

See discussions, stats, and author profiles for this publication at: <https://www.researchgate.net/publication/256118354>

Soy Protein Nanoparticle Aggregates as Pickering Stabilizers for Oil-in-Water Emulsions

ARTICLE in JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY · AUGUST 2013

Impact Factor: 2.91 · DOI: 10.1021/jf401859y · Source: PubMed

CITATIONS

38

READS

174

2 AUTHORS:



Fu Liu

South China University of Technology

16 PUBLICATIONS 164 CITATIONS

SEE PROFILE



Chuan-he Tang

South China University of Technology

102 PUBLICATIONS 1,718 CITATIONS

SEE PROFILE

Soy Protein Nanoparticle Aggregates as Pickering Stabilizers for Oil-in-Water Emulsions

Fu Liu[†] and Chuan-He Tang^{*,†,‡}

[†]Department of Food Science and Technology and [‡]State Key Laboratory of Pulp and Paper Engineering, South China University of Technology, Guangzhou 510640, People's Republic of China

ABSTRACT: In recent years, there have been increasing interests in developing food-grade Pickering stabilizers, due to their potential applications in formulations of novel functional foods. The present work was to investigate the potential of soy proteins to be developed into a kind of Pickering-like stabilizer for oil-in-water emulsions. The nanoparticle aggregates of soy protein isolate (SPI) were formed by sequential treatments of heating at 95 °C for 15 min and then electrostatic screening with NaCl addition. The particle size and microstructure of these aggregates were characterized using dynamic light scattering and atomic force microscopy, indicating that the fabricated nanoparticle aggregates were ~100 nm in size with more surface hydrophobic nature (relative to unheated SPI). The influence of particle concentration (c ; 0.5–6.0%, w/w) and increasing oil fraction (ϕ ; in the range 0.2–0.6) on the droplet size and coalescence and/or creaming stability of the emulsions stabilized by these nanoparticle aggregates was investigated. The results showed that, at $\phi = 0.2$, increasing the c resulted in a progressive but slight decrease in droplet size, and improved the stability against coalescence and creaming; at a specific c , the creaming stability was progressively increased by increasing the ϕ , with better improvement observed at a higher c (e.g., 6.0% vs 2.0%). The improvement of creaming stability was largely associated with the formation of a gel-like network that could entrap the oil droplets within the network. The observations are generally consistent with those observed for the conventional Pickering emulsions, confirming that soy proteins could be applied as a kind of effective Pickering-like stabilizer. The finding may have important implications for the design and fabrication of protein-based emulsion formulations, and even for the development of soy protein products with some unique functions. To the authors' knowledge, this is the first work to report that heat-induced soy protein aggregates exhibit a good potential to act as Pickering-type stabilizers.

KEYWORDS: soy protein isolate (SPI), nanoparticle, protein aggregate, Pickering emulsions, emulsifying property

INTRODUCTION

In recent years, the investigation of novel Pickering emulsions stabilized by food-grade particles has attracted increasing interest in the research field of food and pharmaceuticals, due to their compatibility with food and outstanding stability with respect to coalescence (even when the emulsions are of large size).^{1–3} The Pickering emulsions, stabilized by inorganic or organic particles, e.g., silica particles (or nanoparticles), have been confirmed to exhibit more sustained release of some encapsulated lipophilic drug or ingredients, more stable against lipid oxidation, and even slower in lipid digestibility, as compared with conventional surfactant-based emulsions.^{4–8} This implies that these kinds of emulsions can be developed into effective delivery systems with good functional performance, thus exhibiting a good potential for the development of novel functional foods. Although there is a lot of research addressing the particle-stabilized Pickering emulsions, few of these studies are directly compatible with food, since most of the particles (especially inorganic particles) exhibiting Pickering stabilization are not allowed in foods.¹

To date, only a limited range of colloidal particles compatible with food have been reported as suitable Pickering stabilizers for oil-in-water emulsions. The food-grade Pickering stabilizers, previously reported, included chitin nanocrystal particles,^{8,9} water-insoluble zein,¹⁰ microcrystalline cellulose (MMC),⁶ modified starch,^{6,11,12} some flavonoids,^{13,14} and even solid lipid nanoparticles.¹⁵ One of the common features of these

particles is that they are insoluble or even poorly soluble in water, but can be well dispersed in the system with good surface activity. The importance of surface characteristics of these particles for stabilizing the emulsions has been demonstrated experimentally by Pounov and others.¹⁶ Nevertheless, some limitations frequently exist: the particles are often highly polydisperse and even need additional chemical treatments to warrant interfacial attachment; many particles are poorly characterized, and not approved as food-grade ingredients; and many model systems have a very limited applicability, since the real situation in foods is much more complex. Thus, it would be a challenging issue for food scientists or the food industry to find cheap and effective food-grade particles suitable to stabilize the Pickering emulsions.

Except zein, many food proteins or proteinaceous colloidal particles (e.g., casein micelles) have been considered to be not able to act as Pickering stabilizers.² There are two main arguments for this viewpoint. One is that these colloidal particles usually coexist in the aqueous phase with smaller soluble protein molecules, and the latter species are predominantly responsible for rapidly lowering the interfacial tension during emulsification; the other is that, once the

Received: April 29, 2013

Revised: August 26, 2013

Accepted: August 27, 2013

Published: August 27, 2013

colloidal particles become adsorbed, they start to break down into smaller fragments, and as a consequence, a gel-like adsorbed interfacial layer will be formed.² We do not fully agree with this viewpoint. First, the viewpoint is generally based on proteins from animal sources, e.g., caseins and whey proteins, and it cannot exclude a possibility that, like zein, proteins from other plant sources exhibit an excellent Pickering stabilization. Second, the importance of particle or solid concentration to the emulsifying properties of many proteinaceous colloidal particles is little characterized, and even underestimated. If the concentration of these colloidal particles is high enough, their structural unfolding or breakdown at the interface may be greatly inhibited, since, at high bulk concentrations, fast adsorption of proteins at the interface occurs, giving less time for the adsorbed proteins to orient and spread out at the interface.¹⁷ In this case, the Pickering-like stabilization of the protein colloidal particles would be more distinct than expected. In the case of modified silica stabilized Pickering emulsions, it has been confirmed that increasing silica particle content resulted in formation of a more stable and even gel-like emulsion with smaller droplet size; besides the particle Pickering stabilization, the aggregation of silica particles produced a supplementary stabilization on the emulsions.¹⁸ More importantly, Murray and his colleagues¹⁹ recently found that when the surface active particles (cellulose + ethyl cellulose complexes, modified starch particles, and stable protein-stabilized oil droplets) were applied together with proteins (caseins or whey proteins) to stabilize the emulsions, the interfacial viscoelasticity considerably increased, with concomitant increases in emulsion droplet stability. They attributed the increased stability to the enhanced accumulation of particles at the interface by the presence of the proteins.

Soy proteins are one of the most important food proteins worldwide. In the past decades, they have been applied in a tremendous number of food formulations, due to their good nutritional value and functionality, and even potential health effects. Soy protein isolate (SPI), mainly composed of 11S and 7S globulins, is the most commercially available soy protein product. The globulins in SPI are easily denatured during the commercial production of SPI, and as a result, most of them are present in the aggregated form. Besides their insoluble nature, the aggregated proteins in SPI exhibit good surface active properties, suggesting that this protein exhibits a good potential to be developed into a kind of effective Pickering or Pickering-like stabilizer. Paunov and others¹⁶ for the first time reported that spray-dried soy protein particles with calcium phosphate cores, in a commercial soy protein product (Supro 651), could be effectively adsorbed at the oil–water interface without distinct changes in particle morphology, thus implying a potential to be applied as Pickering stabilizers. Although thermal aggregation and/or gelation of soy proteins have been widely characterized,^{20–22} very few studies have considered the emulsifying properties of thermally induced soy protein aggregates or particles.

There are several reports^{23–26} addressing the influence of thermal treatment or denaturation on the properties of soy protein-stabilized emulsions. In these previous works, the properties of the emulsions are usually understood in a similar manner to the conventional emulsions. Puppo et al.²⁶ observed a gel-like behavior of the emulsions stabilized by heat-acid treated SPI at high protein concentrations (6–10%, w/v). This gel-like behavior has also been observed in the emulsions stabilized by commercial soy protein concentrate^{27,28} and, more

recently, in the emulsions stabilized by a heated SPI at a protein concentration of 6% (w/v).²⁹ The stiffness of the gel-like emulsions could be progressively strengthened by the increases in oil fraction and/or ionic strength in the aqueous phase,²⁹ suggesting importance of interdroplet hydrophobic interactions between adsorbed proteins for the gel-like network formation. In another recent work,³⁰ we further found that the gel-like behavior of these heated soy protein-stabilized emulsions was largely contributed by the thermal aggregates of glycinin (11S globulins); and only when the glycinin content was above a certain value, e.g. 65% relative to total proteins, the formed emulsions exhibited a gel-like behavior. All these works to a large extent support the above hypothesis that soy protein aggregates or particles would be a kind of effective Pickering-like stabilizer, since in most cases for classical particle-stabilized Pickering emulsions, a gel-like behavior could be observed when the solid concentration or oil fraction was high enough.^{9,11,31}

At least two unique advantages impart soy protein particles or nanoparticles to act as Pickering stabilizers. One is that they are nutritional and functional food ingredients themselves, and abundant and derived from natural sources. The other is they can perform “double duty” and not only emulsify but also contribute to steric stabilization. Furthermore, they are suitable for high pressure emulsification, which is superior to many classic Pickering stabilizers, e.g., inorganic particles, since the high abrasion by application of inorganic particles will cause damage to the homogenization devices.³² The crucial point for formulation of Pickering emulsions stabilized by soy proteins is to effectively prepare uniform particles of this protein, especially those of nanoscaled sizes. Thermal treatment seems to be one of the effective techniques to form this kind of SPI particle with insoluble nature. The size of protein colloidal aggregates in this system can be even modulated by variation with ionic strength. Thus, the present work investigated the potential of heat-induced SPI nanoparticle aggregates as suitable Pickering stabilizers. First, the nanoparticle aggregates, formed by heating (at 95 °C for 15 min) the SPI solution at a concentration of 6% (w/v), and then a subsequent NaCl addition, were characterized with respect to particle size and morphology. Second, the emulsions stabilized by these nanoparticle aggregates were characterized in terms of droplet size and emulsion stability, as well as microstructure. In this aspect, the influence of variation with particle (or solid) content and oil fraction (ϕ) on the droplet size and coalescence (and creaming) stability was studied in particular. We demonstrated that the SPI nanoparticle aggregate stabilized emulsions exhibited a similar behavior to many conventional Pickering emulsions, in many ways, e.g., extraordinary stability against coalescence and/or creaming.

MATERIALS AND METHODS

Materials. Freeze-dried SPI was prepared from defatted soy flour according to the same process described in our previous work.²⁹ The protein content of this SPI was about 91% (wet basis), as determined by the Dumas method with a nitrogen conversion factor of 6.25. Soy oil was purchased from a local supermarket in Guangzhou (China). 1, 8-Anilinoanthracene-9-sulfonate (ANS[−]) reagent was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

SPI Nanoparticle Aggregate Preparation. The SPI nanoparticle aggregates were formed by a thermal treatment of SPI solution at a constant concentration of 6% (w/v), followed by addition of varying concentrations of NaCl (to modulate the ionic strength). The SPI

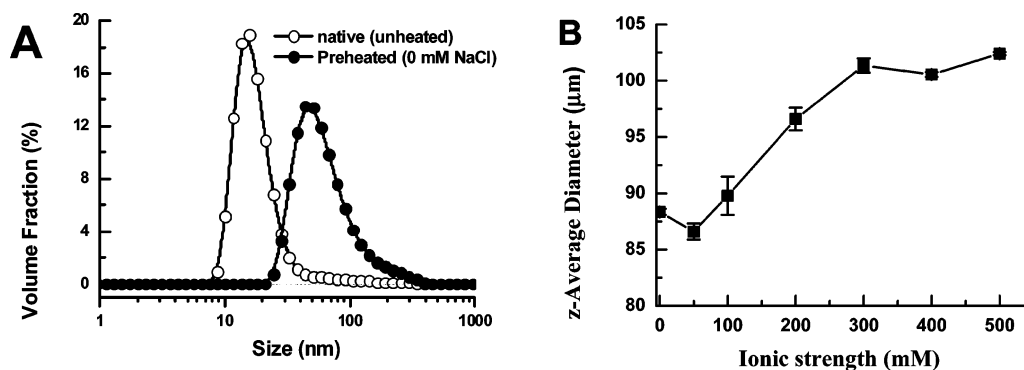


Figure 1. (A) Typical particle size distribution profiles of native and heated (at 0 mM NaCl) SPI. (B) Effects of ionic strength (0–500 mM) on z-average diameter of heat-induced SPI aggregates. The applied protein concentration (c) was 6% (w/v).

solution (6%, w/v) was prepared by dispersing the freeze-dried SPI sample in distilled water for 2 h under a magnetic stirring condition and then left overnight at 4 °C for full hydration of the proteins. Sodium azide (0.02%, w/v) was added to inhibit microbial growth. If necessary, the pH of the SPI solution after the storage was adjusted to 7.0 using 1 M NaOH or 1 M HCl. The SPI solution was heated in a bath at 95 °C for 15 min, and then immediately cooled in an ice bath to room temperature. Next, the heated SPI dispersion was subdivided into several parts for the experiments on the influence of ionic strength (0–500 mM) on the protein aggregate properties. Various heated SPI dispersions with different concentrations of NaCl were prepared by directly adding the salt powder with the needed amount into the dispersions while stirring. During the stirring, the pH of the dispersions was maintained at 7.0, using 1 M NaOH. Finally, the SPI aggregate dispersion (6%, w/v) obtained at 300 mM NaCl was applied for the following emulsion preparation. If needed, the dispersion was diluted to a specific solid concentration (0.5–6.0%) with distilled water (pH 7.0) containing 300 mM NaCl.

Nanoparticle Aggregate Characterization. Particle Size Measurement. The z-average or volume-weighted particle size (D_z) was measured by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, U.K.). To avoid multiple particle effects, particle sizes were measured after diluting samples with purified water of the same pH (pH 7.0) as the original dispersion, to an appropriate particle concentration (e.g., 100 times of dilution, in the present work). All measurements were performed in at least duplicate at room temperature, and the results reported are averaged over three readings.

ζ -Potential Determination. The ζ values of SPI dispersions at varying NaCl concentrations (0–500 mM) were measured by a laser Doppler velocimetry and phase analysis light scattering (M3-PALS) technique using the same Zetasizer (see above), in connection with a multipurpose autotitrator (model MPT-2, Malvern Instruments, Worcestershire, U.K.). The samples were prepared in deionized water containing various concentrations of NaCl (0–500 mM) and diluted to a solid concentration of approximately 0.025% (v/v) with the NaCl solutions with the same concentrations. One milliliter of each diluted sample was put in an electrophoresis cell (model DTS 1060C, Malvern Instruments Ltd., Malvern, Worcestershire, U.K.). The temperature of the cell was maintained at ambient temperature. The data were reported as average values and standard deviations of four measurements performed on three individually prepared emulsions.

Surface Hydrophobicity (H_o). H_o of the proteins was determined with the fluorescence probe ANS[−] according to the method of Haskard and Li-Chan.³³ Serial dilutions in 0.01 M phosphate buffer (pH 7.0) were prepared with the heated SPI dispersions to a final protein concentration of 0.004–0.02% (w/v). ANS[−] stock solution (8.0 mM) was also prepared in the same buffer. Twenty microliters of ANS[−] solution was added to 4 mL of each dilution, and the fluorescence intensity (FI) of the mixture was measured at 370 nm (excitation) and 470 nm (emission) using an F4500 fluorescence-

spectrophotometer (Hitachi Co., Japan). The initial slope of the FI versus protein concentration (mg/mL) plot (calculated by linear regression analysis) was used as an index of H_o .

Atomic Force Microscopy (AFM). AFM images were acquired in tapping mode using a Dimension 3000 microscope (Digital Instruments-Veeco, Santa Barbara, CA, USA), equipped with a “G” scanning head (maximum scan size 10 μm) and driven by a Nanoscope IIIa controller. The heated SPI dispersions at varying concentrations (0–500 mM) of NaCl were diluted with the distilled water of the same concentrations of NaCl, to a final protein concentration of 2 μg/mL. A 2 μL droplet of the diluted samples was immediately spread on a freshly cleaved mica disk and dried in air for 30 min at ambient temperature. For imaging under ambient condition, single-beam uncoated silicon cantilevers (type OMCL-AC, Olympus, and RTESP, Veeco) were used. The drive frequency was set at 300 kHz, and the scan rate was 1.0 Hz. Images were processed by the NanoScope Analysis 1.40r2. For each preparation condition, at least two samples were used for duplication. Approximately 10 images were obtained for each preparation.

Emulsion Preparation. For all emulsions, the SPI nanoparticle aggregates, contained in the dispersion (6%, w/v) that was heated at 95 °C for 15 min in the absence of NaCl, and subsequently added with 300 mM NaCl, were used. The SPI aggregate dispersion (6%, w/v) was diluted with distilled water of the same pH and NaCl concentrations, to a required solid concentration in the range 0.5–6.0%. The emulsions were prepared according to the process described by de Folter et al.,¹⁰ with a few modifications. A total volume of 20 mL of all emulsions was formulated, with relative variation in ϕ from 0.2 to 0.6. As an example, the emulsions at $\phi = 0.2$ were prepared as follows. In brief, 16 mL of the diluted dispersion with a certain solid concentration was brought into a glass vial, and 4 mL of soybean oil was slowly added to the dispersion, while mixing with an Ultra Turrax homogenizer (model IKA-ULTRA-TURRAX T25 basic, IKA Works, Inc., Wilmington, NC) with a dispersing head operating at 10,000 rpm. After all the oil was added, the sample was mixed for 2 additional minutes. The obtained emulsions were directly subject to analysis or storage stability experiments.

Characterization of the Emulsions Stabilized by SPI Nanoparticle Aggregates. Droplet Size Distribution and Volume-Average Droplet Size ($d_{4,3}$). Droplet size distribution of various freshly prepared or stored (7 and 40 days) emulsions was determined using a Malvern MasterSizer 2000 (Malvern Instruments Ltd., Malvern, Worcestershire, U.K.). Deionized water or 1.0% (w/v) SDS solution was used as the dispersant. The relative refractive index of emulsion was taken as 1.095, that is, the ratio of the refractive index of soy oil (1.456) to that of the continuous phase (1.33). Droplet size measurements are reported as volume-average droplet size, $d_{4,3}$ ($=\sum n_i d_i^4 / \sum n_i d_i^3$), where n_i is the number of droplets with diameter d_i . All determinations were conducted at least in duplicate.

Optical Microscopy. The microstructure of the freshly formed or stored (7 days) emulsions was analyzed by optical microscopy using a Leica microscope (DM 2000; Leica Microsystems AG, Wetzlar,

Germany) with a 60× water-immersion objective. The technique did not require any sample preparation. Unless otherwise stated, observations were carried out at ambient temperature.

Visual Observation of Creaming and Determination of Creaming Index. Creaming stability of the emulsions formed at varying conditions was evaluated visually. Ten milliliters of each emulsion was filled into a glass test tube (1.5 cm internal diameter × 12 cm height) and then stored at ambient temperature (placed in a perpendicular state). Height of the serum (H_s) and total height of emulsions (H_t) after specific incubation periods of storage (up to 2 weeks) were recorded at ambient temperature. Mean and standard deviations of three replicates were reported. The percentage of creaming index (CI %) was reported as $(H_s/H_t) \times 100$.

Statistics. An analysis of variance (ANOVA) of the data was performed, and a least significant difference (LSD) with a confidence interval of 95% was used to compare the means.

RESULTS AND DISCUSSION

Characteristics of SPI Nanoparticle Aggregates. Heat-Induced Aggregation at 0 mM Ionic Strength. The particle distribution profile of the native SPI dispersion at $c = 6\%$, applied in the present work, is displayed in Figure 1A. We can see that this SPI exhibited a major monodisperse particle size distribution with sizes ranging from 8 to 100 nm. The D_z of this native SPI was estimated to be approximately 58 nm. Li and others³⁴ observed a polydisperse size distribution profile for an unheated SPI, with two prominent peaks centered at around 10 and 100 nm, attributed to native soy globulins and their aggregates, respectively. The difference might be due to the application of an alcohol washing of defatted soy flake in this previous work. The D_z of this native SPI is basically consistent with those (approximately 50–70 nm) observed by transmission electron microscopy,³⁵ or estimated by size exclusion chromatography in combination with multiangle light scattering detector.³⁶ This diameter is higher than that (~ 34 nm) reported for native SPI at $c = 1\%$,³⁷ but considerably lower than those reported by Fang et al.³⁸ (~ 86 nm), Santiago et al.³⁹ (112 nm), and Boulet et al.⁴⁰ (>200 nm). The extensive variety of particle size for the native SPI confirms the fact that structure (or conformation), surface properties and protein–protein interactions of soy globulins are sensitively influenced by both processing and environmental conditions such as pH, ionic strength and temperature.³⁵

After the SPI dispersion being heated at 95 °C for 15 min, we can see that the particle size distribution profile shifted to higher sizes with a distribution peak centered at around 60 nm (relative to 15 nm for the native SPI), and the polydispersity of the particles distinctly declined (Figure 1A). The observations clearly indicated heat-induced aggregation of the proteins in SPI. The D_z of the aggregate particle was estimated to be about 88 nm. Li et al.³⁴ also observed that heating in boiling water for 15 min resulted in aggregation of native soy protein components in SPI at $c = 1.0\%$, while the initially present soy protein aggregate was nearly unaffected. Wang et al.³⁷ reported that the hydrodynamic diameter of native SPI was slightly but insignificantly affected by heating at 90 °C for 20 min, but could be greatly increased by treatment at 120 °C for 20 min (from 34 to 40 nm). Despite the slight change in diameter, they still observed a prominent increase in amount of the soluble aggregate when the SPI was heated at 90 °C for 20 min, using size exclusion chromatography.³⁷ Interestingly, the particle size distribution profile of the heat-treated SPI in the present work is basically consistent with that observed for defatted soy milk heated in boiling water for 10 min,⁴¹ where it was confirmed

that the protein particles were composed of subunits or polypeptides of glycinin and β -conglycinin, in a complex way.

The influence of the heating (95 °C, 15 min) on the microstructure and morphology of particles in the SPI dispersion was evaluated using AFM, as displayed in Figure 2.

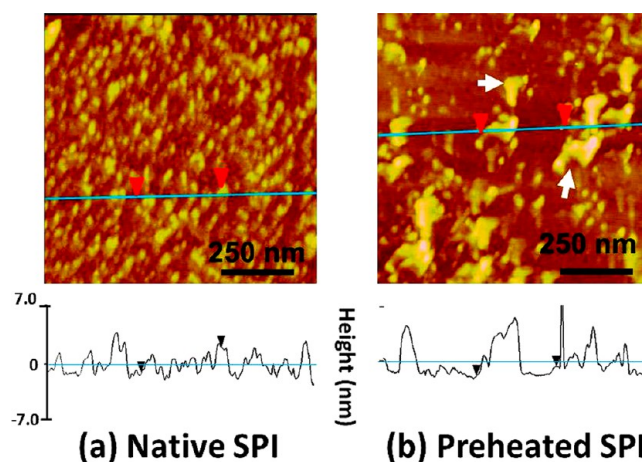


Figure 2. Typical top view AFM images of particles of native (a) and heated (b) SPI dispersions. The height profiles of cross sections at the indicated sites are also included. The heat pretreatment was carried out at 95 °C for 15 min. The arrows indicate the aggregate particles with large sizes.

In the native SPI case, we can observe that most of the particles were present in the form of spherical-shape clusters, with heights ranging from about 2.0 to 4.0 nm and widths of approximately 40–60 nm, though the extent of clustering was relatively low (Figure 2a). The widths for the particles of the native SPI are well in accordance with the D_z as determined by DLS, confirming that the proteins in the native SPI dispersion were mainly in the particle form. The morphology of the particles in the native SPI dispersion is basically similar to that observed by TEM technique, where the diameter of the spherical clusters was approximately in the range of 50–70 nm.³⁵ As expected, the heating resulted in considerable changes in particle morphology of SPI (Figure 2b). For example, the morphology of the particles in the heated SPI dispersion became much more irregular and less uniform, and the particles were much larger in size, relative to the native SPI case. The larger particles had heights of approximately 4–6 nm, and widths varying from 50 to above 200 nm (Figure 2b). The observations clearly confirmed that the heating resulted in a transformation of smaller particles into larger macroaggregate particles.

ζ -Potential and Surface Hydrophobicity (H_o) of Heat-Induced Aggregate Particles: Modulation of Ionic Strength. At 0 mM ionic strength, all the protein particles in the dispersions are to a high extent negatively charged on the surface, and as a consequence, high interparticle electrostatic repulsion can make these particles kinetically kept in the dispersions. The interparticle repulsion can be modulated by variation in ionic strength, thus affecting the size and morphology of the particles in the system. The ζ -potential of aggregate particles in the heated (95 °C, 15 min) SPI dispersion was about -24 mV (Figure 3). The value is similar to that for SPI aggregate dispersion (-20 mV),³⁹ but much lower than those reported for soluble soy protein aggregates at $c = 1\%$ (~ -41 mV)⁴² and the unheated SPI at a comparable

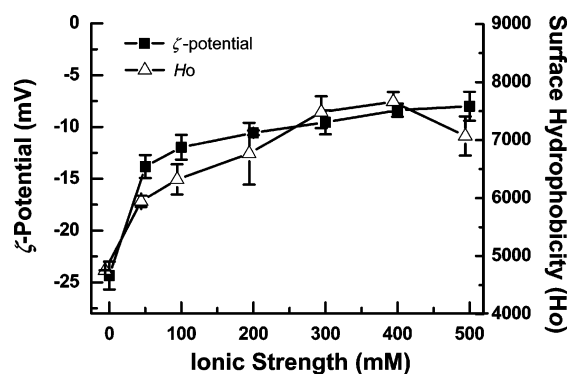


Figure 3. ζ -Potential and surface hydrophobicity (H_0) changes of heat-induced SPI aggregates as a function of ionic strength (0–500 mM). Each data is the mean and standard deviation of triplicate measurements.

pH (−50 mV).³⁹ As expected, increasing ionic strength from 0 to 500 mM resulted in a progressive decrease (up to −8.0 mV) in magnitude of ζ -potential (Figure 3), confirming the effect of electrostatic screening. Li et al.⁴² also observed that increasing ionic strength to 500 mM led to a progressive decrease in ζ -potential from about −41 to −19 mV.

Besides surface charge, surface hydrophobicity (H_0) of proteins is also an important parameter affecting their surface-related functionality. The H_0 of the heat-induced SPI aggregates as a function of ionic strength (0–500 mM) was evaluated using ANS[−] as the probe, and the data are also presented in Figure 3. Unexpectedly, we can see that the H_0 of the aggregate particles in the heated SPI dispersion progressively increased from 4700 to 7600, as the ionic strength increased from 0 to 400 mM (Figure 3). This observation seems to be contrasting from the general viewpoint that increasing ionic strength would lead to formation of more compacted conformation of proteins, with more hydrophobic clusters shifted to the interior of the molecules. One of possible explanations for this inconsistency is that the binding of ANS[−] to the proteins, e.g. through ion pair formation with cationic groups (e.g., lysine, histidine, and arginine groups), might progressively increase, as a result of decreased electrostatic repulsion between the negatively charged groups on the surface of the protein molecules and the organic sulfonate group of ANS[−].⁴³

Influence of Ionic Strength on Size and Morphology of Heat-Induced Aggregate Particles. Figure 1B shows the influence of electrostatic screening (by increasing ionic strength from 0 to 500 mM) on the D_z of the aggregate particles in the heated SPI dispersion at $c = 6.0\%$. We can see that the D_z of the aggregate particles was greatly affected by increasing ionic strength in the range of 0–500 mM (Figure 1B). Despite the remarkable decrease in magnitude of ζ -potential, the addition of 50 mM NaCl, on the contrary, resulted in a significant decline in D_z . As the ionic strength increased from 50 to 300 mM, the D_z progressively increased from about 87 to 102 nm; however, when the ionic strength increased above 300 mM, the D_z basically kept constant. The decline in D_z at an ionic strength of 50 mM might be due to the “salting-in” effect on the aggregate particles. The progressive increase in D_z was clearly attributed to the increased interparticle interactions, and consequently, formation of larger aggregate particles (or macroaggregates). Li et al.³⁴ similarly observed that the hydrodynamic radius (R_h) of heat-induced aggregates of SPI

(1%) at pH 7.0 progressively increased with ionic strength increasing from 0 to 0.4. In another more recent work, they also observed that addition of salt to the aggregates initially formed at 0 mM ionic strength (like in the present work) led to an increase in aggregate size of SPI.⁴²

To further confirm the above argument, we evaluated the influence of increasing ionic strength on the morphology of particles in the heated SPI dispersions using AFM, as shown in Figure 4. We can see that, upon increasing ionic strength, the extent of particle clustering progressively increased, and especially when the ionic strength was above 300 mM, most of the particles became associated and clustered, and were even transformed to much larger irregular particles (Figure 4). The observation is well in agreement with the DLS data (Figure 1B), confirming that the electrostatic screening by increasing ionic strength increased the size of particles in the heated SPI dispersions. The formation of denser clustering of the primary aggregates that were initially formed during the heating by increasing ionic strength was also observed in the cases of other proteins, e.g., ovalbumin and β -lactoglobulin, using light and small-angle X-ray scattering.⁴⁴

Characterization of SPI Nanoparticle Aggregate-Stabilized Emulsions. Influence of Particle Concentration on Droplet Size. We used the SPI nanoparticle aggregates, formed by heating at $c = 6.0\%$ and 0 mM ionic strength and then changing the ionic strength to 300 mM, to stabilize the emulsions. Since the amount or concentration of the particles in the heated SPI dispersion is difficult to specify, in the present work, we applied the total c of the SPI dispersions (undiluted or diluted), e.g., 6% for the undiluted dispersion, as an indicator for the nanoparticle concentration when the influence of particle concentration on the properties of the formed emulsions was characterized. In the case of classical Pickering emulsions stabilized by inorganic nanoparticles, e.g., silica particles, the emulsions are usually formed using an emulsification technique with a low energy level, since for high pressure emulsification these particles are highly abrasive and can damage the plant, especially the pump and valve system, within minutes of processing.³² For the similarity, in the present work, a low energy level of emulsification process with an Ika T25 was applied to form the emulsions.

First, we evaluated the influence of particle concentration in the aqueous phase on the droplet size and microstructure of the fresh emulsions, stabilized by the SPI nanoparticle aggregates at a specific ϕ value of 0.2. Figure 5 shows the typical droplet size distribution profiles of the various fresh emulsions, in water or 1% SDS, and the volume-average diameter ($d_{4,3}$; in water or 1% SDS) data as a function of c are summarized in Figure 6A. In the absence of 1% SDS, all the emulsions formed at varying c values of 0.5–6.0% exhibited a similarly polymodal size distribution profile, with the droplet size widely ranging from 0.6 to 200–300 μm , and a prominent size distribution peak centered at around 60–70 μm (Figure 6A). However, we can still observe that the $d_{4,3}$ of droplets (in water) progressively decreased from about 57 to 50 μm , as the c increased from 0.5 to 2.0%, while a further increase in c up to 6.0% did not result in a significant change in the $d_{4,3}$ (relative to 2%; Figure 6A). On the other hand, the size distribution profiles and the $d_{4,3}$ data at a specific c value were almost unaffected by the presence of 1% SDS (Figures 5 and 6A), indicating that almost all of the droplets were present in the unflocculated form. This can be corroborated by the microstructural observation of the emulsions using optical microscopy (Figure 7, top row).

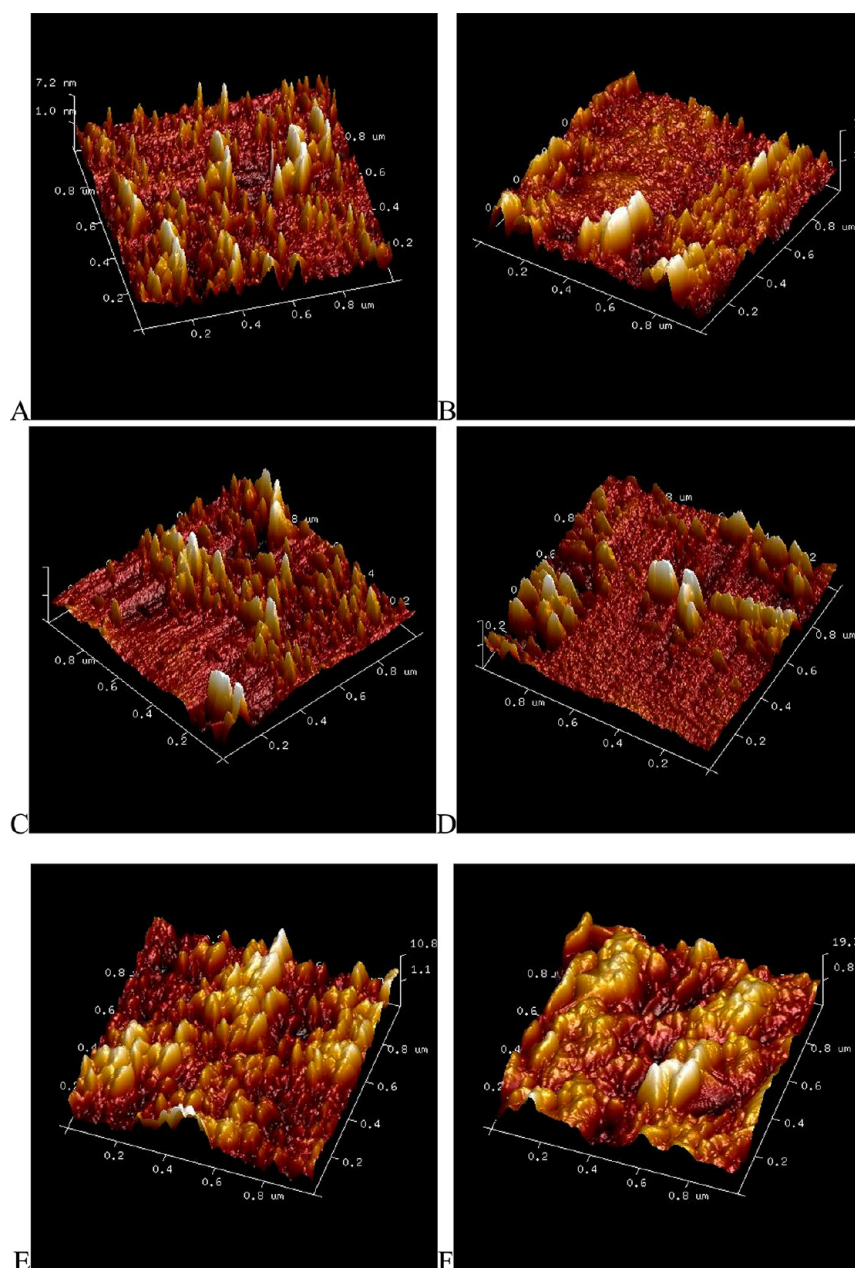


Figure 4. Typical 3D-view AFM images of particles in the heated SPI dispersions, at ionic strength values of 0 (A), 50 (B), 100 (C), 200 (D), 300 (E) and 400 (F) mM, respectively.

The progressive decreases in emulsion size upon increasing the particle concentration have been widely observed for the o/w emulsions stabilized by Pickering stabilizers, such as hydrophobized fumed silica,¹⁸ microcrystalline cellulose (MCC) and modified starch (MS),^{6,11} and chitin nanocrystals.⁹ The emulsion size distribution profiles at $c = 0.5$ –2%, in the present work, are similar to those observed for the emulsions stabilized with MCC and MC at comparable c and ϕ values, using a similar emulsification technique.⁵ The decrease of droplet size with increasing the particle content usually represents an increase in interfacial area, which is consistent with the fact that higher particle content allows the stabilization of larger interfacial area.¹⁸

Influence of Particle Concentration on Emulsion Stability.

All the emulsions, freshly prepared at increasing c values of 0.5–6.0% (w/v), underwent a fast creaming process, indicating

creaming instability. The creaming stability in terms of percentage of creaming index (CI %) upon storage up to 40 days was evaluated visually, and the data are presented in Figure 8. In general, the CI % of all the emulsions fast increased within a storage period of approximately 1 h, followed by a slow increase, and when the storage was prolonged above 3 days, the CI % became almost unchanged (Figure 8). In the steady state of creamed emulsions, we can see that increasing the c from 0.5 to 6.0% resulted in a progressive decrease in CI %, with significant differences observed between the concentrations of 0.5 and 1.0%, or of 4.0 and 6.0%, indicating inhibition of creaming by increasing the particle concentration. Similar phenomena of the influence of particle concentration on creaming stability have been observed for the emulsions stabilized by MCC and MC,⁶ chitin nanocrystal particles,⁹ and zein (at pH 4.0).¹⁰

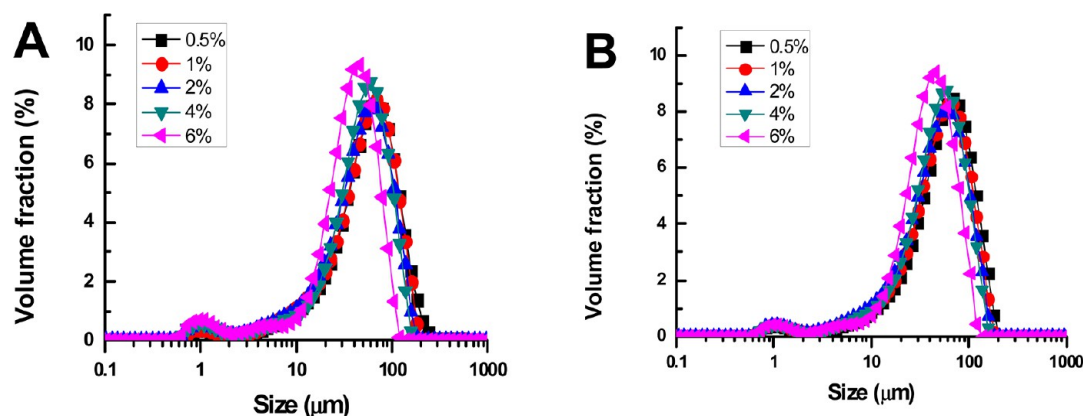


Figure 5. Typical droplet size distribution profiles of the fresh emulsions stabilized by the SPI nanoparticle aggregates at a comparable initial c of 0.5–6.0%, diluted with water (A) and 1% SDS (B).

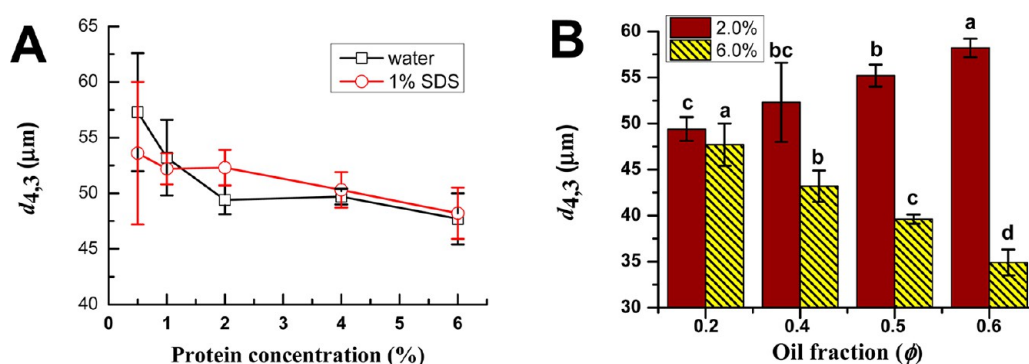


Figure 6. Volume-average diameter ($d_{4,3}$) values of droplets in the fresh emulsions stabilized by the SPI nanoparticle aggregates at varying c or ϕ values. Panel A: the c dependence of the $d_{4,3}$ (in water or 1% SDS), at a specific ϕ value of 0.2. Panel B: the influence of ϕ (0.2–0.6) on the $d_{4,3}$ (in water), at a specific c value of 2.0 or 6.0%. Each data was the mean and standard deviation of triplicate measurements. Different characters (a–d) represent the significant difference at $p < 0.05$ level due to the difference in ϕ (at the same c value).

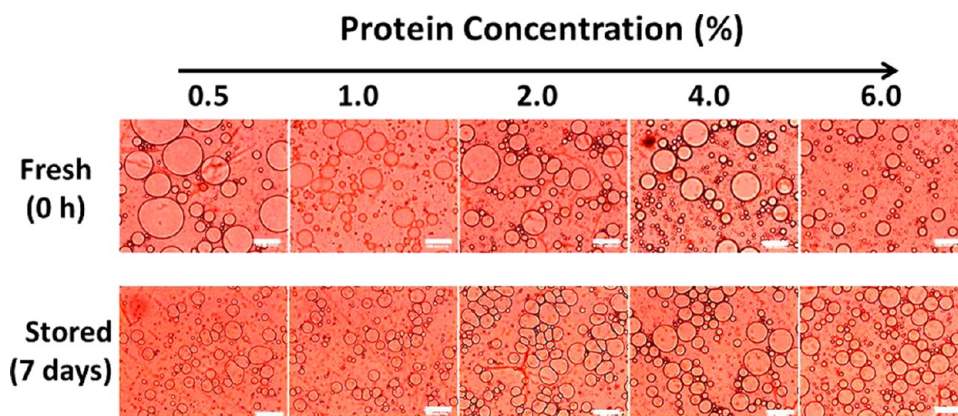


Figure 7. Microstructure of the emulsions stabilized by the SPI nanoparticle aggregates at varying c values of 0.5–6.0%. The applied ϕ was 0.2. Top row: fresh emulsions. Bottom row: emulsions stored after 7 days. The scale bars within the figures are 100 μm in length.

The observations cannot be explained only in terms of decreasing droplet size with respect to increasing the c (Figures 6A and 7), since only the creaming rate of droplets in the system can be affected by the droplet size, while the potential for the creaming instability is not. One of the most reasonable explanations is that, at higher particle concentrations, a higher amount of nanoparticle aggregates per interfacial area is expected to be adsorbed at the interface of oil droplets, and as a consequence, a gel-like network among flocculated oil droplets would be formed, thus leading to inhibition of

creaming to a certain extent. The increase in amount of adsorbed proteins at the interface with increasing the c has been confirmed in our preliminary experiments, where it was shown that, upon increasing the c from 0.5 to 6% (w/v), the amount of adsorbed proteins progressively increased from about 0.08 to 0.62 g/100 mL emulsion, almost in a linear way (data not shown). Formation of a network in the continuous phase of Pickering emulsions, which traps droplets and impedes the drainage of liquid films between them, has been previously confirmed for those stabilized by chitin nanocrystal particles,⁹

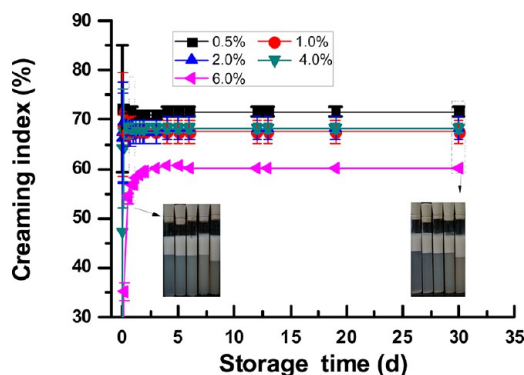


Figure 8. Evolution of percentage of creaming index (CI %) for the emulsions stabilized by the SPI nanoparticle aggregates at a comparable c value of 0.5–6.0%.

and even by silica particle flocs.¹ Reger and others³¹ argued that the formation of gel-like emulsions stabilized by protein–clay complexes was a result of interaction, interpenetration and entanglement of sticky oil droplets (coated with these complexes) with one another.

The coalescence stability of the emulsions upon storage was also evaluated by changes in droplet size ($d_{4,3}$; in 1% SDS), as displayed in Figure 9. We can see that no coalescence occurred

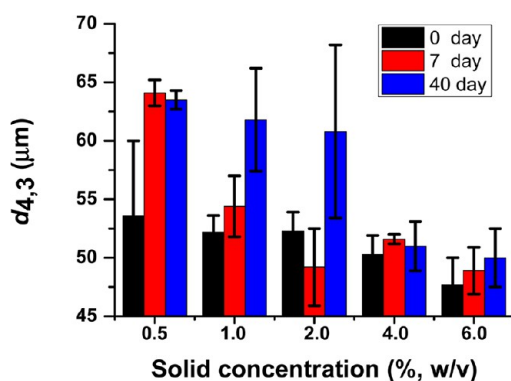


Figure 9. Changes of $d_{4,3}$ of SPI nanoparticle aggregate-stabilized emulsions at varying c values of 0.5–6.0% (w/v), after storage of 0, 7, and 40 days. The size data are determined using 1% SDS as the dispersing solvent. Each data is the mean and standard deviation of triplicate measurements.

for the emulsions formed at $c = 4\%$ or above, even after storage of 40 days, and the emulsions at $c = 1\%$ or above were stable against coalescence upon storage up to 7 days. In the case at $c = 0.5\%$, a significant increase in $d_{4,3}$ (in 1% SDS) was observed when the emulsion was stored up to 7 days, but a further storage up to 40 days did not significantly affect the droplet size any more (Figure 9). The c dependence of the emulsion stability against coalescence was similarly observed for the emulsions stabilized by MCC and MS,⁶ where the emulsions by MCC and MS were stable against coalescence upon storage up to 40 days, at particle concentrations of 1% (or above) and 1.5% (or above), respectively. They attributed the enhancement of coalescence stability to the formation of a thicker interfacial layer around droplets at higher particle concentrations. The high stability against coalescence seems to be one of the unique characteristics of Pickering emulsions. For example, Tzoumaki et al.⁹ confirmed that the chitin nanocrystal particles were quite

effective in stabilizing o/w emulsion coalescence, even at particle concentrations as low as 0.01% (w/v).

Influence of Oil Fraction (ϕ) on Droplet Size. Besides the particle concentration (or c), the properties of the Pickering emulsions at a specific particle concentration in the aqueous phase are closely related to the applied ϕ . We investigated the influence of increasing ϕ on droplet size ($d_{4,3}$; in water) of the emulsions stabilized by SPI nanoparticle aggregates at two specific c values of 2.0 and 6.0% (w/v), and the results are presented in Figure 6B. Interestingly, the droplet size of the emulsions at these two c values changed in a contrasting way with increasing the ϕ from 0.2 to 0.6. For the emulsions at $c = 2.0\%$, the $d_{4,3}$ progressively increased from about 48 to 58 μm with the ϕ increasing from 0.2 to 0.6, whereas in the case at $c = 6.0\%$, the $d_{4,3}$ on the contrary significantly decreased from about 45 to 35 μm (Figure 6B). The changes in droplet size with the ϕ are basically consistent with the differences in emulsion microstructure observed by the optical microscopy (Figure 10). At $c = 2.0\%$, we can observe that the extent of flocculation and/or coalescence of the droplets in the emulsions increased with the increase in ϕ (Figure 10, top row). The observation reflects the fact that, at a relatively low and constant particle concentration, the amount of the particles becomes not enough to fully stabilize oil droplets, as the ϕ increases, and as a result, larger size of droplets will be formed. At this c , it can be still observed that, upon increasing the ϕ , a gel-like network consisting of inhomogeneous droplets and/or flocs became more distinct (Figure 10, top row).

However, the situation at a much higher c value (e.g., 6%) seems to be different. In this case, we can interestingly observe that increasing the ϕ expectedly resulted in a gradual increase in flocculation extent of droplets, but the droplet coalescence on the contrary decreased (as evidenced by decreasing droplet size; Figure 10, bottom row). Especially for the emulsion at $\phi = 0.6$, all the droplets seem to be associated with one another to form a gel-like network; a thick interfacial layer of the SPI aggregates was present at the droplets interface (Figure 11). The influence of increasing the ϕ on the droplet size and emulsion microstructure at $c = 6.0\%$ has been similarly observed for Pickering emulsions stabilized by starch-based nanospheres.¹²

The strengthening of a gel-like network upon increasing the ϕ has also been reported for the emulsions stabilized by heated SPI at the same c value in the aqueous phase, produced by microfluidization as the emulsification technique.²⁹ In this previous work, we largely attributed the strengthening of the gel-like network to enhancement of interdroplet hydrophobic interactions between adsorbed proteins. This explanation seems to be also applicable in the present work, since at higher ϕ values and at a c value above the interfacial saturation concentration, more proteins (or aggregates) were adsorbed to the interface of the oil droplets, and the amount of adsorbed proteins per volume of emulsion increased (data not shown). This is basically in accordance with the viewpoint of Arditty and others⁴⁵ that the formation of a gel-like network for solid-stabilized emulsions is mainly determined by the interfacial elasticity resulting from the strong adhesion (or strong lateral interactions) between the solid particles adsorbed at the oil-in-water interface. Arditty et al.⁴⁵ and Reger and Hoffmann⁴⁶ also observed that the stiffness of gel-like particle-stabilized emulsions progressively increased with the increase in ϕ .

Influence of Oil Fraction (ϕ) on Creaming Stability. The creaming stability of the emulsions stabilized by the SPI

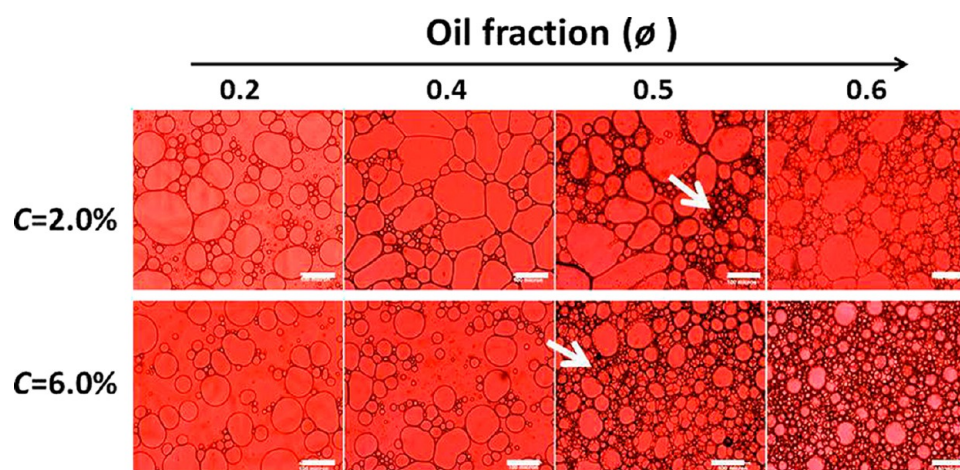


Figure 10. Typical optical micrographs of the emulsions stabilized by SPI nanoparticle aggregates at two specific c values of 2.0% (top) and 6.0% (bottom), and at varying ϕ values (0.2–0.6). The scale bars within the figures is 100 μm in length. The arrows within the figure show flocculated oil droplets with smaller sizes.

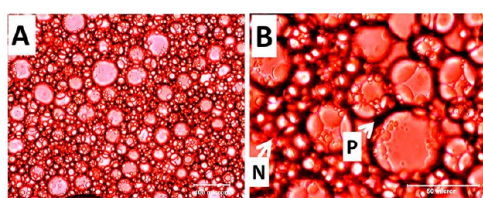


Figure 11. Microstructure at different magnifications (10–40 \times) of the emulsion stabilized by SPI nanoparticle aggregates at $c = 6.0\%$ and $\phi = 0.6$. The scale bars represent 100 and 50 μm in length, for the images with magnifications of 10 \times (A) and 40 \times (B), respectively. The symbol “N” indicates the network composed of flocculated oil droplets, while “P” shows the formation of thick interfacial protein films.

nanoparticle aggregates at $c = 2.0$ or 6.0% , upon storage up to 2 weeks, as affected by varying the ϕ , was evaluated. Figure 11 shows typical visual images of the emulsions after a storage period of 1 day. We can see that, within this storage period, the creaming stability of the emulsions at both c values progressively increased upon the increase in ϕ from 0.2 to 0.6 (Figure 12). When the storage was further prolonged up to 2 weeks, the macroscopic phase separation for all the emulsions was basically unchanged (data not shown), indicating extreme

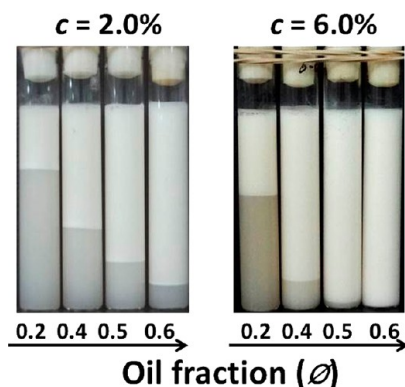


Figure 12. Visual images of the emulsions stabilized by the SPI nanoparticle aggregates at two specific c values of 2.0 and 6.0% and varying ϕ values of 0.2–0.6, after a storage period of 1 day at room temperature.

stability against creaming at prolonged storage periods (e.g., above 1 day). The observations well agree with the above observations (Figure 10), suggesting that the improvement of creaming stability was largely due to the formation of a gel-like network for the emulsions. The importance of the gel-like network formation to the creaming stability has also been suggested for the Pickering emulsions stabilized by chitin nanocrystal particles.⁹

At any applied ϕ , the CI % at any specific storage period (e.g., one day) was significantly lower at $c = 6.0\%$ than at $c = 2.0\%$ (data not shown), further supporting the above argument that increasing the particle concentration was favorable for the stabilization of the emulsions against creaming. In the present work, we can interestingly observe that, at ϕ values of 0.4 or above, the emulsions formed at $c = 6.0\%$ exhibited excellent stability against creaming, with no distinct creaming having occurred even after a storage period of 2 weeks (data not shown). Thus, we can reasonably conclude that the SPI nanoparticle aggregates are excellent Pickering-type stabilizers to formulate emulsions with extreme stability against coalescence and creaming, if appropriate particle concentrations and/or oil fraction are chosen.

In summary, we reported a novel Pickering-like stabilization of SPI nanoparticle aggregates, formed by means of combined treatments of heating and electrostatic screening. The emulsions stabilized by the nanoparticle aggregates exhibited some physicochemical and microstructural characteristics similar to those stabilized by many previously reported Pickering stabilizers. Increasing the particle concentration resulted in formation of the emulsions with smaller droplet size and higher stability against coalescence and creaming. At a higher particle concentration (e.g., 6%), increasing the ϕ was much more favorable for the formation of gel-like emulsions with much smaller droplet size and an extraordinary creaming stability. To the authors' knowledge, this seems to be the first report to show that soy proteins could be developed into a kind of effective Pickering stabilizer, through heat-induced particle formation at appropriate conditions. The findings would be of great importance for the development of soy protein-based emulsion formulations with excellent characteristics, e.g., extraordinary coalescence and/or creaming stability reported for the classic Pickering emulsions.

AUTHOR INFORMATION

Corresponding Author

*C.-H. Tang. Fax (086)20-87114263. E-mail: chtang@scut.edu.cn.

Funding

This work is supported by the National Natural Science Foundation of China (serial number: 31171632), and Program for New Century Excellent Talents in University (NCET-10-0398).

Notes

The authors declare no competing financial interest.

REFERENCES

- (1) Dickinson, E. Food emulsions and foams: Stabilization by particles. *Curr. Opin. Colloid Interface Sci.* **2010**, *15*, 40–49.
- (2) Dickinson, E. Use of nanoparticles and microparticles in the formation and stabilization of food emulsions. *Trends Food Sci. Technol.* **2012**, *24*, 4–12.
- (3) Dickinson, E. Stabilising emulsion-based colloidal structures with mixed food ingredients. *J. Sci. Food Agric.* **2013**, *93*, 710–721.
- (4) Frelichowska, J.; Bolzinger, M.-A.; Pelletier, J.; Valour, J.-P.; Chevalier, Y. Topical delivery of lipophilic drugs from o/w Pickering emulsions. *Int. J. Pharm.* **2009**, *371*, 56–63.
- (5) Kargar, M.; Spyropoulos, F.; Norton, I. T. Microstructural design to reduce lipid oxidation in oil-in-water emulsions. *Procedia Food Sci.* **2011**, *1*, 104–108.
- (6) Karger, M.; Fayazmanesh, K.; Alavi, M.; Spyropoulos, F.; Norton, I. T. Investigation into the potential ability of Pickering emulsions (food-grade particles) to enhance the oxidative stability of oil-in-water emulsions. *J. Colloid Interface Sci.* **2012**, *366*, 209–215.
- (7) Simovic, S.; Prestidge, C. A. Nanoparticle layers controlling drug release from emulsions. *Eur. J. Pharm. Biopharm.* **2007**, *67*, 39–47.
- (8) Tzoumaki, M. V.; Moschakis, T.; Scholten, E.; Biliaderis, C. G. *In vitro* lipid digestion of chitin nanocrystal stabilized o/w emulsions. *Food Funct.* **2013**, *4*, 121–129.
- (9) Tzoumaki, M. V.; Moschakis, T.; Kiosseoglou, V.; Biliaderis, C. G. Oil-in-water emulsions stabilized by chitin nanocrystal particles. *Food Hydrocolloids* **2011**, *25*, 1521–1529.
- (10) de Folter, J. W.; van Ruijven, M. W. M.; Velikov, K. P. Oil-in-water Pickering emulsions stabilized by colloidal particles from water-insoluble protein zein. *Soft Matter* **2012**, *8*, 6807–6815.
- (11) Rayner, M.; Timgren, A.; Sjöo, M.; Dejmek, P. Quinoa starch granules: a candidate for stabilising food-grade Pickering emulsions. *J. Sci. Food Agric.* **2010**, *92*, 1841–1847.
- (12) Tan, Y.; Xu, K.; Liu, C.; Li, Y.; Lu, C.; Wang, P. Fabrication of starch-based nanospheres to stabilize pickering emulsions. *Carbohydr. Polym.* **2012**, *88*, 1358–1363.
- (13) Luo, Z.; Murray, B. S.; Ross, A.-L.; Povey, M. J. W.; Morgan, M. R. A.; Day, A. J. Effects of pH on the ability of flavonoids to act as Pickering emulsion stabilizers. *Colloids Surf., B* **2012**, *92*, 84–90.
- (14) Luo, Z.; Murray, B. S.; Yusoff, A.; Morgan, M. R. A.; Povey, M. J. W.; Day, A. J. Particle-stabilizing effects of flavonoids at the oil-water interface. *J. Agric. Food Chem.* **2011**, *59*, 2636–2645.
- (15) Gupta, R.; Rousseau, D. Surface-active solid lipid nanoparticles as Pickering stabilizers for oil-in-water emulsions. *Food Funct.* **2012**, *3*, 302–311.
- (16) Paunov, V. N.; Cayre, O. J.; Noble, P. F.; Stoyanov, S. D.; Velikov, K. P.; Golding, M. Emulsions stabilized by food colloid particles: role of particle adsorption and wettability at the liquid interface. *J. Colloid Interface Sci.* **2007**, *312*, 381–389.
- (17) Tang, C. H.; Shen, L. Role of conformational flexibility in the emulsifying properties of bovine serum albumin. *J. Agric. Food Chem.* **2013**, *61*, 3097–3110.
- (18) Frelichowska, J.; Bolzinger, M.-A.; Chevalier, Y. Effects of solid particle content on properties of o/w Pickering emulsions. *J. Colloid Interface Sci.* **2010**, *351*, 348–356.
- (19) Murray, B. S.; Durga, K.; Yusoff, A.; Stoyanov, S. D. Stabilization of foams and emulsions by mixtures of surface active food-grade particles and proteins. *Food Hydrocolloids* **2011**, *25*, 627–638.
- (20) German, B.; Damodaran, S.; Kinsella, J. E. Thermal dissociation and association behavior of soy proteins. *J. Agric. Food Chem.* **1982**, *30*, 807–811.
- (21) Petrucci, S.; Añón, M. C. Thermal aggregation of soy protein isolates. *J. Agric. Food Chem.* **1995**, *43*, 3035–3041.
- (22) Renkema, J. M. S.; Knabben, J. H. M.; van Vliet, T. Gel formation by β -conglycinin and glycinin and their mixtures. *Food Hydrocolloids* **2002**, *15*, 407–414.
- (23) Mitidieri, F. E.; Wagner, J. R. Coalescence of o/w emulsions stabilized by whey and isolate soybean proteins. Influence of thermal denaturation, salt addition and competitive interfacial adsorption. *Food Res. Int.* **2002**, *35*, 547–557.
- (24) Palazolo, G. G.; Mitidieri, F. E.; Wagner, J. R. Relationship between interfacial behavior of native and denatured soybean isolates and microstructure and coalescence of oil in water emulsions – Effect of salt and protein concentration. *Food Sci. Technol. Int.* **2003**, *9*, 409–411.
- (25) Palazolo, G. G.; Sorgentini, D. A.; Wagner, J. R. Coalescence and flocculation in o/w emulsions of native and denatured whey soy proteins in comparison with soy protein isolates. *Food Hydrocolloids* **2005**, *19*, 595–604.
- (26) Puppo, M. C.; Sorgentini, D. A.; Añón, M. C. Rheological properties of emulsions containing modified soy protein isolates. *J. Am. Oil Chem. Soc.* **2003**, *80*, 605–611.
- (27) Roesch, R. R.; Corredig, M. Characterization of oil-in-water emulsions prepared with commercial soy protein concentrate. *J. Food Sci.* **2002**, *67*, 2837–2842.
- (28) Roesch, R. R.; Corredig, M. Texture and microstructure of emulsions prepared with soy protein concentrate by high-pressure homogenization. *LWT—Food Sci. Technol.* **2003**, *36*, 113–124.
- (29) Tang, C. H.; Liu, F. Cold, gel-like soy protein emulsions by microfluidization: Emulsion characteristics, rheological and microstructural properties, and gelling mechanism. *Food Hydrocolloids* **2013**, *30*, 61–72.
- (30) Lou, L. J.; Liu, L.; Tang, C. H. The role of glycinin in the formation of gel-like soy protein-stabilized emulsions. *Food Hydrocolloids* **2013**, *32*, 97–105.
- (31) Reger, M.; Sekine, T.; Hoffmann, H. Pickering emulsions stabilized by amphiphile covered clays. *Colloids Surf., A* **2012**, *413*, 25–32.
- (32) Köhler, K.; Santana, A. S.; Braisch, B.; Preis, R.; Schuchmann, H. P. High pressure emulsification with nano-particles as stabilizing agents. *Chem. Eng. Sci.* **2010**, *65*, 2957–2964.
- (33) Haskard, C. A.; Li-Chan, E. C. Y. Hydrophobicity of bovine serum albumin and ovalbumin determined using uncharged (PRODAN) and anionic (ANS[−]) fluorescent probes. *J. Agric. Food Chem.* **1998**, *46*, 2671–2677.
- (34) Li, X.; Li, Y.; Hua, Y.; Qiu, A.; Yang, C.; Cui, S. Effect of concentration, ionic strength and freeze-drying on the heat-induced aggregation of soy proteins. *Food Chem.* **2007**, *104*, 1410–1417.
- (35) Sun, X. S.; Wang, D.; Zhang, L.; Mo, X.; Zhu, L.; Bolye, D. Morphology and phase separation of hydrophobic clusters of soy globular protein polymers. *Macromol. Biosci.* **2008**, *8*, 295–303.
- (36) Keerati-U-Rai, M.; Corredig, M. Effect of dynamic high pressure homogenization on the aggregation state of soy protein. *J. Agric. Food Chem.* **2009**, *57*, 3556–3562.
- (37) Wang, J. M.; Xia, N.; Yang, X. Q.; Yin, S. W.; Qi, J. R.; He, X. T.; Yuan, D. B.; Wang, L. J. Adsorption and dilatational rheology of heat-treated soy protein at the oil-water interface: Relationship to structural properties. *J. Agric. Food Chem.* **2012**, *60*, 3302–3310.
- (38) Fang, Y.; Zhang, B.; Wei, Y.; Li, S. Effects of specific mechanical energy on soy protein aggregation during extrusion process studied by size exclusion chromatography coupled with multi-angle laser light scattering. *J. Food Eng.* **2013**, *115*, 220–225.
- (39) Santiago, L. G.; Maldonado-Valderrama, J.; Martín-Molina, A.; Haro-Pérez, C.; García-Martínez, J.; Malhotra, A.; Coupland, J. N. The

effect of surfactants on the solubility, zeta potential, and viscosity of soy protein isolates. *Food Hydrocolloids* **2004**, *18*, 101–108.

(40) Boulet, M.; Britten, M.; Lamarche, F. Aggregation of some food proteins in aqueous dispersions: effects of concentration, pH and ionic strength. *Food Hydrocolloids* **2000**, *14*, 135–144.

(41) Ren, C.; Tang, L.; Zhang, M.; Guo, S. Structural characterization of heat-induced protein particles in soy milk. *J. Agric. Food Chem.* **2009**, *57*, 1921–1926.

(42) Li, X.; Cheng, Y.; Yi, C.; Hua, Y.; Yang, C.; Cui, S. Effect of ionic strength on the heat-induced soy protein aggregation and the phase separation of soy protein aggregate/dextran mixtures. *Food Hydrocolloids* **2009**, *23*, 1015–1023.

(43) Matulis, D.; Lovrien, R. 1-Anilino-8-naphthalene sulfonate anion-protein binding depends primarily on ion pair formation. *Biophys. J.* **1998**, *74*, 422–429.

(44) Pouzot, M.; Nicolai, T.; Visschers, R. W.; Weijers, M. X-ray and light scattering study of the structure of large protein aggregates at neutral pH. *Food Hydrocolloids* **2005**, *19*, 231–238.

(45) Arditty, S.; Schmitt, V.; Giermanska-Kahn, J.; Leal-Calderon, F. Materials based on solid-stabilized emulsions. *J. Colloid Interface Sci.* **2004**, *275*, 659–664.

(46) Reger, M.; Hoffmann, H. Hydrophobin coated boehmite nanoparticle stabilizing oil in water emulsions. *J. Colloid Interface Sci.* **2012**, *368*, 378–386.