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Compositional mapping of mixed gels using FTIR microspectroscopy

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We have developed a technique to produce compositional maps of phase-separated protein/polysaccharide mixed gels using Fourier transform infrared (FTIR) microspectroscopy. The maps plot out the composition of either the protein, the polysaccharide or the water as a function of position in the sample and are presented in the form of two-dimensional contour plots. Our technique is completely general in nature, since it simply relies on there being some measurable spectral difference between the two biopolymers. However, in this paper we use our technique to study the particular case of aqueous gelatin/amylopectin gels.

Semi-quantitative compositional maps were generated in the first instance by simply plotting the area of the infrared amide II absorption band from the gelatin. Fully quantitative compositional maps, in terms of actual weight percentage concentration of gelatin, amylopectin and water, were also produced by analysing a particular region of the spectra with the method of partial least-squares (PLS).

We recently showed how PLS analysis can be used in conjunction with FTIR spectroscopy to plot the phase diagram of bulk phase-separated solutions which are held above the gel temperature of both components. Thus, our mapping technique allows the concentrations in a gel to be directly compared with those reached at equilibrium in the bulk phase-separated solution, using the same molecular probe, namely, infrared radiation. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Whilst many different techniques have been successfully used to measure concentration in aqueous mixed protein/polysaccharide solutions which have bulk phase-separated into two liquid layers, few attempts have been made to measure concentration in phase-separated mixed protein/polysaccharide gels. This problem has mainly arisen from the lack of suitable techniques capable of probing composition on the length scale present in mixed gels; most phase-separated domains in mixed gels are typically 1–1000 μm in size. We recently showed, however, that it was possible to use Fourier transform infrared (FTIR) microspectroscopy to measure the local concentration in mixed gels (Durrani & Donald, 1994), although in that work we restricted ourselves to the measurement of concentration at just one point in a sample. In this paper we build on our previous work by developing a technique to measure composition over a grid of points in a sample, thus allowing a compositional map of a mixed gel to be constructed.

In the first serious attempt to tackle the question of phase composition in mixed gels, Clark *et al.* (1983) adopted the simple approach that there was no 'cross-contamination' of polymer between the phases, and that the concentrations in the two phases of such gels were just decided by the way in which the water partitioned itself between them. This somewhat naive concept allowed progress to be made, since all that was required was a partition coefficient to quantify how the water split between the two phases.

It now appears from recent work in our group that the final concentrations in a mixed gel actually depend on a number of factors, including the temperature of phase separation, the rate of quenching, the quench depth and the relative rates of phase separation and gelation (Donald *et al.*, 1995). It is also apparent that consideration must be given to the mechanism of phase separation, which may be by either spinodal decomposition or nucleation and growth (Tromp *et al.*, 1995). From our earlier work with FTIR

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microspectroscopy (Durrani & Donald, 1994), it seems that in the case of samples which are two-phase above the gel temperature, equilibrium concentrations may well be reached prior to gelation. But for mixtures which are one phase at high temperature and phase separate on cooling, the final concentration will depend on the relative rates of phase separation and gelation, since the gel time of the polymer which gels first will dictate the extent to which phase separation is arrested (Donald *et al.*, 1995). There is also the possibility, for mixtures which phase separate on cooling, that the gelation of one or other of the two polymers can itself act as an additional driving force to phase separation, since the process of cross-linking affects the entropy of the system.

With this rich variety of behaviour, it is vital to have a technique that can be used to map concentration in phase-separated systems of this kind. In the field of synthetic polymers, Harthcock and Atkin led the way in the use of FTIR microspectroscopy to image mixed polymer blends (Harthcock & Atkin, 1988). They saw their technique as an extension of other methods such as electron microscopy for elemental analysis and Raman microprobing for functional group imaging techniques, which Morris later suggested could be applied to mixed gels (Morris, 1992). However, Harthcock and Atkin only used the intensities of infrared absorption peaks (arising from particular functional groups) to construct semi-quantitative maps. In this paper, we produce not only maps of this kind, but also fully quantitative maps in terms of the actual concentration at each position in the sample.

In this paper we first describe our FTIR imaging technique and then show how it can be applied to a protein/polysaccharide mixture, using the gelatin/amylopectin mixture as an example. D_2O was used as the solvent in all our experiments, since H_2O would otherwise obscure most of the important infrared absorptions from the protein.

MATERIALS AND METHODS

Sample preparation

Commercial food-grade gelatin and amylopectin samples were obtained from Unilever, and D_2O (99.9% isotopic purity) was bought from Aldrich Chemical Company, Dorset, UK. Mixed amylopectin/gelatin/D_2O samples were prepared by weighing out the appropriate masses in a sealed bottle and standing the sample overnight at room temperature to allow the gelatin to swell. The sample was then heated to 85°C using a magnetic stirring bar to ensure adequate mixing. The hot solution, consisting of liquid droplets of one phase in a continuous matrix of the other phase, was then injected into a sample cell held at 51°C. The cell consisted of two 2 mm thick infrared windows separated by a spacer. For semi-quantitative work, calcium fluoride windows with a 12.5 μm tin spacer were used; for fully quantitative maps, silver chloride windows with a 15 μm PTFE spacer were used. The sample was then cooled to room temperature before study. We have previously shown that the composition of such samples does not change with time at room temperature (Durrani & Donald, 1994) and we can, therefore, be confident that, during the course of the mapping, the composition changed only as a function of position.

Data acquisition

Data were collected using a Spectra Tech IR-PLAN FTIR microscope with a deuterated triglycine sulphate (DTGS) detector, attached to a Mattson Galaxy 40-20 FTIR spectrometer. A Prior computer-controlled motorized stage H126PSI was used to move the sample in the x–y plane. Data acquisition was automated using Mattson FIRSTM macro command language to drive the stage in stepwise fashion, in order to cover a two-dimensional grid of points across the sample. At each position in the grid, the FTIR microscope recorded the interferogram over a specified cross-sectional area of the sample, whose dimensions were set using adjustable knife-edge apertures, positioned above and below the sample. In this work, we chose to take data over 40 μm x 40 μm cross-sectional regions of the sample, with a grid step size set to 40 μm. At each position, 1000 interferograms were taken at 8 cm^{-1} resolution, co-added and Fourier transformed using Beer–Norton strong apodization. A spectrum taken over a 40 μm x 40 μm region through a 4 mm thick infrared window was used as background.

Data handling

Because calcium fluoride is an optically transparent material over the wavelengths of visible light, the phase-separated morphology of mixed protein/polysaccharide samples could be seen reasonably well by eye with an FTIR microscope under white light illumination, when the sample was in a calcium fluoride cell. And although calcium fluoride is also transparent over most of the infrared, it becomes opaque below 1200 cm^{-1}. The characteristic absorptions from amylopectin and D_2O, which fall at and below 1200 cm^{-1}, were, therefore, inaccessible. Nevertheless, for samples held between calcium fluoride, the amount of gelatin could be measured by integrating the area of the gelatin amide I band between 1599 and 1711 cm^{-1}. Semi-quantitative maps in terms of the relative amount of gelatin at different positions could, therefore, be plotted out. To supplement the images formed in the FTIR microscope with white light, phase contrast or differential interference contrast (DIC) microscopy has been used. This approach is a far superior way to optically

visualize a mixed biopolymer sample; in this work a Zeiss Axioplan microscope under DIC optics was used to photograph the region of interest studied via the compositional map.

Silver chloride has the advantage as a window material that it is transparent to infrared down to 700 cm^{-1}. This, therefore, made not only the gelatin bands accessible, but also the D_2O and amylopectin absorptions at 1115–1300 cm^{-1} and 987–1120 cm^{-1}, respectively. This allowed us to analyse our spectra with the method of partial least-squares and hence to extract the actual weight percentage concentration of the sample at each position. Full details of this method can be found in our earlier work (Durrani & Donald, 1994). The disadvantage of silver chloride is that it is somewhat opaque to white light and it is harder to identify regions of interest in a sample.

Data plotting

Compositional maps in terms of either peak areas or actual percentage concentrations as a function of position were plotted out using Visual Numerics PV-WAVE software language.

RESULTS

The infrared spectrum of a 40 μm x 40 μm region in a mixed 10% amylopectin/7% gelatin/83% D_2O phase separated mixed sample held between CaF_2 windows is shown in Fig. 1. The large size of the amide I band from the gelatin component between 1599 and 1711 cm^{-1} indicates that this spectrum was taken in a gelatin-rich region of the sample. The area of this amide I band was measured from the spectrum at each position in the sample; the numerical values of these areas were then used to plot the compositional map shown in Fig. 2. The map covers an area of 480 μm by 480 μm, with

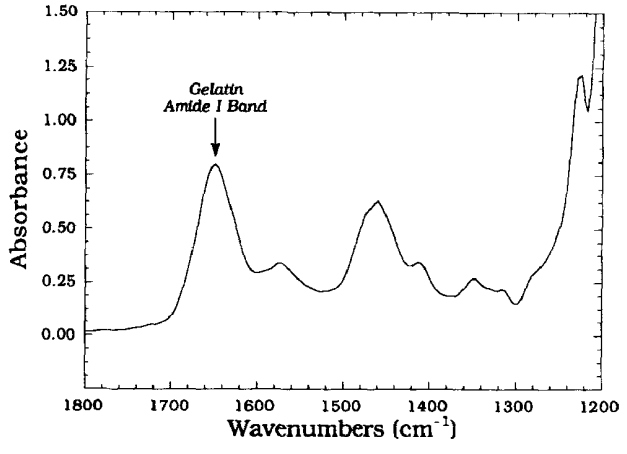

Fig. 1. FTIR spectrum of a 40 μm x 40 μm gelatin-rich region in a 10% (w/w) amylopectin/7% (w/w) gelatin/83% (w/w) D_2O sample held between CaF_2 windows.

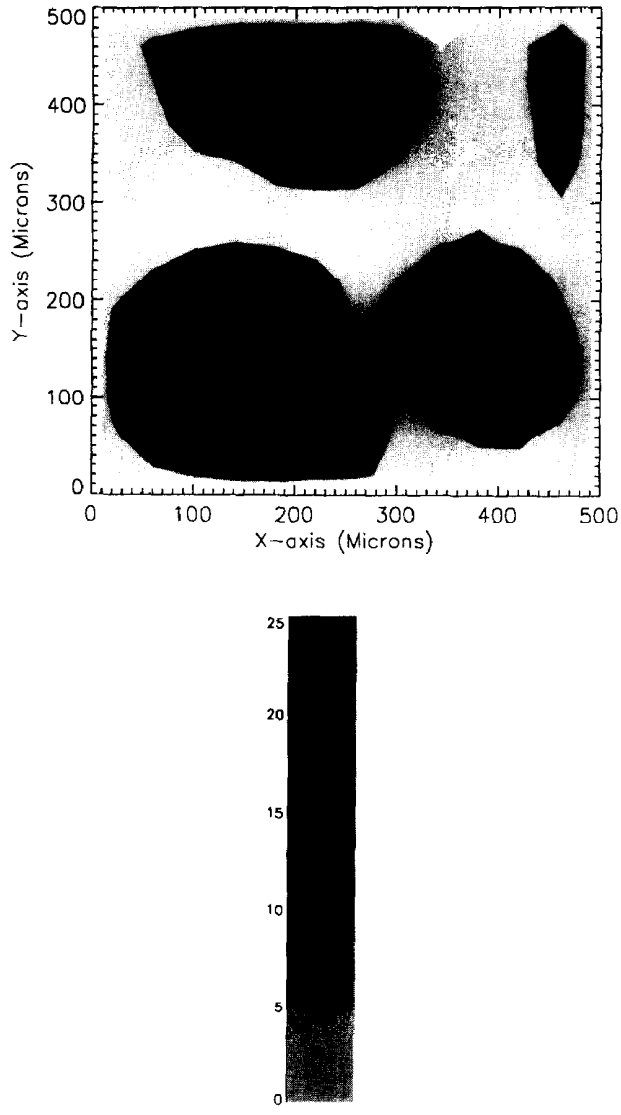

Fig. 2. Compositional map of a 10% (w/w) amylopectin/7% (w/w) gelatin/83% (w/w) D_2O sample, using the area of the gelatin amide I band as a measure of the gelatin composition. The map covers an area 480 μm x 480 μm in size and is based on infrared spectra taken over a grid of 40 μm x 40 μm squares across the sample. The map is represented with the range of areas of the amide I band divided into five levels (see scale bar): <5, 5–10, 10–15, 15–20, >20.

spectra having been taken over 40 μm x 40 μm regions, and can be seen to consist of three distinct gelatin-rich regions. The corresponding optical micrograph of the same region in the sample is shown in Fig. 3. A comparison of Fig. 2 with Fig. 3 clearly shows that the optical and FTIR images correlate with one another, as expected. The gradation in concentration at the edges of each region arise from the fact that the aperture in the FTIR microscope has a finite size which is larger than the width of the boundary between two phases. Therefore, whenever the aperture straddles a phase boundary, the gelatin peak area is a sum of the product of the fraction of the aperture covering each phase with the peak area expected from that phase.

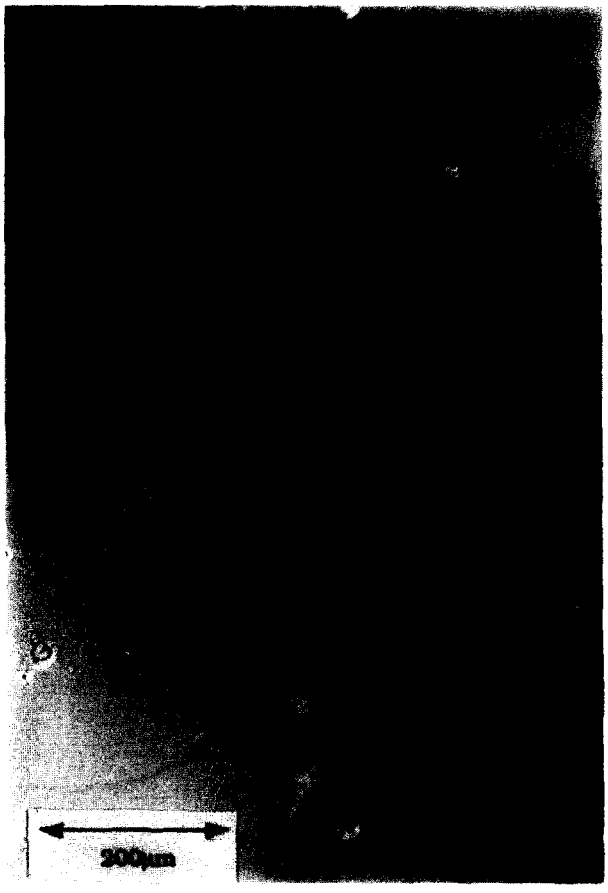

Fig. 3. Optical micrograph (differential interference contrast) of the amylopectin/gelatin/D_2O sample used in Fig. 2, at the centre of which can be seen the region selected for the compositional map of Fig. 2.

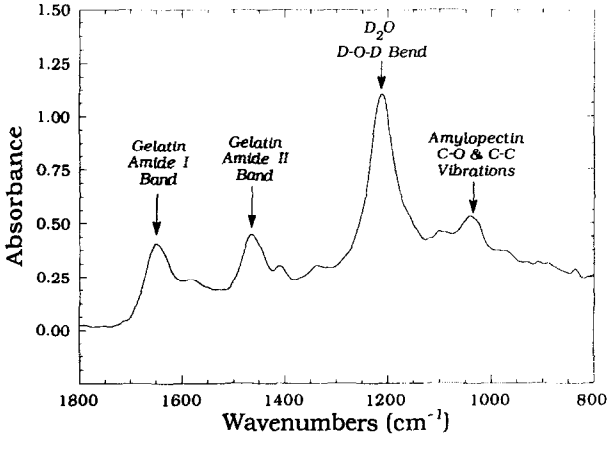

Fig. 4. FTIR spectrum of a 40 μm x 40 μm region in a 10% (w/w) amylopectin/7% (w/w) gelatin/83% (w/w) D_2O sample held between AgCl windows.

When a gelatin/amylopectin mixture was held between two AgCl rather than CaF_2 windows, a greater region of the infrared spectrum was accessible. Figure 4 shows that not only are the gelatin amide I and II bands accessible as before, but so are absorptions from D_2O (centred on 1212 cm^{-1}) and from amylopectin (between

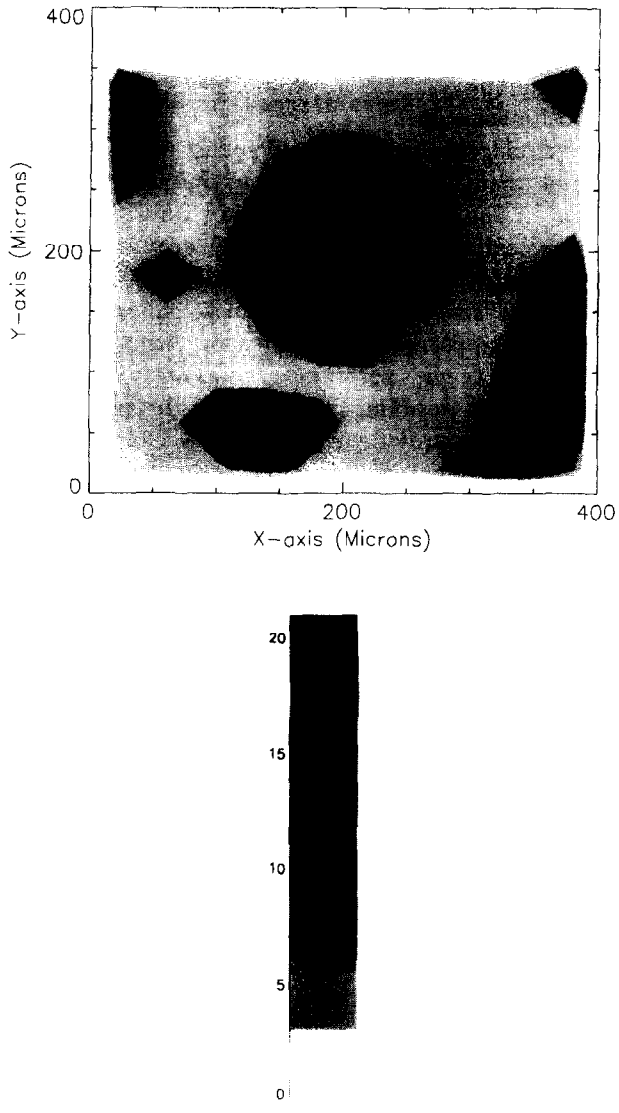

Fig. 5. Compositional map of a 10% (w/w) amylopectin/7% (w/w) gelatin/83% (w/w) D_2O sample, plotted in terms of the percentage concentration of amylopectin. The map covers an area 400 μm x 360 μm in size and is based on infrared spectra taken over a grid of 40 μm x 40 μm regions across the sample. The map is represented with the amylopectin range divided into seven levels (see scale bar): <3%, 3–6%, 6–9%, 9–12%, 12–15%, 15–16%, >16%.

987 and 1120 cm^{-1}). Using partial least-squares analysis of the spectra between 938 and 1726 cm^{-1}, having obtained a series of calibration samples from our earlier work (Durrani & Donald, 1994), the actual weight percentage concentrations at each 40 μm x 40 μm apertured position in the sample were determined using the procedure outlined in that paper. These concentrations were then used to plot the compositional maps in terms of weight percentage amylopectin (Fig. 5), weight percentage gelatin (Fig. 6) and weight percentage D_2O (Fig. 7). Each map covers the same 360 μm x 320 μm region of the sample, with spectra taken over 40 μm x 40 μm regions in each case. It is clear from Figs 5–7 that the region at the centre of the map is rich in

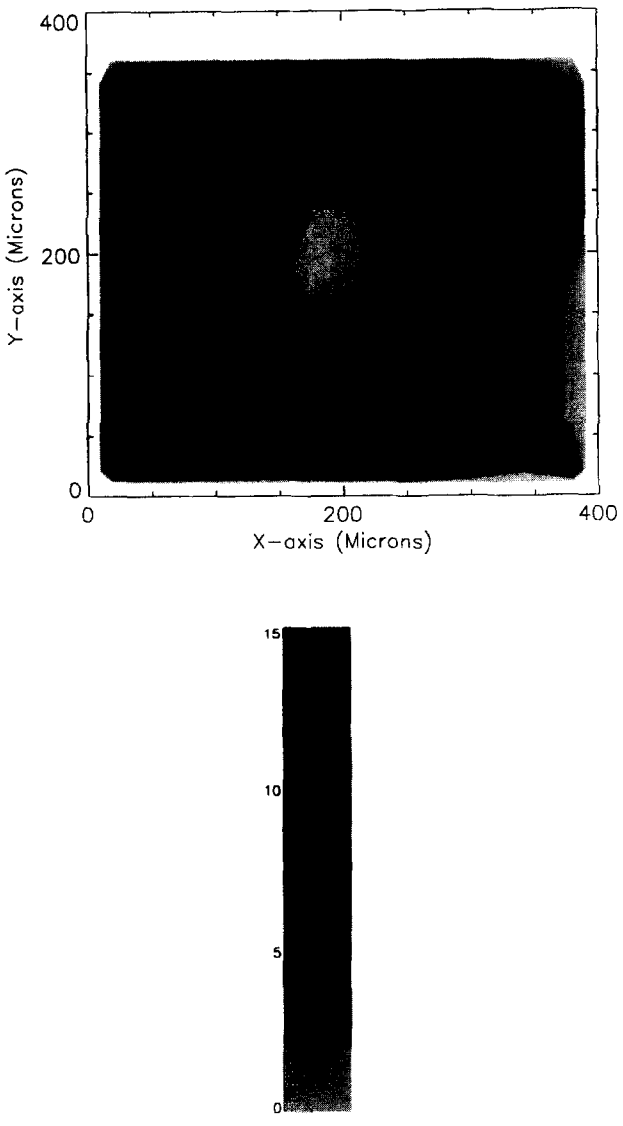

Fig. 6. Compositional map of the same region of the sample shown in Fig. 5, but plotted in terms of the percentage concentration of gelatin. The map is represented with the gelatin concentration range divided into six levels (see scale bar): <2%, 2–4%, 4–6%, 6–8%, 8–10%, >10%.

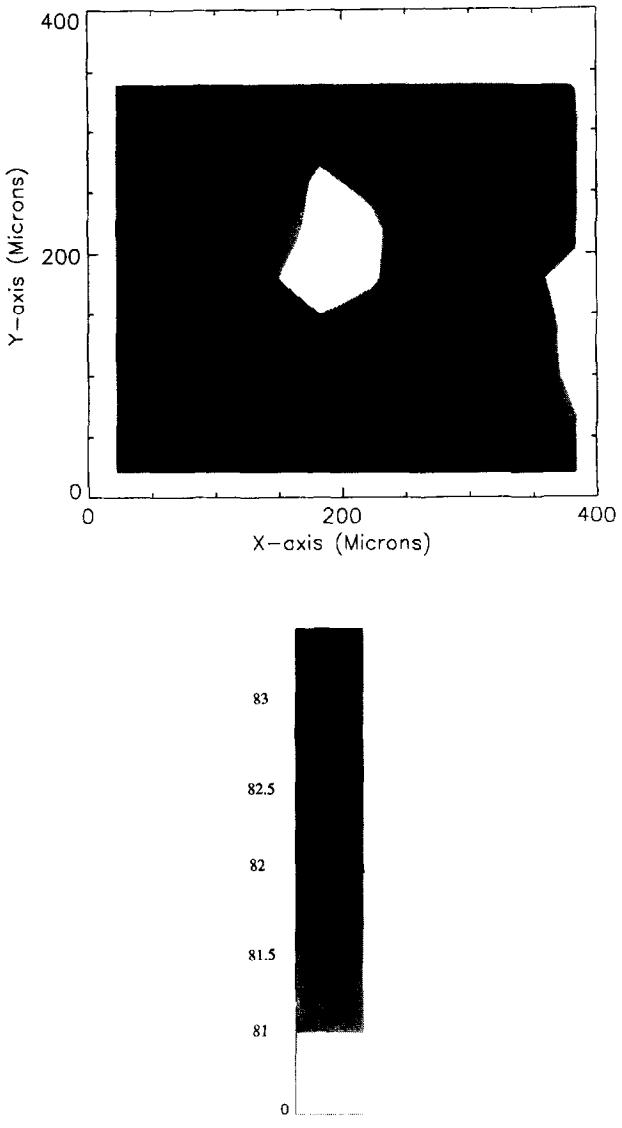

Fig. 7. Compositional map of the same sample shown in Figs 5 and 6, but plotted in terms of the percentage concentration of D_2O. The map is represented with the D_2O concentration range divided into six levels (see scale bar): <81%, 81–81.5%, 81.5–82%, 82–82.5%, 82.5–83%, >83%.

gelatin, deficient in amylopectin and surrounded by amylopectin-rich regions which are deficient in gelatin.

DISCUSSION

The success of our technique is clearly shown by the direct comparison which can be made between the optical micrograph in Fig. 3 and the FTIR image in Fig. 2. Furthermore, the refinement of our method is illustrated by the fact that it was not only able to produce maps of the percentage concentration of protein and polysaccharide, but that it was also able to monitor the D_2O concentration at different positions, most of which lay in a narrow distribution between 81% (w/w) and 84% (w/w).

It is also apparent from Figs 5–7 that regions which are gelatin-rich accommodate more water than amylopectin-rich regions. In order to relate these concentrations in the two phases to equilibrium concentrations, the data from the compositional maps in Figs 5–7 were used to calculate the mean values of the concentrations in the amylopectin-rich and gelatin-rich phases. (Data in transition regions where the aperture straddled the boundary were excluded from these calculations.) The resultant mean concentrations are shown plotted on the equilibrium phase diagram of this system, which we measured in our previous work by allowing the system to bulk phase separate in the absence of gelation and taking the FTIR spectrum of each layer (Durrani *et al.*, 1993). As can be seen for this sample, the

concentrations in the two phases of the gel approximate the concentrations in the phases when the solution was allowed to bulk phase separate to equilibrium in the absence of gelation.

However it must be stressed that this result applies only to this sample which was already two-phase at the temperature of mixing (85°C). If the experiment were repeated on a sample which only phase separated on cooling, it would be likely to produce a different result.

The main limitation to our technique is that an FTIR microscope only works above a minimum length scale; a length which is fixed by the diffraction limit of infrared radiation. This diffraction limit is reached when the aperture size is so small that it approaches the wavelength of the infrared radiation passing through the aperture; infrared radiation is then diffracted outside the designated area of interest specified by the apertures and into unwanted areas (Messerschmidt, 1988). The resultant spectrum then contains spurious information (Sommer & Keaton, 1991). We found the practical minimum length scale to be ~30 μm. This means that mixed gels which phase separate on a length scale smaller than ~30 μm cannot be studied with our approach. Mixed biopolymer samples which have small droplets of this size include those which are one phase at high temperatures (but below 100°C) and then phase separate on cooling. The droplets in such samples are smaller than in those which are already two-phase at these temperatures, because the mixture cannot be quenched as far below the phase boundary in this case. Hence samples which phase separate at lower temperatures during cooling are harder to study with our techniuqe.

One possible way of increasing the resolution of the FTIR microscope, whilst maintaining the chosen aperture size in order to avoid diffraction problems, would be to reduce the step size to some fraction of the aperture dimension and deconvolute the resulting spectra in an appropriate fashion. This would not only narrow the width of the transition zones in the maps, but would also allow samples with smaller phase separated regions to be analysed than is currently possible. However, a smaller step size would increase the number of spectra required to cover a given area of sample, which in turn would lead to significantly longer experimental data acquisition times.

CONCLUSIONS

We have developed a technique to produce compositional maps of phase separated mixed gels. The technique is automated and uses an FTIR microscope interfaced to a motorized stage in order to measure the infrared spectrum at a grid of points across a sample.

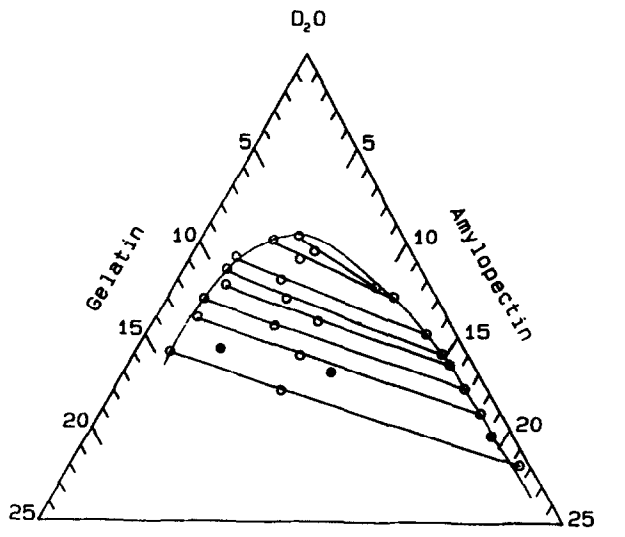

Fig. 8. Mean concentrations in the gelatin-rich and amylopectin-rich phase separated regions, determined from the data used in Figs 5–7 are shown (●) plotted on the ternary equilibrium phase diagram of this system, (○) as measured at 51°C in earlier work (Durrani *et al.*, 1993).

We applied our technique to aqueous gelatin–amylopectin gels and constructed semi-quantitative maps in terms of the gelatin amide I peak area at each position, as well as fully quantitative maps in terms of the percentage concentration of amylopectin, gelatin or water at each position (Fig. 8).

Although we looked at the amylopectin/gelatin system, our mapping technique is of a generic nature and can, therefore, be applied to any other protein/polysaccharide mixture, or indeed to any mixture, provided its component polymers show sufficient spectral difference.

Since we previously used FTIR spectroscopy to measure concentration in liquid mixtures which had bulk phase separated, our mapping technique allows the concentrations at different positions in a mixed gel to be compared with concentrations reached at equilibrium using the same molecular probe, namely, infrared radiation. It is also anticipated that we can use our FTIR technique to complement the information obtained from small-angle light scattering experiments carried out in this group (Donald *et al.*, 1995): light scattering can monitor the course of phase separation and our FTIR mapping technique can look at the final state of the system after gelation. A combination of both techniques ought to provide us with the means to tackle the whole issue of gelation and phase separation in biopolymer mixtures.

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