

Cite this: *Soft Matter*, 2012, **8**, 6807

www.rsc.org/softmatter

PAPER

# Oil-in-water Pickering emulsions stabilized by colloidal particles from the water-insoluble protein zein†

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Received 19th December 2011, Accepted 20th April 2012

DOI: 10.1039/c2sm07417f

Few fully natural and biocompatible materials are available for the effective particle-stabilization of emulsions since strict requirements, such as insolubility in both fluid phases and intermediate wettability, need to be met. In this paper, we demonstrate the first use of water-insoluble proteins, employing the corn protein zein as a representative of this family, as effective particle-stabilizers of oil-in-water emulsions of natural oils and water. For this purpose, we synthesized zein colloidal particles through an anti-solvent precipitation procedure and demonstrated their use in the formation of stable oil-in-water Pickering emulsions as a function of particle concentration, pH and ionic strength. We confirmed that the wetting properties of zein, studied as a function of pH and ionic strength, strongly favor interfacial particle adsorption with oil-in-water three-phase contact angles  $\theta_{ow}$  close to  $90^\circ$ . We found that unmodified zein colloidal particles can produce stable, surfactant-free o/w emulsions with droplet sizes in the range 10–200  $\mu\text{m}$  under experimental mixing conditions (2 min with Ultra Turrax homogenizer at 13 500 rpm) at pH above and below the isoelectric point of zein, for low to moderate ionic strengths (1–10 mM). Under conditions where the particle volume fraction is low (<0.2 wt%) or at low pH, the resulting emulsions are not stable against coalescence. At a higher ionic strength, the zein particles have a tendency to aggregate and the resulting emulsions flocculate, forming an emulsion–gel phase.

## 1. Introduction

Stabilization of fluid-in-fluid dispersions, such as foams and emulsions, can be realized by the addition of surface active particles.<sup>1</sup> Particles with partial wettability can adsorb strongly at the fluid–fluid interface of immiscible fluids. The energy barrier  $\Delta E$  that should be overcome to remove a spherical particle from the interface into one of the bulk phases depends on the three-phase contact angle  $\theta$ , the particle radius  $r$ , and the oil–water interfacial tension  $\gamma_{ow}$ .

$$\Delta E = \pi r^2 \gamma_{ow} (1 \pm \cos \theta)^2 \quad (1)$$

Estimates of this energy show that even for particles as small as a few tens of nanometres, the energy barrier for particle desorption is higher than the kinetic energy of Brownian motion ( $\sim k_B T$ ), which makes the adsorption effectively irreversible.

Although particles can be used as alternative emulsifiers and provide superior stabilization compared to conventional low molecular weight surfactants and proteins, they also have a major drawback. Usually a relatively higher concentration of particles (>1 wt%) is necessary to assure good stabilization against coalescence. The main reasons for this difference are related to the much larger size of the particles in comparison with those of molecular surfactants: the kinetics of adsorption are slower due to the larger amount of material that is needed to cover the same droplet surface, there is a high adsorption barrier and a very high desorption energy.<sup>2</sup>

Although the phenomenon of particle-stabilization in foams and emulsions was initially recognized more than a century ago by Ramsden and Pickering,<sup>3,4</sup> it was not until the last few decades that it received widespread scientific interest. Over this period, the generality of foam and emulsion stability against coalescence provided by the adsorption of solid particles at the fluid–fluid interface has been demonstrated by the use of many different types of particles. Furthermore, many studies have been devoted to the elucidation of the mechanisms behind particle-stabilization by focusing on, among others, the influence of particle size, hydrophobicity and shape.<sup>5–7</sup> The effect of pH on the formation and stability of Pickering emulsions and particle-stabilized foams has also been investigated for several systems.<sup>8–16</sup> The current challenge entails translating the knowledge about emulsion

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† Electronic supplementary information (ESI) available: Water-in-air three-phase contact angle measurements. See DOI: 10.1039/c2sm07417f

stabilization by solid particles into useful industrial applications. Most of the particles used in these studies are, however, synthetic, often inorganic materials (*e.g.* silica) that have limited applicability in, for example, pharmaceutical, agricultural, food and cosmetic formulations.

Therefore, the development of environmentally friendly systems based on fully natural resources is desirable. However, the requirements to serve as a particle-stabilizer, such as insolubility in both fluid phases and intermediate wettability, are so challenging that to date not many available natural materials fulfill them. In a limited number of studies, colloidal particles of biological origin, such as spore particles,<sup>17</sup> chemically modified starch granules<sup>18</sup> and cellulose particles,<sup>19–21</sup> have been employed to stabilize emulsions. Nevertheless, the following drawbacks are frequently encountered and need further research: the particles are often highly polydisperse, need additional chemical treatment to assure interfacial attachment, have colloidal and wetting properties that are poorly characterized and systems that are not fully natural or have a very limited applicability. For instance, the required concentration of food micronutrients, nutraceuticals and additives is usually low and therefore limits their application as particle-based stabilizers.<sup>22</sup> Proteins find wide applications as emulsifiers and can be used in high concentrations. The vast majority of proteins are water-soluble and are usually considered as an emulsifier type distinct from solid particles.<sup>2</sup>

Here, we introduce water-insoluble proteins as a novel class of fully natural particle-stabilizers of emulsions. The class of proline-rich proteins, prolamins, is characterized by a limited solubility in water, but a good solubility in some organic solvents containing water (*e.g.* ethanol–water mixtures).<sup>23,24</sup> These solubility properties, as compared to those of water-soluble proteins, have prevented their use as emulsifiers until now. We used zein as a representative for water-insoluble proteins acting as particle emulsifiers. Zein is an extensively studied, food grade and abundant material extracted from corn and there have been many attempts to commercialize it in various industrial applications.<sup>23,24</sup> The solubility behavior of zein is determined by the high proportion of hydrophobic amino acids, like *e.g.* leucine, proline and alanine, and its relative deficiency of basic or acidic amino acid residues.<sup>25</sup> This makes zein relatively hydrophobic, while its degree of hydrophobicity can be controlled by the degree of ionization of basic and acid amino acid groups. Based on their different solubility behaviors and sequences, there are four classes of zein, known as  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -zein.<sup>26</sup> The low solubility of zein in both water and oil allows for the controlled fabrication of colloidal particles through anti-solvent precipitation. Precipitation of the protein can be controlled by several parameters such as temperature, mixing efficiency, protein concentration and the presence of stabilizers such as surface active molecules and polymers. Zein colloidal particles have been studied for delivery<sup>27,28</sup> and encapsulation, but their use in stabilization of fluid-in-fluid dispersions has not yet been explored.

In this paper, we demonstrate the fabrication of surfactant-free, biodegradable and edible oil-in-water Pickering emulsions based on fully natural renewable resources using relatively monodisperse colloidal zein particles as the emulsifier. As an additional advantage with respect to applicability and scalability,

we show that zein colloidal particles, as opposed to many other natural particle-stabilizers, display intrinsic surface activity. In other words, unmodified particles adsorb at the oil–water interface and require no additional chemical surface modification or pretreatment. The role of parameters such as particle concentration, pH and ionic strength on emulsion formation and stability against coalescence was studied and these observations are related to the colloidal and wetting properties of zein.

## 2. Experimental section

### 2.1. Materials

Zein from corn (batch#: 057K0156) was obtained from Sigma. 1 N HCl and 1 N NaOH solutions were obtained from Merck Co. (Germany) and were used to control the pH. Absolute ethanol (99.9%) was obtained from VWR BDH Chemicals, UK. Sodium chloride was obtained from Sigma and was used to control the ionic strength. Water purified by a MilliQ system was used for all the experiments. Soybean oil was obtained from Fluka (soy oil from *Glycine max*). All ingredients were used without further purification.

### 2.2. Synthesis of zein colloidal particles

In a typical synthesis of zein colloidal particles, 10.0 g zein was dissolved in an aqueous ethanol mixture (80 (v/v)% EtOH), prepared by mixing 320 mL ethanol with 80 mL MilliQ water. 1200 mL MilliQ water was put in a beaker and stirred vigorously. The zein solution was added in 2–3 minutes to this beaker in a controlled way using a syringe. A turbid dispersion with a yellow, milky color was formed with 20 (v/v)% EtOH. To obtain an aqueous dispersion, ethanol was removed under reduced pressure by rotary evaporation. Approximately three quarters of the solvent was removed such that the zein dispersion was concentrated to 2.5 wt%. Finally, the dispersion was centrifuged for 10 minutes at 1000 rpm to remove (possible) larger aggregates. The final dispersion had a pH around 4.0. Dispersions were stored in the refrigerator at 5 °C.

### 2.3. Particle characterization

**2.3.1 Transmission electron microscopy.** The size and shape of the particles were analyzed by taking transmission electron microscopy (TEM) photographs using a Tecnai 200 (FEI Company, The Netherlands), operating at 200 kV. The particles were dispersed in MilliQ water (pH adjusted to 4.0 using HCl solution) and one drop of the diluted dispersion was placed on a 200-mesh carbon-coated copper grid.

**2.3.2 Particle size measurement.** The volume-weighted particle size was measured by dynamic light scattering (DLS) using a Zetasizer Nano (Malvern Instruments Ltd, UK) after appropriate dilution to avoid multiple particle effects. Particle sizes were measured after diluting samples with MilliQ water of the same pH as the original dispersion (*e.g.* by diluting with regular MilliQ water without pH adjustment with HCl, the dispersion starts to flocculate as the isoelectric point is approached). All measurements were carried out at 25 °C and the results reported are averaged over three readings. The apparent

diameter of the particles was also determined from the TEM micrographs by taking the average over measurements on 150 particles.

**2.3.3 Zeta potential measurements.** The  $\zeta$ -potential of the particles was measured with the Zetasizer Nano (sample preparation was similar to that for particle size measurement). The surface charge properties of the particles were investigated by zeta potential measurements as a function of pH, employing cuvettes equipped with an electrode, using the Smoluchowski or Hückel equation for converting measured mobilities. At least two separate measurements were used for each pH value.

**2.3.4 Contact angle measurements.** A 1 wt% zein solution in an aqueous ethanol mixture (90 (v/v)% EtOH) was prepared by magnetic stirring at room temperature. Homogeneous zein films were deposited onto pre-cleaned glass substrates by heating zein solutions at 70 °C for 15 minutes. Before further use, these zein films were stored in a desiccator. The static contact angle was measured using a DataPhysics OCA15 setup. To determine the oil-in-water three-phase contact angles  $\theta_{ow}$ , glass substrates coated with zein films were placed on top of an aqueous subphase with a certain [NaCl] and pH. Next, oil droplets (1–2  $\mu$ L) were formed in the aqueous phase at the tip of a bended needle, which was brought into contact with the substrate. Subsequently, the needle was gently retracted, leaving behind an oil droplet at the zein film. Furthermore, water-in-air three-phase contact angles  $\theta_{wa}$  were measured by depositing water droplets (1–2  $\mu$ L) with a certain [NaCl] and pH onto zein films under an ambient atmosphere. Contact angles were determined automatically, approximating the contour of the imaged droplets with a Laplace–Young fit. Measurements were averaged over at least five droplets.

#### 2.4. Preparation, stability and characterization of emulsions

For all emulsions, zein colloids with a diameter of  $82 \pm 16$  nm were used. To prepare an emulsion, 5 mL of zein dispersion with a certain wt%, pH and [NaCl] was brought into a glass vial. 5 mL of soy bean oil was slowly added to the dispersion, while mixing with an Ultra Turrax homogenizer with a dispersing head operating at 13 500 rpm. After all the oil was added, the sample was mixed for 2 additional minutes. pH adjustments were made with HCl and NaOH solutions. The samples were stored in a refrigerator at 5 °C.

The emulsion type (oil-in-water or water-in-oil) was determined qualitatively by checking the miscibility in a pure water or oil phase. A drop of each emulsion was added to a volume of either pure oil or pure water. Water continuous (oil continuous) emulsions dispersed in water (oil), but remained as drops in oil (water). The emulsion volume fractions were followed in time by measuring the position of the interface 1 hour, 3 days and 2 weeks after sample preparation. Photographs of vessels were taken with a Nikon digital camera. Samples were imaged, both 30 minutes and 2 weeks after preparation, with a Zeiss Axioplan (Carl Zeiss) optical microscope equipped with a video camera (Scion Corp.). A drop of emulsion was diluted with its continuous phase on a microscopic slide and carefully covered with a cover slip. This technique was used to estimate the mean

droplet diameter. The characteristics of the prepared emulsions are summarized in Table 1.

### 3. Results and discussion

#### 3.1. Particle characterization

The synthesis of surfactant-free zein colloidal particles is based on classical antisolvent precipitation, a method widely used for fabrication of various organic colloidal particles.<sup>29</sup> The method comprises the preparation of a solvent mixture containing water and an organic solvent (*e.g.* ethanol and acetone), in which zein powder is dissolved. Next, the zein solution is poured into an aqueous phase under continuous vigorous mixing such that zein precipitates as fine particles in a colloidal dispersion. In the final step the organic solvent is removed by evaporation to obtain an aqueous dispersion containing 0.1–2.5 wt% colloidal particles.

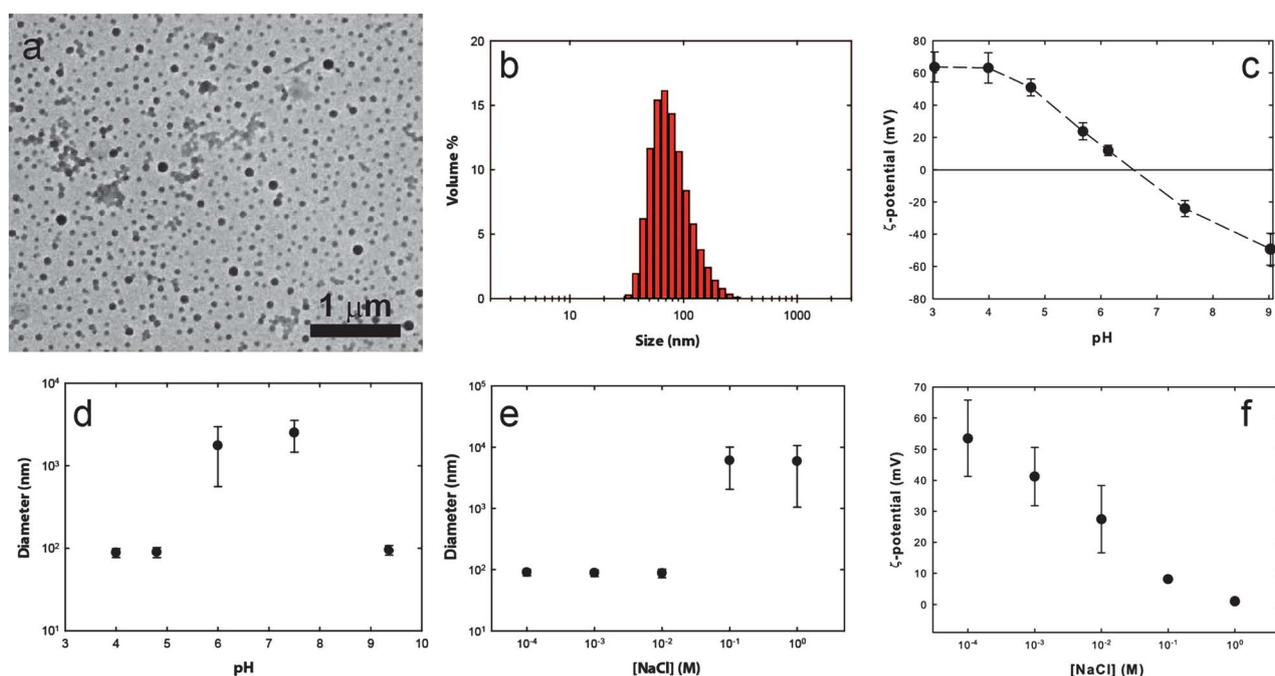
Although this preparation method allows the synthesis of particles with different sizes, to avoid sedimentation in the suspensions we decided to work with particles that were not too large (*i.e.* particles of 500 nm will immediately trigger sedimentation in aqueous dispersions) and not too small in order to have sufficient energy for attachment at the oil–water interface (*i.e.* the energy of attachment should be much higher than the thermal energy  $k_B T$ ). The use of nanoscale colloidal particles, in principle, should also allow stabilization of droplets against coalescence with sizes in the micrometre range, which are comparable to the sizes of most commonly used (industrial) emulsions.<sup>5</sup>

Fig. 1a shows a TEM image of zein colloidal particles with an apparent number-averaged diameter of  $70 \pm 13$  nm, which confirms that they are fairly monodisperse with a spherical morphology. Due to drying of the sample and the vacuum environment needed for electron microscopy, the particles probably have shrunk somewhat. Using dynamic light scattering measurements, a volume weighted particle diameter of  $82 \pm 16$  nm was measured (Fig. 1b). The  $\zeta$ -potentials of the particles, determined by electrophoretic mobility measurements, are plotted against the pH of the aqueous dispersion in Fig. 1c. On lowering the pH to 3, the particles become strongly positively charged ( $\zeta > +60$  mV), with the isoelectric point close to pH 6.5. On increasing the pH, the particles become strongly negatively charged ( $\zeta > -50$  mV). The averaged particle diameters (Fig. 1d) below pH 6 and above pH 8 are constant ( $\sim 85$  nm with a polydispersity of around 15–20%), reflecting that the system is stable and the particles within this pH range are well dispersed and discrete. Close to the isoelectric point of zein (pH  $\approx 6.5$ ), at which the net particle charge is zero and the electrostatic repulsion is decreased, the dispersion becomes unstable. The particle diameters become several micrometres and the polydispersity increases drastically. This apparent increase is due to aggregation of the particles. The displayed particle sizes stem from measurements immediately after pH adjustment and therefore relate to an average aggregated dimension that continues to change until macroscopic aggregation and sedimentation at the bottom of the vessel are observed.

As the ionic strength is varied at a constant pH of 4.0, the apparent diameters (Fig. 1e) initially remain constant ( $\sim 85$  nm with a polydispersity of around 15–20%) up to  $10^{-2}$  M NaCl. Under these conditions, the system is stable and the particles are

**Table 1** Summary of zein-stabilized o/w emulsion characteristics

Wt% at pH 4.0, 1 mM NaCl	Sample name	Emulsion volume		Emulsion characteristics
		1 h	2 weeks	
2	A1	71%	60%	Emulsion-gel
1	A2	74%	74%	Emulsion-gel
0.2	A3	50%	50%	Unstable, macroscopic phase-separation
<i>pH at 1 wt%, 1 mM NaCl</i>				
2.5	C1	60%	55%	Unstable
8.5	C3	63%	63%	Fluid emulsion
9.8	C2	71%	62%	Fluid emulsion
<i>[NaCl] at 1 wt%, pH 4.0</i>				
10 mM	B1	67%	67%	Emulsion-gel
0.1 M	B2	50%	50%	Unstable, transparent aqueous phase
1 M	B3	67%	67%	Emulsion-gel, transparent aqueous phase
<i>[NaCl] at 1 wt%, pH 8.5</i>				
10 mM	D3	70%	70%	Emulsion-gel
0.1 M	D2	75%	65%	Fluid-gel, transparent aqueous phase
1 M	D1	57%	48%	Aggregation, emulsion-gel

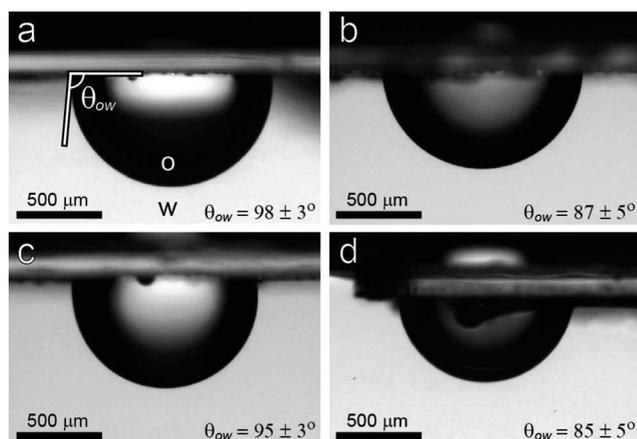


**Fig. 1** (a) Transmission electron micrograph of zein colloidal particles; (b) volume averaged particle size distribution; (c)  $\zeta$ -potential and (d) particle volume averaged diameter of aqueous zein colloidal particles, both as a function of pH at 1 mM NaCl. Close to the isoelectric point of zein (pH  $\approx$  6.5) the particles become unstable and aggregation takes place. (e) Particle volume averaged diameter and (f)  $\zeta$ -potential as a function of [NaCl] at pH 4.0. Above 10 mM the system becomes unstable and aggregation takes place.

well dispersed and discrete. The particles at these ionic strengths are positively charged, as can be seen in Fig. 1f, although the  $\zeta$ -potential decreases with [NaCl] to values below +30 mV at 0.1 M NaCl (Fig. 1f). At ionic strengths above  $\sim$ 10 mM NaCl, the particle diameter increases sharply due to screening of the electrostatic repulsion force at higher ionic strengths, with a further decrease of the  $\zeta$ -potential, forming large, very poly-disperse aggregates of several microns. Again, the measured sizes immediately after ionic strength adjustment relate to an average aggregated dimension that may continue to change until macroscopic aggregation is reached. The aqueous dispersions at this ionic strength become unstable and show signs of sedimentation at the bottom of the vessel.

### 3.2. Wetting properties

Changes in the hydrophobicity of the particles affect their interfacial wetting properties. The changes may be accomplished by pH and ionic strength variations, as studied extensively with the use of macroscopic solid films.<sup>30–35</sup> This effect was explored for zein colloidal particles and used to control their ability to stabilize emulsions. The wetting properties of zein particles were investigated by direct measurements of oil-in-water three-phase contact angles  $\theta_{ow}$  on zein films. Fig. 2 shows images of oil droplets attached to zein films immersed in an aqueous phase with a pH below or above the isoelectric point and an ionic strength of either 1 mM or 1 M NaCl. It is assumed that the zein

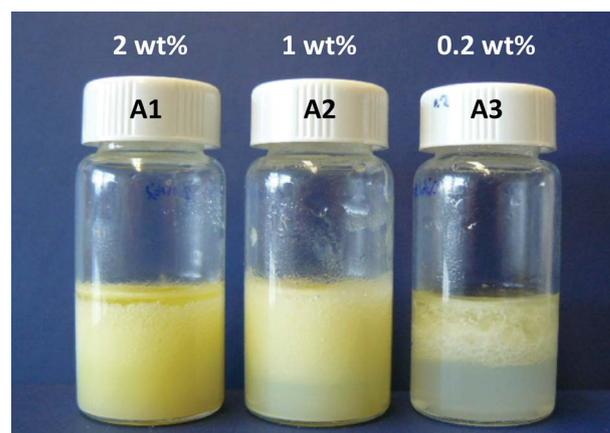


**Fig. 2** Soy bean oil droplets attached to zein films immersed in an aqueous subphase. Oil-in-water contact angles  $\theta_{ow}$  (see insets) were determined for aqueous phases with (a) 1 mM NaCl at pH 4.0, (b) 1 M NaCl at pH 8.6, (c) 1 mM NaCl at pH 8.6 and (d) 1 M NaCl at pH 8.6.

film is fully hydrated by the continuous water phase and therefore closely resembles the microscopic situation at the interface of the emulsions. The measurements of  $\theta_{ow}$  clearly demonstrate that zein is a very suitable material for interfacial adsorption, considering that its value is close to  $90^\circ$ , irrespective of the exact conditions in the aqueous phase. Hence, bare zein colloidal particles could serve well as Pickering emulsifiers. At a low ionic strength (1 mM NaCl), zein has a slight preference for wetting by the aqueous phase. This is the case at both pH 4.0 with  $\theta_{ow} = 98 \pm 3^\circ$  (Fig. 2a) as well as at pH 8.5 with  $\theta_{ow} = 95 \pm 3^\circ$  (Fig. 2c). However, zein becomes somewhat hydrophobic at 1 M NaCl at both pH values, as indicated by oil-in-water contact angles of  $87 \pm 5^\circ$  (pH 4.0, Fig. 2b) and  $85 \pm 5^\circ$  (pH 8.5, Fig. 2d). Moreover, the observed trends are in agreement with measurements of the water-in-air contact angle  $\theta_{wa}$  on zein films. At  $\text{pH} < \text{pI}$  and  $\text{pH} > \text{pI}$ , the  $\theta_{wa}$  values are similar (between  $46^\circ$  and  $50^\circ$ ), as shown in ESI Fig. 1a†. Close to the isoelectric point, however, at pH 6.2, zein becomes more hydrophobic with  $\theta_{wa} = 57 \pm 4^\circ$ . Since the majority of the chargeable groups remain uncharged near pI, the wettability of the zein film by the water droplet is decreased. Furthermore, ESI Fig. 1b† displays an increase in  $\theta_{wa}$  with ionic strength, which again corresponds with an increase in hydrophobicity.

### 3.3. Effect of particle concentration on emulsion stability

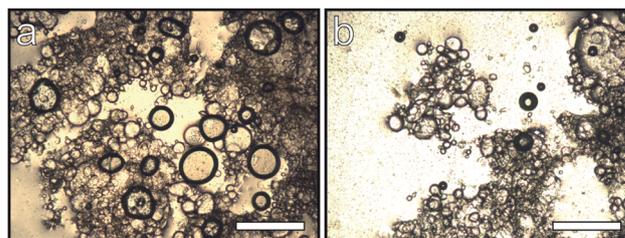
Particle concentration has a profound effect on emulsion stability against coalescence and on the droplet size of the particle covered emulsion droplets.<sup>36</sup> The weight percentage of zein was varied in the range 0.2 to 2 wt%, see Fig. 3. In all the emulsions, the pH of the dispersion was kept constant at 4.0. The zeta potential at pH 4.0 was  $63 \pm 8$  mV, and thus the particles were positively charged. The ionic strength, as adjusted by NaCl, was set to 1 mM. Under these conditions, oil-in-water emulsions were formed. At only 0.2 wt% zein, the volume fraction of the emulsion was 50%; the interface height (determined from the bottom of the sample) at 50% did not change. Due to the low particle concentration macroscopic phase-separation occurred, which led to the formation of very large oil droplets such that



**Fig. 3** Photograph of emulsions stabilized with different concentrations of zein colloidal particles at pH 4.0 and 1 mM NaCl. The photograph was taken three days after preparation.

coalescence into a macroscopic oil phase was observed. Furthermore, some gel-like aggregates were formed in the top phase where the oil droplets creamed. This is an expected phenomenon for polymeric particles which upon compression can aggregate or fuse – in analogy with partial coalescence in emulsions. At higher concentrations of 1 wt% about 74% of the phase volume was occupied by the emulsion phase, which did not change upon storage. A strong emulsion–gel-phase material was obtained that could be put upside down without disturbing the sample. This gelation of the emulsions is again a result of the aggregation of the colloidal particles being compressed between the droplets after creaming. Upon a further increase of the particle concentration to 2 wt%, the interface height was positioned at 29% (71% emulsion volume phase) after 1 h. After 3 days some oil was observed on top of the emulsion phase. After 2 weeks the height was at 40% of the sample volume (60% emulsion volume phase) due to creaming and even more oil was observed on top. Aggregation was observed and the emulsion became gel-like. Optical microscopy pictures of the different emulsions are shown in Fig. 4. The formation of particle-covered flocculated oil droplets is clearly visible and agrees with the hydrophilic nature of the zein film at pH 4.0 with 1 mM NaCl (Fig. 2a). Furthermore, partially flocculated zein particles are observed.

In principle, the formation of an emulsion–gel from particle covered droplets and pure zein particles, which were present in the aqueous phase, could provide additional stabilization against coalescence.<sup>37</sup> The amount of particles adsorbed at the oil–water interface was not quantified. In all cases, however, the aqueous



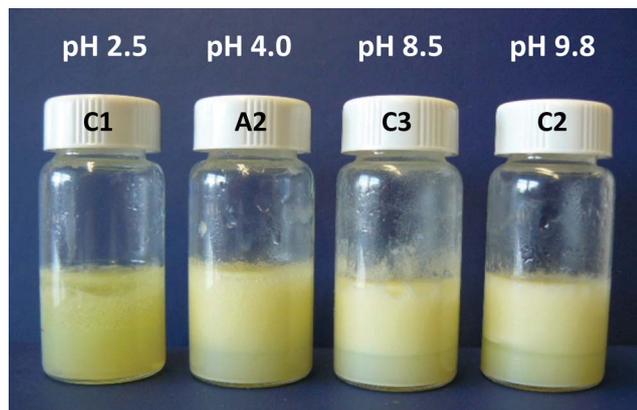
**Fig. 4** Microscopy images (scale bar: 500  $\mu\text{m}$ ) of emulsion samples (a) with 2 wt% zein (A1) and (b) 1 wt% zein (A2), both containing 1 mM NaCl at pH 4.0. Pictures were taken 30 minutes after preparation.

phase was turbid, increasing with particle concentration, indicating that a fraction of the zein colloidal particles was not adsorbed but rather remained dispersed in the continuous water phase due to the slight preference of zein for water.

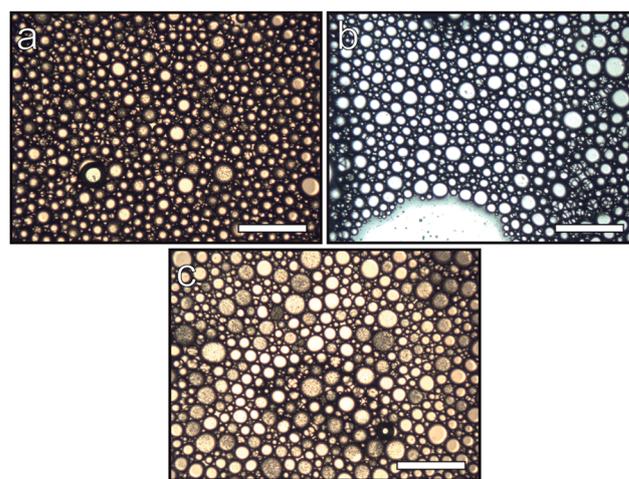
### 3.4. Effect of pH on emulsion stability

pH has a strong effect on the surface charge and stability of colloidal particles.  $\zeta$ -potential measurements confirmed that zein colloidal particles can acquire very high positive surface charges at  $\text{pH} < \text{pI}$  and high negative charges at  $\text{pH} > \text{pI}$  (Fig. 1c). Close to the isoelectric point the particles lose their hydrophilic character and start to aggregate (Fig. 1d). The effect of pH on the emulsion stability against coalescence was studied at a constant zein concentration of 1 wt% and at a constant ionic strength (1 mM NaCl). The pH of the aqueous zein-containing phase was varied and all values (2.5, 4.0, 8.5, 9.8) were chosen to be sufficiently far from the isoelectric point of zein ( $\text{pI} \approx 6.5$ ), since these dispersions were unstable. Thus, in all samples particles were strongly, positively or negatively, charged and oil-in-water emulsions were formed (Fig. 5).

At pH 2.5 the interface was positioned at a height of 40% of the sample volume, corresponding to a 60% volume of the emulsion phase. After 2 weeks the height slightly increased to 45%, decreasing the emulsion phase volume due to creaming. The emulsion was very unstable and some oil was observed on top of the emulsion, increasing with time due to coalescence (Fig. 5). At pH 4.0, already described in the previous section, about 74% of the sample volume was occupied by the emulsion phase. The interface height, which was initially 26%, did not change upon storage. A gel-phase material from oil droplets stabilized by particles and aggregates was obtained that could be put upside down without disturbing the sample. Above the  $\text{pI}$  of zein, at pH 8.5, the interface height was at 37% (63 vol% emulsion phase). The volume of the emulsion phase remained constant with time (measured after 3 days and after 2 weeks). At this pH, no gel structure was formed and the o/w emulsion flowed like a fluid. The oil droplets had a diameter of  $\sim 10$  to  $100 \mu\text{m}$  as estimated from the microscopy pictures. These oil droplets are stable against coalescence, since after 2 weeks a similar droplet size was observed (Fig. 6a and b). At pH 9.8 (Fig. 6c) the situation was



**Fig. 5** Photograph of emulsions stabilized with 1 wt% zein colloidal particles prepared at different pH and at a constant [NaCl] of 1 mM. The photograph was taken three days after preparation.



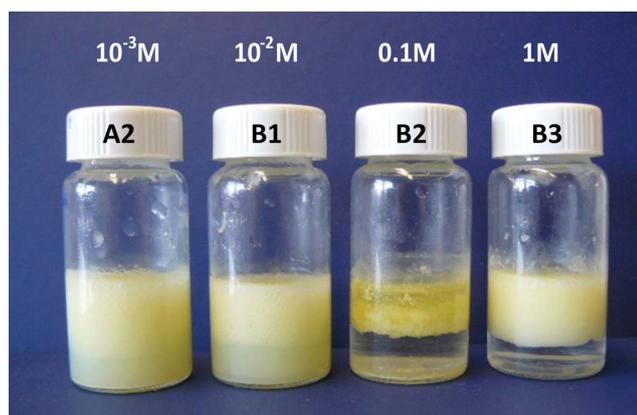
**Fig. 6** Microscopy images of o/w emulsions with 1 wt% zein and 1 mM NaCl. (a) Sample C3 at pH 8.5 30 minutes and (b) 2 weeks after preparation, and (c) C2 at pH 9.8 after 30 minutes. The scale bar corresponds to  $500 \mu\text{m}$ .

very similar. The interface height was 29% after 3 days and 38% (71% and 62% emulsion phase, respectively) after 2 weeks due to creaming. Again, no gel structure was formed and the o/w emulsion flowed like a fluid. The oil droplets had a diameter of  $\sim 10$  to  $100 \mu\text{m}$  as estimated from the microscopy pictures.

Formation of emulsion–gels was observed at  $\text{pH} < \text{pI}$ , but at  $\text{pH} > \text{pI}$  stable fluid emulsions were obtained. This could be explained by the difference in the ratio between acidic and basic groups and between hydrophilic and hydrophobic groups in zein. This may give a different balance between the hydrophilic and hydrophobic properties of the particles and influence their ability to aggregate. Notably, the observation of oil-in-water emulsification agrees with  $\theta_{\text{ow}} > 90^\circ$ , as depicted in Fig. 2a and c.

### 3.5. Effect of ionic strength on emulsion stability

Ionic strength has a strong effect on colloidal stability through influencing the contribution of repulsive electrostatic forces acting between the particles. We have studied the effect of ionic strength on the stabilization of emulsions against coalescence for both positively and negatively charged zein particles, *i.e.* at pH values below and above the isoelectric point. Above 10 mM NaCl the particles start to aggregate (see Fig. 1e) and become more hydrophobic (see Fig. 2b and d and ESI Fig. 1b†). Larger particle sizes increase the energy for detachment from the interface and enhance the stability against coalescence. On the other hand, however, strong particle attraction will lead to the formation of larger aggregates that can sediment very quickly, which obscures their surface activity. The pH of the 1 wt% zein dispersion was kept constant at 4.0, while [NaCl] was varied from 1 mM up to 1 M. Fig. 7 shows a photograph of the samples, in all of which o/w emulsions were formed. At 1 mM a gel-phase material was obtained that was sufficiently strong to be put upside down without disturbing the sample (Fig. 4b). At a higher ionic strength (10 mM) again an emulsion–gel phase was observed, the emulsion volume fraction decreased (67 vol% emulsion phase) and did not change with time. At a higher ionic strength (0.1 M), we observed a decrease in the emulsion volume (50 vol% emulsion phase). At

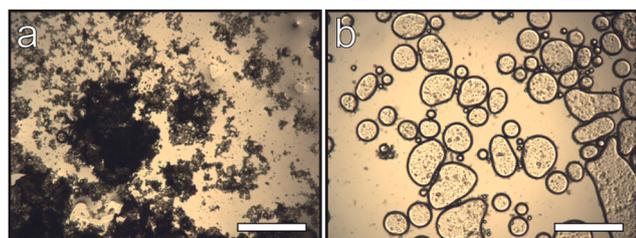


**Fig. 7** Photograph of emulsions stabilized with 1 wt% zein colloidal particles prepared at different [NaCl] and a constant pH of 4.0. The photograph was taken three days after preparation.

this ionic strength zein particles started to aggregate and some sediment at the bottom of the aqueous phase was observed (Fig. 7); hence no stable emulsion was formed but macroscopic phase-separation was observed instead. The water phase is transparent and free of zein particles. The strong aggregation is also evident from the light microscopy pictures (Fig. 8).

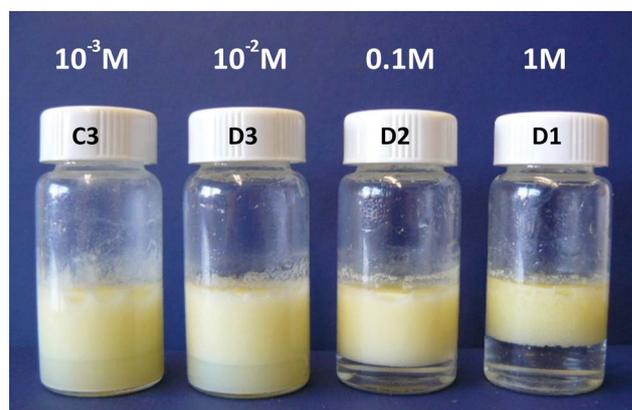
At 1 M NaCl, the highest ionic strength studied, we observed again an increase of the emulsion volume (67 vol% emulsion phase). Here, we also observed some aggregation and formation of a gel structure, while the water phase was again free of particles. The observation of a gel-like emulsion phase may be somewhat unexpected since particle size measurements at different ionic strengths show that particle aggregation was also observed at 1 M NaCl (Fig. 1e), but this could be a result of the delicate balance between hydrophilic and hydrophobic groups of the protein particles and the surface charge of the water–oil interface. It could be a purely kinetic effect: the rate of particle aggregation was slower than the rate at which they adsorbed, which allows the formation of more stable emulsions. In principle, a high concentration of salt can increase the protein solubility (salting in effect) after the initial precipitation (salting out effect). However, for zein such data do not exist. Since at ionic strengths of 0.1 M and higher no particles were present in the transparent aqueous phase, it is very possible that zein particles not only adsorb at the oil–water interface but also migrate to the oil phase.

In a similar way, the effect of ionic strength was studied above the isoelectric point at pH 8.6, where the zein particles are

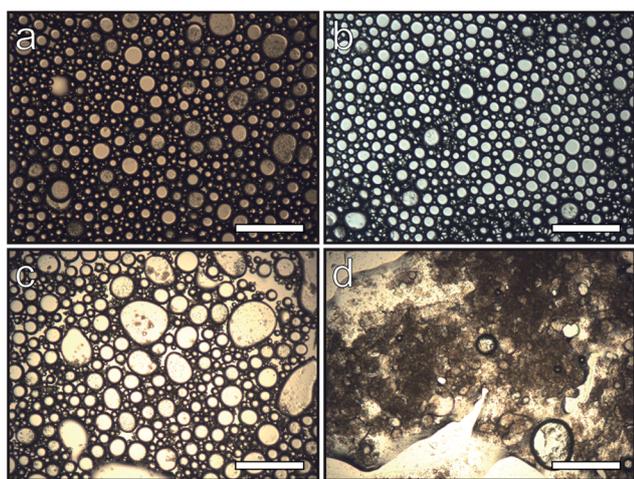


**Fig. 8** Microscopy images of samples with (a) 0.1 M NaCl (B2) showing the presence of aggregated zein particles and (b) 1 M NaCl (B3). All samples were prepared at a pH of 4.0 and 1 wt% zein. The scale bar corresponds to 500  $\mu\text{m}$ .

negatively charged. All emulsions were prepared as described in the experimental section, except that NaCl was added straight after oil addition to assure that the possible zein particle aggregation took place, presumably after the emulsion has already been formed. In all cases o/w emulsions were formed (see Fig. 9). At a low ionic strength (1 mM NaCl), 63% of the sample volume was occupied by the emulsion phase. The volume of the emulsion phase remained constant with time (after 3 days and after 2 weeks). At this pH, no emulsion–gel phase was formed and the o/w emulsion flowed like a fluid. The oil droplets had a diameter of  $\sim 100 \mu\text{m}$  as estimated from the microscopy pictures. These oil droplets are stable against coalescence, since after 2 weeks a similar droplet size was observed, as can be seen from Fig. 10a and b. Upon increasing the ionic strength to 10 mM, 70% of the sample volume consisted of an o/w emulsion phase, which remained constant with time (after 3 days and after 2 weeks). This macroscopic observation agrees with the droplet size estimated from the microscopy pictures shown in Fig. 10. The oil droplets had a typical diameter of  $\sim 100 \mu\text{m}$  and are stable against coalescence, since after 2 weeks a similar droplet size was observed. A gel-like emulsion structure was not formed and the o/w emulsion had a fluid character. At even higher concentrations of salt (0.1 M), the interface height after 3 days was positioned at 25% and changed to 35% after 2 weeks. The water phase is free of zein particles and hence transparent. The structure of the o/w emulsion is not as rigid as that of a gel, but is less mobile than a fluid and droplets with a size of  $\sim 100 \mu\text{m}$  were observed. However, partial coalescence into larger droplets and aggregates of zein particles were found as well, see Fig. 10c. Possibly aggregation of the zein colloids takes place after the emulsion has already been formed, thereby decreasing the interfacial area occupied by the zein particles. This may explain the observed decrease in emulsion volume over time and the partial coalescence into larger droplets. At the highest ionic strength studied (1 M), the volume fraction of the emulsion phase was 57% and decreased only slightly to 48% after 2 weeks. The bottom water phase was clear, indicating that all the particles were adsorbed at the oil–water interface and possibly some particles had migrated to the oil phase. It was found that the zein particles aggregated strongly, leading to the formation of a very stiff emulsion–gel in the top phase (Fig. 9). The large degree of particle aggregation was confirmed by microscopy (Fig. 10). In



**Fig. 9** Photograph of emulsions stabilized with 1 wt% zein colloidal particles prepared at pH 8.6 and different ionic strengths.



**Fig. 10** Microscopy images (scale bar: 500  $\mu\text{m}$ ) of oil-in-water emulsions with 1 wt% zein at constant pH 8.6. (a) Sample D3 containing 10 mM NaCl 30 minutes and (b) 2 weeks after preparation. (c) Sample D2 with 0.1 M NaCl and sample D1 (d) with 1 M NaCl.

accordance with the 0.1 M sample,  $\sim 100\ \mu\text{m}$  sized droplets as well as some very large coalesced oil droplets were observed.

If we now consider the contact angle data, several phenomena that we observed can be explained. First, the fact that particles adsorb at the oil–water interface at low and high pH and at a wide range of ionic strengths follows from  $\theta_{\text{ow}}$  being close to  $90^\circ$ . The formation of oil-in-water emulsions with 1 mM NaCl, at low and high pH, is supported by zein being slightly hydrophilic with  $\theta_{\text{ow}} > 90^\circ$  (Fig. 2a and c). Less expected, however, is o/w emulsification at high ionic strengths since zein becomes somewhat hydrophobic,  $\theta_{\text{ow}} < 90^\circ$  (Fig. 2b and d). We attribute this to the fact that particles were initially dispersed in the aqueous phase. Moreover, the emulsions were formed, while mixing, by slowly adding the oil phase to the aqueous zein dispersion, favoring the formation of an o/w emulsion. These findings suggest that it might be possible to create phase-inverted w/o emulsions. Furthermore, in all the emulsions prepared we found a turbid aqueous phase at low ionic strengths (1–10 mM NaCl), whereas at high ionic strengths (0.1–1 M NaCl) it was free of zein particles and transparent. This corresponds with the wetting properties of zein, as shown Fig. 2 and ESI Fig. 1b†, having a slight preference for water at low ionic strengths and being somewhat hydrophobic at high ionic strengths. In the latter case it might well be possible that, apart from adsorption at the oil–water interface, zein partly migrates to the oil phase.

Additionally, it was found in preliminary experiments that stable o/w emulsions disperse in oil very easily: if a drop of an o/w emulsion was put in a continuous oil phase the drop remained intact (drop test). Upon vigorously mixing, the o/w drop formed many smaller o/w drops that remained dispersed in the oil phase. However, oil-in-water-in-oil emulsification as well as the possibility to create water-in-oil emulsions are topics for future research.

#### 4. Conclusions

In this paper we introduce water-insoluble proteins as a novel class of particle-stabilizers of emulsions, employing colloidal

particles from zein as a representative. The o/w Pickering emulsions formed are biocompatible, edible and based on fully natural renewable resources. It was shown that factors like particle concentration, pH and ionic strength have a pronounced effect on the size, charge and wetting properties of the particles and, hence, on emulsion stability. These were used to tune the ability of zein colloidal particles to stabilize emulsions against coalescence. We found that surfactant-free zein colloidal particles can produce stable oil-in-water emulsions at pH above and below the zein isoelectric point, and for low to moderate ionic strength. Under all of these conditions the oil-in-water three-phase contact angle  $\theta_{\text{ow}}$  was close to  $90^\circ$ , strongly favoring interfacial particle adsorption. As opposed to many other biomaterials, zein colloidal particles are intrinsically surface active and, therefore, require no additional chemical surface modification. We anticipate that our study may trigger deeper research into water-insoluble proteins acting as particle emulsifiers, as this promising class of biomaterials is underutilized, environmentally friendly and scalable. The ability to control particle size-, pH- and ionic strength-dependent emulsion stability, and composition<sup>27,28</sup> relying on natural renewable materials, provides an important link between biology and colloid physics. It opens several new opportunities for the use of particle-based stabilization and structuring in food, pharmaceutical, agricultural, coating, and cosmetic applications. Further research is needed, however, to establish the contribution of particle aggregation on emulsion gelation and employ these systems in real product formulations.

#### Acknowledgements

We thank J. Hazekamp for his assistance with electron microscopy, R. Farr and A. Bot for useful discussions and critical reading of the report. This work is financially supported by Food and Nutrition Delta 2 program (grant FND07002) and The Netherlands Organization for Scientific Research (NWO).

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## Addition and correction

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