Doctoral thesis

Destabilization of membrane raft-mimetic structure on liposomal membrane induced by local anesthetics.

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Chapter1. General introduction

1-1. Mechanism of pain-signal transduction

Initial state of pain-signal transduction starts from depolarization of membrane potential [1–3]. The potential state in and outside of the cell separated by the biomembrane is maintained in negatively charged state due to the function of the ion pump protein present in the membrane. Due to increase of cation near by the membrane, or combining with stimulus, channel protein on the biomembrane will open the small pore which can pass through the cation, and cation in the outside of the membrane flows into inside of membrane. By this mechanism, membrane potential tilts to temporary positive, and these mechanism called membrane "depolarization". When the neighboring membrane region had depolarized, voltage-dependent cation channel will react and form the pore to be pass through cations into inner-membrane, and depolarized the potential of neighboring membrane region. As these membrane depolarization are repeated, the electric signal transfers on the membrane surface (Fig.1-1). Not only pain, but five sense, cooling and warming sense are also transferred by similar mechanisms as explained. In another word to say, transduction of sensation can be inhibited by inactivation of membrane protein which relate on membrane depolarization. As the agents with such abilities, local anesthetics (LAs) were often used as pain killer trough the modern medicine.

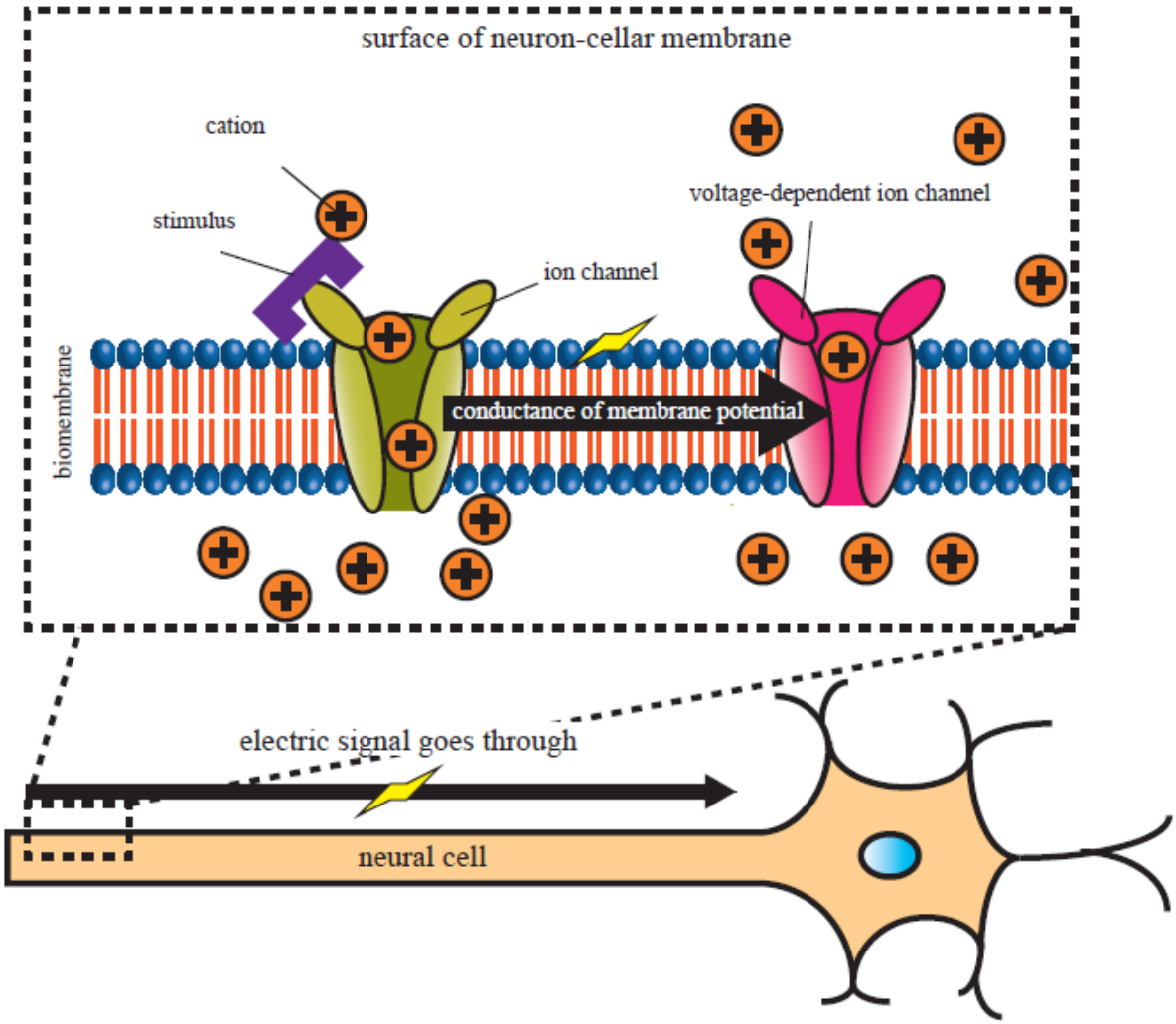

Fig.1-1 Simplified diagram of the mechanism by which an electric signal is transmitted through the surface of the nerve cell membrane

1-2. Mechanisms of local anesthesia

Until recently, local anesthetics (LAs) have been essential drugs especially for modern surgical medicine, and often used to suppress patients' pain. The mechanism of the pain signal suppression is believed that LAs deactivate sodium-ion channel protein and inhibit action potential of neural cell membranes [4–8]. Inhabitation of ion-channels were partially explained by direct interaction with LAs via the binding site by molecular simulations [9,10]. These studies shown the closure mechanisms of cation-gate structures accompanying whom conformational changes. However, the direct interaction hypothesis cannot explain all the mechanisms of interaction diverse ion-channels and LAs. However, location of binding site and their mechanisms how LAs deactivating channel protein have not been fully explained.

On the other hand, some groups expected that these mechanisms of LAs could be explained by the changes of physical properties of biomembranes, where the ion-channel proteins were buried, by the interaction between LAs [11,12]. Since the most of LA molecules have large hydrophobic parts in their structures, LAs may interact with the hydrophobic region of biomembranes, and alter the physical properties of biomembranes. As a result of their interaction, the channel proteins varied in biomembranes can be indirectly influenced by LAs through the physical property changes of biomembranes. In fact, some groups had investigated the correlation on the hydrophobicity of LAs [13]. Furthermore examined the clarified the interaction between phospholipid membranes and LAs by handling nuclear magnetic resonance (NMR) [14,15]. Moreover, some studies reported the function of a

membrane protein like as ion-channels can be affected by physical property changes of membrane and function of ion-channel proteins is also changed consequently [16–18]. Based on the above cases, the relation between the function of the LAs and physical properties of the membrane is not negligible.

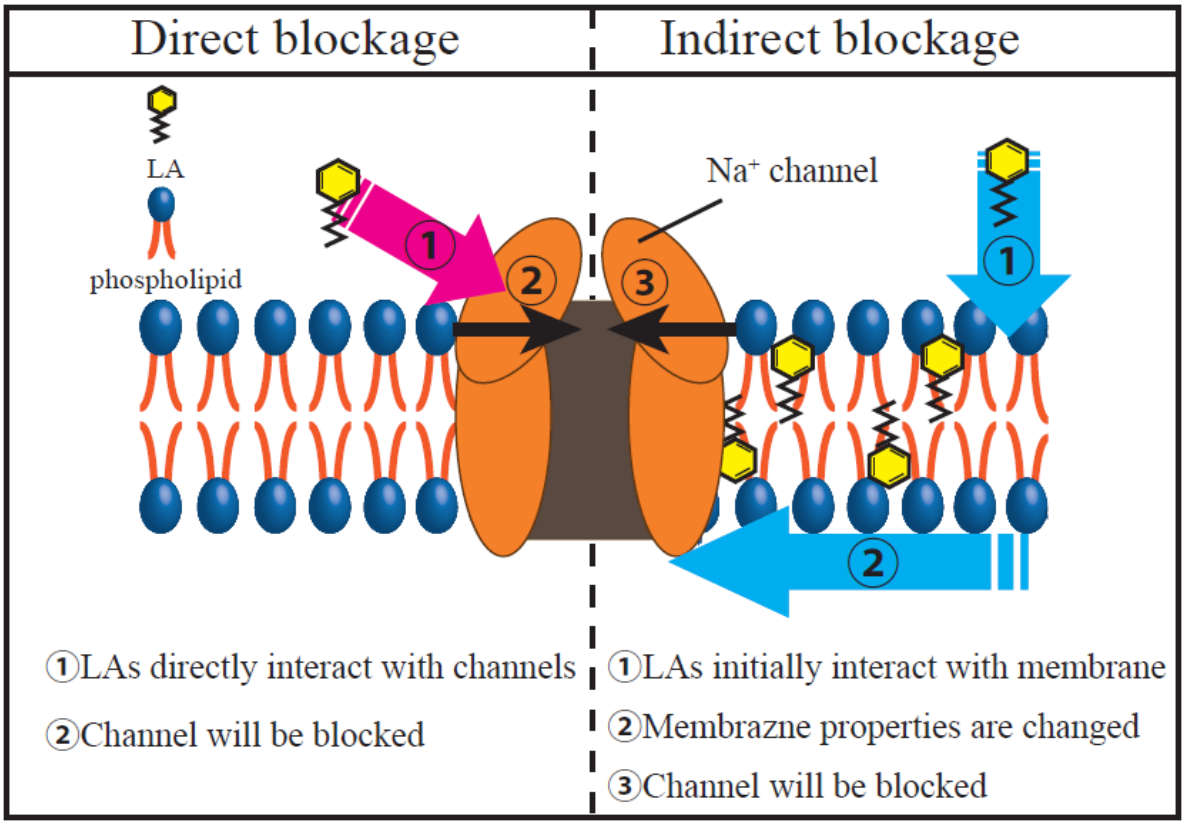

Fig.1-2 Direct (left) and indirect (right) mechanisms of sodium-channel blockage.

1-3. Biomembrane

Inner contents of cell and organelle are separated from outer-environment by biomembranes mainly composed with phospholipids and cholesterol (Chol), and sugars and proteins are distributed [19–23] (Fig.1-3). Molecules constituting a biomembranes are amphipathic molecules having both a hydrophilic group and a hydrophobic group in their structure while directing the hydrophilic group toward the solvent side. These membrane molecules are oriented as fluidic bilayer structure by weak interaction derived from hydrophobicity [24–26]. On the surface of biomembranes, there are various functional molecules are known to be localized [27–29]. The receipt of signal molecules by such receptor proteins exchanges biological signals through the membrane [30–33]. Furthermore, sugar chains which bind to proteins and lipids in membranes and protrude to membrane surface contribute to immune recognition [34]. On the other hand, the opening of the channel protein contributes to the generation and transmission of electrical signals traveling through biological membranes [35,36]. The behaviors and physical property of biomembranes strongly affected to the structure and function of membrane channel proteins [16,30,31]. For these reasons, biomembranes are regarded as an important platform for signal exchange and transduction.

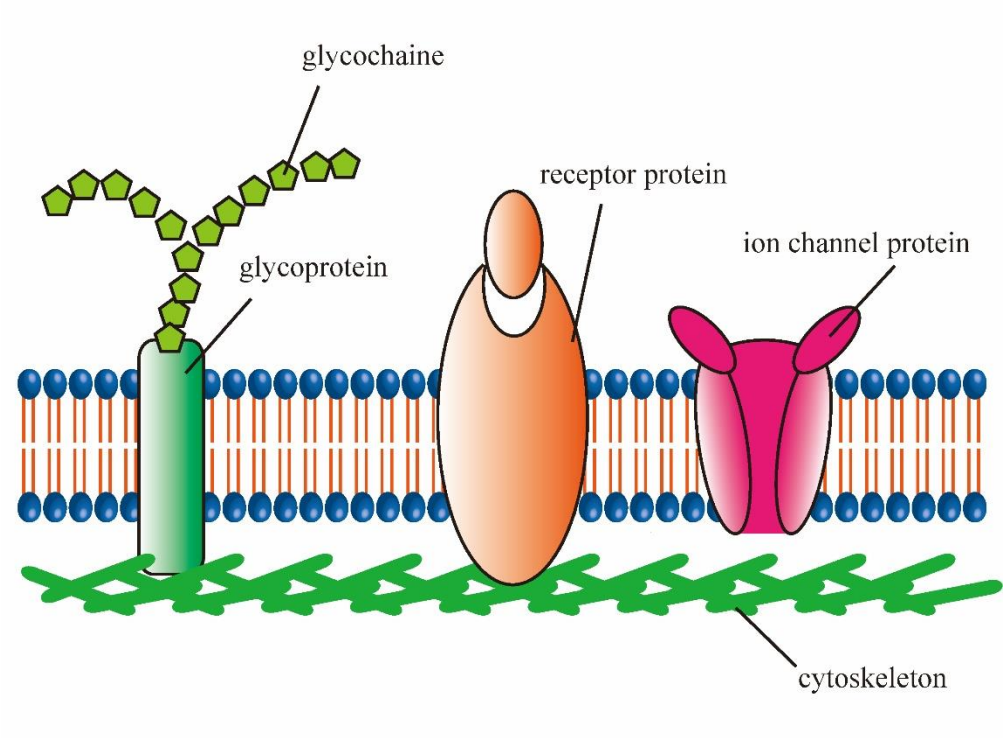

Fig.1-3 Schematic of biomembrane

1-4. Lipid raft: the ordered structure of membrane molecules

Regarding the distribution of membrane molecules, the fluid mosaic model proposed by Singer and Nicolson was mainstream [37,38]. This model suggested the molecules randomly distribute without having the order in the fluid lipid membrane. However, recently, microdomain with well-ordered structure called “lipid raft” was suggested by Simons and Ikonen [39–41]. Lipid raft has relatively less fluidic structure like as phase separation mainly composed with saturated lipid and Chol. This specific structure known to localize the integral proteins such as receptor and ion-channel. In fact, lipid raft localizes receptor protein groups involved in intracellular signals and contributes to signal transmission between nerves [42]. In fact, channel protein which can be influenced by LAs is known to be localized in lipid raft [43]. Thus, this specific microdomain are regarded as the center of signal transduction by function as center organization of functional molecules. Therefore, many studies focus on the behavior and property of lipid raft to clarify the mechanisms of cellar signaling [20].

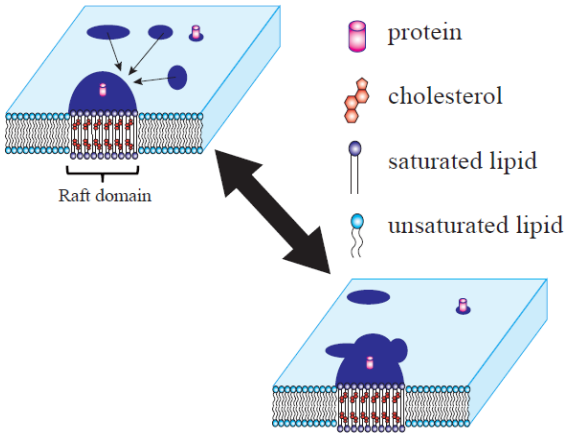

Fig.1-4 Schematic image of biomembrane and lipid-raft.

1-5. Liposome: the biomimetic membrane

Biological membranes are composed of a wide variety of components including proteins and lipids, sugar etc... Moreover, it is necessary to consider the contribution of other organelles and cytoskeleton, it is difficult to discuss physical properties on the biological membrane and phenomena on them. For these reasons, lipid aggregate: liposome are drawing attention as model systems of biological membranes.

Phospholipids, the main component of biomembranes, are amphiphilic molecules composed with hydrophilic head groups and hydrophobic carbon chains (Fig.1-5). When amphiphilic molecules are presented into polar solvents such as water, they self-associate in solution by hydrophobic interaction while directing their hydrophilic head group to solvent side [44,45]. When the solute concentration in the solvent exceeds a certain level, amphipathic molecules form a bilayer membrane structures. Among such bilayer structures, the spherically closed shape is referred to as liposome.

The structure and composition of liposomes are similar to actual biological membranes. At the same time, their structural simplicity makes easier to discuss many phenomena on biomembranes from chemical or physical aspects. Moreover, because they are artifact materials, preparing the experimental environment by adjusting these systems is more prepared than using actual living cells. Especially, the liposomes which has microscale size: the giant unilamellar vesicles (GUVs) are having enough size for microscopic observation. From these reasones, they have been used for many studies as a biomembrane model to discuss [46–48].

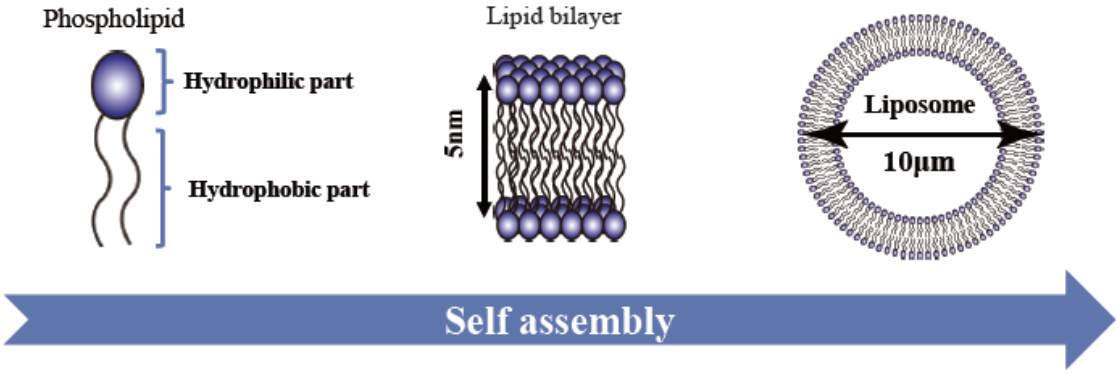

Fig.1-5 Schematic image of phospholipid and its assemblies

1-6. Phase separation on liposome: the raft-mimetic structure

Depending on the composition, it is possible to reproduce the raft-mimetic structure in the liposome membrane [41,49]. The basic structure of raft is said to be resembling phase separation of saturated lipid / cholesterol-rich rich less-fluidity region in the membrane. When a liposome membrane is prepared by mixing a saturated lipid / unsaturated lipid, phase separateds-structure between fluidic liquid-disordered phase (L_d) rich with unsaturated lipids and less-fluidic solid–ordered phase (S_o) rich in saturated lipids. When cholesterol is further added to this composition, a liquid ordered phase (L_o) which behaves liquidly while maintaining an ordered structure appears. In particular, this L_o / L_d phase separation structure is regarded as a mimetic structure of lipid rafts in biological membranes due to compositional similarity and is used in various studies to understand various phenomena occurring on rafts. This phase separation structure is made up of the balance between the interfacial energy functioning between saturated lipid and unsaturated lipid and the entropy of mixture of lipids in the system. The strength of interfacial energy of molecules can be influenced by many factors, but they often discussed with fluidity difference between each molecules [50,51]. If the interfacial energy works strongly, a phase separation structure appears. Conversely, if mixing entropy overcomes interfacial energy, the lipids mix and become a uniform phase state.

The phase separation state of the membrane is an important clue for understanding the physical properties of the membrane as it is involved in the localization of the membrane molecule and can cause a significant change in the local physical properties in the membrane.

If the addition of local anesthetic is involved in the phase state of the membrane, it is presumed that the localization of the membrane molecule and the change of the membrane physical properties will occur at the same time, and it is considered that the function of the channel protein on the membrane will also be affected as a matter of course. For these reasons, we have been studying focusing on the effects of local anesthetics on the phase state of liposome membranes.

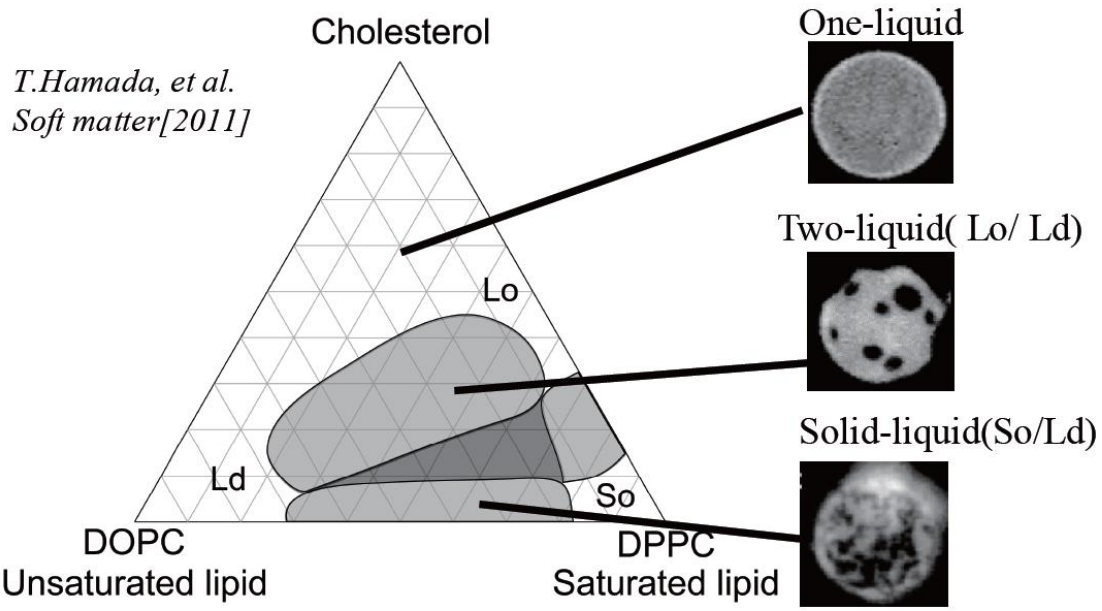

Fig.1-6 Phase diagram of multicomponent-lipid membrane composed with DOPC, DPPC and Chol (left). Images of liposomes which are expressing different phase state (right). Each liposomes are expressing liquid-disordered (L_d) monophase (upper), liquid-ordered (L_o)/L_d 2 phases (middle), and solid-ordered/L_d 2phases (bottom).

1-7. Objective and outline

Several studies had suggested the correlation between behavior of membrane raft and function of LAs. To clarify this question, in previous studies, we focused on the effects of LAs presence on the behavior of reproduced raft-mimetic structure on liposomal membranes. We revealed the influence of LAs on stability of phase-separated structure in liposomal membranes by microscopic observation and handling differential scanning calorimetric technic. We discussed the effect of LAs on stability of raft-mimetic structures on below section.

In the chapter.2, we investigated the effect of LAs (lidocaine, and tetracaine) on formation state of phase-separated structures on saturated lipid (DPPC)/ unsaturated lipid (DOPC) (S_o/L_d) and DPPC/DOPC/Chol (L_o/L_d) liposomes by direct observation with fluorescent microscope. Furthermore, we measured fluidity of LA presented raft-mimetized liposomes by measuring membrane GP values of Laurdan fluorescent in each L_o and L_d phases. From the result of GP value measurement, we discussed correlation between change of fluidity in each phases and stability of phase-separation due to the interaction with LAs.

In the chapter.3, we investigated the thermal stability of the phase-separated structures in biomimetic lipid membranes containing LAs. Initially, the miscibility temperatures in DOPC/DPPC/LAs and DOPC/DPPC/Chol/LAs were measured by fluorescence microscopic observation. Next, we measured the line tension at L_o/L_d interface from the fluctuation of the domain boundary in DOPC/DPPC/Chol/LAs to discuss the stability of the domain. Finally, DSC measurement was used to clarify the lipid transition temperatures in LA-containing lipid membranes. From the results of DSC experiments, the partitioning of LAs in the coexisting

phases was considered. Moreover, we discussed the influence of LAs on thermal stability of phase separation on liposomal membranes.

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Chapter 2. The effect of local anesthetics on stability of raft-mimetic structure and fluidity of liposome

2-1. Introduction

Local anesthetics (LAs) are essential drugs for modern medicine that are often used to suppress patients' pain. LAs are known to target functional protein, voltage-dependent sodium channels, which are located on cellular membranes. The mechanism how LAs shield pain signals was believed that LAs interact with the channel protein directly, and make it be deactivated[1,2]. However, binding sites on the channel proteins had not been elucidated completely. In addition, according to past reports, LAs exhibit toxicity against human cells. The presence of LAs sometimes induces fatal side effects such as allergy, liver malfunction, and analgesia[3]. The mechanisms how these toxicity or diseases inducted by LAs are also not fully understood. Recently, however, some studies are expecting that these mechanisms can be explained by the change of the physical property of lipid membranes where the proteins are buried induced by addition of LAs[4].

Cell membranes which are composed of lipid bilayer structure, are known to relate with signal transductions via membrane dynamics such as vesicular formation. In fact, some studies are providing correlation between physical property changes on biomembrane and behavior of channel proteins[5]. Moreover, "lipid raft model" was proposed by Simons and Ikonen in 1997 [6,7]. 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 lipid raft speculated as a platform for signal transduction.

In order to reveal the mechanism of raft formation, "liposome" which is mainly composed of phospholipids is used as a mimetic model of biomembrane system. Liposome has similar

structure to actual biomembranes, so we can directly observe similar phenomena on liposome with optical microscope. Especially, raft formation can be regarded as the phase separation between saturated lipid and cholesterol-rich and unsaturated lipid-rich phases in model membrane system, so from the biological aspect, it is important to discuss phase behavior of model membrane[8].

In this study, we observed the phase separation in lipid membranes containing typical LAs, lidocaine and tetracaine, by optical microscopes. In order to understand the phase separation, the membrane fluidity is estimated by Laurdan generalized polarization measurement. Finally, we discuss the physiological action of LAs based on the experimental results.

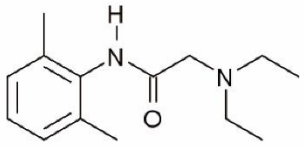
2-1. Materials and Methods

2-2-1. Materials

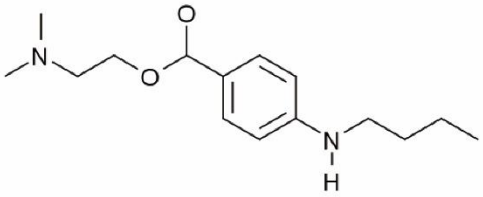
Local anesthetics, 2-(diethylamino)-*N*-(2,6 dimethylphenyl)acetamide (lidocaine) and 2-(dimethylamino)ethyl 4-(butylamino) benzoate (tetracaine) were purchased from Nacalai Tesque (Japan) and Tokyo Chemical Industry (Japan), respectively. Unsaturated lipid, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), saturated lipid, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and cholesterol (Chol) were purchased from Avanti Polar Lipids (USA). Fluorescent probes, Rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine, triethylammonium salt (Rhod-DHPE) and 6-dodecanoyl-2-dimethylaminonaphthalene (Laurdan) were purchased from Invitrogen and Funakoshi, respectively. Rho-DHPE molecules are mainly localized in DOPC-rich liquid-disordered (L_d) phase. Ultrapure water (specific resistance ≥18 MΩ) was obtained using a Millipore Milli-Q purification system. The chemical structures for lipids and LAs are shown in Fig.2-1.

Local anesthetics

(a) lidocaine

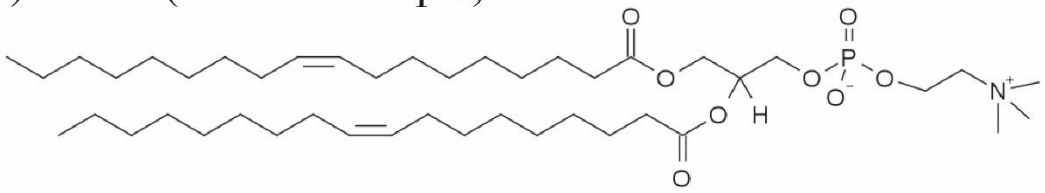


(b) tetracaine

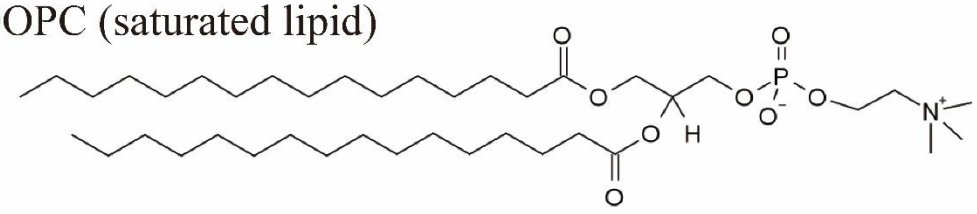


Lipids

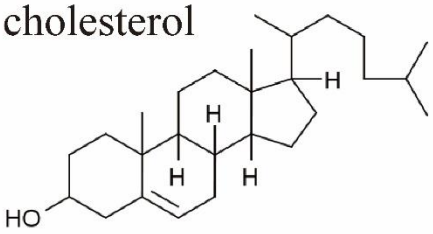
(c) DOPC (unsaturated lipid)



(d) DOPC (saturated lipid)



(e) cholesterol



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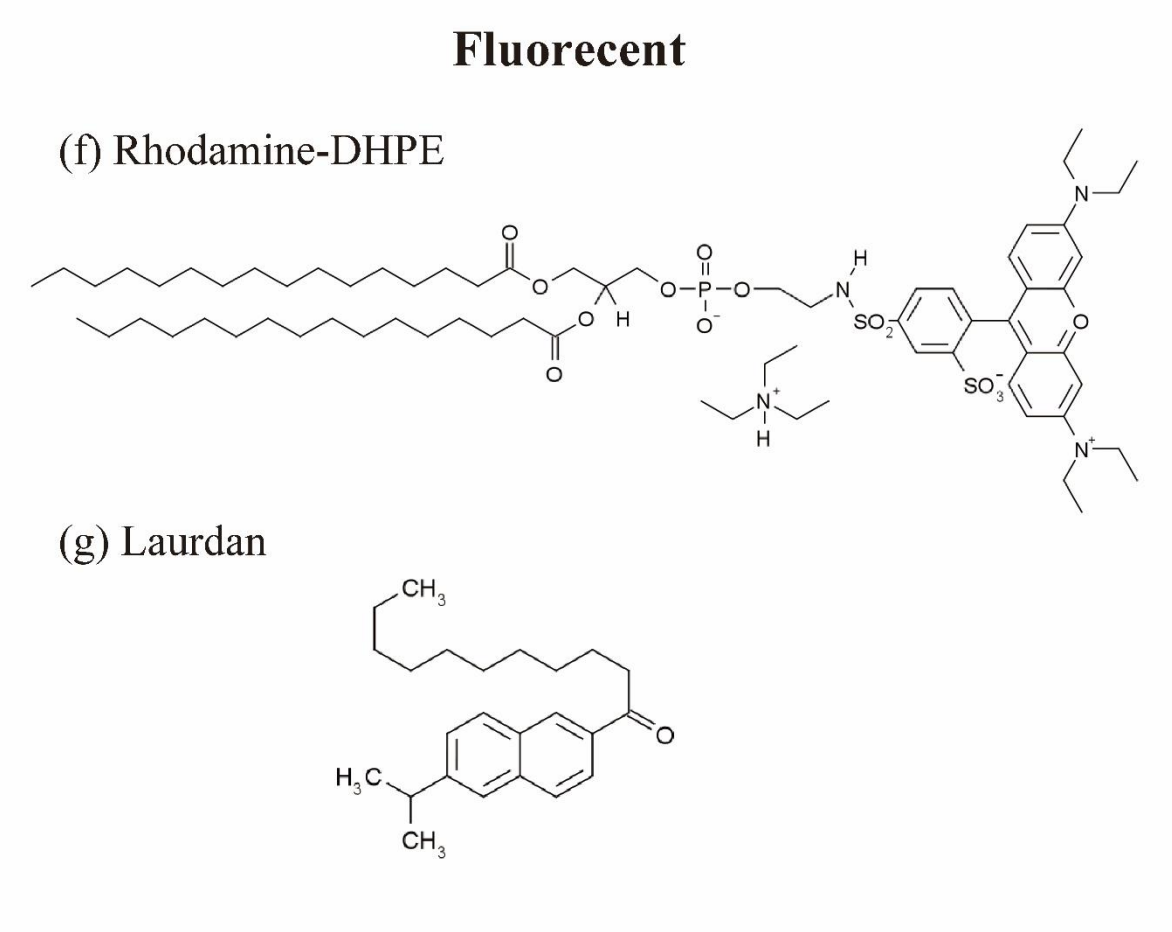

Fig.2-1 Chemical structures of lidocaine (a) and tetracaine (b), DOPC (c) and DPPC (d), cholesterol (e), Rhodamine-DHPE (f), Laurdan (g), respectively.

2-2-2. Preparation of liposome for microscopic observation

Liposomes were prepared by natural swelling method (Fig.2-2). Lipids (DOPC, DPPC, Chol) and LAs (lidocaine, tetracaine), fluorescents (Rhod-DHPE, Laurdan) were dissolved in chloroform, and their concentrations were 2mM, 0.5mM, and 10μM, respectively. 10mM Glucose dissolved in methanol. These stock solutions were put into glass test tubes, and then mixed all of them. These solutions were dried under vacuum for least 3 hours to form thin lipid films. The films were then hydrated overnight with deionized water at 55 °C to produce unilamellar liposomes. The final concentrations were 0.2mM lipids and LAs, 0.6mM glucose, 0.01mM Rhod (10μM in case of GP value measurement), and 0.25μM Laurdan.

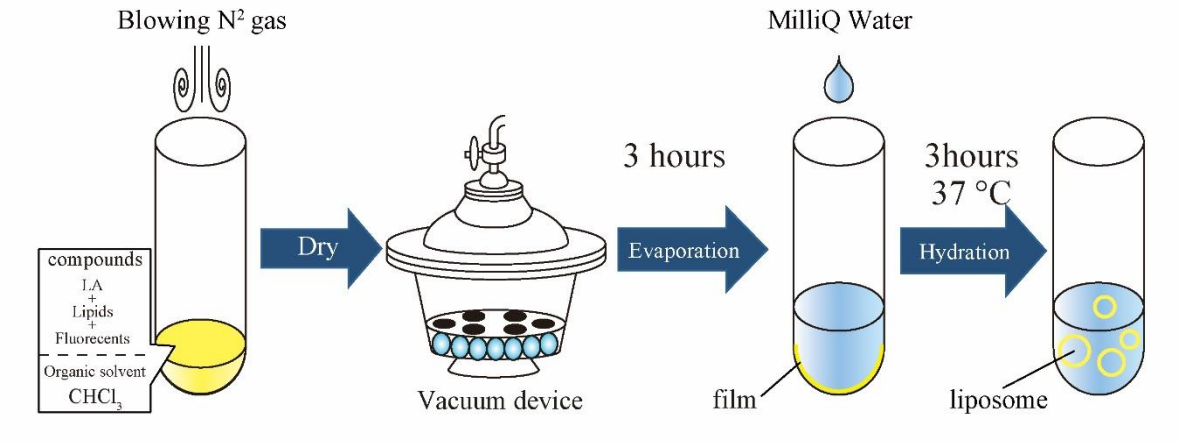

Fig.2-2 Schematic of liposomal preparation

2-2-3. Microscopic observation of phase-separated structure on liposome

We observed effects of LAs on phase separation in the binary (DOPC/DPPC=50/50mol%), and ternary (DOPC/DPPC/Chol=40/40/20mol%) mixtures by using fluorescent microscope (Fig.2-3). The sample temperature was kept at 21.5±1 °C with a microscope stage (MATS-555MORA-BU, Tokai hit). We observed 60 liposomes for each composition, and classified the phase-separated structures based on domain shape and diffusion. In DOPC/DPPC binary system, phase-separated structures mainly formed solid-ordered (S_o) domains having anisotropic shapes surrounded by L_d phase.

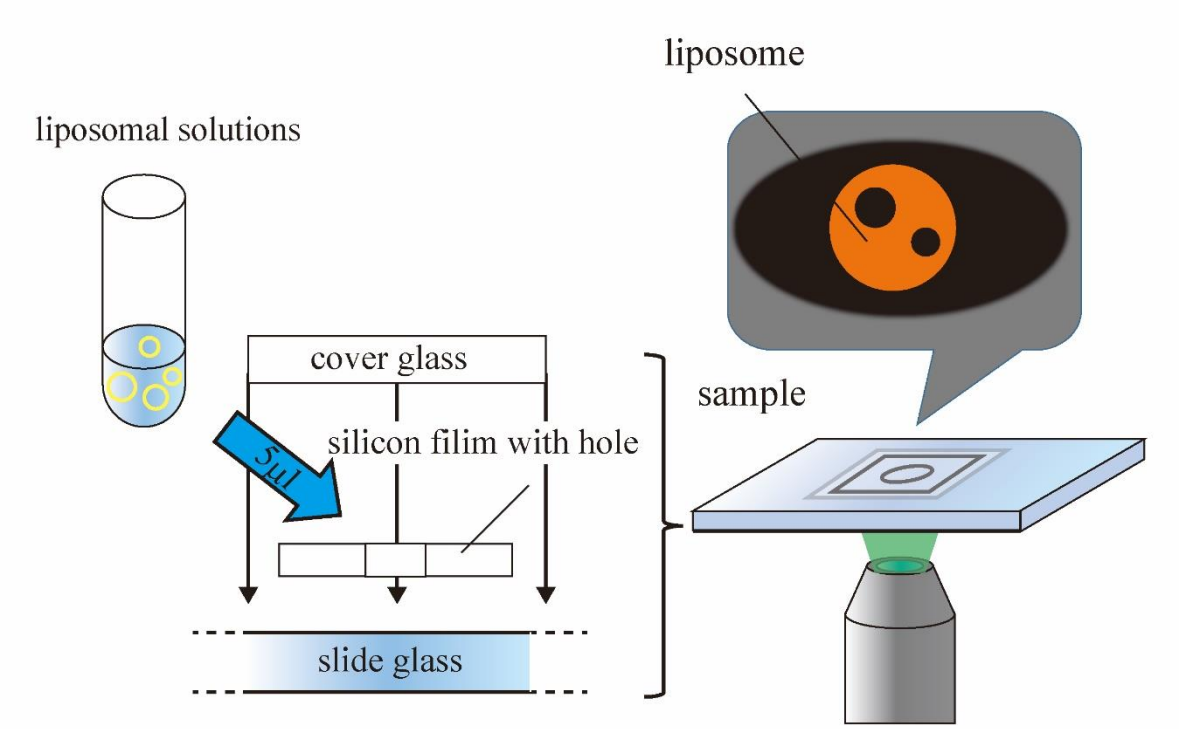

Fig.2-3 Schematic of microscopic observation

2-2-4. Fluidity measurement of phase-separated liposome

Membrane fluidity changes by the presence of LAs were evaluated by using Laurdan (Fig.2-4). The Laurdan generalized polarization (GP) measurement gives degrees of order state of hydrophobic carbon chains of phospholipid molecule [9,10]. 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 dipole relaxation. It is possible to measure the degree of lipid fluidity by this spectrum shift qualitatively. Therefore, when the chains are disordered, as in the liquid phase, the fluorescence shifts toward longer wavelengths. On the other hand, the fluorescence shifts toward shorter wavelength, when the chains are ordered, as in the solid phase. GP value of the membrane can be calculated by the following equation.

$$\text{GP value} = \frac{I_1 - I_2}{I_1 + I_2} \qquad (1)$$

Here, I_1 and I_2 are the fluorescent intensities in the range of 430- 455 nm and 490-540 nm, respectively. The Laurdan GP values were measured in the liposomes consisting of DOPC/DPPC/Chol(40/40/20mol%). We also performed the Laurdan GP measurement in these liposomes containing LAs to evaluate the effect of LAs on the GP values.

In order to discuss membrane fluidities of domain and non-domain regions separately, we set the threshold value determined from Rhod-DHPE fluorescent intensity, and identified the

lower and higher intensity regions as DPPC and Chol-rich liquid-ordered (L_o) phase and DOPC-rich L_d phase, respectively.

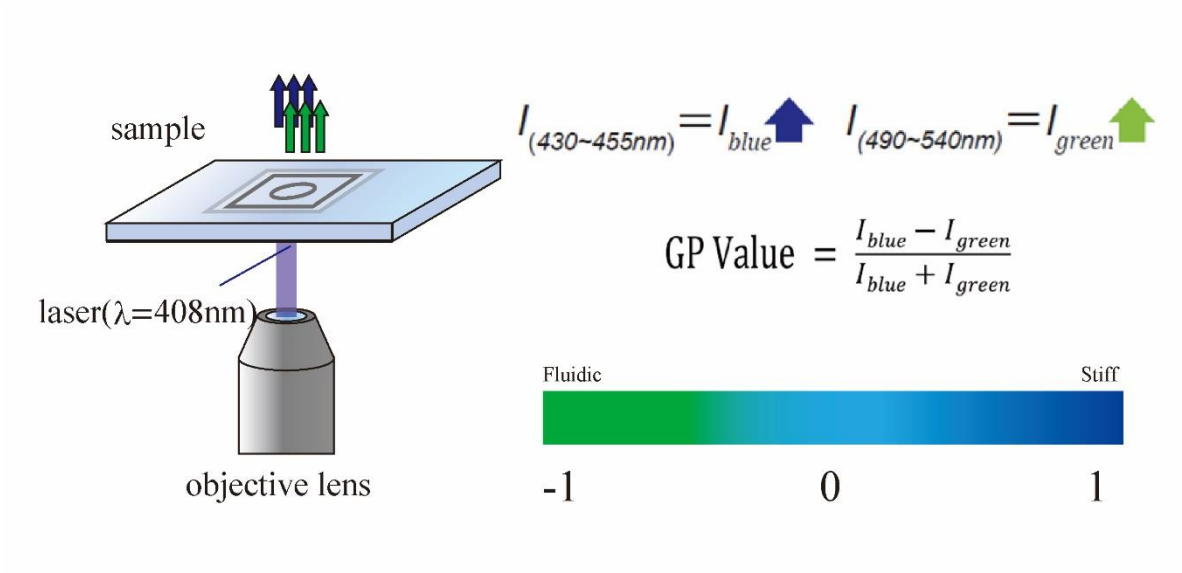

Fig.2-4 Schematic of Laurdan GP value measurment

2-3. Result

2-3-1. Effect of LAs on liposomal phase behavior

We observed 60 liposomes for each composition, and classified the phase-separated structures based on domain shape and diffusion. In DOPC/DPPC binary system, phase-separated structures mainly formed solid-ordered (S_o) domains having anisotropic shapes surrounded by L_d phase (Fig.2-5 (a)). We could not find the significant change in the percentage of phase-separated structures by adding Lidocaine or tetracaine as shown in Fig.2-5(d).

L_o domains having circular shapes surrounded by L_d phase (Fig.2-5(b)), were often observed in DOPC/DPPC/Chol ternary mixture. As increased the Chol molar ratio, the L_o domains were formed easily. Although more than half of liposomes exhibited the phase-separated structures, the ratio of L_o domains was decreased, and that of homogeneous phase (Fig.2-5(c)) was increased by adding lidocaine or tetracaine (Fig.2-5(e)). Since the percentages of phase-separated structures (sum of S_o/L_d and L_o/L_d) are 73% (lidocaine 10%), 65% (lidocaine 20%), 67% (tetracaine 10%), and 60% (tetracaine 20%), tetracaine slightly further suppressed the phase separation compared with lidocaine. These results indicate that the presence of LAs destabilized phase-separated structures, and enhanced mixture of lipids.

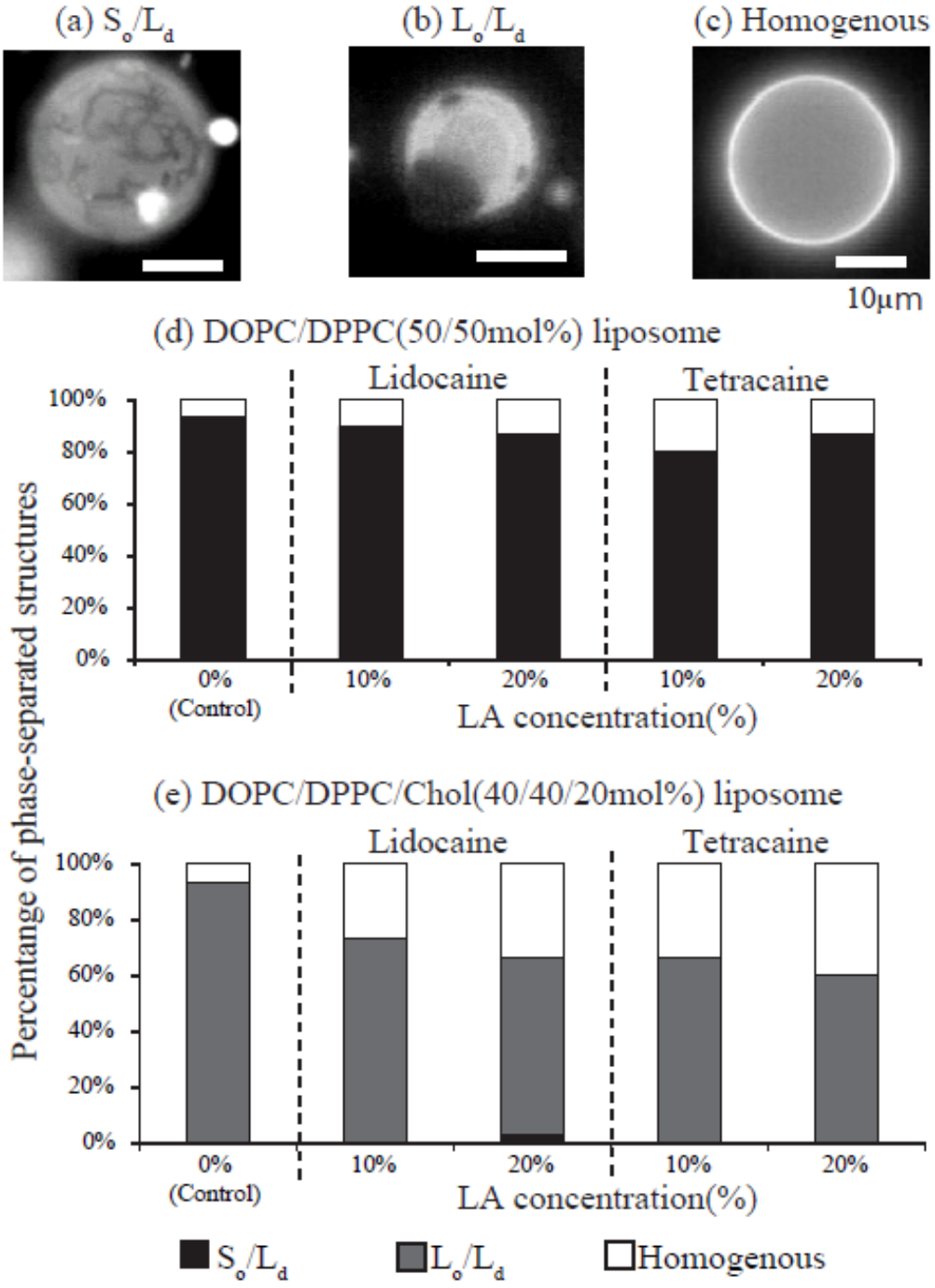

Fig.2-5 Microscopic images of multi-component liposomes; (a)S_o/L_d phase separation, (b)L_o/L_d phase separation, and (c) homogenous phase. Bright region indicates L_d phase, and dark region indicates S_o and L_o phases in (a) and (b), respectively. The percentages of phase-separated structures in DOPC/DPPC/LAs and DOPC/ DPPC/ Chol/LAs, are represented in (d) and (e), respectively. Black, gray, and while bars denote S_o/L_d, L_o/L_d, and homogenous phase, respectively.

2-3-2. Effect of LAs on membrane fluidity

In order to understand the suppression of the domain formation in ternary lipid mixtures with LAs, we measured the GP values of 15 liposomes composed of ternary components (DOPC/DPPC/Chol=40/40/20mol%), and measured GP values of L_o (domain region) and L_d (non-domain region) phases separately based on the fluorescence intensity of Rhod-DHPE; the regions with lower and higher intensities were regarded as L_o and L_d phases, respectively. In Fig.2-6 (a) and (b), the changes of GP values in L_o and L_d phases by adding lidocaine and tetracaine are indicated. The GP values of L_o phase denoted by triangles with solid lines in Fig.2-6 (a) and (b) had not been affected in both cases of lidocaine and tetracaine. However, GP values obtained from L_d (DOPC-rich) phase became higher by presenting LAs (circles with dashed lines in Fig.2-6 (a) and (b)). These results indicate that LAs did not influence to fluidity of L_o phase. On the other hand, L_d phase became less fluidic by LAs. In addition, the difference of fluidity between L_o and L_d phases became smaller as increased the LAs concentration. We will discuss how the phase behavior is governed by the fluidity difference between L_o and L_d phases.

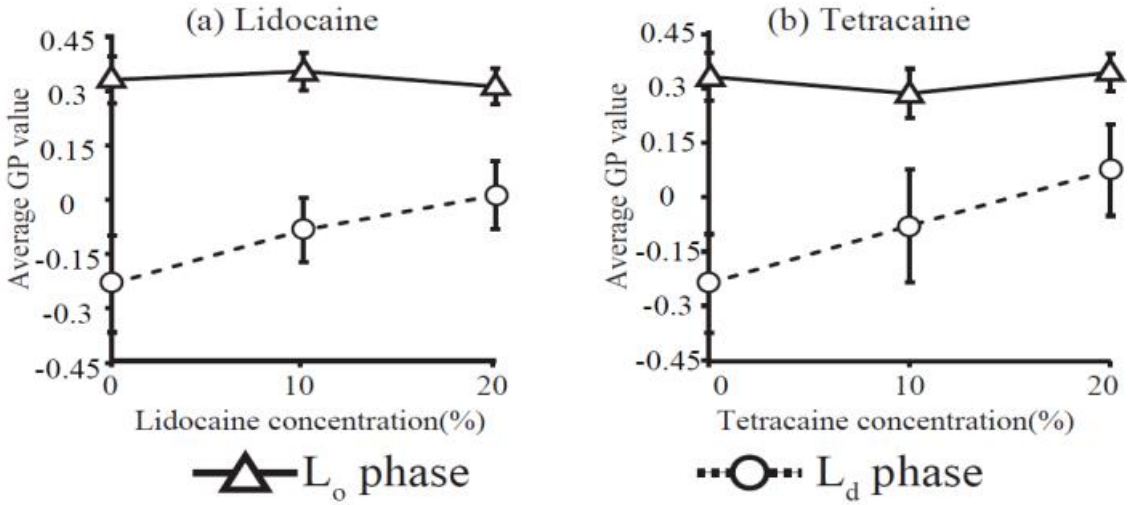

Fig.2-6 Average GP values of LA added membranes (n=15). Triangles with solid lines on (a) and (b) show GP values of DPPC membranes, and circles with dashed lines show GP values of DOPC membranes. Bar on each marker shows standard deviation of GP values of each component.

4. Discussion

We observed the phase separation in binary and ternary lipid mixtures with LAs. In addition, the membrane fluidities were evaluated by the Laurdan GP value measurement. We will discuss the relation between the phase behavior and the membrane fluidity. In general, the phase separation can be understood by the competition between the lipid interaction energy and the mixing entropy of lipids [11]. Here, we focus on the change of the interaction energy by adding LAs for simplicity. Thus, it is important to consider how the line energy at domain boundary is changed by LAs. The line energy is directly influenced by the fluidities of domain and non-domain regions, and the membrane fluidity in each phase is determined by the various physical properties, such as membrane thickness, spontaneous curvature, lipid chain ordering, and dipole density. If the membrane fluidity difference between domain and non-domain regions becomes smaller, we can understand that the line energy at domain boundary is reduced qualitatively and the phase separation is suppressed.

In ternary lipid mixtures with LAs, the phase separation is suppressed by adding LAs. As shown in Fig.2-6, the fluidity of L_d phase was decreased as LAs concentrations increased, whereas that of L_o phase kept almost constant. Since the fluidity difference between domain and non-domain regions became sufficiently smaller, the phase separation is suppressed in the ternary lipid mixtures. We expect that the line tension at domain boundary is reduced sufficiently and will measure it quantitatively.

In binary lipid mixtures with LAs, the phase behavior was not significantly changed as increasing in LAs concentration. Since the scattered or string-like S_o domains were formed

in DOPC/DPPC binary systems, we could not measure the GP values clearly. However, we can expect that the fluidity of the DOPC-rich L_d phase becomes lower by adding LAs as is the case with the ternary system. Since the fluidity of S_o phase was lower than that of L_o phase, the fluidity difference between domain and non-domain regions was larger in the binary system compared with the case of ternary system. Even if the fluidity of L_d phase became lower, the fluidity difference between S_o and L_d phases did not become smaller sufficiently. In other words, the line tension at domain boundary did not become small enough to destabilize the phase-separated structures. Thus, in binary mixture, the phase behavior was not dramatically changed. In future, we will examine the fluidity change between the phases more precisely.

It is worth pointing out that the four important problems still remains. Now, we explain about the phase behavior based on the fluidity change of L_d phase by adding LAs. Although we could not find any effects of LAs on S_o and L_o phases, some researches mentioned about the physical property change of DPPC membranes by LAs [12]. Especially, it is important to reveal the domain formation in LAs-containing membranes as changing the temperature [13]. In such a case, the crucial effects on S_o and L_o phases may be found even in the Laurdan GP value measurement. We will study to understand the role of LAs in the domain formation at different temperatures.

Tetracaine is known to have stronger anesthetics action and be more toxic than lidocaine. In our experimental results, the phase separation was suppressed and the change of membrane fluidity was larger in the case of tetracaine-containing membranes than of lidocaine-containing membrane. However, this difference between tetracaine and lidocaine was too

small to explain the anesthetics action and the toxicity of these LAs. We believe that the obvious difference of phase behavior between lidocaine and tetracaine containing membranes can be found at different temperatures. In addition, it may be possible to reveal the effects of LA molecules on lipid membranes more clearly. It also remains as a future problem.

Our results showed the evidence of interaction between LA molecules and L_d phase that could be regarded as non-raft region in actual cellular membrane. But, even the actual distribution of LA molecules on L_d/L_o phase-separated liposome and the detailed interaction between LA molecules and lipids are still unclear. In order to reveal the LA molecules distribution in heterogeneous membranes and understand the reduction of membrane fluidity of L_d phase by LA molecules based on the molecular interaction between LA molecules and lipids, it is important to perform the additional experiments, such as Nuclear Magnetic Resonance (NMR) measurement.

If LA molecules influence to sodium ion channels known as a target of LAs through the change of the membrane physical properties, we can propose two hypotheses. Since the location of sodium ion channels in actual cellular membrane is not fully understood, we discuss the cases that sodium channels is located in non-raft region and raft region. If sodium channels are located in non-raft region, the action of sodium channels may be suppressed due to the lower membrane fluidity by addition of LAs. On the other hand, when sodium channels are located in raft region, sodium channels cannot function correctly, because raft region becomes unstable by LAs. We consider that it is important to discuss the relation between the location of sodium channels and the physical property change by LAs more precisely.

In this study, we observed the destabilization of phase-separated structures and the membrane fluidity changes in bio-mimetic membranes by adding LAs. Additionally, those effects of LAs showed some correlation with strength of regents.

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Capter.3 Thermal stability of phase-separated domains in multicomponent lipid membranes with local anesthetics

3-1. Introduction

Local anesthetics (LAs) are essential drugs, especially in modern surgical medicine, and are frequently used to suppress pain. The mechanism behind the suppression of pain signals by LAs is believed to result from the deactivation of sodium ion channel proteins and inhibition of the action potentials of neural cell membranes [1–3]. Molecular simulations have partly explained the inhibition of ion channels as direct interactions between LAs and the binding sites on the proteins [4,5]. These studies have demonstrated the closure behavior of cation-gate structures that accompanies conformational change. However, the direct interaction hypothesis does not adequately explain the deactivation of ion channels by LAs. Recent studies have suggested indirect interactions between the channels and LAs [6,7]. Since most LA molecules have large hydrophobic parts in their structures, LAs may interact with the hydrophobic region and alter the physical properties of the biomembranes. As a result, the channel proteins in biomembranes would be indirectly influenced by LAs through changes in the physical properties of the membranes. Some groups have focused on the hydrophobicity of LAs [8] and examined the interactions between phospholipid membranes and LAs using nuclear magnetic resonance (NMR) [9,10]. In addition, it has been reported that the structures of membrane proteins are influenced by the bending rigidity of lipid membranes [11]. Some studies have found that the membrane tension mediated by a mechanical stimulus affects the function of ion channels [12]. As shown in these cases, modulation of the function of membrane proteins via changes in the physical properties of

the membrane, such as bending rigidity and membrane tension, is central to understanding the function and behavior of membrane proteins.

The importance of indirect interactions between channel proteins and LAs has attracted much attention, thus the interactions between biomembranes and LAs are worthy of investigation. Biomembranes, which are mainly composed of a phospholipid bilayer structure, are known to be involved in signal transduction mechanisms, such as vesicle formation [13]. Furthermore, it is believed that compositional heterogeneity emerges spontaneously in biomembranes. These heterogeneous structures are known as a "lipid rafts" [14,15]. The lipid raft is a specific region containing a large amount of cholesterol (Chol) and saturated phospholipids, as well as some functional proteins including ion channel proteins and membrane receptors. Therefore, lipid rafts are thought to be important platforms for signal transduction. Additionally, sodium ion channel proteins, which can be affected by LAs, are also known to exist in lipid rafts [16]. Hence, it is important to clarify the correlation between lipid rafts and biosignal transductions such as those that occur in anesthesia.

Due to the complexities of biomembranes, however, it is difficult to investigate the stability of the lipid raft by adding LAs. Thus, to reveal the interactions between lipid rafts and additive molecules such as LAs, liposomes—regarded as model biomembranes—are widely used. In particular, typical multicomponent lipid membranes consisting of unsaturated lipids, saturated lipids, and Chol exhibit phase separation between the unsaturated lipid-rich liquid-disordered (L_d) phase and the saturated lipid/Chol-rich liquid-ordered (L_o) phase [17–22]. Since the L_o phase is composed of saturated lipids and Chol, it is considered a suitable model of the raft region. The adsorption of LAs onto solid-ordered (S_o) phase, consisting of

saturated lipids and L_o phases, has been investigated by quartz crystal microbalance with dissipation [23,24]. Furthermore, we have observed the suppression of phase separation on raft-mimetic liposomes composed of unsaturated and saturated lipids and Chol via microscopy. Since the fluidity of the L_d phase decreases on the addition of LAs and the fluidity gap between the L_o and L_d phases reduces, these events result in the suppression of phase separation [25]. Conversely, in the case of binary lipid mixtures consisting of unsaturated and saturated lipids, the fluidity difference between the S_o and L_d phases is not adequately reduced. Consequently, separation of the S_o/L_d phase is not suppressed by LAs [25].

Furthermore, from a physiological aspect, anesthesia via LAs can be enhanced by dosing at higher temperatures [26]. Therefore, it is also important to discuss the thermal stability of the L_o phase in the presence of LAs. Differential scanning calorimetry (DSC) measurements have revealed that the thermal stabilities of the S_o and L_o phases are decreased by LAs [23,24,27]. Gray *et al.* revealed that lowering the miscibility temperature, defined as the temperature for the transition between phase separation and the homogeneous phase, via the addition of liquid general anesthetics using giant plasma membrane vesicles isolated from living cells, correlates with the strength of general anesthetics [28]. Although the effects of LAs depend on the presence of Chol, the relationship between LAs and Chol is not fully understood.

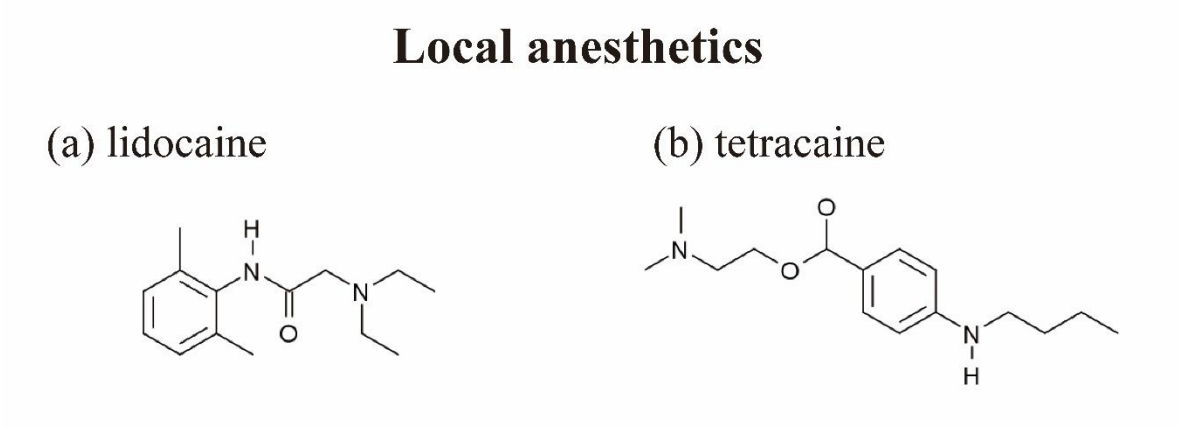
In this study, we investigated the thermal stability of the phase-separated structures in biomimetic lipid membranes containing LAs. First, the miscibility temperatures in unsaturated lipids, dioleoylphosphocholine (DOPC)/ saturated lipids, dipalmitoylphosphocholine (DPPC)/LAs and DOPC/DPPC/Chol/LAs were measured by

fluorescence microscopy. Next, we measured the line tension at the L_o/L_d interface from the fluctuation of the domain boundary in DOPC/DPPC/Chol/LAs to examine the stability of the domain. Finally, DSC measurements were used to clarify the lipid transition temperatures in LA-containing lipid membranes. From the results of the DSC experiments, it was considered that LAs are partitioned as coexisting phases. Moreover, we discussed the influence of LAs on the thermal stability of phase-separated domains in multicomponent lipid membranes.

3-2.Materials and methods

3-2-1. Materials

Unsaturated lipids, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), saturated lipids, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and Chol were purchased Avanti Polar Lipids (Alabaster, USA). Fluorescent probes, Rhodamine B 1,2-dihexadecanoyl-*sn*-glycero-3 phosphoethanolamine, triethylammonium salt (Rhod-DHPE) was purchased from Thermo Fisher Scientific (Waltham , USA). Rho-DHPE molecules are mainly located in the DOPC-rich L_d phase. The LAs 2-(diethylamino)-*N*-(2,6-dimethylphenyl)acetamide (lidocaine) and 2-(dimethylamino)ethyl 4-(butylamino)benzoate (tetracaine) were purchased from Nacalai Tesque (Kyoto, Japan) and Tokyo Chemical Industry (Tokyo, Japan), respectively. Ultrapure water (specific resistance ≥18 MΩ) was obtained using a Millipore Milli-Q purification system. The chemical structures of the lipids and LAs are shown in Fig.3-1.



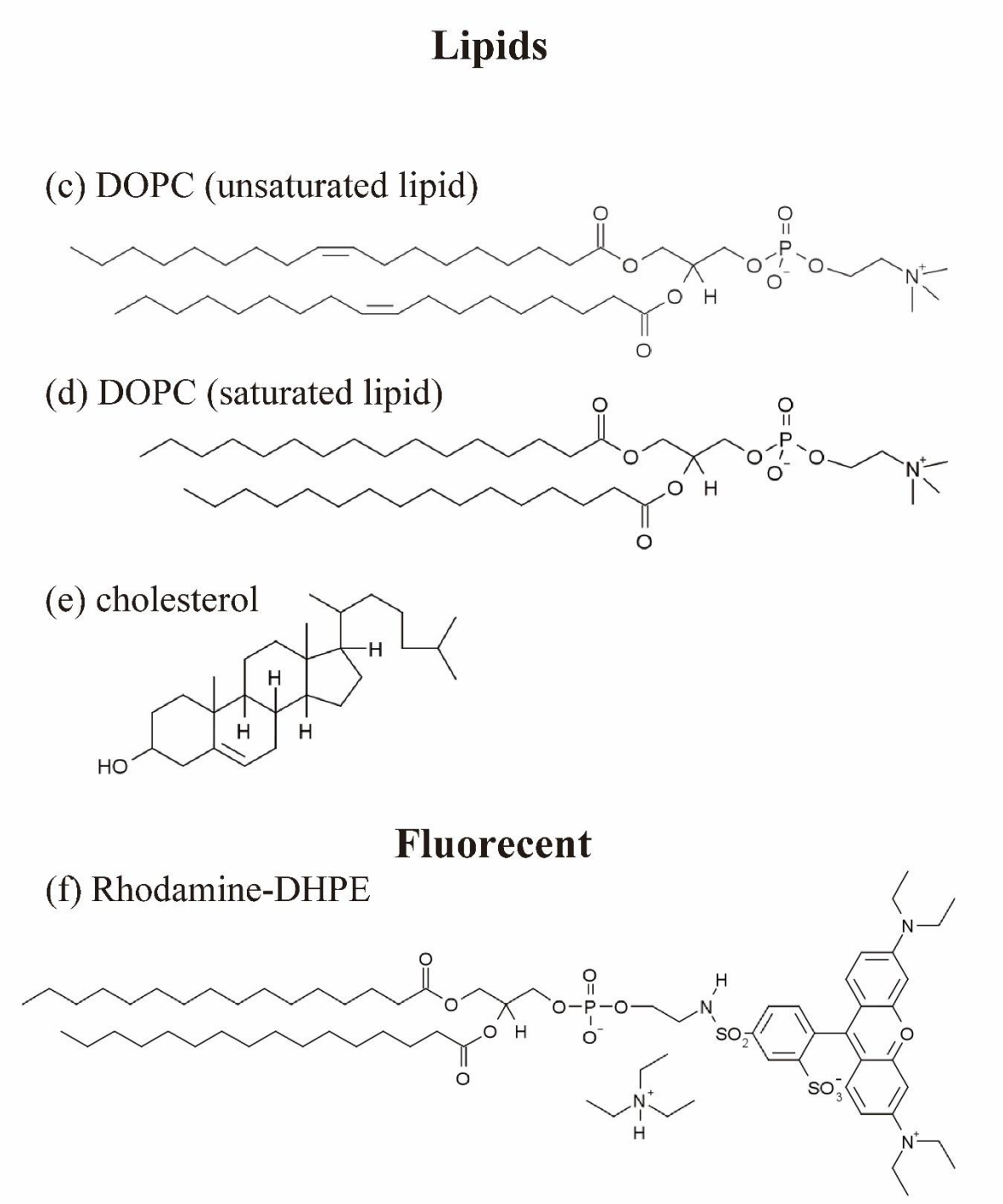


Fig.3-1 Chemical structures of lidocaine (a) and tetracaine (b), DOPC (c) and DPPC (d), cholesterol (e), Rhodamine-DHPE (f) respectively.

3-2-2. Preparation of liposomal solution for microscopic observation

Liposomes for microscopic observation were prepared by the natural swelling method. Lipids (DOPC, DPPC, Chol), LAs (lidocaine, tetracaine), and the fluorescent probe (Rhod-DHPE) were dissolved in chloroform, at concentrations of 2 mM, 0.5 mM, and 0.1 mM, respectively. The stock solutions were transferred into glass test tubes and mixed to the desired compositions. The organic solvent was evaporated under a flow of nitrogen gas, and the lipids were further dried under vacuum for least 3 h to form thin lipid films. The films were then hydrated overnight with Milli-Q water at 55 °C to produce unilamellar liposomes. The final concentrations of lipids and LAs were 0.2 mM, and that of Rhod-DHPE was 1μM.

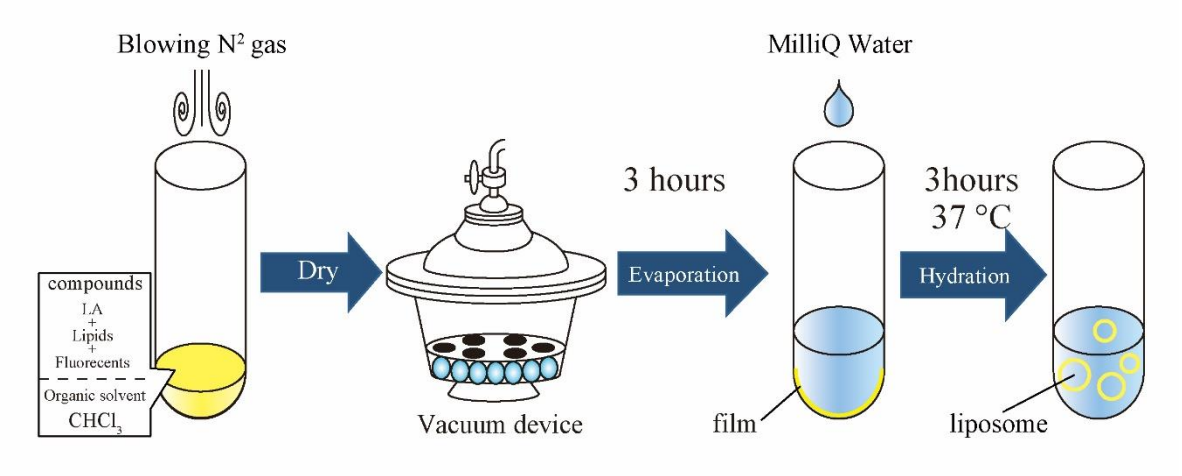


Fig.3-2 Schematic of liposomal preparation for microscopic observation

3-2-3. Microscopic observation of phase separation on liposome

To observe the effects of LAs on phase separation by fluorescent microscopy, we added LAs to DOPC/DPPC lipid mixtures with a DOPC:DPPC ratio of 1:1, and to the DOPC/DPPC/Chol lipid mixtures with a DOPC:DPPC:Chol ratio of 2:2:1. Therefore, the compositions examined were DOPC/DPPC/LAs 50:50:0, 45:45:10, 40:40:20, and DOPC/DPPC/Chol/LAs 40:40:20:0, 36:36:18:10, and 32:32:16:20. The sample temperature was controlled using a microscope stage (MATS-555MORA-BU, Tokai hit) at 20–34 °C.

We counted 30 liposomes in each composition at each temperature, and plotted the fraction of phase-separated liposomes. Based on the experimental plots, we calculated the miscibility temperature (T_{mix}) for the multicomponent lipid membranes. The miscibility temperature is defined as the temperature at which the fraction of phase-separated liposome reaches 50%. To obtain the miscibility temperature, the experimental results were fit with the sigmoidal Boltzmann function:

$$P = \frac{1}{1 + \exp[(T - T_{\mathrm{mix}})/dt]}, \quad -(1)$$

where P is the fraction of phase-separated liposomes, T is the temperature, T_{mix} is the miscibility temperature, and dt is the slope of the sigmoidal curve.

3-2-4 Line tension measurement by flicker spectroscopy of domain boundary fluctuation

We obtained the line tension from the domain boundary fluctuation of a liquid domain [33,34]. Domains were imaged for 1 sec at 30 frames per second. Traces of the domain boundary were obtained from binarized images using Image J. The radius from the center of mass of a domain r as a function of the polar angle γ was represented in terms of a Fourier series expansion,

$$r(\psi) = r_{\mathrm{av}} \left[1 + a_0 + \sum_{k=1}^{\infty} a_k \cos(k\psi) + \sum_{k=1}^{\infty} b_k \sin(k\psi) \right], \qquad -(2)$$

where r_{av} is the average domain radius, k is the mode number, and a_k, b_k are the Fourier coefficients. The excess free energy arises due to the fluctuation and is expressed as

$$\Delta F \simeq \frac{\pi r_{\mathrm{av}}}{2} \gamma \sum_{k=2}^{\infty} (k^2 - 1)(a_k^2 + b_k^2), \qquad -(3)$$

where γ is the line tension. The free energy for each independent mode becomes $k_{\mathrm{B}}T$ from the generalized equipartition theorem, where k_{B} is the Boltzmann constant. Therefore, we obtain the fitting equation,

$$\langle a_k^2 \rangle + \langle b_k^2 \rangle = \frac{2k_B T}{\pi r_{av} \gamma} \left(\frac{1}{k^2 - 1} \right), \qquad -(4)$$

where <…> means the average value of 30 images. The experimental data was fitted with Eq. (4) and we obtained the line tension γ.

3-2-5. Preparation of lipid/LA suspension for DSC experiment

Colloidal solutions for DSC experiment were prepared by natural swelling method and film peeling by ultrasonic wave (Fig.3-3). DPPC and Chol, LAs were dissolved in chloroform, and their concentrations were 300mM, 150mM, 150mM respectively, and conserved in the freezer (-20 °C). These solutions were taken into test tubes, and mixed at the desired composition to give a total volume of 60 μl. The mixed solutions were dried under gentle blowing of nitrogen gas, and formed into lipid films. To evaporate all remaining solvent, samples were dried under vacuum for at least 3 hours. Dried lipid films were hydrated with Milli-Q water (60 μl), and sonicated for 1 hour at least 1 time at 50-60 °C to make them peeled off from the bottom of the test tube. The final concentrations of lipids/LAs mixture in liposomal solution was 150mM. The examined compositions are DPPC/LAs=100:0,

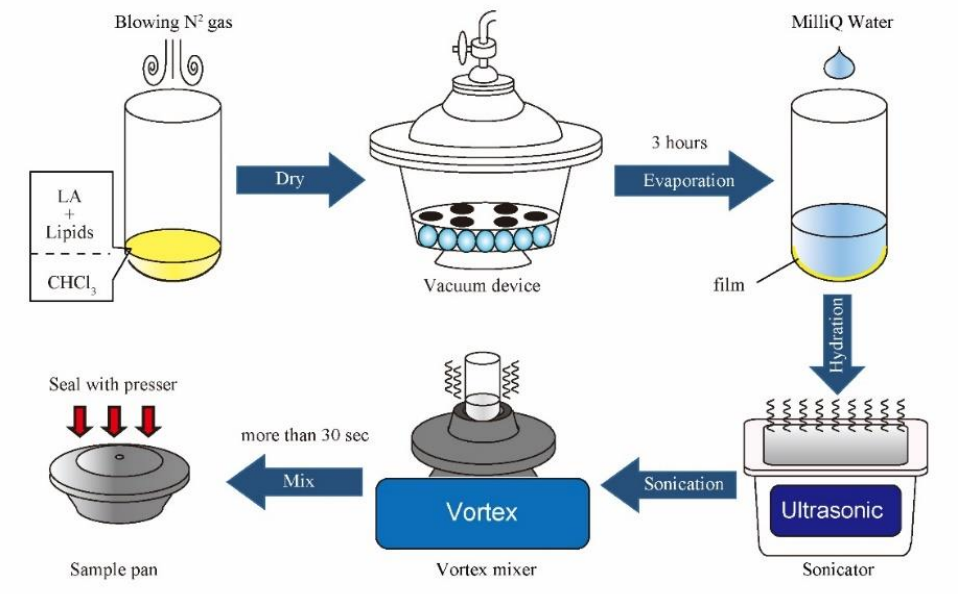


Fig.3-3 Schematic of the colloid solution preparation process

97.5:2.5, 95:5, 92.5:7.5, 90:10 and DPPC/Chol/LAs=90:10:0, 87.75:9.75:2.5, 85.5:9.5:5, 83.25:9.25:7.5, 81:9:10.

3-2-6. DSC experiment

Thermographs were obtained by a DSC-822e (Mettler Toledo International Inc., Switzerland). 12~15μL of liposomal solutions were placed into aluminum sample pans after stirring by vortex mixer for over 30 s. We used sample pans filled with Milli-Q water as reference cells. The weight of the water was identical in both the reference and sample cells. Heating/cooling cycles were performed three times between 20 °C and 60 °C, and the third heating process is shown as a representative thermograph. Heating and cooling rates were set at 5 ° C / min. Each heating/cooling scan was started after a 3–5 min pre-scan incubation at 20/60 °C. All raw data were normalized by the weights of the samples. The same measurements were performed at least three times to ensure reproducibility of the data.We found some asymmetrical peaks at several compositions and they could be described as a linear combination of two independent transitions. An asymmetrical peak is expressed as a linear combination between two Lorentzian functions as shown in Fig.3-4.

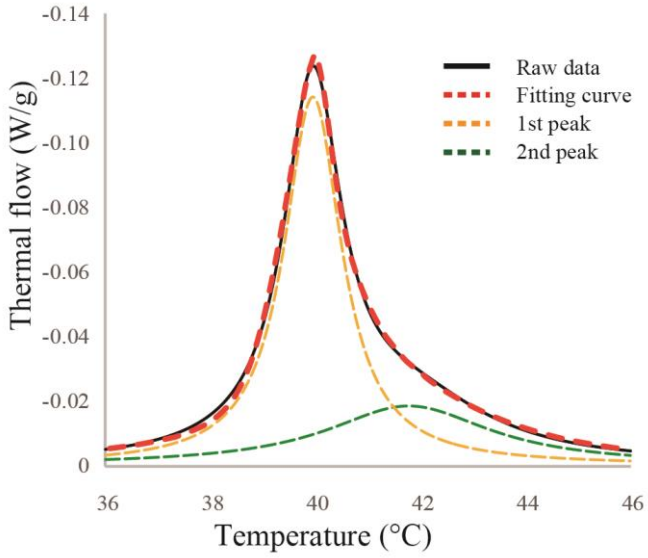


Fig.3-4: An example for curve fitting. Black-solid and red-dashed lines indicate experimental data and calculated fitting curve, respectively. Yellow- and green-dashed lines are the curves for two independent transitions, and the sum of these curves corresponds to the calculated fitting curve (red-dashed line).

3-3. Result

3-3-1. Miscibility temperature measurement in LAs-containing lipid membranes

We observed the effects of LAs on the thermostability of phase-separated structures in multicomponent lipid membranes. First, we investigated the lipid mixtures consisting of DOPC/DPPC/LAs without Chol. In this experiment, we fixed the ratio of DOPC/DPPC to 1:1 and set the LA concentrations to 0%, 10%, and 20%. At lower temperatures, coexistence between the DOPC-rich L_d phase and DPPC-rich S_o phase was observed, as shown in Fig. 3 (a). Rhod-DHPE is localized in the L_d phase; the bright and dark regions correspond to the L_d and S_o phases, respectively. The anisotropic shape domains can be regarded as S_o domains. To examine the thermostability of phase-separated domains, we measured the fraction of phase-separated liposomes as the temperature increased. The results following the addition of lidocaine or tetracaine are shown in Fig. 3-5 (b) and (c), respectively. In all cases, the fractions of the phase-separated liposomes decreased as the temperature increased. This is because the mixing entropy becomes predominant at higher temperatures. The temperature at which the fraction of phase-separated liposomes reaches 50% is the miscibility temperature (T_{mix}). To identify the miscibility temperature of each lipid mixture, we fit the experimental data with Eq. (1). The obtained miscibility temperatures were T_{mix} = 33.1 °C (control), 32.9 °C (lidocaine 10%), 32.1 °C (lidocaine 20%), 32.7 °C (tetracaine 10%), and 31.8 °C

(tetracaine 20%). We did not observe any significant changes in the miscibility temperatures after adding LAs. Therefore, S_o/L_d phase separation was not significantly affected by the LAs. Next, DOPC/DPPC/Chol/LAs lipid mixtures were investigated to gain an understanding of the effects of Chol and LAs on phase separation. We fixed the DOPC/DPPC/Chol ratio to 2:2:1 and set the LA concentrations to 0%, 10%, and 20%. In the presence of Chol, the S_o phase transforms into the L_o phase [17]. Therefore, L_o/L_d phase separation is observed in most of the liposomes at lower temperatures as shown in Fig. 3-5(d). Here, the bright and dark regions correspond to the L_d and L_o phases, respectively. The miscibility temperatures were T_{mix} = 27.7 °C (control), 26.6 °C (lidocaine 10%), 23.0 °C (lidocaine 20%), 23.2 °C (tetracaine 10%), and 21.6 °C (tetracaine 20%). Although we did not find any significant changes in the miscibility temperatures in DOPC/DPPC/LA systems without Chol, they clearly decreased as the LA concentrations increased in lipid mixtures with Chol. The reduction in miscibility temperature with tetracaine was greater than that with lidocaine.

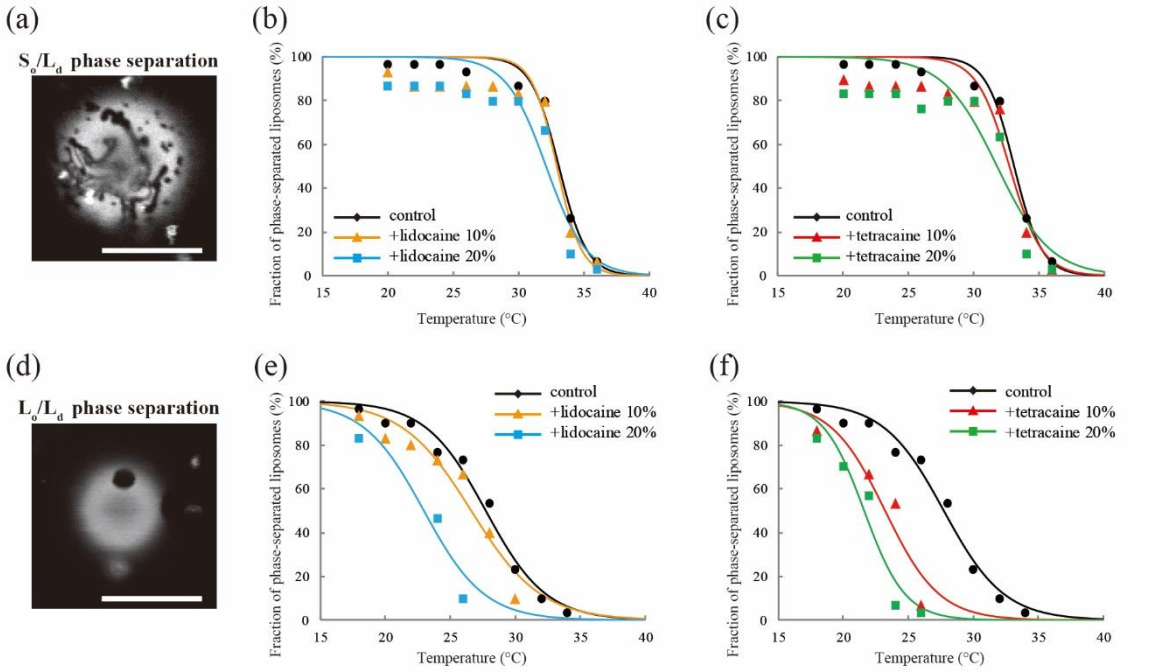


Fig.3-5 Microscopic images of S_o/L_d (a), and L_o/L_d phase-separated liposomes. Fluctuation of phase separation of LA-added DOPC/DPPC (50/50 mol%) (b) (c) and DOPC/DPPC/Chol (40/40/20 mol%) (d) (e) in each temperature. Control in all systems are shown in black-circles, and LA added membranes are shown in colored triangles (LA 10 mol%) and square (LA 20 mol%). Lidocaine-added membranes are colored with orange and blue, and tetracaine-added membranes are colored

3-3-2. Line tension measurement at liquid domain boundary in LAs-containing lipid membranes

Line tension at the domain boundary is one of the most important factors to discuss the stability of phase-separated domains. Generally, the line tension at L_o domain boundary is about ~3 pN [34]. In this subsection, we obtained the line tension at L_o domain boundary in LA-containing lipid membranes by flicker spectroscopy of domain boundary fluctuation. We analyzed several images in which an isolated domain exists at the center of the liposome surface. Because this analysis calculates displacement from the circular shape, we ignore the non-circular shape domain as shown in Fig.3-6(a). The domain boundary fluctuation was imaged for 1 sec at 30 frames per second in Fig.3-6(a), the trace of domain boundary is obtained by image J as shown in Fig.3-6(b), the radial fluctuation is plotted in Fig.3-6(c), the power spectrum calculated from the radial fluctuation is shown in Fig.3-6(d), the averaged Fourier coefficients obtained from 30 images are plotted in Fig.3-6(e), and the line tension can be calculated from the slope of the plot with Eq.(4).

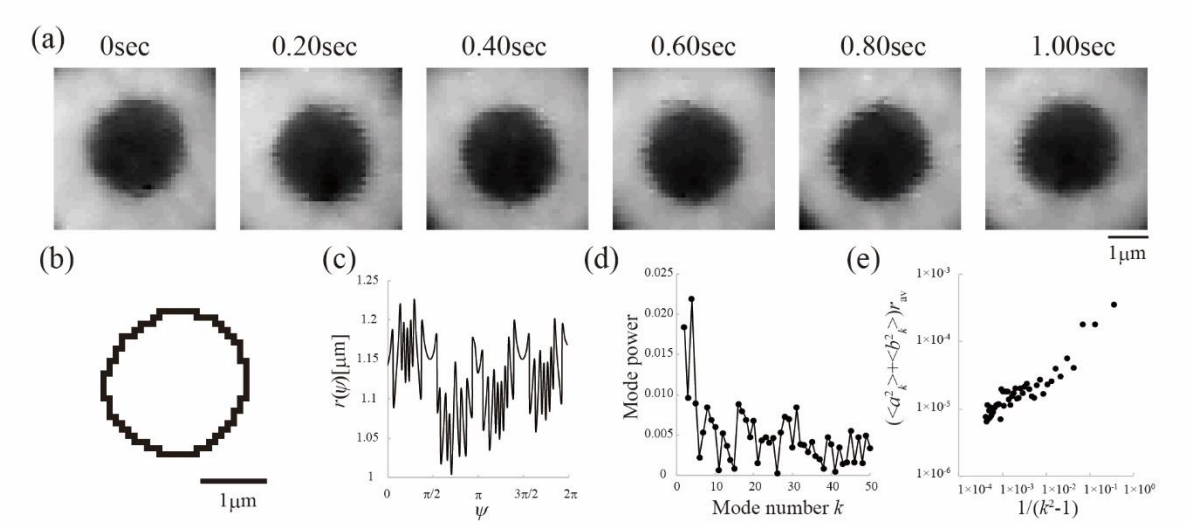


Fig.3-6 Analytical sequence of line tension at L_o/L_d boundary. (a) Sequential images of L_o domain fluctuation on DOPC/DPPC/Chol membrane at 28 °C . (b) Traced outline of domain boundary determined by image banalization. (c) Radial fluctuation as a function of polar angle ψ. (d) Power spectrum calculated from (c). (e) Average Fourier coefficients obtained from 30 images plotted against $1/(k^2-1)$.

The obtained values of line tension are summarized in Fig.3-7. Without LAs, the line tension is about 3 pN at room temperature (20-22°C) and this value is in good agreement with that reported in the previous studies [34]. As the temperature is increased, the line tension is decreased just before the miscibility temperature. When the temperature is increased, and approaches the miscibility temperature, the composition difference between the coexisting two phases becomes smaller generally. Therefore, the line tension is reduced, since the differences in physical properties between the two phases (spontaneous curvature, membrane thickness, and chain ordering) becomes smaller. In the systems with LAs, we can see the same tendency. In particular, the line tension becomes smaller as compared with that obtained in the system without LAs. Moreover, the line tension is decreased in LA-concentration

manner. The line tension reduction for tetracaine-containing membranes is larger than that for lidocaine-containing membranes. This tendency is almost consistent with the result in the miscibility temperature measurement. This measurement clearly shows that LAs strongly decreases the line tension at L_o/L_d domain boundary. We will explain why LAs reduce the line tension in Discussion.

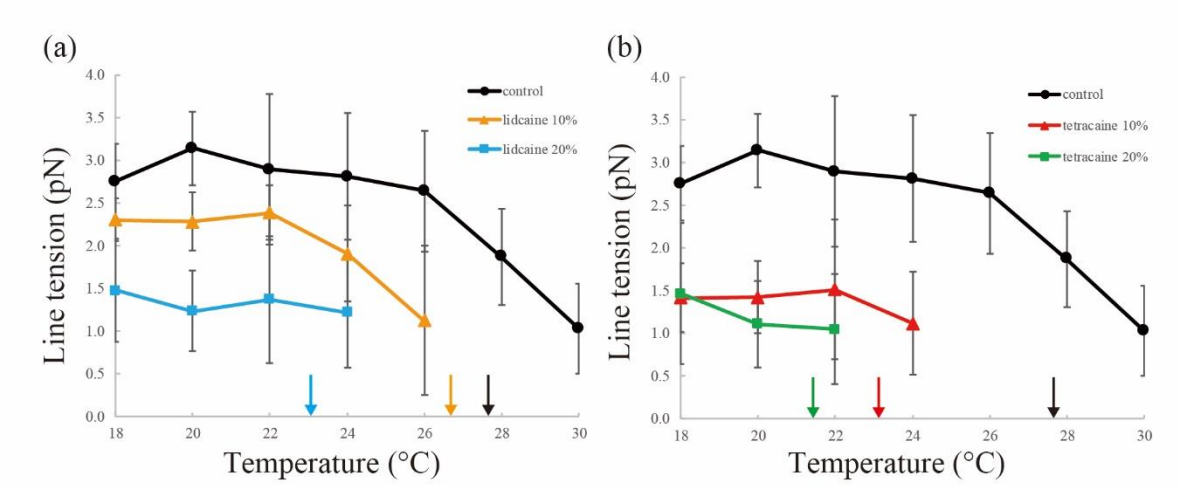


Fig.3-7 Line tension at the boundaries of L_o/L_d phase separation on LA-added DOPC/DPPC/Chol membranes. Circles with black lines show control system, and colored triangles and squares show LA 10 and 20 mol% added membranes. Orange and blue lines show lidocaine-added membranes (a), and red and green lines show tetracaine-added membranes (b). T_{mix} measured by microscopic observations are shown as colored arrows which are correspond to membranes labeled with same colored lines and markers.

3-3-3. DSC measurement in LAs-containing lipid membranes

Next, we discuss the effects of LAs on the thermostability of lipid membranes based on DSC. The representative thermographs for DPPC/LA mixtures are shown in Fig. 3-8. Sharp peaks can be seen at 42 °C that corresponded to the main transition of the lipids between solid (ripple) phase and liquid phase, and the broad peak at 37 °C corresponded to the pretransition state between the solid and ripple phases in the DPPC single component system without LAs, shown as black lines in Fig. 3-8 (a) and (b). This thermograph is consistent with some previous reports [17,21,22]. Since the pretransition peak disappears as the LA concentration increases, we focused on the main transition peak. When both lidocaine and tetracaine were present, the main transition peak shifted toward a lower temperature as the LA concentration increased. Interestingly, the shape of the peak became asymmetrical at lidocaine concentrations of 7.5% and tetracaine concentrations of 5%. We presumed that the asymmetrical peak could be described as a linear combination of two independent transitions. The results of peak deconvolution are shown in Fig. 3-8 (c)–(f). We expressed an asymmetrical peak as a linear combination between two Lorentzian functions. Figure 3-8 (c) and (d) show stronger peaks at higher temperatures, corresponding to the transitions of the DPPC-rich phase. On the other hand, the weaker peaks at lower temperatures, shown in Fig. 3-8 (e) and (f), correspond to the transition of the LA-rich phase. Therefore, as the concentration of the LA increased, some molecules could not dissolve in the DPPC membranes, which led to the formation of an LA-rich phase. This result implies a low affinity

between DPPC and LAs. Moreover, compared with lidocaine, tetracaine seemed to show lower affinity to DPPC.

We also performed DSC measurements for the mixtures of DPPC/Chol/LAs. The thermograph of the DPPC/Chol binary mixture without an LA is denoted by a black line in Fig. 3-9 (a) and (b). A clear peak can be seen around 41.5 °C. It has been reported that phase separation between S_o and L_o phases occurs at a DPPC/Chol ratio of 90:10 [34]. As per the DPPC/LA mixtures, we performed peak deconvolution based on a two-state transition; the strong and weak components are shown in Fig. 3-9 (c, d) and (e, f), respectively. These strong and weak peaks correspond to the DPPC-rich S_o and Chol-rich L_o phases, respectively [34]. In Fig. 3-9 (c) and (d), the stronger peaks shift toward a lower temperature as the LA concentration increased. This behavior resembles that of DPPC-rich phase in DPPC/LA binary mixtures, as indicated in Fig. 6. On the other hand, the positions of the weaker peaks were not significantly changed by the addition of LAs, as shown in Fig. 3-9 (e) and (f). In Fig. 3-10, we summarized the temperature shifts of both the strong and weak peaks. The temperature shift of the S_o phase in the DPPC/LA mixtures was almost identical to that of the S_o phase in the DPPC/Chol/LA mixtures. Since an LA-rich phase is formed in DPPC/LA mixtures, the S_o phase in DPPC/LAs can be considered an LA-poor phase. Therefore, we speculate that the S_o phase in DPPC/Chol/LA mixtures also becomes a LA-poor phase. On the other hand, the clear LA-rich phase was not found in the DPPC/Chol/LA mixtures. In DPPC/Chol/LAs systems, we believe that large amounts of LAs are included in the L_o phase. We further discuss LA localization in heterogeneous membranes based on the DSC results in the Discussion.

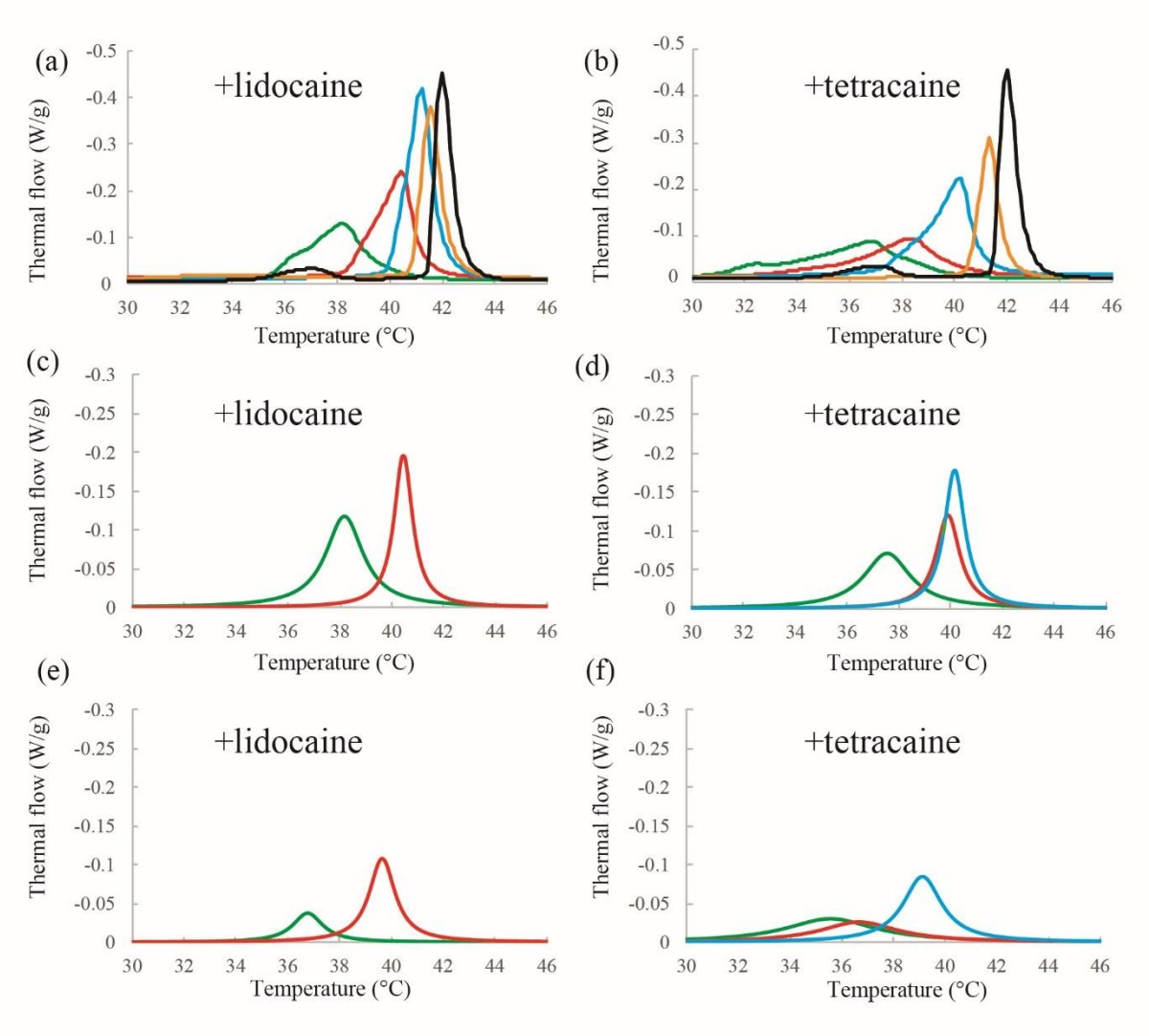


Fig. 3-8 (a, b) Representative DSC thermographs of DPPC/lidocaine and DPPC/tetracaine membranes, respectively. Black, orange, blue, red, and green lines indicate LA at 0, 2.5, 5, 7.5, and 10 mol %, respectively. (c, d) Stronger peaks obtained from peak deconvolution in the DPPC/lidocaine and DPPC/tetracaine membranes, respectively. (e, f) Weaker peaks obtained from peak deconvolution in DPPC/lidocaine and DPPC/tetracaine membranes, respectively. Since the peak shape is almost symmetrical for lidocaine below 5 mol % and tetracaine below 2.5 mol %, we did not perform peak deconvolution in (c, d, e, or f).

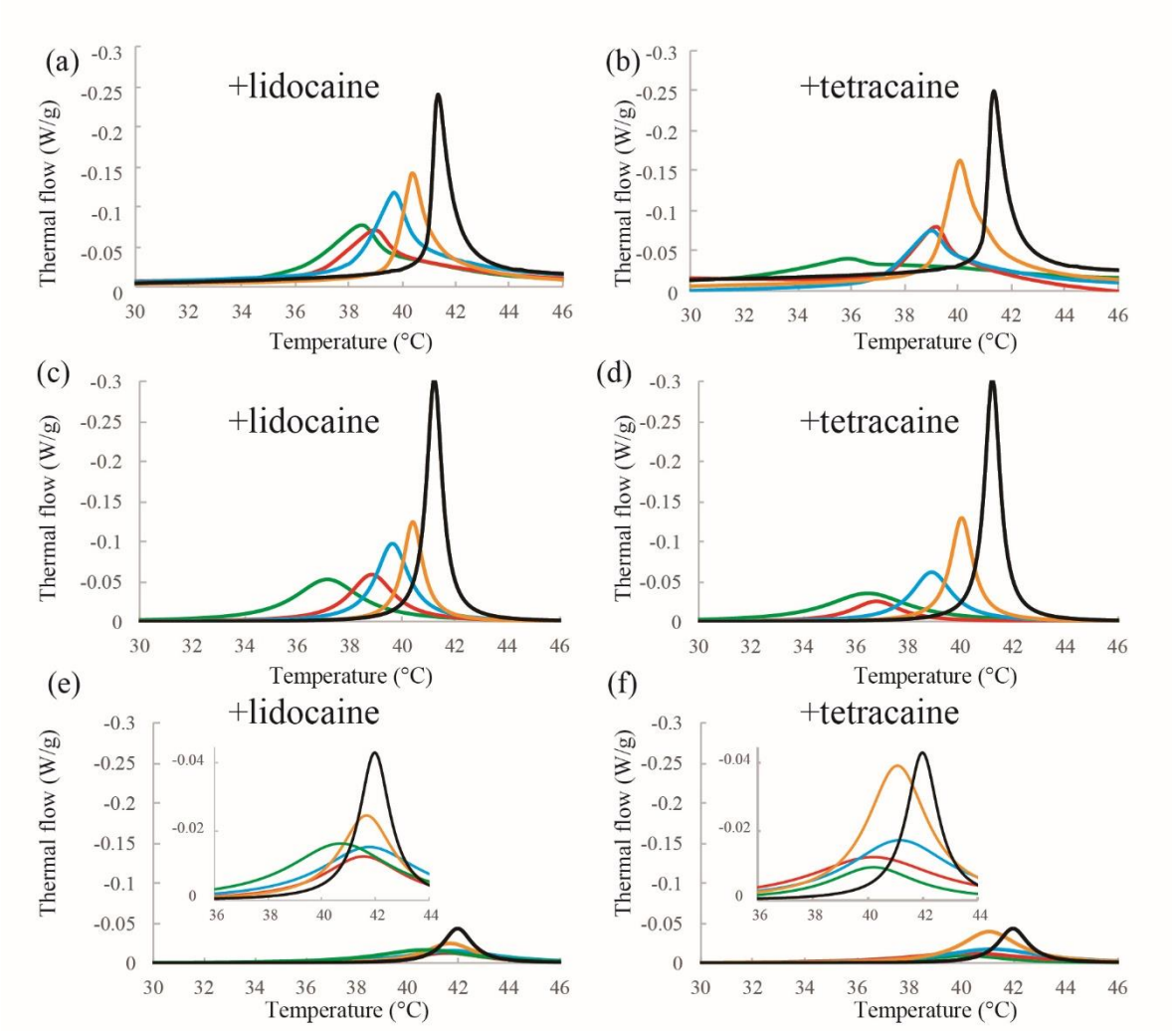


Fig. 3-9 (a, b) Representative DSC thermographs of DPPC/Chol/lidocaine and DPPC/Chol/tetracaine membranes, respectively. Black, orange, blue, red, and green lines indicate LA at 0, 2.5, 5, 7.5, and 10 mol %, respectively. (c, d) Stronger peaks obtained from peak deconvolution in DPPC/Chol/lidocaine and DPPC/Chol/tetracaine membranes, respectively. (e, f) Weaker peaks obtained from peak deconvolution in DPPC/Chol/lidocaine and DPPC/Chol/tetracaine membranes, respectively. Insets show magnifications of the peaks.

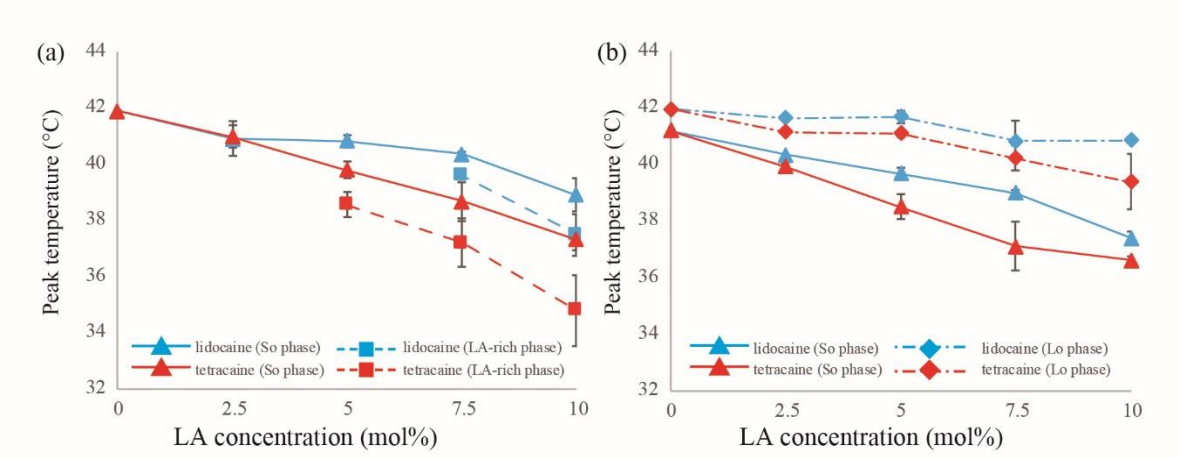


Fig.3-10 (a) The shifts of peak temperatures obtained from the peak deconvolution of the DSC thermographs in DPPC/LAs mixtures as a function of LA concentration. Blue and red lines represent lidocaine and tetracaine-containing membranes, respectively. Solid and dashed lines indicate the temperature shifts of the stronger peak (S_o phase) and the weaker peak (LA-rich phase), respectively. (b) The shifts of peak temperatures obtained from the peak deconvolution of the DSC thermographs in DPPC/Chol/LAs mixtures as a function of LA concentration. Blue and red lines represent lidocaine and tetracaine-containing membranes, respectively. Solid and dot-dashed lines indicate the temperature shifts of the stronger peak (S_o phase) and the weaker peak (L_o phase), respectively.

3-4. Discussion

Microscopic observation revealed that the miscibility temperature (T_{mix}) of the phase-separated structures on DOPC/DPPC membranes was not changed significantly by adding LAs (Fig. 3-5 (b) and (c)). Furthermore, according to the results of the DSC experiment on DPPC/LA mixtures, an LA-rich phase was formed at higher concentrations of LAs (Fig. 3-8 (a) and (b)), which suggests low affinity between DPPC and LAs. Therefore, most LA molecules can be partitioned into DOPC-rich L_d phases in DOPC/DPPC/LAs membranes. LAs did not impart any crucial effects on the DPPC-rich S_o phase, and the miscibility temperature in DOPC/DPPC/LA mixtures was not shifted by them.

On the other hand, as the concentrations of LAs increased, the miscibility temperatures for DOPC/DPPC/Chol/LAs lipid membranes decreased and the thermostability of the L_o phase was lowered (Fig. 3-5(e) and (f)). From the results of the DSC experiments, the shift in the peak temperature of the S_o phase in the DPPC/LA mixtures was almost identical to that of the S_o phase in the DPPC/Chol/LA mixtures (Fig. 3-10). Therefore, we can consider that the amounts of LAs in both S_o phases were almost the same. The DSC results for the DPPC/LA mixtures indicated that the S_o phase corresponded to the LA-poor phase. Based on this finding, the S_o phase for DPPC/Chol/LA mixtures can be also regarded as the LA-poor phase. In addition, we could not find a clear LA-rich phase among the DPPC/Chol/LA mixtures. Therefore, a large amount of LA molecules may be included in the L_o phase in these lipid mixtures. This implies that the presence of Chol promotes the partitioning of LAs. Although a large amount of LAs may have been partitioned into the DOPC-rich L_d phase in

DOPC/DPPC/Chol/LAs membranes, some was included in the L_o phase. The LA molecules in the L_o phase decreased the thermostability significantly. We can think of two mechanisms that explain why the presence of Chol enhances the partitioning of LAs into L_o phase. First, the DPPC-rich phase transitions to the liquid phase from the solid phase in the presence of Chol. Therefore, LA can be partitioned into the L_o phase, because Chol loosens the packing between the DPPC molecules. This hypothesis indicates that Chol promotes the partitioning of LAs into L_o phase indirectly. Second hypothesis is a direct attraction between LAs and Chol. Because LAs and Chol are hydrophobic molecules, they are buried in the hydrophobic region of lipid membranes and may directly interact each other. This mechanism could be revealed by a future NMR study.

The distribution of LAs into the L_o phase is also supported by the line tension measurements. The LAs decreased the line tension at the L_o/L_d domain boundary in DOPC/DPPC/Chol membranes (Fig. 3-7). Some studies have mentioned that the additive molecules adsorb onto the domain interface and reduce line tension [35–40]. Such molecules are called ``linactant" based on an analogy to surfactant [38]. Although the line energy at the domain boundary is decreased by the adsorption of linactants, entropic loss arises instead of line energy gain. Therefore, it is difficult for a molecule to be a linactant, because it would have to possess a structure with an affinity for both coexisting phases. Since the LAs used in this study do not seem to have such structures (see Fig.3-1), they may not be linactants. Therefore, we think that some LAs are incorporated within the L_o phase, as mentioned above. As a result, the chain ordering of the L_o phase is disturbed by the presence of LAs. The differences in physical properties between the two coexisting phases (spontaneous curvature, membrane thickness,

chain ordering) becomes smaller, which decreases the line tension. This mechanism was indicated by electron spin resonance [41], and Laurdan generalized the polarization measurements among different lipid mixtures [42]. On the other hand, according to our DSC experiments, the transition temperatures of lipids in the L_o phase were not significantly changed by adding LAs. A similar tendency has been reported in some other studies [24,27]. However, it is important to investigate the relationship between chain ordering and transition temperature in future studies.

Tetracaine showed more significant effects than lidocaine on the physical properties of the lipid membranes, such as miscibility temperature, line tension at the L_o/L_d interface, and main transition temperature. These findings are consistent with the results of a previous study, which reported that tetracaine suppresses phase separation at room temperature more strongly than lidocaine [25]. Moreover, Paiva *et al*. showed that tetracaine has a stronger effect than lidocaine on the transition temperature of raft-mimetic membranes [23]. Generally, tetracaine is a more powerful and toxic LA than lidocaine [43]. In other words, tetracaine shows anesthetic and cytotoxic effects at lower concentrations than lidocaine. Thus, from these results, the potencies of the LAs and suppression of phase separation may be associated. Furthermore, *n*-alcohol general anesthetics are reported to correlate with pharmacological strength and membrane phase behavior [28]. As the length of the hydrocarbon tail of alcohol becomes longer, the lipid transition temperature decreases and the strength of the anesthesia becomes stronger. Therefore, increased hydrophobicity leads to increased anesthetic action. Moreover, an LA oil/water partitioning experiment showed that the action of the anesthetic and the molecular hydrophobicity are correlated [44]. On the other hand, we suggested the

importance of the affinity between the L_o phase and LAs. Thus, the relationship between the molecular hydrophobicity of LAs and anesthetic action based on the stability of the L_o phase on the addition of LAs should be evaluated in future studies by NMR investigation of the interactions between LAs and the L_o phase.

Since the large part of LAs is hydrophobic, we discussed the phase behavior based on the hydrophobic interaction between lipid membranes and LAs. LAs are medically used as chloride salts to increase the water solubility. Although LAs were included in lipid membranes directly in our study, LAs were added to lipid membranes by dissolving them in water phase in some studies [33]. However, because the experimental results about the main transition temperature shift and phase behavior are almost consistent with our study, the behavior of charged LAs is similar to that of uncharged LAs. Therefore, we believe that the hydrophobic interaction between LAs and lipid membranes is the most important factor. Moreover, in actual living cells, the electric charges on LAs are well screened by many kinds of cations in the vicinity of cell membranes, and the effect of electric charges on LAs may be negligible. On the other hand, some studies mentioned about the importance of electric charges on the phase separation in multicomponent lipid membranes [45]. The studies on the hydrogen bonding between LAs and phospholipid and the effect of LAs on the hydration water near the surface of LAs-containing lipid membrane as well as the electric charge of LAs will be important in near future.

We showed that the raft-mimicking structure (L_o phase) is destabilized by adding LAs. If raft domains are also destabilized by LAs in the cell membranes, the sodium ion channel proteins present may be strongly affected. Voltage-dependent sodium channels form gate structures

that allow selective permeation of specific cations by subunits in the structure-forming multimers [46]. The gate structure is controlled by miniscule structural changes in the proteins of well organized subunits. If the raft structure is altered by LAs, the order, structure, and movement of the subunits forming the gate structure may be impaired. In fact, Brohawn *et al.* found that the function of ion channels placed in membranes isolated from cells can be controlled by external pressure achieved by poking the membranes with a glass capillary [12]. The application of external pressure to membranes is known to change the phase behavior [47], and impact the anesthetic dose. Therefore, the membrane proteins in raft domains are influenced by changes in the physical properties of the membranes, such as rigidity, thickness, fluidity, and phase. Future studies should investigate the mechanisms controlling channel activities via changes in the phase state of the membranes.

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Chapter.4: General conclusion and future perspective

4-1. General conclusion

Through our study, we investigated the effect of LAs presence on phase-separated structure in biomimetic membrane composed with unsaturated phospholipid (DOPC), saturate phospholipid (DPPC), and cholesterol (Chol). Especially, the phase separation on DOPC/DPPC/Chol membrane, which has been regarded as raft-mimetic structure, was destabilized by presence of LAs. We discussed the reasons for destabilization of phase separation by measurement of membrane fluidity and line tension on phase boundaries.

In chaptor.2, initially, we observed formation of phase separation on LAs presented DOPC/DPPC (solid ordered: S_o/liquid disordered: L_d), and DOPC/DPPC/Chol (liquid ordered: L_o/L_d) membranes. This experiment revealed that the presence of LAs suppressed L_o/L_d phase separation in DOPC/DPPC/Chol. On the other hand, S_o/L_d phase separation on DOPC/DPPC liposome was not influenced by LAs. We also clarified the fluidity decrease of DOPC-rich region (L_d) in DOPC/DPPC/Chol liposomes by presence of LAs. Fluidity decrease of L_d phase should make the fluidity difference between DOPC-rich phase and DPPC/Chol-rich phase. This action of LAs should decrease the line energy between L_o and L_d phases, and suppressed phase separation.

In chapter.3, we observed decrease of miscibility temperature of L_o/L_d phase separation in DOPC/DPPC/Chol membrane. Furthermore, based on image analysis, we revealed the line tension decrease at L_o/L_d phase boundary of LA added DOPC/DPPC/Chol liposomes. On the other hand miscibility temperature of S_o/L_d phase separation in DOPC/DPPC liposomes was not influenced by LAs. Finally, we performed Differential Scanning Calorimetry (DSC)

experiments with LA added DPPC and DPPC/Chol membrane to discuss the effect on membrane thermal-stability and localization of LAs in multi-lipid liposomes. Through the DSC experiment with DPPC membrane, addition of LAs decreased the transition temperature of both S_o and L_o membranes in concentration dependent manner. At the same time, in the experiment of DPPC (S_o) membrane with higher LA concentration (5~10 mol%), we observed the shoulder on the lower temperature side on the thermal peaks. On the other hand, we could not observe similar asymmetric shoulder of thermal peaks on DPPC/Chol (S_o/L_o coexist) membranes despite the presence of higher LAs concentration. This phenomena indicates relatively stronger affinity of LAs with L_o phase rather than with S_o phase. Therefore, we discussed localization LAs into DPPC-rich region was enhanced by the contribution of Chol. This localization change of LAs result into decrease of the line tension on phase-boundary, and finally destabilized phase-separated structure.

Interestingly in result of any experiments, relatively stronger and more hydrophobic LA: tetracaine shown stronger impact to expressed membrane properties rather than presence of lidocaine. This fact indicates correlation between LA function and phase behavior of biomembrane. Lacking of heterogeneity on membranes could change various membrane properties like membrane thickness, bending or expansion rigidities, spontaneous curvature, etc. These physical property changes can effect on gaiting or binding abilities of membrane proteins such as ion-channels. Our results are suggesting some correlation between raft destabilization and protein disordering by LAs.

4-2. Future perspective

Our study revealed the destabilization of raft-mimetic structure due to the interaction between LAs. This fact suggested some correlation between physical property change biomembrane and function of LAs. However, we cannot explain all the relation between the action of LA and membrane dynamics from results of our research.

For example, Local anesthetics still have many toxicities and side effects whose mechanisms are not yet clear. Overdose of LA is known to cause painlessness due to cytotoxicity on nerve, allergic reactions, liver dysfunction etc.[1,2], so careful administration by professional technicians is required. Interestingly, it is known that these side effects of local anesthetics are related to the hydrophobicity of each reagent as well as the strength of their pharmacological function [3]. In addition, these toxicities are known to occur on metabolic pathways due to the up taking of LAs into cells. Upon uptake of foreign molecules into cells, biological membranes are involved via vesicular transport [4,5]. Our study also showed an association between the hydrophobicity of the LAs and their impacts on the raft-mimetic structure. It has been shown that the raft structure is involved in molecular transport into cells via endocytosis and the like [6–8]. According to this fact, it is no wonder that the localization of LAs to the biomembrane and the raft structure thereon indirectly leads to toxicity. Therefore, in the future, it is necessary to investigate the relation between up taking of LAs and biomembrane interaction.

First, we examine the influence of local anesthetic addition to actual cells on dynamics such as endocytosis caused by biological membranes with microscopic observation. We study

the effect of LAs on cellular endocytic mechanism by labeling ganglioside GM1 which accumulate in lipid raft [9,10] and tracing its behavior. It has also been found that even on biomimetic membranes, dynamics similar to endocytosis can be induced by the application of osmotic pressure and the surfactant: Triton X-100 [11]. By considering the influence of LAs on dynamics of biomimetic membrane in this way, contribution of LAs can be discussed from the physical aspect. In addition, we will analyze the effect of addition of local anesthetic on cell viability using flow cytometry and consider the relationship between cytotoxicity of LAs.

Furthermore, through our study, correlation between function of membrane protein and membrane dynamics induced by LAs is still unclear. Various signals traveling through the living species such as pain signals are controlled by ionic and cationic cross-membrane flow via the ion channel [12–14]. Structures and functions of these channel proteins were controlled by extremely slight of change of the order and movement of their sub-units [13,15]. Thus, function of channel proteins could be influenced by change of membrane properties, such as its components or fluidity etc., due to the interaction between LAs as we revealed. In fact, Brown *et al*. revealed the function of membrane proteins were influenced by kinetical stimulus conducted through lipid membranes [16]. On the other hand, Booth *et al*. shown the membrane rigidity was influenced on the folding of membrane protein [17]. Thus, the properties of the biomembrane and the stimulation transmitted thereon are related to the structure and function of the membrane protein. Therefore, it is necessary to focus on the protein function and membrane behaviors.

Therefore, for future plan we would like to reveal the effects of physical property change of biomembrane on function of artificial ion-channel which have no binding site for LAs. Furthermore, we need to consider the possibilities of ion-pathways without channel proteins. For examples, we can assume the ion-pathway through the membrane pore formed by application of surfactants or ions [18], and path way via the flip-flop of charged-lipid due to the adsorption on their head groups [19]. Since these membrane pore formation and lipid flip-flop are affected by the rigidity or fluidity of the lipid membrane. Therefore, if formation and ion flow can be clarified using liposomes, it is possible to greatly contribute to elucidation of signal transduction. Therefore, experiments with handling liposomal membranes can give great advance on understanding of correlation between biomembrane and signal transduction.

There is some difficulty to observe the cross-membrane ion-current through liposomal experiment. Therefore, we are planning to handle the membrane potential sensitive fluorescent probe to observe ion-current pass through lipid membrane [20,21]. By this study, we aim to clarify the flow of ions that ultimately dominates signal transmission as well as pain, from the relationship between membrane properties and ion flow.

Firstly, an electrically neutral unsaturated lipid dioleoylphosphatidylcholine (DOPC) and a negatively charged unsaturated lipid dioleoylphosphatidylglycerol (DOPG) will be used for lipids, and NaCl, KCl will be used as salt. When different solutions are used inside or outside the liposome, a solution is prepared using an osmometer so that the liposome is not deformed osmotically. Membrane potential is revealed using di-4-ANEPPS using fluorescence spectrophotometer, fluorescence microscope observation. By using the same salt solution

inside and outside the liposome, the lipid composition is gradually changed from DOPC: DOPG = 10: 0 to 0: 10 to clarify how the static membrane potential changes due to the presence of negatively charged lipid. In addition, by using asymmetric liposome preparation method [22], liposomes whose inner-leaflet are composed with unevenly distributed negatively charged lipids are prepared. By this method, we can generate the liposome which has membrane potential arbitrarily. We are planning to Measure the membrane potential of asymmetric liposomes made in this way, and we also planned to measure the membrane potential when lipid composition or solution salt are changed. Additionally, we are planning to observe the redistribution of membrane potential by flip-flop after standing those membrane for long time.

For next, even in a ternary system having a phase separation structure of neutral saturated lipid dipalmitoylphosphatidylcholine (DPPC), DOPC, Chol, the rest membrane potential will be clarified in various solution conditions and addition of an anesthetic agent. After clarifying the resting membrane potential under each condition, add stimulus and follow the change in potential. As a stimulus, we are planning to add (a) KCl from the outside of the liposome as a model when cells ruptured, (b) the surfactant Triton X-100 as an irritating molecule, and (c) surfactant Tween 20 known as nonirritant to the skin as a negative control [11]. Membrane deformation and change of its potential will be observed in real time with fluorescence microscope when each stimulus are applied. When direct observation with a microscope is difficult, change in membrane potential is quantified by spectroscopic measurement.

Finally, we are planning to perform similar experiment with using the liposome with artificial ion-channel. The research team of Prof. Kimbara, the collaborator of our Lab. in

Tokyo Institute of Technology, had developed the artificial ion-channel that can activate by binding with ligand molecule (2-phenethylamine: PA) [23]. This channel has no specific binding site for LAs. Thus, change of its function due to the application of LAs can be regarded as a result of property change of membrane. First of all, we need to measure resting potential of liposomes with ion-channel. After that, we will add ligand molecules to activate the channels, and measure the membrane potential. Additionally, by adding LAs, we can clarify the contribution of LA application to the behavior of ion channel. Especially, within the membrane system with raft-mimetic structure (phase-separation), we will simultaneously discuss their localization of ion channel on the membrane. We can trace the localization of ion channel because their structure have fluorescence parts. By handling florescence microscope, we are planning to analyze localization change of ion channels due to the interaction between membrane and LAs. Additionally, we are planning to analyze behavior change of ion channel in each different lipid composition, solution, and especially LA application. Cross-membrane permeability of cation can be measured by measurement of fluorescent intensity of di-4-ANNEPS. We are planning to observe changes of cation permeability by the application of LAs. Furthermore, we are also planning to observe cataion permeability of actual ion-channel proteins buried in LA applied membranes. Recently, various of ion-channel proteins modified with green fluorescent protein (GFP) were developed, and used to detect their expression and localization on neural cells [24–27]. We are also planning to observe the influence of membrane physical property change on the function and localization of these channel by measuring membrane potential.

Through these studies, we plan to further discuss the relationship between membrane physical properties and signal transduction in the future.

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