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Diffusion of mixed micelles of bile salt-lecithin in amylopectin gels: A Fourier transform infrared microspectroscopy approach

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Abstract

Using Fourier transform infrared (FTIR) microspectroscopy the average translational diffusion coefficients of bile salt-lecithin mixed micelles diffusing in amylopectin gels of varying concentration were measured, on the assumption that the diffusion coefficient was constant during diffusion. This assumption was examined based on quasielastic light scattering (QLS) measurements of the change in the mixed micellar size on dilution. The size increased significantly on dilution with a buffer without the bile salt, whereas the size was almost constant on dilution with a solution of the bile salt in the same buffer. Because bile salt molecules in intermicellar solution (being much smaller) diffuse much faster than the mixed micelles, the mixed micelles in the diffusing front can be treated as if diluted with the bile salt solution. Therefore their size will be constant during diffusion, validating the assumption of a constant diffusion coefficient. Based on this it is possible to show that the micelles diffuse with a diffusion coefficient of $\sim 10^{-11}$ m^2 s^{-1}, independent (within experimental error) of the amylopectin gel concentration over the range 5%–10% w/w.

Keywords: Diffusion; Unstirred layer; Intestinal absorption; FTIR; Bile salt-lecithin mixed micelle; Amylopectin gel

1. Introduction

Lipid digested products occur primarily within the small intestine by the action of lipase. The products of this lipolysis form mixed micelles with the components of the bile — phospholipids and bile acids [1–6]. For the intestinal absorption of the lipid digested products, the mixed micelles have to cross the unstirred layer, adjacent to the intestinal mucosal membrane. There is a vast literature to support the concept of an unstirred layer separating the bulk luminal fluid from the mucosal surface of the intestine. Several investigators suggest this arises naturally from consideration of the velocity gradients present during peristaltic flow through the intestine. Thus the thickness of the layer will be influenced by the presence of viscous components in the diet, such as non-starch polysaccharides [7,8] and resistant starch [9–11]. Alternative hypotheses have suggested the major component of this layer to be the glycoca-

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lyx or mucous layer on the surface of the microvillus. From previous investigations on the rate-limiting factors for intestinal absorption of the products of lipid digestion, it has been found that the movement of a solute through this barrier is determined by diffusional forces.

The products of lipolysis within the intestinal lumen, monoglycerides and fatty acids, are relatively insoluble within an aqueous environment, but in the presence of bile these lipids exist in mixed micellar aggregates in equilibrium with the monomeric species [12]. Both these forms diffuse from the bulk chyme to the absorptive site, but the micellar phase is the major contributor to the overall flux caused by the limited solubility of the monomers [13]. Reduction in the rate of diffusion of these micellar phases has been cited as one of the mechanisms for the observed in vivo reduction in lipid absorption in the presence of non-starch polysaccharides. However further quantitative investigation is required before its contribution to this reduction can be determined, relative to the other proposed mechanisms, such as the concurrent reduction in intraluminal stirring and sequestration of micellar components [14].

In order to understand and then control the intestinal absorption of lipids, measurements of diffusion of bile salt-lipid mixed micelles in both aqueous solutions and mucosal gels are required. Mazer et al. measured the translational diffusion coefficient of the bile salt-lecithin mixed micelles in aqueous solutions by a quasielastic light-scattering (QLS) method [15]. If a dispersion of micelles is illuminated it will scatter the light. The intensity of the scattered light continuously fluctuates around a mean value because of the thermal motion of the micelles. The time dependence of these fluctuation can be measured and related to the translational diffusion coefficient, D, of the micelles [16]. In addition, from the D value one can deduce a measure of micellar size, the mean hydrodynamic radius, R_h, by using the Stokes-Einstein relation [17]:

$$R_h = kT/6\pi\eta D \qquad (1)$$

where η is the viscosity of solution, k Boltzmann constant, and T the temperature measured on the absolute scale. However, the QLS method cannot be used to study diffusion of the mixed micelles in mucosal gels because of the overwhelming scattering from the gels.

In this study, we used not only QLS, but also Fourier transform infrared (FTIR) microspectroscopy using a methodology similar to that developed to measure diffusion of bovine serum albumin (BSA) in amylopectin gels [18]. FTIR microspectroscopy is a microanalytical technique which interfaces an FTIR spectrometer to an optical microscope [19]. Using this technique, IR spectra can be taken as a function of position along the diffusion capillary. By calibrating the concentration against the integrated area of the peak arising from the vibration of the mixed micelle, using mixed micelle solutions of known concentrations, it is possible to build up the relationship between concentration and position along the capillary. By fitting this to a diffusion equation for diffusion in a semi-infinite medium [20], D can be found using

$$\frac{c(x)}{c(0)} = \mathrm{erfc}\left(\frac{x}{2\sqrt{Dt}}\right) \qquad (2)$$

where $c(x)$ is the concentration at position x along the tube, $c(0)$ the concentration at position $x = 0$, i.e., the concentration in the bulk solution, D the diffusion coefficient and t the time, with the auxiliary conditions: $c = c(0)$ for $x = 0$ and $t > 0$ and $c(x) = 0$ for $x = \infty$. The values of the error function, erfc, were found from tables. From this, the diffusion coefficient, D, can be calculated, assuming that the diffusion coefficient is constant during the process of diffusion. However, Mazer et al. found the mixed micellar size increased on dilution by the QLS method [15]. It would be thought that the mixed micelle in the diffusing front must be diluted, and hence the size might increase. This assumption is examined below.

The main purpose of this article was to use the FTIR microspectroscopic method described previously [18] to study diffusion through a model gel system of varying concentration. The aim was firstly to investigate whether the FTIR microspectroscopic method could be applied to the measurement of diffusion of biologically important mixed micelles in a matrix relevant to that found in vivo, and secondly to quantify the diffusion coefficient for this model system.

2. Experimental section

2.1. Chemicals

Sodium taurocholate, amylopectin from waxy maize, and deuterium oxide (D_2O) of isotopic purity 99.9% were supplied by Sigma (catalogue nos. T 4009, A 7780, and D 4501, respectively). Egg yolk lecithin (average molecular weight 775 g mol^{-1}) was obtained from Lipid Products, South Nuffield, Surrey, UK.

2.2. Preparation of mixed micellar solution

Bile salt-lecithin solutions in D_2O were prepared by the method of coprecipitation [21]. D_2O was used because it does not obscure the infrared ester band of lecithin (unlike H_2O). Lecithin dispersed in chloroform was dried by rotary evaporation under a stream of purified N_2 at 30–40 °C, and then dried in vacuo for at least 48 h, until constant dry weight was obtained. The 0.010 M citric acid-disodium phosphate buffer in D_2O (pH 6.5, containing 0.109 M NaCl and 0.031 M NaN_3) was added to give a 10% w/w lecithin stock, and then flushed with purified N_2, sealed, and covered with foil to protect from light. The 10% w/w lecithin stock was diluted with an equal weight of a 10% w/w sodium taurocholate solution in the same buffer to give a mixed micellar stock (lecithin concentration = 5% w/w, molar ratio L/TC = 0.69). It was then flushed with purified N_2, sealed, and covered with foil.

2.3. Diffusion

Amylopectin was ground with a pestle and mortar for 20 min to reduce particle size. It was then mixed with the D_2O pH 6.5 buffer to form a slurry of the required concentration. The slurry was placed in an ultrasonic bath for 3 min to break up the amylopectin and then heated in a water bath at 100 °C with continuous stirring for 15 min. It was placed in the refrigerator at 4 °C for 15 min to cool, and then injected into a 0.1 mm diameter and 80 mm long special glass capillary (purchased from Pantak Limited, Reading, UK). Both ends of the tube were sealed with paraffin wax and then the tube was kept in the refrigerator at 4°C for 48 h to gel and mature.

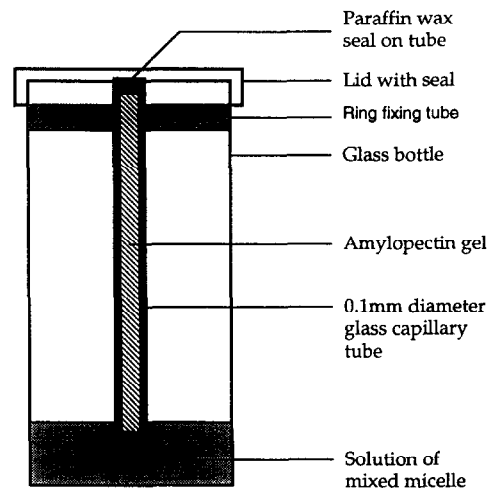

Fig. 1. Schematic figure of the apparatus used for the experiment to measure diffusion of the mixed micelle in amylopectin gels.

As soon as the paraffin at one end of the tube was cut, the tube was suspended in a bile salt-lecithin micelle solution with the exposed end immersed in it. The bottle containing the suspended tube and 2 ml of the solution was flushed with purified N_2, sealed, covered with foil, and placed in a water both at 20°C for a controlled length of time (of the order of a week) to allow diffusion to take place (Fig. 1). At the end of this diffusion time the tube was removed from the solution and the exposed end was sealed with paraffin wax.

2.4. FTIR microspectroscopy

The FTIR instrument used was a Mattson 40-20 Galaxy FTIR spectrometer with a standard DGTS detector, and a narrow-band mercury cadmium telluride (MCT) detector cooled by liquid N_2 dedicated to microspectroscopy, coupled with a Spectra Tech IR-PLAN microscope. A pair of adjustable rectangular apertures were placed above and below the sample tube to delineate a 56×80 μm rectangular section. Thus, spectra were taken from 80 μm length of the tube at 8 cm^{-1} resolution and 1000 scans. The sample tube was moved along the axis of the tube by a motorised stage. In this way, the IR spectra of the sample as a function of position were obtained. A spectrum of the empty tube was used as background, and gels formed by amylopectin-mixed micelle solu-

tion of known concentration were used for calibration; hence the diffusion profile could be derived. The experimental data were fitted to the diffusion equation (Eq. (2)) by a least square non-linear regression method (Genplot, Computer Graphic Service, 52 Genung Circle, Ithaca, NY 14850-8716, USA). From the fitting the diffusion coefficients were calculated. It was found from these measurements that the bulk concentration was constant during the diffusion, as a negligible number of probe molecules were transferred from the big reservoir. No probe molecules were detected at the far end of the tube, indicating that the diffusion occurred as in a semi-infinite medium, without reflection. This view is supported by evaluating how far the micelles would have diffused during the longest experimental runs based on the experimentally determined D, this distance being much shorter than the length of the capillaries used.

2.5. QLS

A Malvern system 4700c sub-micron particle analyser was used in this work. A Spectra-Physics model 2017-04S argon-ion laser ($\lambda = 514.5$ nm) was used in conjunction with a PCS 100 spectrometer and correlator for measuring the autocorrelation function of the scattering light intensity. The temperature of the scattering cell was controlled to 20 ± 0.1 °C by a PCS8 temperature controller/power supply. Cumulant analyses of the autocorrelation functions were executed with the Malvern software, Automeasure. All of the observed autocorrelation functions in the experiments could be fitted using 2 cumulants with a precision of better than 1.0% in each of the data points. Preliminary measurements showed scattering was invariant at various angles from 50° to 130°. Therefore 90° was chosen for the rest of the measurements. In order to investigate the effect of dilution on the mixed micellar size, the mixed micelle stock (lecithin concentration = 5% w/w, molar ratio L/TC = 0.69) was diluted with the pH 6.5 D_2O buffer to give a set of solutions of different lecithin concentration (but the same L/TC). In addition, the mixed micelle stock was diluted with the 0.2% w/w sodium taurocholate solution in the same buffer for comparison.

3. Results

The z-average size of the mixed micelle in the pH 6.5 D_2O buffer (total lipid concentration = 5% w/w, molar ration L/TC = 0.69), obtained from the QLS measurements, was plotted as a function of the total lipid concentration (Fig. 2). This figure shows that the size increases on dilution with the buffer, whereas the size is almost constant on dilution with the 0.2% w/w sodium taurocholate in the same buffer. These results are consistent with those of Mazer et al. [15]. It should be noted that Mazer et al. [15] and Donovan et al. [22,23] have rigorously shown that for an L/TC ratio of 0.7%, 0.2% TC (equivalent to approximately 4 mM) approximates the intermicellar monomeric concentration (IMC). If a mixed micellar system with a lower L/TC ratio were studied, the IMC would have to include the concentration of simple micelles [22,23]. The increase in size on dilution results from the equilibrium between bile salts in the mixed micelles and bile salts in the intermicellar solution, and the increase can be eliminated by using a diluent that contains the appropriate intermicellar bile salt concentration. For the details about analysis of the dilution effects one can refer to the earlier publication [15].

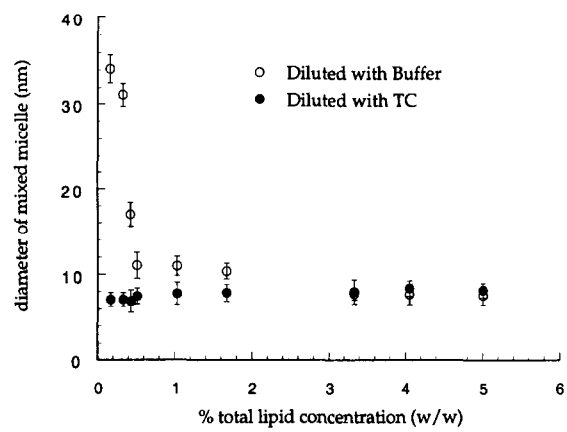

Fig. 2. Effect of dilution on average size of L-TC mixed micelles in the pH 6.5 D_2O buffer (total lipid concentration = 5% w/w, molar ratio L/TC = 0.69) at 20°C. Data points expressed by filled circles represent dilution with the buffer, while opened circles dilution with 0.2% w/w sodium taurocholate solution in the same buffer.

Fig. 3 shows typical FTIR spectra of (a) the bile salt-lecithin mixed micelle solution in the pH 6.5 D_2O buffer (lecithin concentration = 5% w/w, molar ratio L/TC = 0.69), and (b) the 5% w/w sodium taurocholate solution in the same buffer. The spectrum (a) shows the strong carbonyl absorption band from 1710 to 1764 cm^{-1} caused by ester groups in lecithin. This band was chosen for quantification of the mixed micelle concentration because it is independent of sodium taurocholate concentration. The calibration curve of the integrated area of the ester band against the total lipid concentration of the mixed micelle is almost linear, shown in Fig. 4 by the dashed line obtained from a least squares linear regression analysis. Both spectra (a) and (b) show the strong triple bond absorption band at 2044 cm^{-1} caused by sodium azide in the buffer. This band was used as an internal standard band, so that the effect of path length, caused by any slight variation in the tube diameter between tubes, could be removed by subsequent ratioing. The spectra were invariant with time indicating no oxidation of lecithin occurred during diffusion.

Fig. 5 shows a typical diffusion profile of bile salt-lecithin micelles in a 5% w/w total lipid solution diffusing into a 5% w/w amylopectin gel at 20 °C for 122 h, and the corresponding best fit to the diffusion equation (Eq. (2)) expressed by the dashed

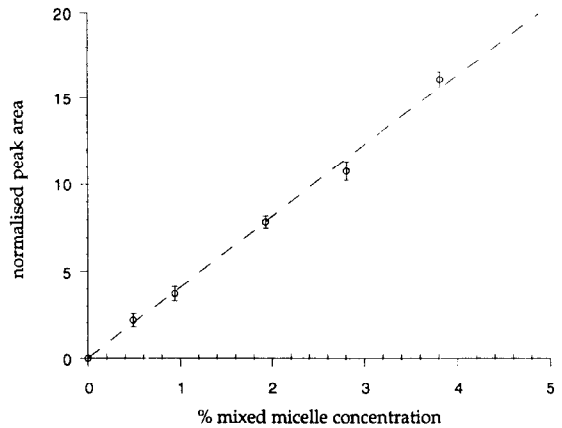

Fig. 4. Calibration curve for the normalised integrated area of the L-TC mixed micelle band from 1710 to 1764 cm^{-1}, against the total lipid concentration of the micelle solution in the pH 6.5 D_2O buffer (molar ratio L/TC = 0.69). Dashed line represents the correlation of these data with a least square linear regression analysis.

line. The diffusion coefficient, D, and the error in the diffusion coefficient, dD, were calculated from this fit. Fig. 6 shows the mean translational diffusion coefficient of bile salt-lecithin mixed micelles (total lipid concentration = 5% w/w, molar ratio L/TC = 0.69) into amylopectin gels (gel concentrations from 5% to 10% w/w) at 20°C. The data are the average value of 3 measurements and the error bars are the

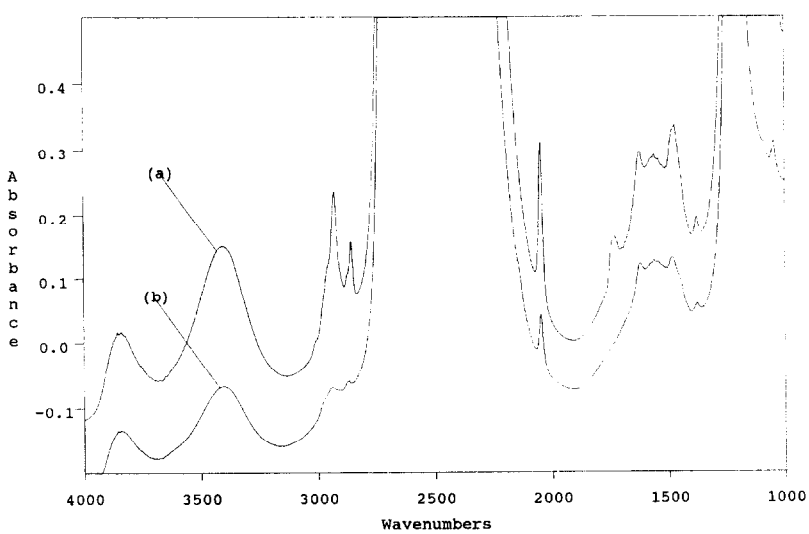

Fig. 3. Fourier transform infrared spectra of (a) L-TC mixed micelles in the pH 6.5 D_2O buffer (total lipid concentration = 5% w/w, molar ratio L/TC = 0.69), and (b) 5% w/w sodium taurocholate solution in the same buffer.

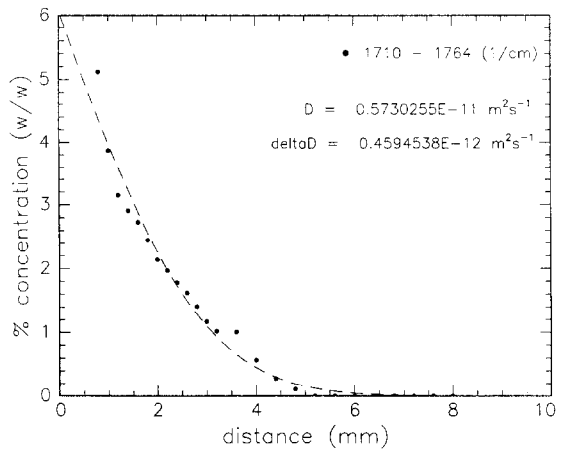

Fig. 5. Diffusion profile of L-TC mixed micelles in the pH6.5 D_2O buffer (total lipid concentration = 5% w/w, molar ratio L/TC = 0.69) in a 5% w/w amylopectin gel at 20°C for 122 h. Dashed line represents result of fitting these data to the diffusion equation (Eq. (2)) by a least square non-linear regression analysis.

standard deviation. The mean diffusion coefficient seems almost constant with only slight fluctuation within the experimental errors, as the gel concentration changes from 5% to 10% w/w amylopectin. Note that for the values of D determined here, the assumption of the boundary condition that there is no reflection is clearly met for capillaries of the length used here.

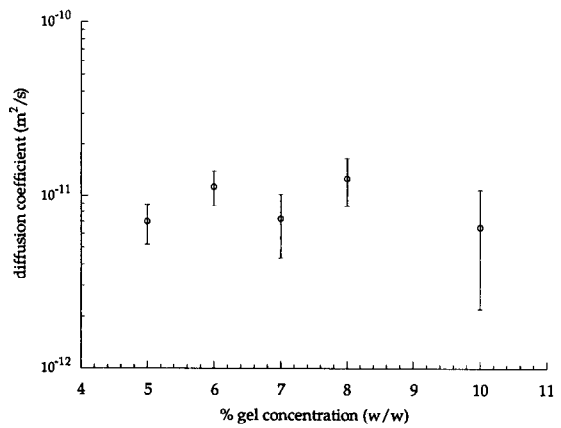

Fig. 6. Mean diffusion coefficient of L-TC mixed micelles in the pH6.5 D_2O buffer (total lipid concentration = 5% w/w, molar ratio L/TC = 0.69) diffusing in amylopectin gels at 20°C. Data are represented by the average values of 3 measurements, and the error bars are the standard deviation.

4. Discussion

The phase diagram (see Fig. 9 in Ref. [15]) for micelles formed in an aqueous mixed dispersion of sodium taurocholate and lecithin is divided into 3 regions. Simple bile salt micelles and mixed micelles coexist at a low L/TC molar ratio in region I; only mixed micelles are present at an intermediate L/TC molar ratio in region II; and bilayer and liposomes are formed at a high L/TC molar ratio in region III. In this study, we investigated the effect of dilution on the diffusion by diluting a bile salt micellar solution of 0.69 L/TC molar ratio and 5% w/w total lipid concentration with the buffer and the bile salt solution. According to the phase diagram, only mixed micelles are present in the starting solution, i.e. region II. Our data (Fig. 2) demonstrated the mean micellar size increases on dilution with the buffer without the bile salt in it, whereas the size was almost constant on dilution with the bile salt solution in the same buffer. These findings are consistent with Mazer et al. (see Fig. 4 in Ref. [15]). The effect of dilution on the micellar size results from the equilibrium between bile salts in the mixed micelles and bile salts in the intermicellar solution. If the system is diluted with the buffer alone, the concentration of bile salt in the intermicellar solution decreases, and hence a certain amount of bile salt must be released from the mixed micelles into the solution to maintain the equilibrium. Thus the size will increase, according to the existing models for the structure of the bile salt-lecithin mixed micelle [15,24]. However, if dilution is carried out with a bile salt solution of the same concentration as already present in the intermicellar solution, the micelle size will be unchanged.

The fit of the concentration profile obtained from the FTIR data to the diffusion equation (Eq. (2)) is based on the assumption that the diffusion coefficient is constant during the diffusion process. In the diffusing front, the mixed micelles are substantially diluted. By the above arguments, this might have increased the micellar size, and thereby decreased the diffusion coefficient. If this occurs there will be

an error introduced into the determination of D. However, the size of the bile salt molecule is much smaller than the mean size of the mixed micelles. Therefore it would be expected that the bile salt molecules should diffuse much faster than the mixed micelles. This means that the mixed micelles in the diffusing front are diluted with the buffer containing bile salt (rather than pure buffer) so that the mean micellar size can be assumed to be constant during diffusion, thus justifying our determination of the diffusion coefficient. This analysis was supported by the fitting results, since if the diffusion coefficient was not constant the experimental data would not be expected to fit an erfc function of the type shown by the dashed line well (Fig. 5).

We found that the mean diffusion coefficients of the mixed micelles diffusing in amylopectin gels were constant as the gel concentration changed from 5% to 10% w/w (Fig. 6). There are few published data on the pore sizes of hydrated starch polysaccharide gels. Leloup et al. [25] measured the accessibility of amylose gels (3%–10% w/w) to a macromolecular probe of a range of hydrodynamic radii. They arbitrarily defined the value chosen to represent an average pore size as the value of hydrodynamic radius for which 50% of the volume of solvent trapped within the gel was accessible to the probe species. Thus, it was calculated that the average pore sizes of amylose gel decreased from 16 to 9 nm while the concentration increased from 5% to 10% w/w. There appear to be no published data on the average pore size of amylopectin gels (5%–10% w/w). If the average size of the mixed micelles is smaller than the mesh size of the gel over all the concentration range, then one would not expect a significant change in the diffusion constant with gel concentration, since the retardation by amylopectin gel network will not be significant. However, Cameron et al. [18] reported that diffusion coefficients of BSA, with a mean hydrodynamic radius of 3.61 nm [25], in amylopectin gels decreased from 2.2×10^{-11} to $1.0 \times 10^{-11}\ m^2\ s^{-1}$ while the amylopectin gel concentration increased from 5% to 10% w/w. The mean hydrodynamic radius of the mixed micelle obtained from QLS (Fig. 2) is similar to the BSAs. However, the mixed micelles studied here are less symmetric than BSA, and with increasing asymmetry the hydrodynamic radius is less appropriate to use as the appropriate dimension [26]; large discrepancies have been observed between the true Stokes radius measured by hydrodynamic methods and the apparent value derived from SEC experiments [27]. Further, the dynamic behaviour of the micelles, e.g. if there are shape and/or volume fluctuations occurring (and for this system the micelles are known to be in dynamic equilibrium with the surrounding liquid, whereas the BSA molecules will be constant entities) might affect the diffusion. Therefore, the effect of amylopectin gel concentration on the mixed micelle diffusion cannot be directly compared with the effect on BSA diffusion, despite the similarity of hydrodynamic radius.

The experimental error in the current study was larger than that observed previously for the BSA diffusion. We cannot rule out the possibility of any change, smaller than the experimental error, in the diffusion coefficient caused by the gel concentration over this range. The precision of the FTIR method depends on a range of factors such as the stability of the micelles and gel medium, sealing of the capillary and spectral analysis affecting the FTIR measurements. Fig. 5 shows that the relative error, expressed by deltaD/D, from the fit of concentration profile to the diffusion equation is about 8%. This error includes the contribution from polydispersity. The standard deviation from three independent measurements is less than 30% (except for the extremely high value of 60% for 10% w/w gel) (Fig. 6).

5. Conclusions

The average translational diffusion coefficient of bile salt-lecithin mixed micelles diffusing in amylopectin gels can be measured by the FTIR microspectroscopic method on the assumption that the diffusion coefficient is constant during the diffusion process. A value of about $10^{-11}\ m^2\ s^{-1}$ is found, independent of amylopectin concentration over the range studied. It may be possible to reduce the error and improve the precision of the FTIR method. These studies show the validity of this approach to study the diffusion of mixed micelles, and it may potentially be used to measure their diffusion coefficient in gels of a protein or mixed protein and

polysaccharide, to correspond more closely with the mucin gel layer in vivo.

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