1 **Enhancing the Bioaccessibility of Hydrophobic Bioactive Agents using**
2 **Mixed Colloidal Dispersions: Curcumin-loaded Zein Nanoparticles plus**
3 **Digestible Lipid Nanoparticles**

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13 **Journal:** Food Research International

14 **Submitted:** December 2015

15 **Revised:**

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Abstract

The potential of mixed colloidal dispersions for increasing the bioaccessibility of a hydrophobic bioactive agent (curcumin) was examined. Curcumin was encapsulated within zein nanoparticles (fabricated by antisolvent precipitation) to obtain a high loading capacity and good chemical stability. These protein nanoparticles were then mixed with digestible lipid nanoparticles (fabricated by microfluidization) designed to increase curcumin bioaccessibility by forming mixed micelles in the small intestine. Changes in particle properties (size, charge, and organization) were measured as the mixed colloidal dispersions were passed through a simulated gastrointestinal tract: mouth; stomach; small intestine. Curcumin bioaccessibility increased with increasing lipid nanoparticle concentration in the mixed colloidal dispersions, which was attributed to an increase in the solubilization capacity of the mixed micelle phase. This study suggests that delivery systems containing mixed colloidal particles (protein and lipid nanoparticles) may be designed to increase the bioaccessibility of lipophilic bioactive agents.

Keywords: nutraceuticals; pharmaceuticals; curcumin; delivery systems; nanoparticles; bioavailability; bioaccessibility; mixed systems

1. Introduction

Turmeric is a natural extract traditionally used as a spice and dye in foods because of its characteristic flavor profile and yellow color (Prasad, Gupta, Tyagi, & Aggarwal, 2014; Syed, Liew, Loh, & Peh, 2015). Turmeric may also be utilized as a bioactive agent because it contains appreciable levels of curcumin, which has been shown to have strong anti-inflammatory, antioxidant, and anti-cancer activities (Aggarwal, Kumar, & Bharti, 2003; Heger, van Golen, Broekgaarden, & Michel, 2014). In nature, curcumin typically exists in three different molecular forms that have varying levels of bioactivity: curcumin, demethoxycurcumin, and bis-demethoxycurcumin (Heger et al., 2014). Clinical trials have shown that curcumin is highly effective against colorectal cancer (Carroll et al., 2011) and pancreatic cancer (Dhillon et al., 2008). Curcumin is believed to exhibit these bioactive effects due to its ability to interfere with various biochemical pathways (Kunnumakkara, Anand, & Aggarwal, 2008). There has therefore been considerable interest in the utilization of curcumin as a bioactive agent in functional foods, supplements, and pharmaceuticals (Schneider, Gordon, Edwards, & Luis, 2015). Nevertheless, there are a number of challenges that currently limit the incorporation of curcumin into commercial products designed to improve human health associated with its low water-solubility, chemical instability, and poor oral bioavailability (Ahmed, Li, McClements, & Xiao, 2012; Fu et al., 2014; Heger et al., 2014).

The challenges associated with utilizing curcumin in commercial products may be overcome by encapsulating it within colloidal delivery systems (McClements, 2012; Patel & Velikov, 2011). Various types of colloidal delivery systems have been studied as a means to enhance the water-dispersibility, chemical stability, and oral bioavailability of curcumin, including casein micelles (Esmaili et al., 2011; Pan, Luo, Gan, Baek, & Zhong, 2014), zein nanoparticles (Gomez-Estaca, Balaguer, Gavara, & Hernandez-Munoz, 2012; Patel, Hu, Tiwari, & Velikov, 2010), curcumin nanoparticles (Margulis, Magdassi, Lee, & Macosko, 2014), colloidosomes (Zhao, Pan, Nitin, & Tikekar, 2014), liposomes (Chen et al., 2015; Niu et al., 2012), nanoemulsions (Ahmed et al., 2012; Yu, Shi, Liu, & Huang, 2012), and emulsions (Tikekar, Pan, & Nitin, 2013). Each of these colloidal delivery systems has certain advantages and disadvantages in terms of its ease of preparation, cost, robustness, and functional performance. An alternative approach developed to increase curcumin bioavailability is to mix a curcumin-rich

material (such as a powder) with an *excipient nanoemulsion* that consists of digestible lipid droplets dispersed within an aqueous medium (Zou, Liu, Liu, Xiao, & McClements, 2015, 2015; Zou et al., 2015). An excipient nanoemulsion may have no health benefits itself, but it increases the health benefits of any bioactive agents consumed with it by increasing their bioavailability. An excipient nanoemulsion may improve the oral bioavailability of co-ingested bioactive agents through numerous mechanisms, such as enhancing the solubility in gastrointestinal fluids, retarding chemical degradation, or increasing absorption (McClements & Xiao, 2014).

In the current study, we investigate the possibility of using mixed colloidal dispersions containing two different types of nanoparticles to increase curcumin bioaccessibility. The main advantage of this kind of delivery system is that one type of nanoparticle can be designed to overcome one problem limiting the bioavailability of a bioactive agent (such as poor chemical stability), while another type of nanoparticle can be designed to overcome another problem (such as low bioaccessibility). In this study, curcumin was encapsulated within protein nanoparticles prepared using an antisolvent precipitation method, and then these nanoparticles were mixed with a dispersion of digestible lipid nanoparticles prepared using high-pressure homogenization. The protein nanoparticles are designed to have a high loading capacity and to protect the curcumin from chemical degradation during storage. On the other hand, the lipid nanoparticles are designed to rapidly digest within the gastrointestinal tract and form mixed micelles that can solubilize and transport the hydrophobic curcumin molecules. Previous studies using curcumin have shown that zein nanoparticles give better stability against chemical degradation than lipid nanoparticles since the diffusion of reactants is more limited by a semi-solid zein matrix than by a fluid lipid matrix (Pan, Tikekar, Wang, Avena-Bustillos, & Nitin, 2015). Other studies have shown that lipid nanoparticles are highly effective at increasing the bioaccessibility of curcumin by forming mixed micelles after they are digested (Ahmed et al., 2012). Thus, mixed colloidal dispersions can be designed to make use of the relative advantages of these two different types of colloidal system. This study therefore provides useful information for the optimization of delivery systems designed to increase the oral bioaccessibility of hydrophobic bioactive agents.

2. Materials and Methods

2.1. Materials

Corn oil purchased from a local supermarket was used as a digestible long chain triglyceride (LCT). The following chemicals were purchased from the Sigma Chemical Company (St. Louis, MO): zein (Lot SLBD5665V), curcumin (SLBH2403V), mucin from porcine stomach (SLBH9969V), pepsin from porcine gastric mucosa (SLBL1993V), lipase from porcine pancreas (SLBH6427V), porcine bile extract (SLBK9078), Tween 80 (BCBG4438V), and Nile Red (063K3730V). Sodium caseinate was purchased from the American Casein Company (Burlington, NJ). Whey protein isolate (WPI) was kind donated by Davisco Foods International Inc. (Le Sueur, MN, USA). All other chemicals were of analytical grade. Double distilled water was used to prepare all solutions and emulsions.

2.2. Preparation of digestible lipid nanoparticles

Initially, an aqueous phase was prepared by dispersing 1% (w/w) Tween 80 into an aqueous buffer solution (5.0 mM phosphate buffer saline (PBS), pH 4.0) and then stirring for at least 2 h. A coarse oil-in-water emulsion was then formed by blending 10% (w/w) corn oil and 90% (w/w) aqueous phase using a high-shear mixer for 2 min (M133/1281-0, Biospec Products, Inc., ESGC, Switzerland). Nanoemulsions containing digestible lipid nanoparticles were then formed by passing the coarse emulsion three times through a microfluidizer (M110Y, Microfluidics, Newton, MA) with a 75 μ m interaction chamber (F20Y) at an operational pressure of 12,000 psi.

2.3. Preparation of curcumin-loaded protein nanoparticles

Curcumin-loaded protein nanoparticles were formed from a hydrophobic protein (zein) using an antisolvent precipitation method. Initially, curcumin (1.32 mg/mL) and zein (26.4 mg/mL) were dissolved in 80% (w/w) ethanol solution at a mass ratio of 1:20. Then, 25 mL of aqueous ethanol solution was rapidly injected into 75 mL of Tween 80 solution (PBS, pH=4.0) that was stirred at 1200 rpm using a magnetic stirring (IKA R05, Werke, GmbH). The resulting colloidal dispersion was then continually stirred for another 30 min at the same speed. The ethanol remaining in the final colloidal dispersions was evaporated using a rotary evaporator

(Rotavapor R110, Büchi Crop., Switzerland), and the same volume of pH 4.0 PBS was added to compensate for the lost ethanol.

2.4. Preparation of mixed nanoparticle colloidal dispersions

The 10% corn oil-in-water nanoemulsions prepared by microfluidization were diluted with different amounts of PBS (5 mM, pH=4.0) to form nanoemulsions containing 2.5%, 5.0%, or 10% corn oil. Nanoemulsions (20 mL) containing different concentrations of lipid nanoparticles were then mixed with dispersions of curcumin-loaded zein nanoparticles (20 mL). The resulting mixtures were then incubated at room temperature while being stirred at 400 rpm for 30 min. Zein-loaded curcumin nanoparticles (20 mL) mixed with PBS (5 mM, pH=4.0) (20 mL) were as a lipid-free control.

2.5. Color analysis of mixtures

The color of the nanoparticle mixtures was measured using an instrumental colorimeter (ColorFlex EZ 45/0-LAV, Hunter Associates Laboratory Inc., Virginia, USA). Color was expressed in CIE units: L^* (lightness/darkness), a^* (redness/greenness), and b^* (yellowness/blueness). An aliquot of sample (15 mL) was placed in a 64-mm path length glass sample cup and then illuminated with D65-artificial daylight (10° standard angle). Three replicate measurements were performed and the results were averaged.

2.6. Particle characterization

The mean particle diameter and particle size distribution of excipient nanoparticle mixtures before and after exposure to gastrointestinal conditions were monitored using static light scattering (Mastersizer 2000, Malvern Instruments Ltd., Worcestershire, UK). Samples were diluted with appropriate buffer solutions (same pH as sample) and stirred in the dispersion unit at a speed of 1200 rpm. The particle size is reported as the surface-weighted (d_{32}) or volume-weighted (d_{43}) mean diameter. The electrical charges (ζ -potential) of the particles in the samples were measured using a micro-electrophoresis instrument (Nano-ZS, Malvern Instruments, Worcestershire, UK). Samples were diluted with appropriate buffer solutions prior to measurements to avoid multiple scattering effects.

The mean particle diameters (Z-average), particle size, and electrical charges (ζ -potential) of micelles collected by centrifuging the raw digesta were measured by a dynamic light scattering instrument and micro-electrophoresis instrument, respectively (Nano-ZS, Malvern Instruments, Worcestershire, UK). Micelles were diluted with buffer solution (5 mM PBS, pH 7.0) prior to measurements to avoid multiple scattering effects. **Three replicate measurements were performed on each sample and the results were averaged.**

2.7. Microstructural analysis

The microstructure of samples was characterized using optical or confocal scanning fluorescence microscopy (Nikon D-Eclipse C1 80i, Nikon, Melville, NY). Samples analyzed by confocal microscopy were dyed with Nile Red (0.1%) and Fluorescein isothiocyanate (FITC) isomer I (0.1%) to highlight the location of the lipid phases and protein phases. All images were captured with a 10 $\times$ eyepiece and a 60 $\times$ objective lens (oil immersion).

2.8. Simulated gastrointestinal digestion

All the nanoparticle mixtures were passed through a three-step GIT model that consisted of mouth, gastric, and small intestine phases, which was slightly modified from our previous study (Zhang, Zhang, Zhang, Decker, & McClements; Zou et al., 2015).

Initial system: The nanoparticle mixtures (**all containing 0.165 mg/mL curcumin**) were placed into a glass beaker in a shaking incubator (Innova Incubator Shaker, Model 4080, New Brunswick Scientific, New Jersey, USA) at **100 rpm and 37 °C for 15 min.**

Mouth phase: A simulated saliva fluid (SSF) containing 3 mg/mL mucin and various salts (**sodium chloride, ammonium nitrate, potassium phosphate, potassium chloride, potassium citrate, uric acid sodium salt, urea, lactic acid sodium salt**) was prepared as described previously (Mao & McClements, 2012). SSF (**20 mL**) was preheated to 37 °C and then mixed with the preheated the nanoparticle mixtures (**20 mL**) at a 1:1 mass ratio. The mixture was then adjusted to pH 6.8 and placed in a shaking incubator at 100 rpm and 37 °C for 10 min to mimic oral conditions

Stomach phase: Simulated gastric fluid (SGF) was prepared by placing 2 g NaCl and 7 mL HCl into a container, and then adding double distilled water to 1 L. The bolus sample from the mouth phase (**20 mL**) was mixed with SGF (**20 mL**) containing 3.2 mg/mL pepsin (**≥ 250**

units/mg) preheated to 37 °C at a 1:1 mass ratio. The mixture was then adjusted to pH 2.5 and placed in a shaker at 100 rpm and 37 °C for 2 hours to mimic stomach digestion.

Small Intestine phase: 30 mL chyme samples from the stomach phase were diluted with 30 mL buffer solution (10 mM PBS, 6.5). The diluted chyme was then incubated in a water bath (37 °C) for 10 min and then the solution was adjusted back to pH 7.0. Next, 3 mL of simulated intestinal fluid (containing 0.5 M CaCl₂ and 7.5 M NaCl) was added to 60 mL digesta. Then, 7 mL bile extract, containing 375.0 mg bile extract (pH 7.0, PBS), was added with stirring and the pH was adjusted to 7.0. Finally, 5 mL of lipase suspension, containing 120 mg of lipase (100-400 units/mg) (pH 7.0, PBS), was added to the sample and an automatic titration unit (Metrohm, USA Inc.) was used to monitor the pH and control it to a fixed value (pH 7.0) by titrating 0.1 M NaOH solution into the reaction vessel for 2 h at 37 °C.

It should be noted that this relatively simple GIT model cannot accurately simulate the complexity of the human GIT. Nevertheless, it is particularly effective at elucidating physicochemical mechanisms that may occur during passage through the GIT, and in rapidly screening samples with different compositions and structures. Once a promising candidate has been identified it would be important to test it using an appropriate animal or human feeding study.

2.9. Curcumin concentration and bioaccessibility after digestion

After *in vitro* digestion, 20 mL raw digesta of each mixture was centrifuged (18,000 rpm, $\approx 38,465 \times g$) using a commercial device (Thermo Scientific, Waltham, MA, USA) at 25 °C for 30 min. The clear supernatant was collected and assumed to be the “micelle” fraction in which the curcumin was solubilized. In some samples, a layer of non-digested oil was observed at the top of the test tubes and it was excluded from the micelle fraction. Aliquots of 5 mL of raw digesta or micelle fraction were mixed with 5 mL of chloroform, vortexed, and centrifuged at 1,750 rpm ($\approx 940 \times g$) for 10 min at ambient temperature. The bottom layer containing the solubilized curcumin was collected, while the top layer was mixed with an additional 5 mL of chloroform and the same procedure was repeated. The two collected chloroform layers were mixed together, and then diluted to an appropriate concentration to be analyzed by a UV–VIS spectrophotometer at 419 nm. The curcumin concentrations in the overall raw digesta and in the

mixed micelle phase were calculated from the absorbance measurements using a standard curve and the known dilution factor.

The bioaccessibility was calculated from this data using the following equation:

$$\text{Bioaccessibility} = 100 \times (C_{\text{Micelle}} / C_{\text{Raw Digesta}}) \quad (1)$$

Here, C_{Micelle} and $C_{\text{Raw Digesta}}$ are the concentration of curcumin in the micelle fraction and in the overall raw digesta after the pH-stat experiment, respectively.

2.10. Statistical analysis

All experiments were carried out on two freshly prepared samples, with three measurements being made on each sample for the light scattering and ζ -potential instruments (see above). The results are expressed as means $\pm$ standard deviations (SD). Data were subjected to statistical analysis using SPSS software (version 18.0). Means were subject to Duncan's test and a P -value of <0.05 was considered statistically significant.

3. Results and Discussion

3.1. Initial properties of nanoparticle mixtures

The antisolvent precipitation method used in this research was able to produce relatively small curcumin-loaded zein nanoparticles ($d_{32} \approx 100$ nm; $d_Z \approx 120$ nm) with a monomodal particle size distribution (**Figure 1a, Table 1**). Similarly, the high-pressure homogenization (microfluidizer) method used was able to produce relatively small lipid nanoparticles ($d_{32} \approx 175$ nm; $d_Z \approx 191$ nm) with a monomodal particle size distribution. The mixed colloidal dispersions contained particles with mean diameters within the range of the two individual systems (**Figure 1a, Table 1**). These results suggest that the different types of nanoparticles were compatible with each other, *i.e.*, each type of nanoparticle did not promote aggregation of the other type when they were mixed together.

The visual appearance of the colloidal dispersions was recorded using digital photography (**Figure 1b**), and the change in their color was quantified by colorimetry (**Table 1**). The suspension of curcumin-loaded zein nanoparticles was transparent, but had a strong yellow color due to selective absorption of light by curcumin (Anand et al., 2008). The high optical clarity of

this system can be attributed to the small size of the zein nanoparticles relative to the wavelength of light (McClements, 2002). The suspension of lipid nanoparticles was opaque and had a whitish appearance, which can be attributed to stronger scattering of light by the larger lipid droplets. The opacity of the mixed systems increased as the concentration of lipid nanoparticles was increased from 2.5 to 10 wt%, while the color intensity (“yellowness”) decreased. Again, these effects can be attributed to the ability of the lipid nanoparticles to strongly scatter light, which leads to an increase in lightness and to color fading (McClements, 2002).

Confocal fluorescence microscopy images showed that the suspensions of lipid nanoparticles (lipid dyed red), curcumin-loaded zein nanoparticles (protein dyed green), and their mixtures contained relatively small particles that were fairly evenly spread throughout the system (**Figure 1c**). Nevertheless, there did appear to be a few clumps of protein-rich particles within the mixtures, which may have been evidence that these systems were not fully mixed or that some association of the zein nanoparticles had occurred. Nevertheless, large particles were not detected by the light scattering instrument (**Figure 1a**), which suggested that any flocs formed were held together by relatively weak attractive forces, which were disrupted when the samples were diluted for particle size analysis. In addition, we did not observe any phase separation due to gravitational separation (creaming or sedimentation) in the samples.

The initial electrical characteristics of the nanoparticle suspensions were also measured (**Table 1**). The curcumin-loaded zein nanoparticles had a relatively high positive charge ($\zeta \approx +20$ mV) because their pH (4.0) was below the isoelectric point of the zein molecules ($pI \approx 6.5$). Conversely, the lipid nanoparticles had a net charge close to zero ($\zeta \approx -1.0$ mV) because they were coated by a non-ionic surfactant (Tween 80). All mixed nanoparticle suspensions had net charges around zero, which suggests that the lipid nanoparticles dominated the overall signal used to calculate the ζ -potential. This may have been because the lipid nanoparticles scattered light more strongly than the protein nanoparticles due to their larger size, as evidenced by their higher opacity (**Figure 1b**).

3.2. Influence of gastrointestinal conditions on system properties

The various colloidal dispersions were passed through a simulated gastrointestinal tract (GIT) that contained mouth, stomach, and small intestine regions, and changes in system properties were measured.

As mentioned in the previous section, all of the nanoparticle suspensions initially contained relatively small particles ($d_{43} < 200$ nm) highlighting that antisolvent precipitation was appropriate for forming stable protein nanoparticles and microfluidization was suitable for forming stable lipid nanoparticles (**Figures 2 and 3**). After exposure to the oral phase, there was evidence of extensive particle aggregation in all the colloidal dispersions containing zein nanoparticles as seen in the light scattering data (**Figures 2 and 3**) and the confocal microscopy images (**Figure 4**). Particle aggregation can be attributed to bridging and/or depletion flocculation associated with the presence of mucin in the simulated saliva. Mucin is a complex glycoprotein that may bind to the surfaces of nanoparticles through electrostatic or hydrophobic forces, thereby linking them together (Vingerhoeds, Blijdenstein, Zoet, & van Aken, 2005). In addition, sufficiently high levels of non-adsorbed mucin may promote aggregation by generating a depletion attraction between the nanoparticles. The large flocs formed in the oral phase appeared to partially dissociate after exposure to the gastric phase (**Figure 4**), although there was still evidence of some larger particles in the systems. These effects can be attributed to changes in the pH, ionic strength, and enzyme activity of the aqueous phase surrounding the nanoparticles in the simulated gastric juices. There was also evidence of relatively large aggregates within the small intestine phase. These aggregates may have been partially-digested protein or lipid particles and/or particles formed from protein or lipid digestion products (such as mixed micelles formed from free fatty acids, monoacylglycerols, and bile salts). The changes in the particle size distribution of the zein-nanoparticles in the absence and presence of lipid nanoparticles is compared in **Figure 3**. There was evidence of a population of small particles within the mouth and stomach phases for the samples containing lipid nanoparticles that were not seen in their absence. This suggests that some of the lipid nanoparticles did not aggregate because they were coated by a layer of non-ionic surfactant molecules that was more stable to pH changes, *e.g.*, by generating a strong steric repulsion.

As discussed in Section 3.1, the curcumin-loaded zein nanoparticles had a relatively high positive charge because the initial pH was below the isoelectric point of the proteins. On the other hand, the mixed systems had a net charge close to zero, which suggested that the lipid nanoparticles (stabilized by a non-ionic surfactant) dominated the light scattering signal used to calculate the charge. The electrical charge on all the nanoparticle suspensions became fairly negative after exposure to simulated oral conditions (≈ -10 mV), which is probably due to the increase in pH to around 7, as well as the adsorption of anionic mucin molecules to the surfaces of the nanoparticles (**Figure 5**). The magnitude of the negative charge decreased appreciably after the nanoparticle suspensions were exposed to gastric conditions (≈ -3 mV), which can at least be partly attributed to changes in protein charge. The proteins would be expected to be positively charged under the highly acidic conditions present in the stomach, but the negatively charged mucin molecules may have formed complexes with them leading to a net negative charge. In addition, some of the proteins will have been digested by the pepsin in the gastric fluids, leading to molecular species with different charge characteristics. All of the nanoparticle systems became highly negatively charged after exposure to simulated intestinal conditions, which may be due to the change in pH to around neutral and to formation of anionic free fatty acids resulting from lipid digestion.

3.3. Digestibility

The digestion of the nanoparticles in the colloidal dispersions was measured during the small intestine phase using an automatic titration (pH stat) method. The volume of alkaline solution required to maintain the pH at neutral conditions was recorded and then converted into the amount of free fatty acids released (**Figure 6**). In general, there was a rapid initial increase in the calculated FFAs released during the first 5 minutes, followed by a more gradual increase at longer times, indicating that the digestion process was relatively rapid. In the absence of lipid nanoparticles, there was only a slight increase in the calculated FFAs released over time, as would be expected because they did not contain lipids. This slight increase was probably due to the presence of some undigested protein in the sample that was broken down by proteases arising from the pancreatin present in the simulated intestinal fluids. One would have expected that most of the protein would have already been digested within the gastric phase due to the presence of pepsin in the simulated gastric fluids. Nevertheless, the protein may not have been

fully digested before reaching the small intestine phase. As expected, the amount of FFAs released during the course of the small intestine phase increased as the amount of lipid nanoparticles present in the initial colloidal dispersions was increased. As a result, there would be more free fatty acids and monoacylglycerols available to form mixed micelles in the intestinal fluids at the end of the small intestine phase.

3.4. Bioaccessibility

After the small intestine phase, the samples were centrifuged and the mixed micelle phase was collected. The particle size and charge of the particles in the mixed micelle phase was measured (**Table 1**), and the bioaccessibility of the curcumin was calculated (**Figure 7**). The mixed micelle phase contained particles that were around 100 to 300 nm in all systems. These particles may have been micelles or vesicles comprising of bile salts and phospholipids from the bile extract, as well as free fatty acids and monoacylglycerols from the lipid digestion products. In addition, there may have been some colloidal particles arising from undigested lipids or proteins remaining in the samples.

The presence of the lipid nanoparticles clearly improved curcumin bioaccessibility (**Figure 7**). **In the absence of lipids, the bioaccessibility was much lower (< 36%) than in the presence of lipids (61 to 91%).** Curcumin is known to have low water-solubility, and so the modest bioaccessibility observed in the absence of lipid nanoparticles may have been due to curcumin solubilized within micelles/vesicles formed from bile salts and phospholipids, or due to association of curcumin with some of the proteins/peptides in the system. The bioaccessibility increased with increasing lipid nanoparticle concentration, which can be attributed to the fact that the lipid nanoparticles were rapidly digested within the small intestine phase and formed FFAs and monoacylglycerols. These lipid digestion products would have been assembled into mixed micelles that could solubilize the hydrophobic curcumin molecules within their non-polar interiors. The lipid nanoparticles may therefore be able to overcome one major limitation of curcumin - its low solubility in gastrointestinal fluids. Nevertheless, additional strategies may be needed to overcome other limitations such as chemical/biochemical transformation in the GIT. **For example, it may be necessary to include additives (such as buffers, antioxidants, or enzyme inhibitors) that can inhibit chemical degradation or metabolic enzymes.**

3.5. Comparison with other approaches

In this study, we used a mixed colloidal delivery system to improve the bioavailability characteristics of curcumin: digestible lipid nanoparticles mixed with curcumin-loaded protein nanoparticles. Numerous other approaches based on colloidal delivery systems can also be used to improve curcumin bioavailability, which have been reviewed recently (Flora, Gupta, & Tiwari, 2013; Hani & Shivakumar, 2014; Mohanty, Das, & Sahoo, 2012; Sun et al., 2012; Wang et al., 2014; Yallapu, Nagesh, Jaggi, & Chauhan, 2015). These approaches usually involve trapping the curcumin in one type of colloidal particle. For example, in a recent study we compared the efficacy of three food-grade colloidal delivery systems for improving the bioavailability of curcumin using an *in vitro* GIT model: protein nanoparticles (zein); lipid nanoparticles (nanoemulsions); and phospholipid nanoparticles (nanoliposomes) (Zou et al., 2016). The zein nanoparticles had a considerably higher curcumin loading capacity than the nanoemulsions and nanoliposomes, while the nanoemulsions led to a considerably higher curcumin bioaccessibility than the other two systems. Thus, the mixed colloidal systems developed in the current study take advantage of the favorable features of two different types of food-grade nanoparticles: protein-nanoparticles can be used to encapsulate high levels of curcumin and help protect it from chemical degradation, while lipid nanoparticles help to increase curcumin bioaccessibility by forming a source of mixed micelles.

4. Conclusions

This study has shown that the bioaccessibility of curcumin, an important hydrophobic nutraceutical, can be improved considerably using mixed nanoparticle systems. The curcumin was initially encapsulated inside zein nanoparticles, which have previously been shown to protect this chemically labile bioactive substance from degradation. These curcumin-loaded zein nanoparticles were then mixed with a suspension of digestible lipid nanoparticles. The high surface area of the lipid nanoparticles means that they are rapidly digested under small intestine conditions and form mixed micelles. These mixed micelles are capable of incorporating the hydrophobic curcumin molecules within their non-polar interiors thereby increasing the solubility of the curcumin in the intestinal fluids. Our results suggest that mixed colloidal delivery systems may be specifically designed to increase the chemical stability and

bioaccessibility of hydrophobic nutraceuticals and pharmaceuticals. One type of nanoparticle may be designed to overcome one challenge (such as poor chemical stability), whereas another type of nanoparticle may be designed to overcome another challenge (such as low bioaccessibility).

The current study used a relatively simple gastrointestinal tract model to study the influence of mixed particle colloidal delivery systems on curcumin bioaccessibility. In future studies it would be useful to carry out a more detailed investigation of their impact on the bioaccessibility, absorption, and transformation characteristics of curcumin using both in vitro and in vivo methods. In particular, it would be useful to use more advanced analytical tools (such as chromatography and mass spectrometry) to analyze the various types of metabolites formed by curcumin within the GIT, as well as to assess their bioactivities relative to the parent compound.

5. Acknowledgements

This material was partly based upon work supported by USDA, NRI Grants (2011-03539, 2013-03795, 2011-67021, and 2014-67021). This project was also partly funded by the Deanship of Scientific Research (DSR), King Abdulaziz University, Jeddah, under grant numbers 330-130-1435-DSR, 299-130-1435-DSR, 87-130-35-HiCi. The authors, therefore, acknowledge with thanks DSR technical and financial support. This project was also partly supported by the National Natural Science Foundation of China (NSFC31428017).

6. References

- Aggarwal, B. B., Kumar, A., & Bharti, A. C. (2003). Anticancer potential of curcumin: preclinical and clinical studies. *Anticancer Res*, 23(1a), 363-398.
- Ahmed, K., Li, Y., McClements, D. J., & Xiao, H. (2012). Nanoemulsion- and emulsion-based delivery systems for curcumin: Encapsulation and release properties. *Food Chemistry*, 132(2), 799-807.
- Anand, P., Thomas, S. G., Kunnumakkara, A. B., Sundaram, C., Harikumar, K. B., Sung, B., Tharakan, S. T., Misra, K., Priyadarsini, I. K., Rajasekharan, K. N., & Aggarwal, B. B. (2008). Biological activities of curcumin and its analogues (Congeners) made by man and Mother Nature. *Biochemical Pharmacology*, 76(11), 1590-1611.

413 Carroll, R. E., Benya, R. V., Turgeon, D. K., Vareed, S., Neuman, M., Rodriguez, L., Kakarala, M., Carpenter,
414 P. M., McLaren, C., Meyskens, F. L., Jr., & Brenner, D. E. (2011). Phase IIa clinical trial of
415 curcumin for the prevention of colorectal neoplasia. *Cancer Prev Res (Phila)*, 4(3), 354-364.

416 Chen, X., Zou, L.-Q., Niu, J., Liu, W., Peng, S.-F., & Liu, C.-M. (2015). The Stability, Sustained Release and
417 Cellular Antioxidant Activity of Curcumin Nanoliposomes. *Molecules*, 20(8), 14293-14311.

418 Dhillon, N., Aggarwal, B. B., Newman, R. A., Wolff, R. A., Kunnumakkara, A. B., Abbruzzese, J. L., Ng, C. S.,
419 Badmaev, V., & Kurzrock, R. (2008). Phase II trial of curcumin in patients with advanced
420 pancreatic cancer. *Clin Cancer Res*, 14(14), 4491-4499.

421 Esmaili, M., Ghaffari, S. M., Moosavi-Movahedi, Z., Atri, M. S., Sharifzadeh, A., Farhadi, M., Yousefi, R.,
422 Chobert, J.-M., Haertlé, T., & Moosavi-Movahedi, A. A. (2011). Beta casein-micelle as a nano
423 vehicle for solubility enhancement of curcumin; food industry application. *LWT - Food Science
424 and Technology*, 44(10), 2166-2172.

425 Flora, G., Gupta, D., & Tiwari, A. (2013). Nanocurcumin: A Promising Therapeutic Advancement over
426 Native Curcumin. *Critical Reviews in Therapeutic Drug Carrier Systems*, 30(4), 331-368.

427 Fu, S., Shen, Z., Ajlouni, S., Ng, K., Sanguansri, L., & Augustin, M. A. (2014). Interactions of buttermilk
428 with curcuminoids. *Food Chemistry*, 149(0), 47-53.

429 Gomez-Estaca, J., Balaguer, M. P., Gavara, R., & Hernandez-Munoz, P. (2012). Formation of zein
430 nanoparticles by electrohydrodynamic atomization: Effect of the main processing variables and
431 suitability for encapsulating the food coloring and active ingredient curcumin. *Food
432 Hydrocolloids*, 28(1), 82-91.

433 Hani, U., & Shivakumar, H. G. (2014). Solubility Enhancement and Delivery Systems of Curcumin a Herbal
434 Medicine: A Review. *Current Drug Delivery*, 11(6), 792-804.

435 Heger, M., van Golen, R. F., Broekgaarden, M., & Michel, M. C. (2014). The Molecular Basis for the
436 Pharmacokinetics and Pharmacodynamics of Curcumin and Its Metabolites in Relation to
437 Cancers. *Pharmacological Reviews*, 66(1), 222-307.

438 Kunnumakkara, A. B., Anand, P., & Aggarwal, B. B. (2008). Curcumin inhibits proliferation, invasion,
439 angiogenesis and metastasis of different cancers through interaction with multiple cell signaling
440 proteins. *Cancer Letters*, 269(2), 199-225.

441 Mao, Y., & McClements, D. J. (2012). Influence of electrostatic heteroaggregation of lipid droplets on
442 their stability and digestibility under simulated gastrointestinal conditions. *Food & Function*,
443 3(10), 1025-1034.

444 Margulis, K., Magdassi, S., Lee, H. S., & Macosko, C. W. (2014). Formation of curcumin nanoparticles by
445 flash nanoprecipitation from emulsions. *Journal of Colloid and Interface Science*, 434(0), 65-70.

446 McClements, D. J. (2002). Theoretical prediction of emulsion color. *Advances in Colloid and Interface
447 Science*, 97(1-3), 63-89.

448 McClements, D. J. (2012). Advances in fabrication of emulsions with enhanced functionality using
449 structural design principles. *Current Opinion in Colloid & Interface Science*, 17(5), 235-245.

450 McClements, D. J., & Xiao, H. (2014). Excipient foods: designing food matrices that improve the oral
451 bioavailability of pharmaceuticals and nutraceuticals. *Food & Function*, 5(7), 1320-1333.

452 Mohanty, C., Das, M., & Sahoo, S. K. (2012). Emerging role of nanocarriers to increase the solubility and
453 bioavailability of curcumin. *Expert Opinion on Drug Delivery*, 9(11), 1347-1364.

454 Niu, Y. M., Ke, D., Yang, Q. Q., Wang, X. Y., Chen, Z. Y., An, X. Q., & Shen, W. G. (2012). Temperature-
455 dependent stability and DPPH scavenging activity of liposomal curcumin at pH 7.0. *Food*
456 *Chemistry*, 135(3), 1377-1382.

457 Pan, K., Luo, Y., Gan, Y., Baek, S. J., & Zhong, Q. (2014). pH-driven encapsulation of curcumin in self-
458 assembled casein nanoparticles for enhanced dispersibility and bioactivity.
459 [10.1039/C4SM00239C]. *Soft Matter*, 10(35), 6820-6830.

460 Pan, Y., Tikekar, R. V., Wang, M. S., Avena-Bustillos, R. J., & Nitin, N. (2015). Effect of barrier properties
461 of zein colloidal particles and oil-in-water emulsions on oxidative stability of encapsulated
462 bioactive compounds. *Food Hydrocolloids*, 43, 82-90.

463 Patel, A., Hu, Y., Tiwari, J. K., & Velikov, K. P. (2010). Synthesis and characterisation of zein-curcumin
464 colloidal particles. *Soft Matter*, 6(24), 6192-6199.

465 Patel, A. R., & Velikov, K. P. (2011). Colloidal delivery systems in foods: A general comparison with oral
466 drug delivery. *Lwt-Food Science and Technology*, 44(9), 1958-1964.

467 Prasad, S., Gupta, S. C., Tyagi, A. K., & Aggarwal, B. B. (2014). Curcumin, a component of golden spice:
468 From bedside to bench and back. *Biotechnology Advances*, 32(6), 1053-1064.

469 Schneider, C., Gordon, O. N., Edwards, R. L., & Luis, P. B. (2015). Degradation of Curcumin: From
470 Mechanism to Biological Implications. *J Agric Food Chem*.

471 Sun, M., Su, X., Ding, B. Y., He, X. L., Liu, X. J., Yu, A. H., Lou, H. X., & Zhai, G. X. (2012). Advances in
472 nanotechnology-based delivery systems for curcumin. *Nanomedicine*, 7(7), 1085-1100.

473 Syed, H. K., Liew, K. B., Loh, G. O. K., & Peh, K. K. (2015). Stability indicating HPLC–UV method for
474 detection of curcumin in Curcuma longa extract and emulsion formulation. *Food Chemistry*,
475 170(0), 321-326.

476 Tikekar, R. V., Pan, Y., & Nitin, N. (2013). Fate of curcumin encapsulated in silica nanoparticle stabilized
477 Pickering emulsion during storage and simulated digestion. *Food Research International*, 51(1),
478 370-377.

479 Vingerhoeds, M. H., Blijdenstein, T. B. J., Zoet, F. D., & van Aken, G. A. (2005). Emulsion flocculation
480 induced by saliva and mucin. *Food Hydrocolloids*, 19(5), 915-922.

481 Wang, S., Su, R., Nie, S. F., Sun, M., Zhang, J., Wu, D. Y., & Moustaid-Moussa, N. (2014). Application of
482 nanotechnology in improving bioavailability and bioactivity of diet-derived phytochemicals.
483 *Journal of Nutritional Biochemistry*, 25(4), 363-376.

484 Yallapu, M. M., Nagesh, P. K. B., Jaggi, M., & Chauhan, S. C. (2015). Therapeutic Applications of Curcumin
485 Nanoformulations. *Aaps Journal*, 17(6), 1341-1356.

486 Yu, H., Shi, K., Liu, D., & Huang, Q. (2012). Development of a food-grade organogel with high
487 bioaccessibility and loading of curcuminoids. *Food Chemistry*, 131(1), 48-54.

488 Zhang, R., Zhang, Z., Zhang, H., Decker, E. A., & McClements, D. J. Influence of emulsifier type on
489 gastrointestinal fate of oil-in-water emulsions containing anionic dietary fiber (pectin). *Food*
490 *Hydrocolloids*(0).

491 Zhao, Y., Pan, Y., Nitin, N., & Tikekar, R. V. (2014). Enhanced stability of curcumin in colloidosomes
492 stabilized by silica aggregates. *LWT - Food Science and Technology*, 58(2), 667-671.

493 Zou, L., Liu, W., Liu, C., Xiao, H., & McClements, D. J. (2015). Designing excipient emulsions to increase
 494 nutraceutical bioavailability: emulsifier type influences curcumin stability and bioaccessibility by
 495 altering gastrointestinal fate. *Food & Function*, 6(8), 2475-2486.

496 Zou, L., Liu, W., Liu, C., Xiao, H., & McClements, D. J. (2015). Utilizing Food Matrix Effects To Enhance
 497 Nutraceutical Bioavailability: Increase of Curcumin Bioaccessibility Using Excipient Emulsions.
 498 *Journal of Agricultural and Food Chemistry*, 63(7), 2052-2062.

499 Zou, L., Zheng, B., Liu, W., Liu, C., Xiao, H., & McClements, D. J. (2015). Enhancing nutraceutical
 500 bioavailability using excipient emulsions: Influence of lipid droplet size on solubility and
 501 bioaccessibility of powdered curcumin. *Journal of Functional Foods*, 15, 72-83.

502 Zou, L., Zheng, B., Zhang, R., Zhang, Z., Liu, W., Liu, C., Xiao, H., & McClements, D. J. (2016).
 503 Encapsulation, protection and delivery of curcumin: Comparison of lipid, protein, and
 504 phospholipid nanoparticles under simulated gastrointestinal conditions. *RSC Advances*, *In press*.

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Figure 1 (a). Particle size distributions of curcumin-loaded zein nanoparticles (Zein-nPs), lipid nanoparticles (L-nPs), and mixtures of curcumin-loaded zein nanoparticles with different levels of lipid nanoparticles (Z-nLPs + L-nPs);

Figure 1 (b). Photographs of suspensions of lipid nanoparticles, curcumin-loaded zein nanoparticles, and their mixtures.

Figure 1 (c). Microstructure of lipid nanoparticles, curcumin-loaded zein nanoparticles, and their mixtures.

Figure 2. Influence of simulated gastrointestinal conditions on the mean droplet diameter (d_{43}) of curcumin-loaded zein nanoparticles (Z-nP), and mixtures of these nanoparticles with different levels of lipid nanoparticles (Z-nP + L-nP). Different lowercase letters mean significant differences ($p < 0.05$) of the droplet diameter of an emulsion between digestion phases; Different capital letters mean significant differences ($p < 0.05$) of the droplet diameter between curcumin-loaded zein nanoparticles (Z-nP), or mixtures of these nanoparticles with different levels of lipid nanoparticles (Z-nP + L-nP).

Figure 3a. Influence of simulated gastrointestinal conditions on the particle size distributions of a suspension initially containing curcumin-loaded zein nanoparticles.

Figure 3b. Influence of simulated gastrointestinal conditions on the particle size distributions of a mixed suspension containing curcumin-loaded zein nanoparticles and 5% lipid nanoparticles.

Figure 4. Influence of simulated gastrointestinal conditions on microstructure of curcumin-loaded zein nanoparticles (Z-nPs), and mixtures of these nanoparticles with different levels of lipid nanoparticles (Z-nPs + L-nPs) determined by confocal fluorescence microscopy. The scale bars represent a length of 20 μm , and the red regions represent lipids.

Figure 5. Influence of simulated gastrointestinal conditions on the particle charge of mixed colloidal dispersions containing curcumin-loaded zein nanoparticles and different levels of lipid nanoparticles. Different lowercase letters mean significant differences ($p < 0.05$) of the particle charge of an emulsion between digestion phases; Different capital letters mean significant differences ($p < 0.05$) of the particle charge in curcumin-loaded zein nanoparticles (Z-nP), or mixtures of curcumin-loaded zein nanoparticles with different levels of lipid nanoparticles within the same digestion phase.

Figure 6. Influence of incubation temperature on calculated free fatty acids release profile for mixtures containing curcumin-loaded zein nanoparticles and different levels of lipid nanoparticles.

Figure 7. Influence of lipid content (concentration of lipid nanoparticles) on the bioaccessibility of curcumin from mixed nanoparticle systems.

Table 1. The mean droplet diameter (d_{32}), ζ -potential, and tristimulus color coordinates of curcumin-loaded zein nanoparticles (Z-nP), and Z. Samples designated with different letters (a, b, c) were significantly different (Duncan, $p < 0.05$).

	L-nP	Z-nP	Z-nP+2.5% L-nP	Z-nP+5% L-nP	Z-nP+10% L-nP
Mean diameter (nm)	191 $\pm$ 2 ^b	120 $\pm$ 12 ^a	181 $\pm$ 2 ^b	183 $\pm$ 1 ^b	190 $\pm$ 3 ^b
PDI	0.161 $\pm$ 0.001 ^a	0.252 $\pm$ 0.005 ^b	0.151 $\pm$ 0.023 ^a	0.173 $\pm$ 0.001 ^a	0.152 $\pm$ 0.014 ^a
ζ -potential (mV)	-1.0 $\pm$ 0.0 ^a	19..9 $\pm$ 0.8 ^c	-0.3 $\pm$ 0.2 ^a	-0.5 $\pm$ 0.1 ^a	0.9 $\pm$ 1.0 ^b
d_{32}	0.175 $\pm$ 0.013 ^c	0.100 $\pm$ 0.001 ^a	0.148 $\pm$ 0.005 ^b	0.153 $\pm$ 0.007 ^b	0.152 $\pm$ 0.006 ^b
d_{43}	0.245 $\pm$ 0.007 ^c	0.125 $\pm$ 0.003 ^a	0.224 $\pm$ 0.006 ^b	0.228 $\pm$ 0.007 ^b	0.226 $\pm$ 0.007 ^b
L	93.1 $\pm$ 0.9 ^e	27.3 $\pm$ 0.4 ^a	80.3 $\pm$ 0.2 ^b	85.8 $\pm$ 0.2 ^c	89.6 $\pm$ 0.1 ^d
a	-1.4 $\pm$ 0.2 ^e	-15.7 $\pm$ 0.1 ^a	-12.1 $\pm$ 0.4 ^d	-12.9 $\pm$ 0.5 ^c	-13.7 $\pm$ 0.2 ^b
b	2.9 $\pm$ 0.3 ^a	36.9 $\pm$ 0.9 ^b	86.2 $\pm$ 1.7 ^e	82.3 $\pm$ 2.3 ^d	73.0 $\pm$ 0.5 ^c

Table 2. Bioaccessibility (BA), curcumin concentration in the raw digesta ($C_{\text{Raw digesta}}$), and curcumin concentration (C_{Micelle}), mean particle diameter, polydispersity index (PDI), and ζ -potential of the micelle phase isolated from raw digesta of curcumin-loaded zein nanoparticles (Z-nP), and mixtures of these nanoparticles with different levels of lipid nanoparticles (L-nP). Samples designated with different letters (a, b, c) were significantly different (Duncan, $p < 0.05$).

	Z-nP	Z-nP + 2.5% L-nP	Z-nP + 5% L-nP	Z-nP + 10% L-nP
BA (%)	35.2±8.0 ^a	61.0±4.8 ^b	80.6±9.1 ^c	91.2±4.6 ^c
$C_{\text{Raw digesta}}$ (µg/mL)	100.9±6.7 ^a	100.7±5.4 ^a	89.2±3.0 ^a	120.9±10.3 ^b
C_{Micelle} (µg/mL)	34.9±5.7 ^a	54.0±5.7 ^b	71.6±5.7 ^c	109.8±3.8 ^d
Mean diameter (nm)	203±16 ^b	314±43 ^c	134±7.8 ^a	115±15 ^a
PDI	0.598±0.042 ^c	0.336±0.004 ^b	0.285±0.002 ^{ab}	0.262±0.051 ^a
ζ -potential (mV)	-10.3±2.1 ^c	-14.8±1.7 ^{bc}	-19.6±1.1 ^{ab}	-22.3±4.2 ^a