Surfactant-induced membrane dynamics and skin irritation

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Abstract

Background: We have been studying clarification of the correlation between the irritation of surfactants and the membrane dynamics of cell-sized liposomes. However, regarding the surfactant-induced membrane dynamics, the characteristics of each contributing surfactant and the specific mechanism at the molecular level have not yet been clarified, and an alternative method for the Draize test has not been developed.

Method: Cell-sized liposomes composed of unsaturated phospholipids (1,2-dioleoyl -sn-glycero-3-phosphocholine) were prepared, and the membrane dynamics of liposomes after the addition of various surfactants was observed using laser confocal microscopy and types of morphological changes of liposomes were classified.

Results and Discussion: Eight different types of morphological changes could be classified dependent on intensity of stimuli induced by various surfactants. An increase in excess surface area of liposomes occurred for the addition of strongly irritating surfactants (ex. Triton X-100), while they shrinkage was observed by adding low or no irritating surfactants (ex, Tween20). Shrinkage could be observed for amino acid surfactants depending on the intensity of stimulation. Therefore, we concluded that our method is applicable as an alternative method to animal test such as Draize test. In order to acquire the excess surface area, the surfactant molecules added from outside of liposome should be included in the inner leaflet of bilayers. If the surfactants in the outer leaflet moved to the inner leaflet (flip-flop), the bilayer obtained the excess surface area. Therefore, we considered that membrane dynamics depends on the rate of Vflip-flop, and Vout. We further used our method to characterize low irritating mixed systems of surfactants such as sodium cocoyl glutamate and mixture of anionic surfactant and amphoteric surfactant by comparing the result with stinging test.

Key word: Liposome, Membrane dynamics, Surfactants, Irritation, Alternative method

Introduction

Draize test is a method to evaluate the skin and eye irritation, and is that the surfactants are instilled into rabbits' eyes directly and the score (Draize score) is determined by evaluators [1]. The Draize test is the most famous method for evaluating the irritancy of a surfactant, in which the rabbit is instilled with the surfactant and the degree of irritation is visually judged and scored by a person. The subject to be evaluated was inflammation sore of the rabbit eye after addition of a surfactant, and the cornea was rated at 80 points, the iris at 10 points, and the conjunctiva at 20 points, totaling 110 points (Table 1).

Table 1: Relationship of Draize score and irritation

Draize score	Irritation	Brevity code
100～110	Maximally irritating	Mx
80～100	Extremely irritating	E
50～80	Severely irritating	S
25～50	Moderately irritating	M3
15～25	Mildly irritating	M2
2.5～15	Minimally irritating	M1
0.5～2.5	Practically nonirritating	PN
0.0～0.5	Nonirritating	N

The results obtained in the Draize test are effective for the development of cosmetics and detergents, but in particular, hypoallergenic surfactants with a Draize score of 0 to 15 (non-irritating/substantially non-irritating/minimally irritating) are very difficult to subdivide because they are less likely to cause apparent inflammation in the Draize score.

The Draize test is also controversial because the test substance is administered directly to the eye of the live rabbits. Due to the functional and structural differences between human and rabbit eyes, the Draize test method is considered non-scientific and a cruel test on experimental animals. In order to solve the above problems, the Organization for Economic Cooperation and Development (OECD) adopted two methods, the bovine corneal enucleation test method and the isolated chicken eyeball test, as alternative methods to the Draize test. These alternative methods have strong correlation with the Draize test and are considered effective alternatives. However, since these methods also use animals, they are still cruel tests on experimental animals [2]. From the above points, there is a demand for the development of an alternative method for the Draize test that can obtain highly quantitative results even for low-irritating surfactants without sacrificing animals.

It is the cell membrane that initiates inflammation and sores caused by surfactants. Surfactants exert a solubilizing effect through membrane permeation, thereby damaging biological membranes [3]. Damage to the cell membrane causes calcium ions to flow into

the cells. This calcium ion binds to calmodulin and activates phospholipase A2 on the cell membrane. Phospholipase A2 esterifies the phospholipid of biological membrane to release arachidonic acid. Arachidonic acid is oxidized by cyclo- oxygenase and produces various prostaglandins that are the causative agents of pain and inflammation (Fig. 1). In particular, prostaglandin E2 and prostaglandin I2 enhance the inflammatory action by the plasma kinin through enhancement of vascular permeability. This series of pathway is called the arachidonic acid cascade, and the damage of cell membrane is the starting point of the cascade [3].

Therefore, in order to understand mechanism of irritation at molecular level and evaluate strength of irritation caused by surfactants, it will be important to study interaction between surfactants and cell membrane.

Phospholipid molecules are the main components of biological membranes and are amphipathic molecules that have both hydrophilic and hydrophobic groups. It is known that, like a surfactant, it self-assembles in a medium to form various higher-order structures. One of the typical higher-order structures of lipids is a lamella structure (bilayer structure) in which molecules self-assemble in a film shape. The characteristic size of such a higher-order structure is determined by the interaction between molecules. A structure in which such a bilayer membrane is closed is called a liposome (Fig.2). Nanometer-sized liposomes are called SUV (Small unilamellar vesicle), and micrometer-order liposomes are called LUV (Large unilamellar vesicle) or GUV (Giant unilamellar vesicle). In liposomes, lateral diffusion of lipid (lateral diffusion) and Flip (movement from outer membrane to inner membrane)-Flop (movement from inner membrane to outer membrane) are constantly performed. Since liposomes can reproduce a composition and size similar to those of biological membranes, they are used as biological cell mimetic membranes (Fig. 2) [4].

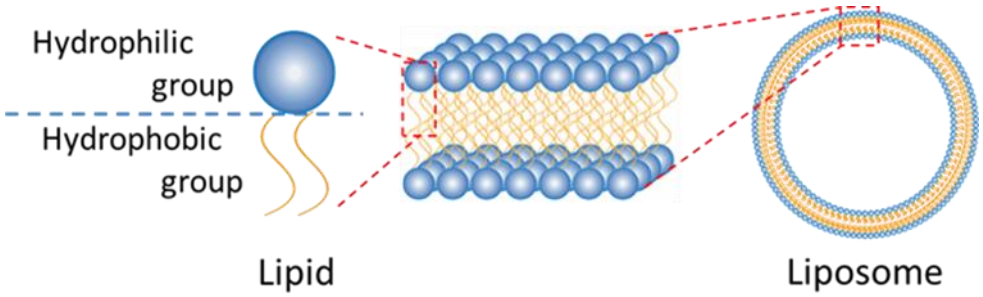


Fig. 1: Liposome

In our laboratory, we have been studying clarification of the correlation between the irritation of surfactants and the membrane dynamics of cell-sized liposomes. However, regarding the surfactant-induced membrane dynamics, the characteristics of each contributing surfactant and the specific mechanism at the molecular level have not yet been clarified, and an alternative method for the Draize test has not been developed.

In this study, we focused on low irritating surfactants in order to evaluate hypoallergenic surfactants more precisely than current alternative methods.

Materials and Methods

Chemicals

Phospholipid, 1,2-dioleoyle-*sn*-glycero-3-phosphocholine (DOPC) was purchased from Avanti Polar Lipids. Rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3- phosphoethanolamine (Rhodamine-DHPE) used as a fluorescent probe, was purchased from Invitrogen. Used surfactants are summarized in Tables. 2 and 3. TritonX-100, Tween20, and Tween80 were provided from Nacalai Tesque, Santa Cruz Biotechnology, Kanto Chemical Co., Inc., respectively. The amino-acid surfactants listed in Table 3 and amphoteric surfactant, cocamidpropyl betaine were provided from Asahi Kasei Chemicals.

Table.2 List of nonionic-surfactants

Surfactants	Figure	Draize score (5%)
TritonX-100	2	32.3
Tween20	3	0
Tween80	4	0
Baby soap	5	-

Table.3 List of anionic-surfactants

Surfactant (Trademark)	Figure	Draize score (5%)
Sodium cocoyl glutamate (ACDS-L)	6(X=Na^+)	＜15
TEA cocoyl glutamate (ACMT-L,CMT-L)	6 (X=H^+TEA)	＜15
Sodium lauroyl aspartate (FLDS-L)	7 (X=Na^+)	＜15
TEA lauroyl glutamate (LT-12)	8 (X=H^+TEA)	＜15
Sodium lauroyl glutamate (LK-11)	8 (X=Na^+)	＜15

Preparation of liposomes

Liposomes were prepared by the natural swelling method. DOPC and Rhodamin-DHPE were dissolved in chloroform/methanol (2:1,v/v), and the concentrations were 2 mM and 0.1 mM, respectively. Glucose was dissolved in methanol and the concentration was 10mM. Chloroform 10μl, DOPC 20μl, Rhodamine-DHPE 8μl, and glucose 12μl were mixed and the mixed solution was dried under vacuum for 3 hours to form thin lipid films. The films were

hydrated with 200 μl deionized water at 37 ℃ for over 3 hours. Deionized water was obtained from a Millipore Milli-Q purification system.

Microscopic observation

The prepared liposomal solution was diluted to 1/10 concentration with Milli-Q water. The liposomal solution 8μl was placed in the lower compartment, and 32 μl of surfactant solution was added to the upper compartment. The confocal laser microscope was used, and the wavelength of laser for excitation was 532nm [4].

Stinging test

Stinging means "tingling," and the test looks for a transient reaction that disappears without other inflammatory symptoms such as tingling, burning, or itching, which are usually accompanied by erythema or edema. The test was conducted by the following process: (1) Wipe off the back of the neck with a tissue soaked in purified water. (2) Apply non-woven gauze permeated with 1 mL of the sample with surgical tape. (3) Hearing was conducted every minute immediately after application. (4) Record the situation up to 10 minutes later.

Results

Membrane dynamics induced by various surfactants

We observed the membrane dynamics induced by the addition of the surfactants. Some examples of the observed membrane dynamics were shown in Figs 2 (Triton X-100) and 3 (Sodium cocoyl-glutamate).

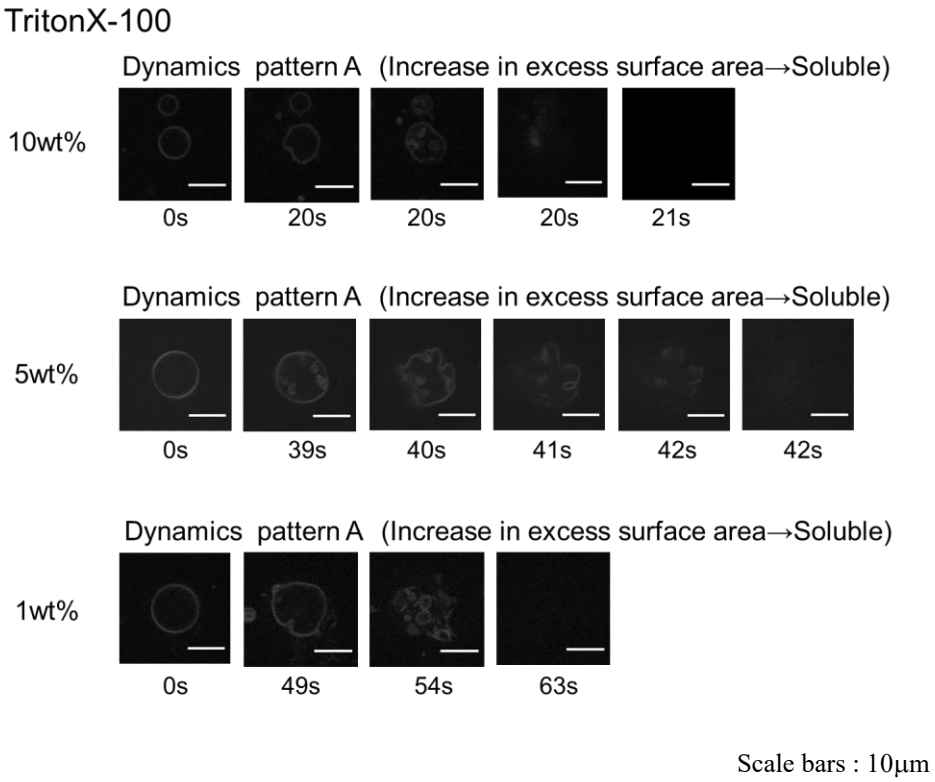


Fig. 2: Membrane dynamics induced by Triton X-100

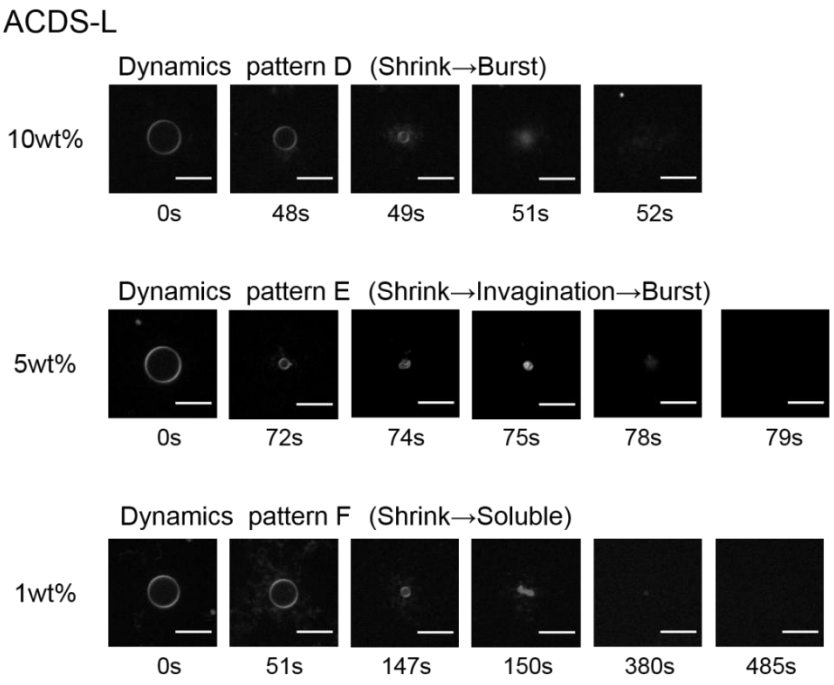


Fig. 3: Membrane dynamics induced by Sodium cocoyl glutamate (ACDS-L)

From the observed membrane dynamics, we classified eight types of membrane dynamics as shown in Fig. 4.

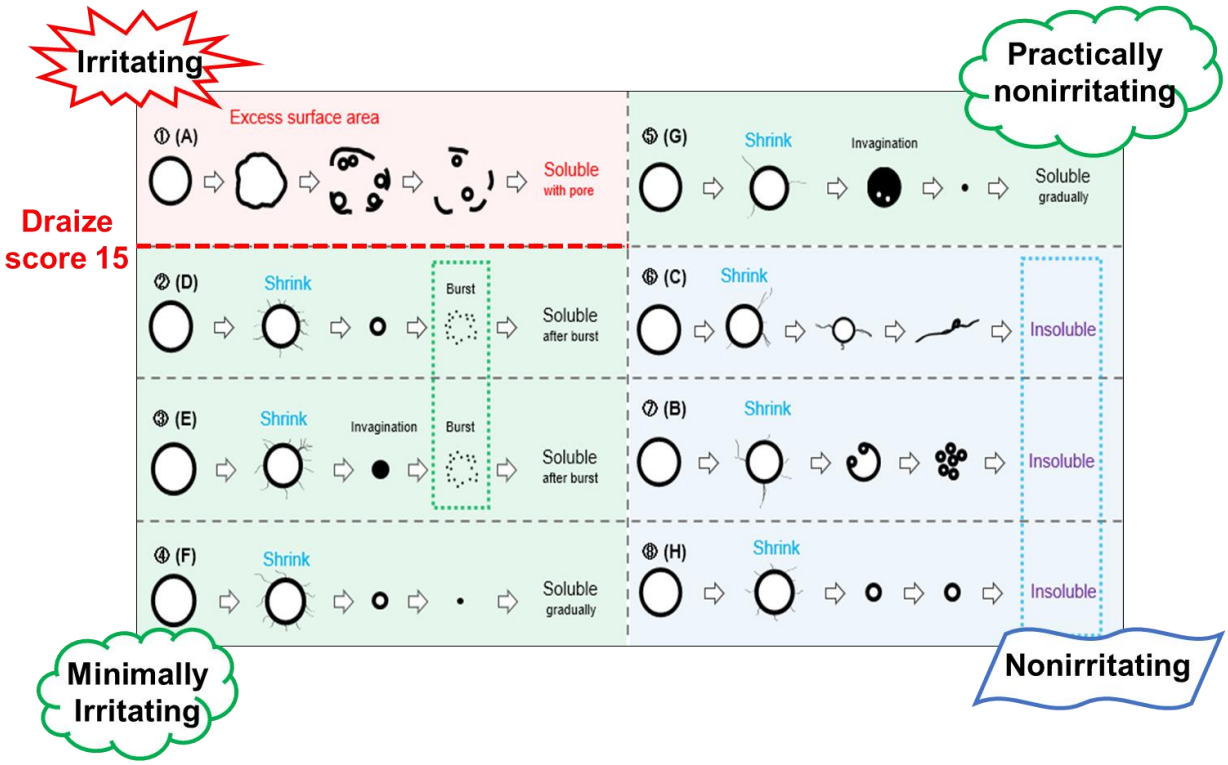


Fig. 4: Eight different membrane dynamics corresponding to strength of irritation

Comparison between sodium cocoyl-glutamate and sodium lauryl-glutamate

Coconut oil is extracted from the fruit of the coconut palm. Its origin was believed to be from Polynesia to tropical Asia, but today it is grown in tropical regions around the world. Coconut oil is composed of about 90% saturated fatty acids, most of which are "medium-chain" fatty acids mainly lauric acid (C12)(Table 4).

Table 4

Fatty acid composition of coconut oil			
Fatty acid	Types of Fatty acid	Carbon number	Ratio(%)
Oleic acid	Unsaturated	18	6.9
Linoleic acid	Unsaturated	18	0.2
Caproic acid	Saturated	6	0.4
Caprylic acid	Saturated	8	7.7
Capric acid	Saturated	10	6.2
Lauric acid	Saturated	12	47.0
Myristic acid	Saturated	14	18.0
Palmitic acid	Saturated	16	9.5
Stearic acid	Saturated	18	2.9

Sodium cocoyl-glutamate is an anionic surfactant composed of coconut oil fatty acids and glutamic acid. This biodegradable surfactant is excellently suited for sensitive and allergic skin and has an extremely low irritation potential. In order to clarify low irritation potential of sodium cocoyl-glutamate, irritation induced by sodium lauroyl-glutamate was evaluated by both membrane dynamics and human stinging test (Fig. 5).

Stinging test	Maximum Stimulus score	Stinging test	Maximum Stimulus score
Sodium Cocoyl Glutaminate (28mM)	17.0	Sodium Lauroyl Glutamate (28mM)	17.5

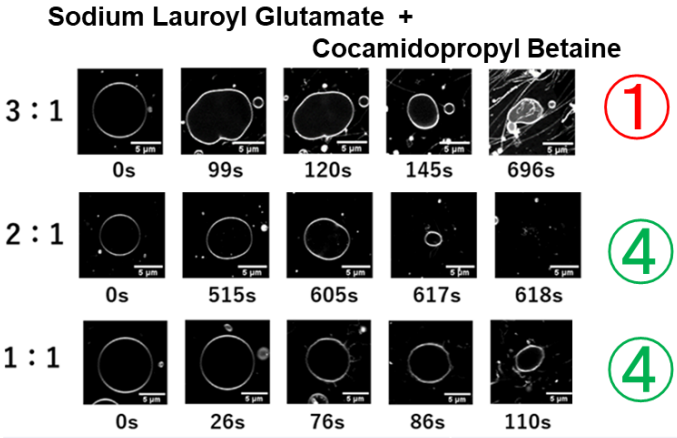
Fig. 5: Evaluation of sodium cocoyl glutamate and sodium lauroyl glutamate based on membrane dynamics and stinging test.

Results of both membrane dynamics and stinging test indicated that sodium cocoyl glutamate is less irritating than sodium lauroyl-glutamate. Therefore, it was suggested that surfactants other than C12 (lauroyl-glutamate) may be effective to reduce irritation.

Mixture of anionic surfactant and amphoteric surfactant

Cocamidopropyl betaine is amidopropyl dimethylamino acetate betaine chemically synthesized from fatty acids obtained from coconut oil. It is an amphoteric surfactant with a molecular weight of 342.5. Cocamidopropyl betaine is often used in cosmetics to increase detergency and improve foaming in combination with anionic surfactants and also to relieve irritation from strong anionic surfactants.

We tested irritation caused by mixture of sodium lauroyl glutamate and cocamidopropyl betaine at different mixing ratio, 3:1. 2:1 and 1:1 using both membrane dynamics and stinging test (Fig. 6).



Stinging test	Maximum Stimulus score
Sodium Lauroyl Glutamate +Cocamidopropyl Betaine (3:1)	11.0
Sodium Lauroyl Glutamate +Cocamidopropyl Betaine (2:1)	10.0
Sodium Lauroyl Glutamate +Cocamidopropyl Betaine (1:1)	9.0

Fig. 6: Evaluation of irritation caused by mixture of sodium lauroyl glutamate and cocamido propyl betaine.

Both membrane dynamics and stinging test indicated that amphoteric surfactant, cocamido propyl betaine can significantly reduce stimuli caused by sodium lauroyl glutamate.

Discussion

We classified the eight surfactant-induced membrane dynamics in order of the irritation. As a result, the low-irritating surfactants could be classified in order of the irritation by our newly developed method. We observed the membrane invagination and the pore formation for the highly-irritating surfactant such as TritonX-100. On the other hand, the large deformation could not be found in the case of non-irritating surfactant such as Tween20 and minimally-irritating surfactants. Importantly, the membrane dynamics of high-irritating surfactant and four relatively higher-irritating surfactants in low- and non-irritating surfactants showed the solubilization at the final state (Dynamics 2-5). On the other hand, we could not find the solubilization in the cases of three dynamics of relatively lower-irritating surfactants in low- and non-irritating surfactants (Dynamics6-8). Therefore, the solubilization process is important to evaluate the degree of irritation roughly. Additionally, two dynamics out of four membrane dynamics of relatively higher-irritating surfactants in low- and non-irritating surfactants (Dynamics 2 and 3) showed the burst process. The surfactants which showed such burst behavior might have higher irritation. Therefore, the burst can be an important process in the evaluation of the surfactant irritation. We considered that the solubilization and the burst processes will become crucial indicators to evaluate the degree of irritation of surfactants.

The deformation was determined by the acquisition of the excess surface area by addition of surfactants. In order to acquire the excess surface area, the surfactant molecules added from outside of liposome should be included in the inner leaflet of bilayers. If the surfactants in the outer leaflet moved to the inner leaflet (flip-flop), the bilayer obtained the excess surface area (Fig. 7).

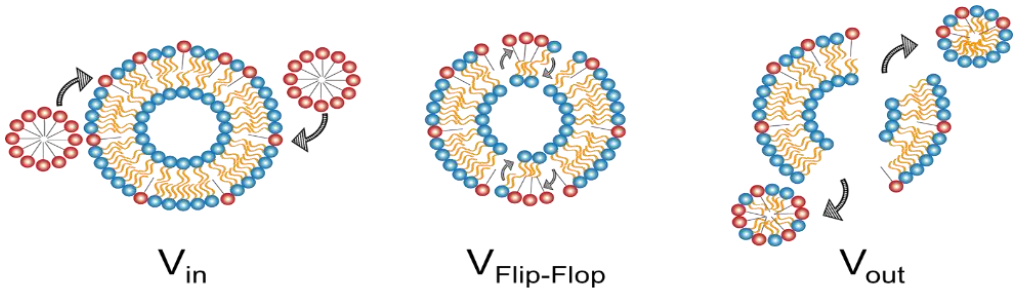


Fig. 7: Three different velocities in movement of surfactants and lipids

Therefore, since the membrane area increases as in the case of the highly stimulant surfactant, it can be said that the amount of the surfactant molecule sticking into the membrane is larger than the molecule released as a micelle in the movement of the molecule (Vout <Vin). Also, the surfactant molecule initially sticks into the outer leaflet of the lipid bilayer. If Flip-Flop does not occur, it is expected that the membrane will be distorted and the area expansion will not occur clearly. Therefore, the stabbed surfactant molecules must be evenly distributed between the inner and outer leaflets ($V_{Flip\text{-}Flop} \approx$ Vin). It can be said that the condition that causes the expansion of the liposome by integrating the two conditions is Vout $< V_{Flip\text{-}Flop} \approx$ Vin (Fig. 7).

The shrinkage of liposomes can be said to be a phenomenon in which surfactant molecules stick to the membrane, surfactant molecules and lipids form micelles, are released out into water, and the amount of surface molecules on the membrane becomes smaller. Therefore, the amount of molecules released as micelles is larger than the amount of molecules piercing the membrane (Vin <Vout). Further, it was suggested that the liposome does not shrink unless the amount of the surfactant molecule penetrating the membrane is smaller than the amount of the molecule migrating to the inner membrane due to Flip-Flop (Vin $\leqq V_{Flip\text{-}Flop}$). The condition that causes the liposome shrinkage can be understood by Vin $\leq V_{Flip\text{-}Flop}$ <Vout.

Liposome rupture is the phenomenon that when micelles are released from water into liposomes, they become unstable due to the exposure of the hydrophobic part of the lipid, making it impossible to maintain the spherical shape. Therefore, it can be said that the release of micelles is more intense than contraction (Vin <<Vout). Similarly to contraction, it can be said that the liposome does not rupture unless the amount of the surfactant molecule penetrating the membrane is smaller than that of the molecule that migrates to the inner membrane (Vin $\leqq V_{Flip\text{-}Flop}$). It can be said that the condition that causes the liposome rupture by integrating the two conditions is Vin $\leq$ VFlip-Flop << Vout.

Flip-Flop rates of surfactant-containing biomimetic membranes can be measured by using asymmetric nano-sized liposomes in which only the fluorescent dye of the outer leaflet. The ratio of the fluorescence intensity of the outer leaflet to the total was examined by fluorescence spectrophotometer and the data was fixed to obtain the Flip-Flop rate.

According to the results of membrane dynamics using mixture of surfactants (Sodium cocoyl glutamate, Sodium lauroyl glutamate and Cocamido propyl betaine), our alternative method to animal test based on membrane dynamics is consistent to the results of stinging test. Amphoteric surfactants (Cocamido propyl betaine) is effective to reduce irritation of an anionic surfactant, possibly because of ionic interaction and enlargement of apparent molecular weight.

We considered that the method is very promising as a new evaluation method.

Conclusion

【1】There are two types of membrane dynamics, inflate (Draize score higher than 15) and shrinkage (Draize score less than 15).

【2】Membrane dynamics can be characterized by 3 velocities (Vin, Vout and $V_{Flip\text{-}Flop}$).

【3】There was a good correlation between the results of the stinging test and the liposome

【4】Lauroylglutamate (main composition of cocoyl glutamate) is more irritating than sdium cocoyl glutamate, indicating that surfactants other than C12 may be effective to reduce irritation.

【5】Amphoteric surfactants is effective to reduce irritation of an anionic surfactant, possibly because of ionic interaction and enlargement of apparent molecular weight (Coacervation)

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Conflict of Interest Statement

There is no conflict of interest.

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