1 Surface Modification of Zein Colloidal Particles with Sodium Caseinate to Stabilize Oil-in-Water
2 Pickering Emulsion

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Abstract

Zein colloidal nanoparticles can adsorb at the oil-water interface to form Pickering emulsion. However, zein Pickering emulsion is usually not stable due to the poor wettability of zein colloidal nanoparticles. The objective of this study was to modify the surface of zein nanoparticles using sodium caseinate (NaCas) and assess the properties of zein/NaCas nanocomplexes and the resultant oil-in-water Pickering emulsions. One percent (w/w) of zein/NaCas colloidal nanocomplexes were formed, with the zein:NaCas ratios (w/w) ranging from 10:1 to 10:4 at pH=3 by an ultrasound treatment. The zeta-potential of the zein/NaCas nanocomplexes showed altered surface charges, indicating that NaCas adsorbed on the surface of the zein colloidal nanoparticles. Three-phase contact angle measurements suggested that the original zein colloidal nanoparticles were preferentially wetted in water. The incorporation of 0.1%-0.2% (w/w) NaCas significantly enhanced its wettability in the oil, and intermediate wettability was achieved at a zein:NaCas ratio of 10:3. Confocal laser scanning microscopy (CLSM) showed that the incorporation of NaCas improved the interfacial coverage of the Pickering emulsions. When the zein:NaCas ratio ranged from 10:1 to 10:3, the interface was composed of zein/NaCas nanocomplexes. At a zein:NaCas ratio of 10:4, NaCas can competitively adsorb to the interface and formed a hybrid interfacial structure. The Zein/NaCas nanocomplexes stabilized the Pickering emulsions and exhibited greater centrifugal stability than plain zein emulsions at most pHs and ionic strengths. The underlying mechanisms of the improved emulsion stability are discussed in this paper. This study explored a novel approach to stabilizing Pickering emulsions via the surface modification method using a food-grade protein.

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Key words

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1. Pickering emulsion

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2. Zein colloidal nanoparticles

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3. Sodium caseinate

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4. Surface modification

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5. Wettability

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6. Limited coalescence

1. Introduction

Emulsions are commonly used in the food and pharmaceutical industries for the encapsulation and delivery of bioactive compounds (McClements, 2012). Conventional food emulsions are stabilized by biopolymers and low-molecular-mass (LMM) surfactants, which have been extensively studied in the past few decades (A. Bos & van Vliet, 2001; Wilde, Mackie, Husband, Gunning, & Morris, 2004). Alternatively, colloidal particle-stabilized Pickering emulsions have attracted considerable interest in recent years due to their superior stability against coalescence and irreversible adsorption to the oil-water interface (Dickinson, 2010).

Unlike biopolymers and LMM surfactants, colloidal particles can adsorb at the interface to form a rigid shell that is a potentially thicker layer, resulting in reduced mobility, which may offer enhanced stability to encapsulants (Tikekar, Pan, & Nitin, 2013). To improve the compatibility of Pickering emulsifiers in pharmaceutical and food applications, many pioneering studies have derived Pickering emulsifiers from biological sources, e.g., pea protein (Liang & Tang, 2014), soy protein aggregates (Liu & Tang, 2014, 2013), whey protein microgel particles (Destribats, Rouvet, Gehin-Delval, Schmitt, & Binks, 2014) and zein protein nanoparticles (de Folter, van Ruijven, & Velikov, 2012; Gao et al., 2014).

It is widely recognized that the wettability of colloidal nanoparticles in the oil-water interface can substantially affect the emulsion stability. The wettability is usually expressed using the oil-water three-phase contact angle θ . An appropriate θ angle is a hinge that ensures the type of emulsion (O/W or W/O) and irreversible adsorption. Generally, the θ angle to achieve irreversible adsorption is too close to neither 0° nor 180° (Dickinson, 2012). However, most of the known inorganic and biological colloidal particles have been found to be too hydrophilic, thus resulting in slow diffusion and low interfacial coverage during emulsification (Gao et al., 2014; Qi et al., 2014). Several approaches have been explored to improve the surface properties of both inorganic and biological materials, including utilizing small molecular surfactants as well as lecithin and hydrophobin, to synergistically stabilize Pickering emulsions

(Binks, Rodrigues, & Frith, 2007; Gao et al., 2014; Ghouchi Eskandar, Simovic, & Prestidge, 2007; Nesterenko, Drelich, Lu, Clausse, & Pezron, 2014). To be compatible with the food and pharmaceutical industries, there is a need to minimize the usage of surfactants due to their toxicity. As an alternative, recent studies have applied processing techniques such as heating and sonication to generate colloidal particles without using surfactants (Destribats et al., 2014; Liu & Tang, 2015). Zein colloidal nanoparticles have been found as a potential food grade Pickering stabilizer (de Folter et al., 2012; Gao et al., 2014). Zein emulsions also suffer from stability issues due to the creaming and coalescence that occur at pHs close to the isoelectric point. To improve the feasibility of zein as a Pickering emulsifier, surface modification with sodium stearate has been proposed (Gao et al., 2014). However, the toxicity of a surfactant such as sodium stearate can still be a problem when applying the Pickering emulsion for food.

Sodium caseinate (NaCas) has been utilized to improve the stability and dispersibility of zein colloidal particles in the aqueous phase through surface adsorption, and this technique has also been applied for encapsulation purposes (Chen & Zhong, 2014; Li et al., 2014; Patel, Bouwens, & Velikov, 2010; Ye, 2008). Two mechanisms (electrostatic coacervation and direct molecular interactions) have been proposed to interpret the process (Patel et al., 2010), but there is still a lack of mechanistic information. A recent study suggested that β -casein can act as a molecular chameleon and adsorb to a variety of surfaces by changing its own molecular arrangement according to pH, ionic strength and the specific surface types (Evers, Andersson, Lund, & Skepö, 2012; Svensson, Kurut, & Skepö, 2014). It is still unknown whether NaCas behaves like β -casein when it interacts with a zein colloidal surface, but the adsorption implies the potential alternation of the surface composition. This surface modification could also tailor the surface roughness, which was proved to substantially affect a particle's wettability (San-Miguel & Behrens, 2012). To date, there has been no systematic study that has investigated the adsorption of NaCas on zein colloidal particles, in addition to the concomitantly formed zein/NaCas nanocomplexes.

Food-grade Pickering emulsions have recently been a subject of renewed interest, but there are still many challenges to their application. The main problem with biological Pickering emulsifiers is that they are inherently hydrophilic, resulting in insufficient droplet coverage, and they require surfactants as synergistic stabilizers (Gao et al., 2014). Herein, we present novel research on the surface properties of zein/NaCas colloidal nanocomplexes. The objective of this study is to investigate the potential of modifying the surface properties using an amphiphilic protein, sodium caseinate (NaCas). Furthermore, we study the surface properties of zein/NaCas colloidal nanocomplexes, including the wettability and surface charge, as a function of pH and the zein:NaCas ratios. The zein/NaCas nanocomplexes were used to prepare oil-in-water Pickering emulsions, and the macroscopic stability of the Pickering emulsions against environmental stresses as well as the interfacial properties of the Pickering emulsions are characterized and discussed.

2. Methods and materials

2.1 *Materials.*

Zein (Z3625, with the total protein concentration $92.01 \pm 0.58\%$), sodium caseinate (C8654, with the total protein concentration $89.28 \pm 0.18\%$), protein assay kits including Biuret reagent (B3934) and Folin and Ciocalteu's phenol reagent (F6678), pure water (320072), sodium dodecyl sulfate (L3771) and sodium azide (S2002) were obtained from Sigma Aldrich Inc. (St. Louis, MO, USA). Sodium chloride (S271-3) was purchased from Fisher Scientific (Pittsburgh, PA, USA). Canola oil (Crisco Inc., Orrville, OH, USA) was purchased from a local store. Ethanol (anhydrous denatured 200 proof) was obtained from Cole-Parmer Inc. (Vernon Hills, IL, USA). The other chemicals used were all analytical grade.

2.2 Preparation and characterization of zein/NaCas nanocomplexes

2.2.1 *Preparation of zein colloidal nanoparticles.* Zein colloidal nanoparticles were prepared following the liquid-liquid dispersion method. Six grams of zein was dissolved into 100 ml 70% ethanol solution to form a stock solution. The stock solution was then dispersed to 150 ml DI water under high-speed (12,000 rpm) homogenization (IKA-ULTRA-TURRAX® T25 basic, IKA® Works, Inc., Wilmington, NC, USA) to form a dispersion of zein colloidal nanoparticles. To remove large zein aggregates, the dispersion was centrifuged (model IEC CENTRA CL2, Thermo Scientific Inc., Waltham, MA, USA) at 100 x g for 10 min. Excessive ethanol and water were evaporated by a rotary evaporator (HaakeBuchler Instruments Inc., Saddle Brook, NJ, USA) at 65°C, and the sample was centrifuged at 100 x g for 10 min again to ensure the removal of aggregates formed during the evaporation. The final concentration of the zein colloidal nanoparticle dispersion was 1% (w/w).

2.2.2 *Formation of zein/NaCas nanocomplexes.* To avoid aggregation, the pH of the zein colloidal dispersions was adjusted to be approximately 2.8 using HCl. The zein/NaCas nanocomplexes were prepared at pH=3 by adding various amounts of NaCas powder into zein colloidal nanoparticle dispersions under constant magnetic stirring at 500 rpm for 10 min (IKA RO10, Works, Inc., Wilmington, NC, USA). In total, zein/NaCas nanocomplexes with four different ratios were prepared: 10:1, 10:2, 10:3 and 10:4 (zein: NaCas, w/w). The samples (40 ml) were then treated with a sonicator equipped with a standard 1/8" diameter probe (Ultrasonic processor Q55, Qsonica, Newtown, CT, USA) at 50 % power for 30 seconds to facilitate the NaCas adsorption. The final pH of the zein/NaCas nanocomplex dispersions was adjusted to 3.0. Sodium azide (0.01%, w/w) was added as a preservative.

2.2.3 *Particle size and zeta-potential.* The zeta potential measurements were conducted using a zeta potential analyzer (model ZetaPALS, Brookhaven Instruments Corporation, Brookhaven, NY, USA) at various pHs ranging from 3.0 to 9.0. All samples were diluted by 100 times to minimize the interaction between particles and prevent isoelectrostatic precipitation. The pH was adjusted using HCl or NaOH solution, and an average value of three measurements was

reported for each sample. The hydrodynamic sizes of the zein colloidal nanoparticles were also determined using dynamic light scattering (DLS).

2.2.4 Wettability. The three-phase contact angle (θ) was measured using a VCA Optima system (AST Products Inc., Billerica, MA, USA). The methodology and sample preparation were modified from previous studies (Qi et al., 2014; L.-J. Wang et al., 2015). Briefly, freeze-dried protein samples were compressed into a thin film. This protein film was then immersed in canola oil. A droplet (2 μ L) of pure water was placed on the protein film. After equilibrium was reached, contact angles were determined using the instrumental software. All measurements were replicated for at least six times.

2.2.5 Morphology. The morphological structure of zein colloidal nanoparticles, zein/NaCas nanocomplexes and NaCas were obtained using a scanning electron microscope equipped with a field emission electron gun (model Philips XL30 ESEM-FEG, FEI Company, Hillsboro, OR, USA). Samples were subjected to freeze-drying (Labconco Freezone 6, Labconco Corp., Kansas city, MO, USA) for 48 hours, followed by adhering to a conductive carbon tape and sputter coating (model Dest-1 TSC, Denton Vacuum LLC., Moorestown, NJ, USA) with a gold layer before imaging.

2.3 Preparation and characterization of emulsions

2.3.1 Preparation of emulsions. All emulsions were prepared using the same emulsifier concentration (1%, w/v), oil fraction ($\Phi=0.5$, v/v) and pH (pH=3.0). Canola oil was added slowly to the aqueous phase while mixing with a high-speed homogenizer at 12,000 rpm (IKA-ULTRA-TURRAX® T25 basic, IKA® Works, Inc., Wilmington, NC, USA). The samples were homogenized for another 2 min after all the oil was added. The pH and ionic strength of the emulsions were adjusted using HCl, NaOH and NaCl solutions. The emulsion type was confirmed by a drop test. Briefly, a drop of each emulsion sample was added to pure canola oil or pure water. If the emulsion droplet remained intact in the oil phase, it indicated an oil-in-water emulsion.

2.3.2 *Centrifugal Stability*. An accelerated stability test was conducted to check the stability of the emulsions. One ml of freshly prepared emulsions with different levels of pH (3.0, 5.0, 7.0 and 9.0) and salt (NaCl) concentrations (0M, 0.006M, 0.06M and 0.6M) were vortexed and placed into micro-centrifuge tubes. The samples were then centrifuged at 9400 x g for 10 min (model MiniSpin® plus, Eppendorf, Westbury, NY, USA). The amount of released oil was quantified by weighing, and the weight was further converted to volume in accordance with its density. The percentage of the released oil during centrifugation over the total amount of oil was reported. All measurements were conducted in triplicate.

2.3.3 *Emulsion morphology and droplet size*. The morphology was characterized using an optical microscope equipped with a camera (Axio Imager A1, Zeiss, Germany). The digital images were taken at 20X magnification. The size distributions were analysed using a laser diffraction particle sizer (SALD-2300, Shimadzu, Kyoto, Japan). Emulsion samples were gently re-dispersed into 5mM SDS (sodium dodecyl sulfate) solution for 40 min in the circulator before each measurement. A refractive index of 1.450 was used for proteins. The surface average diameter ($d_{3,2}$) was calculated by the following equation:

$$D_{3,2} = \frac{\sum n_i d_i^3}{\sum n_i d_i^2} \quad (1)$$

where n_i is the total number of droplets with diameter d_i .

2.3.4 *Interfacial structure*. A Confocal Laser Scanning Microscope (CLSM) was used to visualize the interfacial structure of the emulsion droplets. Emulsion samples (1 ml) were stained with a mixture (40 μ L) of Nile blue (0.1%) and Nile red (0.1%) for 2 hours. A Zeiss LSM 700 confocal microscope (Zeiss, Germany) was operated using two excitation lines (488 nm for the Nile blue dye and 634 nm for the Nile red dye) to visualize the emulsions. Images were taken at a magnification of 20x and further processed using the instrumental software.

2.3.5 *Surface protein loading (τ)*. The surface protein loading (τ) was quantified using a modified procedure that was described in a previous study (Liang & Tang, 2014). The freshly

prepared emulsions were centrifuged at 100 x g (model IEC CENTRA CL2, Thermo Scientific Inc., Waltham, MA, USA) for 20 min. This speed was chosen to allow the complete separation of the creaming phase and the serum phase, without the precipitation of non-adsorbed protein. The protein concentration in the serum phase was determined using the Lowry method (Lowry, Rosebrough, Farr, & Randall, 1951). The surface protein loading τ (mg/m²) can be calculated by the following equation:

$$\tau(\text{mg}/\text{m}^2) = (C_0 - C_f) * d_{3,2}/6\Phi \quad (2)$$

where C_0 is the initial protein concentration in the aqueous phase, C_f is the final concentration of protein in the serum fraction determined by the Lowry method, $d_{3,2}$ is the surface average diameter of emulsion droplets, and Φ is the volume fraction of oil.

2.4 Statistical analysis. All the measurements were conducted at least in triplicate. Data were analyzed using OriginPro 2015 software (OriginLab Corp., Northampton, MA) and Microsoft Excel 2010 (Microsoft Corp., Seattle, WA). Analysis of variance (ANOVA) and least significant difference (LSD) were used to compare the means. The significance level was set at 0.05. The linear regression line with the 95% prediction band was analyzed by OriginPro.

3. Results & Discussion

3.1 *Mechanisms of the zein/NaCas interactions.*

3.1.1 *Morphology of zein/NaCas nanocomplexes*

The SEM images shown in Fig. 1 provide the visualization of zein/NaCas nanocomplexes, zein colloidal nanoparticles and NaCas prepared from pH 3.0. The surface of the plain zein particles was smooth, while the addition of NaCas was able to change the surface roughness, indicating surface adsorption. At the zein:NaCas ratio of 10:4, there was no visible smooth zein surface in Fig 1E, which indicates complete surface coverage by NaCas. Fig. 2 shows the effect of pH 3 and 5 on the NaCas adsorption. At the same zein:NaCas ratio (10:3), the adsorption of NaCas is

observed at pH 3.0 (indicated by the arrow) but not at pH = 5.0. Thus, the adsorption of NaCas on the zein colloidal particles is a pH-dependent process and did not occur at pH 5.0, which may be due to the isoelectric coagulation of NaCas. It has been suggested that the driving force of this type of adsorption is either direct electrostatic interaction or molecular interaction (Patel et al., 2010). The results from this experiment suggest that direct molecular interaction is the driving force. At pH 3.0, both zein and NaCas are positively charged, but the adsorption occurred regardless of their repulsive electrostatic forces. Therefore, we consider that the adsorption is mainly driven by hydrophobic interaction. The specific molecular rearrangement of NaCas involved during this process is not discussed in this paper, as it is beyond the scope of the current study.

3.1.2 *Dynamic light scattering (DLS) and Zeta-potential.*

The mean hydrodynamic particle size of the zein colloidal nanoparticles was found to be 136 ± 6.2 nm, with a monomodal size distribution (polydispersity = 0.121 ± 0.028). The size of nanoparticles is in agreement with previous studies (Patel et al., 2010; Zhong & Jin, 2009). Fig. 3 shows the zeta-potential (mV) as a function of pH for zein/NaCas nanocomplexes with different zein:NaCas ratios. A rapid shift in the isoelectric point was observed from the plain zein (approximately 6.5) to the nanocomplexes with a zein:NaCas ratio of 10:1 (approximately 5.6), indicating that NaCas at this ratio was able to cover a considerable portion of the zein surface. However, even at the zein:NaCas ratio of 10:4, where complete surface coverage is observed in Fig 1, the isoelectric point of the zein/NaCas nanocomplexes was 5.3, which does not coincide with the isoelectric point of NaCas at 5.0. This disparity may be ascribed to the molecular rearrangement of NaCas at the zein colloidal surface, resulting in an uneven surface charge distribution of NaCas between the exposed and adsorbed sides.

At pH = 3.0, the zeta-potential of the zein/NaCas nanocomplexes was surprisingly higher than that of the plain NaCas. One possibility could be the folding flexibility of sodium caseinate upon adsorption and its amphiphilic nature. Caseinate comprises two distinct regions, one

hydrophilic side with a high surcharge density due to the rich amount of phosphoserine residues and one hydrophobic side with far fewer surface charges (Singh, 2011). In the aqueous phase at different pHs, the zein/NaCas nanocomplexes are usually at their lowest surface energy state due to the rearrangement or folding of sodium caseinate, the maximization of their surface charge exposure and their becoming hydrophilic. For example, at pH=3, the surface charge of sodium caseinate is approximately +10 mV, while the zein/NaCas nanocomplexes are +20 mV. In this case, the adsorbed NaCas is likely to orient its low electronic charge side towards zein and expose its high electronic charge side to interact with water, which leads to an elevated zeta-potential. The uneven adsorption due to the nanoparticle polydispersity could also be a reason because smaller particles could be better covered than larger ones.

3.1.3 *Wettability.*

The wettability of zein has been extensively studied in previous studies, and various results have been obtained. In general, zein, as an amphiphilic molecule (Y. Wang & Padua, 2012), has surface properties that are highly dependent upon the processing conditions and sample preparation methods that are applied. To date, many studies have indicated that the zein surface is moderately hydrophilic (de Folter et al., 2012; Gao et al., 2014; Gezer, Brodsky, Hsiao, Liu, & Kokini, 2015; Muthuselvi & Dhathathreyan, 2006; Yin et al., 2014), while few have reported it as hydrophobic (L.-J. Wang et al., 2015).

Samples derived through the evaporation-induced self-assembly (EISA) process exhibit intermediate wettability, due to the formation of a self-assembled and orderly arrayed lamellar structure (Wang & Padua, 2012). Because Pickering emulsions are stabilized by the intact zein colloidal particles instead of lamellar zein molecules, it would be more reasonable to report the wettability of zein particles in this study. In addition to the molecular morphology, drying techniques to prepare zein film also make difference (Gao et al., 2014; Wang et al., 2015). In this study, we decided to choose the freeze-drying method for improved retention of the

original microstructure. The wettability of plain zein colloidal particles, zein/NaCas nanocomplexes with different zein:NaCas ratios, and the plain NaCas was determined following the methodology described in previous studies (Qi et al., 2014; L.-J. Wang et al., 2015).

Fig. 4 illustrates the change of the oil-water three-phase contact angle as a function of the zein:NaCas ratio. The results confirm that plain zein colloidal particles were preferentially wetted by the aqueous phase, while the incorporation of NaCas significantly enhanced their wettability in the oil phase. Intermediate wettability ($\theta \sim 80^\circ$) was achieved at the zein:NaCas ratios of 10:2, 10:3 and 10:4. There was no statistically significant difference in the contact angle between zein/NaCas nanocomplexes with the zein:NaCas ratio of 10:4 and the plain NaCas, indicating complete surface coverage. The increase in wettability upon NaCas adsorption was caused by the change in surface composition. However, the enhancement of surface roughness could also contribute to the alteration of wettability (San-Miguel & Behrens, 2012).

3.1.4 *Zein:NaCas ratio.*

The adsorption of NaCas on zein colloidal particles has recently been studied for the purposes of improving the colloidal stability, protein digestibility and spray-drying redispersibility. The produced zein/NaCas nanocomplexes were mainly applied as wall materials for encapsulation (Chen & Zhong, 2014; Li et al., 2014; Patel et al., 2010; L. Wang et al., 2013). In previous studies, zein/NaCas nanocomplexes were formed through dispersing a zein ethanol/water binary solution into deionized water containing NaCas under high-speed shearing (Chen, Zhang, & Zhong, 2015; Patel et al., 2010; Zhang et al., 2014). The essential zein:NaCas ratio to achieve monolayer coverage was estimated at 1:0.29 (w/w) (Patel et al., 2010). However, another study observed the precipitation of zein/NaCas nanocomplexes when the zein:NaCas ratio reached 1:0.35-1:0.5, and the unsaturated surface coverage was proposed as the reason (Zhang et al., 2014). Therefore, to investigate the zein:NaCas ratio that forms monolayer coverage and to ensure that the precipitation of zein/NaCas nanocomplexes was not induced by the isoelectric

coagulation of NaCas, the pH was controlled below the isoelectric point of NaCas ($pI = 5.0$ was obtained from our experiment) throughout the adsorption process. At $pH = 3.0$, all the samples with different zein:NaCas ratios ranging from 10:0 to 10:4 were macroscopically stable, indicating that the precipitation of zein/NaCas nanocomplexes observed in previous studies could have been induced by the isoelectric aggregation of NaCas. According to the contact angle measurements, surface coverage was achieved when the zein:NaCas ratio was between 10:3 and 10:4, as discussed above. This result is close the previously estimated ratio of 1:0.29 (Patel et al., 2010).

3.2 Characterization of Pickering emulsions

3.2.1 *Centrifugal stability.*

The emulsion stability was evaluated via the centrifugal method because of its many advantages, such as improved reproducibility and accuracy (Qi et al., 2014). Moreover, the centrifugal stability is a characteristic of the emulsion itself (Tcholakova, Denkov, & Lips, 2008). Typically, Pickering emulsions exhibit poor centrifugal stability when compared with conventional emulsions stabilized by proteins and small molecular surfactants, primarily due to the differences in the emulsion droplet sizes and the destabilization mechanisms (Tcholakova et al., 2008; Tcholakova, Denkov, Ivanov, & Campbell, 2002, 2006). To ensure the reliability of using the centrifugal method, two compared emulsions systems have to have a similar droplet size. Generally, larger droplets are more susceptible to coalescence, and many studies have reported a linear correlation between the critical osmotic pressure and the drop radius (Tcholakova et al., 2002, 2006). Therefore, we will not discuss the centrifugal stability of emulsions stabilized by the plain NaCas due to the significant size difference, but the data is still presented here for validation purposes. Table 1 demonstrates the percentage of oil released after the centrifugation ($9400 \times g$ for 10 min) at different pHs and ionic strengths.

3.2.1.1 *The effect of pH and ionic strength*

Four levels of pH (3.0, 5.0, 7.0 and 9.0) and four levels of ionic strengths (0M, 0.006M, 0.06M, and 0.6M) were selected to study the emulsion stability with the aim of mimicking the conditions in food systems. Overall, the emulsions at pH 3.0 and pH 9.0 showed better stability than those at pH 5.0 and pH 7.0 due to the isoelectric coagulation of zein and NaCas at approximately pH 7.0 and 5.0, respectively. It was noted that samples with zein:NaCas ratios of 10:0, 10:1 and 10:2 were not stable at pH 7.0, probably due to the isoelectric coagulation of zein particles. However, the zein:NaCas ratios of 10:3 and 10:4 provided superb stability at pH 7.0, suggesting that isoelectric aggregation of zein was suppressed, probably due to the greater extent of surface coverage by NaCas.

In general, it can be concluded that increasing the amount of NaCas in the zein/NaCas nanocomplexes to ratios of 10:3 or 10:4 could help improve Pickering emulsion stability. Nevertheless, the effect of pH and ionic strength did not show a clear trend on the centrifugal stability due to the counteraction and synergism of various factors, such as electrostatic balance, rheological properties, and emulsion surface coverage.

Although it is not the focus of this study, we interestingly found that the 0:10 samples exhibited superior stability at all pH levels and ionic strengths tested, including pH 5.0, the isoelectric point of NaCas. One explanation is that the initially existing electrostatic repulsion diminishes when the pH approaches the isoelectric point, which leads to an enhanced hydrophobic interaction. Hence, an intensive flocculation between emulsion droplets induces the formation of a gel network structure (Dickinson & Eliot, 2003).

3.2.1.2 Effect of NaCas on the Pickering emulsion stabilization

The effect of NaCas in stabilizing Pickering emulsions can be attributed to the following mechanisms. First, the adsorption of NaCas on zein nanoparticles helps balance the hydrophilic and hydrophobic groups on the surface and inhibits the self-aggregation of zein in the bulk

phase. Second, the NaCas improves interfacial properties by facilitating particle adsorption. Fig. 5 shows that the surface coverage was enhanced when adjusting the zein:NaCas ratio from 10:0 to 10:3. Third, if the amount of NaCas reaches a critical ratio, the excessive NaCas can competitively adsorb to the interface and minimize interstitial distance. It was reported that the critical osmotic pressure decreases substantially with an increase in the interparticle distance (Tcholakova et al., 2008). The evidence and underlying mechanisms of enhanced interfacial adsorption and competitive adsorption will be discussed further in this paper. Furthermore, the excessive NaCas that is not adsorbed gives rise to the depletion flocculation and bridging to provide the extra physical stability. In general, the presence of non-adsorbed NaCas in the gap between emulsion droplets is able to create an attractive force (depletion force), which helps stabilize the emulsion (Dickinson, 2015). It has been reported that the magnitude of depletion force is proportional to the number density of NaCas sub-micelles (Dickinson & Golding, 1997).

3.2.2 *Droplet size and morphology.*

Fig. 6 shows the optical microscopy pictures of the emulsion samples. ImageJ was utilized to analyze the images and calculate the area average droplet size ($d_{3,2}$). The results are shown in Table 2. The droplet size initially increased with increasing NaCas fraction until reaching the maximum at the zein:NaCas ratio of 10:3, followed by a decrease at the zein:NaCas ratio of 10:4. When an emulsion system includes two or more different types of emulsifiers, the behaviour of each component during the emulsification process is complicated. In this study, Pickering emulsions were prepared using zein colloidal particles and zein/NaCas nanocomplexes. There could be two possible behaviours of NaCas during the emulsification process: 1) it could remain on the surface of the zein colloidal particles or 2) it could migrate to the oil-water interface to stabilize the oil droplets. It is widely recognized that when there is more than one emulsifier or surface active compound, there is competitive adsorption between the emulsifiers (Dickinson, 2011; Ghouchi Eskandar et al., 2007; Niu et al., 2015). On the other hand, the interactions between different components should also be considered. Surfactant

was found to anchor on a colloidal particle surface to form an agglomeration, followed by the adsorption of such agglomerated particles at the oil-water interface (Nesterenko et al., 2014).

Different from those small-sized emulsifiers driven by Brownian motion, hydrodynamic or convective forces are recognized as the driving force for the Pickering emulsions (Destribats et al., 2014; Tcholakova et al., 2008). We assumed that zein/NaCas nanocomplexes act as intact emulsifier units during emulsification using hydrodynamic forces, and we used the so-called “limited coalescence” or “partial coalescence” model to verify this assumption. The limited coalescence model infers that dispersing droplets experience coalescence upon formation until its surface reaches a threshold emulsifier adsorption, τ^* (Tcholakova et al., 2008). Generally, the limited coalescence theory is only suitable in an emulsifier-poor regime, where the emulsifier concentration predominately influences the droplet size, rather than the power density during energy dissipation. However, a recent study confirmed the limited coalescence phenomenon for those emulsions containing excessive emulsifiers (Destribats et al., 2014), and our data with zein show similar results (Fig. 7).

A mathematical model was built to study the limited coalescence quantitatively (Tcholakova, Denkov, Sidzhakova, Ivanov, & Campbell, 2003):

$$d_{3,2} \approx \frac{6\phi}{(1-\phi)} \frac{\tau^*}{C_{INI}} \quad (3)$$

Here, C_{INI} is the initial emulsifier concentration (wt%), τ^* is the threshold surface adsorption or coverage to hamper droplets from coalescence, and ϕ is the volume fraction of the dispersed phase. In many studies, a proportional correlation between $d_{3,2}$ and $1/C_{INI}$ has been found in the emulsifier-poor regime and has recently been proved in the presence of excessive emulsifier after emulsification (Destribats et al., 2014; Golemanov, Tcholakova, Kralchevsky, Ananthapadmanabhan, & Lips, 2006; Tcholakova, Denkov, & Banner, 2004; Tcholakova et al., 2003). In this experiment, we were able to identify a linear region where the zein:NaCas ratio is between 10:0 and 10:3, where $d_{3,2}$ was found to be proportional to the $1/C_{zein\ ini}$ (the initial concentration of zein, wt%). This is in agreement with the emulsion size derived from plain zein Pickering emulsions, despite that the overall emulsifier concentration remained at 1% (wt%)

(Fig. 7). However, the emulsion sample with the zein:NaCas ratio of 10:4 significantly deviated from the linear regression with a 95% prediction band. The plot indicates that NaCas did not participate in the interfacial adsorption until the zein:NaCas ratio reached 10:4, at which point we consider it to entail excessive NaCas that originally existing as a multilayer deposition on the zein surface. During the emulsification, those multilayer NaCas acted as free emulsifier units, which were able to diffuse and competitively adsorb to the oil-water interface. This resulted in the formation of a smaller droplet size with a multi-component interface that consists of both zein/NaCas nanocomplexes and NaCas sub-micelles (Fig. 8). This result was further confirmed by two additional data points at the zein:NaCas ratios of 10:6 and 10:10.

3.2.3 *Interfacial properties*

The interfacial properties were characterized quantitatively by the surface protein loading and visually using the confocal laser scanning microscope (CLSM). A distinct feature of Pickering emulsions is the formation of a densely packed layer at the oil-water interface, which provides the steric barrier to hinder droplet coalescence (Ghouchi Eskandar et al., 2007). The surface protein loading data is presented in Table 2. The results strongly suggest that zein/NaCas nanocomplex stabilized emulsions are Pickering emulsions due to their significantly higher protein concentration at the interface compared to the NaCas emulsion, whereas the dense packing of emulsifier at the interface is a distinct feature of Pickering emulsions (Dickinson, 2010; Ghouchi Eskandar et al., 2007; Song et al., 2015). The incorporation of NaCas at zein:NaCas ratios from 10:1 to 10:2 significantly increased the surface protein concentration, yet the further addition of NaCas caused the surface protein to decrease ($p < 0.05$).

In a previous study that prepared Pickering emulsions using zein/sodium stearate complexes, interfacial adsorption or accumulation was enhanced as a result of the alteration of wettability (Gao et al., 2014). Nevertheless, in this study, the surface loading significantly decreased when the zein:NaCas ratio increased to 10:4, a trend that is contrary to the observed wettability. This

may indicate that the adsorption of NaCas occupied the interface, thus hindering the further adsorption of zein/NaCas nanocomplexes.

Fig. 5 presents a visualization of the droplet interface using CLSM. Fig. 5A shows an emulsion droplet stabilized by the plain zein colloidal nanoparticles, where large aggregates of zein colloidal particles can be observed at the oil-water interface, with the droplet surface sparsely covered by the emulsifiers. Our supplemental material indicates that zein colloidal particles have the tendency to agglomerate reversibly in the aqueous phase. This agglomeration substantially reduced the efficacy of the zein colloidal particles as Pickering emulsifiers. According to Fig. 5B, C, D and E, the incorporation of NaCas gradually changes the interfacial structure to be smooth and homogeneous, perhaps for the following three reasons: 1) zein agglomeration was inhibited by the presence of NaCas (shown in the supplemental information); 2) surface modification of zein colloidal particles with altered wettability improved the interfacial adsorption; and 3) the excessive NaCas is able to cover the interstitial space between particles. Fig. 8 illustrates three types of interfacial structures, which vary in accordance with the zein:NaCas ratios.

4. Conclusions

Adsorption of NaCas onto zein colloidal nanoparticles was able to modify the surface the zein particles and form zein/NaCas nanocomplexes. The wettability and surface charge of the nanocomplexes were tuneable through adjusting the zein:NaCas ratio. Zein colloidal particles are inherently hydrophilic, while the incorporation of NaCas can significantly modify the three-phase contact angle. The monolayer surface adsorption of NaCas and the intermediate wettability were both found to be close to 10:3. The obtained zein/NaCas nanocomplexes were then used to prepare Pickering emulsions. The emulsions using nanocomplexes with zein:NaCas ratios of 10:3 and 10:4 exhibited enhanced surface coverage and centrifugal stability. The zein/NaCas nanocomplexes stabilized the oil droplets, following the limited coalescence theory, when the zein:NaCas ratio was between 10:0 and 10:3. However, after the monolayer adsorption was achieved, the excessive NaCas competitively adsorbed to the interface to form a particle-polymer hybrid interfacial structure.

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Graphical abstract

Figure Captions

Figure 1. Scanning electron microscopy (SEM) pictures of the plain zein colloidal particles (A), the zein/NaCas nanocomplexes with the zein:NaCas ratio of 10:1 (B), 10:2 (C), 10:3 (D), 10:4 (E), and the plain NaCas (F) prepared at pH 3.0.

Figure 2. Scanning electron microscopy (SEM) pictures of the zein/NaCas nanocomplexes with the zein:NaCas ratio of 10:3 at pH 3.0 (A), and 5.0 (B).

Figure 3. Zeta-potential versus pH curves at different zein:NaCas ratios.

Figure 4. Three-phase contact angle (θ) of protein films with different zein:NaCas ratios.

Figure 5. Confocal laser scanning microscopy (CLSM) pictures of emulsion droplet prepared with the plain zein colloidal particles (A), the zein/NaCas nanocomplexes with the zein:NaCas ratio of 10:1 (B), 10:2 (C), 10:3 (D), 10:4 (E), and the plain NaCas (F) at pH 3.0.

Figure 6. Optical microscopy images of emulsions prepared at pH 3.0 using 1% plain zein colloidal particles (A), 1% zein/NaCas nanocomplexes with the zein:NaCas ratio of 10:1 (B), 10:2 (C), 10:3 (D), 10:4 (E), and plain NaCas (F).

Figure 7. Emulsion droplet mean diameter, $d_{3,2}$ (μm), as a function of the inverse initial concentration of zein ($\text{wt}\%^{-1}$). The linear regression line with 95% prediction band was derived using plain zein Pickering emulsions with different zein concentration.

Figure 8. Schematic diagram of different interfacial arrangements at various zein:NaCas ratios.

Table Captions

Table 1. Volume percentage of released oil (%) after high-speed centrifugation at 9400 x g for 10min. Emulsions were prepared using emulsifiers with different zein:NaCas ratios, followed by the adjustment of the pH and ionic strengths.

Table 2. Effect of the zein:NaCas ratio on the emulsion droplet size ($d_{3,2}$) and the surface protein concentration τ (mg/m²).

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Figure 7.

Figure 8.

Table 1. Centrifugal stability as a function of Zein:NaCas ratio, pH and ionic strength

pH 3.0					pH 5.0				
Zein:NaCas ratio	Ionic strength [NaCl]				Zein:NaCas ratio	Ionic strength [NaCl]			
	0M	0.006M	0.06M	0.6M		0M	0.006M	0.06M	0.6M
10:0	25.71 ^{ab}	65.79 ^b	83.05 ^b	75.43 ^{ab}	10:0	81.75 ^a	80.69 ^a	86.42 ^a	89.62 ^a
10:1	28.44 ^a	63.46 ^b	65.64 ^c	64.42 ^{bc}	10:1	67.90 ^b	66.86 ^b	67.93 ^b	60.90 ^b
10:2	19.05 ^{abc}	79.47 ^a	92.00 ^a	80.22 ^{ab}	10:2	62.87 ^{bc}	78.45 ^a	81.50 ^a	88.42 ^a
10:3	2.57 ^c	3.79 ^c	32.46 ^d	58.51 ^c	10:3	61.09 ^c	66.77 ^b	52.14 ^c	3.03 ^d
10:4	5.10 ^{bc}	7.07 ^c	68.42 ^c	77.59 ^{ab}	10:4	84.49 ^a	80.99 ^a	79.03 ^a	78.34 ^c
0:10	0.15 ^c	0.38 ^c	0.00 ^e	1.13 ^d	0:10	0.00 ^d	0.00 ^c	0.00 ^d	0.00 ^d

pH 7.0					pH 9.0				
Zein:NaCas ratio	Ionic strength [NaCl]				Zein:NaCas ratio	Ionic strength [NaCl]			
	0M	0.006M	0.06M	0.6M		0M	0.006M	0.06M	0.6M
10:0	82.12 ^a	74.73 ^b	79.83 ^a	77.78 ^a	10:0	7.76 ^a	17.07 ^a	78.74 ^a	87.93 ^a
10:1	94.47 ^a	99.53 ^a	94.50 ^a	87.46 ^a	10:1	4.52 ^b	7.00 ^b	34.84 ^b	76.21 ^b
10:2	85.09 ^a	45.87 ^c	36.66 ^b	24.91 ^b	10:2	4.77 ^b	6.59 ^b	6.84 ^c	15.88 ^c
10:3	1.59 ^b	1.87 ^d	3.33 ^c	2.37 ^c	10:3	1.46 ^c	1.27 ^b	1.11 ^c	1.74 ^d
10:4	5.65 ^b	6.67 ^d	5.41 ^c	8.17 ^c	10:4	3.76 ^{bc}	4.30 ^b	4.53 ^c	6.42 ^d
0:10	3.01 ^b	3.62 ^d	4.05 ^c	3.27 ^c	0:10	1.44 ^c	1.59 ^b	1.94 ^c	2.92 ^d

Different superscript letters indicate the significant difference within the same column (P<0.05).

Table 2. $d_{3,2}$ and surface protein concentration (τ) as a function of zein:NaCas ratio.

Zein:NaCas ratio (w/w)	$d_{3,2}$ (μm)	Surface protein concentration τ (mg/m^2)*
10:0	45.91 ^d	108.89 ^a
10:1	50.69 ^c	99.83 ^b
10:2	52.88 ^b	109.11 ^a
10:3	55.18 ^a	95.88 ^c
10:4	52.81 ^b	86.47 ^d
0:10	22.42 ^e	3.15 ^e

* Different superscript letters indicate the significant difference within the same column ($P < 0.05$).