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Structuring and Functionalization of Dispersions Containing Egg Yolk, Plasma and Granules Induced by Mechanical Treatments

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In this study, the impact of mechanical treatments on the physicochemical and emulsifying properties of hen egg yolk and its fractions plasma and granules has been assessed. Yolk, plasma, and granule dispersions at pH 4.0 and 0.75 M NaCl were subjected to rotor-stator and high-pressure pretreatments at different dynamic pressure levels: 30, 100, and 200 bar at 20 °C. Physicochemical characteristics (protein solubility, rheological behavior, and micro- and ultra-structures) and emulsifying properties (oil/water 60:40 emulsions: droplet size and flocculation, protein adsorption) of control dispersions and dispersions subjected to mechanical pretreatments (rotor-stator or high pressure) were compared. Homogenization at high pressures (100 and 200 bar) led to a decreased protein solubility and to an increase in apparent viscosity of yolk and plasma dispersions. These pressures certainly disrupted low-density lipoproteins (LDL) particles and generated aggregates of proteins liberated from LDL and livetins in the plasma fraction, and led to a moderated reorganization of the microstructure of granules. Despite the modifications observed in the pretreated plasma and granules dispersions, the oil droplet diameter and the bridging flocculation obtained in emulsions made with these dispersions were similar to that obtained with untreated dispersions. Results concerning interfacial protein adsorption suggested that preformed or natural aggregates at least partially persist at the oil–water interface.

KEYWORDS: Yolk; granules; plasma; mechanical treatments; protein aggregation; emulsifying properties

INTRODUCTION

Hen egg yolk is widely used as an ingredient in many food emulsions. It provides appreciated organoleptic characteristics, and as a very efficient emulsifying agent, it stabilizes emulsified foods such as mayonnaises, salad dressings, and creams. At the same time, yolk can be seen as a good example of a natural supramolecular assembly of lipids and proteins with different organization levels.

Yolk can be fractionated into its two main fractions, plasma and granules, by a mild centrifugation process without causing any denaturation to the proteins (*1*). The supernatant (plasma), representing 75–81% of the yolk dry matter, accounts for 52–58% of the proteins and 85% of the phospholipids. The precipitated granules, which make the remaining 19–25% of the yolk dry matter, account for 42–48% of the proteins and for 15% of the phospholipids (*2*–*4*). Plasma is composed of 85% low-density lipoproteins (LDL) and 15% livetins. Granules comprise 70% high-density lipoproteins (HDL), 16% phosvitin, and 12% LDL (*3*). Plasma constituents (LDL and livetins) are remarkably soluble in the common pH and NaCl range of food emulsions (*4*).

The complexity of the composition and the structure of yolk renders difficult to understand clearly its functionality. In particular, the different levels of supramolecular structures (LDL and granules) are key points to better apprehend yolk functionality. LDL (2/3 of yolk dry matter) are spherical nanoparticles (17–60 nm diameter) with a lipid core (triglycerides and cholesterol esters) surrounded by a phospholipid and protein film (*5*). This nanostructure allows the transport through the aqueous phase to the interface of these amphiphilic species that would be insoluble in another form.

Unlike plasma, granules consist in protein aggregates ranging in diameter from 0.3 μm to several micrometers, depending on environmental conditions (*6*) According to Causeret et al. (*7*), granules are insoluble at pH from 4.3 to 6.5 because they form HDL–phosvitin complexes linked by phosphocalcic bridges as

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HDL and phosvitin contain a high proportion of phosphoserin amino acids able to bind calcium. The numerous phosphocalcic bridges make the granules microstructure very compact, poorly hydrated, and weakly accessible to enzymes and lead to an efficient protection against thermal denaturation and heat gelation. At neutral pH, the solubility of the granules is improved with the ionic strength (0.55 M NaCl leads to dissociation of granules) but at pH 4 high ionic strength has a poor effect (*7*). Consequently, pH 4 and high ionic strength confer a granule microstructure very similar to that of native conditions.

According to Anton et al., (*2*) plasma exhibits better emulsifying activity and granules have the best emulsion stabilization properties. At the same time, it was demonstrated that plasma was more sensitive to heat treatments (55–76 °C) than granules. More precisely, emulsifying activity of plasma severely decreased after a previous heating at 72 °C, whereas that of granules remained steady for the same treatment (*8*).

Thus, these two fractions have different composition, structures, emulsifying properties, and sensibilities to heat treatments. These differences observed between plasma and granules indicated that there is a scope for a more efficient or specific use of these fractions as a function of industrial processes, compared to whole egg yolk. Presently, even if some patents (*9*) concerning their preparation at an industrial scale exist, these fractions are not used in the industrial production of O/W emulsions because of a lack of knowledge concerning their sensibilities to thermomechanical treatments encountered during industrial processes. Furthermore, there is little understanding available on the effect of mechanical treatments (homogenization processes) on their structure and functionality. Thus, in this work, an effort was made to better understand the effect of such pretreatments (such as rotor-stator and high-pressure homogenization) on the ability of yolk fractions to produce O/W emulsions.

Consequently, dispersions of yolk, plasma, and granules have been subjected to mechanical shear via rotor-stator (20000 rpm/1.5 min) and high-pressure homogenizer at three different pressures (30, 100, and 200 bar) at pH 4.0, 0.75 M NaCl concentration and 20 °C, followed by measurement of their physicochemical (protein solubility, rheological behavior, and micro- and ultra-structures) and emulsifying properties (droplet size, flocculation, coalescence, and adsorbed proteins).

MATERIALS AND METHODS

Preparation of Egg Yolk, Plasma and Granules Dispersions. Fresh hen eggs were manually broken, and the albumen was discarded. Each yolk was carefully rolled on a filter paper (Whatman, Maidstone, England) to remove albumen and chalazes adhering to the vitellin membrane. This membrane was then punctured to collect the pure (unadulterated) egg yolk in a beaker cooled in ice–water. Yolk was fractionated into plasma and granules according to the method of McBee and Cotterill (*10*). Yolk was diluted with an equal volume of a 0.17 M NaCl solution and stirred with a magnetic stirrer for 1 h at 4 °C. This solution was then centrifuged at 10000*g* for 45 min at 4 °C, and the supernatant (plasma) was separated from the sediment (granules). Plasma was centrifuged again under the same conditions for a complete separation of plasma and granules. The granules were washed with a 0.17 M NaCl solution and collected. Once the separation achieved, samples were stored at 4 °C and were kept during a maximum time of a week and a half-because of the growth of microorganisms. Dispersions of yolk, plasma, and granules were prepared at pH 4 (0.05 M acetate buffer) and 0.75 M NaCl concentration (4.4%). The protein concentration was standardized at 30 mg/mL (3%). Consequently, samples have the same protein quantities but have different lipid quantities: 56, 87, and 14 mg/mL, respectively, for yolk, plasma, and granules dispersions.

Mechanical Treatments. Mechanical treatments were carried out with a rotor-stator PT 3000 homogenizer (Kinematica, Switzerland) and a high-pressure homogenizer (Stansted Fluid Power Ltd., Stansted, England). Dispersions submitted to rotor-stator were homogenized for 1.5 min at 20000 rpm using a 12 mm diameter head. This shear corresponds to the treatment used in the preparation of emulsions. Dispersions subjected to high-pressure processing were treated at 30, 100, and 200 bar for 5 min under controlled temperature (20 °C) to avoid an increase of temperature during pressure processing. Dispersions were then stored at 8 °C overnight.

Microstructure Determination by Confocal Laser Scanning Microscopy (CLSM). To study the three-dimensional arrangement of complex structures, samples were analyzed under a confocal laser scanning microscope (CLSM) using a Zeiss LSM 410 (Zeiss, Le Pecq, France). A molecular probe Alexa Fluor 546 (Molecular Probes, Leiden, Netherlands) was used for staining proteins green (green laser 543 nm). This molecular probe was added to dispersions, and then the blend was deposited in a cavity slide (containing 25 μL). The samples were examined after one night diffusion at 10 °C with a 63 × oil immersion objective (with a 1× zoom). All the fluorescence images have a resolution of 512 × 512 pixels. The superimposition of the pictures shows the relative distribution of the proteins in the system.

This technique can not show phenomena that occur in plasma dispersions because of the small size of its constituents. Consequently, in this report, only CLSM images of granules will be shown.

Ultrastructure Determination by Transmission Electron Microscopy (TEM). The plasma dispersions were observed using transmission electron microscopy with a JEOL JEM 1230 (Tokyo, Japan) at acceleration tension of 80 kV. The plasma dispersions were diluted with an equal volume of 2% sodium phosphotungstate (pH 7.4), then a small droplet (50 μL) of this mixture was placed on a Formvar carbon-coated grid and air-dried before the observation with the electron microscope.

Protein Solubility. Treated and untreated dispersions (30 mg protein/mL) were equilibrated for 1 h at room temperature under continuous stirring with a magnetic stir bar. Five mL of each dispersion was taken for the determination of the initial protein content. The remaining solution was centrifuged for 20 min at 10000*g*, at 10 °C. Then, 1 mL of supernatant was collected for protein content determination. Protein content of dispersions was determined by the procedure of Markwell et al. (*11*). Calibration of the assay was performed with standard bovine serum albumin. Protein solubility in percent was calculated as follows: solubility (%) = protein in supernatant in mg/mL/protein initial in mg/mL × 100.

Particle Size Measurements. Particle size distributions of nanoparticles (low-density lipoproteins) of plasma dispersions were determined by dynamic light scattering using a Zetasizer Nano Instrument (Malvern, UK). Samples were diluted by 1/30 with acetate buffer to standardize the protein concentration to 1 mg/mL. Sample analyses were conducted in the zetasizer in a 1 cm spectroscopic plastic cell thermostated at 20 °C. Measurements were conducted at least in triplicate. Data analysis was performed using the CONTIN algorithm. Each size measurement corresponds to 5 autocorrelation functions recorded during 30 s. The size distributions are given in intensity percentage as a function of particle diameter, in the range of 1–10000 nm.

Concerning granules, as dynamic light scattering is not able to detect aggregates that exceed 6 μm, particle size distributions of granules microparticle dispersions were measured by laser light diffraction using a Saturn DigiSizer 5200 (Micromeritics Instrument Corp., USA). Samples were not diluted. Histograms of size distribution are given in volume percentage as a function of particle diameter, in the range of 1–200 μm. Relative refractive indices between dispersed phase (protein aggregates) and water, 1.503 (imaginary part was fixed at 0.01), and 1.33, respectively, were used for the analysis.

Rheology Characterization. Viscosity measurements were carried out at 20 °C in a Contraves Low Shear 40 rheometer with a controlled rotation speed, equipped with coaxial cylinders of DIN 412 geometry. Flow curves were determined over an increasing shear rate: from 10 to 100 s^{-1}. This rheometer enabled accurate determination of the flow behavior ranging from Newtonian fluid (low viscosity) to materials

exhibiting plastic behavior. To give comparable ranges of shear rate in the different samples, a measure of the apparent viscosity was given for a shear rate of 20 s^{-1}.

Preparation of the Emulsion. Oil-in-water emulsions were prepared with 60 mL of sunflower oil and 40 mL of the different dispersions. Sunflower oil and the aqueous phase were homogenized for 1.5 min at 20000 rpm using a rotor-stator PT 3000 homogenizer (Kinematica, Switzerland) equipped with a 12 mm diameter head. Homogenization of the emulsion premix was then achieved with a high-pressure homogenizer at 100 bar (Stansted Fluid Power Ltd., Stansted, England). Recirculation of each emulsion was made during 5 min at a flow rate of 340 mL/min.

Oil Droplet Size Measurement. The volume-surface area average diameter distribution ($d_{3,2}$) and the volume frequency distribution ($d_{4,3}$) of the emulsion droplets were determined by laser light diffraction using a Saturn DigiSizer 5200 (Micromeritics Instrument Corp., USA). The refractive index of oil was 1.475 and the imaginary part of refractive index (due to absorption) was fixed at 0.01. After homogenization, a 1/10 emulsion dilution in a solution containing 1 wt/vol % of sodium dodecyl sulfate (SDS) was made to determine the size of the deflocculated emulsion droplets.

Interfacial Protein Concentration (Γ) and Adsorbed Protein Percentage (AP %). Oil droplets were washed from the nonadsorbed proteins according to the method described by Patton and Huston (*12*). Two mL of fresh emulsion were diluted into 2 mL of sucrose solution (500 g/L in 0.1 M pH 4 acetate buffer). Two mL of this mix were carefully deposited at the bottom of a centrifuge tube containing 10 mL of the sample buffer solution. The tubes were centrifuged at 3000*g* during 2 h at 10 °C. After centrifugation, two phases were observed: the creamed oil droplets at the top of the tube and the aqueous phase of the emulsion at the bottom. The tubes were frozen at –20 °C and then cut to separate the phases. Adsorbed proteins at the creamed phase were desorbed by adding 20 mL of 1% SDS, and the dispersion was then centrifuged at 10000*g* during 20 min at 10 °C. The bottom aqueous phase, which contained the nonadsorbed proteins, was carefully extracted with the aid of a micropipette. Adsorbed and nonadsorbed protein concentration was determined by the method used by Markwell et al. (*11*). Interfacial protein concentration was calculated as:

$$\Gamma(\mathrm{mg/m^2}) = \text{adsorbed protein concentration (mg/mL emulsion)}/\mathrm{Sv(m^2/mL\ emulsion)}$$

Adsorbed protein percentage (AP (%)) was calculated as the adsorbed protein respect to initial protein concentration.

Flocculation index. Emulsions were kept at ambient temperature (20 °C) for 4 h, and then the volume frequency distribution was measured as indicated previously. To detect the flocculation process, emulsions were diluted in buffers with and without SDS. The flocculation index was evaluated as follows: FI($d_{4,3}$ of flocs/$d_{4,3}$ of droplets) × 10 where $d_{4,3}$ of flocs is the volume frequency mean of emulsion without SDS and $d_{4,3}$ of droplets is the volume frequency mean of emulsion in the presence of 1 wt/vol % SDS.

Depletion Flocculation. The presence (or degree) of depletion flocculation can be deduced by steady-state flow measurements through viscosity versus shear stress assays. The principle of the measure was to eliminate any flocculation (bridging and depletion) by using an intense strain. Then, the reformation of droplets aggregates is highly influenced by depletion. Modification of emulsion flocs can then be characterized by viscosity variation measurements. A first step consisted of deformation of flocs present in the emulsion by application of a shear stress of 280 Pa during 2 min. Flocs become aligned with the shear field, which decreases their resistance to flow. Thereafter, the emulsion was left 15 min to enhance droplet interactions. Finally, an increasing shear stress from 0.02 to 280 Pa in a 4 min period was applied to the emulsions for floc disruption (*13*). Measurements were performed at 20 °C in a CARRIMED CSL50 (TA Instruments Ltd., Leatherhead, UK) equipped with a plate/cone sensor system (40 mm diameter, 4.01° angle).

Coalescence Index. The stability of the emulsions toward coalescence was assessed by comparison between droplets diameters after the preparation of the emulsion and after 7 days. Emulsions were stored at 4 °C without stirring, and coalescence was measured after 7 days by estimation of the specific surface area (m^2/ml oil, $SS=6/d_{3,2}$) by laser light diffraction using a Saturn DigiSizer 5200 (Micromeritics Instrument Corporation, USA). The coalescence index was calculated as follows, $CI = (SS_o - SS_7/SS_o)100$, where SS_o is the specific surface area of fresh emulsion, and SS_7 the specific surface area 7 days after emulsion preparation. For this type of experiments emulsions were diluted in a buffer containing 1 wt % SDS.

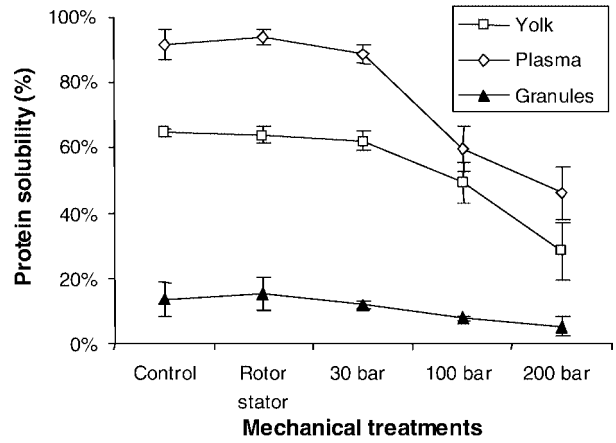

Figure 1. Protein solubility of dispersions (30 mg of protein/mL, pH 4, 0.75 M NaCl) as a function of mechanical treatments measured after centrifugation at 10000*g* for 20 min. Measurements were repeated three times.

STATISTICAL ANALYSES

Three measurements were conducted for each of the following: protein solubility, particle size measurements (dynamic light scattering and laser light diffraction), rheology characterization, average droplet diameter, flocculation index, and depletion flocculation measurement. Statistical analysis was performed using a one-way analysis of variance according to the general linear model procedure with least-square mean effects to determine significant differences between treatments. Multiple range test was applied to determine which means were significantly ($P < 0.05$) different according to Fisher's Least Significant Differences (LSD). Statistical analysis was carried out using Statgraphics plus version 2.1 software (Statistical Graphics Corp., Princeton, NJ).

RESULTS

Protein Solubility. Untreated plasma dispersions exhibit high protein solubility (about 92%) at pH 4.0 and 0.75 M NaCl (**Figure 1**). Plasma proteins seems largely soluble whatever the physicochemical conditions (pH and ionic strength) because they are completely incorporated into LDL which does not sediment when centrifuged because of their low density (0.98) (*2*). So it is not a real molecular solubility but rather a dispersibility due to their incorporation into LDL.

Solubility of granules proteins is low compared to that of plasma: 14% versus 92%. The low protein solubility of granules in acidic conditions (pH 4.0) is related to their structure. Independent of ionic strength, at pH 4.0, granules made up of HDL and phosvitin aggregated via calcium cations (Ca^{2+}) bridging negatively charged phosphoserine residues, remain as insoluble microparticles (*7*).

The protein solubility of yolk dispersions lies between that of plasma and granules, reflecting the relative contribution of each fraction to the total protein content of yolk, approximately 55% of yolk proteins recovered in plasma and 45% in granules.

Concerning plasma, the protein solubility of all samples was not modified by the rotor-stator and 30 bar treatments. On the

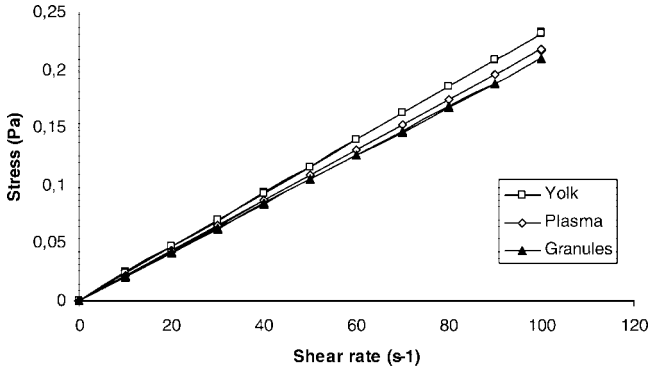

Figure 2. Flow curves of yolk, plasma, and granule untreated dispersions (30 mg of protein/mL, pH 4, 0.75 M NaCl). Measurements were repeated three times.

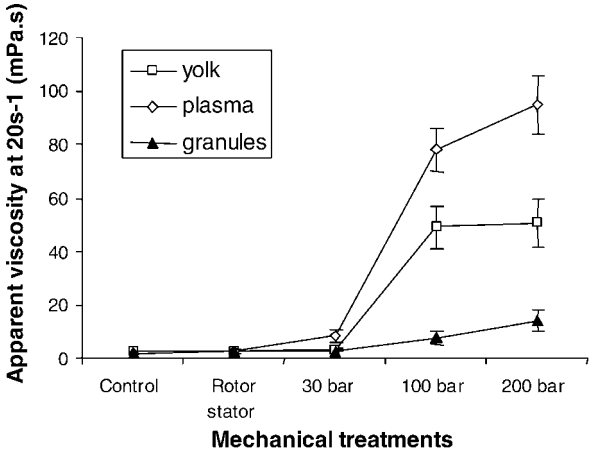

Figure 3. Apparent viscosity of yolk, plasma, and granule dispersions (30 mg of protein/mL, pH 4, 0.75 M NaCl) as a function of mechanical treatments. Shear rate = 20 s^{-1} at 20 °C. Measurements were repeated three times.

other hand, for 100 and 200 bar, the protein solubility of yolk and plasma decreased significantly. The plasma protein solubility fell to 60% at 100 bar and to 45% at 200 bar. That of yolk protein fell to 50% at 100 bar and to 28% at 200 bar. This change was observed visually by the increase of the dispersion opalescence (results not shown). The treatments had only a small effect on the granules' protein solubility which was already very low.

Apparent Viscosity. The flow curves (**Figure 2**) for untreated yolk, plasma and granule dispersions were characteristic of Newtonian liquids with low viscosity (2×10^{-3} Pa.s). We observed the same behavior for dispersions submitted to rotor-stator and to 30 bar high-pressure treatment, where the apparent viscosity was similar to that of control dispersions (**Figure 3**). In contrast, at 100 and 200 bar, dispersions exhibited a low yield stress with flow behavior. At these pressures, the apparent viscosity of yolk and plasma dispersions increased dramatically (compared to that of the control dispersions) whereas that of granules dispersions slightly increased: respectively 49 and 78 mPa.s for yolk and plasma in contrast to 8 mPa.s for granules at 100 bar, and 51 and 95 mPa.s for yolk and plasma in contrast to 14 mPa.s for granules at 200 bar.

Particle Size Measurements. Dynamic light scattering was performed to assess the aggregation state of plasma LDL due to mechanical treatments via the measurement of apparent hydrodynamic diameters (**Figure 4**). For the untreated (control) plasma dispersion, two defined populations with different average diameters were observed: 40 nm for the major popula-

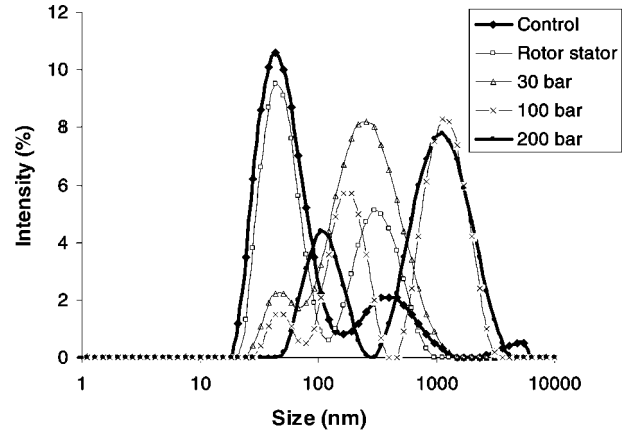

Figure 4. Dynamic light scattering of plasma dispersions (30 mg of protein/mL, pH 4, 0.75 M NaCl) as a function of mechanical treatment. Measurements were repeated three times.

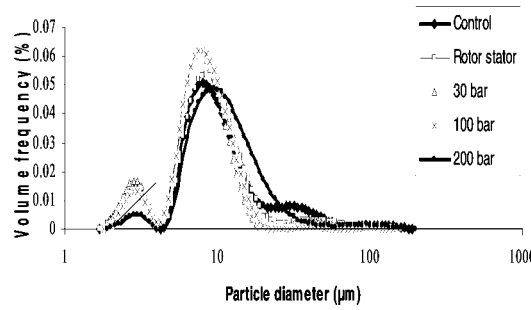

Figure 5. Particle size distributions of microparticles of granules dispersions (30 mg of protein/mL, pH 4, 0.75 M NaCl) as a function of mechanical treatment. Measurements were repeated three times.

tion (about 80% in intensity) and 350 nm for the second population. These results are in agreement with Martinet et al. (*13*) who observed by electron microscopy LDL diameters ranging from 17 to 60 nm. Moreover, the second population at 350 nm seems to be the very low-density lipoproteins as observed earlier by Martin et al. (*14*).

No changes in the average diameter distribution of LDL size were observed for rotor-stator treatment (50 nm for the major population and 340 nm for the second one) whereas high-pressure had an effect on LDL diameter. We observed a progressive shift of the major population from 40 to about 200 nm for the 30 and 100 bar pressures where the percentage of population at 40 nm decreases to about 10% in intensity. There is also a shift of the second population from 350 to 1100 nm for 100 and 200 bar. The percentage of the population at 1100 nm increases to 60% and 70% respectively for 100 and 200 bar. It seems that at 30 bar, structural changes first start to appear and while 100 bar is the first level that initiates a consequent aggregation of LDL (about 1μm size aggregates).

Laser light diffraction was performed to assess the particle size distributions of microparticles of granules dispersions (**Figure 5**). The untreated granules dispersions exhibit two populations (3 and 8 μm) that remain independent of treatment. Contrary to plasma dispersions, the different treatments have no effect on the size of granules microparticles.

Microstructural Analysis. Confocal micrographs (**Figure 6**) show the microstructure of untreated granules and treated granules at 100 and 200 bar. In acidic (pH 4.0) and high NaCl concentration (0.75 M) conditions, granules form insoluble complexes where the majority of HDL and phosvitin are bound together by phosphocalcic bridges between their seryl residues. In comparison with the untreated dispersion, high-pressure

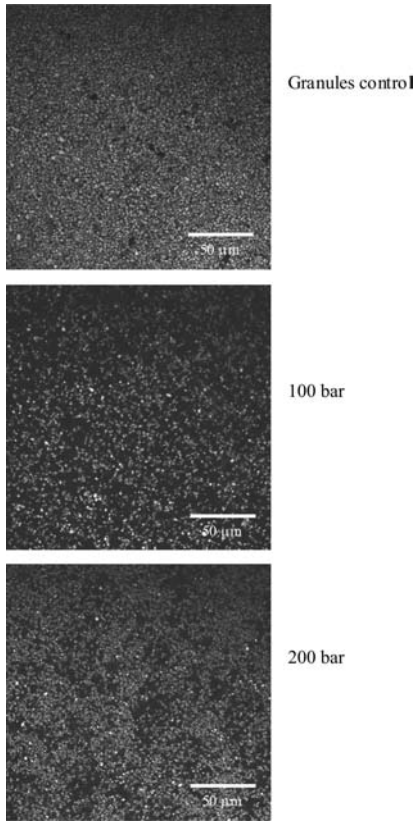

Figure 6. Confocal micrographs showing the structure of granule dispersions (30 mg of protein/mL, pH 4, 0.75 M NaCl): (a) untreated dispersions; (b) 100 bar; (c) 200 bar.

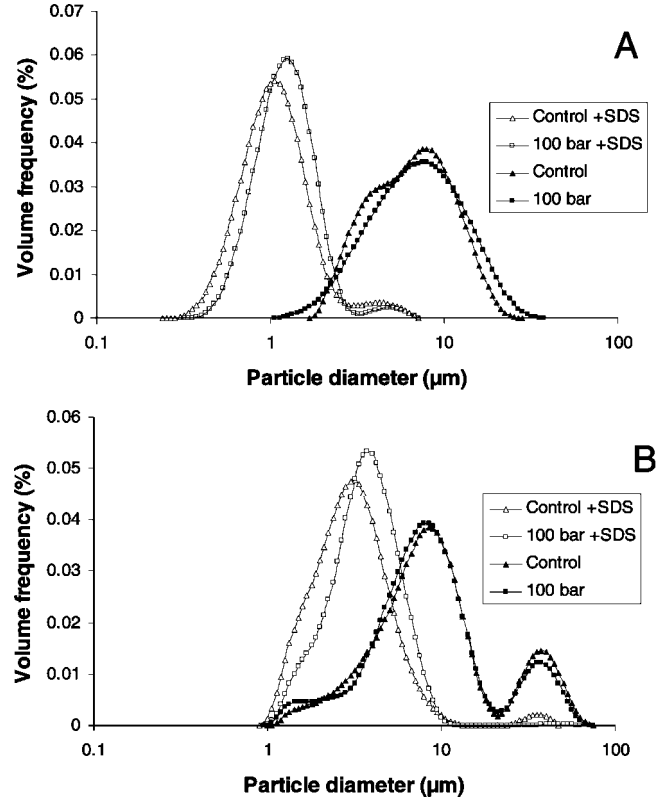

Figure 7. Volume frequency of plasma (A) and granule (B) emulsions (30 mg of protein/mL, pH 4, 0.75 M NaCl) prepared with 100 bar treated and untreated dispersions. Measurements were made in the presence and absence of SDS. Measurements were repeated three times.

treatments (100 and 200 bar) caused major changes in the granules microstructure. At 100 bar pressure, confocal micrographs showed an open microstructure with large protein clusters and big pores. When granules dispersions were homogenized at 200 bar, the microstructure became finer and more condensed with a decrease in pore size and an increase in the degree of interconnectivity between clusters.

Characterization of Emulsions. To compare the effect of these pretreatments on the capacity of dispersions to stabilize emulsions, we have chosen the 100 bar pretreated dispersions for each fraction because it was the critical level leading to significant structural modifications. Droplet size distribution (calculated on volume) of oil-in-water emulsions made with untreated and treated (100 bar) dispersions are shown in **Figure 7A** and **B**.

Both plasma (**Figure 7**A) emulsions (previously treated or not), under deflocculation conditions (with SDS addition), exhibited a quasimonomodal distribution with a similar $d_{4,3}$ of about 1.1 μm (**Table 1**). All plasma emulsions were characterized by a distribution of oil droplets in the range of 0.2–7 μm. It is apparent that prior high-pressure treatment (100 bar) on plasma suspensions does not alter its capacity to stabilize freshly formed oil–water interfaces.

In the absence of SDS addition (no deflocculation), emulsions presented higher oil droplet size than those observed with SDS, suggesting the presence of flocculated oil droplets in all emulsions. Emulsions presented a similar major population of particles of 6.4 and 6.9 μm, respectively for control and previously treated emulsions. The emulsions made with pressure treated plasma dispersions presented monomodal distributions contrary to what was observed for the control emulsion. The observed flocculation seems to be bridging flocculation of droplets since depletion cannot occur because of the fluid motion in the granulometer. As a result, we assume that the prior 100 bar treatment did not significantly modify the droplet size distribution of the plasma emulsion.

Table 1. Plasma and Granules Emulsion Characteristics: Diameter Expressed in Volume ($d_{4,3}$), Flocculation Index (FI), and Coalescence Index (CI)[a]

	$d_{4,3}$ (μm)	FI	CI
	plasma pH 4, 0.75 M		
control	1.1 ± 0.1 a	6 ± 1.9 a	1.1 ± 0.1 a
100 bar	1.2 ± 0.1 a	5.7 ± 0.9 a	0.2 ± 0.1 b
	granules pH 4, 0.75 M		
control	3.1 ± 0.2 a	2.9 ± 0.2 a	0.2 ± 0.3 a
100 bar	3.5 ± 0.2 a	2.4 ± 0.2 a	0.2 ± 0.2 a

[a] Measurements were repeated three times. Significant differences ($P < 0.05$) between control and 100 bar samples were assigned by different letters.

Both emulsions made with granules (**Figure 7B**), under deflocculation conditions, exhibited a monomodal distribution with a bigger $d_{4,3}$ than that of plasma ($d_{4,3}$ about 3 μm). Moreover, granulometric index shows a non significant increase of the oil droplet size made with treated granules dispersion: 3.5 μm versus 3.1 μm for control emulsion (**Table 1**). Without a deflocculation process, emulsions presented similar droplet distribution with a large percentage of particles of 9 μm and a minor fraction of particles of 37 μm, suggesting no changes in bridging flocculation at 100 bar in spite of higher droplet size under deflocculated conditions.

On the basis of these results, we have calculated a flocculation index (FI) presented in **Table 1**. For plasma emulsions, the FI values were not significantly modified with 100 bar pretreatment. Concerning granules emulsions, a nonsignificant decrease of FI

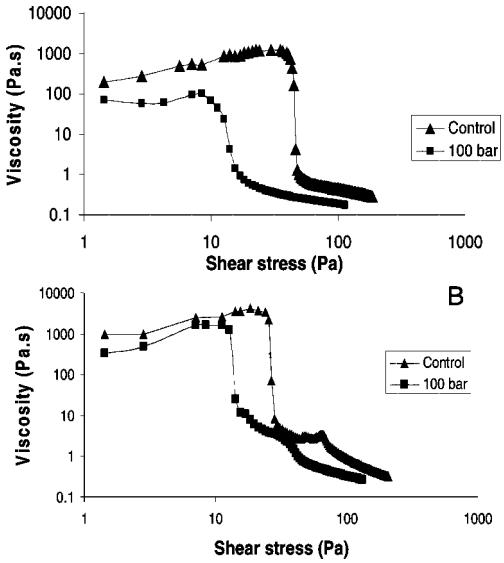

Figure 8. Viscosity versus shear stress of plasma (A) and granules (B) emulsions (30 mg of protein/mL, pH 4, 0.75 M NaCl) prepared with 100 bar treated and untreated dispersions. Measurements were repeated three times.

was observed for the pressure treatment (2.9 for control emulsion and 2.4 for emulsion with treated dispersion).

Furthermore, we did not notice any coalescence (**Table 1**) whatever the sample studied.

Figure 8 shows viscosity of emulsions as a function of shear stress application. The principle of the measurement was first to eliminate any flocculation (bridging and depletion) by using an intense strain. Then reformation of droplet aggregates is highly influenced by depletion. Modification of emulsion flocs can then be characterized by viscosity variation measurements.

Control plasma emulsions (**Figure 8A**) exhibited a mild viscosity (about 200 Pa's) at low shear stress, and then a rapid drop of viscosity from a threshold shear stress (50 Pa). A complete disruption was observed from 55 Pa. The apparent viscosity observed at low shear stress demonstrated the presence of a flocculated system. The initial viscosity is dependent on the size of droplet aggregates and, consequently, on the number of droplets associated with these aggregates. Furthermore, the stress value corresponding to the drop in apparent viscosity gave an estimation of the force required to break the flocs (deflocculation). High-pressure treatment of plasma did not drastically change this behavior. Nevertheless, it can be seen that the threshold for deflocculation decreased with the treated emulsions (10 Pa) indicating weaker interactions between oil droplets.

Depletion flocculation was also observed for emulsions made with granules (**Figure 8B**) but at a higher initial level than for plasma emulsions (1000 Pa's). However, interactions between droplets seem less important given the threshold for deflocculation (25 Pa). In this case too, high-pressure treatment of granules seemed to decrease the threshold (12 Pa) of deflocculation.

DISCUSSION

Mechanical Pretreatments Affect More the Constituents of Plasma than That of Granules. Our results clearly show that the constituents of plasma are more sensitive to mechanical treatments than those of granules. We have evidenced that from 100 bar pretreatment, solubility of plasma proteins decreased, apparent viscosity of dispersion increased and size of the particles was enhanced. These phenomena seem to reflect a mechanism of protein aggregation. However, even if the general

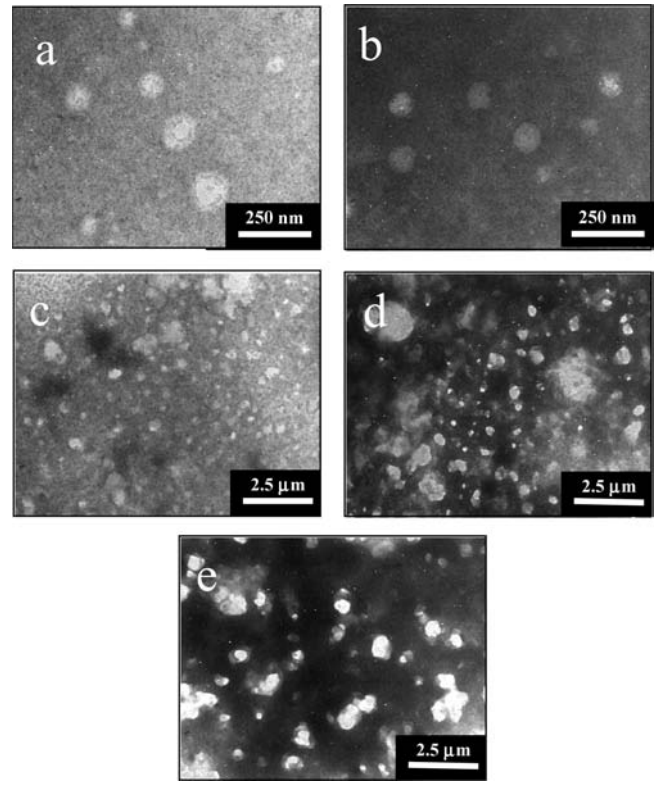

Figure 9. Transmission electron microscopy of LDL dispersions (30 mg of protein/mL, pH 4, 0.75 M NaCl): (a) untreated dispersions; (b) rotor-stator, (c) 30 bar, (d) 100 bar; (e) 200 bar.

theoretical model concerning network formation during aggregation of protein solutions can be applied to describe mechanism of plasma protein aggregation (*15*), we have to keep in mind that plasma is not a pure protein solution but a dispersion of particles (LDL) where neutral lipids are surrounded by a monofilm of phospholipids and proteins. So the first step before to envisage protein aggregation is the destabilization and/or disruption of the supramolecular structure of LDL. To assess this fact we have made an additional study to check the morphology of LDL after the different treatments (**Figure 9**).

In our study it is obvious that LDL, at the concentration studied and at pH 4 and 0.75 M NaCl (conditions endured for industrial mayonnaises) are disrupted and reorganized in big heaps with a non spherical shape from 100 bar pretreatment. Whereas, for the control and the rotor-stator treatment, whitish spherical shapes of native LDL are observed. This additional study gives the indubitable evidence that the first step specific to plasma (disruption of LDL supramolecular structure after treatment) is real under high-pressure pretreatment of at least 100 bar and re-equilibration time. After that, the liberated constituents like neutral lipids and proteins can reorganize and aggregate probably through different type of interactions. Interestingly, it appears that these effects are very dependent of physicochemical conditions as it was demonstrated previously (*13*) that the integrity of purified LDL at pH 7 and low ionic strength is not altered by several passes through a high-pressure homogenizer at up to 250 bar.

Furthermore, we have to recall that plasma contains an important quantity of soluble proteins: livetins (15% of plasma dry matter but 55% of plasma proteins) coexisting with LDL. These proteins are available to interact with LDL proteins liberated during disruption of LDL. And it was also previously found that livetins are the most labile egg yolk proteins in relation to thermomechanical treatments (*16*). Consequently, the

presence of livetins in plasma should enhance the quantity of proteins able to denature and aggregate, enhancing the pressure effect observed on plasma constituents. Medium conditions (pH 4, 0.75 M NaCl) are favorable to protein aggregation as the high quantity of salt induces a high ionic strength synonymous of a decrease of the interaction electrical potential due to an electrostatic screening by the counterions. Furthermore, these conditions of pH are very near of the isoelectric point of the livetins (4.3–5.5) favoring also their interactions.

Additionally, a heat treatment at 70 °C and above during heating at 4% w/v (*8*) provokes the disruption of LDL entities. At more elevated temperature (75 °C), LDL dispersions form strong cohesive gels in the same conditions (*17–19*). Heating provokes weakening of lipid–protein interactions due to protein unfolding, favoring structural disruption, after which interactions between proteins are increased leading to gel formation. Tsutsui et al. (*20*) assumed that lipids are included in the structure of LDL aggregates.

An other point is that heat treatments cause a gelation of LDL dispersions (depending on concentration, medium conditions and temperature), whereas we have seen in our study that high-pressure treatments occurring in a homogenizer only provoke thickening (at a concentration > at the critical gelation concentration (*21*)). Mechanical treatment as provided by high-pressure homogenizers is often accompanied by a substantial increase of temperature (up to 75 °C in the mixing chamber). Consequently, a moderate mechanical treatment (up to 250 bar) combined with an increase of temperature (70–75 °C) could provoke a gelation of the system. In the present study, the temperature of plasma dispersions was maintained at 20 °C to avoid these possible thermal effects and to focus exclusively on the observation of the impact of mechanical processes.

Mechanical pretreatments have slight effect on the constituents of granules. We have evidenced very limited increase of viscosity and decrease of solubility and narrow structural changes in the dispersions visualized by confocal microscopy. Under experimental conditions used here (pH 4.0 and 0.75 M NaCl), the constituents of granules (HDL and phosvitins) are in form of microparticles (0.3–8 μm) bounded together by phosphocalcic bridges between their seryl residues to form insoluble complexes (*7*). In this study, it appears that mechanical pretreatment (up to 200 bar) increased the density of microstructure joining the granule subcomponents without altering it.

These Modifications Only Slightly Impact Emulsifying Properties. In the present study, pressure treatment of plasma and granules dispersions prior to emulsification appeared to only slightly affect emulsifying properties. Indeed, independent of the egg yolk fraction, oil droplet diameters and flocculation of emulsions made with treated and untreated dispersions were similar. We can just notice the decrease of coalescence index in emulsions made with 100 bar treated plasma but even if they are significantly different the two values reflect practically no coalescence.

Concerning plasma, for which we have evidenced modifications during treatments, two hypotheses are proposed to explain our results. First, the created particles (increase of particle sizes) are disrupted during the homogenization process and they are adsorbed in a non aggregated form at the oil–water interface. Or, second, the created particles (probably aggregates) are slightly modified and they adsorb in this form at the oil–water interface. According to the theory of isotropic turbulence, convective mass transport rate increases with the size and the density of molecules. Consequently, the turbulence experienced in high-pressure homogenization would favor the adsorption of large protein aggregates over individual molecules (*22*). We have assessed (results not shown) that the percentage of plasma and granules adsorbed proteins were increased when we used the samples pretreated at 100 or 200 bar: 70%, 84%, and 84% plasma adsorbed proteins. The corresponding protein interfacial concentration increased from 2 to 3 mg/m^2 at the same time. So this moderate increase of protein adsorption yield indicates that adsorption of plasma aggregates at the oil–water interface is possible but not so important as regards of the size of created particles.

Similar findings have been made by Campbell et al. (*23*) on egg yolk heated in the presence of NaCl and sugar. They suggested that thermally aggregated egg yolk proteins actually adsorb at the oil–water interface. In our study, we have to keep in mind that treated dispersions were stored at 8 °C overnight before measurement of physicochemical characteristics and fabrication of emulsions. Consequently, in dynamic conditions like those occurring during homogenization it is likely that preformed aggregates are partly disrupted and have not enough time to be reformed before adsorption.

Anton et al. (*23*) and Aluko and Mine (*24*) also showed that granules were partially disrupted by mechanical shear forces, leading to the formation of granules fragments, notably during high-pressure homogenization. The hydrophobic interactions binding proteins (*25*) are quite weak, so they can probably be dissociated by shear forces present in the conditions used for emulsification (100 bar). In our study, homogenization processes induce forces that may partially disrupt the protein aggregates formed during mechanical treatment and therefore liberate a multitude of smaller aggregates. Such fragments could adsorb at the oil–water interface. It can then be suggested that, during homogenization, the protein aggregates formed during mechanical treatments are adsorbed at the oil–water interface as whole aggregates or as fragments of these aggregated structures. We have checked this assertion and controlled that 80%, 92% and 91% of granules proteins were adsorbed at the oil–water interfaces, respectively for control, 100, and 200 bar pretreatment, corresponding to an increase from 6 to 10 mg/m^2 for granules proteins. This highlights the fact that in our conditions, granules exhibit naturally an important presence of protein aggregates, that these aggregates are slightly modified during pretreatments (densification of the microstructure) and that numerous aggregates are present (significant interfacial concentration) at the oil–water interfaces whatever the pretreatment.

CONCLUSION

This work has suggested that mechanical treatments of egg yolk, plasma and granules dispersions before emulsification cause some modifications of physicochemical characteristics, especially for plasma. We have deduced that high-pressures (100 and 200 bar) have a significant effect on plasma fraction, leading to disruption of LDL followed by an aggregation between liberated proteins and livetins, but have a small effect on granules microstructure because of its native structure. These modifications only slightly affected the emulsifying properties of plasma as we have observed the same particle diameter and bridging flocculation for emulsions made with both untreated and treated dispersions. The effects of pretreatments of granules on their emulsifying properties led to a slight decrease in bridging and depletion flocculation. These results suggest that the constituents of treated dispersions can adsorb at the oil–water interface as whole aggregates or as fragments disrupted during the homogenization process. Further work is required to

elucidate the formation of pretreated aggregates during homogenization and to understand especially the impact of prior mechanical treatment on the interfacial properties.

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LITERATURE CITED

(1) Schmidt, G.; Bessman, M. J.; Hickey, M. D.; Thannhauser, S. J. The concentrations of some constituents of egg yolk in its soluble phase. *J. Biol. Chem.* **1956**, *262*, 1027–1031.

(2) Anton, M.; Gandemer, G. Composition, solubility and emulsifying properties of granules and plasma from egg yolk. *J. Food Sci.* **1997**, *62*, 484–487.

(3) Burley, R. W.; Cook, W. H. Isolation and composition of avian egg yolk granules and their constituents α- and β-lipovitellins. *Can. J. Biochem. Phys.* **1961**, *39*, 1295–1307.

(4) Saari, A.; Powrie, W. D.; Fennema, O. Isolation and characterization of low-density lipoproteins in native egg yolk plasma. *J. Food Sci.* **1964**, *29*, 307–315.

(5) Cook, W. H.; Martin, W. G. Egg lipoproteins. In *Structural and Functional Aspects of Lipoproteins in Living Systems*; Tria, E., Scanu, A. M., Eds.; Academic Press: London and New York, 1969; pp 579–615.

(6) Chang, C. M.; Powrie, W. D.; Fennema, O. Microstructure of egg yolk. *J. Food Sci.* **1977**, *42*, 1193–1200.

(7) Causeret, D.; Matringe, E.; Lorient, D. Ionic strength and pH effects on composition and microstructure of yolk granules. *J. Food Sci.* **1991**, *56*, 1532–1536.

(8) Le Denmat, M.; Anton, M.; Gandemer, G. Protein denaturation and emulsifying properties of plasma and granules of egg yolk related to heat treatment. *J. Food Sci.* **1999**, *64* (2), 194–197.

(9) Causeret, D.; Lorient, D. Procédé de fractionnement des constituants du jaune d'oeuf. EP Patent no. 0 430 757 A1, **1991**.

(10) McBee, L.; Cotterill, O. Ion exchange chromatography and electrophoresis of egg yolk. *J. Food Sci.* **1979**, *44*, 656–660.

(11) Markwell, M. A.; Hass, S. M.; Bieber, L. L.; Tolbert, N. E. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **1978**, *87*, 206–210.

(12) Patton, S.; Huston, G. E. A method for isolation of milk fat globules. *Lipids* **1986**, *21*, 170–174.

(13) Martinet, V. Relationships between structure and function of hen egg yolk lipoproteins: physico-chemical characterisation and emulsifying properties. Ph.D. Thesis, University of Nantes, 2003, 218 pp.

(14) Martin, W. G.; Augustyniak, J.; Cook, W. H. Fractionation and characterization of the low-density lipoproteins of hen's egg yolk. *Biochim. Biophys. Acta* **1964**, *84*, 714–720.

(15) Clark, A. H. *Functional properties of food macromolecules*; Hill, S. E., Ledward, D. A., Mitchell, J. R., Eds.; Aspen: Gaithersburg, MD, 1998; pp77–142.

(16) Dixon, D. K.; Cotterill, O. J. Electrophoretic and chromatographic changes in egg yolk proteins due to heat. *J. Food Sci.* **1981**, *46* (990), 981–983.

(17) Nakamura, R.; Fukano, T.; Tanigushi, M. Heat induced gelation of hen's egg yolk low-density lipoprotein dispersion. *J. Food Sci.* **1982**, *47*, 1449–1456.

(18) Kojima, E.; Nakamura, R. Heat gelling properties of hen's egg yolk low-density lipoprotein (LDL) in the presence of other proteins. *J. Food Sci.* **1985**, *50*, 63–66.

(19) Tsutsui, T. Functional properties of heat-treated egg yolk low-density lipoprotein. *J. Food Sci.* **1988**, *53*, 1103–1106.

(20) Tsutsui, T.; Obara, T. Hydrophobic components in delipidated granule of egg yolk. *Agric. Biol. Chem.* **1982**, *46*, 2587–2589.

(21) Le Denmat M. Oil-in-water food emulsions made with yolk and its fractions: physical properties and thermal treatment. Ph.D. Thesis, University of Rennes, 2000, 213 pp.

(22) Walstra, P. Formation of emulsions. In *Encyclopedia of Emulsion Technology: Basic Theory*; Becher, P. , Ed.; Marcel Dekker, Inc.: New York, 1983; Vol. 1, pp 57–127.

(23) Campbell, L.; Raikos, V.; Euston, S. Heat stability and emulsifying ability of whole egg and egg yolk as related to heat treatment. *Food Hydrocolloids* **2005**, *19*, 533–539. Anton, M.; Beaumal, V.; Gandemer, G. Adsorption at the oil/water interface and emulsifying properties of native granules from egg yolk: Effect of aggregated state. *Food Hydrocolloids* **2000**, *14*, 327–335.

(24) Aluko, R. E.; Mine, Y. Characterization of oil-in-water emulsions stabilized by hen's egg yolk granules. *Food Hydrocolloids* **1998**, *12*, 203–210.

(25) Kiosseoglou, V. D.; Paraskevopoulou, A. Molecular interactions in gels prepared with egg yolk and its fractions. *Food Hydrocolloids* **2005**, *19*, 527–532.

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