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Sameh Obeid, Fanny Guyomarc'H, Gregory Francius, Herve Guillemain, Xiaoxi Wu, et al.. The surface properties of milk fat globules govern their interactions with the caseins: Role of homogenization and pH probed by AFM force spectroscopy. *Colloids and Surfaces B: Biointerfaces*, 2019, 182, pp.110363. 10.1016/j.colsurfb.2019.110363 . hal-02215870

HAL Id: hal-02215870

<https://hal.science/hal-02215870>

Submitted on 31 Jul 2019

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The surface properties of milk fat globules govern their interactions with the caseins: Role of homogenization and pH probed by AFM force spectroscopy

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ARTICLE INFO

Keywords:

Interfacial properties
Emulsion
Lipid droplet
Casein micelle
Adhesion force
Atomic force microscopy

ABSTRACT

The surface of milk fat globules consists of a biological membrane rich in polar lipids and glycoproteins. However, high shear stress applied upon homogenization disrupts the membrane and leads to the adsorption of casein micelles, as the major protein fraction of milk. These changes in the interface properties could affect the interactions between native or homogenized milk fat globules and the surrounding protein matrix, at neutral pH and upon acidification. In this study, macroscale rheometry, microscopic observations, nanoscale AFM-based force spectroscopy and physico-chemical analysis were combined to examine the interfacial composition and structure of milk fat globules and to evaluate their interactions with casein micelles. We showed that the surface properties of milk fat globules (biological membrane vs. caseins) and pH govern their interactions with casein micelles. The adhesion between individual fat globules and casein micelles was higher upon homogenization, especially at acid pH where the work of adhesion increased from 3.3×10^{-18} to 14×10^{-18} J for native and homogenized fat globules, respectively. Consequently, casein-coated homogenized fat globules yield stiffer milk acid gels. These findings cast light on the importance of colloidal particle's surface properties and pH on their connectivity with the surrounding matrix, which modulates the bulk microstructure and rheological properties with potential functional consequences, such as milk lipid digestion.

1. Introduction

The interfacial properties of lipid droplets govern the droplet-matrix interactions with consequences on the microstructure, rheological and functional properties of emulsion-filled gels, such as those found in food, pharmaceutical or cosmetic formulations. Lipid droplets behave as filler particles and can be classified as either active when bound to the gel matrix with increase in the gel's modulus or as inert when they have little affinity for the surrounding matrix [1,2]. The interactions between the surface of lipid droplets and the surrounding matrix require further scientific investigations to be fully understood and controlled.

Among dietary emulsions, milk is of special interest since it is a natural oil-in-water emulsion secreted by mammary cells in which the lipids are dispersed as 4- μm colloidal assemblies called the milk fat globules (MFG) [3]. MFG are the unique lipid delivery assemblies

providing energy and bioactive molecules to mammal newborns. MFG are composed of a core of triacylglycerols (TAG) surrounded by a biological membrane, called the milk fat globule membrane (MFGM), organized as a trilayer of polar lipids, proteins and cholesterol [3]. Many of the MFGM membrane proteins are heavily glycosylated, forming a thick glycocalyx that ensures physical stability [4]. The MFGM polar lipids consist mainly of zwitterionic or anionic glycerophospholipids (phosphatidylcholine, PC; phosphatidylethanolamine, PE; phosphatidylinositol, PI; phosphatidylserine, PS) and sphingolipids (mainly sphingomyelin). At the pH of milk, i.e. pH 6.7, the MFG bear a negative ζ -potential of -10 mV [5]. The main proteins found in the aqueous phase of bovine milk are the κ -, β -, α_{s1} - and α_{s2} -caseins (80% wt total proteins) organized as hydrated colloids, called casein micelles, maintained by intermolecular hydrophobic interactions and calcium phosphate bridges [6]. The glycosylated end of the peripheral κ -caseins forms a "hairy-layer" at the surface of the micelle, providing steric stability [7].

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<https://doi.org/10.1016/j.colsurfb.2019.110363>

Received 3 March 2019; Received in revised form 3 July 2019; Accepted 13 July 2019

Available online 14 July 2019

0927-7765/ © 2019 Published by Elsevier B.V.

In milk, casein micelles have a hydrodynamic diameter of 100–200 nm and a ζ -potential of -15 to -20 mV [8,9]. Acidification of milk induces the neutralization of casein micelles at about pH 4.6 and the collapse of the “hairy-layer”, both participating to their precipitation into acid gels [10]. Among other factors, acidification participates to the destabilization of milk that is used in the preparation of dairy products, e.g. in yoghurt or cheese making, and that occurs upon digestion in the stomach [11,12].

Homogenization is a mechanical process used in the dairy industry to decrease the size of MFG and improve their physical stability. Homogenization leads to the disruption of the MFGM with the adsorption of casein micelles at the surface of processed MFG to stabilize the newly formed interface [13]. Hence, the surface composition of the homogenized MFG is drastically different from that of the native MFG. Consequently, homogenized MFG behave as active fillers upon milk gelation, in contrast to native MFG [14,15]. The differences in the interfacial properties between native and homogenized MFG that affect the fat droplet-protein matrix interactions and the microstructure of milk at acid pH can have consequences on the mechanisms of MFG digestion [16,17]. It has been proposed that the microstructure and rheological properties of gels depend on the strength of interactions at the colloidal particle level [18]. However, little is known about the interactions involved at the nanoscale between native or homogenized MFG and the casein network formed upon acidification of milk. The main reason is the lack of experimental techniques allowing information to be obtained directly from complex colloidal systems at the relevant level.

Atomic force microscopy (AFM) senses interaction forces in the pN to nN range, acting between a tip at the end of a flexible cantilever and a sample surface [19]. The advantages of AFM are its ability to operate under physiological conditions and the possibility to combine force spectroscopy and imaging. In order to probe specific interactions, the AFM tip is functionalized with the molecule of interest. To date, investigations performed by AFM force spectroscopy to measure interaction forces between individual objects involved in food applications were essentially conducted on bacteria interacting with proteins [20–22] or MFGM [23], or on interacting bubbles and/or droplets [24–26]. However, the link between nanoscale force spectroscopy and bulk properties is not investigated, albeit identified as important [27].

The present study was designed to determine whether the surface composition of native vs. homogenized MFG and pH affect their interaction with caseins. For the first time, AFM-based force spectroscopy was used to reach, at the colloidal particle scale, quantitative analysis of the adhesion between individual MFG and casein micelles. Comparison with rheological assessment of the bulk elasticity of native or homogenized milk was meant to set a link between individual and macroscopic behaviors.

2. Materials and methods

2.1. Materials

Native raw bovine milk was provided by Gaec des Fougères (La Chapelle des Fougères, France). NaN_3 (0.02% wt/v) was added to prevent bacterial growth. Milk samples were stored at 20 ± 1 °C and used within 24 h. Casein micelles were isolated from milk at the dairy pilot plant of INRA-STLO as previously described [9]. PIPES buffer (1,4-piperazinediethane sulfonic acid) contained PIPES 10 mM, NaCl 50 mM and CaCl_2 2 mM, all purchased from Sigma-Aldrich. The buffer's pH was adjusted to pH 6.7 or 4.5. Glucono-delta-lactone (GDL), Fast-Green FCF and Nile-Red fluorescent dyes were purchased from Sigma-Aldrich. D (+)-sucrose was purchased from VWR-Chemicals (St Louis, USA).

2.2. Homogenization of milk

Native milk was warmed to 50 °C and homogenized twice at 20 MPa

using an Emulsifex-C3 homogenizer (Avestin, Ottawa, Canada).

2.3. Size measurements

The MFG size distribution was measured at 20 °C by laser light scattering (Mastersizer 2000, Malvern Instruments, Orsay, France). The refractive indexes were 1.333 for water, and 1.458 or 1.460 for MFG at 633 or 466 nm respectively. About 0.2 mL of the sample was diluted in 100 mL of water in the measurement cell in order to reach 8% obscuration. The size of MFG was characterized by the mode diameter, e.g. the most abundant diameter of the frequency distribution. The specific surface area $S = 6\phi/d_{32}$ was calculated (ϕ is the volume fraction of milk fat and d_{32} is the volume/surface average diameter defined as $\sum n_i d_i^3 / \sum n_i d_i^2$, where n_i is the number of fat globules of diameter d_i). The average hydrodynamic diameter of casein micelles was determined by dynamic light scattering (NanoSizer-ZS, Malvern Instruments). Measurements were carried out at a scattering angle of 173 degrees and a wavelength of 633 nm. The viscosity and the refractive index of the buffer were respectively 1.003 mPa.s and 1.33 at 20 °C. Measurements were performed in triplicate.

2.4. Zeta potential measurements

Electrophoretic mobility of MFG and that of casein micelles were measured by dynamic light scattering at 20 °C, using Zetasizer Nano ZS (Malvern Instruments) and the apparent zeta-potential ζ was deduced using the Smoluchowski relation. Samples were diluted 1/100 v/v in PIPES buffer and the pH was adjusted to pH 6.7 or 4.5. Measurements were performed in triplicate.

2.5. Washing of MFG

MFG were washed to remove un-adsorbed proteins from the samples. Milk samples were warmed to 50 °C then 10 g of milk were mixed with 5 g of PIPES buffer pH 6.7 containing 50% (wt/wt) sucrose. The mix was then deposited, using a syringe, at the bottom of 50 mL centrifuge tubes filled with 30 g of PIPES buffer pH 6.7 containing 5% (wt/wt) sucrose. Centrifugation was performed at 1600g during 20 min for native milks or 2500g during 30 min for homogenized milks in order to form a layer of washed MFG at the top of the tubes that have been collected for further investigations (gel electrophoresis, AFM).

2.6. Rheological measurements

Rheological measurements were carried out at 50 °C by small amplitude oscillatory rheology using DHR-2 instrument (TA Instruments, Guyancourt, France) with coaxial cylinders. The storage modulus, G' (in Pa), was monitored as function of the pH, upon addition of 1.65% (wt/wt.) GDL in milk samples, at a frequency of 1 Hz and at 1% strain. The gelation pH was established as the intercept of a linear extrapolation of the rapidly rising elastic modulus G' with the pH axis; the final G' value was taken at pH 4.5. The gels at pH 4.5 were then subjected to a stress-sweep test from 10^{-4} to 10 Pa at a frequency of 1 Hz to measure the yield stress value. Measurements were performed in triplicate.

2.7. Transmission electron microscopy

The samples were solidified in agar 1.5% (wt/v), cut into 1 mm pieces, fixed overnight with 25 g.kg⁻¹ glutaraldehyde in 0.1 M Na-cacodylate and post-fixed for 1 h in 1% osmium tetroxide [28]. The pieces were then dehydrated through increasing ethanol concentrations, infiltrated and embedded in Epon resin mixture (Electron Microscopy Sciences), then finally polymerized at 60 °C for 24 h. Ultra-thin sections were cut using Leica ultra-microtome and stained with 4% uranyl-acetate for 1 h. Sections were observed with JEOL-1400 transmission electron microscope (TEM) operating at 120 kV; images were recorded

on camera Gatan Orius SC-1000.

2.8. Confocal laser scanning microscopy

The microstructural analysis of milks and acid gels was performed with an inverted Eclipse-TE2000-C1 si microscope (NIKON, Champigny-sur-Marne, France). Confocal laser scanning microscopy (CLSM) experiments were performed using a He-Ne laser operating at 543 nm (emission detected between 565–615 nm), a laser diode operating at 642 nm, a long pass filter > 650 nm, and $\times 100$ (NA 1.4) oil immersion objective. The TAG core of MFG was stained with Nile-Red fluorescent dye (exc. 543 nm). The proteins were stained using Fast-Green FCF (exc. 642 nm) [29]. After labeling, the samples were kept 30 min at 20 °C prior to CLSM observations. The labeled milk samples were either observed at pH 6.7 or acidified by adding GDL (1.65% wt/wt; stirred for 1 min, incubated at 50 °C) and then observed when the milk gel pH reached 4.5.

2.9. Atomic force microscopy

AFM probes with attached borosilicate glass particles (5 μm diameter), coated with gold and modified with NH_3 groups (Novascan, Ames, USA; nominal spring constant 0.01 N.m^{-1}) were incubated overnight in a 20 g.kg^{-1} casein micelle solution at 4 °C, and then washed and kept in PIPES buffer (pH 6.7) until use. CLSM experiments confirmed the successful grafting of caseins on the probes (Supplementary information-SI.1).

Washed MFG were diluted to 1:10 in PIPES buffer (pH 6.7) and immobilized on Isopore 0.6- μm pore-size Millipore filters by gently passing the MFG suspension through the filter until blockage. The filter membrane was then gently rinsed with buffer, to remove poorly immobilized MFG, attached inside a Petri dish using double-sided adhesive tape, then immersed in PIPES buffer.

AFM imaging was performed at 22 ± 1 °C in PIPES buffer using an MFP-3D Bio AFM (Asylum Research, Santa Barbara, USA), silicon MSNL10 probes (nominal spring constant $0.01\text{--}0.03 \text{ N.m}^{-1}$ – Bruker Nano Surfaces, Santa Barbara, USA) and the force-mapping mode, with a typical scan rate of 1 Hz on $10 \times 10 \mu\text{m}^2$ and at least 64×64 pixels. The adhesion force measurements were also performed through the force-mapping mode by using the casein micelles-modified probes as illustrated in Fig. 1-A. The cantilever spring constant was determined prior to each experiment using the thermal noise method. For each sample, adhesion force measurements were first performed in PIPES buffer at pH 6.7. Then, the buffer was replaced by PIPES buffer at pH 4.5 and the set-up was incubated for about 30 min at this pH before starting again the measurements. The force maps were recorded on $50 \times 50 \mu\text{m}^2$ surface and 30×30 pixels with an applied force of 500 pN, a probe velocity of $1 \mu\text{m.s}^{-1}$ and a contact time of 1 s. Four independent experiments were performed to measure the adhesion between grafted casein micelles and either native MFG or homogenized MFG, at pH 6.7 and at pH 4.5.

Interactions between the casein micelles-modified probes and the immobilized MFG are expected to produce a random series of adhesion peaks on the AFM retraction force curves, evidencing multiple intermolecular interactions (e.g. several casein molecules adhering to MFG) and/or polymer stretching (e.g. uncoiling of macromolecules - Fig. 1-B) [30,31]. Force curves showing non-specific adhesive events or deformed baseline were discarded. In each experimental condition, force curves showing significant adhesion events were analyzed in order to determine the maximal adhesion forces (F_{adh}), the adhesion work (area below the baseline, W_{adh}) and the rupture distance (D_{adh} - Fig. 1-B). Because the measured adhesion involves objects (casein micelles, MFG) whose dimensions are not themselves normally distributed, the distributions of F_{adh} , W_{adh} or D_{adh} were not Gaussian. The AFM experimental data were therefore statistically analysed using the Wilcoxon rank-sum test adapted for non-normally distributed data. Differences

were considered statistically significant when P value < 0.001.

3. Results and discussion

3.1. Impact of homogenization on the surface properties and structure of MFG

TEM images of native and homogenized MFG are shown in Fig. 1-A. The TAG core of native MFG was surrounded by a thin layer of dark material corresponding to the MFGM. The electron dense material in MFGM corresponds to unsaturated fatty acids stained by osmium and membrane proteins [28]. Gel electrophoresis experiments showed the presence of MFGM proteins at the surface of native MFG, mainly the glycoproteins forming the glycocalyx (supplementary information-SI.2). Furthermore, TEM images showed independent casein micelles with a rough surface and a size between 100–200 nm [8,9]. In contrast, the homogenized MFG imaged by TEM (Fig.1-A) showed that the small TAG core was surrounded by a thick layer of milk proteins corresponding mainly to caseins, as confirmed by gel electrophoresis (supplementary information-SI.2).

In native milk, the size distribution of MFG ranged between 1.5 and 8 μm with a mode diameter of 3.7 μm (Table 1). As expected, MFG in homogenized milk were smaller than in native milk. Their size distribution ranged from 0.04 to 2 μm and was bimodal with mode diameters at 0.17 (range: 0.04 – 0.3 μm) and 0.6 μm (range: 0.3–2 μm) [13,32]. Homogenization of milk induced an increase in the MFG surface area from 1.9 ± 0.1 to $32.5 \pm 4.9 \text{ m}^2.\text{g}^{-1}$ fat. The average hydrodynamic diameter of casein micelles was $173 \pm 2 \text{ nm}$ [9]. At pH 6.7, the ζ -potential of native MFG was -12.2 mV (Table 1), due to the negatively charged phosphatidylserine and phosphatidylinositol located in the outer leaflet of the MFGM and to the membrane-specific proteins [5,29]. In contrast, the ζ -potential of homogenized MFG was $-16.5 \pm 1.0 \text{ mV}$, i.e. similar to that of the casein micelles alone ($-15.6 \pm 0.9 \text{ mV}$; Table 1). Therefore, the increase in negativity of the homogenized MFG was due to the adsorption of milk proteins, especially the negatively-charged casein micelles [33]. The size distributions and ζ -potential values of native or homogenized MFG after washing did not significantly differ to those of MFG in native or homogenized milk samples (Table 1). At pH 4.5, the ζ -potential absolute values of the washed native or homogenized MFG and that of casein micelles significantly decreased (Table 1), as a result of protonation of the acido-basic groups such as organic and inorganic phosphate and carboxylic residues [34]. As a result, casein micelles and the homogenized MFG were almost neutralized, while native MFG exhibited a slightly electronegative ζ -potential value of -6.6 mV.

3.2. Microstructure and acid gelation behavior of native or homogenized milk as a function of pH

CLSM images (Fig. 2-A) showed that at pH 6.7 MFG and milk proteins were homogeneously distributed in the volume of both native and homogenized milk samples. At pH 4.5, the casein micelles aggregated in both milk samples to form a protein network [34,35]. In native milk, MFG were trapped in the serum pockets and did not seem to interact with the protein network (Fig. 2-A, bottom-left). Meanwhile, homogenized MFG were fully embedded within the protein network and were not located in the serum pockets (Fig. 2-A, bottom-right). Microstructural investigations therefore showed that, at acid pH, the homogenization process affected the microstructure of the gels and the connectivity between MFG and the protein network.

Rheological experiments performed upon acidification (Fig. 2-B) showed that the gelation pH of native milk was higher than that of homogenized milk with values of 5.10 ± 0.02 and 4.90 ± 0.02 , respectively. At least two hypotheses can be formulated to explain the difference in gelation pH. Our first hypothesis is that the decrease in gelation pH measured for homogenized milk could be due to the

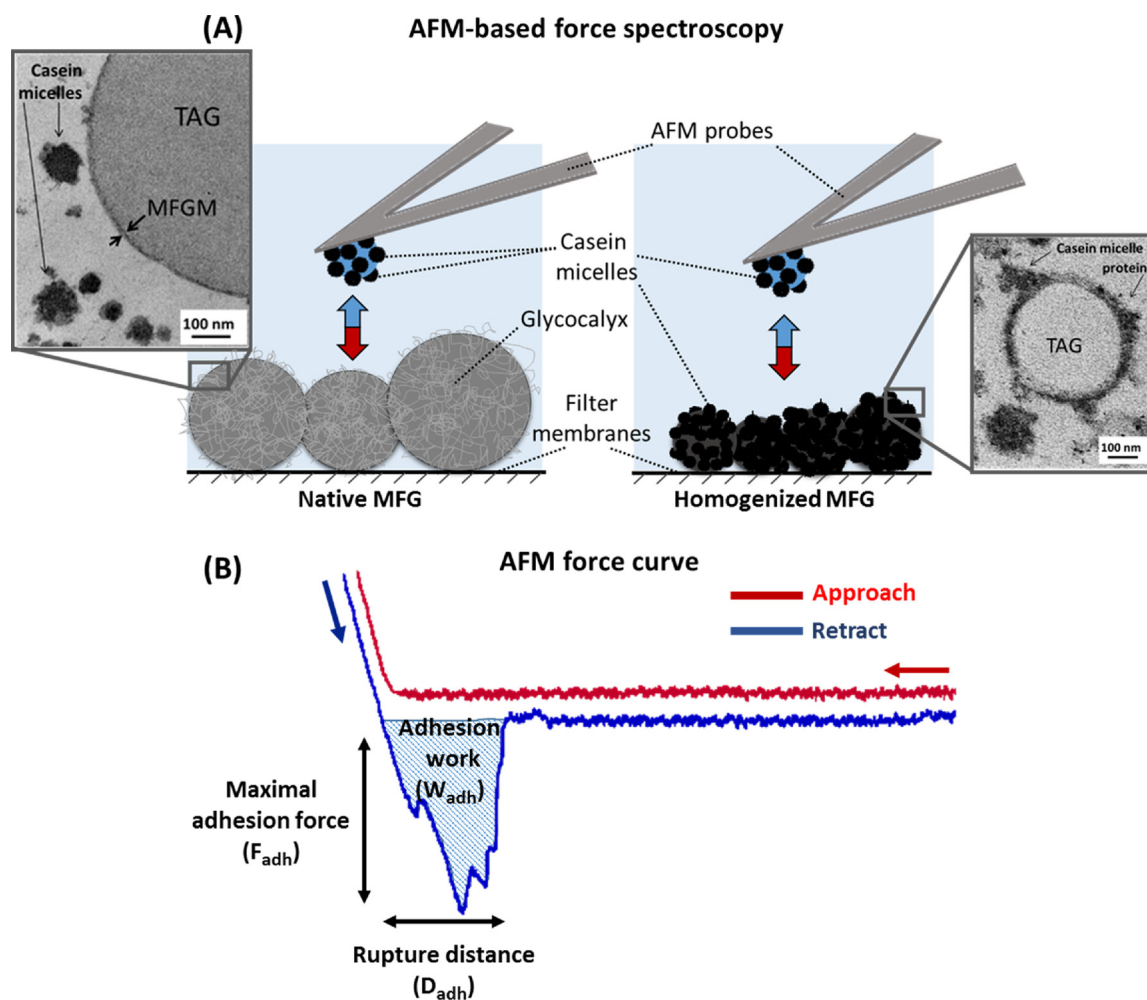


Fig. 1. (A) Schematic illustration of the AFM-based force spectroscopy experiments, performed in PIPES buffer at 22 °C, to measure the adhesion forces between casein micelles attached to the AFM probes and milk fat globules (MFG) immobilized on filter membranes. The insets show TEM images of native or homogenized MFG. MFGM: milk fat globule membrane; TAG: triacylglycerol. (B) Example of an AFM force curve where the maximal adhesion force, the adhesion work and the rupture distance are determined from the retraction curve.

Table 1

Mode diameters of native and homogenized MFG in milk or after washing of MFG, hydrodynamic diameter of casein micelles and zeta-potential values determined at pH 6.7 or 4.5. * indicates samples with bimodal size distribution. ND: not determined due to the precipitation of caseins at acid pH. Mean values calculated from 3 independent experiments.

Samples	Diameter (μm)	Zeta-potential (mV)	
		pH 6.7	pH 4.5
Native milk	3.7 ± 0.3	-12.2 ± 0.6	ND
Homogenized milk*	0.17 ± 0.01	-16.5 ± 1.0	ND
Washed native MFG	0.6 ± 0.1	-11.8 ± 0.5	-6.6 ± 0.2
Washed homogenized MFG*	0.13 ± 0.01	-15.6 ± 0.9	-4.7 ± 0.6
Casein micelles	0.173 ± 0.002	-15.6 ± 0.9	-2.3 ± 0.5

displacement of a part of casein micelles from the aqueous phase of milk to the surface of MFG after homogenization. The adsorption of caseins at the surface of homogenized MFG decreases the amount of micellar casein in solution, and then the amount of casein micelles able to induce the gelation. A second hypothesis is that the caseins adsorbed at the surface of homogenized MFG exhibit more negative charges toward the aqueous phase (the ζ -potential shows a higher negative value after homogenization of milk as shown in Table 1, due to the adsorption

of the caseins at the surface of MFG) that could lead to a lower pH of gelation. The final G' value of homogenized milk gel was significantly higher than that of native milk gel (16.5 ± 3.4 versus 2.4 ± 0.1 Pa, respectively; Fig. 2-B). Moreover, the yield stress of the homogenized milk gels was higher than that of the native milk gel (2.70 ± 0.04 versus 0.5 ± 0.2 Pa, respectively; not shown). Therefore, homogenized milks formed stiffer acid gels compared to native milks [36,37].

3.3. AFM imaging and adhesion force measurements

AFM topography images in buffer at pH 6.7 showed that immobilized native or homogenized MFG formed a continuous layer of juxtaposed individuals (Fig. 3-A, B). Homogenized MFG appeared smaller than native MFG, in agreement with Table 1 and Fig. 2. Increased surface roughness was also observed on homogenized MFG, in agreement with the adsorption of casein micelles [38]. The appearance of the individual MFG did not seem to be modified upon acidification to pH 4.5 (supplementary information-SI.3).

The adhesion forces (F_{adh}) were measured between the casein micelles-modified probes and the immobilized MFG. Values lower than 45 pN were regarded as noise (deep-blue pixels in Fig. 3-A, B). Native MFG samples at pH 6.7 showed the lowest percentage (27%) of force curves with significant adhesion events. In contrast, this percentage was the highest (83%) in the case of homogenized MFG at pH 4.5. In between, 61% of the force curves obtained on homogenized MFG at pH

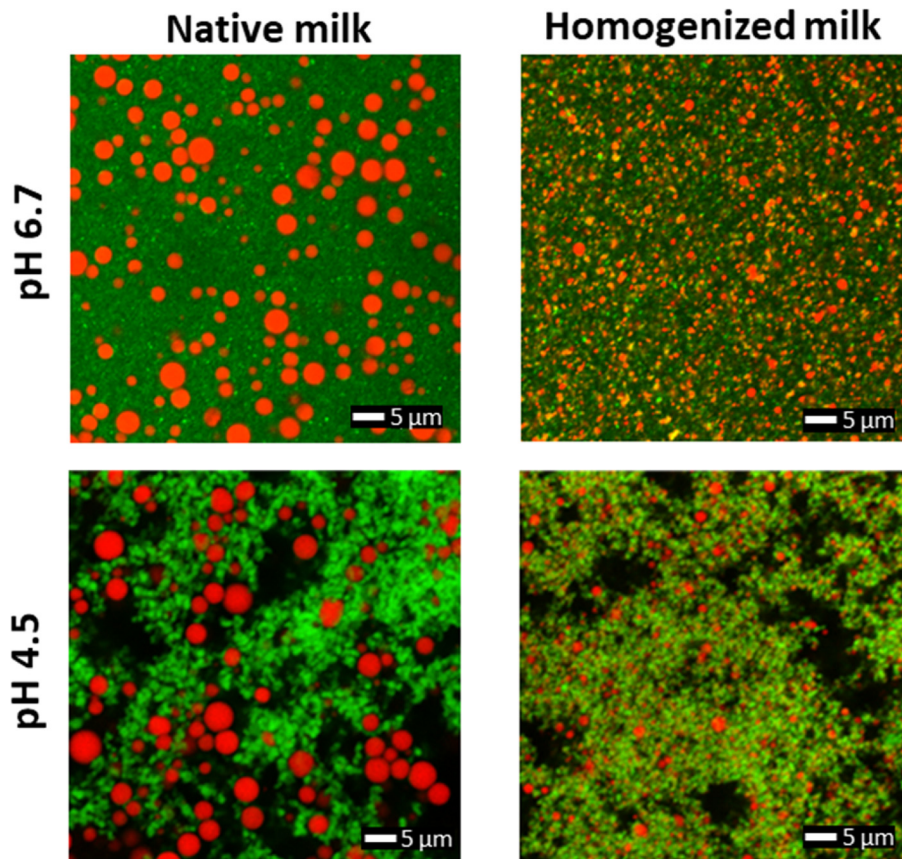
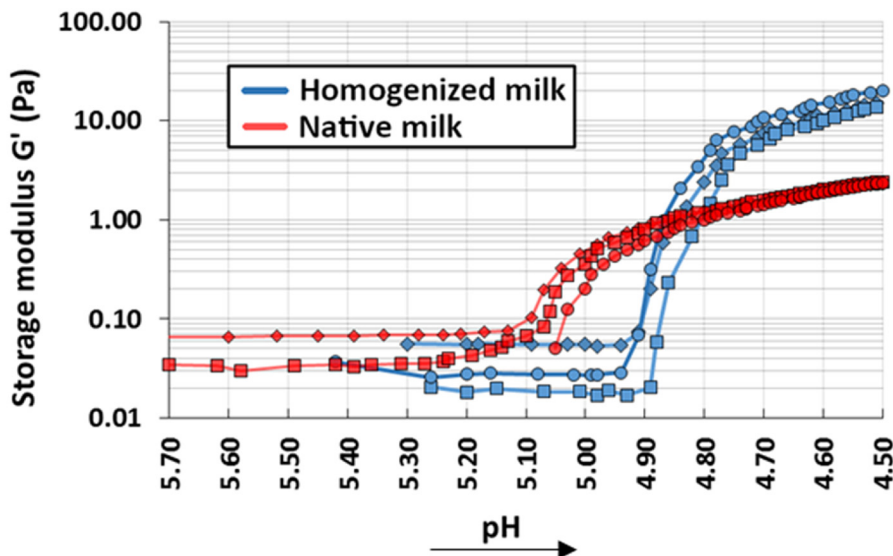
(A) Microstructure of milks (pH 6.7) and gels (pH 4.5)**(B) Rheological properties as a function of pH**

Fig. 2. (A) Confocal microscopy images of native (left) or homogenized (right) milk at pH 6.7 (top) and 4.5 (bottom). Proteins are in green and triacylglycerides of the MFG are in red. Serum pockets appear in black in the gels. (B) Evolution of the storage modulus (G') as a function of pH in native (red) or homogenized (blue) milks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

6.7 or on native MFG at pH 4.5 exhibited significant adhesion events. The Worm-like chain (WLC) model, that describes the mechanical stretching response of polypeptides under a pulling force [39], fitted the adhesion events visible on the retraction force curves (Fig. 3-A, B, bottom images) with satisfactory χ^2 values in the $2 \cdot 10^{-3}$ to 10^{-2} range.

Although the WLC model is adapted to study the stretching of single molecules, it provides supplementary insights on the integrity and flexibility of molecules during multiple interactions which is likely to occur when using colloidal probes [23]. Adhesion events were therefore the signature of the stretching of polypeptides molecules, i.e. the casein

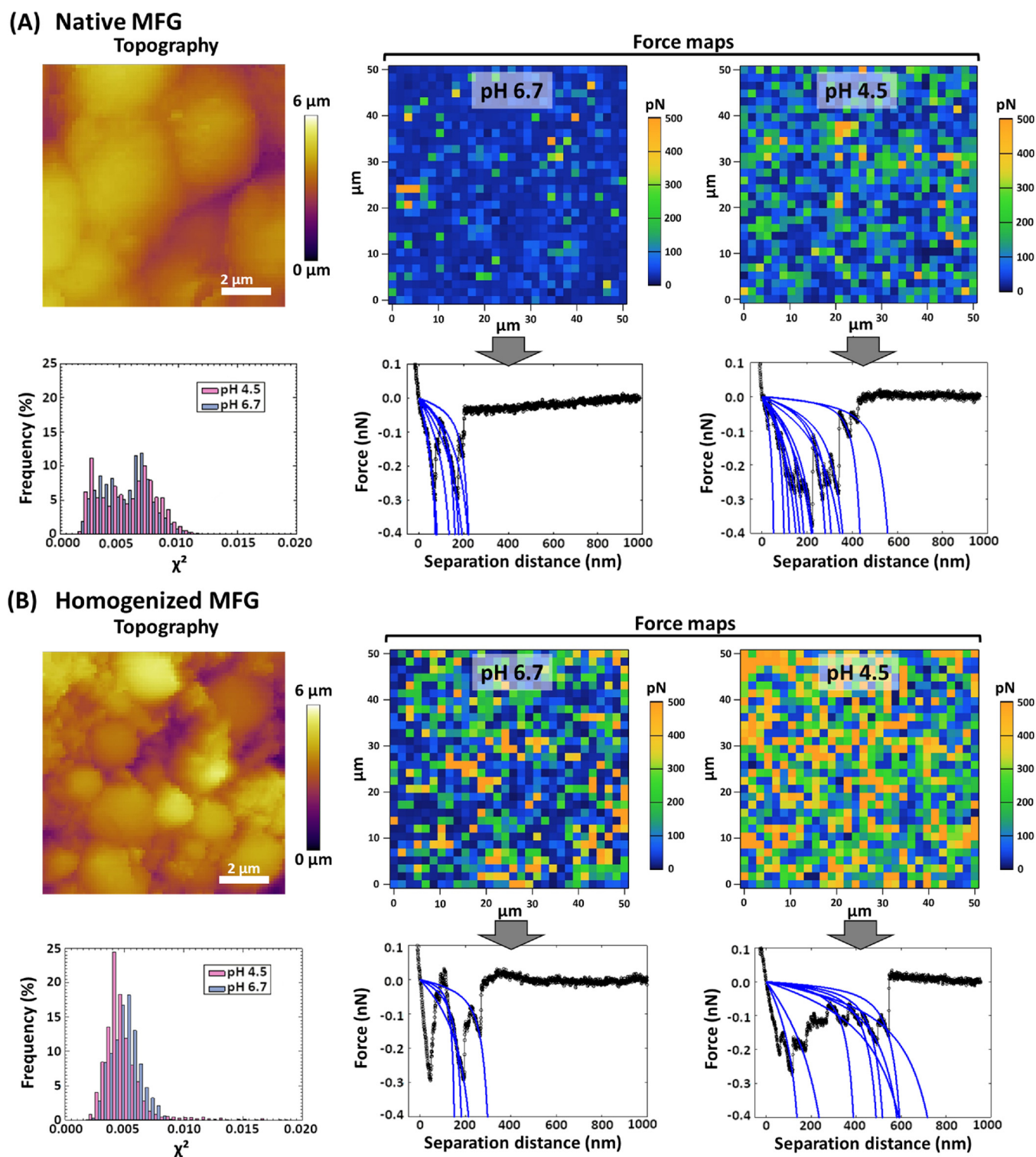


Fig. 3. Force spectroscopy measurement of the adhesion between casein micelles and (A) native or (B) homogenized MFG. Top-left images for each panel: AFM topography images of immobilized MFG. Top-middle and right images: Adhesion force maps recorded on (A) native or (B) homogenized MFG, using casein-micelles modified probes, at pH 6.7 (middle images) or 4.5 (right images). Bottom-middle and right images: examples of retraction force curves fitted with the WLC model ($n = 900$). The distributions of χ^2 values are shown for each experimental condition (A & B, bottom-left images). All experiments were performed at 22 °C in PIPES buffer. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

micelles. The contour length (L_c), indicative of the maximal elongation of the polymer, did not significantly vary with pH, with values in the 50–400 nm range. This highlighted that the primary structure of polypeptide chains (i.e. the casein molecules) was unaltered by acidification. For native MFG, there was no pH effect on the persistence length

(l_p) of the pulled polypeptides, indicative of their stiffness, with values of 0.1–0.5 nm. Meanwhile, l_p slightly decreased with pH for the homogenized MFG, with more frequent values below 0.2 nm (supplementary information-SI.4). The processing of our experimental data with the WLC model was done for all events/peaks in each force curve

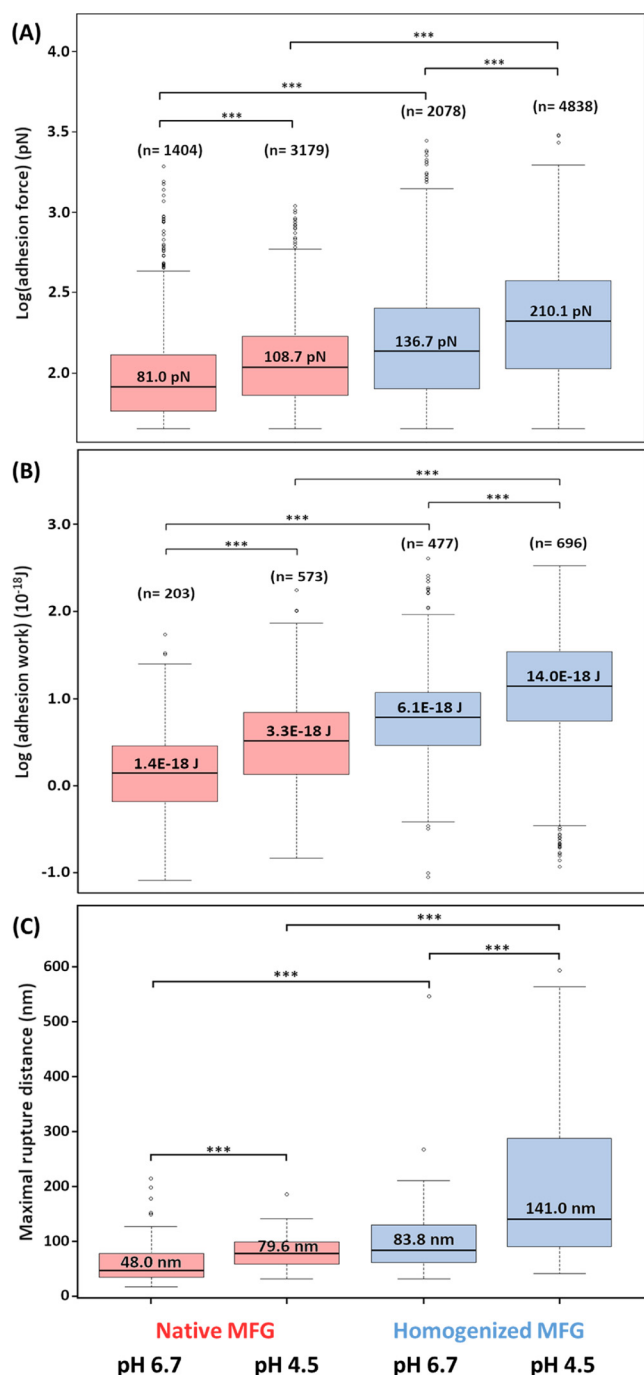


Fig. 4. Distributions of (A) the maximal adhesion forces, (B) the adhesion work and (C) the rupture distance between casein micelles and native (red) or homogenized (blue) MFG, investigated at pH 6.7 and 4.5 using AFM. The number of analyzed individuals (n) is shown for each condition in (A) and (B). In (C): $n = 60$ in each condition. The bold lines in the boxplots indicate the median values. (***) indicates $P < 0.001$, using the Wilcoxon test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

but also for the very last stretching event only that contains mainly the enthalpic contribution rather than the entropic one mainly considered in the WLC model. The data obtained from the last stretching event (adhesion force, L_{max} , l_p), exhibited similar tendencies as function of pH and homogenization as those reported for all the events/peaks (L_c , l_p and F_{adh} – shown in supplementary information SI-4).

The results found in this study highlighted that casein molecules

were more flexible at pH 4.5. This effect is more visible when caseins are present on both sides of the interaction (i.e. in homogenized MFG) than when the bulky glycocalyx is involved (i.e. in native MFG). Concomitantly, an increase of F_{adh} was measured between MFG and the casein micelles upon acidification to 4.5 (Fig. 3), which suggested that interactions between proteins became stronger [22,40].

At both pH 6.7 and 4.5, the F_{adh} values were significantly higher for the homogenized MFG than for the native MFG, as evidenced by the increased area of green or orange pixels in the corresponding force maps (compare Fig. 3-B to 3-A). At pH 6.7, the median F_{adh} was 137 pN for the homogenized MFG, against 81 pN for the native MFG (Fig. 4-A). At pH 4.5, the F_{adh} values for homogenized MFG were also significantly higher than for native MFG, with median values of 210 pN and 109 pN, respectively. Furthermore, these results showed that the F_{adh} values between casein micelles and the MFG were significantly higher at pH 4.5 than at pH 6.7 ($P < 0.001$). The enhancement of adhesion magnitude when pH is decreased should be related to stronger attractive interactions and/or to less electrostatic repulsive forces (Table 1) between the components present at the surface of MFG (MFGM in native MFG or caseins in homogenized MFG) and the casein micelles grafted on the AFM probe [22,40].

The adhesion work (W_{adh}) and the rupture distance (D_{adh}) showed the same tendencies as F_{adh} (Fig. 4-B, C) and as the frequency of adhesion events. Lowest median values were obtained upon interaction of casein micelles with native MFG at pH 6.7, with $W_{adh} = 1 \times 10^{-18}$ J and $D_{adh} = 48$ nm. Highest values were recorded upon interaction of casein micelles with homogenized MFG at pH 4.5, with $W_{adh} = 14 \times 10^{-18}$ J and $D_{adh} = 141$ nm. In between, homogenized MFG at pH 6.7 showed median $W_{adh} = 6 \times 10^{-18}$ J and $D_{adh} = 84$ nm, while native MFG at pH 4.5 showed median $W_{adh} = 3 \times 10^{-18}$ J and $D_{adh} = 80$ nm.

Altogether, AFM measurements clearly showed that more force, more energy and longer pulling distance are required to detach casein micelles from homogenized MFG than from native MFG. Moreover, this is all the truer when pH is set at 4.5, the isoelectric pH of casein micelles.

3.4. Changes in adhesion forces between lipid droplets and the protein matrix impact the microstructure and the rheological properties

Homogenization is a common technological process used in dairy industries that alters the structure and surface properties of MFG and their behavior as a function of pH. The multiscale approach used in this study allowed to increase knowledge of the adhesion forces involved at the nanoscale that govern the lipid droplet – matrix interactions, the microstructure and the rheological properties, as discussed below from the colloidal particle to the bulk behavior (Fig. 5).

At pH of 6.7, native MFG and casein micelles are both negatively charged (Table 1). Furthermore, native MFG exhibit a stabilizing glycocalyx whereas casein micelles exhibit a stabilizing brush of κ -casein [3,10]. Electrostatic and steric repulsions thus prevent contact between native MFG and casein micelles (Fig. 2-A & 5-A). Moreover, casein micelles do not spontaneously interact with the polar lipids located in the biological membrane surrounding native MFG [8]. This is explained by i) the repulsions due to the presence of anionic polar lipids (PI and PS) in the fluid matrix of the MFGM, and ii) the condensed state (low intermolecular distances) of the ordered lipid domains in the gel or liquid-ordered phases that are rich in zwitterionic polar lipids, mainly milk sphingomyelin [3,5,29,8]. In the case of homogenized MFG, steric hindrance at the vicinity of the MFG is lower as less or no glycocalyx is longer present due to the replacement of the MFGM by casein micelles upon homogenization (Fig. 1; Fig. 5-B). Furthermore, caseins exhibit a more flexible structure than that of the highly structured MFGM glycoproteins, since they have low secondary and tertiary structures [41]. Hence, when casein micelles (i.e. those attached to the AFM probe and those covering the homogenized MFG) are forced into contact during

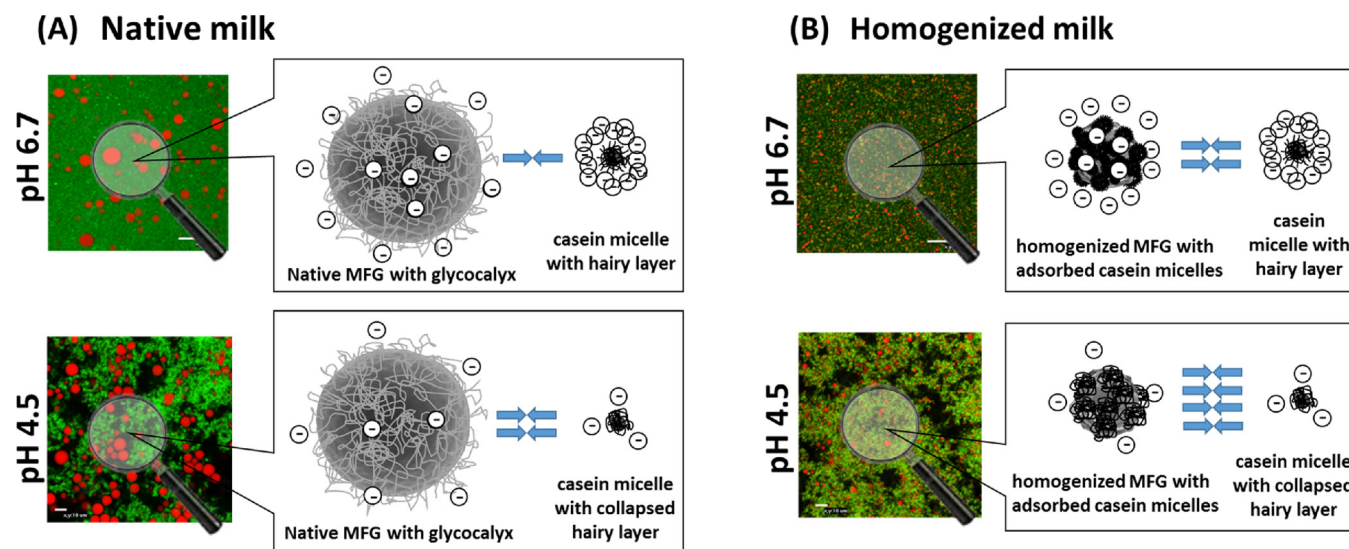


Fig. 5. Multiscale interactions between MFG and casein micelles depending on the MFG's surface properties. Schematic representation of the interactions between casein micelles and (A) native or (B) homogenized MFG, at pH 6.7 (top) and pH 4.5 (bottom). The number of negative symbols is proportional to the respective ζ -potential of objects. The number of arrows is proportional to the adhesion force between objects as determined by AFM. CLSM images show the proteins (in green) and the MFG (in red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

AFM measurements, hydrogen bonds and hydrophobic interactions can be involved, as in the micellar assembly [6]. Therefore, at pH 6.7, the adhesion was higher for homogenized MFG compared to native MFG.

Under acidic conditions (pH 4.5), the electrostatic repulsions between casein micelles and MFG decrease, in agreement with the decrease in the ζ -potential values (Table 1), and the κ -casein "hairy-layer" collapses [34,42]. These changes in the surface properties of both MFG and the casein micelles accounted for the increased adhesion at pH 4.5 compared to pH 6.7 (Fig. 3 & 4). However, the steric effect of the glycocalyx prevents native MFG from establishing strong hydrophobic interactions with casein micelles at pH 4.5 [5]. Consequently, adhesion between casein micelles and MFG is greater for homogenized MFG than for native MFG no matter the pH value. Also, the D_{adh} measured by AFM was higher in the case of homogenized MFG since casein micelles are present on both sides of the interaction.

The AFM-based force measurements recorded at the nanoscale account for the inert vs active filler behavior upon gelation of native vs homogenized MFG, respectively (Fig. 2-A). They were in good agreement with the acid gelation behaviors of native or homogenized milk measured at the macroscale, since higher adhesion forces between homogenized MFG and caseins were related to stiffer acid gels compared to native MFG (Fig. 2-B).

4. Conclusion

This study showed that the interfacial properties of MFG impact their interactions with the surrounding caseins which govern the bulk microstructure and rheological properties. Homogenized MFG were able to establish higher adhesion forces with casein micelles, thanks to the caseins adsorbed at their TAG/water interface, especially at pH 4.5 near the isoelectric pH of casein micelles. Thus, in contrast to native MFG, homogenized MFG interacted with the casein network formed upon acidification, acting as active fillers and increasing the stiffness of milk acid gels. In this study, AFM has proved a valuable technique to examine, at the nanoscale, the adhesion between MFG and casein micelles. To the authors' knowledge, this is an original application to food colloids of the correlation between individual adhesion force and bulk elasticity. This study therefore opens perspectives for the closer evaluation of the individual colloid-colloid interactions in relationship with the bulk rheological and physical properties of gels.

Acknowledgements

Sameh Obeid's postdoctoral fellowship was funded by CNIEL (French Dairy Interbranch Organization, Paris, France) in the Moon project (scientific coord. C. Lopez, INRA). The authors thank all the members of CNIEL's committee on dairy lipids for stimulating discussions. Maryvonne Pasco and Jordane Ossemond (INRA-STLO, Rennes) are acknowledged for their contribution in gel-electrophoresis analysis of MFG surface proteins and CLSM observations of AFM tips, respectively. The authors thank Florence Rousseau (INRA-STLO, Rennes) for help in particle size and zeta potential measurements. Christelle Lopez warmly thanks Mr and Mrs Denoual from Gaec des Fougères for providing milk samples. TEM images were done at the Microscopy Rennes Imaging Center (University Rennes 1, France). The MFP3D-BIO atomic force microscope was funded by the FEDER, MESRI, INRA, Conseil Général 35 and Rennes Métropole.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.colsurfb.2019.110363>.

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