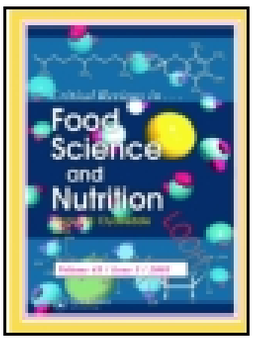

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Emulsifying Properties of Soy Proteins: A Critical Review with Emphasis on the Role of Conformational Flexibility

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Running title: Emulsifying properties of soy proteins

ABSTRACT: Soy proteins as important food ingredients exhibit a great potential to be widely applied in food formulations, due to their good nutrition, functional properties and health effects. The knowledge about the structure-function relationships of these proteins is crucial for their applications, but still very scanting, especially that on their molecular mechanism of emulsification. The purpose of this review is to present a comprehensive summary of the knowledge about emulsifying and interfacial properties of soy proteins, achieved during the past decades, and particularly, to put an insight in understanding the role of conformational flexibility in their emulsifying properties. The interplays between the emulsifying and interfacial properties are also elucidated. For these proteins, the conformational flexibility rather than the surface hydrophobicity is the crucial parameter determining their emulsification performance. On the other hand, evidence is fast growing to indicate that because of the insoluble nature, soy proteins are a kind of unique materials to perform as food-grade Pickering stabilizers. The knowledge about the Pickering emulsion stabilization is distinctly different from that for conventional emulsions stabilized by soy proteins. Thus, different strategies should be taken to develop soy proteins into a kind of effective emulsifiers, depending on the preference of emulsification performance or emulsion stability.

Keywords: Soy protein isolate (SPI); glycinin; *β*-conglycinin; structure-function relationship; Pickering stabilizers; interfacial adsorption

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INTRODUCTION

Soy proteins are a kind of important food proteins commercially available in the market, and have been widely recognized to show good nutritional value, functional properties and even health effects, including serum-cholesterol lowering effects (Friedman & Brandon, 2001; Kinsella, 1979; Nishinari et al., 2014). Soy protein isolate (SPI) is the most important soy protein product, produced by a conventional process of alkali extraction-acid precipitation. Due to their amphipathic nature (hydrophilic and hydrophobic), these proteins exhibit a good ability to diffuse and/or adsorb to and stabilize interface of oil droplets during emulsification, thus acting as effective emulsifiers to form and stabilize oil-in-water emulsions. However, although the emulsification of soy proteins has been recognized in the literature for several decades, their application as emulsifiers in practical formulations is still very limited. One of the major reasons causing this is the poor solubility and/or dispersion of proteins in many commercial soy protein products. Good solubility of proteins is usually needed for their optimal performance, for example, as emulsifiers to stabilize emulsions, though there are no direct relationships between their solubility and emulsifying properties (Damodaran, 1996). Another major limitation is the highly variable conformational characteristics of soy proteins, which is even complicated by the fact that soy proteins are a complex mixture of different proteins with different structural and physicochemical properties. The properties and structural characteristics of soy proteins are highly dependent on the protein and non-protein composition of the products, processing history (especially thermal treatment and drying), and even a number of environmental parameters, including ionic strength, pH and temperature.

During the past decades, great effort has been made in understanding the relationships between the emulsifying properties and structural characteristics of soy proteins, but, on the whole, the knowledge is unsatisfactory. The knowledge about their structure-function relationships is still basically within the general consensus that, for example, the emulsification performance or ability of proteins is closely related to their surface hydrophobicity (Kato &

Nakai, 1980). Although Damodaran (1996) had suggested that the conformational flexibility (ability to undergo conformational rearrangement) at the interface might play a more crucial role in the emulsifying properties of proteins, than the surface hydrophobicity, the knowledge in this aspect is very scanting, even in the case of well-recognized monomeric globular proteins, e.g. bovine serum albumin (BSA). With the accumulating knowledge about the conformational characteristics of globular proteins, in the past decade, this situation seems to be changing. Furthermore, the knowledge about the interfacial properties of globular proteins at quiescent interfaces is increasingly accumulating in the colloid and interface field, which may also provide an insight in understanding the relationships between the emulsifying and structural properties of soy proteins.

On the other hand, incorporation of soy proteins in many food formulations has becomes a remarkably increasing need, from the health food industry and the food science of academic research. Many novel technologies, e.g. nanotechnology, emerged in the other fields provide a very promising opportunity to develop novel foods with some unique characteristics, e.g. as controlled-release delivery systems for nutraceuticals (Neethirajan & Tayas, 2011; Rashidi & Khosravi-Darani, 2011; Weiss et al., 2006). More interestingly, the insoluble nature of soy protein on the contrary imparts it a good possibility to readily formulate protein particles in nano sizes, which may act as promising Pickering stabilizers to formulate emulsions, or as nanocarriers to deliver lipid-soluble nutraceuticals.

This review aims to present a comprehensive understanding in emulsifying properties of soy proteins, with emphasis to understanding the importance of structural characteristics. To achieve this objective, the structural characteristics, including the conformations at quaternary, tertiary and secondary levels, of different kinds of soy proteins, are first summarized in the first section. In the next section, the interfacial and emulsifying properties of soy proteins, including emulsifying performance, emulsion stability, as well as interfacial properties (dynamics of adsorption and interfacial rheology at the interface) are systemically reviewed. Especially the

relationships between the emulsifying and interfacial properties of these proteins are also discussed. The third section mainly concentrates on the recent findings of ours about the potential of soy proteins to be formulated as a kind of effective Pickering stabilizers, in which the interfacial and emulsifying properties of soy protein nanoparticles were characterized in particular. The last section is to provide an insight in the understanding the role of conformational flexibility at different levels of conformations in the emulsifying properties of soy proteins.

SOY PROTEINS: COMPOSITION AND STRUCTURAL CHARACTERISTICS

In soybean seeds, albumins and globulins are the main storage proteins, with the latter accounting for about 50-90% of total seed proteins. The major protein fractions in soy proteins are listed in **Table 1**, which are subdivided into 15S, 11S, 7S and 2S fractions according to their sedimentation coefficients (Utsumi et al., 1997). The 11S and 7S globulins, also named as glycinin and β-conglycinin, are the two major globulins in soy proteins. The ratio of soy 11S to 7S globulins is about 0.5-1.7 depending on the type of cultivars. For example, Cai & Chang (1999) examined 13 soybean genotypes and found that the variety had a great influence on the 7S (17.2-23.5%) and 11S (36.3-51.3%) contents, as well as the 11S:7S ratio. The genotypic variation in protein composition has been confirmed to affect the emulsifying properties of soy proteins (Pesic et al., 2005), in which it was suggested that the 11S:7S ratio strongly reflects the emulsifying ability of soy proteins, whereas the ratio of 11S:7S form for glycinin might be crucial for the stability of soy protein emulsions.

The 11S globulin of soybean, glycinin, is a hexamer with a molecular mass of 300-380 kDa, which has five types of subunits. Each subunit is composed of an acidic polypeptide (A_x, x=1a, 2, 1b, 4 and 5) with a molecular mass of ~35 kDa and a basic polypeptide (B_y, y=1a, 1b, 2 and 3) with a molecular mass of ~20 kDa, which are linked together by a disulfide bond (SS). These subunits are divided into two groups (I and II) based on their amino acid sequence. Group I consists of A1aB1b, A1bB2 and A2B1a, whereas the group II includes A3B4 and A5A4B3.

Besides the disulfide bridge between the acidic and basic polypeptides (e.g. A1aB1b), there is another conserved disulfide bridge within the acidic polypeptide (Tandang-Silvas et al., 2010). The 7S globulins of soybean include three major fractions, namely β-conglycinin, γ-conglycinin and basic 7S globulin. β-Conglycinin, the most prevalent 7S globulins of soybean, is a glycoprotein, and accounts for 30-50% of the total seed proteins. It is a trimer with a molecular mass of 150-200 kDa, which consists of three major subunits, namely α' (72 kDa), α (68 kDa) and β (52 kDa) (Utsumi et al., 1997). The α'- and α-subunits consist of the extension region (α', 141 residues; α, 125 residues) and the core region (418 residues), while the β-subunit has only the core region (416 residues). The core regions share high sequence identities among them (75-90%), and the extension regions of α'- and α-subunits exhibit about 57% similarity in sequence (Maruyama et al., 1998). The β-conglycinin is usually lacking in cysteine residues, and thus devoid of disulfide bridges.

The composition of soy proteins has been well recognized in the two book chapters by Utsumi et al. (1997) and Kilara & Harwalkar (1996), and more recently, a review by Tandang-Silvas et al. (2010). The following mainly covers the present knowledge about the structural characteristics of glycinin and β-conglycinin, as well as soy protein isolate (SPI).

Quaternary structure of glycinin and β-conglycinin

Native glycinin and β-conglycinin are a combination of different isoforms with a high heterogeneity. Each native isoform possesses a quaternary structure. For glycinin, electrostatic and hydrophobic bondings are involved in stabilization and maintenance of its quaternary structure consisting of six AB subunits (**Figure 1**), while for β-conglycinin (trimer), the quaternary structure is mainly associated by hydrophobic interactions, electrostatic and hydrogen bondings (Peng et al., 1984). These non-covalent interactions are not only readily affected by the changes in environmental conditions such as pH and ionic strength (μ), but also very susceptible to processing and storage stresses, including freezing, drying and heating. As a result, the quaternary structure state of these oligomeric globulins considerably varies with the processing

history of defatted soy flour, isolation and preparation processes. This is the main underlying reason causing the difficulty in fully understanding the molecular mechanisms of functional properties of these globulins.

In fact, the quaternary state of any oligomeric globulin is a result of balance between inter- and intro-molecular interactions, attractive or repulsive. The balance is deliberately modulated by pH and μ in the medium. As expected, the quaternary dissociation and/or re-association occur as the pH and μ changes. It has been well recognized that at pH 7.6 and at μ = 0.5 glycinin is mainly present in a hexameric form (11S form), and decreasing ionic strength to 0.01 at the same pH causes its dissociation of the 11S-form into the 7S-form (Lakemond et al., 2000). This transformation is clearly due to enhanced intra-molecular electrostatic repulsion (especially at the subunit level). The quaternary dissociation from the 11S to 7S or 3S form also occurs when the pH is decreased from 7.6 to a value below the isoelectric point (pH 4.5), e.g., 3.8-2.0 at which all the proteins are positively charged (Lakemond et al., 2000). The dissociation would inevitably result in the exposure of basic polypeptides, initially buried within the interior of the compact 11S-form molecules, to the aqueous solvent. The 'exposed' basic polypeptides may thus undergo some kind of structural reorganization, e.g. structural changes in secondary and tertiary levels, but to what extent these changes occur is still uncertain (Lakemond et al., 2000). In a more recent work, Ruiz-Henestrosa et al. (2012) applied the dynamic light scattering technique to evaluate the hydrodynamic diameter of different assembled forms of glycinin at different pH or ionic strengths, and confirmed the existence of an equilibrium between different self-assembled forms of glycinin as follows:

$3S(AB) \leftrightarrow 7S((AB)_3) \leftrightarrow 11S((AB)_6) \leftrightarrow 15S((AB)_{12}) \leftrightarrow >15S$ (aggregates)

In contrast with glycinin, the quaternary structure of β-conglycinin seems to be much less complex. At pH 7.0 and at μ = 0.5, it possesses a 7S form with a cyclic structure. As the μ is decreased to 0.1, it is mainly present in the 9S form that is composed of two identical cyclic ensembles facing each other (Thanh & Shibasaki, 1978 a). The 7S-form structure can be

dissociated by increasing concentrations (like 6 M) of urea into isolated subunits; the isolated subunits can be reassociated to form a 7S-form structure upon removal of urea by dialysis (Thanh & Shibasaki, 1978 b). The dissociation-association reversibility of β-conglycinin is largely associated with the high reserved structure of its subunits, especially at the tertiary conformation level. The presence of N-glycans in the subunits, with two N-glycans within the core regions of α'- and α-subunits and one in the core region of β-subunit (Maruyama et al., 2002 a), may help maintain the high reserved subunit structure, and further facilitate the correct folding and assembly of the trimeric form of β-conglycinin (Maruyama et al., 1998). Not only the number of N-glycans but also the N-linked position within the subunits produces an influence on the structural and physicochemical properties of 7S globulins. Kimura et al. (2008) indicated that the 7S globulin (phaseolin) from French bean possessing two N-glycans within each subunit (with two carbohydrate moieties in close positions), exhibits higher solubility than soybean 7S globulin also with the two N-glycans that are located in separate positions (**Figure 2**).

In usual, glycinin (11S) or β-conglycinin (7S) with different subunit or polypeptide constituents exhibits a similar quaternary structure. Besides the environmental conditions, e.g. pH and μ, the stability of quaternary structure of these assembled globulins is also highly variable depending on the heterogeneity and amino acid composition of their subunit or polypeptide constituents. In this regard, the elucidation of the influence of subunit composition on the quaternary structure stability of glycinin seems to be impossible, due to the difficulty in obtaining the glycinin with a homogenous subunit constituent. By employing the *Escherichia coli* expression system, the Utsumi group had succeeded in cloning cDNAs encoding individual subunit precursors of glycinin, though in the form of proglycinin, and compared the structural properties of these recombinant proglycinins (Park et al., 2005). It was found that the order of emulsifying ability of these proglycinin was not of the same for the surface hydrophobicity (Park et al., 2005), implying that the differences in emulsifying ability might be largely due to the differences in structural stability (especially at the quaternary level). In contrast, the differences

in quaternary structure stability of 7S globulins that is mainly maintained by the inter-subunit hydrophobic interactions are much readily elucidated. Using differential scanning calorimetry (DSC), Kimura et al. (2008) compared the denaturaion temperatures (T_d) of various 7S globulins from cowpea, French bean, fava bean, pea and soybean at two μ values of 0.5 and 0.08, and observed that the extent of the decrease in T_d values upon decreasing μ from 0.5 to 0.08 was higher in the case of cowpea, pea and soybean, than in the others. The observations indirectly indicated that the inter-subunit hydrophobic interactions play a more important role in the maintenance of the quaternary structure of 7S globulins from cowpea, pea and soybean than those of French bean and favabean. In a more recent work, Tang & Sun (2011) compared the amino acid composition and structural characteristics of three 7S or 8S globulins from French bean, red bean and mungbean, and confirmed that the 7S globulin (phaseolin) from French bean exhibited highest flexibility at the quaternary level among all the test globulins. The higher quaternary flexibility of phaseolin is closely dependent on its higher relative acidic/basic charged amino acid ratio, and percentage of polar uncharged amino acid (relative to other vicilins; Tang & Sun, 2011).

It is generally considered that the quaternary structure of glycinin (11S) is more rigid and compact than that of β-conglycinin (7S), which is consistent the facts that 1) the quaternary structure of the parallel hexagonal rings for the 11S globulin (**Figure 1**) would facilitate the maximal packing of globulins which would in turn maximize its hydrophilicity (Macone, 1999); 2) the glycinin is more susceptible to many physicochemical stresses, e.g., urea (Clara Sze et al., 2007), high pressure treatment (Molina et al., 2001); 3) the glycinin subunits is basically devoid of the presence of N-linked glycans that would favor the intra-subunit attractive interactions (Tandan-Silvas et al., 2010). However, it still needs to be verified that the much poorer emulsifying ability or surface properties of soy glycinin is largely ascribed to the lower flexibility of its quaternary structure than soy β-conglycinin.

One point should be to note that for glycinin, the equilibrium between different isoforms as

affected by pH and μ may be irreversible, due to the sulfhydryl group(SH)/SS exchange (especially at low μ and at acidic pH). To date, most of the works regarding purified soy glycinin and β-conglycinin are based on the methods of Thanh & Shibasaki (1976) and Nagano et al. (1992). In both the processes of isolation, a reducing agent, sodium bisulfate (0.098%, w/v) or 2-mercaptoethanol (2-ME; 0.01 M), was applied, clearly in order to inhibit the occurrence of intermolecular SS formation (of glycinin) during the extraction. In most of the cases, the reducing agent together with the salts (e.g. NaCl applied in the Nagano et al.'s process) was removed by dialysis to obtain the freeze-dried glycinin products. To my surprise, no information has been available elucidating whether this treatment (combined applications of dialysis and lyophilization) would produce an influence on the structural properties (especially the quaternary structure and aggregated state) of glycinin. In fact, Wolf (1993) had pointed out that the presence of free acidic and basic polypeptides in glycinin prepared with 0.01 M of 2-ME was not surprising. Once the 0.01 M 2-ME is removed by the dialysis, the SH/SS interchange occurs in the last obtained glycinin, which would be more favored by the decrease in μ (up to 0). Thus, the lyophilized glycinin prepared with both the processes is not really 'native glycinin', and instead, it may be a polymerized or aggregated glycinin, partly due to the intermolecular SS formation. One clever strategy to avoid the occurrence of this situation is to keep the extracted glycinin in solution state in the presence of 0.4 M NaCl and 0.01 M 2-ME, as applied by Lakemond et al. (2000).

To testify the above hypothesis, the lyophilized glycinin prepared with the process of Nagano et al. (1992) was resolubilized in water at pH 7.0 and at a protein concentration (c) of 1.0% (w/v). The morphology and particle size of the proteins in the glycinin solution was evaluated using atomic force microscopy (AFM) and dynamic light scattering (DLS), as displayed in **Figures 3** and **4** (Liu & Tang, 2014 b). AFM observations indicated that most of proteins in the glycinin solution were present in the form of nanoparticles, exhibiting an elliptic morphology with countour sizes of (125-150)×(40-60) nm and the height ranging from 2.5 to 5.0 nm (**Figure 3 A,**

A´). DLS data showed that these nanoparticles had a *z*-average diameter (D_z) of about 70 nm (**Figure 4 A**), which is comparable to the radius of gyration (R_g; 26.9-39.7 nm) values observed for the dialyzed and lyophilized glycinin (resolubilized in a pH 7.4 buffer), using size exclusion chromatography coupled with multiangle laser light scattering (SEC-MALLS) (Keerati-U-Rai & Corredig, 2009 a). Using the same process of Nagano et al. (1992) but without dialysis prior to the lyophilization, Ruiz-Henestrosa et al. (2012) found that most of the lyophilized and resolubilized glycinin was present in the native state, with the D_z comparable to the dimension of 11S globulin reported in the literature [(10.4-12.6) nm×(10.4-12.6) nm×(7.5-9) nm; Utsumi et al., 1997]. The comparison of the D_z between these works confirmed that the dialysis treatment prior to lyophilization might lead to severe aggregation, possibly associated with the progressive decrease in μ. The glycinin nanoparticles, obtained by dialysis and lyophilization, could be disrupted by the presence of 6 M urea, or 0.5% SDS, but seemed to be resistant to the presence of 30 mM dithiothreitol (DTT) alone (**Figure 4 A**), indicating the hydrophobic interactions mainly involved in the aggregation. On the contrary, the D_z of SG nanoparticles in the presence of DTT was significantly higher than that of the control (without addition of any perturbing-agent; **Figure 4 A**), possibly indicating loosing (but without disruption) of the global structure of the nanoparticles. The observations suggest that both the hydrophobic interactions and disulfide bonds are involved in the maintenance of the global structure of the nanoparticles.

Furthermore, it should be pointed out that the balance between the inter- or intra-molecular interactions of proteins in the solution is highly dependent on the *c*. The variation in *c* may thus greatly change the quaternary structure of proteins, depending on the surface nature of the proteins. Recently, Liang & Tang (2013) interestingly observed that the D_z of a typical 7S globulin (phaseolin, from red kidney bean) at pH 7.0 increased from 8.1 to 8.4~8.6 nm, as the *c* increased from 0.1% (w/v) to a value within 0.25-1.0% (w/v). The observation indicated that within the test *c* range, increasing the *c* led to enhanced intermolecular attractions, and further formation of less compacted quaternary structure.

Tertiary structure of glycinin and β-conglycinin

For a monomeric globular protein, e.g., BSA, its structural stability is mainly conferred from hydrogen bonding, hydrophobic interactions and even disulfide bonds. If the magnitude of hydrogen bonds between the protein and aqueous phase, or that of intra-molecular hydrophobic interactions and disulfide bonds is high, the conformational flexibility of this protein is considered to be low. The conformational stability or flexibility of a monomeric globular protein can thus be evaluated using DSC (mainly to detect the disruption of hydrogen bondings maintaining the structure of the protein, during heating), or spectroscopic techniques (to detect the changes in polarity of microenvironment of hydrophobic amino acids, e.g. Trp or Tyr). For 7S or 11S oligomeric globulins, the conformational stability/flexibility of their subunits or polypeptides (at the tertiary level) is difficult to evaluate, due to the complexity of composition and structure of their subunit constituents, and the influence of inter-subunit interactions. For these oligomeric globulins, the unfolding and denaturation of their subunits requires disruption of additional inter-subunit interactions, especially those maintaining their quaternary structure (Sanchez-Ruiz, 1992). Thus, upon heating of DSC determination, the denaturation of a typical 7S globulin usually consists of three steps: 1) the trimeric 7S globulin is reversibly dissociated into subunits (at quaternary level); 2) the dissociated subunits are further irreversibly unfolded and denatured (at tertiary level); 3) the completely denatured subunits are irreversibly aggregated (**Figure 5**). The steps 1 and 2 are endothermic (ΔH_1 and ΔH_2), which address the conformational changes at quaternary and tertiary levels, while the last step is exothermic.

Due to the difficulty to exclude the contribution of the reversible thermal event (step 1) from the total event, most of the previous works directly applied the peak temperature (T_d) of the endothermic event as an indicator for thermal stability of 7S globulins (Damodaran, 1988; Meng & Ma, 2001; Tang et al., 2006). An effective strategy proposed to precisely evaluate the denaturation of subunits or polypeptides in oligomeric proteins is that the DSC determination should be carried out in the presence of appropriate concentrations of urea. Urea can disrupt the

inter-subunit interactions (e.g., hydrophobic and hydrogen bonds) of oligomeric proteins, without affecting their intra-subunit interactions. Banerjee & Kishore (2004) reported a DSC study on the irreversible thermal unfolding of concanavalin A (dimer), in the presence 2 M urea, though in the work they did not mention why this concentration of urea was applied. More recently, Liang & Tang (2013) found that the application of urea with a concentration up to 6 M can result in complete disruption of 7S-form phaseolin into isolated subunits, without distinctly affecting the tertiary structure. In this work, it was interestingly found that the T_d and ΔH of the phaseolin was progressively deceased from 92.5 to 83.2 ºC, and from 16.60 to 6.4 J/g, respectively, with the urea concentration increasing up to 6 M; if the urea (at 6 M) was removed by dialysis, the dissociated subunits were re-associated into the 7S-form globulin. Therefore, it is reasonably hypothesized that the quaternary conformation plays a much more important role in stabilizing the global structure of oligomeric globulins than their tertiary conformation (subunits).

Since the early 1990s, the Professor Utsumi's group has pioneered the structural characterization of legumin storage proteins in general and *β*-conglycinin in particular by means of protein engineering technique, with the aim to develop crops with modified genes for production of storage protins with enhanced functionalities (Tandang-Silvas et al., 2011). Maruyama et al. (1998) compared the DSC scans of the recombinant normal and mutant subunits (*α*, *α´* and *β*; in fact, they would be self-assembled into homotrimers), and native *β*-conglycinin at $\mu = 0.5$ and at pH 7.6, and observed that the *β*-subunit (or *β* homotrimer) exhibited the highest T_d (90.8 ºC), followed by the *α´* (82.7 ºC) and *α* (78.6 ºC) subunits, while native *β*-conglycinin gave two endothermic peaks at 79 and 83.1 ºC (which were close to those of the *α* and *α´* subunits). From these observations, they concluded that the thermal stability of heterotrimers is conferred by the subunit having the lowest denaturation temperature among the constituent subunits (e.g., *α* for α_3, $\alpha_2\alpha'$, $\alpha_2\beta$, $\alpha\alpha'2$, *α*, $\alpha\alpha'\beta$ and $\alpha\beta_2$; *α´* for $\alpha'3$, $\alpha'_2\beta$, $\alpha'\beta_2$; Maruyama et al., 1998). If the μ was changed to 0.08, or the pH was decreased to 3.8, the T_d of all the individual subunits (or homotrimers) was decreased (Maruyama et al., 1999), further indicating the importance of

inter-subunit hydrophobic interactions for the overall structure stability. The importance of inter-subunit hydrophobic interactions was further confirmed by Maruyama et al. (2001) by means of accessible surface area estimation on soy *β*-conglycinin *β* homotrimer.

Besides the hydrophobic interaction, hydrogen bonds and one salt bridge are also involved in the trimerization (Maruyama et al., 2001). Furthermore, it was indicated that the carbohydrate moieties of *α* and *α´* subunits do not influence the thermal stability of their homotrimers (Maruyama et al., 2002), further confirming that the thermal stability of these subunits relies on their core region, and is independent of the acidic extensive regions. Despite all these exciting findings, it still remains uncertain whether the structural stability or flexibility of different subunits (*α*, *α´* and *β*) in a dissociated state (e.g. in the presence of urea) follows the order of thermal stability of their homotrimers, e.g. *β* >*α´*> *α*. To unravel the structural basis causing the differences in thermal stability of various homotrimers (*α*, *α´* and *β*), the Utsumi's group interestingly suggests that the differences in total cavity volume might be one of the main structural features, based on the analyses of their crystal structures (Tandang-Silvas et al., 2011). In general, the thermal stability of these oligomeric globulins is inversely related to their total cavity volume. In fact, this explanation is consistent with our above hypothesis that the magnitude of inter-subunit interactions (especially hydrophobic interactions) dominates the overall structure of these oligomeric globulins during the heating, over those maintaining the structure at the tertiary level, since the stronger inter-subunit hydrophobic interactions, like in the *β* homotrimer, would be expected to result in formation of more compact trimeric structure than in *α´* or *α* homotrimers.

In contrast with soy 7S globulins (*β*-conglycinin), the situation of soy 11S globulins (glycinin) is more complex, since in this case, it may also involve the irreversible and reversible 11S-to-7S dissociations. Soy glycinin contains 21 disulfide bonds, of which 15 are intra-subunit and 6 are inter-subunit bonds, while *β*-conglycinin is devoid of intra- and inter-subunit disulfide bond linkages (Damodaran, 1988). The extensive intra- and inter- subunit disulfide bonds in the

glycinin may provide much greater stability against thermal denaturation, and as a consequence, the T_d and ΔH are considerably higher for glycinin than those for β-conglycinin at same test conditions (Damodaran, 1988; Kimura et al., 2008). For example, Kimura et al. (2008) reported that at pH 7.6 the glycinin and β-conglycinin exhibited the T_d of 93.5 and 78.5 ºC at μ = 0.5, and of 81.2 and 65.7 ºC at μ = 0.08, respectively. In this work, it was interestingly observed that the extent of ΔH increase for the glycinin, as the μ was increased from 0.08 to 0.5, was much greater than that for the β-conglycinin, implying that the hydrophobic interaction plays a more important role in the maintenance of the quaternary structure of glycinin. Maruyama et al. (2004) compared the thermal stability of four mutant glycinins composed of only group I (A1aB1b, A1bB2 and A2B1a) or group II (A3B4 and A5A54B3) with those of the normal glycinin (11S) composed of five kinds of subunits, and found that the T_d was similar to each other except that of A3B4-glycinin (A3B4-glycinin was least thermally stable among all test samples). However, in another work investigating the structure-function relationships of soybean proglycinins (trimers), they observed a contrasting phenomenon, that is, the A3B4-proglycinin was the most stable proglycinin trimer among all test five proglycinins (Prak et al., 2005). This inconsistency confirms the above argument that the overall structure stability is affected by a number of variables, e.g., the intra-subunit hydrophobic interaction, the cavity size and the number of hydrogen bonds (Tandang-Silvas et al., 2010).

In a more recent work, Clara Sze et al. (2007) investigated the susceptibility of the structure of soybean glycinin and β-conglycinin, obtained by Nagano et al.'s process (with dialysis), to many protein perturbants including urea and GuHCl. It was observed that, 1) increasing urea concentration up to 4 M progressively increased the tryptophan accessibility of glycinin to the fluorescence quenchers (acrylamide or iodide), while the tryptophan accessibility of β-conglycinin was not affected by the presence of urea; 2) GuHCl was more effective to result in exposure of typtophan resides in glycinin and in β-conglycinin. The observations confirmed that a higher extent of hydrophobic interactions was involved in the intra-subunit interactions of

glycinin than of β-conglycinin. In other words, this observation together with the observations of Kimura et al. (2008) indicates that the tertiary conformational flexibility of glycinin is greater than that of β-conglycinin, though the quaternary conformational flexibility is considerably lower.

Secondary structure of soy proteins

The globulins from different plant sources, including soybean, buckwheat seeds, rice and pea, share a similar and highly conserved feature of secondary structure, that is, they typically possess low levels of α-helix and very high β-sheet secondary structure (Choi & Ma, 2005; Ellepola et al., 2005; Marcone, 1999). Thus, these globulins are often considered to be a kind of β-type proteins. In contrast with the globulins, the albumins (another kind of storage proteins) are rich in α-helix but lower in β-sheet. For example, the albumins from buckwheat seeds contain α-helix and β-sheet of 15.4% and 31%, respectively, while for the globulins, it is 6.4% and 36%, respectively (Tang & Wang, 2010). The secondary structure composition of SPI is basically similar to that of 7S or 11S globulins, with a large quantity of extended strands and low α-helix content (Tang & Ma, 2009).

Structural characteristics of soy protein isolate (SPI)

Soy protein isolate (SPI) is the most commercially available soy protein product, which usually contains 85-90% protein (dry basis). The major proteins in SPI are glycinin and β-conglycinin accounting for about 70% of total protein. In general, the production of SPI consists of an aqueous extraction of protein at alkaline pH, selective recovery of the solubilized protein, separation, washing and neutralization, and last, drying (Moure et al., 2006). The most frequently used extraction solvent is water with sodium hydroxide to adjust the pH, and the most applied recovery of the protein is acid precipitation at around its isoelectric point (~pH 4.5). Commercial SPI is usually produced using a spray-drying technique, while for the preparation in the lab or at plant pilot scale, SPI is obtained by freeze-drying. Prior to the freeze-drying, a dialysis to remove the salts in the protein dispersion is often applied. All these processes with a

variety of processing variables influence the yield, structure and even functionalities of the last obtained protein products. Of course, the properties of the SPI are also affected by the soy meal or flour material from which the protein is produced.

In the practical case for the commercial production of SPI, it is impossible to precisely and evenly adjust the pH in the system to the required values, e.g. pH 8.0 (alkali solublization) and 4.5 (acid precipitation). A portion of proteins in commercial SPI might suffer a severe damage of structure by alkali or acids, added during the alkali solubilization and/or acid precipitation process. The pH-related modifications in structure for soy proteins, especially glycinin and β-conglycinin, mainly occur at the quaternary and/or tertinary levels (Jiang et al., 2009; Lakemond et al., 2000; Petruccelli & Añón, 1996; Wagner & Guéguen, 1995; Wagner et al., 1996). The glycinin is much more sensitive to pH changes than the β-conglycinin, for example, the T_d of glycinin decreased by 10ºC when the pH increased from 6.0 to 11.0, while that of β-conglycinin did not change (Petruccelli & Añón, 1996). Wagner et al. (1996) indicated that decreasing the pH of acid treatment from 3.5 to 1.0 led to a progressive increase in extent of denaturation, and subsequently, loss of solubility of the glycinin, while the β-conglycinin was much less affected. The conformational changes caused by the extreme pH usually result in a progressive subunit dissociation, exposure of hydrophobic clusters initially buried within the interior of the proteins to the aqueous solvent, and aggregation of unfolded and/or denatured proteins (Jiang et al., 2009; Petruccelli & Añón, 1996; Wagner et al., 1996). Furthermore, the production of commercial SPI involves a mild thermal treatment before drying and the spray-drying process itself that also leads to denaturation of the proteins. Thus, most of proteins in commercial SPI are denatured and present in the aggregated form, and even insoluble (Lee et al., 2003). This is one of the major reasons causing low solubility of commercial SPI. From the solubility profiles as a function of pH and NaCl concentrations, Lee et al. (2003) divided various test commercial SPI products into three groups: the 1st group had high solubility near the *p*I (around 4.5); the 2nd group had low solubility near the *p*I, but high solubility at pH 11; the last

group had low solubility even at pH 11. Among these three groups of commercial SPI products, the 2nd group seems to be the most prevailing one. The 2nd group showed decreased solubility with increasing NaCl concentration (Lee et al., 2003), indicating the prominence of salting-out effects.

Besides the solubility, the surface hydrophobicity (H_o) of proteins is another important physicochemical parameter that can to a large extent reflect the tendency of protein molecules to aggregate. Wagner et al. (2000) systematically investigated the influence of preparation conditions (including extraction, centrifugation conditions, pH of redissolution, and initial concentration for freeze-drying), and treatments inducing protein denaturation (e.g., thermal treatment, addition of reducing agents or urea, dialysis or base or acid treatment prior to freeze-drying, and initial concentration for freeze-drying) on the solubility and H_o of laboratory SPI products at pH 7.0, and evaluated the solubility of these SPI products as a function of the H_o of their proteins. They interestingly identified three well-defined groups of laboratory isolates: (A) native with high solubility and relatively low H_o, (B) partially or totally denatured with high solubility and H_o, and (C) totally denatured with low solubility and H_o (**Figure 6**). The two principal modifications of these isolates are represented by (I) denaturation and (II) aggregation-insolubilization, which is highly related to the applied concentrations or the presence of calcium. If the protein concentration is less than 6%, the denaturation (induced by the addition of reducing agents or urea, base or acid treatment prior to freeze-drying, as well as heating; process I) increases the H_o of these isolates, without distinctly decreasing their solubility of the isolates. In contrast, if the protein concentration is > 8% and/or the salts are present, the insolubilization process of the isolates (process II) would be favored. For commercial SPI products, the relationship between the solubility and H_o could not be included in any of the three groups of laboratory counterparts; instead, they could be approximately divided into two groups: partially native (A´) and totally denatured (C´) (**Figure 6**). Solubility values in these two groups were similar to those of the group C of laboratory isolates, but the H_o levels were much lower

(Wagner et al., 2000). The decreased solubility for the group A´ (relative to the group A) might be largely attributed to the application of a mild thermal treatment before drying or by the spray-drying process itself (process III), during the production of commercial SPI; compared to the group C, the shift of the group C´ to zones of lower surface hydrophobicity could be as a result of the usual addition of phospholipids (process IV) (Wagner et al., 2000).

Due to the fact that the denatured and/or aggregated state of proteins in SPI, obtained in laboratory or commercial available, is highly variable, depending not only on the nature of proteins in the starting materials, but also on a lot of processing conditions, the present knowledge about the structural characteristics of SPI, in some cases, is inconsistent and even contradictory. For example, the solubility of totally denatured proteins in SPI (e.g., the groups C, A´ or C´) progressively increases with their H_o (**Figure 6**), which is distinctly contrasting from the consensus that the proteins with higher surface hydrophobicity tend to more readily aggregate, due to enhanced intermolecular hydrophobic interactions. This seemly abnormal inconsistency can be well explained if the differences in aggregation extent or size of the proteins between these isolates are taken into account. In fact, the insoluble precipitates or macro-aggregates in the isolates are mainly a result of enhanced hydrophobic interactions between 'small aggregates'. Besides the hydrophobic interaction, sulphydryl oxidation or SS/SH exchange may be also involved in the formation of protein aggregates (Visschers & de Jongh, 2005), which has been confirmed in extremely low or high pH-denatured SPI (Jiang et al., 2009). The situation for native or partially denatured proteins in SPI seems to be distinctly different. In this situation, a negative relationship between the solubility and H_o can be basically observed (**Figure 6**; groups A and B), confirming higher tendency to aggregate for the proteins with higher H_o.

To date, the aggregated state or particle size of proteins in SPI products, especially those with low solubility, is little understood, though that of the major globulins (glycinin and β-conglycinin) in native state has been well recognized. A number of previous works investigating the

emulsifying properties of laboratory SPI are generally performed assuming that all of the proteins in SPI are in native state. However, the actual situation seems to be different. In many cases of freeze-dried isolates, especially those treated by a dialysis before drying, the proteins are partially denatured and even present in the aggregated form. For example, using size exclusion chromatography in combination with multiangle light laser scattering (SEC-MALLS) technique, Keerati-U-Rai & Corredig (2009) indicated that in the dialyzed-lyophilized-resolubilized SPI solution (at pH 7.4; c = 0.2 wt%), there were three major SEC elution peaks with average molecular mass and R_g of 3.3×10^6 Da (aggregate peak), 875 and 456 kDa, and of 38.5, 29.3 and 27.3 nm, respectively. With the same technique, Tang and Ma (2009) observed a similar SEC elution profile of laboratory SPI (treated by dialysis), with a soluble aggregate peak of mean molecular mass and R_g of 7.63×10^6 Da and 27.4 nm, respectively. In another work with dynamic light scattering (DLS) technique, Liu & Tang (2013) showed that the freeze-dried SPI at pH 7.0 exhibited a monodisperse particle size distribution with sizes ranging from 8 to 100 nm, with the *z*-average diameter of about 58 nm. These reported molecular masses (MW) or sizes in the laboratory SPI solutions are considerably higher or greater than those for native glycinin [hexamer; with MW and dimension of 300-380 kDa and (12.6×12.6×7.5 nm), respectively] and β-conglycinin [trimer; with MW and dimension of 180-200 kDa and (12.5×12.5×3.75 nm), respectively; Utsumi et al., 1997], thus confirming the prominence of protein aggregates.

The size of insoluble aggregates, or precipitates in commercial SPI cannot be estimated, except that they can be kinetically dispersed in a medium. Jong (2013) applied a homogenization process (at 10,000 rpm for 10 min) to disperse a commercially spray-dried SPI in distilled water (at c = 7.0 wt%), and indicated that most of the proteins had particle sizes ranging from 2.5 to 30 μm, with a number average size of around 6.5 μm. If a high energy level of homogenization (microfluidization) was further applied, the size of SPI aggregates in commercially SPI could be considerably decreased; after 26 passes of microfluidization treatment, the hydrodynamic radius (R_h) of the aggregates was reduced to 69-132 nm depending on the c and pH (Jong, 2013). The

observations indicated that the precipitates in commercially SPI could be transformed by a high energy level of homogenization into a kind of soluble aggregate nanoparticles. A similar transformation of insoluble proteins to soluble aggregates in commercial SPI has also been reported in Tang et al. (2009), by means of combined homogenization and ultrasonic treatments, whereby it was shown that both non-covalent and covalent interactions, e.g. hydrophobic interactions, hydrogen bonds and disulfide bonds, were involved in the formation of soluble aggregates.

Structural changes upon adsorption at the interface

Very little information on the structural changes of soy proteins (especially 7S or 11S globulins) upon adsorption at interfaces is available in the scientific literature. Actually, it is not much known about how the structural changes occur even for well-recognized monomeric globular proteins, e.g., BSA or α-lactalbumin. Catelain & Genot (1994) indicated that, when BSA is adsorbed at the dodecane-water interface, it undergoes distinct conformational changes (at the tertiary level) that involve the shift of aromatic amino acids (especially of tryptophanyl resides) to a more hydrophobic environment. In another work, Zhai et al. (2012) confirmed that besides the considerable conformational changes at the tertiary level, native α-lactalbumin also suffered a dramatic change of secondary structure when adsorbed at the oil-water interface, and specifically, the α-helix content increased from about 30% in solution to 46-59% at the interface, thus providing insight into the understanding of conformational changes of globular proteins at the interface.

By comparison, the secondary structure in many oligomeric proteins, including soy glycinin and β-conglycinin, is highly ordered and preserved, due to the strong restrictions from the presence of their quaternary structure (Subirade et al., 1994). For these oligomeric proteins in native state, the destruction of the quaternary structure seems to be a prerequisite for the conformational changes at the tertiary and even secondary levels, when adsorbed at the interfaces. The structural restrictions become more outstanding, when these proteins are present in the

aggregated state, e.g. for SPI. Recently Herrero et al. (2011) have provided some evidences to indicate that adsorbed at the oil-water interface, SPI might also suffer a significant change in secondary structure, with increases in α-helix and unordered coil contents at the expense of β-sheet. This seems to be consistent with the observations of X-ray scattering and direct imaging on the morphology of SPI at the interfaces (Fayad et al., 2011), wherein, for example, it was observed that the mean radius of gyration for SPI increased from 20 nm (unadsorbed) to 30 nm (adsorbed at the interface). However, in another recent work using fluorescence spectroscopy and microcalorimetry, Keerati-u-rai et al. (2012) indicated that for the globulins heated at 75 or 95ºC, or SPI, minor structural changes were observed upon adsorption at the oil-water interface. In contrast, the native glycinin or β-conglycinin suffered a significant change in tertiary conformation upon adsorption at the interface, as evidenced by red shifts of tryptophan fluorescence (Keerati-u-rai et al., 2012).

INTERFACIAL AND EMULSIFYING PROPERTIES OF SOY PROTEINS

The ability of proteins to aid formation and stabilization of oil-in-water emulsions is of vital relevance for many food applications, e.g. in chopped and comminuted meats, cake batters, coffee whiteners, milks, mayonnaise, salad dressings and frozen desserts (Kinsella, 1979). In general, proteins stabilize oil droplets in an emulsion system, mainly through two main aspects: lowering the interfacial tension between oil and water; retarding droplet coalescence during emulsification, by forming an interfacial film (thus generating repulsive interactions, e.g. steric and electrostatic) (Damodaran, 2005; McClements, 2004). This indicates that, the emulsification activity of proteins is highly dependent on their effectiveness to decrease the interfacial tension, and the ease with which they can diffuse to, penetrate, unfold and rearrange at the interface. For a protein as a good emulsifier, it should meet three basic requirements: i) fast adsorption to interface; ii) high ability of its structure to unfold at the interface; iii) formation of a cohesive and viscoelastic interfacial film (Damodaran, 2005).

Although soy proteins have been recognized to exhibit good surface activities, e.g. high

surface hydrophobicity, it still remains a controversy whether they are a kind of effective emulsifiers or surfactants. Before addressing this issue, we should first make sure what targeted emulsification effects we want to achieve, when soy proteins are used to stabilize and formulate the emulsions. If the efficiency of emulsification is favored, like for small surfactants, such as lecithins, these proteins cannot be considered to be a kind of good emulsifiers, due to their complex, compact and even aggregated structure. However, if the stability of the emulsions stabilized by soy proteins, e.g. against coalescence and/or creaming, is a preferable aspect of emulsification to be considered, they would be an excellent kind of emulsifiers or emulsion stabilizers. Interestingly, soy proteins exhibit a high tendency to form a kind of gel-like emulsions with extraordinary stability against creaming (and coalescence), which is for the first time observed for the emulsions stabilized by soy globulins (especially those heated at 95ºC; Aoki, Taneyama & Inami, 1980), and further confirmed for the emulsions by soy protein concentrate (SPC; Roesch & Corredig, 2002, 2003), and more recently, by heated SPI (Tang & Liu, 2013). The formation of gel-like emulsions has been ascribed to a novel Pickering stabilization of nanoparticles in heated SPI case (Liu & Tang, 2013, 2014). The findings would impart these proteins a promising potential to be applied in a number of soy protein-based emulsion formulations with a gel-like microstructure, especially as delivery systems for heat-labile lipid soluble bioactives.

In the literature, there are basically three categories of works about the emulsifying properties of soy proteins. The first category is to investigate the potential of individual protein components (e.g. purified glycinin and β-conglycinin) in soy proteins to form the emulsions. The second is carried out on a few important soy protein products, such as SPI (laboratory or commercial), with the aim to further utilize these proteins in the applications that require emulsification functions of proteins. The last but not less importantly, is to investigate the techniques to improve the emulsifying properties of soy proteins. Unfortunately, to date, the knowledge about the emulsifying and interfacial properties of soy proteins, obtained by different workers or in

different labs, is not always consistent, and in some cases, even contradictory. This is consistent with the following facts: i) soy proteins is a mixture of various protein constituents with complex structures and diverse physicochemical properties; ii) the structure and properties of soy proteins (and globulins in particular) are highly susceptible to processing parameters with which they are obtained, and dependent on environmental conditions (e.g., pH, μ and temperature); iii) in most cases, soy proteins are partially or totally denatured, and thus present in the aggregate form.

In this section, we mainly summarized the previous works about the emulsifying and interfacial properties of different soy proteins, as well as their relationships (As for the novel Pickering stabilization of soy proteins, see Section 5).

Emulsifying properties

Emulsifying performance

The emulsifying performance is an important aspect for emulsifying properties of proteins, which is usually evaluated by two relatively simple empirical indices: emulsifying capacities (EC) and emulsifying activity index (EAI). EC of a protein is usually defined as the maximum amount of oil that can be dispersed in an aqueous solution containing a specific amount of the protein without breaking down or reverting the emulsion (McClements, 2009). The greater the volume of oil incorporated into the emulsion, the higher the EC of the protein. Instead, EAI is a more widely applied parameter evaluating the emulsifying ability or efficiency of proteins. In general, EAI is determined from measurements of the turbidity of a dilute emulsion, which is defined as the total interfacial area of droplets in emulsions stabilized by a specific amount of proteins (Cameron et al., 1991; Pearce & Kinsella, 1978). However, both the EC and EAI are empirical and qualitative indices reflecting the emulsifying properties of proteins, which are highly dependent on the type of applied homogenizers, homogenization conditions, the applied concentration (for EC) and/or oil fraction (for EAI), and even the type of applied oil (Gu et al., 2009; McClements, 2009). For example, Gu et al. (2009) observed that at pH 6.9 the EAI of SPI decreased with increasing the oil concentrations from 5% to 20% for sunflower and soy oil

emulsions, while it contrarily increased for palm sterin emulsion; at 10 or 20% concentrations, the EAI for palm oil emulsion was considerably higher than that for the other two emulsions.

In more recent times, droplet size analysis is a more preferable and accurate technique to evaluate the emulsifying performance of proteins, than the EAI. Using this technique, one point needs to be cautious that all the droplets in a dilute emulsion should be separate from one another (in order to reach this, the droplet size measurements are usually performed in the presence of SDS). The smaller the droplet size of an emulsion stabilized by a protein, the greater the emulsifying ability of the protein is.

Previous works have reported the EC of different soy protein preparations, including SPI, SPC and their globulin fractions (7S and 11S) (Aoki et al., 1980; Elizalde et al., 1996; Kinsella, 1979). During the initial stage of emulsification, soy proteins can be fast adsorbed and packed at the interface of oil droplets and as a consequence, thick interfacial films of multilayers are formed. As the amount of incorporated oil increases, the interfacial area progressively increases with the progress of emulsification, and accordingly, the amount of unadsorbed proteins on the contrary decreases. Once there are not enough proteins in the aqueous phase to cover the newly created interfacial area, the droplet size of the emulsion increases, and the thickness of initially formed interfacial films also diminishes. At comparable conditions, SPI exhibits a much higher EC than SPC (Kinsella, 1979; Wang & Johnson, 2001). The difference in EC between SPI and SPC is associated with the differences in effective protein content, protein solubility, and extent of denaturation and/or aggregation. The EC of soy proteins is closely related to the pH of their solutions or dispersions. The pH dependence of the EC of laboratory SPI and soy 7S or 11S fractions is basically in accordance with their pH-solubility profiles, with EC minimal at around the isoelectric point (*p*I) and increasing at pH deviating from the *p*I (Aoki et al., 1980), reflecting the importance of protein solubility to the emulsifying properties of soy proteins. However, the situation for commercial SPI seems to be a bit different. Elizalde et al. (1996) indicated that over the test pH range of 2.0-7.0, maximal EC is observed at pH near *p*I, e.g. pH 3.0 or 5.5, among

different commercial SPI samples; the EC is linearly and negatively related to the balance between hydrophile and lipophile (WOAI), calculated as the ratio between water and oil adsorption capacities. Thus, the differences in pH-dependent behavior of EC between the laboratory and commercial SPIs just reflect that besides the solubility, surface hydrophobicity (H_o; another important physicochemical parameter) is also vital for the emulsifying properties of soy proteins.

The importance of solubility and H_o on the emulsifying ability or activity of food proteins has been elucidated (Nakai, 1983). Interestingly, a significant correlation ($p < 0.01$) has been established between the EAI and the H_o of a number of experimentally tested proteins (Nakai, 1983). The argument is mainly based on the fact that all the test food proteins exhibited a good solubility, or the solubility is not the limiting factor affecting their emulsification. For proteins of low solubility, the solubility is the more important parameter in determining their EAI (Li-Chan et al., 1984). Thus, it is generally recognized that a balance of hydrophobicity and hydrophilicity of a protein is an important factor for its emulsifying ability (Damodaran, 1997). The general knowledge about the relationships between the emulsifying ability and H_o and/or solubility of food proteins can be also applicable to the case of soy proteins, though in the latter case, the practical situation would be more difficult. The major storage proteins in soy proteins, glycinin (SG) and β-conglycinin (SC), have an oligomeric and quaternary structure that is highly susceptible to many processing and environmental conditions, and once their structure is altered, the inter- or intra-molecular interactions will change accordingly, which may lead to considerable changes in their surface and even emulsifying properties. Besides the surface properties, the conformational flexibility of these compact oligomeric globulins is also a characteristic molecular feature markedly affecting their emulsifying properties (Schwenke, 2001). In contrast with sodium caseinate (at low concentrations), the conformational flexibility of soy proteins is totally much poorer, thus causing relatively lower ability or capacity to stabilize the emulsions (Palazolo et al., 2003, 2005).

Glycinin (SG) and β-conglycinin (SC) exhibit a distinctly different behavior of emulsifying performance. In general, the emulsifying performance of the SC is much superior to that of the SG under the same test conditions, in terms of EC (except at pH 2.0; Aoki et al., 1980), and emulsion size (Keerati-U-Rai & Corredig, 2010; Kimura et al., 2008; Luo et al., 2013). For example, Keerati-U-Rai & Corredig (2010) compared the volume-average droplet size ($D_{4,3}$) of the emulsions pH= 7.4 and μ = 0.1, stabilized by SG and SC. The emulsions were prepared at ϕ = 0.1 and increasing *c* values of 1.0-3.0%, by means of microfludization as the emulsification technique. The results indicated that the $D_{4,3}$ of the fresh SG emulsions progressively decreased from 17.9 to 1.4 μm, as the *c* increased from 1.0 to 2.5%, and a further increase in *c* did not lead to a significant increase in $D_{4,3}$; in contrast, the $D_{4,3}$ (0.3-0.5 μm) of the SC emulsions slightly changed over the whole *c* range, and was considerably lower than that of the SG emulsions even at the highest *c* (Keerati-U-Rai & Corredig, 2010). This suggests that under the investigated conditions, a minimal *c* value at which the oil droplets are just fully covered by the protein is below 1% for SC, while for SG, it needs a minimal *c* value of around 2.5%. In our recent work using the same microfluidization, we observed that for the emulsions stabilized by non-treated soy globulins (SG + SC), formed at a constant *c* of 1.0 % (w/v) and ϕ = 0.1, increasing the SG content from 0 to 80% (relative to total protein) resulted in a progressive increase in $D_{4,3}$ (in 1% SDS) from 0.48 to 0.82 μm (Luo et al., 2013), confirming the better emulsification performance of the SC. The differences in emulsifying ability between these two globulins can result from the following two aspects: (a) the SC (trimeric form) has a lower molecular weight than the SG (hexameric form), and diffuses more rapidly to the interface; (b) the 7S-form SC exhibits higher flexibility, and therefore, can more readily spread and anchor at the interface.

As for the role of SC or ratio of SC to SG in the emulsifying ability of SPI, it still remains a debate. Chove et al. (2001) pointed out that the SPI samples rich in SC subunits exhibited a high functionality in terms of EAI and ESI. However, in another work investigating the influence of genotypic variation in protein composition on the emulsifying properties of soy proteins, it was

on the contrary shown that the EAI of soy proteins was strongly and negatively correlated with their concentration of SC (Pesic et al., 2005).

Both glycinin and β-conglycinin are multisubunit globulins, and for SC, the subunits are even N-glycosylated. The emulsifying properties of these proteins are highly dependent on the composition and heterogeneity, and extent of N-glycosylation of their subunits. In this aspect, the Utsumi's group has pioneered some works investigating the relationships between the emulsifying ability and structural features of SC and/or SG, by protein or gene engineering, with the aim to provide a direction for soybean breeding to select the varieties containing SC and/or SG with targeted subunit compositions (Maruyama et al., 1999, 2002 a, b, 2004; Park et al., 2005). The main findings of SC are as follows: (a) at pH 7.6 and μ =0.5, the emulsifying ability of α subunit is close to that of BSA, and much better than that of α' and β subunits; in contrast, the emulsifying ability of the deletion mutants α_c and α'_c subunits (without carbohydrate moieties) is much poorer than that of their counterpart ones (Maruyama et al., 1999); (b) the order of emulsifying ability for native homotrimers consisting of α, α' and β subunits follows the same order for their subunits (Maruyama et al., 2002 a); (c) as for SC heterotrimers, the emulsifying ability is mainly determined by the primary constituent subunits, e.g. the ability of the heterotrimers containing one β subunit is similar to that of the α or α' homotrimers, whereas that of the heterotrimers containing two β subunits is close to that of the β homotrimer (Maruyama et al., 2002 b). In contrast, the importance of subunit composition on the emulsifying ability of glycinin is much more complicated. By comparison among different mutant and native glycinins, it is found that the emulsifying ability of these glycinins is related to the length of their hypervariable regions that are rich in acidic amino acids (and have a disordered structure) (Maruyama et al., 2004). Besides this relationship, there are no significant relationships between the emulsifying ability and structural features of mutant glycinins or proglycinins at subunit levels (Maruyama et al., 2004; Park et al., 2005).

In fact, most of proteins in many soy protein preparations are present in the aggregated form.

The protein aggregation and even precipitation greatly delays the rate and effectiveness of adsorption of these proteins at the interface, thus restricting their emulsifying performance, or verse vice (Keerati-u-rai et al., 2011; Palazolo et al., 2004, 2005; Wang & Johnson, 2001). For example, Palazolo et al. (2005) observed that at c = 0.1% (w/v) and without NaCl, the average size of the emulsion stabilized by denatured SPI (obtained by heating the native SPI) is lower than that by the native SPI, indicating that in this case the thermal denaturation and/or aggregation led to a decrease in emulsifying performance. A similar influence of heating on the emulsion size has been observed for the emulsions stabilized by laboratory (unheated and freeze-dried) and pilot plant (heated and spray dried) SPI, at c = 2.0% (w/v) or less (Keerati-u-rai et al., 2011). For the proteins with larger size, more protein will be needed to fully cover the interface of oil droplets created during the emulsification. However, it should be strengthened that how the thermal denaturation or aggregation affects the emulsification performance of proteins depends on the nature of the proteins and the extent of protein unfolding and/or aggregation (Raikos, 2010).

Last, one point should be to clarify that the evaluation of the emulsifying performance is usually performed at a low c value (e.g. 0.2-1.0%, w/v). At high bulk c values, e.g. > 1% (w/v), the importance of many structural characteristics (e.g., solubility and/or H_o, and conformational flexibility) for the emulsifying performance of proteins becomes less important, due to the fast adsorption and saturation of proteins at the interface. For the emulsions (at pH 8.0) formed at c = 7% (w/v) and ϕ = 0.3, Puppo et al. (2011) observed that there was no significant difference between the $D_{4,3}$ of the emulsions stabilized by native SG and SC (1.3 μm), which is distinctly different from that observed at lower c and ϕ values (Keerati-U-Rai & Corredig, 2010). Furthermore, it is also vital to choose the mode or process of an emulsification when evaluating the emulsification performance of a protein, since the emulsification with a higher energy level input will produce an initial emulsion with a higher interface area (and a higher interfacial tension), and as a consequence, the rate of fast adsorption (during the most initial period of

adsorption) may become the limiting factor determining the emulsification performance (Damodaran, 2005).

Emulsion stability

The protein-stabilized emulsions are essentially lyophobic colloidal dispersions that are never truly in a state of thermodynamic equilibrium (Damodaran, 2005). The knowledge about the kinetic stability of emulsions is vital for the development and utilization of food formulations based on the emulsions. Generally, the term 'emulsion stability' or 'emulsion instability' is a relative concept reflecting the ability of an emulsion to resist changes in its physicochemical properties upon storage (McClements, 2007). The kinetic stability of a protein-stabilized emulsion is mainly determined by density difference between the oil and aqueous phases, inter-droplet colloidal interactions, and the structure and properties of interfacial protein films (Damodaran, 2005). The instability mechanisms of emulsions include gravitational separation (creaming/sedimentation), flocculation (bridging or depletion), coalescence, Ostwald ripening and phase inversion. For protein-stabilized emulsions, the main issues of the instability are creaming, flocculation and coalescence (**Figure 7**). To date, a great progress has been achieved in the in-depth understanding about many aspects of the (in)stability of protein-stabilized emulsions. Many excellent reviews have been available addressing (a) the adsorption of proteins at the interface and the relationship of adsorbed layer structure to emulsion stability (Dalgleish, 1997 a, b; Dickinson, 1994; Lam & Nickerson, 2013), (b) molecular mechanisms involved in the formation and stability of protein-stabilized emulsions (creaming, flocculation and coalescence) (Damodaran, 2005; Dickinson, 2010 a), (c) factors affecting the stability of globular protein-stabilized emulsions (including a number of environmental stresses and solution conditions; McClements, 2004; Tcholakova et al., 2006), and (d) techniques and methodologies for characterization of emulsion stability (McClements, 2007). However, it should be to note that most of the knowledge is based on milk proteins in general and caseins or whey proteins in particular. It still remains to be confirmed whether the knowledge about the emulsion stability of

these milk proteins can be applicable to the situation of more complex and compact plant storage proteins. Actually, the properties and stability mechanisms of emulsions stabilized by plant proteins in general and soy proteins in particular are far away from being understood and in some cases, the knowledge is inconsistent and even contradictory.

On the other hand, we have to realize a fact that except a few cases, where the proteins are basically kept native, like for purified β-conglycinin and SPI obtained without dialysis (prior to lyophilization), most of the proteins in a number of soy protein preparations are present in the denatured or partially denatured, and aggregated form, though to a varying extent. This unique structural feature of soy proteins, which is determined by their intrinsic physical, chemical and structural properties, is distinctly contrasting from that of whey proteins and caseins that are primarily hydrophilic in nature (Dickinson, 2010 a). The high susceptibility of soy proteins to interact with one another or aggregate, to a large extent, determines the highly unstable nature of the emulsions stabilized by these proteins. Besides the nature of the proteins, another important variable determining the properties and stability of an emulsion is the ϕ of the dispersed oil phase. In usual, for a dilute stable emulsion ($\phi < 0.01$), the mean distance between individual droplets is much greater than the average droplet diameter; for a moderately concentrated emulsion ($\phi \sim 0.1$) these two length scales are of approximately similar magnitude; and for a highly concentrated emulsion (e.g., $\phi > 0.5$), the mean distance will be much smaller than the average droplet diameter (Dickinson, 2010 a). It has been reported that for any applied oil, increasing ϕ from 0.05 to 0.2 led to a progressive reduction in emulsion stability of SPI (Gu et al., 2009).

For the destabilization of a protein-stabilized emulsion, three different processes (creaming, flocculation and coalescence) may be concomitantly involved. There are a wide variety of different analytical techniques and methodologies to characterize the different aspects of emulsion stability (McClements, 2007). Among all these, the application of emulsion stability index (ESI), which was developed by Pearce & Kinsella (1978), is a relatively simple and widely used approach, based on the measurements of the turbidity of a dilute emulsion at a specific

wavelength. The higher the ESI for an emulsion, the greater the emulsion stability is. Another simple method to evaluate emulsion stability or instability (ES or EI) is to determine the relative changes in moisture content after storage of a specific period (e.g. 24 h) (Aoki et al., 1980; Elizalde et al., 1996). Based on the emulsions formed at c = 0.2% and ϕ = 0.35, Aoki et al. (1980) observed that both SPI and β-conglycinin (SC) show a similar ES profile with a minimal value occurring at pH close to the *p*I, and their ES at pH 7.0 or above is remarkably greater than that of the glycinin (SG). This observation suggests that the SC fraction might dominate the behavior of emulsion stability of SPI, which is basically in accordance with the findings of Chove et al. (2001). A similar pH-dependence of emulsion (in)stability to that of SPI or SC emulsions has been also observed for the emulsions stabilized by commercial SPI products, formed at c=2.0% and ϕ = 0.5 (Elizalde et al., 1996).

The much poorer emulsion stability of SG (relative to SC) cannot be explained by the differences in their solubility, though in the SC emulsion case, the solubility seemed to be vital for its emulsion stability (Aoki et al., 1980). The differences in ES between these two globulins might be largely due to the differences in mechanical or viscoelastic properties of their interfacial films. It has been recognized that besides the surface properties, the molecular flexibility of a protein is also important for its emulsion stability (and in some cases, the conformational flexibility of proteins is even more important than their surface characteristics, e.g., H_o) (Damodaran, 1997; Kato & Nakai, 1980). From this viewpoint, the differences in emulsion stability can thus be ascribed to the higher molecular flexibility of the SC than the SG (see the foregoing section). It has been shown that increasing the flexibility of proteins resulted in enhanced emulsion stability of glycinin or soy proteins (Jiang et al., 2009; Pesic et al., 2005; Wagner & Guéguen, 1995).

Flocculation. Flocculation refers to association of two or more oil droplets without changing their individual integrities, which is facilitated by a net force of inter-droplet attraction (**Figure 7**). The flocculation occurs when the attractive interactions between individual droplets (e.g. van

der Waals forces) dominate the long-range repulsive interactions (including electrostatic and steric forces), but not the short-range repulsive interactions (McClements, 2007). In the emulsions stabilized by proteins, some kinds of additional interactions, such as hydrophobic interactions, between the adsorbed interfacial films of individual oil droplets may also become important, and even dominant in the promotion of flocculation of oil droplets (Damodaran, 2005). The state and extent of droplet flocculation in a protein-stabilized emulsion is highly affected by a number of different factors, including the ϕ, the bulk c, structure and properties of the protein, and environmental conditions (e.g., temperature, pH, μ) (Dickinson, 2010 a). For soy proteins, the flocculated state of oil droplets in freshly prepared emulsions is even heavily dependent on the emulsification process or energy input (Liu & Tang, 2014 a). There are two kinds of flocculation: bridging and depletion. Both bridging and depletion flocculation can occur in protein-stabilized emulsions containing no other hydrocolloids, but what kind of flocculation dominates the global process depends on the c and/or ϕ, and the size of the proteins. In the dilute emulsions and at low c values, the bridging flocculation is more likely to occur, while at high c values at which the amount of proteins is much higher than that needed to fully cover the interface of oil droplets, depletion flocculation also occurs, due to the excluded volume effect of polymeric materials.

Although the mechanisms and affecting factors of the flocculation (in)stability of the emulsions stabilized by milk proteins have been well recognized (Damodaran, 2005; Dickionson, 2010; McClements, 2007), it is only during the recent years that the knowledge about the flocculation or flocculated state of droplets in the emulsions stabilized by soy proteins has been rapidly accumulating (Keerati-u-rai & Corredig, 2010; Keerati-u-rai et al., 2011; Liu & Tang, 2013, 2014; Luo et al., 2013; Palazolo et al., 2005, 2011; Puppo et al., 2005; Tang & Liu, 2013). The first thing that needs to be paid particular attention to is that, the flocculation behavior of soy protein-stabilized emulsions is complicated by a variety of their complex structural and physicochemical properties that are highly dependent on the protein composition, processing

history of these proteins, emulsification conditions (e.g., c and/or ϕ, the type and energy input level of emulsification), and even environmental conditions. Nevertheless, the factors affecting the flocculation (in)stability and/or flocculated state of oil droplets in these emulsions can be still investigated under specific conditions.

In many cases, most of droplets in the freshly prepared emulsions stabilized by soy proteins (and SPI in particular) are already present in the flocculated state (Palazolo et al., 2011; Puppo et al., 2005; Tang & Liu, 2013). This is remarkably different from that for the emulsions stabilized by sodium caseinate, or whey proteins, whereby almost no bridging flocculation might occur when the amount of the proteins is enough to fully cover the oil droplets in the system (Palazolo et al., 2011). The difference reflects that the intrinsically hydrophobic/hydrophilic nature of proteins to a large extent determines the global inter-droplet interactions of protein-stabilized emulsions. The flocculation degree of oil droplets in fresh soy protein emulsions is heavily dependent on the applied c and/or ϕ, and extent of protein denaturation and/or aggregation. Puppo et al. (2005) observed that the fresh emulsion stabilized by untreated SPI (at pH 8.0), formed at c =1% (w/v) and ϕ = 0.3, exhibited a polymodal droplet size distribution with the major peak centered at about 10 μm, in the absence of SDS, while in the presence of 1% SDS, the droplet size distribution became almost monomodal (with the peak at around 1.0 μm), indicating severe bridging flocculation. Increasing c and/or decreasing the ϕ can inhibit the occurrence of bridging flocculation in the emulsions stabilized by unheated or native SPI (Keerati-u-rai et al., 2011; Palazolo et al., 2011; Tang & Liu, 2013). For example, all the emulsions stabilized by unheated SPI, formed at c =1.5-2.0% (w/v) and ϕ =0.1 (Keerati-u-rai et al., 2011), or at c = 1.0% (w/v) and ϕ = 0.2 (e.g., at 0 mM NaCl; Tang & Liu, 2013; **Figure 8**), exhibited a monomodal droplet size distribution profile with the peak centered at around 0.6-1.0 μm, in the absence of SDS. The inhibition of bridging flocculation can be largely due to the fact that at higher c and/or lower ϕ values, more proteins are available to fully cover the interface of oil droplets, newly created during the emulsification, and the long-range electrostatic repulsion

dominates inter-droplet interactions between the droplets.

Generally, thermal treatments of soy proteins, especially at temperatures above the denaturation temperatures of the major components (e.g., glycinin), can remarkably affect the balance of their surface hydrophobicity/hydrophilicity, and even their aggregated state (or particle sizes), subsequently leading to the changes in emulsion stability, e.g. flocculated state of droplets in the emulsions. The changes in flocculation degree (FD%; promotion or inhibition) induced by the thermal denaturation seem to be dependent on the applied c and/or ϕ, and even the emulsification process. Palazolo et al. (2011) reported that at $\phi = 0.25$, the heating (at 90 ºC for 5 min) of the protein solutions prior to the emulsification decreased the FD% of the SPI emulsions at c values of 0.5 or 1.0% (w/v), but slightly increased the FD% at c = 2.0% (w/v). A similar decrease in FD% of SPI emulsions at comparable c and ϕ values, by a thermal treatment at 95ºC for 30 min has been observed by Li et al. (2011). Keerati-u-rai et al. (2011) observed that at $\phi = 0.1$, the emulsions stabilized by the thermally-treated SPI (pilot plant) at c =1-1.5% (w/v) exhibited a kind of bridging flocculation, while those by native SPI (laboratory) were basically not flocculated. A similar promotion in bridging flocculation is also similarly observed for the emulsions at c = 1.0% (w/v) and ϕ =0.2 (Tang & Liu, 2013; **Figure 8 A, C**). Like in the case of the emulsions stabilized by unheated SPI, increasing the c (at a specific low ϕ, e.g., 0.2) resulted in a progressive inhibition of bridging flocculation for the emulsions stabilized by thermally denatured SPIs (Li et al., 2011; Palazolo et al., 2011; Tang & Liu, 2013).

In another recent work of ours investigating the emulsifying properties of heated SPI (Liu & Tang, 2014 a), it is interestingly found that the flocculated state of oil droplets in the fresh emulsions at $\phi = 0.2$ was not only dependent on the applied c (in the range 0.5-6.0%, w/v), but also greatly affected by the energy input level of emulsification. Different from the previous work where the heating was performed directly on the protein solution at c = 1.0% (Tang & Liu, 2013), the heat treatment at same conditions, in this work, was carried out first on the SPI solution (pH 7.0) at c = 6.0% (w/v), and the heated solution was then further diluted to the

required *c* values of 0.5-6.0% (w/v) for the next experiments. At low *c* values, e.g. 0.5 or 1.0% (w/v), the flocculation degree in the fresh emulsions progressively increased with increasing the energy input level of emulsification (**Figure 9**), which is generally consistent with the consensus that when the *c* is the limiting parameter, or the amount of the protein is not enough to fully cover the interface of oil droplets, the increased new interface area during the emulsification, as a result of enhanced emulsification strength, will be favorable for the occurrence of bridging flocculation. When the *c* was increased above 1 or 2% (w/v), the bridging flocculation was completely inhibited in the emulsions produced by low energy input levels of emulsification (stirring, or stirring + ultrasonication), where in the emulsions produced by combined stirring and microfluidization, a severe bridging flocculation still occurred (**Figure 9**).

Interestingly, a gel-like network structure with the predominantly elastic in nature, in the emulsions at $c > 0.5\%$ (w/v) by the microfluidization, has been confirmed using dynamic rheological measurements (Liu & Tang, 2014 a). The flocculation-induced gelation of emulsions stabilized by denatured soy proteins, or whey proteins has also previously been reported in the literature (Liu & Tang, 2011; Manoi & Rizvi, 2009; Roesch & Corredig, 2002, 2003; Tang & Liu, 2013). A mechanism for the gel-like network formation in the emulsions stabilized by heated SPI has been proposed, in which the hydrophobic interactions between the denatured (and aggregated) proteins adsorbed on individual oil droplets play a vital role in the gel-like network formation (Tang & Liu, 2013; **Figure 10**).

The flocculated state of droplets in protein-stabilized emulsions is particularly sensitive to pH and μ, since when the pH approaches the *p*I of the adsorbed proteins, or when the μ exceeds a critical value, the electrostatic repulsion between the droplets will be greatly lessened, and even no longer strong enough to resist the various attractive inter-droplet interactions, e.g., van der Waals, hydrophobic or depletion (Dickinson, 2010 a; McClements, 2004). **Figure 8** also shows that as the μ increased from 0 to 500 mM, the droplet size distribution profiles (in the absence of SDS) of both unheated and heated SPI emulsions (pH 7.0; formed at $c = 1\%$, w/v and $\phi = 0.2$)

gradually shifted to higher sizes, while those in the presence of SDS were basically unchanged, indicating the promotion of bridging flocculation by the electrostatic screening. The emulsions stabilized by the heated SPI were much more susceptible to the changes in μ than those by the unheated SPI (Tang & Liu, 2013), indirectly confirming the importance of hydrophobic interactions between the adsorbed proteins on individual oil droplets for the flocculation. On the other hand, increasing the μ is also favorable for the gel-like network formation in the heated SPI-stabilized emulsions, formed at high c and/or ϕ values, e.g., c = 6% (w/v) and ϕ = 0.3 (Tang & Liu, 2013; **Figure 11**).

The role of individual soy globulins (SG and SC) in the bridging flocculation and network formation of the gel-like emulsions stabilized by SPIs, particularly those thermally denatured, is distinctly different (Luo et al., 2013). To unravel the relative importance of SG and SC, we investigated the influence of the SG content (0-100%; in total soy globulins, SG + SC) on the flocculated state of droplets in the unheated and heated (at 85ºC for 15 min) soy globulin-stabilized emulsions at c = 1.0% (w/v) and ϕ = 0.1, as well as rheological behavior of these emulsions at c = 6.0% (w/v) and ϕ =0.3, as indicated in **Figure 12**. As displayed, the flocculation degree of droplets in the emulsions was closely dependent on the glycinin content in total soy globulins, and the dependence was much more distinct for the emulsions stabilized by heated soy globulins than that of unheated counterparts (**Figure 12 A**). For the unheated case, the droplet size (in water) of the emulsions distinctly deviated from that in the presence of 1% SDS, as the glycinin content was above 50%, while in the heated case, the presence of SG at a glycinin content of 25% had already resulted in a remarkable deviation. If the c and ϕ were increased to 6% (w/v) and 0.3, we can observe that the apparent viscosity (and storage modulus; data not shown) of heated soy globulin-stabilized emulsions progressively increased, as the glycinin content was above 65%, while in the unheated case, only a slight increase in apparent viscosity was observed at the glycinin content above 90% (**Figure 12 B**). The observations clearly indicated that thermally denatured SG plays a vital role in the bridging flocculation and gel-like network

formation of the heated soy protein-stabilized emulsions. The main inter-droplet interactions involved in the formation and maintenance of the gel-like network in these soy protein emulsions are mainly hydrophobic interactions (Lou et al., 2013), as also previously proposed by Floury et al. (2002).

In contrast, the long-period stability of flocculation of droplets or initially present flocs in a soy protein-stabilized emulsion is more difficult to characterize, since it is affected by a number of variables, including the size, shape and internal structure of initially present flocs. There is no close relationship between the flocculated state of oil droplets (in fresh emulsions) and the flocculation stability upon storage of a long period. In general, it can be recognized that the emulsions stabilized by denatured soy proteins, formed at low c and high ϕ values, exhibit a greater flocculation stability upon storage (e.g., 24 h) than those by native ones, irrespectively of μ (Keerati-u-rai et al., 2011; Palazolo et al., 2005). The underlying mechanism might be related to the hydration nature and structure of flocs (initially present in the emulsions), and the structural changes of adsorbed proteins at the interface. Last, it should be to note that the flocculation stability upon storage for a protein-stabilized emulsion is completely different from that under some stresses, e.g. freezing, heating or high-pressure treatment. For example, Palazolo et al. (2011) reported that a freeze-thawing treatment resulted in a severe bridging flocculation in the emulsions stabilized by denatured SPI (at c = 1-2%, w/v), but the flocculated state of oil droplets in the emulsions stabilized by native SPI (at comparable c values) was nearly unaffected.

Coalescence. Coalescence leads to an increase in size of droplets in an emulsion, as a result of droplet fusion, and may eventually result in oiling off (phase separation) (**Figure 7**). Generally, larger droplets coalescence more readily than smaller ones; and the coalescence occurs when the droplets are in proximity for elongated periods, e.g. in concentrated, flocculated or creamed emulsions (Damodaran, 2005; McClements, 2010). In a protein-stabilized emulsion, coalescence is a relatively slow process in comparison with creaming and flocculation, but it can be greatly

accelerated when shear forces are applied (Britten & Giroux, 1991). The coalescence stability of the emulsions stabilized is primarily determined by the steric repulsion between adsorbed protein layers and the viscoelastic properties of the interfacial protein films that are highly dependent on the bulk *c* and the nature of the proteins (Damodaran, 2005).

The actual thickness of interfacial films in a protein-stabilized emulsion is very difficult to determine. Using dynamic light scattering, Keerati-u-rai & Corredig (2010) evaluated the thickness of interfacial layer of different unheated soy proteins (SG, SC and SPI), by adsorbing these proteins at increasing concentrations from 0.06 to 1.4% (at pH 7.4) on latex microspheres (polystyrene), and obtained the layer thickness in the order: SPI (about 45 nm) > SG (37 nm) > SC (31 nm). No matter what aggregated state it is for these proteins, these values indicate that adsorbed at the interface, they formed a multilayer corresponding to that of several native molecules (e.g., 10-12 nm for native hexameric SG). The layer thickness for these unheated soy proteins is considerably greater than that of an entangled layer for β-casein at the *n*-hexane-water interface (7-8 nm) and of spread monolayer for BSA at the air-water interface (approximately 3 nm) (Dickinson, 1994). If the proteins in SPI are denatured by a thermal treatment and then present in the highly aggregated state, the thickness of the adsorbed interfacial layers consisting of large protein aggregates will be greatly increased (**Figure 13**). Therefore, although the structural and physicochemical properties of soy proteins considerably vary with the protein composition (glycinin, SG and β-conglycinin, SC), processing history and even environmental parameters (e.g., pH and μ), it is generally recognized that the emulsions stabilized by soy proteins exhibit excellent stability against coalescence, due to their high thickness and/or viscoelasticity of interfacial layer films.

Using stirring to accelerate the coalescence, Mitidieri & Wagner (2002) investigated the influence of thermal denaturation and μ on the coalescence of the emulsions stabilized by SPI (at pH 7.0), formed at a very low *c* of 0.1% and $\phi = 0.25$. They indicated that 1) although the emulsion stabilized by denatured SPI had a significantly higher interfacial protein concentration

(Γ; 2.9 mg/m^2) than that by native SPI (2.2 mg/m^2), the latter exhibited a remarkably higher stability against coalescence under agitation (in the absence of NaCl); 2) the addition of increasing concentrations (0-0.15 M) of NaCl, irrespectively before or after emulsification, progressively decreased the coalescence stability of native SPI emulsions, while the stability of denatured SPI emulsions was slightly affected by the NaCl addition (Mitidieri & Wagner, 2002). The results suggest that in this case, the viscoelastic properties of interfacial films play a more important role in the coalescence stability of SPI emulsions (than the thickness of adsorbed protein layers), however, when the inter-droplet electrostatic repulsion is screened, the situation seems to be the reversal. In another work, they further investigated the influence of the bulk *c* (0.1-1.0%, w/v) on the coalescence stability of the emulsions stabilized by native and denatured SPI, as compared with sodium caseinate (a well recognized protein emulsifier) (Palazolo et al., 2003). It is shown that 1) although the equilibrium interfacial pressure (π_e) or Γ at a specific *c* value was different between the native SPI and sodium caseinate emulsions, their coalescence stability was similar that was considerably higher than that of denatured SPI emulsions; 2) increasing *c* led to a progressive increase in coalescence stability of denatured SPI, and the increases in stability of the native SPI or sodium caseinate emulsions was much less in magnitude (Palazolo et al., 2003). If the flocculation is also taken into account when evaluating the global destabilization of SPI emulsions, as analyzed by backscattering in quiescent storage, the situation seems to be different. In this case, the global stability against both coalescence and flocculation for denatured SPI emulsions was contrarily higher than those prepared with native proteins, where the better stability had been attributed to formation of a hydrated and gel-like structure of cream phase in the emulsions stabilized by denatured SPI (Palazolo et al., 2005). The differences in the results could be ascribed to the disruption of droplet flocs formed in the system upon the stirring (applied in the previous works).

Creaming. In an oil-in-water emulsion, creaming is essentially as a consequence of the difference in the density between the dispersed oil droplets and the bulk aqueous phase. In a

dilute system, the creaming rate of oil droplets (υ) can be generally described by the Stokes law,

$$\upsilon = 2r^2(\Delta\rho)g/9\eta_o,$$

where r is the radius of the oil droplets, $\Delta\rho$ is the difference in the densities of the oil and the aqueous phase, g is acceleration due to gravity, and η_o is the viscosity of the medium (Damodaran, 2005). From this equation, it is known that the rate of creaming can be slowed down by decreasing the size of oil droplets, and/or increasing the viscosity of the aqueous phase. The creaming for an emulsion is usually accelerated by occurrence of flocculation and coalescence (**Figure 7**). It should be emphasized that for concentrated emulsions, the Stokes law cannot be well applicable, since in these systems, the complex inter-colloidal interactions to a large extent dominate the global creaming behavior.

Since the size and flocculated state of oil droplets in soy protein-stabilized emulsions is highly affected by the applied ϕ and/or c, composition and denaturation/aggregation of the proteins, emulsification process, and even many environmental conditions (e.g., μ and pH), the creaming behavior of these emulsions is difficult to predict based on the Stokes law. Palazolo et al. (2005) indicated that using a shearing (20000 rpm) as the emulsification technique, the emulsion stabilized by thermally denatured SPI at c = 0.1% (w/v) and ϕ = 0.33 exhibited a higher stability against creaming upon 24 h of storage than that by unheated SPI, regardless of μ. In this case, the creaming stability for the emulsion by denatured SPI was even similar to that for sodium caseinate-stabilized emulsion. However, in our recent work on unheated and heated (85ºC, 15 min) soy globulin (SG+SC)-stabilized emulsions at c = 1% (w/v) and ϕ = 0.1 (produced by microfluidization), we observed that no matter what the glycinin content in the globulins (0-100%) was applied, all the unheated emulsions were very stable against creaming upon storage up to 13 days, while in the heated case, the emulsions fast developed into cream layer (top) and a diluted emulsion, or even serum layer (bottom), within a short period of storage, when the glycinin content was above 60% (Luo et al., 2013). The differences in creaming behavior as affected by the protein denaturation might be related to the differences in size and

structure of droplets or flocs, and the nature of inter-droplet interactions. In the previous work (Palazolo et al., 2005), the protein concentration was very low and ϕ relatively high, and as a consequence, the heat treatment might be favorable for the formation of an emulsion with smaller sizes of droplets; whereas in our recent work (Lou et al., 2013), the amount of proteins was distinctly enough to fully stabilize the oil droplets, and the heating led to more denatured proteins adsorbed at the interface of oil droplets (as evidenced by enhanced interfacial protein concentrations for the emulsions stabilized by heated soy globulins), which greatly favored the bridging flocculation (and further creaming).

Irrespective of the emulsification process or energy input level, the creaming stability of the emulsions stabilized by heated SPI (at pH 7.0) generally increases with increasing the *c* from 0.5 to 6.0% (at a constant ϕ of 0.2, or 0.6), or with increasing the ϕ from 0.2 to 0.6 (at a specific *c* value, e.g. 2 or 6%, w/v) (Liu & Tang, 2013, 2014; Tang & Liu, 2013). Especially for the emulsions produced by the microfluidization, no creaming occurred upon storage up to 30 days, when the *c* was above 0.5%, though a severe droplet flocculation occurred (Liu & Tang, 2014 a). The extreme stability against creaming is largely related to the gel-like network formation involving droplet bridging flocculation (see the ongoing). The importance of the formation of a gel-like network for the creaming stability has also been previously observed for the emulsions stabilized by soy protein concentrate (with highly thermally denatured proteins) (Roesch & Corredig, 2002, 2003). This unique characteristic of soy protein emulsions is of vital relevance for the development of soy protein emulsion formulations in the food industry.

Interfacial properties (dynamics of adsorption and interfacial rheology)

For a protein, the behavior of its adsorption and the properties of adsorbed protein layers at the oil-water interface largely determine its emulsification performance, as well as the properties (and the stability in particular) of its emulsions. Thus, the knowledge about the interfacial properties of proteins is vital in understanding of their emulsifying properties, at the microscopic and molecular level. The interfacial properties are generally studied on planar oil-water (O-W)

interfaces, rather than in an emulsion formed under nonequilibrium conditions of homogenization, based on the assumption that the properties of the adsorbed interfacial proteins are similar on planar interfaces and in emulsion droplets (Dalgleish, 1997 b; Murray, 2011). There are primarily two categories of interfacial studies, one investigating the dynamics of adsorption at the interface, and the other characterizing the rheology of adsorbed interfacial films. The dynamic adsorption of a protein at the interface is complex and dynamic process, involving diffusion of the molecule from the aqueous phase to the subsurface (a layer adjacent to the fluid interface), penetration and unfolding of the protein at the interface, and the structural rearrangement of adsorbed proteins at the interface (Tang & Shen, 2014). Although, in a homogenized emulsion system, the adsorption of a protein to the interface of droplets is not diffusive, the adsorption behavior of the protein and the ease of structural changes of adsorbed proteins at the interface (within a very short period) play a vital role in its emulsifying properties. Besides the adsorption effects, the lateral interactions between adsorbed proteins that are indicative of the interfacial shear viscosity or dilatational elasticity/viscosity are also important for the stability of the corresponding emulsions.

The literature addressing the interfacial properties of soy proteins (and soy globulins in particular) has been greatly accumulating in the past decade (**Table 3**). However, most of the works in this aspect were performed at the air-water (A-W) interface, with the aim proposed to help understand the foaming properties of these proteins. Although the emulsifying and foaming properties of a protein are not always accordant, it is generally recognized that the adsorption behavior and interfacial properties of proteins between the A-W and O-W interfaces are basically similar, despite the fact that some differences may exist (Williams & Prins, 1996). One main difference between these two interfaces is the difference in magnitude of interfacial affinity between the protein and the interface, or ‘dispersion interactions’ between protein and the dispersed phase (e.g., oil or gas phase; Damodaran, 2005; Sengupta & Damodaran, 1998). The dispersion interactions between protein and the oil phase are much stronger than between protein

and the gas phase, with the former always attractive and the latter generally repulsive (Sengupta & Damodaran, 1998). For soy proteins, it has been confirmed that the interfacial affinity for these proteins is distinctly higher at the O-W interface than at the A-W interface (Sangtiago et al., 2008). Due to this difference, proteins are adsorbed at the O-W interface, at a much greater rate, than at the A-W interface. **Figure 14** shows the adsorption isotherms of SPI at the A-W and O-W interfaces, with the surface pressure (π) plotted against the initial protein concentration in the bulk (c). From this figure, it can be distinctly observed that at low c values, the same bulk concentrations have higher π values at the O-W interface than at A-W interface, while both isotherms overlap at the high c values (Sangtiago et al., 2008). Another difference is that the time needed to reach the equilibrium state of adsorption (after which the interfacial tension or π will not distinctly change any more) is different between the two interfaces. For the O-W interface, it is difficult to reach a complete equilibrium state of adsorbed protein films, e.g. more than 15 h is needed for the adsorption of plant proteins including soy globulins (Ducel et al., 2004). In contrast, the needed time for the A-W interface seems to be much less. The difference might be largely ascribed to the difference in conformational state and ease of conformational changes of the adsorbed proteins.

In this section, we primarily summarized the works about the dynamics of adsorption at interface for soy globulins (SG and SC), including diffusion of the proteins to the interface, penetration and unfolding of the protein molecules at the interface, and structural rearrangement of adsorbed proteins at the interface. The changes in π and dilatational interfacial characteristics upon adsorption are usually applied to characterize the adsorption kinetics. The interfacial rheology of the soy protein films is also summarized. Last, the possible relationships between the interfacial and emulsifying properties of soy proteins are discussed.

Dynamics of adsorption

The dynamics of adsorption of proteins at the A-W or O-W interface can be monitored by measuring the changes in surface pressure (π) or surface dilatational characteristics (e.g., surface

dilatational modulus, E or its phase angle, ϕ) as a function of adsorption time. In usual, the π for an adsorbed protein film in any specific test system increases with adsorption time, with the rate of adsorption decreasing upon increasing time, at least during the initial period of adsorption, which reflects the adsorption and coverage of proteins at the interface (e.g., Damodaran & Song, 1988; Perez et al., 2009; Tang & Shen, 2014; Xu & Damodaran, 1994). Besides the interfacial coverage, the dynamics of adsorption for the E can also be related to the lateral interactions of adsorbed proteins at the interface. Generally, in a diffusion-controlled adsorption, the rate of adsorption highly depends on the concentration gradient, which can be described using a modified form of the Ward and Tordai' equation (Ward & Tordai, 1946):

$$\pi = 2C_0KT(Dt/3.14)^{1/2},$$

where C_0 is the initial bulk concentration, D the diffusion coefficient, K the Boltzmann constant, T the absolute temperature, and t is the time. If the diffusion at the interface controls the adsorption process, a plot of π against $t^{1/2}$ will then be linear and the slope of this plot will be the diffusion rate constant (k_{diff}) (Perez et al., 2009; Ruíz-Henestrosa et al., 2007; Xu & Damodaran, 1994). **Figure 15 A** shows typical time evolution profiles of π for the adsorbed films of heated SPI at the O-W interface, with varying c values of 0.01-1.5% (w/v). From this figure, we can see that only at a very low c (=0.01%, w/v) the adsorption of the protein at the interface during the initial period (denoted as stage ❶; up to about 300 s) was diffusion-controlled, while at c values of 0.05% or above, a jump in π was observed, even at zero time (indicating that the adsorption at these c values was too fast to be detected). In fact, it can be still seen that the extent of the jump in this case progressively increased with increasing the c from 0.01 to 1.5% (w/v) (**Figure 15 A**), reflecting that increasing the c greatly facilitated the most initial diffusion and/or adsorption of the protein from the bulk to the interface. Similar phenomena have been previously observed for the adsorption of native SPI at the O-W interface (Wang et al., 2012), of BSA at the O-W interface (Miller et al., 2001; Tang & Shen, 2014). By comparison, the π values at the end of adsorption (about 10800 s; 14-16 mN m^{-1}) for the heated SPI adsorbed films at c values of

0.01-1.5% (w/v) are distinctly greater than those (8.7-10.9 mN m^{-1}) for the BSA films at comparable *c* values (Liu & Tang, 2014 a; Tang & Shen, 2014), indicating more effective adsorption of heated SPI at the interface.

After the diffusion (if a diffusion process exists), the rate of adsorption gradually declines with time, due to the presence of an energy barrier caused by the initially adsorbed proteins. This reflects that the penetration of the protein into the interfacial films, and unfolding and rearrangement of the adsorbed proteins at the interface (as denoted as the stages ❷ and ❸) become to be 'rate-limiting' (**Figure 15 A, B**; Perez et al., 2009; Ruíz-Henestrosa et al., 2007). The ease of the penetration and rearrangement for the adsorbed proteins can be evaluated by applying a first-order phenomenological equation as follows (Perez et al., 2009; Suttiprasit et al., 1992):

$$\text{In}\ [(\pi_f-\pi_t)/(\pi_f-\pi_0)] = -\ k_i \cdot t,$$

where π_f, π_t and π_0 are the surface pressures at the final adsorption time (e.g., 10800 *s*) of each step, at any time (*t*), and at the initial time, respectively; and k_i is the first-order rate constant. **Figure 15 C** shows a typical plot of In $[(\pi_f-\pi_t)/(\pi_f-\pi_0)]$ against the time for the heated SPI at *c* = 0.5% (w/v).

In practice, this plot can yield two or more linear regions, with the first slope taken as a first-order rate constant of penetration (k_P) and the next slope referring to as a first-order rate constant of molecular rearrangement (k_R) for the adsorbed protein. The magnitude in k_P and k_R is usually indicative of the ease of penetration and structural rearrangement, respectively.

Due to the differences in physicochemical and structural properties, different soy protein preparations (including their two purified fractions, SG and SC) may vary in their interfacial properties, though in a very complex way. For a specific protein, its interfacial properties, e.g. dynamics of adsorption, are not only affected by the *c*, but also, possibly to a higher extent, dependent on many environmental factors, such as μ and pH, and even temperature. Many previous works had investigated the influence of varying pH and/or μ and *c* on the interfacial

properties of SG and/or SC, especially their dynamics of adsorption at the A-W interface (Martin et al., 2002; Patino et al., 2003, 2004; Ruíz-Henestrosa et al., 2007). According to the fact the 11S-form of SG can be transformed into the 3S/7S form, as the pH is changed from a neutral to acidic value (e.g., from 6.7 to 3.0), Martin et al. (2002) found that when the SG was present in the 3S/7S form, it adsorbs much faster at the A-W interface than that in the 11S-form, consequently producing a higher surface concentration and a higher *E*. Although the 3S/7S form of SG exhibited much greater *E* and surface shear viscosity (η^s) values of adsorbed protein films at initial periods of adsorption than the 11S-form, but the at long-term periods, these values between the two forms became similar (Martin et al., 2002), indicating that both the amount of adsorbed proteins and the structural rearrangement of adsorbed proteins at the interface might contribute to the rheological properties of the adsorbed films. Similar findings about the influence of conformational state or characteristics on the adsorption of SG have been reported by Wagner & Guéguen (1995), and Patino et al. (2004). For example, Wagner & Guéguen (1995) indicated that the structural changes of SG, induced by dissociation, deamidation and reduction, remarkably improved the initial adsorption (up to about 30 min) of the protein at the A-W interface, but slightly affected the equilibrium π. More interestingly, they argued that the molecular size of the proteins and unfolding at the interface (rather than the surface hydrophobicity) rule the interfacial adsorption of SG. Besides the conformational factor, Patino et al. (2004) also confirmed the importance of solubility for the interfacial properties of soy proteins, including SG and SC.

If the *c* is extremely low, e.g., at c = 0.001% (w/v), or when the pH was close to the *p*I for both SG and SC, there may exist a lag period after which the π distinctly increased with time (Patino et al., 2004). The existence of a lag period was similarly observed for the adsorption of soy globulins at the A-W interface, at c = 0.1 wt% and at low μ values (0.05 M) (Ruíz-Henestrosa et al., 2007). However, the lag period cannot be observed in the adsorption of unheated SPI or heated SPI at the A-W and/or O-W interface, even at a lower *c* value, e.g.,

0.0003% (Santiago et al., 2008; Liu & Tang, 2014 a). The difference may be related to the differences in molecular characteristics between the purified soy globulin fractions and the SPI.

In contrast, the influence of μ on the dynamics of adsorption for soy globulins is in a more complex way, depending on the type of the proteins and pH (Ruíz-Henestrosa et al., 2007). At pH 5.0 (close to the *p*I), it was observed that increasing the μ from 0.05 to 0.5 M remarkably improved the dynamics of adsorption for both the SG and SC, including the absence of lag period, increased k_{diff} (and shortened period of diffusion), and even higher π and E values (at the end of adsorption, e.g., 180 min) (Ruíz-Henestrosa et al., 2007). By comparison, at pH 7.0 the μ has a significant complex influence on the k_{diff} and π (and/ E) values (at 180 min). The SC at pH 7.0 was more rapidly adsorbed at μ = 0.5 M than at μ = 0.05 M, while for SG, the opposite was observed. On the other hand, the π values (at 180 min) for both the SG and SC were slightly affected by increasing μ, but much lower E values were observed at the higher μ value (Ruíz-Henestrosa et al., 2007). The improvement of adsorption dynamics at pH 5.0 by increasing I may be largely associated with the improved solubility. The significant reduction in E (at 180 min), in combination with slight changes in π values (at pH 7.0) by increasing μ just reflects that in this case, the conformational flexibility might play a major role in the lateral interactions of adsorbed proteins at the interface.

Last, it should be to note that the time evolution of E of soy protein adsorbed films can also be monitored using a similar first-order kinetic equation to that in the π case, to provide information about the diffusion of the protein to the interface and the penetration and/or unfolding of the molecules at the interface, as well as structural rearrangement of adsorbed proteins (Patino et al., 2003). Different from the π that usually depends on the interfacial adsorption, the E of adsorbed protein films is dependent not only on the interfacial adsorption, but also on interfacial interactions (Ruíz-Henestrosa et al., 2007). If the E is only related to the amount of proteins adsorbed at the interface, all E data should be normalized in a single master curve of E versus π (Lucassen-Reynders et al., 1975; Patino et al., 2003, 2005; Ruíz-Henestrosa

et al., 2007; Tang & Shen, 2014). For soy globulins, this normalization is only observed for SG or reduced SG, no matter what the pH or μ (Patino et al., 2003; Ruíz-Henestrosa et al., 2007). It is thus reasonably hypothesized that the surface dilatational properties of SG at the A-W interface are predominantly determined by the amount of adsorbed proteins, while in the SC case both the adsorption and lateral molecular interactions contribute to the *E* development. On the other hand, the *c* dependence of the *E* at a specific time of adsorption (e.g., 120 min) is different between the SG and SC adsorbed films (Patino et al., 2003). In the SC adsorbed films, it was surprisingly observed that at higher bulk *c* values (e.g. 1% versus 0.1%) the *E* value significantly decreased. Patino et al. (2003) attributed this seemly abnormal decrease in *E* to the formation of more aggregated proteins at the interface at higher *c* values. Furthermore, the slope of the *E*-π plots for both SG and SC at different conditions is higher than 1 (characteristic of the behavior of an ideal gas), indicating a non-ideal behavior with higher molecular interactions as the π increases.

Interfacial rheology of adsorbed proteins

Interfacial rheological properties of proteins that are generally related to various aspects of colloidal stability for the corresponding emulsions have been extensively studied (Bos & van Vliet, 2001; Murray, 2002). There are two kinds of interfacial rheological properties, shear and dilatational. The principal difference between dilatational and shear interfacial rheology is that dilatational rheology is much more sensitive to the kinetics of adsorption. Both the dilatational and shear rheology can be performed in a dynamic or steady state, close to or far away from equilibrium (Bos & van Vliet, 2001). The interfacial rheological properties of a protein are closely related to the structure and lateral interactions of adsorbed proteins at the interface that highly depend on the adsorption time and/or ageing time (Lucassen-Reynders et al., 2010). In general, the *E* or η^s of adsorbed protein layers is far greater than that of adsorbed small molecular surfactant layers, and among different proteins, the *E* or η^s of adsorbed films of flexible proteins (e.g. caseins) is much lower in magnitude than those of the globular proteins (e.g., whey

proteins).

In an early work, Rivas & Sherman (1984) studied the interfacial shear rheology of different soy protein products (SG, SC and SPI) at the O-W interface, as affected by variation in pH, μ and even ageing time. The results indicated that the surface viscoelasticity (using an instantaneous elastic modulus, $E_{o(s)}$ as an indicator that can reflect the film strength at very short times after applying the constant stress and before any lateral interactions are disrupted) of the adsorbed films considerably varied with the type of proteins, pH and μ, and even the ageing time. The main findings of this important work include: 1) Adjusting pH near the *p*I of the proteins, or the presence of NaCl (0.5 M) greatly facilitates the $E_{o(s)}$ development of adsorbed films from all the test soy proteins, which is consistent with the fact that decreased repulsion forces are favorable for the packing and interactions of adsorbed proteins at the interface; 2) Irrespectively of pH or addition of NaCl, the SC adsorbed films exhibit much higher surface viscoelasticity than the SG counterparts, indicating that the SC molecules tend to form more ordered interfacial films with stronger lateral interactions between adsorbed proteins at the interface; 3) the viscoelasticity of SPI adsorbed films is similar to that of the SC films, implying that the SC might largely determine the viscoelasticity development of adsorbed films from SPI. Since the molecular conformation and aggregated state of soy proteins in solution are highly dependent on the pH and μ, it is thus reasonably suggested that associated and/or aggregated state of soy proteins play a vital role in determining the viscoelasticity of adsorbed films.

Besides the pH and μ, the surface rheological properties of soy proteins may be also affected by the bulk *c* and the denatured/aggregated state. Recently, Wang et al. (2012) investigated the surface dilatational rheology of unheated and heated (at 90 and 120 ºC) SPIs at the O-W interface, at pH 7.0 and varying *c* values of 0.001-1.0% (w/v). The heat treatment remarkably increased the gyration radius and surface hydrophobicity of the SPI molecules, possibly as a result of heat-induced unfolding and aggregation. In all the cases, the *E* progressively increased with time, with its elastic modulus (E_d) greater than the loss modulus (E_v) (Wang et al., 2012),

indicating the strengthening of lateral interactions between adsorbed proteins, and the predominantly elastic in nature for the adsorbed proteins. The development of E or E_d for the adsorbed SPI films at a test c value was nearly unaffected by the heating at 90 ºC, while in the case of heating at 120 ºC, a distinct fast increase in E or E_d was observed during the initial adsorption, but the E or E_d at long-term periods of adsorption was similar for unheated and heated SPI films (Wang et al., 2012). They attributed this improvement to the increase in conformation flexibility. This explanation seems to contradict from the general view that the heating at higher temperatures of a protein leads to formation of more compacted aggregates with larger sizes, as also evidenced by the higher gyration of radius or hydrodynamic radius at 120 ºC (relative to that at 90 ºC; Wang et al., 2012). Although not specifically characterized in this work, it might be largely associated with the differences in breakdown of charged groups (e.g., deamidation and/or decarboxylation) from the proteins between these two temperatures. They also interestingly observed that for unheated and heated SPI maximal E values of adsorbed films at long-term periods of adsorption were observed at c = 0.01% (w/v) (Wang et al., 2012), indicating weakening of lateral interactions between adsorbed proteins at higher c values. This seemly abnormal collapse-type phenomenon at high c values has also observed for the adsorbed films at the A-W interface from the SC at pH 8.0 (Patino et al., 2003), or from both the SG and SC at pH 2.0 (Patino et al., 2.0), which seems to be common for a number of other proteins (e.g., β-lactoglobulin, BSA, ovalbumin and β-casein) at both the O-W and A-W interfaces (Bos & van Vliet, 2001). In a more recent work investigating the emulsifying and interfacial properties of plant (including potato, soy and pea) and milk proteins at pH 7.0 and 10.0, Amine et al. (2014) also observed a general reduction in complex modulus (or Gibbs elasticity) at the O-W interface as the c in the aqueous phase progressively increased, especially in the case of pea and potato proteins. This is possibly due to slower structural rearrangements of adsorbed proteins in multilayers (at higher c values).

In contrast, the surface dilatational or shear moduli of adsorbed protein films at the A-W

interface are generally higher than that at the O-W interface (Bos & van Vliet, 2001), which implies that the ease of unfolding and structural rearrangement of the proteins is more important for the interfacial rheological properties at the A-W interface. The difference may be caused by the difference in magnitude of protein affinity between the two interfaces.

Besides the differences in adsorption behavior, Martin et al. (2002) noticed that the differences in the present form (11S or 7S/3S) for SG also resulted in a considerable variation in its surface rheological properties at the A-W interface. Much greater E or η^s of the adsorbed films from 7S/3S-form SG was observed at relatively short periods of adsorption (e.g., 1.7 h) than that from the 11S-form SG, though at long-term periods (e.g., 24 h), the η^s of SG in the different forms was approximately the same (Martin et al., 2002). It should be to note that in this case, the ellipometric layer thickness for the 11S-form SG was greater than that for the 7S/3S-form one at a comparable c value, thus suggesting that the interfacial rheological properties of SG is mainly determined by the restructuring and lateral interactions of the proteins at the interface, and hardly by the increase in amount of adsorbed proteins from the bulk. Similar to the observations at the O-W interface (Rivas & Sherman, 1984), Ruíz-Henestrosa et al. (2007) showed that the adsorbed films from SC at the A-W interface have higher E values than those from the SG (at pH 7.0 and at a same c of 0.1 wt%), and the addition of 0.5 M NaCl progressively improved the E development. The observations confirm that soy globulins with different conformational characteristics are adsorbed at the interface with different degrees of association depending on the environmental factors. The importance of the conformational characteristics of soy globulins for their surface rheological properties has been confirmed in a previous work (Patino et al., 2003). All the formed adsorbed films of soy globulins at the A-W interface can be considered to be practically elastic in nature (Patino et al., 2003, 2005). This is basically consistent with the view that the adsorbed protein film is suitable to be considered as a thin three-dimensional network of molecules interacting through different kinds of interactions, e.g., hydrogen bonds, hydrophobic interactions and electrostatic repulsion (Murray, 2002).

Relationships between the emulsifying and interfacial properties

The emulsifying properties of a protein are usually characterized using a dynamic system, in which the energy barrier of adsorption can be readily overcome by the hydrodynamic forces produced during emulsification (Day et al., 2009; Dickinson, 2001), while in the case of interfacial measurements (mostly at quiescent conditions), the adsorption behavior is highly affected by the energy barrier, e.g. the electrostatic barrier caused by the initially adsorbed proteins at the interface. Nevertheless, it can be still generally recognized that, the emulsification performance or efficiency of a protein is closely related to its adsorption characteristics at the interface, including diffusion (or adsorption during the earliest period) to the interface, and ease of anchoring and structural unfolding and/or rearrangement of adsorbed proteins at the interface, while the properties of adsorbed protein films at the interface, e.g., film thickness and viscoelasticity, largely dominates the stability against coalescence (and in some cases, against flocculation) of the emulsions. The relationships between the emulsification performance and interfacial adsorption for a protein can be easily understood. For example, for a protein, the faster the rate of diffusion and/or adsorption, or the more ease of unfolding and rearrangement of adsorbed proteins at the interface, the better its emulsification performance is. In contrast, the relationships between emulsion stability (including those against flocculation and creaming) and the surface rheological properties seem to be more complex and difficult to be understood, though a lot of effort has been made in this field of colloid and interface science (Bos & van Vliet, 2001; Dalgleish, 1997 a, b; Dickinson, 1999, 2001; Muray, 2002).

On the other hand, we should realize that most of these relationships, particularly those experimentally established between the interfacial rheological parameters (at a macroscopic surface) and specific aspects of emulsion stability are based on the proteins with well-recognized structural and physicochemical characteristics, e.g. whey protein or caseins. Due to the different physicochemical and structural properties from those milk proteins, plant proteins (and soy proteins in particular) may exhibit a different pattern of the relationships between the interfacial

properties and emulsification functions. It can be generally considered that soy protein emulsions show excellent stability against coalescence, due to the formation of thick interfacial films from these proteins. On the other hand, the highly hydrophobic nature of soy proteins usually imparts the corresponding emulsions a high tendency to flocculate one another, through the bridging way. Thus, it seems to be of more interest to establish the possible relationships between the interfacial properties (of soy proteins) and flocculation stability, than to uncover the importance of interfacial properties to the coalescence stability.

Relationships between emulsifying performance and interfacial adsorption. In theory, an ideal emulsifier is the one that can diffuse to and stabilize the new interface created during the emulsification, at a rate faster than that at which the recoalescence occurs between different droplets with bare interfaces. The rate of diffusion that highly depends on the concentration gradient is of vital relevance to inhibit the occurrence of re-coalescence, thus improving the emulsification performance. At low bulk *c* values, the diffusion process is usually the limiting step of adsorption affecting the emulsification, while at high *c* values, the ease of unfolding or arrangement of initially adsorbed proteins may become predominant.

Very few studies are available providing solid evidences to suggest a direct relationship between the emulsification performance and the interfacial adsorption of soy proteins, or other globular proteins (Cui et al., 2014; Li et al., 2011; Liang & Tang, 2013; Liu & Tang, 2014 a; Tang & Shen, 2014). An effective strategy to unravel these relationships is to synchronously modulate the emulsifying and interfacial properties of the proteins at specific conditions, by means of changing one or more parameter, since all the emulsifying and interfacial properties are characterized only in a relative way. Li et al. (2011) investigated the influence of a heating (at 95ºC for 30 min) of SPI dispersions at concentrations ranging from 0.2-1.2% (w/v) on its emulsifying ability (with EAI as an indicator) at pH 7.0, as well as the adsorption of the proteins at the interface under the equilibrium state. They observed that the heating significantly improved the emulsifying ability of SPI, and increased the percentage of adsorbed proteins in the

emulsions, but the interfacial protein concentration was distinctly decreased (Li et al., 2011), suggesting that the improvement of emulsification performance might be due to enhanced adsorption and spreading of proteins at the interface. In a further work, they confirmed similar changes in emulsification performance and adsorption behavior of proteins at the interface of oil droplets in the heated SPI emulsions, by means of increasing the concentration (1-5%, w/v; at which the SPI was heated), or increasing the energy input of emulsification (Cui et al., 2014).

Recently, we investigated the emulsifying properties and adsorption dynamics at the O-W interface of a heated SPI at pH 7.0, as affected by variation in *c* (0.5-6.0% and 0.01-1.5% for the emulsifying and interfacial experiments, respectively) and/or emulsification process (Liu & Tang, 2014 a). The heated SPI solutions at varying *c* values were obtained by heating a SPI solution at *c* = 6.0% (w/v), at 95ºC for 15 min, and then diluting it to the required *c* in the test range. We interestingly observed that, 1) for a specific emulsification process, there was approximately a critical *c* value above which the emulsion size (in the presence of SDS) was basically unchanged upon increasing the *c*, and the critical *c* value was much lower when the emulsification with a higher level of energy input was applied; 2) concurrently, increasing the *c* from 0.01 to 1.5% led to a progressive increase in rate of diffusion, or the rate of the most initial adsorption (which was undetectable in the experiments), and there was also a critical *c* value (in this case, it was around 0.5%, w/v) above which the behavior of the diffusion or adsorption during the initial period was unchanged upon a further increase in *c*; in contrast, the adsorption behavior of the proteins following the diffusion, including penetration, unfolding and structural rearrangement at the interface, was slightly affected by changing the *c* (**Figure 15 A**; Liu & Tang, 2014 a). The observations about the *c* dependence of the emulsification performance and dynamic interfacial adsorption at the interface clearly confirm that the emulsification performance of SPI (particularly heated) is primarily related to the rate of diffusion, or rate of initial adsorption at the interface. A similar relationship has been previously established between the rate of diffusion (at the A-W interface) and the overall foam capacity for soy globulins (SG and SC) or their

hydrolysates at pH 5.0 or 7.0 (Ruíz-Henestrosa et al., 2007, 2009). Poon et al. (1999) also observed a similar relationship between the interfacial adsorption (at initial adsorption period) and the emulsifying activity for apomyoglobin (a highly folded globular protein; without disulfide bonds) and its derived peptides (e.g., the peptide (1-55)).

Besides the diffusion process, the ease of penetration, unfolding or structural rearrangement of adsorbed proteins at the interface may be also important for their emulsification performance. In this aspect, the conformational characteristics seem to play a crucial role in affecting the adsorption behavior of the proteins. In another recent work of ours, we investigated the emulsifying and interfacial adsorption properties of phaseolin (an ideal 7S-form vicilin from kidney bean) at pH 7.0 and a specific *c* value of (0.5%, w/v), as modulated by urea with increasing concentrations of 0-8 M (Liang & Tang, 2013). Interestingly, the results indicated that the emulsification performance of this 7S globulin progressively increases with urea concentration increasing from 0 to 8 M, as evidenced by a progressive reduction in surface-average droplet size ($d_{3,2}$; in 1% SDS) from 19.9 to 1.8 μm, while the adsorption at the O-W interface was remarkably improved at an urea concentration of 2 M, and the π development was on the contrary progressively inhibited by increasing urea concentration at concentrations above 2 M (Liang & Tang, 2013). By analyzing the kinetics of adsorption, we also observed that the first-order rate constant of rearrangement (k_R) also progressively increased from -7.4×10^{-4} to -33×10^{-4} s^{-1} with urea concentration over the whole concentration range, while the rate constant of penetration and unfolding (k_P) was slightly affected (Liang & Tang, 2013). The observations clearly indicated that the improvement of emulsification performance of this 7S globulin by the presence of 2 M urea was mainly due to the enhanced rate of diffusion, while that at urea concentrations above 2 M was more determined by the improved ease of structural rearrangement of adsorbed proteins at the interface. The importance of conformational characteristics to the emulsification and interfacial adsorption of those globulins will be discussed in the following section.

Another strategy to indicate the relationships of soy proteins is to compare different soy proteins with different emulsification performance and the interfacial properties. Although it is well recognized that the SC exhibits a much better emulsification performance than the SG (Keerati-u-rai & Corredig, 2010; Lou et al., 2013), it is uncertain which aspect of adsorption at the O-W interface cause the difference between these two globulins. To testify this, we investigated the dynamics of adsorption of soy globulin mixtures (SG + SC) at the O-W interface, at a total bulk *c* value of 0.5% (w/v) but with different SG contents in the mixtures (0-100%), as displayed in **Figure 16**. From the figure, it can be seen that the π of all the test samples suffered a dramatic jump at the most initial adsorption (e.g. at zero time of adsorption), with the magnitude in π at zero time progressively decreasing with the SG content (from 0 to 100%), and after that, the π progressively increased with the time up to 3 h; dramatic changes in adsorption behavior were observed when the SG content was increased up to above 90% (**Figure 16**). The dependence of adsorption behavior of these globulins on the SG content is interestingly similar to the *c* dependence for the adsorption of heated SPI (**Figure 15 A**; Liu & Tang, 2014 a), indicating that the SC exhibited higher rate of diffusion or rate of adsorption at the initial adsorption periods. The observations further confirm the close relationships between the emulsification performance and the rate of diffusion (or rate of adsorption at the earliest adsorption period) of soy proteins.

Relationships between emulsion stability and interfacial rheology. As aforementioned, protein-stabilized emulsions are essentially unstable and tend to destabilize upon storage, or under many stresses, such as freezing, heating, changing in environmental conditions (e.g., pH and μ), and shearing. For soy protein-stabilized emulsions, the flocculation, coalescence and creaming are the three main physical mechanisms of destabilization. Among these mechanisms of destabilization, the flocculation is the one dominating their global destabilization, which is closely associated with the hydrophobic nature of the proteins adsorbed at the interface of droplets. However, the flocculation or flocculated state of droplets in the emulsions is the most

subtle and complicated one to control, since it can be sensitively affected by a lot of different variables, and also because it largely determines the emulsion structure and influences the other destabilization mechanisms (Dickinson, 2010 a). Thus, the relationships between the emulsion flocculation stability and interfacial characteristics of soy proteins are difficult to establish, and will not be discussed below.

Coalescence is the most investigated destabilization mechanism for protein-stabilized emulsions in relation to the interfacial rheological characteristics (Bos & van Vliet, 2001). It is well recognized that the coalescence stability highly depends on the structure (e.g., steric structure) and viscoelastic properties of interfacial adsorbed films at the interface of oil droplets (Damodaran, 2005). The steric repulsion between adsorbed protein layers can retard flocculation that is the initial step for the coalescence occurring. In usual, the higher the interfacial protein concentration, and/or the stronger the viscoelasticity of interfacial films, the better the coalescence stability is for a protein-stabilized emulsion. For example, for isolated wheat protein (an acidic deamidated wheat protein with few hydrophobic regions on the molecular surface), it formed a relatively weak fluid-like film at the O-W interface, but the correspondingly formed emulsions exhibited excellent coalescence stability (even in the highly flocculated state), due to the thick interfacial layer to provide a barrier to droplet coalescence (Day et al., 2009), confirming the importance of interfacial layer structure for the coalescence stability. Very rare direct studies are available to measure the coalescence of individual oil droplets with a planar O-W interface. In this regard, Kim et al. (2005) had made an interesting investigation on native and heated *β*-lactogloublin-coated oil droplets, and found that heating improved the interfacial surface elasticity and viscosity, and the improvement was closely related to the increased resistance to coalescence of the single droplets. So, one tends to expect that there is a possibility to establish a casual relation between the interfacial rheological characteristics (determined at macroscopic surfaces) and the coalescence stability of the emulsions. Based on the fact that in many cases, when a small molecular surfactant is added to a preformed protein-stabilized

emulsion, the protein adsorbed at the interface of oil droplets will be replaced by the surfactant, especially at high surfactant to protein ratios, it can be reasonably considered that packing of protein molecules in the interfacial films at the interface is inhomogeneous, and there are some void spaces or 'holes' in the interfacial films (Bos & van Vliet, 2001; Damodaran, 2005). The coalescence stability is highly associated with the inhomogeneous state or the holes of the films, and subsequently, the susceptibility to the film rupture. To help understand the importance of interfacial film characteristics to the coalescence stability, the protein-coated oil droplets with different structure and rheological properties can be approximately subdivided into four types (I-IV), depending on the type and nature of proteins (as illustrated in **Figure 17**):

(I) The surface of oil droplets is covered by a thick and highly viscoelastic interfacial film from flexible proteins, e.g., β-casein, and in this case, the droplets are usually highly stable to resist the film rupture and occurring of coalescence, due to strong steric stabilization and high film viscoelasticity;

(II) The interfacial film on the droplet surface is thin but with high elasticity, which is susceptible to coalescence due to the high 'brittle' nature of the films, especially when a shearing stress is applied, like in the case of the emulsions stabilized by BSA or whey proteins;

(III) In the case of oligomeric globulins with relatively low tendency to aggregate (due to their highly hydrophilic nature), e.g., β-conglycinin, a dense and viscoelastic interfacial film can be formed at the interface of oil droplets, in which the structure of adsorbed proteins (or subunits) is slightly changed (relative to that of unadsorbed ones in the bulk), but strong lateral interactions between adsorbed proteins may occur. This kind of droplets also exhibits high coalescence stability, largely as a result of high film surface elasticity/viscosity, and viscoelascity.

(IV) For the proteins with high tendency to aggregate, which are usually present in the form of aggregated particles with large sizes, e.g., soy glycinin and SPI, they will

form very thick but inhomogeneous interfacial films at the interface of oil droplets, and in this case, the oil droplets seem to be more like entrapped within a network consisting of aggregated particles. Although in theory some 'holes' exist on the surface of oil droplets covered by these aggregated proteins, they still exhibit excellent stability against coalescence, due to the strong steric stabilization and the presence of the aggregated protein network at the interface.

Rivas & Sherman (1984) first established a close correlation between the rate of droplet coalescence in corn oil-in-water emulsions stabilized by soy proteins and the instantaneous surface elastic modulus ($E_{o(s)}$) of the protein films at the O-W interface, irrespective of the type of soy proteins (SG, SC or SPI). The coalescence stability of the emulsions formed at c =2.0% (w/v) and ϕ=0.5, upon storage up to 24 h, progressively decreased as the $E_{o(s)}$ increased. By comparison, the SC-stabilized emulsions exhibited higher coalescence stability than those stabilized by the SG, no matter what the pH or addition of NaCl was applied, which is consistent with the higher surface viscoelasticity parameters of the SC interfacial films (Rivas & Sherman, 1984). This is also in accordance with the observations of dynamics of adsorption at the O-W interface for the SC and SG (**Figure 16**), or at the A-W interface for SG with different forms (11S or 7S/3S) (Martin et al., 2002), indicating that the fast adsorption at the initial adsorption period for the SC was favorable for the close and ordered packing of the proteins at the interface, especially at the long-term periods of adsorption, thus imparting higher stability against coalescence (than that for the SG).

In the previous work by Rivas & Sherman (1984), all the emulsions were formulated at a relatively higher c value (2%), at which relatively sufficient proteins would be present to fully stabilize the droplets. The observed relationships between the coalescence stability and the interfacial viscoelasticity may not be applicable to other situations at low c values where the amount of proteins is insufficient for the emulsion formation. In the latter situations, the interfacial rheological properties may become a parameter dominating the coalescence stability

of the emulsions, over the steric stabilization. For example, Mitidieri & Wagner (2002) compared the coalescence stability (under stirring conditions) of native and denatured (treated at 90 ºC) SPI-stabilized emulsions, formed at c = 0.1% (w/v) and ϕ = 0.25, and observed that the native SPI emulsion exhibited much greater coalescence stability than the denatured SPI counterpart. Considering that the emulsion size was similar between the two emulsions and the interfacial protein concentration of the denatured SPI emulsion was even higher (Mitidieri & Wagner, 2002), it can thus be deduced that the only reason causing the higher coalescence stability for the native SPI emulsion (relative to the denatured SPI emulsion) might be the much greater interfacial viscoelasticity. This has been confirmed by Wang et al. (2012), who observed that at a comparable c (0.1%, w/v) the unheated SPI shows an improved adsorption at the O-W interface in regard to the SPI heated at 90ºC, especially at long-term periods of adsorption (during which the unfolding and rearrangement of adsorbed proteins plays a role). The better agitation-coalescence stability for the native SPI emulsions (relative to the denatured SPI counterparts at a comparable c) has been similarly observed at higher c values up to 1.0% (w/v), though among all the denatured SPI emulsions, it was still observed that increasing the c led to a progressive increase in coalescence stability (Palazolo et al., 2003). Amine et al. (2014) also observed a good correlation between the interfacial elasticity and emulsion stability against coalescence (after storage of 24 h) for milk and plant proteins (including soy proteins), by comparison of their emulsifying and interfacial characteristics between at pH 7.0 and 10.0, based on the assumption that increasing the protein charge by increasing the pH above the *p*I could partially unfold the structure of these proteins, thus improving their emulsifying and interfacial properties.

However, in a more recent work of Palazo et al. (2005), a contrary phenomenon about the differences in coalescence stability between the native and denatured (heated at 90ºC) SPI stabilized emulsions (at c =0.1%, w/v and ϕ = 0.33) was observed. The coalescence stability of the denatured SPI emulsions was even similar to that of the sodium caseinate emulsions,

regardless of the μ (e.g., 0.5 M; Palazo et al., 2005). To note that, in this work, the coalescence stability was evaluated under quiescent conditions (different from the agitated conditions in the previous work), while the former work (Palazolo et al., 2003) the stability was investigated under agitated conditions. Thus, it is possible that the destabilization mechanism of coalescence might be different between the quiescent and agitated conditions. The authors contributed the improved coalescence stability (at quiescent conditions) to the formation of a gel-type structure of cream phase. The explanation has been supported by the observations of Roesch & Corredig (2002, 2003), and recently confirmed by our group in a series of works revealing that the heat-induced nanoparticles of soy proteins can act as a kind of effective Pickering stabilizers to form the emulsions with a gel-like microstructure (Luo et al., 2013; Liu & Tang, 2013, 2014; Tang & Liu, 2013).

Besides the Pickering stabilization against coalescence, the formation of a gel-like structure in soy protein-stabilized emulsions also imparts an excellent stability against creaming to these emulsions (Liu & Tang, 2013, 2014; Roesch & Corredig, 2002, 2003; Tang & Liu, 2013). In this case, the gel-like network formation is highly associated with the serious bridging flocculation of droplets, as a result of enhanced hydrophobic interactions between the adsorbed proteins on individual oil droplets.

SOY PROTEIN NANOPARTICLES: A PROMISING KIND OF FOOD-GRADE PICKERING STABILIZERS

Food-grade Pickering particles

During the past few years there has been surprisingly increasing interest by scientists in food science and colloid and interface science in the development of food-grade nanoparticles or microparticles for use in the Pickering emulsions, due to their potential applications in the formulations of novel foods and medicine (Dickinson, 2010 b, 2012, 2013). For example, the particle-stabilized emulsions can be developed into a new functional food or drug system for more effective incorporation and controlled-release delivery of neutraceuticals (Augustin &

Hemar, 2009; Frelichowska et al., 2009; Garrec et al., 2012; Simovic & Prestidge, 2007). Furthermore, the lipid oxidation in oil-in-water emulsions can be significantly reduced by the Pickering stabilization (Kargar et al., 2011).

A common unique feature for the Pickering emulsions is their excellent stability against droplet coalescence, provided by a physical (steric) barrier of particles adsorbed at the interface. The outstanding coalescence stability, which is very impressive especially at very low interfacial particle coverage, is a general feature for a variety of Pickering emulsions stabilized by different types of Particles (Destribats et al., 2010). The extent of the Pickering stabilization depends on the density of particle packing at the interface, and on the extent how difficult it is to remove the adsorbed particles from the interface (Dickinson, 2012). In usual, the free energy of desorption, or the binding energy (ΔG_d) of one particle is considerably higher than the thermal energy (kT; where k is Boltzmann's constant and T is the absolute temperature), which is related to the particle size (i.e., $\Delta G_d \sim r^2$, where r is the radius of particles). Even for relatively small nanoparticles ($r\sim$ 5-10 nm), the adsorption of the particles at the O-W interface is irreversible ($\Delta G_d >> 10\ kT$), and if the r is increased by 10-fold (to 50~100 nm), the ΔG_d will be considerably greater than 1000 kT. However, it should be kept in mind that the large size of particles is unfavorable for their emulsification efficiency, which is the main reason why the Pickering emulsions are usually large in droplet size and coarse. Thus, there should be a deliberate balance between the Pickering stabilization and the formation of fine emulsions when choosing a kind of Pickering stabilizers in practice.

To date, an increasing number of food-grade particles as effective Pickering stabilizers for oil-in-water emulsions have been available in the literature, including modified starch particles (Rayner et al., 2012 a, b; Tan et al., 2014; Timgren et al., 2013; Yusoff & Murray, 2011), chitin nanocrystals (Tzoumaki et al., 2011, 2012), cellulose nanocrystals (Kalashnikova et al., 2011), flavonoids (Luo et al., 2011), corn zein (de Folter et al., 2012), cocoa particles (Gould et al., 2013), and, more recently, nanoparticles or microparticles from whey proteins (Destribats et al.,

2014; Meshulam & Lesmes, 2014; Nguyen et al., 2013; Schmitt et al., 2010; Shimoni et al., 2013), soy proteins (Paunov et al., 2007; Liu & Tang, 2013, 2014) and even pea proteins (at acidic pHs; Liang & Tang, 2014). These particles are usually highly variable in sizes from several nanometers to several micrometers. Despite of the different sources, one common feature is shared for these Pickering stabilizers that they are basically insoluble or poorly soluble in nature, but can be kinetically dispersed and kept stable in the aqueous phase. For example, water-insoluble zein has to be transformed into a kind of colloidal particles through an anti-solvent precipitation technique when applied as Pickering particles to stabilize oil-in-water emulsions (de Folter et al., 2012).

Protein-based particles as the Pickering stabilizers seem to be much advantageous over those from other sources, since due to the amphiphilic nature, many proteins exhibit good surface-related activities themselves, and thus do not need any chemical treatments any more (to improve the surface properties). For the highly hydrophilic starch particles, chemical grafting with hydrophobic moieties (e.g., octenyl succinic anhydride) on their surface are usually needed to improve their surface activities (Rayner et al., 2012 a, b; Timgren et al., 2013). On the other hand, it should be mentioned that, except the water-insoluble zein, some physical treatments, e.g. thermal heating, are sometimes necessary to ensure the good Pickering stabilization for many proteins, including whey proteins and soy proteins (Destribats et al., 2014; Liu & Tang, 2013, 2014; Nguyen et al., 2013; Schmitt et al., 2010). In the case of whey proteins, it has been indicated that the action of heating is to facilitate the covalent bond (e.g., disulfide bonds) formation within the whey protein microgels (Schmitt et al., 2010). The intra-particle disulfide bonding has been also confirmed to play a vital role for the SPI nanoparticles as the Pickering stabilizers (Liu & Tang, 2014 a). Therefore, it can be reasonably hypothesized that except zein, the presence of strong intra-particle interactions (especially disulfide bonds) may be a prerequisite for many food proteins to be developed into effective Pickering stabilizers.

Compared with whey proteins, soy proteins as potential Pickering stabilizers are more

promising in several aspects as follows: (i) they are abundantly and commercially available; (ii) in some cases, like SPI, the proteins in many soy protein preparations are present in the aggregated form with nano- or micro-sizes, and even do not need a thermal treatment; (iii) soy protein-based particles are more appropriate to form stable and gel-like Pickering emulsions; (iv) the last but not less importantly, soy proteins exhibit many health effects, including serum cholesterol-lowering effects, and thus can play a 'double-duty' in the formulation of functional foods. Paunov et al. (2007) first experimentally observed that a commercial spray-dried soy protein product (Supro 651), with calcium phosphate as the particle cores, which consists of soy protein aggregates with sizes of 1-30 μm, could be adsorbed at the O-W interface without distinct changes in morphology (**Figure 18 A, B**). They also successfully formulated the Pickering oil-in-water emulsions by this spray-dried soy protein product, at a concentration of 3.2 wt% (with respect to the oil phase) and $\phi = 0.5$ or 0.9, with homogenous structure and good stability (with the structure of the emulsion at $\phi = 0.5$ as illustrated in **Figure 18 C, D**), indicating the potential of soy protein products to act as Pickering stabilizers.

Interfacial adsorption and emulsification performance

For a given emulsifying agent, the droplet size of the formed emulsions is mainly determined by the magnitude in interfacial tension, when sufficient emulsifier is present under dynamic conditions of emulsification. For (nano)particles with large sizes, the dynamic interfacial tension at the bare O-W interface is usually high, thus causing the difficulty to form Pickering emulsions with smaller droplet sizes (Dickinson, 2010 b). In general, the droplet size of O/W Pickering emulsions stabilized by particles, e.g., hydrophobic silica, is highly dependent on the solid particle to oil fraction ratio, no matter what differences in emulsification performance between different particles (Chevalier & Bolzinger, 2013). At low solid to oil fraction ratios, only limited interfacial area can be stabilized, so that coarse emulsions are formed with large droplet sizes. In this situation, increasing the solid to oil fraction ratio may result in a progressive decrease in droplet size of the emulsions, since more particles are available to cover higher interfacial area.

When the solid/oil fraction ratio reaches a critical value (C_o), the droplet size will not be changed by increasing the ratio any more. The magnitude of C_o is largely determined by the nature of particles, and even the emulsification process. Under the same conditions of emulsification, the lower the C_o for a particle, the better the emulsification performance is. For hydrophobized fumed silica, a minimal solid concentration of about 6 wt% was needed for the formation of emulsions with a specific oil content of 20 wt% (by a ultrasonic treatment), above which the droplet size was independent of the silica content (Frelichowska et al., 2010). In contrast, much lower concentrations of particles (< 0.6 wt%) were reported for the emulsions (at pH 3-7) stabilized by whey protein microgels, or SPI nanoparticles (induced by a thermal treatment), obtained by a shearing emulsification, even at a much higher ϕ of 0.5 (Destribats et al., 2014; Liu & Tang, 2014 a), indicating much better emulsification performance for the protein-based Pickering stabilizers. In the case of SPI nanoparticles, it was also indicated that when the energy input level of emulsification was increased, the minimal protein concentration needed for the full coverage of interfacial area increased, e.g., up to 1.0% (w/v) (Liu & Tang, 2014 a), indicating that more particles would be needed to provide a timely stabilization of oil droplets with small sizes, newly created during the emulsification.

The high emulsification performance of heat-induced whey protein or SPI nanoparticles is closely related to the fast diffusion or initial adsorption at the O-W interface. As indicated in **Figure 15A**, we can see that even at a very low *c* value of 0.01% (w/v), the π of the interfacial film of SPI nanoparticles (induced by heating) progressively and linearly increased up to approximately 7.5 mN/m within a short period of 100 *s*, which is more than 10 times that of N-isopropylacrylamide (NIPAM) microgels at the same solid concentration (a typical kind of well recognized Pickering particles; Pinaud et al., 2014), indicating fast diffusion at the interface. The rate of diffusion for these nanoparticles was progressively increased by increasing the *c* up to 0.5% (w/v), as evidenced by the progressive increase in the π at zero time (**Figure 15 A**), suggesting the importance of concentration-dependent diffusion to the emulsification

performance. By comparison, the penetration, unfolding and structural rearrangement of these nanoparticles following the diffusion was limited, and slightly dependent on the applied c in the range 0.01-1.5% (Liu & Tang, 2014 a), suggesting that no distinct conformational changes and lateral interactions occurred between these nanoparticles adsorbed at the interface. This also implies that once adsorbed at the interface, these Pickering nanoparticles might be packed into a monolayer with relatively strong electrostatic repulsion and steric stabilization.

Based on the previous findings of ours that both the heated glycinin (SG) played a crucial role in the formation of gel-like emulsions stabilized by soy proteins (Tang & Liu, 2013; Luo et al., 2013), which, we know at the present, are essentially a kind of structured Pickering emulsions, we tend to consider that SG is a more appealing and appropriate material to formulate the Pickering stabilizers. In fact, most of the proteins in unheated SG are already in the nanoparticle state (**Figure 3 A, B**). If the SG was further heated at a temperature higher than its denaturation temperature (e.g., 90 and 100 ºC), larger sizes of SG particles would be formed, and the particle morphology became more irregular (**Figure 3**). More interestingly, the heat treatment resulted in enhanced surface properties and strengthened intra-particle interactions (especially covalent disulfide bonds; **Figure 4**), which have great implications for the Pickering stabilization of this kind of SG nanoparticles. Based on these, in a more recent work we investigated the potential of the unheated and heated (at both 90 and 100ºC) SG as Pickering stabilizers to form emulsions at *c* values of 0.5-6.0% (w/v) and a specific ϕ of 0.5, and their interfacial adsorption behavior at *c* values in the range 0.01-1.5% (w/v), and observed that the heating improved the rate of diffusion of SG at the O-W interface at *c* values > 0.05%, and the ability to stabilize the emulsions (or emulsification performance) at $c > 0.5\%$ (Liu & Tang, 2014 b). More interestingly, in all the emulsions stabilized by the unheated and heated SG at $c = 0.5\%$ (w/v) a structure of bridging droplets was observed (with microscopic observations displayed in **Figure 19**), which is clearly as a result of insufficient proteins to cover the interface of oil droplets. This phenomenon is common in the Pickering emulsions stabilized by particles at low solid concentrations

(Destribats et al., 2013).

The emulsification performance and adsorption behavior at the interface of protein-based Pickering particles can be also significantly influenced by pH and/or μ, since these environmental parameters greatly affects the surface charge, and indirectly, surface hydrophobicity of protein particles. For example, for SG nanoparticles induced by heating at 100 ºC, their zeta potential at pH 7.0 progressively changed from -36 to -6 mV, as the μ was increased from 0 to 500 mM (Liu & Tang, 2014 c), indicating the electrostatic screening of surface charge on these nanoparticles. Accordingly, it was observed that the droplet size (in 1% SDS) of the emulsions stabilized by these nanoparticles at c = 0.5-1.0% (w/v) and ϕ = 0.5, progressively decreased with increasing the μ from 0 to 500 mM, while at c = 2% (%), significant reduction in droplet size was observed only at μ values less than 100 mM (Liu & Tang, 2014 c). On the other hand, it was also shown that for these nanoparticles the rate of diffusion (as evidenced as the magnitude of π at zero time) at the interface progressively increased with the increase in μ (Liu & Tang, 2014 c), confirming the close relationship between the interfacial adsorption and the emulsification performance, especially at low particle concentrations.

Nanoparticle packing at the interface and limited coalescence

For Pickering emulsions, the droplet size usually decreases with increasing particle concentration, when the solid-to-oil fraction ratio is low. This so-called limited coalescence process can allow the production of a wide range of the Pickering emulsions with a highly monodisperse size distribution and mean droplet sizes ranging from micron to millimeter (Arditty et al., 2003, 2004). For the emulsions following limited coalescence, the surface particle coverage (C%) can be directly deduced from the following equation (assuming all the particles are adsorbed at the interface; Destribats et al., 2011):

$$\frac{1}{d_{3,2}} = \frac{S_{eq}}{6CV_d} \ ,$$

where $d_{3,2}$ (specifically in 1% SDS) is the surface-averaged droplet size, S_{eq} is the interfacial area covered by the proteins at the equatorial section, and V_d is the oil volume. If we plot the

average inverse droplet diameter ($1/d_{3,2}$) against ($\frac{S_{eq}}{V_d}$), the parameter C (%) can be directly obtained from the slope of the experimental curve $\frac{1}{d_{3,2}} = f(\frac{S_{eq}}{V_d})$ (Destribats et al., 2011). Suppose that all the particles at the interface of one droplet are evenly distributed in a 2-D square area with a square or regular hexagonal array, we can deduce the particle center-to-center distance at interface ($D_{c\text{-}c}$) as following:

$D_{c-c} = \sqrt{\frac{\pi}{4C}}\, d_H$ (square 2D-array)

or

$D_{c-to-c} = \sqrt{\frac{\sqrt{3}\pi}{6C}}\, d_H$ (regular hexagonal 2D-array)

where d_h the hydrodynamic diameter of the particles.

From the plots of the inverse droplet diameter ($1/d_{3,2}$) against ($\frac{S_{eq}}{V_d}$) (**Figure 20**), we obtained the C% values for different nanoparticles from unheated and heated SG, and found that considerable differences existed between the unheated and heated SG nanoparticles (**Table 4**). The C% of unheated SG nanoparticles was approximately 7 times a theoretical value based on hexagonal array of particles at interface without deformation (Destribats et al., 2014). The reasonable explanation for this is that a multilayer of nanoparticles other than a monolayer was formed at the interface of oil droplets, as illustrated in **Figure 21 A**. Interestingly, the C% of heated SG nanoparticles (at 90 ºC) is basically the same as the theoretical value (~90%) of a hexagonal 2D-array for the particle packing at the interface, and in this case, the calculated $D_{c\text{-}to\text{-}c}$ with the hexagonal array model was also more (but insignificantly) close to that of the hydrodynamic diameter of the particles (**Table 4**), suggesting that the nanoparticles was packed in monolayer at interface, with a hexagon 2D-array way (**Figure 21 B**). The situation for the heated SG nanoparticles at 100 ºC is slightly different from that at 90 ºC, in which the nanparticle packing at interface could be more appropriately described using a square array model with a lower C% (**Table 4** and **Figure 21 C**). The differences in nanoparticle packing at

interface between different heated SG samples might be largely due to the differences in surface properties, especially charge density. The stronger inter-particle electrostatic repulsion would be more favorable for the particle packing at interface with lower interfacial coverage. This argument is indirectly corroborated by the microscopic observations that upon emulsification, soft uncharged microgels made of NIPAM formed a monolayer with a hexagonal 2D-array (Destribats et al., 2011, 2013).

We also evaluated the influence of electrostatic screening (with NaCl addition) on the surface coverage (C%) and $D_{c\text{-}to\text{-}c}$ of SG nanoparticles (induced by heating at 100 ºC), and interestingly observed that the presence of NaCl (50-500 mM) remarkably decreased the C% (from ~89% to 36%), and concurrently, the $D_{c\text{-}to\text{-}c}$ increased accordingly, irrespectively of the applied array models (Liu & Tang, 2014 c). Accordingly, the emulsification performance at any test *c* value (0.5-2.0%, w/v) progressively increased with increasing the extent of electrostatic screening. This observation indicated that under reduced electrostatic repulsion conditions, much less particles were needed to provide a full stabilization of the interface. The underlying mechanism for this improvement might be associated with the occurrence of bridging flocculation, and to a less extent, enhanced conformational flexibility of the nanoparticles, but needs to be confirmed in the future.

UNDERSTANDING THE ROLE OF CONFORMAITONAL FLEXIBILITY IN THE EMULSIFYING AND INTERFACIAL PROPERTIES OF SOY PROTEINS

Conformational flexibility: Definition and characterization

Although it is well recognized that the conformational flexibility, sometimes also referred to as conformational rigidity, of a protein, plays a crucial role in its emulsifying and interfacial properties (Damodaran, 1996, 1997), there is no discernible definition for the concept ‘conformational flexibility’.). The flexibility of a protein can be defined as the relative movement of various domains in the protein or the reorientational relaxation rates of amino acid residues in its polypeptide chain (Damodaran, 1997). The conformation of a protein, e.g., in the solution,

generally corresponds to a state of various domains, or amino acid residues in its polypeptide chains, associated in a certain (and possibly, complex) manner. According to a specific meaning of the state, the conformations can be understood in terms of secondary, tertiary or quaternary levels for the proteins. For example, for a protein with only one polypeptide, the conformational flexibility at the tertiary level may reflect the ease of their tertiary conformational changes, from an initial state to another one, when they suffer a change in a physicochemical environment (e.g., pH and denaturing agent), or other stresses. When referring to a protein consisting of several polypeptides, e.g., 7S or 11S globulin, the situation is more complex. In this case, the conformational flexibility of one polypeptide will be greatly restricted by the presence of other polypeptides. Thus, the flexibility of a multi-polypeptide protein will involve two levels of conformational changes: tertiary and quaternary (the contribution of the conformational changes at the secondary level can be considered to be minor). In usual, for oligomeric globulins the conformational changes at the tertiary level occur, only when the molecules are dissociated into subunits, to a certain extent.

There is another situation often occurring in plant proteins, e.g., SPI or SPC, in which the proteins are usually present in the aggregated state. This situation is also applicable to other proteins when they are subject to thermal or other denaturing treatments. Herein, we must note that for the aggregated proteins, the physicochemical properties (e.g., particle size and low solubility) rather than the conformational flexibility will dominate their functionalities, especially surface-related functionalities. In fact, we can still understand the conformational flexibility of these aggregated proteins, in a similar way to that of unaggregated proteins (e.g., monomeric or oligomeric globular proteins), if a conformational state beyond the quaternary level is introduced. There are basically two kinds of aggregated proteins that consist of partially and completely denatured proteins, respectively. For the former, the partially denatured proteins are usually associated together, mainly through non-covalent intermolecular interactions, and thus, less energy is needed for the dissociation of the aggregated proteins into ‘native proteins’.

Whereas in the case of highly denatured proteins, the global structure of the aggregated proteins would be maintained by strong intermolecular interactions, including covalent crosslinks like disulfide bonds. Furthermore, we should also note that once a protein is partially or completely denatured, its surface properties considerably vary, and it is very probable that the enhanced surface hydrophobicity of these aggregated proteins might be favorable for their surface functionalities, due to fast adsorption and Pickering stabilization.

The conformational flexibility of proteins is a relative concept, which is meaningful only when the conformational changes of two or more different proteins, or the conformational changes of one specific protein between different conditions, are compared. For example, we can generally consider that the flexibility of β-casein (at tertiary conformational level) is greater than that of β-lactoglobulin (monomeric). There are several techniques to successfully detect the conformational changes of proteins in the solution, upon changes in environmental conditions (e.g., pH, μ and addition of protein perturbants), or even chemical and physical treatments (e.g., heating or high pressure treatment), including spectroscopic techniques and thermal analyses (especially DSC), and even chromatographic techniques (Gorinstein et al., 1996; Schwenke et al., 2001; Subirade et al., 1994; Tang & Shen, 2013). Based on the fact that the proteins with greater flexibility are usually more easily digested, Kato et al. (1985) applied the susceptibility of various proteins to protease digestion as an indication of relative conformational flexibility, and observed good relationships between the foaming power or emulsifying ability and the digestion velocity of proteins. The susceptibility of globulins to protease digestion was reported to be closely related to their protein denaturation that can reflect, to a large extent, the conformational stability of the proteins (Gorinstein et al., 1996).

The conformational state of a protein is highly dependent on the applied concentration in the bulk, the nature of the protein, and even environmental parameters, e.g., μ and pH. This may be one of the reasons causing the difficulty in defining and understanding the conformational flexibility of proteins. In many cases, the changes in surface hydrophobic/hydrophibilic

properties (and solubility) of proteins can be understood as a result of the changes in their conformational changes. For example, when an oligomeric globulin is dissociated into separate subunits or polypeptides, the H_o of the globulin will be expected to increase, due to increased exposure (to aqueous phase) of hydrophobic clusters initially buried within the interior of the molecules; in this case, the emulsifying properties of the protein are also expected to be improved. From the conventional viewpoint, the improvement of the emulsifying properties of a protein would be largely attributed to the enhanced H_o, but in fact, the enhanced H_o is just an indication for the increased conformational flexibility of this protein particularly at the quaternary level. Thus, the conformational flexibility might play a more crucial role in the emulsifying properties of proteins than as expected.

Role of conformational flexibility in interfacial adsorption of globular proteins or aggregated proteins

When a globular protein adsorbs at the O-W interface, it may suffer a change in conformation from native state, with extent of the changes depending on its conformational flexibility. If the conformation of adsorbed proteins, e.g., tertiary conformation for monomeric proteins, is highly flexible, the adsorbed proteins will readily become unfolded and spread at the interface (to cover the interfacial area as much as possible). The conformation state of proteins adsorbed at the interface is difficult to be characterized and understood. However, there are increasing evidences to support the viewpoint that in most cases, the conformation state or conformational flexibility of proteins is similar between in the solution (unadsorbed) and at the interface (adsorbed). To better understand the structure and dynamics of proteins at the interface, for example, Dickinson & Matusumura (1994) introduced the concept of ‘molten globule state’ (which has been well confirmed in the solution for a protein with similar secondary structure but different tertiary conformation, as compared with its native one) to describe the state of an adsorbed globular protein at the liquid interface. It is generally recognized that a protein with higher conformational flexibility or greater ease of conformational changes in solution exhibits

better interfacial and emulsifying properties (Tang & Shen, 2013).

On the other hand, we have known that 1) the conformational state of proteins is highly dependent on its concentration in the aqueous solution; 2) the adsorption behavior of proteins at the interface is also affected by the protein concentration; 3) there are close relationships between the structural characteristics of proteins in solution and properties of adsorbed interfacial films; 4) soy proteins mainly consist of globular proteins, and their properties and structural characteristics are highly variable with their composition, processing history and even environmental conditions. At low concentrations, the adsorption of the proteins at the interface is relatively slow, and there is more space or time for the initially adsorbed proteins to spread out at the interface and change their conformations (to cover maximal surface area) (Dickinson, 1999; Wüstneck et al., 1999). In this case, the conformational flexibility or ease of conformational changes of the proteins may determine their unfolding and arrangement at the interface, and even the properties of formed interfacial films. At high concentrations, the proteins would be fast adsorbed at the interface, and packed not in an optimal way, and as a consequence, the unfolding and further structural arrangement of the proteins at the interface might be greatly restricted, even from the very beginning of adsorption. Despite of the restriction, the lateral interactions between adsorbed proteins may still be strengthened due to close packing and formation of multi-layers at the interface.

Globular proteins can be present in various forms depending on their associated state and extent of denaturation and/or aggregation, including monomeric, dimeric or oligomeric (trimeric and hexameric) and aggregated (partially or completely denatured). The role of conformational flexibility in the adsorption behavior of the globular proteins with different forms can be approximately elucidated as follows (as illustrated in **Figure 22**):

(a) *Monomeric globular proteins*: Most of the knowledge about the interfacial properties of proteins in the literature is based on this kind of globular proteins, e.g. BSA, monomeric β-lactoglobulin and ovalbumin. Due to unlimited restrictions of quaternary conformation,

these proteins can be readily adsorbed and packed at the interface, and effectively stabilize the interface. Thus, it is generally recognized that these proteins exhibit good interfacial and emulsifying properties. At low concentrations, these proteins adsorbed at the interface are allowed to fully unfold and rearrange their conformations, and as a result, a well-organized monolayer of proteins will be favorably formed. By comparison, multilayers of proteins at the interface will be favorably formed at high concentrations (**Figure 22 (a)**). In a recent work, we have successfully elucidated the importance of conformational flexibility (at the tertiary level) to the different aspects of adsorption of BSA at the interface, and interestingly indicated that at a very low concentration (e.g., 0.01%, w/v), increasing flexibility led to improved adsorption of BSA, mainly by means of enhanced initial adsorption (at the interface) as well as ease of structural arrangement (Tang & Shen, 2014).

(b) *Oligomeric globular proteins (trimeric 7S)*: The adsorption behavior of trimeric globular proteins (7S) at the interface seems to be more dependent on the concentration and their conformational state than that of monomeric globular proteins. For these proteins, the adsorption behavior seems to be predominantly determined by the conformational flexibility at the quaternary level. In another recent work of ours, we found that at a concentration of 0.5% (w/v), increasing flexibility, especially at quaternary level, progressively improved the emulsifying ability of phaseolin (an ideal 7S globulin from kidney bean), mainly by means of improved structural arrangement of adsorbed proteins (Liang & Tang, 2013). This has been also confirmed in the work comparing the emulsifying and structural properties of different vicilins (trimeric 7S/8S globulins; Tang & Sun, 2011). At low concentrations the proteins would be preferentially adsorbed at the interface with three subunits in contact with the interface (if not, the strong affinity and enough interfacial space allow them anchor at the interface, in the optimal manner). In this case, the conformational flexibility at the quaternary level might largely determine the fast and even coverage of interfacial area. If the trimeric globular proteins are readily dissociated (once adsorbed at the interface), their adsorption

behavior at low concentrations will be similar to that of monomeric proteins. This might be one of the reasons for observations that at low concentrations, many vicilins (7S globulins) exhibit comparable and even better emulsifying ability than caseins and albumin (Rangel et al., 2003). By comparison, at high concentrations the adsorption of these proteins at the interface is fast, and the restrictions of quaternary conformation will limit their anchoring and ordered packing at the interface. Even for the proteins with good quaternary conformational flexibility, a much prolonged period will be needed for the full arrangement of adsorbed proteins (directly at the interface) to form ordered interfacial films (**Figure 22 (b)**).

(c) *Oligomeric globular proteins (hexameric 11S)*: In contrast with trimeric globular proteins, the unfolding and arrangement of hexameric globular proteins adsorbed at the interface are much more limited, due to strong intra-molecular attractive interactions. Larger sizes of these globular proteins also lead to slower adsorption at the interface, and decrease the efficient coverage of interfacial area (relative to trimeric ones). Another point needs to be to note that for most of 11S globulins (including those from soybean), they tend to aggregate at high concentrations, which will further limit their anchoring and arrangement at the interface (**Figure 22 (c)**).

(d) *Aggregated globular proteins (partially denatured)*: The situation for the adsorption behavior of these aggregated proteins is basically similar to that of hexameric globular proteins. In usual, much more amount of the proteins is needed to fully cover the interfacial area than those with small sizes (e.g. monomeric globular proteins; **Figure 22 (d)**). Even at high concentrations, their adsorption rate or emulsifying ability is greatly limited by the large sizes. However, when adsorbed at the interface, they tend to form thick interfacial layers that will provide a good steric stabilization for the emulsions.

(e) *Aggregated globular proteins (completely denatured)*: When globular proteins are completely denatured, they tend to be present in the aggregated form with strong intermolecular interactions. For example, covalent disulfide bonds are often involved in the

thermally-induced aggregate particles from soy proteins and whey proteins (Liu & Tang, 2014; Schmitt et al., 2010). Besides the higher surface hydrophobicity, the surface hydrophilicity of these particles may also be strengthened (relative to their original proteins), thus exhibiting a different adsorption behavior from the aggregates from partially denatured proteins. Once adsorbed at the interface, these particles with a strong internal structure do not undergo a distinct change in the structure (or conformation), and more importantly, there may be strong lateral electrostatic repulsion between adsorbed particles. As a consequence, a monolayer even with a relatively low interfacial coverage is usually formed, which seems to be slightly dependent on the particle concentration (**Figure 22 (e)**). In this case, increasing the concentration is favorable for the fast adsorption at the interface and stabilization of interfacial area, but does not change the structure of interfacial films.

No matter what levels of conformational changes occur at, it can be generally considered that the global flexibility of globular proteins decreased in the order: monomeric > dimeric > trimeric > hexameric > aggregated (partially denatured) > aggregated (completely denatured). If only the emulsification performance of soy proteins is considered into account, which is generally evaluated at a low concentration, it will follow the same order as the global flexibility. For example, Liu et al. (1999) observed that the acidic subunits (AS11S; a dimer species) from soy glycinin exhibited a much better adsorption behavior at the O-W interface, as well as higher emulsifying ability than soy glycinin. The improvement in adsorption and emulsifying ability of soy glycinin can be well explained in terms of increased conformational flexibility, especially at the quaternary level. In contrast with this, the situation at high concentrations seems to be a bit different, wherein the interface is saturated by the proteins, at a much faster rate, and the unfolding and arrangement of adsorbed proteins are limited. In this case, the emulsification performance of the proteins may be largely determined by the rate of diffusion or rate of adsorption at the earliest periods. The surface hydrophobicity and size, rather than the conformational flexibility, of the proteins would become crucial parameters determining their

emulsification performance.

On the other hand, the properties and structure of interfacial protein films for soy proteins are also dependent on their initial concentration and conformational flexibility. In general, the unfolding and arrangement of adsorbed proteins more readily occur for the proteins with greater global flexibility, and the correspondingly formed interfacial films exhibit higher elasticity and/or viscoelasticity. From this viewpoint, it can be reasonably concluded that increasing the global flexibility is favorable for the emulsion stability of soy proteins. Herein, however, another issue should be considered that besides the rheological properties of interfacial films, the stability of the emulsions stabilized by soy proteins is also related to the thickness, or steric hindrance of their interfacial films. The steric stabilization, also called as 'Pickering stabilization', of particles of globular proteins, generally increases with increasing their particle sizes, which is on the contrary reversal to the order of global flexibility (see above). Although the aggregated particles of completely denatured soy proteins are rigid, they exhibit excellent stability against coalescence.

Understanding the modifications in emulsifying properties soy proteins: An insight from conformational flexibility

The investigation of structure-functionality relationships of food proteins is always the subject of researches in the food science field during the past decades. There are several conventional strategies available to increase or decrease the surface functionalities, and specifically emulsifying properties, of food proteins, e.g., i) to modify surface charge density of proteins, ii) to modify surface hydrophobicity; iii) to modify molecular size (Nakai, 1996). These strategies can be achieved by a number of treatments or techniques, including physical, chemical, enzymatic and even genetic (Damodaran, 2005; Nakai, 1996; Schwenke, 2001). The classification of these strategies is not on a strict basis. In fact, even a simple physical treatment, e.g. heating, may lead to considerable changes in surface charge and hydrophobicity, as well as molecular size of proteins. The surface charge (and/or hydrophobicity) and molecular size are the

fundamental parameters of structural properties of proteins, that can be more easily understood and manipulated in studying their structure-function relationships, than molecular or conformational flexibility, though the latter structural factor may be more appropriate for their surface functionalities. This is supported by the fact that of all the investigated molecular characteristics of 19 different proteins, only the molecular flexibility was confirmed to indicate a close correlation with their surface activities at the A-W interface (Damodaran, 2005; Razumovsky & Damodaran, 1999).

Heating and high pressure treatments are two kinds of important physical modifications for food proteins. The heat-induced modifications involve structural changes at different conformational levels (secondary, tertiary and quaternary), depending on the conditions of heating, protein concentration, environmental conditions (e.g., pH, ionic strength), and even the composition and properties of the proteins themselves. This is the reason why this kind of modifications is essentially referred to as a trial-and-error approach (Damodaran, 2005). There are basically two situations occurring during the heating of globular proteins. In the first situation, the partial denaturation and/or structural unfolding of the proteins occur upon heating. The partially denatured and/or unfolded proteins, can be generally considered to exhibit enhanced global flexibility of the molecules (relative to native ones), which would be favorable for their emulsifying properties, especially the emulsification performance. However, when the extensive heating is performed, the proteins will completely unfold and denature, and as a result, the denatured proteins with high surface hydrophobicity tend to aggregate one another, and even form insoluble aggregates. The aggregation and/or polymerization of proteins, from the view of molecular flexibility, may impair their adsorption at the interface (see the above), and emulsifying properties. Similar modifications in structural and emulsifying properties of soy proteins, as affected by a number of factors, can also be observed in the case using high pressure treatments (Keerati-U-Rai & Corredig, 2009 a; Puppo et al., 2005; Tang & Ma, 2009; Wang et al., 2008). To date, few works tried to modify the emulsifying properties of soy proteins with

these physical treatments, in a way of monitoring the molecular flexibility.

Chemical treatments, e.g., by means of acylation and glycation on ε-amino groups of proteins, have been widely shown to effectively modify the emulsifying properties of soy or other legume proteins (e.g., Achouri & Zhang, 2001; Baniel et al., 1992; Fan et al., 2006; Franzen & Kinsella, 1976). Succinylation is more effective than acetylation in modifying the surface characteristics, e.g. the changes in charge from positive to negative for the former. Besides the charge, the acylation can lead to other intensive changes, e.g. hydrophobicity (especially when anhydrides of long chain carboxylic acids were applied) and molecular sizes (Nakai, 1996). In general, the acylation (especially succinylation) of soy or other legume proteins improves their solubility (e.g. at neutral pH), emulsifying properties (emulsifying ability and emulsion stability), which have been largely attributed to the changes in their surface characteristics (Franzen & Kinsella, 1976; Schwenke, 2001; Yin et al., 2009). Succinylation and/or acetylation usually results in a stepwise dissociation of 11 S/7S globulins into smaller sizes of subunits (Barman et al., 1977; Knopfe et al., 1998). The acylation-induced dissociation is an indication of remarkable increase in quaternary conformation flexibility of the proteins. Thus, the improvement of the emulsifying properties of these proteins by the acylation can be largely ascribed to the enhanced flexibility of the molecules, especially at quaternary level. Another point of concern is that, sometimes, the acetylation at high extents of modifications may cause aggregation of the proteins, due to markedly enhanced intermolecular attractive interactions (Sckwenke, 2001), which is supposed to be unfavorable for their interfacial and emulsifying properties. This may to a great extent explain why, in some cases, the succinylation rather than acetylation remarkably improve the emulsifying properties of soy or other legume proteins (Franzen & Kinsella, 1976; Yin et al., 2009). In fact, the differences in modifications between succinylation and acetylation indirectly support the above argument that the conformational flexibility plays a crucial role in the interfacial and emulsifying properties of soy proteins.

Glycosylation with reducing carbohydrates or sugars is another effective chemical treatment

to modify the emulsifying properties of food proteins, including soy proteins, based on the fact that the glycosylation affects the hydrophilic/hydrophobic balance and favors the water-protein interactions (Achouri et al., 2005; Liu et al., 2012; Oliver et al., 2006). The modifications of the emulsifying properties of food proteins are highly variable depending on the type and amount of applied reducing agents, the extent of modifications, and even the nature of the proteins. No matter what these affecting factors, it can be generally recognized that for the proteins with low solubility or large molecular sizes, the glycosylation can increase their solubility, and concurrently, their emulsifying properties (Baniel et al., 1992; Oliver et al., 2006). The improved solubility is indicative of dissociation and/or structural unfolding of protein molecules, thus suggesting that in this case, the improvement of emulsifying properties might be largely attributed to the changes in molecular flexibility. In fact, this can be supported by the fact that having higher flexibility (mainly at the quaternary level), the SC with a certain extent of glycation itself exhibits much better emulsification performance than the SG. In a recent work investigating the influence of glycation on the emulsifying properties of phaseolin (a typical 7S globulin), it has been further confirmed that besides surface properties (e.g., PS and H_o), the flexibility in tertiary and/or quaternary conformations plays a major role in the emulsifying properties of glycated vicilins (Tang et al., 2011).

Enzymatic hydrolysis with different kinds of proteases is one of the most promising approaches to improve the emulsifying properties of proteins. It is well recognized that only limited enzymatic hydrolysis improves the emulsifying properties of soy proteins, including SPC and SPI (Jung et al., 2005). The improvement of emulsifying properties is largely associated with the increased solubility and H_o. If the comparison of emulsifying properties is performed on the untreated SPI, and their hydrolysates (obtained after a centrifugation to remove the insoluble), a contrary result will be often observed (Achouri et al., 1998; Tsumura et al., 2005). Similar phenomena have been observed in the case of modification of the 11S globulins by limited proteolysis, wherein it has been suggested that the presence of a high portion of undigested core

(denoted as ‘glycinin-T’ or ‘legumin-T’ which still maintains a similar quaternary structure of the native molecules) is a perquisite for the improvement of emulsifying properties (Schwenke, 2001). It is even stated that both the released water-soluble peptides and the undigested cores might exhibit a co-operative effect in the forming and stabilizing the emulsions. In this situation, the enzymatic breakdown of the protein molecules (or aggregated proteins) as well as the increased H_o of the resultant undigested ‘cores’ are indicative of the enhanced molecular flexibility (especially at the quaternary level) by the limited hydrolysis, further confirming the importance of conformational characteristics to the emulsifying properties of soy proteins.

CONCLUDING REMARKS

The fast growing interest and demand of consumers in soy protein formulations as an important part of health foods push both the food science scientists and industrial producers to utilize the potential functionalities of soy proteins as maximal as possible. The good emulsifying properties of soy proteins impart a high potential for them as a kind of food-grade emulsifiers to be formulated into a wide range of emulsion-based foods or food ingredients. Despite the fact that the emulsifying properties of soy proteins have been studied for several decades, their structure-functionality relationships, molecular mechanism for the emulsification, as well as properties of the correspondingly formed emulsions are still far away being well understood. This greatly limits the scope of the applications for soy proteins in the food or other fields. This review provided an extensive and critical insight into the emulsifying and interfacial properties of soy proteins, with an emphasis on the importance of conformational flexibility to their emulsifying properties.

Consistent with the fact that the structural characteristics of soy proteins are highly variable depending on a stream of intrinsic and extrinsic factors, such as composition and properties of starting materials (and proteins), history of processing or treatments, and environmental conditions (e.g., pH, ionic strength and temperature), the emulsifying properties of these proteins also considerably vary. These proteins, especially those in SPI, exhibit a high tendency to

aggregate, thus basically resulting in limited solubility and poor emulsifying properties, from a conventional viewpoint that good solubility is necessary for the proteins to show good emulsification performance. However, if from another viewpoint of Pickering stabilization of particles for the emulsions, the insoluble nature of soy proteins on the contrary imparts them a great potential in formulating a kind of novel food-grade Pickering emulsions with some unique characteristics, e.g. extraordinary stability against coalescence and even creaming, and formation of emulsions with a gel-like structure. These findings may greatly advance the knowledge about the about the emulsification functions of soy proteins, thus providing a great potential for these proteins to be applied in novel food formulations.

In this review, an extensive understanding about the importance of conformational flexibility at different conformational levels to the emulsifying and interfacial properties of soy proteins has been provided. If the emulsification performance is a preferred parameter of emulsifying properties, soy proteins with high conformational flexibility, especially at the quaternary level, usually exhibit good emulsifying properties. The conformational flexibility plays a much more important role in the emulsifying properties of soy proteins than as expected. Thus, it would provide a novel strategy to modify the emulsifying properties of soy and other proteins, by monitoring the conformational flexibility rather than the conventional parameters, e.g., surface charge and/or hydrophobicity. The broad application of many advanced spectroscopic techniques to characterize the conformational changes of proteins can ensure the successful implementation of this novel strategy, though a big ongoing challenge still remains.

On the other hand, the insoluble nature of soy proteins imparts them a great potential to serve as a kind of Pickering stabilizers for oil-in-water emulsions. Based on the Pickering stabilization, a novel kind of soy protein-based emulsions or emulsion gels can be produced with some unique characteristics, e.g. as encapsulation and/or delivery systems of bioactive ingredients. The properties and microstructure of these colloidal systems can be well modulated by changing the particle concentration and/or oil fraction, and even the emulsification energy level. More

evidences have been available to indicate that soy proteins exhibit a higher potential than other animal or milk proteins in formulating novel colloidal systems to be applied in functional foods. However, many issues in this area related to soy protein emulsions should be further elucidated, such as structure-Picking stabilization function relationship of soy protein nanoparticles, the behavior of different soy protein emulsions or emulsion gels during *in vitro* or *in vivo* digestion, the stabilization and release behavior of heat-labile bioactives encapsulated in these colloidal systems, and effective ways to formulate soy protein Pickering particles.

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Table 1. Major protein fractions in soybean (Kilara & Harwalkar, 1996)

Fractions	Total protein (%)	Components	Molecular masses (kDa)
2S (α-Conglycinin)	22	Trypsin inhibitors	8~21.5
		Cytochrome *c*	12
7S (β- and γ-Conglycinin)	37	Hemagglutinin	110
		Lipoxygenase	102
		β-Amylase	61.7
		7S globulin	180-210
11S (glycinin)	31	11S globulin	350
15S	11		~600

Table 2. Representative works on the emulsifying properties of soy proteins

Protein type [a]	Emulsifying properties	Items characterized	Emulsifying conditions [b]	Main findings or conclusions	References
SPI, glycinin (SG) and β-congly cinin (SC)	Emulsifying capacity; emulsion stability	EC; emulsion stability (ES)	Stirring (16,000 rpm); ϕ = 0.35-0.7 (for ES); c = 0.2-1.2%; pH 2.0-10.0; μ (uncontrolled)	1) The EC of all samples is minimal at near *p*I, and increases as the pH deviates from the *p*I; 2) In general, SC shows higher EC and ES values than SG in the pH range of 2-10; 3) Increasing ϕ (at c=0.4%; from 0.4 to 0.7), or c (from 0.3 to 1.2%; at critical ratio of oil phase to water phase) progressively increases the breaking stress of the emulsions (SG and SC), with the SG emulsions more affected by the heating.	Aoki, Taneyama & Inami (1980)
Commer cial SPI	Emulsifying capacity; emulsion	EC; emulsion instability	Stirring (6000 rpm); ϕ = 0.5 (for	1) EC of these SPIs is maximum near the *p*I (except the hydrolysed	Elizalde et al. (1996)

	stability	(EI)	EI); c = 2.0 wt%; pH 2.0-7.0; μ (uncontrolled)	isolate); 2) Variation in pH affects the EC, by means of changing the hydrophilic/lipophilic balance; 3) The pH dependence of the EI is opposite to that of EC, which is closely related to the water adsorption capacity.	
Native SG + its acidic subunits (AS)	Emulsifying ability; emulsion stability	EAI; droplet size ($d_{3,2}$)	Sonication; ϕ = 0.166; c = 0.1-1.0% (w/v); pH 7.0; μ = 0.05-0.50	1) The AS exhibits much better emulsifying ability than native or heated SG; 2) The emulsions stabilized by AS are very stable upon storage, below 0.15 M ionic strength.	Liu, Lee & Damodaran (1999)
Native SC (or its subunits)	Emulsifying ability	Droplet size	Stirring (22,000 rpm); ϕ ~0.14; c = 0.5 g/L; pH 7.6 or 3.7; μ= 0.08-0.5	1) The emulsifying ability of SC or its subunits is highly related to the hydrophilic nature and composition of the subunits (e.g. presence of carbohydrate moieties); 2) The emulsifying ability at pH 7.6 is better than at pH	Maruyama et al. (1999, 2002 a, b)

				3.7.	
Native and modified SG	Emulsion stability	Flocculation -creaming rate constant; coalescence stability	Stirring (20,000 rpm); ϕ = 0.25; c = 0.3-3.0 g/L; pH 7.6; μ= 0.02-1.5	1) At high I (>0.2) increasing I progressively destabilizes the native SG emulsions; at specific I (0.02 or 0.5) increasing c gradually increases the emulsion stability; 2) Deamidated and reduced SG exhibits poor emulsion stability at high I.	Wagner & Guéguen (1999)
Commercial SPC	Emulsion stability	Droplet size; creaming stability	High pressure homogenization (70-80 MPa); ϕ = 0.1 or 0.2; c = 1-10 % (w/v); pH 7.5; μ= 0.1	The emulsions formed at high c and/or ϕ values show excellent stability against creaming, which is closely related to the formation of a gel-like network in the emulsions.	Roesch & Corredig (2002, 2003)
Native and denatured SPI (or whey soy	Emulsifying ability; Emulsion stability	EAI; Coalescence and/or flocculation percentage	Stirring (20,000 rpm); ϕ ~ 0.25; c = 0.1~1.0 % (w/v); pH 7.0; μ =	1) All soy proteins show similar EAI, but the emulsions stabilized by native SPI have highest coalescence stability; 2) Increasing I progressively	Mitidieri & Wagner (2002); Palazolo et al. (2003)

proteins)			0.02-0.17	decreases EAI of native SPI (prior to emulsification), as well as the coalescence stability; 3) Increasing *c* leads to enhancement of coalescence stability of all emulsions;	
Modified SPI	Emulsifying ability; emulsion stability	EAI; ESI	Stirring (13,500 rpm); ϕ = 0.25; *c* = 10.0 g/L; pH 7.0; μ (unspecified)	Both the acid and alkaline pH-shifting treatments markedly improve the emulsifying ability and emulsion stability of SPI, with highest extents of improvement observed at extreme pHs.	Jiang et al. (2009)
SG, SC	Emulsifying ability; Emulsion stability	Droplet size	Microfludization; $\phi \sim 0.1$; *c* = 0.5-3.0% (w/v); pH 7.4; μ= 0.1	1) A much higher amount of SG is needed to produce emulsions with small sizes and with slight changes in sizes (after 7-day storage) than SC; 2) Heating before emulsification greatly increases the SG emulsion sizes, while the size of the	Keerati-u-rai & Corredig (2010)

				SC emulsions is slightly affected.	
Native or heated and spray-dried SPI	Emulsifying efficiency; Emulsion stability	Droplet size	Microfludization; $\phi \sim 0.1$; c = 1.0-2.5% (w/v); pH 7.4; μ= 0.1	1) At a low c native SPI exhibits higher emulsifying efficiency than the heated one, while at high c values, their efficiency is similar; 2) Dialysis prior to drying greatly enhances the emulsifying efficiency of SPI; 3) The formation of heat-induced protein aggregates improves the emulsion storage stability.	Keerati-u-rai et al. (2011)
Non- and heat-treated SPI or soy globulins (SG + SC)	Emulsion stability	Droplet size; creaming behavior; microstructure	Microfludization; ϕ=0.2 (SPI) or 0.1 (SG+SC); c = 0.5-4.0% (w/v); pH 7.0; μ= 0~0.5 (SPI), or 0 (SG+SC)	1) Increasing the μ led to a progressive increase in extent of droplet flocculation of SPI emulsions, with higher increasing extent observed for heated SPI emlusions; 2) For heated SPI emulsions, increasing the bulk c was favorable for the creaming	Lou et al. (2013); Shao & Tang (2014); Tang & Liu (2013)

				stability; 3) Thermal denatured SG plays a vital role in the emulsion stability against flocculation and creaming.	
Heated SPI	Emulsifying efficiency; emulsion stability	Droplet size; creaming behavior; microstructure	Stirring, sonication or microfluidization; ϕ = 0.2-0.6; *c* =0.5-6.0% (w/v); pH 7.0; μ= 0.3	1) Increasing the *c* results in formation of emulsions with smaller droplet size and better stability against coalescence and creaming; 2) At a specific *c*, the creaming stability of the emulsions at higher ϕ is much better than at lower ϕ; 3) Increasing the energy input level of emulsification greatly improves the emulsifying efficiency, extent of droplet flocculation, as well as the emulsion coalescence and creaming stability.	Liu & Tang (2013, 2014)
Native and	Emulsifying efficiency	Droplet size	Stirring (10,000-22,0	1) Larger sizes of heated SPI exhibited higher	Cui et al. (2014)

heated SPI	or ability	00 rpm); ϕ = 0.1; c = 0.3% (w/v); pH 7.0; μ = 0-0.4	2) The emulsifying efficiency of heated SPI was related to the homogenization conditions and the μ.

[a] Symbols: SPI-soy protein isolate; SPC-soy protein concentrate; SG: soy glycinin; SC: soy β-conglycinin; EC-emulsifying capacity; EAI-emulsifying activity index; ESI-emulsion stability index (ESI);

[b] c: protein concentration in the aqueous phase; ϕ: oil fraction; μ: ionic strength. All the experiments were performed at 20-25ºC.

Table 3. Summary of mechanistic studies on adsorption and interfacial rheology of soy proteins at the oil-water (O-W) and/or air-water (A-W) interface.

Protein type [a]	Sample preparations	Items investigated	Test conditions [b]	Main findings or conclusions	References
SPI, SG and SC	Thanh & Shibasaki (1976)	Interfacial rheology at O-W interface	*c* (unspecified); pH 2.5-7.5; μ = 0 or 0.5	1) Surface rheological parameters (e.g. viscoelasticity) were maximum at around pI or in the presence of NaCl; 2) At any test pH the viscoealsticity of both SPI and SC is similar, and greater than that of SG; 3) The rate of droplet coalescence in the emulsions is closely related to the instantaneous surface elastic modulus of the interfacial films.	Rivas & Sherman (1984)
Native and modified SG	Thanh & Shibasaki (1976); dialysis prior to lypophilizat	Adsorption kinetics at A-W interface	*c* = 0.02-0.08 g/L; pH 8.0; μ =0.2	1) Modifications (dissociation, deamidation and reduction) greatly enhance the rate of adsorption at the initial process, but slightly affect the equilibrium surface pressure	Wagner & Guéguen (1995)

	ion			(π_e); 2) The adsorption of native SG is heavily affected by ionic strength, while the effect of ionic strength is limited for modified SG.	
Native SG+ Acidic subunits (AS)	Thanh & Shibasaki (1976)	Adsorption kinetics at A-W interface	c = 0.001 g/L; pH 7.0; μ= 0.1	Higher rate of adsorption and final surface pressure (π) for AS than native SG	Liu et al. (1999)
Native SG	Thanh & Shibasaki (1976); without drying	Adsorption kinetics at A-W interface; surface shear viscosity measurements	c = 0.01 or 0.1 g/L; pH 3 or 6.7; μ= 0.03	1) The SG in the 3S/7S form (at pH 3) adsorbs much faster at the interface than in the 11S form (at pH 6.7); 2) The network at pH 3 is more resistant to deformation than at pH 6.7.	Martin et al. (2002)
SC, SG, and reduced SG		Dynamic surface dilatational modulus (E) kinetics at	c = 0.001-1.0 wt%; pH 2.0, 5.0 or 8.0; μ= 0.1	1) The E increases with time, which has been related to protein adsorption; 2) The dilatational properties of the adsorbed films are	Patino et al. (2003, 2005)

		A-W interface; Surface visocoelasticity of adsorbed films		elastic in nature, depending on the molecular structure of the protein.	
SC, SG, reduced SG	ibid	Adsorption kinetics at A-W interface	c = 0.001~1.0 wt%; pH 2.0, 5.0 and 8.0; μ= 0.1	1) The adsorption increases with the concentration (c), depending on the protein and pH; 2) There is a lag period at low c values; 3) The initial adsorption is diffusion-controlled, while the penetration and unfolding of the protein controls the long-term adsorption	Patino et al. (2004)
SC and SG	Nagano et al. (1992); lyophilization without dialysis	Dynamics of adsorption (π or E) at A-W interface	c = 0.1 wt%; pH 5.0 or 7.0; μ= 0.05 or 0.5 M	1) The interfacial characteristics of these globulins are much improved at the high ionic strength; 2) There exist close relationships between foaming and the rate of diffusion of the	Ruíz-Henestrosa et al. (2007)

				globulins to the interface, and between the foam stability and the π or E at long-term adsorption.	
SPI	Obtained in a Pilot plant	Adsorption kinetics at A-W or O-W interface	c = 0.001-10.0 g/L (for A/W), or 0.00001-1.0 g/L (for O/W); pH 7.4; 0.05 M Tris buffer	1) At a low c (0.01 g/L) the SPI exhibits lower rate of diffusion and π (than purified SG or SC); 2) The SPI exhibits much higher interfacial affinity at the O/W interface than at the A/W interface; 3) The nanoparticles in SPI hardly unfold at the interfaces.	Santiago et al. (2008)
SG and its hyolysates	Nagano et al. (1992) for SG	Dynamics of protein adsorption at A-W interface	c = 0.1-1.0 wt%; pH 5.0 or 7.0; μ= 0.05 M	1) The adsorption dynamics of the SG and its hydrolysates depend on the pH; 2) The enzymatic hydrolysis much improves the interfacial characteristics of SG.	Ruíz-Henestrosa et al. (2009)
SPI (+ heated)	Obtained in laboratory; dialysis prior to lypophilizat ioin	Dynamics of protein adsorption at O-W interface	c = 0.001-1.0%, w/v; pH 7.0; μ= 0.01	1) The heat treatment at 120 ºC improves the adsorption of SPI at the interface; 2) Both the unheated and heated SPI show a maximum in E at c = 0.01%.	Wang et al. (2012)

Heated SPI	Obtained in laboratory; dialysis prior to lypophilizat ioin	Adsorption kinetics at O-W interface	*c* = 0.01-1.5%, w/v; pH 7.0; μ= 0.3	1) Increasing *c* progressively accelerated the initial diffusion and/or adsorption of heated SPI; 2) The penetration and subsequent rearrangement of adsorbed proteins is slightly dependent on the *c*.	Liu & Tang (2014)

[a] Symbols: SPI-soy protein isolate; SG: soy glycinin; SC: soy β-conglycinin;. [b] All the experiments were performed at 20-25ºC.

Table 4. Summary of surface coverage (*C*%) and particle center-to-center distance at interface ($D_{c\text{-}to\text{-}c}$) for the emulsions stabilized by the nanoparticles from unheated and heated (at 90 and 100ºC) SG preparations, as determined by limited coalescence process. Each datum is the means and standard deviation of duplicate calculations performed on different separate measurements. (Liu & Tang, 2014 b)

Nanoparticles	Hydrodynamic diameter (nm)	Surface coverage, *C* (%)	Particle center-to-center distance at interface ($D_{c\text{-}to\text{-}c}$) (nm)	
			Square array model ($D_{c-to-c} = \sqrt{\frac{\pi}{4C}}\, d_H$)	Regular hexagonal array model ($D_{c-to-c} = \sqrt{\frac{\sqrt{3}\pi}{6C}}\, d_H$)
Unheated SG	58.1±0.7	632±51	20±7	22±1
Heated SG (at 90 ºC)	102.3±6.1	94±17	93±1	98±8
Heated SG (at 100 ºC)	134.5±7.3	81±6	133±3	143±5

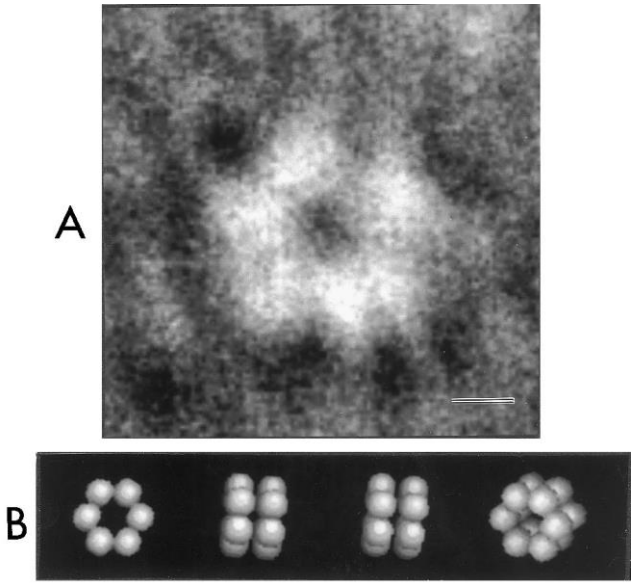


Figure 1. (A) Electronic micrograph of a single globulin complex exhibiting six stain-excluding regions and a central stain-filled depression or hole (protein white, stain dark). Scale bar represents 2.5 nm. (B) Three-dimensional model corresponding to symmetrized characteristic views of globulin derived by single particle analysis. (Marcone et al., 1994)

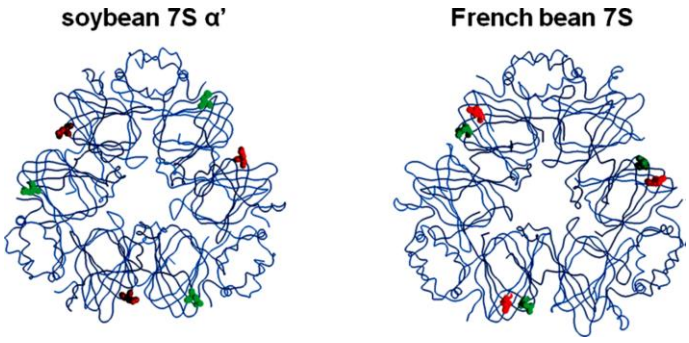


Figure 2. Comparison of the N-glycosylation sites of soybean 7S globulin *α′* and those of French bean 7S globulin. This figure was prepared by MOLSCRIPT and Raster3D. Carbohydrate moieties are colored by red and green. (Kimura et al., 2008)

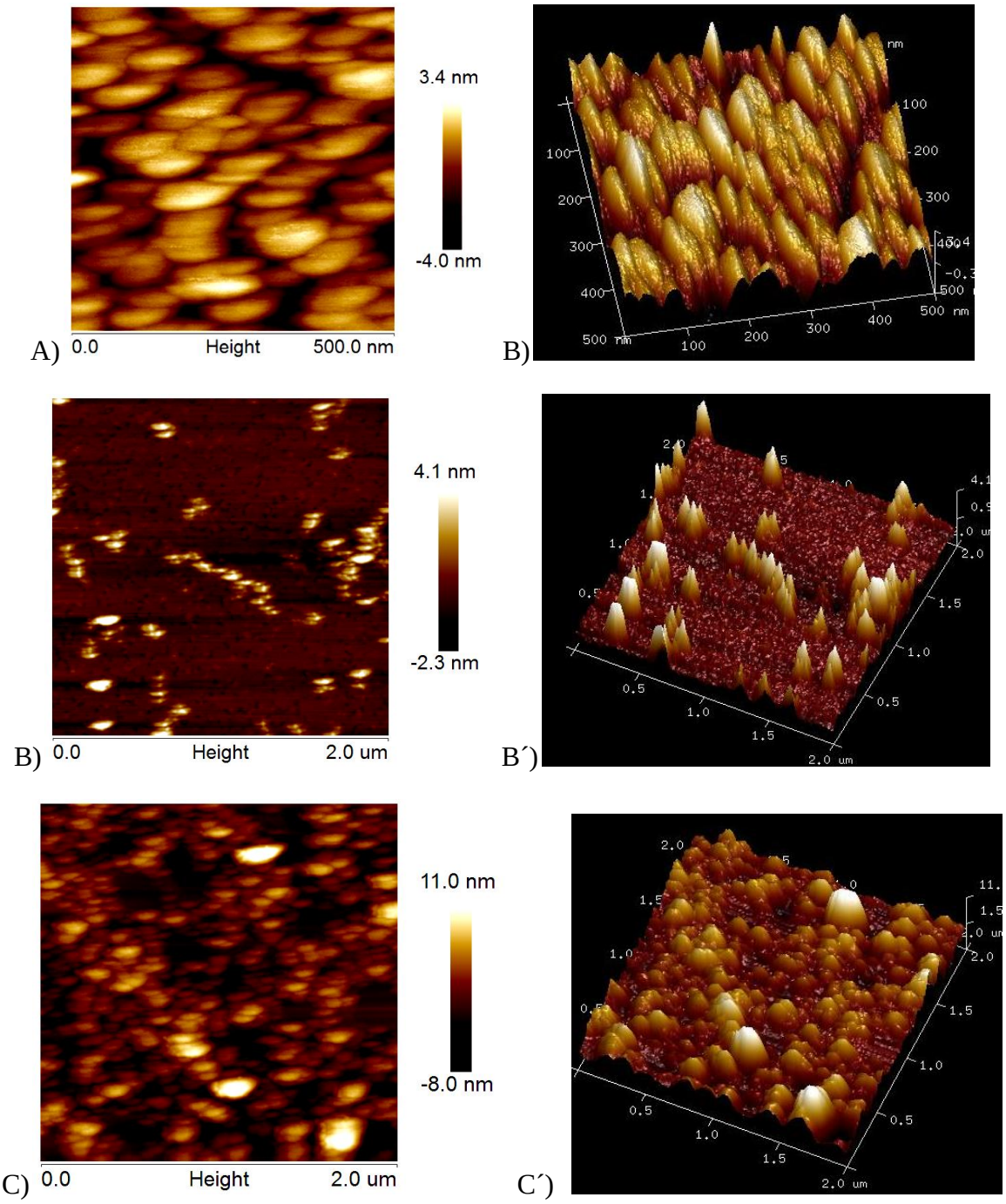


Figure 3. Typical top view (A-C) and 3D-view (A´-C´) AFM images of nanoparticles in non- and heat- treated soy glycinin dispersions in water (1%, w/v) at pH 7.0. Panel A and A´: non-treated SPI; Panel B and B´: SPI treated at 90ºC for 15 min; Panel C and C´: SPI treated at

100ºC for 15 min. (Liu & Tang, 2014 b)

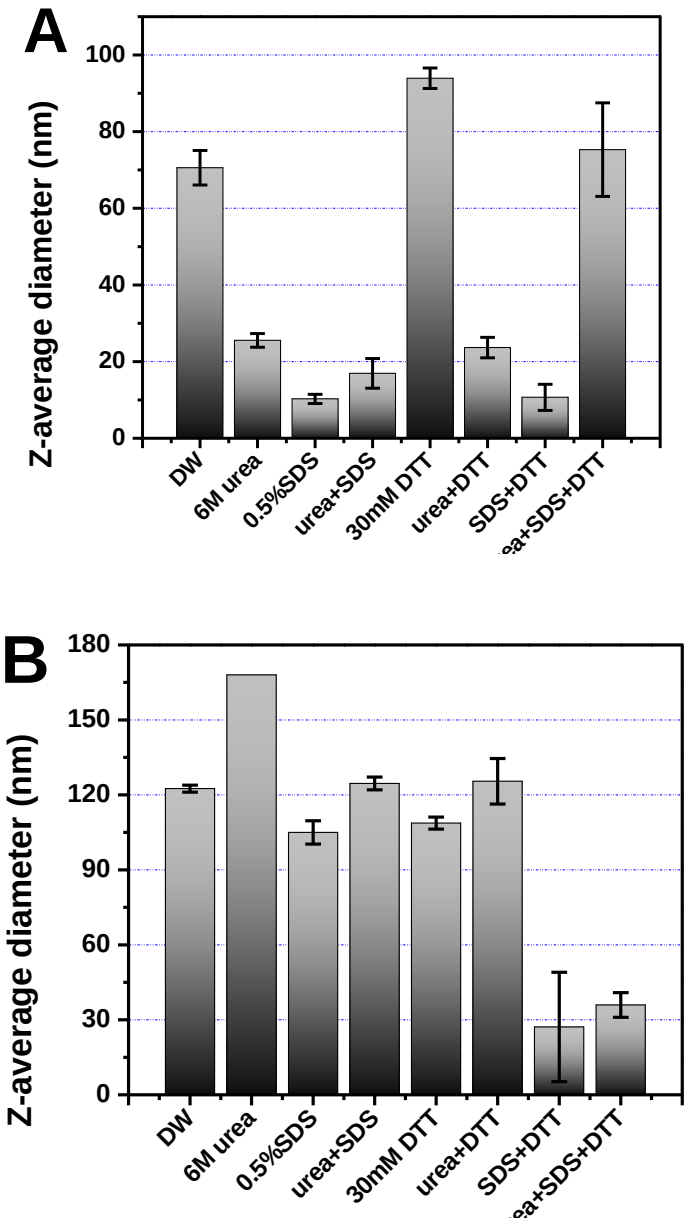


Figure 4. Influence of various protein-perturbing solvents on the *z*-average diameter (D_z) of nanoparticles in unheated (A) or 100^o C-heated (B) soy glycinin dispersions at pH 7.0. Each data is the means and standard deviation of duplicate measurements. (Liu & Tang, 2014 b)

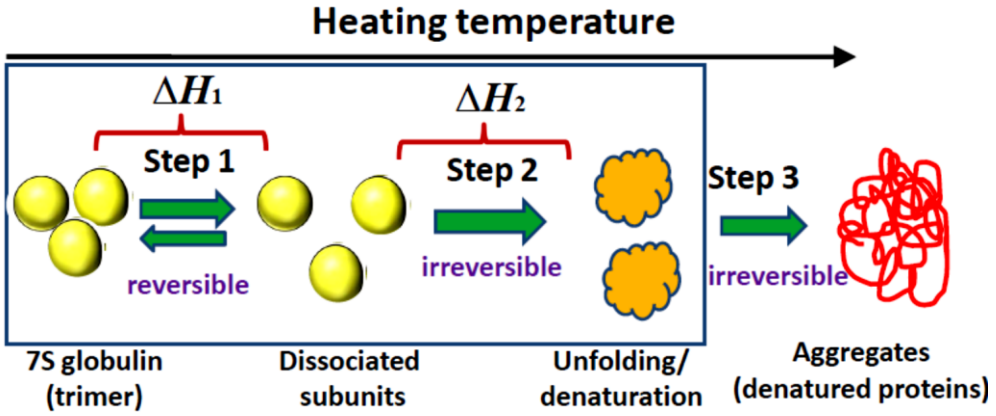


Figure 5. A typical illustration for the heat-induced denaturation and/or aggregation of 7S globulins (trimer), as detected by DSC. The thermal event consists of three main steps: 1) the disruption of 7S globulin (trimer) with quaternary structure into individual subunits; 2) further structural unfolding and denaturation of dissociated subunits; 3) aggregation of denatured proteins. The step 1 is reversible (the dissociated subunits can be re-associated to form the 7S form, if cooled), while the steps 2 and 3 are irreversible. In usual, the enthalpy change (ΔH), widely applied in DSC analysis to indicate the energy needed for the full protein denaturation, is contributed from two aspects: the energy needed for the quaternary structure dissociation (ΔH_1), and that needed for the structural unfolding of dissociated subunits (at the tertiary level; ΔH_2).

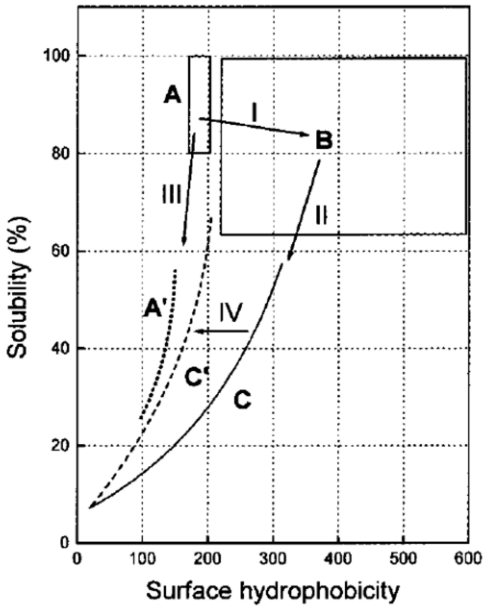


Figure 6. Laboratory and commercial SPI products sorted as a function of the relationship between water solubility and surface hydrophobicity (H_o). According to the solubility-H_o profiles, laboratory SPI products can be divided into three groups: (A) native with high solubility and relatively low H_o, (B) partially or totally denatured with high solubility and H_o, and (C) totally denatured with low solubility and H_o. The commercial SPI can be approximately subdivided into two groups: partially (A´) and totally denatured (C´). The processes I-IV represent: (I) denaturation; (II) aggregation-insolubilization; (III) application of a mild thermal treatment or spray-drying process itself (instead of freeze-drying process); (IV) the addition of phospholipids (Wagner et al., 2000).

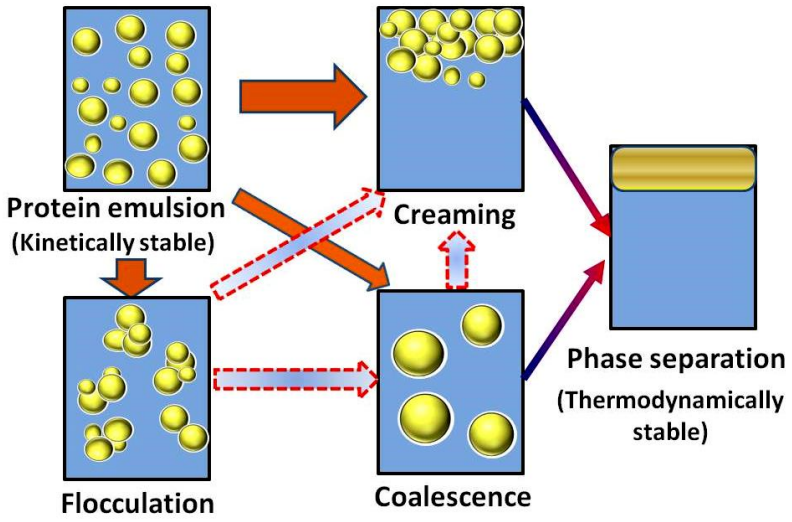


Figure 7. Schematic diagram of the main instability mechanisms that occur in protein-stabilized emulsions: flocculation, coalescence and creaming. In usual, the flocculation of oil droplets in the emulsions is favorable for the occurrence of droplet coalescence, and both flocculation and coalescence accelerate the process of creaming.

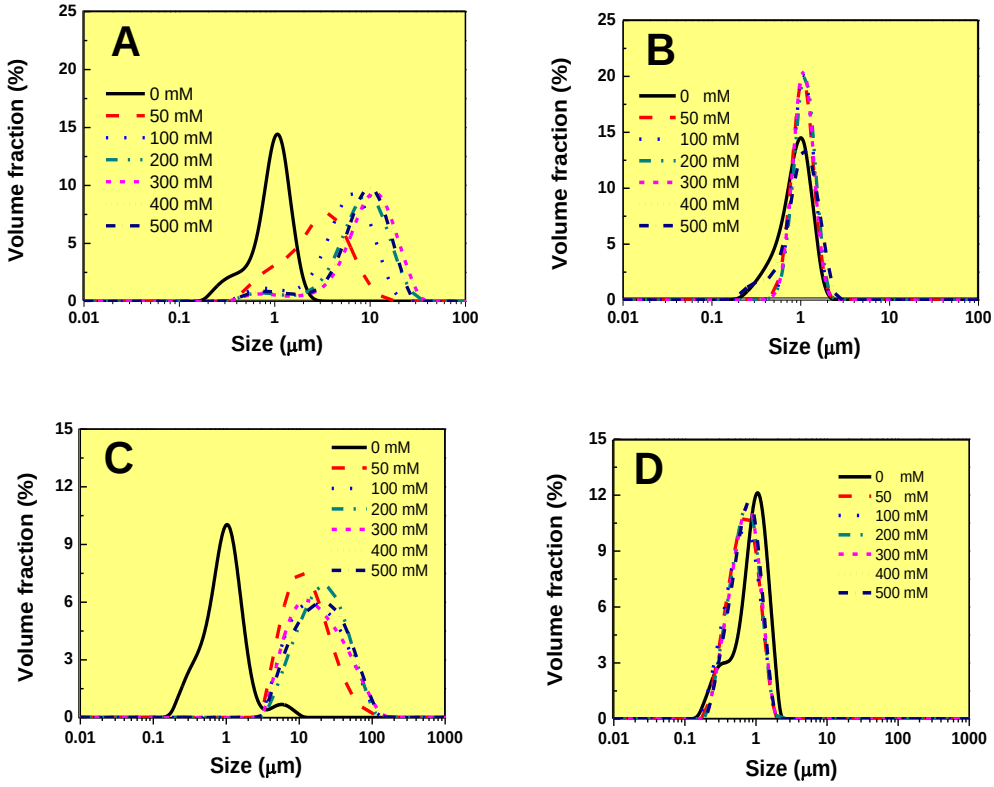


Figure 8. Size distribution profiles of freshly prepared emulsions (at pH 7.0) stabilized by untreated (A, B) and preheated (C, D) SPIs under various NaCl concentrations (0-500 mM). A and C: diluted in water; B and D: diluted in 2% SDS. The emulsions were obtained using a microfludization process as the emulsification technique. The heat pretreatment was carried out at 95ºC for 15 min. The protein concentration in continuous phase was 1% (w/v), and the oil fraction 0.2. (Tang & Liu, 2013)

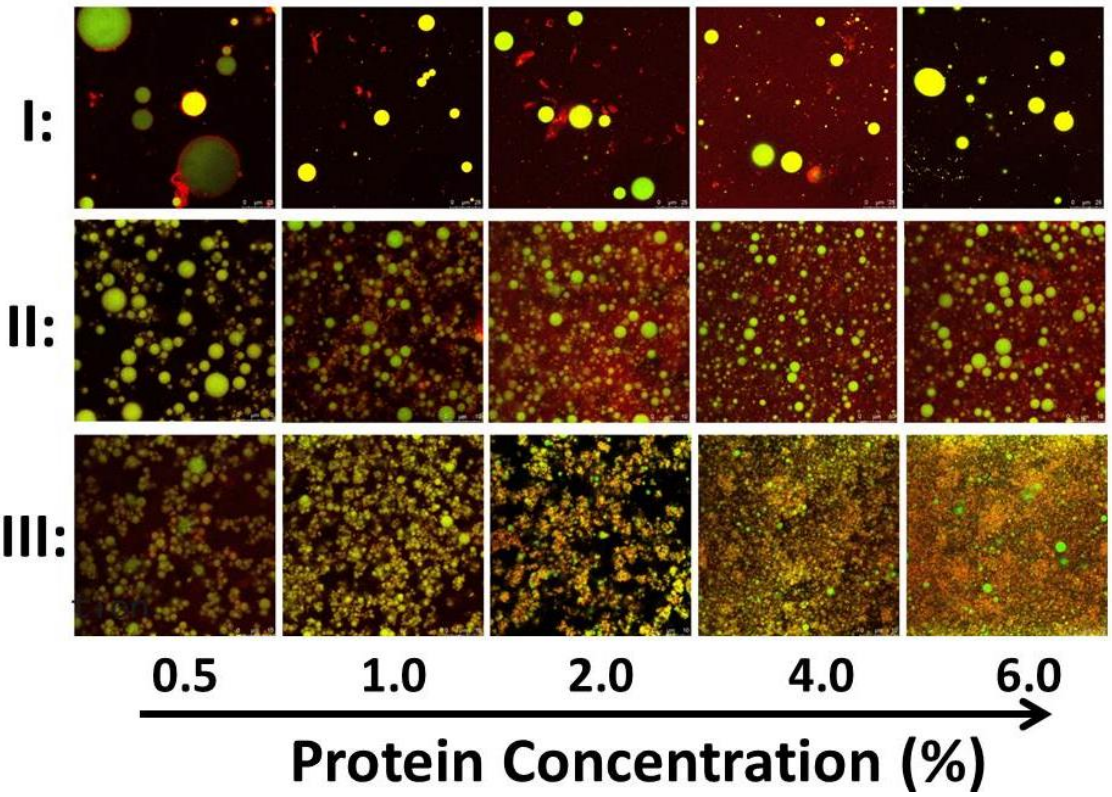


Figure 9. Typical CLSM images of the emulsions (I-III; at pH 7.0 and μ = 300 mM) stabilized by heated SPI at a comparable initial *c* of 0.5-6.0% (w/v). The emulsions (I-III) correspond to those produced by the three emulsification processes: (I) emulsification with an Ika T25; (II) emulsification with an Ika T25 in combination with ultrasonic equipment; (III) emulsification with an Ika T25 in combination with a microfluidizer. (Liu & Tang, 2014 a)

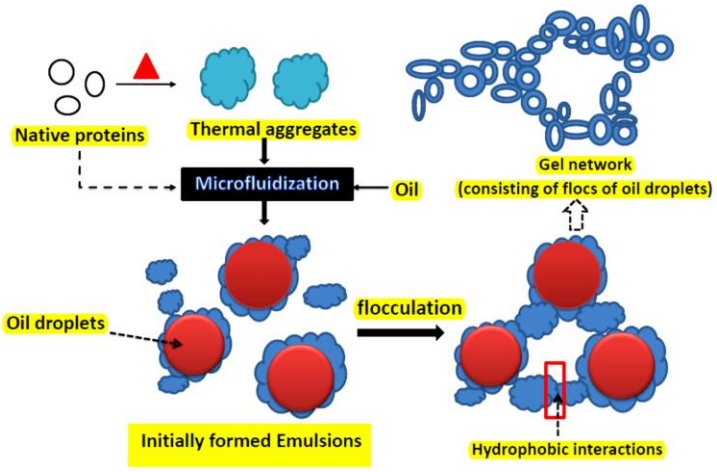


Figure 10. Proposed scheme for the formation process of a gel-like network structure in emulsions stabilized by preheated SPI, obtained by means of microfluidization. In this scheme, hydrophobic interactions between denatured proteins adsorbed on individual oil droplets play a vital role in the droplet flocculation, and subsequently, a gel-like network formation. (Tang & Liu, 2013)

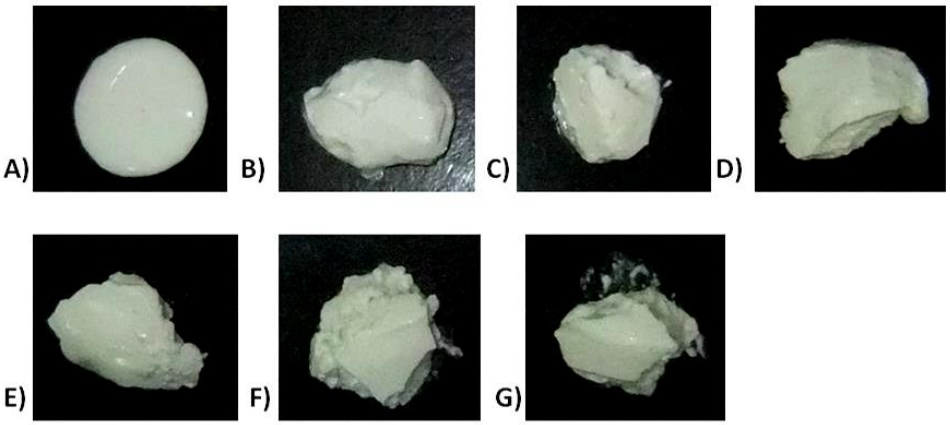


Figure 11. Visual observations of gel-like emulsions from preheated SPI at various NaCl concentrations of 0-500 mM. The protein concentration and ϕ were 6% (w/v) and 0.3, respectively. A-G present NaCl concentrations of 0, 50, 100, 200, 300, 400 and 500 mM, respectively. (Tang & Liu, 2013)

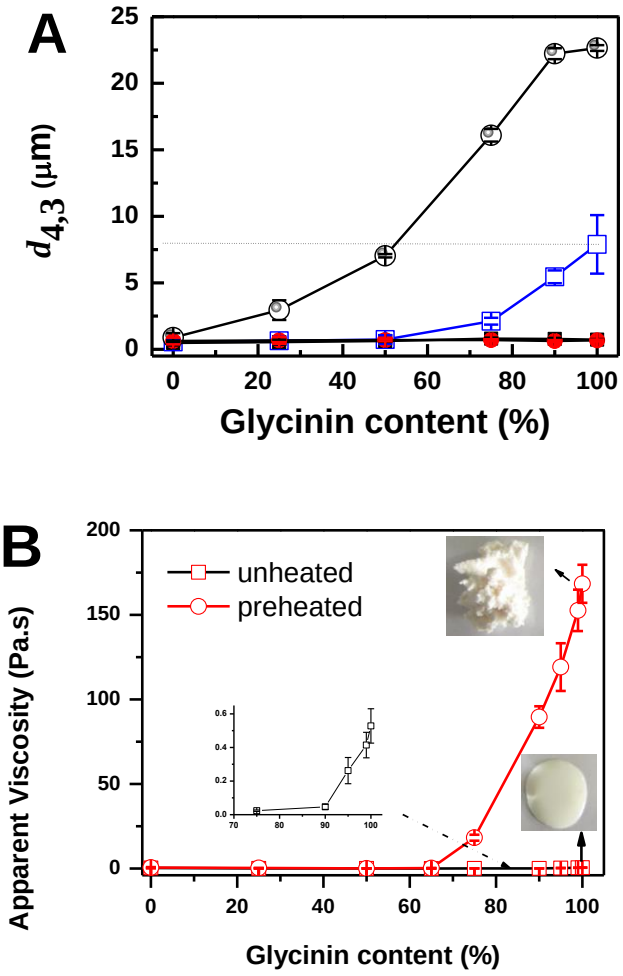


Figure 12. A) Glycinin content dependence of volume mean diameter ($d_{4,3}$) of unheated (□,■) and preheated (○, ●) soy globulin-stabilized emulsions (pH 7.0), determined with deionized water (hollow symbol) and 1% SDS (filled symbol) as the dispersing solvents. The emulsions were formed at c = 1.0% (w/v) and ϕ = 0.1, by means of microfluidization. The heat pretreatment was performed at 85 ºC for 15 min. Each data point consists of the means and standard deviations of at least duplicate measurements. B) The glycinin content dependence of apparent viscosity (η; at a shear rate of 2.3 s^{-1}) of unheated and preheated soy globulin-stabilized emulsions (at pH 7.0), formed at c = 6.0% (w/v) and ϕ = 0.3. Visual images of the emulsions stabilized by glycinin alone are also included in the figure. (Luo et al., 2013)

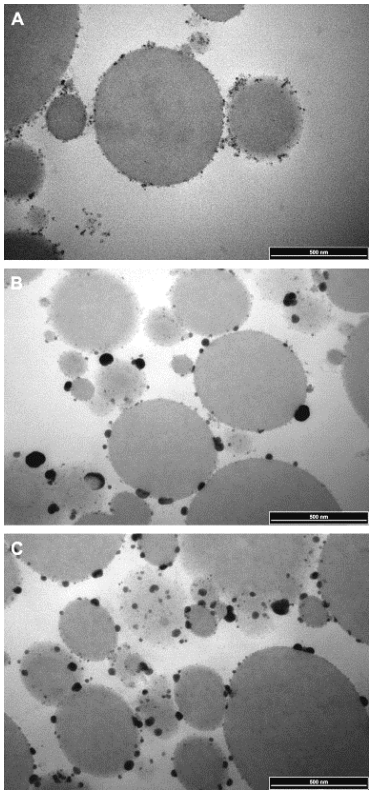


Figure 13. Transmission electron micrographs of SPI-stabilized emulsions (at pH 7.4), formed by a microfluidization as the emulsification technique at a bulk protein concentration of 1.0% (w/v) and oil fraction of 0.1. Panel A: unheated SPI; Panels B and C correspond to SPI heated at 75 and 95ºC for 15 min, respectively. (Keerati-u-rai & Corredig, 2009 b)

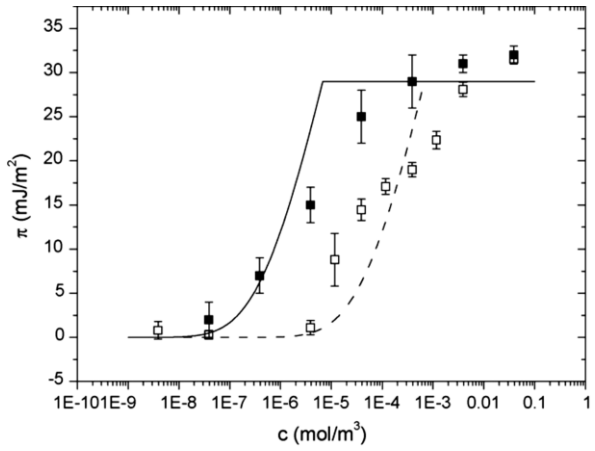


Figure 14. Adsorption isotherms of soy protein isolate at the air–water interface (□) and at the tetradecane–water interface (■). The surface pressure (π) at the interface was plotted against the bulk protein concentration (c). (Santiago et al., 2008)

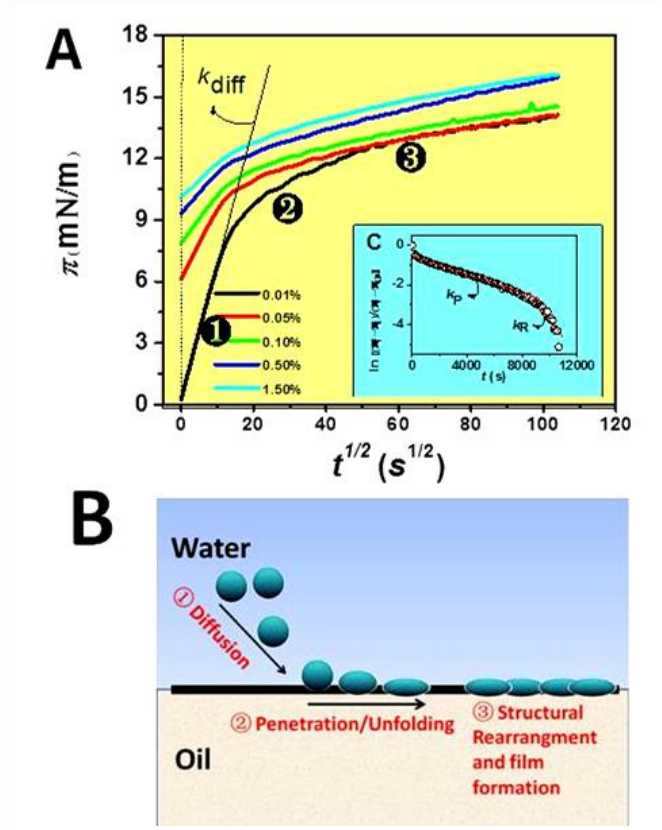


Figure 15. A) Time evolution of the surface pressure (π) for the adsorption of heated SPI at the O-W interface at *c* values of 0.01-1.5% (w/v). The SPI solution (6%, w/v) was heated at 95ºC for 15 min, and then diluted to the required *c* values in the test range. (Adapted from the data of Liu & Tang (2014)). B) An illustration for the adsorption of a globular protein at the interface, which consists of three main stages: (1) diffusion of the protein to the interface; (2) penetration and unfolding at the interface; (3) structural rearrangement of adsorbed proteins at the interface and formation of cohesive and viscoleastic films. These three stages are also illustrated in Figure 14 A. C) Typical profile of the molecular penetration and configurational rearrangement steps at the interface for the heated SPI at *c* = 0.5% (w/v). The k_P and k_R represent first-order rate constants of penetration and rearrangement, respectively.

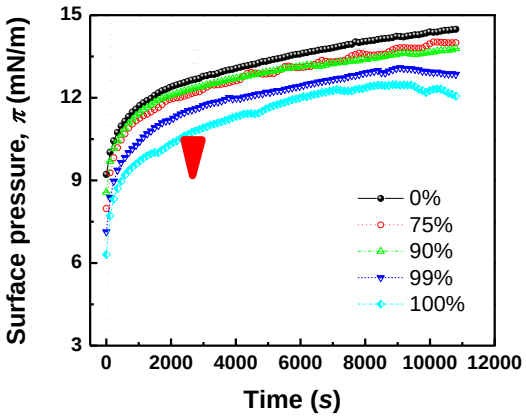
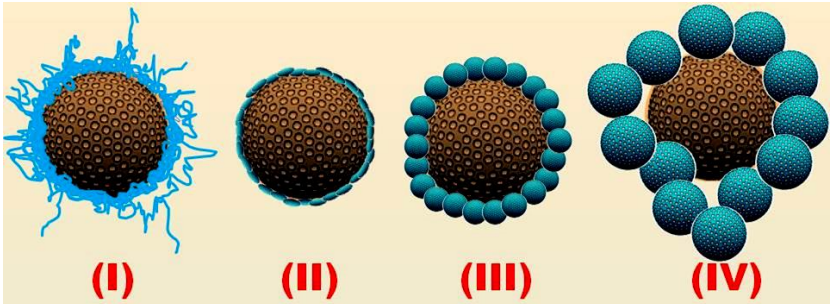


Figure 16. Time evolution profiles of the surface pressure (π) for the adsorption of soy globulin mixtures (SG + SC) at the O-W interface, as a function of the glycinin content in the mixtures (0-100%). The total protein concentration in the aqueous phase is 0.5% (w/v). (Unpublished data)



Items	(I)	(II)	(III)	(IV)
Film thickness	thick	thin	intermediate	thick
Viscoelasticity	high	elastic	high	mainly elastic
Examples	β-casein	BSA	β-conglycinin	Glycinin, SPI

Figure 17. Illustration for four representative interfacial structures on surface of oil droplets with different structural and rheological properties.

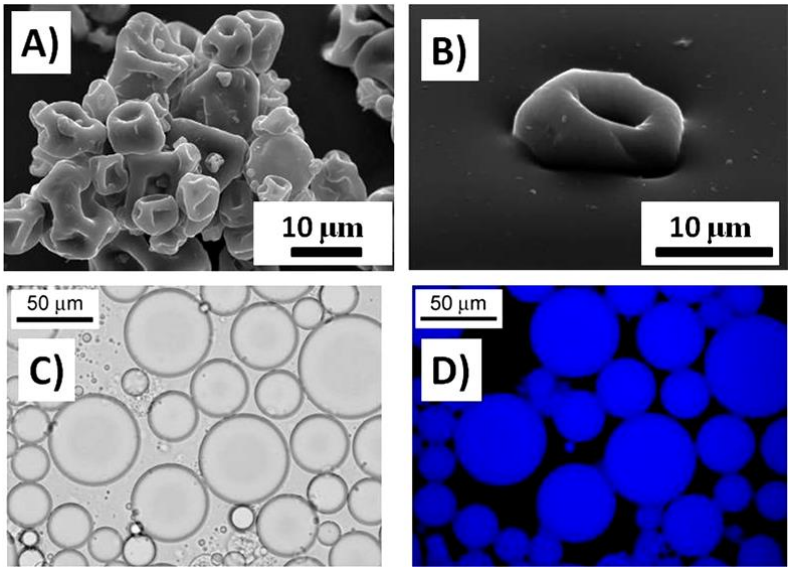


Figure 18. A) SEM image of the spray-dried soy protein particles (Supro 651). B) SEM image of soy protein particles (Supro 651) trapped on PDMS from the decane-water interface (in contact with the water phase in the system) with the gel trapping technique. C-D) Optical (C) and fluorescence microscope (D) images of the emulsions stabilized by 3.2 wt% Supro 651 particles (with respect to the decane phase) at a decane volume fraction of 0.5. (Adapted from the results of Paunov et al. (2007))

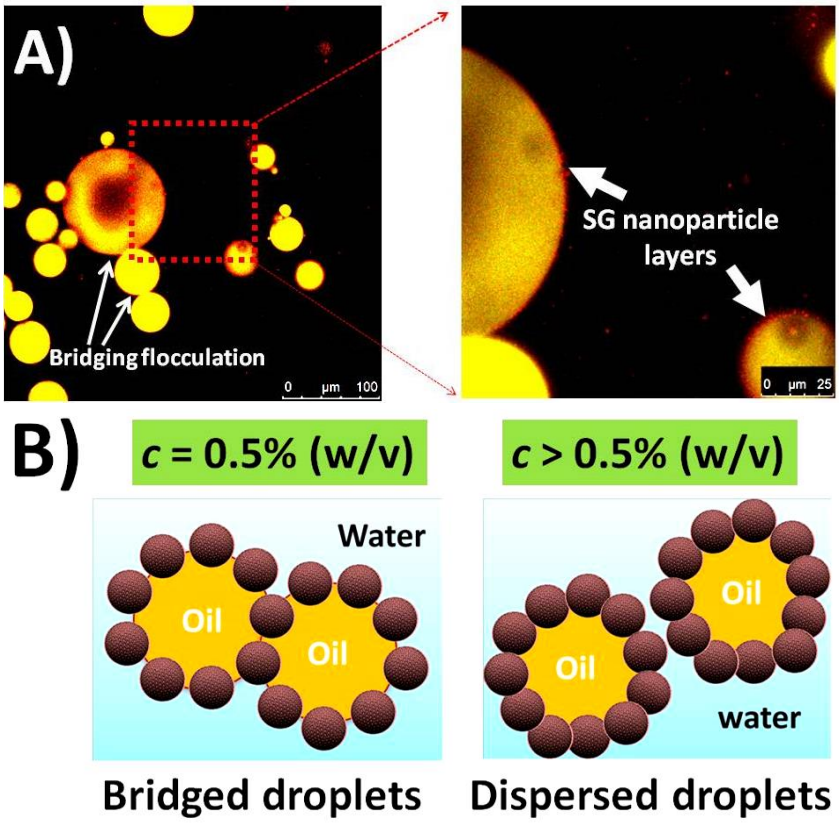


Figure 19. A) Typical CLSM observations of the emulsion stabilized by the heated (at 100 ºC) SG nanoparticles, at *c* = 0.5% (w/v) and ø of 0.5. B) Scheme illustration for the bridging and dispersed emulsions stabilized by SG nanoparticles, formed at different solid concentrations (*c*). (Liu & Tang, 2014 b)

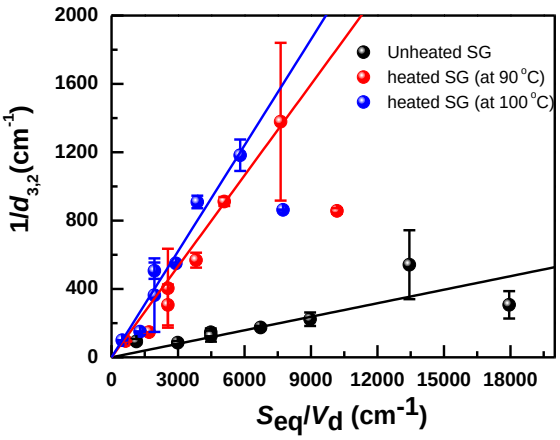


Figure 20. Evolution of the inverse droplet diameter ($1/d_{3,2}$) of the unheated and heated SG-stabilized Pickering emulsions as a function of the total equatorial surface area (S_{eq}) normalized by the oil volume (V_d). The heated SG nanoparticles were obtained by heating at 90 and 100 ºC for 30 min, respectively. Each datum is the means and standard deviation of two measurements on separate samples. (Liu & Tang, 2014 b)

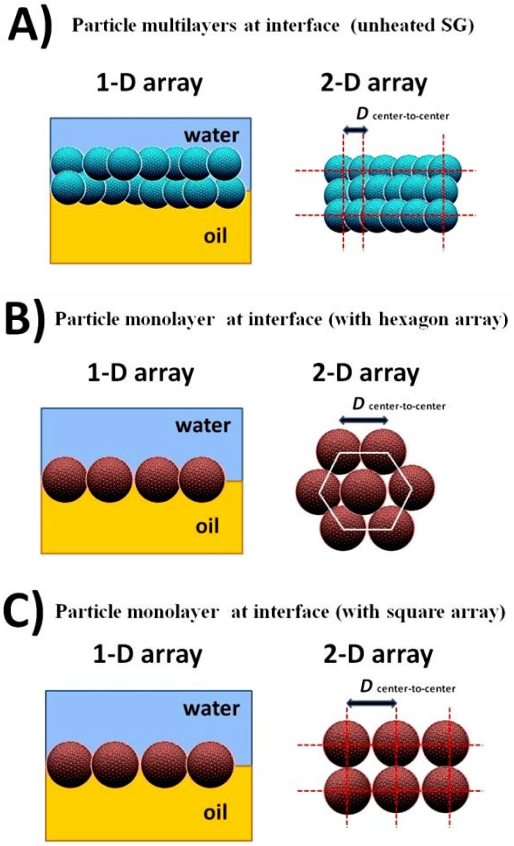


Figure 21. Scheme illustrations of SG nanoparticle packing and arrangement (1-D and 2-D arrays) at interface for unheated SG (A), SG heated at 90 ºC (B) and 100 ºC (C). For unheated SG, multilayers of SG nanoparticles would be formed at interface, while in the cases of heated SG, the nanoparticles at interface was arranged into a compact monolayer, in a square or regular hexagonal array. $D_{c\text{-}to\text{-}c}$ is the particle center-to-center distance at interface between two neighboring particles. (Liu & Tang, 2014 b)

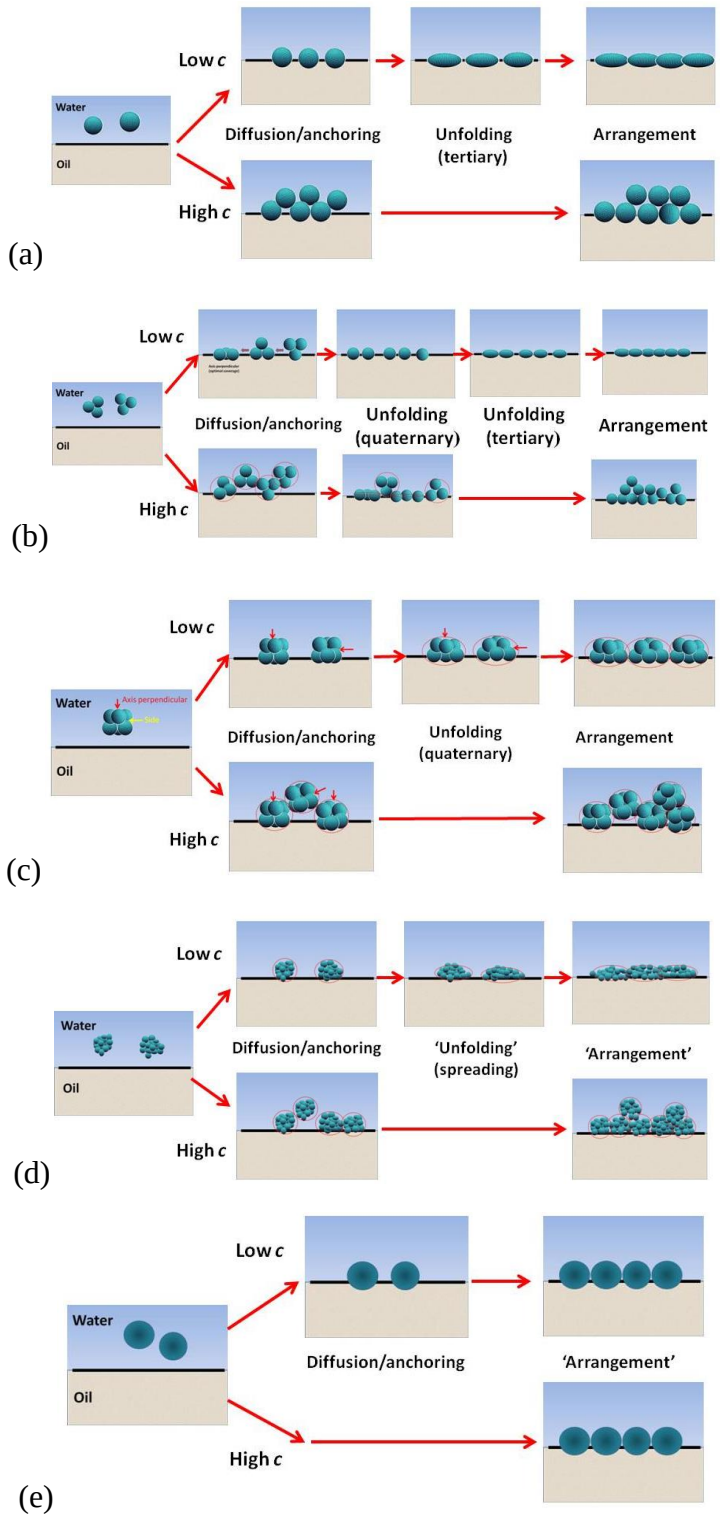


Figure 22. Illustration for the importance of conformational flexibility for the adsorption of

different kinds of globular proteins (with different conformations or structures) at the O-W interface. The adsorption to be considered mainly include i) diffusion (to the interface)/anchoring at the interface, ii) unfolding, and iii) arrangement of adsorbed proteins.

