

# Adsorption of hydrophobin / $\beta$ -casein mixtures at the solid-liquid interface

I M Tucker<sup>1</sup>, J T Petkov<sup>1†</sup>, J Penfold<sup>2,3,\*</sup>, R K Thomas<sup>3</sup>, A R Cox<sup>4</sup>, N Hedges<sup>4</sup>

1. Unilever Research and Development, Port Sunlight, Quarry Road East, Bebington, Wirral, CH62 4ZD, UK
2. ISIS, STFC, Rutherford Appleton Laboratory, Chilton, Didcot, Oxon, OX1 0QX, UK
3. Physical and Theoretical Chemistry Laboratory, Oxford University, South Parks Road, Oxford, OX1 3QZ, UK
4. Unilever Research Laboratories, Sharnbrook, Beds, MK44 1LQ, UK

\* Corresponding Author: [jeff.Penfold@stfc.ac.uk](mailto:jeff.Penfold@stfc.ac.uk)

† Current address: KLK Oleo, SDN BHD, Menara KLK, Muliara Damansara, 47810 Petaling, Jaya Selanger, Malaysia

## ABSTRACT

The adsorption behaviour of mixtures of the proteins  $\beta$ -casein and hydrophobin at the hydrophilic solid-liquid surface have been studied by neutron reflectivity. The results of measurements from sequential adsorption and co-adsorption from solution are contrasted. The adsorption properties of protein mixtures are important for a wide range of applications. Because of competing factors the adsorption behaviour of protein mixtures at interfaces is often difficult to predict. This is particularly true for mixtures containing hydrophobin as hydrophobin possesses some unusual surface properties. At  $\beta$ -casein concentrations  $\geq 0.1$  wt%  $\beta$ -casein largely displaces a pre-adsorbed layer of hydrophobin at the interface, similar to that observed in hydrophobin-surfactant mixtures. In the composition and concentration range studied here for the co-adsorption of  $\beta$ -casein – hydrophobin mixtures the adsorption is dominated by the  $\beta$ -casein adsorption. The results provide an important insight into how the competitive adsorption in protein mixtures of hydrophobin and  $\beta$ -casein can impact upon the modification of solid surface properties and the potential for a wide range of colloid stabilisation applications.

**Keywords:** protein adsorption, mixed proteins, hydrophobin,  $\beta$ -casein, hydrophilic surface.

### Highlights:

- Co-adsorption of hydrophobin /  $\beta$ -casein at hydrophilic solid-liquid interface
- At relatively high  $\beta$ -casein concentrations,  $\beta$ -casein displaces pre-adsorbed hydrophobin layer
- In hydrophobin /  $\beta$ -casein co-adsorption from solution  $\beta$ -casein dominates the adsorption
- Some limited evidence for co-adsorption under certain conditions

## INTRODUCTION

Most proteins are at least partially surface active, and are often described as surface active polymers. Hence proteins have been extensively exploited in stabilising foams, emulsions, and colloidal dispersions (1-4). In the many diverse applications which exploit the surface activity of proteins, the proteins are frequently used in combination with other proteins (5-7) or with surfactants (8-11). Their surface activity can also result in some less favourable properties, such as those associated with bio-fouling (12-14), and strategies to minimise protein adsorption are often adopted.

The subject of this paper is the study of the sequential and co-adsorption of two quite different proteins, hydrophobin and  $\beta$ -casein, at the solid-solution interface. Hydrophobin is a small globular protein ( $\sim 7$  to 10 kDa) produced by filamentous fungi (15, 16). Its globular structure has a well-defined hydrophobic patch, arising from leucine side chains, and is compact and tightly bound due to four intra-molecular disulphide bridges. The protein is highly surface active and forms a dense monolayer at the air-water interface with exceptionally high surface shear elastic and viscous moduli (17, 18). Its surface properties give rise to especially stable bubbles, foams and emulsions (19), and its adsorption at a variety of interfaces has been extensively studied (17, 20-22). In contrast  $\beta$ -casein is a more disordered protein which lacks a well-defined tertiary structure. This results in a greater ability and flexibility to adopt different conformations at interfaces (23), and its adsorption has been extensively studied under a variety of conditions (24-28).

Predicting the adsorption pattern of mixtures of proteins with quite different structures, such as hydrophobin and  $\beta$ -casein, and how they compete for the surface or interact at the interface is often difficult. Depending upon the nature of the interface and the proteins both competitive and co-adsorption have been reported (5, 6, 18, 29). In particular, at the air-water interface Radulova et al (7) interpreted changes in the surface rheology of adsorbed layers of hydrophobin in the presence of  $\beta$ -casein as associated with the incorporation of  $\beta$ -casein into a predominantly hydrophobin layer. This was subsequently confirmed and quantified by some more direct surface adsorption and surface structural measurements by Tucker et al (30). Using a variety of

surface sensitive techniques Marinova et al (6) and Mackie et al (5, 10) concluded that homogeneous mixed surface layers of  $\beta$ -casein and  $\beta$ -lactoglobulin were formed. Sengupta and Damodoran (29) concluded that  $\beta$ -casein and BSA were incompatible and formed inhomogeneous phase separated films.

Although a wide range of systems have been studied, studies of mixed protein adsorption at the liquid-solid interface incorporating hydrophobin are less common. However given the unusual and attractive surface properties of hydrophobin and the demonstrated ability of co-surfactants or other proteins to modify those properties at the air-water interface, the study of mixed protein adsorption involving hydrophobin is an important area. In this paper we present results of hydrophobin /  $\beta$ -casein adsorption at the hydrophilic solid surface using neutron reflectivity, NR, and the measurements were made by co-adsorption and sequential adsorption.

## **EXPERIMENTAL DETAILS**

### **(i) Neutron Reflectivity**

The neutron reflectivity, NR, measurements were made at the solid-solution interface on the D17 reflectometer (31) at the Institute Laue Langevin, Grenoble, France. The reflectivity,  $R(Q)$ , was measured as a function of the wave vector transfer normal to the surface,  $Q$ , (where  $Q$  is defined as  $Q=4\pi \sin\theta/\lambda$ ,  $\theta$  is the grazing angle of incidence, and  $\lambda$  is the neutron wavelength). A  $Q$  range  $\sim 0.009$  to  $0.26 \text{ \AA}^{-1}$  is covered using a neutron wavelength range of 2.5 to 20  $\text{\AA}$  and two grazing angles of incidence of 0.8 and 3.0  $^\circ$ , with a resolution in  $Q$  of  $\Delta Q/Q \sim 4\%$ . The NR data were normalised to the incident neutron beam spectral distribution, measurement time and variation in the detector efficiency, and set on an absolute scale by reference to the direct beam intensity and the scattering from a water sample.

The neutron beam is incident at the solid-liquid interface by transmission through the solid crystalline silicon in the vertical plane, at grazing incidence with an illuminated area  $\sim 30 \times 30 \text{ mm}^2$ , as described elsewhere (32). The silicon blocks (supplied by Crystran) were polished in the  $\langle 111 \rangle$  direction to a surface roughness  $\leq 5 \text{ \AA}$  rms. The volume of solution in contact with the surface is  $\sim 5 \text{ mL}$ , and exchange  $\sim 20$  to  $30 \text{ mL}$  of solution at a rate of  $5 \text{ mL / min}$  using a chromatography pump provides efficient sample

changing and rinsing. The measurement time for the full reflectivity profile was  $\sim 30$  to 60 mins.

The neutron reflectivity data are modelled using the exact optical description of reflectivity for thin films (33, 34). The simplest model, that is, the model with the least number of layers, that provides an adequate description of the data, is used; and assessed by least squares. The variation in neutron refractive index depends upon the scattering power of the different components or media through its scattering length density  $\rho$ , (where  $\rho=b/V$ ,  $b$  is the neutron scattering length and  $V$  the molecular volume, with different  $b$ ,  $V$  values as summarised in table 1), as described in detail elsewhere (35).

**Table 1.** Neutron scattering length and molecular volumes for the different components

Component	$b (\times 10^{-3} \text{ \AA})$	Molecular volume, $V (\text{\AA}^3)$	Scattering length density, $\rho$ ( $\times 10^{-6} \text{ \AA}^{-2}$ )
hydrophobin	16.2 <sup>39</sup>	9190	1.76
B-casein	53.3 <sup>36,37</sup>	29600	1.8
D <sub>2</sub> O	0.192	30	6.35

The scattering length values take into account the amount of exchangeable protons (36-38) in hydrophobin and  $\beta$ -casein for solutions prepared in D<sub>2</sub>O

In the analysis of the NR data an oxide layer at the solid silicon ( $\rho=2.08 \times 10^{-6} \text{ \AA}^{-2}$ ) surface is included in the modelling, with a thickness  $d$  of 10  $\text{\AA}$  and a scattering length density  $\rho$  of  $3.5 \times 10^{-6} \text{ \AA}^{-2}$ . Due to the sequence of measurements it was not possible to measure this separately, but the parameters used are in the typical range of parameters arising from the surface preparation method (30, 39, 40). A roughness of 5  $\text{\AA}$  is included at each interface, and a background of  $2 \times 10^{-6}$  is included in each calculation.

## (ii) Materials and measurements made.

The  $\beta$ -casein was obtained from Sigma-Aldrich (99.9% pure) and used as supplied. The class II hydrophobin, HFBII, was produced using a yeast fermentation and subsequently

purified by a two phase extraction at Unilever Research, Vlaardingen (17, 38). High purity water (Elga-Ultrapure) was used and D<sub>2</sub>O was obtained from Sigma-Aldrich. All the NR measurements were made in D<sub>2</sub>O at 25°C. All glassware and sample cells were cleaned in Decon90 alkali detergent solution and rinsed thoroughly in high purity water. Stock solutions of 1 wt%  $\beta$ -casein and 0.08 wt% (0.8 mg/ml) hydrophobin were prepared in D<sub>2</sub>O, and fully equilibrated before the final solution compositions and concentrations were fixed by the chromatography mixing pump, which delivered the solutions to the sample cell in-situ.

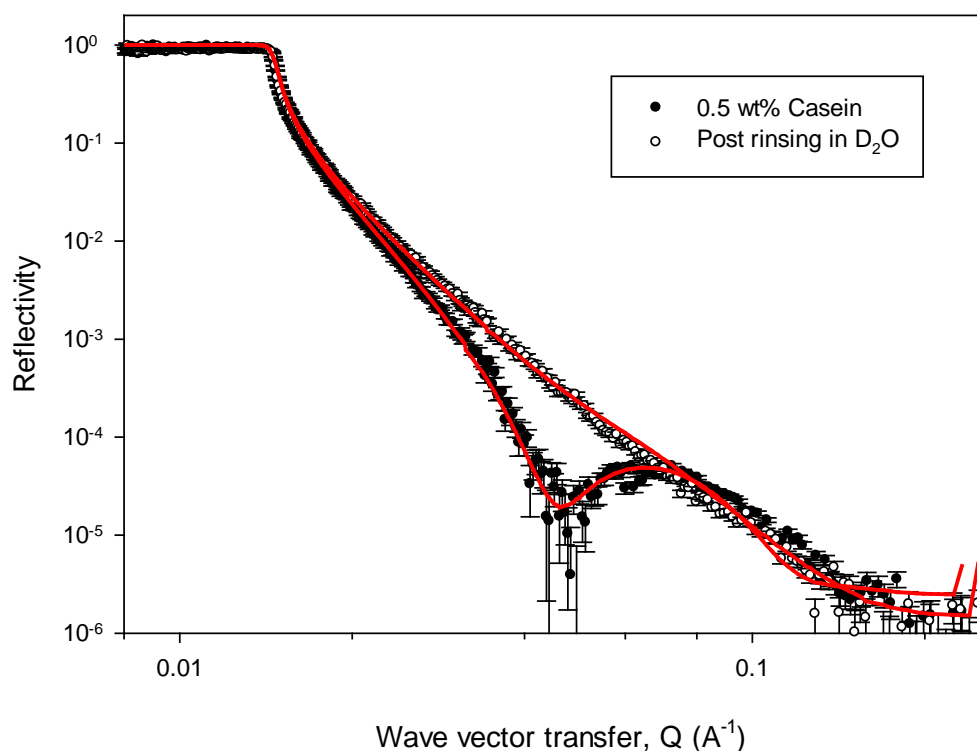
All the silicon surfaces were prepared using a mild piranha treatment, as described in detail elsewhere (39), to ensure a hydrophilic oxide surface of well-defined thickness, density and hydrophilicity.

The following sequence of measurements was made at the hydrophilic silica surface. B-casein adsorption from D<sub>2</sub>O was measured for  $\beta$ -casein concentrations of 0.05, 0.1, 0.2, 0.3 and 0.5 wt%, prior to its combination with hydrophobin. This was followed by rinsing in D<sub>2</sub>O. Hydrophobin was then adsorbed onto the same surface from a 0.2 mg/ml solution in D<sub>2</sub>O, followed by  $\beta$ -casein adsorption onto the hydrophobin coated surface for the same  $\beta$ -casein concentrations as described above. This sequence of measurements was completed by rinsing in D<sub>2</sub>O. A fresh sequence of measurements was then made for the co-adsorption of 0.02 wt% (0.2 mg/ml) hydrophobin /  $\beta$ -casein in D<sub>2</sub>O (for  $\beta$ -casein concentrations from 0.005 to 0.5 wt%, as described earlier), followed by rinsing in D<sub>2</sub>O. The final measurements in these sequences were two repeated measurements of 0.1 wt%  $\beta$ -casein in D<sub>2</sub>O.

## **RESULTS and DISCUSSION**

### **(i) B-casein adsorption.**

The adsorption of  $\beta$ -casein onto the hydrophilic silica surface was measured by NR in the  $\beta$ -casein concentration range of 0.01 to 0.5 wt% in D<sub>2</sub>O. Although  $\beta$ -casein adsorption at the hydrophilic solid-solution interface has been well characterised (26, 28) the measurements here provide an important reference point for the subsequent sequential and co-adsorption measurements with hydrophobin. The data at a  $\beta$ -casein concentration of 0.5 wt% and the associated model fit are shown in figure 1, along with data for the surface post  $\beta$ -casein adsorption and rinsing in D<sub>2</sub>O.



**Figure 1.** NR data for (o) Si / D<sub>2</sub>O (post adsorption and rinsing in D<sub>2</sub>O) and (●) 0.5 wt%  $\beta$ -casein / D<sub>2</sub>O. The solid lines are model fits as described in the text and using the model parameters summarised in the text and in table 2.

The appearance of a pronounced interference fringe with the addition of  $\beta$ -casein is consistent with significant  $\beta$ -casein adsorption. The adsorption at a  $\beta$ -casein concentration in the range 0.01 to 0.05 wt% is relatively weak. For concentrations  $\geq 0.1$  wt% it is more pronounced and is relatively constant over the concentration range 0.2 to 0.5 wt%. The data are relatively well described by a single layer of uniform composition, with the model parameters summarised in table 2.

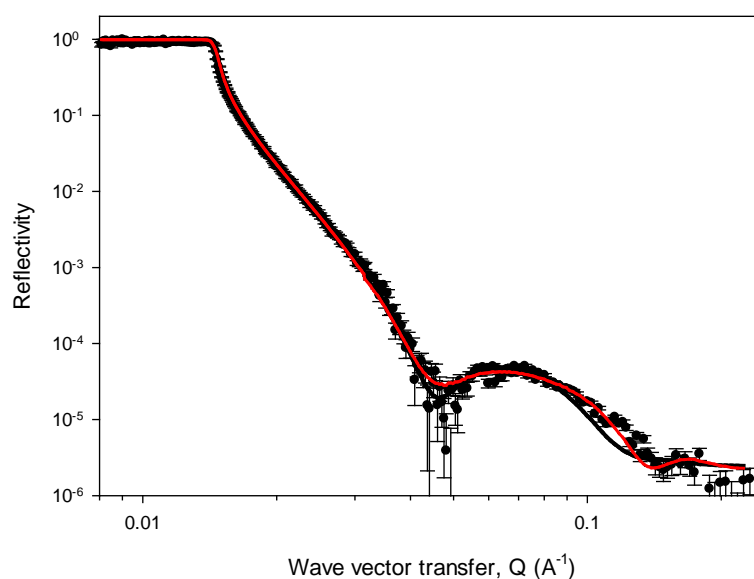
**Table 2.** Key model parameters from analysis of NR data for  $\beta$ -casein adsorption onto hydrophilic silica.

<b><math>\beta</math>-casein concentration (wt%)</b>	<b>d (<math>\pm 5</math> Å)</b>	<b><math>\rho</math> (<math>\pm 0.4 \times 10^{-6}</math> Å<sup>-2</sup>)</b>	<b><math>\Phi_{\beta\text{-casein}}</math> (<math>\pm 0.05</math>)</b>	<b>Adsorbed amount, <math>\Gamma</math> (<math>\pm</math> <math>0.05 \times 10^{-10}</math> mol cm<sup>-2</sup>)</b>
0.01	62	5.8	0.12	0.42
0.05	47	5.0	0.30	0.78
0.1	53	4.7	0.37	1.08
0.2	56	4.5	0.42	1.27
0.3	61	4.5	0.42	1.39
0.5	63	4.5	0.42	1.40

The volume fraction of  $\beta$ -casein at the interface is estimated from  $\rho = \rho_s (1 - \phi) + \rho_a \phi$ , where  $\rho_s$  and  $\rho_a$  are the scattering length densities of the D<sub>2</sub>O solvent ( $6.35 \times 10^{-6}$ ) and  $\beta$ -casein ( $1.8 \times 10^{-6}$ ), and  $\phi$  is the volume fraction of  $\beta$ -casein. The adsorbed amounts are relatively low compared to those reported by Tiberg et al (26), but it is noted that the studies of Tiberg et al (26) were made in buffer solution and this and the variability in surface preparation will both have an impact on the adsorption.

The NR data for the  $\beta$ -casein adsorption is slightly better modelled by a two layer model, as shown in figure 2.





**Figure 2.** NR data for 0.5 wt%  $\beta$ -casein /  $D_2O$ . The solid lines are model fits for one (black) and two layer (red) models, for the key model parameters as summarised in the text.

The key model parameters for the two-layer model are summarised in full in table S1 in the Supporting Information. The model consists of a dense inner layer,  $d_1$ , (adjacent to the solid surface)  $\sim 55$  Å similar to that obtained in the single layer fits and a dilute outer layer,  $d_2$ , (adjacent to the solution phase) with a thickness  $\sim 40$  Å. The key model parameters for the data at 0.5 wt%  $\beta$ -casein are  $d_1$ ,  $\rho_1$  of 61 Å and  $4.6 \times 10^{-6}$  Å $^{-2}$ , and  $d_2$  and  $\rho_2$  of 44 Å and  $6.1 \times 10^{-6}$  Å $^{-2}$ ; with the corresponding volume fractions of  $\beta$ -casein in the layers of 0.37 and 0.05 respectively.

Tiberg et al (26) measured  $\beta$ -casein adsorption at the hydrophilic silica surface by NR in the presence of 0.1 mg/mL imidazole buffer solution. Their data were described by three layers which varied in thickness and composition over a period  $\sim 5$  hrs. However the total thickness is relatively constant after  $\sim 3$  hrs at  $\sim 100 \pm 10$  Å. The outermost layer (adjacent to the solvent) is relatively dilute and diffuse, and so the general pattern of adsorption is broadly similar to that reported here. In the measurements reported here the surface is exposed to an increasing concentration of  $\beta$ -casein, from 0.05 to 0.5 wt%, over a similar timescale. The variations in thickness with time reported by Tiberg et al (26) result in final values that are broadly similar to those measured here. Tiberg et al (26) reported a total equilibrium thickness  $\sim 105$  Å and this compares with a value in the range 86 to 105 Å at the higher  $\beta$ -casein concentrations in table S2 in the Supporting

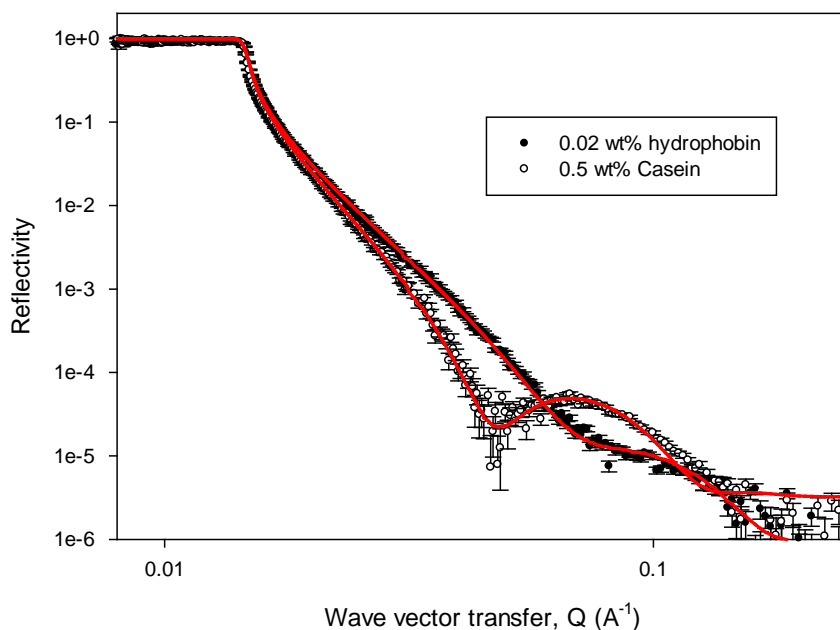
Information. Any detailed differences are probably associated with difference in the surface preparation and the differences in solution conditions (impact of the imidazole buffer), as discussed earlier in the context of the adsorbed amounts. This latter point is highlighted in the variation in the adsorbed layer characteristics at the air-water interface (24-27, 29, 30). Tucker et al (30) reported an adsorbed layer of  $\beta$ -casein at the air-water interface described by two layers with a total thickness  $\sim 80$  to  $90 \text{ \AA}$  (at pH 4-7), with a more dense layer adjacent to the air phase and a more dilute diffuse phase adjacent to the solvent phase. Although the two layer description is broadly similar to that presented here the outer layer was more dense than the dilute diffuse outer layer in this data. Hence this would imply the  $\beta$ -casein at the air-water interface adopts a different structure. Although the differences imply that the nature of the interface plays a role, the differences encountered are not massive. The results of Tucker et al (30) also however indicate the importance of the solution conditions as the surface structure at pH 4 to 7 is quite different to that at pH 3, where the data are consistent with a thinner more compact layer  $\sim 40 \text{ \AA}$ .

Rinsing in  $D_2O$  partially removed the  $\beta$ -casein from the surface. The NR data in figure 1, post  $\beta$ -casein adsorption and rinsing in  $D_2O$ , is modelled as a single layer  $\sim 56 \text{ \AA}$  and a scattering length density  $\sim 5.5 \times 10^{-6} \text{ \AA}^{-2}$ . This corresponds to a volume fraction of  $\beta$ -casein remaining at the surface  $\sim 0.19$ , and this is of order half the value at the maximum adsorption observed here. It is generally observed that  $\beta$ -casein is irreversibly adsorbed at the hydrophobic surface. At the hydrophilic surface Tiberg et al (26) reported only a slight desorption from the hydrophilic surface in the presence of buffer. The desorption upon rinsing here is larger and is a further indication of the sensitivity of the adsorption to the exact nature of the surface and the solution properties.

## **(ii) Sequential $\beta$ -casein /hydrophobin adsorption**

The initial NR measurements on hydrophobin /  $\beta$ -casein mixed adsorption at the hydrophilic silica surface were made as sequential measurements; that is, adsorption of  $\beta$ -casein onto a pre-adsorbed hydrophobin coated surface. Hydrophobin was adsorbed onto a bare hydrophilic silica surface from a 0.02 wt% (0.2 mg/mL) solution in  $D_2O$ . The resulting reflectivity is best described as a single layer with a thickness  $\sim 36 \pm 3 \text{ \AA}$  and a

scattering length density  $\sim 3.1 \pm 0.2 \times 10^{-6} \text{ \AA}^{-2}$  (see figure 3). This is similar to that reported previously by Zhang et al (38) and is consistent with a dense bilayer of hydrophobin adsorbed at the interface with a volume fraction  $\sim 0.7$ , and an adsorbed amount of  $0.47 \times 10^{-10} \text{ mol cm}^{-2}$ .



**Figure 3.** NR data for (●) 0.02 wt% (0.2 mg/ml) hydrophobin / D<sub>2</sub>O and (○) 0.5 wt% β-casein / D<sub>2</sub>O post hfb2 adsorption. The solid lines are model fits as described in the text and for the key model parameters in the text and in table 3.

Post hydrophobin adsorption a series of β-casein adsorption measurements were made in D<sub>2</sub>O with β-casein concentrations varying from 0.05 to 0.5 wt%, the same sequence as was made for β-casein onto the bare hydrophilic surface. The NR data for 0.5 wt% β-casein and its associated model fit are also shown in figure 3, and the key model parameters are summarised in table 3.

The sequence of measurements summarised in tables 2 and 3 indicate that there is no pronounced time dependence, over the time scale of the measurements, in the adsorption; and this is further confirmed later in the paper with some specific measurements

**Table 3.** Key model parameters from analysis of NR data for  $\beta$ -casein adsorption onto pre-adsorbed hydrophobin surface.

$\beta$ -casein concentration (wt%)	$d (\pm 5 \text{ \AA})$	$\rho (\pm 0.4 \times 10^{-6} \text{ \AA}^{-2})$	$\Phi_{\beta\text{-casein}} (\pm 0.05)$	$\Gamma (\pm 0.05 \times 10^{-10} \text{ mol cm}^{-2})$
0.05	42	3.8	0.57	1.32
0.1	49	4.7	0.36	1.01
0.2	52	3.9	0.55	1.56
0.3	57	3.8	0.55	1.79
0.5	61	3.9	0.53	1.83

The evolution in the adsorbed layer thickness and scattering length density in table 3 is broadly similar to that in table 2 for  $\beta$ -casein adsorption onto hydrophilic silica. Interpreting the scattering length density values as arising from  $\beta$ -casein adsorption results in broadly similar volume fractions of  $\beta$ -casein at the interface. However there are some notable systematic differences. At the lowest  $\beta$ -casein concentration the effective volume fraction is much higher,  $1.32 \times 10^{-10} \text{ mol cm}^{-2}$  compared to  $0.79 \times 10^{-10} \text{ mol cm}^{-2}$ . At the higher  $\beta$ -casein concentrations the effective adsorption is also larger, 1.56 to  $1.83 \times 10^{-10} \text{ mol cm}^{-2}$  compared to 1.27 to  $1.43 \times 10^{-10} \text{ mol cm}^{-2}$ . The results are consistent with the  $\beta$ -casein largely displacing the hydrophobin at the interface, and are interpreted as volume fractions and adsorbed amounts in terms of  $\beta$ -casein adsorption. However the systematically higher adsorption implies that there may be some co-adsorption may be occurring which is most pronounced at the lowest  $\beta$ -casein concentration. The similarity of the scattering length densities of the two components makes it difficult to quantify the individual contributions. It may also be the result of the apparent higher effective adsorption at the higher  $\beta$ -casein concentrations, but there is no apparent reason for the greater adsorption.

The displacement of hydrophobin from the hydrophilic surface (22) and the air-water interface (5, 10, 41) by surfactant at surfactant concentrations greater than the critical micellar concentration, cmc, has been reported, and the observation here for hydrophobin and  $\beta$ -casein is similar. This implies that above a critical concentration of  $\beta$ -casein (as was deduced for surfactants) hydrophobin /  $\beta$ -casein mixed aggregates form in solution and that the co-adsorption of hydrophobin into mixed solution

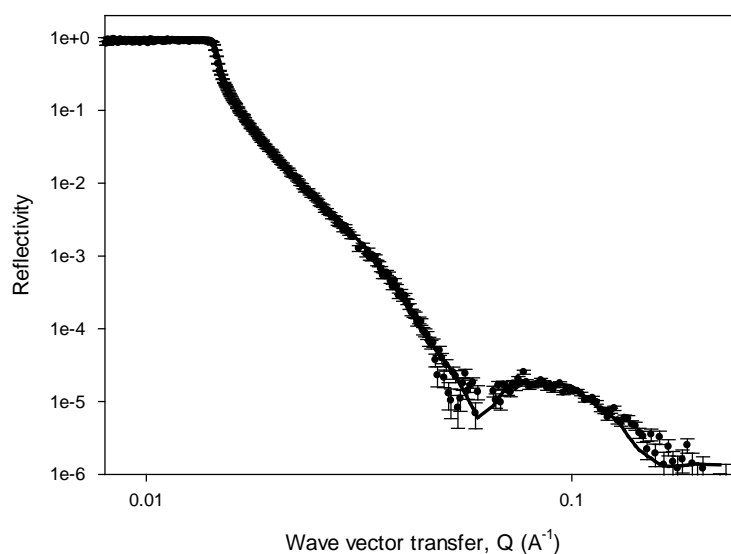
aggregates is favoured over adsorption at the interface. It is difficult to distinguish between  $\beta$ -casein and hydrophobin at the interface, as their corresponding scattering length density values ( $\rho=b/v$ ) are similar (see table 1). Sometimes it is feasible to distinguish in terms of the thickness of the adsorbed layer, as applied here. However in mixed layers it is difficult to separate the contributions from the two components.

As observed for the initial  $\beta$ -casein adsorption the NR data for the  $\beta$ -casein post hydrophobin adsorption is slightly better modelled as two layers (see key model parameters in table S2 in the Supporting Information). The values are similar to those in table S1 in the Supporting Information. Hence they provide no additional insights into the structure or composition of the layers. Overall the results are less consistent with coadsorption and more consistent with a surface dominated by  $\beta$ -casein.

Rinsing in D<sub>2</sub>O removes the  $\beta$ -casein and any residual hydrophobin to some extent, as was observed in the previous sequence of measurements for  $\beta$ -casein alone. That is, the NR data post adsorption and rinsing in D<sub>2</sub>O are modelled as a layer  $\sim 52$  Å and a scattering length density  $\sim 5.3 \times 10^{-6}$  Å<sup>-2</sup>, corresponding to a residual adsorption with a volume fraction  $\sim 0.2$ . It should be noted that in a previous study (39) hydrophobin adsorption onto hydrophilic silica was reversible to rinsing in D<sub>2</sub>O.

### (iii) $\beta$ -casein / hydrophobin co-adsorption

The co-adsorption of  $\beta$ -casein and hydrophobin was studied at the hydrophilic silica surface from a combination of 0.02 wt% (0.2 mg/mL) hydrophobin in D<sub>2</sub>O with three different  $\beta$ -casein concentrations, 0.05, 0.1 and 0.2 wt%. The NR data for 0.02 wt% (0.2 mg/mL) hydrophobin / 0.1 wt%  $\beta$ -casein / D<sub>2</sub>O are shown in figure 4.



**Figure 4.** NR data for 0.02 wt% (0.2 mg/mL) hydrophobin / 0.1 wt%  $\beta$ -casein /  $D_2O$ . the solid line is a model fit as described in the text and for key model parameters summarised in table 4.

The data are adequately described by a one or two layer model, and the two layer model is marginally better. The key model parameters for the two-layer model are summarised in table 4. The corresponding single layer model parameters are summarised in table S3 in the Supporting Information.

**Table 4.** Key model parameters from analysis of NR data for  $\beta$ -casein / hydrophobin coadsorption onto hydrophilic silica.

Hydrophobin concentration wt% (mg/ml)	$\beta$ -casein concentration (wt%)	$d_1$ ( $\pm 5$ Å)	$\rho_1$ ( $\pm 0.2 \times 10^{-6}$ Å <sup>-2</sup> )	$d_2$ ( $\pm 5$ Å)	$\rho_2$ ( $\pm 0.2 \times 10^{-6}$ Å <sup>-2</sup> )
0.02 (0.2)	0.05	43	4.7	43	6.1
0.02 (0.2)	0.1	50	4.6	51	6.1
0.02 (0.2)	0.2	58	4.6	54	6.0

In order to further eliminate any time dependent effects and issues relating to the reproducibility of the surface, two further measurements of  $\beta$ -casein adsorption onto the rinsed hydrophilic silica surface were made, for a concentration of 0.1 wt%  $\beta$ -casein

and over a time period of 2 hours. Like the previous data the reflectivity is best described as two layers, as summarised in table 5a; the equivalent single layer model parameters are summarised in table S4 in the Supporting Information.

**Table 5.** Key model parameters from analysis of NR data for  $\beta$ -casein adsorption onto hydrophilic silica (a) post hydrophobin adsorption and coadsorption (b) pre and post hydrophobin adsorption.

**(a) 0.1 wt%  $\beta$ -casein**

Time	$d_1$ ( $\pm 5$ Å)	$\rho_1$ ( $\pm 0.2 \times 10^{-6}$ Å <sup>-2</sup> )	$d_2$ ( $\pm 5$ Å)	$\rho_2$ ( $\pm 0.2 \times 10^{-6}$ Å <sup>-2</sup> )
0.0	53	4.0	49	6.1
+ 2 hrs	55	4.4	43	6.0

**(b)  $\beta$ -casein**

$\beta$ -casein concentration (wt%)	Conditions	$d_1$ ( $\pm 5$ Å)	$\rho_1$ ( $\pm 0.2 \times 10^{-6}$ Å <sup>-2</sup> )	$d_2$ ( $\pm 5$ Å)	$\rho_2$ ( $\pm 0.2 \times 10^{-6}$ Å <sup>-2</sup> )
0.5	Initial adsorption (see table 2)	61	4.6	44	6.1
0.5	Post hydrophobin adsorption (see table 3)	61	4.7	45	6.2
0.2	Co-adsorption with 0.2 mg/ml hydrophobin	58	4.6	54	6.1

The parameters in table 5a indicate that there is no significant time dependence over the timescale of these measurements, and affirm the inferences on time dependence from the earlier results summarised in tables 2 and 3.

In principle the data in figure 4 and table 4 can be interpreted as arising from  $\beta$ -casein adsorption, adsorption of a mixed hydrophobin /  $\beta$ -casein layer or  $\beta$ -casein adsorption onto a hydrophobin layer or vice-versa. However the similarity with the data in figures 1 and 3 and the parameters in tables 2 and 3 strongly imply that the adsorption is predominantly associated with  $\beta$ -casein and that hydrophobin does not compete effectively with  $\beta$ -casein for the surface. This is reinforced by the comparison of the parameters in table 5b, which directly compare the initial  $\beta$ -casein adsorption, the adsorption post hydrophobin adsorption and the coadsorption of  $\beta$ -casein / hydrophobin. Furthermore rinsing in D<sub>2</sub>O does not entirely remove the surface layer, consistent with the previous observations that  $\beta$ -casein is only partially desorbed by rinsing. A single layer, with a thickness  $\sim 52$  Å and a scattering length density  $\sim 5.3 \times 10^{-6}$  Å<sup>-2</sup> and corresponding to a volume fraction  $\sim 0.2$ , remains at the surface. In contrast, rinsing in D<sub>2</sub>O will completely remove hydrophobin from a hydrophilic surface (40). Hence the results indicate that, in the co-adsorption measurements (see table 4) and at the hydrophobin /  $\beta$ -casein concentrations and compositions studied,  $\beta$ -casein is preferentially adsorbed at the interface.

It appears, from the  $\beta$ -casein / hydrophobin combinations studied here, that in coadsorption from  $\beta$ -casein / hydrophobin mixtures the adsorption is dominated by the  $\beta$ -casein at all the  $\beta$ -casein concentrations studied. This is slightly different to what was observed in the sequential adsorption studies described earlier, where some evidence for coadsorption was evident. In both case there is a similarity with the behaviour for hydrophobin / surfactant adsorption at the air-water interface (22) and liquid / solid interface (40); where for surfactant concentrations  $>$  cmc the surfactant is preferentially adsorbed, and the hydrophobin is displaced from the surface. At the air/water interface (30) the coadsorption of hydrophobin and  $\beta$ -casein is slightly different. Coadsorption occurs at high pH, but at low pH the adsorption is dominated by the hydrophobin. For  $\beta$ -lactoglobulin / hydrophobin mixtures the surface adsorption is dominated by the hydrophobin (30).



#### **(iv) Discussion**

The pattern of adsorption in surface active mixtures depends upon their relative surface activities, the degree to which they undergo specific interactions at the surface or interface and their ability to form complexes and self-assemble in solution.

In the sequential adsorption measurements the data are interpreted in terms of the  $\beta$ -casein largely displacing the hydrophobin at the interface. Although the apparent measured adsorption of the  $\beta$ -casein can be interpreted to some degree as co-adsorption, especially at the lowest  $\beta$ -casein concentration. However as discussed earlier it is not possible to quantify the extent of co-adsorption with any certainty. The behaviour reported is analagous to that observed in hydrophobin / surfactant mixtures. At the air-water interface at surfactant concentrations  $>$  cmc hydrophobin was displaced at the interface in favour of surfactant / hydrophobin complex formation in solution (22). Similar behaviour was reported for hydrophobin / surfactant mixtures at the solid-solution interface (40). Although the results present here for the co-adsorption are also consistent with the results in reference 40, the results further show that for the range of parameters studied here the surface adsorption is dominated by the  $\beta$ -casein.

In protein mixtures the patterns of adsorption are more complex and depend also upon the nature of the interface and whether the proteins can interact or compete at the interface, and both competitive adsorption and co-adsorption are observed (5, 6, 18, 29). At the air-water interface co-adsorption occurs between  $\beta$ -casein and hydrophobin (7, 30). From the evidence in the literature the nature of the protein structure is clearly important. Marinova et al (6) reported the adsorption of mixed films of  $\beta$ -casein and BSA. Others reported inhomogeneous phase separated film formation in  $\beta$ -casein / BSA mixtures (29),  $\beta$ -casein /  $\beta$ -lactoglobulin films at the air-water interface (5, 10).

From the results presented here and consideration of the trends reported in the adsorption of protein / surfactant and protein / protein mixtures, it is proposed that the dominance of the  $\beta$ -casein in the sequential adsorption and co-adsorption of  $\beta$ -casein and hydrophobin results from a combination of the greater affinity of  $\beta$ -casein for the hydrophilic surface and the ability of the disordered nature of the  $\beta$ -casein structure to effectively complex with hydrophobin in solution.

## SUMMARY

The nature of protein and mixed protein adsorption is important for a range of applications (41, 42). The unusual surface properties of hydrophobin has attracted much recent attention (7, 15-22, 40, 41) in the context of food related formulations, and the manipulation of those properties by the addition of different types of cosurfactant is potentially important. Although protein and mixed protein adsorption has been extensively studied at the air-water interface (5, 6, 10, 11, 23-29, 45) and solid-solution interfaces (26-28, 44), there has been relatively little reported on the adsorption of hydrophobin with other proteins and surfactants (7, 18, 22, 30, 40, 44). We have partially addressed this here with a study of the adsorption of hydrophobin and  $\beta$ -casein at the hydrophilic solid-solution interface using NR. Measurements were made for sequential adsorption and co-adsorption. Although previously well characterised the adsorption of  $\beta$ -casein and hydrophobin were also measured for a direct comparison with the data for the mixed adsorption. The  $\beta$ -casein and hydrophobin adsorption onto the hydrophilic surface were broadly similar to that previously reported (26, 28, 40). For the sequential adsorption of  $\beta$ -casein onto a pre-coated hydrophobin surface, above a critical  $\beta$ -casein concentration,  $\sim 0.1$  wt %, the hydrophobin is largely displaced from the surface by the  $\beta$ -casein. This is similar to what is observed generally in protein / surfactant mixtures (45), and especially in hydrophobin / surfactant mixtures (22, 30, 40). Over the range of  $\beta$ -casein concentrations studied there is some evidence of coadsorption, especially at  $\beta$ -casein concentrations  $\leq 0.1$  wt% but the evidence is inconclusive. In the coadsorption studies the data strongly imply that the adsorption is predominantly associated with  $\beta$ -casein adsorption, and that under the conditions studied hydrophobin does not compete effectively for the surface.

## **SUPPORTING INFORMATION**

Some tables of the model parameters from the model fits to the NR data are included

## **AUTHOR INFORMATION and CONTRIBUTIONS**

Corresponding author email address: [jeff.penfold@stfc.ac.uk](mailto:jeff.penfold@stfc.ac.uk)

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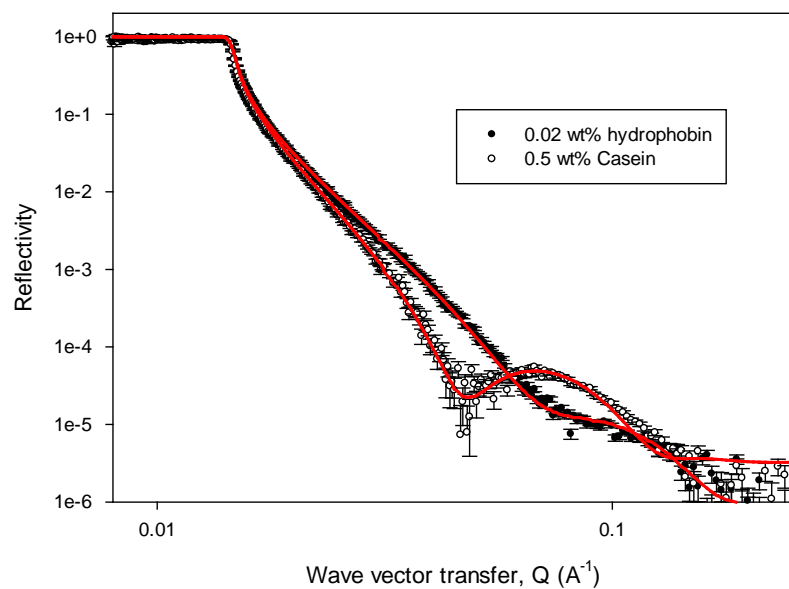
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## GRAPHICAL ABSTRACT



**Contrasting NR data for  $\beta$ -casein and hydrophobin adsorption,**