1   Structural Characterization of Solid Lipoproteic Colloid Gels by Ultra-Small-Angle X-ray
2   Scattering and the Relation with Sodium Release

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Abstract

Sodium reduction in protein/lipid-based products such as cheese is becoming increasingly important to the food industry. Understanding the structure critical to sodium release is one of the keys to effectively controlling the sensory quality of the product while lowering the sodium content. In this study, ultra-small-angle X-ray scattering (USAXS), novel to food research, was used to characterize the structure of solid lipoproteic colloid (SLC) gels as a model food. The SLC gels were made via heat-induced gelation of emulsions of whey protein isolate and anhydrous milk fat. The gels varied in the contents of protein, fat, and NaCl and homogenization pressures. The gyration radii of the protein aggregates ($r_{g,p}$) and the fat globules ($r_{g,f}$) of the samples before and after the gelation were obtained via fitting the USAXS profiles to inspect the structure formation of the SLC gels. The effects of formulation and processing on the gel $r_{g,p}$ and the gel $r_{g,f}$ were analyzed. In addition, the gel $r_{g,f}$ and the hydrodynamic radius of the droplets ($r_{h,e}$) in the emulsions were correlated with sodium release. The correlation suggested that gel $r_{g,f}$ is a better indicator of sodium release than the emulsion $r_{h,e}$. The findings from this study indicated that USAXS is feasible for the structural investigation of protein/lipid-based foods.

Key words

1. Ultra-small-angle X-ray scattering (USAXS)
2. Solid lipoproteic colloid gel
3. Sodium release
4. Sodium reduction
5. Whey protein isolate
6. Structure

1. Introduction

Reducing the sodium content of food products based on lipid/protein emulsion structures such as cheese and processed meat has gained increasing attention in the food industry (Desmond, 2007; Desmond, 2006; Johnson, Kapoor, McMahon, McCoy, & Narasimmon, 2009). Currently, industrial and experimental strategies for sodium reduction include stealth reduction (Phelps et al., 2006), sodium replacement (Eoin, 2006; Sinopoli & Lawless, 2012), saltiness potentiation (Brewer, Gills, & Vega, 1995; Heidolph et al., 2011; Yamaguchi & Takahashi, 1984), multisensory application (Djordjevic, Zatorre, & Jones-Gotman, 2004; Dotsch et al., 2009), and physical modification of salt crystals (Heidolph et al., 2011). More recently, the new concept of structural engineering has been proposed to enhance the sodium release and/or saltiness perception of the food matrix (Busch, Yong, & Goh, 2013; Jiménez-Colmenero, 2013; Stieger & van de Velde, 2013). The hypothesis underlying this strategy is that the structure of the food matrix dominates the physicochemical properties of the foods and hence the sodium release and saltiness perception of the food products (Kuo & Lee, 2014a). Therefore, at a given salt level, the sodium release and saltiness perception of the food matrix can potentially be maximized by an optimized food structure.

Various mechanisms have been proposed in the literature to enhance sodium release via food structure modification. Sodium release is greater in foods with more available sodium, with a higher diffusion coefficient of sodium, with more serum release (i.e., the amount of squeezable liquid), or with a higher degree of fragmentation (Kuo and Lee, 2014a). In lipoproteic foods, the availability of sodium has been increased by lowering the protein contents (Boisard, et al., 2014; Lauverjat, et al., 2009; Ruusunen, et al., 2005). Additionally, the diffusion coefficient of sodium in lipoproteic foods has been increased by lowering the fat or dry matter content (Chabanet, et al., 2013; Flourey, et al., 2009; Hughes, et al., 1997; Lauverjat, et al., 2009; Panouille, et al., 2011; Phan, et al., 2008). In addition, lowering the degree of protein crosslinking lowered the diffusion coefficient of sodium (Panouille, et al., 2011). In a model polysaccharide/protein gel, the serum release was found to be higher in a bi-continuous microstructure than in a protein-continuous one (Stieger, 2011). Lastly, the fragmentation degree of lipoproteic foods is greater with higher fat content (de Loubens, et

al., 2011), lower protein content (Panouille, et al., 2011), or smaller particle size of the emulsion droplets (Kuo and Lee, 2014b). The increased sodium release in lipoproteic foods associated with smaller emulsion droplets was ascribed to the higher surface area after the food breakdown (Kuo & Lee, 2014b). When emulsion gels undergo deformation, the initiation of fracture more likely starts at the interface between fat and protein than between protein and protein. This tendency is because the fat droplets are structural defects, and defects increase the stress concentration of the gel matrix (Sala, van Vliet, Cohen Stuart, Aken, & van de Velde, 2009; van Vliet, Luyten, & Walstra, 1993). This property has been further demonstrated by reductions in the gel fracture strain, stress or energy and by increases in sodium release with higher fat contents or lower emulsion droplet particle sizes in lipoproteic gels (de Loubens et al., 2011; Kuo & Lee, 2014b; Panouille, Saint-Eve, de Loubens, Deleris, & Souchon, 2011; Phan et al., 2008). Our previous study on model solid lipoproteic colloid (SLC) gels revealed that a higher homogenization pressure led to a lower hydrodynamic radius of the emulsion droplets ($r_{h,e}$), which implied an SLC gel network with more randomly dispersed structural defects. More structural defects thus led to easier fracture and higher surface area of the fractured debris. However, the increase in sodium release with reduced $r_{h,e}$ was not universally significant (Kuo & Lee, 2014b). Because the $r_{h,e}$ was obtained from dynamic light scattering (DLS) of the emulsions prior to the heat-induced gelation, we hypothesized that the particle size of the actual fat globules within the lipoproteic gel correlates better than the emulsion $r_{h,e}$ to the fragmentation degree and thus the release of sodium. An ideal analytical tool to obtain the particle size of fat globules in solid gels is necessary to validate the hypothesis.

Currently, microscopy is the most relevant tool for analyzing the nano- and microstructures of solid foods, and electron microscopy has been extensively used for its suitable characterization range (Dudkiewicz, Luo, Tiede, & Boxall, 2012; Kalab, Allan-Wojtas, & Miller, 1995). However, electron microscopy usually requires sample pre-treatment, which can take several days and potentially damage the original food structure. In addition, electron microscopy is limited to 2-D imaging a thin layer or a surface (Harada & Matsuoka, 2004) and thus may not represent the bulk sample well.

A small but increasing number of studies have been reported on using ultra-small-angle X-ray scattering (USAXS) to characterize biomaterials (Agrawal, Sanabria-DeLong, Jemian, Tew, & Bhatia, 2007; Nave, Diakun, & Bordas, 1986). Even fewer studies have used USAXS to study food systems (Peyronel, Pink, & Marangoni, 2014; Peyronel, Quinn, Marangoni, & Pink, 2014; Väänänen et al., 2003). Depending on the scattering angle, the family of X-ray scattering, including wide-, small-, and ultra-small-angle X-ray scattering, can provide structural information such as chemical composition, crystallography, size, shape and association of particles at different scales (Dudkiewicz, Luo, Tiede, & Boxall, 2012). Typical pinhole small-angle X-ray scattering (SAXS) characterizes structures at 1 - 100 nm. With advanced USAXS instruments, the range can be extended to 1 nm - 1 μ m (Ilavsky, Allen, Long, & Jemian, 2002; Ilavsky et al., 2013; Ilavsky et al., 2009). This range ideally covers the wide size range of individual components in foods and the associated structures of the components. Requiring minimal or no pre-treatment, USAXS provides ensemble 3-D structural information from a bulk sample while minimizing the damage of the structure. In addition, USAXS can be applied to turbid systems, such as solid fat and denatured protein, that light scattering is unable to characterize (Harada & Matsuoka, 2004; Peyronel, Quinn, Marangoni, & Pink, 2014). Using a synchrotron X-ray radiation source rather than a conventional source provides higher flux and thus higher contrast, so that normally low-contrast biomaterials or foods can be studied more effectively (Harada & Matsuoka, 2004).

The objective of the present study is to use USAXS/pinhole SAXS to investigate the structure of SLC gels and to correlate the USAXS-derived structural properties in the gel with sodium release. The structure formation during the heat-induced gelation of the samples and the effects of formulation and treatment on the gyration radii of the protein aggregates ($r_{g,p}$) and the fat globules ($r_{g,f}$) are discussed. Two radius values, the USAXS-derived gel $r_{g,f}$ and the DLS-derived emulsion $r_{h,e}$, are compared for how well each correlates to sodium release from the SLC gels.

2. Materials and Methods

2.1 Materials and Sample Preparation

Whey protein isolate (WPI, Hilmar 9000, Hilmar Ingredients, Hilmar, CA, USA) and anhydrous milk fat (AMF, Berkshire Dairy & Food Products, LLC, Wyomissing, PA) were used to prepare the SLC gels. The SLC gels are coded according to their formula and homogenization pressure as Protein(%)-Fat(%)-NaCl(%)-pressure (MPa) (Table 1). The SLC gels in this study contained 2 levels of protein (8 and 16%, w/w), 4 levels of fat (0, 11, 22 and 33%, w/w), and 2 levels of NaCl (1.5 and 3.5%, w/w), and they were prepared under 2 different homogenization pressures (14 or 55 MPa). The formula of the samples in this study was chosen to approximate the contents and texture of commercial lipoproteic foods. The two homogenization pressures can result in significantly different emulsion $r_{h,e}$ values (Kuo & Lee, 2014b), which enables investigating the effects of fat globule particle size on the sodium released from the SLC gel. The SLC gel preparation was previously described in detail (Kuo & Lee, 2014b). Briefly, WPI was suspended in a NaCl aqueous solution at 45°C and incubated at 6-8°C overnight. Then, the WPI suspension was pre-homogenized with pre-melted AMF at 45°C using an IKA T-25 Digital High-Speed Homogenizer (IKAWorks Inc., Wilmington, NC, USA). The pre-emulsion was pressure homogenized using an APV 2-stage homogenizer (15 MR, SPX Flow Technology, Soeborg, Denmark) and then heated at 90°C for 30 min to form the SLC gels. Three to five batches were made for each SLC gel sample.

2.1 USAXS Measurement

The USAXS combined with pinhole SAXS was carried out using the Bonse-Hart double-crystal USAXS instrument at beamline 15-ID-D operated by ChemMatCARS at the Advanced Photon Source, Argonne National Laboratory, Argonne, IL, USA (Ilavsky et al., 2009). The 1 mm thick gel sample was sealed in a silicone isolator (JTR20R-A2-1.0-Press-To-Seal, Grace Bio-Labs, Inc., Bend, Oregon, USA). After temperature equilibration for 3 h, the specimen was measured at the scattering vector range $Q = 0.0001$ to $1.35 \text{ \AA}^{-1}$ at 20°C, where Q is equal to $4\pi\sin(\theta/2)/\lambda$, and θ and λ are the scattering angle and beam wavelength, respectively. The USAXS measurement was carried out for all samples listed in Table 1 except for one sample

due to instrument operational error. Background and absorption corrections for the 1-D profile and subsequent data analyses were performed by IGOR Pro v6.12 with the Irena package (Ilavsky & Jemian, 2009). The Modeling II macro of the Irena package was used to fit the profile of the slit-smeared data (slit length 0.02677 and 0.028503 Å⁻¹ for the samples with 1.5% and 3.5% NaCl, respectively). A spheroid with an aspect ratio of one was used as the structure for the fittings of both $r_{g,p}$ and $r_{g,f}$ in this study. Selected SLC emulsions (Fig. 1) were also measured prior to the heating to compare the structural change before and after the gelation.

2.2 Hydrodynamic Radius, Gel Morphology, Sodium Release and Textural properties

To investigate the correlations of the gel $r_{g,f}$ and the emulsion $r_{h,e}$ with sodium release from the gel, ten SLC samples (8-22-1.5-55, 8-33-1.5-55, 8-33-1.5-14, 16-11-1.5-55, 16-11-1.5-14 and their five counterparts with 3.5% NaCl) were selected for characterizations of the emulsion $r_{h,e}$, gel morphology, sodium release and textural properties. Our previous study (Kuo & Lee, 2014b) showed wide variations in sodium release across the SLC gels with 1.5% NaCl listed above. The samples with 3.5% NaCl were also selected to compare the effects of the gel $r_{g,f}$ and the emulsion $r_{h,e}$ at different levels of sodium.

The emulsion $r_{h,e}$ was observed via DLS using a ZetaPALS Zeta potential analyzer (Brookhaven Instruments Co., Holtsville, N.Y., U.S.A.) at 25°C. After the vacuum procedure, the freshly made emulsions were diluted with DI water to reach the appropriate intensity for the DLS measurement. The $r_{h,e}$ values were collected from the intensity-weighted particle size distribution, which was averaged from 3 instrumental readings. Three to five DLS measurements were completed for each sample.

The internal morphology of the SLC gels was examined using environmental scanning electron microscopy with a field emission electron gun (ESEM, FEI Co., Hillsboro, Oreg., U.S.A.). The gels were cut into 3 × 3 × 7 mm³ sticks and then freeze fractured in liquid nitrogen. The freeze-fractured gels were immediately mounted on the stage, and ice in the samples was allowed to sublime (i.e., freeze etching) at 1 Torr using the wet mode in the ESEM chamber. The fractured face was then observed with an accelerating voltage of 20 kV.

The sodium release from the SLC gels was measured by simultaneously compressing the gel in DI water and measuring the conductivity of the water at room temperature (23 °C) (de Loubens and others 2011a). The samples were compressed using a texture analyzer (TA-XT2i, Texture Technologies Corp., Scarsdale, N.Y., U.S.A.). The conductivity was measured using a conductivity probe (Orion DuraProbe 4-Electrode Conductivity Cells 013005MD) connected with an Orion VERSA STAR Multiparameter Benchtop Meter (Thermo Fisher Scientific Inc., Waltham, Mass, U.S.A.). A cylindrical sample of SLC gel (25.4-mm dia., 25.4-mm length) was placed in a 500 mL jar under a TA-25 cylinder probe (50-mm dia., aluminum) of the texture analyzer. First, 400 mL of DI water was added into the jar. After 25 seconds, the texture analyzer began compressing the gel at a crosshead speed of 1.0 mm/s until 80% strain. The conductivity of the DI water was read every 5 s for a total of 365 s while the water was stirred at 200 rpm by a mechanical stirrer. Three parameters were extracted from the curves of the *in vitro* sodium release (Fig. 2). The maximum rate of sodium release, $R_{\max}$, is the greatest slope calculated from any 3 continuous data points along the concentration–time curve. The maximum concentration of sodium release, $C_{\max}$, is the sodium concentration at the end of the measurement. The area under the curve of sodium release, AUC, is the integrated area under the concentration–time curve.

The textural properties of the SLC gels were measured via a compression test using the texture analyzer (TA-XT2i, Texture Technologies Corp., Scarsdale, N.Y., U.S.A.) at room temperature (23 °C). A thin layer of mineral oil (Sigma 330779, Sigma-Aldrich, St. Louis, Mo., U.S.A.) was spread on top of the gel. The gels were then compressed under the TA-25 cylinder probe (50-mm dia., aluminum) at a speed of 1 mm/s to a maximum strain of 80%. The serum release from the gels during the compression test was collected by a pre-weighed filter paper (Whatman 42, Maidstone, Kent, U.K.) placed underneath the gel. The serum release is the difference in the weight of the filter paper before and after the compression of the gel.

2.3. Statistical analyses

228 Pearson correlation was used for all the correlation analyses in this study. For all the SLC
229 gels, the gel $r_{g,p}$ and the gel $r_{g,f}$ were correlated with the gel formula and treatment. For the
230 ten further characterized samples (listed in Section 2.2), the USAXS-derived gel $r_{g,f}$ and the
231 DLS-derived emulsion $r_{h,e}$ were correlated with the sodium release and textural properties
232 of the SLC gels.

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3. Results and Discussion

3.1. Structure of the SLC gels formed via heat-induced gelation

3.1.1. Structural formation of the non-fat gels

Figure 1A shows the USAXS profiles of the non-fat dispersions before the heat gelation process and their corresponding gels. The non-fat dispersions (Fig. 1A, 8-0-1.5-14, 8-0-1.5-55, and 16-0-1.5-55 dispersions) presented knee-like patterns, with the knee at approximately $0.06 \text{ \AA}^{-1}$. Based on the Guinier approximation, these curves represented the particles with the gyration radius (r_g) between 1.7 – 2.2 nm. These sizes corresponded to the r_g s of the native beta-lactoglobulin (bLG) monomers or dimers (1.4-1.7 and 2.1-2.3 nm, respectively) (Barteri, Gaudiano, Rotella, Benagiano, & Pala, 2000; Gottschalk, Nilsson, Roos, & Halle, 2003; Moitzi et al., 2011; Verheul, Pedersen, Roefs, & de Kruif, 1999; Witz, Luzzati, & Timasheff, 1964). These sizes also corresponded to the r_g of the native alpha-lactalbumin (aLA) (1.6 nm) (Arai et al., 2002). In WPI, bLG is the most abundant component, followed by aLA (typically ranging from 44–70% w/w and 14–22% w/w, respectively) (Farrell Jr. et al., 2004; Foegeding, et al., 2011; Lucey, 2014). Therefore, the $r_{g,p}$ s of the above non-fat dispersions may be an average of the r_g s of the bLG and the aLA present in the sample.

The USAXS profiles of the non-fat gels (Fig. 1A, 8-0-1.5-14, 8-0-1.5-55, and 16-0-1.5-55 gels) also displayed the knee-like patterns, but they were shifted toward the lower Q range compared with those of their corresponding dispersions. The Guinier approximation resulted in the $r_{g,p}$ s of the gels ranging between 7.2 and 9.2 nm, much higher than the corresponding $r_{g,p}$ s of the dispersions. These particles in the gels could be aggregates consisting of bLG and aLA. Previous research revealed that bLG formed aggregates with aLA via disulfide bonds and hydrophobic interactions when heated in an aqueous solution (Dagleish, Senaratne, & Francois, 1997). Protein aggregates with a radius of 29 nm were reported when a WPI solution (3% w/w protein, 54 mM NaCl) was heated at 90°C for 5 min (Ryan et al., 2012). The much smaller WPI aggregates found in this study as compared with those in previous studies could be due to the homogenization treatment prior to heating. The shearing force of the homogenization may have dispersed the protein particles,

disrupting the inter-particle interactions and thus reducing the degree of aggregation. Our previous work has shown that the particle size of protein aggregates decreases with increasing homogenization pressure (Kuo & Lee, 2014b). Additionally, differences in the concentrations of protein and NaCl and in the heating conditions could also lead to the varying particle size of the protein aggregates.

At the lower Q range ($Q < 0.005 \text{ \AA}^{-1}$), the non-fat dispersions (Fig. 1A, 8-0-1.5-14, 8-0-1.5-55, and 16-0-1.5-55 dispersions) did not show any scattering signals, indicating a lack of larger structures at this scale (approximately 100 nm to 5 μm). The non-fat gels (Fig. 1A, 8-0-1.5-14, 8-0-1.5-55, and 16-0-1.5-55 gels) showed a linear log-log decay in the Q range of $0.0002 - 0.002 \text{ \AA}^{-1}$. A power law fitting in this Q range on the de-smeared data provided exponents of approximately 4. This power law decay is attributed to Porod scattering by the smooth surfaces of larger scatterers (Feigin & Svergun, 1987; Yoshida, Fukushima, & Yamaguchi, 2014). The Guinier regions of these larger scatterers are beyond the lowest Q (corresponding to 2 μm) in the USAXS measurement in this study. These larger scatterers are believed to be the protein particulates in the SLC gels. These protein particulates, with sizes above 2 μm and smooth surfaces, can be identified in the ESEM image of the SLC gel 8-0-1.5-14 (Fig. 3A).

3.1.2. Structure formation of the fat-containing SLC gels

Fig. 1B shows the USAXS profiles of the fat-containing SLC emulsions before the heat gelation process and their corresponding gels. At $Q = 0.15 \text{ \AA}^{-1}$, a peak was observed in the patterns of the fat-containing samples. This peak is the diffraction of the 2L (bilayered stacking) form of the crystalline AMF within the fat globules in the emulsion structure (Lopez, Lavigne, Lesieur, Bourgaux, & Ollivon, 2001). Similar to the non-fat dispersions (Fig. 1A), the fat-containing emulsions (Fig. 1B, 8-33-1.5-14, 8-33-1.5-55, 16-33-1.5-14 and 16-33-1.5-55 emulsions) exhibited scattering at approximately $Q = 0.06 \text{ \AA}^{-1}$, suggesting that this pattern was from the protein scattering. The $r_{g,p}$ s of these particles, however, range between 2.4 and 4.6 nm, slightly higher than those in the non-fat emulsions (1.7 – 2.2 nm). The higher emulsion $r_{g,p}$ s suggests more aggregation of the bLG or the aLA into oligomers. For example, the bLG octamer has an r_g of 3.4 nm (Timasheff & Townend, 1964; Witz, Luzzati, & Timasheff, 1964). The higher degree of protein aggregation could be attributed to the relatively higher proportion of protein to water in the fat-containing emulsions than in

the non-fat dispersions. For example, the proportion of protein to water in the emulsion 8-33-1.5-14 and the dispersion 8-0-1.5-14 are 0.12 and 0.09, respectively.

As in the non-fat samples (Fig. 1A), the protein scattering patterns of the fat-containing samples shifted toward lower Q s after the gelation process (Fig. 1B, 8-33-1.5-14, 8-33-1.5-55, 16-33-1.5-14 and 16-33-1.5-55 gels). The $r_{g,p}$ s are again higher than their non-fat counterparts, ranging between 23 and 67 nm. This difference could be the net result from higher concentrations of NaCl and protein in the aqueous phase of the fat-containing samples. Previous studies on heating WPI and bLG solutions showed that the aggregate diameters increased with increasing NaCl concentrations due to lower intermolecular repulsion, enhanced chemical and physical aggregation, and lower protein solubility (Ryan et al., 2012; Verheul, Roefs, & de Kruif, 1998). Additionally, the aggregate association rate increased with increasing concentrations of either NaCl or protein (Bryant & McClements, 2000; Marangoni, Barbut, McGauley, Marcone, & Narine, 2000; Ryan et al., 2012; Wu, Xie, & Morbidelli, 2005).

At a Q of approximately $0.001 \text{ \AA}^{-1}$, all the fat-containing samples (Fig. 1B) showed clear knees in the scattering profiles, which were used to calculate the $r_{g,f}$. This pattern was attributed to the fat globules for the following reasons. First, this pattern was not observed in the profiles of the non-fat samples. Second, compared with $r_{g,p}$, this pattern showed relatively moderate changes in size after the gelation, which better reflected the status of the fat globules rather than the protein aggregates during heating. Third, as will be discussed in section 3.3.1., ESEM observation of the cross-sectional area of the SLC gels revealed fat particles with a radius of approximately 500 nm, corresponding to the $r_{g,f}$ range between 77 and 711 nm (Table 2).

3.2. Effect of formulation and treatments on the SLC gel structure

Fig. 4 shows the USAXS profiles of the SLC gels made with different formulas and at different homogenizations pressures. All the non-fat gels in Fig. 4 shared similar profile features, including knee-like scattering from the protein aggregates and a power law decay representing the 3-D networking of the aggregates. Likewise, all the fat-containing gels in Fig. 4 shared similar profile features, including an AMF diffraction peak and two knee-like scatterings from the protein aggregates and the fat globules. The AMF peak intensity

increased with the increasing fat content in the formula, implying a successful incorporation of fat into the SLC gels. Additionally, the AMF peaks are broader in the gels made with higher homogenization pressure (Fig. 4, C vs. A and D vs. B, 55 vs. 14 MPa). According to the Scherrer formula, wider diffraction peaks imply smaller crystallites (Patterson, 1939). Hence the broader AMF peaks indicated smaller fat crystals in the fat globules. This finding confirmed that the higher pressure effectively dispersed the emulsion structure. Table 2 lists the values of the gel $r_{g,p}$ and $r_{g,f}$ derived from the Guinier approximation of the USAXS profiles. Overall, both the gel $r_{g,p}$ and $r_{g,f}$ decreased with increasing homogenization pressure ($p < 0.01$). This correlation is much stronger when only the fat-containing gels were analyzed ($p < 0.001$). Higher homogenization pressures have been shown to induce partial denaturation of proteins, improving protein adsorption on the fat globules and thus resulting in a more stable emulsion system (Lee, Lefèvre, Subirade, & Paquin, 2009). Within the group of fat-containing gels made at the low homogenization pressure, the $r_{g,p}$ was positively correlated ($p < 0.01$) with the protein content and negatively correlated ($p < 0.05$) with fat content. A similar positive correlation between the gel $r_{g,p}$ and the protein content was found within the group of fat-containing gels made with the high homogenization pressure ($p < 0.05$). These correlations between the $r_{g,p}$ s and the protein and fat contents indicated the counteracting behaviors between the protein and fat during the emulsification and gel formation. A higher protein content may promote the probability of protein-protein interaction, leading to larger protein aggregates. A higher fat content may interfere with the protein association, preventing the growth of the protein aggregates. On the other hand, a higher fat content may require a greater fraction of protein for emulsification, reducing the available amount of protein for protein-protein interaction. In the group of fat-containing samples made with the higher homogenization pressure, the gel $r_{g,p}$ did not significantly depend on the fat content. This lack of dependence may be due to the more thorough dispersion of the protein at the higher pressure, which overcame the effect of fat content on the gel $r_{g,p}$.

3.3. Dependence of sodium release on the gel $r_{g,f}$ or emulsion $r_{h,e}$

3.3.1. Correlations between the gel $r_{g,f}$ and sodium release

To understand the influence of the gel $r_{g,f}$ and the emulsion $r_{h,e}$ on sodium release from the SLC gels, ten fat-containing SLC samples (listed in Section 2.2) were further characterized for

sodium release, the texture and morphology of the gels, and the emulsion size, $r_{h,e}$. The sodium release by the gel as it was compressed by the texture analyzer was measured in water. However, the behavior of the textural properties of the samples acquired during the sodium release measurements was found to be consistent with the behavior observed in the actual textural analysis (data not shown). Hence, the breakdown properties of the gels observed from the textural analysis can be used to explain the differences in their sodium release properties. Table 3 shows the sodium release and textural properties of the SLC gels with 3.5% NaCl and the $r_{h,e}$ values of the corresponding emulsions. The properties of the SLC samples with 1.5% NaCl can be found in our previous study (Kuo and Lee, 2014b). Table 4 shows some of the correlation coefficients between the above properties and the gel $r_{g,f}$ derived from USAXS. For the SLC gels with 3.5% NaCl, the gel $r_{g,f}$ was negatively correlated with the C_{max} (the maximum concentration of released sodium) and the AUC (area under the curve of sodium release vs. time plot) at a highly significant level ($p < 0.005$). These correlations can be attributed to the effect of fat on the breakdown properties of the SLC gels. Previous studies on SLC gels showed that, at the same total fat content, smaller fat globules implied a gel network with a higher number of randomly dispersed fat particles (Kuo & Lee, 2014b; Phan et al., 2008). As fat interfered with the integrity of the protein network, the higher number of dispersed fat particles could lead to a greater extent of breakdown and more debris when compressed. This hypothesis can be supported by the positive correlation found between the gel $r_{g,f}$ and the strain at the maximum stress of the SLC gels with 3.5% NaCl (Table 4). In other words, the required strain to break the gels decreased as the gel $r_{g,f}$ decreased. The greater extent of breakdown and more debris, which results in more total surface area for the broken gels, would favor the enhanced release of sodium from the gel in a given period of time (de Loubens et al., 2011). This hypothesis can be supported by the negative correlations of the strain at maximum stress with the C_{max} and with the AUC in the SLC gels with 3.5% NaCl (Table 4). The negative correlation between the gel $r_{g,f}$ and the sodium release, however, was not significant in the SLC gels with 1.5% NaCl. This lack of significance may be due to a difference in the force of inter-protein interaction among the SLC gels with varying NaCl contents. A higher amount of NaCl in the SLC gels implied greater charge screening on the repulsive force between protein molecules. Previous studies showed that this improved screening favored the aggregation, branching, and densification of whey protein particles (Langton & Hermansson,

1996; Pouzot, Nicolai, Durand, & Benyahia, 2004; Verheul, Roefs, & de Kruif, 1998). Hence, an overall stronger protein-protein interaction was expected in the protein networks with higher NaCl contents (Hussain, Gaiani, Jeandel, Ghanbaja, & Scher, 2012; Lorenzen & Schrader, 2006). As a result, the SLC gels with 3.5% NaCl exhibited relatively stronger inter-protein interaction than the SLC gels with 1.5% NaCl, and this stronger interaction made the initiation of break upon compression more likely to occur at the protein-fat interface. In other words, the properties of the protein-fat interface dominated the breakdown properties of the SLC gels with 3.5% NaCl. Decreased gel $r_{g,f}$ led to higher sodium release due to increases in the surface area, the probability of break initiation, and the extent of breakdown. In contrast, the protein-protein interaction in the SLC gels with 1.5% NaCl was weaker than that with 3.5% NaCl, and thus the breakdown at the protein-fat interface might not have been dominant. This difference explained the lack of correlation between the gel $r_{g,f}$, the strain at maximum stress, and the sodium release in the SLC gels with 1.5% NaCl. The above hypothesis can be supported by the ESEM images of the SLC gels shown in Fig. 3. The SLC gels with 3.5% NaCl displayed a denser and more aggregated network morphology than that of the SLC gels with 1.5% NaCl, which suggested greater protein-protein interaction at higher NaCl contents. Furthermore, for the two SLC gels with 3.5% NaCl made with the lower homogenization pressure (8-33-3.5-14 and 16-11-3.5-14, Fig. 3J and L), some bright spherical fat particles stood out from the protein network. In the images of the corresponding 1.5% NaCl gels (8-33-1.5-14 and 16-11-1.5-14, Fig. 3D and F), these fat globules were less visible and more embedded within the protein network. This difference in the appearance of the fat globules among the protein matrix indicated a possibly stronger protein-protein interaction and weaker protein-fat interaction in the SLC gels with the higher NaCl content.

3.3.2. Correlations between the emulsion $r_{h,e}$ and sodium release during compression

No significant correlation was found between the emulsion $r_{h,e}$ and the sodium release from the SLC gels with either 1.5 or 3.5% NaCl (Table 4). This lack of correlation could be because the emulsion $r_{h,e}$ was measured in the SLC emulsions before the heat-induced gelation. Among the 10 samples that underwent the correlation analysis, most of them showed less than $\pm 10\%$ difference between the gel $r_{g,f}$ and the emulsion $r_{h,e}$ values. This consistency

427 implied that the emulsion droplets were relatively stable during the heating process.
428 However, for the samples 16-11-1.5-14 and 16-11-3.5-14, the gel $r_{g,f}$ values were 61% and
429 101% greater than the emulsion $r_{h,e}$ values. This increase suggested that the emulsion
430 droplets in these two samples were less stable and might have coalesced more drastically
431 during the heating.

432 According to Fig. 1B showing some selected SLC samples with 1.5% NaCl, the $r_{g,f}$ s grew by 10
433 to 50% after the gelation. Hence, the fat globules in both SLC gels made with 1.5% and 3.5%
434 NaCl exhibited inconsistent degrees of coalescence as a result of the heating. In the case of
435 3.5% NaCl, because the gel breakdown and accordingly the sodium release were directly
436 related to the size of the fat globules in the gel, the USAXS-derived gel $r_{g,f}$ proved to be more
437 predictive of sodium release than the DLS-based emulsion $r_{h,e}$.

438

4. Conclusions

In this study, USAXS was successfully used as a novel technique to investigate the structure of SLC gels and as a demonstration of its potential to predict the sodium release properties of the gels. The protein in the SLC emulsions underwent heat-induced aggregation during the gelation process, resulting in significant increases in the particle sizes of the protein aggregates. Additionally, the fat-containing gels had much larger protein aggregates compared with those in the non-fat gels. The sizes of the fat globules were comparatively more stable than those of the protein aggregates throughout the gelation. The gel $r_{g,p}$ and the gel $r_{g,f}$ were negatively correlated with the homogenization pressure, and the protein and fat contents counteractively affected the protein aggregation. For the SLC gels made with the higher NaCl content, the USAXS-derived gel $r_{g,f}$ predicted the sodium release better than the DLS-based emulsion $r_{h,e}$ did. With sodium being the primary chemical for salty stimuli, present findings in the literature do not agree on whether saltiness perception depends primarily on the maximum rate (de Loubens, et al., 2011), maximum intensity or area under the curve (Morris, et al., 2009) of sodium delivery. Future studies evaluating the saltiness perception of the SLC gels with respect to sodium release and gel structures may help identify the governing parameters for saltiness perception. This approach can provide industrial insights and practical strategies for sodium reduction via engineering the structure of lipoproteic foods.

459

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461

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643

Figure captions

Fig. 1. Slit smeared USAXS profiles of the SLC emulsions and their counterpart gels. A. Non-fat samples and B. Fat- containing samples. The curves are vertically shifted to avoid overlap. The positive numbers are the r_g values of the particles in nanometer, with bolded numbers being those of the gels. The negative numbers are the slope of the power law decay exponents. Sample code represents protein(%w/w)-fat(%w/w)-NaCl(%w/w)-homogenization pressure(MPa).

Fig. 2. Representative curves of the *in vitro* sodium release of two SLC gels. The maximum rate of sodium release, R_{max} , the maximum concentration of sodium release, C_{max} , and the area under the curve of sodium release, AUC were derived from each curve as indicated in the graph. Sample code represents protein (%w/w)-fat (%w/w)-NaCl (%w/w)-homogenization pressure (MPa).

Fig. 3. Environmental scanning electron microscopy (ESEM) images of the cross-sections of frozen-fractured lipoproteic emulsion gels. Sample code represents protein(%w/w)-fat(%w/w)-NaCl(%w/w)-homogenization pressure(MPa).

Fig. 4. Slit smeared USAXS profiles of the SLC gels made with 1.5% (A, C) or 3.5% (B, D) NaCl at homogenization pressures of 14 (A, B) or 55 (C, D) MPa. The curves are vertically shifted to avoid overlap. Sample code represents protein(%w/w)-fat(%w/w)-NaCl(%w/w)-homogenization pressure(MPa).

Figure 1

Figure 2

Figure 3

Figure 4

Tables

Table 1

Formula and homogenization pressure of the solid lipoproteic colloid (SLC) gels. Sample code represents protein(% w/w)-fat(% w/w)-NaCl(% w/w)-homogenization pressure(MPa).

Protein (%, w/w)	Fat (%, w/w)	Sample code			
		1.5 % (w/w) NaCl		3.5% (w/w) NaCl	
		Homogenization at 14 MPa	Homogenization at 14 MPa	Homogenization at 14 MPa	Homogenization at 55 MPa
8	0	8-0-1.5-14	8-0-1.5-55	8-0-3.5-14	8-0-3.5-55
8	11	8-11-1.5-14	8-11-1.5-55	8-11-3.5-14	8-11-3.5-55
8	22	8-22-1.5-14	8-22-1.5-55	8-22-3.5-14	8-22-3.5-55
8	33	8-33-1.5-14	8-33-1.5-55	8-33-3.5-14	8-33-3.5-55
16	0	16-0-1.5-14	16-0-1.5-55	16-0-3.5-14	16-0-3.5-55
16	11	16-11-1.5-14	16-11-1.5-55	16-11-3.5-14	16-11-3.5-55
16	22	16-22-1.5-14	16-22-1.5-55	16-22-3.5-14	16-22-3.5-55
16	33	16-33-1.5-14	16-33-1.5-55	16-33-3.5-14	16-33-3.5-55

Table 2

Radii of gyration of the protein aggregates ($r_{g,p}$) and the fat globules ($r_{g,f}$) in the SLC gels derived from USAXS measurement. Sample code represents protein(%w/w)-fat(%w/w)-NaCl(%w/w)-homogenization pressure(MPa).

Sample (n for NaCl%)	$r_{g,p}$ (nm)		$r_{g,f}$ (nm)	
	n = 1.5	n = 3.5	n = 1.5	n = 3.5
8-0-n-14	9.2	3.1	-	-
8-11-n-14	87.0	59.8	449.3	371.5
8-22-n-14	54.9	53.6	484.6	387.3
8-33-n-14	40.0	37.6	319.3	291.2
16-0-n-14	8.8	2.8	-	-
16-11-n-14	125.1	155.5	457.5	711.0
16-22-n-14	91.5	103.0	325.2	407.8
16-33-n-14	67.0	N.D. ^a	274.0	N.D. ^a
8-0-n-55	7.2	7.5	-	-
8-11-n-55	27.5	24.4	120.5	125.2
8-22-n-55	22.2	21.6	125.8	121.6
8-33-n-55	22.5	31.7	121.2	110.1
16-0-n-55	8.2	20.7	-	-
16-11-n-55	45.5	35.5	153.6	91.2
16-22-n-55	35.0	24.3	113.6	77.4
16-33-n-55	33.5	30.2	101.0	66.3

a. Sample not measured due to operation error.

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16 **Table 3**

17 Sodium release and textural properties of the SLC gels with 3.5% NaCl, and the hydrodynamic radius of the droplets in the corresponding
 18 emulsions ^a.

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Sample	R _{max} ± SD ^b (ppm Na/s)	C _{max} ± SD (ppm Na)	AUC ± SD (10 ³ ppm Na.s)	Serum release ± SD (g)	Max stress ± SD (Kpa)	Strain at maximum stress ± SD (%)	Emulsion r _{h,e} ± SD (nm)
8-22-3.5-55	2.38 ± 0.77ab ^c	181.21 ± 5.88a	43.13 ± 1.52a	0.49 ± 0.08b	112.25 ± 15.80ab	54.08 ± 3.42c	127.88 ± 22.42b
8-33-3.5-55	2.59 ± 0.49ab	166.53 ± 10.15ab	39.54 ± 2.54ab	0.30 ± 0.07c	166.29 ± 29.46ab	56.41 ± 1.12c	134.62 ± 10.40b
8-33-3.5-14	1.76 ± 0.30b	150.44 ± 9.70b	35.51 ± 2.44b	0.47 ± 0.01b	101.22 ± 3.89b	60.28 ± 1.98b	329.18 ± 13.17a
16-11-3.5-55	2.90 ± 0.12a	178.09 ± 17.75a	43.39 ± 4.50a	0.47 ± 0.07b	179.14 ± 16.04a	46.30 ± 2.00d	296.16 ± 3.34b
16-11-3.5-14	2.09 ± 0.31ab	95.18 ± 20.13c	24.41 ± 5.66c	0.90 ± 0.04a	172.75 ± 83.98a	69.61 ± 2.72a	352.12 ± 33.19a

21 a. See Kuo and Lee (2014b) for the properties of the SLC gels with 1.5% NaCl.

22 b. R_{max}, maximum rate of sodium release; C_{max}, maximum concentration of sodium release; AUC, area under the curve of sodium release.
 23 Serum release, amount of liquid expelled from the sample during the compression test. r_{h,e}, hydrodynamic radius of the emulsions
 24 particles measured using dynamic light scattering (DLS).

25 c. The numbers followed by the same letters are not significantly different.

26

27

Table 4

Correlation coefficients between sodium release, textural properties and particle sizes of fat globules of the SLC gels at constant NaCl contents ^a.

NaCl % in SLC gels	$C_{\max}$ (ppm Na)		AUC (10 ³ ppm Na.s)		Strain at maximum stress (%)		Emulsion $r_{h,e}$ from DLS (nm)		Gel $r_{g,f}$ from USAXS (nm)	
	1.5	3.5	1.5	3.5	1.5	3.5	1.5	3.5	1.5	3.5
$R_{\max}^b$ (ppm Na/s)	0.989 *** ^c	0.553	0.994 ****	0.610	-0.924 *	-0.749	-0.151	-0.412	-0.498	-0.578
$C_{\max}$ (ppm Na)			0.999 ****	0.996 ****	-0.957 *	-0.913 *	-0.170	-0.652	-0.551	-0.987 ***
AUC (10 ³ ppm Na.s)					-0.948 *	-0.940 *	-0.163	-0.632	-0.537	-0.979 ***
Serum release (g)					0.773	0.705	0.599	0.642	0.821	0.923 *
Strain at maximum stress (%)							0.415	0.399	0.761	0.902 *
Emulsion $r_{h,e}$ from DLS (nm)									0.883 *	0.660

a. Refer to Section 2.2 for the list of the samples. The correlation was carried on each of the five samples with the same NaCl contents.

b. $R_{\max}$, maximum rate of sodium release; $C_{\max}$, maximum concentration of sodium release; AUC, area under the curve of sodium release. Serum release, amount of liquid expelled from the sample during the compression test. $r_{h,e}$, hydrodynamic radius of the emulsions particles measured using dynamic light scattering (DLS).

c. The numbers with the superscripts of one, two, three or four asterisks indicate significant correlations for $P < 0.05$, 0.01, 0.005 and 0.001, respectively.

**Solid lipoproteic
colloid gels**

Gel made at higher homogenization pressure

- Smaller fat globules in gel
- More extensive breakdown upon compression in water
- Greater sodium release