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EFFECTS OF A NON-IONIC SURFACTANT ON SMALL UNILAMELLAR LECITHIN VESICLES

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Abstract The effects of Triton X-100 on small unilamellar lecithin vesicles has been studied by means of quasielastic light scattering, NMR, sedimentation and stopped flow techniques. At low surfactant/lipid ratios an increase in membrane permeability and a moderate growth of the vesicle is observed. Higher Triton concentrations induce the formation of large structures with a radius of up to 800 Å. Small surfactant-lipid mixed micelles are obtained by further increase of the Triton concentration. The molar ratios at which the formation of the different types of aggregates takes place was found to be highly dependent on the total concentration of detergent and lipid.

INTRODUCTION

Vesicles or liposomes are fascinating structures which have rapidly come into widespread use, not only as favourite model membranes in the life sciences but also in applications where encapsulation and protection of substances are required, mainly for drug delivery[1,2,3]. The basic physicochemical knowledge about the vesicles and their formation, stability and interaction with small molecules is, however, rather sketchy. It is not even known if a vesicle constitutes a thermodynamically stable structure[4].

Small surface active agents often have a profound effect on the vesicle membrane. Already at very low concentrations the surfactant causes the membrane to become leaky. Higher concentrations lead to disruption and dissolution of the vesicle structure and to the formation of mixed micelles. The effect is observed for bile salts and, due to their biological significance, the lecithin - bile salt systems have been thoroughly studied[5,6] and there are phase diagrams available[7]. Much less is known about interactions with other surface active agents.

The nonionic surfactant Triton X-100 is a polydisperse preparation of [p-(1,1,3,3,-tetramethyl-butyl)phenyl]poly(oxyethylene), with an average of 9.5 oxyethylene units per molecule. It is used widely to disrupt membranes and solubilize membrane components and also to open vesicles.

We have chosen Triton X-100 for a descriptive study of the interaction of a nonionic surfactant with egg-lecithin vesicles.

MATERIALS AND METHODS

MATERIALS

Egg yolk lecithin of grade 1 was purchased from Lipid Products, Nutfield, UK. Triton X-100 was bought from MERCK, Darmstadt, West Germany and 6-carboxy fluorescein from Eastman Kodak Company, Rochester N.Y., USA.

METHODS

Preparation of vesicles

The lipid vesicles were prepared by ultrasonic irradiation of samples containing about 40 mg Lecithin in 3 ml water or 0.1 M 6-carboxy fluorescein. An MSE Soniprep 150 was used for the sonication and afterwards the samples were diluted with water to obtain the final concentrations.

Leakage studies

The vesicle leakage was studied by monitoring the fluorescence increase due to the release, and subsequent dilution, of the vesicle entrapped self-quenching probe 6-carboxy fluorescein[8]. Vesicles containing 6-carboxy fluorescein (6-CF) were pre-

pared by sonication of lecithin dispersed in a 0.1 M water solution of the probe. At this concentration the fluorescence of the probe is very low due to self-quenching. To remove untrapped solutes the sample was run through a Sephadex G-50 column using water as the eluent. After additional dilution with water the vesicle preparation was filtered through a 0.22 μm Millex-GV filter and transferred to one of the driving syringes of a high-speed stopped flow apparatus[9]. The Triton X-100 solution was likewise filtered and put into the second driving syringe. The experiment was performed using a halogen lamp as the light source and during each shot the fluorescence was focused onto a photomultiplier. The increase in fluorescence, due to the mixing of vesicles and detergent, was followed by recording the signal from the photomultiplier in a transient recorder, Datalab, DL901, England. A computer was used to read and further process the signal. The averaged result from 5 consecutive shots was presented on a digital plotter.

Quasielastic Light Scattering, QELS

Triton X-100 was added to the sonicated and diluted vesicle solution whereafter the sample was left to equilibrate for 24 hours. All samples were filtered through a 0.22 μm Millex-GV filter. The light scattering experiments were performed at 25°C. Incident irradiation of 488 nm was from a Coherent Super Graphite 4-W Ar ion laser, equipped with a quarts-etalon frequency stabilizer. 10 mm precision-bore

NMR tubes (Wilmad Glass Co., N.J., U.S.A.) were used as scattering cells. The detector system comprised an ITT FW 130 photomultiplier, the output of which was digitized by a Nuclear Enterprises amplifier/discriminator system. A Langley-Ford 128 channel autocorrelator was used to generate the full autocorrelation function of the scattered intensity.

The time correlation function was analyzed by the method of cumulants[10]. Thus $\ln | g^{(2)}(\tau) - 1 |$ versus τ was fitted with appropriate weighting in a linear regression program to a second order equation. The first coefficient is $-2\bar{\Gamma}_1$ and the second μ_2, where $\bar{\Gamma}_1$ is the average decay rate ($=\bar{D}\, q^2$ where q is the scattering vector $\frac{4\pi n}{\lambda} \sin \frac{\Theta}{2}$ and $\bar{D}$ the diffusion coefficient) and its variance is $\mu_2 / \bar{\Gamma}^2$.

Measurements were initially made at a series of angles in the range 30° to 120° and (Γ_1/q^2) found to be independent of q indicating a diffusive process. Subsequent measurements were made at $\Theta = 90^{\circ}$.

The value of $(\mu_2 / \bar{\Gamma}^2)$, is freqently used as a measure of the polydispersity of the scattering particles. In the present case this parameter was small (~0.1) and the correlation function close to a single exponential.

Mean values of the hydrodynamic radius, $\bar{R}_H$, were evaluated using the Stokes-Einstein equation:

$$R_H = k_B T / 6\pi\eta_0 \bar{D}$$

where η_o is the solvent viscosity, k_B is Boltzmann's constant and T absolute temperature.

For a more detailed description of the data collection and analysis see [11].

Nuclear Magnetic Resonance, NMR

The samples were prepared and treated in the same way as described for the QELS experiments the ^1H NMR self-diffusion measurements were performed on a Varian XL 300 spectrometer equipped with a custom-built 5 mm field gradient probe, using internal D_2O lock for field/frequency stabilization. The measurement procedures have recently been described in detail [12]. The interval, τ, between the 90-180° r.f. pulses was kept constant at 140 ms and the length of the time, δ, during which the nonspinning sample was subjected to a linearly inhomogeneous magnetic field, ranging from 20 to 90 ms. The interval between the magnetic field gradient pulses, Δ, was kept constant equal to τ, throughout. All diffusion measurements were performed at 25± 0.2°C.

The observed self-diffusion coefficients are population weighted time-averages of intrinsic self-diffusion coefficients in different molecular environments over a time span of the order of 100 ms. In the present case the intrinsic diffusion coefficients are those of Triton X-100 or lecithin in bound (aggregate associated) and free states, respectively. If the surfactant aggregates can be characterized with a single self-diffusion coefficient, and there is a rapid exchange between

bound and free molecular sites on the timescale of the experimental method, the observed self-diffusion coefficient is given by a two-site model [13];

$$D_{app} = pD_{agg} + (1-p)D_{free}$$

where D_{app} is the observed self-diffusion coefficient, D_{agg} and D_{free} the self-diffusion coefficients in the bound and free state, respectively, and p the mean fraction of associated moieties. For surfactant compositions where small surfactant aggregates coexist with larger assemblies, the two-site model must be extended to give account for different D_{agg} values, i.e. a three-site model has to be used. In surfactant mixtures of lecithin and Triton X-100 the concentration of free lecithin molecules is negligible, and therefore the observed self-diffusion coefficient of lecithin is equal to D_{agg}. Thus, if the two-site model is applicable, the concentration of free Triton X-100 (C_{tot}*p) molecules can be directly determined from the self-diffusion measurements. When measuring the free surfactant concentration of pure Triton X-100 the diffusion coefficient of the micellar entity was obtained by monitoring the diffusion of a solubilized probe molecule, hexamethyldisiloxane (HMDS) which is assumed to have a negligible solubility in water.

Sedimentation

All samples were prepared and filtered as described

in the Light Scattering section. The sedimentation experiments were performed in an MSE analytical ultracentrigue, Centriscan 75. The cells, having 10 mm thick epoxy resin centerpieces, were positioned in an MSE titanium rotor. A schlieren system of the photoelectric scanning type was used to record the position of the sedimenting boundary. The speed of the rotor was 800 rps in all experiments and the temperature was kept at 25°C. The position $\bar{x}$ (distance from the centre of rotation) of the boundary between solution and solvent was recorded as a function of time, t. The sedimentation coefficient, S, is defined by

$$S = \frac{d\bar{x}/dt}{\omega^2 \bar{x}}$$

Where ω^2 is the angular velocity.

RESULTS

LEAKAGE STUDIES

Triton x-100 increases the membrane permeability and thereby enhances the release of trapped fluorescent marker. Two different courses can be distinguished dependent on the detergent/lipid molar ratio. As shown in figure 1A, at low detergent concentrations the release is slow and can be followed as a linear increase of inten-

sity with time. The time required for release of all probes depends strongly upon the amount of Triton added. At sufficiently low detergent concentration the release proceeds for hours whereas at a higher molar ratio the release may take place within a few seconds. Figure 1B gives an example of a different type of probe release which sets in above a certain molar ratio, specific for the lipid concentration used.

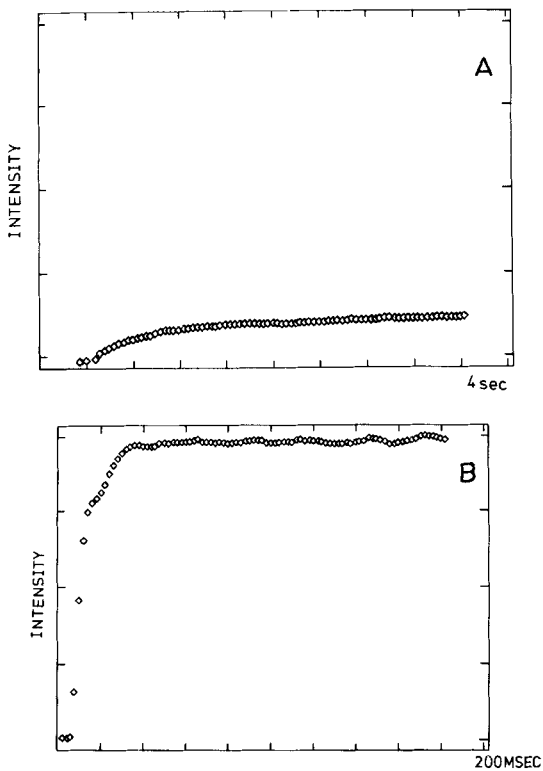

FIGURE 1.A,B. Fluorescence intensity (in arbitrary units) versus time after addition of Triton X-100 to sonicated vesicles containing 6-carboxy fluorescein.

Although still dependent on the amount of Triton added, see fig.2, the release takes place on a different timescale. Within 500 ms or less a constant level of fluorescence intensity, corresponding to a total release of vesicle content, was reached.

Figure 1A shows the increase in intensity for a sample with a Triton concentration of 19 mol% whereas figure 1B shows the increase in a sample containing 43 mol% Triton. In both cases the lecithin concentration is 1.2 mM.

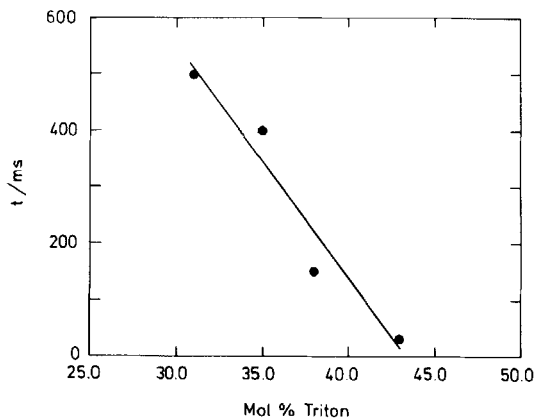

FIGURE 2. Time before total leakage is obtained versus Triton X-100 concentration.

QELS

Fig.3A shows the average hydrodynamic radius obtained after addition of different amounts of detergent to a vesicle preparation containing 1.2 mM lecithin. The particle size in-

creases slightly with increasing Triton concentration, until a critical surfactant/lipid molar ratio is reached. After this point the curve rises steeply, passes through a maximum and thereafter falls rapidly. The radius of 112 Å, at 0 mol% Triton, corresponds to the vesicle size after sonication. The maximum radius of 833 Å was obtained at 56 mol% surfactant and at 72 mol% addition the radius was found to be 54 Å. QELS was also used to determine the particle size in a pure Triton solution, with a concentration above the c.m.c.. Here the hydrodynamic radius was estimated as 38 Å. Figure 3A also shows the change in aggregate size as a function of mol% Triton when the lipid concentration is decreased to 0.32 mM. The most striking

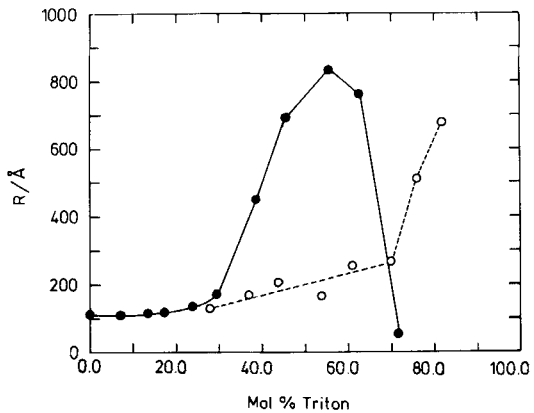

FIGURE 3A. Hydrodynamic radius of lecithin-Triton aggregates as a function of Triton concentration for samples containing 1.2 mM (●) and 0.32 mM (○) lecithin respectively.

effect is that a considerably higher detergent/lipid molar ratio is needed to produce the large structures. For a sample containing 0.32 mM lecithin, 76 mol% Triton is required to attain the radius achieved with 45 mol% Triton with a lipid concentration of 1.2 mM.

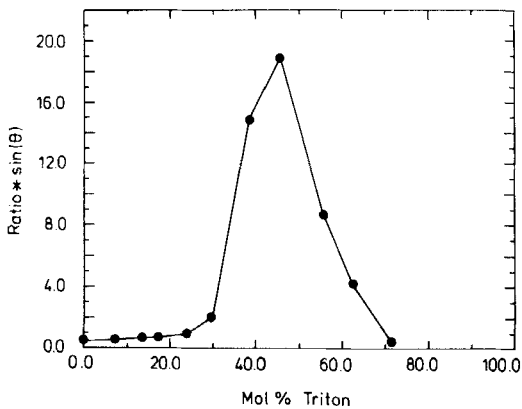

FIGURE 3B. Light scattering intensity (corrected for scattering volume) versus Triton concentration for samples containing 1.2 mM lecithin.

Figure 3B shows the light scattering intensity of the samples having 1.2 mM lecithin content. The values peak at about 46 mol% Triton, considerably lower than the concentration for the minimum in QELS diffusion coefficient.

Two stock solutions containing sonicated lecithin and Triton X-100, were diluted to make two series of solutions with the same detergent/lipid ratio but different total concentrations.

In both stock solutions the initial lecithin concentration was close to 1.2 mM. QELS was again used to determine the aggregate size and Figure 4 shows the radius after dilution to different total concentrations. By adequate dilution of a sample containing 73 mol% detergent, the aggregate radius changed from about 60 Å to between 600 and 700 Å, Fig. 4A. This change took place at a lecithin concentration of 0.40 mM and less. Figure 4B shows dilution of a solution consisting of lecithin and 56 mol% Triton. Here, the sample being diluted containes aggregates with a radius of around 850 Å, and dilution down to 0.20 mM lecithin does not lead to the formation of structures of significantly different size.

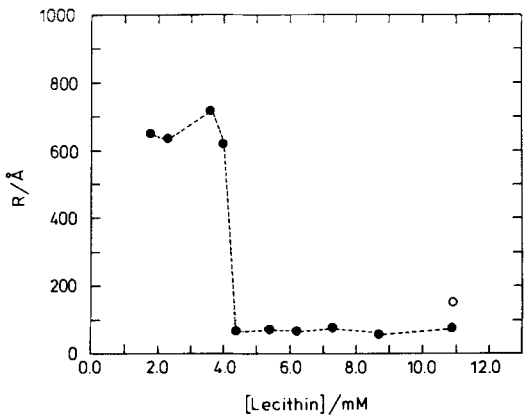

FIGURE 4A. Hydrodynamic radius after dilution of a stock solution consisting of 1.1 mM lecithin and 73 mol% Triton versus final lecithin concentration (●). (○) shows the hydrodynamic radius of the vesicles when no detergent is added.

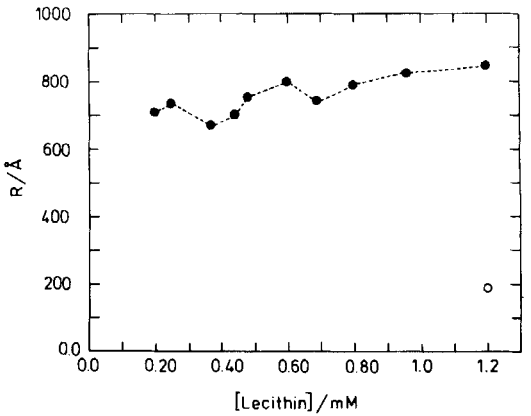

FIGURE 4B. Hydrodynamic radius after dilution of a stock solution consisting of 1.2 mM lecithin and 56 mol% Triton versus final concentration of lecithin (●). (○) shows the hydrodynamic radius of the vesicles when no detergent is added.

NMR

The NMR measurements, carried out on solutions containing 1.065 mM Lecithin and different amounts of Triton, showed that the diffusion coefficient for the lipid remains relatively constant until the detergent concentration is about 65 mol%. After this point a significant increase of the diffusion coefficient is noted. Data for Triton shows that the diffusion coefficient diminishes with increasing mol% detergent in the sample. Diffusion measurements were also carried out for a Triton solution, with a concentration above c.m.c.. The result,

together with the results from the above described experiment, can be seen in Figure 5. For comparison, the diffusion coefficients from QELS experiments are also shown. The most striking difference is that the very marked decrease in diffusion coefficient, between 30 and 62 mol% Triton, obtained in the QELS experiment can not be seen for lecithin in the NMR measurements.

The two-site model was used to estimate the concentration of free Triton from the NMR self-diffusion data. The results are shown in Table 1. The self-diffusion coefficient of Triton X-100 was determined as 24×10^{-11} m^2 s^{-1} from measurements below the cmc. For a solution with 4.2 mM Triton X-100 and trace amounts of HMDS the free surfactant concentration was obtained as 0.26 mM, close to a reported c.m.c-value of 0.24 mM[15]. The straight-forward application of the two-site model leads to free surfactant concentrations that are higher than the c.m.c. We will return to this problem in the discussion.

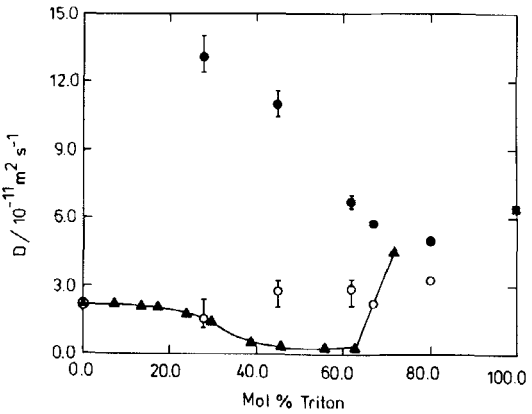

FIGURE 5. Self-diffusion coefficients of Triton (●), self-diffusion coefficients of lecithin (○) and diffusion coefficients calculated from QELS (▲) versus Triton concentration for samples containing 1.065 mM lecithin. (■) shows the experimental self-diffusion coefficient calculated for a 4.2 mM pure Triton X-100 solution. Error bars corresponds to estimated 80% confidence intervals. Absence of bars indicates that the confidence interval is less than the diagram.

Table 1

Total and free concentrations of Triton, as calculated with the two-site model from NMR self-diffusion measurements on systems with 1.065 mM lecithin.

Mol% Triton	[Triton]/mM	$[Triton]_{free}$ /mM
29	0.44	0.22
45	0.88	0.34
62	1.76	0.28
67	2.10	0.38
80	4.20	0.35
100	4.20	0.26[a]

a. Calculated from measurements on a system consisting of Triton and trace amounts of HMDS.

SEDIMENTATION

Ultracentrifuge measurements were carried out on five samples containing 6.5 mM Lecithin and different concentrations of Triton. Figure 6 shows the result. At the lowest molar ratio a peak with a calculated sedimentation coefficient of 6.0×10^{-13} s was observed. At 15 mol% Triton the sedimentation coefficient had increased to 6.5×10^{-13} s. When the amount of Triton was increased to 26 mol% the sedimentation coefficient was 38×10^{-13} s and the shape of the curve reveals

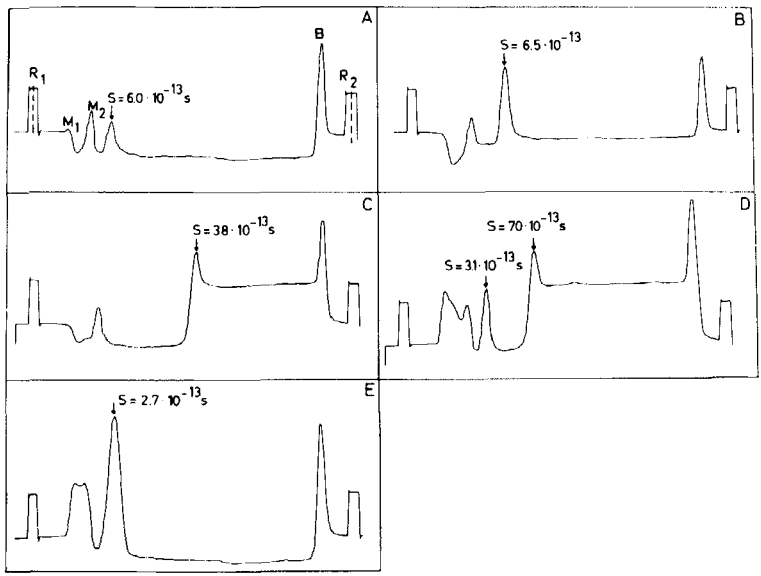

FIGURE 6. Sedimentation spectra, with dc/dr on the vertical- and radius on the horisontal axes, obtained from ultracentrifuge measurements on samples containing 6.5 mM lecithin and 8 mol% Triton, (A), 15 mol% Triton, (B), 26 mol% Triton,(C), 41 mol% Triton, (D), 61 mol% Triton, (E). The value of the sedimentation coefficient, calculated for each peak, is also shown in the figure. In (A) the reference marks R_1 and R_2, the air/liquid meniscus M_1 and M_2 for the reference and sample cells and the bottom of the cell, B, are marked. The varying appearance of M_1 and M_2 in the spectras shown is due to the different sample cells used in the experiment.

that the sample contains large, polydisperse aggregates. 41 mol% detergent gives two distinct peaks corresponding to sedimentation coefficients of 3.1×10^{-13} and 70×10^{-13} s respectively. At 61 mol% finally, only one peak giving a sedimentation coefficient of 2.7×10^{-13} s, is seen.

DISCUSSION

Most of the experiments were performed at a lipid concentration of 1.2 mM. Unless otherwise stated, the following discussion pertains to this concentration.

At the lowest concentrations of Triton X-100, up to about 30 mol%, the addition of surfactant results in an increased leakage rate, and a small change in vesicle size. At concentrations of above 30 mol% Triton, leakage becomes fast and results in complete release within 500 ms. The rate increases with the surfactant concentrations. The QELS data show that the size of the structures obtained, after 24h, starts to increase strongly just at 30 mol% Triton, and reaches a maximum at around 50-60 mol%. The disruption of the membrane thus seems to be accompanied by a fusion or flocculation of the vesicles. The NMR self-diffusion measurements, however, give results indicating a rather rapid self-diffusion of lecithin in the range where large structures should be present. Our interpretation is that we have a mixture of

large structures and small mixed micelles in this region; the sedimentation results are consistent with such a view.

Some problems arise, however, when the NMR data are interpreted through the two-site model, Table 1. For all but the first lecithin solution, the free surfactant concentration obtained is larger than the cmc. In the composition region where the results suggest coexistence between mixed micelles and larger structures the two-site model should obviously not apply, but similar values are obtained also in the region where only mixed micelles should be present. We have no simple explanation for this inconsistency.

At very high Triton X-100 concentrations only mixed micelles are present. Large structures are obtained by dilution of a stock mixed micellar solution. The size of the aggregates formed in this way is equal to the maximum size observed when surfactant is added to vesicle solutions. QELS suggests the similar large structures also when the micellar stock solution is diluted to total concentrations where small vesicles are expected from the data obtained when the region is approached from the vesicle side by addition of surfactant. No significant change in size can be observed when stock solutions of the large aggregates are diluted even to very low total concentrations. It is interesting to compare these results with the observations made by Schurtenberger et al

for the glycocholat-lecithin system[6]. They did not observe any change in the aggregate size upon dilution of a preequilibrated solution of large mixed vesicles; a result, consistent with our observations for the Triton-lecithin system, indicating that the aggregates can exist in a metastable state. They did, however, obtain small mixed glycocholat-lecithin vesicles by means of dilution of a mixed micellar stock solution. This result is contrary to ours but the difference may be due to the different concentrations of the mixed micellar stock solutions used. Whereas in both of the dilution experiments we used stock solutions of comparable concentrations (0.8 mg/ml lipid, 1-2 mg/ml Triton) Schurtenberger et al used a highly concentrated mixed micellar stock solution with a total lipid concentration of 50 mg/ml.

The exchange of lecithin molecules between ordinary vesicles is known to be slow[16] compared to the timescale of the NMR self-diffusion measurements. This fact and the observed kinetic stability of the large structures, also when they coexist with mixed micelles, suggests that the exchange of lecithin is slow also between these two structures. The NMR spin-echo signals obtained for the lecithins, in the region where large structures coexist with mixed micelles, then mainly represent only lecithin in mixed micelles, assuming that the signal from the large structures is broadened and hidden in the noise due to the shorter T_2 values[17].

With such complications in the interpretation of the NMR results it is not surprising that the straight-forward application of the two-site model gives rise to the inconsistent results in Table 1, as discussed above. Our results give a good qualitative description of the transformations that occur when a surfactant is added to a vesicle solution and also points out two questions for further investigations:

- what is the mechanism behind the increased leakage rate at low surfactant concentrations?
- what is the structure of the large aggregates and how do they transform in the region of coexistence with mixed micelles?

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