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Front-Face Fluorescence Spectroscopy Study of Globular Proteins in Emulsions: Displacement of BSA by a Nonionic Surfactant

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The displacement of a globular protein (bovine serum albumin, BSA) from the surface of oil droplets in concentrated oil-in-water emulsions by a nonionic surfactant (polyoxyethylene sorbitan monolauarate, Tween 20) was studied using front-face fluorescence spectroscopy (FFFS). This method relies on measurement of the change in intensity (I_{MAX}) and wavelength (λ_{MAX}) of the maximum in the tryptophan emission spectrum. A series of oil-in-water emulsions (21 wt % *n*-hexadecane, 0.22 wt % BSA, pH 7.0) containing different molar ratios of Tween 20 to BSA ($R = 0-131$) were prepared. As the surfactant concentration was increased, the protein was progressively displaced from the droplet surfaces. At $R \geq 66$, the protein was completely displaced from the droplet surfaces. There was an increase in both I_{MAX} and λ_{MAX} with increasing Tween 20 concentration up to $R = 66$, which correlated with the increase in the ratio of nonadsorbed to adsorbed protein. In contrast, there was a decrease in I_{MAX} and λ_{MAX} with Tween 20 concentration in protein solutions and for $R \geq 66$ in the emulsions, which was attributed to binding of the surfactant to the protein. This study shows that FFFS is a powerful technique for nondestructively providing information about the interfacial composition of droplets in concentrated protein-stabilized emulsions in situ. Nevertheless, in general the suitability of the technique may also depend on protein type and the nature of the physicochemical matrix surrounding the proteins.

KEYWORDS: BSA; Tween 20; competitive adsorption; emulsions; fluorescence

INTRODUCTION

Emulsifiers are widely used in the food industry to facilitate emulsion formation and to improve emulsion stability (*1*, *2*). Emulsifiers rapidly adsorb to the surface of the droplets formed during homogenization, where they facilitate further droplet disruption by lowering the interfacial tension and retard droplet aggregation by forming protective membranes around the droplets (*3*−*5*). A wide variety of substances are available for use as emulsifiers in foods, including fatty acid derivatives, phospholipids, proteins, and polysaccharides (*1*−*3*, *6*, *7*). The effectiveness of a particular emulsifier at improving emulsion formation and stability depends on its chemical structure and the environmental conditions under which it operates, e.g. molecular environment, temperature, and pressure.

In many food emulsions there are a number of different types of surface-active molecules that can compete for the oil−water interface (*8*−*10*). The composition of the interface therefore depends on the type and concentration of surface-active molecules present in the system, as well as the history of the system, e.g. time of ingredient addition, previous thermal treatments (*11*−*15*). The interfacial composition has a major impact on the sign, magnitude, and/or range of many of the colloidal interactions responsible for emulsion stability, e.g., van der Waals, electrostatic, steric, hydrophobic, and hydration interactions (*5*−*16*). The type of emulsifier molecules present at an oil−water interface also determines its viscoelastic properties, which influences the stability of emulsion droplets to coalescence (*17*). By controlling the type of emulsifiers present at the oil−water interface, a food manufacturer therefore has some control over the physicochemical properties and stability of food emulsions (*5*, *12*, *18*−*20*). In some situations it may be possible to use combinations of emulsifiers to create emulsions with properties that are better than those that can be achieved using individual emulsifiers. In other situations it may be possible to avoid using combinations of emulsifiers that adversely affect emulsion properties. Consequently, it is important for food manufacturers to understand the factors that

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determine interfacial composition in emulsions. To achieve this goal it is necessary to have analytical techniques that provide information about the composition and properties of the interfacial membranes surrounding the emulsion droplets.

A wide variety of analytical techniques has been developed to provide information about the composition of interfacial layers separating bulk liquids. Information about interfacial composition and emulsifier organization at a planar interface can be obtained from measurements of the interfacial tension, interfacial rheology, radiolabeling, Brewster angle microscopy, or atomic force microscopy (*15*, *21*−*27*), as well as from techniques that utilize reflection of electromagnetic radiation or subatomic particles from the interface (*28*−*32*). Most of these techniques cannot be used to provide information about the composition of the interfaces surrounding emulsion droplets, and so different types of analytical techniques have to be used. One of the most widely used methods of determining interfacial composition is to centrifuge an emulsion and measure the concentrations of the different types of emulsifier present in the serum phase. The interfacial composition is then inferred from knowledge of the total concentration of emulsifiers present in the emulsion (*33*). This technique is time-consuming and destructive, and the preparation procedure may alter the interfacial composition. There is therefore a need for analytical techniques that can provide information about the interfacial composition of emulsions in situ. Fourier transform infrared (FTIR) can be used to provide information about changes in the conformation of protein emulsifiers adsorbed to droplet surfaces in concentrated emulsions (*34*). It may therefore be possible to utilize this technique to distinguish between adsorbed and nonadsorbed proteins in emulsions. Nevertheless, the IR absorbance of H_2O overlaps that from proteins, so emulsions usually have to be dispersed in D_2O rather than water (*34*). Recently, a novel approach has been developed to monitor the properties of adsorbed protein emulsifiers in concentrated emulsions. In this approach an emulsion is made optically transparent by adding a solute to the aqueous phase so that its refractive index becomes the same as that of the oil phase (*35*). The resulting refractive index matched emulsion (RIME) can then be analyzed using standard spectroscopy techniques, such as fluorescence, FTIR, circular dichroism, or UV−visible spectrometry. It may therefore be possible to utilize this approach to provide information about interfacial composition in concentrated emulsions. Nevertheless, there are concerns about the influence of the solutes added to the emulsions to increase the refractive index of the aqueous phase (e.g., glycerol, poly(ethylene glycol), or sucrose) on the structural properties of the proteins, since many solutes change protein conformation and aggregation at the high concentrations needed to create RIMEs (*36*).

The objective of this study is to examine the possibility of using front-face fluorescence spectroscopy (FFFS) to noninvasively monitor the displacement of a globular protein (BSA) from the surface of oil droplets by a nonionic surfactant (Tween 20) in concentrated emulsions. Previous studies have shown that FFFS is sensitive to the molecular environment of proteins in emulsions, i.e. adsorbed vs nonadsorbed state (*37*−*39*). This information is inferred from measurements of changes in the intensity (I_{MAX}) and/or wavelength (λ_{MAX}) of the maximum in the protein tryptophan emission spectrum resulting from the interaction of electromagnetic radiation with an emulsion. The value of λ_{MAX} is appreciably smaller in the adsorbed state than in the nonadsorbed state of BSA in emulsions, which means that it should be possible to obtain information about the relative proportions of adsorbed and nonadsorbed protein. The major advantage of the FFFS technique would be that information about the interfacial composition of the droplets in emulsions could be obtained directly, without any sample preparation.

MATERIALS AND METHODS

Materials. Analytical grade hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium azide (NaN_3), Tween 20, and *n*-hexadecane were purchased from the Sigma Chemical Co. (St. Louis, MO). Powdered BSA (Lot 7602E, Fraction V) was obtained from ICN Chemicals Inc., Aurora, OH). Deionized water was used for the preparation of all solutions (<10 MΩ, resistivity, MilliQ Water Purification System).

Emulsion and Solution Preparation. An emulsifier solution was prepared by dispersing 5 g/L of BSA and 0.2 g/L sodium azide (as an antimicrobial) in deionized water. The resulting solution was stirred for 3 h to ensure complete dissolution of the protein and then the pH was adjusted to 7.0 with 1 M NaOH. The protein solution was then stored at 4 °C for 20 h before use. An oil-in-water emulsion was prepared by homogenizing 30 wt % *n*-hexadecane oil and 70 wt % emulsifier solution at room temperature. The oil and emulsifier solution were blended using a high-speed blender at 20000 rpm for 4 min (Polytron PT 6100, Kinematica, Littan, Switzerland) and then recirculated through a one-stage high-pressure valve homogenizer for 20 min at 7000 Pa (one-stage laboratory homogenizer, Stansted Fluid Power, UK). The temperature of the emulsions never exceeded 30 °C during the homogenization process. There was a slight decrease in the pH (~0.1 unit) of the emulsions after homogenization, so it was adjusted back to 7.0 using 1 M NaOH solution. One hour after homogenization, a series of emulsions was prepared by diluting the 30 wt % emulsion with different ratios of water and Tween 20 solutions to obtain 21 wt % *n*-hexadecane emulsions with 3.1 g/L BSA and 0−7.6 g/L Tween 20 in the aqueous phase ($R = 0-131$). These emulsions were stored in a temperature-controlled environment at 20 °C for 24 h before further analysis. A series of protein solutions was also prepared by diluting the initial emulsifier solution with different ratios of water and Tween 20 solution to obtain protein solutions containing 3.1 g/L BSA and 0−7.6 g/L Tween 20 ($R = 0-131$).

Particle Size Determination. The particle size distribution of the emulsions was measured using a laser diffraction instrument (Malvern, Worcs, UK). This instrument measures the angular dependence of the intensity of light scattered from a stirred dilute emulsion and then indicates the particle size distribution that gives the closest fit between theoretical calculations and experimental measurements. A refractive index ratio of 1.08 and an absorption of 0.001 was used in the particle size calculations, corresponding to an instrument presentation code of 0403. To avoid multiple scattering effects, the emulsions were diluted with water prior to making the measurements to obtain obscuration values between 10 and 15%. The emulsions were stirred continuously throughout the measurements to ensure that the samples were homogeneous. Dilution and stirring may have partially disrupted weakly flocculated droplets, although it is unlikely that they will have disrupted any strongly flocculated droplets. The theory used to calculate the particle size distribution assumes that the particles are spherical and homogeneous, and therefore, the data obtained on emulsions that contained flocs should be treated with caution, because they are nonspherical and nonhomogeneous. Particle size measurements are reported as volume-surface mean diameters, d_{32} ($=\sum n_i d_i^3/\sum n_i d_i^2$, where n_i is the number of particles with diameter d_i). The measured mean particle diameters were highly reproducible (<5% difference) and are reported as the average of measurements made on two samples.

Creaming Index. Six grams of emulsion was transferred into a plastic test tube and then stored for 24 h at 20 °C. The total height of the emulsions (H_E) and the height of the droplet-depleted lower layer (H_L) were measured. The extent of creaming was characterized by a *creaming index:* % creaming $= 100 \times (H_L/H_E)$. The creaming index provides indirect information about the extent of droplet flocculation in an emulsion. Flocs move upward more rapidly than individual particles because of their greater size (provided a three-dimensional network of aggregated particles does not form); hence, the creaming index is higher.

Front-Face Fluorescence Measurements. Fluorescence emission spectra of 21 wt % *n*-hexadecane oil-in-water emulsions were measured using a spectrofluorometer (4800C, SLM Instruments, Urbana, IL). This apparatus was fitted with excitation (35°) and emission (vertical) polarizers and a variable angle front-surface accessory set at 56°. Emulsion samples were poured into quartz cells with an optical path length of 10 mm. Each individual emission spectrum was the average of two runs recorded from 300 to 380 nm using a 1 nm step, with the excitation wavelength set at 290 nm. Excitation and emission slits were set at 4 nm. All spectra were corrected for instrumental distortions in excitation by using a reference cell containing rhodamine B (2 g/L) in ethanol. Spectrums were measured on two or three different aliquots of each emulsion at 20 ± 1 °C.

Free Protein Measurements. The free protein concentration in the aqueous phase of the emulsions was determined using a modified Lowry method (*40*). Emulsions that had been stored for 2 days after homogenization at 20 °C were placed in plastic tubes and centrifuged for 30 min at 1000*g* to separate them into a creamed layer and a serum layer. The serum layers were then collected by making small holes in the bottom of each of the plastic (Nalgen) tubes. A portion of the serum phase was diluted 1:30 with water, and then the necessary reagents were added according to the instructions described previously (*40*). The absorbance of the solutions was then measured at 660 nm using a UV−visible spectrophotometer. The protein concentrations in the serum phases were determined using a calibration curve prepared using BSA solutions of known concentration.

Circular Dichroism Measurements. The CD spectra of the serum phase of the emulsions collected by centrifugation and of protein solutions were recorded (CD6, Jobin Yvon, Longjumeau, France). Information about secondary structure was obtained using wavelengths in the far-UV (180−250 nm, 1 nm increment) with a quartz cell with a path length of 0.1 mm. Information about tertiary structure was obtained using wavelengths in the near-UV (250−350 nm, 1 nm increment) with a quartz cell with a path length of 5 mm.

RESULTS AND DISCUSSION

Particle Size and Creaming Measurements. At all surfactant-to-protein ratios ($R = 0-131$) the particle size distribution of the emulsions was monomodal and the mean particle diameter was similar ($d_{32} = 0.97 \pm 0.05$ μm), indicating that the emulsions were stable to droplet flocculation. The emulsions were also relatively stable to gravitationally induced creaming, with the creaming index being less than 5% at all surfactant concentrations.

Centrifugation and Free Protein Concentration Measurements. The emulsion separated into two layers after centrifugation at 1000*g* for 30 min: an opaque creamed layer at the top and a transparent serum layer at the bottom. The thickness of the creamed layer formed after centrifugation was similar in all of the emulsions, being about 30% of the total emulsion height. The serum layer collected from the centrifuged emulsions was analyzed to determine the concentration of nonadsorbed protein. In the absence of surfactant, around 40% of the BSA in the emulsions remained in the aqueous phase of the emulsions after homogenization (**Figure 1**), indicating that around 60% was adsorbed to the droplet surfaces. As the ratio of surfactant-to-protein was increased, the BSA concentration in the aqueous phase increased, indicating that protein was displaced from the droplet surfaces. Little BSA displacement occurred when *R* was increased from 0 to 20, extensive displacement occurred when *R* was increased from 20 to 66, and the protein was completely displaced at higher *R* values.

Information about the physicochemical processes that occur when globular proteins are displaced from planar surfaces by nonionic surfactants have recently been obtained using atomic force microscopy, Brewster angle microscopy, interfacial tension, and interfacial rheology techniques (*26*, *27*, *31*, *32*). When a protein-coated interface is brought into contact with an aqueous solution containing nonionic surfactant, a sequence of events occurs that depends on the surfactant concentration. At relatively low surfactant concentrations, surfactant molecules adsorb to the interface and form small islands of surfactant located within the protein network. As the surfactant concentration is increased, the size of the surfactant-rich regions expands, restricting the protein network to a smaller surface area. At relatively high surfactant concentrations, the protein region increases appreciably in thickness and eventually the protein molecules are completely displaced from the interface. The two-dimensional phase separation of the interface into a protein-rich and a surfactant-rich region can clearly be observed using microscopy techniques (*26*, *27*, *31*, *32*). These findings would account for our observation that there was negligible displacement of the protein from the emulsion droplet surfaces at low surfactant concentrations ($R < 20$) but a sharp loss of protein at higher surfactant concentrations. The fact that the globular proteins form thick layers at the interface immediately prior to desorption suggests that they have become aggregated, probably as a result of surface denaturation (*41*).

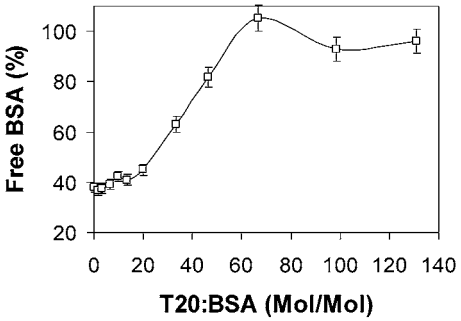

Figure 1. Dependence of the percentage of nonadsorbed protein in an emulsion on the surfactant-to-protein molar ratio (*R*) for 21 wt % *n*-hexadecane oil-in-water emulsions stabilized by BSA (pH 7.0).

Circular Dichroism Measurements. Circular dichroism (CD) measurements were made on protein solutions (3.1 g of BSA/L) or on serum phases separated from emulsions (1.2−3.1 g of BSA/L, depending on *R*) in the absence and presence of surfactant ($R = 0$ and 131). The resulting spectra were normalized with respect to the protein concentration in the aqueous phase so that they could be directly compared with each other. Far-UV CD spectra were similar for all samples analyzed, indicating that there were no major alterations in the secondary structure of the BSA molecules due to adsorption−desorption from the surface of the emulsion droplets or due to interaction with Tween 20 (**Figure 2a**).

Near-UV CD is sensitive to the contributions made by aromatic side chains to the spectra and can provide valuable information about changes in protein conformation and ligand binding (*42*). There were appreciable differences in the near-UV CD spectra of the samples (**Figure 2b**), which suggested that there were alterations in either the structure or interactions of the BSA molecules. In the absence of Tween 20 ($R = 0$), the optical rotation of the BSA was more negative in the serum phase extracted from the emulsion than when the protein had been dispersed directly in aqueous solution. The origin of this effect could have been that the BSA in the aqueous phase of the emulsion had either undergone a change in conformation and/or interactions as a result of the presence of the emulsion droplets. These changes could have been the result of exchanges between adsorbed and nonadsorbed protein states during the lifetime of the emulsion (*33*) or due to denaturation of the protein during the homogenization process (*41*). Nevertheless, it is more

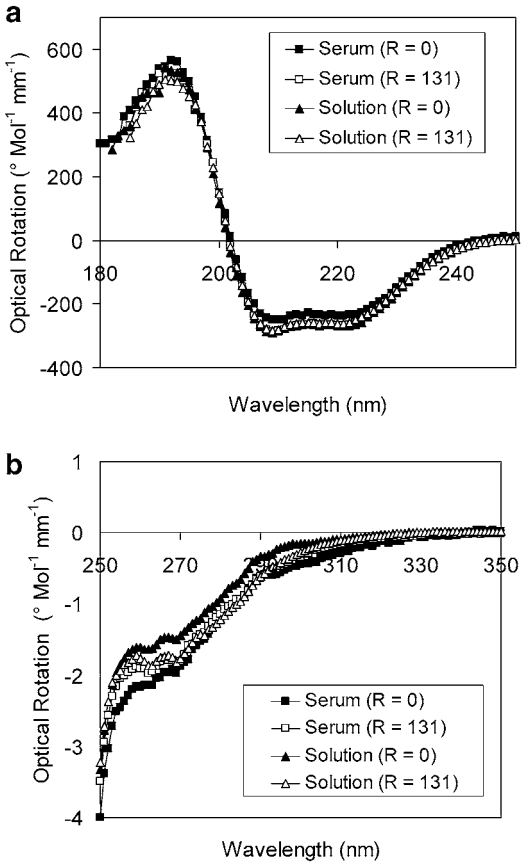

Figure 2. (a) Normalized far-UV circular dichroism spectra of BSA dispersed in either aqueous solution or in serum phase extracted from emulsions. Measurements were made in the absence ($R = 0$) and presence ($R = 131$) of Tween 20. (b) Normalized near-UV circular dichroism spectra of BSA dispersed in either aqueous solution or in serum phase extracted from emulsions. Measurements were made in the absence ($R = 0$) and presence ($R = 131$) of Tween 20.

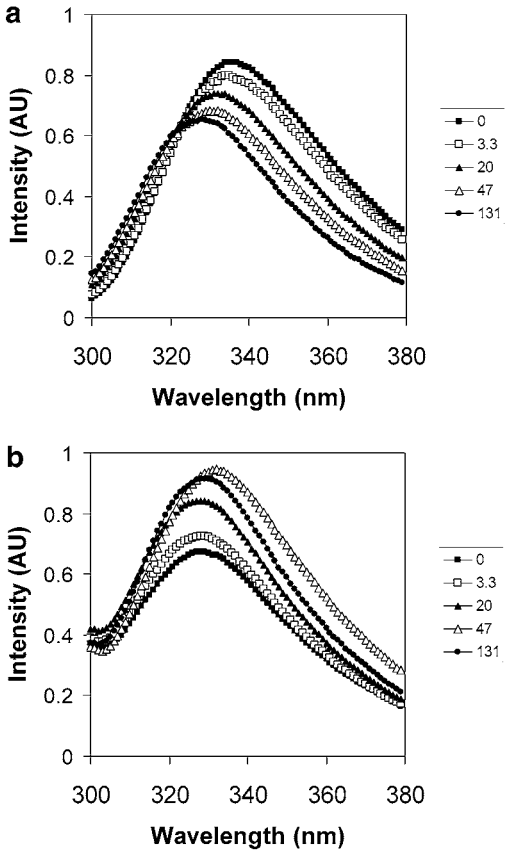

Figure 3. (a) Fluorescence emission spectra of BSA (3.1 g/L water) dispersed in aqueous solutions containing different concentrations of Tween 20 ($R = 0$–131). An excitation wavelength of 290 nm was used. (b) Fluorescence emission spectra of BSA (2.3 g/L emulsion) dispersed in emulsions containing different concentrations of Tween 20 ($R = 0$–131). An excitation wavelength of 290 nm was used.

likely that the observed differences in CD spectra are due to scattering of light by the presence of aggregates in the serum (*43*). The turbidity of the solution at 600 nm (<0.01 cm^{-1}) was appreciably less than that of the serum phase (~ 0.15 cm^{-1}), probably due to the presence of some small emulsion droplets or protein aggregates that were not removed from the serum during the centrifugation of the emulsion. Indeed, we found that the CD spectra of the solution became more similar to that of the serum when a small quantity of oil droplets was added to the solution to make its turbidity similar to that of the serum.

There was an appreciable difference between the near-UV CD spectra of the BSA dissolved directly in aqueous solution in the presence and absence of Tween 20 (**Figure 2b**). The presence of the surfactant ($R = 131$) caused the optical rotation to decrease slightly, indicating that there was a change in the conformation and/or interactions of the protein. Previous studies have shown that a nonionic surfactant (Tween 80) was capable of preventing aggregation of BSA molecules in aqueous solutions (*44*). It was postulated that the surfactant molecules bound to nonpolar patches on the protein surface through hydrophobic interactions, thereby reducing the attractive interactions between the proteins. The change in the CD spectra as a result of the presence of the nonionic surfactant observed in our study may therefore have been because of dissociation of protein aggregates and/or due to surfactant binding. Alternative analytical techniques, such as chromatography, light scattering, or electrophoresis, would be needed to distinguish between these possibilities. Interestingly, there was little difference between the spectrum of BSA in the serum layer at $R = 131$ and the spectrum of BSA dissolved directly in aqueous solution at $R = 131$. There are a number of possible explanations for this observation. First, the surfactant may have interacted with the protein molecules to breakdown any aggregates formed, thus making adsorbed and nonadsorbed proteins more similar in structure and/or reducing turbidity effects. Second, the surfactant was added to the emulsions approximately 1 h after preparing them; hence, the proteins may have been displaced from the droplet surfaces before they had sufficient time to undergo extensive changes in conformation and aggregation.

Fluorescence Measurements on Emulsions and Solutions. The influence of surfactant concentration on the fluorescence spectra of BSA dispersed in aqueous solutions and in emulsions was measured (**Figure 3**). The presence of surfactant had an appreciable impact on both the height (I_{MAX}) and wavelength (λ_{MAX}) of the fluorescence emission maximum of the solutions and emulsions. Nevertheless, the influence of surfactant on λ_{MAX} and I_{MAX} was qualitatively different for BSA in the two systems (**Figure 4**). Addition of surfactant to the protein solutions caused a relatively steep decrease in λ_{MAX} from $R = 0$ to 33, followed by a slower decrease at higher R values (**Figure 4a**). On the other hand, in the emulsions, there was little change in λ_{MAX} from $R = 0$ to 20, followed by a steep increase from $R = 20$ to 46, followed by an appreciable decrease at higher R values. The influence of surfactant on I_{MAX} was also appreciably different in the emulsions and solutions (**Figure 4b**). The values were

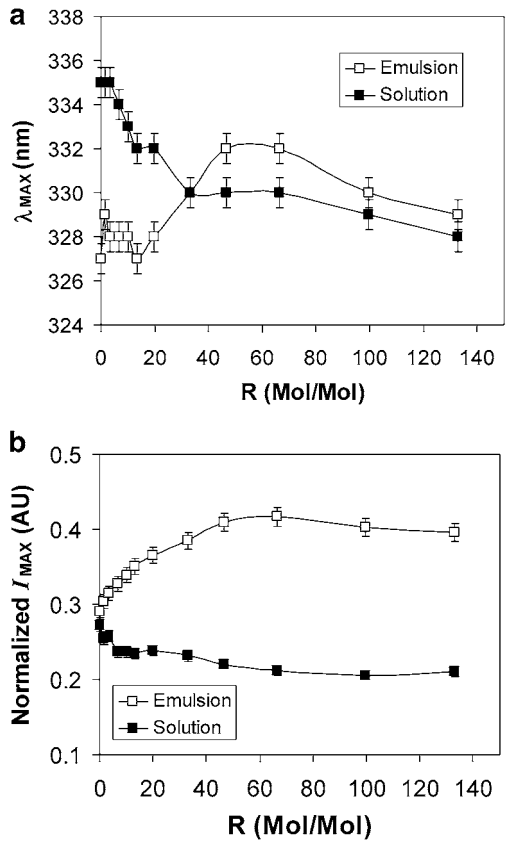

Figure 4. (a) Dependence of the wavelength of the maximum in the fluorescence emission spectra on surfactant-to-protein molar ratio for BSA dispersed in either aqueous solutions or emulsions. (b) Dependence of the normalized height of the maximum in the fluorescence emission spectra on surfactant-to-protein molar ratio for BSA dispersed in either aqueous solutions or emulsions. The fluorescence signal was normalized with respect to the total BSA concentration in the samples: 3.1 g/L for the solutions and 2.3 g/L for the emulsions.

normalized with respect to the protein concentration in the samples so that they could be compared on an equal protein basis. Increasing the Tween 20 concentration in the solutions from $R = 0$ to 10 caused an appreciable decrease in the height of the fluorescence emission maximum. At higher surfactant concentrations, there was only a slight decrease in I_{MAX} with increasing R. On the other hand, the addition of surfactant to the emulsions caused an appreciable increase in I_{MAX} for R between 0 and 66, after which it caused a slight decrease.

In the solutions, the blue shift (decrease) of λ_{MAX} with increasing Tween 20 concentration suggested that the tryptophan residues of BSA moved into a more hydrophobic environment at higher R values. The most likely explanation of this observation is the binding of nonionic surfactant molecules to hydrophobic patches on the protein surfaces, thus screening tryptophan residues from the polar solvent (*44*). It should be noted that the cmc of Tween 20 is 0.06 mM; hence, the surfactant was present predominantly as micelles at surfactant-to-protein ratios greater than about 2, since the concentration of BSA in the solutions was 0.047 mM. In the emulsions, the value of λ_{MAX} was relatively insensitive to Tween 20 at low surfactant concentrations ($R < 20$), which suggests that there was little change in the polarity of the tryptophan environment. It is interesting to note that there was little displacement of BSA from the droplet surfaces over the same R range (**Figure 1**). The red shift (increase) of λ_{MAX} with increasing Tween 20 concentration observed in the emulsions above $R > 20$ suggests that the tryptophan residues of BSA moved into a less hydrophobic environment at higher R values. When the surfactant concentration was increased above $R > 20$, the proteins were displaced from the surface of the emulsion droplets and moved into the aqueous phase (**Figure 1**). The observed red-shift could therefore be accounted for by a change in the polarity of the environment of the BSA tryptophan groups after desorption. When the protein is adsorbed to the oil−water interface, the tryptophan residues are likely to be in contact with the hydrophobic oil phase, but when they are displaced into the continuous phase, they are more likely to be exposed to polar water. Alternatively, the change in the environment of the tryptophan residues may have been the result of an alteration in the conformation of the BSA molecules upon desorption. For example, it is possible that one or more tryptophan residues in the adsorbed state were buried in the hydrophobic interior of the BSA molecule, but they became more exposed to the hydrophilic exterior in the desorbed state. It is not possible to determine which of these physiochemical mechanisms is responsible for the observed changes from the fluorescent measurements alone. The slight decrease of λ_{MAX} observed at high surfactant concentrations in the emulsions ($R = 66-132$) may have been due to binding of Tween 20 to hydrophobic patches on the desorbed proteins (*44*). The fact that the λ_{MAX} values are similar for the BSA in the aqueous solutions and in the emulsions at high R values suggests that the proteins have similar conformations and binding properties, which supports the findings of the near-UV CD measurements discussed above.

The major reason that normalized I_{MAX} values are not the same for BSA in emulsion and in solution is due to light scattering by the emulsion droplets, i.e. "inner filter" effects (*37*, *45*). The incident and emitted electromagnetic waves are scattered extensively by the emulsion droplets, which changes the intensity of the radiation reaching the fluorescence detector. It is usually difficult to correlate changes in I_{MAX} with precise molecular processes, since many factors contribute to the quantum yield of proteins (*45*). Nevertheless, we found that the I_{MAX} vs R curves exhibited similar trends to the λ_{MAX} vs R curves; i.e., as I_{MAX} increased, λ_{MAX} tended to increase (compare parts a and b of **Figure 4**). The main exception to this trend was the emulsion at low surfactant concentrations ($R < 20$), where I_{MAX} increased but λ_{MAX} remained relatively constant. Thus, it would seem that in our system I_{MAX} tended to increase with an increase in the polarity of the tryptophan environment and decrease with an increase in the hydrophobicity of the tryptophan environment. In solutions, the decrease in I_{MAX} with increasing R (**Figure 4b**) probably occurred because of an increase in the hydrophobicity of the molecular environment of the tryptophan residues associated with binding of surfactant molecules to the proteins (*44*). It is possible that I_{MAX} could have been reduced because of adsorption of light by the surfactant in the UV−visible region. Nevertheless, there was little change in I_{MAX} with surfactant concentration from $R =$ 10 to 131, which suggests that light absorption by the surfactant did not have a major effect on the fluorescence intensity. In emulsions, the increase in I_{MAX} with increasing R (**Figure 4b**) probably occurred because of the increase in polarity of the tryptophan residues' environment resulting from the movement of BSA molecules from the surface of the oil droplets into the aqueous phase (**Figure 1**). Nevertheless, other physicochemical phenomenon may also have contributed to the observed dependence of I_{MAX} on R in the emulsions, e.g., surface denaturation of the proteins (*33*), binding of surfactant molecules to the proteins, and/or dissociation of protein aggregates by

surfactant (*44*). Unfortunately, it is not possible to disentangle these different potential contributions to the fluorescence emission spectrum.

Ideally, we would like to have been able to estimate the fraction of adsorbed and nonadsorbed proteins in the emulsions from the fluorescence data. Previous studies suggest that measurements of λ_{MAX} can be used to determine the percentage of adsorbed BSA (P_{ads}) in an emulsion (*37*, *38*):

$$P_{ads} = 100 \times (\lambda_{MAX,E} - \lambda_{MAX})/(\lambda_{MAX,E} - \lambda_{MAX,S}) \quad (1)$$

where, $\lambda_{MAX,S}$ and $\lambda_{MAX,E}$ are the wavelengths of the maximum in the emission spectrum for 100% protein dispersed in an aqueous solution and for 100% protein adsorbed to emulsion droplet surfaces, respectively. Previous studies using hydrocarbon oil-in-water emulsions stabilized by BSA have found values of $\lambda_{MAX,S} = 335$ nm and $\lambda_{MAX,E} = 320$ nm (*38*). These values were used in our calculations of the percentage of adsorbed protein in the emulsions described below.

In the absence of surfactant, there was a relatively large difference in λ_{MAX} between the BSA in the emulsion ($\lambda_{MAX} = 327$ nm) and the BSA in aqueous solution ($\lambda_{MAX} = 335$ nm). The lower value of λ_{MAX} in the emulsions suggests that the tryptophan residues were in a more hydrophobic environment in the adsorbed state, presumably because they were in direct contact with the oil droplets or because of surface denaturation of the protein. The percentage of adsorbed protein calculated from eq 1 was 53%, which is fairly close to the value of 60% found from protein displacement experiments (**Figure 1**).

Unfortunately, it was not possible to use the above approach to quantify the percentage of adsorbed protein in our surfactant displacement experiments, because the presence of the surfactant changed the value of λ_{MAX} appreciably (**Figure 4a**). It may be possible to quantify the relative proportions of proteins in different adsorption states in other emulsions where surfactant−protein interactions do not cause large changes in λ_{MAX}. Despite these limitations, the fluorescence technique did provide some valuable qualitative information about the interactions of globular proteins with nonionic surfactants in concentrated emulsions. In particular, it showed that there was a direct interaction between protein and surfactant, and it indicated the surfactant concentration where all the protein was displaced from the droplet surfaces.

Fluorescence Measurements of Creamed and Serum Layers. We also examined the influence of surfactant on λ_{MAX} and I_{MAX} for the creamed and serum phases obtained after centrifugation of the emulsions 48 h after homogenization (**Figure 5**). In the absence of surfactant, the value of λ_{MAX} was higher in the serum phase ($\lambda_{MAX} = 333$ nm) than in the creamed layer ($\lambda_{MAX} = 324$ nm), indicating that the tryptophan of the adsorbed proteins was in a more hydrophobic environment than that of the nonadsorbed proteins. The value of λ_{MAX} of the creamed layer was less than that of the whole emulsion ($\lambda_{MAX} = 327$ nm) because a greater fraction of protein was at the interface in the creamed layer. The fraction of protein adsorbed to the droplet surfaces in the creamed layer was predicted to be 73% using eq 1. We found that λ_{MAX} of the serum layer ($\lambda_{MAX} = 333$ nm) was significantly less than that of the protein in solution ($\lambda_{MAX} = 335$ nm), which suggested that there was some change in the conformation of the BSA during its lifetime in the emulsions, e.g., surface denaturation during homogenization. This phenomenon may be another factor limiting the application of eq 1 to the determination of protein concentration in emulsions, since the value of $\lambda_{MAX,S}$ in the equation would have to be changed.

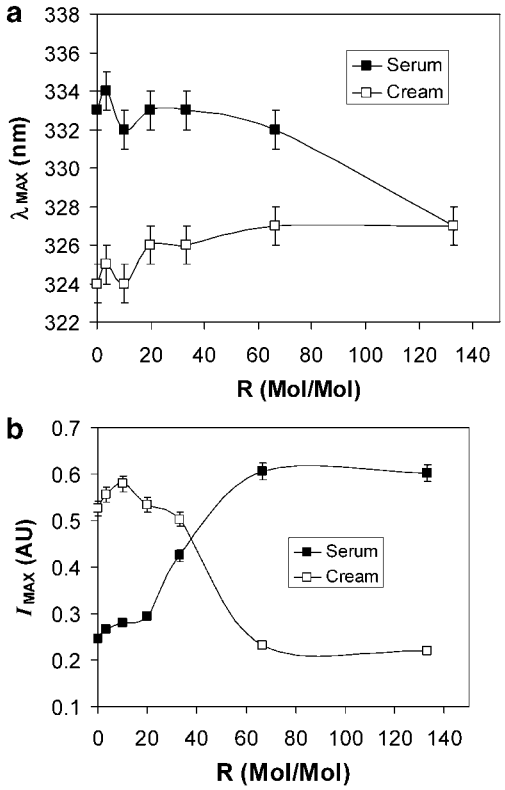

Figure 5. (a) Dependence of the wavelength of the maximum in the fluorescence emission spectra on surfactant-to-protein molar ratio for BSA dispersed in either serum or creamed phases separated from emulsions by centrifugation after 48 h storage. (b) Dependence of the height of the maximum in the fluorescence emission spectra on surfactant-to-protein molar ratio for BSA dispersed in either serum or creamed phases separated from emulsions by centrifugation after 48 h storage.

As the concentration of surfactant in the emulsions increased, there was an increase in λ_{MAX} of the creamed phase, probably because the protein moved from the droplet surfaces into the serum phase. There was also a decrease in λ_{MAX} of the serum phase as R increased, probably due to the interaction of the protein with Tween 20 (**Figure 4a**). In the absence of surfactant, there was an appreciable difference in the values of I_{MAX} in the serum and creamed layers. This difference was mainly due to the different protein concentrations in the two phases. We measured a protein concentration of 1.2 g/L in the serum phase and calculated a protein concentration of 4.4 g/L in the creamed phase from measurements of the thickness of the creamed layer and the fraction of total protein in the serum phase. In addition, some of the differences in I_{MAX} in the serum and in the cream could be attributed to differences in protein environment and light scattering in the two media. As the surfactant concentration in the emulsions was increased, there was little change in I_{MAX} of the serum or cream from $R = 0$ to 20, followed by an appreciable increase in I_{MAX} for the serum, a decrease for the cream from $R = 20$ to 66, and a leveling off at higher surfactant concentrations. The most likely origin of these changes is the increase in protein concentration in the serum phase and the decrease in protein concentration in the creamed phase as R is increased due to displacement of protein from the droplet surfaces. Indeed, the form of the I_{MAX} vs R curve (**Figure 5b**) was very similar to the form of the free protein vs R curve (**Figure 1**). It should be noted that a fluorescence emission peak was still observed in the creamed layer at high surfactant concentrations when all of the protein was desorbed from the

droplets, which was presumably due to the protein present in the aqueous phase surrounding the droplets.

CONCLUSION

This study has shown that front-face fluorescence spectroscopy is a powerful tool for nondestructively monitoring the displacement of BSA from the surfaces of emulsion droplets by a nonionic surfactant. The technique is sensitive to the molecular environment of the BSA molecules in the system, as well as their adsorption−desorption history. At relatively low surfactant concentrations ($R = 0-66$) the droplet interface is comprised of a combination of protein and surfactant. At relatively high nonionic surfactant concentrations ($R \geq 66$), the majority of BSA is displaced from the droplet surfaces. The major advantage of the FFFS technique is that it can be used to study concentrated emulsions in situ. It should therefore be possible to use this technique to gain a better understanding of the role that protein−surfactant interactions play in determining emulsion properties. Nevertheless, we should mention that the technique is only suitable for studying proteins in which there is an appreciable change in the fluorescence emission spectra in the adsorbed and nonadsorbed states. In other studies within our laboratory, we have observed that there is a much smaller change in the emission spectra between the adsorbed and nonadsorbed states for proteins other than BSA, e.g., β-lactoglobulin and casein. The FFFS technique may therefore have limited application to some protein systems.

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