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A new CSLM-based method for determination of the phase behaviour of aqueous mixtures of biopolymers

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On mixing different types of high molecular weight (bio)polymers in an aqueous solution, phase separation often occurs. In some cases, the occurrence of phase separation may be readily observed, because due to density differences the heavier of the two phases is accumulated at the bottom of the vessel in which the mixture is contained. By using classical techniques, the composition of the two phases may then be determined. In the case where the density differences are not so large, and the viscosity of the system is high, the two phases remain intimately mixed. An alternative route to determine the phase behaviour of these systems might be a microscopic technique (Confocal Scanning Laser Microscopy, CSLM), using the fluorescence intensity of labelled biopolymers to quantify their concentration and phase volume in the system. Experiments were performed with several mixtures of sodium alginate, labelled with fluorescein, and sodium caseinate, fluorescently labelled with Texas Red. The viscosity of the mixtures studied was low enough to allow bulk phase separation of the phases by using an ultracentrifuge. Results of the phase volumes, and the composition of the phases, obtained independently by applying the two different methods (CSLM, or analysis of the separate phases after centrifugation) were compared and found to be in reasonable agreement. Copyright © Elsevier Science Ltd

INTRODUCTION

Nowadays the fat in food products like spreads and dressings is being (partly) replaced by biopolymer solutions to produce low caloric foods. Where traditionally the fat present in most foods determined the physical properties of the products to a large extent, now these specific product properties have to be obtained by biopolymers such as proteins and carbohydrates.

Generally mixtures of biopolymers are applied to mimic the specific properties of the fat in the food systems (Kasapis *et al.*, 1993; Cain *et al.*, 1989). As the biopolymers used are large molecules with a high molecular weight, the mixing behaviour of the biopolymers is quite different from that of low molecular weight species. Dissimilar high molecular weight substances generally do not mix on a molecular scale but rather give rise to phase separation. In that case a dispersion consisting of two different types of aqueous phases is formed in which each phase mainly contains one type of biopolymer. Variation of the type and concentration of the polymers in such mixtures affects the phase volume and composition of the separate phases, and thus provides a means for manipulating the rheological properties of the system.

In order to understand better the properties of biopolymer systems, their phase behaviour should be known. Knowledge of this behaviour would be helpful in the design of new low/zero fat foods.

Biopolymer mixtures of interest for application in food products generally contain highly viscous and gelled phases. The phase behaviour of such systems cannot be investigated either by the classical method of centrifugal separation of the phases and subsequent analysis of them (Tolstoguzov, 1990), or by determination of the osmotic pressure of the biopolymer solutions (Koning *et al.*, 1993). However, an alternative method for analysing the phase behaviour of these mixtures might be a method which employs Confocal Scanning Laser Microscopy (CSLM) (Blonk & van

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Aalst, 1993). This technique probes & local concentration of biopolymers in the dispersed and continuous phases by means of fluorescent labels that are coupled to the biopolymer molecules. The use of different fluorescent labels for different types of biopolymers enables discrimination of different biopolymers. The CSLM technique can be applied to a gelled biopolymer system *in situ*, i.e. the biopolymers in the dispersed and continuous phase can be identified and quantified without the need for bulk phase separation as is usual in the classical method. To explore whether the application of CSLM provides a suitable method for determining phase diagrams of viscous and gelled biopolymer mixtures, experiments were performed on aqueous solutions of mixtures of fluorescently labelled sodium caseinate and sodium alginate. These mixtures were selected because of previous publication of data on their phase behaviour (Antonov *et al.*, 1980), and because the limited viscosity of these systems allowed bulk phase separation by centrifugation. Verification of the phase diagram established by CSLM was arrived at by comparing the CSLM data for concentrations and phase volumes with data obtained by application of the classical method.

CSLM and phase separated biopolymer mixtures

In the confocal microscope a laser beam is focused into a small volume element in a sample (Wilson, 1990). When the sample contains suitable chromophores, the laser light may be absorbed. Part of the energy absorbed in this way may subsequently be released in the form of fluorescence having a longer wavelength than that of the laser beam. Fluorescence originating from this irradiated volume element is separated from the scattered laser light by a suitable filter, and then focused into a diaphragm in front of a detector. The laser beam passes through the confocal plane step by step, and the fluorescence intensity in each volume element is monitored. The value of the fluorescence intensity is stored as a pixel value in a digital image which is constructed from the scanned area in the sample. The image can be stored in a computer and analysed later on. By reducing the size of the diaphragm the thickness of the sample slice from which light is collected is reduced, resulting in a sharper image of the 'optical section'. Depending on diaphragm size and numerical aperture of the objective used, the thickness of the optical section ranges from a few tenths up to a few micrometres.

In order to detect and quantify the biopolymers with the CSLM, they are coupled with fluorescent labels, having a different emission spectrum so that by using appropriate filters, they can be discriminated. Aqueous mixtures are made of the two types of labelled biopolymers and phase separation is allowed to take place. After the system has reached equilibrium, the sample is positioned onto the CSLM and two images are acquired, one for each of the two fluorescent labels. Assuming a linear relation between the detected fluorescence signal and the concentration of the fluorescent species, the ratio of the fluorescence intensities in the dispersed and continuous phase is a measure of the ratio of the concentrations of the biopolymer in the dispersed and continuous phase.

MATERIALS AND METHODS

Materials

Sodium alginate (Manucol DM) was obtained from Kelco International. Sodium caseinate was obtained from DMV; its protein content amounts to approximately 90%. Fluorescein isothiocyanate (FITC) was obtained from Janssen Chimica, 4'-amino fluorescein and Texas Red were obtained from Aldrich and Molecular Probes, respectively. The excitation and fluorescence spectra of fluorescein and Texas Red are shown in Fig. 1.

Sodium alginate was labelled with fluorescein according to the following procedure. Sodium alginate (5 g) was dissolved in 400 ml water. To the stirred solution concentrated sulphuric acid (96%) was added in drops in order to precipitate the alginic acid. The mixture was centrifuged and the pellet was washed and centrifuged twice with water and twice with acetone. The faintly brown powder was dried *in vacuo* in the presence of diphosphorpentoxide.

The resulting 3 g of alginic acid (18 mmol monosaccharide) was mixed with 80 ml water, 30 ml 1,4-dioxane, 50 mg (0.14 mmol) 4'-aminofluorescein and 1 g (7.8 mmol) EDC (1-[3-dimethylaminopropyl]-3-ethyl-carbodiimide hydrochloride; ex Janssen Chimica). The mixture was stirred for 3 h and left overnight. The colour slowly turned orange. Not all alginic acid had

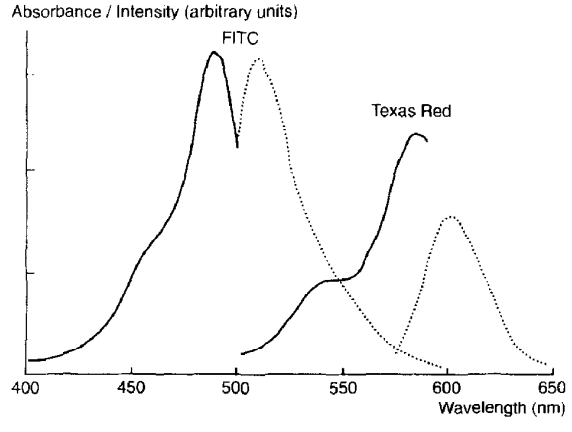

Fig. 1. Excitation (solid lines) and fluorescence (dotted lines) spectra of the fluorescent labels used in this study. The wavelengths of the Ar/Kr mixed gas laser are 488 and 568 nm.

dissolved when the reaction was stopped. The mixture was filtered and washed with acetone until the acetone was colourless (three times). The powder was dried to yield 3 g product. The degree of substitution was determined using UV–vis spectroscopy (490 nm, Perkin Elmer Lambda 3B equipment), and found to be 0.008, i.e. 8 molecules of fluorescein per 1000 sugar units. The labelled alginate was applied at a concentration of 0.3% (w/w) to the Sodium-alginate solutions, which corresponds to $1*10^{-5}$%(w/v) fluorescein.

Sodium caseinate was labelled with FITC or Texas Red by addition of an aqueous solution of the fluorescent label to the protein solution at 45°C, just before mixing the protein and alginate solutions. FITC was applied at a concentration of 0.01% (w/v), while Texas Red was used at a concentration of 0.002% (w/v). These concentrations were kept constant during the experiments irrespective of the protein concentration used.

Preparation of the biopolymer mixtures

Sodium caseinate was dissolved in 0.1 M NaOH at 45°C; sodium alginate was dispersed in 0.1 M NaOH at room temperature and then heated to 70°C.

Both biopolymer solutions were mixed in a ratio of 1:1 at 45°C. The solution of sodium alginate was stirred with a turbine stirrer, while the solution of sodium caseinate was slowly added within about 15 min, meanwhile increasing the stirrer speed. Stirring was continued for another 15 min at 45°C and additionally 30 min at room temperature. The size of the sample was typically 100 g.

After mixing, 6 Beckman centrifuge tubes of 10 ml each were filled with the solution. These tubes and the remaining liquid were stored for at least 24 h at room temperature before centrifuging and CSLM analysis.

Classical analysis of the biopolymer mixtures

The two aqueous phases in the biopolymer mixtures were separated by ultracentrifugation using a Beckman centrifuge and a swing-out type of rotor (SW41TI). The separation took place at room temperature at a rotor speed of 36,000 rev/min corresponding to 100,000–225,000 *g*. Complete separation took from 1–16 h dependent on the viscosity of the mixture. After centrifugation the volume of both phases was determined by making a mark on the outside of the tubes, one at the interface of the liquids, and one at the upper liquid level. Using these marks the volumes of both phases were established — after the tubes had been emptied and filled with water — by weighing the amount of water corresponding to the marked volumes of the two phases.

The concentration of sodium caseinate and that of sodium alginate in the separated phases of the mixtures were determined in the following way. The protein content was measured using the Kjeldahl method (Kjeldahl, 1883). This implies determination of the nitrogen content of the samples, and multiplication of the number obtained by 6.38. The sodium alginate concentration was determined using the Clegg–Anthron method (Clegg, 1956).

CSLM analysis of the biopolymer mixture

Observations were made using a BioRad MRC600 Confocal scanning Laser Microscope equipped with an Ar/Kr mixed gas laser emitting at 488 and 568 nm. Suitable filter settings were used to perform a double labelling experiment. Based on the size of the dispersed phase, microscope objectives were used having magnifications of 20 or 40 times. The detection system of the CSLM was used in photon counting mode which enables sensitive and accurate measurement of the fluorescence signals. A cavity in a glass sample holder was filled with the biopolymer mixture and a cover glass was put on the sample. The mixtures were stirred gently prior to sampling.

In Fig. 2 two images are shown which were obtained for a mixture containing 7.2% Texas Red-labelled sodium caseinate and 2.0% fluorescein-labelled sodium alginate (sample code J in Table 2). The image on the right-hand side of Fig. 2 shows the distribution of sodium alginate. The grey level histograms of the images in Fig. 2 are given in Fig. 3. Both grey level histograms exhibit two well-separated peaks representing the concentrations of the biopolymers in the different phases. The grey level histogram in each of the phases does not show one distinct value for each of the two concentrations in the two phases, but rather shows a distribution around a mean value.

Near the interface of the dispersed droplets in Fig. 2, a transition region from light to dark can be observed. The occurrence of this transition region is due to the limiting resolution in the *z*-direction rather than to a gradual change in concentration of biopolymers on the interface of the dispersed droplets and the continuous phase. Optical sections obtained near the poles of the dispersed droplets will show a broad diffuse interface (compare the small feature right of the large droplet in Fig. 2). As a consequence of the limited resolution, the fluorescence intensity of the individual phases has to be determined in regions at a sufficient distance from the interface and in an optical section that is not close to the pole of a dispersed droplet. The intensity is measured in a number of regions per image. The average fluorescence intensities of the different labels in the distinct phases are used to calculate the concentration of the biopolymers.

Calculation of the relative volumes of the two phases from the experimentally obtained images and grey level histograms involves estimation of the pixel intensity

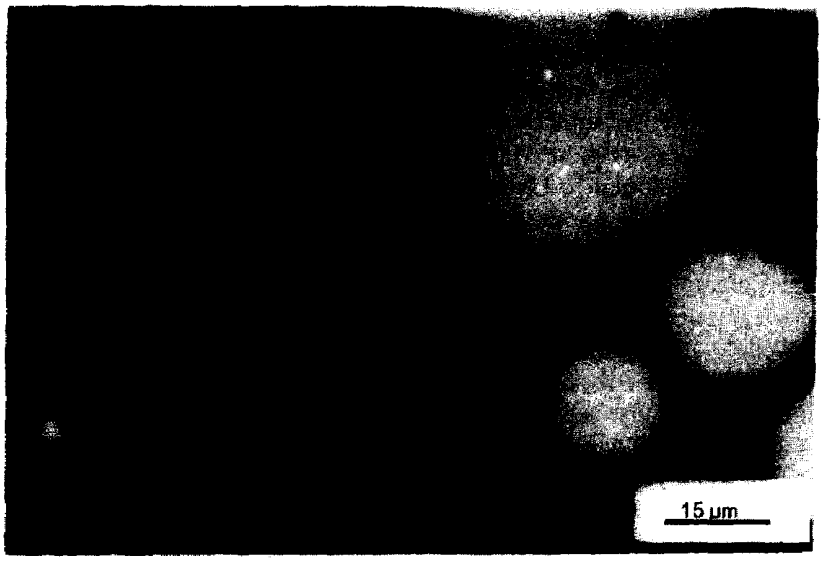

Fig. 2. CSLM images of a phase separated system containing 7.2% (w/w) Na-caseinate and 2.0% (w/w) Na-alginate in water. Left image shows the fluorescence intensity of Texas Red (= Na-caseinate); Right image shows the fluorescence of fluorescein (= Na-alginate).

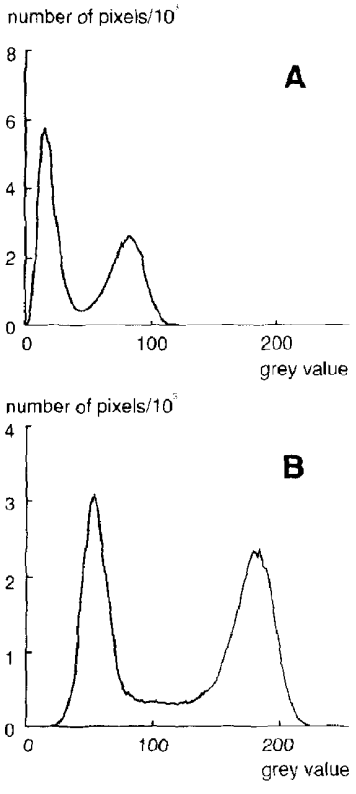

Fig. 3. Grey level histograms of the two images in Fig. 2. A. Intensity distribution of the Texas Red fluorescence, obtained from the left image. B. Intensity distribution of the fluorescein fluorescence, obtained from the right image.

which can be considered as the threshold between the two phases. Calculation of the relative volumes of the phases then follows by attributing all pixels having a grey level intensity below the threshold value to one phase, and all pixels having a grey level intensity above the threshold value to the other phase. The threshold is set manually by shifting the threshold value between the two peaks in the grey level histogram until, from judging the image, a proper selection is made in which the threshold value corresponds to the interface between the dispersed and continuous phase.

In the CSLM image a dispersed phase (d) and a continuous phase (c) may be distinguished. The volume fraction of both phases is denoted by Φ_d and Φ_c, respectively. A linear relation between the concentration C of the fluorescent label and its fluorescence intensity I is assumed: $C = K*I$, where K is a constant. The concentration of the fluorescent label in the dispersed and continuous phase and its average value in the mixture is denoted by C_d, C_c and C_a, respectively.

The concentration of the fluorescent label, which is considered to be proportional to the concentration of the biopolymer to which it is coupled, in the individual phases is given by:

$$\begin{aligned} C_d &= K*I_d \\ C_c &= K*I_c \\ C_a &= K*I_a \end{aligned} \tag{1}$$

The value of the constant K can be calculated from:

$$K = \frac{C_a}{I_a} \tag{2}$$

I_a is given by

$$I_a = (I_d*\Phi_d) + (I_c*\Phi_c) \tag{3}$$

Combination of Equations (1)–(3)

$$C_d = \frac{I_d}{(I_d*\Phi_d) + (I_c*\Phi_c)} * C_a \tag{4}$$

RESULTS

Phase diagram established by the centrifugation procedure

Two series of experiments were performed. In the first series, FITC-labelled caseinate and unlabelled alginate were used. In all experiments the overall concentration of sodium alginate was kept constant at 2.0% (w/w), whereas the concentration of sodium caseinate was varied between 1.8 and 10.6% (w/w). Results are given in Table 1.

In the mixture containing 2% (w/w) alginate and 1.8% (w/w) caseinate, phase separation does not take place; the biopolymers are compatible at these low concentrations. Increasing the caseinate concentration leads to phase separation, whereby the volume of the lower (heavier, caseinate-rich) phase increases. In the columns marked 'total' the concentrations given are calculated by multiplying the measured concentrations in the respective phases with the corresponding phase volume. The calculated values for the total concentrations agree well with the known concentrations.

In Fig. 4 the results are presented in a phase diagram, together with literature data for a comparable alginate/caseinate system (Antonov *et al.*, 1980). The phase volume fractions which can be calculated from the data in Fig. 4 correspond well with the values given in Table 1. The phase volume ratio for the volume fraction of the caseinate-rich phase (upper part) and the alginate-rich phase (lower part) in Fig. 4 can be obtained from the relative length of the tie line on both sides of the total concentration (represented by the point of intersection of the tie lines and the horizontal line which corresponds to the constant alginate concentration of 2% (w/w) used in the experiments).

In Table 2 the results of the second series of experiments in which Texas Red-labelled sodium caseinate was mixed with fluorescein-labelled sodium alginate. For comparison, a mixture containing only non-labelled biopolymers was also prepared, and the results are given in Table 2.

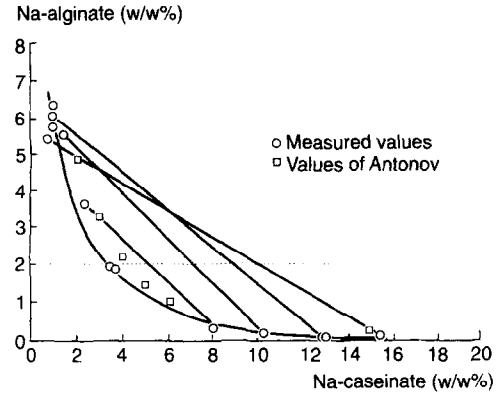

Fig. 4. Phase diagram of Na-alginate/Na-caseinate. The tie lines connect the points representing the compositions of the two coexisting phases. The points of intersection of the tie lines with the dotted line correspond with the overall concentration of the biopolymers.

Phase diagram determined by the CSLM method

Results of the calculations of phase volumes and concentrations of caseinate in the separate phases are given in Table 3. A number of the systems showed separation of phases at macroscopic scale (sample codes marked with an asterisk in Table 3). In this case phase volume determination by CSLM could not be carried out properly. Therefore the concentrations of caseinate in the distinct phases were calculated using the phase volume data obtained by centrifugation.

The sample containing 5.3% (w/w) caseinate (D) showed phase separation during microscopical observation. In Fig. 5 a series of 16 images of this system is shown obtained from various focal planes in the sample; every following image is obtained 10 μm higher in the sample. The lower part of the sample (first

Table 1. Results of phase diagram measurements of the system Na-caseinate/2.0 w/w% Na-alginate in 0.1 M NaOH (fluorescein-labelled sodium caseinate).

Code	Total conc. Na-cas (w/w%)	Phase separation (%)		Conc. Na-cas measured (w/w%)			Conc. Na-alginate measured (w/w%)		
		upper phase	lower phase	upper phase	lower phase	total	upper phase	lower phase	total
A	1.8	100	0	1.8		1.8	2.0		2.0
B	3.5	99	1	3.5	5.0	3.5	2.0	n.d.	2.0
C	4.0	90	10	3.7	6.6	4.0	1.9	n.d.	
D	5.3	49	51	2.4	8.0	5.3	3.6	0.4	2.0
E	7.1	36	64	1.4	10.3	7.1	5.6	0.1	2.1
F	8.8	33	67	0.9	12.7	9.8	6.3	0.0	2.1
G	8.8	35	65	0.9	12.8	8.7	5.8	0.1	2.1
H	10.6	33	67	0.6	15.3	10.4	5.4	0.1	1.8

n.d. not determined.

Table 2. Results of phase diagram measurements of the system Na-caseinate/Na-alginate in 0.1 M NaOH (Texas Red-labelled sodium caseinate and fluorescein-labelled sodium alginate.

Code	Total conc (w/w%)		Phase separation %		Conc. Na-caseinate (w/w%)			Conc. Na-alginate (w/w%)		
	cas.	alg.	upper phase	lower phase	upper phase	lower phase	total	upper phase	lower phase	total
I	9.0	2.0	38	62	0.9	13.8	8.9	5.2	0.8	2.5
J	7.2	2.0	41	59	1.1	11.3	7.1	4.2	0.6	2.1
K	7.2	2.0	39	61	1.2	10.9	7.1	4.2	0.7	2.0
L	3.6	3.0	77	23	2.1	9.3	3.8	3.0	n.d.	n.d.

Note: Biopolymers in sample K are not labelled.
n.d. not determined.

Table 3. Phase volumes and concentrations of FITC-labelled caseinate in mixtures with 2.0% (w/w) alginate. Comparison of the results obtained by CSLM and centrifugation followed by chemical analysis. Sample codes followed by * show results calculated using phase volume data from centrifugation

Code	Bulk conc. cas. (w/w%)	Phase volume (%)		Centrifugation		CSLM		Remark
		upper phase	lower phase	% cas upper	% cas lower	% cas cont.	% cas disp.	
A	1.8	100	0	1.8				1
B*	3.5		1		5.0		5.5	
C*	4.0		10		6.6		4.6	
D*	5.3	49		2.4			3.1	2
E*	7.1	36		1.4			1.6	
G	8.8	39		0.9			2.8	3

1 No phase separation.
2 No stable system; separation in two phases. CSLM results of the casein continuous layer.
3 Duplex system.
Shaded results relate to the continuous phase.

Fig. 5. Z-series of images of the system (coded D) containing 5.3% (w/w) labelled caseinate and 2.0% (w/w) alginate. Images collected at 10 μm intervals in the z-direction starting from the bottom of the sample.

set of 10 images) contains a dispersed concentrated caseinate phase, while in the upper part of the sample (last set of 6 images) the caseinate-rich phase is continuous and the fraction of dispersed phase is very low. The values for the concentrations of caseinate and alginate in the two phases, given in Table 3, represent the mean values resulting from measurement of a number of images collected soon after the sample had been put onto the microscope.

At a concentration of 8.8% (w/w) caseinate in the mixture (G), dispersed caseinate-rich droplets were observed in the dispersed caseinate-poor phase (see Fig. 6). These emulsion systems are called duplex emulsions. Since the specific mass of the inner emulsion droplets will be comparable to that of the continuous phase, it is expected that during centrifugation the inner emulsion droplets will migrate to the caseinate-rich continuous phase.

Results of the second series in which both caseinate and alginate have been labelled are shown in Table 4.

Representative images of these three systems are given in Fig. 7.

DISCUSSION

Quantitative measurements using (confocal) fluorescence microscopy may be influenced by a non-linear relationship between the measured intensity and the concentration of the fluorescent species. The range of fluorochrome concentration, where there is a linear relation between fluorescence intensity and concentration of the fluorescer, has to be established, and on this basis a suitable loading of the component of interest has to be chosen. According to Heertje *et al.* (1990), a linear relation exists between the local fluorescence intensities and the local concentrations of the fluorescing solutes, under the experimental conditions applied in this work.

Since the aqueous alginate–caseinate systems are liquid, the emulsion droplets exhibit a tendency to coalesce and in some cases even to phase separate on a macroscopic scale. When applying a microscopic observation technique to obtain quantitative information on the system, these instabilities in the system have to be recognized. Where spontaneous separation on a macroscopic level occurs, microscopy

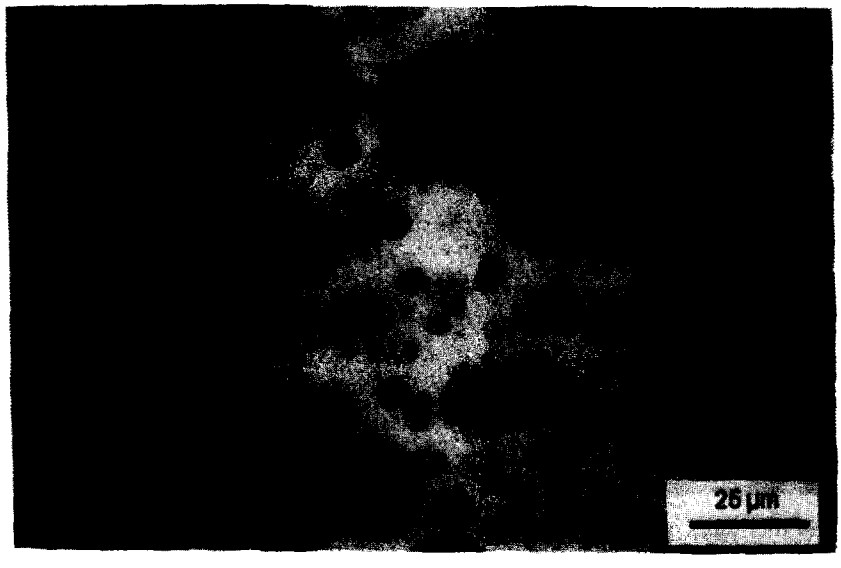

Fig. 6. CSLM image of a phase separated system containing 8.8% (w/w) Na-caseinate labelled with FITC and 2.0% Na-alginate in water. Note the presence of dispersed droplets in the dispersed phase (duplex system).

Table 4. Phase volumes and concentrations of Texas Red-labelled caseinate in mixtures with fluorescein-labelled alginate. Comparison of the results obtained by CSLM and centrifugation followed by chemical analysis. Sample code followed by * show results calculated using phase volume data from centrifugation

Code	Bulk conc. (w/w%)		Disp. phase (%)	Disperse phase conc. (%w/w)				Continuous phase conc. (%w/w)			
				caseinate		alginate		caseinate		alginate	
	cas.	alg.		centr.	CSLM	centr.	CSLM	centr.	CSLM	centr.	CSLM
I	9.0	2.0	38	0.9	2.1	5.2	5.1	13.8	13.2	0.8	0.2
J*	7.2	2.0	40	1.1	2.6	4.2	3.5	11.3	10.3	0.6	1.0
L	3.6	3.0	23	9.3	7.7	n.d.	1.8	2.1	2.4	3.0	3.4

n.d. not determined.

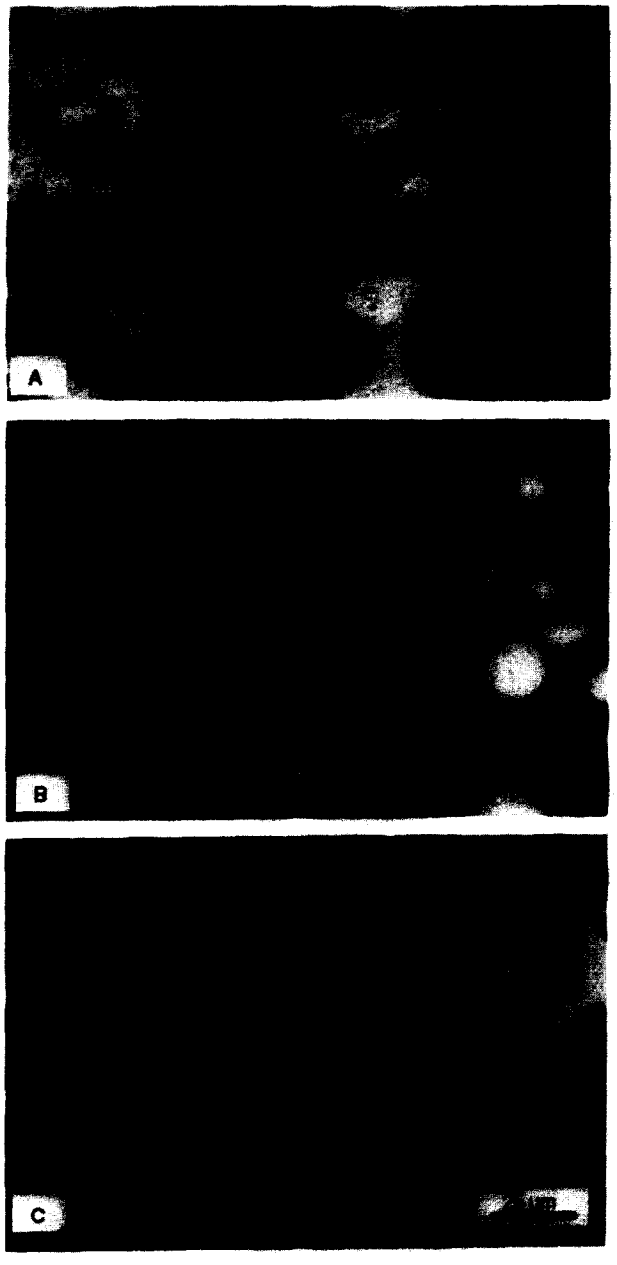

Fig. 7. Galleries consisting of four pairs of CSLM images of a phase separated system. Left image of a pair shows the fluorescence intensity distribution of Texas Red (= Na-caseinate); right image of a pair shows the fluorescence distribution of fluorescein (= Na-alginate). a: 9.0% (w/w) Na-caseinate and 2.0% (w/w) Na-alginate in water; b: 7.2% (w/w) Na-caseinate and 2.0% (w/w) Na-alginate in water; c: 3.6% (w/w) Na-caseinate and 3.0% (w/w) Na-alginate in water.

cannot be used to determine the phase volumes, but these data have to be obtained from centrifugation. Gentle stirring of the separated systems prior to the observation ensures the presence of both phases in the confocal image so that the fluorescence intensities can be measured.

The results of the first and second series obtained by classical analysis and those of the second series using CSLM are plotted in a phase diagram (see Fig. 8). In

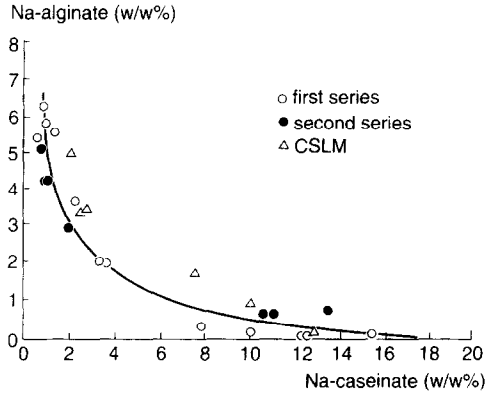

Fig. 8. Phase diagram of fluorescein-labelled Na-caseinate/Na-alginate (centrifugation method) and fluorescein-labelled Na-alginate/Texas Red-labelled Na-caseinate (centrifugation and CSLM methods).

general there is a reasonably good fit of data of the two techniques. Discrepancies between results obtained by the two methods may be explained by the limited accuracy of both methods. It was found that the Clegg–Antron method for the determination of the alginate concentrations is affected by the concentration of the protein in the system. A small, not well reproducible contribution of protein to the measured alginate concentration was found. The alginate concentrations were corrected for this contribution. A better method for the determination of alginate was not available at the time of the experiments. The accuracy of the CSLM data is limited by resolution in these experiments. The boundaries between continuous and dispersed phase are not sharp, which causes uncertanties in the determination of the phase volumes.

A great advantage of the CSLM method is that it offers information about the microstructure of the mixed biopolymer systems which cannot be obtained from the centrifugation method. It can be verified which of the two phases is the dispersed and which is the continuous one. The size distribution of the dispersed droplets and the occurrence of duplicity in the system can be observed. Under proper temperature control, changes in phase volumes and concentrations of the components may be studied.

CONCLUSIONS

It was found that CSLM is a promising technique to study the phase behaviour of highly viscous aqueous mixtures of biopolymers. By labelling the biopolymers with a fluorescent marker, the different types of biopolymers in the dispersed and continuous phases can be distinguished and their relative concentration quantified.

The volume ratio of the dispersed and continuous phase can be determined satisfactorily for stable systems. For sodium alginate/sodium caseinate mixtures

(stable with respect to their microstructure), the data obtained for the phase behaviour of the mixture by CSLM were in reasonably good agreement with the values obtained using the classical method.

For less viscous systems, the classical method has advantages over the CSLM method. Less viscous systems exhibit a higher tendency to phase separate on a macroscopic scale, which causes uncertainties as to the relevance of the microscopically observed pictures. The phase volume ratio is especially prone to errors.

Localization by CSLM of labelled biopolymers in a mixed aqueous system also allows observation of dynamical and structural features of the particular system under study; in one case duplex emulsions were found, and in another inversion of phases was observed when moving the focal plane from the bottom to the top of the sample.

Suitable fluorescent labelling of the biopolymers is the most time-consuming aspect of using the CSLM method. It should be carefully checked that the label is and remains bound to the biopolymer of interest. Recording of the data and image analysis are fast.

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