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Unique Incorporation Behavior of Amino Acid-Type Surfactant into Phospholipid Vesicle Membrane

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Abstract

The incorporation behavior of some anionic surfactants, including amino acid-type surfactants, on phospholipid vesicles was investigated. This was done by measuring the release of a vesicle-entrapped fluorescence probe and the scattered light intensities of vesicle particles in the surfactant solution as a function of surfactant concentration and time. Sodium dodecyl sulfate, sodium dodecanesulfonate, sodium dodecanoyl sarcosinate, and sodium dodecanoyl glutaminate were employed in this study. All surfactants ruptured the phospholipid vesicle at around each critical micelle concentration by mixed micelle formation with phospholipid. While leakage of the fluorescence probe took place at a very low concentration in the sulfate- or sulfonate-type surfactant systems, it occurred at the concentration just below the CMC in the amino acid-type surfactant systems. Kinetic analysis of the release of the probe from the vesicles showed that the former surfactants adsorbed independently and homogeneously onto the phospholipid vesicles, while the latter surfactants were cooperatively incorporated.

Keywords : amino acid-type surfactant • phospholipid vesicle • incorporation of surfactant • 5(6)-carboxyfluorescein • cooperative adsorption

Introduction

The interaction between phospholipid vesicle membranes and surfactants is of great interest from the standpoints of the solubilization and the reconstitution of membrane proteins [1]. In particular, the sugar-type and the oxyethylene-type nonionic surfactants have been widely used for this purpose, and thus the nonionic surfactant-induced transition from phospholipid vesicles to micelles has been well studied [2-11]. Although

studies using anionic [12-17], cationic [12,18], and zwitterionic surfactants [19] have also been published, they have received less attention. Since anionic surfactants are used as detergents, the interaction of anionic surfactants with phospholipid vesicles might be a good model for investigating the influence of detergent on skin [17]. *N*-Acyl amino acid surfactants, which are anionic amino acid-type surfactants, are very useful because of their biodegradability and low toxicity [20]. The low toxicity of amino acid-type surfactants has been confirmed by *in vivo* experiments [21,22]. In addition, there has been interest in *N*-acyl amino acid surfactants with respect to their chirality [23-29]. We clarified the chiral effect on the Krafft temperature of *N*-acyl amino acid surfactants systematically [28,29]. In spite of the interest in their low toxicity from the standpoint of their physicochemical properties, there are few studies on the interaction between amino acid-type surfactants and liposomes, except for those concerning their antiviral activity [30-32].

In this study, incorporation behavior of some surfactants, including amino acid-type surfactants, into phospholipid vesicles was investigated. This was done mainly by measuring the release of a vesicle-entrapped fluorescence probe and the scattered light intensities of vesicle particles in the surfactant solution as a function of both the surfactant concentration and the time after mixing the surfactant solution with the vesicle dispersion. In addition, the release via phospholipid membrane was analyzed kinetically. The so-called three-stage model has been used to describe the variations in vesicles brought about by addition of surfactant [33,34]. In stage I, surfactant monomers adsorb and distribute on the vesicle membrane until saturation. In stage II, the mixed vesicles and the mixed micelles coexist. Finally, all the phospholipid molecules are present as

mixed micelles in stage III. This work focuses on the first stage of this model. In addition, the difference between the incorporation behavior of the amino acid-type surfactants and that of the sulfate-type surfactants is discussed from the viewpoint of cooperative adsorption, in order to clarify the specific character of the amino acid-type surfactants.

Experimental

Materials

Sodium dodecyl sulfate (SDS), sodium dodecanesulfonate (SDSfo) and sodium *N*-dodecanoyl sarcosinate (SDSar) were purchased from Tokyo Kasei Kogyo Co. Ltd. *N*-Dodecanoyl glutamic acid was provided by AJINOMOTO Co., Inc. Sodium *N*-dodecanoyl glutamate (SDG) was prepared from dodecanoyl glutamic acid by addition of an equivalent quantity of sodium hydroxide. All the above substances were purified by recrystallization from ethanol solution. Owing to the high Krafft temperature of SDSfo, some of the supersaturated aqueous solutions had to be used at 25 °C. L-α-Dipalmitoyl- phosphatidylcholine (DPPC) and 5(6)-carboxyfluorescein (CF) were purchased from Wako pure chemical industries Ltd. and Kanto Chemical Co. Inc., respectively, and were used as received. In this experiment, CF was used as a mono-sodium salt. For all vesicle solution preparations, 0.1 M tris(hydroxymethyl)aminomethane (tris)-HCl buffer at pH = 7.4 was employed. The CMC values were determined by measuring the surface tensions of their aqueous solutions in 0.1 M tris-HCl buffer at 25 °C, and 1.1 mM, 5.8 mM, 3.5 mM, and 14.0 mM, for SDS, SDSfo, SDSar, and SDG, respectively.

Preparation of vesicles

Vesicle solutions were prepared by hydration of a dry DPPC film (20 mg) with 2 ml of tris-HCl buffer with CF (0.1 M) or fluorescence probe-free buffer. The CF concentration was high enough for self-quenching. Solutions were stirred at 50 ºC, which is above the gel-liquid crystal transition point of DPPC vesicles (43.9 °C). In addition, five freeze-thaw cycles were carried out at –196 and 50 °C. Finally, the vesicle dispersions were extruded into 100 nm polycarbonate filter on an extruder (Avanti Polar Lipids Mini-Extruder) at 50 ºC [35]. The CF encapsulating vesicles were separated from untrapped CF molecules by gel filtration. The concentration of DPPC was constant for all measurements (C_{Lipid} = 0.14 mM).

Fluorescence measurement

Aliquots (10 μl) of vesicle suspension were added to 1 ml portions of several surfactant solutions. Fluorescence measurements were performed using a Hitachi F-3010 fluorescence spectrometer at 25 ºC. The fluorescence intensity of leaked CF was measured at 515 nm by excitation at 490 nm. Excitation and emission band-passes were 1.5 nm. At the end of the measurements, 100 μl of Triton X-100 was added to all fractions in order to rupture all of the vesicles, and the fluorescence intensity was measured again.

Scattered light measurement

Mixtures of 10 μl of CF-free vesicle dispersion and 1 ml of several surfactant solutions were used for the scattered light measurements. The scattering intensity was measured at 500 nm and 25 ºC. The excitation and emission band-passes were 3 nm.

Distribution of particle size measurement

The size distribution of the vesicle dispersion was measured by dynamic light scattering using a Malvern instruments HPP-5001 at 25 ºC. Samples were filtered through a membrane filter with 200 nm pores prior to processing.

Results and Discussion

The scattered light intensity from vesicle dispersion is shown as a function of the reduced concentration, where the concentration was converted using each critical micelle concentration ($C^* = C_S$ / CMC : C_S is the surfactant concentration), at several time points in Fig. 1, for the four surfactant systems. It is considered that the intensity reflects the number and/or the size of DPPC vesicles in solution. Since no distinct change in the intensity was detected below $C^* = 0.1$ for any of the systems, the vesicle particles were not destroyed by these surfactants in the very low concentration range. For SDS and SDSfo systems, the intensity decreased with increasing concentration and time at around $C^* = 0.5$. This result shows that these surfactant molecules ruptured the DPPC vesicles by the mixed micelle formation, and SDS ruptured vesicles much more readily than SDSfo. It is seen, furthermore, that the intensities in the SDS system show a small maximum under the CMC. This might imply the existence of intermediate structures during the vesicle to micelle transition such as mixed micelles in the vesicle membrane [11,14,16,17]. Similarly, the vesicle particles were destroyed at the CMC for the SDSar and SDG systems. Another important point for the SDG system is that the intensity increases gradually with time at just below the CMC. This result suggests

that the adsorbed SDG molecules in the monomer state assist the fusion or coagulation of the DPPC vesicles. The rate of rupture of vesicles was slower for SDSar than for SDS and SDG molecules.

Therefore, the vesicle size distribution in surfactant solutions was examined. Figure 2 indicates the results obtained for several concentrations of surfactants at one hour (I) and 24 hours (II) after mixing the DPPC vesicle dispersion with each surfactant solution. For the SDS system, although the peaks near 100 nm were not altered at one hour after mixing, they became sharper and shifted a little to a smaller size after 24 hours, compared with the surfactant-free system. These results suggest that vesicle particles in these surfactant solutions are not able to aggregate together because of the electrostatic repulsion caused by the increased surface charge of vesicles due to the adsorbed surfactant. There are no distinct differences between the distributions of the monomer and micellar solutions. Furthermore, a tiny peak appeared at about 5 nm in the system with the highest concentration $C^* = 2$ after 24 hours for the SDS system. This clearly shows that lipid-surfactant mixed micelles coexisted with the vesicles. The results for the SDSfo system were similar to those of the SDS system except for the absence of the mixed micelle peaks in the micellar solution. On the other hand, the size distribution of the SDSar system hardly changed with concentration and time. Unfortunately, the existence of mixed micelles was not confirmed by these measurements. In contrast to other systems, in the SDG system the behavior of the particle size distribution was strongly dependent on concentration and time. Two new peaks appeared at around 5 nm and 300 nm in the micellar solution ($C^* = 2$), even after one hour. These peaks may reflect the appearance of mixed micelles and aggregation of vesicles, respectively. Furthermore,

after 24 hours, larger particles were produced in the monomer solution (C^* = 0.4) as well as the micellar solution. This result strongly supports the above suggestion from the scattered light measurements that the DPPC vesicles may be gathered together by the adsorbed SDG molecules. It is suggested that the rupture and the aggregation of DPPC vesicles occurs simultaneously in the SDG micellar solution.

Next, the values of the leakage ratio of CF molecules, which were estimated from the fluorescence intensity of the samples, were plotted against C^* at several time points as shown in Fig. 3. The leakage ratio L was defined by

$$L\ (\%)\ = \frac{I - I_{\mathrm{bg}}}{I_{\mathrm{triton}} \times r - I_{\mathrm{bg}}} \times 100 \qquad [1]$$

where I and I_{triton} are the fluorescence intensities of the samples during the measurements, and at the end of the measurements when all vesicles were completely ruptured by Triton X-100, respectively, and r is the dilution factor due to the addition of Triton X-100; in this case the r value was 1.1. The background intensity I_{bg} was estimated by

$$I_{\mathrm{bg}} = I_{\mathrm{triton}} \times \frac{I_{\mathrm{buffer}}}{I_{\mathrm{triton+buffer}}} \qquad [2]$$

where I_{buffer} is the fluorescence intensity of the surfactant-free sample and $I_{\mathrm{triton+buffer}}$ is that of the mixture of the sample and Triton X-100. It is seen for both the SDS and SDSfo systems that the leakage ratio increases even at very low concentrations below C^* = 0.1, and is almost constant near the CMC. This concentration was identical to the concentration at which the scattered intensity started to decrease, as shown in Fig. 1. Therefore, it was considered that the mixed micelles between DPPC and SDS or SDSfo were formed above this concentration, though unfortunately the

corresponding peaks were not obtained in the size distribution measurement. Below $C^* = 0.2$, it seems that the SDS or SDSfo molecules adsorb onto the surfaces of vesicles, and the adsorbed surfactants bring about slow leakage of CF from the inner phase of the vesicles. Furthermore, both leakage ratios increased with increasing time over the whole experimental range. From the comparisons of both the fluorescence and scattering experiments between SDS and SDSfo systems, it is concluded that the SDS molecules interact with DPPC vesicles more strongly than SDSfo molecules do, although both systems interact in a similar manner.

The leakage ratio of the SDSar system was saturated at about $C^* = 1$, and this concentration corresponded to the point at which the scattered intensity started to decrease. Above this concentration, mixed micelles of DPPC and SDSar molecules might be formed, as well as the cases of the SDS and SDSfo systems. However, these results differed from those of the SDS and SDSfo systems in the following two points: first, the leakage ratio was very small below $C^* = 0.1$, and second, it was relatively large around the CMC even at the moment of mixing, and almost saturated after three hours. These results are clearly shown in Fig. 4, where the leakage ratios at $C^* = 0.1$ and at $C^* = 1$ are plotted against time for both the SDS and the SDSar systems. It is presumed from this result that the SDS molecules incorporate homogenously on the vesicle membranes, resulting in the slow leakage of CF, while the SDSar molecules adsorb cooperatively and result in rapid leakage. The leaking behavior of CF was more remarkable for the SDG system than the SDSar system from the standpoint of the cooperative adsorption on the vesicle surface. The values of the leakage ratio were not changed by variations in either the concentration or

the time at lower concentrations below C^* = 0.1. This result clearly shows that the SDG molecules hardly affect the vesicles in the monomer state. Just below the CMC, however, the leakage ratio increased steeply. It should be noted that the collapse of vesicles was observed at just above the CMC, as shown in Fig. 1. This suggests that SDG molecules also bring about rapid leakage of CF from the DPPC vesicles, similarly to SDSar. It has been verified that the sudden fall in the leakage ratio above the CMC was due to the quenching of CF fluorescence by solubilization into SDG micelles.

Thus, the time dependence of the CF leakage was strongly affected by the type of polar head group on the anionic surfactant. In order to investigate the interaction between DPPC vesicles and the surfactants in detail, we considered the kinetics of the release of CF from vesicles according to the model of Nagawa and Regan [5]. As mentioned above, our experimental results confirm that most of the release of CF from the vesicle is due to leakage, not rupture. It was then assumed that the leakage rate would be proportional to the nth power of the surface concentration of occupied sites and the gradient of CF concentration between the inner phase of vesicles and the bulk solution. It seems reasonable to assume that the former is proportional to the surfactant-lipid concentration ratio X_S $(= C_S / C_{Lipid})$ and the latter is connected with the CF retention ratio of vesicles R $(= 1 - L/100)$. Therefore, the kinetics of the release of CF would be expected to obey the following rate law.

$$-dR/dt = kRX_S^n \qquad [3]$$

Here k is the intrinsic rate constant which includes the term for the affinity of the surfactant to the vesicles. If the release of CF follows pseudo-first-order kinetics in terms of R, the integrated rate equation is

$$\ln R = -k_{\mathrm{exp}} t \qquad [4]$$

where

$$k_{\mathrm{exp}} = kX_{\mathrm{S}}^{n}, \text{ or } \ln k_{\mathrm{exp}} = n \ln X_{\mathrm{S}} + \ln k . \qquad [5]$$

Applying eq. 4 to the experimental results under 15 minutes, the value of k_{exp} was obtained as a function of X_{S} for each surfactant system. These results are shown by logarithmic plots in Fig. 5. It is seen that the values of $\ln k_{\mathrm{exp}}$ increase with increasing surfactant concentration and are then constant above a certain concentration of surfactant for all systems. These points almost correspond to the CMCs. From this fact, it was confirmed that the rate of CF release was barely affected by the rupture of vesicles, owing to the mixed micelle formation. Furthermore, taking eq. 5 into account, the values of n and k were obtained from the slope and intercept of the fitted line in the lower concentration range, and are summarized in Table 1. The order of the reaction n is representative of the type of adsorption of the surfactant on the vesicle surface. Since the n values of both the SDS and SDSfo systems were less than unity, it was confirmed that these surfactants adsorbed independently and homogeneously on DPPC vesicles. On the other hand, cooperative adsorption on vesicles must have occurred for amino acid-type surfactant systems whose n values are greater than two. These results support our depiction, in which the amino acid-type surfactant molecules are organized on the DPPC vesicle membrane. The value of k seems to relate to an interaction between the surfactant and DPPC. Thus, the amino acid-type surfactants, which have smaller values of k, may act more weakly on lipid membranes than SDS or SDSfo. These results suggest that the amino acid-type surfactants adsorb cooperatively on the vesicle surface, and as a result, the surfactant

molecules organize to make a kind of channel which is large enough for the CF molecules to pass through easily. The CF molecules might pass through the leakage channel as soon as it is constructed. From these facts, it is possible to conclude that most of the release of CF from the vesicles was due to leakage via the channels, and that the contribution by vesicle rupture was relatively small.

Figure 6 was drawn to summarize the vesicle behaviors triggered by addition of the surfactants against the concentration of the surfactants. In the case of the SDS system, the surfactant molecules were adsorbed homogeneously even at very low concentrations below $C^* = 0.1$, and then they ruptured the vesicle at around the CMC. The manner for the interaction of SDSfo with DPPC vesicles resembles that of the SDS system, however the interaction is weaker than that for SDS. For the SDSar system, although rupture of the vesicles was also observed at around the CMC, similarly to in the SDS and SDSfo systems, the concentration at which mixed micelle formation took place was a little higher than in those systems. Adsorption of SDSar molecules onto vesicles occurred cooperatively. As a consequence, the release of CF took place rapidly compared to both the SDS and the SDSfo systems. The SDG molecules hardly acted on the DPPC vesicles in monomer solution with a low concentration, and they adsorbed onto the vesicle surface drastically just below the CMC. Furthermore, the destruction of DPPC vesicles only occurred above the CMC of SDG. Consequently, the amino acid-type surfactants might form leakage channels on the vesicle surface.

In summary, the disparity in the effect on the vesicle membrane between the sulfate- or sulfonate-type surfactants and the amino acid-type surfactants is caused by the different forms of adsorption, i.e., SDS and

SDSfo adsorbed uniformly on the vesicles even at very low concentrations, while the amino acid-type surfactants localized on part of the vesicle membranes at concentrations just below the CMC.

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Figure Captions

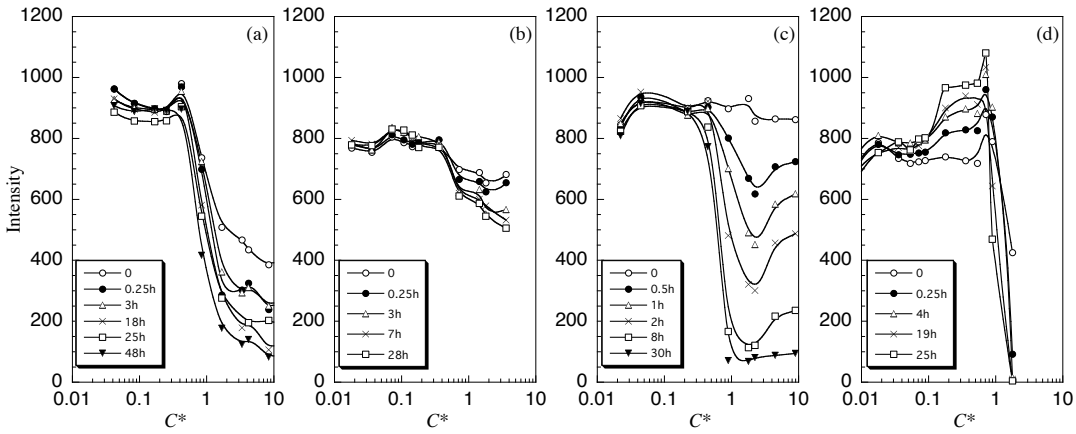

Fig. 1 Scattering intensity of DPPC vesicles in each surfactant solution as a function of the reduced surfactant concentration at various times after mixing. (a) SDS, (b) SDSfo, (c) SDSar, (d) SDG.

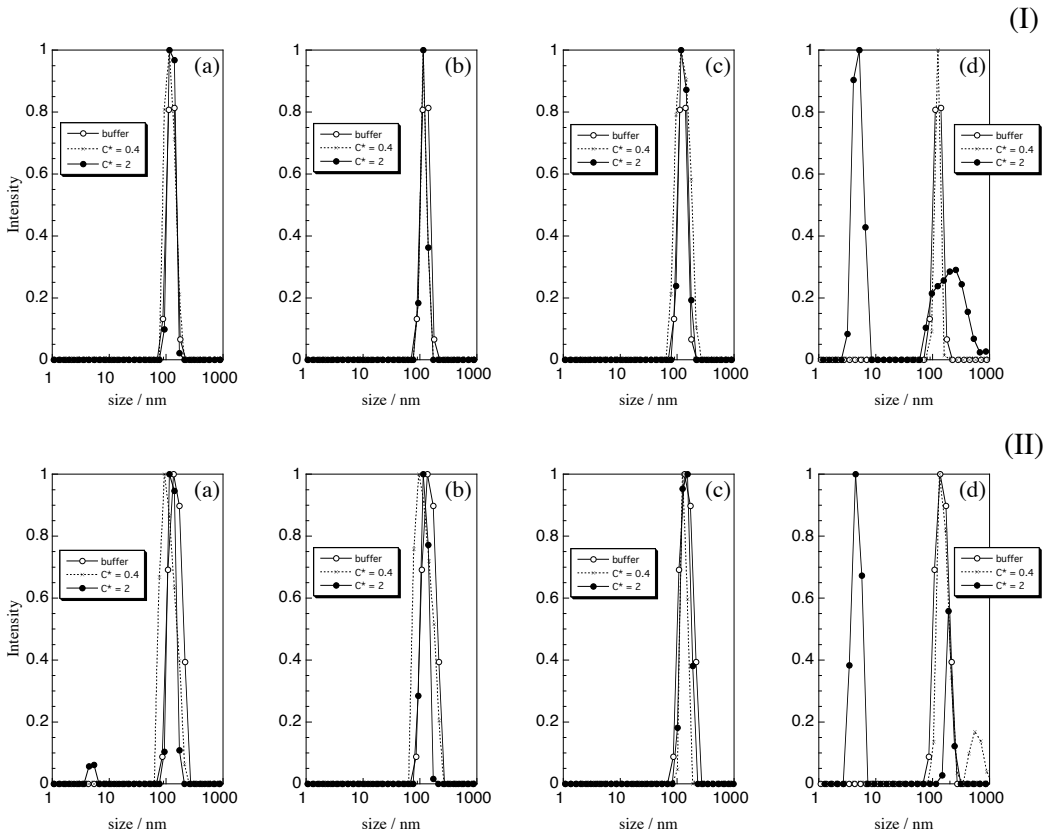

Fig. 2 Size distribution of DPPC vesicles in each surfactant solution. (I) after 1 hour, (II) after 24 hours; (a) SDS, (b) SDSfo, (c) SDSar, (d) SDG.

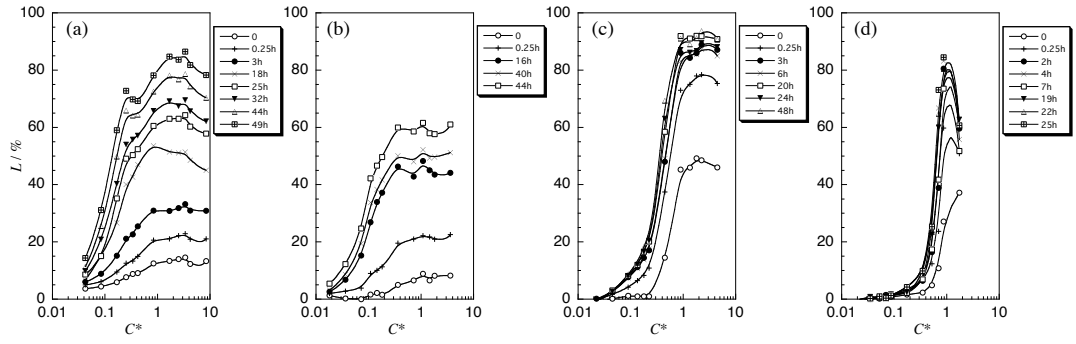

Fig. 3 Leakage ratio of CF from DPPC vesicles vs reduced surfactant concentration curves in each surfactant solution at various times after mixing. (a) SDS, (b) SDSfo, (c) SDSar, (d) SDG.

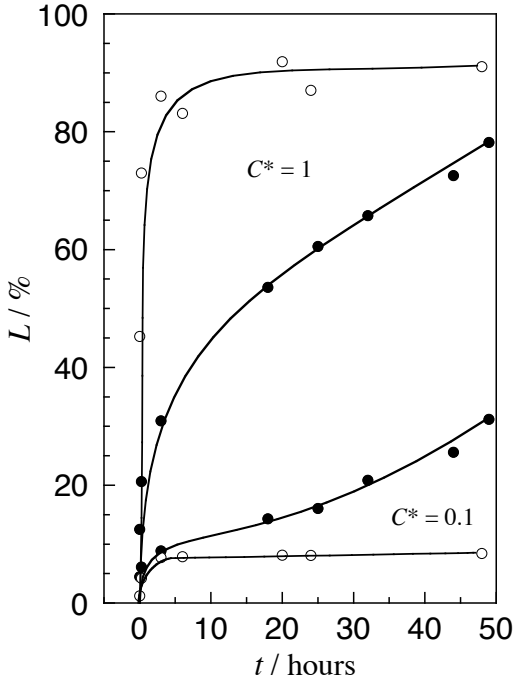

Fig. 4 Leakage ratio of CF vs time for each surfactant solution at C^* = 0.1 and at C^* = 1. Full circles, SDS; open circles, SDSar.

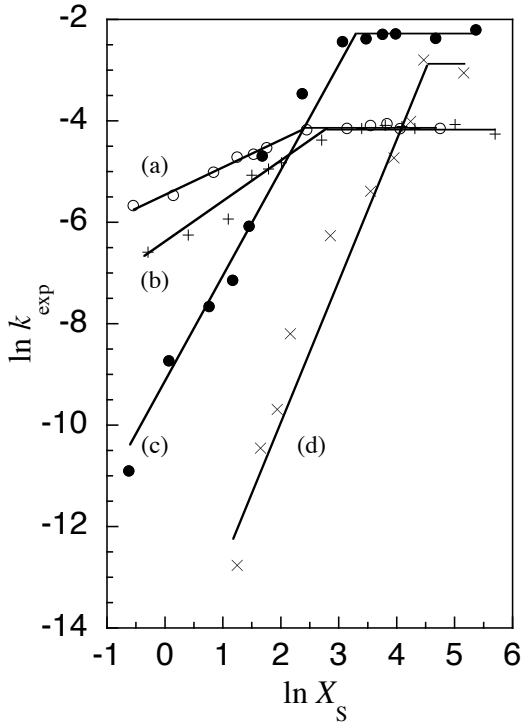

Fig. 5 ln k_{exp} vs ln X_S plots for each surfactant solution. (a) SDS, (b) SDSfo, (c) SDSar, (d) SDG.

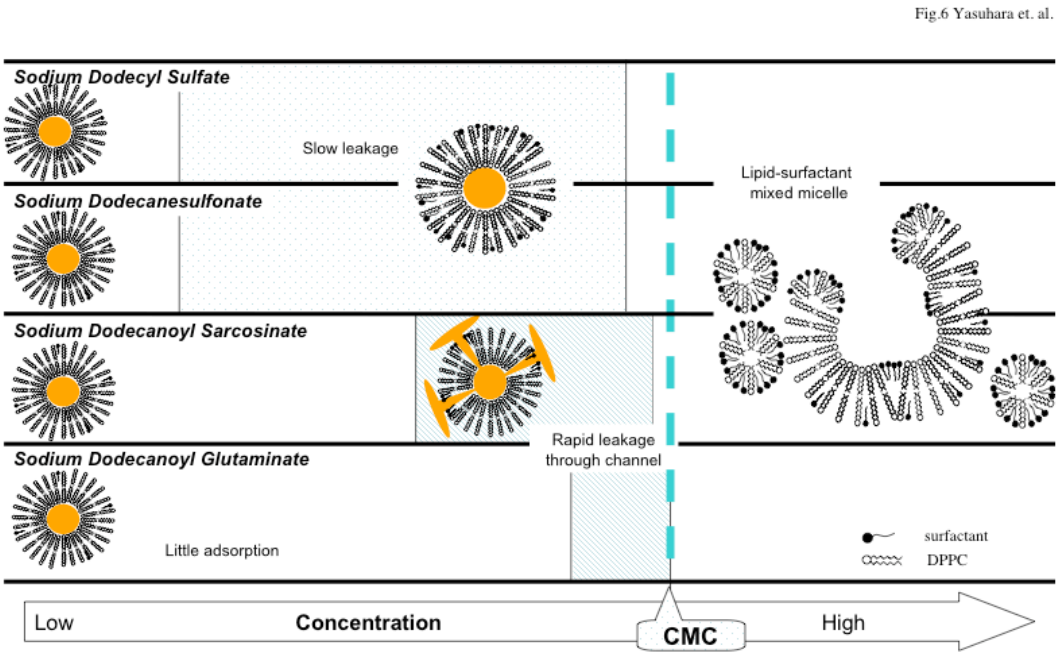

Fig. 6 Model for the action of each surfactant on DPPC vesicles.

Table 1 Calculated kinetic parameters.

surfactant	k / min^{-1}	n
SDS	4.3×10^{-3}	0.5
SDSfo	1.5×10^{-3}	0.8
SDSar	9.2×10^{-5}	2.3
SDG	3.1×10^{-7}	2.7