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Author(s)	Sugahara, Ko
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Description	Supervisor:高木昌宏 教授, マテリアルサイエンス研究科, 修士

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Japan Advanced Institute of Science and Technology

Membrane interaction and dynamics of local anesthetics

By

Ko Sugahara

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Written under the direction of

Professor Masahiro Takagi

Assistant Professor Naofumi Shimokawa

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［1.Introduction］

Local anesthetics (LAs) are essential drugs for modern medicine. Although they are often used as painkiller, the mechanisms how they suppress the pain are still unclear.

LAs are known to target voltage-dependent sodium channels which located on cellular membrane. Until recently, the mechanism how LAs shield pain signals was believed that LAs interact with the channel protein directly. However, because of the relation between hydrophobicity and strength of LA, the indirect effect on channel proteins through a change in physical properties of the cellular membrane had been suggested (Fig.1) [1].

Cell membranes which are composed of lipid bilayer structure, are known to relate with signal transductions via membrane dynamics such as vesicular formation. Moreover, "lipid raft model" was proposed by Simon and Ikonen in 1997 [2]. Lipid raft is the region where the large amount of saturated lipids and cholesterols are included and some functional molecules such as ion channels and membrane receptors can be localized, and they are speculated as a platform for signal transduction (Fig.2).

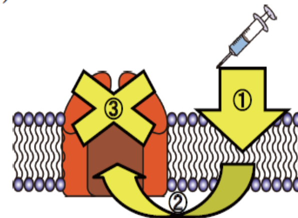
In order to reveal the mechanism of raft formation, "liposome" which is mainly composed of phospholipids is used as a model of biomembrane system (Fig.3). Liposome has similar structure and component to actual biomembranes, so we can directly observe similar phenomena on liposome with optical microscopes. Especially, raft formation can be regarded as the phase separation between saturated and cholesterol-rich and unsaturated lipid-rich phases in model membrane system.[3]

Objectives of this thesis is to observe physical property changes of liposomes that are derived from interactions between lipid membranes and LAs, and discuss relation with physiological action of LAs. First, to confirm the direct interaction between phospholipid membranes and LAs, we observe the membrane fluctuation and analyze the thermo-responses of liposomes. Next, the phase separation in membranes containing LAs is observed and we discuss how LAs affect on the domain formation. Finally, the membrane fluidity change by addition of LAs is evaluated using Laurdan which is sensitive to the membrane fluidity. Based on these experimental results, we discuss how LAs interact with lipid membranes.

(a) Direct effect to channel

① LA affects to channel conformation.
② Close and shut out flux of NA+.

(b) Indirect effect to channel



① LA changes membrane fluidity
② Fluidity change affects to channel conformation
③ Close and shut out flux of Na+

Fig.1 Different mechanism of local anesthetics (LAs) on sodium chann

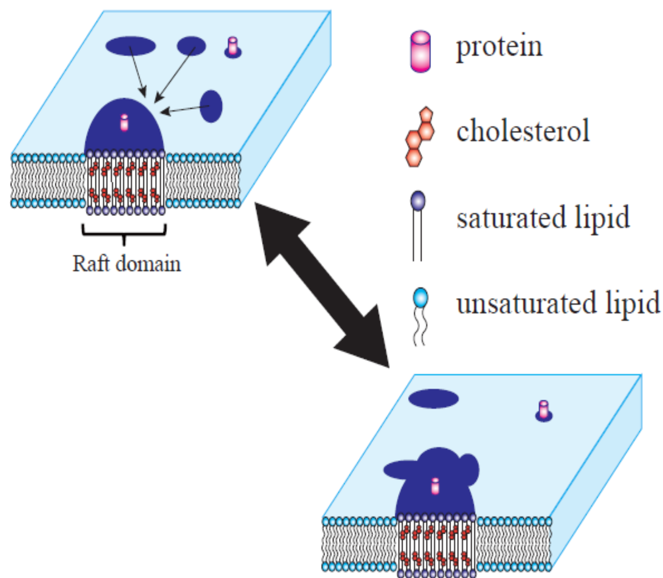


Fig.2 Schematic image of membrane raft

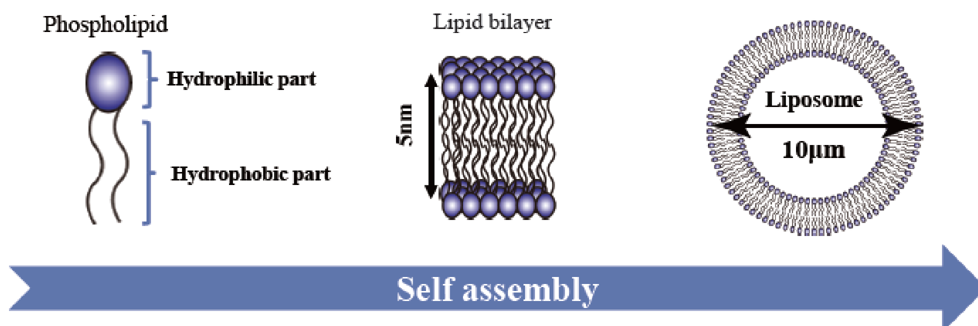


Fig.3 Schematic image of phospholipid and its assemblies

［2. Materials and Methods］

2-1. Materials

Unsaturated lipid, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), saturated lipid, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and cholesterol (Chol) were purchased from Avanti Polar Lipids. Fluorescent probes, Rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Rhod) and 6-Dodecanoyl-2-Dimethylaminonaphthalene (Laurdan) were purchased from Invitrogen and Funakoshi, respectively. Local anesthetics, lidocaine and tetracaine were purchased from Nacalai Tesque and Tokyo Chemical Industry, respectively.

2-2. Preparation of liposomes

Liposomes were prepared by natural swelling method as shown in Fig.4. Lipid mixtures (DOPC, DPPC, Chol, LAs, Rhod, Laurdan) dissolved in chloroform/methanol (2:1, v/v), and glucose dissolved in methanol in glass test tubes, and they mixed each other. These solutions were dried under vacuum for 3 h to form thin lipid films. The films were then hydrated overnight with deionized water at 37 ^{o}C to produce unilamellar liposomes. The final concentrations were 0.2mM lipids 0.6mM glucose, 0.01mM Rhod, and 0.5μM Laurdan.

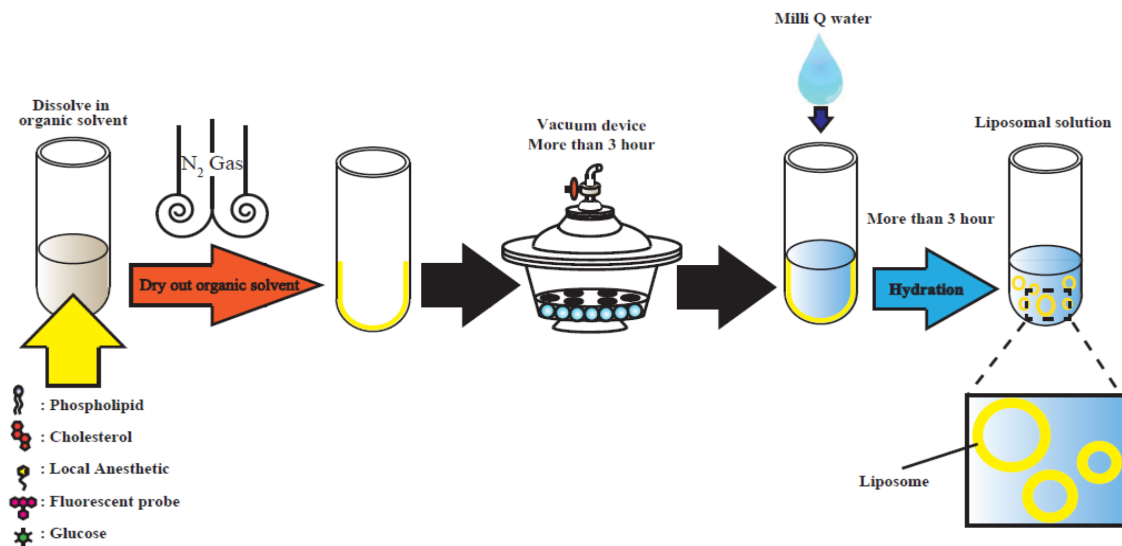


Fig.4 Schematic image of natural swelling method

2-3. Method

2-3-1. Analysis of thermo-responses of liposomes

We observed membrane fluctuation of DOPC liposomes with/without LAs by using phase contrast microscope with rising temperature from T_0=21.5°C at a rate of 1.0°C/min.

2-3-2. Raft-like domain observation

We can realize raft-like structure as a phase separation on multicomponent liposomes. We observed effects of LAs on phase separation on surface of binary（DOPC/DPPC）, and ternary（DOPC/DPPC/Chol）liposomes by using fluorescent microscope at T=21.5±1°C.

2-3-3. Measuring GP values of liposomes

The Laurdan generalized polarization (GP) measurement gives the lateral packing of lipid bilayers [4]. The wavelength of the fluorescent emission from Laurdan is affected by the number of water molecules near Laurdan. The fluorescent spectrum shifts toward longer wavelengths due to solvent relaxation. It is possible to measure the degree of lipid packing by this spectrum shift. Therefore, when the lipid packing is loose like in liquid phase, the fluorescent wavelength shifts toward longer wavelength. On the other hand, the fluorescent wavelength shifts toward shorter wavelength, when the lipid packing is tight like in solid phase. This degree is expressed as

$$GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}$$

and is called generalized polarization (GP) value. Here, I_{440} and I_{490} are the fluorescent intensities at 440nm and 490nm, respectively.

［3.Results］

3-1. Thermo-responsiveness of liposomes containing LA

We observed 30 liposomes for each composition. Compared with control (DOPC liposome without LA), liposomes with LA started membrane fluctuation with smaller temperature increase than liposomes without LA (Fig.5)(Fig.6). We listed up needed ΔT for fluctuation 50% of samples on Table.1. The membranes fluctuated at higher temperature. After fluctuation, we could see that the liposomes recovered their shapes spherical as decreasing in temperature. This result implies that this membrane fluctuation is not caused by the dissolution of LAs in the membranes to the water bulk region. The more membrane fluctuations in temperature change was observed in concentration dependent manner of the LAs. In addition, tetracaine containing liposomes fluctuated with smaller temperature increase than lidocaine containing liposomes.

3-2. Influence of LA to phase separation on surfaces of liposomes

We observed 60 liposomes for each composition, and classified the phase-separated structures with domain shape and diffusion. Phase-separated structures on liposomes with two components, DOPC and DPPC, mainly formed solid-like domains having anisotropic shapes. Distribution of formed domains did not show the significant change by adding LAs to the membrane components.

Liquid-like domains having circular shape, were often observed in the liposomes formed with ternary components, DOPC/DPPC/Chol. As increasing in the Chol molar ratio, the liquid-like domains appeared. The ratio of liquid-like domains was decreased, and that of no domain was increased by adding LAs. The ratio of solid-like domain was not significantly affected by LAs. Tetracaine containing liposomes showed the relatively larger effects on the phase separation compared with lidocaine containing liposome, but this difference was very small.(Fig.7)

3-3. Effect of LA on membrane fluidity of liposomes

We measured GP values of 15~20 liposomes for each composition. DOPC liposomes have relatively lower GP values than DPPC liposomes. This means that DOPC liposomes are more fluidic. We arranged GP values in ascending order; DOPC<DOPC/Chol<DPPC≒DPPC/Chol.

GP values of DOPC and DOPC/Chol liposomes became larger as increasing in the LAs concentration. These results showed that the fluidities of DOPC and DOPC/Chol liposomes became lower by adding LAs (Fig.8). On the other hand, GP values of DPPC and DPPC/Chol liposomes did not show significant change by LAs. Also, we cannot find the GP value difference between DPPC and DPPC/Chol liposomes. In general, Chol is known to increase the membrane fluidity of DPPC. We may not observe the membrane fluidity of DPPC-rich region and the effect of LAs precisely.

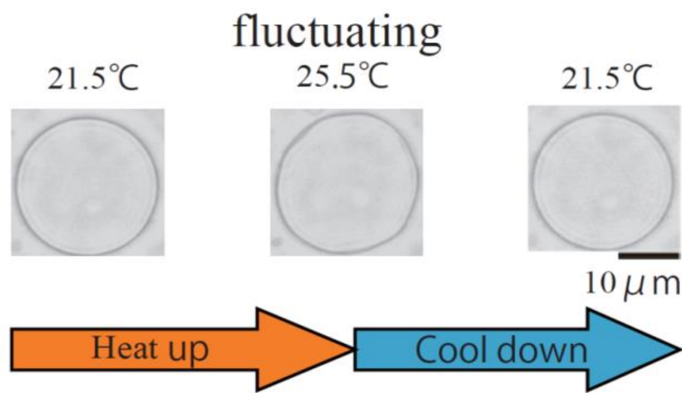


Fig.5 Microscopic images of fluctuating DOPC liposome

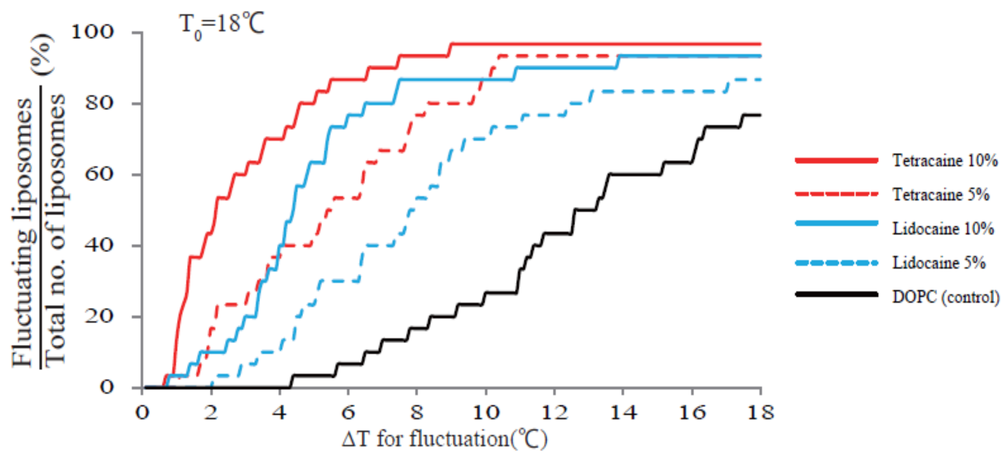


Fig.6 Thermal responses of liposomes against temperature changes

	DOPC(control)	Lidocaine+5%	Lidocaine+10%	Tetracaine+5%	Tetracaine+10%
ΔT for fluctuation(℃)	12.8	8	4.2	5	2.2

Table.1 Temperature change for fluctuation of 50% liposomes

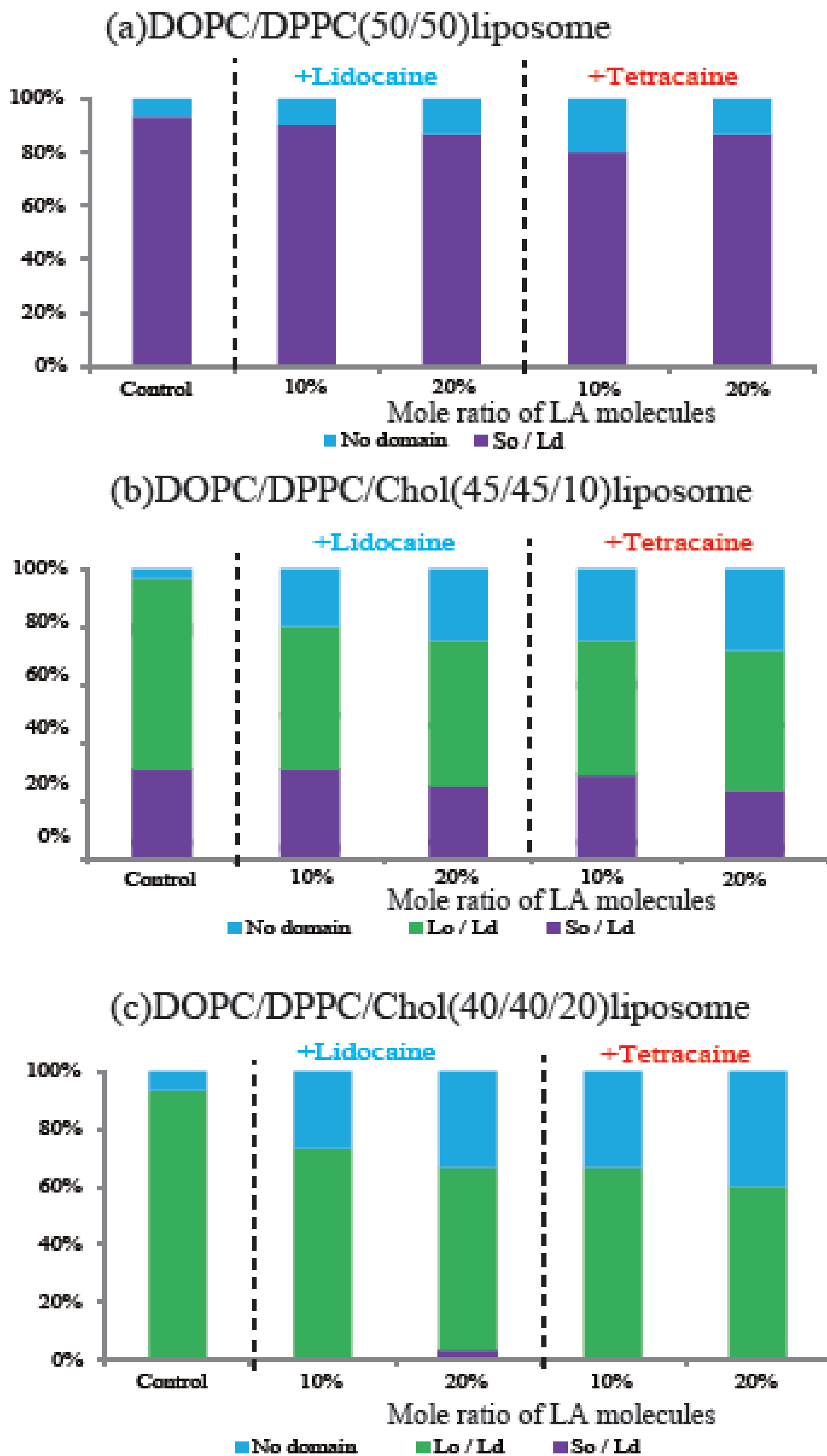
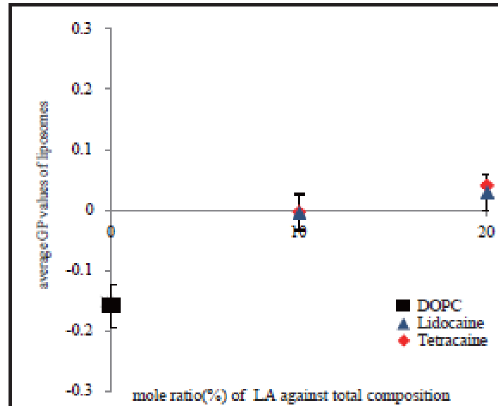
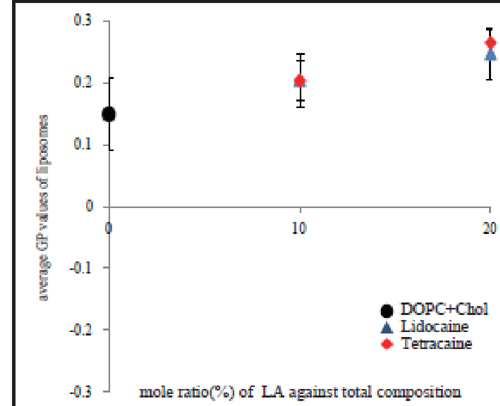


Fig.7 Distribution of liposomal phase separation for each components

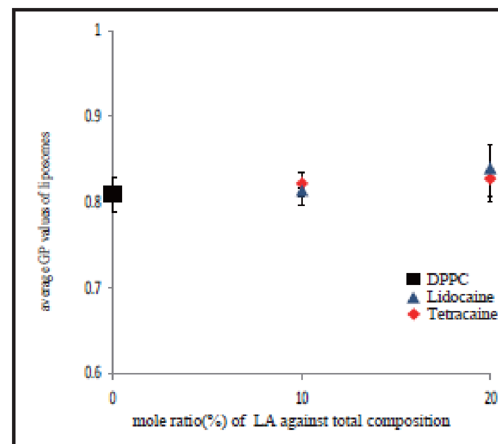
(a) DOPC membrane



(b) DOPC/Chol.(80/20) membrane



(c) DPPC membrane



(d) DPPC/Chol.(80/20) membrane

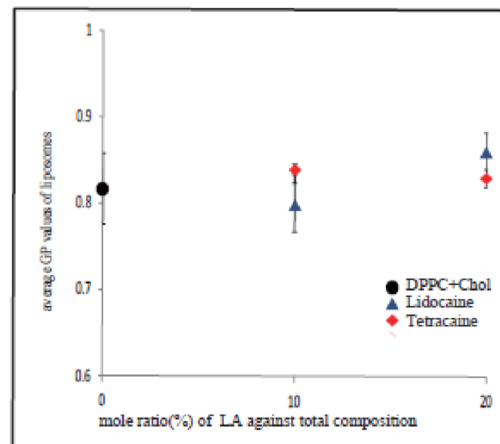


Fig.8 Changes of GP values of liposomes by addition of LAs

［4.Discussion］

4-1. Hydrophilic interaction between LA and phospholipid

LA-containing liposomes represents the high thermo responses compared with DOPC liposomes, and this result implies the direct interaction between LAs and DOPC membranes. Interestingly, the liposomes containing tetracaine that has relatively stronger anesthetics action than lidocaine, showed higher thermo sensitivity. Yoda *et al.* reported that the liposomes containing oxidized Chol also showed higher thermo sensitivities[5]. This is because water molecules are incorporated in the membrane hydrophilic region due to the hydrophilicity of oxidized Chol. These water molecules form the networks via hydrogen bonds among the hydrophilic parts of phospholipids and water molecules. Bonding energies of these networks are relatively weaker compared with other attractions such as van der Waals attraction and hydrophobic interaction between hydrophobic tails. Therefore, these weak hydrogen bonds and networks could easily collapse by slight increase in temperature. As a result, the membrane structure becomes loose and unstable. Furthermore, based on NMR studies Weizenmann *et al.* reported that LA molecules are inserted in nearby hydrophilic head group region of phospholipid membranes [6]. Tetracaine has more number of hydrophilic parts rather than Lidocaine. This should be related on membrane thermo-sensitivities.

4-2. Effects of LAs on membrane fluidity and domain phase separation

Addition of LAs affected on the phase separation in the DOPC/DPPC/Chol ternary lipid mixtures, but not in DOPC/DPPC binary lipid mixtures. This should be related with localization of LA molecules. According to the results from measuring GP values, DOPC region becomes stiffer by LAs. We could not find significant change of GP values in DPPC and DPPC/Chol liposomes. Some experimental studies mentioned that GP value measurement is not suitable for the evaluation of the membrane fluidity in DPPC-rich region [6]. However, Yamanaka *et al.* showed that LAs should decrease fluidity of DPPC region [7]. Therefore, in binary lipid mixtures, the fluidity difference between DOPC and DPPC regions did not significantly change, so that phase separation did not change meaningfully. In ternary lipid mixtures, however, Chol is known to be localized in DPPC-rich region, and make DPPC region fluidic. Hata *et al.* reported that the partitioning of Chol into DPPC-rich region makes LA solubility into membrane decrease. According to this report, LAs should be localized mainly in DOPC region to avoid Chol, and make DOPC region stiffer[8]. Both effects of LAs and Chol makes the fluidity difference between DOPC and DPPC regions decreased, and these two regions were mixed each other consequently.

[5.Conclusion]

Addition of LAs to DOPC liposomes gives rise to the membrane fluctuation by slight increase in temperature. Moreover, the stronger LA, Tetracaine containing membranes showed relatively higher thermo sensitive. The phase-separated structures on surface of ternary lipid liposomes had disappeared by addition of LAs. In binary lipid mixtures, however, the change of phase separation was not observed. Membrane GP values of DOPC and DOPC/Chol membranes became higher as increasing in the amount of LAs. On the other hand, DPPC and DPPC/Chol membranes did not show significant GP value changes. These results proved the existence of interaction between phospholipid liposome and LAs. Moreover, these interactions gave physical properties changes to liposomes.

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