Current Opinion in Colloid & Interface Science 16 (2011) 412–420

Contents lists available at ScienceDirect

Current Opinion in Colloid & Interface Science

journal homepage: www.elsevier.com/locate/cocis

Structures and stability of lipid emulsions formulated with sodium caseinate

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ARTICLE INFO

Article history:
Received 30 July 2010
Accepted 9 June 2011
Available online 7 July 2011

Keywords:
Emulsion
Sodium caseinate
Stability
Structure

ABSTRACT

The physicochemical properties of emulsions play an important role in food systems as they directly contribute to texture, sensory and nutritional properties of foods. Sodium caseinate (NaCas) is a well-used ingredient because of its good solubility and emulsifying properties and its stability during heating. One of most significant aspects of any food emulsion is its stability. Among the methods used to study emulsion stability it may be mentioned visual observation, ultrasound profiling, microscopy, droplet size distribution, small deformation rheometry, measurement of surface concentration to characterize adsorbed protein at the interface, nuclear magnetic resonance, confocal microscopy, diffusing wave spectroscopy, and turbiscan. They have advantages and disadvantages and provide different insights into the destabilization mechanisms. Related to stability, the aspects more deeply investigated were the amount of NaCas used to prepare the emulsion, and specially the oil-to-protein ratio, the mobility of oil droplets and the interactions among emulsion components at the interface. It is known that the amount of protein required to stabilize oil-in-water emulsions depends, not only on the structure of protein at the interface, and the average diameters of the emulsion droplets, but also on the type of oils and the composition of the aqueous phase. Several authors have investigated the effect of a thickening agent or of a surface active molecule. Factors such as pH, temperature, and processing conditions during emulsion preparation are also very relevant to stability. There is a general agreement among authors that the most stable systems are obtained for conditions that produce size reduction of the droplets, an increase in viscosity of the continuous phase and structural changes in emulsions such as gelation. All these conditions decrease the molecular mobility and slow down phase separation.

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1. Introduction

The term “emulsion stability” refers to the ability of an emulsion to resist changes in its properties over time. Physical instability results in an alteration in the spatial distribution or structural organization of the molecules. Creaming, flocculation, coalescence, partial coalescence, phase inversion, and Ostwald ripening are examples of physical instability. The development of an effective strategy to prevent undesirable changes in the properties of a particular food emulsion depends on the dominant physicochemical mechanism(s) responsible for the changes. In practice, two or more of these mechanisms may operate in concert. It is therefore important for food scientists to identify the relative importance of each mechanism, the relationship between them, and the factors that influence them, so that effective means of controlling the stability and physicochemical properties of emulsions can be established [1•].

Nowadays the food industry has a growing interest in the replacement of synthetic emulsifiers by natural ones, such as polysaccharides and proteins. Casein is of particular importance as an emulsifier because of its ability for rapidly conferring a low interfacial tension during emulsification and because of the strongly amphiphilic characteristics of the major individual caseins. Casein in milk is strongly aggregated into polydisperse protein particles called “casein micelles”. The micelles may be dispersed by adding a calcium chelator or also by urea, SDS, high pH or ethanol, indicating that hydrogen bonds, hydrophobic and electrostatic interactions are also involved in micelle integrity [2]. Sodium caseinate is composed of a soluble mixture of disordered hydrophobic proteins having a strong tendency to associate into small protein particles which coexist in equilibrium with the free casein molecules. In the casein micelle system, the micelle state may be the lowest free-energy state of the system. Of particular interest will be micelle structure and the mechanisms which operate in determining micelle size [3•].

The present review summarizes key and recent research describing behavior and structure of oil-in-water (O/W) emulsions stabilized by sodium caseinate. The most employed and most innovative methods were also discussed.

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doi:10.1016/j.cocis.2011.06.003

2. Stability and rheology

Addition of the polymer to a dispersion can promote stability or destabilize the dispersion, depending on the nature of interactions between the polymer and the solvent and between the polymer and the dispersed particles [4•]. Some of the possible effects of polymer chains on a dispersion may be summarized as follows: in the case of very low polymer concentrations, bridging flocculation may occur as a polymer chain forms bridges by adsorbing on more than one particle. At higher concentrations distances among particles may be higher and mask van der Waals attraction between the particles causing steric stabilization. At moderate to high polymer concentrations, the free polymer chains in the solution causes depletion flocculation. At even higher polymer concentrations the effect is known as depletion stabilization. The polymer-depleted regions between the particles can only be created by demixing the polymer chains and solvent. In good solvents the demixing process is thermodynamically unfavorable, and under such conditions one can have depletion stabilization [4•].

Food O/W emulsions were studied with the aim of providing experimental evidence in support to polymer theory. Dickinson's findings may be summarized as follows: stability and rheology of emulsions made with NaCas depend on two main factors, the structure and composition of the adsorbed protein layer at the oil–water interface, and the state of self-assembly and aggregation of the protein in the aqueous phase. In emulsions containing protein at concentrations well below that required for monolayer saturation coverage, the system exhibits time-dependent bridging flocculation and coalescence. At protein contents around that required for saturation monolayer coverage, the system is unflocculated, and is very stable towards creaming and coalescence. At higher protein contents, the creaming stability of the system is greatly reduced due to depletion flocculation of protein-coated droplets by unadsorbed sub-micellar caseinate. And, at even higher protein contents, there is partial restabilization of the flocculated emulsion in the form of a strong particle gel network. The rheological behavior of caseinate emulsions were largely determined by the interactions between droplets and especially by the nature and strength of the interparticle attractive forces which were dependent on the structure and composition of the adsorbed layer at the oil/water interface [3•]. The rheological behavior of those systems demonstrated that the droplet networking effect was very relevant since with the casein systems even up to protein concentrations of 8 wt.% there was relatively little contribution to the emulsion rheology from the viscoelastic properties of the continuous phase. So, the structural mechanism influencing the rheology of the casein-rich emulsions can apparently be considered as entirely attributable to interdroplet depletion interactions.

In agreement with Dickinson [3•], Berli et al. (2002) found that the rheological response of the emulsion was highly dependent on the concentration of caseinate. When the concentration of free proteins in the bulk solution was low, emulsions were Newtonian while for higher caseinate concentration emulsions became shear-thinning [5]. According to these authors, the variation of emulsion viscosity with protein concentration could not be explained by the variation of the suspending fluid viscosity. The strength of interparticle interactions and a genuine variation in the radius of the sub-micelle with formulation had a key role in rheological behavior.

3. Non perturbing studies

Emulsions have been studied by numerous techniques [6]. Most of them involved some form of dilution. This dilution disrupts some structures that play an important role in stability. The ability to study the stability of food emulsions in their undiluted forms may reveal subtle nuances about their stability [7]. Diffusing wave spectroscopy (DWS), laser scanning confocal microscopy (LSCM), nuclear magnetic resonance (NMR), and Turbiscan are among the more powerful, non-perturbing techniques used to characterized emulsions.

Diffusing wave spectroscopy (DWS) is a light scattering technique that is highly sensitive to changes in the local particle dynamics. A major advantage of light scattering over conventional rheology is that it is a "zero-stress" technique since the signal responds to changes in behavior driven by the spontaneous thermal fluctuations. The apparatus employs a bifurcated multi-fibre bundle as light guide. Half of the optical fibres carry light to the sample from a He-Ne laser, and the other half take the back-scattered light back from the sample to the photomultiplier. There are two parameters that can be extracted from the experiments: the characteristic relaxation time of the system (τ) and the correlation function amplitude (CFA). The parameter τ characterizes the decay of the particles motions giving rise to the fluctuating light-scattering intensity. τ is influenced by system viscosity, particle size, particle concentration, and particle interactions. CFA (the mean-square scattered intensity minus the square of the mean intensity) depends on the equipment used (system geometry, laser power, photomultiplier, voltage) and the scattering properties (particle size, concentration and state of aggregation) [8]. Hemar et al. [9] studied the stability of flocculating NaCas emulsions. The structure of an emulsion undergoes several stages during flocculation by the addition of a polymer. Initially the oil droplets are brought together to form flocs because of the difference in osmotic pressure between the inter-droplet region poor in polymer and the surrounding region rich in polymer. These flocs start to migrate to the top of the emulsion due to density differences between the flocs and the continous phase, an effect known as creaming. According to Hemar et al. [9] the information obtained from back-scattering DWS is related both to the effective particle size of the flocs and to the viscosity of the flocculated emulsion. Their results confirmed that the creaming process was delayed at high oil concentrations, presumably due to the formation of oil droplets networks. Eliot et al. [8] also evaluated changes in dynamical properties of NaCas emulsions (4 wt.% protein, 30 vol.% vegetable oil) by diffusing wave spectroscopy (DWS). In their work, the changes in the DWS relaxation time and correlation function amplitude have been compared with changes in small deformation rheological parameters and confocal microscopy observations over the same temperature range. The general trend of behavior was that changes in DWS signal occur at significantly higher temperatures than might be expected on the basis of earlier rheological measurements. The complexity of the DWS response is due to the separate contributions to the correlation function from both the mobility of the emulsion droplets themselves and the dynamical motion of the casein aggregates loosely associated with those droplets [8]. According to Eliot et al. [8] the correlation between DWS and small-deformation rheology is imperfect probably as a reflection of the fact that the two techniques are sensitive to different aspects of the structural and dynamic changes taking place in NaCas-stabilized-emulsions on heating. Because structure is an important parameter for many food products and many food products are processing under shear Ruis et al. (2008) studied the effect of shear on the acidification of a sodium caseinate-stabilized emulsion using DWS and rheometry [10]. Combining these techniques they determined the sol-gel point of emulsions and the radius of the aggregates at this point as a function of the shear rate. Their results indicated that the radius of the aggregates at the sol-gel transition decreased with increasing shear rate and suggested that shear induced the creation of a more open structure of the network formed by aggregates.

The advantage of using NMR is that it can be applied to concentrated emulsions without pre-treatment or dilution of the sample. Acquisitions of the measurement data are usually fast and do not require excessive sample volumes. Both the dispersed and the continuous phase of emulsions can be characterized using NMR [11]. NMR has also been used to study lipid-protein interactions. Day et al. [7] used NMR to provide further information that would complement that obtained by other conventional techniques used for characterizing emulsions. They

studied fish O/W emulsions prepared using varying amounts of sodium caseinate (0.1 to 1.0 wt% protein) and 25 wt% oil giving and oil-to-protein ratio of 250 to 25. In their systems, the T_2 values obtained from low field ^{1}H NMR measurements were sensitive to the structure of the bulk emulsions, with shorter T_2 being obtained in flocculated emulsions. The high-field NMR spectra had increased line broadening with the increase in protein content, due to increased interaction between oil molecules and protein at the interface. ^{31}P NMR spectra, which reflect the mobility of the casein component only, showed that at the lower protein content (0.1 wt.%) the proportion of protein immobilized at the interface of the oil droplets was higher compared to that in the continuous phase.

In a recent work we analyzed the stability of emulsions formulated with 0, 20, 30 or 40 wt.% solution of trehalose as aqueous phase, 10 wt.% of a commercial oil, sunflower (SFO), olive (OO) or a concentrated from fish oils (CFO) and NaCas at 0.5, 1, 2, 3, 4, or 5 wt.%, giving oil-to-protein ratios of 20–2 [12••]. The emulsion stability was analyzed using a vertical scan analyzer (Turbiscan MA 2000) which allows the optical characterization of any type of dispersion [13••]. The reading head is composed of a pulsed near-IR light source ($\lambda = 850$ nm) and two synchronous detectors. The transmission detector receives the light, which goes through the sample (0°), while the backscattering detector receives the light back-scattered by the sample (135°). The samples were scanned from the bottom to the top in order to monitor the optical properties of the dispersion along the height of the sample placed in the cell. In this way, the physical evolution of this process is followed without disturbing the original system and with good accuracy and reproducibility [13••]. Thus, by repeating the scan of a sample at different time intervals, the stability or the instability of dispersions can be study in detail. The profiles allow calculation of either creaming, sedimentation, or phase separation rates, as well as flocculation, and the mechanism making the dispersion unstable can be deduced from the transmission or the backscattering data [14••]. The curves obtained by subtracting the BS profile at t = 0 from the profile at t ($\Delta BS = BS_t - BS_0$), display a typical shape that allows a better quantification of creaming, flocculation and other destabilization processes. In our systems, the serum phase was still optically opaque, no light reached the transmission detector, and all emulsions remained fully turbid along the tube during a week of storage at 22.5 °C. This means that they would have been considered stable by visual observation. However, the back scattering (BS) profiles showed destabilization that could not be detected by other means. In agreement with previous findings, the main mechanism of destabilization in the formulations we studied depended on NaCas concentration [3•]. Emulsions formulated with 0.5 and 1 wt.% NaCas destabilized mainly by creaming. For the 2 wt.% NaCas emulsion, both creaming and flocculation mechanisms, were involved. The BS-profile changes with time showed that the main destabilization mechanism for emulsions stabilized by 3, 4 or 5 wt.% NaCas was flocculation. Concentrations below 0.5 wt.% NaCas seemed to be below the ones required for saturation monolayer coverage since creaming rate was greater for 0.5 wt.% than for 1 wt.% NaCas. Further addition of protein led to high instability. The 2–4 wt.% NaCas range was the worst situation. At these protein contents, there was flocculation and migration of flocculates. Although it was reported that at protein contents around that required for saturation monolayer coverage, the NaCas systems should be very stable towards creaming and coalescence [3•,15], there was no such oil-to-protein ratio in ours systems between creaming of small particles and flocculation destabilization processes. At even higher protein contents (5 wt.% NaCas concentration), emulsions were very stable especially when the aqueous phase contained trehalose or sucrose. In these conditions, the systems remained in the liquid state for at least a week, fully turbid, that is no changes were noticed by visual analysis and no creaming or flocculation was detected by the Turbiscan. Although there was no gel formation when emulsions were kept at 22.5 °C for a week, they still remained stable [12••].

As an example Fig. 1 shows the backscattering and ΔBS curves for the emulsion formulated with 10 wt.% OO and 5 wt.% NaCas. The sample was mixed using an Ultra-Turrax high speed blender giving a coarse emulsion (Fig. 1a and b), and then was further homogenized using an ultrasonic liquid processing resulting in a fine emulsion (Fig. 1c and d). Both, coarse and fine emulsions had a monomodal particle size distribution, with a volume-weighted mean diameter ($D_{4,3}$) of 15.734 and 0.692 μm, and a wide (W = 90%–10%, volume percentiles) of 22.940 and 1.333, respectively. Fig. 1a shows a typical profile of a sample which main destabilization mechanism was creaming of individual particles. Creaming was detected using the Turbiscan as it induced a variation of the concentration between the top and the bottom of the cell. The droplets moved upward because they had a lower density than the surrounding liquid. When creaming of individual particles take place in an emulsion, the ΔBS curves show a peak at heights between 0–20 mm and a quite Gaussian shape peak at the top (Fig. 1b). The variation of the peak wide, at a fixed height, during the studied time, can be related to the kinetics of migration of small particles [13••]. The creaming destabilization kinetics was evaluated by measuring the peak thickness at 50% of the height at different times (bottom zone). The slope of the linear part of a plot of peak thickness vs. t gives an indication of the migration rate. The coarse emulsion shown in Fig. 1a and b destabilized by creaming of individual particles. The process started after 12 min with a creaming rate of 46.28 mm/h (correlation coefficient R = 0.9953).

Flocculation was followed by measuring the BS_{av} as a function of storage time in the middle zone of the tube (Fig. 1c and d). As was theoretically demonstrated by Mengual et al. [13••], the BS intensity decreased as the particle size increased (when particle size is higher than the wavelength (λ) of the incident light). It should be mentioned that if the particle size is lower than λ of the incident light, BS increase with particle size. The relevant point is that the BS value always change (moves up or down) with particle size [13••]. This phenomenon was used by several authors for quantifying flocculation kinetics [12••,14••,16••,17••]. The optimum zone to evaluate flocculation was the one no affected by creaming (bottom and top of the tube), that is, the 20–50 mm zone. Fig. 1c shows as an example backscattering profiles of a sample which main destabilization mechanism is flocculation and the ΔBS curves (reference mode) corresponding to these profiles are reported in Fig. 1d. The absence of a creaming peak in the 0–20 mm zone indicates that creaming of small particles is negligible in this emulsion. The variation of BS (ΔBS) with time for emulsion in Fig. 1c and d is shown in Fig. 2. There were almost no changes in BS for the first 4 h. Then, there was a rapid flocculation that took place during the first 21 h of storage. Finally, the ΔBS with time reached an asymptotic value. The flat-top wide peak that appeared at the top of the tube after 10 h of storage was indicative of creaming caused for migration of flocculates. In our systems, creaming that took place in coarse or fine-particles emulsion started at different times. For emulsion in Fig. 1a,b the delay was 12 min while for emulsion in Fig. 1 c and d it was 10 h. This difference in creaming behavior was probable due to the fact that creaming occurred as a result of two different phenomena: migration of individual particles or migration of flocculates. Both emulsions were formulated in the same way, that is they have the same oil-to-protein ratio, but were prepared using different processing conditions, giving very different particle size and therefore showed different creaming behavior.

4. Effect of pH, preparation and additives

The overall stability and rheology of fine O/W emulsions made with NaCas as the sole emulsifier and stabilizer are sensitive to various combinations of factors such as temperature, pH, ionic strength, calcium ion content, and protein-to-oil ratio [18]. According to Eliot and

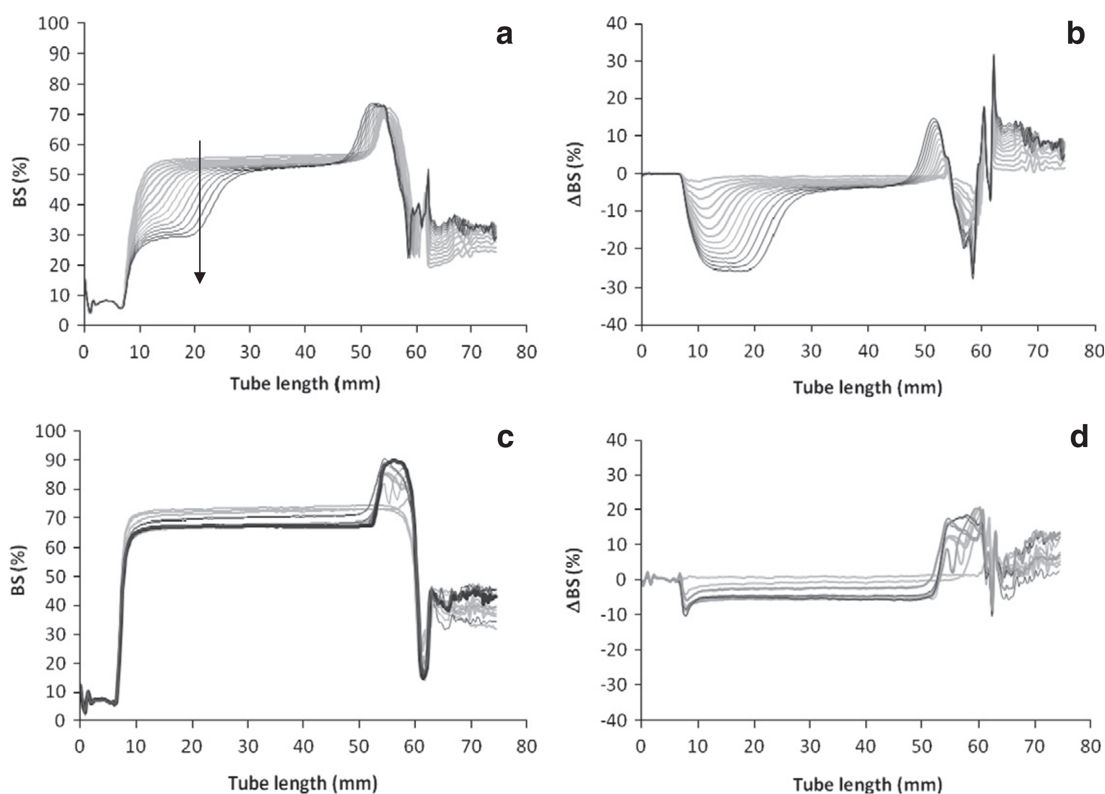

Fig. 1. Changes in back scattering (BS), actual and in reference mode profiles, as a function of the tube length with storage time (samples were stored for 1 week, arrow denotes time) in quiescent conditions for emulsions with 10 wt.% olive oil (OO) as fat phase, no trehalose added to the aqueous phase, and a concentration of sodium caseinate (NaCas) of 5.0 wt.%. (a) actual profiles of coarse emulsion, (b) profiles in reference mode of coarse emulsion, (c) actual profiles of emulsion further homogenized by ultrasound treatment, (d) profiles in reference mode of emulsion further homogenized by ultrasound treatment. Tube length 65 mm.

Dickinson [19] a stable emulsion sample has a monomodal particle size distribution, a Newtonian rheological behavior, and it gives no discernible creaming after 2 days. Destabilization of casein micellar dispersion may be induced by acidification, enzyme action, severe thermal processing (e.g. in sterilization) or by high-pressure treatment (a few thousand atmospheres), [20]. Eliot and Dickinson (2003) reported that for concentrated emulsions of constant protein content, there is a certain range of conditions of pH and calcium content for which the interdroplet pair interaction is net repulsive at around 20 °C, but became substantially net attractive at higher temperature (>35–40) [19,21]. It would therefore appear that some calcium ion binding combined with pH reduction is required to provide the optimum balance of intermolecular forces conducive to heat-induced caseinate-stabilized emulsion thickening behavior [7].

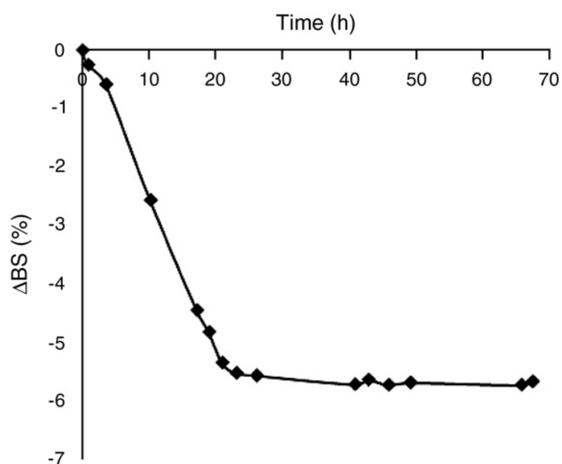

Fig. 2. Variation in BS in the 20–50 mm zone monitored over 70 h, for a sample stored in quiescent conditions at 22.5 °C. The kinetics correspond to the emulsion formulated with 10 wt.% OO as fat phase and 5.0 wt.% of NaCas as stabilizer (Fig. 1d). Tube length: 65 mm. Abbreviations as in Fig. 1.

Since the pioneering work of Dickinson other scientist has been working on the stability and structure of NaCas emulsions [22–31]. Liu et al. [32] brought the first evidence of the stabilization effect of a soybean-soluble polysaccharide (SSPS) in acidified NaCas-emulsions. While destabilization occurred at low polysaccharide concentrations, probably via bridging flocculation, acid-induced aggregation of the oil droplet was completely prevented by 0.2% SSPS or high-methoxyl pectin (HMP). However, the interaction behavior of SSPS during acidification was different from that of HMP. At comparable concentrations, HMP was more effective than SSPS. The adsorption of HMP changed the droplet surface as well as the interaction between droplets, resulting in the structural rearrangement of droplets. While HMP stabilized the emulsion droplets via steric and electrostatic repulsion, SSPS may provide stabilization mainly through its adsorption at the interface and steric repulsion [32]. Sosa-Herrera et al. [33*] analyzed the relationship between rheological properties of caseinate-stabilized O/W emulsions and the physicochemical characteristics of the aqueous phase. Their systems were formulated with 30 wt.% sunflower oil, 2% wt.% NaCas and with or without 0.06 and 0.1 wt.% gellan gum. Emulsions with gellan gum were stable against both flocculation and creaming, while the rheological behavior strongly depends on the structural state of the polysaccharide matrix, which is influenced by the presence of oil droplets and casein aggregates acting as filler particles. According to these authors the interaction between casein-covered oil droplet and gellan chains appears to be the crucial aspect to understand. They are not clear yet and needs further investigation.

Perrechil and Cunha [34*] characterized O/W emulsions with an oil-to-protein ratio of 30 by visual observation, microstructure and

rheological measurements. They evaluated coarse and fine neutral emulsions stabilized by NaCas, caseinate emulsions with addition of locust bean gum (LBG), and acidified caseinate emulsions. As a summary of their study it may be mentioned that several factors led to an increase in the stability of emulsions: high-pressure homogenization that produced a reduction in the droplet size and therefore a decrease in the creaming velocity, the increase in LBG content in the initial solution which caused an increase of viscosity, and a low pH (3.7) closer but lower to the pI (4.6) since in this condition the adsorb protein molecules had a tendency to interact which resulted in the formation of a gel network.

Using combined static and dynamic light scattering, Semenova et al. [35] studied systems with 0.5 wt/v% NaCas and 0.01, 0.1 or 1.0 wt/v % dextran sulfate (DS). The complexes were prepared by mixing together the two biopolymer components in bulk solution or bringing them together at the interface in a protein-stabilized foam. A significant difference was observed between properties of complexes formed in solution and those formed at the interface. The "mixed" emulsions made from NaCas-DS complexes had smaller and more monodisperse oil droplets, and also better long-term stability, than the equivalent "bilayer" emulsions [36]. In particular, they inferred that the greater hydrophilicity and the more open/bulky architecture of complexes formed in the bulk aqueous phase are better able to provide effective steric/electrostatic stabilization of the so-called "mixed" emulsions, as compared with the interfacial complexes formed in the so-called "bilayer" emulsions [35].

Schokker and Dalgleish found a big difference in flocculation behavior between emulsions prepared with different homogenizers [22]. In a previous work in our lab [17••] we study the effect of emulsion stability on initial retention of a low-trans fat encapsulated in a trehalose matrix. The emulsion formulated with SFO and NaCas in an oil-to-protein ratio of 8 was very stable after 195 h at 22.5 °C. No creaming and coalescence occurred and, as a result of that, it remained fully turbid and no clarification was detected by the transmission detector. This emulsion also had a 20 wt.% trehalose dissolved in the aqueous phase. In Cerdeira et al. work the pre-emulsion was further passed through a two-stage valve high pressure homogenizer giving a $D_{4,3}$ of 1.10 $\pm$ 0.13 μm [17••]. In the Álvarez Cerimedo et al. (2010) study, the pre-emulsions were homogenized for 20 min using an ultrasonic liquid processing. The oil-to protein ratios selected varied from 20 to 2 giving $D_{4,3}$ values from 0.2 to 0.5 μm. Emulsions were stable only when the oil-to-protein ratio was 2 [12••]. This means that the oil-to-protein ratio that gave stability for at least a week, that is no changes were detected, depended on processing conditions.

In Álvarez Cerimedo et al. (2010) study we also evaluated the effect of a disaccharide (trehalose) on emulsion stability following destabilization with a Turbiscan [12••]. As for emulsions without additives in the aqueous phase, emulsions with trehalose showed a monomodal distribution but the shapes were more Gaussian than the formulations without trehalose, which is indicative of a more homogeneous population of droplets. $D_{4,3}$ was always smaller than for the emulsions without sugar in the aqueous phase showing that trehalose may have interacted with the protein influencing droplet size [12••]. Similar results were obtained formulating emulsions with SFO or OO, or using a different disaccharide (sucrose). Coarse emulsions with sucrose or trehalose also had smaller $D_{4,3}$ than emulsions without sugar in the aqueous phase. For example, the 20 wt.% sucrose emulsion formulated with SFO and 5 wt.% NaCas had a $D_{4,3}$ of 9.950 μm. When sugar was trehalose $D_{4,3}$ was 12.706 μm. For the emulsion without sugar $D_{4,3}$ was 16.350 μm, a value significantly greater than with sugar addition. As in the case of emulsions without trehalose, the main mechanism of destabilization in a given formulation depended on NaCas concentration. However, the rate of destabilization was markedly lower with addition of sugar. The 5 wt.% NaCas emulsion did not flocculate during a week at 22.5 °C. Its BS remained unchanged during storage showing a great stability. This sample remained fully turbid and in the liquid state [12••]. Similar results were found analyzing emulsions with SFO or OO or adding sucrose instead of trehalose to the aqueous phase. Coarse emulsions also showed slower migration rate for formulations with sucrose or trehalose in the aqueous phase than without sugars. For example, for emulsions formulated with OO and 5 wt.% NaCas migration rates were 48.07 mm/h without sugar addition and 23.26, 24.90, and 23.91 mm/h for 20 wt.% sucrose, 20 wt.% trehalose, and 30 wt.% trehalose, respectively. Fig. 3 shows confocal laser scattering microscopy (CLSM) images of emulsions with 10 wt.% OO as fat phase, 20 wt.% trehalose solution as aqueous phase, and different concentrations of NaCas kept in quiescent conditions at 22.5 °C for 24 h. Structure of emulsions varied with NaCas concentration. The image corresponding to the emulsion stabilized with 0.5 wt.% NaCas showed a homogeneous droplet distribution. The main destabilization mechanism for this emulsion was creaming of individual particles. When protein concentration was 3 or 4 wt.% emulsions presented aggregates/flocculates which were absent in 0.5 wt.% emulsions. In these emulsions there were higher protein concentrations in the aqueous phase. However, flocculation behavior was not in agreement with bulk protein concentration since flocculation rate was slower for higher protein concentrations [12••]. This result was in agreement with those of Dickinson who found that for flocculating emulsions the "lag" time between emulsion preparation and apparent onset of creaming was seen to increase with protein concentration [3•]. With ultrasound homogenization, the average particle size of fat globules is reduced leading to an increase of the interfacial area and may also lead to a modification of the protein structure at the interface. Surprisingly, a concentration of 5 wt.% NaCas produced an emulsion with a very homogeneous droplet distribution and a lower $D_{4,3}$ (0.281 μm). In addition to a higher concentration of NaCas in the aqueous phase this emulsion most likely had more protein for the coverage of oil droplet surface. The absence of aggregates/flocculates is in agreement with the great stability of this emulsion as studied by turbiscan (Fig. 3).

To further describe the behavior and structure of NaCas emulsions we analyzed our system using small angle X-ray scattering (SAXS). Fig. 4 shows the normalized intensity vs. reciprocal lattice spacing q, where $q = 2\pi/d = 4\pi \sin(\theta)/\lambda$, where d is the interplanar spacing and 2θ is the Bragg angle, for the emulsion formulated with 5 wt.% NaCas, 20 wt.% trehalose, and 10 wt.% CFO and prepared using an Ultraturrax high speed blender (coarse emulsion) or further homogenized by ultrasound treatment (fine emulsion). As may be noticed, intensity for q values lower than 0.3 nm^{-1} varied according to the preparation method. This may be indicative of structural differences between coarse and fine emulsions. Kalnin et al. reported that in aqueous NaCas solutions values of q_{max} increased with NaCas concentration showing that the aggregation state of the protein changed as a function of its concentration [37•]. The variation of this scattering maximum, q_{max}, is not linear and beyond a certain NaCas concentration the shift observed becomes weaker. In their systems they observed two regimes up to about 3 wt.% and beyond 3 wt.%. According to these authors [37•], peak maxima values for emulsions patterns were very similar to the values for aqueous patterns indicating that micelles organization did not change by the presence of fat phase. Our systems behaved in the same way as their emulsions. In the example in Fig. 4 both samples had 5 wt.% NaCas concentration and a q_{max} value of 0.227 nm^{-1}. However, the differences in patterns suggest a different organization of particles. According to Berli et al. the total caseinate in an emulsion is distributed between the O/W interface and the bulk aqueous phase [5]. The concentration of caseinate adsorbed at the interface can be calculated as $C_{ad} = 6\Gamma\phi_{oil}/d_{3,2}$, where Γ is the surface protein coverage and ϕ_{oil} is the volume fraction of oil and $d_{3,2}$ is the Sauter diameter. The protein concentration in the bulk solution is then $C_b = (C - C_{ad})/(1 - \phi_{oil})$. Kalnin et al. have reported a similar expression for caseinate distribution in an emulsion [38•]. According to these authors, both, interface and aqueous NaCas, increased with

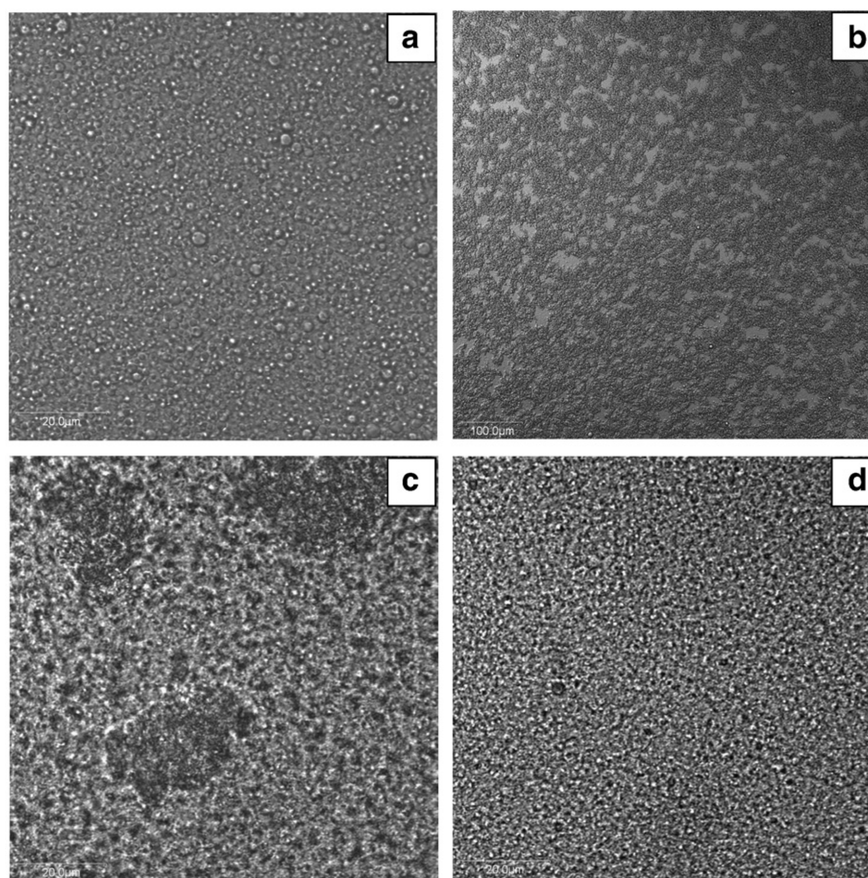

Fig. 3. Confocal laser scattering microscopy (CLSM) images of emulsions with 10 wt.% olive oil (OO) as fat phase, 20 wt.% trehalose solution as aqueous phase, and different concentrations of sodium caseinate (NaCas) kept at 22.5 °C for 24 h: (a) 0.5 wt.%, (b) 3.0 wt.%, (c) 4.0 wt.%, and (d) 5.0 wt.%.

increase of total NaCas. However, aqueous concentration increased in a quite constant linear way while NaCas at the interface increased at a higher rate when particle size diminished significantly. As there was a great difference in particle size among coarse and fine emulsions it is likely that interfacial NaCas was much greater in fine than in coarse emulsions. For example, for SFO emulsions formulated with 5 wt.% NaCas and 30 wt.% trehalose $D_{4,3}$ were 11.231 and 0.201 for coarse and fine emulsions, respectively. Coarse emulsions had more protein in the bulk phase and lower interfacial area and always destabilized by creaming of small particles. On the other hand, fine emulsions with 5 wt.% NaCas are flocculating emulsions. These results suggest that there should be a minimum interfacial area for flocculation. It may be notice that for the same processing conditions increased in NaCas concentration leads to smaller particle size ($D_{4,3}$), that is, greater superficial area [12••].

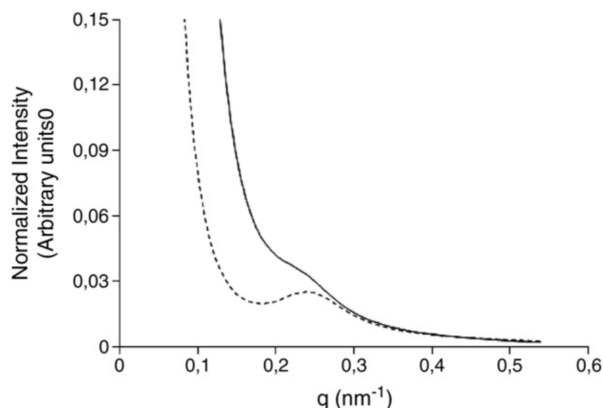

Fig. 4. Normalized intensity vs. reciprocal lattice spacing q, where $q = 2\pi/d = 4\pi \sin(\theta)/\lambda$, where d is the interplanar spacing and 2θ is the Bragg angle, for the emulsion formulated with 5 wt.% NaCas, 20 wt.% trehalose, and 10 wt.% CFO. Solid line: emulsion further homogenized by ultrasound treatment; dotted lines: coarse emulsion. CFO: concentrated from fish oils.

Fig. 5 reports the effect of sugar addition (trehalose or sucrose) on the maximum reciprocal lattice spacing, q_{max}, for emulsions with different NaCas concentration and 10 wt.% of CFO, SFO, or OO as fat phase analyzed at 8 °C. Some aqueous phase components such as hydrocolloids proved to stabilize emulsions because they increase viscosity. The slightly increased of q_{max} values with trehalose or sucrose addition might suggest that trehalose had an effect further than viscosity changes since the aggregation state of the protein changed with the aqueous phase formulation. These results were in agreement with the small particle size found when trehalose was added to aqueous phase. In food emulsions particle size is usually modified by interactions among emulsion components. Addition of alcohol to the aqueous phase of caseinate emulsions at concentrations well below that causing protein precipitation showed that the presence of ethanol can enhance emulsion stability. This is because ethanol causes a significant reduction in the interfacial tension between the oil phase and aqueous protein solution, and so the homogenization of an emulsion premix containing alcohol produces oil droplets with a significantly lower average size than for the equivalent alcohol-free system. The reduction in droplet size leads to reduction of creaming rate [39]. Flocculation was also slowed by the presence of alcohol [40]. Alcohol modifies the average size and composition of the unadsorbed caseinate submicelles which are putatively responsible for the depletion flocculation. Dickinson and Davies (1999) demonstrated that the effect of ionic calcium on the stability of caseinate emulsions of controlled droplet size and protein-

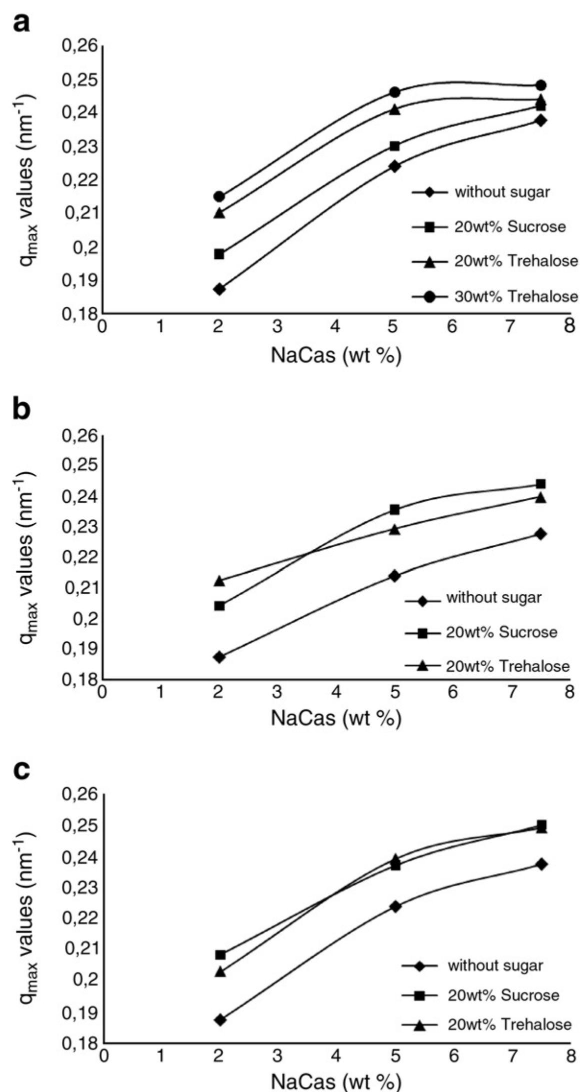

Fig. 5. Reciprocal lattice spacing q vs. sodium caseinate concentration for emulsions formulated with 10 wt.% of (a) concentrated from fish oils (CFO), (b) sunflower oil (SFO), and (c) olive oil (OO).

to-oil ratio is sensitive to whether the calcium salt is introduced before or after homogenization. According to these authors these different behaviors can reasonably be attributed to changes in adsorbed layer structure and protein surface coverage caused by changes in charge density and state of aggregation of the protein [41]. In a later work, Radford et al. (2004) found that some combinations of ionic calcium and ethanol may give stable emulsions. The narrow zone of stability that they identified was indicative of cooperation between calcium ions and ethanol [15]. Medina-Torres et al. also studied the influence of alcohol on the stability and rheology of emulsions containing excess of unabsorbed NaCas. For some NaCas batches there were almost no changes when alcohol was added to the samples up to 45 d storage time [42•]. In addition to these findings, it was reported that polysaccharides which are not particularly surface active such as xanthan gum and which are usually added to the aqueous phase of emulsions as thickening agents to retard instability mechanisms did not affect the size of the emulsion droplets [43•]. On the contrary, the fact that particle size diminished for sugar addition does not allow disregarding interactions. According to Garti droplet size can be smaller if polysaccharides are present with the protein during homogenization, so the rate of creaming can be reduced as long as there is no bridging flocculation [44]. Interactions between polysaccharides and proteins are based on hydrogen bond, and dipole-dipole associations, in which the presence of OH-groups plays a predominant role. Beside, in the Biochemistry field, strong interactions between peptides and short oligosaccharides have been recently reported as playing an important role in protein recognition [45]. All the above indicates that sugar-protein interactions are very common. Regarding trehalose special properties described in literature it is not unreasonable to think that trehalose may work as coadjutant in micelle formation and, also, may change the quality of water as a solvent, improving the solvent-protein interaction, leading to smaller particles sizes and more stable emulsions. Although sucrose has a different structure with the monosaccharides units bound C_1–C_4, it seems to have the same properties. Belyakova et al. reported that there was a pronounced dissociation of sodium caseinate sub-micelles in the presence of sucrose at a pH above the protein´s isoelectric point due, most likely, to direct hydrogen bonding between sodium caseinate and sucrose [46•]. The dissociation of sodium caseinate sub-micelles was in excellent agreement with the more homogeneous microstructure and the formation of smaller compact protein structures as detected by confocal laser scanning microscopy.

NMR relaxation properties depend on the spectral density functions, which in turn are sensitive to molecular motion. Spectrometer frequency, molecular size, and internuclear distances are also important parameters. In fact, spectral densities are related to the motional correlation times, which measure the rate of molecular tumbling. These parameters are the basic longitudinal (T_1) and transversal (T_2) relaxation times. NMR relaxation measurements may be used to probe the change of mobility due to structural changes of the sample. Fig. 6 shows the effect of sucrose or trehalose addition on relaxation longitudinal time T_1 (a) and on relaxation transversal time T_2 (b) for emulsions formulated with 0.5, 2 or 5 wt.% Nacas and 10 wt. OO. The proton signal from the mobile molecules made the most important contributions to the relaxation times. The protein components that stabilized the interface made only a negligible contribution to the NMR signal. T_1 and T_2 values from low- field NMR appeared to be dominated by the structure of the bulk emulsion. The results showed that sugar displayed surface activity in addition to effects on hydrodynamic characteristics of emulsions, and modified emulsion structure. If sugar had acted only as a thickening agent, shorter T_2 and greater T_1 values would have been expected with addition of sugar for all protein concentrations. The fact that the behavior of T_1 was unexpected, that is, curves obtained for emulsions with sugars crossed the ones for formulations without sugars and had very close values for all NaCas concentrations, indicated changes in structure in the bulk emulsion since T_1 is strongly affected by matrix mobility. T_2 had also an unexpected behavior. Values of T_2 were always higher for emulsions with addition of sugar showing a less restricted mobility, which was the opposite result expected for a thickening agent. Emulsions formulated with SFO or CFO showed similar behaviors.

5. Conclusions

The development of new techniques that allow analyzing fully turbid samples looking stable in their undiluted form revealed subtle nuances about their stability. There are two different creaming processes: the creaming of individual particles and the migration of flocculates. Migration of individual particles takes place for emulsions with high oil-to-protein ratio or with big particles sizes (around 10 μm) while migration of flocculates occurred at low ratio and with sizes below 1 μm. Both processes have different kinetics that can be followed by repeating the scan of a sample at different time intervals. Although it was reported that at protein contents around that required for saturation monolayer coverage, the sodium caseinate systems should be very stable towards creaming and coalescence, new studies with non disturbing techniques showed that there is no such a ratio in several caseinate emulsions.

There is a general agreement among authors that there is a protein concentration beyond which emulsions become flocculating systems.

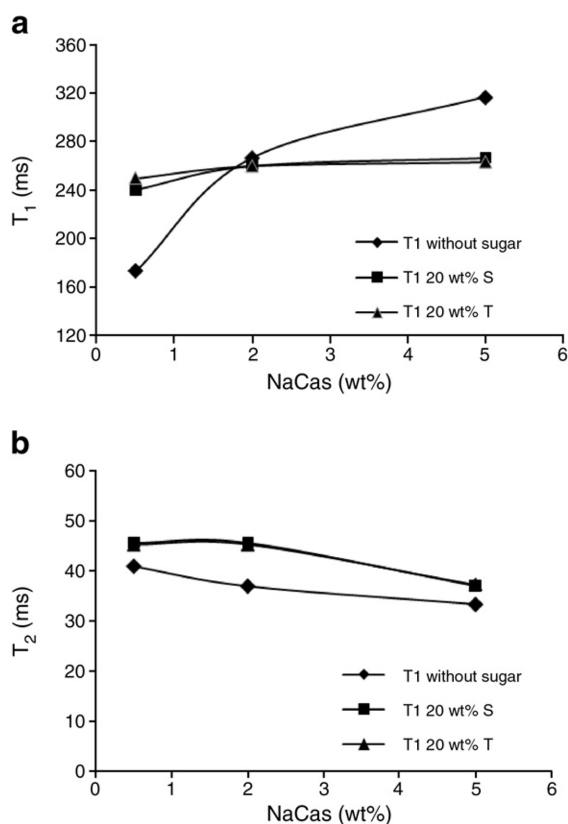

Fig. 6. Relaxation times as a function of NaCas concentration for emulsions formulated with 10 wt.% olive oil. (a) T_1 (b) T_2 S : sucrose; T : trehalose. Emulsions were evaluated immediately after preparation.

However, emulsions with the same formulation and processed in different ways may have very different behaviors. Coarse emulsions always destabilized by migration of individual particles for any protein content. Application of small angle X-ray scattering (SAXS) techniques demonstrated the importance of the size of interfacial area. It may be said that there is a critical value of interfacial area for flocculation instead of a critical concentration of protein.

Early works have shown some evidence about the role of interactions among emulsion components on stability. Recent studies using SAXS have proved that the reciprocal lattice spacing (q), which changes with protein structure, is modified by the presence of active components such as sugars. These interactions change the structure of the protein at the interface and the emulsion structure itself modifying stability. Further evidence of interactions was recently provided by nuclear magnetic resonance. Addition of sugar has proved to have an effect further than viscosity changes.

From our results and others discussed in literature, it is clear that the stability of caseinate emulsions was strongly affected not only by the oil-to-protein ratio but also by processing conditions and composition of aqueous phase. The structure of the protein affected by factors such as pH, processing treatments, and ionic strength, and the interactions protein–sugar or with other additives such as alcohol played a key role in creaming and flocculation processes of emulsions stabilized by sodium caseinate. These interactions modified emulsion structure and therefore its behavior. Further work to characterize structure of sodium caseinate emulsions should be done in order to understand and control stability.

Acknowledgment

María L. Herrera and Roberto J. Candal are researchers of the National Research Council of Argentina (CONICET). This work was supported by CONICET through Project PIP 11220080101504, by the National Agency for the Promotion of Science and Technology (ANPCyT) through Project PICT 0060, and by the University of Buenos Aires through Project X 451. The authors wish to thank to the Synchrotron Light National Laboratory (LNLS, Campinas, Brazil) for the use of X-ray facilities and the SPES company for providing the concentrated from fish oils.

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