by this method; this method should be sensitive to cell structure, providing an alternative to conventional MRI (which uses relaxation-weighted spin density). Experiments of this type can be performed with any NMR spectrometer if variable linear magnetic-field gradients are available. Although a large spin concentration is required to generate the dipolar demagnetizing field, this condition is always fulfilled in aqueous solution.

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Neutron Reflection Study of Bovine β-Casein Adsorbed on OTS Self-Assembled Monolayers

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Specular neutron reflection has been used to determine the structure and composition of bovine β-casein adsorbed on a solid surface from an aqueous phosphate-buffered solution at pH 7. The protein was adsorbed on a hydrophobic monolayer self-assembled from deuterated octadecyltrichlorosilane solution on a silicon (111) surface. A two-layer structure formed consisting of one dense layer of thickness 23 ± 1 angstroms and a surface coverage of 1.9 milligrams per square meter adjacent to the surface and an external layer protruding into the solution of thickness 35 ± 1 angstroms and 12 percent protein volume fraction. The structure of the (β-casein) layer is explained in terms of the charge distribution in the protein.

The mechanism of adsorption of proteins at interfaces and the structure and homogeneity of the adsorbed layer are important prerequisites for a full understanding of the role of proteins in the stabilization of foams and emulsions and provide essential information for research in protein chromatography (*1*), biomedical materials (*2*), and cellular adhesion (*3*). There are therefore a large number of studies involving protein adsorption at both solid-liquid interfaces, such as on colloidal particles (*4–7*), on metal surfaces (*8, 9*), on silica surfaces (*10, 11*), and on polymer surfaces (*12*), and at air-liquid interfaces (*13–15*). The adsorption is so sensitive to the nature of the substrate that is it difficult to construct theoretical models, and it is therefore desirable to work with well-defined interfaces.

We have used neutron reflectivity (*16*) to determine the structure of the milk protein β-casein adsorbed on a chemically modified hydrophobic silicon surface. A hydrophobic self-assembled monolayer (SAM), formed on the silicon surface from octadecyltrichlorosilane (OTS) solution, was first characterized and then studied with a monolayer of protein. A hydrophobic surface was chosen because the conformation of proteins is, in most cases, determined by hydrophobic interactions in the nonpolar residues of the peptide chains.

The molecule β-casein is a single-chain protein with a known sequence of 209 residues (*17*) and a molecular mass of ~24,000 daltons. It has a 21-residue amino terminal sequence that contains one-third of the charged residues that the molecule has at pH 7. The remainder of the polypeptide side chains are mostly nonpolar and hydrophobic, and so the molecule is amphiphilic and surface-active. Sedimentation field-flow fractionation and dynamic light scattering of β-casein adsorbed on colloidal polystyrene latices (*5, 6*) indicate that this protein is a flexible molecule with a highly hydrophobic "tail," which may be the site of adsorption to nonpolar surfaces, and a hydrophilic portion that penetrates deeply into the aqueous environment. Prime and Whitesides have studied the adsorption of various proteins on SAMs of ω-functionalized long-chain alkanethiolates on gold (*9*) and found that they are excellent model systems for studying the interactions of proteins with organic surfaces.

We used the specular reflectivity of neutrons, which has recently proved to be a successful technique for studies of solid-liquid interfaces (*18*), to obtain valuable complimentary information on β-casein adsorption at a hydrophobic silicon surface. The experimental details have been given elsewhere (*19*). The technique has been used for the characterization of monolayers formed from OTS on silicon blocks (*20*) and offers several advantages over traditional methods. In a neutron reflection experiment, the specular reflection R is measured as a function of the wave vector transfer κ perpendicular to the reflecting surface, where $\kappa = (4\pi/\lambda)\sin\theta$ (θ is the glancing angle of incidence and λ is the wavelength of the incident neutron beam). The relation of R to the scattering length density across an interface, $\rho(z)$, is given by

$$R(\kappa) = \frac{16\pi^2}{\kappa^2} \mid \tilde{\rho}(\kappa) \mid^2 \qquad (1)$$

where $\tilde{\rho}(\kappa)$ is the one-dimensional Fourier transform of $\rho(z)$, that is

$$\tilde{\rho}(\kappa) = \int_{-\infty}^{+\infty} \exp(-i\kappa z)\rho(z)dz \qquad (2)$$

(ρ is a function of the distance z perpendicular to the interface).

In a typical analysis, the measured data are compared with a reflectivity profile calculated according to the optical matrix method (*21*) for different model density profiles. A model consists of a series of layers, each with a scattering length density ρ and thickness t. An additional parameter σ, the interfacial roughness between any two consecutive layers, may also be included in the matrix calculation. By variation of ρ and t for each layer, the calculated profile may be compared with the measured profile until the optimum fit to the data is found. Although any one profile may not provide a unique solution, one can obtain an unambiguous model of the interface by using different isotopic contrasts.

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Contrast variation relies on the fact that different nuclei scatter neutrons with quite different amplitude and, in the case of protons and deuterons, with opposite phase. Use of a combination of protonated and deuterated materials allows the reflectivity profile of a system to be substantially changed while maintaining the same chemical structure of the interface. Moreover, it is possible, by adjustment of the H/D ratio, to prepare solvents that are contrast-matched to the substrate. The contrast between the substrate and the solvent is then zero, so a reflectivity profile arises only from the adsorbed material. In this study we prepared water of different "contrasts" to match the silicon substrate (water CMSi) or the oxide layer (water $CMSiO_2$) by mixing H_2O and D_2O in the mass ratio 0.595/0.405 and 0.401/0.599 to give scattering length densities of 2.07×10^{-6} and 3.41×10^{-6} Å^{-2}, respectively (see Table 1 for scattering length densities and some physical dimensions of the materials used) (*22*).

To characterize the oxide layer, we measured reflectivity profiles at the interface between the silicon block and water at different isotopic compositions: D_2O, H_2O, water CMSi, and water $CMSiO_2$. We analyzed the data by using the optical matrix method and obtained information about both the thickness and the chemical composition of the layer. A model consisting of a single uniform layer of material was used for all four profiles, defined by a thickness t_0, which is the same for all four profiles, and a scattering length density ρ_0. We found it necessary to vary the scattering length density of the oxide layer, which can only be explained by assuming that some water is present in this layer. The oxide layer was found to have a thickness of 15 ± 1 Å and to be formed by SiO_2 and 28% water (by volume). This result is in good agreement with previous measurements on blocks treated in a similar manner (*19*, *20*). Although this simple model implies that the water is uniformly distributed in the oxide layer, it is likely that it is distributed more toward the bulk water side of the layer, indicating roughness of the surface. During the pretreatment of the substrate, we made the block hydrophilic and hence more reactive toward the silanating reagent by soaking it in a mixture of NH_4OH-H_2O_2-H_2 (5:1:1 by volume) at 70°C for 10 min. Upon removal from this solution [called "basic peroxide" or "RCA standard clean 1" treatment (*23*)] the block was hydrophilic because of the presence of silanol groups on the surface. The oxide may have been made unusually porous by the "basic peroxide" treatment of the substrate. The fitted profiles and the parameters used in the fitting model are described more fully in (*19*).

Oriented SAMs can be formed on polar solid surfaces by chemisorbing amphiphiles from organic solutions at a solid-fluid interface (*24*, *25*). The organochlorosilane OTS is commonly used to form such monolayers on a number of substrates, including glass (*24*–*26*). These OTS films have good physical and chemical stability, the hydrocarbon portion of the OTS forming densely packed chains, oriented nearly perpendicular to the surface.

After the characterization of the oxide layer, the block was immersed for 1 hour in a solution of deuterated OTS (d-OTS) (*19*) in CH_2Cl_2 at a concentration of 1.5×10^{-3} M, removed, and successfully rinsed in CH_2Cl_2, ethanol, and water. A deuterated hydrophobic agent was chosen to give a better contrast between the surface and the protonated casein. Reflectivity measurements were taken after the treatment, and again four profiles were measured in the presence of D_2O, H_2O, water CMSi, and water $CMSiO_2$.

Most of the studies undertaken on OTS SAMs show the formation of closely packed crystalline-like layers, oriented nearly perpendicular to the surface with a thickness equal to or very close to the extended chain length of the octadecyl group. Our results gave a slightly different model. In fact, we could fit the profiles obtained with the contrasts H_2O, water CMSi, and water $CMSiO_2$ with a two-layer model (oxide and OTS) where the OTS layer had effectively a crys-

Table 1. Properties of materials used.

Material	Density* (g cm^{-3})	Volume (Å^3)	Length† (Å)	b‡ (10^{-4} Å)	ρ (10^{-6} Å^{-2})
H_2O	0.9975	30		−0.168	−0.56
D_2O	1.105	30		1.905	6.35
Water CMSi	1.038	30		0.621	2.07
Water $CMSiO_2$	1.059	30		1.023	3.41
Si	2.32	20		0.415	2.07
SiO_2	2.16	47		1.585	3.41
$-C_{18}D_{37}$	0.7768	542	24.3	36.65	6.76
β-Casein	1.365	29,594	640	532.6	1.80

*Taken from (*31*) except for the β-casein density, which is from (*5*). The value for $-C_{18}D_{37}$ was calculated from the density of liquid octadecane and the molecular weight of the deuterated octadecyl chain. †OTS (*32*) and β-casein (*7*) (the length of β-casein is the "brush" thickness for the protein tail). ‡From (*33*).

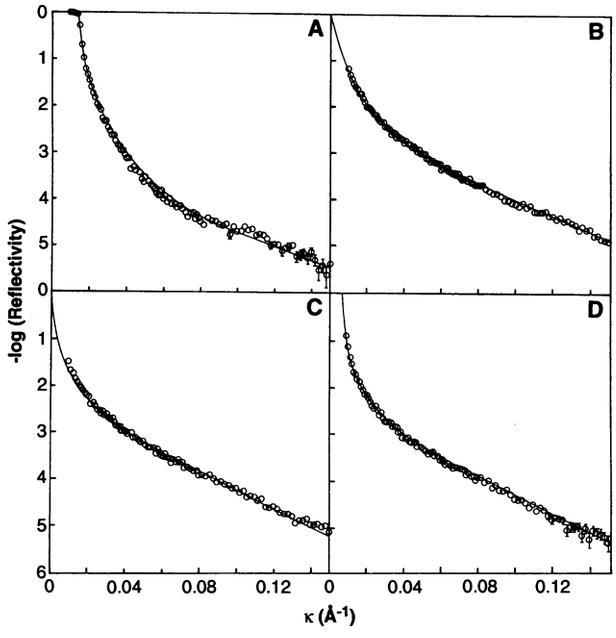

Fig. 1. Neutron reflectivity profiles measured at the Si–SiO_2–deuterated (d-OTS) OTS–water interface: (**A**) Si–SiO_2–d-OTS–D_2O; (**B**) Si–SiO_2–d-OTS–H_2O; (**C**) Si–SiO_2–d-OTS–CMSi; and (**D**) Si–SiO_2–d-OTS–$CMSiO_2$. The continuous line shown with the data is calculated from a double-layer description of the hydrophobic layer: the first layer is a crystalline-like layer with a thickness of 16 Å and an area per molecule of 20 Å^2, and the second layer is a liquid-like layer with a thickness of 11 Å and an area per molecule of 29 Å^2.

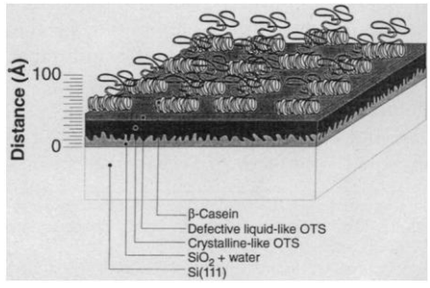

Fig. 2. Schematic representation of the adsorbed layer of β-casein at the interface between a modified hydrophobic silicon surface and an aqueous solution.

talline-like structure and a thickness (within error) equal to the extended chain length of the octadecyl molecule, but the profile measured in the presence of D_2O could not be fitted with this model. The only model that could fit the four profiles simultaneously was a three-layer model, where it was necessary to assume that the OTS layer formed partly by a crystalline-like structure and partly by a liquid-like structure. The fitted profiles are shown in Fig. 1, and the parameters used are summarized in Table 2.

The first feature of the chosen model that we noticed is that the apparent thickness of the oxide after the treatment with the hydrophobic agent increases from 15 ± 1 to 19 ± 1 Å, while the percentage of water drops from 28 to 20%. This change can be explained by the presence of the siloxane cross-linking of the silanating agent, which occurs at the surface during the film preparation. The chemical composition of this moiety is similar to that of the underlying silicon oxide, and its presence has the effect of apparently increasing the thickness of the oxide layer.

The second layer in the model is formed by that part of the octadecyl chains with a crystalline-like density (see Fig. 2). The thickness of this layer is 16 ± 1 Å and corresponds to ~10 $-CH_2-$ groups of the molecule. The density of the layer is 0.920 g cm^{-3} which, being ~15% higher than the liquid normal-alkane density, corresponds to the density of a crystalline phase (*27*). The area per molecule is 21 ± 1 $Å^2$ and the coverage (*18*) is 7.9 ± 0.4 μmol m^{-2}, which agree well with reported data and, combined with the thickness result, correspond to a layer of closely packed molecules almost perpendicular to the surface. It was also calculated that there are five molecules of water for every three octadecyl chains, which are presumably associated with defects in the layer. [For the calculations of the density, the area per molecule, and the number of water molecules per octadecyl chain, see (*19*).]

The third layer has a density of 0.667 g cm^{-3}, which corresponds to a defective layer, possibly liquid-like. The layer thickness was 11 ± 1 Å, corresponding to approximately eight $-CH_2-$ groups. The area per molecule is greater than for the previous layer, whereas the surface coverage is lower, equal to 28 ± 1 $Å^2$ and 5.9 ± 0.2 μmol m^{-2}, respectively, as would be expected from the more defective nature of the layer. In this layer, there is nearly one molecule of water per hydrocarbon chain.

The concentration chosen for the β-casein (Sigma, used as received) was 0.05 mg cm^{-3}, well below the critical micelle concentration of the protein [~0.5 mg cm^{-3} at room temperature (*28*)] and in the plateau region of the adsorption isotherm at the air-liquid interface (10^{-4} to 10^{-2} % by weight) (*29*). We prepared solutions by dissolving the protein sample in 20 mM sodium phosphate buffer at pH 7.0. Three neutron reflectivity measurements were carried out, and solutions were made in D_2O, H_2O, and water CMSi. We made the first measurement only after the solution had been in contact with the surface for 10 hours. We believe, from preliminary experiments (*30*), that β-casein needs time to reach its final conformation on the surface. On this occasion we could not detect any variations of conformation with time and therefore we believe that by the start of the experiment the molecules probably had already adopted their final conformation.

The data were well fitted by a model in which the casein was divided into two layers. The first is a dense layer, adjacent to the surface, with a thickness of 23 ± 1 Å and a volume fraction of protein of 61%. The second layer has a thickness of 35 ± 1 Å and is 12% protein by volume. Figure 3 shows the reflectivity profiles fitted with the parameters of β-casein given in Table 2. The four structural parameters are coupled, and it is possible to fit the two-layer model with up to a 10% variation in each of the four parameters. However, it has proved impossible to fit fundamentally different models to the data, which makes us confident that the above interpretation is unique. For example, a single monolayer will not fit the observations, nor will a

Table 2. Parameters used to fit the oxide, hydrophobic, and protein layers; ρ^i is the scattering length density of the material in water of isotopic composition *i*.

Layer	t ±1 (Å)	ρ^{D_2O} (10^{-4} $Å^{-2}$)	ρ^{H_2O} (10^{-4} $Å^{-2}$)	ρ^{CMSi} (10^{-4} $Å^{-2}$)	ρ^{CMSiO_2} (10^{-4} $Å^{-2}$)
Oxide	19	4.01	2.59	3.13	3.41
OTS					
Crystalline	16	6.80	5.70	6.11	6.33
Defective	11	5.10	4.60	4.80	4.88
β-Casein					
Inner	23	3.57	0.889	1.90	
Outer	35	5.80	−0.275	2.04	

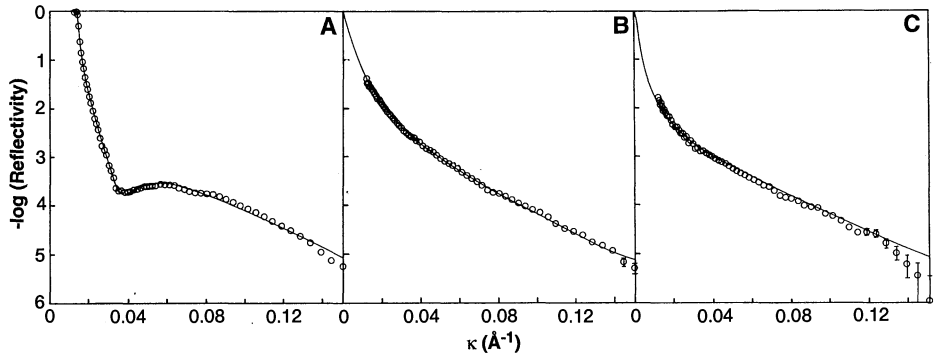

Fig. 3. Neutron reflectivity profiles measured at the Si-SiO_2-d-OTS-β-casein-water interface: (**A**) Si-SiO_2-d-OTS-β-casein-D_2O; (**B**) Si-SiO_2-d-OTS-β-casein-H_2O; and (**C**) Si-SiO_2-d-OTS-β-casein-CMSi. The continuous line shown with the data is calculated from a double-layer description of the protein layer: the first layer is a denser layer with a thickness of 23 Å and an area per molecule of 7.88 × 10^{-3} $Å^2$, the second layer is a less dense layer with a thickness of 35 Å and an area per molecule of 2.37 × 10^{-3} $Å^2$.

Table 3. Properties of the hydrophobic layer and β-casein derived from the parameters from the model fit. For the method of calculation of these parameters see (*19*).

Layer	Area ($Å^2$)	Coverage (μmol m^{-2})	Volume fraction	Number of waters	Density (g cm^{-3})
OTS					
Crystalline	21 ± 1	7.9 ± 0.4	0.857	1.5	0.920
Defective	28 ± 1	5.9 ± 0.2	0.926	0.85	0.667
β-Casein					
Inner	(2.1 ± 0.1) × 10^3	(7.9 ± 0.4) × 10^{-2}	0.61	630	1.349
Outer	(7.0 ± 0.1) × 10^3	(2.37 ± 0.04) × 10^{-2}	0.12	7173	1.349

structure with the two layers reversed.

Finally, we assessed the reversibility of the adsorption by measuring the neutron reflectivity profile in the presence of D_2O, after rinsing the surface copiously with water. This profile was almost identical to that obtained in the presence of the protein, but totally different from that with a clean surface, indicating that the protein is not easily removed. However, prolonged soaking in a solution of a nonionic surfactant eventually removed the β-casein.

The results from the reflectivity experiment are summarized in Table 3. These structural parameters can be explained in terms of the charge distribution of the protein molecule. The protein β-casein resembles a surfactant with a polar head and a nonpolar tail. The high concentration of negatively charged amino acids in positions 15 to 25 effectively makes this portion a negatively charged head. The first 40 to 50 residues of the amino-terminal polypeptide are predominantly hydrophilic, thus allowing this part of the molecule to extend into the aqueous phase (Fig. 2). If we assume that the first layer is formed only by the hydrophobic part of the protein molecule, which will consist of 160 to 170 residues, the area per amino acid residue, calculated from the area per protein molecule assuming a uniform distribution of the protein on the surface, is 12 to 13 $Å^2$. On the basis of the scattering length density of this inner layer, the number of water molecules per protein molecule is ~630. If the second layer is formed by the first 40 to 50 amino acids of the protein chain, then the area per amino acid fragment in this layer would be 130 to 165 $Å^2$.

The protein adsorption process and the structure of the adsorbed layer depend strongly on the nature of the substrate, and for this reason results from different experiments may not easily be compared. The closest comparable results are those of Nylander and Wahlgrem (*11*), who studied the interfacial structure of β-casein on hydrophobized silica surfaces by ellipsometry. They found that the amino-terminal part of an adsorbed β-casein molecule is likely to protrude into the solution and that, at a protein concentration of 0.1 mg cm^{-3}, the area per molecule of the adsorbed protein was 1.66×10^3 $Å^2$ and the coverage was 0.10 μmol m^{-2}. These values indicate a slightly higher coverage than in our system. This may be because our solution was less concentrated, which might lead to less adsorption, or because the ability of neutron reflection to determine the layer structure directly should lead to a more accurate measurement of the coverage.

A two-layer structure has been suggested for β-casein at other surfaces. At the most comparable surface, negatively charged polystyrene spheres, Mackie *et al.* (*7*) found by small-angle x-ray scattering that most of the protein was in a 20 Å layer near the surface with the remainder extending 100 Å into the solution. Caldwell *et al.* (*5*), using dynamic light scattering, deduced an overall thickness of 150 Å. Both of these values are considerably greater than that observed here, which is surprising because the higher adsorbed amount that we observe might be expected to be associated with a more brush-like conformation of the hydrophilic part of the protein and consequently a greater extension of this part of the molecule away from the surface. The difference may, of course, simply be the change in the surface.

Dickinson *et al.* (*13*) have used neutron reflection to study β-casein adsorbed at air-water and oil-water interfaces and were also able to fit a two-layer model, but with 94% protein in a first layer of thickness 20 Å, and 14 to 21% in a second layer of thickness 70 to 50 Å. They observed only small differences for the two types of interfaces. Although similar in dimension to our layer, the coverage is completely different. This is surprising given that our surface should be much more hydrophobic than the air-water interface and, indeed, the coverage in their first layer is physically improbable and much larger than the coverages reached by Gan *et al.* (*15*) for a monolayer of β-casein spread on water.

There is an intrinsic difficulty in assessing the coverage of protein from such an experiment because it is not possible to prepare a deuterated protein, which means that the experiment relies on a rather small contrast of protein with air or the precise amount of D_2O missing from the layer or both. The coverage is an ill-conditioned quantity under these circumstances. Gan *et al.* have studied the surface viscoelasticity of β-casein monolayers at the air-liquid interface and found that both static and dynamic monolayer properties undergo a major change at surface concentration of ~1.0 mg m^{-2}, where amino acid residues can no longer completely cover the surface and some extension of residues into the underlying solution occurs (*15*). As our surface concentration was 1.9 mg m^{-2}, we should expect some protrusion of the protein into the aqueous phase, as we observed.

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