calculated from the following equation：

$$DH\ (\%) = B \times N_b \times \alpha^{-1} \times (M)^{-1} \times (h_{tot})^{-1} \times 100$$

where B = base consumption (mL); N_b = base concentration (2N);

α^{-1} = average degree of dissociation of α-amino groups =1.01; M = mass of protein (g); h_{tot}= total number of peptide bonds in the protein substrate = 9.2 milli-equivalents/gram protein.

Native zein and its hydrolysates derived from specified time points were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli.[23] Aliquots of 5μL samples were loaded in each well on the gel. A molecular weight (MW) standard composed of a cocktail of proteins (14.4-97.40 kDa) (Bio-Rad Laboratories) was also run.

Self-assembly properties and interfacial activity of ZH

Critical micelle concentration (CMC) was determined based on intensity ratio of the first peak to the third (I1/I3) of pyrene fluorescence spectrum during its entrapment in hydrophobic domains forming upon micellization.[24] A series of decimal dilutions of ZH (0.01 up to 20 mg/mL) were prepared using phosphate buffer solution (PBs, pH 7.2, 10 mM). The final concentration of pyrene in

each sample was 1.0 μM. Each spectrum was measured in the wavelength range 350-500 nm with the excitation wavelength being 335 nm. The excitation and emission slit widths were set at 5 and 2.5 nm, respectively. The intensity ratio of I1 to I3 of the pyrene fluorescence spectrum showed the micropolarity where the probe exists. The intensity ratios of I1to I3 were plotted as a function of logarithm of ZH concentrations. The data were fitted using the nonlinear fitting with Boltzman's Curve. The CMC was obtained from the in flection point of the nonlinear fitting. As contrasts, ZHs obtained after 1 h (ZH1) and 3 h (ZH3) enzymolysis were all carried out.

Dynamic interfacial adsorption of ZH at oil-water interface was determined by monitoring the evolution of surface tension. An optical contact angle meter, OCA-20 (Dataphysics Instruments GmbH, Germany) was used in a dynamic mode for measuring surface tension at the oil-water interface at 25 °C. Details of this apparatus were given elsewhere.[25] The samples were diluted to 1 mg/mL with PBs (10 mM, pH 7.2), and placed in the syringe to reach the desired constant temperature. Then a drop was delivered into a purified corn oil and allowed to stand for 2 h to achieve protein adsorption. As contrasts, ZH1, ZH3, and Na-Cas at the same concentration (1 mg/mL) were all carried out.

Fabrication and characterization of ZH-Cur nanocomplexes

Unless otherwise specified, ZH obtained after 3 h hydrolysis time was selected in the following sections, to simplify the experimental process. 0.1 mL stock solution of Cur (3 mg/mL in ethanol) was respectively added into 2.9 mL of ZH solutions (1, 2.5 and 5 mg/mL). The mixtures were centrifuged at 10000×g, 4 °C for 20 min to pellet the unbound Cur, and the supernatants containing Cur nanocomplexes were preserved in a light-resistant container at 4 °C for determination. As the contrasts, ZH without Cur and Cur without ZH in the same PBs solution with homologous

concentration were also prepared.

UV-vis spectra were measured by UV-spectrophotometer (UV2300, ECHCOMP). Cur ethanol solution (20 μL, 3 mg/mL) was added into ZH solution (2.98 mL, 2.5 mg/mL) at room temperature. Appropriate contrasts of ZH without Cur and of Cur without ZH were also tested.

To quantify the bound Cur in ZH solutions, the methodology according to Ma *et al.*[26] was adopted with some modifications. Briefly, 100 μL of freshly prepared nanocomplexes dispersion was added to 900 μL methanol in a tube. After a violent shaking, each sample was centrifuged at 15, 000 × g, 4℃ for 30 min, and the extracted supernatant was used for Cur quantification. Each sample was filtered through a 0.22 μm filter (Millipore, Billerica, MA, USA) prior to HPLC determination, then aliquots of 20 μL sample was injected to mobile phase (methanol) using a reversed column (C_{18} column, 250×4.5 mm, 5μm, Waters), and a UV detector at 425 nm. The flow rate was 0.5 mL/min. The solubility of Cur in various zein hydrolysate solutions was quantified by the Cur standard curve ($Y = 0.44 X + 0.0015$, $R^2 = 0.9994$). Encapsulation efficiency (EE) was calculated as the percentage of Cur in the supernatant with respect to the total added Cur. Analysis report was based on the results obtained from three replicates of each sample.

The chemical stability of Cur was evaluated based on the methodology of Ma *et al.*[26]. The freshly prepared nanocomplexes dispersions containing sodium azide (0.002%, w/v) were incubated under ambient conditions. As a contrast, free Cur was also dispersed in PBs by the same method. At specified time points, samples (100 μL) were taken out and added to 900 μL of methanol for quantitative analysis of Cur by RP-HPLC, as described above. The results were represented by retained ratio of Cur, which was calculated as the percentage of retained Cur at certain time point with respect to the initial value.

The particle size and surface charge of the nanocomplexes and ZH alone (2.5 mg/mL) were measured using a commercial dynamic light scattering and micro-electrophoresis device (Malvern Instruments Co. Ltd., Worcestershire, UK). The size distributions were calculated from the scattered light intensity fluctuations by cumulant analysis of the autocorrelation function. Zeta potential was reported on the basis of three replicates. All measurements were carried out at 25 °C.

The morphology of the nanocomplexes prepared in ZH solution (2.5 mg/mL) was observed using XFlash 5030T transmission electron microscopy (Bruker, Gremany). As contrasts, ZH alone and free Cur were all carried out. To eliminate the effect of introduced ethanol, equivalent volume of ethanol was added in each case. The freshly prepared samples were diluted 100 times with deionized water. One drop of a diluted sample was placed on a freshly glow-discharged carbon film on a 400-mesh copper grid and then stained with 1% uranyl acetate.

Study of the interactions by spectroscopy

The fluorescence spectra were recorded using an F7000 fluorescence spectrophotometer (Hitachi Co., Japan). The fluorescence of Cur was measured by fixing its concentration at 2 μg/mL and varying the concentrations of ZH from 0 to 5 mg/mL. The emission spectra were recorded from 450 to 650 nm with an excitation wavelength of 425 nm. Similarly, intrinsic fluorescence of ZH was measured by fixing the concentration at 1 mg/mL and varying the concentrations of Cur from 0 to 30 μg/mL. Emission spectra were recorded from 290 to 450 nm at an excitation wavelength of 280 nm. Quenching of protein fluorescence due to energy transferred from the tyrosine (Tyr) residue to Cur served to determine the binding affinity. Fluorescence quenching is described according to the Stern-Volmer equation (Eq.1) [27]: $F_0/F = 1+k_q\tau_0[Q]=1+K_{SV}[Q]$. In this equation F_0 and F are the fluorescence intensities in the absence and presence of Cur, respectively, [Q] is the Cur concentration,

K_{SV} is the Stern-Volmer quenching constant, k_q is the bimolecular quenching rate constant, and τ_0 is the lifetime of fluorescence in the absence of a quencher. Hence, Eq. 1 was applied to determine K_{SV} by linear regression of a plot of F_0/F versus [Q]. Fluorescence quenching can be further classified as dynamic or static quenching. In the case of static quenching, the quenching data can be analysed according to a modified Stern-Volmer model (Eq. 2)[27]: $F_0/\Delta F = F_0/(F_0-F) = 1/(faKa[-Q]) + 1/fa$. In this equation *fa* is the fraction of accessible fluorescence, and *Ka* is the effective quenching constant for the accessible fluorophores, and can be treated as an associative binding constant between a quencher and an acceptor. The linear regression between $F_0/(F_0-F)$ and 1/[*Q*] enables the determination of *1/faKa* (slope) and *1/fa* (intercept), and therefore *Ka.*

The freshly prepared nanocomplexes dispersion prepared in ZH solution (2.5 mg/mL) was lyophilized under light-resistant condition by covering with foil paper before FTIR determination. As contrasts, the powdered samples of Cur monomer and ZH were also prepared. FTIR spectra were measured on a Nicolet Avatar 360 FTIR spectrometer with a 4 cm^{-1} resolution and 64 scans between wavenumbers of 4000 cm^{-1} and 400 cm^{-1}. The powdered samples were prepared as KBr disks with 1 mg of the samples in 100 mg of KBr.

Statistical Analysis

Unless otherwise specified, all measurements were carried out in triplicate, and an analysis of variance (ANOVA) of the data was performed using the SPSS 19.0 statistical analysis system. The Duncan Test was used for comparison of mean values among the three treatments using a level of significance of 5%.

Results and discussion

Preparation and Characterization of ZH

The enzymolysis efficiency plays a key role in preparation of ZH. As an alkaline endopeptidate, *Alcalase* have high efficiency and low cost operation in proteins digestion. Thus, *Alcalase* has been widely used in the enzymolysis of zein or corn protein meal.[20, 28, 29] Water solubility of zein at different incubation time is shown in Fig. 1A. With incubation time increasing, the water solubility of zein gradually increased, and a plateau was achieved after 2 h hydrolysis. This liquefaction process of zein as a function of incubation time can be clearly seen from Fig.1A (inset). After 1 h incubation, zein was almost completely liquefied. Additionally, the hydrolysis treatment made zeaxanthin released, which led to the hydrolysate solutions showed salmon pink and the color became deeper with incubation time increasing.

This hydrolysis process was further monitored by DH (Fig.1B) and the electrophoretic patterns (inset of Fig.1B). Native zein was composed of two polypeptides, which matched the MW of α-zein (21 and 25 kDa).[30] The result indicates that commercial zein is composed primarily of α-zein. This finding is well supported by previous report.[31] The two polypeptides of α-zein are all susceptible to *Alcalase* (Fig. 1B), showing a steady degradation over the incubation time. In the first 1 h，DH was rapidly increased accompanying with incubation time and the electrophoretic patterns were quickly receded. Over the next 2 h, the DH was increased from 14.4% to 19%, and native proteinic bands completely disappeared. The HPLC-MS results further indicate that the molecular weight of ZH is almost all below 1000 Da (see supporting information). These findings are in line with the previous report, where *Alcalase*-treated ZH yielded three major fractions with estimated MMs of 640, 354 and 251 Da.[32] Additionally, the enzymolysis of *Alcalase* don't appreciably change the percentage of most

amino acids in the soluble fractions of ZH.[20]

Amphiphilic characteristics of ZH

Pyrene fluorescence probe was used for studying self-assembly properties of ZH. Pyrene is an uncharged hydrophobic molecule, whose fluorescence is strongly influenced by the polarity of its local environment. Pyrene has a very low solubility in water, and its concentration in saturated aqueous solutions has been reported to be either 3.8×10^{-7} M or 7.0×10^{-7} M.[33, 34] Pyrene could selectively solubilize in hydrophobic regions or microphases existing in aqueous medium. The vibration fine structure of its monomer fluorescence spectra in solution makes pyrene an excellent probe to monitor the changes of local environment polarity.[24] The intensity ratio of the first peak to the third (I1/I3) of fluorescence spectrum of pyrene shows the micro-environmental polarity where the probe exists.[35] The abrupt change of I1/I3 as a function of surfactant concentrations has been commonly used to determine CMC of the surfactant solution.[24] Fig. 2A shows the intensity ratios of I1/I3 against ZH concentrations in PBs buffer. The value of I1/I3 was gradually decreased with increasing ZH concentrations, and a plateau in the I1/I3 *vs* ZH concentrations plots appeared beyond 10 mg/mL. The CMC of ZH1 and ZH3 (hydrolysates acquired from 1 or 3 h hydrolysis) in aqueous solution were found to be 1.14 and 1.63 mg/mL. This result means that ZH obtained at different hydrolysis time all have the micelles formation capacity. Additionally, the CMC of ZH1 was slightly less than that of ZH3, suggesting stronger micelles formation capacity for ZH1. This results may be attribute to the stronger hydrophobic properties of ZH1, which can be confirmed by the peaks time of RP-HPLC（see supporting information）

The micelles formation of ZH in water may be attributed to special amino acid sequence of α-zein. α-Zein contains a large number of hydrophobic amino acids (>60%), and its subunits (Z19,

Z22) are well-known for highly repeated homologous sequence[36]. *Alcalase* is an endoproteinase, which has a high specificity for aromatic (Phe, Trp, and Tyr), acidic (Glu), sulfur-containing (Met), and aliphatic (Leu and Ala).[37] This suggests that the proteolysis of zein by *Alcalase* could result in plenty of amphipathic polypeptide fragments appearances, which likely lead to the formation of ZH micelles in water solution.

Excellent interfacial properties (foaming and emulsification) for many peptides or protein hydrolysates (e. g., corn gluten hydrolysates[28] and rice bran protein hydrolysates[38]) have been reported. Surfactin, which refers to a bacterial cyclic lipopeptide, can reduce the surface tension of air-water from 72 mN m^{-1} to 27 mN m^{-1} at a low concentration.[39] The time evolution of surface tension for ZH1, ZH3 and Na-Cas at the oil-water interface are shown in Fig.2B. The surface tensions of ZH1, ZH3 and Na-Cas were gradually decreased with the increase of adsorption time in 2 h, which can be associated with surfactant substance adsorption at the interface. Na-Cas is an excellent surfactant with good emulsifying property in practice, and this can be proved by the reduction of surface tension at oil-water interface. ZH1 and ZH3 both showed prominent surface tension reduction capacity, although they cannot compare with Na-Cas. It indicates that ZHs prepared in different hydrolysis time have the same strong interfacial active.

Water solubility and chemical stability of ZH-Cur nanocomplexes

ZH displays great potential applications in solubilization of hydrophobic actives and in nanoparticles delivery systems due to its amphiphilic characteristics. Hence, Cur was chose as a model to study the complexation between ZH and Cur. The absorption spectra of free Cur, ZH alone and ZH-Cur mixture in PBs are presented in Fig. 3A. Because of poor solubility, free Cur in PBs has only a weak absorption peak at 425 nm. However, the absorbance intensity of ZH-Cur mixture is

considerably increased. Additionally, the results also showed that ZH has no absorbance at this wavelength. These results indirectly indicate that Cur could be solubilized hugely due to the existence of ZH. The appearances of Cur in pure PBs and ZH solutions can be observed in Fig. 3B (inset). The free Cur in PBs was very turbid due to its poor water solubility. However, the existence of ZH tremendously solubilized Cur, and the ZH-Cur mixture solutions displayed yellow and highly transparent appearances. The water solubility of Cur could reach up to 90 μg/mL (Fig. 3B) when the ZH concentration is above 2.5 mg/mL, which is increased about 32 times compared with that of free Cur in PBs (2.9 μg/mL). However, according to previously reported results (11 ng/mL),[40, 41] Cur solubility is increased 8200-fold by this anti-solvent process in ZH solutions. It needs to point out that Cur solubility also has a great increase in PBs after this anti-solvent process. A similar result has been previously reported, in which the Cur solubility in water is 3.14 μg/mL after a similar anti-solvent treatment.[8] Additionally, it can be calculated that the encapsulation efficiency of Cur can reach up to 90% when ZH concentration is above 2.5 mg/mL. Therefore, ZH could be utilized as a solubilizer for Cur, and it may be suitable for development of Cur-enriched clear beverages. The solubilization of Cur in ZH solutions could attribute to the entrapment of Cur in hydrophobic core of ZH micelles, which hugely limited intermolecular aggregations of Cur.

Fig.3C shows the short-time storage stability of Cur in ZH solutions with different concentrations. The result showed that free Cur in PBs was very unstable, and only 4% Cur was retained after 24 h storage at ambient conditions. This result is well supported by previous report that the degradation of free Cur is usually very fast under neutral-basic conditions.[42, 43] As expected, the stability of Cur in the presence of ZH was greatly improved. Cur was very stable in the initial 12 h, in spite of the stability slowly decreased with further extension of storage time. Final retained ratio of Cur was still

above 60% in ZH solution with a concentration of 5 mg/mL. In fact, after 15 days storage at 4 °C under light resistant conditions, the retained ratios of Cur in ZH solutions (2.5 and 5 mg/mL) were all above 90% (data not shown). The possible mechanisms for stabilization of Cur in ZH solutions may be explained as follows. Firstly, the "immobilization" of Cur by binding to ZH is decreased its mobility and consequently its chemical reactivity. Another one is the physical barrier against penetration of oxidizing agents and UV-light, whose accessibility to Cur is hindered by the entrapment in ZH micelles. A similar report can be found in the literature, in which the author suggest that the presence of a hydrophobic zein matrix around Cur provides protection against the damaging effects of UV radiations by minimizing the interaction of light rays with the entrapped molecules.[44] Besides, the prominent antioxidative effect of ZH may also play a role in protection of Cur from degradation.

Colloidal characteristics of ZH-Cur nanocomplexes

The colloid stability of ZH-Cur nanocomplexes can be characterized by their particle size and zeta potential. Generally, smaller size and higher zeta potential mean prominent colloid stability. The volume-weighted particle size distribution and zeta potential of ZH alone and ZH-Cur nanocomplexes are shown in Fig. 4. ZH had a wide size distribution from tens to hundreds of nanometers in PBs and its zeta potential was approximate to -40 mV. The particle size of free Cur was micron-sized in PBs and its zeta potential was about -25 mV, which indicate that the Cur particles are unstable in long time storage. In fact, after hours standing at ambient conditions, Cur aggregation and precipitation happened. However, Cur in ZH solutions resulted in the formation of monodispersed nanoparticles (diameters <50 nm), and the zeta potential was increased from -25 to -45 mV based on ZH concentrations (Fig. 4B). The results indicate that Cur particles in ZH solutions

become more uniform and stable. In fact, the particle size of ZH-Cur nanocomplexes in ZH solutions with the concentrations of 2.5 and 5 mg/mL had no any change in 15 days (data not shown), after stored at 4 °C and light resistant conditions. A similar result has been found for Na-Cas stabilized Cur nanoparticles.[45] However, the size scale of ZH based Cur particles is far smaller comparing with the Na-Cas based particles (150 nm). This may be attributed to the fact that the molecular weight of ZH is much smaller than that of Na-Cas.

The morphologies of free Cur, ZH alone and ZH-Cur nanocomplexes in water solution were observed by TEM (Fig. 5). Due to low water solubility, larger aggregates (Fig. 5A) of free Cur could be easily observed. The sphere-like ZH aggregates (Fig. 5B) with size scales of 20 to 100 nm, displayed a high degree of polydispersity, which could be regarded as the morphologies of ZH micelles. The image of ZH-Cur nanocomplexes is shown in Fig. 5C. Interestingly, the nanocomplexes were transparent in TEM image and showed regular spherical structures with small diameters (<50 nm). This result is very in line with the previous DLS results (Fig. 4A). In addition, it can be observed that a plenty of smaller black spots appeared around the transparent particles in the image (Fig. 5C). This phenomenon may be attributed to the adsorption of small Cur aggregates on the surface of ZH-Cur nanocomplexes.

Complexation of ZH with Cur

The intrinsic fluorescence of proteins has been widely used to investigate the binding of drug molecules to proteins in solutions. Fluorescence of Tyr residues can be emitted at the excitation wavelength of 280 nm. Our previous study indicates that ZH have a strong fluorescence emission peak at around 305 nm upon excitation at 280 nm. The fluorescence emission spectra of ZH in the presence of different Cur concentrations are shown in Fig. 6A. The intensity of the fluorescence

emission of ZH at around 305 nm was gradually decreased with the increase of Cur concentrations, and the maximum emission wavelength remained unchanged. These results indicate that there are no changes in the local dielectric environment of Tyr residues, and Cur molecules might bind to any or all of Tyr residues through hydrophobic interactions. The previous report on fluorescence study of Cur-casein micelles complexation well support these results.[46]

The use of fluorescence in binding study requires attention to the possibility of non-binding induced (dynamic) quenching.[1] To judge quenching models, the fluorescence quenching data were analysed using the Stern-Volmer Eq.1. The values of *Ksv* and k_q for the system were calculated to be 1.54×10^8 M^{-1} and 1.54×10^{13} $M^{-1}s^{-1}$, respectively, which were much higher than maximal dynamic quenching constant (2.0×10^{10} M^{-1} s^{-1}). This indicates that the quenching process between ZH and Cur is mainly due to static quenching by the formation of the ZH-Cur complexes. The effective quenching constant *Ka* was calculated using the modified Stern-Volmer Eq. 2, and the corresponding *Ka* value (1.35×10^4 M^{-1}) is given in Fig. 6A (inset), suggesting the strong interactions between ZH and Cur in aqueous phase. Similar results about the interactions between proteins and Cur have been reported previously.[46, 47]

The fluorescence of Cur is very sensitive to the polarity of its surrounding environment. We used intrinsic fluorescence spectroscopy of Cur to study the photophysical properties of Cur in the presence of ZH (Fig. 6B). The results showed that only a low-intensity broad fluorescence peak at around 550 nm appeared, when free Cur was excited at 425 nm in absence of ZH. Small increments of ZH resulted in a sharper fluorescence peak with increased intensity and a blue-shift in maximum emission wavelength of Cur (from ~550 nm to ~ 500 nm). This phenomenon could be further support by previous report, in which the incorporation of pigment (Cur) in colloidal particles result in a blue

shift by 10 nm and a prominent hypochromic effect.[44] The blue-shift and increment of fluorescence intensity indicate the movement of Cur from a polar to a less polar micro-environment. The results are in good agreement with previous reports, where Cur is bound with various proteins, such as α_{S1}-casein, casein micelles and human serum albumin.[43, 46, 48] The authors all suggest that the increment and blue-shift of fluorescence signal is attributed to the binding of Cur with the hydrophobic regions of protein molecules. Additionally, some previous study also indicate that proline-rich protein (PRP) could interact with polyphenol by hydrophobic interactions with the pyrrolidine ring of proline.[49] Zein is a PRP too, and its hydrolysate contains 12% proline residuals.[20] Hence, there is thus a possibility that Cur could bind to the pyrrolidine ring of proline.

The FTIR spectra of Cur monomer, ZH alone and the complexes of ZH and Cur are shown in Fig. 6C. The characteristic absorbance peak around 3510cm^{-1}, corresponding to the -OH stretching vibration of Cur, disappeared after the binding of ZH with Cur. Moreover, the number and intensity of absorbance peaks in the fingerprint region of Cur obviously reduced after the complexation between ZH and Cur. Compared with ZH alone, there was a shift of the absorbance peak from 1540 to 1542 cm^{-1}after binding with Cur, and the peak of ZH at 1658 cm^{-1} also shifted to 1655cm^{-1}. The results suggest that Cur possibly bind together with the amide I group of ZH. A similar result has been reported by *Kang Pan, et al*.[45]

Conclusions

With amphiphilic characteristic, ZH tend to self-assemble into micelles in water solution, leading to strong hydrophobic complexation of ZH with Cur molecules, as confirmed by the spectroscopy analyses. The complexation remarkably increased the water solubility of Cur, moreover, the chemical stability of Cur during storage under ambient conditions also considerably improved. The ZH-Cur

nanocomplexes are nano-scale (diameter <50 nm) in water solution, its dispersions are transparent and displayed a good colloidal stability. Compared with native zein, ZH maybe turn into a better vehicle to delivery water-insoluble bioactive compounds, because ZH itself has a beneficial effect on bodies. Thus, ZH could be used as a new nano-delivery vehicle for Cur, which gives a solution for preparation of Cur-enriched functional beverages.

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Referances

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Figure captions

Fig. 1 A: Water solubility of zein at different incubation time. Different letters (a-e) above the bars indicate significant difference ($p<0.05$) in mean. The inset shows the appearances of zein suspensions at specified incubation time. B: Hydrolysis degree as a function of incubation time. The inset shows the SDS-PAGE of zein in different incubation time (lane 1 is native α-zein, and lane 2-6 are ZH after 10, 30, 60, 120, and 180 min incubation). The arrows in the inset indicate the subunits of α-zein (21 and 25 kDa).

Fig. 2 A: Pyrene fluorescence ratio of I1:I3 as a function of ZH1 and ZH3 concentrations. ZH1 and ZH3 represent ZHs obtained after 1 and 3 h hydrolysis. B: Surface tensions of ZH1, ZH3 and Na-Cas at oil-water interface against adsorption time. The concentration of each sample is 1 mg/mL.

Fig. 3 A: UV-vis absorbance spectra of ZH alone (red), free Cur (black), and ZH-Cur mixture (blue). B: Solubility of Cur in ZH solutions with different concentrations. Different letters (a-c) above the bars indicate significant difference ($p<0.05$) in mean. Inset: Appearances of Cur dispersions in ZH solutions with different concentrations. C: Residual ratios of Cur as a function of storage time at ambient conditions.

Fig. 4 Typical size distribution (A) and zeta potential (B) of ZH alone and ZH-Cur nanocomplexes in ZH solutions with different concentrations. Different letters (a-d) above the bars indicate significant difference ($p<0.05$) in mean.

Fig. 5 Typical TEM images of free Cur (A), ZH alone (B) and ZH-Cur complexes (C) with 3.0 k×, 15.0 k×, and 25.0 k× magnification respectively.

Fig. 6 A: Intrinsic fluorescence of ZH in the presence of different concentrations of Cur. Inset: Modified Stern-Volmer plots describing fluorescence quenching of ZH in the presence of Cur. B: Fluorescence spectra of Cur with varying concentrations of ZH. C: FTIR spectra of free Cur (blue), ZH alone (red), and ZH-Cur complexes (ZH-Cur, black). Arrows (in Fig. A and B) indicate the concentration increasing trend of ZH or Cur.

Fig. 1

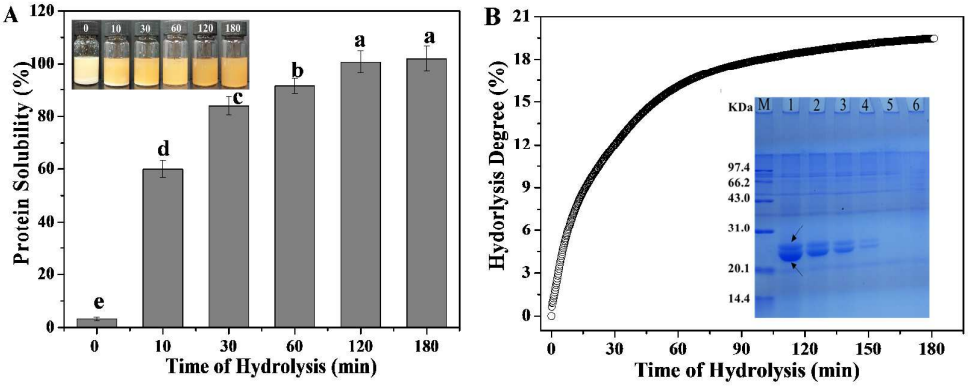


Fig. 2

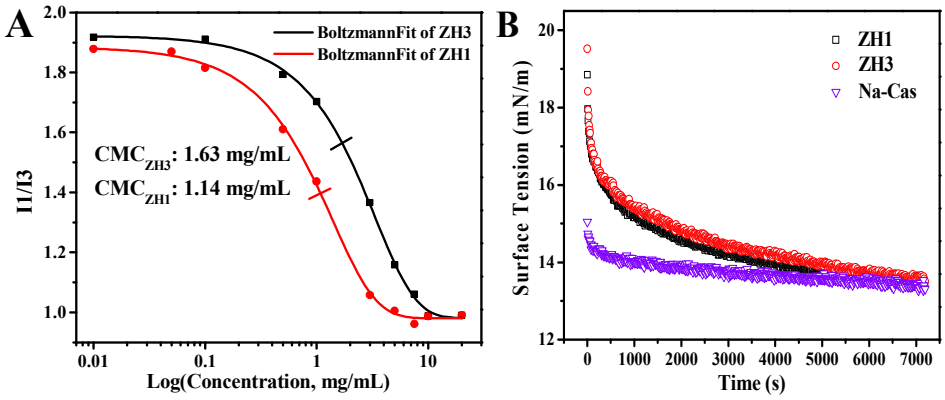


Fig. 3

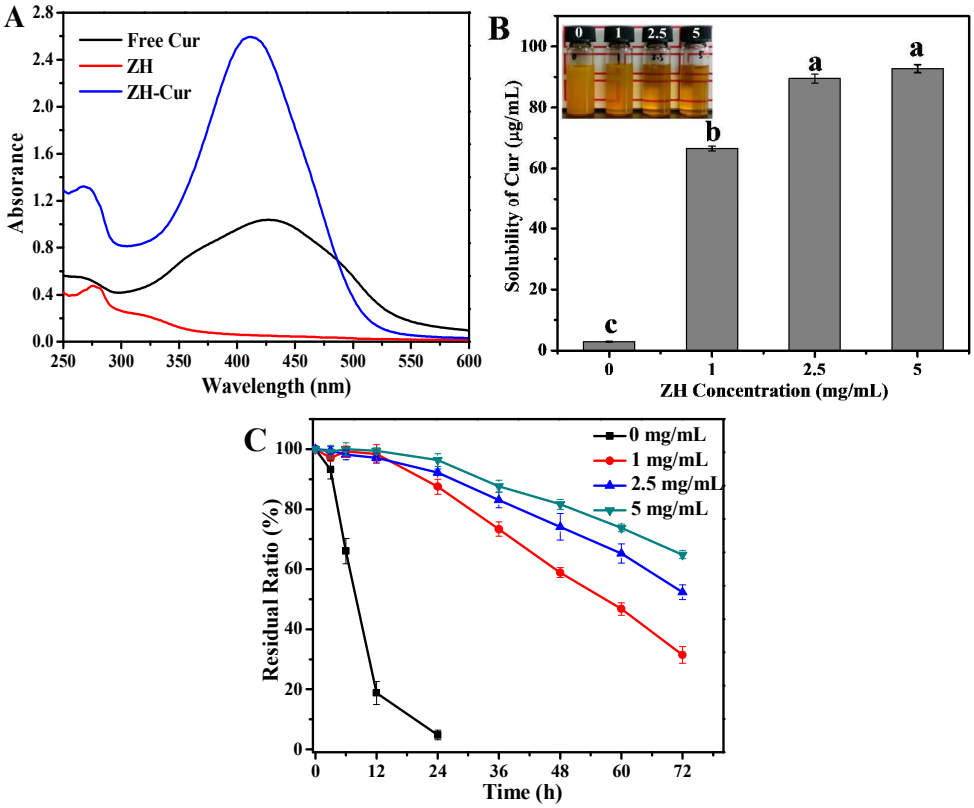


Fig. 4

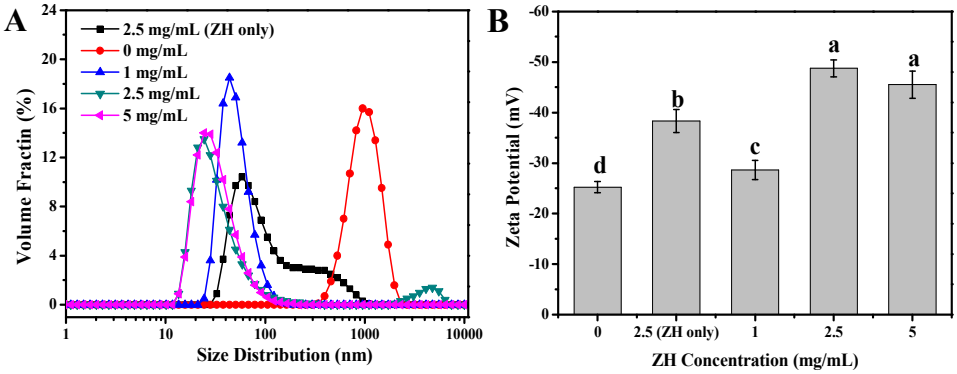


Fig. 5

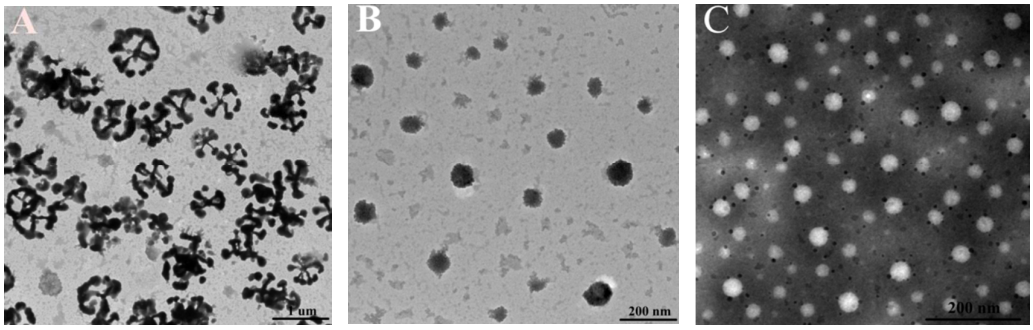
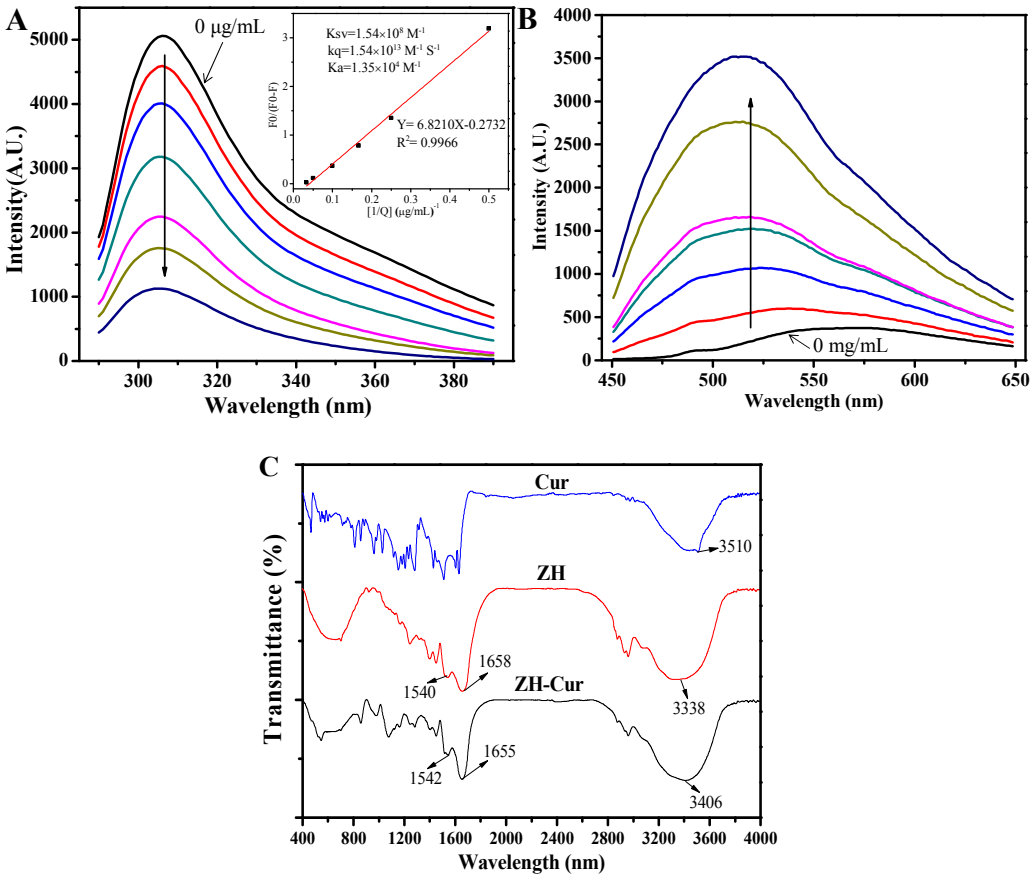


Fig. 6



Graphical Abstract

In this paper, we developed amphiphilic zein hydrolysate (ZH) as a novel delivery vehicle, which could be used for preparation of curcumin (Cur)-loaded nanoparticles by a simple anti-solvent method. These particles have a small size (<50 nm) and its dispersion in aqueous solution is transparent, which could have a great application potential in nutraceutical-fortified food and clear beverages. Spectroscopic studies showed that the formation of these nanoparticles mainly attribute to strong hydrophobic complexation between ZH and Cur.