Investigation of particle dynamics in gels involving casein micelles: A diffusing wave spectroscopy and rheology approach

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Abstract

The different physical properties observed by rheology and light scattering have been studied in detail for three different gelling systems, namely acidified heated skim milk, heated skim milk plus κ-carrageenan and heated skim milk plus agar. The use of diffusing wave spectroscopy (DWS) permitted the study of undiluted mixtures of skim milk and polysaccharides. The results of this investigation show that, for the particular sample conditions used in this study, rheological measurements find the G' value of the agar plus milk system to be the largest while placing the acidified milk system as the weakest gel. DWS, in contrast, shows a fluid system for the agar plus milk case but greatly restricted dynamics in the acidified milk system. This work is not concerned with the strengths of the gels per se, but rather with the relation between the gel macrorheology and the dynamics of the particles in the gel. These results demonstrate the differences in the basic physical principles between rheology, which follows the viscoelastic response of system to an applied stress, and DWS which follows the short-term dynamics of a scattering particle. The results obtained here highlight the importance of a multi-technique approach when studying complex systems.
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1. Introduction

The interactions between proteins and polysaccharides have been extensively studied and their understanding is important in the food industry for a number of reasons. Primarily, polysaccharides are used as stabilizing agents and texture modifiers. They can be added to thicken the dispersion medium and slow down creaming or aggregation, or they can be added to form a self-sustaining gel to keep ingredients in dispersion (De Vries, 2002; Grindrod & Nickerson, 1986; Xu, Stanley, Goff, & LeMaguer, 1992). The physical and sensorial characteristics of the final products are largely determined by the type of polysaccharide and also by the specific interactions present, if any, between polysaccharides and the emulsion droplets or casein micelles. The determination of these interactions is not trivial and although it has been investigated exten-

sively, it still remains elusive, primarily due to the difficulty of studying interactions in a usually turbid and concentrated system. As is often the case, most experimental methods such as rheology, microscopy or chromatography can be rather invasive, requiring modification of states which are sometimes critical for the interaction itself (such as destruction of weak gels, dilution or drying).

In this work, we look at three different colloidal gelling systems in which the same colloidal particles play different roles in the final gel. The first system of study is acidification of heated skim milk in which the gels themselves are made up of the casein particles. The second system is κ-carrageenan and heated skim milk, where the negatively charged κ-carrageenan can attach to the surfaces of the casein micelles via specific attractive interactions with a positively charged region of the κ-casein (Snoeren, Payens, Jeunink, & Both, 1975) and the ensuing gel incorporates both polysaccharide and casein micelles in its structure. The third system is made up of agar and heated skim milk. In this case, the agar has little or no

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affinity to the milk proteins, so the final gel will mostly be made up of polysaccharide with a few casein micelles playing an active part in the structure. We use a combination of techniques, namely diffusing wave spectroscopy (DWS), rheology and scanning electron microscopy (SEM) to study the differences between these systems.

1.1. Acid gelation of milk

Skimmed cows' milk is a suspension of casein micelles dispersed in serum. These particles are large protein complexes containing about 80% of the protein in the milk and taking up about 10% of the volume. They are colloidal particles with mean radii of about 100 nm that are at least quasi-stable under a wide range of temperatures and pH values above 5.5 (Holt, 1992).

The acidification of milk is a classical colloidal destabilization which results in the formation of a gel. In solution, the casein micelles are stabilized mainly by steric and electrostatic mechanisms (de Kruif & Holt, 2003). They are surrounded by a "hairy" layer of κ-casein extending several nanometres into solution from the micellar surface (Dalgleish & Holt, 1988; de Kruif & Zhulina, 1996; Walstra, Bloomfield, Wei, & Jenness, 1981). These slightly negatively charged structures (ζ-potential of around -20 mV) (Dalgleish, 1984) sterically stabilize the casein micelles. The removal or collapse of this layer by enzymatic reaction or acidification causes destabilization of the casein micelles. Heating of the milk causes denaturation of the milk serum proteins and their subsequent interaction with the micellar surface (Lucey & Singh, 1998; Lucey, Teo, Munro, & Singh, 1997; Vasbinder, Alting, & de Kruif, 2003) as well as the formation of soluble whey protein/κ-casein complexes. During acidification of heated milk, the modified casein micelles interact with each other as well as with the soluble complexes to form aggregates, though the exact mechanism of gel formation is still not fully understood. Eventually a space-filling gel develops (Alexander, Donato, & Dalgleish, 2007; Horne, 2003; Lucey et al., 1997).

1.2. Carrageenan interactions with casein micelles

Carrageenans are algal polysaccharides extracted from red seaweed, consisting of partly sulphated galactose/anhydrogalactose chains. κ-carrageenan contains three sulphate groups per disaccharide repeating unit (Chronakis, Doublier, & Piculell, 2000). Its practical utility derives mainly from its ability to form cold-setting, reversible gels in aqueous environments, especially in the presence of cations such as K$^+$ and Ca^{2+} (Doyle, Giannouli, Philip, & Morris, 2000). Carrageenans must undergo conformational ordering as a condition for gelation (Hermansson, 1989; Hermansson, Eriksson, & Jordansson, 1991). The gels are thermally reversible and exhibit hysteresis. At high temperature (above 50 °C), the κ-carrageenan molecules exist in an unordered form, but as they cool, they first adopt a helical conformation and then form gels. Experimental evidence shows that the ordered conformation of κ-carrageenan in solution and gels is that of a double helix formation (Rees, Steele, & Williamson, 1969). κ-carrageenan aggregates consist of "bundles" of aligned helices (Piculell, Hakansson, & Nilsson, 1987). The formation of a network of linear polymers requires both association and branching. The double helix formation provides a mechanism for association. Rees and Welsh (1977) have further suggested that the branching is caused by kinks in the chain which inhibit helix formation and therefore force the individual chains to combine with more than one partner.

In milk, κ-carrageenan can cause gelation by complexing with the casein micelles. Unlike agar, carrageenan has high milk specificity (Towle, 1973). It interacts with the casein micelles under specific conditions of temperature, ionic strength and concentration (Hemar, Hall, Munro, & Singh, 2002; Puvanenthiran, Goddard, McKinnon, & Augustin, 2003), despite the fact that both κ-carrageenan and casein micelles are negatively charged at the natural pH of milk (Snoeren et al., 1975). Increases in the diameters of casein micelles in the presence of κ-carrageenan have been interpreted as being a result of the binding of the polysaccharide to the micelles (Spagnuolo, Dalgleish, Goff, & Morris, 2005), and direct evidence for the formation of micelle/polysaccharide networks has been demonstrated by electron microscopy (Martin, Goff, Smith, & Dalgleish, 2006; Spagnuolo et al., 2005).

1.3. Agar interactions with casein micelles

Agar is also an algal polysaccharide, with a main structure of repeating units of D-galactose and 3-6 anhydro-L-galactose. It is composed of two major fractions, namely agarose which is a neutral gelling polymer, and agaropectin which is a non-gelling fraction (Araki, 1956) and is sometimes sulphated, the relative abundance of each being dependent on the original raw material and the method of extraction (Araki, 1966). Agar has few anionic sites and does not interact strongly with proteins (Selby & Wynne, 1973).

Agar is strongly hydrophilic and requires to be heated to dissolve into aqueous solution. Its melting point is usually around 85 °C or higher and when cooled it sets into a gel. The setting temperature is much lower than the melting temperature (Selby & Wynne, 1973).

The gelation of agar in milk involves only the agarose fraction. Gelling occurs via association of a double helix formation interacting via hydrogen bonds (Rees, 1969) much like κ-carrageenan. Due to this unique gelling ability, agar gels are fully reversible. Furthermore, agar gels are fairly open structures with a large mesh size which can span from a few nanometres to a few micrometres depending on agar concentration and can hold a large amount of unbound water which can move freely throughout the network. Globular proteins of 30×10^6

Da, for example, can traverse a 2% agarose gel (Rees, 1969).

1.4. DWS theory

An in-depth treatment of DWS theory can be found in Alexander and Dalgleish (2004, 2006). In general terms, DWS treats the scattering of light in a turbid medium as a diffusive process. Just as dynamic light scattering, DWS detects and measures the intensity fluctuations of a speckle

where T and T_0 are the intensity of the scattered and the laser light respectively and L is the sample thickness. An exhaustive treatment of the theory can be found elsewhere (Alexander & Dalgleish, 2004).

The MSD can be calculated from the DWS measured correlation functions and fitted to Eq. (2) and inputting the sample's $l*$, where $<\Delta r^2(\tau)>(m^2)$ is calculated, *point by point in time* using the relation:

$$\langle \Delta r^2(\tau) \rangle = \frac{6}{k_0^2} \frac{t}{\tau}. \tag{4}$$

$$g_{(2)}(t) \approx \left[\frac{\left(\frac{L}{l*}+\frac{4}{3}\right)\sqrt{k_0^2 \langle \Delta r^2(\tau) \rangle}}{\left(1+\frac{4}{9}k_0^2 \langle \Delta r^2(\tau) \rangle\right)\sinh\left[\frac{L}{l*}\sqrt{k_0^2 \langle \Delta r^2(\tau) \rangle}\right]+\frac{4}{3}\sqrt{k_0^2 \langle \Delta r^2(\tau) \rangle}\cosh\left[\frac{L}{l*}\sqrt{k_0^2 \langle \Delta r^2(\tau) \rangle}\right]} \right]^2. \tag{5}$$

of scattered light. Because each scattering event along the photon path is responsible for the cumulative randomization of the scattered light, we can follow the average particle dynamics at very short length scales, much shorter than the wavelength of the laser light. As a result, each scattering particle needs only to move a relatively small amount, on the order of a few tens of nanometres, to affect the DWS signal. This ability to follow fast dynamics enables DWS to measure the time dependence of the mean square displacement (MSD) of the scattering particles.

All the measurements in this work were carried out in the transmission mode, where all the photons must traverse the whole length of the sample, L, to reach the detector. The field autocorrelation function $g_1(\tau)$ is related to the intensity autocorrelation function via the Siegert relation:

$$g_{(2)}(t) = 1 + |g_{(1)}(t)|^2. \tag{1}$$

In this work, the sample thickness $L \gg l*$ (see below); therefore, the field autocorrelation function can be quite accurately described by (Weitz & Pine, 1993)

$$g_{(1)}(t) \approx \frac{\left(\frac{L}{l*}+\frac{4}{3}\right)\sqrt{\frac{6t}{\tau}}}{\left(1+\frac{8t}{3\tau}\right)\sinh\left[\frac{L}{l*}\sqrt{\frac{6t}{\tau}}\right]+\frac{4}{3}\sqrt{\frac{6t}{\tau}}\cosh\left[\frac{L}{l*}\sqrt{\frac{6t}{\tau}}\right]}, \tag{2}$$

where $\tau = (Dk_0^2)^{-1}$, D is the particle diffusion coefficient and $k_0 = 2\pi/\lambda$ is the wave vector of the light. The other important parameter in DWS is known as $l*$, the photon transport mean free path length. This is the length scale over which the direction of light is randomized and it is directly proportional to the turbidity of the system. It can be measured by transmission measurements with respect to a well-defined model colloidal suspension with known $l*$ value as follows:

$$\frac{T}{T_0} = \frac{5l*/3L}{1+4l*/3L}, \tag{3}$$

This would yield a functional description of $g_2(t)$ as a function of MSD of the form:

Romer, Scheffold, and Schurtenberger (2000), have shown that, when a high-density colloidal gel is formed and the network spans over the whole sample chamber, the average MSD can be described for all τ by a stretched exponential of the form:

$$\langle \Delta r^2(\tau) \rangle = \delta^2 \left[1 - e^{-(\tau/\tau_c)^p} \right], \tag{6}$$

where δ^2 is the plateau height and related to the distance that an individual particle can move away from its average position and τ_c is the characteristic decay time which determines the spectrum of excited modes in the gel. The parameter p is the exponent related to diffusion, which becomes 1 for freely diffusing particles and drops to 0.7 for fully fractal gels. This equation was first derived by Krall and Weitz (1998) for low-volume-fraction colloidal gels, who, using DLS, proved that the extended exponential shown in Eq. (6) can be used both for ergodic and non-ergodic systems.

2. Materials and methods

Fresh milk was collected from the Ponsonby Research Station of the University of Guelph (Guelph, Ontario) and sodium azide (0.02% w/v) was immediately added to prevent bacterial growth. The raw milk was skimmed at $6000g$ for 20 min at 5 °C in a Beckman-Coulter centrifuge (model J2-21, Beckman Coulter, Mississauga, Ontario, Canada) and filtered three times through Whatman glass fiber filters (Fisher Scientific, Mississauga, Canada) and stored at 4 °C until required.

2.1. Heat treatments and acidification

Skimmed milk was heated in 15 ml tubes placed in a water bath at 85 °C, allowing 2–3 min for the samples to reach the final temperature, and then leaving for 10 min before removing and cooling rapidly to room temperature

in an ice bath. The milk was then stored in a refrigerator at 4 °C until further use. For acidification, 1.0% (w/w) of glucono-δ-lactone (GDL) was added to the milk which had been preheated to 30 °C. The mixture was agitated for 1 min to dissolve the acidulant. The sample for DWS or rheological measurements was placed in the measuring equipment. The rest of the sample was used for monitoring of the pH, which was followed continuously until the pH had decreased to below 4.8. To prepare samples for SEM, after the addition of GDL the milk was poured into Petri dishes and immediately an activated carbon planchet (see below) was immersed into the sample.

2.2. Mixing of polysaccharide with milk

Different amounts of κ-carrageenan (ex Kappaphycus alvarezii (cottonii), Danisco, Germantown International Ltd., USA) or agar (Sigma-Aldrich, St Louis, MO, USA) were added to the skimmed milk to achieve final concentrations ranging from 0.005% to 0.1% (w/w) for κ-carrageenan and from 0.01% to 1.0% (w/w) for agar. The polysaccharides were added in powder form to defined volumes of fresh skim milk and the mixtures were stirred at room temperature for approximately 5 min. These mixtures were then heated to 85 °C in a water bath and held for 10 min (while stirring), after which they were transferred directly to the cuvettes of the DWS equipment and held at 25 °C until the measurement (between 30 and 50 min later). All samples were previously checked for time stability as explained in Alexander and Dalgleish (2007). For rheological measurements, the sample cylinder was kept at 85 °C while the samples were poured in. Subsequently, the temperature was ramped down at 3 °C per minute until it reached 25 °C to start the measurements. Samples for the SEM (final concentrations of 0.2% and 0.5% (w/w) for agar and 0.02% and 0.05% (w/w) for κ-carrageenan) were poured into Petri dishes and cooled to 25 °C. All runs were done in duplicate or triplicate.

2.3. Rheology

A controlled-stress rheometer (AR 2000, TA instruments) equipped with a Peltier temperature controller with a Couette device consisting of two concentric cylinders of diameters 30 and 28 mm was used. For the acid gelation of milk experiment, time sweep oscillatory measurements were performed at a frequency of 0.1 Hz with a stress of 0.02 Pa. For the milk + polysaccharide experiments, a frequency sweep from 10 to 0.01 Hz was performed with the same controlled stress of 0.02 Pa. The rheological behaviour of the systems was also checked to ensure stability throughout the duration of a DWS measurement.

2.4. Scanning electron microscopy (SEM)

Samples for SEM were prepared as described by Martin et al. (2006), but some changes were made. Clean and dry

polished carbon planchets (diameter 12.7 mm; Canemco-Marivac, Canada) were sputter coated with thickness of ∼35 nm of Au/Pd (ratio 60:40) (using an Emitech K550, Ashford, Kent, UK), directly immersed into a 2 mM solution of 11-mercaptoundecanoic acid in 100% ethanol and left for at least 18 h to form a self-assembled monolayer (SAM). The terminal carboxyl groups of the SAM were modified by carbodiimide chemistry using *N*-hydroxysuccinimide and *N*-ethyl-*N*-(dimethyl-aminopropyl) carbodiimide (Martin et al., 2006). The activated carbon planchet was immersed in the sample for 60 min (samples with polysaccharides) or until a defined pH was attained (acidified heated milk), then carefully removed to disturb the gels as little as possible and rinsed with 10 mM imidazole buffer (containing 1 mM Ca; at the same pH as the sample) and immersed in a 1.5% glutaraldehyde solution in 10 mM imidazole buffer at the same pH as the sample for 30 min. After this fixation process, the carbon planchet was rinsed by MilliQ water and subjected to dehydration and critical point drying. Prior to SEM, the sample was sputter coated by a thin layer (∼15 nm) of Au/Pd (60:40). The sample was inspected by Hitachi S-4500 FE-SEM (Tokyo, Japan), using an acceleration voltage of 10 kV and an emission current of 10 μA. All images were acquired digitally using Quartz PCI software (Vancouver, BC, Canada).

3. Results

Fig. 1 shows G' and G'' values at 0.1 Hz for the three systems studied in this work: (A) acidified heated skim milk (AHSM); (B) heated skim milk + κ-carrageenan (HSM + κ-carrageenan) and (C) heated skim milk + agar (HSM + agar). The gelling point for the AHSM systems, as defined by a crossover of G' and G'' (or tan δ = 1), is around pH 5.3 (see inset Fig. 1) and at the final pH of 5.2, the maximum G' attained is about 2 Pa, although the gel continues to develop as the pH deceases further (Lucey et al., 1997). In the case of HSM + κ-carrageenan, the formation of a gel (tan δ < 1) occurred at a κ-carrageenan concentration between 0.02% and 0.03%. In this case, the maximum value of G' obtained for the highest concentration of κ-carrageenan was 91 Pa. Lastly, for the HSM + agar, gelation occurred at an agar concentration of below 0.5% with the maximum G' value being 514 Pa at the agar concentration of 1.0%. For agar concentrations above 0.5%, the mixtures exhibited phase separation due to the thermodynamic incompatibility between the two components leading to the formation of a protein-rich and polysaccharide-rich phase (Bourriot, Garnier, & Doublier, 1999; Tolstoguzov, 2002). In general, it is important to note that we are not comparing HSM + κ-carrageenan gels with HSM + agar gels, as the concentration range of polysaccharides probed in this work is different for both. At this stage, it is simply necessary to note that the different samples had low G'(AHSM), low-to-moderate G'
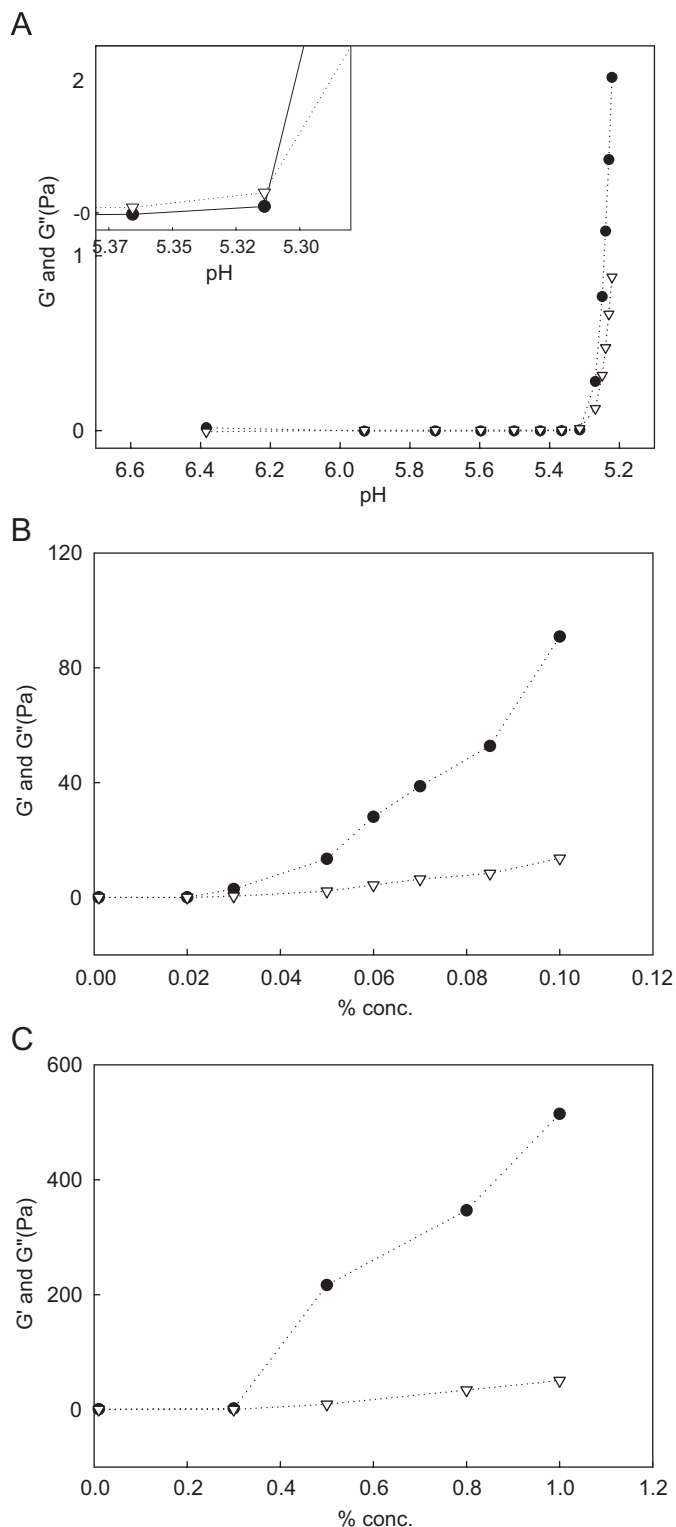
Fig. 1. Rheological data showing the storage modulus G' (closed symbols) and the loss modulus G'' (open symbols) for (A) acidified heated skim milk system as a function of pH (the inset is a detailed view of the G', G'' crossover), (B) heated skim milk system plus κ-carrageenan and (C) heated skim milk system plus agar. Plots (B) and (C) are as functions of polysaccharide concentration. The dotted lines are an aid to the eye. Note the differences in the scale of the y-axis for the three panels.
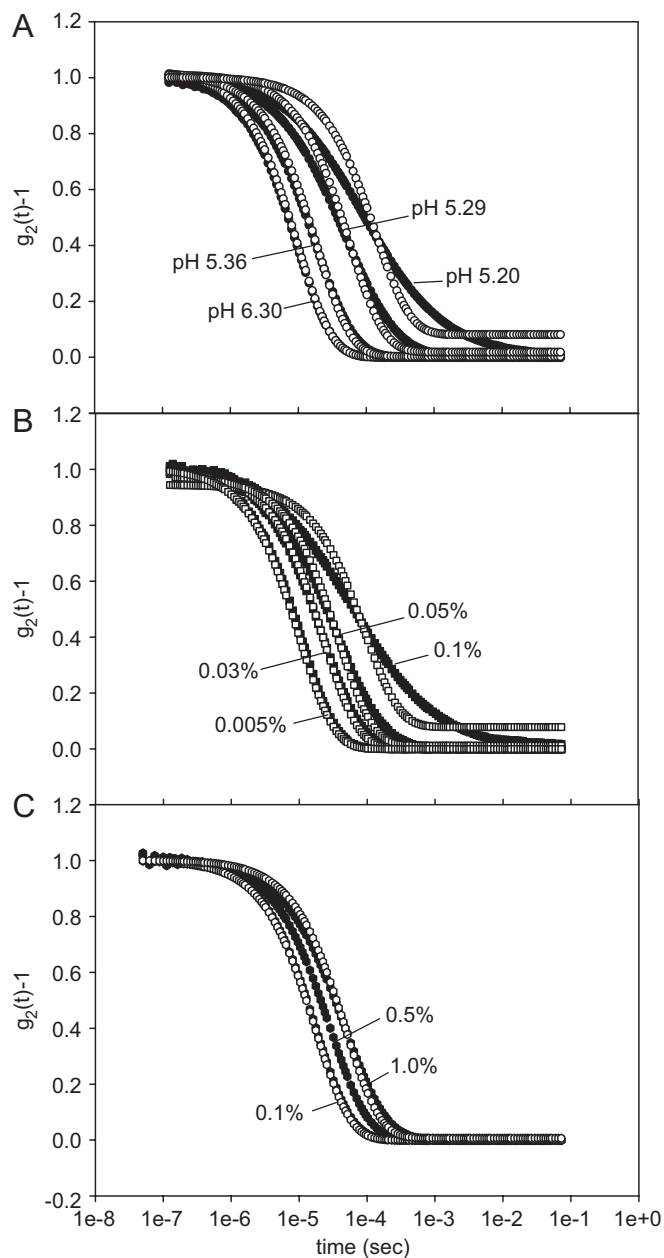

Fig. 2. DWS correlation functions for heated skim milk at different pH (A) and different concentrations of κ-carrageenan and agar (B and C respectively) (filled symbols). Superimposed are fits (open symbols) to the experimental data generated using Eq. (2). The text indicates pH or polysaccharide concentration for the selected curves.

(HSM + κ-carrageenan) and low-to-high G' (HSM + agar); these will be compared later with the results from DWS.

Fig. 2 shows selected correlation functions obtained by DWS measurements for the three systems of study. All the correlation functions obtained in this work fully decorrelate to zero at long times, indicating that the samples are fully ergodic. The superimposed curves (open symbols) are fits to the experimental data generated using Eq. (2). In all the cases, the autocorrelation functions show a slowing down of the characteristic decay time τ, evidenced by a shift of the curves to higher times as the gels are formed

and become stronger. AHSM (Fig. 2A) shows a large change in τ as a function of pH, from the pH of the milk of 6.3 where the milk is completely liquid (left-most curve), to the pH of 5.2 where the milk is already gelled (right-most curve), as measured by rheology. At high pH, the fit of the correlation function to Eq. (2) is very good. By the time the pH drops to 5.36 (second curve from left) the fit starts to deviate slightly, although it is still well within an acceptable range. However, when the pH reaches 5.29 (third correlation function from left), it is clear that Eq. (2) is no longer an appropriate fit. This is around the pH of gelation according to the rheology measurements (5.3). At pH 5.2, where rheology measurements indicate a gel strength of around 2 Pa, the fit to Eq. (2) is completely inapplicable. Thus, once the milk starts to gel, fitting with a single τ value cannot be justified (Alexander & Dalgleish, 2004).

Fig. 2B demonstrates that HSM + κ-carrageenan also shows a slowing down of the dynamics as the concentration of κ-carrageenan increases (left-most to right-most curve), albeit not as pronounced as for the case of AHSM. In this instance, up to a κ-carrageenan concentration of 0.03%, the theoretical fit to Eq. (2) works very well. This is also the concentration where the gelation point of the HSM + κ-carrageenan system is found, as measured by rheology. For the concentrations of κ-carrageenan of 0.05% and 0.1%, it can be seen that Eq. (2) is not an appropriate fit. The system clearly cannot be represented by a single exponential decay and the presence of a gel restricts the application of Eq. (2). In contrast to the mixtures containing carrageenan, HSM + agar (Fig. 2C) shows very little change in the characteristic decay time as a function of agar concentration (here again, left-most to right-most curve) and the completely gelled system does not seem to differ very much dynamically from the original, fluid one. All curves are well represented by a simple exponential decay fit, even at the final agar concentration of 1.0% where a strong gel is formed as defined by rheology ($G' = 514$ Pa). For all the milk systems shown here, the correlation functions span from fluid to gelled cases.

Fig. 3 displays the evolution of the MSD of our three systems during the different gelation processes. These values were calculated from Eq. (4) and superimposed is the fit generated by the stretched exponential shown in Eq. (6) (solid line), calculated only for the last experimental measurement (pH 5.2 for the AHSM system, 0.1% κ-carrageenan concentration for the HSM + κ-carrageenan and 1.0% concentration for the HSM + agar system). It is important to mention here that the MSDs are calculated point by point in correlation time and do not require a single fit with a constant decay time spanning the whole range of time accessible in the experiment (as those shown in Fig. 2). When a sample begins to gel, the different clusters formed within the growing gel contribute a wide span of characteristic decay times to the sample. This is the reason why Eq. (2), with a single decay time τ, ceases to be valid upon gelation. However, the correlation function can
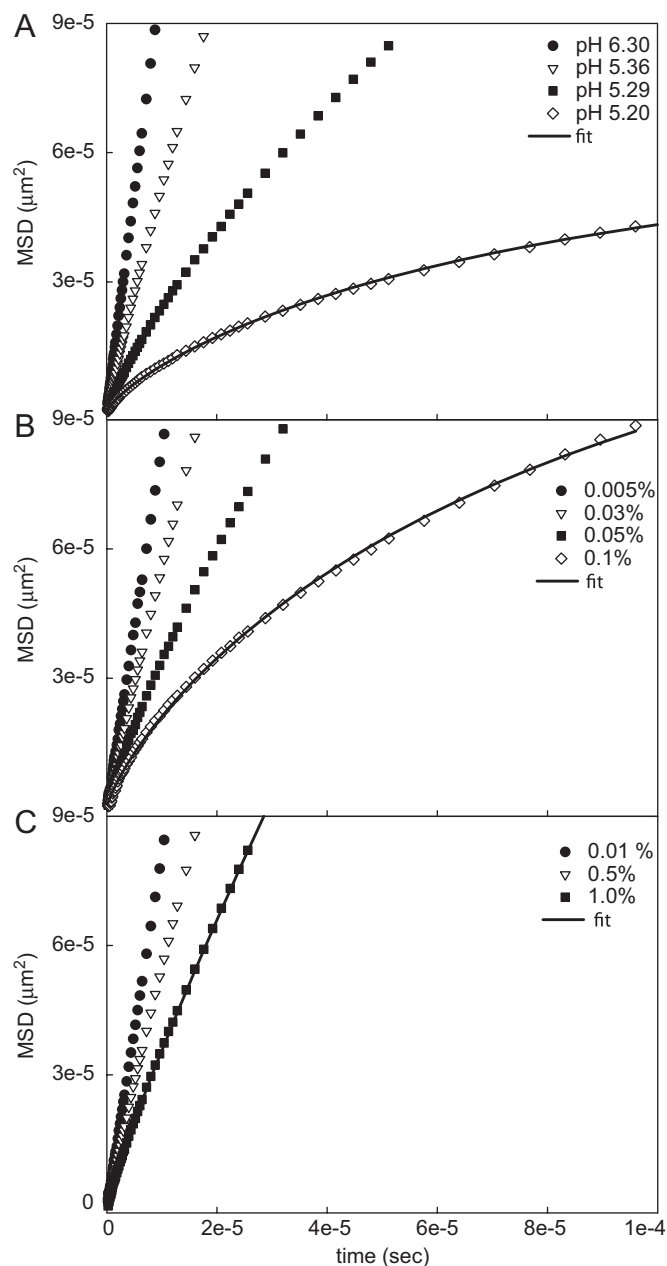

Fig. 3. Mean square displacement curves for acidified heated skim milk system (A), heated skim milk system plus κ-carrageenan (B), and heated skim milk system plus agar (C). The selected pH and concentrations are the same as shown in Fig. 2. Also shown is the fit to the last curve as generated by Eq. (6) (solid line).

still be fitted at each point with a different decay time. This is what gives rise to the MSD curves shown in Fig. 3. Thus a single correlation function can be treated to give an MSD/time plot as shown. It should be understood that the time referred to is the decay time within a correlation run, and does not mean the time from the onset of the experiment, or the formation of gels.

For the initial stages of the experiments (high pH or low concentrations of polysaccharides), the casein micelles are freely diffusive over the accessible time range of our experiments. This is manifested by the linear relation of
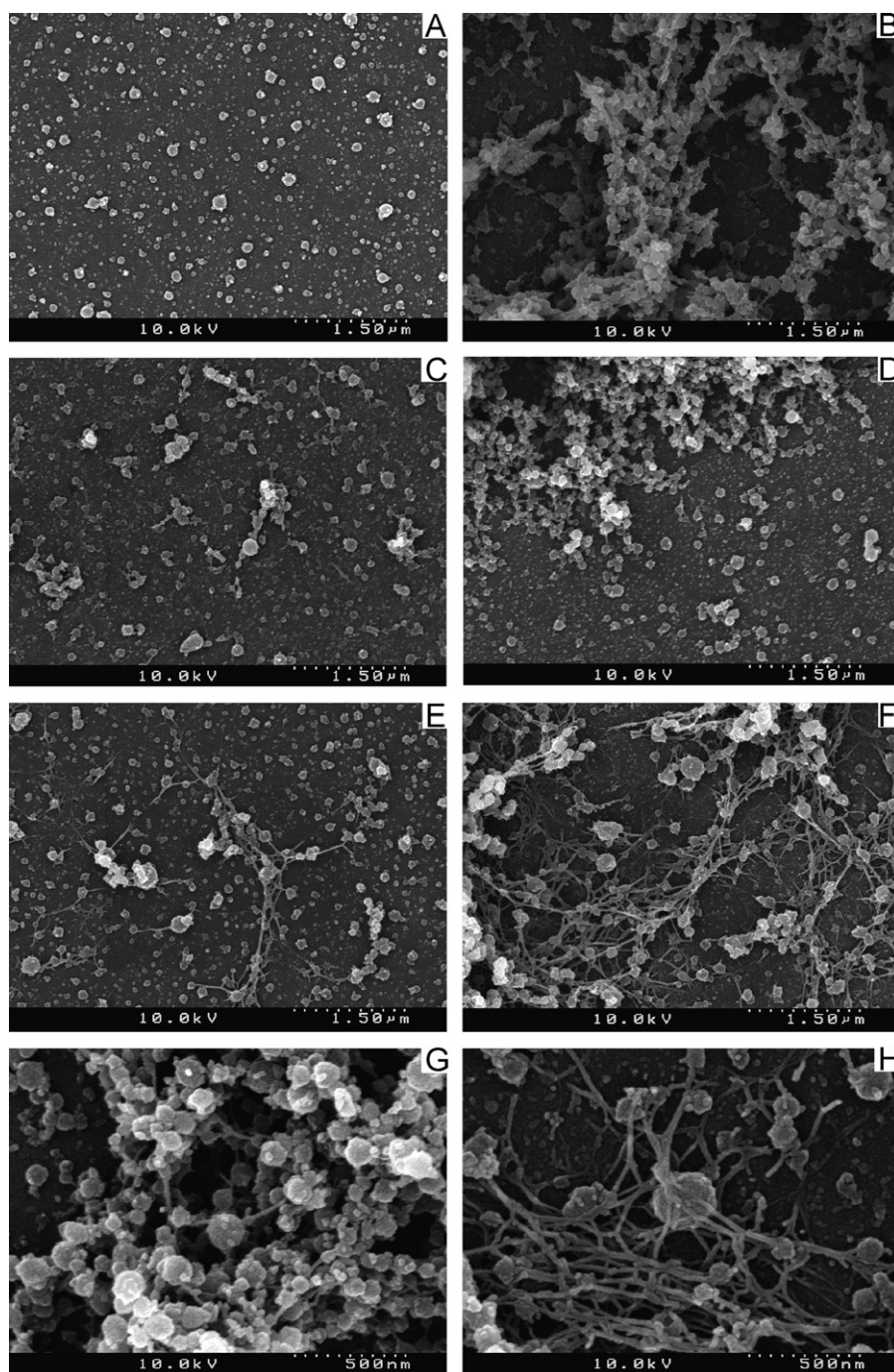
Fig. 4. Microstructure of heated skim milk at pH 6.70 (A) and acidified heated skim milk at pH 5.20 (B), heated skim milk plus 0.02% (C) and 0.05% (D) (w/w) κ-carrageenan and heated skim milk plus 0.2% (E) and 0.5% (F) (w/w) agar, respectively. (G,H) Detailed microstructure of the systems containing 0.05% and 0.5% of κ-carrageenan and agar, respectively.

the MSD with time. However, as the pH drops in the AHSM or the concentration of polysaccharide increases in the κ-carrageenan system, the plot of MSD against time is no longer linear (Figs. 3A and B). This can be interpreted as a spatial constraint in the long-time behaviour of the micelles. Nevertheless, the fits show that the systems can be well represented by a sub-diffusive model. The third system of HSM + agar does not show this arrested behaviour even at the highest agar concentration. There is a linear

dependence of MSD with time for all the concentrations of agar tested.

Fig. 4A is an electron micrograph showing the distribution of casein micelles from heated skim milk which have been linked to the substrate at pH 6.70. The casein micelles appear relatively spherical with diameters of approximately 100–150 nm. As the pH decreases during acidification, aggregates begin to form. Fig. 4B shows a micrograph of the milk system at pH 5.20 (same magnification as 4A). In

this figure, we can see groups of individual casein micelles closely packed together forming long strands of about 1.5 μm in length and 300–400 nm in diameter. These strands are also grouped together to form large aggregates. This is the beginning of the gelation phenomenon as shown by rheology and DWS.

Figs. 4C and D show SEM micrographs of mixtures of heated skim milk with 0.02% and 0.05% (w/w) κ-carrageenan, respectively. The κ-carrageenan seems to be a thin and relatively long polymer (up to 300 nm in length according to Fig. 4G which is in agreement with structure described by Spagnuolo et al. (2005)). At lower concentration (0.02%) the system forms small aggregates which are spatially distinct from each other. At higher concentration (0.05%, Fig. 4D), huge aggregates are formed. The κ-carrageenan seems to be bound onto the surface of the casein micelles, as reported by Spagnuolo et al. (2005). Carrageenan chains are observed with associated groups of caseins along their length as well as carrageenan-micellar complexes.

According to the micrographs, agar is a thin polymer with branched strands longer than κ-carrageenan. The length of an agar strand could be up to 1 μm (Fig. 4E). At low concentrations of agar (0.2% (w/w)) just a few casein micelles are involved in interactions with agar while most of them are still seen as individual micelles in the background. At higher concentrations of agar (Fig. 4F), more casein micelles appear to participate in structure formation. As shown by the micrograph, agar creates a more open structure than that produced by κ-carragenan at the concentrations probed in this work. Though some direct interactions between agar and caseins can been seen, there are relatively few casein micelles in the complexes. The casein micelles seem to be far apart from each other (compared with the types of tightly-packed network seen in AHSM and HSM + carrageenan). According to the de-tailed structure of the agar + milk system (Fig. 4H), it seems that the casein micelles are surrounded by agar polymers or trapped into the agar network compared to the system with κ-carrageenan (Fig. 4G) where it seems that the casein is more strongly interacting and therefore attached onto the network. These micrographs show very interesting differences in the detailed interaction phenomenon.

4. Discussion

In this work we have used DWS, rheology and microscopy to try to detect differences in the role of the casein micelles in the three different gels. The acid gels in milk are formed from the association of linked casein micelles and soluble complexes. It is also accepted that κ-carrageenan is a gelling polysaccharide that has high specificity for the casein micelles in milk. If cooled under its coil-helix transition, κ-carrageenan forms a gel in which the casein micelles are active components and hence part of the gel network. On the other hand, agar is also a gelling polysaccharide when cooled below its conformational transition; however, it has low milk reactivity and when gelling will incorporate very few of the casein micelles into the gel network. In this case, the casein micelles are expected to be an inert filler of an otherwise agar gel. The electron micrographs confirm these three different types of gels.

We should point out that in any light scattering experiment the scattering signal is dominated by particles having higher contrast and larger size. In our three different systems the casein micelles are the only scatterer-ing particles since whey proteins, κ-carrageenan and agar are too small and/or lacking in contrast to be detected by the light. Even under gelling conditions, DWS will detect the effect of the polysaccharide gel on the casein micelle mobility and not the polysaccharide itself. In the case of acidified milk, the scattering signal will of course be from the gel because the micelles themselves are forming it. In the case of the phase separating agar, care must be taken that we are indeed probing all domains of the sample. The values of $l*$ (not shown) for the agar system are one order of magnitude larger than the typical void in the gel as seen by the micrographs in Fig. 4F and H (around 400 vs. 2 μm). Since DWS uses the diffusive path of photons, it inherently averages over many independent volumes of a sample; so in this case, all accessible volume is being probed (Meller, Gisler, Weitz, & Stavans, 1999). Bulone and San Biagio (1995) have shown, using dynamic light scattering, that a 1.5% solution of agarose behaves like entangled polymers arranged in a mesh with a time scale longer than the diffusion of latex spheres which were incorporated in it. This allowed a continuous rearrangement of the sample which remains macroscopically liquid. As stated above, the scattering signal collected arises mostly from the casein micelles mainly due to contrast dominance. Therefore, we can conclude that as these micelles get pushed together and trapped in microdomains, they will dominate further the scattering signal collected.

For the rheological experiments shown in this work and for these specific concentrations, the HSM + agar gels are the stiffest, followed by the HSM + κ-carrageenan and lastly by the AHSM system; however, little can be said about the microstructure of the different gels due to the nature of the experimental method. If we examine the light scattering data, the DWS correlation functions (Fig. 2) show the complete opposite trend in terms of dynamics. The most arrested motion of the micelles corresponds to the AHSM system, followed by the HSM + κ-carrageenan system and lastly by the HSM + agar system. At this point, it is necessary to stress that due to the cooperative effect of all scattering events in the photon path along the sample, each particle need only move a small amount, of the order of a few tens of nanometres, to contribute to the dephasing of the scattered light wave. Therefore, we are really probing the short-time diffusion of the particles. This is quite opposite to rheometric measurements since, due to the macroscopic nature of rheology, in order to detect a

$\tan \delta = 1$, a self-supporting network must be formed, and all the components of the system are involved.

During acidification, casein micelles and denatured whey proteins form structures (Alexander & Dalgleish, 2005; Donato, Alexander, & Dalgleish, 2007; Guyomarc'h, Law, & Dalgleish, 2003). The final structure formed during acidification seems to be relatively rigid. Casein micelles are attached to each other either directly or via interaction with denatured whey proteins, and their movement is severely restricted by the fact that a casein particle will be surrounded closely by neighbours which will confine it to a fixed average position. After gelation, there is a spectrum of excitation modes as evidenced by the inapplicability of the single exponential fit. This is corroborated by the micrograph of Fig. 4B, which indeed shows a close packing and tight restriction of the caseins in the network. Calculations on the data shown by Fig. 3 show that, at a pH of 5.2, the micelles are in simple diffusive motion (initial straight part of the MSD curve) on length scales of $\sqrt{\langle \Delta r^2 \rangle} \cong 3\,\text{nm}$, after which their movement is restricted by the close neighbouring micelles, limiting their range of movement and resulting in average excursions (parameter δ in Eq. (6)) of around 8 nm. The same calculations, for the case of κ-carrageenan and milk, yield a diffusive motion of the order of 4 nm, and an average displacement of around 10–12 nm; this is an estimate since the equation works better for fully arrested systems. Though the caseins are still trapped in space, their excursions are larger than in the acidified milk by about 50%. DWS also shows a dramatic decrease in the dynamics of the micelles as the concentration of κ-carrageenan increases (Fig. 2B). There is definitely a sign of a progressing gelling mechanism as evidenced by the poor fit to Eq. (2). Though the constraint in movement is not as evident as for acidified milk, the micromolecular characteristics are those of a gel. Lastly, for the agar plus milk system, light scattering data show no sign of the formation of a gel (as judged by micellar motion) in our accessible time scales. The casein micelles barely slow down their motion in solution as indicated by the small increase in the correlation time, and from the MSD data (Fig. 3C); it can be concluded that they are mostly freely diffusive (MSD shows a linear relation between displacement and time) throughout the whole experiment. This is extremely interesting since rheology of this system shows the most elastic gel of them all. However, at the microstructural level, there is no sign of arrested motion. This can be clearly understood from Fig. 4F. Though some caseins are attached to the agar gel, most are free to move about. This micrograph shows a self-supporting network formed mainly by the agar which is what gives the gel its large stiffness as shown by rheology.

These results give us a direct measurement of the different roles played by the casein micelles in each of the three gels. In acidified milk, the caseins are themselves part of the network and have very close interactions with other caseins, which greatly restrict their movement, even when the gel is relatively inelastic. In HSM + κ-carrageenan,

though the caseins are obviously part of the gel themselves, as demonstrated by the slowing down of the particle dynamics and FE-SEM, they are much freer to move around their average positions than in the acid gel, even though the gel itself is much more elastic than the acid gel. This can be interpreted as the existence of interactions between the gelling carrageenan and the casein micelles. As the polysaccharide forms a gel, it immobilizes the caseins with it. However, as microscopy shows, these strands of polysaccharide trapping the micelles are long and from DWS data we can determine that they must be quite flexible, allowing the micelles to move semi-freely at short time scales. For HSM + agar, the results are quite surprising and really show the importance of a multi-technique approach of study. The MSD measurements show that the micelles are not severely spatially restricted. Therefore, it can be concluded that due to the low binding capacity of casein micelles to agar, the particles reside mostly freely inside agar gel pockets. As shown by microscopy, some casein micelles interact with agar but they are few in number and the attachment seems to be in a much looser form than that of acidified milk and carrageenan (Fig. 4H). It has already been established that agar gels are very hydrated and of open structure (see introduction); therefore, we can conclude that the micelles are mostly simply trapped in this gel without actually being part of it and as such, they are able to move quite freely at short time range yielding free diffusing type of dynamics.

5. Conclusions

The work presented here clearly demonstrates the different types of interactions that can be present in complex gelling systems. Although through rheology measurements we can assess the elasticity of a gel, there is no microstructural information. By studying the dynamics of the casein micelles, more information about particle–polysaccharide interactions can be extracted. In particular, we were able to detect the affinity of κ-carrageenan to casein micelles and the non-affinity of agar to them. However, this work raises a word of caution about the use of tracer particles for gelation studies, as well as the use of microrheology in multi-component complex systems. Erroneous conclusions about interactions and gel properties might be reached if only narrow time scales are probed. As we can see, DWS using casein micelles as tracer particles would not detect the formation of the strong agar gel. Lastly, DWS was capable of detecting differences in the scattered signal whether it was arising from active network particles or gel fillers. We are able to quantitatively measure the average excursion of a particle without disruption of the structure.

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