Structure modifications of AOT reverse micelles due to protein incorporation

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Abstract

Structural modifications of AOT/water/isooctane reverse micelles due to incorporation of proteins were studied at various water contents and protein concentrations, using small-angle X-ray scattering (SAXS) experiments under static conditions, rheometry analysis, and SR-SAXS experiments under induced shear flow. Two proteins, lysozyme (pI 11.1, \( M_w \) 14,300 Da) and BSA (pI 4.3, \( M_w \) 66,700 Da), were chosen as models. SAXS analysis of protein-containing reverse micelles at low water content detected minima in the average micelle size versus protein concentration curve, for both proteins, below and above their isoelectric point. This minimum was attributed to changes in the size distribution of the reverse micelles. SAXS measurements of reverse micelles at high water content have shown them to have a cylindrical form. Incorporation of lysozyme at pH 7 into the cylindrical micelles induced a shape transition to spherical micelles, which was associated with a decreased viscosity. SR-SAXS measurements under induced shear flow and dynamic conditions revealed alignment of the cylindrical micelles in the flow direction. The anisotropy parameter, a measure of the degree of the spatial order, was found to increase with increasing shear rate and to decrease with increasing lysozyme concentration.

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1. Introduction

Reverse micelles are thermodynamically stable surfactant aggregates with a polar core, which are formed spontaneously when certain types of surfactants are dissolved in organic solvents. Water and host macromolecules can be solubilized in that core, protected from direct contact with the organic solvent; therefore their biological activity remains unharmed [1,2]. Due to this property there is growing interest in reverse micelles for future applications in biotechnological industry, such as selective extraction of proteins or enzymatic catalysis in organic media. Proteins can be selectively extracted by manipulating parameters such as surfactant concentration, salt concentration in the aqueous solution, water acidity, or water content. The water content \( W_0 \), expressed in terms of the ratio of water molar concentration to surfactant molar concentration, is one of the important parameters in this system, as it determines the size of the water core and thus the size of the reverse micelles.

Several groups have investigated the process of protein incorporation into reversed micelles in terms of the driving forces for protein solubilization, protein localization, and size or shape perturbations induced by the protein [3–15]. Additionally, the structure of the protein, which is crucial if an enzymatic reaction is to be carried out using reverse micellar system, has been examined. A strong relation between the activity of the incorporated protein and the reverse micelle size has been reported [16–18]. When the micelle size is comparable to the protein dimensions, its structure is more affected by the water content. Protein structure and activity in reverse micelles also depend on the protein localization. Proteins that are located in the water core tend to maintain their activity, while localization near the surfactant interface will often lead to lower activity [16]. As for the size of the micelles, the literature reports somewhat contradictory results. Pileni and co-workers suggested a model [5,9,14,15] according to which hydrophilic proteins such as \( \alpha \)-chymotrypsin and ribonuclease tend to be located in the water core, avoiding contact with the surfactant...
head groups. This localization causes an equivalent increase in the water core volume and therefore increased radii. On the other hand, proteins such as cytochrome c anchor to the surfactant molecules at the interface or near it, due to electrostatic or hydrophobic interactions with the surfactant. This localization leads to increased interfacial area and therefore decreases the water core radii [9]. An opposite observation was reported by Melo et al. [3,4], who observed swelling of reverse micelles due to incorporation of the protein cutinase that attaches to the micelle wall. This phenomenon was attributed to conformational changes and unfolding of the protein.

The current research focuses on structural modifications due to protein incorporation in water-containing reverse micelles formed by the anionic surfactant sodium bis-(2-ethylhexyl) sulfosuccinate (AOT) in the organic solvent isooctane. One of our objectives was to evaluate whether the influence of the protein stability on the size of the reverse micelles, observed by Melo et al. [3,4] for cutinase, is a general phenomenon that could be observed with other proteins as well. Therefore two model proteins were chosen, lysozyme and bovine serum albumin (BSA), which differ in their stability in reverse micelle systems. Lysozyme changes its conformation markedly and denatures in the AOT/isooctane/H2O system at all water contents [17,19], due to strong specific interaction with AOT molecules in the micellar interface. In contrast, BSA maintains its structure to a large extent during extraction with AOT reverse micelles [20,21]. We were further interested in evaluating the influence of protein concentration, a parameter that has not been studied thoroughly before [18], on the micelle structure. As described in this paper, our results revealed an interesting and unexpected dependence of the micelle size on the protein concentration. Finally, we have characterized the micelle structure under shear. In addition to scientific interest, this study has practical significance since industrial utilization of reverse micelle solutions, either as a solvent for liquid–liquid extraction or for enzymatic catalysis, must be performed under stirring conditions.

2. Materials and methods

2.1. Materials and solutions preparation

AOT (Fluka), isooctane (Carlo Erba or Scharlau), and lysozyme and BSA (Sigma) were used as received. Lysozyme and BSA aqueous solutions were prepared using Milli-Q water. If needed, water acidity was adjusted by adding NaOH or HCl 0.1 M solutions. For the preparation of reverse micelle solutions with incorporated proteins, measured volumes of water, lysozyme, or BSA aqueous solutions were injected into 100 mM solutions of AOT in isooctane using a Hamilton syringe. The injected volume was calculated for the water-containing reverse micelles to give a desired water content

$$W_0 = W_{0,app} = [H_2O]/[AOT].$$

In each series of experiments identical injected volumes were used, even though water was replaced with protein solution, in order to evaluate the influence of the protein–micelle interactions.

2.2. Rheology

Rheology measurements were performed using a Rheometric Scientific (ARES) strain-controlled shear rheometer fitted with a cup-and-bob (Couette) fixture covered with an anti-evaporation cover. Steady rate sweep measurements were performed in a shear rate range of 1–250 Hz. Calculations and processing of the results were performed using the RSI Orchestator software package version 6.5.1.

2.3. Small angle X-ray scattering (SAXS)

SAXS measurements under static conditions were performed with CuKα radiation using a compact Kratky camera having a linear position-sensitive detector system (Raytech) with pulse-height discrimination and a multichannel analyzer (Nucleus). The entrance slit to the collimation block was 20 µm, and the slit length delimiters were set at 15 mm. The sample detector distance was 26.4 cm. Reverse micelle solutions were placed in cylindrical quartz cells (A. Paar Co., 1 mm path length). The sample temperature was kept at 25 °C by means of a temperature controller (A. Paar). Primary beam intensities were determined using the moving slit method of Stabinger and Kratky [22] and subsequently using a thin quartz monitor as a secondary standard. The scattering curves, as a function of the scattering vector

$$q = 4\pi \sin \theta/\lambda,$$

where $2\theta$ and $\lambda$ are the scattering angle and the wavelength, respectively, were corrected for counting time and for sample absorption. The background scattering (isooctane-filled capillary) was measured separately and subtracted from the scattering curve. The correction of the effect of the beam dimension (desmearing) was performed according to the indirect transformation method [23,24] using the program ITP. Data analysis was based on fitting the desmeared curve to an appropriate theoretical model using a least-squares procedure.

SAXS measurements under shear force were performed at the beam line ID02, European Synchrotron Radiation Facility, Grenoble, France. The wavelength of the incident photons was 1 Å, and the sample-to-detector distance was either 1 or 2 m. The measurements were performed using a two-dimensional multiwire proportional gas counter. The samples were studied in a polycarbonate coquette cell having a 1-mm annular gap between the rotor (outer) and stator (inner) cylinders, mounted on motorized translation stages, which allowed the sample to be aligned in both the radial and tangential positions [25]. In the radial configuration the incident beam was perpendicular to the flow direction but parallel to the velocity gradient. In the tangential configuration the incident beam was parallel to the flow direction or the shear velocity. The raw data were corrected to account for the transmission of the 2-D detector response.

3. Modeling of the SAXS intensities

The analysis of scattered X-ray intensities requires consideration of the existence of an inherent polydispersity in the size of the reverse micelles, which arises from a balance between several contributions to the free energy of the micellar solution [7].
The scattered X-ray intensity, \( I(q) \), is expressed as [6,8,9]

\[
I(q) = n_p \bar{P}_s(h) S(h),
\]

where \( n_p \) is the number of micelles per unit volume, \( \bar{P}_s(q) \) is the average polydisperse form factor, which is related to the structure of the reversed micelles (shape and size), and \( S(h) \) is the structure factor, which provides information on the structural arrangement of the reversed micelles (intermicellar interactions). In dilute solutions, the structure factor is approximately equal to unity, due to negligible interparticle interference.

At low water content, AOT reverse micelles are commonly described using a model of spherical “core and shell” aggregates [8]. This model describes an aggregate with an outer diameter of \( R_s \), built from a core having a radius of \( R_c \) and electron density of \( \rho_c \), surrounded by a thin spherical shell of surfactant molecules having an electron density of \( \rho_m \). The form factor for such an aggregate is given by

\[
P_s(q) = \left[ \frac{4\pi}{3} R_s^3 (\rho_m - \rho_m) \Phi(q R_s) + \frac{4\pi}{3} R_c^3 (\rho_c - \rho_m) \Phi(q R_c) \right]^2
\]

(2)

with

\[
\Phi(q R_s) = 3 \left( \frac{\sin(q R_s) - q R_s \cos(q R_s)}{(q R_s)^3} \right),
\]

\[
\Phi(q R_c) = 3 \left( \frac{\sin(q R_c) - q R_c \cos(q R_c)}{(q R_c)^3} \right),
\]

where \( \rho_m \) is the electron density of the medium.

The polydisperse form factor, \( \bar{P}_s(q) \), was obtained from \( P_s(q) \) by assuming a Gaussian (normal) size distribution [9] with \( \sigma \) as the root-mean-square deviation from the mean water core radius \( R_c \), and then evaluated as

\[
\bar{P}_s(q) = \frac{1}{\sqrt{2\pi}\sigma} \int P_s(q) \exp \left( -\frac{(R_c - \bar{R}_c)^2}{2\sigma^2} \right) dR_c.
\]

In fitting Eqs. (1)–(3) to the experimental SAXS curves it was assumed that the shell has a constant thickness of 0.4 nm [6]. The electron densities of the shell, the water core, and the solvent were fixed as \( \rho_s = 550 \text{ e}^{-/\text{nm}^3} \), \( \rho_c = 334.6 \text{ e}^{-/\text{nm}^3} \), and \( \rho_m = 240.8 \text{ e}^{-/\text{nm}^3} \), respectively. The electron density of aqueous protein solution was measured and found to be almost equal to that of water only; therefore the electron density of the water core was kept constant. Fit was obtained by varying the values of \( \bar{R}_c \), \( \sigma \), and \( n_p \).

4. Results and discussion

The two proteins selected for this research differ greatly in size. BSA (MW 66 kDa, pI 4.9) is a bulky hydrophilic protein having radius of about 3.5 nm [20,21], while lysozyme (\( M_w \) 14 kDa, pI 11.1) has a much smaller radius of about 2 nm. To allow us to examine different scenarios, we chose to work at two water contents: \( W_0 = 40 \), for which the average reverse micelle size (4.9 nm [26]) is larger than both proteins, and \( W_0 = 20 \), where the reverse micelles (2.5 nm) could be large enough to accommodate the lysozyme but not the BSA. For each protein the experiments were conducted at two pH values: below the isoelectric point, where the positively charged protein strongly interacts with the AOT headgroups, and above the pI. It should be noted, thus, that while lysozyme does not interact with AOT above its isoelectric point [27], BSA produces weak interactions with the surfactant both below and above the pI [20].

Structural studies of AOT reverse micelles in the presence of the proteins were first performed using the Kratky camera under static conditions. The water content was kept constant at either \( W_0 = 20 \) or \( W_0 = 40 \) while the protein concentration was varied. Experimental SAXS curves for various lysozyme concentrations (0–200 \( \mu \)M, obtained from injecting protein solutions having concentrations of 0–40 g/l) at \( W_0 = 40 \) and pH 7, are shown in Fig. 1. As AOT reverse micelles at low water content are expected to have an isotropic, core–shell spherical shape, the experimental data were fitted to Eqs. (1)–(3), assuming \( S(q) = 1 \). The resulting fits, shown as solid lines in Fig. 1, were calculated from the best-fit parameters given in Table 1. As can be seen, the model gave a good fit to all the experimental scattering curves. Fits with the same qualities were obtained for all other experiments presented in this manuscript.
The average core radius $\bar{R}_c$ is plotted in Fig. 2 as a function of the solubilized lysozyme concentration. For the whole measured concentration range, the average size of the protein-containing micelles is smaller than the size of the empty ones. Yet a clear minimum is observed at pH 7 both for $W_0 = 40$ (Fig. 2a) and for $W_0 = 20$ (Fig. 2b). A minimum is still observed, although it is less pronounced, at pH 12, which is above the isoelectric point of lysozyme (dashed line in Fig. 2). Similar SAXS measurements and analysis were performed for BSA-containing AOT reverse micelles, and their results are summarized in Fig. 3. As for the lysozyme, a minimum value of $\bar{R}_c$ is revealed at water content of $W_0 = 40$ (Fig. 3a) and $W_0 = 20$ (Fig. 3b), both below the pI (pH 3) and above it (pH 8).

A previous work by Pileni and co-workers [12] did not detect minima in the graph describing the reverse micelle size vs cytochrome c concentration. Rather, the micelle size was found to decrease with an increase in the protein concentration. A decrease in the average micelle size was also observed in other studies [16,17,19] in which a single protein concentration was used. The decrease in the micelle size was attributed to the anchoring of the positively charged protein molecules to the surfactant headgroups, due to electrostatic interactions between them. The protein was claimed to perform as a substitute for additional surfactant, causing the effective water content $[H_2O]/[AOT]$ to decrease and therefore the mean size of the water pool also to decrease [16,17,19]. The concentration range used in the work of Pileni and co-workers was narrower than the one used in the current study. Therefore, it is possible that a minima was not observed experimentally but it do exist. Still, the model suggested by Pileni and co-workers cannot explain the minima in the $R_c$ vs concentration curve. Moreover, since this minimum appears both for positively charged proteins and for negatively charged ones, it is unlikely to attribute it to electrostatic interactions.

An alternative explanation to the changes observed in the average micelle size could be realized by inspecting the fit parameters (Table 1) and the entire size distribution, such as the ones shown in Fig. 4 for reverse micelles with incorporated lysozyme. First, it can be noticed from Table 1 that the number of micelles is not constant, but rather it reaches a maximum value at the same concentration where the minimum radius is observed. In other words, the number of micelles that are formed in a solution containing a relatively low protein concentration is larger than the number of micelles formed without the presence of protein. Since more micelles are formed, without the AOT concentration changing, their average size must become smaller. Beyond a critical protein concentration, the number of micelles decreases and therefore the average size increases. Another interesting observation is that the size distribution in the presence of protein is broader compare to that
of the “empty” micelles (Fig. 4). In particular, it seems that the fraction of smaller micelles is larger in the presence of the protein. In an attempt to quantify these changes, we have calculated a differential size distribution $\Delta n(R_c)$, defined as the difference between the number of micelles in the presence and in the absence of protein. Several representative differential size distributions are shown in Figs. 5 and 6. For $W_0 = 40$, two clear positive peaks are seen, one around 3 nm and the other at about 7.5 nm (marked with an arrow in Fig. 5). For $W_0 = 20$, again two peaks appear; however, one of them is negative, indicating a decrease in the number of micelles having an average radius of about 2.5 nm. Although the full size distribution can be predicted theoretically, this task is beyond the scope of this manuscript. Still, our results qualitatively demonstrate that adding a protein shifts the entire size distribution rather than causing a contraction or expansion of each individual micelle. Moreover, a preference for formation of micelles with favorable size in the protein-containing reverse micelles seems to support the previously suggested mechanism [3,4] according to which two populations of micelles are formed, protein-containing micelles and empty ones.

The effect of protein incorporation on the reverse micelle size at low water content led to the study of the influence of added lysozyme on the flow characteristics and viscosity of the reverse micelles system using rheometry experiments. This study was also performed as preliminary work for dynamical SR-SAXS microstructure measurements of the reverse micelles system in the presence of the protein. Viscosities of reverse micelle solutions, formed by injecting lysozyme at different initial...
aqueous concentrations (3, 5, and 10 g/l) into 100 mM AOT reverse micelle solutions, were measured at a range of water content ($W_0$) of 0–100, and are shown in Fig. 7, in comparison to similar measurements without protein incorporation. Low lysozyme concentration (3 g/l) does not affect the viscosity. In contrast, increasing the lysozyme concentration (5 or 10 g/l) hindered the sharp leap in viscosity that occurred in water volume fractions equivalent to that of the percolation threshold [10]. This sharp leap occurs at a higher water content of $W_0 = 80$ in the presence of lysozyme, compared to $W_0 = 70$ without it. Incorporation of lysozyme at all concentrations also hindered the transition in the flow characteristics of the reverse micelle solution from a Newtonian fluid to a shear-thinning fluid at high water content. This transition occurred at $W_0 = 90$ in protein-containing reverse micelles, in comparison to $W_0 = 80$ without the protein. These results contradict the observation of Huruguen et al. [10] who reported that the presence of cytochrome c expedited the percolation threshold and the sharp leap in viscosity.

Since the changes in the rheological behavior may indicate that the structure is altered due to the shear flow and the protein incorporation, we have studied the microstructure of the AOT/water/isooctane reverse micelles under shear flow using SR-SAXS. Reverse micelles solutions with 100 mM AOT in isooctane at different water contents ($W_0$) of 20, 40, 70, 80, and 90 with incorporated lysozyme at different concentrations (0–40 g/l) in the injected aqueous solution at pH 7 were examined in a shear rate range of 0–900 rpm. At water contents lower then $W_0 = 70$ or less, when compared to similar measurements performed using the Kratky camera at static conditions. Contrary, reverse micelles at higher water content of $W_0 = 80$, without added lysozyme, have demonstrated an anisotropic scattering patterns at all shear rates (Fig. 8). Accordingly, the model of core–shell spherical aggregates failed to give a reasonable fit to the experimental data. Huruguen et al. [11] suggested a model of cylindrical objects, forming a network due to micelle entanglement, to describe their SAXS data. The form factor $P_C(q)$ of such an object is given by [28]

$$P_C(q) = \frac{\pi^{1/2}}{q} \left[ \frac{\Delta \rho}{q R \sin \alpha} \frac{2 J_1(qR \sin \alpha)}{q L \cos \alpha/2} \right]^2 \sin \alpha d\alpha,$$

where $\Delta \rho$ is the electron density difference between the cylinder and the medium, $J_1$ is the first-order Bessel function, $L$ the cylinder length, and $R$ the radius of its cross-section. In the current work, we have followed the approach of Huruguen et al. but used a form factor of core–shell cylindrical rodlike objects. Following preliminary calculations, we were able to justify the use of a simplified expression that is applicable in the limit of very long core–shell cylinders [29],

$$P_C(q) = \frac{\pi L}{q} \left\{ (\rho_s - \rho_m) \pi R_s^2 \frac{2 J_1(qR_s)}{q R_s} \right\}^2 + (\rho_c - \rho_s) \pi R_c^2 \frac{2 J_1(qR_c)}{q R_c} \right\}^2,$$

where $\rho_c$ and $R_c$ are the electron density and the radius of the polar core of the cylindrical reverse micelle, $R_s$ is the outer radius of the micelle, $\rho_s$ is the electron density of the cylindrical shell surrounding the core, and $\rho_m$ is the electron density of the medium. In addition, we have taken into account the polydispersity of the cylinder’s radius. The average form factor was calculated assuming a Gaussian size distribution by replacing
\( P_s(q) \) with \( P_c(q) \) in Eq. (3). Finally, the scattering intensity was calculated from Eq. (1), assuming \( S(q) = 1 \) (as applying the structure factor given by Huruguen et al. did not improve the fit). The best fit to this model, which was calculated from the best-fit parameters \( R_c = 5.42 \text{ nm} \) and \( \sigma = 1.29 \) and is shown as a dashed line in Fig. 9, agrees well with the experimental data at small scattering angles. However, the fits at higher angles are rather poor, indicating the coexistence of smaller objects. Indeed, a good fit to the experimental data was obtained by suggesting a two-component model that takes into account the coexistence of small spherical micelles having a diameter of 1 nm (solid line in Fig. 9) [26]. The ratio of surfactant molecules forming cylindrical micelles to those forming spherical micelles, \( N_c/N_s \), which was calculated from the fit assuming an identical area per headgroup in both types of aggregates was found to be equal to 223. It is evidence that most surfactant molecules are found in the cylindrical micelles.

At high water content of \( W_0 = 90 \), the anisotropy subjected under shear force reduces with increasing lysozyme concentration and increases with an increase in the shear rate, as shown in Fig. 10. The anisotropy is noticeable at low lysozyme concentrations of 1–5 g/l and is most pronounced with no lysozyme addition at all (0 g/l). At higher lysozyme concentrations of 10–40 g/l anisotropy does not appear at any shear rate. As before, the anisotropic scattering patterns have shown good agreement with the theoretical fitting model of cylindrical reverse micelles (Eq. (5)), whereas isotropic patterns, obtained at higher lysozyme concentrations of 10–40 g/l, showed good agreement with a model of spherical reverse micelles. The different shapes and mean water core radius of reverse micelles at \( W_0 = 90 \) and different lysozyme concentrations are summarized in Table 2. As can be seen from the data given in Table 2, the reverse micelles at \( W_0 = 90 \) change their shape from a cylindrical micelle to a spherical one due to the lysozyme incorporation. The shape change is gradual, as evident from the gradual decrease in the cylinder radii at lysozyme concentrations of 1–5 g/l, followed by formation of spherical reverse micelles at lysozyme concentrations of 10 g/l and higher.

The changes in the micelle shape correlates well with the viscosity measurements. With the empty micelles, the transition from spheres at \( W_0 = 70 \) to cylinders at \( W_0 = 80 \) increases the aspect ratio (L/D) and therefore raises the resistance of the reverse micelles to shear force [30], thus causing the sharp leap in viscosity observed in the rheometry experiments (Fig. 7). This sharp viscosity leap was hindered to \( W_0 = 80 \) by incorporating lysozyme concentration of 3–5 g/l into the reverse micelle solution, which changes their shape back from cylinders spheres.

The transition from nonisotropic to isotropic scattering patterns was quantified using the anisotropy parameter \( A(q) \), which is defined as the ratio between the scattering intensities in perpendicular and parallel to the flow direction in the detector plane H–V and is given by [31]

\[
A(q) = \frac{I_V(q)}{I_H(q)} = \frac{I(qe_z)}{I(qe_x)},
\]

where \( I_V(q) \) and \( I_H(q) \) are the scattering intensities measured in the vertical and in the horizontal axes of the detector plane, in which the scattering vector is perpendicular and
parallel to the cylinder axes, respectively. The 2-D scattering pattern, measured in the radial X-ray beam position, was cross-sectioned in both detector plane primary axes in order to get one-dimensional scattering curves of \( I(\theta) \) and \( I(\phi) \). Anisotropy parameter was calculated for the maximum change in the ratio between those two scattering curves \( I(\theta)/I(\phi) \) and illustrated for reverse micelles with water content of \( W_0 = 90 \) in Fig. 11.

The anisotropy parameter indicates the degree of spatial order of the cylindrical reverse micelles in the flow direction due to the induced shear force. The anisotropy parameter increases with increasing shear rate, as shown in Fig. 11 for \( W_0 = 90 \). This suggests a better alignment of the cylinders in the flow direction at higher shear rates, and therefore correlates well with the shear-thinning phenomenon observed in the rheometry experiments. The shear force causes a reduction in the shear resistance by straightening and loosening the cylindrical micellar aggregates along the flow direction thus the micelles tend to slide over each other instead on entangling in each other [32–34]. The degree of anisotropy also increases with increasing the lysozyme concentration in the range of 1–5 g/l. This observation indicates that the aspect ratio \( (L/D) \) of the micelles increases with increasing protein concentration.

5. Conclusions

The influence of protein incorporation on the microstructure of the reverse micelles was studied by varying the water content \( W_0 \), the protein concentration, and its net charge. It was found that the presence of proteins induced changes in the micelle size distribution, leading to a minimum in the average radius vs protein concentration curve. At high water content, a shape change from spherical to cylindrical micelles was observed. The shape transformation was associated with an increased viscosity of the reverse micelle solution, and with rheological characteristics of a shear thinning liquid. Adding a protein to the reverse micelles hindered both the shape transformation and the viscosity changes by shifting the percolation to higher water contents.

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