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Micellar-casein–κ-carrageenan mixtures. I. Phase separation and ultrastructure

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Abstract

The phase behaviour of micellar-casein (MC)–κ-carrageenan mixtures was investigated using different ionic forms of the carrageenan (Na^+, K^+) in different ionic conditions and at two temperatures. At 50°C, that is above the conformational transition temperature of the carrageenan, a macroscopic phase separation was clearly evidenced. Accordingly, ternary phase diagrams were established as a function of the biopolymer concentration for each system. By analogy with other particulate systems, it was assumed that this phase separation originated from a depletion–flocculation mechanism.

The ultrastructure of the MC–carrageenan mixtures is described at 20°C using confocal laser scanning microscopy and phase contrast microscopy. All observations showed that phase separation has taken place. A casein-rich phase was dispersed in a carrageenan-rich phase at a low casein content (below 3%); above this casein concentration in the mixtures, the phase separation process yielded a casein continuous network within the carrageenan network. It was suggested that the phase separation process initiated at 50°C was almost immediately inhibited by gelation upon cooling, resulting in a biphasic system the structure of which depended upon the initial composition.

Keywords: κ-carrageenan–micellar-casein mixtures; Phase behaviour; Confocal microscope

1. Introduction

Carrageenans are widely used as thickening and stabilizing agents in the dairy industry (Heertje, 1993; Hood & Allen, 1977; Snoeren, 1976), and particularly in milk desserts, in which casein constitutes the major protein component (Swaisgood, 1982). However, the mechanism by which these polysaccharides react with milk components is not fully understood, limiting the development of stabilized milk products.

Casein, in the so-called micellar form, is composed of relatively large entities with a rather complex structure (diameter between 20 and 600 nm), (Schmidt, 1982). This colloidal assembly is a supramolecular association of individual casein molecules: α_{S_1}, α_{S_2}, β and κ casein. These fractions are organized within the micelle according to their hydrophobic and hydrophilic character. The κ-casein fraction, due to its very hydrophilic nature, is known to be mainly present on the surface of the micelle, providing a steric and electrostatic stabilizing outer layer (Visser, 1992). Indeed, Thomsen, Jakobsen, Nielsen, Peterson and Rasmussen (1995) have shown by ^{31}P NMR that the C terminal part of κ-casein (the third C terminal part is called the glycomacropeptide) has a considerable conformational mobility. The integrity of the micelle is maintained by colloidal calcium phosphate bridges (Van Dijk, 1990). The micelle is thus in dynamic equilibrium with its ionic environment (Holt, 1992; Visser, 1992).

Carrageenans are sulphated polysaccharides extracted from red seaweed. They are linear polymers, with a backbone structure of alternating α-1,4 and β-1,3 linked galactose residues and varying proportions and positions of sulphate groups. κ-carrageenan is the lowest sulphated carrageenan fraction. It forms thermoreversible gels in aqueous solution and in the presence of cations. At a temperature above the helix–coil transition temperature, κ-carrageenan molecules exist as random coils. Gelation, which occurs on cooling, has been attributed to a two-step phenomenon involving helix formation followed by aggregation (Rochas & Rinaudo, 1984). The transition temperature is strongly dependent on the nature of the ions (Rochas & Rinaudo, 1980) gelation being promoted in the following order:

$$Rb^+ > K^+ > NH_4^+ > Ca^{2+} > Na^+ > Li^+$$

Biopolymer mixtures are often used in the food industry

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Table 1
Ionic form of carrageenan and ionic composition of the medium used in the study

System	Ionic form of κ-carrageenan	Medium	Helix–coil transition temperature of κ-carrageenan (°C)[a]	$[\eta]$ (dl/g)[b]
1	Na^+	0.25 M NaCl	44	5.3
2	K^+	0.25 M NaCl	43.9	6.9
3	K^+	0.05 M NaCl–0.01 M KCl	43.2	8.7

[a] From microDSC measurements: maximum peak temperature upon heating.
[b] Intrinsic viscosity at 50°C.

because of their synergistic properties; however, mixed systems containing micellar casein (MC) have been the subject of very few studies despite the fact that the behaviour of other protein–polysaccharide mixtures has been extensively investigated. As many as eighty protein–polysaccharide–water ternary systems have been described by Tolstoguzov and co-workers (Tolstoguzov, 1986; 1991). Most of the systems exhibited phase separation due to thermodynamic incompatibility between the biopolymers, leading to the formation of a protein-rich phase and a polysaccharide-rich phase in the mixture. However, such mechanisms have not been described in the case of casein–carrageenan systems. A few studies have been reported but with casein fractions or caseinates rather than with casein micelles (Hansen, 1968; Snoeren, 1976; Hood & Allen, 1977; Lynch & Mulvihill, 1996). Whatever the type of carrageenan used, a specific interaction between the sulphate groups of the carrageenan and a very short, positively charged region, situated between residues 97 and 112, of the κ-casein (globally negatively charged) was evoked. This specific region is situated at the boundary between the hydrophobic (residues 1–105) and hydrophilic (residues 106–169) parts of the κ-casein. When dealing with α_S and β-casein mixed with ι-carrageenan, an excluded volume effect is evoked to explain the rheological changes induced by the presence of the protein (Lynch & Mulvihill, 1996). Actually, since κ-casein is assumed to be situated on the outer layer of the micelle, most of these studies extrapolate the results obtained on fractions and imply a specific interaction between carrageenan and κ-casein to explain the properties of MC–carrageenan mixtures. Some studies, however, have been directly performed either on carrageenan–MC mixtures (Drohan, Tziboula, McNulty & Horne, 1997) or on carrageenan in skim milk (Langendorff, Cuvelier, Launay & Parker, 1997). Drohan et al. (1997) have suggested that the gelation of dairy products with κ-carrageenan could be due to the presence of K^+ and Ca^{2+} ions in milk, because of the importance of these cations for the gelation of the polysaccharide. They found that, for a carrageenan concentration higher than 0.018%, gel formation involved mainly carrageenan–carrageenan cross-linkages and not carrageenan–casein or casein–casein linkages. However, they did not totally exclude the possibility of an electrostatic interaction between κ-casein and κ-carrageenan. In their study of ι-carrageenan–milk mixtures, Langendorff et al. (1997) considered the possibility of adsorption of the carrageenan chains on the outer part of the micelle, and proposed a mechanism involving coating of the micelles by ι-carrageenan chains, leading to a stable system. Upon addition of carrageenan in excess, a depletion–flocculation secondary process would occur, leading to the sedimentation of the ι-carrageenan-covered micelles. However, they mentioned that a depletion–flocculation mechanism in itself could also explain the behaviour of these systems.

The aim of the present study was to understand whether the MC–carrageenan system follows the general behaviour of the casein–polysaccharide mixtures (Bourriot et al., 1997; Bourriot et al., 1999a; Bourriot et al., 1999b) or if carrageenan behaves in a specific way in the presence of casein as it is generally accepted in the literature. For this purpose, (i) the phase behaviour of these mixtures has been described when the carrageenan exist as random coil in solution, i.e. at a high temperature, and (ii) their ultrastructure has been observed by Confocal Laser Scanning Microscopy (CLSM) and Phase Contrast Microscopy (PCM) at room temperature.

2. Materials and methods

2.1. Materials

MC was a native calcium phosphocaseinate sample purified by ultrafiltration and then freeze-dried. This was kindly supplied by Laboratoire de Recherches et de Technologie Laitière (INRA Rennes, France). It had the following characteristics: total protein content 90.7%; non-casein protein 5.0%; lactose 0.5%; salts 8% (calcium: 3.2%).

The κ-carrageenan (KC) sample was provided by SKW Biosystems (France). The pure sodium or potassium forms of the carrageenan sample were obtained by ion exchange of a hot 2% carrageenan solution with a commercial ion exchange resin (Amberlite IR 120) and followed by freeze-drying (Morris & Chilvers, 1983). Intrinsic viscosities were determined at 50°C using a highly sensitive

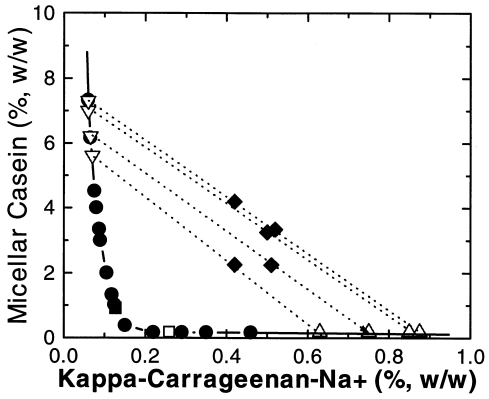


Fig. 1. Phase diagram of the MC–κ-carrageenan-Na^+ mixture (0.25 M NaCl, 50°C). Binodal (solid curve, points ●); tie lines (dotted lines); initial mixtures (◆); upper phase (△); lower phase (▽); critical point (■); threshold concentration (□).

coaxial cylinders viscosimeter (Low Shear 40, Contraves, Switzerland) and are reported in Table 1.

2.2. Preparation

MC (10%, w/w) was dispersed in 0.25 M NaCl or in 0.05 M NaCl–0.01 M KCl at 20°C, pH 7 by stirring with a paddle at 1300 rpm for 5 min and then sonicated for 8 min at 50 W. The particles size distribution was checked using a Malvern Mastersizer IP laser granulometer. The average diameter was 0.25 μm which is in agreement with literature data (Schmidt, 1982; Swaisgood, 1982).

κ-carrageenan solutions (1%, w/w) were prepared at 90°C in 0.25 M NaCl or in 0.05 M NaCl–0.01 M KCl under magnetic stirring for 30 min.

Casein–carrageenan mixtures were prepared at 70°C under magnetic stirring for 15 min at appropriate ratios. The total biopolymer concentration was below 10% (w/w).

Three different mixed systems containing MC were studied, depending on the ionic form of the carrageenan and the ionic strength of the medium. Their respective composition is detailed in Table 1. These different medium conditions were chosen because the conformational transition upon heating was nearly at the same temperature (43–44°C).

2.3. Methods

2.3.1. Phase diagrams

Phase diagrams were established according to the biopolymer concentrations at 50°C, in 0.25 M NaCl or in 0.05 M NaCl–0.01 M KCl. The 24 h-aged mixtures were centrifugated at 1000*g* for 15 min. Phase separation boundary was then detected only by eye since the two phases were clearly separated when phase separation occurred. The upper phase was a carrageenan-rich phase while MC was concentrated in the lower phase. For the upper phase, the casein content was obtained by measuring the absorbance at 277 nm and the carrageenan concentration was determined by the dry matter method. The composition of the lower phase was obtained by calculating the concentrations from the volumes.

2.3.2. Flame emission spectroscopy

The repartition of the calcium in each phase after phase separation has been measured with an atomic absorption spectrometer (Z8200 Hitachi) at 422.7 nm using a fuel lean air–acetylene flame.

2.3.3. Microscopic observations

Microscopic observations were carried out using CLSM and PCM.

CLSM was performed with a Zeiss LSM 410 Axiovert microscope with a water immersed × 40 objective. It was used in fluorescence mode. MC was labelled with 8-anilino, 1-naphthalene sulphonic acid (ANS) which is thought to be confined in the hydrophobic zones of the proteins (Fitzgerald & Swaisgood, 1989). It is to be emphasized that ANS does not covalently bind to casein micelles. When it is adsorbed onto proteins, ANS is fluorescent in the UV light. The excitation using the UV laser was performed at 364 nm and the emission of fluorescence was recorded between 450 and 470 nm. κ-carrageenan was covalently labelled with rhodamine isothiocyanate (RITC), according to the slightly modified method of De Belder and Granath (1973). RITC can be excited by a second laser, at a wavelength of 543 nm. The emission of fluorescence was then recorded above 570 nm, so that no superimposition of the two recorded emissions occurred.

PCM was used to complete information obtained by CLSM. PCM uses light diffraction and does not require any modification of the initial samples. PCM observations were performed using a Zeiss Axiophot microscope, with a × 40 objective and an oil immersed × 100 objective.

The samples were placed at 70°C between a preheated (concave for CLSM) slide and a coverslip and sealed to prevent evaporation. Blends were examined at room temperature.

3. Results

3.1. Phase diagrams

The phase diagrams of the different systems were established at 50°C, which is above the conformational transition temperature of the κ-carrageenan in the different ionic media (43–44°C), so the polymer will behave like a random coil in solution. Phase separation was observed 24 h after preparation and subsequent centrifugation.

Fig. 1 illustrates the main features of the phase diagram of a MC–κ-carrageenan-Na^+ mixture in 0.25 M NaCl, pH 7 and at 50°C. The binodal (solid curve) separates the single-phase region from the two-phase domain and was built after direct observation of the phase separation in test tubes (points ●). Upon mixing the two biopolymer systems well

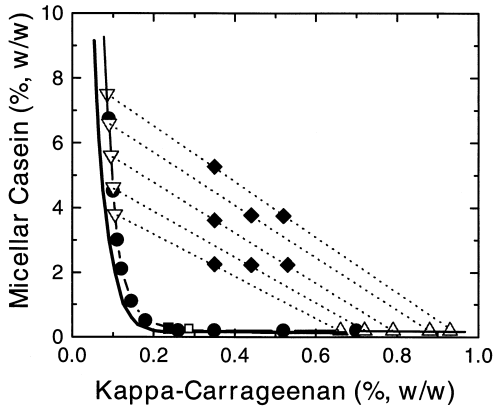


Fig. 2. Phase diagram of the MC–κ-carrageenan-K^+ mixture (0.25 M NaCl, 50°C). Binodal (solid curve, points ●); tie lines (dotted lines); initial mixtures (◆); upper phase (△); lower phase (▽); critical point (■); threshold concentration (□). Comparison with κ-carrageenan-Na^+ (thicker solid curve).

beyond the binodal (points ◆), it was possible to draw the tie-line (dotted line) from the respective composition of the lower phase (points ▽) and the upper phase (points △). Whatever the composition of mixed systems taken on the same tie-line, the composition of the separated phases remains the same, only the respective volumes of the phases change (Tolstoguzov, 1992). The coordinates of the critical point (obtained from the intersection of the binodal to the line joining the middle of the tie lines) were 1.00% of casein and 0.12% of carrageenan. This point (symbolized by ■) represents the composition of a system separating into two phases of the same volume and composition. The threshold concentration (symbolized by □) is defined as the lower total polymer concentration (Cs) leading to the phase separation. In this system it was equal to 0.40% (0.15% casein and 0.25% carrageenan).

Fig. 2 displays the phase diagram obtained with the potassium form of κ-carrageenan, in 0.25 M NaCl. In the presence of the carrageenan-K^+, the area of the single-phase region slightly widened with respect to the sodium

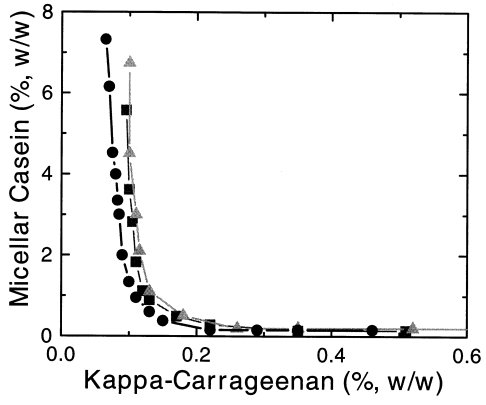


Fig. 3. Comparison of binodals of MC–κ-carrageenan-Na^+ (0.25 M NaCl) (●), MC–κ-carrageenan-K^+ (0.25 M NaCl) (▲) and MC–κ-carrageenan-K^+ (0.05 M NaCl–0.01 M KCl) (■) mixtures at 50°C.

Table 2
Repartition of calcium ions after phase separation

	MC (%)	KC (%)	Ca (mM/g KC)	Ca (mM/g MC)
Initial mixture	2	0.5	3.05	0.76
Upper phase	0.2	0.75	0.21	0.78
Lower phase	5.9	0.08	58.91	0.80
Initial mixture	3	0.3	7.46	0.75
Upper phase	0.2	0.6	0.25	0.76
Lower phase	5.4	0.09	45.69	0.76
Initial mixture	1	0.7	1.06	0.75
Upper phase	0.18	0.8	0.18	0.78
Lower phase	6.7	0.07	73.93	0.77
Initial mixture	1	0.5	1.50	0.75
Upper phase	0.2	0.58	0.27	0.78
Lower phase	5.3	0.09	45.97	0.78

form of carrageenan (thick line). Phase separation occurs thus at higher concentration with the potassium form than with the sodium form, as confirmed by the threshold concentration which corresponds in this case to 0.50% (0.22% of casein and 0.28% of carrageenan) compared to 0.40% for the sodium form. The slope of the tie-lines was slightly lower than the one obtained with the sodium form of the polysaccharide.

Fig. 3 shows the binodal obtained upon mixing carrageenan in the potassium form with MC, but in a different medium, i.e. in 0.05 M NaCl–0.01 M KCl. In the same figure the two previous binodals obtained with the respective sodium and potassium forms of κ-carrageenan in 0.25 M NaCl are represented. It is clearly seen that phase separation did occur in a similar way with the potassium form in both solvents.

Whatever the ionic form of the polyelectrolyte and the ionic composition of the solvent, mixing κ-carrageenan with MC at 50°C leads to a phase separation phenomenon, yielding a casein-rich phase and a κ-carrageenan-rich phase. In the case of κ-carrageenan concentration higher than 0.2%, demixing of the system will occur whenever the casein concentration is above 0.15%. For casein concentration higher than 3%, a low amount of carrageenan (around 0.1%) will induce phase separation and hence the destabilization of the system.

3.2. Repartition of the calcium ions

Casein micelles are known to contain a large amount of calcium which is mostly bound to the micelles via calcium phosphate bridges (Van Dijk, 1990). However, it is possible to complex calcium by a sequestrant, like EDTA (Griffin, Lyster & Price, 1988) leading to partial modification of the structure of the micelle. Indeed, it has been suggested that carrageenan could act as a sequestrant in milk-carrageenan systems, explaining the so-called milk reactivity of carrageenan (Drohan et al., 1997). We estimated the repartition of calcium ions in each phase after phase separation by absorption spectroscopy in order to check whether the

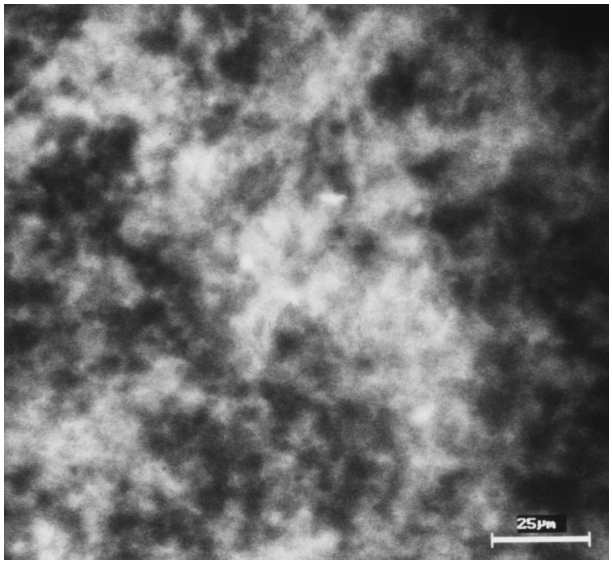


Fig. 4. CLSM observation (364 nm) of a 3% MC (+ ANS)–1% RITC-κ-carrageenan-Na^+ mixture in 0.25 M NaCl at 20°C.

calcium content of casein micelles changes upon demixing. Table 2 shows that the ratio calcium–casein is the same for the initial mixtures as well as for the two separated phases, indicating an even repartition of calcium ions between phases. Moreover, in the upper phase (rich in carrageenan), the calcium content did not vary with the carrageenan concentration, which implies that there is no 'pumping' of the calcium by the carrageenan chains.

3.3. Microstructure of the mixtures

3.3.1. Confocal laser scanning microscopic observations

CLSM acts as an optical scanner, being able to focus on only one plane, of well-defined thickness, in the whole sample, which means that the emission of fluorescence of the rest of the sample does not interfere with the structural information coming from the focal plane.

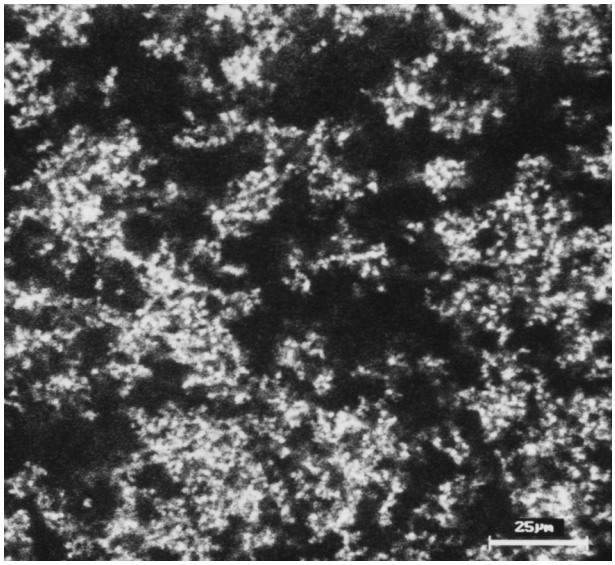


Fig. 5. CLSM observation (543 nm) of a 3% MC (+ ANS)–1% RITC-κ-carrageenan-Na^+ mixture in 0.25 M NaCl at 20°C.

Figs. 4 and 5 correspond to a mixture containing MC (3%) and κ-carrageenan-Na^+ (1%). The double labelling technique was employed, casein being probed with ANS, and carrageenan being covalently labelled with RITC. Fig. 4 was obtained by observation of the system at a wavelength of 364 nm, which is close to the wavelength of the maximum excitation of ANS (372 nm). Clear areas correspond therefore to the fluorescence of ANS, revealing the presence of MC. By contrast, dark areas correspond to the localization of κ-carrageenan, RITC not being fluorescent at this wavelength. In Fig. 5, the same focal plane of the same mixture was observed at the excitation wavelength of RITC (543 nm). Clear areas correspond then to the fluorescence of RITC, allowing localization of carrageenan in the mixture; by contrast, dark zones correspond to the localization of proteins.

The comparison between the two photographs shows that clear areas in Fig. 4 corresponded to the dark ones in Fig. 5 and inversely. It is thus possible to localize very precisely each biopolymer in the mixture according to the chosen wavelength. Moreover, we can observe that the two macromolecular components are distributed into two separated phases. There are no areas devoid of polymer or areas containing both biopolymers.

The following observations have only been performed in the UV light, indicating directly the presence of casein micelles in three different casein–carrageenan systems.

Fig. 6(a), (b) and (c) correspond to the observations of mixtures containing 0.5% of κ-carrageenan-Na^+ and respectively, 0.1%, 3% and 5% of MC labelled with ANS in 0.25 M NaCl at 20°C. In Fig. 6(a) an irregular repartition of the casein micelles within the mixture was exhibited. Proteins appeared concentrated in small droplets of size ranging from 1 to 5 µm. Carrageenan, in dark areas, constituted the continuous phase of the system. When the casein content was higher (3%, Fig. 6(b)), the size of the fluorescent zones increased up to 10–100 µm. In Fig. 6(c) (MC 5%), areas concentrated in casein were spread all over the picture. Two phases coexisted obviously in all these mixtures, one containing mainly MC whereas the other one was enriched in κ-carrageenan.

The ultrastructure at 20°C of mixtures in 0.25 M NaCl containing 0.5% κ-carrageenan-K^+ and increasing concentrations of casein (0.5, 3 and 4%) is shown in Fig. 7(a)–(c), respectively. In Fig. 7(a), the repartition of casein was similar to the system containing the sodium form of the carrageenan at a low casein content (Fig. 6(a)). Caseins were organized in small droplets (1–5 µm), regularly distributed in the mixture. At higher casein contents (Fig. 7(b) and (c)), the structure of the casein-rich areas was slightly different from the previous ones. Casein was located in droplets, as for the previous system, but they did not tend to form larger areas, rather giving rise to a more 'granular' structure to the

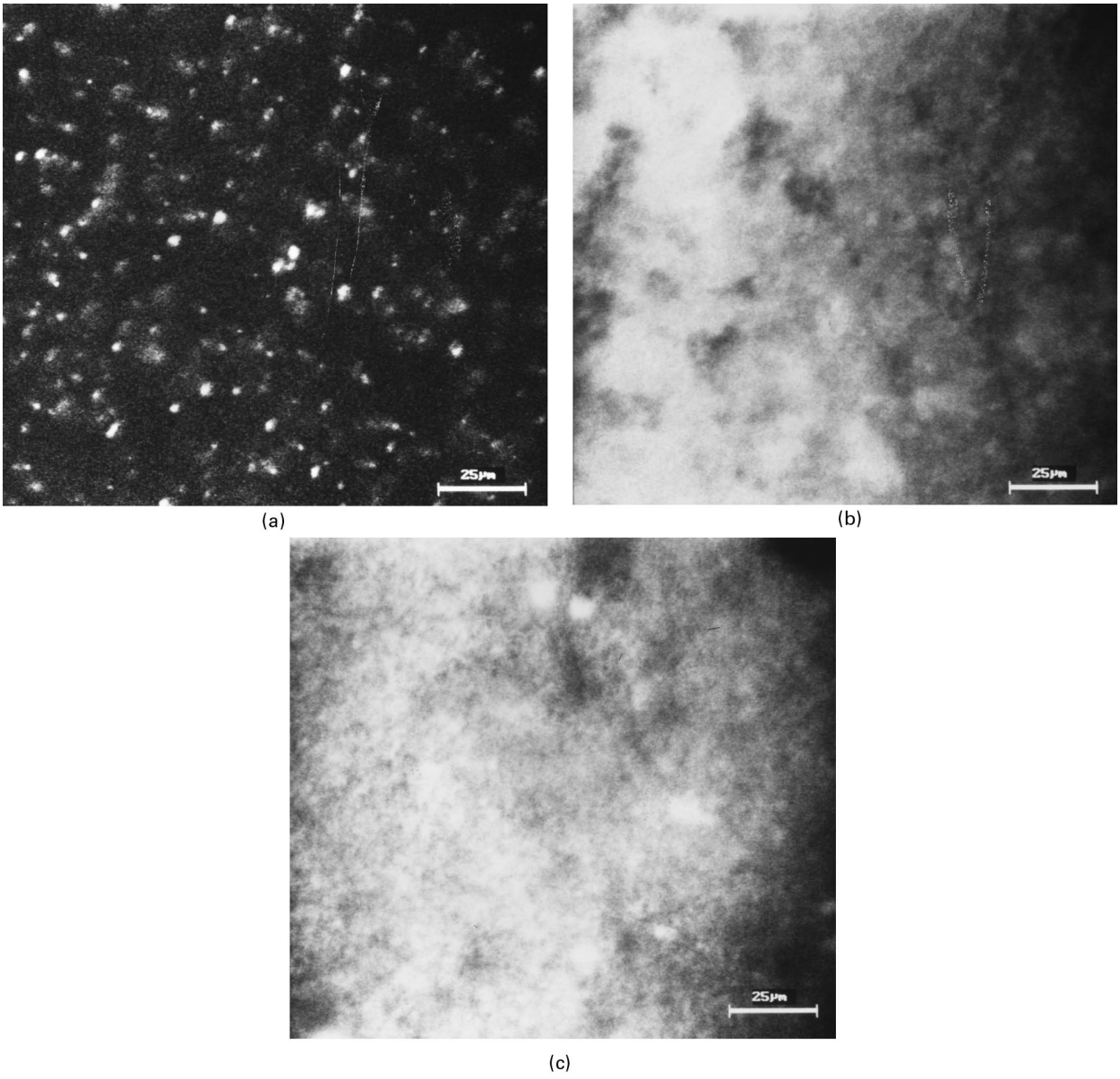


Fig. 6. CLSM observations (364 nm) of 0.5% κ-carrageenan-Na^+ mixtures containing 0.1% (a), 3% (b) and 5% (c) of MC (+ANS) in 0.25 M NaCl at 20°C.

picture. In the same way, the two biopolymers were distributed into two separated phases, each being enriched in one of the components.

Fig. 8 (a)–(c) correspond to the observation of MC–0.5% κ-carrageenan-K^+ mixtures in 0.05 M NaCl–0.01 M KCl, containing 0.1, 3 and 5% of casein, respectively. The structures appeared to have the same features as for the mixtures prepared with the same ionic form of the polysaccharide in 0.25 M NaCl and two phases obviously coexisted in the system.

3.3.2. Phase contrast microscopic observations

CLSM observations required labelling of the MC and/or of the carrageenan by a fluorescent probe. To determine if the labelling of the sample disturbed the ultrastructure, PCM observations were performed. Since ANS is adsorbed in the hydrophobic zones of the casein, labelling allowed the respective distributions of the two biopolymers to be determined but the structural details of the protein organization could not be described. Since PCM is based on the difference between refractive indices of the structures in presence, the global structure of the system across the whole sample thickness is characterized by this technique.

Fig. 9 shows a 3% casein suspension in 0.25 M NaCl at 20°C, which is roughly the concentration of casein in milk. At this concentration, casein, which appears in relief in the picture, is quite regularly dispersed in the medium. However, it should be noticed that individual micelles, having sizes ranging from 20 to 600 nm, cannot be

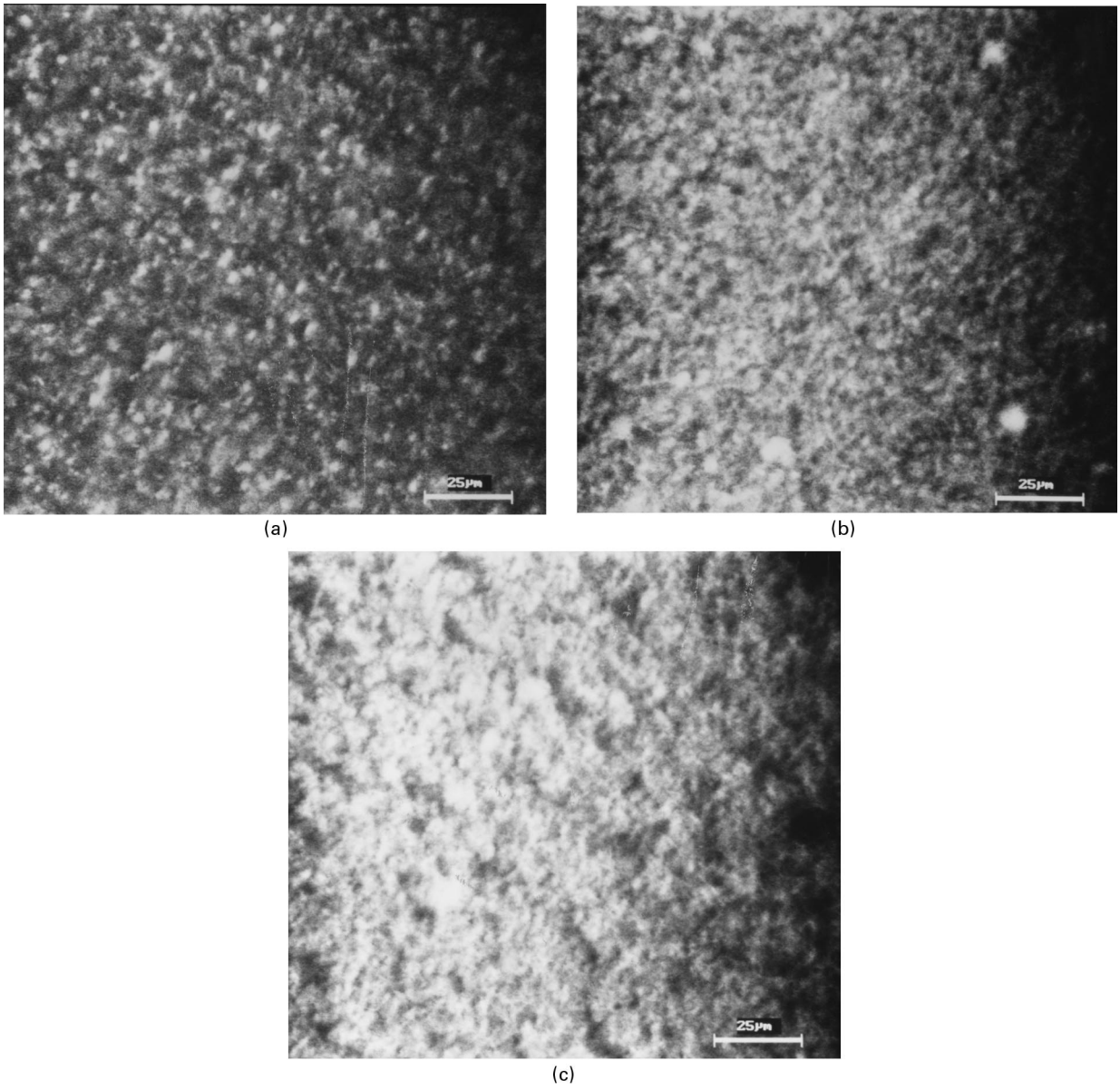


Fig. 7. CLSM observations (364 nm) of 0.5% κ-carrageenan-K^+ mixtures containing 0.5% (a), 3% (b) and 4% (c) of MC (+ANS) in 0.25 M NaCl at 20°C.

distinguished in the picture, because of the resolution of the method (in the range of μm).

The effect of addition of 0.5% κ-carrageenan-Na^+ to the casein suspension is shown in Fig. 10(a). This deals with the same system as the one presented in Fig. 6(b). In the same way, we can observe an irregular repartition of casein micelles in the medium. These appeared concentrated in areas of various sizes (10–100 μm). Zones devoid of casein appeared in the picture as 'smooth zones'. The comparison of this picture with Fig. 6(b) allows these areas to be attributed to the presence of carrageenan. Fig. 10(b) corresponds to the same mixture, observed at a higher magnification. From this picture, it can be seen that casein is closely packed in the concentrated areas, suggesting that they are aggregated. These photographs confirm that two phases coexisted in the mixture, one containing mainly casein and the other being enriched with carrageenan.

The ultrastructure of casein-0.5% κ-carrageenan-K^+ mixtures in 0.25 M NaCl containing 0.5% of casein (Fig. 11(a)) and 3% of casein (Fig. 11(b) and (c)) is presented. Fig. 11(a) (MC 0.5%) corresponds to the same mixture as that shown in Fig. 7(a). Casein appeared concentrated in small aggregates (1–5 μm) which were regularly dispersed in the carrageenan continuous phase. In Fig. 11(b), the aggregation phenomenon was more pronounced and led to the formation of very large areas enriched with casein that seemed to form a continuous network in the mixture. The granular structure observed by CLSM (Fig. 7(b)) is confirmed. Fig. 11(c) shows the same mixture at a higher magnification. It can also be clearly seen that casein micelles were concentrated in areas that were dispersed

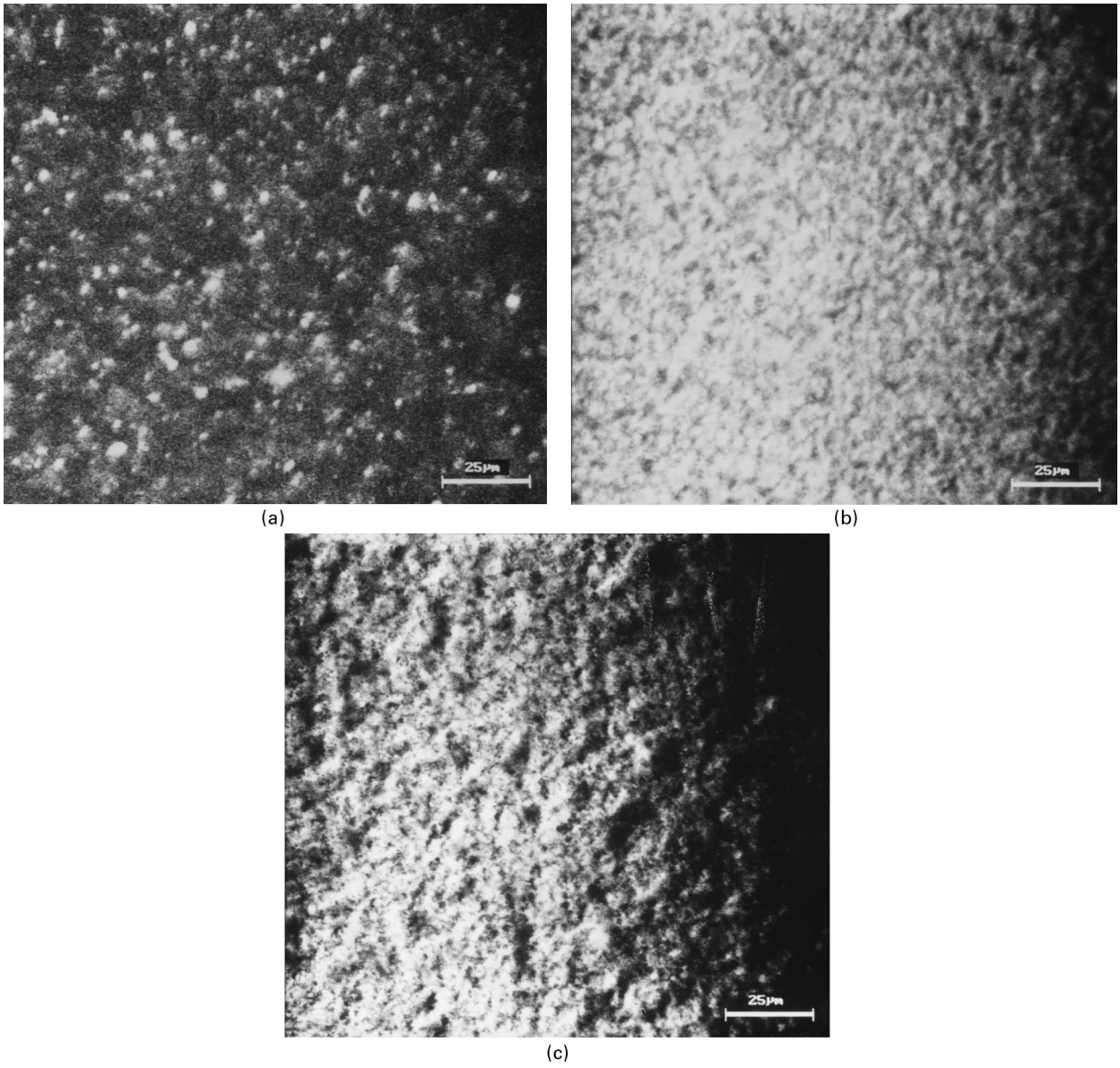


Fig. 8. CLSM observations (364 nm) of 0.5% κ-carrageenan-K^+ mixtures containing 0.1% (a), 3% (b) and 5% (c) of MC (+ANS) in 0.05 M NaCl–0.01 M KCl at 20°C.

Fig. 9. PCM observation of a 3% MC suspension in 0.25 M NaCl at 20°C.

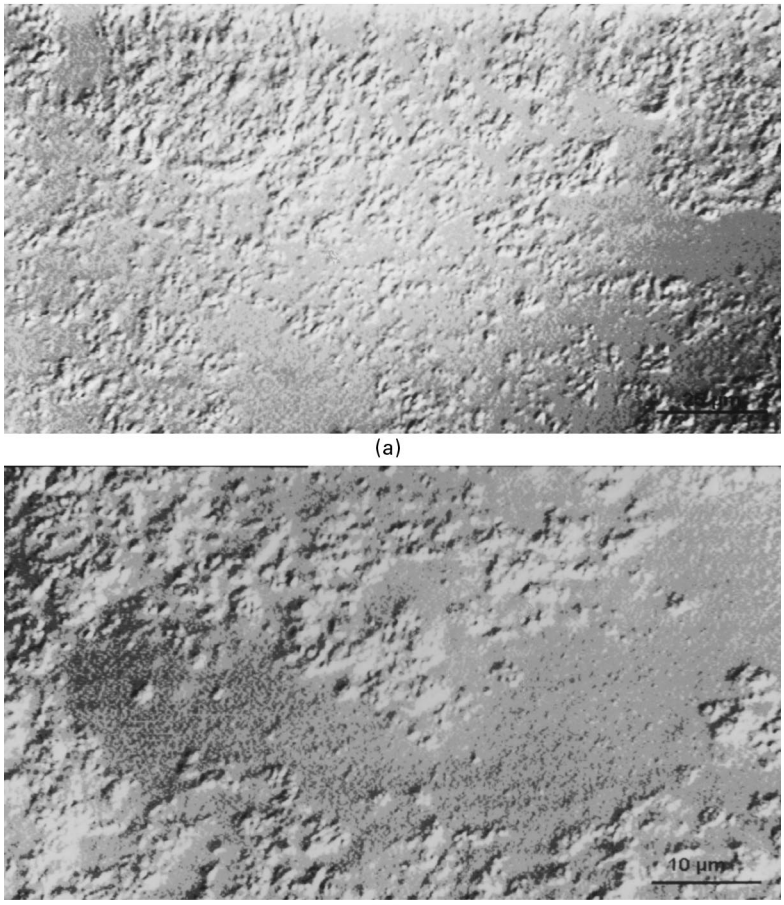


Fig. 10. PCM observations of a 3% MC–0.5% κ-carrageenan-Na^+ mixture in 0.25 M NaCl at 20°C.

in the carrageenan phase, devoid of casein. As previously observed, phase separation had obviously occurred.

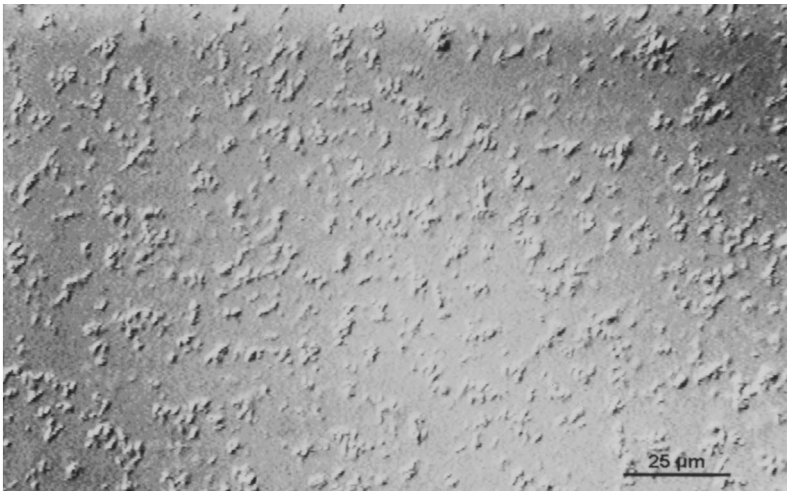
Fig. 12 corresponds to the observation of a 3% casein suspension in 0.05 M NaCl–0.01 M KCl. As in 0.25 M NaCl, casein micelles appeared to be regularly dispersed in the medium: the change in ionic strength of the medium does not seem to modify the stability of the suspension.

The ultrastructure of a 3% casein–0.5% κ-carrageenan-K^+ mixtures in 0.05 M NaCl–0.01 M KCl is illustrated in Fig. 13(a) and (b). It can be seen that the change in ionic strength did not affect the global structure of the mixture, which is consistent with the CLSM observations (Fig. 8(b)). Casein micelles were aggregated in large areas, yielding a continuous network in the system.

These observations show that labelling of the biopolymers does not change the global structure of the system. It is obvious that casein–carrageenan mixtures tend to be phase separated at 20°C, for all the ionic forms of κ-carrageenan and ionic strengths of the medium studied. Moreover, above a certain casein content in the mixture, casein micelles seem to form the continuous phase of the system.

4. Discussion

At 50°C, for the different systems investigated, segregative phase separation occurred (Figs. 1–3), leading to the formation of a casein-rich phase and a κ-carrageenan-rich phase. At this temperature, the sodium or potassium forms of κ-carrageenan behave as random coil macromolecules, whatever the ionic strength. Clearly, casein and carrageenan do not coexist in the same phase within the mixture when κ-carrageenan molecules are in the disordered state. It can be noticed that the binodal of the two systems containing K^+-κ-carrageenan are superimposed, in spite of the difference in total ionic strength. The binodal corresponding to the system containing Na^+-κ-carrageenan was very close to the previous ones but the area of the monophasic zone was slightly smaller. Demixing may be ascribed to a depletion–flocculation process, since the casein micelles can be regarded as spherical particles. This process is induced by the presence of carrageenan molecules in the casein suspension, as it has been already shown when other polysaccharides, charged or not, are added to a casein suspension (Bourriot et al., 1997; 1999a,b; Tuinier & De Kruif, 1997). Phase separation by depletion–flocculation is due to the difference in osmotic pressure between the solvent



(a)

(b)

(c)

Fig. 11. PCM observations of 0.5% κ-carrageenan-K^+ mixtures containing 0.5% (a), 3% (b and c) of MC in 0.25 M NaCl at 20°C.

and the space between the particles from which the polymer has been excluded. The high osmotic pressure in the bulk polymer solution leads the particles to be pushed together and thus aggregated (Asakura & Oosawa, 1954; 1958). According to this mechanism, κ-carrageenan would be excluded from the space between the micelles when the casein concentration is high enough. A simple way to confirm that particles are weakly flocculated is to dilute an

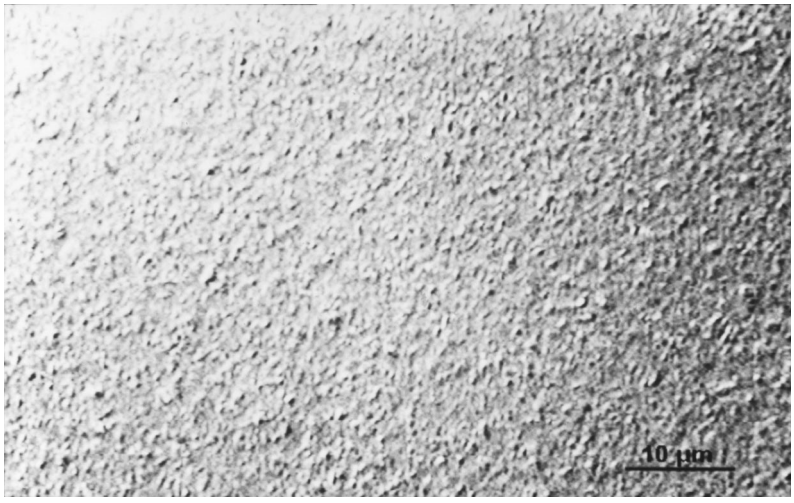


Fig. 12. PCM observation of a 3% MC suspension in 0.05 M NaCl–0.01 M KCl at 20°C.

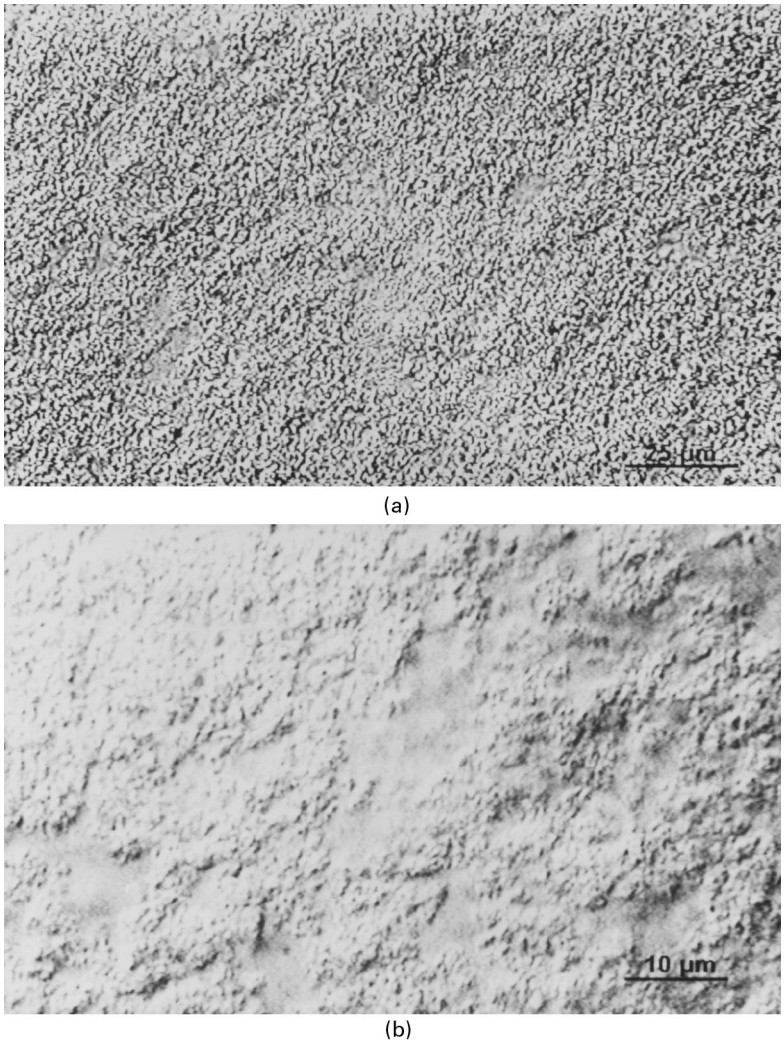


Fig. 13. PCM observations of a 3% MC–0.5% κ-carrageenan-K^+ mixture in 0.05 M NaCl–0.01 M KCl at 20°C.

aggregated sample after macroscopic phase separation (Patel & Russel, 1989). Indeed, in the case of a phase separated casein–carrageenan system, this led to a single-phase system, indicating that the process is reversible, which is consistent with the depletion–flocculation mechanism.

Synergism at 20°C between carrageenan and MC is generally ascribed in the literature to specific interactions between the two biopolymer species. This is the case of a specific linkage between κ-carrageenan and casein micelles via electrostatic bond that was first proposed by Snoeren (1976). He assumed that the positive zone of the κ-casein was easily accessible. However, it has been evidenced since that this region is located in the hydrophobic zone of the κ-casein. It is to be emphasized that this model implies the penetration of the κ-carrageenan chains into the hairy layer of the micelle (globally negatively charged as the carrageenan) to allow the polysaccharide to interact with the positively charged region of the κ-casein. This appears unlikely for electrostatic and steric reasons. In the case of casein–ι-carrageenan mixed systems, it has been suggested (Langendorff et al., 1997) that casein micelles must be first covered by carrageenan chains before phase separation takes place, which would imply both binding through specific interactions and a depletion–flocculation mechanism. Dalgleish and Morris (1988) have reported the occurrence of a precipitation when milk was added to κ-carrageenan chains in the ordered form, which may be consistent with a depletion–flocculation mechanism. However, they suggested that this precipitation would arise from extensive cross-linkages of the casein micelles with the ordered polysaccharide chains. However, it seems unlikely that flexible κ-carrageenan could adsorb onto casein micelles at 50°C, and thus it is all the more difficult to imagine that κ-carrageenan molecules could bind to casein micelles when they are more rigid and form double helices. It is therefore of primary interest to describe the ultrastructure of casein–carrageenan systems at room temperature. This allows the distribution of macromolecular components in the medium to be described with more accuracy. If at 50°C, phase separation clearly took place in the mixture, the mixed system appeared homogeneous at 20°C and stable from a macroscopic point of view. According to the present observations by CLSM as well as by PCM, it appears however that the mixtures are also phase separated with a casein-rich phase and a κ-carrageenan-rich phase, provided the polymer concentration is high enough. It is likely that the phase separation process initiated when the carrageenan was in the disordered state could have been halted by the gelation of the polysaccharide upon cooling below its coil–helix transition temperature. This explains why a macroscopic phase separation was not obtained. The kinetics of cooling, and then of carrageenan gelation, will compete with the kinetics of the phase separation process and will determine to a large extent the structure of the system and hence its properties. Although the gelation temperature could not be determined exactly by rheological means because of the phase separation process, this is expected to be around 26–30°C, whatever the ionic conditions (from DSC measurements, to be published). The mixtures being prepared at 70°C and cooled down at 20°C quite slowly, this gives time for the phase separation to take place before the system freezes in.

Our microscopic observations did not show large amounts of casein micelles in the κ-carrageenan-rich phase. This was observed through CLSM which allowed both biopolymers to be detected (Figs. 4 and 5). This showed that the two biopolymers were located inside two different microscopic phases. Also, PCM observations indicated that the κ-carrageenan-rich phase contains very little casein, with the presence of the ‘smooth’ and the ‘rough’ zones, corresponding, respectively, to the carrageenan phase and the casein phase. This can be related to the phase diagram obtained at 50°C, where the κ-carrageenan-rich phase was nearly devoid of casein, with a protein content lower than 0.15%. Therefore, the present observations do not provide any direct information on the postulated occurrence of specific interactions between casein and κ-carragenan, but it appears clear that the major event taking place in these mixed systems is related to phase separation phenomena.

5. Conclusions

In view of the present results, it is clear that phase separation occurred in different casein–carrageenan systems at 50°C as well as at 20°C, provided the biopolymer concentration was high enough. Above the transition temperature of the κ-carrageenan, when chains are disordered, the macroscopic phase separation is completed whereas it is limited at room temperature due to the gelation of the carrageenan. In both cases, phase separation leads to the formation of a casein-rich phase and a κ-carrageenan-rich phase. Above a certain amount of casein in the mixture (3%), the casein-rich phase forms a continuous network within the system. Demixing can be ascribed to the aggregation of casein micelles probably due to a depletion–flocculation phenomenon induced by the presence of the κ-carrageenan chains in the suspension of MC. The effect of such mechanisms on the rheology and thermal behaviour of casein–κ-carrageenan systems will be discussed in a forthcoming paper.

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