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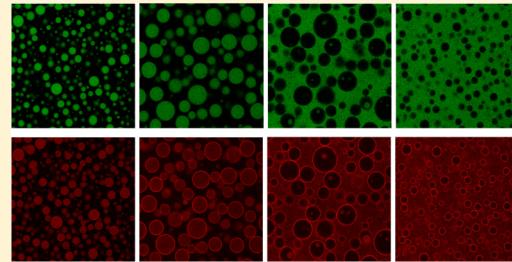
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## Stabilization of Water-in-Water Emulsions by Addition of Protein Particles

Bach T. Nguyen, Taco Nicolai,\* and Lazhar Benyahia

LUNAM, Université du Maine, IMMM UMR CNRS 6283, PCI, 72085 Le Mans cedex 9, France

**ABSTRACT:** The effect of the addition of protein particles was investigated on the stability of water-in-water emulsions formed by mixing aqueous dextran and poly(ethylene oxide) solutions. Protein particles with hydrodynamic radii ranging from 15 to 320 nm were produced by heating globular proteins in controlled conditions. The structure of the emulsions was visualized with confocal laser scanning microscopy using different fluorescent probes to label the dextran phase and the protein particles. It is shown that contrary to native proteins, protein particles adsorb at the interface and can form a monolayer that inhibits fusion of emulsion droplets. In this way, water-in-water emulsions could be stabilized for a period of weeks. The effect of the polymer composition and the protein particle size and concentration was investigated.



### INTRODUCTION

When two incompatible liquids are mechanically mixed, generally an emulsion is formed of small droplets of one liquid embedded in the second liquid. At rest, the droplets will grow by fusion or Oswald ripening until finally two distinct macroscopic phases are formed. Emulsions can be stabilized by adding surfactants that adsorb to the interface and inhibit fusion of colliding droplets. Most often, surfactants are amphiphilic molecules or polymers, but it has been shown that solid particles adsorbed at the interfaces can be exceptionally efficient stabilizers forming so-called Pickering emulsions.<sup>1,2</sup> When a particle enters the interface, the free energy is reduced by an amount that depends on their radius ( $R$ ), the contact angle with the interface ( $\theta$ ), and the interfacial tension ( $\gamma$ ):

$$\Delta G = \pi R^2 \gamma (1 - |\cos \theta|)^2 \quad (1)$$

In the past, mainly oil-in-water Pickering emulsions have been studied and their stability can be understood by the fact that for  $R > 10$  nm particles, the binding energy is orders of magnitude larger than the kinetic energy.

Water-in-water emulsions can be produced by mixing aqueous solutions of incompatible polymers.<sup>3</sup> Such emulsions have been stabilized in the past by gelling one or both of the phases as they cannot be stabilized by molecular or polymeric surfactants. Recently, it was shown that submicrometer particles adsorb irreversibly to the interface of the two aqueous phases opening up the possibility to create stable water-in-water emulsions without gelling one of the phases.<sup>4–6</sup> A detailed quantitative investigation showed that also in this case, the adsorption of particles at the interface could be explained by the reduction of the free energy even though the interfacial tension between two aqueous polymer solutions is orders of magnitude smaller than between oil and water.<sup>7</sup> In that study, fusion of droplets was observed leading to expulsion of particles

from the interface and macroscopic phase separation, most likely because the mechanical forces during fusion were sufficient to drive the particles from the interface. However, for other systems, the adsorption of particles at the interface significantly stabilized the systems.<sup>4–6</sup>

The objective of the present investigation was to produce and characterize stable water-in-water emulsions using protein particles. Such types of emulsions could potentially be useful for applications, e.g., in cosmetics or food products, possibly as an alternative to oil-in-water emulsions or to deliver ingredients with preferred solubility in the dispersed phase. Model emulsions were formed by mixing dextran and poly(ethylene oxide) (PEO) for which the phase diagram and the interfacial tension have been reported elsewhere.<sup>8</sup> The protein particles were produced by heat-induced aggregation of the globular whey protein  $\beta$ -lactoglobulin ( $\beta$ -lg) under specific conditions, where the aggregation leads to the formation of stable suspensions of well-defined protein particles.<sup>8,9</sup> We will show that protein particles occupy the interface and can stabilize water-in-water emulsions for a period of weeks, whereas native proteins did not enter preferentially the interface. The effects of the polymer composition and thus the interfacial tension, and the protein particle concentration and size were investigated.

### MATERIALS AND METHODS

**Materials.** The dextran and PEO samples used for this investigation were purchased from Sigma-Aldrich. The nominal weight average molar mass was  $M_w = 5 \times 10^5$  g/mol for the dextran and  $M_w = 2 \times 10^5$  g/mol for the PEO. For this study rather high molar masses were chosen in order to delay creaming or sedimentation of the emulsion droplets. Dextran labeled with the fluorophore fluorescein isothiocyanate (FITC) ( $M_w = 5 \times 10^5$  g/mol) was purchased from

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Sigma-Aldrich. Dextran was used without further purification, but the PEO sample contained a small amount of silica particles which were removed by filtration and centrifugation before use. Solutions of dextran and PEO were prepared by dissolving the powder in salt free water (Milli-Q) at neutral pH while mildly stirring. Concentrations are indicated as weight percentages.

$\beta$ -lactoglobulin (Biopure, lot JE 001-8-415) was purchased from Davisco Foods International, Inc. (Le Sueur, MN, U.S.). Stable suspensions of protein particles were prepared by heating aqueous solutions of  $\beta$ -lg at a concentration of 40 g/L in pure water at pH 5.8 or at pH 7 with different amounts of  $\text{CaCl}_2$  (0–6 mM). The solutions were heated in airtight vials in a water bath at 85 °C for 10 h until the reaction was completed. The z-average hydrodynamic radius ( $R_h$ ) of the particles was determined by dynamic light scattering. In the absence of added salt, small strand-like  $\beta$ -lg aggregates were formed, while larger spherical particles were produced after adding controlled amounts of  $\text{CaCl}_2$ . A detailed description of the formation of the protein particles and their characterization using light scattering can be found in refs 8 and 9.

The emulsions were prepared by mixing aqueous solutions of PEO (0–8 wt %), dextran (0–14 wt %), and  $\beta$ -lg (0–1 wt %) at pH 7 in the required amounts using a mini shaker. Trials showed that the order of mixing or the speed of mixing did not significantly influence the structure of the emulsion. In fact, vigorous shaking by hand or using an Ultratorax with gave equivalent results.

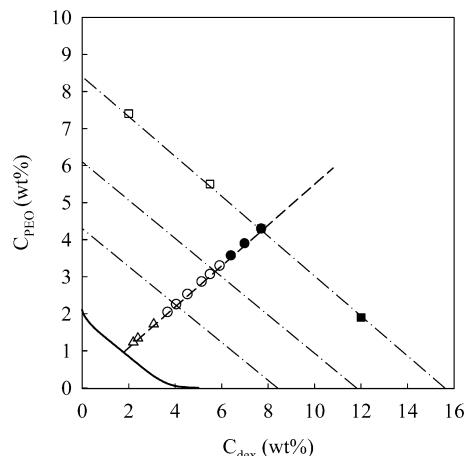
**Methods.** The proteins and the dextran were visualized separately with a confocal laser scanning microscope (CLSM) by utilizing different fluorescent labeling. The proteins were labeled with the fluorochrome rhodamine B isothiocyanate, by adding 5 ppm rhodamine to the solutions. A small fraction of the dextran was labeled with Fluorescein isothiocyanate (FITC). CLSM observations were made with a Leica TCS-SP2 (Leica Microsystems Heidelberg, Germany). Images of 512 × 512 pixels were produced at different zooms with two different water immersion objectives: HC× PL APO 63× NA = 1.2 and 20× NA = 0.7. The solutions were inserted between a concave slide and a coverslip and hermetically sealed. The incident light was emitted by a laser beam at 543 nm and/or at 488 nm. The fluorescence intensity was recorded between 560 and 700 nm. It was verified that the use of labeled dextran and proteins had no influence on the emulsions.

Care was taken not to saturate the fluorescence signal so that it was proportional to the concentration of the probes. It was furthermore verified that the rhodamine signal was proportional to the protein concentration. This allowed us to deduce the protein concentration in each phase from the rhodamine fluorescence signal. The proportionality between the fluorescence intensity and the concentration was calculated from the average intensity and the known average protein concentration.

The criterion for stability of the emulsions was taken as the absence of a visible layer of the pure dispersed phase. We considered the system unstable as soon as a thin (<1 mm) layer became noticeable. We stress that the formation of a layer of the pure continuous phase is not a sign of destabilization of the emulsion droplets, but of creaming or sedimentation of droplets of the dispersed phase.

## RESULTS AND DISCUSSION

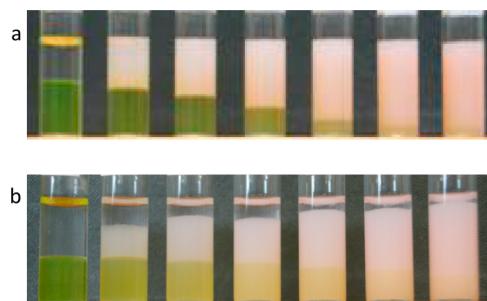
The phase diagram of PEO/dextran mixtures used for this study has already been reported<sup>7</sup> and is reproduced in Figure 1. The two phases are practically pure semidilute PEO and dextran solutions except very close to the critical point situated at  $C_{\text{PEO}} = 1.0\%$  (w/w) and  $C_{\text{dex}} = 1.7\%$ . The dashed line in Figure 1 indicates the compositions where the phases occupy approximately the same volume fraction. The interfacial tension ( $\gamma$ ) increases with increasing polymer concentration, but remains orders of magnitude smaller than for oil-in-water emulsions even at the higher polymer concentrations studied here. For this system  $\gamma$  has the following power law dependence on the tie line length (TLL):<sup>7</sup>  $\gamma \approx 10^{-3} \cdot \text{TLL}^{3.9} \mu\text{N}/\text{m}^2$ . We



**Figure 1.** Phase diagram for aqueous mixtures of PEO and dextran. The solid line indicates the binodal. A few tie lines are drawn for illustration as dashed dotted lines. The dashed line indicates the compositions where the volume fraction of the two phases is equal. Open and filled squares indicate compositions leading to dextran droplets or PEO droplets, respectively. Circles indicate compositions at which protein particles ( $R_h = 150$  nm) adsorb to the interface of PEO droplets that remained stable for a period of at least one week (filled), or that showed signs of destabilization within that period (open). The triangles indicate compositions that led to phase separation without adsorption of protein particles at the interface.

verified that the phase diagram was not influenced by addition of protein particles up to at least  $C_{\text{pro}} = 1\%$ .

**Stability.** Pure mixtures of PEO and dextran completely phase separated within 1 h and formed a dextran-rich phase at the bottom and a PEO-rich phase at the top. Addition of native protein did not have a significant effect on the behavior of the mixtures. However, addition of protein particles led to stabilization of the emulsions, i.e., the droplets did not merge even though they creamed or sedimented at various rates, see below. This is illustrated in Figure 2a, where we show emulsions formed by mixing 3.3% PEO and 9.5% dextran in the presence of different concentrations of protein particles with hydrodynamic radius  $R_h = 150$  nm after having been left standing for one week. At this composition, PEO droplets are formed in the continuous dextran phase. For  $C_{\text{pro}} > 0.1\%$ , we

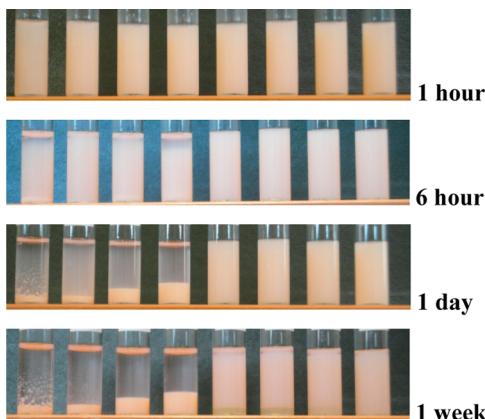


**Figure 2.** Emulsions of PEO/dextran mixtures in the presence of different concentrations of protein particles with  $R_h = 150$  nm ( $C_{\text{pro}} = 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6\%$ , from left to right) after one week standing. (a)  $C_{\text{PEO}} = 3.3\%$  and  $C_{\text{dex}} = 9.5\%$ . (b)  $C_{\text{PEO}} = 3.3\%$  and  $C_{\text{dex}} = 5.5\%$ . Creaming of the PEO droplets gives rise to an opaque emulsion top layer, while destabilization causes a transparent PEO top layer. The dextran bottom phase is colored by the presence of labeled dextran. Excess protein particles render the dextran phase increasingly turbid with increasing protein concentrations.

did not visually observe destabilization of the PEO droplets for a period of at least a week. However, we did observe creaming of the droplets leading to an opaque top layer. The rate of creaming decreased with increasing protein concentration and will be discussed below. The increase of the turbidity of the bottom dextran layer was caused by the presence of excess protein particles that prefer to be situated in the dextran phase, see below.

At lower dextran concentrations ( $C_{dex} = 5.5\%$ ), the emulsions were not completely stable for a week. Destabilization of the PEO droplets manifested itself macroscopically by the appearance of a clear homogeneous PEO phase at the top, see Figure 2b. The stability decreased further with decreasing protein concentrations at low protein concentrations ( $C_{pro} < 0.2\%$ ). For  $C_{pro} = 0.05\%$ , the formation of a thin clear top layer signaling destabilization visually could be observed even at  $C_{dex} = 9.5\%$  after two days. A systematic study showed that at higher protein concentrations the stability of the emulsions was controlled by the interfacial tension and depended little on the composition. The duration for which the emulsions were stable at rest increased with increasing  $\gamma$  up to at least one week for  $\gamma > 30 \mu\text{N}$ .

The evolution with time of emulsions at different compositions on the same tie-line (maximum TLL in Figure 1,  $\gamma = 75 \mu\text{N}$ ) containing 0.5% protein particles with  $R_h = 150 \text{ nm}$  is shown in Figure 3. At this interfacial tension, the



**Figure 3.** Evolution with waiting time of emulsions formed by dextran/PEO mixtures at different compositions on the same tie-line containing 0.5% protein particles with  $R_h = 150 \text{ nm}$ .  $C_{PEO}/C_{Dex} (\%)$  from left to right: 8/1; 7.4/2; 6.3/4; 5.5/5.5; 4.3/7.7; 3.3/9.5; 1.9/12; and 0.8/14. The 4 samples on the left formed dextran droplets in the continuous PEO phase and the 4 samples on the right formed PEO droplets in the continuous dextran phase.

emulsions were stable for at least one week at all compositions. CLSM images taken a few minutes after preparation of the suspensions showed that for  $C_{PEO} > 5\%$  droplets of the dextran phase formed in a continuous PEO phase, while at lower PEO concentrations, PEO droplets were formed in the continuous dextran phase, see Figure 4. Phase inversion occurred when the volume fraction ( $\phi$ ) of the dextran phase became larger than about 0.4. Creaming of PEO droplets was hardly visible after one week, while sedimentation of dextran droplets was complete after one day. The reason for the relatively rapid sedimentation of the dextran droplets is that they aggregated, which is also the reason why the front of the sedimenting emulsion is not very distinct. We do not know why the dextran

droplets aggregated, whereas the PEO droplets did not. In both cases, the droplets are covered with a layer of protein particles. Depletion interactions or attractive interactions between the protein layers would not be expected to differ much in the two situations. The principal difference is that the protein particles prefer to reside in the dextran phase, see below, but why that should induce aggregation of the dextran droplets is not obvious to us.

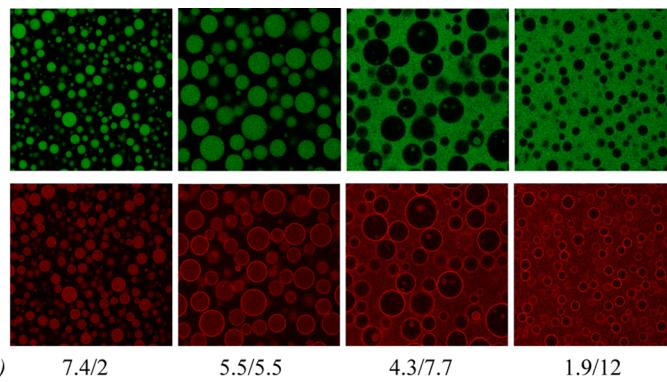
**Microscopic Structure.** Figure 4 shows CLSM images of emulsions formed at different compositions on the same tie-line ( $\gamma = 75 \mu\text{N}$ ) for which the evolution in time was shown in Figure 3. As was mentioned in the Materials and Methods section, the structure did not depend on the mixing procedure. We believe that the reason that the same structures are obtained is that in each case, the 3 components are much more finely dispersed during mixing. The droplets grow quickly by fusion until they have reached within minutes the metastable state size shown in the images. We could observe the latter stages of this ripening process with CLSM, but it was too quick for a systematic investigation with the methods at our disposal. At low dextran concentrations, droplets of the dextran phase were formed in a continuous PEO phase and vice versa for high dextran concentrations. It can be clearly seen that protein particles are adsorbed at the interface and that the excess protein prefers to reside in the dextran phase rather than the PEO phase.

According to eq 1, the binding energy of protein particles at the interface depends on the interfacial tension that can be varied by varying the polymer composition. In order to test the effect of  $\gamma$  on the adsorption of protein particles at the interface, an emulsion prepared at  $C_{PEO} = 4.3\%$  and  $C_{dex} = 7.7\%$  with 0.4% protein particles was diluted progressively with water containing 0.4% protein toward the critical point.

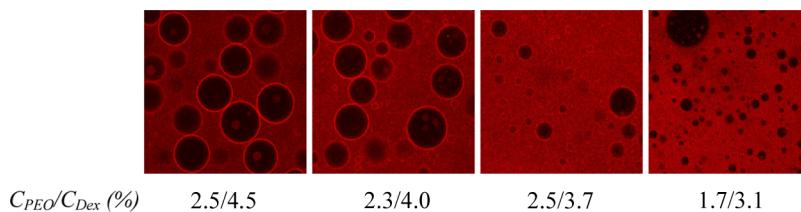
Figure 5 shows CLSM images of the mixtures at different dilutions. Coverage of PEO droplets by protein particles was observed down to  $C_{PEO} = 2.05\%$  and  $C_{dex} = 3.7\%$  ( $\gamma \approx 4 \mu\text{N}/\text{m}^2$ ), but at  $C_{PEO} = 1.7\%$  and  $C_{dex} = 3.1\%$  ( $\gamma \approx 2 \mu\text{N}/\text{m}^2$ ) they no longer adsorbed at the interface. Unfortunately, we cannot quantify the reduction of the free energy for the present system, because the protein particles are polydisperse and we do not know the contact angle. Notice that the correlation length of the semidilute polymer phases is of the order a few nanometers so that the polymer solutions may be considered continuous on the length scale of the protein particles, but not on the length scale of native proteins.

The critical interfacial tension needed to drive adsorption of the protein particles at the interface did not depend significantly on the protein concentration nor on the polymer composition. However, the droplet size did depend on these parameters, see Figure 6. For a given composition the size of PEO droplets decreased with increasing protein concentration. The number average droplet radius ( $R$ ) was determined by manually measuring for several images the radii of the droplets that were in focus.

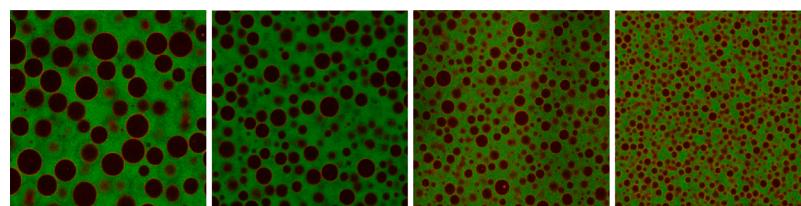
The results as a function of the protein concentration are shown in Figure 7 for two different compositions. For both compositions,  $R$  decreased with increasing  $C_{pro}$ , but the droplets were systematically smaller when the ratio  $C_{PEO}/C_{dex}$  was smaller. For Pickering emulsions, a decrease of the droplet size with increasing particle concentration is expected as incompletely covered small droplets will coalesce until the interface is fully stabilized. However, we do not find  $R^{-1} \propto C_{pro}$  as would be expected if the coverage at steady state was independent of the



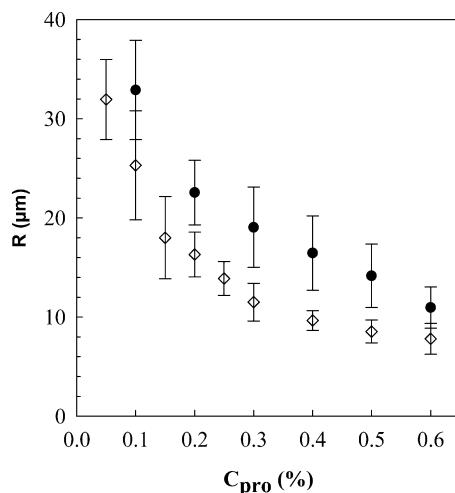
**Figure 4.** CLSM images ( $160 \times 160 \mu\text{m}$ ) of the dextran signal (top) and the protein signal (bottom) for PEO/dextran mixtures in the presence of 0.5% protein particles ( $R_h = 150 \text{ nm}$ ) for different polymer compositions on the same tie-line indicated.



**Figure 5.** CLSM images ( $130 \times 130 \mu\text{m}$ ) of the protein signal for PEO/dextran mixtures in the presence of 0.4% protein particles with different polymer compositions.



**Figure 6.** CLSM images of the dextran signal ( $500 \times 500 \mu\text{m}$ ) showing the effect of the protein particle ( $R_h = 150 \text{ nm}$ ) concentration on the droplet size for a mixture containing 3.3% PEO and 9.5% dextran: from left to right  $C_{pro} = 0.1, 0.15, 0.2, and 0.4%.$



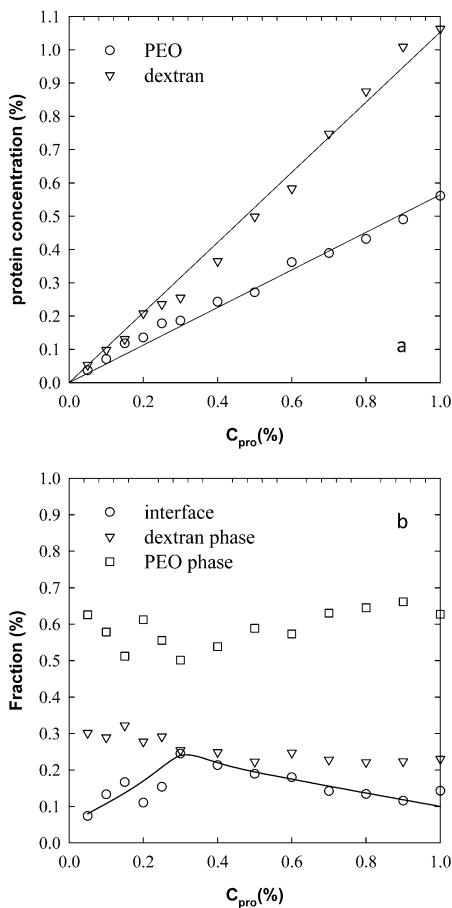
**Figure 7.** Dependence of the number average droplet radii on the protein concentration for two compositions:  $C_{PEO} = 3.3\%$  and  $C_{dex} = 5.5\%$  (closed symbols) or  $C_{PEO} = 3.3\%$  and  $C_{dex} = 9.5\%$  (open symbols). The error bars represent the standard deviation of the size distribution.

protein concentration,<sup>10</sup> see below. For a given protein concentration, the droplet size decreased for compositions

with a smaller volume fraction of the dispersed phase, which may also be explained by an increase of the ratio protein particles/interfacial area for a given droplet size.

The concentration of protein in the two phases was determined for mixtures containing 3.3% PEO and 9.5% dextran with different protein concentrations by measuring the fluorescence intensity of the two phases, see Figure 8a. The protein concentration was systematically higher in the dextran phase than in the PEO phase as can already be seen from the images taken with the protein signal shown in Figures 4 and 5. Knowing the volume fraction of each phase ( $\phi_{dex} = 59\%$  and  $\phi_{PEO} = 41\%$ ), we can calculate the amount of proteins in each phase and by comparing the sum with the total amount of proteins, we can deduce the amount of proteins at the interface per unit of volume ( $C_{int}$ ). The partitioning of the proteins between the two phases and the interface is shown in Figure 8b. The fraction of proteins at the interface was found to increase with  $C_{pro}$  from about 10% to 25% at  $C_{pro} = 0.3\%$  and then to weakly decrease at higher  $C_{pro}$ .

Using the average radius of the droplets, we can calculate the droplet surface area per unit of volume as follows:  $S = 3 \cdot \phi_{PEO} / R$ . The surface area occupied by an adsorbed spherical protein particle is  $\pi R_h^2$  and number of particles that is needed per unit of volume to form a dense monolayer is  $S / (\pi R_h^2)$ . The mass of a protein particle depends on the protein density within the

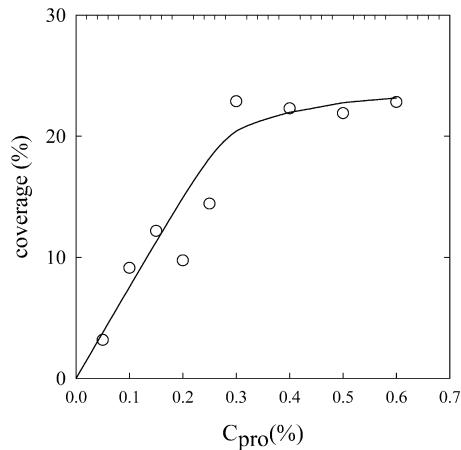


**Figure 8.** Part (a) shows the weight fraction of proteins in the dextran phase and the PEO phase for mixtures with  $C_{\text{PEO}} = 3.3\%$ ,  $C_{\text{dex}} = 9.5\%$  as a function of the total concentration of protein particles with  $R_h = 150 \text{ nm}$ . Part (b) shows the partitioning of protein particles between the dextran phase, the PEO phase and the interface for mixtures. The solid line is a guide to the eye.

particles ( $\rho \approx 15\%$ ):  $m_{\text{pro}} = \rho \cdot 4\pi R_h^3 / 3$ . Thus, the concentration of proteins that is needed to create a dense monolayer at the interface is equal to  $C_{\text{int}} = m_{\text{pro}} \cdot S / (\pi \cdot R_h^2)$ , i.e.,  $C_{\text{int}} = \rho \cdot R_h \cdot \phi_{\text{PEO}} \cdot 4/R$ .

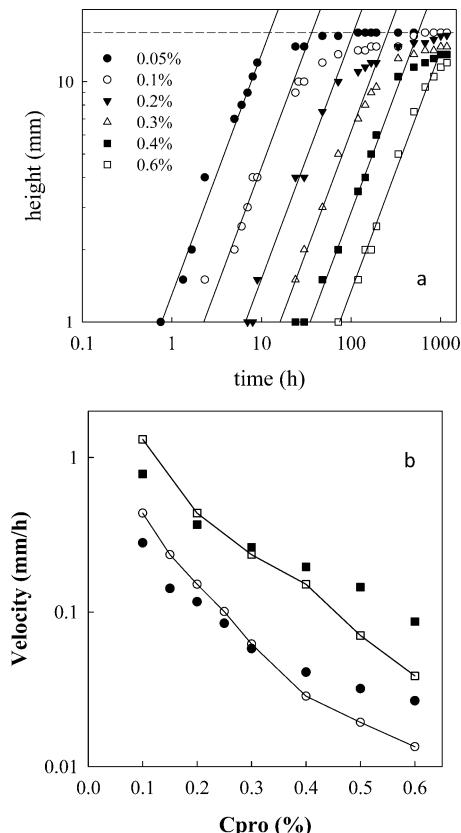
The coverage of the interface is obtained by normalizing the experimentally observed amount of proteins at the interface with that expected for a dense monolayer, see Figure 9. The coverage increased at low protein concentrations until it stabilized for  $C_{\text{pro}} > 0.3\%$  at approximately 23%. Notice, however, that there is considerable uncertainty in the calculated value of the coverage for a dense monolayer because the protein particles and the droplets are polydisperse and the protein particles are not perfect spheres. Our use of the number average droplet radius and the z-average protein particle radius leads to an overestimation of  $C_{\text{int}}$  for a dense monolayer and thus an underestimation of the coverage. Therefore, it is difficult to draw firm conclusions about the packing of the proteins at the saturated interface, but it is highly unlikely that multilayers were formed.

**Droplet Creaming or Sedimentation.** PEO droplets creamed and dextran droplets sedimented, because in all cases, the dextran phase was denser than the PEO phase. For a given polymer composition, the creaming velocity ( $v$ ) of PEO droplets decreased rapidly with increasing protein particle concentration. The velocity of creaming or sedimentation was

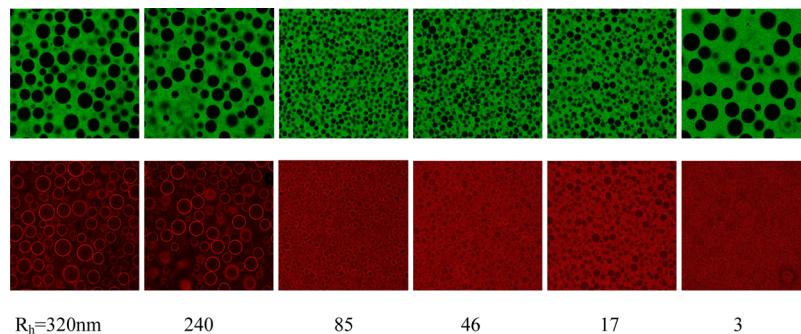


**Figure 9.** Percentage of the surface of the PEO droplets covered by protein particles with  $R_h = 150 \text{ nm}$  as function of the total protein concentration for mixtures with  $C_{\text{PEO}} = 3.3\%$  and  $C_{\text{dex}} = 9.5\%$ . The solid line is a guide to the eye.

quantified by measuring the height of the emulsion ( $h$ ) as a function of time. Figure 10a shows the results for creaming of PEO droplets in mixtures containing  $C_{\text{PEO}} = 3.3\%$ ,  $C_{\text{dex}} = 9.5\%$ , and different concentrations of protein particles. The height of



**Figure 10.** (a) Evolution of the height of the emulsion layer with time during standing for mixtures with  $C_{\text{PEO}} = 3.3\%$ ,  $C_{\text{dex}} = 9.5\%$ , and different concentrations of protein particles with  $R_h = 150 \text{ nm}$ . The straight lines have a slope of one. (b) Creaming velocity as a function of the protein particle concentration for mixtures with  $C_{\text{PEO}} = 3.3\%$ ,  $C_{\text{dex}} = 9.5\%$  (circles) and  $C_{\text{PEO}} = 3.3\%$ , and  $C_{\text{dex}} = 5.5\%$  (squares). Velocities calculated from the measured droplet sizes are shown as filled symbols.



**Figure 11.** CLSM images of the dextran signal (top) and the protein particles with different sizes indicated in the figure (bottom) taken within a few minutes after preparation. The emulsions contained 3.3% PEO, 9.5% dextran, and 0.2% protein.

the emulsion layer decreased initially approximately linearly with time implying that the emulsion creamed with constant velocity:  $h(0) - h(t) = v \cdot t$ . The velocity strongly decreased with increasing protein concentration, see Figure 10b.

The velocity of a droplet under gravity is a function of the viscosity of the continuous phase ( $\eta$ ), the density difference between the two phases ( $\Delta\rho$ ), and the radius of the droplet:

$$v = g \cdot \Delta\rho \cdot 2 \cdot R^2 / (9 \cdot \eta) \quad (2)$$

$\Delta\rho$  was calculated from the polymer concentrations in each phase using the specific volumes of PEO (0.831 mL/g) and dextran (0.626 mL/g)<sup>11</sup> yielding  $\Delta\rho = 50 \text{ kg/m}^3$ . The viscosity of the continuous dextran medium was determined as 0.09 Pa·s and was not significantly different when 1% protein particles were added. The velocities calculated using the measured droplet sizes are compared with the observed velocities in Figure 10b. The agreement is reasonable considering the uncertainty in the measured droplet sizes. Similar experiments done at a lower dextran concentration (5.5%) showed faster creaming, because the viscosity of the continuous dextran medium was lower (0.04 Pa·s), but also because the average droplet size was slightly larger.

The thickness of the creamed emulsion layer at steady state was close to that of the pure PEO phase layer obtained in the absence of proteins, implying that the PEO droplets pack densely, leaving little space for the continuous dextran phase. The volume fraction of randomly closed packed monodisperse spherical droplets is about 0.63, but for polydisperse and deformable droplets it can be closer to unity.

Sedimentation of dextran droplets was much faster than predicted from their size. The reason is that these droplets stick together and form large flocs that can even be observed macroscopically. Creamed PEO droplets could be easily dispersed by gently shaking, but sedimented dextran droplet required strong shaking to redisperse.

#### Effect of the Size and Nature of the Protein Particles.

The PEO droplet size in the emulsions depended on the size of the protein particles. This is illustrated in Figure 11 for mixtures at  $C_{\text{PEO}} = 3.3\%$  and  $C_{\text{dex}} = 9.5\%$  containing 0.2% proteins. The droplet size decreased with decreasing size of the protein particles down to  $R_h = 85 \text{ nm}$ . It was similar for  $R_h = 46 \text{ nm}$  and  $R_h = 85 \text{ nm}$ , but increased for  $R_h = 17 \text{ nm}$  and  $R_h = 3 \text{ nm}$  (native proteins). We note that the particles with  $R_h = 17 \text{ nm}$  are not spherical, but curved strands with a length of about 50 nm and a diameter of about 5 nm.<sup>12</sup> Down to  $R_h = 46 \text{ nm}$ , a protein layer adsorbed at the interface could be clearly seen, but for  $R_h = 17 \text{ nm}$ , it was not obvious. Native proteins could not be detected at the interface and did not show a marked

preference for the dextran phase. The decrease of the droplet size with decreasing particle size can be explained by the fact that at the same weight concentration of protein, the number of particles increases with decreasing size. The smallest protein particles did not effectively inhibit fusion of PEO droplets, which explains why the droplet size was larger.

The macroscopic evolution of the samples as a function of time showed that the emulsions were not stabilized by native proteins and phase separated at approximately the same rate as protein free mixtures. Systems containing small strands with  $R_h = 17 \text{ nm}$  phase separated slightly slower indicating that the particles did adsorb at the interface, but did not effectively inhibit fusion of the droplets. Particles with  $R_h = 46 \text{ nm}$  stabilized the emulsion for up to 7 h, but after one day clear signs of destabilization were observed. It should be noted, however, that this system is in fact a mixture of smaller strand-like particles and larger spherical particles as explained in refs 8 and 9. The larger spherical protein particles ( $R_h \geq 85 \text{ nm}$ ) stabilized the emulsions for a period of up to about a week. For these mixtures, the velocity at which the PEO droplets creamed increased with increasing particle size, which can be explained by the increase of the droplet size.

The protein particles we have used in this study were produced by heating. However, milk naturally contains protein particles with similar size and density that are called casein micelles. It might be of interest to know if these particles can also be used to stabilize water-in-water emulsions. Casein micelles are approximately spherical complexes of different types of casein proteins with an average radius of about 150 nm held together by colloidal calcium phosphate.<sup>13</sup> We have done preliminary measurements on emulsions in the presence of casein micelles and found that casein micelles do adsorb to the interface, but that the emulsions destabilized within a few hours.

It is clear that in order to effectively stabilize water-in-water emulsions with protein particles, one needs to use particles with a radius larger than 50 nm. However, the particles should not be too large in order to maximize the number of particles at a given protein concentration. The shape and composition of the particles may also be important, as already indicated by the preliminary tests with casein micelles. Here we have used relatively dense spherical particles, but it would be interesting to test the stabilization capacity of protein particles with different morphologies.

## CONCLUSIONS

Water-in-water emulsions formed by mixing dextran and PEO solutions could be stabilized by addition of protein particles,

while keeping both phases in the liquid state. The stability of the emulsions increased with increasing interfacial tension and particle size and the time during which no visible layer of the dispersed phase was formed could last for a period of weeks, which may be useful for applications.

The emulsions were stabilized by the formation of a monolayer of protein particles at the water–water interface that was driven by the reduction of the free energy when particles adsorb to the interface. Native proteins did not adsorb to the interface, because they were too small and therefore could not stabilize water-in-water emulsions via this so-called Pickering effect even though they are excellent stabilizers of oil-in-water emulsions. The droplet size of the dispersed phase was found to decrease with increasing protein concentration and when the difference between the volume fractions of the two phases was larger. With increasing protein concentration, the surface coverage increased initially, but saturated when it reached about 30%. Best results were obtained with protein particles with a radius of around 100 nm, but the structure and the composition of the particles may also be important to consider.

Gravity caused creaming of PEO droplets in the continuous dextran medium and sedimentation of dextran droplets in the continuous PEO phase. In the former case the rate of creaming was determined by the droplet size and the viscosity of the continuous medium and could be extremely slow; less than 1 mm per week. Sedimentation of dextran droplets in the continuous PEO phase was relatively rapid at all conditions due to aggregation of the droplets.

## AUTHOR INFORMATION

### Corresponding Author

\*E-mail: Taco.Nicolai@univ-lemans.fr.

### Notes

The authors declare no competing financial interest.

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