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ORIGINAL CONTRIBUTION

Diffusing wave spectroscopy investigations of acid milk gels containing pectin

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Abstract The influence of the polysaccharide pectin on the gelation of acidified milk is studied in concentrated, undiluted, quiescent systems, primarily using diffusing wave spectroscopy. For pectins with a low degree of methylesterification (DM), interactions with milk-serum calcium yielded precipitated polysaccharide aggregates, even without acidification, that subsequently did not interact with casein micelles. However, high DM fine structures do not interact significantly with serum-calcium and absorb onto casein micelles as the pH is reduced below 5. A limited surface coverage of high DM pectin facilitates efficient bridging which enhances the rate of micelle aggregation and subsequent gelation and produces a clear signature in the shape of the measured MSD. The work highlights the fact that the behaviour of pectin in milk systems depends not only on the interaction of different polymeric fine structures with casein micelles, but also to a large extent on the interactions with calcium.

Keywords Milk · Casein micelles · Pectin · Acidification · Diffusing wave spectroscopy

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Introduction

Casein moieties in milk are assembled into micelles during biosynthesis and despite the fact that the detailed arrangement of the protein variants is complex, it is well established that these entities are stabilised in solution by kappa-casein molecules forming an entropy rich steric barrier at their surface [1]. Enzymatic cleavage or electrostatic collapse of this barrier destabilises the micelles and triggers assembly, yielding the formation of networks that consist primarily of aggregated casein micelles. While the micellar integrity depends in detail upon the nature of the environmental conditions employed in destabilisation, these systems nevertheless tend to exhibit the microstructural appearance of particulate networks—a fact that has not escaped the attention of physicists interested in colloidal assembly per se [2, 3]. Thus, acid milk gels (those triggered by the collapse of the stabilising protein layer owing to changes in the polymeric charge brought about by lowering the pH) are built of a three dimensional network of chains and clusters of milk proteins that at a smaller scale retain some of the integrity of the particulate micellar form [4]. They do, however, form a heterogeneous and complex system. The heterogeneity manifests at two levels: in the network itself and in the presence of voids in the colloidal system. Acid milk gels have been extensively studied by bulk rheology [5] and more recently by microrheological techniques [2, 6–9]. In particular, diffusing wave spectroscopy (DWS), a non-invasive multiple scattering technique, has found considerable utility in studying the underlying dynamics of these systems. Here, autocorrelation functions resulting from the intensity fluctuations of light that has been multiply scattered by the sample are analysed and information on the dynamics of the scatterers is thus extracted. In these systems the scattering is dominated by casein micelles and protein

aggregates, so that the introduction of probe particles, common in such microrheology experiments is not required. Via DWS, rheological behaviours at high frequency can be obtained, that contain information additional to that obtainable with conventional rheology, with the potential to give insights into the network structure.

Pectin, an ionic polysaccharide extracted from the plant cell wall, is commonly added to acid milk preparations in order to stabilize them as the pH is reduced. Indeed for acid milk drinks, which have low solids content (2–5% *w*/*w*), pectin is able to inhibit casein micelles coagulation, yielding a macroscopically homogeneous viscous solution at pH values below 5, in contrast to the precipitation of milk proteins that is observed in its absence. Pectin is composed of three pectic polysaccharides [10]: homogalacturonan (HG), rhamnogalacturonan I (RGI) and rhamnogalacturonan II (RGII). HG is a linear polymer of (1-4)-linked α-D-galacturonic acid and its methylesterified counterpart with the ratio of uncharged methylesterified residues to the total galacturonic acid content (the degree of methylesterification or DM) playing a major role in determining the polymers functionality. RGI has a backbone consisting of the repeating dissacharide rhamnose-galacturonic acid, where the rhamnose residues provide potential sites for the attachment of glycan side-chains. RGII has a backbone of (1-4)-linked α-D-galacturonic acid but with many conserved complex sugar side chains. Typical extraction processes modify the in vivo pectin fine structure (which is still a matter of debate to some extent), notably by decreasing the number of side-chains, and commercially available pectins are routinely found to consist of primarily linear chain homogalacturonan (around 90%).

The interaction between pectin and casein micelles has been widely studied by dynamic light scattering (DLS) in diluted systems [11–13] and by rheology and microscopy [14–16]. It has been suggested that, at sufficient concentrations, pectin can interfere with the aggregation of milk upon acidification by two mechanisms: firstly, DLS studies have shown that below pH 5 certain pectin fine structures can adsorb onto casein micelles via electrostatic interactions [17]. These pectin layers increase the steric repulsion between casein micelles [18], replacing the stabilizing effect that was produced by the κ-casein at higher pH, and limiting their sedimentation at low protein concentration e.g. in acid milk drink. It has also been suggested that pectin may play a role in forming a weak gel in the voids of the micellar particulate network [19] and indeed confocal microscopy studies using fluorescence recovery after photobleaching (FRAP) have provided evidence for a reduced pectin mobility in these systems, although the removal of the "serum pectin" by centrifugation did not affect the stability of the system [20]. Other studies with systems of lower pectin concentration (0.12% *w*/*w*) located pectin in stabilised systems by using a monoclonal pectin antibody [21] and found it to be present predominantly in, or at the surface of, the protein aggregates—rather than in the voids of the network. Pectin fine structure, notably the DM (and also the distribution of the methylester group on the backbone) will strongly influence not only the polymers interaction with calcium, as is well-known, but also with casein. Indeed, high DM pectins have been shown to stabilize casein dispersions more effectively than those of low DM [12, 22–23].

In this work we are interested in the following questions: (1) if indeed, in the course of acidification, pectin adsorbs around the casein micelles prior to significant micellar aggregation even in more highly concentrated quiescent systems, then how would such a polymer coating effect the mechanical properties of the resultant gel? And (2) what understanding of the interaction might be gained by carrying out these experiments with a judicious choice of polysaccharide fine structures? To answer these questions, we use diffusing-wave spectroscopy, a technique which does not require sample dilution, to investigate the micro-rheological behaviour of these systems.

Materials and methods

Materials

Acid milk gel preparation

Low-heat skim milk powder (NZMP, New Zealand) was used to prepare reconstituted skim milk with 20% *w*/*w* milk solids. With a low-heat milk powder, only the caseins will be part of the network (in contrast to that which has undergone a more severe heat treatment in which the whey proteins can also play a significant role). A 0.2% *w*/*w* pectin solution was prepared in parallel. Pectins with different fine structures were used, the degree of methylesterification, molecular weight and origin of which are reported in Table 1. The two dispersions were stirred overnight at 4 °C to ensure full hydration. Subsequently, pectin and skim milk powder solutions were mixed in equal quantities and sheared for 1 h with a magnetic stirrer before any analysis. Acidification of the final sample was achieved by the addition of between 1 and 2.3% *w*/*w* glucono-δ-lactone (GDL) at 20 °C. For all samples studied, the pH was measured every 5 min during the acidification process.

Methods

Diffusing wave spectroscopy

Diffusing wave spectroscopy is a dynamic light scattering method that is designed to be used with turbid samples,

Table 1 Characteristics of pectin samples used in this study

	DM/%	Mw/g mol^{-1}	Origin and treatment
R77.8	77.8	120,000	Copenhagen pectin fungal PME
R78	78	30,000–100,000	Fluka Biochemica, Switzerland
R31.1	31.1	120,000	Copenhagen pectin fungal PME
R30	30	44,000	Homemade by alkali treatment [41]
E30	30	44,000	Homemade by treatment with a processive enzyme (PME) [41]

where each photon encounters multiple scattering events between entering the sample cell and being detected. In such systems the photons path can be considered a random walk and, as such, the decay of the autocorrelation function of the scattered light owing to the motion of the scatterers can be calculated from the solution to a well-known diffusion problem. Owing to the multiple scattering nature of the technique each individual scattering particle need only move a small amount in order to generate significant de-phasing effects when summed over the entire photon trajectory. Thus, DWS can measure motions at high frequency and the technique has found great utility in studying the motion of tracer particles added to systems to probe their microrheological properties over a broad frequency range. In this work the casein micelles themselves act as the probes with the evolution of the correlation function during gelation primarily reflecting changes in their dynamics. The measured temporal autocorrelation of intensity fluctuations of the scattered light was measured as:

$$g_1(t) \equiv \frac{1}{\beta}\left(\frac{\langle I(t)I(0)\rangle}{\langle I\rangle} - 1\right)^{1/2} \quad (1)$$

Where β is a constant, characteristic of the optics, and $I(0)$ and $I(t)$ are the intensity of the detected light at the time zero and t. While DWS can be carried out in transmission or backscattering modes, transmission is preferred here owing to the increased simplicity of the boundary conditions: each detected photon has clearly traversed a distance equivalent to the width of the sample cell. Under these conditions the calculated temporal autocorrelation function for the transmitted light can be written as [24]:

$$g_1(t) = \frac{\frac{L/l^*+4/3}{z_o/l^*+2/3}\left\{\sinh\left[\frac{z_0}{l^*}\sqrt{k_0^2\langle\Delta r^2(\tau)\rangle}\right] + \frac{2}{3}\sqrt{k_0^2\langle\Delta r^2(\tau)\rangle}\cosh\left[\frac{z_0}{l^*}\sqrt{k_0^2\langle\Delta r^2(\tau)\rangle}\right]\right.}{\left(1+\frac{8t}{3\tau}\right)\sinh\left[\frac{L}{l^*}\sqrt{k_0^2\langle\Delta r^2(\tau)\rangle}\right] + \frac{4}{3}\sqrt{k_0^2\langle\Delta r^2(\tau)\rangle}\cosh\left[\frac{L}{l^*}\sqrt{k_0^2\langle\Delta r^2(\tau)\rangle}\right]} \quad (2)$$

where l^* is the transport mean free path, z_0 the penetration depth (considered equal at l^* in these experiments), L thickness of the sample (4 mm), $k_o=2\pi n/\lambda$, the wave vector of the light and $<\Delta r^2(t)>$ is the mean square displacement (MSD) of the particle. Hence, when l^* is known, the experimentally determined correlation function can be turned into a plot of MSD versus lag time, by inverting Eq. 2 with a zero-crossing routine.

l^* is obtained by performing an experiment on a model dispersion of latex beads in water and fitting l^* using the accepted viscosity. Subsequently l^* for future samples is obtained by scaling the value obtained for the latex beads dispersion, based on the change in transmitted intensity when the sample is introduced. It is known that for non-absorbing slabs of thickness L, the transmitted intensity is directly proportional to $(5l^*/3L)/(1+4l^*/3L)$, so that by measuring the change in transmittance, the change in l^* can be calculated.

The experimental set up has been fully described elsewhere [25, 26]. Briefly, laser light with a wavelength of 633 nm (35 mW He Ne Melles Griot laser) diffused through the sample, contained in a plastic cuvette of 10-mm width, 50-mm height and 4-mm path length. The transmitted scattered light was collected using a single-mode optical fibre (P1-3223-PC-5, Thorlabs, Germany) and was detected with a photomultiplier tube module (Hamamatsu HC120-08). The auto-correlation analysis was performed using a Malvern 7132 correlator. Tests were run for 3 min to ensure low noise intensity autocorrelation functions.

Bulk rheology

The viscoelasticity of the systems was analysed by dynamic oscillatory rheometry using a Paar Physica UDS 200 instrument in the strain-controlled manner. Immediately after the addition of GDL and stirring for 1 min, the sample was loaded into a measurement cell with a cone geometry. The storage (G') and loss moduli (G'') were recorded during 3 h at a constant frequency of 1 Hz and a constant strain of 0.5%. The sample was maintained at 20 °C during all measurements.

Confocal microscopy

A Leica confocal laser scanning microscope (TCS SP5 DM6000B) was used in fluorescence mode with a DPSS 561 laser (excitation wavelength of 561 nm, emission spectrum 565–659 nm) and an oil-immersion objective (×100). The number of pixels per image was 2,048×2,048. The protein network was dyed with Fast-green prior to

acidification by addition of 6 μl of dye, from a 0.2% *w*/*w* mother solution, to 1 ml of sample.

^{31}P NMR

^{31}P NMR experiments were carried on a Bruker 400 Ultrashield spectrometer (^{31}P operating frequency 161.97 MHz) at a temperature of 20 °C. Samples were measured in a 5-mm-diameter thin wall tube (Wilmad LabGlass, USA). A 1-mm diameter capillary containing phosphoric acid (10 mM) was first inserted into the NMR tube to provide a chemical shift reference (the phosphoric acid peak was set to 0 ppm). The capillary was then removed and spectra (256 scans, 65,000 data points) were recorded for each sample. The glycerophosphoycoline peak which was not affected by the presence of pectin was used as a concentration reference.

Results and discussion

Acid milk gels

Systems containing 10% *w*/*w* milk solids non-fat (MSNF), corresponding to around 13% volume fraction of casein micelles [27, 28], were reconstituted and were acidified at 20 °C, using GDL. DWS was used to record the evolution of the correlation function of transmitted multiply-scattered light as described in the experimental section and thereby report on the dynamics of the scatterers; the casein micelles and aggregates thereof formed during gelation. Figure 1a shows the light autocorrelation function $g_1(\tau)$ as a function of the time lag τ, as measured by DWS, during the acidification process. The sol-gel transition is clearly reflected in the dynamics of the scatterers. While initially $g_1(\tau)$ is characteristic of a Newtonian fluid, i.e. goes to zero at long times, as acidification proceeds the correlation function does not decay to zero, showing that a high viscosity fluid or visco-elastic gel is formed. At long times, and at low pH values, $g_1(\tau)$ tends to be quite flat corresponding to the presence of a quasi-elastic material.

Typically, in microrheological studies of soft materials, stable scatterers are artificially introduced into the system, l^* is measured, and hence the mean square displacement of the particles can be obtained by simple inversion of Eq. 2, with a zero-crossing routine. Such a numerical approach to the extraction of the MSD dispenses with the assumption that MSD=6*Dt*, i.e. that the medium is purely viscous; although in cases where this is known to be the case the substitution of this expression into Eq. 2 yields an expression to which data can be directly fitted using standard non-linear least squares algorithms. The data in Fig. 1a are analyzed by inversion, although it should be

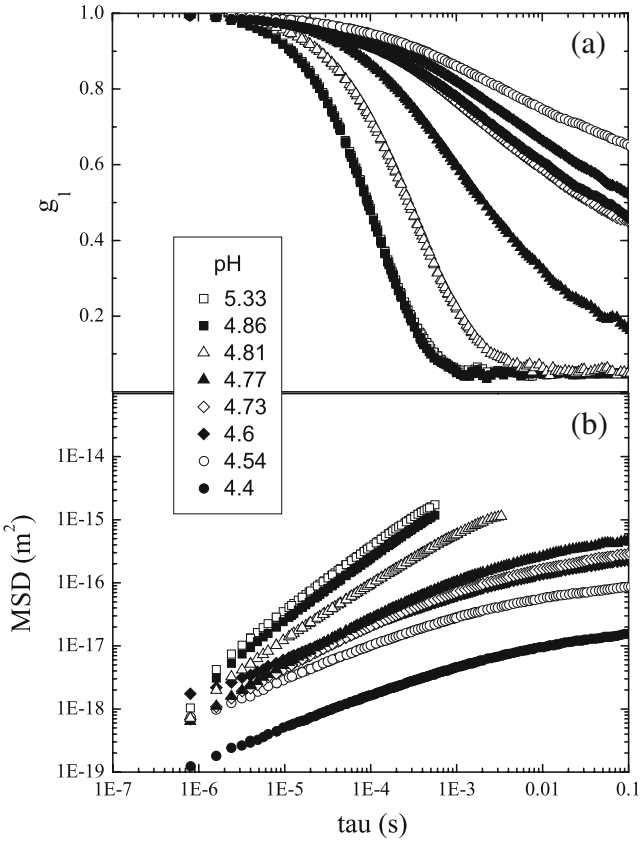

Fig. 1 **a** Temporal autocorrelation function evolution for milk (10% *w*/*w* MSNF) during acidification and **b** the corresponding evolution of the measured MSDs

stressed that the integrity and size of these omnipresent scatterers are possible functions of pH. Although l^* is re-measured along with each corresponding correlation function this means that the actual MSD values measured after the gelation point should be taken as estimates whose accuracy depends upon the extent of these scatterer modifications. Nevertheless, microscopy evidence suggests that micelles are not completely dissociated and an intact particulate gel is a reasonable model of the system [4, 29]. Furthermore, we still expect the variation of MSD with time lag τ to capture the essential physics of the gel properties.

Micelle mean square displacements as a function of lag time obtained by inversion of the correlation functions recorded during the acidification process are shown in Fig. 1b. Results from the starting solution yielded a linear dependence of the MSD with time, with a slope of 1 evident from a double logarithmic plot, indicative of a viscous medium. Upon acidification, the slope of the mean square displacement versus time plot retained its power law dependence at high frequency, but the relevant exponent decreased with decreasing pH. As the high frequency behaviour corresponds to small displacements, of the order of 1–10 nm, it predominantly probes the nature of the

network's constituent elements. In order to get some information about the nature and the density of the cross-links, the study of the long-time behaviour is essential, giving access to the state where large displacements of the beads can significantly strain the network, and indeed the acidification-induced evolution of a gel is clearly reflected at lower frequencies.

As the high frequency behaviour contains fundamental information about the network constitution and had received considerable attention previously focused on the comparison of the acidified-milk system with other colloidal gels and physical models [2, 3, 8, 30], we initially concentrated on this region. Figure 2a shows the high frequency exponent revealed by the slope of the double logarithmic MSD versus lag time plot, as a function of time during acidification, carried out at different rates as a consequence of different added GDL concentrations. By adjusting the concentration of added GDL, it was possible to obtain a stable weakly gelled system that exhibited an exponent close to 0.7 at high frequency, as has been found previously [3], and attributed to the percolation of a fractal network. However, in order to achieve this result the terminal pH in this system had to be such that the micelles were just able to interact and percolate without their

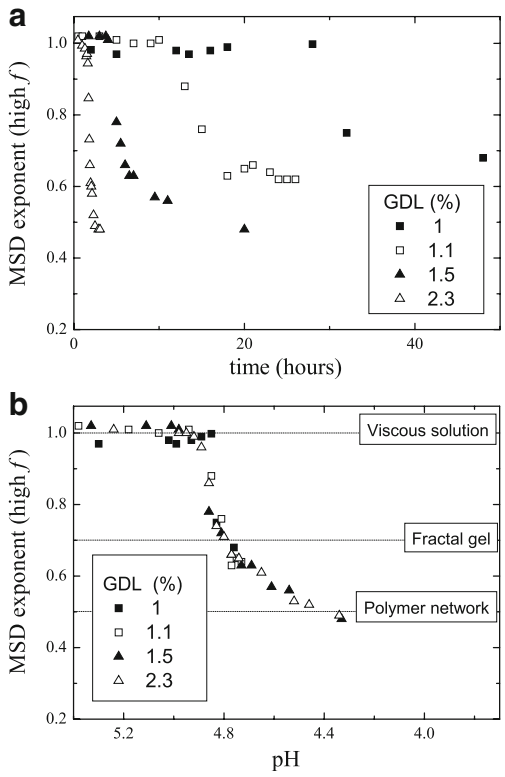

Fig. 2 **a** High frequency exponents revealed by the slope of double logarithmic MSD versus lag time plots, as a function of time during acidification (carried out at different rates as a consequence of different GDL concentrations) and **b** the data mapped onto pH instead of time, using the varying rates of GDL hydrolysis

significant disruption. In general, when the pH continued to decrease below this value (around 4.8) the slope in the logarithmic MSD plot continued evolving and ultimately achieved a value of around 0.4–0.5; indicating that while the physics of the colloidal fractal network is captured at the point of percolation, further evolution of the micellar structure results in a structure that is more suggestive of the mechanical properties being dominated by a continuous flexible polymer network. While the detailed analysis is complicated by the fact that it is the scatterers themselves that are losing their integrity, it is clear at least that, at this temperature, the widely described 0.7 exponent is, in general, fleeting and reports the contact of largely intact micelles beginning to interact, while further changes reflect the loss of micelle integrity. It is possible that in previous work [8] such a regime has been more prevalent owing to the fact that many of the prior studies where carried out at 30 °C; where the micelle is known to retain more integrity to lower pH values [31].

In order to achieve a high enough pH in the system at 20 °C so as to capture the fractal regime, it was necessary to use a GDL concentration that meant that the actual time-course of acidification was extremely slow; on the order of 48 h. Using other GDL concentrations ensured different end pH values but also generated different rates of acidification. Nevertheless, when the data were mapped onto a plot showing the slope of the logarithmic MSD plot versus pH, all experiments fell onto the same curve; within experimental uncertainties, as shown in Fig. 2b. This clearly indicates that our hypothesis is not complicated by kinetic issues and that the same percolated fractal structure (indicated by the 0.7 signature) is reached transiently by systems acidified at all rates used in this study—if the pH is held at around 4.8 then this state persists, but on reducing the pH further changes to the structure of the micelles are facilitated, and accordingly, the high-frequency mechanical properties are modified.

Addition of pectin

It has previously been clearly shown in systems containing low micelle concentrations compared to those reported here that upon the reduction of pH to around 5, in the presence of the anionic plant polysaccharide pectin, polymers with certain fine structures are able to interact with casein micelles, and that such an adsorption can help to stabilise acidified milk against precipitation of the micelles upon subsequent reduction of pH [13, 17, 18].

Here, two highly methylesterified pectins with random distributions of esterified groups and the same high DM (R78 and R77.8), but different molecular weights, were added at a variety of concentrations to the studied acid-milk system. The observed behaviour was compared with that

found when a pectin with only (randomly distributed) 31% methylesterification (R31.1) was used. This low DM sample was of similar molecular weight to one of the high DM pectins used, and indeed was generated from the same mother pectin. In addition, two further pectins of a lower molecular weight and similar low DM value were investigated; one with a random charge distribution (R30) and one with a more blockwise intramolecular charge distribution (E30), generated by the action of a processive demethylating enzyme.

Figure 3 shows the scatterer mean square displacements as a function of lag time, obtained as described previously, by inversion of the correlation functions, recorded at (a) pH 5.10 and (b) pH 4.40; during the acidification with 1.5% *w*/*w* GDL; of 10% *w*/*w* MSNF systems containing 0.1% *w*/*w* of the examined pectin samples. These samples were all homogeneous one-phase at the starting pH. The high DM (R78 and R77.8) pectins both clearly influenced the shape of the correlation function after acidification, compared with all the other pectins investigated (including a low DM pectin (R31.1) which was produced from the same mother pectin as one of the 78% samples (R77.8) and had an equivalent molecular weight). We hypothesise then that this change reflects the interaction of this fine structure with the micelles. Exploratory experiments were carried out at 3% MSNF and indeed only the structures that caused the change of MSD shape in the more concentrated systems studied here were found to stabilise those systems against precipitation upon acidification. For all other samples the MSD versus time followed roughly the same shape as the data recorded during the acid-induced gelation of milk alone. Indeed, taking the high frequency slope of the MSD in a double logarithmic plot and plotting this against pH, the data is indistinguishable (within experimental uncertainties established from three repeat experiments) from the results obtained from milk alone, for all but the 78% DM samples, as shown in Fig. 4.

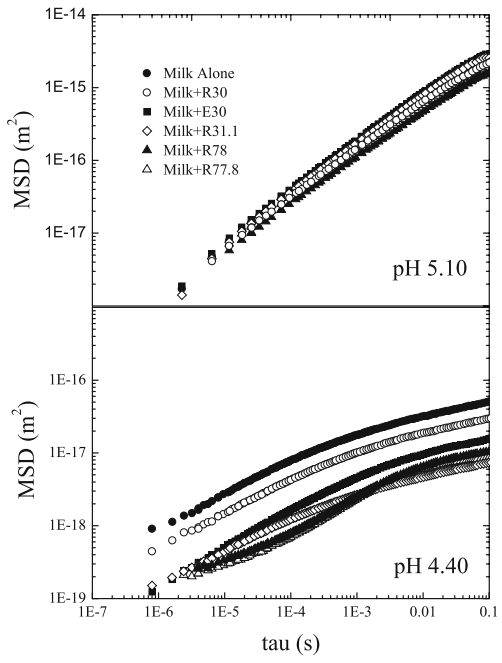

Fig. 3 Scatterer mean square displacements as a function of lag time, obtained as described previously, by inversion of the correlation functions, recorded at **a** pH 5.10 and **b** pH 4.40; during the acidification with 1.5% *w*/*w* GDL; of 10% MSNF systems containing 0.1% *w*/*w* of the examined pectin samples

Figure 4 also shows that for the 78% DM samples (R78 and R77.8), not only is the end slope different in the high frequency region, but additionally an enhanced rate of slowing of the dynamics is observed in time, that persists when the data is mapped onto the variation with pH. Bulk rheological measurements of the gelation of the acidified-milk system with and without 0.1% *w*/*w* R78 also demonstrates the rate enhancement in the system with the added pectin, as shown in Fig. 5. This suggests that the polymer adsorption that we suggest is indicated by change of shape of the MSD also enhances the rate of micellar aggregation, consistent with the idea of bridging. (Depletion can be ruled out on the basis that; firstly, the adsorption has been shown to be active at pH values above the unperturbed aggregation of the milk so there is not likely to be significant polymer concentration in solution at the relevant pH, and secondly, that lower DM pectins of the same molecular weight as a DM 78% sample, had no measurable effect). This, in turn, suggests that the surface coverage is incomplete.

Taking an approximate radius of 100 nm for a casein micelle and considering a volume fraction of 13% of casein micelles, we can estimate that 1 l of milk contains around 3.1×10^{16} casein micelles. The addition of 0.1% *w*/*w* of pectin (R77.8 with a Mw of 120,000 mol g^{-1}) represents therefore around 160 molecules of pectin per micelle or one molecule of pectin per 7.8×10^{-16} m^2 of the surface of the casein micelle; giving an average pectin molecule an area

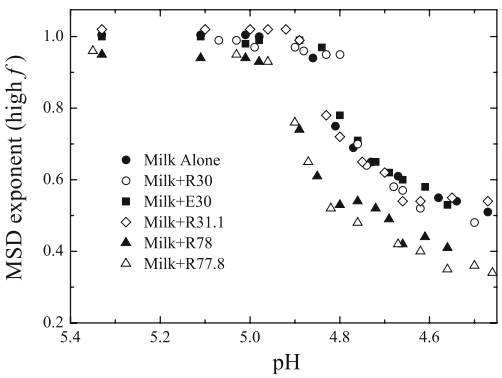

Fig. 4 The high frequency exponents revealed by the slope of double logarithmic MSD versus lag time plots, as a function of pH with different pectic polymers added

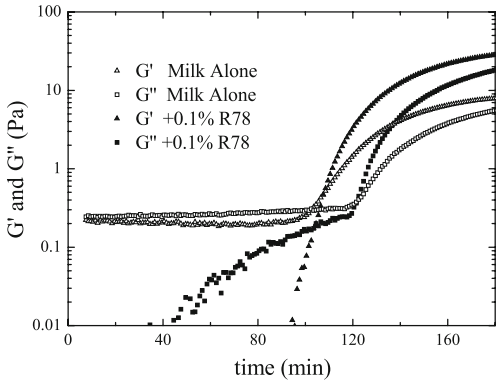

Fig. 5 Bulk rheological measurement showing the formation of an acidified milk gel and the repeat experiment in the presence of 0.1% *w*/*w* DM 78% pectin; i.e. the bulk rheological properties measured at 1 Hz, carried out on the equivalent systems of interest examined by the microrheological analysis

of around 28 nm×28 nm of surface to occupy. Since a fully extended pectin molecule is around 350 nm in length and 1 nm diameter, and the most probable conformation has a radius of gyration of the order of 10 nm, the hypothesis of incomplete coverage certainly seems reasonable. Furthermore, the current estimate of full coverage [32] is 1 mg of pectin per m^2 of casein micelle which corresponds to more than twice the pectin concentration used in this study.

In previous work the estimation of the relevant surface coverage of adsorbed polymer has been complicated by the fact that if pectin is added to casein micelles in sufficient concentrations then even prior to acidification depletion flocculation drives the system to phase separate [33]. In order to circumvent this problem, systems are generally homogenised by shearing devices during acidification [12, 18] until the evolved visco-elasticity of the system is capable of arresting the demixing (stirred yoghurts). However, in such systems the particulates being stabilised are protein aggregates whose size is determined by the homogenisation process, leaving surface coverage estimates difficult. In addition, there is some evidence [20] that suggests that the added polymer has some functionality during this period that does not rely on it being adsorbed in the stabilised system (at least not tightly enough that it cannot be removed by centrifugation). In order to avoid the inherent complications in studying such homogenised systems, we chose to work at pectin concentrations that were miscible with our chosen casein micelle concentration. While this limited the concentration range examined, it meant that neither demixing nor the role of shear forces had to be considered in the interpretation of the results.

Returning to the fact that the addition of all polysaccharide samples, with the exception of the high DM samples, had a negligible effect on the acidified milk system (Fig. 4), we report a crucial observation. It should be noted that on addition of the low DM pectins to the milk systems aggregate/microgel formation was visually apparent in these quiescent systems to varying extents even prior to acidification (being most severe for the PME-generated structure).

While it has been clearly demonstrated that the pectin–casein micelle interaction is largely electrostatic in nature [34, 35], the details of how the polymer charge distribution might influence its adsorption have received relatively scant attention. In the absence of a detailed molecular model of the interaction that could potentially reveal the pectin epitope with the largest binding energy it has tacitly been assumed that unesterified blocks would fulfil this role more efficiently than those punctuated with methyl groups, although there seems to be minimal experimental evidence of this. It is also worth noting that in terms of the functionality of the adsorbed polymer as a steric barrier the problem is significantly more complex than selection of the polymer with the highest binding, as it is after all the distribution of loops and trains that ultimately generates stabilisation, even if the binding is possibly multilayered. Assuming that the preferential binding epitope is completely unesterified it would seem to be a reasonable hypothesis that indeed a pectin of as high as possible DM interspersed with unesterifed blocks of sufficient length to bind the micelle would provide the idealised fine structure. Unfortunately, the value of such a sufficient length is unknown and, although it might possibly be approximated by studying the effect of pectins of very high DM, presently, the only way that one might in any case introduce more than the random compliment of a certain blocklength into the chain would be to use enzymatic processing. Such processing may well yield pectins with a greater propensity to bind the micelle but such polymers would also possess a greatly enhanced calcium sensitivity. While a recent study [36] has shown that low DM pectins can be gelled with serum extracted from milk at increasing stages of acidification, and hence containing increasing amounts of calcium ions, the possibility of pectins modifying the calcium phosphate equilibrium when present, in an analogous way to EDTA, has not been addressed. In fact we have found in the current study that, even without acidification, calcium-induced microgel formation of low DM (or otherwise calcium sensitive) pectins could be observed.

In order to gather further evidence of this phenomena we carried out ^{31}P NMR experiments. High-resolution liquid-state ^{31}P NMR spectroscopy is a non-invasive technique, which has been extensively used to study dairy systems [37]. It allows the detection of the resonances from the mobile ^{31}P atoms, such as those of the inorganic phosphorus and the individual phosphoproteins, but not the colloidal calcium phosphate (CCP) or the aggregated casein molecules which are not detected due to their low mobility. It is this NMR technique which is used in this work to

investigate quantitatively the effects of pectin of differing fine structures on the inorganic phosphorus and phosphoproteins in milk.

The measured ^{31}P NMR spectra of 10% MSNF solutions with or without the addition of pectins are reported in Fig. 6. The spectra show four main peaks, which are assigned in the literature [38] as inorganic phosphate (P_i; peak 1), phosphoproteins (SerP; peak 2), glycerophosphoylcoline (peak 3), glycerophosphorylethanoline (peak 4). When high DM pectin (R77.8) was added there was no discernable change in the spectrum while with the addition of the low DM sample (R31.1), the P_i peak increases noticeably, and is slightly shifted. This behaviour is entirely consistent with that observed on the addition of EDTA [39] and is an indication that the low DM pectin indeed removes calcium from the micelle.

To summarise, we suggest our results can be explained in the following way: the lower DM fine structures (regardless of molecular weights and intramolecular distribution), interact with, and even enhance the concentration of, serum calcium (through the dissociation of the colloidal calcium phosphate present inside the casein micelle), effectively precipitating a substantial fraction of them. This accounts for the visual appearance of precipitates, the observed changes in the ^{31}P NMR spectrum, and subsequently the fact that there is not enough functional polymer left to bind to the micelle. Repeat experiments carried out using reduced amounts of the effective R78 polymer, shown in Fig. 7, indicate that in order to have any influence on the systems behaviour at this concentration of micelles a concentration of above 0.025% of functional polymers is required. In contrast, the high DM (random intermolecular distribution) fine structures do not interact significantly with the serum calcium and are more likely to adsorb onto the casein micelles as the pH is reduced below 5. Their limited surface coverage (dictated in this study by the desire to start acidification from a one-phase system) leads to

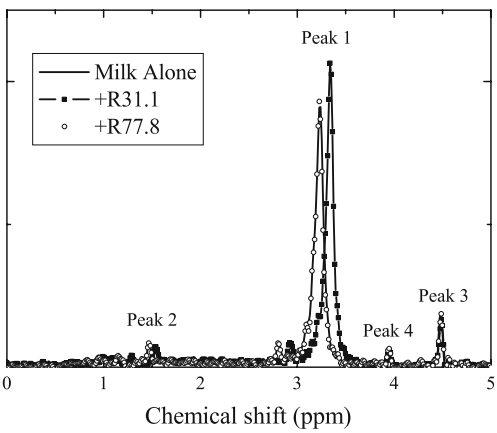

Fig. 6 ^{31}P NMR spectra of 10% milk solutions, and with the addition of high (R77.8) and low (R31.1) DM pectin samples

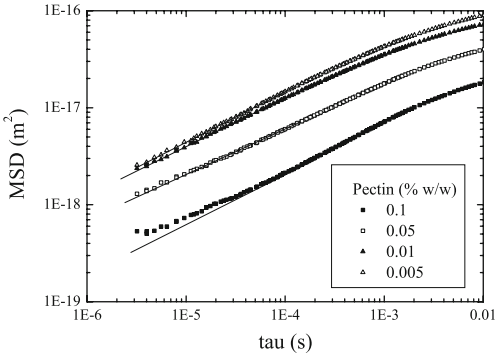

Fig. 7 Scatterer mean square displacements as a function of lag time, obtained as described previously, by inversion of the correlation functions, recorded at pH 4.62 as a function of pectin concentration; for the R78 pectin sample

efficient bridging which enhances the rate of micelle aggregation and subsequent gelation. While the network appears similar upon visualisation of the resultant milk protein network, as shown in the images displayed in Fig. 8, the small amount of adsorbed pectin contributes to the high frequency microrheology is a complex manner; providing a change of high frequency slope as a consequence of an observed shape change in the MSD. It is worth noting that similar exponents at high frequency and hints of such a sigmoidal shape have been found previously in studies of colloidal gels embedded in polymer solutions of varying visco-elasticity [40]. While these previous studies were carried out at considerably higher polymeric concentrations and focused on the consideration of how the network and the surrounding medium each contributed to the elastic and viscous components of the system, it could be that in the system studied here the local concentration of the bridging pectin around the micelles is sufficient, even below full coverage, to influence the relaxation modes of the network in a similar manner. It must, however, be remembered that the colloidal network in our system is itself evolving as the pH is reduced.

Thus, for pectic polymers to exhibit in situ stabilising functionality in acid-milk systems, they must be able to adsorb to the casein micelle surface in a manner providing steric stabilisation upon acidification and be molecularly available to do so. For these requirements to be fulfilled at least three factors are crucial: (1) at least one casein micelle binding epitope is available per chain, (2) a significant portion of the chain does not adsorb to the surface of the casein micelle and (3) the polymer should be sufficiently calcium insensitive not to form microgels pre-acidification. The reasons for the preponderance of high DM and amidated pectins in the industrial stabilisation of acid-milk drinks are then clear. The investigation of whether a more efficient pectin fine structure exists awaits a better molecular model of the adsorption of anionic polysacchar-

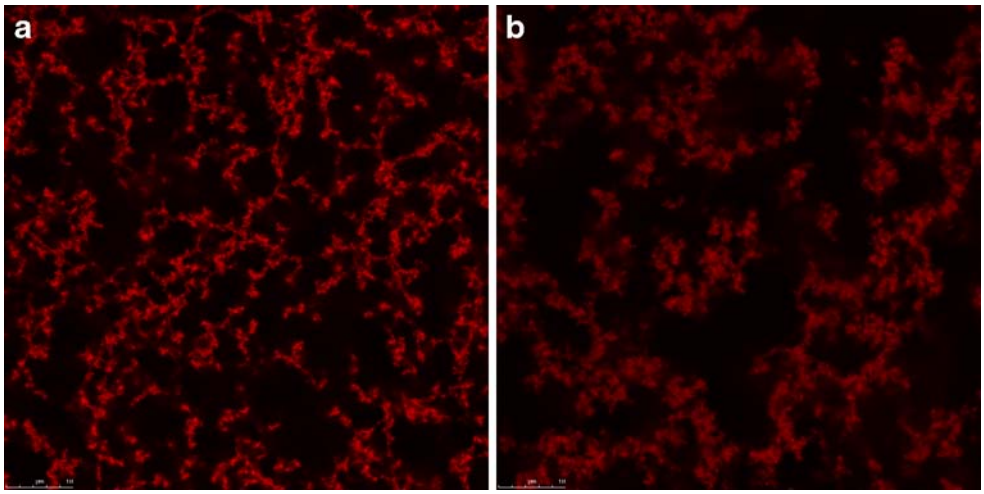

Fig. 8 Micrographs showing an acidified milk gel and a gel made in the presence of 0.1% R78; i.e. the microstructures pertaining to the systems examined with the bulk rheological measurements in Fig. 5

ides onto casein micelles that will, among other things, allow the surface coverage to be estimated in a more realistic way, considering the orientation of molecules at the surface. At present, all estimates [17] are order of magnitude estimates based on standard polymer texts [32].

Conclusions

Upon acidification of sufficiently concentrated milk systems a percolated stress bearing fractal structure, based on associated casein micelles, is transiently established. This state has a high frequency microrheological signature; specifically that the slope of the MSD with lag time follows a power law with an exponent of 0.7 that has been observed in other colloidal systems and discussed theoretically [3, 30]. If the pH of this system does not fall significantly below 4.8 then this state is long-lived. However, on reducing the pH further subsequent changes to the structure of the elementary building blocks are facilitated, and accordingly, the slope in the logarithmic MSD plot evolves, ultimately achieving a value of around 0.4–0.5, indicating that while the physics of the colloidal fractal network is captured at the point of percolation further evolution of the micellar structure results in a structure in which the mechanical properties are more suggestive of a continuous flexible polymer network.

When pectin was added to such milk systems, at concentrations nominally corresponding to approximately half coverage of the micelles, an interaction of the added anionic polymers with serum calcium was observed visually for all low DM (or otherwise calcium sensitive) pectin fine structures, even without acidification. Further evidence that the appearance of precipitated polysaccharide aggregates was directly linked to interactions with calcium was obtained from phosphorous NMR studies, which clearly showed that changes in the colloidal calcium balance occurred on the addition of such polymers. We hypothesise that this effective precipitation is the reason that the calcium sensitive polymers are found to be ineffectual in interacting with the casein micelles and therefore that the effect of fine structure on the polysaccharide–protein interaction per se is not accessible in experiments of this type. However, high DM (random intermolecular distribution) fine structures do not interact significantly with the serum calcium and absorb onto casein micelles as the pH is reduced below 5. At low micelle concentrations such adsorption generates steric stabilisation of the micelles, as the κ-casein brush collapses, and protects the system against precipitation. At higher casein concentrations, but still in thermodynamically compatible systems, the limited surface coverage of the high DM pectins leads to efficient bridging which enhances the rate of micelle aggregation and subsequent gelation and produces clear signatures in the shape of the measured MSD.

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