

Journal Pre-proof

Structural characterization of nanoemulsions stabilized with sodium caseinate and of the hydrogels prepared from them by acid-induced gelation

Juan Manuel Montes de Oca-Avalos, Cristián Huck-Iriart, Virginia Borroni, Karina Dafne Martínez, Roberto Jorge Candal, María Lidia Herrera



PII: S2665-9271(20)30017-4

DOI: <https://doi.org/10.1016/j.crfs.2020.03.010>

Reference: CRFS 32

To appear in: *Current Research in Food Science*

Received Date: 23 December 2019

Revised Date: 30 March 2020

Accepted Date: 30 March 2020

Please cite this article as: Montes de Oca-Avalos, J.M., Huck-Iriart, C., Borroni, V., Martínez, K.D., Candal, R.J., Herrera, M.L., Structural characterization of nanoemulsions stabilized with sodium caseinate and of the hydrogels prepared from them by acid-induced gelation, *Current Research in Food Science*, <https://doi.org/10.1016/j.crfs.2020.03.010>.

This is a PDF file of an article that has undergone enhancements after acceptance, such as the addition of a cover page and metadata, and formatting for readability, but it is not yet the definitive version of record. This version will undergo additional copyediting, typesetting and review before it is published in its final form, but we are providing this version to give early visibility of the article. Please note that, during the production process, errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

© 2020 The Author(s). Published by Elsevier B.V.

Juan Manuel Montes de Oca-Avalos: Performing experiments. Data modeling

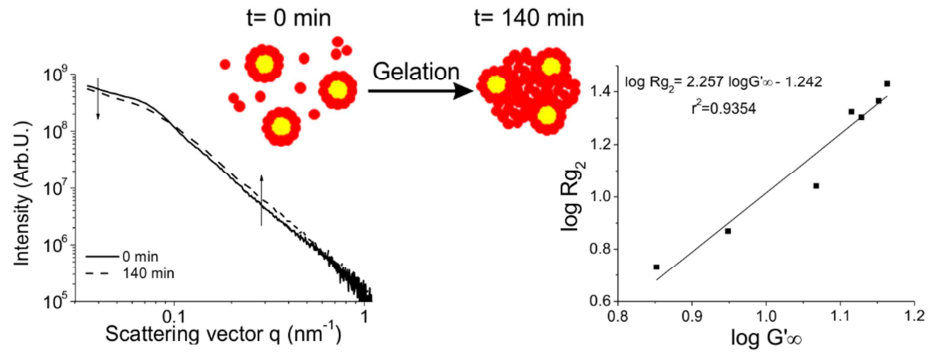
Cristián Huck-Iriart: Software Programming. Data modeling

Virginia Borroni: Statistical analysis. Help in writing the initial draft

Karina Dafne Martínez: Help in writing the initial draft

Roberto Jorge Candal: Conceptualization. Methodology design. Data interpretation

María Lidia Herrera: Conceptualization. Methodology design. Original Draft Preparation.



Journal Pre-proof

Structural characterization of nanoemulsions stabilized with sodium caseinate and of the hydrogels prepared from them by acid-induced gelation

Juan Manuel Montes de Oca-Avalos^{a1}, Cristián Huck-Iriart^b, Virginia Borroni^a, Karina Dafne Martínez^a, Roberto Jorge Candal^c, and María Lidia Herrera^{a*}

^a Institute of Polymer Technology and Nanotechnology, University of Buenos Aires- CONICET, Facultad de Arquitectura, Diseño y Urbanismo, Intendente Güiraldes 2160, Pabellón III, C1428EGA Ciudad Autónoma de Buenos Aires, Argentina

^b Laboratorio de Cristalografía Aplicada, Escuela de Ciencia y Tecnología, Universidad Nacional de San Martín (UNSAM), Campus Miguelete, 25 de Mayo y Francia, 1650 San Martín, Provincia de Buenos Aires, Argentina

^c Instituto de Investigación e Ingeniería Ambiental, Universidad Nacional de San Martín (UNSAM), Campus Miguelete, 25 de Mayo y Francia, 1650 San Martín, Provincia de Buenos Aires, Argentina

Juan Manuel Montes de Oca-Avalos: jmontesdeocaav@gmail.com. Performing experiments. Data modeling; Cristián Huck-Iriart: chuck@unsam.edu.ar. Software Programming. Data modeling; Virginia Borroni: mvirborroni@gmail.com. Statistical analysis. Help in writing the initial draft; Karina Dafne Martínez: karinadafnem@yahoo.com.ar. Help in writing the initial draft; Roberto Jorge Candal: ricandal@gmail.com. Conceptualization. Methodology design. Data interpretation; María Lidia Herrera: * corresponding autor. mlidiaherrera@gmail.com.

Conceptualization. Methodology design. Original Draft Preparation.

¹ Permanent address: Center for the Development of Advanced Materials and Nanotechnology, Universidad Nacional de Ingeniería, Av. Túpac Amaru 210, Rimac, Lima, Perú.

Abstract

Hydrogels obtained by acidification with glucono- δ -lactone (GDL), starting from nanoemulsions formulated with different concentrations of sodium caseinate (1 to 4 wt.%) or 4 wt.% sodium caseinate and sucrose (2 to 8 wt.%), were prepared with the aim of quantifying structural parameters of both, initial nanoemulsions and hydrogels after 2.5 h of GDL addition, using the Guinier-Porod (GP) or the generalized GP models. Gelation process was followed by performing in situ temperature-controlled X-ray small angle scattering experiments (SAXS) using synchrotron radiation. In nanoemulsions, the calculated radius of gyration for oil nanodroplets ($R_{g_{oil}}$) decreased with increasing protein concentration and for the 4 wt.% protein nanoemulsion, with increasing sucrose content. Calculated values of $R_{g_{oil}}$ were validated correlating them with experimental Z-average values as measured by dynamic light scattering (DLS). For hydrogels, radii of gyration for the sphere equivalent to the hydrogel scattering object ($R_{g_{sph}}$) were close to 3 nm while correlation distances among building blocks (R_{g2}) were dependent on formulation. They increased with increasing contents of sodium caseinate and sucrose. R_{g2} parameter linearly correlated with hydrogel strength (G'_{∞}): a more connected nanostructure led to a stronger hydrogel.

Key words: nanostructure, SAXS, Guinier-Porod Model, nanoemulsion, hydrogel

1. Introduction

Milk can be considered to be a colloidal emulsion of casein particles, historically called micelles. The native casein micelles are composed of a complex of associated protein and calcium phosphate of about 200 nm. The protein fraction of the casein micelles, which represents ~93% of its dry mass, is composed of four individual sub-micelles, called α s1-, α s2-, β - and κ -casein [1]. Sodium caseinate (NaCas) is a commercial milk product obtained by removing most of calcium phosphate. The final product contains disaggregated sub-micelles of approximately 10–12 nm. Owing to its high nutritional value, health benefits, and desirable functional properties such as emulsification, water and fat-binding, thickening and gelation, sodium caseinate is now extensively used as an ingredient in food products. Fermentation of milk is of importance during the processing of a number of dairy products such as yoghurt-like desserts [2]. This process can be mimicked by acidifying sodium caseinate-stabilized nanoemulsions with GDL. Acid gelation occurs as the pH slowly decreases.

A hydrogel may be defined as a nonfluid colloidal or three-dimensional hydrophilic polymeric network with the capacity of imbibing large amounts of water [3]. Hydrogels may be from synthetic, semi-synthetic, or natural origins. Natural biopolymers are preferred because they are non-toxic and are friendly to the environment. Hydrogels have applications in numerous fields. In the food industry, they performed a variety of functions, including nutrient and flavor carriers [4, 5], calorie reduction [6], trans and saturated fat replacements in desserts [7], and oral delivery of lipophilic active ingredients [8], among others. The structural properties of those hydrogels were closely related to their performance for an intended application.

Nanoemulsions are usually defined as a thermodynamically unstable colloidal dispersion consisting of two immiscible or partially miscible liquids, with one of the liquids being dispersed as small spherical droplets (discontinuous phase) in the other liquid (continuous phase), with radius sizes of droplets smaller than 100 nm [9].

Nanoemulsions present several advantages over conventional emulsions due to the

small droplets size they contain: high optical clarity, good physical stability against gravitational separation or droplet aggregation, and enhanced bioavailability of encapsulated substances [10]. Films or hydrogels prepared from sodium caseinate-stabilized nanoemulsions showed advantages compared to the ones obtained from conventional systems. The enhancement in properties was explained by higher homogeneity in the structure, in terms of distribution of dispersed phase and absence of droplets aggregates as analyzed by confocal laser scanning microscopy [7,11]. In nano-based sodium caseinate films, this homogeneity led to improved physical properties such as water vapor permeability and stability with respect to oil release [11], while in nano-based hydrogels, led to lower porosity as described by X-ray microtomography and improved rheological properties [7].

Small-Angle X-ray Scattering (SAXS) is a technique that has been increasingly used in the past ten years to describe food structure [7,12-18], to name a few. SAXS technique has the advantage over other very established methods such as microscopy approaches (data are collected on a focal plane) that the obtained results give averaged information on the samples, analyzed in bulk, evaluating the whole volume with statistical significance [19,20]. The high photon flux of the synchrotron source allows in situ characterization of hydrogel formation in a sample holder and as samples suffer no manipulation or treatment, structure is analyzed without disturbing the systems. Besides, turbid samples such as nanoemulsion-based hydrogels are successfully analyzed [11]. SAXS is a technique by which nanoscale density differences in a sample can be quantified by analyzing the elastic scattering behavior of X-rays when travelling through it. SAXS has been used for characterizing nanoscale electron density correlations and/or the shape of nanoscopic objects with sizes up to ~100 nm. Quantifying structure in nanoscale is of great importance since it has been proved in several systems that structural characteristics correlated with macroscopic properties such as rheological behavior under small or large deformation [12,17].

Traditionally, small angle X-ray scattering (SAXS) data are characterized by the Guinier

and Porod regions. The model used in this work for fitting SAXS data is an empirical model that allows describing scattering objects of different geometries, from spherical (the Guinier-Porod model) to nonspherical objects such as rods, platelets or lamellae (the generalized Guinier-Porod model), [21].

The aim of the present work was to quantify structural elements of sodium caseinate hydrogels prepared by acidification with GDL and of the nanoemulsions from which they were prepared. The Guinier-Porod and the generalized Guinier-Porod models were used to calculate radii of gyration and structural parameters. The calculated data were compared with data measured with experimental techniques based on different principles than those of SAXS.

2. Materials and methods

2.1 Materials

Sodium caseinate (NaCas), sucrose (S), glucono delta-lactone (GDL), and sodium azide were purchased from Sigma (Sigma–Aldrich, St. Louis, Mo., USA). Ethyl acetate was obtained from Sintorgan (Sintorgan S.A., Buenos Aires, Argentina). All reagents used were of analytical grade. No further purification was performed. Bidistilled water was used in all experiments (18 mΩ; Milli-Q Water System, Millipore Corporation, Billerica, MA, USA). The lipid phase selected was commercial sunflower seed oil (SFO) with the following fatty acids composition: palmitic (5.7 wt.%), stearic (4.3 wt.%), arachidic (1.6 wt.%), behenic (1.1 wt.%), oleic (82.6 wt.%), and linoleic (3.9 wt.%) acids.

2.2 Hydrogels preparation

Hydrogels were obtained by acidifying nanoemulsions with GDL. Nanoemulsions were prepared using a combination of a high-energy homogenization and evaporative ripening methods previously reported for whey protein-stabilized systems, with minor changes [22]. For studying the effect of protein and sucrose concentrations, eight

nanoemulsions were prepared. Solutions with concentrations of 1, 2, 3, or 4 wt.% of NaCas in water were prepared. The 4 wt.% NaCas-stabilized nanoemulsion may also contain S added to the aqueous phase in concentrations of 2, 4, 6, or 8 wt.%. The lipid phase was a solution of 15 wt.% SFO in ethyl acetate. In all nanoemulsions prepared, the ratio NaCas/SFO was kept constant at 0.6. Sodium azide was used in a percentage of 0.01 wt.% to prevent microbial contamination. Nanoemulsions preparation method comprises three stages: the formation of a coarse emulsion (droplets in the microns), further homogenization to obtain a fine emulsion (droplets from 0.2 to 0.6 μm in diameter) and solvent evaporation to give nanoemulsions. In the first step, lipid and aqueous phases were mixed using an Ultra-Turrax (UT) T18 high-speed blender (S 18N-19G dispersing tool, IKA Labortechnik, Janke & Kunkel, GmbH & Co., Staufen, Germany), operated at 20,000 rpm for 1 min. Samples were kept in an ice bath (0 °C) during processing. The process was repeated three times. In the second step, the resultant coarse emulsions were further homogenized for 20 min using an ultrasonic liquid processing (US), VIBRA CELL, VC750 (power 750 W, frequency 20 kHz) model (Sonics & Materials, Inc., Newtown, CT, USA), with a 13-mm-diameter and 136-mm-length tip. The selected amplitude was 30%. The temperature of the sample-cell was controlled by means of an ice bath (0 °C) with a temperature cut down control of 20 ± 1 °C during ultrasound treatment. After ultrasound treatment, fine emulsions were obtained, but droplets were still in the conventional emulsions range. In the third step, the prepared fine emulsions were placed in a rotary evaporator Buchi model R 100 (Buchi, Postfach, Switzerland) connected to a vacuum pump and a recirculating chiller to eliminate ethyl acetate. The degree of ethyl acetate evaporation was determined by carrying out a mass balance of emulsions before and after solvent evaporation [22]. The process was performed at 45 °C for 20 min. Then, the samples were cooled quiescently to room temperature (22.5 °C). The pHs of the SFO emulsions were 6.66 ± 0.05 , close to 7. No buffer was added to the emulsions. Nanoemulsions were analyzed for droplet size distribution immediately after preparation. The average diameter of

droplets (Z-average) was obtained from the distribution in volume using a DLS device NanoBrook 90Plus Particle Size Analyzer (Brookhaven Instruments Corporation, New York, USA), equipped with a laser and operated at room temperature. Nanoemulsions were diluted in distilled water until the final concentration of NaCas was 0.1 wt%. The measurements were performed in duplicate with a scattering angle of 90° . For hydrogel formation, GDL was added to nanoemulsions at a GDL/NaCas ratio of 0.2. This GDL/NaCas ratio was chosen because it was the one that allowed obtaining the best rheological properties of all the ratios tested [7]. The final pH value was 4.75 ± 0.20 . The required amount of GDL was accurately weighed and added to 10 g of nanoemulsions. GDL was dissolved by stirring tubes with a vortex for 10 s and nanoemulsion was immediately placed in a SAXS cell for liquids previously described [23].

2.3 SAXS measurements

Small angle X-Ray scattering (SAXS) measurements were performed in the SAXS1 beamline of the Brazilian National Laboratory of Synchrotron Light (LNLS, Campinas, Brazil). For all experiments, the selected wavelength was 1.548 \AA . A Pilatus 300K detector was used with a distance sample/detector of 3090.7 mm. The scattering intensity distributions as a function of scattering vector q were obtained in the q range between 0.035 and 1.33 nm^{-1} , with $q = (4\pi/\lambda) \sin(\theta) = 2\pi/d$, where λ is the radiation wavelength, 2θ is the scattering angle, and d is the interplanar distance. The SAXS patterns were recorded with exposure times of 1 min and a resting time of 1 min. Samples were analyzed for up to 2.5 h. Temperature in the liquid cell was controlled at 22.5°C using a water bath. One-dimensional curves were obtained by integration of the 2D data using the program FIT-2D [24]. The SAXS normalized patterns were fitted using the Guinier-Porod and the generalized Guinier-Porod model [21]. Samples were analyzed by duplicate.

2.3.1 Nanoemulsions characterization: the Guinier-Porod (GP) model

In nanoemulsion systems (initial state of gelation process), three objects contributed to the scattered intensity, $I(q)$: nanodroplets (I_{oil}), protein adsorbed on the interface (I_{ads}), and protein dispersed on the aqueous phase (free protein, I_{free}). The total intensity may be expressed as:

$$I(q) = I_{oil} + I_{ads} + I_{free} + back \quad (1)$$

where $back$ is a constant background parameter.

To take into account the relative contribution of each scattering element, a scale factor (SC) was included:

$$I(q) = SC_{oil}P_{oil}(q) + SC_{ads}P_{ads}(q) + SC_{free}P_{free}(q) + back \quad (2)$$

with P_{oil} , P_{ads} , and P_{free} the form factors of oil nanodroplets, adsorbed protein, and free protein, respectively.

To evaluate P , a Guinier–Porod empirical model developed by Hammouda was selected [21]. The form factor is given by two contributions, the Guinier (P_1) and Porod (P_2) terms:

$$P_1(q) = \frac{1}{q_c^s} \exp\left(-\frac{q^2 R_g^2}{3-s}\right) \quad \text{for } q \leq q_c \quad (3)$$

$$P_2(q) = \frac{D}{q^d} \quad \text{for } q \geq q_c \quad (4)$$

where R_g is the radius of gyration, s is a parameter related to object symmetry, d is the Porod exponent, and D is a scaling factor. Parameter s is equal to 0 for spherical objects, to 1 for rods or rigid cylinders, and to 2 for flat objects or lamellas. In addition, a Porod exponent of 4, points to particles with smooth surfaces while a value of 3, points to very rough surfaces [25].

With the requirement that the values of the Guinier and Porod terms and their slopes (derivatives) be continuous at a value q_c , the following relationships are obtained:

$$q_c = \frac{[(d-s)(3-s)/2]^{1/2}}{R_g} \quad (5)$$

$$D = \exp\left(-\frac{q_c^2 R_g^2}{3-s}\right) q_c^{d-s} \quad (6)$$

and therefore, including D , equations (3) and (4) may be written as follows:

$$P_1(q) = \frac{1}{q_c^s} \exp\left(-\frac{q^2 R_g^2}{3-s}\right) \quad \text{for } q \leq q_c \quad (7)$$

$$P_2(q) = \exp\left(-\frac{q_c^2 R_g^2}{3-s}\right) \frac{q_c^{d-s}}{q^d} \quad \text{for } q \geq q_c \quad (8)$$

Due to the large oil/water interface area of nanoemulsions, free NaCas lose the casein-casein correlation, which is visible as a broad peak at intermediate angles [13], in consequence, no structure factor was employed to interpret the systems studied in this work.

2.3.2 Gels characterization: the Generalized Guinier-Porod (GP) model

The model in 2.3.1 is not suitable for nonspherical scattering objects [21]. To evaluate protein contribution in hydrogels, the selected model was the generalized Guinier-Porod approach, which is applicable for quantifying structures with scattering objects such as rods or lamellae [26, 27, 21]. NaCas was the matrix in hydrogels, and therefore, adsorbed (molecules at the interface oil/water) and free (micelles dispersed in the aqueous phase) protein cannot be distinguished since molecules are connected to form a tridimensional network. In the case of hydrogels, equations 1 and 2 became:

$$I(q) = I_{oil} + I_{prot} + back \quad (9)$$

where I_{prot} is the scattered intensity of total protein.

$$I(q) = SC_{oil} P_{oil}(q) + SC_{prot} P_{prot}(q) + back \quad (10)$$

As in the case of nanoemulsions, in hydrogels, the scattering parameters of nanodroplets were evaluated from P_{oil} using the Guinier-Porod approach. Protein scattering objects were quantified from I_{prot} using the generalized Guinier-Porod model, with the following equations:

$$P_1(q) = \frac{1}{q^{s_2}} \exp\left(-\frac{q^2 R_{g2}^2}{3-s_2}\right) \quad q \leq q_2 \quad (11)$$

$$P_2(q) = \frac{G}{q^{s_1}} \exp\left(-\frac{q^2 R_{g1}^2}{3-s_1}\right) \quad q_2 \leq q \leq q_1 \quad (12)$$

$$P_3(q) = \frac{D}{q^d} \quad q \geq q_1 \quad (13)$$

where G and D are scaling factors, S_1 and S_2 are two slopes in the Guinier zone, and R_{g1} and R_{g2} are distances corresponding to the scattering object, with $R_{g1} < R_{g2}$.

According to [21], “ $3 - s_2$ and $3 - s_1$ are the dimensionality parameters, and R_{g2} and R_{g1} are the radii of gyration for the short and overall sizes of the scattering object”. In this work, interpretation of R_{g2} parameter was the same as the cutoff distance for the fractal model [13]. This approach was previously reported by Pink et al., 2013 and Quinn et al., 2014. In those articles, parameters obtained from other X-ray techniques such as ultra-small X-ray scattering (USAXS) were also interpreted in a fractal way [28, 29]. Considering that the global function and their slopes (derivatives) must be continuous at q_1 and q_2 , the following relationships are obtained:

$$q_2 = \left(\frac{s_1 - s_2}{\frac{2}{3-s_2} R_{g2}^2 - \frac{2}{3-s_1} R_{g1}^2} \right)^{1/2} \quad (14)$$

$$G = \exp \left[-q_2^2 \left(\frac{R_{g1}^2}{3-s_1} - \frac{R_{g2}^2}{3-s_2} \right) \right] q_2^{(s_2-s_1)} \quad (15)$$

$$q_1 = \frac{1}{R_{g1}} \left[(d - s_1) \frac{3-s_1}{2} \right]^{1/2} \quad (16)$$

$$D = G \exp \left(-\frac{q_1^2 R_{g1}^2}{3-s_1} \right) q_1^{(d-s_1)} \quad (17)$$

Including these expressions in P , the generalized Guinier-Porod model may be written as follows:

$$P_1(q) = \frac{1}{q^{s_2}} \exp \left(-\frac{q^2 R_{g2}^2}{3-s_2} \right) \quad q \leq q_2 \quad (18)$$

$$P_2(q) = \frac{q_2^{(s_2-s_1)}}{q^{s_1}} \exp \left[-\frac{q^2 R_{g1}^2}{3-s_1} - q_2^2 \left(\frac{R_{g1}^2}{3-s_1} - \frac{R_{g2}^2}{3-s_2} \right) \right] \quad q_2 \leq q \leq q_1 \quad (19)$$

$$P_3(q) = \frac{G q_1^{(d-s_1)}}{q^d} \exp \left(-\frac{q_1^2 R_{g1}^2}{3-s_1} \right) \quad q \geq q_1 \quad (20)$$

The values of R_{g1} do not correspond to scattering objects with spherical symmetry. To compare the parameters calculated for hydrogels with that calculated for

nanoemulsions, radius of gyration of the sphere equivalent to the hydrogel scattering object (R_{gsph}) were obtained with the following equation:

$$R_{gsph} = \frac{2\sqrt{3}R_{g1}}{[(4-s_1)(3-s_1)]^{1/2}} \quad (21)$$

The Guinier-Porod model allows calculating droplet coverage (C), that is total surface occupied by adsorbed protein, by the limit in the Porod region as follows [25]:

$$C \propto \frac{SC_{ads} [P_{GP}^{ads}(q) q^4]_{q \rightarrow \infty}}{\Delta\rho_{ads}^2} \quad (22)$$

where, $\Delta\rho_{ads}$ is the difference in electronic density between adsorbed protein and the background. Protein density was set at $1,1 \times 10^{-5} \text{ \AA}^2$, taken into account literature values, and density change due to sucrose addition was considered as reported [30].

Replacing the expression for Porod region in Ec. 22, and considering that $d = 4$ and $s = 0$, it becomes:

$$C \propto \frac{SC_{ads}}{\Delta\rho_{ads}^2} \frac{36 e^2}{R_g^4} \quad (23)$$

2.4 Rheological measurements

Gelation under shear was followed by dynamic oscillatory rheology using a Paar PhysicaMCR 300 rheometer (Anton Paar Inc., Ashland, USA). After adding GDL, approximately 1.2 g of emulsion was immediately loaded into parallel plates (PP30/S) separated by 1 mm and changes in G' and G'' moduli with time were monitored. Data were taken at 22.5 °C, every 2 min, and were recorded for up to 3 h. Studies were performed with a 0.01 % strain at a constant frequency of 1 Hz. Asymptotic values of G' (G'_∞) were calculated from G' vs. time curves using values of time higher than the time at which $G' = G''$, with the following empirical equation:

$$\log G' = \log G'_\infty \left[\frac{k_1 t}{1+k_1 t} \right] \quad (24)$$

where k_1 is a constant and t is the time. Values of G'_∞ are the average of three determinations.

2.5 Statistical analysis

For the SAXS model, the parameter “*R* value” was selected to provide an indicator of the fit quality [31]. It was calculated using the following equation:

$$R \text{ value} = \frac{\sum_{i=1}^N |I_{exp}(q_i) - I_{mod}(q_i)|}{\sum_{i=1}^N I_{exp}(q_i)} \quad (25)$$

A *R* value ranging between 0 and about 0.1 indicates a good to acceptable fit, whereas large values (up to infinity) denote a poor fit.

3. Results

3.1 Quantification of nanoemulsions structure

3.1.1 SAXS data

Figure 1 shows the results obtained when all nanoemulsions were analyzed by SAXS. As may be observed, for all *q* values, total intensity increased with increasing concentrations of protein. In a previous work, Z-average of these nanoemulsions was reported to decrease with increasing NaCas concentrations [10]. In other words, the percentage of droplets in the nano range (not invisible for SAXS technique) increased for the 4 wt.% NaCas nanoemulsions compared to the 1 wt.% NaCas system. Changes in total intensities were in agreement with these findings. The number of scattering objects with sizes within SAXS measurements range (~100 nm) increased and therefore intensity was the highest for the more concentrated formulation (4 wt.% NaCas nanoemulsion). The increase in sucrose concentration led to an increase in intensity only in the low *q* values, showing a different behavior than for protein concentration. The effect of sucrose on total intensity could be expected to involve other mechanisms of action (see below results and discussion sections).

3.1.2 Nanoemulsions fitted by GP model

Table 1 reports the values of Guinier-Porod model parameters selected for fitting nanoemulsions SAXS curves. All scattering objects, oil droplets, protein micelles (free

in bulk), and protein adsorbed at the interface were considered spherical, and therefore, parameter s was set at 0. All surfaces were considered smooth with parameter d equal to 4. Values of calculated parameters corresponded to average values of scattering objects. A lower limit of 15 nm for R_g values of oil droplets was selected taken into account previous DLS results [10]. Radius of protein objects adsorbed at the interface was considered 3 nm as previously reported [13].

As an example, Figure 2 shows the results of fitting the 4NaCas0S-nanoemulsion SAXS curve with the GP model. The experimental data, the calculated total intensity, and the contributions of the three scattering objects (nanodroplets, adsorbed protein, and free protein) are reported. The sum of the three contributions fitted experimental values very well as may be observed from values of parameters R (Table 2). As expected, from the analysis of all curves (see supplementary materials), I_{oil} increased with increasing protein and sucrose contents since nanodroplets number also increased in both cases (lower z-average for higher protein and sucrose contents). I_{ads} and I_{free} increased when NaCas concentrations increased from 1 to 4 wt.%. However, addition of sucrose to the 4 wt.% NaCas nanoemulsion diminished both protein-intensities, indicating that protein/sucrose interactions were strong and able to modify protein/protein interactions.

The calculated radii of gyration for oil nanodroplets ($R_{g_{oil}}$) and free protein ($R_{g_{free}}$) are reported in Table 2. $R_{g_{free}}$ slightly increased with increasing protein concentration up to 4 wt.% NaCas, while $R_{g_{oil}}$ always decreased; for the 4 wt.% NaCas nanoemulsion $R_{g_{free}}$ significantly diminished. When protein content was 4 wt.%, both radii diminished with increasing sucrose concentration. Taken into account that sphere radius (R_s) and R_g are related by $R_s = \sqrt{\frac{5}{3}} R_g$ [12], since each system presented a given size distribution, R_s is a weighted average radius (larger particles scatter X-ray more than small ones), the radius of a sphere composed of a nanodroplet covered by a layer of spherical protein particles, and therefore the diameter of droplet/covering scattering element,

may be calculated. Figure 3 reports the diameters calculated by fitting SAXS data with the GP model and the diameters (Z-average) obtained by measuring droplets size distribution using DLS for all emulsions studied. As may be observed calculated values were always smaller than values measured by DLS. However, tendencies were similar: values significantly diminished with increasing concentrations of protein, and for the same protein concentration, with increasing sucrose concentrations ($p < 0.05$). Although significant, differences due to the addition of sucrose were smaller than the ones obtained increasing NaCas concentration. It was expected that DLS values were greater than values calculated from SAXS curves. DLS measures not only droplets/coverage size but also hydration layer that moves with nanodroplet. In addition to R values (Table 2), the fact that values from two different techniques correlated indicated that SAXS model assumptions regarding geometry and particle surface were correct.

3.1.3 Droplet coverage

Figure 4 reports droplet coverage (C), as total surface occupied by adsorbed protein, for all samples. C significantly increased with increasing protein content ($p < 0.05$). C value for the 1 wt.% NaCas-stabilized nanoemulsion was 4 times smaller than C value for the 4 wt.% nanoemulsion since there is a higher concentration of total protein in the nanoemulsion and a higher dispersed oil concentration that must be coated to be stabilized. Thus, the molecules preferred to be located at the interface than to remain in solution forming micelles. On the other side, it might be expected that sucrose did not have an effect on droplet coverage since it is not a surfactant. However, for the 4 wt.% NaCas-stabilized nanoemulsion, C slightly increased with increasing sucrose content: 2% coverage for every 2% increase in sucrose concentration. Sucrose slightly increased the tendency of being at the interface most likely because it interacted with protein molecules modifying protein/protein interactions and protein molecules affinity for the interface.

3.2 Quantification of nanogels structure

3.2.1 Gelation kinetics

Figure 5 shows, as an example, gelation kinetics of the nanoemulsion formulated with 4 wt.% NaCas and 8 wt.% S. SAXS patterns reported were obtained every 20 min. As time passed, the intensity in the Guinier zone diminished (lower inset) while in the Porod zone increased (upper inset), indicating that there were structural changes confirming the occurrence of sol/gel transition. Changes in patterns indicated that the size of scattering elements and connections of matrix building blocks changed as the gelation progressed. These changes in the structure were very complicate and therefore only initial and final states were characterized.

3.2.2 Structural characterization of gels

SAXS curves for gels measured after 2.5 h of GDL addition were fitted by the GP model for nanodroplets and the generalized GP model for protein matrix. Table 3 summarizes the selected values for the models parameters. For oil nanodroplets, as was done when fitting nanoemulsions SAXS curves, s was set at 0 (sphere) and d at 4 (smooth surface). For protein scattering elements, s_2 was set at 0 since the Guinier region was successfully fitted using only one slope, and s_1 value was fitted by the program. In all cases, results were very close and therefore a fixed value of 2.6, which was the average value calculated for all SAXS curves, was selected for parameter s_1 . As the radius of gyration of the sphere equivalent to the hydrogel scattering object (R_{gsph}) depends on s_1 and Rg_1 , by fixing s_1 , R_{gsph} only depended on Rg_1 . Distances Rg_1 and Rg_2 varied freely and were fitted by the program because the behavior during sol/gel transition was not known and therefore these values were hard to predict. Rg_1 corresponded to the size of the scattering object, which was not spherical since protein formed a network and no isolate protein objects can be identified in this matrix. Rg_2 was related to the length of interactions among building blocks and it might be expected that the higher the Rg_2 value the stronger the gel.

Figure 6 reports the intensity vs. q curve for the hydrogel prepared from the 4NaCas8S nanoemulsion. Figure 6 shows experimental data (I_{exp}), global fit of the model (I_{model}), and the contributions of the two scattering elements considered: oil nanodroplets (I_{oil}) and protein (I_{prot}). The calculated intensity fitted the experimental data very well. Parameter R also confirmed the goodness of the fit (Table 4). For all samples, both contributions, I_{oil} and I_{prot} increased with increasing contents of protein and sucrose, indicating that more scattering elements were present in hydrogels containing a higher solid mass (see supplementary materials). Table 4 reports values of parameters obtained from the GP model for nanodroplets, from the generalized GP model for protein scattering objects, and values of radius of gyration of the sphere equivalent to the hydrogel scattering object (R_{gsph}). As mentioned before, Rg_1 and Rg_2 may be interpreted as radius of gyration for building blocks and correlation length between building blocks, respectively. Rg_1 slightly increased with increasing protein concentration and for the 4 wt.% NaCas hydrogel, no significant differences were found with increasing sucrose concentration, indicating that building blocks were similar for different formulations ($p < 0.05$). Rg_2 significantly increased with increasing protein concentration and for a fixed protein content, slightly increased with increasing sucrose content. Connections among building blocks were improved for hydrogels containing a higher solid mass. Radius of gyration of oil nanodroplets (R_{goil}) significantly diminished with increasing protein and sucrose concentrations. According to these calculations, nanodroplets kept their original nanosize, which slightly increased from nanoemulsion to gel. These results showed that droplet coalescence was negligible during the sol/gel transition and that scattering objects of hydrogel had nanosizes.

3.3 Sol/gel transition by rheology

Figure 7 reports the evolution of G' and G'' moduli as a function of time for the nanoemulsion/hydrogel transition. The starting nanoemulsion selected as example was the one formulated with 4 wt.% NaCas and 8 wt.% sucrose. At the beginning of the run,

the behavior of G' and G'' with time was characteristic of a liquid system. G' and G'' values varied around zero. As the hydrogel formed, both moduli increased sharply. There is a time at which G' is equal to G'' and after this point, G' is always significantly higher than G'' . There is a general agreement among authors considering gelation time as the time at which both moduli have the same value. After that time hydrogel was structured until reached a plateau value for G' and G'' . As was reported the degree of stiffness of gels may be inferred from asymptotic values of G' (G'_{∞}), [32]. Figure 8 shows a plot of $\log G'_{\infty}$ vs. $\log R_{g2}$. As may be noticed both parameters correlated. Longer distances of interactions among building blocks corresponded to greater gel strength. The hydrogel prepared from the 4 wt.% NaCas/8 wt.% sucrose nanoemulsion was not included in Figure 8 because for this sample there was a notorious increase in G'_{∞} , greater than the one expected from the linear tendency showed for the other formulations. This hydrogel had the greatest strength of all systems studied and the highest solid mass of all. In this formulation, in addition to building blocks connections, solid mass also contributed to rheology.

4. Discussion

Previous work on sodium caseinate/trehalose conventional emulsions showed that the protein-to-oil ratio modified droplets size of discontinuous phase and strongly influences stability and destabilization mechanisms [33]. In this article, the protein-to-oil ratio was fixed at 0.6. However, for the prepared nanoemulsions, Z-average and polydispersity index as measured by DLS decreased while stability as evaluated by Turbiscan notoriously increased [34], indicating that droplets size and stability depended on more than one parameter. Other factors such as total solid mass were also important since they affected droplet coverage (C), influencing the affinity of protein molecules for the interface, and also the interactions among protein molecules, as may be inferred from parameter C . This increase in droplet coverage was in agreement with the fact that radius of gyration of free protein ($R_{g_{free}}$) significantly

diminished for the 4 wt.% NaCas-stabilized nanoemulsion while droplets size notoriously decreased, indicating that more protein was at the interface and less molecules interacted in solution to form micelles. As previously reported this formulation was stable for 2 months [33]. The effects of total solid mass were also noticeable in the nanoemulsion-based hydrogels as evidenced by the behavior of parameters R_{g2} and G'_{∞} . The highest the solid mass the greater the correlation distances and final strength of hydrogels.

Increasing sucrose content had a lower effect on systems parameters than increasing protein concentration. However, sucrose modified interactions among protein molecules increasing parameter C , and decreasing $R_{g_{free}}$ and $R_{g_{oil}}$ values. In conventional systems, addition of 20 wt.% sucrose to a 5 wt.% NaCas emulsion modified the long-term adsorption of NaCas at oil/water interface, and significantly changed interfacial dynamic characteristics, indicating that because of sucrose addition, NaCas became a better surfactant. In those systems, the interactions between sucrose and NaCas modified protein functionality, which led to a dramatic change in emulsion microstructure and stability [35]. SAXS calculated parameters supported this effect on nanosystems too. However, as stability of nanoemulsions is notoriously higher than the one of conventional emulsions with the same formulations, it occurred in less extent. Microtomography analysis of hydrogels prepared from nanoemulsions showed that porosity decreased and wall thickness increased with increasing sucrose content [7]. Sucrose was part of hydrogel structure, indicating that protein/sucrose interactions are strong and that these interactions modified the affinity among protein molecules. These findings are in agreement with the fact that R_{g2} distance increased with increasing sucrose concentration, indicating interactions among a higher number of building blocks. Rheological parameter G'_{∞} showed good correlation with R_{g2} distance as reported in Figure 8. A more connected nanostructure led to a stronger hydrogel.

The curve I_{prot} vs. q was also fitted using a fractal approach (see supplementary materials). The fractal dimension obtained was 2.5, a value very close to s_1 value, a Guinier slope in the medium zone of I vs. q curve, showing that all nano-based hydrogels had the same symmetry for connections between building blocks. The linear correlation between R_{g2} and G'_{∞} reported in Figure 8 confirmed that all hydrogels were fractal in nature. The calculated slope was 2.3, a value close to the fractal dimension calculated by the fractal model. The correlation distances calculated from the fractal model were in agreement with R_{g2} distances, which supported the physical interpretation chosen for parameter R_{g2} .

5. Conclusions

Hammouda approach [21] was successfully used to interpret nano-based sodium caseinate/sucrose hydrogels and the nanoemulsions that were used to prepare them. In hydrogels, two different types of objects were described together: oil droplets and protein building blocks. These objects had different geometries, however, they were quantified from a curve that described the whole q range. The model had the advantage of allowing protein building blocks quantification without assigning a geometry to them. Guinier zone was fitted with only one slope with low error, which indicated that all analyzed hydrogels had the same symmetry for building blocks connections. In both, nanoemulsions and nano-based hydrogels, model parameters correlated with macroscopic physical properties: calculated values of radius of gyration of nanodroplets ($R_{g_{oil}}$) showed the same tendencies as Z -average values measured by DLS, and long distances, assigned to connections between building blocks, (R_{g2}), correlated with G'_{∞} values, indicating that the connections between building blocks (nano scale) strongly influenced hydrogel strength (macroscopic system).

Acknowledgments

This work was supported by the University of Buenos Aires [grant number 20020170100039BA]; the Synchrotron Light National Laboratory (LNLS), Campinas, Brazil [grant numbers D11A-SAXS1-14296, SAXS1-20150056]; and the Argentinian National Agency for the Promotion of Science and Technology (ANPCyT) [grant number PICT 2018-00868].

References

- [1] C. G. De Kruif, T. Huppertz, V. S. Urban, A. V. Petukhov, Casein micelles and their internal structure, *Advances in colloid and interface science*. 171 (2012) 36-52. <https://doi.org/10.1016/j.jcis.2012.01.002>.
- [2] P. Myllärinen, J. Buchert, K. Autio, Effect of transglutaminase on rheological properties and microstructure of chemically acidified sodium caseinate gels. *International dairy journal*, 17(7), (2007) 800-807. <https://doi.org/10.1016/j.idairyj.2005.10.031>.
- [3] M. M. Elsayed, (2019). Hydrogel preparation technologies: relevance kinetics, thermodynamics and scaling up aspects. *Journal of Polymers and the Environment*, 27(4), 871-891. <https://doi.org/10.1007/s10924-019-01376-4>.
- [4] J. Guo, Y. C. Jin, X. Q. Yang, S. J. Yu, S. W. Yin, J. R. Qi, Computed microtomography and mechanical property analysis of soy protein porous hydrogel prepared by homogenizing and microbial transglutaminase cross-linking. *Food Hydrocolloids*, 31(2), (2013) 220-226. <http://dx.doi.org/10.1016/j.foodhyd.2012.10.023>.
- [5] L. López-Hortas, E. Conde, E. Falqué, H. Domínguez, M. D. Torres, Preparation of Hydrogels Composed of Bioactive Compounds from Aqueous Phase of Artichoke Obtained by MHG Technique. *Food and Bioprocess Technology*, (2019)1-12. <https://doi.org/10.1007/s11947-019-02301-2>.
- [6] C. Chung, B. Degner, E. A. Decker, D. J. McClements, Oil-filled hydrogel particles for reduced-fat food applications: Fabrication, characterization, and

properties. *Innovative food science & emerging technologies*, 20, (2013) 324-334.

<https://doi.org/10.1016/j.ifset.2013.08.006>.

[7] J. M. Montes de Oca-Ávalos, V. Borroni, C. Huck-Iriart, A.S.Navarro, R.J. Candal, M.L. Herrera, Relationship between Formulation, Gelation Kinetics, Micro/Nanostructure and Rheological Properties of Sodium Caseinate Nanoemulsion-Based Acid Gels for Food Applications (2019) *Food and Bioprocess Technology*, 13, (2020) 288-299. <http://dx.doi.org/10.1007/s11947-019-02394-9>.

[8] Z. Zhang, R. Zhang, Q. Tong, E. A. Decker, D. J. McClements, Food-grade filled hydrogels for oral delivery of lipophilic active ingredients: Temperature-triggered release microgels. *Food Research International*, 69, (2015) 274-280.

<https://doi.org/10.1016/j.foodres.2015.01.004>.

[9] D. J. McClements, Edible delivery systems for nutraceuticals: designing functional foods for improved health. *Therapeutic delivery*, 3(7), (2012) 801-803.

<https://doi.org/10.4155/tde.12.56>.

[10] J. M. Montes de Oca-Ávalos, R. J. Candal, M. L. Herrera, Nanoemulsions: Stability and physical properties. *Current Opinion in Food Science*, 16, (2017) 1-6.

<http://dx.doi.org/10.1016/j.cofs.2017.06.003>.

[11] J. M. Montes-de-Oca-Ávalos, D. Altamura, R. J. Candal, F. Scattarella, D. Siliqi, C. Giannini, M. L. Herrera, Relationship between nano/micro structure and physical properties of TiO₂-sodium caseinate composite films. *Food Research International*, 105, (2018) 129-139. <https://doi.org/10.1016/j.foodres.2017.11.011>

<https://doi.org/10.1016/j.foodres.2017.11.011>

[12] P. R. R. Ramel Jr, F. Peyronel, A. G. Marangoni, Characterization of the nanoscale structure of milk fat. *Food chemistry*, 203, (2016) 224-230.

<http://dx.doi.org/10.1016/j.foodchem.2016.02.064>.

[13] C. Huck-Iriart, J. Montes-de-Oca-Ávalos, M. L. Herrera, R. J. Candal, C. L. Pinto-de-Oliveira, I. Linares-Torriani, New insights about flocculation process in sodium caseinate-stabilized emulsions. *Food Research International*, 89, (2016) 338-346.

- [14] L. H. Fasolin, M. A. Cerqueira, L. M. Pastrana, A. A. Vicente, R. L. Cunha, Thermodynamic, rheological and structural properties of edible oils structured with LMOGs: Influence of gelator and oil phase. *Food structure*, 16, (2018) 50-58. <https://doi.org/10.1016/j.foostr.2018.03.003>.
- [15] Z. Li, Z. Yang, D. Otter, C. Rehm, N. Li, P. Zhou, Y. Hemar, Rheological and structural properties of coagulated milks reconstituted in D2O: Comparison between rennet and a tamarillo enzyme (tamarillin). *Food hydrocolloids*, 79, (2018) 170-178. <https://doi.org/10.1016/j.foodhyd.2017.12.004>.
- [16] F. Muneer, E. Johansson, M. S. Hedenqvist, T. S. Plivelic, K. E. Markedal, I. L. Petersen, R. Kuktaite, The impact of newly produced protein and dietary fiber rich fractions of yellow pea (*Pisum sativum* L.) on the structure and mechanical properties of pasta-like sheets. *Food research international*, 106, (2018) 607-618. <https://doi.org/10.1016/j.foodres.2018.01.020>.
- [17] Y. Wang, B. Eastwood Z. Yang, L. de Campo, R. Knott, C. Prosser, Y. Hemar, Rheological and structural characterization of acidified skim milks and infant formulae made from cow and goat milk. *Food Hydrocolloids*, 96, (2019) 161-170. <https://doi.org/10.1016/j.foodhyd.2019.05.020>.
- [18] A. J. Martins, M. A. Cerqueira, L. M. Pastrana, R. L. Cunha, A. A. Vicente, Sterol-based oleogels' characterization envisioning food applications. *Journal of the Science of Food and Agriculture*, 99(7), (2019) 3318-3325. <https://doi.org/10.1002/jsfa.9546>.
- [19] D. Costa da Silva, C. Huck-Iriart, G. Kellermann, L. J. Giovanetti, A. F. Craievich, F. G. Requejo, In situ study of the endotaxial growth of hexagonal CoSi₂ nanoplatelets in Si (001). *Applied Physics Letters*, 107(22), (2015) 223101. <https://doi.org/10.1063/1.4936377>.
- [20] E. M. Anitas, Small-Angle Scattering from Weakly Correlated Nanoscale Mass Fractal Aggregates. *Nanomaterials*, 9(4), (2019) 648. <https://doi.org/10.3390/nano9040648>

- [21] B. Hammouda, A new Guinier–Porod model. *Journal of Applied Crystallography*, 43(4), (2010) 716-719. <https://doi.org/10.1107/S0021889810015773>.
- [22] S. J. Lee, D. J. McClements, Fabrication of protein-stabilized nanoemulsions using a combined homogenization and amphiphilic solvent dissolution/evaporation approach. *Food Hydrocolloids*, 24(2010) (6-7), 560-569. <https://doi.org/10.1016/j.foodhyd.2010.02.002>.
- [23] L. P. Cavalcanti, I. L. Torriani, T. S. Plivelic, C. L. P. Oliveira, G. Kellermann, R. Neuenschwander, Two new sealed sample cells for small angle x-ray scattering from macromolecules in solution and complex fluids using synchrotron radiation. *Review of scientific instruments*, 75(2004) (11), 4541-4546. <https://doi.org/10.1063/1.1804956>.
- [24] A. P. Hammersley, S. O. Svensson, M. Hanfland, A. N. Fitch, D. Hausermann, Two-dimensional detector software: from real detector to idealised image or two-theta scan. *International Journal of High Pressure Research*, 14(1996) (4-6), 235-248. <https://doi.org/10.1080/08957959608201408>.
- [25] A. Guinier, G. Fournet, K. L. Yudowitch, In Small-angle scattering of X-rays. New York, 1955 John Wiley and Sons (Eds). 268 pp. <https://doi.org/10.1002/pol.1956.120199326>.
- [26] V. Luzzati, Interprétation des mesures absolues de diffusion centrale des rayons X en collimation ponctuelle ou linéaire: Solutions de particules globulaires et de batonnets. *Acta Crystallographica*, 13(1960) (11), 939-945. <https://doi.org/10.1107/S0365110X60002284>.
- [27] R. P. Hjelm Jr, P. Thiyagarajan, H. Alkan-Onyuksel, Organization of phosphatidylcholine and bile salt in rodlike mixed micelles. *The Journal of Physical Chemistry*, 96(1992) (21), 8653-8661. <https://doi.org/10.1021/j100200a080>.
- [28] D. A. Pink, B. Quinn, F. Peyronel, A. G. Marangoni, Edible oil structures at low and intermediate concentrations. I. Modeling, computer simulation, and predictions for X ray scattering. *Journal of Applied Physics*, 114(2013) (23), 234901. <http://dx.doi.org/10.1063/1.4847996>.

- [29] B. Quinn, F. Peyronel, T. Gordon, A. Marangoni, C. B. Hanna, D. A. Pink, Aggregation in complex triacylglycerol oils: coarse-grained models, nanophase separation, and predicted x-ray intensities. *Journal of Physics: Condensed Matter*, 26(2014) (46), 464108. <http://dx.doi.org/10.1088/0953-8984/26/46/464108>.
- [30] A. W. Perriman, D. J. McGillivray, J. W. White, Reactions of isolated monomolecular protein films. *Soft Matter*, 4(2008) (11), 2192-2198. <https://doi.org/10.1039/B804711A>.
- [31] I. Breßler, J. Kohlbrecher, A. F. Thünemann, SASfit: a tool for small-angle scattering data analysis using a library of analytical expressions. *Journal of applied crystallography*, 48(2015) (5), 1587-1598. . <https://doi.org/10.1107/S1600576715016544>
- [32] N. Raak, C. Schöne, H. Rohm, D. Jaros, Acid-induced gelation of enzymatically cross-linked caseinate in different ionic milieus. *Food hydrocolloids*, 86, (2019) 43-49. <https://doi.org/10.1016/j.foodhyd.2018.01.037>.
- [33] M. S. Á. Cerimedo, C. H. Iriart, R. J. Candal, M. L. Herrera, Stability of emulsions formulated with high concentrations of sodium caseinate and trehalose. *Food Research International*, 43(2010) (5), 1482-1493. . <https://doi.org/10.1016/j.foodres.2010.04.008>
- [34] J. M. de Oca-Ávalos, R. J. Candal, M. L. Herrera, Colloidal properties of sodium caseinate-stabilized nanoemulsions prepared by a combination of a high-energy homogenization and evaporative ripening methods. *Food Research International*, 100, (2017) 143-150. <http://dx.doi.org/10.1016/j.foodres.2017.06.035>.
- [35] C. Huck-Iriart, V. M. P. Ruiz-Henestrosa, R. J. Candal, M. L. Herrera, Effect of aqueous phase composition on stability of sodium caseinate/sunflower oil emulsions. *Food and bioprocess technology*, 6(2013) (9), 2406-2418.

Figure captions

Figure 1. Normalized scattered intensity (Arb.U.) as a function of scattering vector q (nm^{-1}) for nanoemulsions prepared with a) 1, 2, 3 or 4 wt.% NaCas without sucrose and b) 4 wt.% NaCas with 0, 2, 4, 6, or 8 wt.% sucrose.

Figure 2. Results of fitting the 4NaCas0S-nanoemulsion SAXS curve with the Guinier-Porod (GP) model. The plot shows experimental data (I_{exp}), global fit of the model (I_{model}), and the contribution of the three scattering objects considered: oil nanodroplets (I_{oil}), adsorbed protein (I_{ads}) and free protein (I_{free}).

Figure 3. Diameters obtained by fitting small angle X-ray scattering (SAXS) data with the Guinier-Porod (GP) model, (empty bars), and diameters measured by dynamic light scattering (DLS Z-average), (grey bars), for nanodroplets present in the emulsions studied. Different letters among each data set indicates significant differences ($p < 0.05$).

Figure 4. Total area occupied by the adsorbed protein (C , in arbitrary units), calculated from the Porod zone. Different letters indicates significant differences ($p < 0.05$)

Figure 5. Scattered intensity obtained every 20 min after glucon- δ -lactone (GDL) addition to the nanoemulsion with 4 wt.% sodium caseinate (NaCas) and 8 wt.% Sucrose, (GDL/NaCas ratio = 0.2). Lower inset: zoom of the Guinier zone. Upper inset: zoom of the Porod zone.

Figure 6. Results of fitting small angle X-ray (SAXS) data with the generalized Guinier-Porod (GP) model for the 4 wt.% sodium caseinate (NaCas) 8 wt.% sucrose nano-based hydrogel. The plot shows experimental data (I_{exp}), global fit of the model (I_{model}),

and the contribution of the two scattering elements considered: oil nanodroplets (I_{oil}) and protein (I_{prot}).

Figure 7. Elastic (G') and loss (G'') moduli as a function of gelation time for the nanoemulsion formulated with 4 wt.% sodium caseinate (NaCas) and 8 wt.% sucrose. Black symbols: G' , grey symbols G'' .

Figure 8. Plot of $\log G'_{\infty}$ vs $\log R_{q2}$. Data were linearly fit, equation and R^2 are shown in the graph.

Table 1. Values of Guinier Porod model parameters selected for fitting nanoemulsions small angle x-ray scattering (SAXS) curves.

	Oil nanodroplets (oil)	Adsorbed Protein (ads)	Free Protein (free)
Scaling Factor	Free	Free	Free
R_g	Free (>15 nm)	Fix at 3 nm	Free
d	Fix at 4 (smooth surface)	Fix at 4	Fix at 4
s	Fix at 0 (sphere)	Fix at 0	Fix at 0

Table 2. Gyration radii for oil nanodroplets ($R_{g_{oil}}$) and free protein ($R_{g_{free}}$) for all nanoemulsions

Sample	Protein	Oil nanodroplets	Goodness of fit
	$R_{g_{free}}$ (nm)	$R_{g_{oil}}$ (nm)	R
1NaCas0S	10.18 ± 0.05^a	36.0 ± 0.2^a	0.041
2NaCas0S	10.65 ± 0.05^b	35.0 ± 0.2^b	0.053
3NaCas0S	11.74 ± 0.05^c	32.6 ± 0.02^c	0.037
4NaCas0S	10.74 ± 0.05^d	26.44 ± 0.09^d	0.026
4NaCas2S	10.33 ± 0.05^e	25.63 ± 0.02^e	0.027
4NaCas4S	9.7 ± 0.2^f	24.84 ± 0.02^f	0.026
4NaCas6S	9.0 ± 0.1^g	24.80 ± 0.02^g	0.021
4NaCas8S	8.8 ± 0.2^g	23.9 ± 0.06^h	0.031

\pm Error of calculated parameter

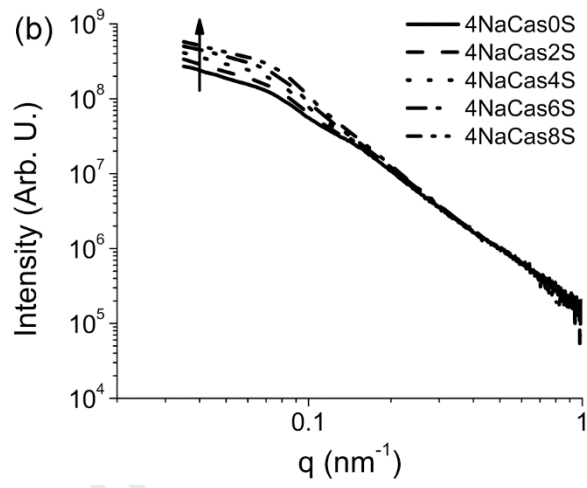
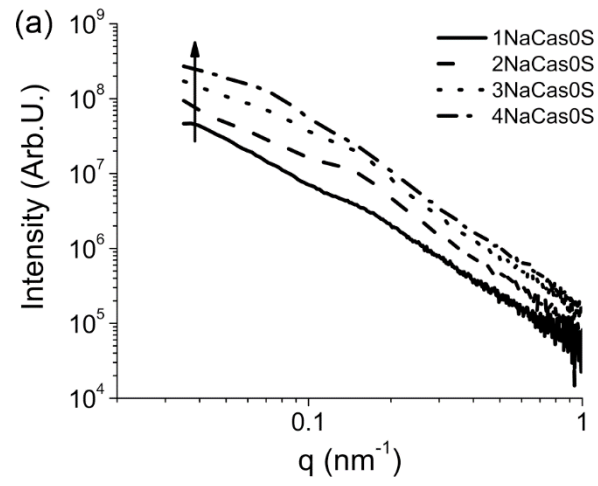
Table 3. Values of the GP and the generalized GP models parameters selected for fitting nanogels SAXS data

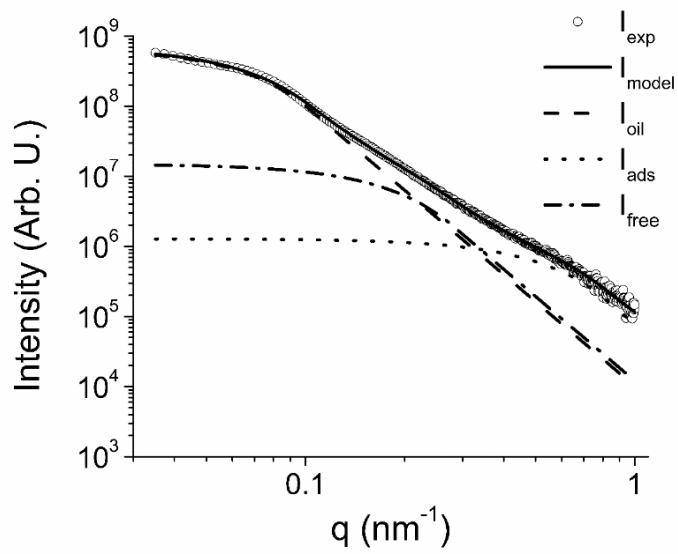
	Oil nanodroplets (GP)	Protein (Generalized GP)
Scale Factor	Free	Free
Gyration radius	R_g Free (>15 nm)	R_{g1} and R_{g2} free
d	Fix at 4 (smooth surface)	Fix at 4
s	Fix at 0 (sphere)	s_2 fix at 0 s_1 fix at 2.6

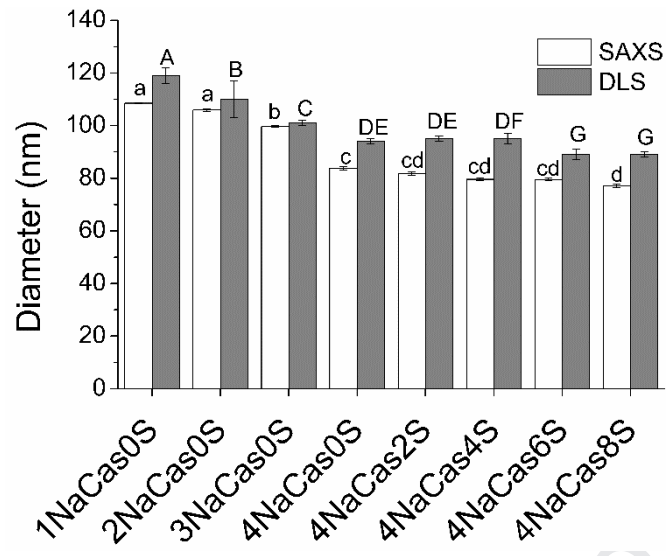
Table 4. Gyration radii for oil nanodroplets (R_g) and protein (R_{g_2} and R_{g_1}) as well as the radius of the equivalent sphere for all nanogels

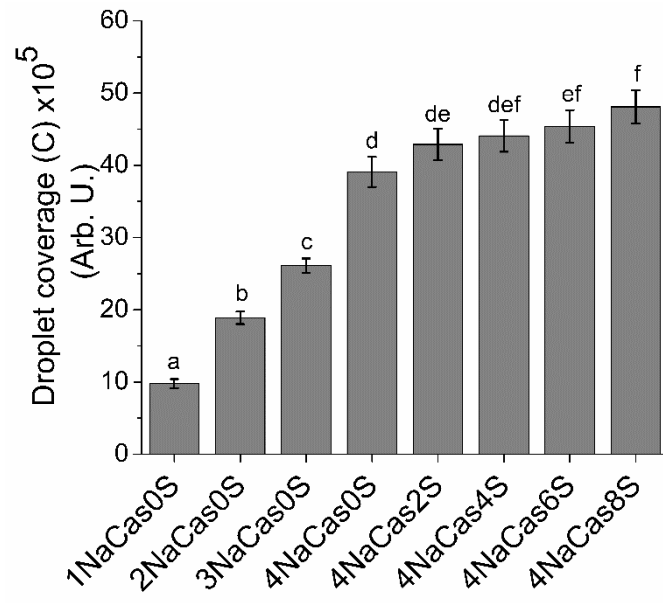
Sample	Protein			Oil Nanodroplets	Goodness of fit
	R_{g_2} (nm)	R_{g_1} (nm)	$R_{g_{sph}}$ (nm)	R_g (nm)	R
N1NaCas0S	7.11 ± 0.05^a	0.30 ± 0.002^a	1.80 ± 0.01^a	43.57 ± 0.3^a	0.061
N2NaCas0S	8.88 ± 0.06^b	0.43 ± 0.003^b	2.56 ± 0.02^b	42.88 ± 0.3^b	0.039
N3NaCas0S	11.68 ± 0.08^c	0.42 ± 0.003^b	2.50 ± 0.02^b	48.74 ± 0.4^c	0.018
N4NaCas0S	13.03 ± 0.09^d	0.56 ± 0.004^c	3.33 ± 0.02^c	46.90 ± 0.3^d	0.014
N4NaCas2S	13.44 ± 0.09^e	0.55 ± 0.003^c	3.28 ± 0.02^c	39.99 ± 0.3^e	0.019
N4NaCas4S	14.18 ± 0.1^f	0.55 ± 0.003^c	3.31 ± 0.02^c	33.45 ± 0.2^f	0.026
N4NaCas6S	14.56 ± 0.1^g	0.60 ± 0.004^c	3.56 ± 0.03^c	32.38 ± 0.2^g	0.027
N4NaCas8S	14.64 ± 0.1^g	0.52 ± 0.003^c	3.11 ± 0.02^d	27.34 ± 0.2^h	0.030

± Error of calculated parameter

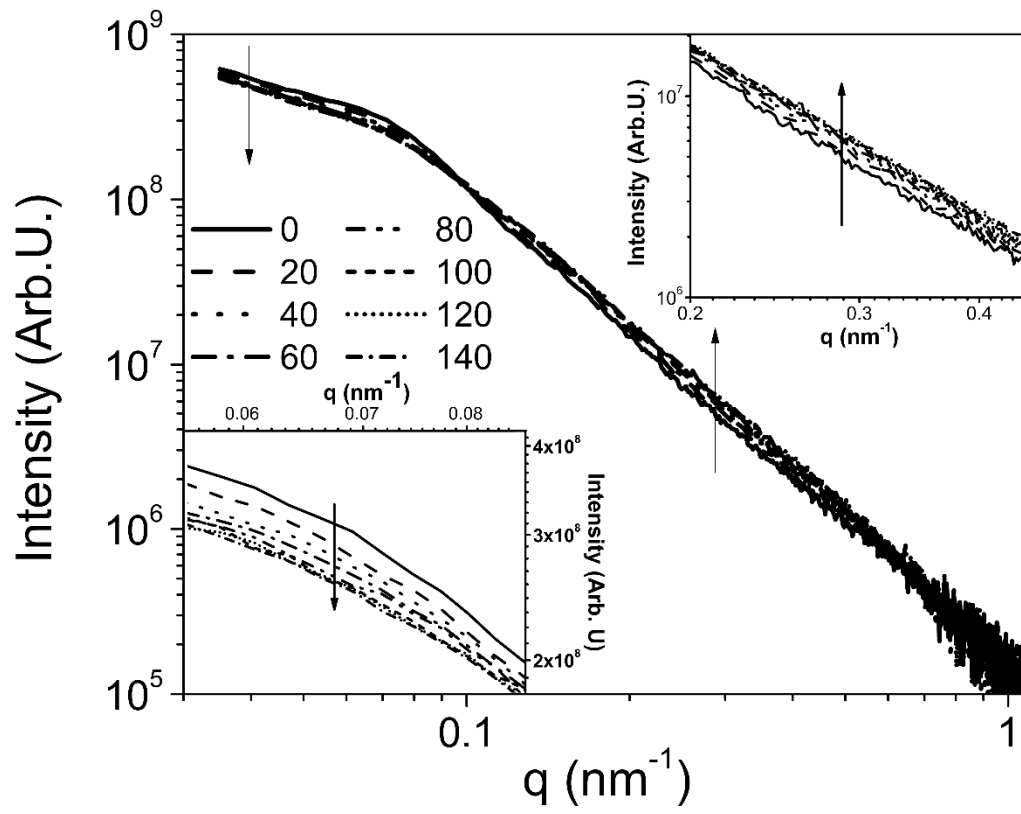


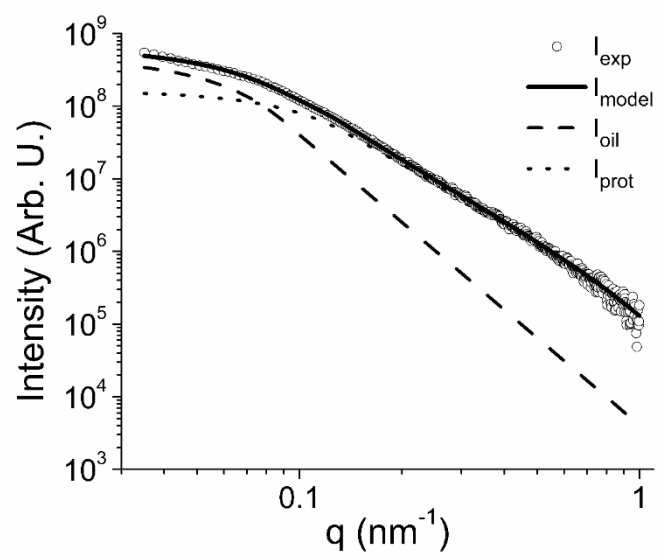


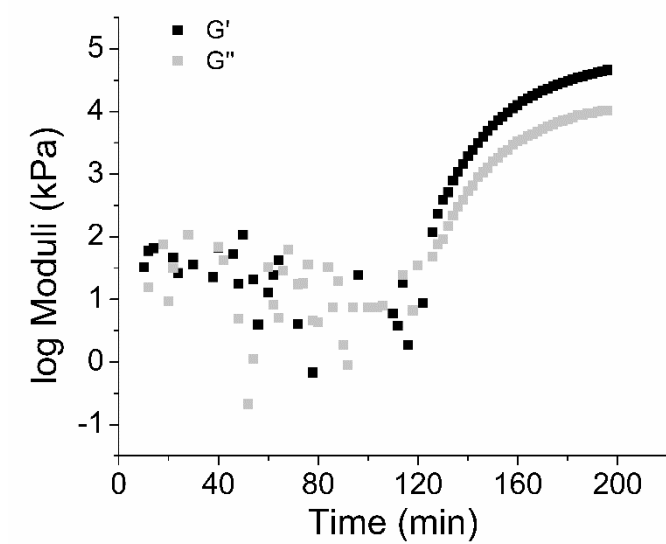


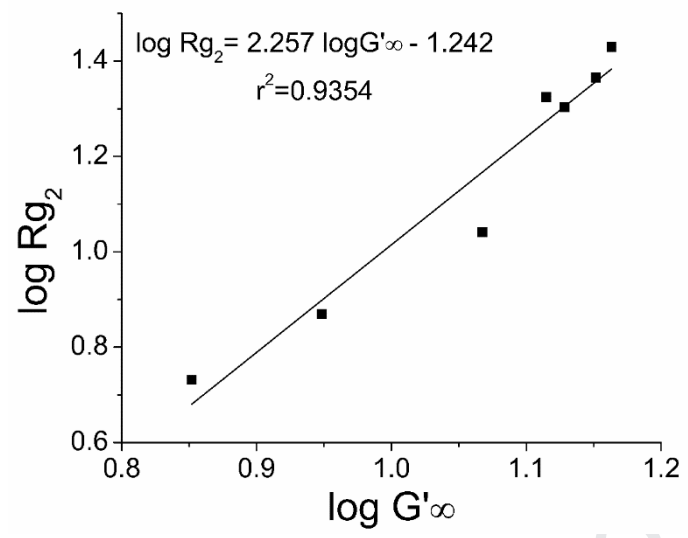


Journal Pre-proof









Journal Pre-proof

- Nanodroplets gyration ratio decreased with increasing protein and sucrose contents
- Nanodroplets coverage increased with increasing protein and sucrose contents
- Building blocks gyration ratio of gels were independent on composition
- Building blocks correlation distances were significantly affected by composition
- R_{g2} distances correlated with asymptotic values of G' (G'_{∞})

Journal Pre-proof

AUTHOR DECLARATION

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the Corresponding Author and which has been configured to accept email from mlidiaherrera@gmail.com.

Signed by all authors as follows:

23/12/2019

Juan Manuel Montes de Oca-Avalos



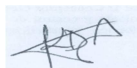
Cristián Huck-Iriart



Virginia Borroni



Karina Dafne Martínez



Roberto Jorge Candal



María Lidia Herrera

