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Front-Face Fluorescence Spectroscopy Study of Globular Proteins in Emulsions: Influence of Droplet Flocculation

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Measurement of the intensity (I_{MAX}) and/or wavelength (λ_{MAX}) of the maximum in the tryptophan (TRP) emission spectrum using front-face fluorescence spectroscopy (FFFS) can be used to provide information about the molecular environment of proteins in nondiluted emulsions. Many protein-stabilized emulsions in the food industry are flocculated, and therefore, we examined the influence of droplet flocculation on FFFS. Stock oil-in-water emulsions stabilized by bovine serum albumin were prepared by high-pressure valve homogenization (30 wt % *n*-hexadecane, 0.35 wt % BSA, pH 7). These emulsions were used to create model systems with different degrees of droplet flocculation, either by changing the pH, adding surfactant, or adding xanthan. Emulsions (21 wt % *n*-hexadecane, 0.22 wt % BSA) with different pH (5 and 7) and molar ratios of Tween 20 to BSA ($R = 0-131$) were prepared by dilution of the stock emulsion. As the surfactant concentration was increased, the protein was displaced from the droplet surfaces, which caused an increase in both I_{MAX} and λ_{MAX}, because of the change in TRP environment. The dependence of I_{MAX} and λ_{MAX} on surfactant concentration followed a similar pattern in emulsions that were initially flocculated (pH 5) and nonflocculated (pH 7). Relatively small changes in FFFS emission spectra were observed in emulsions (21 wt % *n*-hexadecane, 0.22 wt % BSA, pH 7) with different levels of depletion flocculation induced by adding xanthan. These results suggested that droplet flocculation did not have a major impact on FFFS. This study shows that FFFS is a powerful technique for nondestructively providing information about the molecular environment of proteins in concentrated and flocculated protein-stabilized emulsions. 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: Emulsions; fluorescence; flocculation; BSA; Tween 20; competitive adsorption

INTRODUCTION

Physicochemical properties of oil-in-water food emulsions, such as texture, shelf life, and taste, are strongly influenced by the characteristics of the interfacial membrane surrounding the lipid droplets (*1*−*3*). These characteristics depend on the type and concentration of surface-active molecules in the system, the ability of other components within the system to modify the interfacial characteristics of the surface-active molecules (e.g. salts, acids, bases, biopolymers), and the processing treatments that the product experiences during its lifetime (e.g. temperature, pressure, shear forces). The creation of food products with desirable physicochemical characteristics depends on the selection of the most appropriate emulsifier or combination of emulsifiers for each specific product (*4*−*7*). To make a rational selection it is important to have a thorough understanding of the factors that determine interfacial characteristics and to understand the relationship between interfacial characteristics and bulk physicochemical properties. Analytical techniques are therefore needed to provide information about the properties of emulsifiers in emulsions.

Previous studies have shown that front-face fluorescence spectroscopy (FFFS) can be used to noninvasively provide information about the molecular environment of proteins in concentrated emulsions (*8*−*10*), as we discussed in the accompanying paper in which we studied BSA displacement by a nonionic surfactant using the same technique (*11*). The major advantage of the FFFS technique is that information about the interfacial composition of the droplets in emulsions can be obtained directly, without sample preparation, e.g. dilution.

The droplets in many food emulsions are flocculated (*1*, *2*), which could limit the application of the FFFS technique, because the spatial distribution of particles within a colloidal dispersion influences the scattering of electromagnetic radiation (*12*, *13*).

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The purpose of the present study was therefore to examine the influence of droplet flocculation on the FFFS signal from proteins in emulsions, so as to determine whether front-face fluorescence spectroscopy could be used to provide valuable information about protein properties in flocculated emulsions.

MATERIALS AND METHODS

Materials. Analytical grade hydrochloric acid (HCl), sodium hydroxide (NaOH), Tween 20, and *n*-hexadecane were purchased from the Sigma Chemical Co. (St. Louis, MO). Sodium azide (NaN_3) was obtained from Merck Chemicals (Darmstadt, Germany). Powdered BSA (Lot 7602E, Fraction V) was obtained from ICN Chemicals Inc. (Aurora, OH). Powdered xanthan gum (Rhodigel Clear 80) was obtained from Rhodia Pharmaceutical Ingredients (Lyon, France). Deionized water was used for the preparation of all solutions (<10 MΩ, resistivity, MilliQ Water Purification System).

Emulsion Preparation. An emulsion containing 30 wt % *n*-hexadecane and 70 wt % BSA solution (5 g/L) as emulsifier was prepared as indicated in the preceding paper (*11*). The emulsion was divided into two portions and the pH of one portion was adjusted to 7.0 (to avoid flocculation) and that of the other to 5.0 (to induce flocculation). pH adjustment was carried out using 1 M NaOH and 1 M HCl solutions approximately 30 min after the initial emulsion left the homogenizer. The particle size distribution of these emulsions was measured after the emulsions had been stored at 20 °C for 24 h using laser diffraction (Malvern, Worcs, UK) as described earlier (*11*). The measured mean particle diameters were highly reproducible (<5% difference) and are reported as the average of measurements made on two samples. There was little evidence of droplet flocculation in the pH 7 emulsion but extensive droplet flocculation in the pH 5 emulsion.

Preparation of Emulsions with Different Tween 20 Concentrations. Emulsions were prepared in which the degree of droplet flocculation and the fraction of adsorbed protein was varied by adding Tween 20 to displace the proteins from the interface and disrupt floc formation. A series of emulsions was prepared by diluting the 30 wt % emulsions (pH 5 and 7) with aqueous Tween 20 solutions to obtain 21 wt % *n*-hexadecane emulsions with 0.31 wt % BSA and 0−0.76 wt % Tween 20 in the aqueous phase (molar ratio, $R = 0-131$). This procedure was carried out about 1 h after the initial emulsion left the homogenizer. These emulsions were stored in a temperature-controlled environment at 20 °C for 24 h before further analysis. The results for the pH 7 emulsion have been fully reported in another paper (*11*), which focused on the ability of FFFS to provide information about the TRP environment of proteins in nonflocculated emulsions. In this paper, we focus on the pH 5 data and only compare it with the pH 7 data in order to highlight differences between the behavior of flocculated and nonflocculated emulsions.

Preparation of Emulsions with Different Xanthan Concentrations. Emulsions were prepared in which the degree of droplet flocculation was varied, but the fraction of adsorbed proteins was unchanged, by adding xanthan gum to induce depletion flocculation. A series of emulsions was prepared by diluting the 30 wt % emulsions (pH 7.0) with aqueous xanthan solutions to obtain 21 wt % *n*-hexadecane emulsions with 0.31 wt % BSA and 0−0.04 wt % xanthan in the aqueous phase. This procedure was carried out about 1 h after the initial emulsion left the homogenizer. These emulsions were stored in a temperature-controlled environment at 20 °C for 24 h before further analysis.

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 (*11*).

Front-Face Fluorescence Measurements. Front-face fluorescence emission spectra were measured in duplicate or triplicate using a spectrofluorometer (4800C, SLM Instruments, Urbana, IL) from 300 to 380 nm with the excitation wavelength set at 290 nm, as described earlier (*11*).

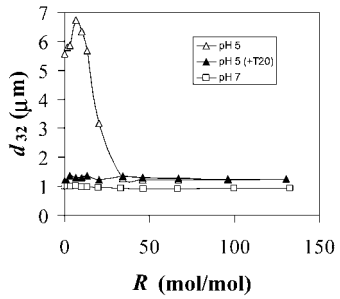

Figure 1. Dependence of the mean particle diameter (d_{32}) on the surfactant-to-protein molar ratio (R) for 21 wt % *n*-hexadecane oil-in-water emulsions stabilized by BSA. Data are shown for pH 5 and 7 emulsions and for pH 5 emulsions to which 1 wt % Tween 20 was added before making the laser diffraction measurements to disrupt any flocs.

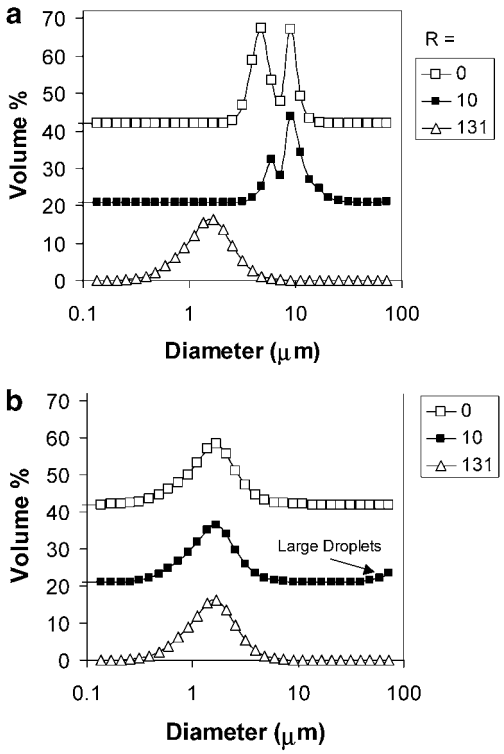

Figure 2. Particle size distributions of 21 wt % *n*-hexadecane oil-in-water emulsions (pH 5) stabilized by BSA with different surfactant-to-protein molar ratios (R, see box). Data are shown for emulsions in the (a) absence and (b) presence of 1 wt % Tween 20 added before making the laser diffraction measurements to disrupt any flocs.

Free Protein Measurements. Emulsions were centrifuged for 30 min at 1000*g* to separate them into a cream layer and a serum layer. The free protein concentration in the aqueous phase (serum layer) of the emulsions was determined using a modified Lowry method (*14*), as reported earlier (*11*).

RESULTS AND DISCUSSION

Particle Size and Creaming Measurements of Emulsions Containing Tween 20. The mean particle diameter (d_{32}) and particle size distribution of *n*-hexadecane oil-in-water emulsions (pH 5) incubated in the presence of different molar ratios of Tween 20 to BSA were measured (**Figures 1** and **2**). In the absence of Tween 20, the pH 5 emulsion was highly susceptible to droplet aggregation, with the mean particle diameter being around 5.6 μm (**Figure 1**). The particle size distribution of this emulsion was bimodal and consisted of particles that were

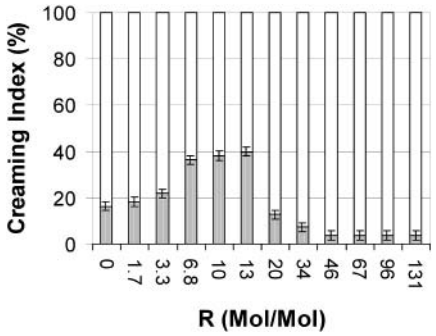

Figure 3. Influence of surfactant-to-protein molar ratio (*R*) on the creaming stability of 21 wt % *n*-hexadecane oil-in-water emulsions stabilized by BSA. The height of the serum layer (gray) was measured after the emulsions were stored for 24 h at 20 °C.

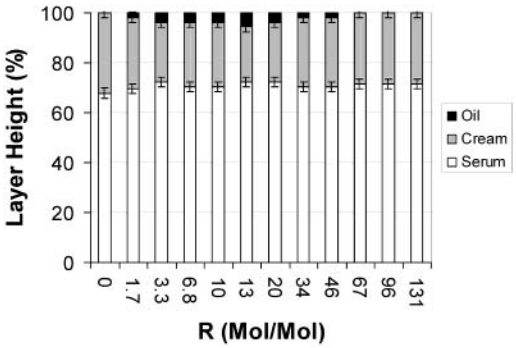

Figure 4. Influence of surfactant-to-protein molar ratio (*R*) on the stability of 21 wt % *n*-hexadecane oil-in-water emulsions stabilized by BSA to centrifugation at 1000*g* for 30 min.

considerably larger than those observed in the presence of high surfactant concentrations (**Figure 2**). Presumably, aggregation occurred because at this pH the electrical charge on the droplets was not large enough to generate a sufficiently strong electrostatic repulsion between the droplets to prevent droplet aggregation (*2*). The particle diameter increased slightly from $R = 0$ to 7 and then decreased steeply as the surfactant concentration was increased further ($R = 7-34$). At *R* values ≥ 34 there was little evidence of flocculation in the emulsions (**Figure 1**). An increase in droplet flocculation upon the addition of relatively low concentrations of nonionic surfactant has also been reported in previous studies (*15*), where it was attributed to the ability of the surfactant to partially displace the protein and increase the hydrophobicity of the droplet surface. The decrease in droplet flocculation observed at high surfactant concentrations can be attributed to the ability of the Tween 20 to displace the protein molecules from the droplet surface and form an interfacial membrane that prevents droplet flocculation (*1*).

For all *R* values, the addition of 1 wt % Tween 20 to the emulsions prior to laser diffraction measurements caused the mean particle diameter to return to values (1.2−1.4 μm) slightly higher than that of the pH 7 stock emulsion immediately after it left the homogenizer (0.93 μm). This finding suggested that the observed increase in particle size was primarily due to flocculation, because the aggregates could be largely disrupted by surfactant, but that there was some coalescence because of the small increase in droplet size compared to the original droplets (**Figure 1**). In addition, the particle size distribution measurements indicated that there was a small number of very large particles in the pH 5 emulsions at intermediate surfactant concentrations ($R = 3-46$) that could not be broken down by addition of 1 wt % Tween 20 prior to making the laser diffraction measurements, e.g., see **Figure 2b** for $R = 10$. These particles were probably large oil droplets formed due to coalescence (the centrifugation experiments discussed below support this).

After 24 h storage at 20 °C, a transparent serum layer was observed at the bottom of the pH 5 emulsions for $R = 0-34$ (**Figure 3**). The thickness of this layer increased from about 18% to about 40% of the emulsion height as the surfactant concentration was increased from $R = 0$ to 13, indicating that these emulsions were highly unstable to gravitationally induced creaming (**Figure 3**). When the surfactant concentration was increased further, there was a steep decrease in the creaming index, and at high surfactant concentrations ($R \geq 46$) only thin turbid serum layers were formed (creaming index < 5%). The data from the creaming experiments therefore supports the data from the laser diffraction experiments, which showed that the addition of surfactant to the emulsions promoted droplet flocculation at low levels ($R \leq 20$) but retarded flocculation at high levels ($R > 20$). By contrast, at all surfactant-to-protein ratios ($R = 0-131$), there was little evidence of droplet flocculation in pH 7 emulsions (*11*), as indicated by a monomodal particle size distribution, little change in d_{32} (0.97 ± 0.05 μm), and good creaming stability (creaming index < 5%).

Centrifugation and Free Protein Measurements of Emulsions Containing Tween 20. Emulsions were centrifuged at 1000*g* for 30 min to extract their serum layers for analysis. The pH 5 emulsions separated into a cream and a serum layer after centrifugation at low ($R = 0$) and high ($R \geq 67$) surfactant concentrations (**Figure 4**). However, they separated into three layers at intermediate surfactant concentrations: a transparent oil layer at the top, an opaque cream layer in the middle, and a transparent serum layer at the bottom (**Figure 4**). These results show that intermediate surfactant concentrations can have an adverse effect on the coalescence stability of emulsions. Microscopy studies of proteins at planar interfaces have shown that there is a two-dimensional phase separation of the system into a protein-rich and a surfactant-rich region when nonionic surfactants are introduced into the system (*16*−*19*). As the surfactant concentration is increased, the proteins become restricted to a smaller surface area and form relatively thick regions of aggregated protein. It would appear that droplets surrounded by these phase-separated protein−surfactant membranes are more prone to coalescence than droplets surrounded by either pure protein or pure surfactant membranes. In contrast, the pH 7 emulsions separated into two layers after centrifugation at 1000*g* for 30 min at all surfactant concentrations: an opaque cream layer at the top (30%) and a transparent serum layer at the bottom (70%) (*11*). Hence, there was no evidence of droplet coalescence in emulsions at neutral pH. The origin of this effect may be due to the stronger electrostatic repulsion between emulsion droplets at pH 7 than at pH 5, which would prevent them from coming into close enough proximity to coalesce.

The serum layer collected from the centrifuged emulsions was analyzed to determine the concentration of nonadsorbed protein. The behavior of pH 5 and 7 emulsions was fairly similar (data not shown), suggesting that the ability of the nonionic surfactant to displace the protein was not particularly sensitive to differences in electrical charge or conformation of the protein resulting from pH changes.

Fluorescence Measurements on Emulsions and Solutions Containing Tween 20. The influence of surfactant concentration on the fluorescence spectra of BSA dispersed in aqueous solutions and in emulsions at pH 5 is shown in **Figures 5** and **6**.

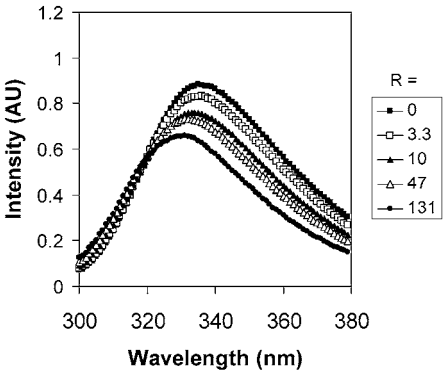

Figure 5. Fluorescence emission spectra of BSA (3.1 g/L water) dispersed in aqueous solutions (pH 5) containing different concentrations of Tween 20 ($R = 0$ to 131). An excitation wavelength of 290 nm was used.

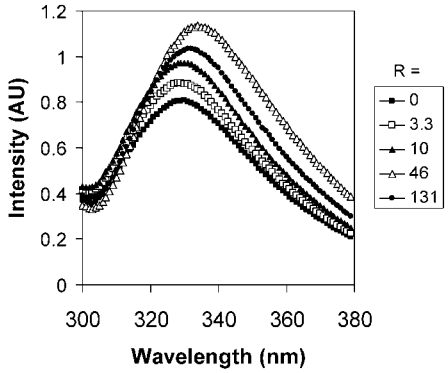

Figure 6. Fluorescence emission spectra of BSA (2.3 g/L emulsion) dispersed in 21 wt % *n*-hexadecane oil-in-water emulsions (pH 5) containing different concentrations of Tween 20 ($R = 0$–131). An excitation wavelength of 290 nm was used.

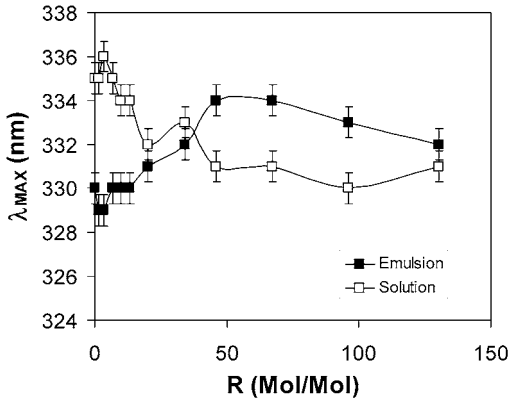

Figure 7. Dependence of the wavelength of the maximum in the fluorescence emission spectra (λ_{MAX}) on surfactant-to-protein molar ratio (R) for BSA dispersed in either emulsions or solutions at pH 5.

The wavelength (λ_{MAX}) of the fluorescence emission maximum was determined from these spectra (**Figure 7**) and were similar to those observed for pH 7 emulsions and solutions (*11*). In the protein solutions, the overall decrease in λ_{MAX} with increasing surfactant concentration suggests that the TRP environment became more hydrophobic (*20*), probably because of binding of surfactant molecules to the protein surface (*21*). In the protein-stabilized emulsions, the increase in λ_{MAX} at intermediate surfactant concentrations is probably because the TRP environment became more hydrophilic when the protein was desorbed from the droplet surfaces. The decrease in λ_{MAX} at higher surfactant concentrations is probably because the TRP

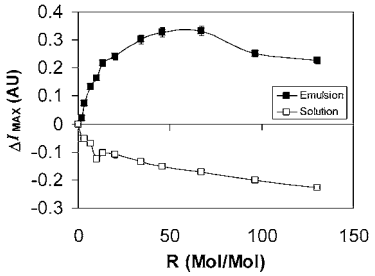

Figure 8. Dependence of the increment in the height of the maximum in the fluorescence emission spectra (ΔI_{MAX}) with increasing surfactant-to-protein molar ratio (R) for BSA dispersed in either aqueous solutions or emulsions at pH 5.

environment became more hydrophobic due to surfactant binding. The similarity of the results with pH 7 experiments (*11*) suggests that λ_{MAX} measurements were not particularly sensitive to the degree of droplet flocculation in the emulsions.

The height (I_{MAX}) of the fluorescence emission maximum were also determined from the spectra of BSA dispersed in aqueous solutions and in emulsions at pH 5 (**Figure 8**). The data are plotted as the difference between the height of the intensity maximum in the presence and absence of surfactant (ΔI_{MAX}), to highlight the differences caused by the addition of surfactant. The results were also similar to those observed for pH 7 emulsions and solutions (*11*) with the value of ΔI_{MAX} in the flocculated emulsions being only slightly higher than that in the nonflocculated emulsions. The dependence of ΔI_{MAX} on surfactant concentration was previously attributed to changes in the fluorescence quantum yield of the TRP residues caused by alterations in protein structure, location, or binding (*11*). ΔI_{MAX} decreases with increasing hydrophobicity, since ΔI_{MAX} tends to increase when λ_{MAX} increases (with the exception of the $R = 0-20$ data in the emulsions). The similarity of the results with pH 7 experiments suggests that measurements of ΔI_{MAX} are also not particularly sensitive to the degree of flocculation of the emulsion droplets.

Measurements in Emulsions and Solutions Containing Xanthan Gum. The above experiments suggest that the FFFS technique was not particularly sensitive to the degree of droplet flocculation in the emulsions. To further examine this hypothesis, we prepared a series of emulsions in which the degree of droplet flocculation was different, but the protein environment was the same by adding xanthan gum to induce depletion flocculation. These emulsions contained 21 wt % *n*-hexadecane emulsions and had 0.31 wt % (3.1 g/L) BSA and 0−0.04 wt % (0−0.4 g/L) xanthan in the aqueous phase (pH 7). The particle diameter of these emulsions was measured after 24 h storage and found to be the same for all xanthan concentrations ($d_{43} = 1.05 \pm 0.05$ μm); this is not surprising, since the driving force for depletion flocculation is decreased by emulsion dilution (*2*). Nevertheless, we observed extensive creaming in the emulsions at xanthan concentrations $\geq$ 0.047 g/ L, as evidenced by the formation of a clear serum layer at the bottom of the emulsions and a turbid cream layer at the top (**Figure 9**). Emulsions with xanthan concentrations below this critical flocculation concentration are nonflocculated, whereas those above are flocculated (*22*). Nevertheless, we observed no significant change in λ_{MAX} (**Figure 10**) and only a slight increase in ΔI_{MAX} (**Figure 11**) when the xanthan concentration was increased from 0 to 0.4 g/L, providing support that the FFFS technique is not particularly sensitive to droplet flocculation. The fact that ΔI_{MAX} was slightly higher in the pH 5 emulsions than in the pH 7 emulsions in the presence of Tween 20 may therefore have been caused by

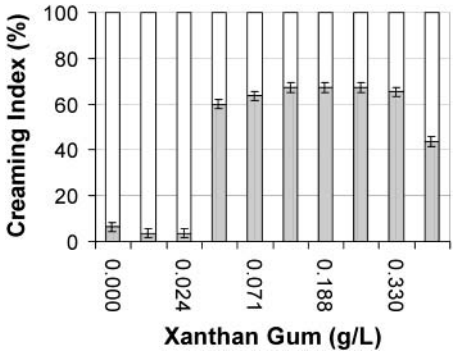

Figure 9. Influence of xanthan concentration on the creaming stability of 21 wt % *n*-hexadecane oil-in-water emulsions stabilized by BSA. The height of the serum layer (gray) was measured after the emulsions were stored for 24 h at 20 °C. Extensive creaming at higher xanthan concentrations is indicative of depletion flocculation.

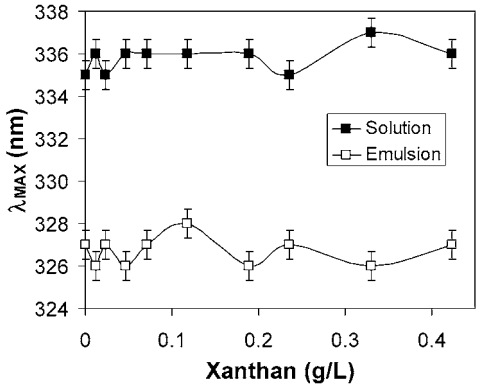

Figure 10. Dependence of the wavelength of the maximum in the fluorescence emission spectra (λ_{MAX}) on xanthan concentration for BSA dispersed in either emulsions or solutions (pH 7).

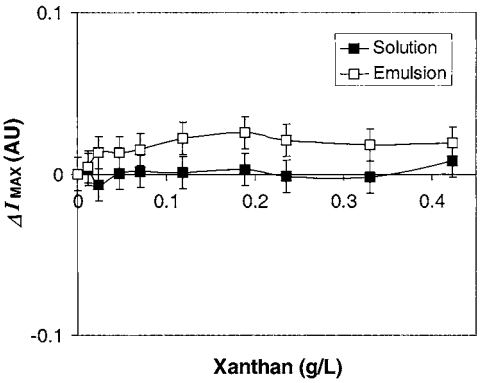

Figure 11. Dependence of the increment in the height of the maximum in the fluorescence emission spectra (ΔI_{MAX}) on xanthan concentration for BSA dispersed in either aqueous solutions or emulsions (pH 7).

droplet flocculation altering the degree of light scattering. We observed no significant change in λ_{MAX} (**Figure 10**) or ΔI_{MAX} (**Figure 11**) with xanthan concentration in the protein solutions, which indicated that xanthan did not cause changes in the protein TRP environment.

Studies of the influence of droplet flocculation on the reflectance of light from concentrated oil-in-water emulsions also found that droplet flocculation only had a minor effect on emulsion lightness and reflection coefficient (*23*). In dilute emulsions, droplet flocculation has a major impact on the angular profile of the scattered light, which is why laser diffraction instruments can be used to monitor flocculation in dilute, strongly flocculated systems (**Figure 1**). The most likely reason that droplet flocculation does not have a major impact on the optical properties of concentrated emulsions is because the radiation is multiply scattered and that the droplets are already in fairly close proximity.

CONCLUSION

Previous studies have shown that front-face fluorescence spectroscopy is a powerful tool for nondestructively providing information about the molecular environment of proteins in concentrated oil-in-water emulsions. In this study we have shown that FFFS measurements are not strongly affected by the degree of droplet flocculation in the emulsions. Thus, FFFS can be used to probe the environment of proteins in emulsions that are both concentrated and flocculated without the need of any sample preparation. The technique may therefore be suitable as an on-line monitoring technique for certain applications in the food industry, e.g. in situ monitoring of the displacement of proteins from emulsion droplets by nonionic surfactants in ice cream production.

Finally, we should note that there may be certain systems where the FFFS technique is sensitive to droplet flocculation, e.g. if there was an appreciable change in the TRP environment after flocculation. This situation could occur if the tryptophan residues of the adsorbed proteins were directed toward the aqueous phase, rather than being directed toward the oil phase or buried in the protein interior. Then, TRP residues might move from a more polar to a less polar environment as a result of droplet flocculation.

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