OUKA

Osaka University Knowledge Archive

Title	Study on Characterization Method of Membrane Surface of Surfactant-Vesicles and Control of Membrane-Membrane Interaction
Author(s)	Hayashi, Keita
Citation	大阪大学, 2013, 博士論文
Version Type	VoR
URL	https://hdl.handle.net/11094/27495
rights	
Note	

Osaka University Knowledge Archive : OUKA

https://ir.library.osaka-u.ac.jp/

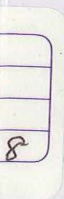
Osaka University

工博 16312

Study on Characterization Method of Membrane Surface of Surfactant-Vesicles and Control of Membrane-Membrane Interaction

KEITA HAYASHI

MARCH 2013



Study on Characterization Method of Membrane Surface of Surfactant-Vesicles and Control of Membrane-Membrane Interaction

A dissertation submitted to

THE GRADUATE SCHOOL OF ENGINEERING SCIENCE

OSAKA UNIVERSITY

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN ENGINEERING

BY

KEITA HAYASHI

MARCH 2013

Preface

This dissertation work was carried out under the supervision of Prof. Dr. Hiroshi Umakoshi at the Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University from 2010 to 2013.

The objective of this thesis is the establishment of the systematic design of the interaction between the different vesicles membrane based on the key properties of the membrane. The interest is focused on (1) characterization of key parameters of surfactant vesicles, (2) analysis of the interaction between detergent vesicle and lipid vesicle, and (3) possible application to control of the membrane-related phenomena (such as drug-delivery system).

The author hopes that this research could contribute to the rational design of the novel drug carrier in drug delivery system, together with the deeper understanding of all phenomenon relating to the membrane.

Keita Hayashi

Keita Hayashi

Division of Chemical Engineering
Graduate School of Engineering Science
Osaka University
Toyonaka, Osaka 560-8531, Japan

Contents

General Introduction 1

Chapter 1 Characterization of Membrane Properties of Surfactant-Vesicles ~Composition of Span 80 Vesicle and Phospholipid Vesicle (Liposome)~

1. Introduction 13
2. Materials and Methods 19
   2.1. Materials. 19
   2.2. Preparation of liposome 19
   2.3. Preparation of Span 80 vesicle. 20
   2.4. Measurement of membrane fluidity. 21
   2.5. Dielectric dispersion analysis. 22
   2.6. Evaluation of membrane polarity. 22
3. Results and Discussion 24
   3.1. Characterization of Membrane Fluidity. 24
   3.2. Characterization of Headgroup Mobility Using Dielectric Dispersion Analysis. 28
   3.3. Characterization of Membrane Polarity. 29
   3.4. Fluid, Flexible, and Wet as a novel index for Membrane Property. 34
4. Summary 39

Chapter 2 Effect of Membrane Properties on Membrane–Membrane Interaction

1. Introduction 40
2. Materials and Methods 42
   2.1. Materials 42
   2.2. Preparation of liposome 42
   2.3. Preparation of Span 80 vesicle. 42

2.4. Calcein leakage 43

2.5. Fluorescence resonance energy transfer (FRET) experiment 44

2.6. Laurdan monitoring 45

2.7. Evaluation of the DOX encapsulation efficiency 47

3. Results and Discussion 48

3.1. First Step of Membrane-Membrane Interaction: Membrane Perturbation. 48

3.2. Second Step of Membrane-Membrane Interaction: Lipid Mixing and Hemifusion. 56

3.3. Effect of the Diffusion of Span 80 vesicle/molecule on the liposome membrane. 61

3.4. Design of the Surfactant-Vesicle Membrane for the Efficient Drug Capsulation in to the Drug Carrier Based on the Membrane Polarity. 67

4. Summary 71

Chapter 3 Control of Membrane-Membrane Interaction between Vesicle and Cell Based on Membrane Properties

1. Introduction 74

2.1. Materials 77

2.2. Preparation of liposome 77

2.3. Preparation of Span 80 vesicle. 77

2.4. Remote loading method. 78

2.5. Cells and cell culture. 78

2.6. Evaluation of the cellular uptake of doxorubicin hydrochloride (DOX). 79

2.7. MTT assay. 79

2.8. Observation of blebbing formulation. 80

2.9. Detection of the alteration of plasma membrane properties. 81

2.10. Annexin 5 -PE staining (Observation by fluorescence microscopy). 82

2.11. Observation of F-actin 82

2.12. Modification of ESA on the vesicle membrane 83

3. Results and Discussion 84

3.1. Design Scheme of Drug Carrier for Effective Drug Delivery 84

3.2. Direct Drug Delivery Based on "Flexible" Nature of Span 80 Vesicle. 86

3.3. Partial Transformation of Plasma Membrane to induce Cellular Response through the Fusion of Span 80 vesicle ~Induction of "Pseudo-Apoptosis"~ 90

3.4. Vesicle Delivery Considering the Targeting to the Organelle Inside Cells 97

3.5. Design of Antibody – Displayed vesicle ~ the Modification of Span 80 Vesicle Membrane by Novel Lectin *Eucheuma serra* Agglutinin ~. 103

4. Summary 107

General Conclusion 109

Suggesting for Future Work 115

1. Application of the membrane characterization for the surfactant vesicle 115

2. Induction of membrane-membrane interaction by the difference of membrane properties 116

3. Design of the drug carrier ~ more complex drug delivery strategy ~ 117

Nomenclature 119

List of Abbreviation 120

References 122

List of Publications 134

Acknowledgement 135

Summary

Surfactant molecules can form a variety of self assembles in an aqueous environment. The vesicle membrane is known to induce various kinds of unique properties (membrane properties), which differs from those of the single molecule.. However, a clear relationship between membrane properties and membrane behaviors (i.e. aggregation, fusion, and interaction with molecule) has not been clarified yet. In this thesis, the method to characterize the membrane properties was established through the systematic investigation of various properties in addition to the conventionally-used property (membrane fluidity). Various kinds of surfactant vesicles were categorized according to the obtained membrane properties. Based on the relationship between membrane properties and "membrane-membrane" interaction, the method to control the interaction of different surfactant vesicles was finally presented.

In chapter 1, the surfactant vesicle (liposome or Span 80 vesicle) was analyzed by using various fluorescence probes (DPH, TMA-DPH, Laurdan, and ANS) and by using dielectric dispersion analysis. Both interior and surface of the surfactant vesicle membrane were systematically characterized, especially focusing on three kinds of properties, such as "*Fluid*" (membrane fluidity), "*Flexible*" (headgroup mobility) and "*Wet*" (membrane polarity). The interior property of Span 80 vesicle was similar to that of liposome, while there is a difference in the surface property. The dielectric dispersion analysis revealed the high intense headgroup mobility of Span 80 vesicle as compared with liposome. This property was also found to affect the insertion of water molecules into the Span 80 vesicle membrane.

In chapter 2, the interaction between different vesicles was investigated based on their membrane properties. Span 80 vesicle with intense headgroup mobility could easily interact with liposome, resulting in the perturbation of liposomal membrane. The perturbation effect was dependent on the membrane properties of liposome. Phospholipids at gel phase are tightly arrayed on the membrane, resulting in the intense perturbation after the insertion of Span 80 vesicle into the liposome membrane. The above results show that the liposome membrane cannot be reconstructed after the fusion of Span 80 vesicle owing to the incompatibility of phase state between two vesicles. In addition, the membrane penetration of doxorubicin hydrochloride was also found to be dependent on the membrane polarity. These results show that the characterized membrane properties can contribute to the understanding of "membrane-membrane" interaction.

In chapter 3, a design scheme for the efficient drug delivery was finally proposed based on the above findings. The headgroup mobility was first studied as the first step of interaction between surfactant vesicle and plasma membrane. Span 80 vesicle with high headgroup mobility could effectively deliver the drug to cells as compared with liposome. After the fusion with plasma membrane, Span 80 vesicle with high fluidity diffuses immediately on the plasma membrane. The plasma membrane fused with Span 80 vesicle resulted in the induction of the apoptotic like cell death (blebbing, and exposure of phosphatidylserine). On the other hand, it has been shown that hybrid vesicle (vesicle-in-vesicle system) can deriver the inner second vesicle into the cytoplasm. Moreover, the accumulation of drug into the cells was improved by modifying the surfactant vesicle with protein targeting a cancer cell.

As a whole, it has been shown that the "membrane-membrane" interaction between different vesicles can be controlled based on the characterized membrane properties by using the method presented in this thesis. It is expected that the clarified relationship between membrane properties and "membrane-membrane" interaction can contribute not only to the design of effective drug carrier, but also to the understanding of phenomenon on/in the membrane.

General Introduction

Surfactant molecule that has both hydrophobic and hydrophilic groups in its structure can form various kinds of "self-assembly" structures in aqueous or organic environment, such as micelle, disc, hexagonal cylinder, and vesicle (**Fig. 1**). The conformation of the self-assemble is also known to be related with the structure of the surfactant molecule. The surfactant with large headgroup and small acyl chain (Type: Inverted cone) constructs the normal micelle in an aqueous solution. The micelle is the simplest and smallest structure among the self-assemblies (less than 100 nm). The hydrophobic part of the surfactant interacts with each other by the hydrophobic effect and its hydrophilic part is exposed to the surface in contact with water solution. A vesicle is one of the self-assemblies, composed of the surfactant with a spatial geometry. For example, a phospholipid has a headgroup and the acyl chain with the same size (Type: Cylinder). The vesicle size is the larger than the micelle size (50 nm ~ 100 μm). A vesicle forms lipid bilayer with the inner aqueous phase.

Surfactant vesicle shows the unique feature by forming the lipid bilayer (**Table 1**). The membrane properties are known to affect the "membrane-related" behavior,

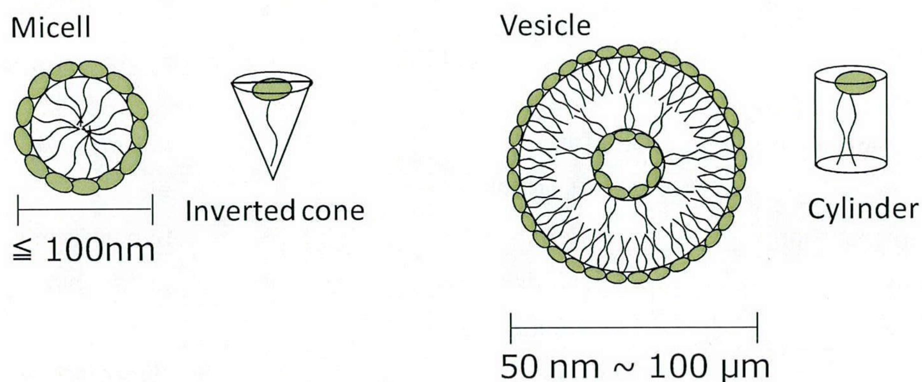


Fig. 1 Schematic illustration of self-assembly structures, such as micelle and vesicle.

such as membrane fusion, and the membrane interaction with other molecules or other membranes. It is expected that the understanding of these membrane properties can contribute to the control of the membrane-related behaviors. A vesicle composed by the phospholipids is especially known as the liposome. The membrane properties of the liposome are regulated, depending on the composition of the phospholipids. The following characteristics are known as common properties of the liposome. (1) The membrane fluidity is reduced when the liposome is prepared by not unsaturated lipid, but saturated lipid. (2) The phase separation of the liposome is induced by the mixing unsaturated lipid with saturated lipid or steroid (i.e. phospholipid with cholesterol). The ratio of these lipids affects the size of the phase separation and the phase separation temperature. (3) When the anionic lipid such as phosphatidylserine is inserted into the liposomal membrane, the surface charge of the liposomal membrane is turned to become anionic. It is important to characterize the membrane properties which can be evaluated based on different kinds of principle.

Table 1 Membrane properties and its measuring method

Membrane property	Material/Method	Measuring mechanism
Particle size	light scattering photometer (DLS)	Light scattering
Phase transition temperature	differential scanning calorimeter (DSC)	Differential scanning calorimetry
Zeta potential	Zeta potential measurement system	Electrophoresis method
Membrane fluidity	DPH	Anisotropy of fluorescence
	TMA-DPH	
Phase separation	Pyrene	Dimer/Excimer fluorescence ratio
Polarity	Laurdan	Stokes shift
	Prodan	
	ANS	Fluorescence intensity

The cells are surrounded by the plasma membrane composed of the phospholipids (**Fig. 2**), which can also be used for the liposome preparation. The plasma membrane has also been characterized in relation to the membrane properties. The plasma membrane contains many kinds of membrane protein, resulting in its complex structure as compared with the liposome membrane. The plasma membrane also functions as the "foothold" of the membrane protein for its work. The plasma membrane can thus accumulate various kinds of relating proteins (e.g. Fas and FADD), on its surface. Membrane protein complex can induce its function in the hydrophobic environment provided by the lipid bilayer membrane. Moreover, phospholipids are modulated by the sugar chains, where the type of sugar chain is varied among the cell lines. The properties of the plasma membrane are thus related well with the molecular assembly structure at the hydrophobic-hydrophilic interface of the lipid bilayer membrane.

The cells also regulate the membrane properties by themselves. (1) The most important role of the plasma membrane is the compartmentation of various

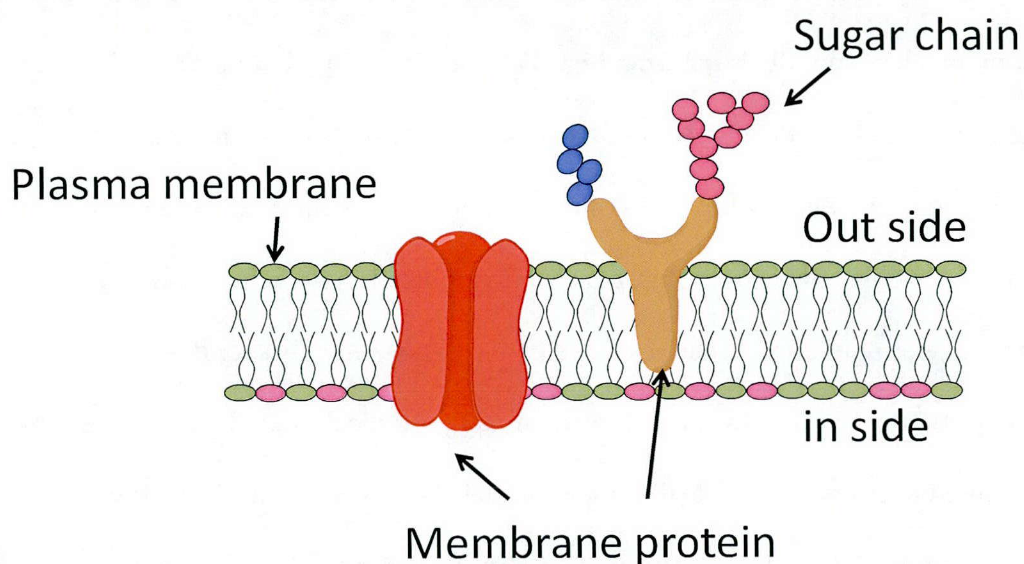


Fig. 2 Schematic illustration of simple scheme of the plasma membrane.

biomolecules against the extracellular environment. The existence of the plasma membrane can retain all the biomolecules into the cytoplasm, and can protect them from external reagent (ion, water molecule, or toxin). When cationic ion inflows into the cytoplasm by the injury of the plasma membrane, the cells are attempt to reconstruct the plasma membrane in order to inhibit the inflow of the cationic ion. Therefore, the plasma membrane is necessary for cells to live. (2) The plasma membrane is the platform which proteins can work. A lipid raft, a tiny domain containing large amounts of cholesterol or sphingolipid, is well-known as the protein accumulation site (e.g. G-protein and receptor). The membrane fluidity of the lipid raft is locally reduced as compared with that of other part of the plasma membrane. The acylation of G-protein can induce the accumulation in the lipid raft. The signal transduction, relayed by this G-protein receptor, is known to be localized into the same lipid raft. The function at/on the plasma-membrane can thus be related with the properties of lipid membrane, and its function can be modulated there.

The membrane properties are related not only to the membrane function, but also to the cell death. There are various reports on the feature of cell death induced by various mechanisms (**Table 2 and Fig. 3**). The membrane is one of the key factors to determine the cell death. Apoptosis has previously been investigated, especially focusing on its relationship with the alteration of membrane properties. Apoptotic process is well-known as the programmed cell death, and is also one of the cellular response relating well with the response of the plasma membrane. It is known that there are two typical responses in the case of apoptotic cell membrane; (i) exposure of phosphatidylserine (PS), and (ii) apoptotic bleb (Kepp et al., 2011, Melino et al., 2005, Orrenius et al., 2011). PS usually localizes in the inner leaflet of the plasma membrane

Table 2 Relationship between cell death mechanism and the feature

	Necrosis	Apoptosis
Genetic Program	None	Yes
Membrane	Lysed	Intact PS[1)] exposure
Organelles	Lysed	Intact
Mitos	Blown	Intact
Nucleus		Chr. condens DNA fragm.
Enzymes	None	Caspase
Receptors		Death Rec.
Regulators		Bcl family IAP

1) PS: phosphatidylserine

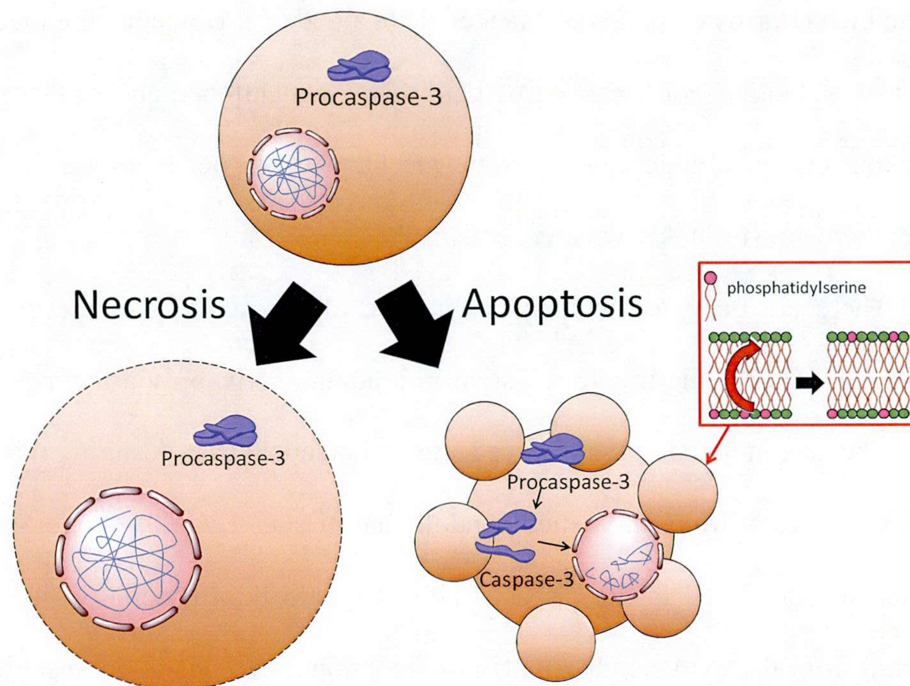


Fig. 3 Schematic illustration of typical cell death (necrosis/apoptosis)

(Leventis and Grinstein, 2010). Membrane protein, flippase, maintains the asymmetry of membrane component between inner and outer leaflet by using ATP (Hirt and Leist, 2003, Krijnen et al., 2010). However, the exposure of PS may result in not only the inhibition of the flippase, but also the calcium-dependent activation of scramblase (Krijnen et al., 2010, Suzuki et al., 2010). PS can thus be regarded as a marker of "corpse" (Fadeel and Xue, 2006). Macrophage can immediately engulfs the apoptotic cells with the exposure of PS to outer leaflet, and can release anti-inflammatory cytokines (i.e. TGF-β, PGE2, IL-10) in order to inhibit the inflammation (Nagata et al., 2010). Apoptotic bleb can also be observed as a morphological alteration (Kepp et al., 2011, Orrenius et al., 2011). In recent years, it has been reported that the kinase (ROCK-I, MLCK) and the serine threonine kinase (p38MAPK) can be related with the apoptotic bleb (Barros et al., 2003, Mackenzie et al., 2005). However, the mechanism of the apoptotic bleb has not been clarified entirely. As mentioned above, the relationship between plasma membrane and apoptotic mechanism has been studied for long time, although there are still unknown mechanisms.

There are many difficulties in the case of the delivery of the drug into the diseased cells. Due to the immune system in a human body, only a few percentages of drugs can be accumulated in the diseased site in a practical trial, resulting that almost all drugs are excreted from the reticuloendothelial tissue. Even if the drug could be fortunately accumulated in the diseased site, it would be difficult for the drug to be transferred from the extracellular matrix to the cytoplasm. This is because the cell was enveloped by the plasma membrane composed by phospholipids. It is hence difficult to deriver the drugs, especially hydrophilic drug, or large drugs with higher molecular weight. The drugs need to be directly delivered to diseased cells in order to achieve an

effective chemotherapy. The use of the drug carrier can solve this problem. Many kinds of drug carrier were developed in order to effectively deriver many kinds of substances, such as drug, DNA, RNA, and protein to the diseased cells (**Table 3** and **Fig. 4**).

The interface of the drug carrier is an important target to be designed for the regulation of the pharmacokinetics (**Fig. 5**). There have been many reports on various kinds of drug carriers. (i) Retrovirus vector, used as the effective gene delivery carrier,

Table 3 Summary of drug carrier

Name	Carrier	Drug	Clinical Stage	Citation
NK105	Micelles	Paclitaxel	P2	(Kato et al., 2012)
NC-6004	Micelles	Cisplatin	P1/2	(Baba et al., 2012)
NK012	Micelles	SN-38	P2	(Nagano et al., 2009)
Doxil	Pegylated liposome	Doxorubicin	Approved	(Lasic, 1996)
Myocet	Liposome	Doxorubicin	Approved	(Mross et al., 2004)
LE-SN-38	Liposome	SN-38	P2	(Zhang et al., 2004)
AmBisome	Liposome	Amphotericin B	Approved	(Hiemenz and Walsh, 1996)
Span 80 vesicle	Niosome	ESA		(Omokawa et al., 2010)
Rexin-G	Retrovirus	mutant form of the cyclin G1 gene	Approved	(Morse, 2005)
Abraxane	Protein aggregation	Paclitaxel	Approved	(Kratz and Elsadek, 2012)

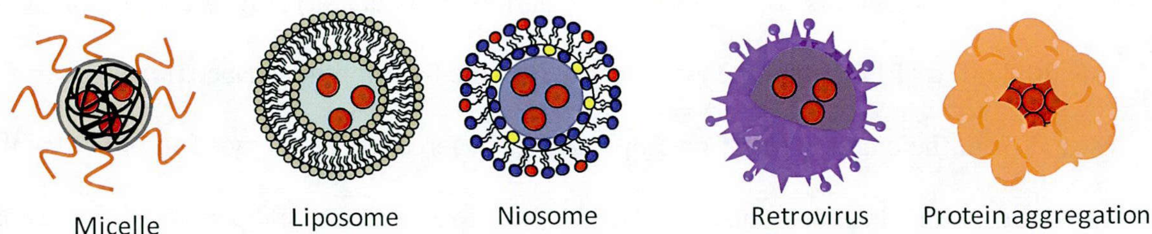


Fig. 4 Schematic illustration of various drug carriers

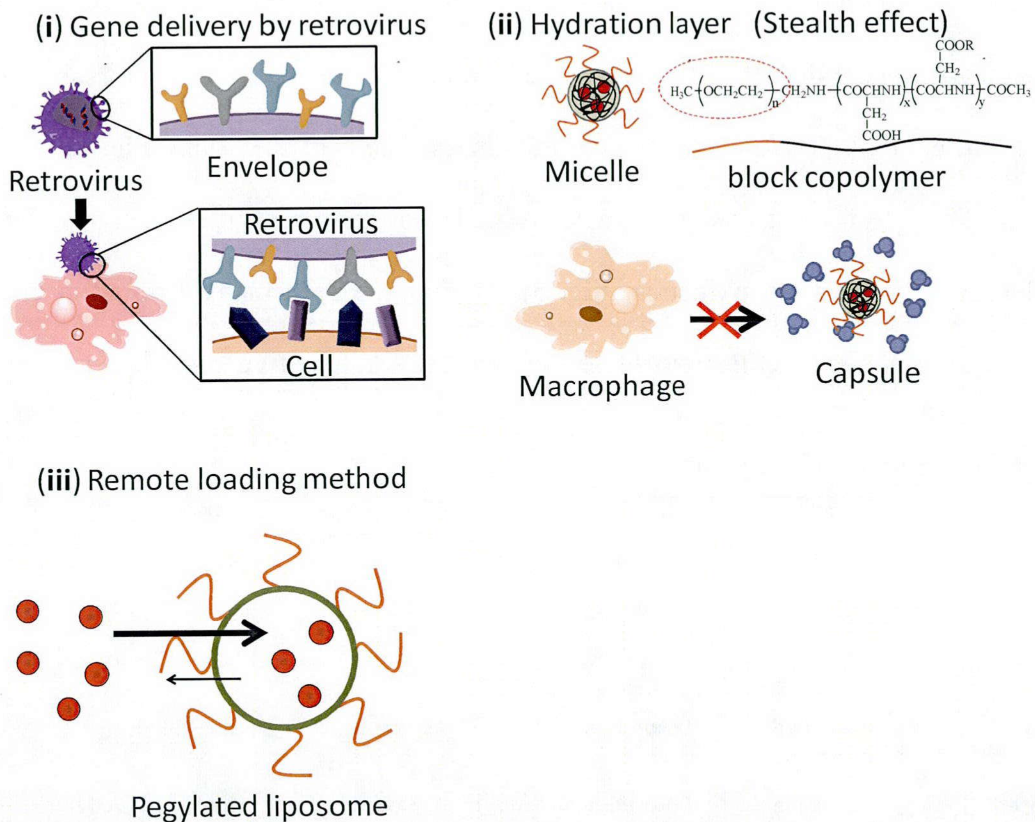


Fig. 5 Schematic illustration of surface of drug carrier and strategy in the drug delivery system

has a viral envelope. This viral envelope is mainly composed by the phospholipids derived from donor, and contains proteins to be derived from viral gene and to promote the membrane fusion. As a result, the retrovirus vector finally releases DNA or RNA into the cytoplasm. (ii) Polymer micelle can often be used as a drug carrier, where the outside of the micelle is composed by the poly(ethylene glycol) (PEG) unit to induce its "stealth" effect. PEG alters the drug carrier into the hydrophilic surface, resulting in the escape from the engulfment of macrophage. (iii) Moreover, the pegylation effect for the circulation time has demonstrated by the comparison between pegylated liposomal product (Doxil®) and non-pegylated liposomal product (Myocet™). The pegylation

dominantly extends the drug half life in the blood from 2-3 hours to >55 hours (Rivera, 2003). (iv) The entrapped efficiency can also be regulated by the surface properties of the drug carrier. Doxil®, which is especially famous liposomal product, is the doxorubicin hydrochloride (DOX) encapsulated by pegylated liposome with higher entrapping efficiency because of the pH gradient across the membrane.

Thus, there are various kinds of drug carriers, where new types of drug carriers are still being designed and developed now by "trial-and-error" strategy. These drug carriers have been evaluated in relation to the individual properties, such as drug encapsulation efficiency, or drug permeability or rationally. However these drug carriers are not designed systematically. It is important to design the drug carrier according to various indexes analyzed based on the different principles. The membrane properties are hence expected to be used as the index of the design of drug carrier. The membrane properties are thus shown to be the key factor to decide the membrane-related behavior, suggesting their relation with the "membrane-membrane" interaction. A variety of strategies to increase the accumulation of drugs into the disease site have been reported by modification of the protein, focusing on the function of protein. However, the important role of "membrane itself" in the drug carrier design has not been proposed yet.

The final purpose of this study is to establish the strategy to design an efficient membrane surface by using a surfactant vesicle for a novel drug delivery system. The membrane properties of surfactant vesicles were first characterized in order to clarify the key parameters relating to the "membrane-related" phenomena. After the method to control the "membrane-membrane" interaction was clarified among different kinds of surfactant vesicles, the methodology has been applied to the design and development of

different kinds of drug delivery system using surfactant vesicles.

The framework and flowchart of this thesis are shown in **Figs. 6 and 7** respectively.

In chapter 1, various kinds of surfactant vesicles were characterized by using fluorescence probe method and dielectric dispersion analysis method. These surfactant vesicles were characterized, focusing on various kinds of membrane properties, "*fluid*", "*flexible*" and "*wet*", which differ in their principle. After the membrane properties of various kinds of surfactant vesicles were characterized, the above vesicles were categorized based on the above three properties.

In chapter 2, the relationship between the membrane properties and "membrane-membrane" interaction was systematically investigated. The membrane properties, obtained in chapter 1, were correlated with the interaction phenomena. According to these results, the scheme for the design of drug carrier was finally established.

In chapter 3, the possible application of the designed surfactant vesicle was studied based on the above findings. A design scheme on the appropriate membrane surface of drug carrier was proposed considering the membrane-membrane interaction between the vesicle and biological cells. Some case studies, such as (i) DOX delivery using Span 80 vesicle, (ii) induction of Pseudo-Apoptosis of cancer cell, (iii) intracellular organelle targeting using vesicle-in-vesicle, and (iv) active targeting to cancer cells, were employed to show the significance of this scheme.

The results obtained in this work are summarized in the General Conclusion section. Suggesting for Future Work is described as extension of the present thesis.

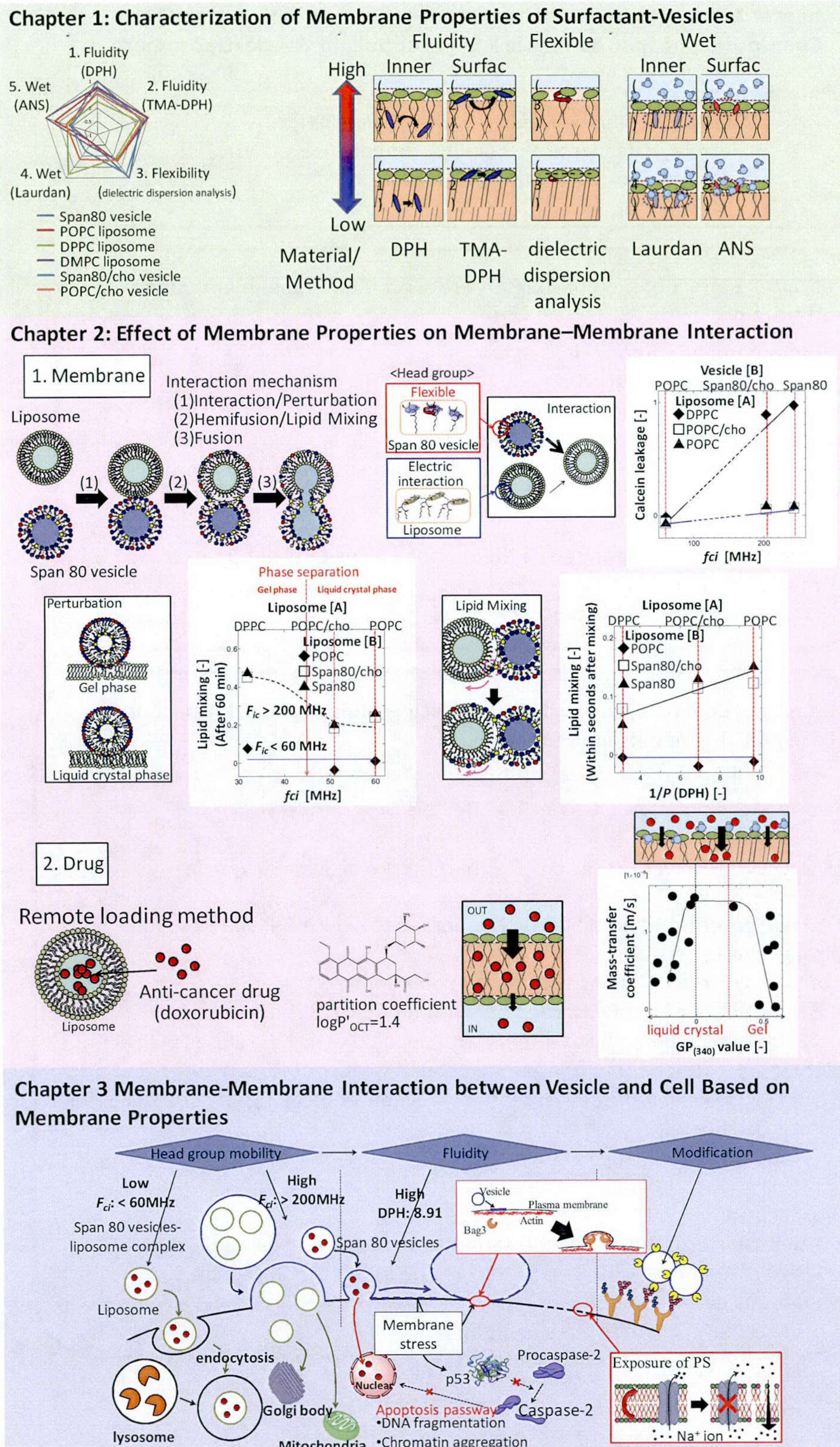


Fig. 6 Framework of the present study

Chapter 1 Characterization of Membrane properties of Surfactant-Vesicles ~Composition of Span 80 Vesicle and Phospholipid Vesicle (Liposome)~

Characterization of

1. "Fluid" (Membrane Fluidity) (DPH, TMA-DPH)
2. "Flexible" (Headgroup Mobility) (impedance analyzer)
3. "wet" (Membrane Polarity) (Laurdan, ANS)

Chapter 2 Effect of Membrane Properties on Membrane–Membrane Interaction

・"Membrane-membrane" interaction
 1. Membrane Perturbation
 2. Lipid Mixing and Hemifusion

・Effect of the Diffusion of Span 80 vesicle/molecule on the liposome membrane

・Design of the Surfactant-Vesicle Membrane for the Efficient Drug Capsulation in to the Drug
Carrier Based on the Membrane Polarity

Chapter 3 Control of the Surfactant-Vesicle – Biological Cell Interaction Based on The Membrane Properties

・Design Scheme of Drug Carrier for Effective Drug Delivery

・Direct Drug Delivery Based on "Flexible" Nature of Span 80 Vesicle.

・Partial Transformation of Plasma Membrane to induce Cellular Response through the Fusion
of Span 80 vesicle ~Induction of "Pseudo-Apoptosis"~
 (1) General Cellular Response after Fusion of Span 80 vesicle
 (2) Bleb formulation by treatment with Span 80 vesicle is related with actin filament.
 (3) Pseudo-Apoptotic Response; the Induction of Blebbing and Cell Death for Cancer Cells
 by Treated with Span 80 Vesicle

<**Application**>
・Vesicle Delivery Considering the Targeting to the Organelle Inside Cells
・Design of Antibody – Displayed vesicle ~ the Modification of Span 80 Vesicle Membrane by
Novel Lectin *Eucheuma serra* Agglutinin ~

Fig. 7 Flowchart of the present study

Chapter 1 Characterization of Membrane Properties of Surfactant-Vesicles ~Composition of Span 80 Vesicle and Phospholipid Vesicle (Liposome)~

1. Introduction

Drug delivery composes of many elemental steps: the drug permeation (Ashley et al., 2011); the accumulation of drug carrier to the disease site (Omokawa et al., 2010); the retention time in the blood vessel (Lasic, 1996); and uptake (**Fig. 1-1**). Recently, those phenomena have withdrawn much attention as the membrane-relating phenomena. This is because the above phenomena are largely affected by the membrane properties and states. It is therefore implied that one can design the drug delivery system by tuning the membrane properties to control the drug permeability from drug carrier, its accumulation to the disease site, and the retention time in the blood vessel.

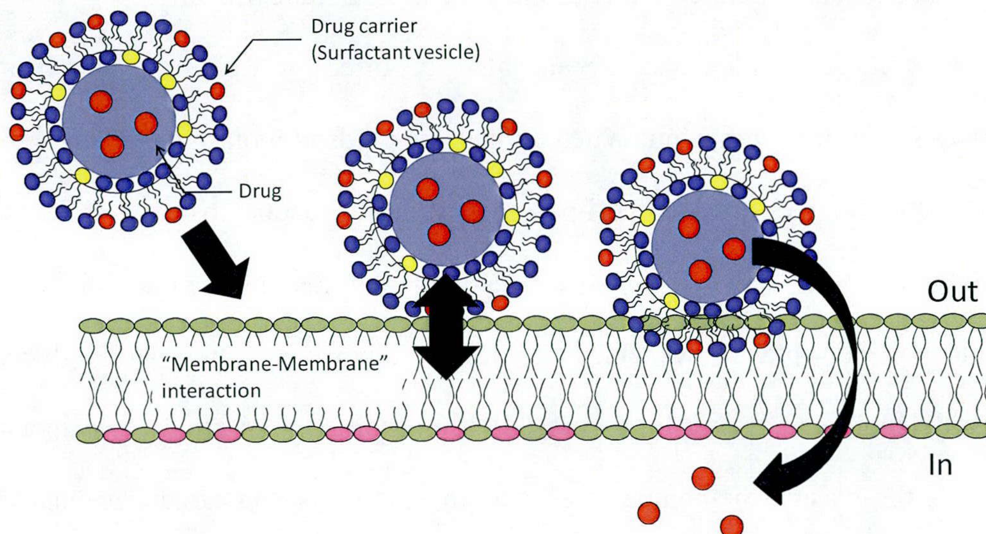


Fig. 1-1 Scheme of drug delivery ("membrane-membrane" interaction)

It is the *membrane fluidity* that has been first investigated from the viewpoint whether the membrane property would be a key factor for a control of membrane-relating phenomena. Membrane fluidity is one of well-known indexes of the lateral diffusion of lipid molecule, and has been reported to be related with the drug permeability (Shimanouchi et al., 2009). Doxil®, a commercial drug using lipid vesicle modified with PEG, is rationally designed based on the membrane properties. Doxil® is usually prepared by hydrogenated soy phosphatidylcholine (saturated lipids) and cholesterol (Lasic, 1996). It has been reported that the saturated lipids can form liposome at gel phase, while the unsaturated lipids do at liquid crystalline phase (Koynova and Caffrey, 1998). A liposome at gel phase is known to show the lower membrane fluidity as compared with that at liquid crystalline phase (Bakht et al., 2007). The insertion of cholesterol into the lipid membrane contributes to the physical structure of the bilayer membrane, resulting in the significant reduction of the permeability of drug from liposome (Pugh et al., 1989). Thus, it is a common finding that the membrane fluidity is an adequate parameter to characterize the membrane property.

Membrane fluidity has been also reported to be related with the membrane-membrane interaction. When a liposome binds to a plasma membrane of the biological cell, the cell uptakes the liposome by fusion, endocytosis, or phagocytosis (Düzgüneş and Nir, 1999). It has been reported that the membrane fluidity could contribute to the uptake mechanism by cells. Liposome (phosphatidylglycerol : phosphatidylcholine = 1:9) at liquid crystalline phase has been reported to induce its fusion with the plasma membrane to an extreme extent as compared with that at gel phase (Papahadjopoulos et al., 1973). This previous finding clearly shows that the membrane fluidity rather than the electrostatic interaction might dominate the

membrane-membrane interaction. From another point of view, when a liposome is injected into the blood vessel, the liposome binds to the blood proteins, such as albumin, globulin, or opsonin protein. It has been reported that the amount of rat serum albumin bound to the liposome can be increased by the increase of liposomal membrane fluidity (Dimitrova et al., 2000). The binding affinity of opsonin, biomolecule that targets on antigen, to liposome is especially important to understand the pharmacokinetics. Opsonization can promote the engulfment of macrophage, resulting in the reduction of the concentration of the drug in the blood vessel (Chonn et al., 1992). It has also been reported that there could be a relationship between the membrane fluidity of its liposome and its pharmacokinetics (Moghimi and Patel, 1989). Therefore, the control of membrane fluidity is important for the design of membrane-related phenomena, including the effective drug carrier.

Along with the reports based on the spectroscopic study using the variety of fluorescence probes or the direct imaging, it has been revealed that the membrane could be characterized by the physicochemical properties in addition to the membrane fluidity.

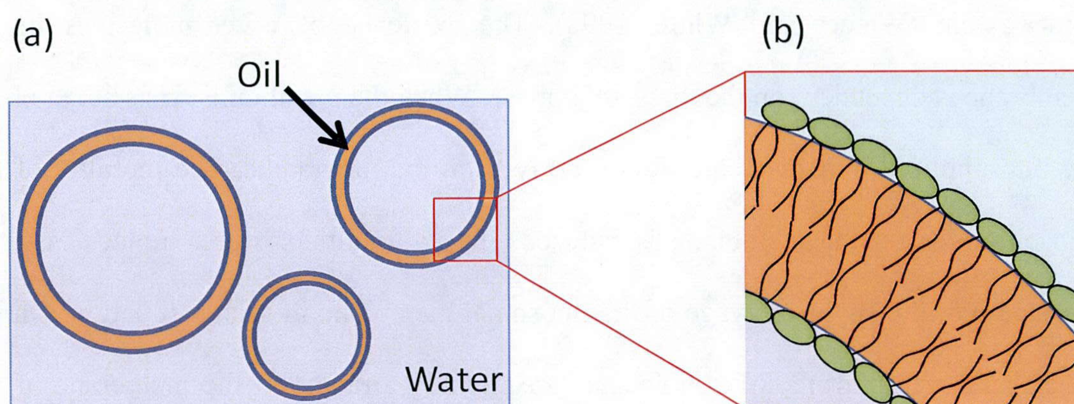


Fig. 1-2 Structure of (a) surfactant vesicle and (b) lipid bilayer.

Membrane-related phenomena are not always explained only by membrane fluidity. For example, the headgroup mobility on the membrane surface directly affects the surface property that could govern the membrane-membrane phenomena. Usually, vesicles composed by the amphiphiles, such as surfactant, may expose their hydrophilic headgroup to aqueous phase surrounding the vesicle (**Fig. 1-2**). It is suggested that the headgroup mobility can directly affect the interaction with the plasma membrane or proteins. Among the possible surfactants, a phosphocholine has a permanent dipole in its headgroup structure. The headgroup of phosphocholine molecules on the membrane interact with each other, resulting in the formation of highly-ordered structure on the surface. The lipid membrane also shows its fluid nature, caused by intense mobility of the headgroup. It has been reported that the headgroup mobility is well correlated with the membrane fluidity (Shimanouchi et al., 2011). Alternatively, the dynamics of the membrane can affect the polarity of the membrane. Vesicular membrane is composed by hydrophilic part (headgroup region) and hydrophobic part (acyl chain region). Water molecules can be inserted hardly into the vesicular membrane of hydrophobic part, while some water molecules exist in the hydrophilic part of the vesicular membrane to some extent (Wiener and White, 1992). The existence of water molecules in the membrane is dependent on the liposomal phase. When the membrane exists at gel phase, the phospholipid molecules are tightly arrayed on the membrane. The mobility of the phospholipids on the membrane is reduced. It is difficult for water molecules to be inserted into the slight cleavage of the liposomal membrane in such a situation. On the other hand, the phospholipid molecules are flexibly arrayed on the membrane in the case of the liquid crystalline phase (Koynova and Caffrey, 1998), and can move dynamically on the membrane surface (Shimanouchi et al., 2011). It is easy for water

molecules to be inserted into the membrane of not only hydrophilic part, but also hydrophobic part in this case as compared with liposome at gel phase (Stepniewski et al., 2011).

In this chapter, vesicles were characterized according to the membrane properties, such as membrane fluidity, headgroup mobility, and polarity, by contrasting them with other formulations such as water-in-oil emulsion (**Table 1-1** and **Fig. 1-3**). The fluidity of the membrane was evaluated by using both fluorescence probe, 1,6-diphenyl-1.3.5-hexatriene (DPH) and trimethlyammonium-DPH (TMA-DPH). Membrane fluidity can herewith be defined as the resolution of fluorescence anisotropy. It has been reported that DPH and TMA-DPH localized into the inner and surface of the membrane, respectively (Borenstain and Barenholz, 1993). The previous findings indicate that DPH and TMA-DPH show the membrane fluidity of inner and surface membrane, respectively. The flexibility is defined the dipole moment of headgroup of surfactant vesicles. The headgroup mobility was evaluated by using the dielectric dispersion analysis. The polarity is defined the existence of water molecule, which can

Table 1-1 Analytical method of membrane properties

Region	Material/Method	
Inner Surface	DPH TMA-DPH	Membrane Fluidity was evaluated by the fluorescence anisotropy of DPH or TMA-DPH.
Surface	Impedance analyzer	Head group mobility was evaluated by the specific frequency of head group structure.
Inner Surface	Laurdan ANS	Membrane polarity was evaluated by the fluorescence intensity or spectra

be evaluated by environmentally-responsive fluorescence probe. 8-Anilino-1-naphthalenesulfonic acid (ANS), and 6-dodecanoyl-N,N-dimethyl-2-naphthylamine (Laurdan) have been previously reported to be inserted into the membrane surface, and inner membrane, respectively (Bagatolli et al., 1999, Slavik, 1982). The existence of water molecules was analyzed form the alteration of the fluorescence intensity and the peak sift of both fluorescence probes (ANS and Laurdan). Based on the results obtained here, the novel viewpoint to characterize surfactant vesicles indicated that the membrane property could be categorized by ***fluid***, ***flexible***, and ***wet***. These viewpoints would give the better understanding to design the effective drug carrier.

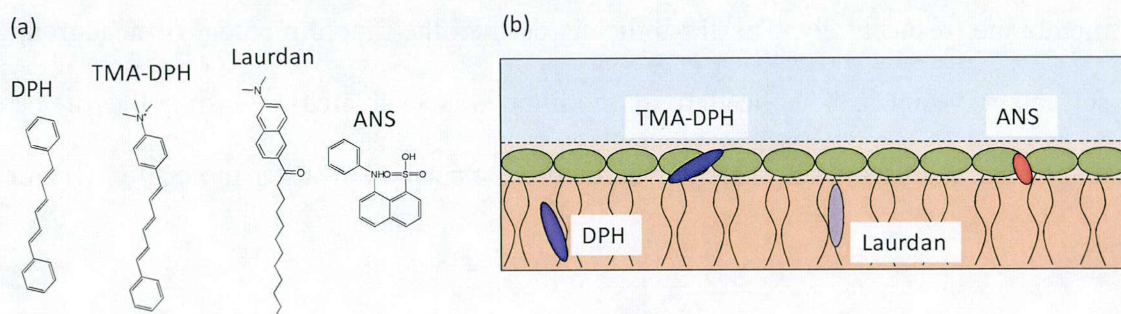


Fig. 1-3 (a) The chemical structure and (b) location into the membrane of the fluorescence probe, DPH, TMA-DPH, Laurdan and ANS. DPH and Laurdan were inserted into the inner membrane. TMA-DPH and ANS were inserted into the membrane surface.

2. Materials and Methods

2.1. Materials.

Sorbitan monooleate (Span 80) and polyoxyethylene sorbitan monooleate (Tween 80) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Phospholipids such as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1,2-dimirystoyl- *sn*-glycero-3-phosphocholine (DMPC), and 1,2-dipalmitoyl-*sn*-glycer-o-3-phosphocholine (DPPC) were purchased from the NOF Corporation (Tokyo, Japan). 8-Anilino-1-naphthalenesulfonic acid (ANS) and 1,6-diphenyl-1,3,5-hexatriene (DPH) were obtained from Molecular Probes Inc. (Eugene, OR, U.S.A.). 6-Dodecanoyl-N,N-dimethyl-2-naphthylamine (Laurdan), N,N,N-trimethyl-4-(6-pheny-l-1,3,5-hexatrien-1-yl) phenylammonium p-toluenesulfonate (TMA-DPH), and cholesterol were purchased from Sigma-Aldrich and AnaSpec Inc. (Fremont, CA, USA), respectively. Lysozyme was obtained from Seikagaku Co. (Tokyo, Japan).

2.2. Preparation of liposome

Phospholipids and cholesterol were dissolved in a chloroform solution. The organic solvent was removed by evaporation in a rotary evaporator. The residual lipid film, after drying under a vacuum overnight, was hydrated with the inner-phase liquid (distributed water or phosphate-buffered saline (PBS; 137mM NaCl, 2-9mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4; pH 7.3). The suspension was subjected to five cycles of freezing and thawing and then extruded. The lipid concentration was determined by phosphorous analysis.

2.3. Preparation of Span 80 vesicle.

Span 80 vesicle was prepared by the two-step emulsification method (Kato et al., 2006) , as shown in **Fig. 1-4**. 6 ml of n-hexane solution containing Span 80 and cholesterol was added to 0.6 ml of inner-phase liquid (distributed water or PBS), followed by the first emulsification for 6 min at 17,500 rpm using a micro-homogenizer NS-310E 2 (Microtec Co., Ltd., Funabashi, Japan). The solvent obtained from the water-in-oil emulsion was evaporated in a rotary evaporator at 28 °C under reduced

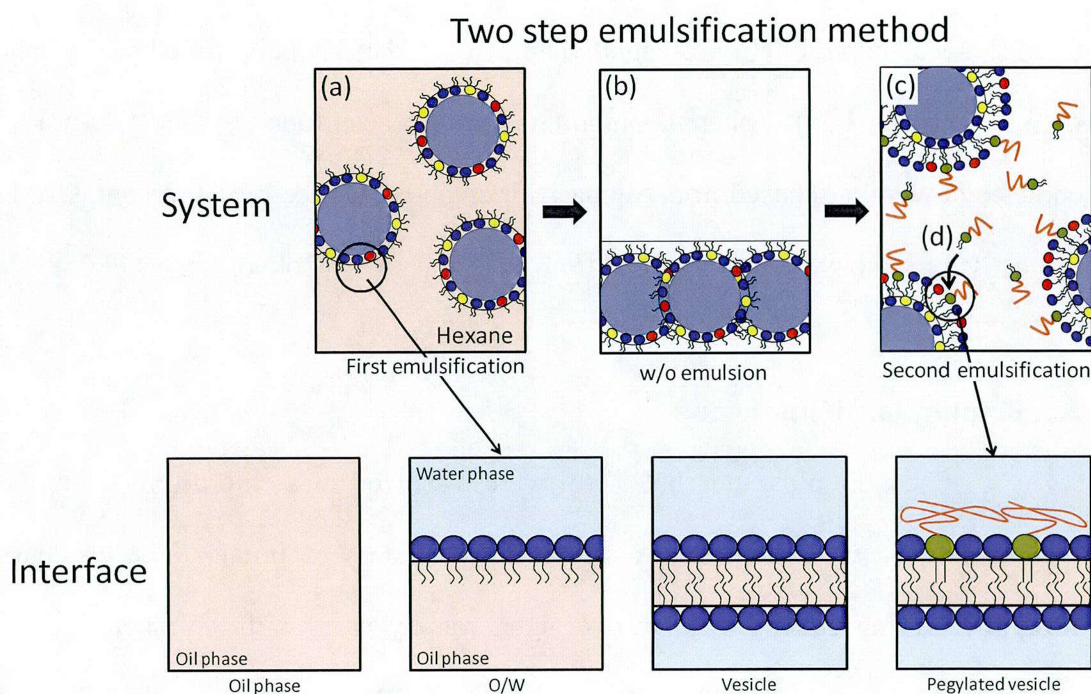


Fig. 1-4 Schematic illustrations for two step emulsification technique and the interface of various systems. (a) First emulsification. Span 80 was homogenized with water droplet in the n-hexane solution. (b) The n-hexane solution was removed by evaporator. (c) w/o emulsion was suspended and homogenized into the water solution containing Tween 80, resulting in the formulation of vesicle. (d) Pegylation. When Tween 80 solution contain pegylated lipid, the pegylated lipid enter the vesicular membrane.

pressure, yielding a water-lipid emulsion to which 6 ml of outer-phase liquid (distributed water or PBS) containing 96 mg of Tween 80 was added, followed by mixing with the homogenizer for 2 min at 3,500 rpm to obtain the heterogeneous Span 80 vesicle suspension. The heterogeneous vesicle suspension was stirred with a magnetic stirrer for 3 hours at room temperature, followed by storage overnight at 4 °C. The vesicle was then purified by ultracentrifugation (50,000 rpm at 4 °C for 120 min) in a Himac centrifuge CR15B (Hitachi Koki Co., Ltd., Tokyo, Japan). The purified Span 80 suspension was passed through 100-nm nucleopore track-etch polycarbonate membranes and purified twice by ultracentrifugation.

2.4. Measurement of membrane fluidity.

Membrane fluidity was evaluated with the anisotropy of the fluorescence of DPH and TMA-DPH (Hayashi et al., 2011, Kato et al., 2008). DPH or TMA-DPH solubilized in ethanol was added to a pre-formed vesicle suspension to give a lipid/probe molar ratio of 250 ($[(\text{TMA-})\text{DPH}]_{final}$ = 1 μM). Then, the mixture was incubated at least for 30 min at room temperature under gentle stirring. The fluorescence polarization of the samples was measured with an FP 6500 spectrofluorometer (JASCO, Japan) equipped with a polarizer. The sample was excited with vertically polarized light (360 nm), and the emission intensity (430 nm) both parallel and perpendicular to the excited light was recorded. The temperature was kept at 35 °C. Then, the polarization of DPH and TMA-DPH was calculated from the following equation:

$$\mathrm{P} = \frac{(I_{0°0°} - \mathrm{G}I_{0°90°})}{(I_{0°0°} + \mathrm{G}I_{0°90°})} \tag{1-1}$$

$$G = \frac{I_{90°0°}}{I_{90°90°}} \tag{1-2}$$

Since polarization is inversely proportional to fluidity, membrane fluidity is expressed as $1/P$.

2.5. Dielectric dispersion analysis.

An impedance analyzer (RF impedance analyzer 4291B; Agilent Technologies, Palo Alto, CA) equipped with a handmade brass electrode cell was used to measure the dielectric spectra of liposome suspension in the frequency range between 1.0 MHz and 1.0 GHz. The dispersion was analyzed from the following equation consisting of fourth- or fifth-type Debye's equation (Shimanouchi et al., 2011).

$$\varepsilon'' = \Sigma \frac{\Delta\varepsilon_1 (f/f_{ci})}{\{1+(f/f_{ci})^2\}} \tag{1-3}$$

It has been reported that the liposome suspension has four different characteristic relaxations: the lateral diffusion of ionic species (i = 1, first-step, several MHz); the mobility of lipid headgroup (i = 2, second-step, ~50 MHz); the water bound to liposome membranes (i = 3; third-step, 200~500 MHz); the bulk water (i = 4, fourth-step, ~20 GHz). Therefore, equations (2) and (3) were assumed to be written by a summation of four relaxation terms. In general, the large f_{ci} represents the rapid mobility of target materials.

2.6. Evaluation of membrane polarity.

Hydrophilic/hydrophobic characterization of the membrane of vesicles was evaluated by the environmentally sensitive fluorophore ANS and Laurdan at 35 °C

(Parasassi et al., 1994, Slavik, 1982). ANS or Laurdan solubilized in ethanol was added to a pre-formed vesicle suspension to give a lipid/probe molar ratio of 100 ($[ANS]_{final}$, $[Laurdan]_{final}$ = 1 μM). Then, the mixture was incubated at least for 30 min at room temperature under gentle stirring. ANS was excited at 350 nm, and the emissions were observed from 375 nm to 600 nm; Laurdan was excited at 340 nm and the emission spectra were observed from 425 nm to 550 nm with FP-6500 fluorometer at 35 °C.

The Laurdan emission spectrum has a red shift due to dielectric relaxation (Stokes shift). The emission GP spectra were calculated by measuring the GP value for each emission wavelength as follows:

$$GP_{(340)} = \frac{(I_{440} - I_{490})}{(I_{440} + I_{490})} \tag{1-4}$$

I_{440}: The fluorescence intensity of Laurdan at 440 nm

I_{490}: The fluorescence intensity of Laurdan at 490 nm

3. Results and Discussion

3.1. Characterization of Membrane Fluidity.

In the first series of experiments, the interface structure of each system was investigated by using rod-like fluorescence probes because their intramolecular rotational motion was sensitive to the ordered-structure of the interface. The intramolecular rotational motion of two fluorescence probes, DPH and TMA-DPH, was herein quantified with their polarization (*P*) whose reciprocal value represents the mobility of both (Lentz, 1993, Shimanouchi et al., 2011).

Figure 1-5 shows the (1/*P*) value of DPH and TMA-DPH in the variety of systems described in **Fig. 1-4**. Overall, the order of (1/*P*) value of DPH and TMA-DPH were (1,2) > (3-6). Since Span 80 molecules can move at the interface between water and hexane, the intramolecular mobility of DPH and TMA-DPH can be allowed, which

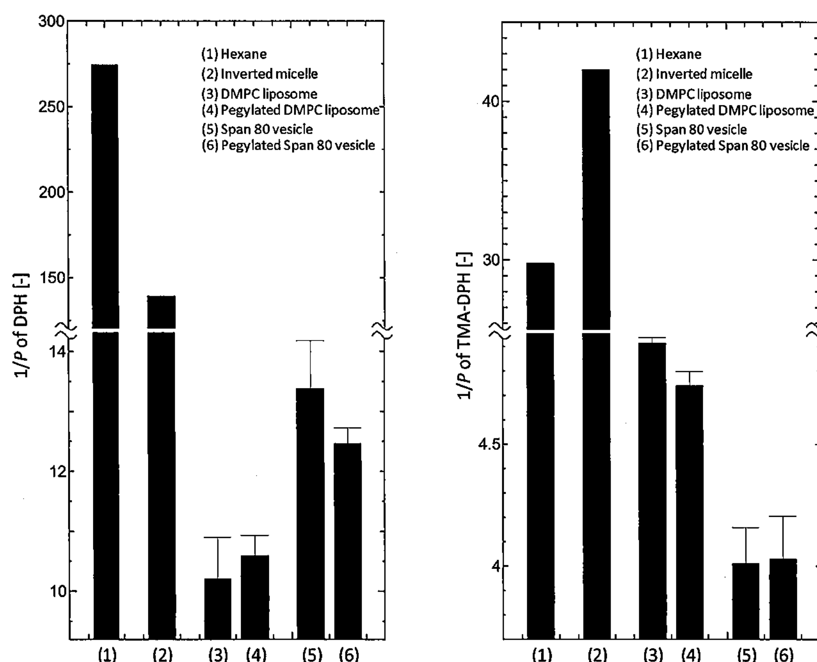


Fig. 1-5 Mobility of (a) DPH and (b) TMA-DPH in various systems (37 °C). The value was evaluated by the polarization.

is consistent with their large value of $(1/P)$ in the system of (1) and (2). In contrast, the supramolecular assemblies, such as phospholipid vesicle (liposome) and Span 80 vesicle, indicated the low mobility of DPH and TMA-DPH. This is because the mobility of DPH and TMA-DPH orientated into the acyl chain region of phospholipids or Span 80 molecule was easily restricted. Furthermore, comparing the DMPC liposome (3,4) with the Span 80 vesicle (5,6), the mobility of DPH was the following order: (3, 4) < (5, 6). In contrast to DPH, the mobility of TMA-DPH was (3, 4) > (5, 6). The interface of Span 80 vesicles is composed of sorbitol as headgroup. It is therefore considered that TMA-DPH orientated into the deeper region. In addition, the significant effect of induction of PEG layer to liposome and Span 80 vesicle was not observed. It is suggested that the mobility of lipids was restricted in the formation of vesicle from the inverted micelle.

Alternatively, cholesterol was used to clarify the effect on membrane structure. POPC liposome (liquid crystalline phase), DPPC liposome (gel phase), and Span 80 vesicle were examined because cholesterol was considered to affect the membrane structure, depending on the phase state of membranes. The membrane fluidity measured by DPH is shown in **Fig. 1-6(a)**. DPPC liposome shows low membrane fluidity ($(1/P)$ of DPH = 3.10) with DPH, while POPC liposome shows high membrane fluidity ($(1/P)$ of DPH = 9.71). The membrane fluidity of the Span 80 vesicle ($(1/P)$ of DPH = 13.3) shows higher than other vesicles. The membrane fluidity measured by TMA-DPH is shown in **Fig. 1-6(b)**. The obtained results on the membrane fluidity of the Span 80 vesicle by using TMA-DPH was found to be not similar with the membrane fluidity using DPH, in contrast to the case of DPPC and POPC liposome. The membrane fluidity of DPPC liposome ($(1/P)$ of TMA-DPH = 3.19) is lower than

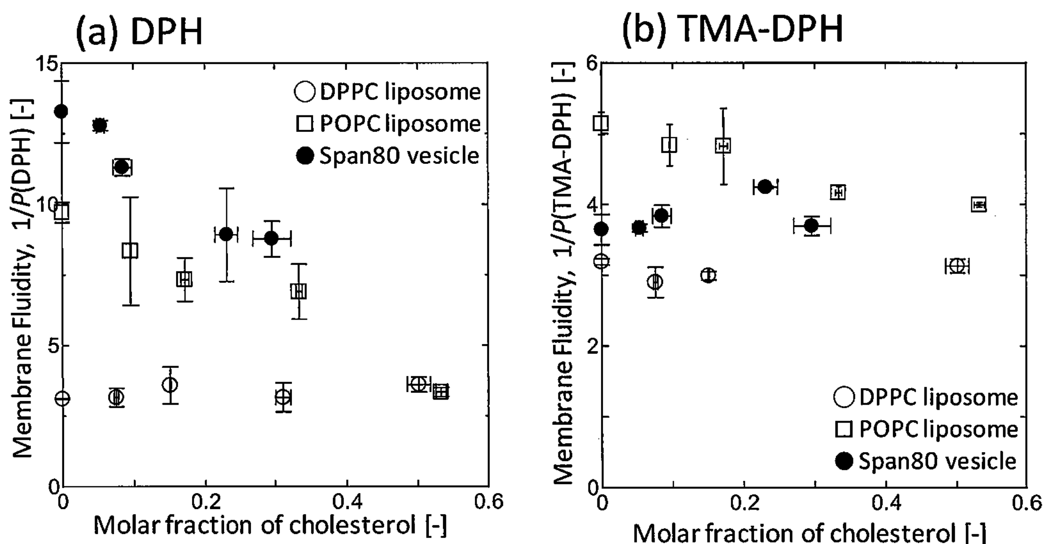


Fig. 1-6 Membrane fluidity measurements carried out at 35 °C. The membrane fluidity was evaluated as a reciprocal of polarity (P). The molar ratio of DPH and TMA-DPH to vesicles 1:250. The data with standard error were obtained from three independent experiments.

that of POPC liposome (($1/P$) of TMA-DPH = 5.14). However, the membrane fluidity with TMA-DPH of Span 80 vesicle is lower than that of POPC liposome, in spite of the highest membrane fluidity of Span 80 vesicle with DPH. In order to evaluate the membrane fluidity more in detail, the alteration of the membrane fluidity of vesicle was observed by modifying the vesicle membrane with cholesterol. The alteration of membrane fluidity with DPH is similar to that of TMA-DPH. No alteration in the membrane fluidity of DPPC liposome was observed, while the membrane fluidity of POPC liposome was reduced in the case of cholesterol modification. On the other hand, there is a difference of the effect of cholesterol on the membrane fluidity of Span 80 vesicle in the case of DPH and TMA-DPH measurements. The addition of cholesterol was found to reduce the membrane fluidity when DPH was applied to the measurement

in the case of Span 80 vesicle. On the contrary, the membrane fluidity with TMA-DPH of Span 80 vesicle shows a similar value regardless of the content of cholesterol.

Membrane fluidity is dependent on the lipid composition of liposome, such as hydrophobic groups saturated or unsaturated acyl-chain (Koynova and Caffrey, 1998). DPPC is saturated lipid, and can form a liposome at gel phase. POPC is unsaturated lipid, and can form a liposome at liquid crystalline phase. Therefore, the membrane fluidity of DPPC liposome is lower than that of POPC liposome. The membrane fluidity of Span 80 vesicle is also higher than that of DPPC liposome, suggesting that the Span 80 vesicle membrane exist at liquid crystalline phase. However, there is a difference of the membrane properties between POPC liposome and Span 80 vesicle, in spite that they both exist at the same liquid crystalline phase. It is well known that the cholesterol can reduce the membrane fluidity of liquid crystalline phase. The membrane fluidity with TMA-DPH of POPC liposome is reduced by adding cholesterol, while no reduction in the membrane fluidity with TMA-DPH was observed in the case of Span 80 vesicle. It is suggested that the above phenomena can be to be related with the localization of TMA-DPH molecule in the membrane. It has been reported that the DPH and TMA-DPH molecules localize into the inner and surface of liposome membrane, respectively (Borenstain and Barenholz, 1993). The inner membrane structure of Span 80 vesicle is similar to that of liposome (acyl chain region), while the headgroup structure of Span 80 vesicle (sorbitol) is different from that of liposome (phosphocholine). It is suggested that the difference of headgroup structure affects the role of cholesterol for the membrane and localization of TMA-DPH on the Span 80 vesicle membrane, resulting in no alteration of membrane fluidity with TMA-DPH of Span 80 vesicle by adding cholesterol.

3.2. Characterization of Headgroup Mobility Using Dielectric Dispersion Analysis.

Headgroup mobility of vesicle at liquid crystalline phase (POPC liposome and Span 80 vesicle) was analyzed by using an impedance analyzer, as shown in **Fig. 1-7**. The dispersion of the dielectric spectra of liposomes was observed at a specific frequency region from 20 MHz to 300 MHz (Shimanouchi et al., 2011). The above result attributed to the motion of the zwitterionic headgroup of lipid inside the liposome membrane. A specific frequency of POPC liposome was observed at 60 MHz. Cholesterol was added to the membrane to alter the membrane properties, as described above. The dielectric dispersion that attributed to the headgroup of lipid molecule was shifted to a low-frequency range with the increase in the cholesterol content. This result suggests the restriction of the headgroup mobility of the POPC molecule via its interaction with cholesterol. A different result was obtained, when Span 80 vesicle or

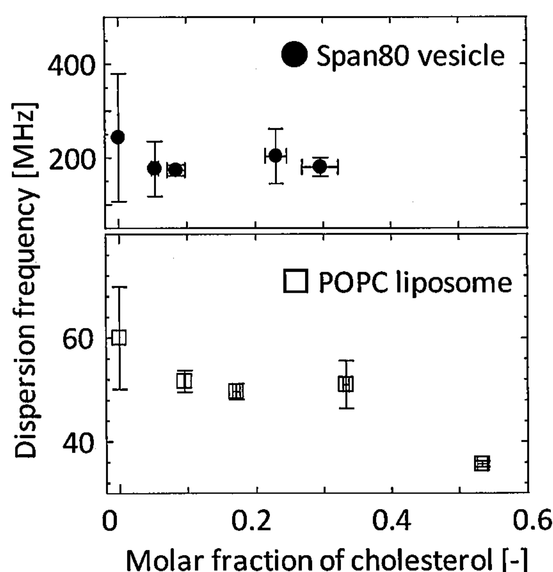


Fig. 1-7 Dielectric dispersion parameters of Span 80 vesicle and liposome. The relaxation frequency of Span 80 vesicle was constant with cholesterol concentration against cholesterol dependence on POPC liposome.

Span 80/cholesterol vesicle were analyzed. The specific frequency of Span 80 vesicle headgroup was observed at 243 MHz, being higher than that of POPC liposome headgroup. This value was not varied when the cholesterol content was changed.

These results correspond well with the results on the membrane fluidity of the membrane surface, as described above (**Fig. 1-6**), where the membrane fluidity with TMA-DPH of POPC liposome was reduced, and the specific frequency of POPC liposome headgroup was shifted to a low-frequency range by adding cholesterol. The addition of cholesterol reduces the mobility of the phospholipid itself (lateral diffusion and headgroup mobility). The obtained higher value in the specific frequency of the headgroup on Span 80 vesicle suggests the high mobility of headgroup on the vesicular membrane. It is considered that Span 80 vesicle has an induced-dipole on the headgroup (sorbitol structure), resulting in a weak electrostatic interaction between the molecules as compared with the case of liposome. The effect of cholesterol on Span 80 vesicle also supported the high headgroup mobility. Cholesterol can affect the inner membrane of Span 80 vesicle similarly in the case of liposome (**Fig. 1-6(a)**), while the headgroup region is not affected in spite of the cholesterol coexistence by the contribution of high headgroup mobility, different from the case of liposome (**Figs. 1-6(b) and 1-7**).

3.3. Characterization of Membrane Polarity.

The intense mobility of lipid at the interface of membranes resulted in the exposure of hydrophobic environment of membranes (Flanagan and Hesketh, 1973). It has been reported that the hydrophobic fluorescence probe ANS can emit the large fluorescence intensity if ANS exists in the hydrophobic environment. The ANS fluorescence intensity for various systems was therefore measured as shown in **Fig. 1-8**.

The order of ANS intensity was (1, 2) > (3, 4) > (5, 6), indicating that Span 80 vesicles have the quite hydrophilic surface. Meanwhile, the pegylation of liposome and Span 80 vesicle indicated no remarkable change in hydrophobicity, implying no binding of ANS to PEG layer or no difference in hydrophobicity between PEG layer and bulk aqueous phase.

Similarly in the case of (TMA-)DPH, the effect of cholesterol to the membrane structure was investigated in terms of the hydrophobicity. Herein, POPC liposome (liquid crystalline phase), DPPC liposome (gel phase), and Span 80 vesicle were examined because the cholesterol is consider to affect the membrane structure, depending on the phase state of membranes. The difference of ANS fluorescence intensity is shown in **Fig. 1-9**. The fluorescence intensity of ANS in the POPC liposome (I = 80.8) was higher than that of ANS in the other vesicle. Cholesterol was found to reduce the fluorescence intensity of ANS in the POPC liposome. The fluorescence intensity

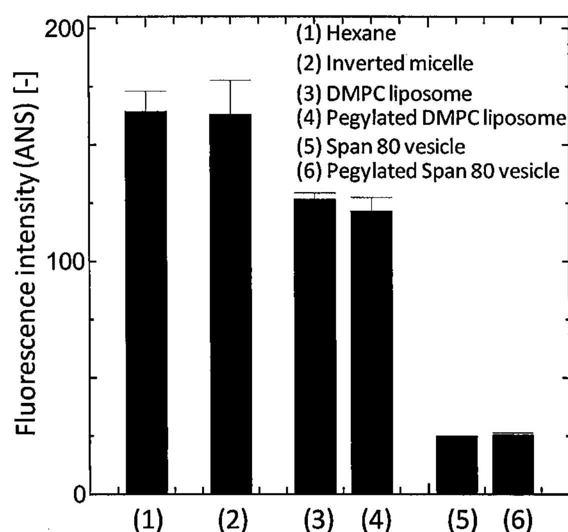


Fig.1-8 ANS fluorescence intensity for various systems. Measurements were performed at 37°C.

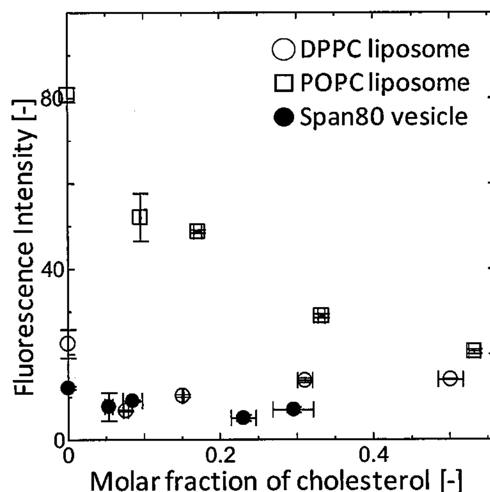


Fig.1-9 Hydration as a function of cholesterol ratio increasing. Fluorescence spectra of ANS probe in Span80 vesicle and liposome membrane at different amount of cholesterol containing at 35 °C.

of ANS in the both of DPPC liposome (I = 22.5) and Span 80 vesicle (I = 12.1) was lower than that of POPC liposome. Cholesterol did not reduce the fluorescence intensity of ANS in the both DPPC liposome and Span 80 vesicle. ANS inserts into the membrane surface of vesicle, and it has been reported that ANS exists at headgroup region (Slavik, 1982). ANS monitors the polarity at the headgroup region of the vesicle membrane. It has been reported that water molecule inserts the liposome at liquid crystalline phase membrane more easily than that of liposome at gel phase (Stepniewski et al., 2011), while ANS fluorescence intensity of POPC liposome with liquid crystalline phase is higher than that of DPPC liposome at gel phase. It seems that the tight array of lipid on the membrane at gel phase prevents the insertion of ANS into the vesicle membrane. The fluorescence intensity of Span 80 vesicle was lower than that of POPC liposome, in spite of similar phase state (liquid crystalline phase). The headgroup of Span 80 vesicle has more dynamic mobility as compared with that of liposome owing to the difference of the headgroup structure, as described above (**Section 3.2.**). It is

suggested that this property can promote the insertion of water molecule into the membrane of Span 80 vesicle. Cholesterol has known to form the water accessible site, resulting in the reduction of ANS fluorescence intensity of POPC liposome (Slavik, 1982). Span 80 vesicle with intense headgroup mobility was not affected by the cholesterol. Span 80 vesicle membrane is already accessible for water molecule either with or without cholesterol. In the following, the different probe sensitive to the polar environment was used to reveal the polarity of Span 80 vesicle as mentioned above.

The polarity of each system was then investigated by using Laurdan (Bagatolli et al., 1999). The spectrum of Laurdan for system (1) and (2) showed the peak at 435 nm (**Fig. 1-10 (a)**). In contrast, the peak of liposomes (3, 4) and Span 80 vesicles (5, 6) was shifted into the longer wavelength range, suggesting that DMPC liposome and Span 80 vesicles were more hydrophilic than hexane and inverted micelle (W/O emulsion). This result is compatible with that obtained in ANS binding experiment (**Fig. 1-9**). To compare the polarity of the interface of Span 80 vesicle with liposome, the generalized polarization (GP) values were then evaluated. The order of GP value was same as that of

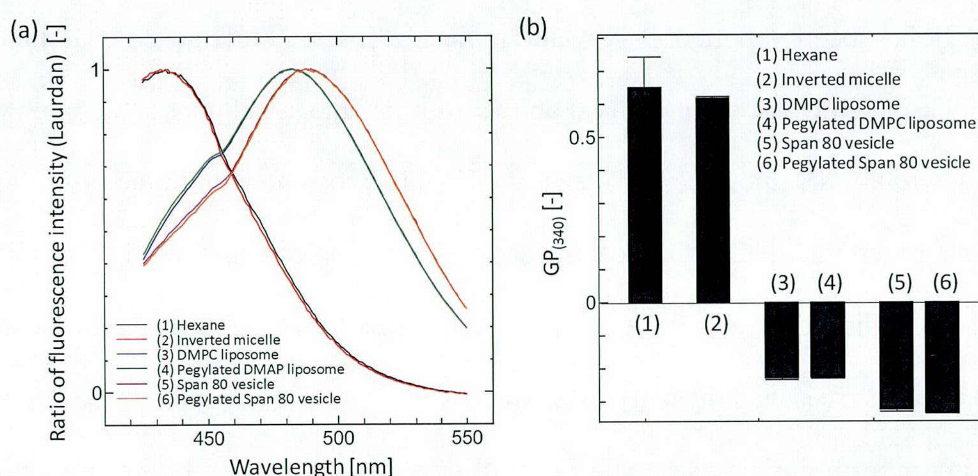


Fig. 1-10 (a) Fluorescence spectra for laurdan in the variety of systems. (b) Generalized polarization value at 340 nm. Measurements were performed at 37 °C.

ANS binding experiment, indicating the large polarity (hydrophilicity) of Span 80 vesicle. Meanwhile, the induction of PEG layer resulted in no variation in GP value in both cases of DMPC liposomes and Span 80 vesicles.

The fluorescence spectra of Laurdan in the (a) DPPC liposome, (b) POPC liposome, and (c) Span 80 vesicle were shown in **Fig. 1-11**. When Laurdan incubated into DPPC liposome suspension, the peak of Laurdan fluorescence intensity was observed at 440 nm either with or without cholesterol. The peak of Laurdan fluorescence intensity was observed at 490 nm in POPC liposome and Span 80 vesicle membrane. Cholesterol increased the fluorescence intensity at 440 nm and reduced the value at 490 nm. It has been reported that Laurdan inserts deeply into the vesicle membrane as compared with ANS (Bagatolli et al., 1999). Laurdan monitors the inner membrane polarity. Both peak at 440 nm and 490 nm show hydrophilic and hydrophobic environment, respectively (Bagatolli et al., 1999). In this study, the polarity of vesicle membrane was quantitatively evaluated, by calculating $GP_{(340)}$ value (**Fig. 1-12**) (Parasassi et al., 1994). DPPC liposome membrane is the most hydrophobic as compared with POPC liposome and Span 80 vesicle. DPPC liposome at gel phase

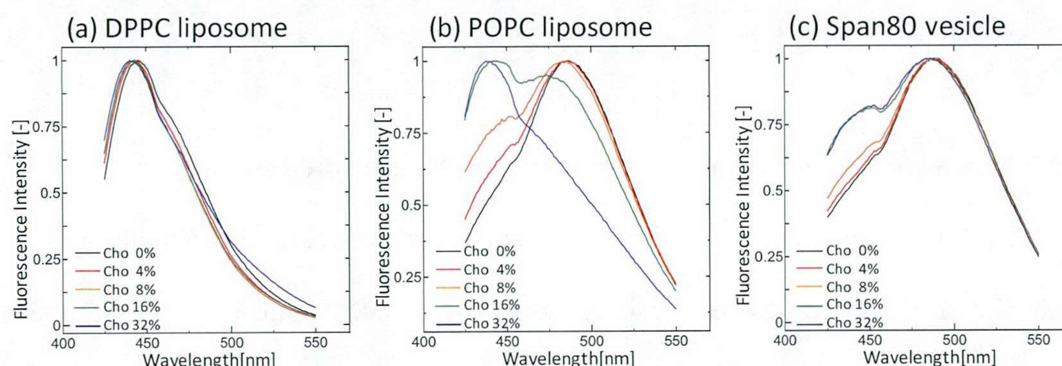


Fig. 1-11 Hydration as a function of cholesterol ratio increasing. Fluorescence spectra of Laurdan probe in (a) DPPC liposome, (b) POPC liposome, and (c) Span80 vesicle membrane at different amount of cholesterol containing at 35 °C.

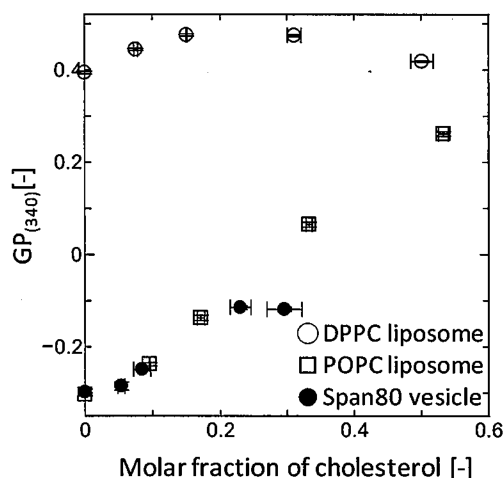


Fig. 1-12 Dipolar reorientation of solvent during Laurdan. Laurdan $GP_{(340)}$ values at various cholesterol concentrations containing. The percent values represent mol % of cholesterol in total mol of lipids.

can prevent the insertion of water molecule into the membrane as described by ANS regardless with or without cholesterol. The membrane of POPC liposome and Span 80 vesicle is more hydrophilic than DPPC liposome. Cholesterol increased the $GP_{(340)}$ value of POPC liposome and Span 80 vesicle. The inner membrane was composed by the unsaturated acyl chain in the case of POPC liposome and Span 80 vesicle. The hydrophilic property of inner membrane in case of POPC liposome is similar to that in case of Span 80 vesicle.

3.4. Fluid, Flexible, and Wet as a novel index for Membrane Property.

To fully characterize the variety of surfactant vesicles, the alteration of the physicochemical properties of vesicles was evaluated. Cholesterol is known to contribute to the maintenance of homeostasis through the modulation of the membrane properties and its diffusion (Maxfield and Tabas, 2005), and to function as a "hardener" of the lipid bilayer (Pugh et al., 1989, Shimanouchi et al., 2009). PEG layer is also

known as the inducer of the hydrophilic layer. However, the effects of cholesterol and PEG are different, depending on the membrane phase, concentration, temperature, and membrane component. In this chapter, the membrane polarity, fluidity and headgroup mobility were characterized in order to reveal differences in the membrane properties of surfactant vesicles.

As mentioned in introduction of this chapter, the membrane fluidity is one of the important properties to discuss the variety of membrane-relating phenomena. The membrane fluidity evaluated by DPH and TMA-DPH was dependent on the phase state, cholesterol concentration, and component of the membrane (**Figs. 1-5 and 1-6**). These results suggest that the dynamic property at the surface of the membrane was different from that at the interior (**Figs. 1-13(1) and (2)**). This dynamics is caused by the lateral diffusion of surfactant arrayed in the membrane. At the surface, the lateral diffusion was restricted by the dipole-dipole interaction between headgroups of surfactant and by the van der Waals interaction between acyl chains of surfactant in membrane. Therefore, "***Fluid***" property is considered to be an indispensable property of the membrane.

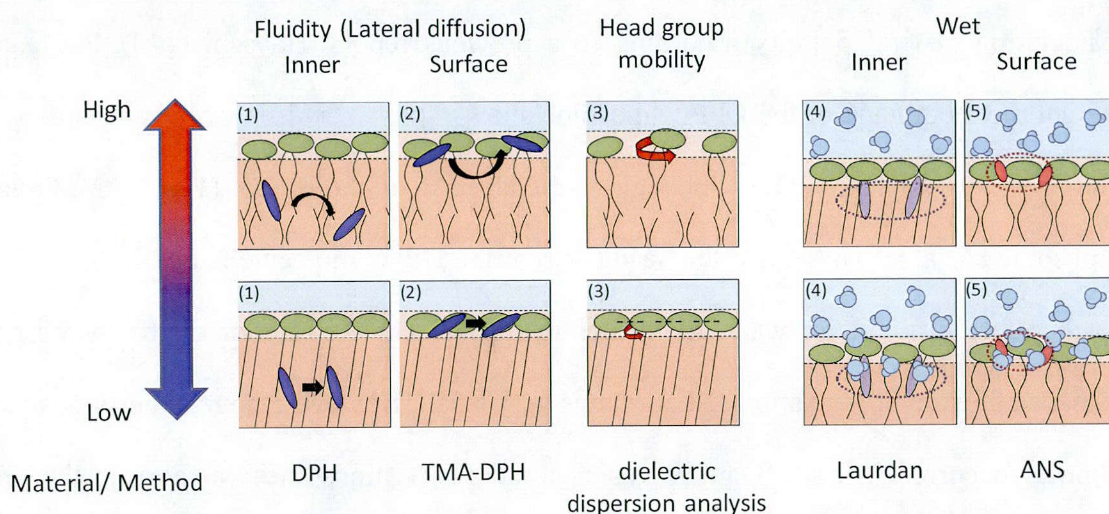


Fig. 1-13 Total view of the membrane characterization.

Likewise, the headgroup mobility is an index for the dynamics of membrane interface. Especially, the headgroup of Span 80 (sorbitol) could move with an intense mobility (**Fig. 1-7**), due to the weak interaction between sorbitol. This is caused by the hydroxyl group of sorbitol could induce the weak dipole-dipole interaction. In contrast, the phospholipid with a permanent dipole formed the strong interaction between phospholipids, inducing the reduction of headgroup mobility. As previously reported (Shimanouchi et al., 2011), the headgroup mobility can be correlated with the interfacial dynamics estimated by the TMA-DPH (membrane fluidity at the surface of vesicle membranes). In such a sense, it is considered that the headgroup mobility composed of the flexibility of membrane interface, which is considered as "***Flexible***" property of surfactant vesicles (**Fig. 1-13(3)**).

By using Laurdan and ANS, the polarity of membrane interface can be evaluated. Laurdan has a more hydrophobic structure than ANS, implying that it exists in a deeper region of the vesicle membrane. Actually, the results measured using Laurdan show a different tendency in the polarity of the membrane as compared with those measured using ANS (**Fig. 1-8** to **Fig. 1-12**). The fluorescence of the Laurdan was evaluated by calculating general polarity according to a previous report (Hayashi et al., 2011), in which the membrane of the DPPC liposome showed higher hydrophobicity than that of the Span 80 vesicle or POPC liposome in the absence of cholesterol (**Fig. 1-12**). DPPC liposome has a gel phase, and the headgroup of the DPPC molecule is tightly packed. It is therefore difficult for water molecules to invade into the interior of the membrane because the rate of relaxation of Laurdan is very small. However, the relaxation of water dipoles occurred in both Span 80 vesicles and POPC liposomes, suggesting that the vesicle in the liquid crystalline phase was able to entrap small water molecule in the

inner membrane. Thus, these surfactant vesicles were considered to have membranes with "***Wet***" characteristics (**Figs. 1-13(4)** and **(5)**).

From the above discussion, the *fluid*, *flexible*, and *wet* was expressed in the xyz-space as shown in **Fig. 1-14**. Surfactant vesicles might be one of formulations in a style of W/O/W emulsion. The discrepancy in such formulation between surfactant vesicles including emulsion should be derived from the *Wetness* of the interface. Therefore, *xy*-plane defined as fluid and flexible was adopted as the fundamental plane. In addition, wetness was adopted as *z*-axis to discuss the variation of formulation of surfactant. The organic phase and W/O emulsion were observed on *xy*-plane. In contrast, the formulation such as liposomes and Span 80 vesicle were scattered in *xyz*-space (*z* being not 0). Especially, Span 80 vesicle obviously differs from other formulations,

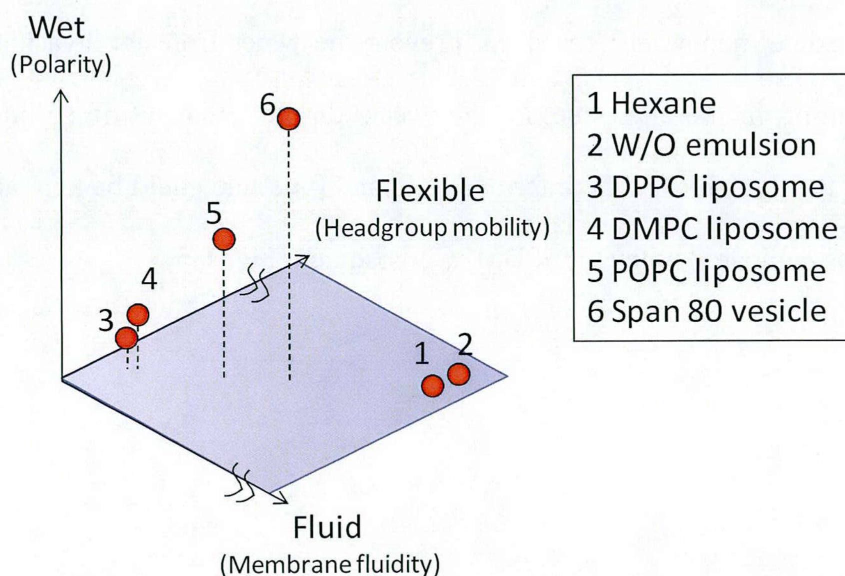


Fig. 1-14 Schematic illustration on categorization of surfactant formulations in membrane properties (Fluid/Flexible/Wet).

which implies the discrepancy of vesicle preparation. In the following, the influence of the two-step emulsification on the formulation of Span 80 was discussed based on **Fig. 1-14**.

It has been reported that Span 80 is capable of forming emulsions with several hundred micron in diameter, in water / n-tetradecane system under the high Span 80 concentration (Cornec et al., 1996), suggesting the stable partitioning of Span 80 into the interface of water / hexane system at the first emulsification as shown in **Fig. 1-4(b)**. The variation of the formulation for Span 80 along with the two-step emulsification process induced the reduction of hydrophobicity (**Figs. 1-8** and **1-10**), possibly resulting from the removal of hexane from the formulations of Span 80 at the second emulsification process. Taking into account the intense mobility of headgroup (sorbitol) for Span 80 (Hayashi et al., 2011), water molecule was likely to be present at the deeper region of vesicle membranes. This was probably because the sorbitol (headgroup) of Span 80 in vesicle membranes could not prevent the water from its invasion into the deeper region of membranes due to the weak dipole moment of sorbitol. As a consequence, the dynamics at the interface of Span 80 vesicle could be kept at the high extent level, as compared with that of DMPC liposomes (**Fig. 1-5**).

4. Summary

A surfactant vesicle is formed by the assembly of molecule, resulting in the induction of unique membrane properties. Membrane properties are dependent on the lipid composition of the surfactant vesicle. An unsaturated phospholipid, POPC molecule forms a liposome at liquid crystalline phase. POPC molecule dynamically moves on the liposome membrane, resulting in the easy insertion of water molecules as compared with DPPC liposome at gel phase. Span 80 molecules also forms Span 80 vesicle at liquid crystalline phase. The inner membrane properties of Span 80 vesicle are similar to the POPC liposome. However, the headgroup mobility of Span 80 vesicle is more intense than that of POPC liposome. The headgroup of POPC molecule with permanent dipolar moment contributes to the electrical interaction of headgroup region. On the other hand, Span 80 vesicle composed by non-ionic surfactant, Span 80 molecule. The headgroup interaction of Span 80 vesicle is lower than that of POPC liposome. The headgroup mobility of Span 80 vesicle is not prevented by the headgroup interaction as compared with POPC liposome. It is suggested that the low interaction of the headgroup induces the dynamic membrane in the case of niosome (non-ionic surfactant vesicle) like Span 80 vesicle. This dynamic membrane is expected to induce the strong interaction with the plasma membrane. The characterization of membrane properties contribute to the prediction of pharmacokinetics of the drug encapsulated drug carrier (**Fig. 1-14**).

Chapter 2 Effect of Membrane Properties on Membrane–Membrane Interaction

1. Introduction

Recently, the "membrane-membrane" interaction has been assigned to several kinds of steps. Considering the drug delivery system, the membrane of drug carrier, such as surfactant and phospholipid vesicles, can bind to the biomembrane. Some vesicles are internalized via an endocytosis, as shown in **Fig. 2-1**. Other vesicles can fuse with the biomembrane. Alternatively, the membrane properties affect not only their interaction with the cells, but also the encapsulation efficiency of drugs. It has been reported that the permeability of drugs from surfactant vesicle can be related with the membrane fluidity of surfactant vesicle (Shimanouchi et al., 2009). It has been also reported that there are various kinds of interactions between surfactant vesicle membrane and drugs (Alves et al., 2011, Hao and Li, 2011, Okamura and Yoshii, 2008). Therefore, it is expected that the membrane properties could be related with the retention capability of surfactant vesicle. In phenomenological studies, it has been found that the lipid composition dominated the "membrane-membrane" interaction and the "membrane-molecule" interaction.

In contrast to the phenomenological investigations, the molecular mechanistic

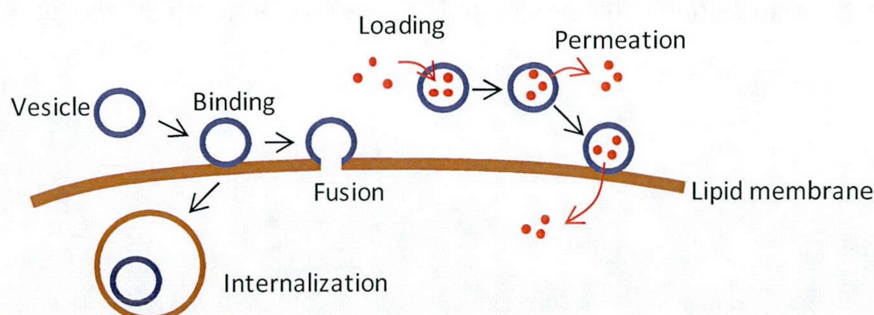


Fig. 2-1 Possible interaction modes of membrane-membrane interaction

studies have been reported. To fully understand the "membrane-membrane" interaction from the view point of "molecule" and "molecular assembly", the interaction properties have been characterized by using the variety of measurement methods, such as the leakage of molecular probe or the fluorescence energy resonance transfer. Based on the numerous reports, the binding of vesicle with the membrane can be assigned to a membrane perturbation. The process of fusion has been discussed from the viewpoints of lipid mixing/hemifusion. Among the possible surface properties, membrane fluidity and hydrophobicity of the liposome has been reported to be a key factor to improve the pharmacokinetic behaviors, such as the drug permeation (Komizu et al., 2006, Poste and Papahadjopoulos, 1976, Uchiyama et al., 1995), the binding of proteins to the liposomes (Kuboi et al., 2004), and the fusion (Kuboi et al., 2000). Meanwhile, the relationship between the phenomenological process of membrane-membrane interaction and the molecular mechanism has been still unclear. By clarifying the above relationship, the strategy of drug delivery system using surfactant-vesicles would become clear from the view point of molecule.

In this chapter, the "membrane-membrane" interaction was investigated from the viewpoints of (i) membrane perturbation, (ii) a lipid mixing/hemifusion, and (iii) a diffusion of lipid on the membrane. In addition, considering the small molecule favoring to distribute the lipid membrane as the alternative to the membrane, a distribution of small materials into the vesicle was investigated. Finally, the possible predominating factors for the interaction between membranes were elucidated from the results obtained herein, together with the results with respect to the membrane propertied discussed in **chapter 1**. The investigation performed herein would give the relationship between the phenomenological process and the molecular mechanistic factor.

2. Materials and Methods

2.1. Materials

Sorbitan monooleate (Span 80), polyoxyethylene sorbitan monooleate (Tween 80), and doxorubicin hydrochloride (DOX), and ammonium surface were purchased from Wako Pure Chemical Industries (Osaka, Japan). Cholesterol, calcein, and Triton®X-100 were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Phospholipids such as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were purchased from the NOF Corporation (Tokyo, Japan). 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl) (NBD-PE) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (rhodamine-PE; (Rh-PE)) was purchased from Avanti Polar lipids (Alabaster, AL, U.S.A.).

2.2. Preparation of liposome

Phospholipids and cholesterol were dissolved in a chloroform solution. The organic solvent was removed by evaporation in a rotary evaporator. The residual lipid film, after drying under a vacuum overnight, was hydrated with the inner-phase liquid (155 mM $(NH_4)_2SO_4$ or PBS; 137mM NaCl, 2-9mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4). The suspension was subjected to five cycles of freezing and thawing and then extruded. The lipid concentration was determined by phosphorous analysis.

2.3. Preparation of Span 80 vesicle.

Span 80 vesicle was prepared by the two-step emulsification method (Kato et al., 2006). 6 ml of n-hexane solution containing Span 80 and cholesterol was added to

0.6 ml of inner-phase liquid (155mM $(NH_4)_2SO_4$ or PBS), followed by the first emulsification for 6 min at 17,500 rpm using a micro-homogenizer NS-310E 2 (Microtec Co., Ltd., Funabashi, Japan). The solvent obtained from the water-in-oil emulsion was evaporated in a rotary evaporator at 28 °C under reduced pressure, yielding a water-lipid emulsion to which 6 ml of outer-phase liquid (PBS) containing 96 mg of Tween 80 was added, followed by mixing with the homogenizer for 2 min at 3,500 rpm to obtain the heterogeneous Span 80 vesicle suspension. The heterogeneous vesicle suspension was stirred with a magnetic stirrer for 3 hours at room temperature, followed by storage overnight at 4 °C. The vesicles were then purified by ultracentrifugation (50,000 rpm at 4 °C for 120 min) in a Himac centrifuge CR15B (Hitachi Koki Co., Ltd., Tokyo, Japan). The purified Span 80 suspension was passed through 100-nm nucleopore track-etch polycarbonate membranes and purified twice by ultracentrifugation (When Span 80 vesicle was prepared by $(NH_4)_2SO_4$, Outer liquid phase was exchanged from $(NH_4)_2SO_4$ to PBS).

2.4. Calcein leakage

Calcein (100 mM) was encapsulated into the liposome. The liposome (8.1 μM) was mixed with Span 80 vesicles (0.45 mM) at 37 °C. When Span 80 vesicle could interact with the liposome membrane, the entrapped calcein is leaked from the liposome, resulting in the recovery of its fluorescence from the self-quenching state (Bárány-Wallje et al., 2007). At the end of the above experiment, Triton®X-100 was added to this liposome suspension at the final concentration of 0.83 vol%, in order to disrupt the structure of the liposome completely. The perturbation effect of Span 80 vesicles on liposome was evaluated by the amount of calcein released, as described

below:

$$\text{RF} = \frac{(I_t - I_0)}{(I_{\max} - I_0)} \tag{2-1}$$

RF is the fraction of calcein released. I_0, I_t and I_{max} are the fluorescence intensities measured at the beginning of the experiment, at any incubation time t, and after the Triton®X-100 addition to give a maximal fluorescence intensity, respectively.

2.5. Fluorescence resonance energy transfer (FRET) experiment

The liposome used for the FRET experiment was prepared by mixing 0.4 mol% NBD-PE and 0.2 mol% Rhodamine-PE. A fluorescence labeled liposome (1.5 mM) was added to the Span 80 vesicle (25 mM). The alteration of the fluorescence of NBD and Rhodamine (Rho) was observed with a fluorescence spectrophotometer, FP-6500 (JASCO Co., Tokyo, Japan) at 37 °C. Lipid mixing was evaluated by the energy transfer efficiency (ET), as described below:

$$\text{ET} = \frac{I_{\text{Rho}}}{I_{\text{NBD}}} \tag{2-2}$$

where I_{Rho} and I_{NBD} indicate the fluorescence intensities at the respective emission, for Rho-PE (584 nm) and NBD-PE (527 nm) after excitation at 472 nm. The percentage of lipid mixing, %ET, was calculated by the following equation:

$$\%\text{ET} = \frac{(\text{ET}_0 - \text{ET}_t)}{(\text{ET}_0 - \text{ET}_{\max})} \tag{2-3}$$

where ET_t indicates the transfer efficiency at any incubation time t after vesicle mixing, ET_0 refers to the vesicles just after mixing, and $\text{ET}_{\max}$ is the value after the addition of

Triton®X -100.

2.6. Laurdan monitoring

Hemifused vesicle: A hydrophobic fluorescence probe, Laurdan (1.0 μM), was added to the liposome suspension (0.1 mM), and the mixed suspension was incubated for more than 30 min. In another experiment, the Span 80 vesicle suspension was stepwisely added as shown in **Fig. 2-2**. The ratio of lipid component used in this study is summarized in **Table 2-1**.

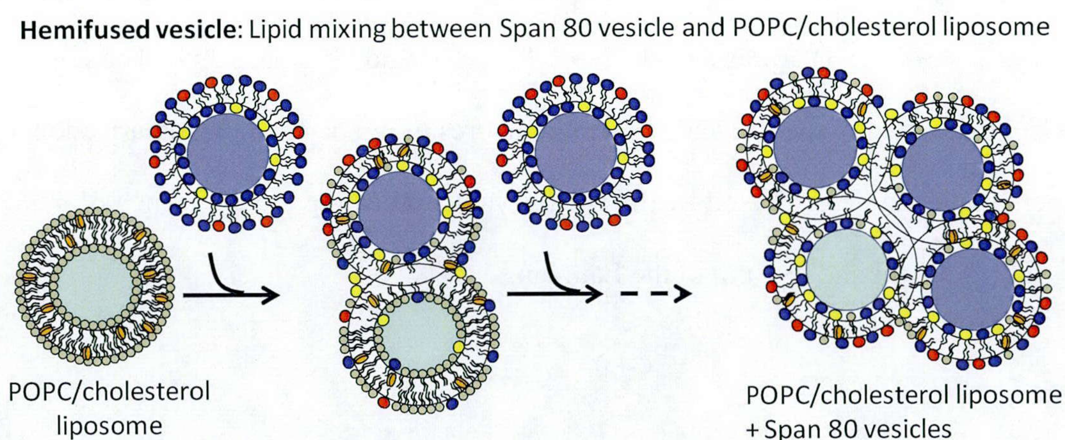


Fig. 2-2 Preparation method of **hemifusion vesicle**.

Table 2-1 Ratio of lipid composed in **hemifused vesicle** suspension.

	Molar ratio [%]		
Span 80 vesicles suspension [μl]	POPC	Cholesterol	Span 80
0	75.0	25.0	0.00
200	62.5	20.8	16.7
400	53.6	17.9	28.6
600	46.9	15.6	37.5
800	41.7	13.9	44.4
1000	37.5	12.5	50.0

Blended vesicle: POPC/cholesterol/Span 80 (37.5:12.5:50) were dissolved in chloroform and were used for the preparation of vesicles by the thin film method (**Fig. 2-3**), as described before (**2.2. Preparation of liposome**), followed by incubation with Laurdan (final concentration: 1.0 μM) for more than 30 min. The Laurdan fluorescence spectra were measured with the fluorescence spectrophotometer FP-6500 (JASCO Co., Tokyo, Japan) at 37 °C.

The Laurdan is the one of the environmentally-responsive fluorescence probe, the fluorescence spectra of Laurdan show the distinct double peak at 440 nm and 490 nm by the difference of the polarization around the Laurdan molecule when Laurdan is excited at 340 nm (Parasassi et al., 1994, Parasassi and Gratton, 1995). The Laurdan can easily insert into the vesicular membrane, resulting in that the polarization of the membrane can be evaluated by the $GP_{(340)}$ value indicating the difference of the double peak at 440 nm and 490 nm of the Laurdan.

$$GP_{(340)} = \frac{(I_{440} - I_{490})}{(I_{440} + I_{490})} \tag{2-4}$$

I_{440}: The fluorescence intensity of Laurdan at 440 nm

I_{490}: The fluorescence intensity of Laurdan at 490 nm

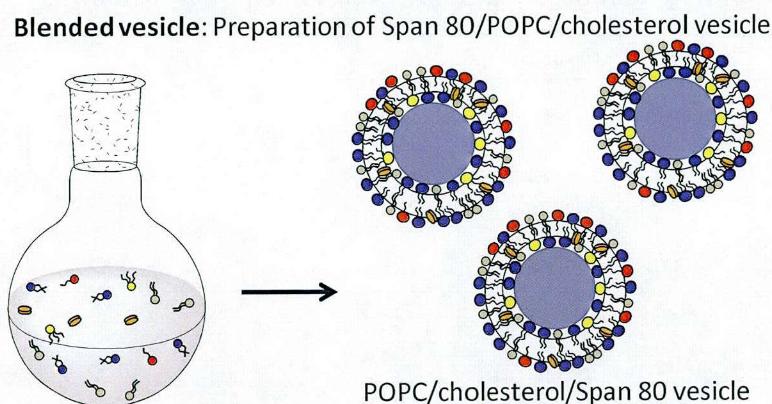


Fig. 2-3 Preparation method of **blended vesicle**.

2.7. Evaluation of the DOX encapsulation efficiency

DOX (0.5 mM) was mixed with the liposome suspension (inner phase: $(NH_4)_2SO_4$) (5 mM) at 20 °C, 40 °C or 60 °C. After the incubation for each time (0 ~ 150 min), this suspension was added to the aliquot of NaOH solution. The DOX solution could observe the absorption at 590 nm at the basic condition. The suspension was measured the absorption at 590 nm, calculating the amount of DOX encapsulated into the surfactant vesicle. A mass-transfer coefficient was calculated from the following equation:

$$-\frac{dC_{\mathrm{out}}}{dt} V_{\mathrm{out}} = S \times N \quad (2\text{-}5)$$

$$N = K \times C_{\mathrm{out}} \quad (2\text{-}6)$$

C_{out}: Concentration of the DOX in the outer phase [mol·m^{-3}]

V_{out}: Total volume of vesicle [m^{3}]

S: Total surface of vesicle [m^{2}]

N: Material flow rate [mol·m^{-2}·s^{-1}]

K: Mass-transfer coefficient [m·s^{-1}]

3. Results and Discussion

3.1. First Step of Membrane-Membrane Interaction: Membrane Perturbation.

In order to confirm the interaction between Span 80 vesicle and phospholipid vesicle (liposome), the leakage of calcein entrapped inside the liposome was first analyzed in the presence of excess of Span 80 vesicle in contrast to the target liposome. In this experiment, the degree of the intensity of the interaction between the different kinds of vesicles (Span 80 vesicle and liposome) could be monitored because the leakage can be induced through the membrane perturbation caused by their interaction. The perturbation of the liposome membrane after the addition of the Span 80 vesicle was evaluated through the analysis of calcein leakage from the liposome. In the first series of experiments, the liposome encapsulating the calcein (**liposome [A]**) (a simplified model of plasma membrane) and the **vesicle [B]** (a drug carrier) were prepared as schematically shown in **Fig. 2-4(a)**. **Figure 2-4** and **Table 2-2** show the time course of the calcein leakage from the **liposome [A]** (RF value), induced by the addition of **vesicle [B]**. The addition of Span 80 vesicle (**vesicle [B]**) to DPPC liposome (**liposome [A]**) immediately induced a significant increase in the RF value, indicating that almost all the calcein was leaked from the DPPC liposomes. It is considered that this is caused by the strong interaction of the Span 80 vesicle with the DPPC liposome membrane. Furthermore, a similar tendency for the RF value was also observed in the case of POPC or POPC/cholesterol liposome (**Figs. 2-4(b)** and **(c)**). It was thus demonstrated that the Span 80 vesicle could perturb the membrane of all kinds of liposomes tested here.

The Span 80 vesicle used as a **vesicle [B]** was replaced by phospholipid vesicles in order to confirm the possible interaction between different liposomes

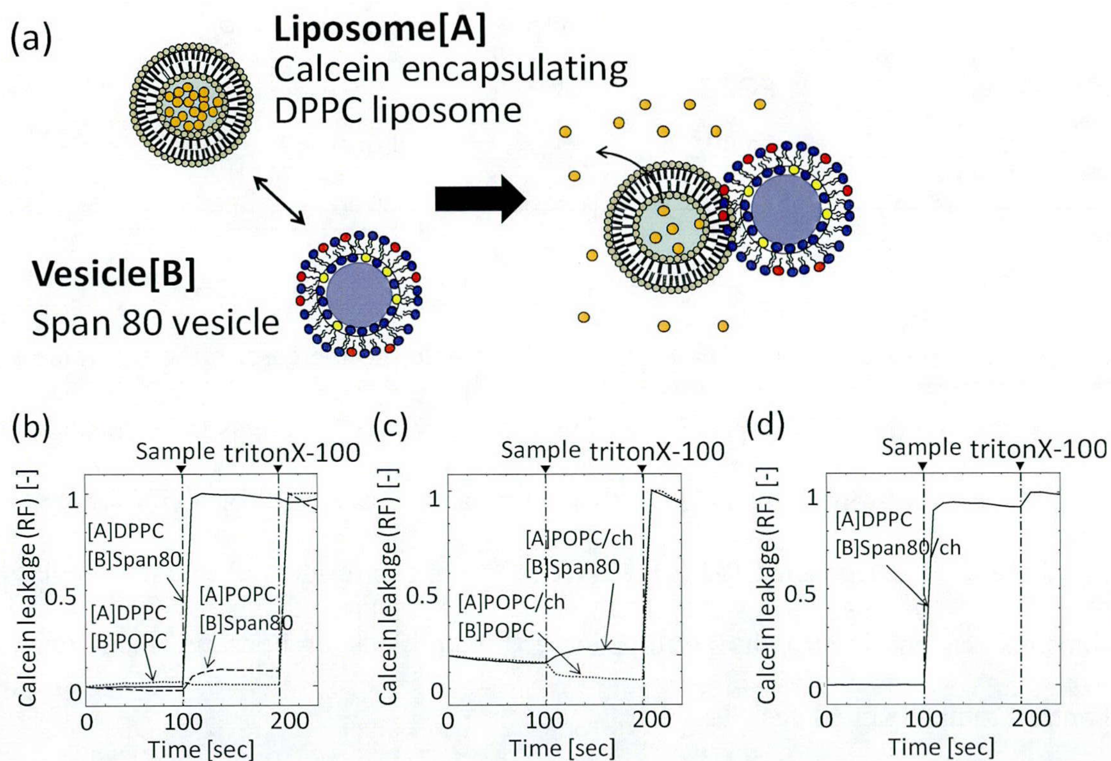


Fig. 2-4 Calcein leakage from liposome, caused by Span 80 vesicle. (a) Calcein encapsulating liposome (**liposome [A]**) was added to Span 80 vesicle (**vesicle [B]**), and the membrane interaction was evaluated from the amount of calcein leakage. (b) Calcein was leaked from **liposome [A]** (DPPC or POPC) by adding **vesicle [B]** (Span 80 or POPC), or (c) from **liposome [A]** (POPC/cholesterol) by adding **vesicle [B]** (Span 80 or POPC). The molar ration of POPC/cholesterol and Span80/cholesterol was set at 75 : 25 and at 67 : 33, respectively. **Abbreviation:** ch, cholesterol

Table 2-2 Calcein leakage from **liposome [a]** by adding **vesicle [B]**.
Abbreviation: ch, cholesterol

		Liposome [A]		
		DPPC	POPC/ch	POPC
Vesicle [B]	POPC	—	—	—
	Span 80/ch	High	Low	Low
	Span80	High	Low	Low

themselves. In contrast to the case of Span 80 vesicle, no increase in the RF value was observed when the POPC liposome was used as **vesicle [B]** and was mixed with DPPC liposome as **liposome [A] (Fig. 2-4(b))**. The same was true for POPC/cholesterol liposome used as **liposome [A] (Fig. 2-4(c))**. The above results indicate that the POPC liposome cannot induce the perturbation in other kinds of liposome membrane, in contrast to the Span 80 vesicles.

When the Span 80 vesicle was used as **vesicle [B]**, the calcein leakage from POPC or POPC/cholesterol liposome was less than that from DPPC liposome (**Figs. 2-3(b)** and **(c)**). The above results imply that the calcein leakage from **liposome [A]** can be correlated with the phase state of **liposome [A] (Fig. 2-5)**. The phospholipid molecules have been previously reported to be tightly arrayed on the membrane in the case of DPPC liposome at gel phase liposome (Koynova and Caffrey, 1998). The liposome membrane at the gel phase is also known to be hard as compared with that at the liquid crystalline phase (Shimanouchi et al., 2009). It has been reported that the tight phospholipid array is hence disturbed by the insertion of the extra lipids such as cholesterol (Ipsen et al., 1987). Some researchers have reported that the low flexibility of the DPPC inhibits the construction of the hard membrane when DPPC is mixed with

other lipids on membrane (Alipour et al., 2008, Carrillo et al., 2003). The observed leakage of almost all calcein from DPPC liposome after the addition of Span 80 vesicle could be related with the previous finding that Span 80 vesicle disrupted the tight phospholipid array and inhibited the reconstruction of hard membrane of the DPPC liposome. On the other hand, the phospholipids array of the POPC or POPC/cholesterol liposome is not so tight as compared with that of the gel phase liposome (Koynova and Caffrey, 1998, Thewalt and Bloom, 1992), indicating that the membrane of the POPC or POPC/cholesterol liposome is rather "*fluid*" than "*hard*" (Shimanouchi et al., 2009). The leakage of the calcein from POPC or POPC/cholesterol liposome was inhibited by the flexible membrane reconstruction of liposomal membrane when Span 80 vesicle interacted with liposome.

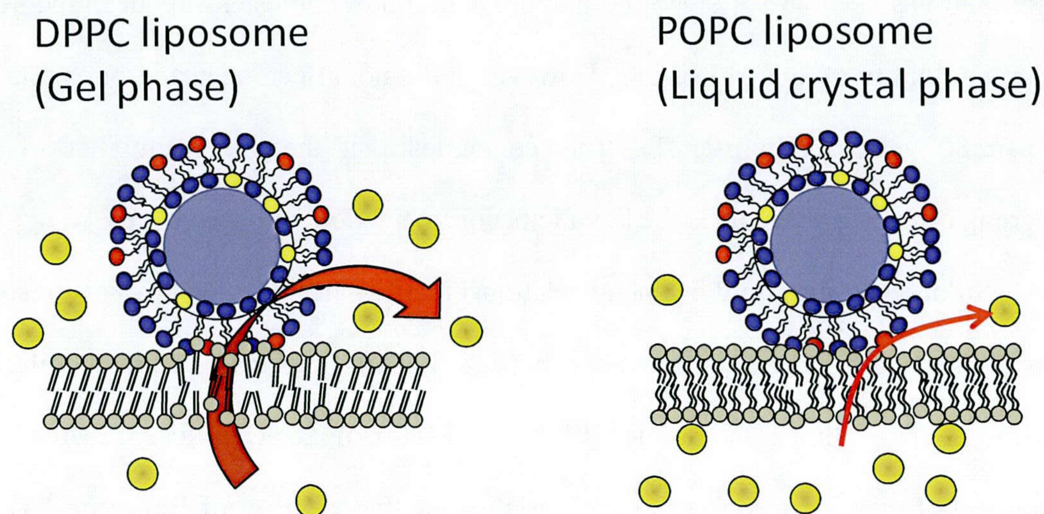


Fig. 2-5 Schematic illustration of the interaction between Span 80 vesicle and liposomes. The amount of calcein leakage from DPPC liposome is more than that from POPC liposome.

The observed difference between the Span80 vesicle and POPC liposome can be caused by the "*flexible*" nature on the Span 80 vesicle (**Chapter 1**). The headgroup of phosphatidylcholine as a component of the liposome membrane possesses a dipole moment, with both a positively charged group (N^+-$(CH_3)_3$) and a negatively charged one (-PO_4^--), which provide a relatively strong electrostatic interaction between phosphatidylcholine molecules (Shimanouchi et al., 2011). This interaction at the membrane surface can restrict the interaction with the membrane of the apposed liposomes. In contrast to the phosphatidylcholine, the headgroup of Span 80 is sorbitol that has no permanent dipole moment, implying that the interaction between the headgroup of Span 80 is relatively weak. The larger value in the calcein leakage after the addition of Span 80 vesicle might be caused by the larger mobility of the headgroup of Span 80 in contrast to phospholipid. This opinion is also supported by **Fig. 2-4(d)**. **Figure 2-4(d)** shows the strong interaction between Span 80/cholesterol vesicle and liposome. It has been already reported that the addition of cholesterol reduced the inner membrane fluidity of Span 80 vesicle. However, it did not affect the headgroup mobility of Span 80 vesicle (**Chapter 1**). It is concluded that the larger mobility of the headgroup of Span 80 vesicle is the key factor for interaction with liposome.

In order to discuss the general relationship, the calcein leakage from **liposome [A]** is plotted against its headgroup mobility (**Fig. 2-6(a)**). In the case of low mobility of the headgroup of lipids in **vesicle [B]** (< 60 MHz), no calcein leakage from the **liposome [A]** was induced despite the variation of the mobility of **liposome [A]**. In contrast, the **vesicle [B]** with high mobility (> 200 MHz) could induce the calcein leakage from the **liposome [A]**. The critical mobility of **liposome [A]** can be observed at 30 MHz. Alternatively, the calcein leakage from **liposome [A]** is plotted against the

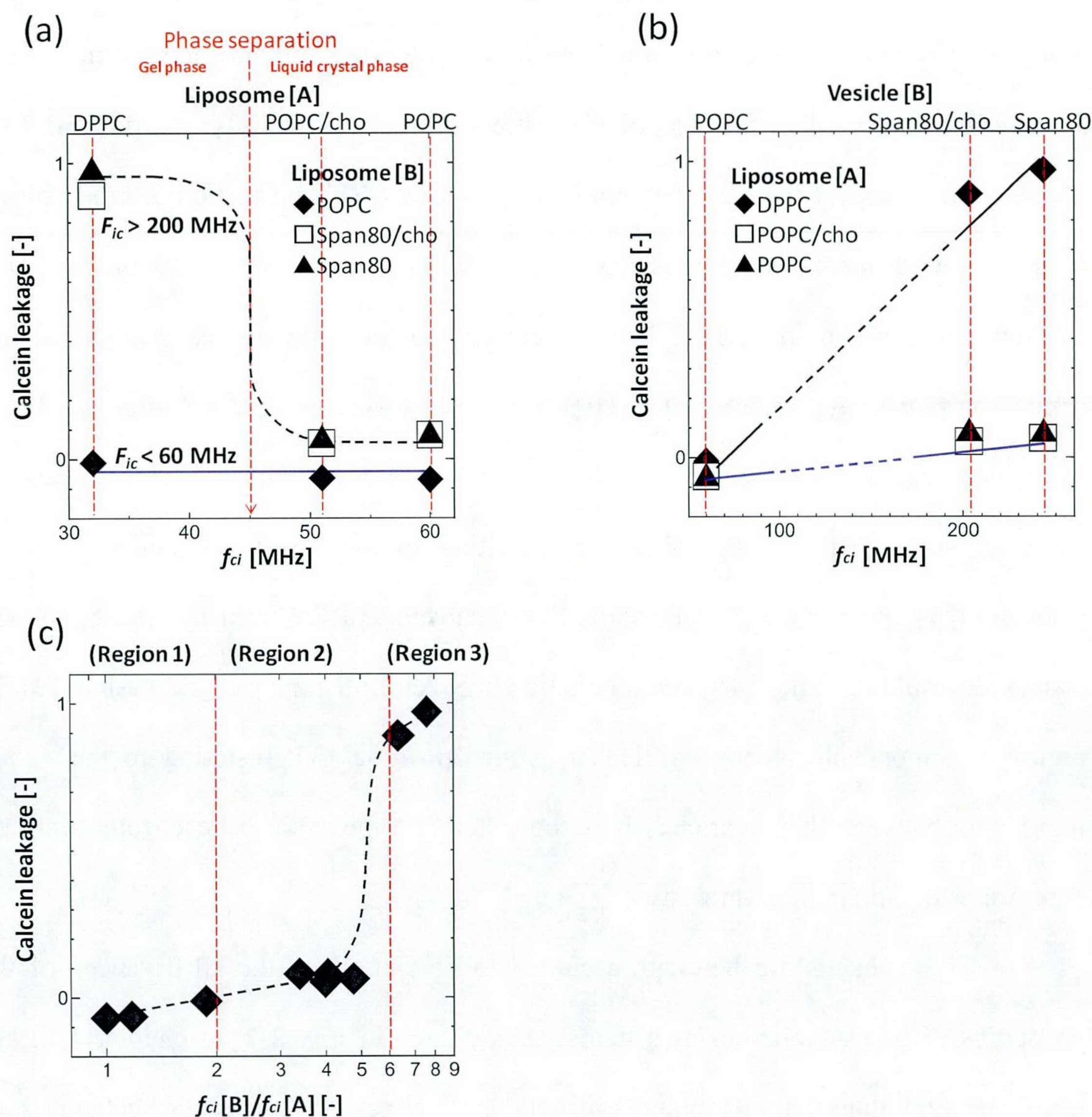


Fig. 2-6 Relationship between calcein leakage and headgroup mobility of (a) **liposome [A]**, and (b) **vesicle [B]**. (c) The calcein leakage was systematically analyzed according to the ratio headgroup mobility between **liposome [A]** (f_{ci} [A]) and **vesicle [B]** (f_{ci} [B]). **Abbreviation:** ch, cholesterol

headgroup mobility of **vesicle [B]**, as shown in **Fig. 2-4(b)**. Below the critical headgroup mobility of **liposome [A]**, an increase in headgroup mobility of **vesicle [B]** induced no remarkable increase in calcein leakage. Meanwhile, an increase in calcein leakage was observed in the case of the critical headgroup mobility of **liposome [A]**. These results suggest that the stiff lipid membrane was subject to be perturbed by its interaction with membrane possessing on interface with high mobility. Moreover, this relationship was shown in **Fig. 2-6(c)** according to the ratio of headgroup mobility (**liposome [A]**: f_{ci} [A], **vesicle [B]**: f_{ci} [B]). These plots were categorized into three kinds of region (Region 1: < 2, Region 2: 2~6, Region 3: > 6). In region 1, the calcein leakage from **liposome [A]** was not observed, resulting in the lower interaction between **liposome [A]** and **vesicle [B]**. In region 2, the calcein leakage from **liposome [A]** was observed, resulting in the adequate interaction between **liposome [A]** and **vesicle [B]**. In region 3, almost all calcein was leaked from **liposome [A]**, resulting in the strong interaction between these vesicles. It is suggested that the ratio of headgroup mobility determines the interaction intensity.

These results are herewith assumed to be caused by the "difference" of the properties of two membranes as schematically shown in **Fig. 2-7**. In chapter 1, it has been clarified that the membrane property can reflect the molecular behaviors of membrane component. After the different membranes can be contacted with each other, the difference of the molecular behaviors can be a driving force to trigger the next step of the "membrane-related phenomena", such as membrane perturbation, lipid mixing, and membrane fusion. In order to confirm the above assumption, the differences in membrane properties were plotted against the calcein leakage as shown in **Fig. 2-8**. The differences in membrane fluidity, head group mobility and polarity are herewith

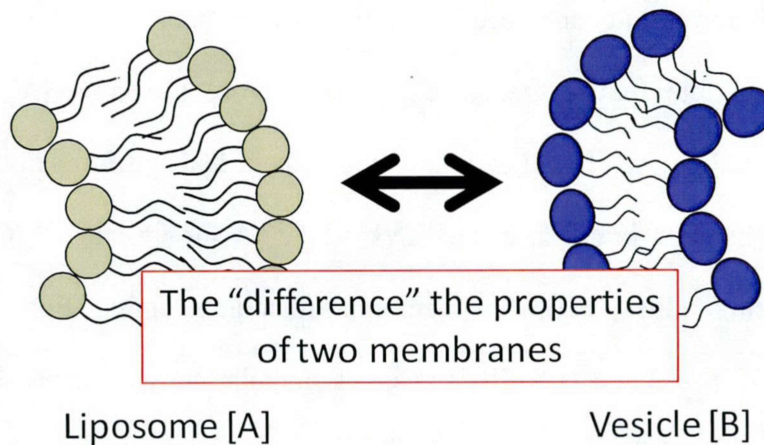


Fig. 2-7 "Difference" the properties of two membranes induce the "membrane-related phenomena".

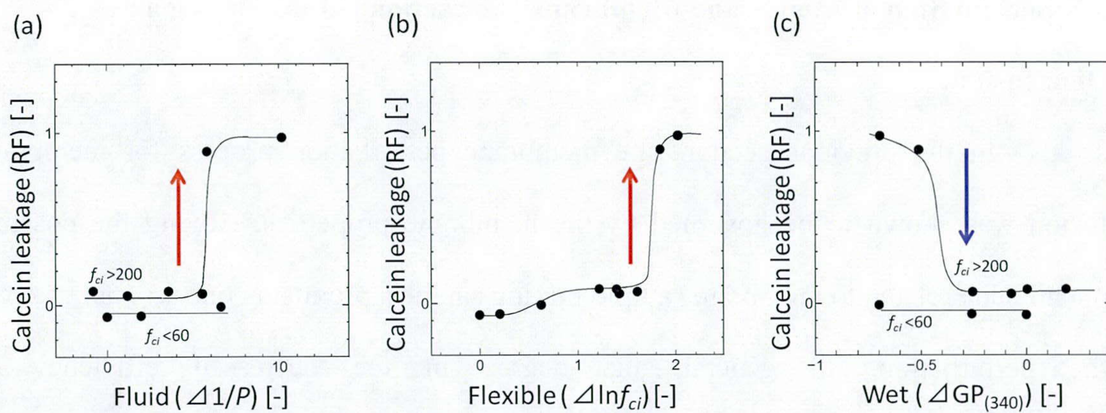


Fig. 2-8 Relationship between calcein leakage and differences in membrane properties, (a) fluid, (b) flexible, and (c) wet.

calculated from the following equation;

Membrane fluidity: $\varDelta\, {}^{1}/_{P} = {}^{1}/_{P_{[B]}} - {}^{1}/_{P_{[A]}}$ (2-7)

Head group mobility: $\varDelta \ln f_{\mathrm{ci}} = \ln f_{\mathrm{ci[B]}} - \ln f_{\mathrm{ci[A]}}$ (2-8)

Polarity: $\varDelta \mathrm{GP}_{(340)} = \mathrm{GP}_{(340)} - \mathrm{GP}_{(340)}$ (2-9)

In case of membrane fluidity and head group mobility, the increase of both $\varDelta 1/P$ and $\varDelta \ln f_{ci}$ was found to induce the calcein leakage from **liposome [A]**. Instead, the decrease of $\varDelta GP_{(340)}$ was found to induce the calcein leakage from **liposome [A]**. A threshold was observed in all cases ($\varDelta 1/P = 5.5$, $\varDelta \ln f_{ci} = 1.8$, $\varDelta GP_{(340)} = 3.5$). The above results show that the membrane-membrane interaction was induced especially when the value of difference of membrane properties between two membranes (called as "membrane potential") was overpassed above the threshold of the differences in membrane properties. It was thus found that the "membrane potential" could be a key factor to explain the membrane-related phenomena.

3.2. Second Step of Membrane-Membrane Interaction: Lipid Mixing and Hemifusion.

In the previous section, the membrane perturbation process for membrane fusion was shown to be governed by the membrane properties. Among the possible phenomena related to membrane, a lipid mixing can be a promising one as a next step of the perturbation. In general, the degree in the decreased efficiency of proximity-dependent fluorescence resonance energy transfer (FRET) has been considered to be correlated with lipid mixing (Marsden et al., 2011, Struck et al., 1981). A liposome incorporating two different fluorescence-labeled lipids has been used to evaluate the efficiency of FRET between them (%ET), which is a quantitative index for the lipid mixing. In this section, the interaction between **liposome [A]** and **vesicle [B]** is discussed in terms of %ET value, by using the **liposome [A]** labeled with both NBD-PE and Rho-PE (**Fig. 2-9(a)**). When the Span 80 vesicle was used as **vesicle [B]**, the %ET value for the DPPC liposome as **liposome [A]** increased after the addition of the Span

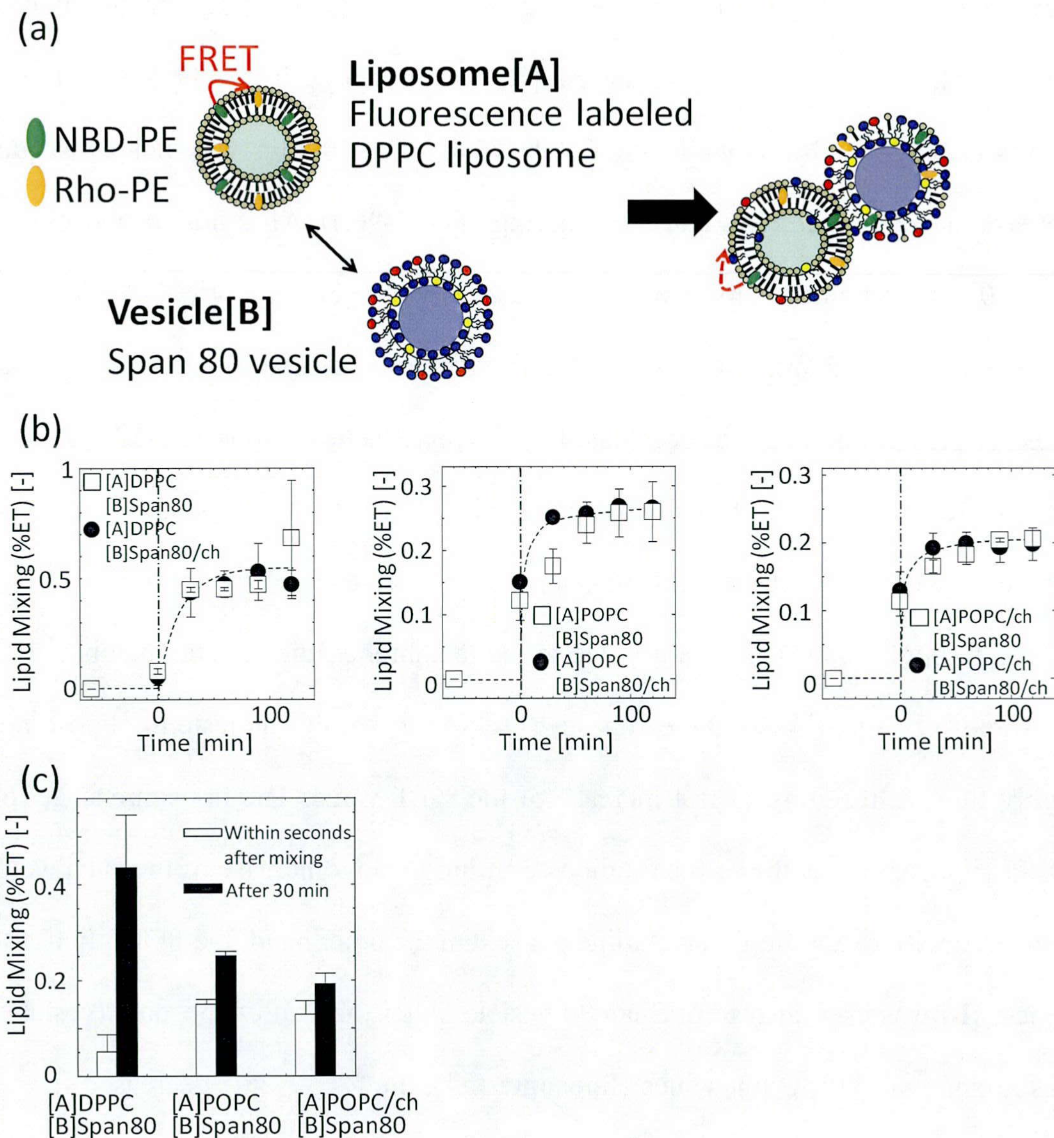


Fig. 2-9 Lipid mixing between Span 80 vesicle and liposome. (a) Span 80 vesicle (**vesicle [B]**) was added to fluorescence labeled liposome (**liposome [A]**), and lipid mixing was evaluated by FRET method. (b) Lipid mixing of **liposome [A]** by adding **vesicle [B]**. (c) Comparison of lipid mixing: right seconds after mixing vesicle, and 30 min later. Mean values (± standard deviation) are given for measurements carried out with 3 times. The molar ration of POPC/cholesterol and Span80/cholesterol was 75 : 25 and 67 : 33, respectively. **Abbreviation:** ch, cholesterol

80 vesicle **(Fig. 2-9(b))**. The %ET value after 30 min was consequently increased as compared with that after mixing **(Fig. 2-9(c))**. The %ET value for the POPC or POPC/cholesterol liposome as **liposome [A]** also increased just after adding the Span 80 vesicle **(Fig. 2-9(b))**, while the %ET value after 30 min was not consequently increased as compared with that after mixing **(Fig. 2-9(c))**. All kinds of liposomes and Span 80 vesicle immediately interact with each other as shown in **Fig. 2-4** (Calcein was immediately leaked from liposome after the addition of Span 80 vesicle), while the speed of the lipid mixing was dominated by the phase state of liposome **(Liposome [A])** (i.e. The diffusion speed of Span 80 molecule is slower at DPPC liposome as compared with POPC or POPC/cholesterol liposome). The difference of the increasing speed of the %ET (lipid mixing) value attributed to the membrane fluidity. The membrane of the liposome at liquid crystalline phase (POPC or POPC/cholesterol liposome) is highly-fluided, resulting in fast increase of the %ET value. The liposome at gel phase (DPPC liposome) has the lower fluidity, resulting in slow increase of the %ET value as compared with that at liquid crystalline phase. On the other hand, the POPC liposome as **vesicle [B]** was used instead of Span 80 vesicle. After the POPC liposome **(vesicle [B])** was mixed with DPPC liposomes **(liposome [A])**, the %ET value decreased from 0.44 to 0.1 in contrast to the case of the mixture of DPPC **(liposome [A])** with Span 80 **(vesicle [B])** **(Fig. 2-9(c))**. It is therefore considered that the abnormal properties of Span 80 vesicle, such as flexible surface and molecular structure of lipids, are important to increase the %ET value.

When cholesterol is incorporated into the Span 80 vesicle, the lateral diffusion of Span 80 in the membrane can be reduced, while keeping the intense mobility of the headgroup of the Span 80 **(Chapter 1)**. The Span 80/cholesterol vesicle was herein used

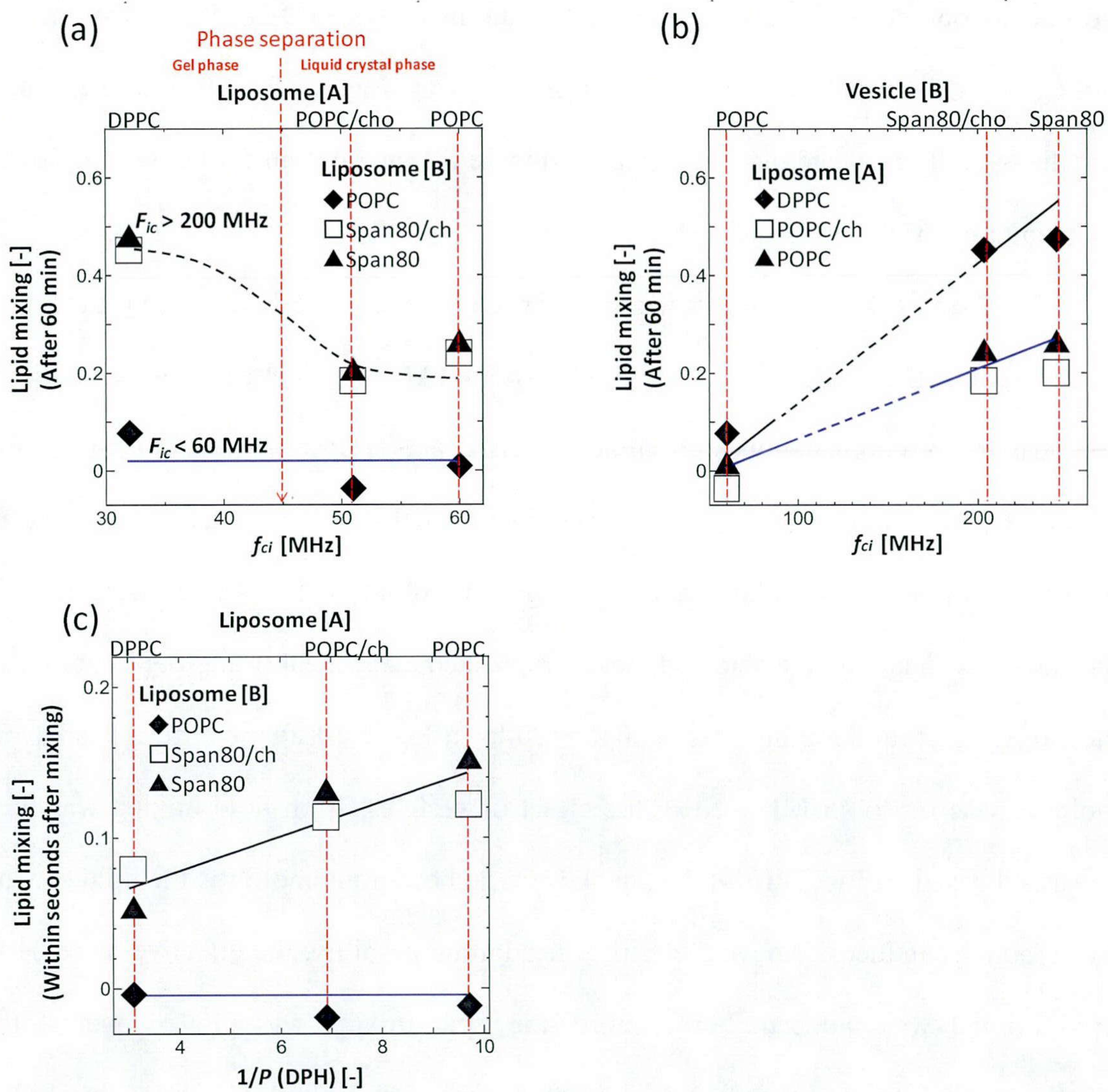


Fig. 2-10 Relationship between lipid mixing and headgroup mobility of (a) **liposome [A]**, (b) **vesicle [B]**. (c) Relationship between lipid mixing and the membrane fluidity. **Abbreviations:** ch, cholesterol

as **vesicle [B]**, in order to examine the significance of headgroup mobility throughout the interaction between vesicles. The %ET value increased to 0.2 ~ 0.4 in the case of DPPC, POPC, and POPC/cholesterol as **liposome [A] (Fig. 2-9(b))**. It was thus found that the Span 80/cholesterol vesicle could promote the energy transfer between lipids in the membrane of the **liposome [A]**.

In order to discuss the effect of headgroup mobility, the lipid mixing ratio was plotted against the headgroup mobility of **liposome [A] (Fig. 2-10(a))**. **Vesicle [B]** with low headgroup mobility induced no lipid mixing against **liposome [A]**. In contrast, the **vesicle [B]** with high headgroup mobility could induce the lipid mixing and a significant increase in lipid mixing in the range of headgroup mobility below 45 MHz. As well as the calcein leakage, it is considered that there existed the critical headgroup mobility in the induction of lipid mixing. Based on the results in **Fig. 2-10(a)**, the critical headgroup mobility was set to 45 MHz. Next, the effect of **vesicle [B]** on lipid mixing was then discussed based on **Fig. 2-10(b)**. Below the critical headgroup mobility, the lipid mixing was gradually induced. Above the critical headgroup mobility, the effective increase in lipid mixing was induced. Furthermore, the lipid mixing was plotted against the membrane fluidity as shown in **Fig. 2-10(c)**, the membrane fluidity of **liposome [A]** is related with the lipid mixing within seconds after mixing. This suggests that the lipid mixing requires the intense headgroup mobility and the lateral diffusion.

Similarly in the case of section 3.1 **(Fig. 2-7** and **Fig. 2-8)**, the value of the lipid mixing between two vesicle membranes was also plotted against the "membrane potentials", defined as Eq (2-7) ~ (2-9), as shown **in Fig. 11**. In the case of membrane fluidity, the increase of each $\triangle 1/P$ was found to induce the lipid mixing in **liposome [A] (Fig. 2-10 (a))**. On the contrary, the decrease of $\triangle GP_{(340)}$ was found to induce the

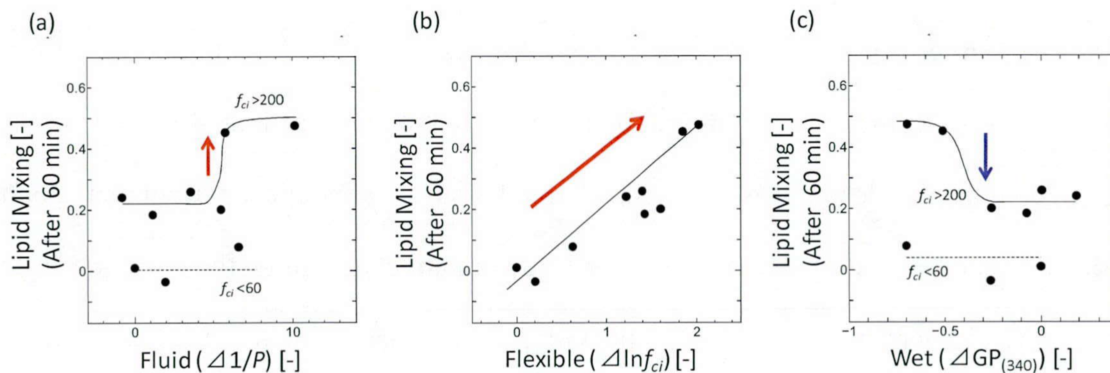


Fig. 2-11 Relationship between lipid mixing and differences in membrane properties, (a) fluid, (b) flexible, and (c) wet.

lipid mixing in **liposome [A] (Fig. 2-10 (c))**. A threshold was observed in both cases (⊿ $1/P = 5.5$, ⊿ $\ln f_{ci} = 1.8$, ⊿ $GP_{(340)} = 3.5$). A clear dependence of the lipid mixing on the ⊿ $\ln f_{ci}$ was observed as shown in **Fig. 2-10(b)**. The above results show that the lipid mixing, followed by membrane perturbation, can be strongly dependent on the molecular motion of the membrane.

3.3. Effect of the Diffusion of Span 80 vesicle/molecule on the liposome membrane.

It was examined whether the Span 80 vesicle could alter the polarity of the liposome membrane by lipid mixing. A **hemifused vesicle** was first prepared by mixing Span 80 vesicle with POPC/cholesterol (75:25 in molar ration) for 30 min of incubation (**Fig. 2-2**). POPC/cholesterol/Span 80 vesicle (37.5: 12.5: 50 in molar ration) was also prepared as ideally hemifused vesicles (**blended vesicles**) (**Fig. 2-3**). The membrane polarity of the **hemifused vesicle** was compared with that of the **blended vesicle**. The fluorescence spectra for the Laurdan-embedded POPC/cholesterol liposome are shown

in **Fig. 2-12(a)**. A peak derived from in nonpolar environment was observed at around 440 nm (curve: 0 μl). The addition of Span 80 vesicle resulted in the increase of the peak derived from in polar environment around 490 nm (curves: 400 and 800μl), together with the decrease of the peak derived from in nonpolar environment around 440 nm (Parasassi et al., 1998). In order to quantitatively monitor the variation in the polar environment of the membrane, the $GP_{(340)}$ value was estimated from the observed spectra according to the literature (Parasassi et al., 1994), and was plotted against of the molar ratio of Span 80, as shown in **Fig. 2-12(b)**. The $GP_{(340)}$ value linearly decreased with the increase in the ratio of Span 80, which was compatible with the presence of an isosbestic point at around 470 nm, as observed in **Fig. 2-11(a)**, and with the finding of $GP_{(340)}$ (Span 80) < $GP_{(340)}$ (POPC/cholesterol). These results suggest that the variation

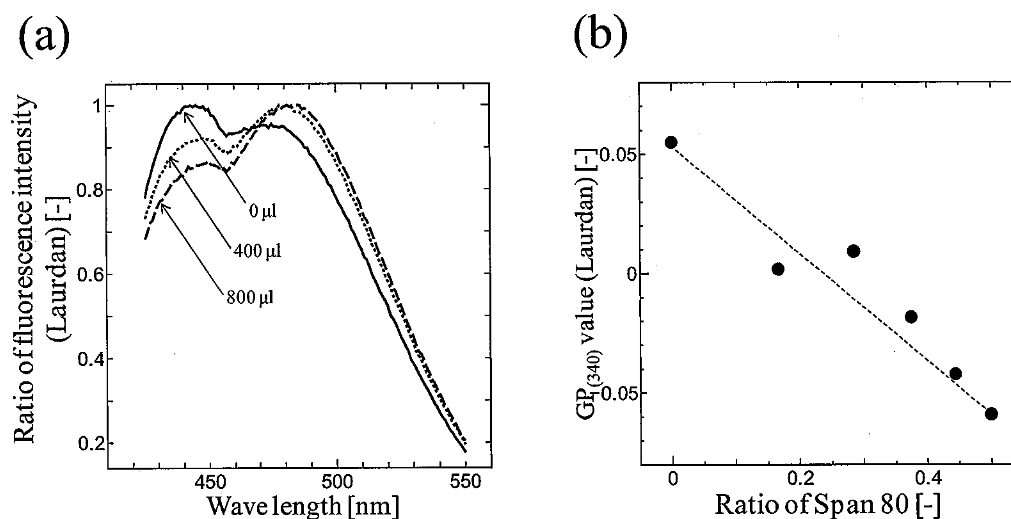


Fig. 2-12 Alteration of liposomal membrane property by Span 80 vesicle. (a) Fluorescence spectra of Laurdan after adding Span 80 vesicle by 0 to 1000 μl in volume. (b) The relationship between the $GP_{(340)}$ value and the ratio of Span 80 vesicle

of the polar environment of the vesicle membranes is related with the interaction between vesicles.

In addition, the variation in the polar environment of the **hemifused vesicle** was compared with that of the **blended vesicle** prepared as ideal **hemifused vesicle.** The fluorescence spectra of the Laurdan bedded in the membrane of the Span 80/POPC/cholesterol vesicle **(blended vesicle)** was slightly different from those of the **hemifused vesicle (Fig. 2-13(a))**. The $GP_{(340)}$ value for the **hemifusion vesicle** was higher than that of the **blended vesicle (Fig. 2-13(b))**. From the experiments using Laurdan, it can be concluded that the Span 80 vesicle did not considerably hemifuse with the POPC/cholesterol liposome, though some of Span 80 vesicle slightly hemifuse

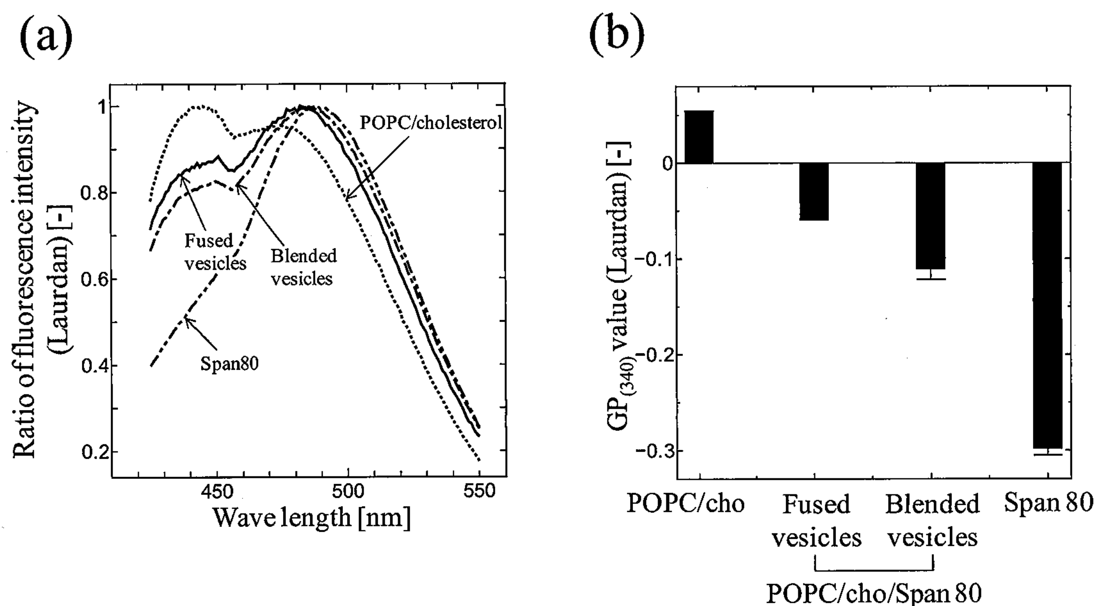


Fig. 2-13 Composition of membrane polarity between **hemifused vesicle** and **blended vesicle** (a) Fluorescence spectra of laurdan for Span 80 vesicle, POPC/cholesterol liposome, **hemifused vesicle**, and **blended vesicle. Blended vesicle** is the vesicle composed of POPC, cholesterol, and Span 80. (b) The comparison of the $GP_{(340)}$ value for the **hemifused vesicle** and that of the **blended vesicle.**

with themselves. However, Span 80 vesicle hemifuses with liposome at large extent enough to alter the polarity of the liposome membrane. These results are also supported by the hemifusion between Span 80 vesicle and liposome.

In order to discuss the mechanism underlying in vesicle-vesicle interaction phenomena, the lipid mixing of various vesicles was compared with the calcein leakage. As shown in **Fig. 2-14**, biphasic trend was observed in their relationship. First, a significant increase in lipid mixing was induced by the small incremental change in calcein leakage. The calcein leakage requires the defect on the lipid membrane, so that calcein pass through there. It is therefore considered that the membrane perturbation of stable lipid membrane due to the outer environmental condition could give the space for lipid molecules to diffuse laterally. In contrast, in the high calcein leakage region, the gradual increase of lipid mixing was observed. This is because there is a considerable

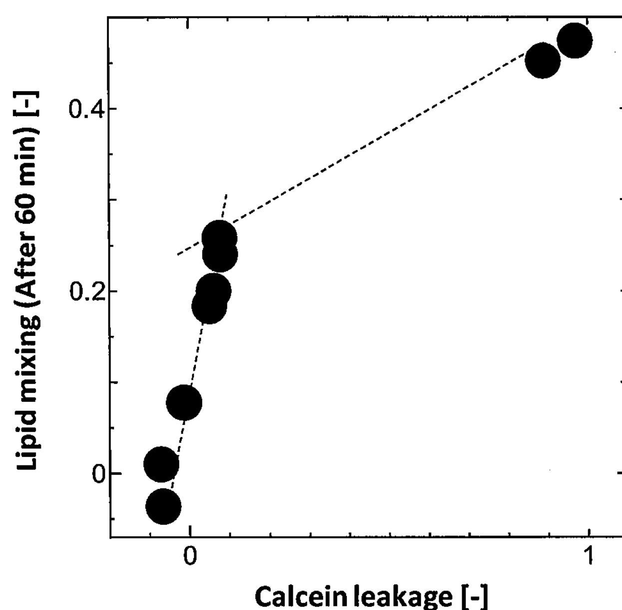


Fig. 2-14 Relationship between calcein leakage and lipid mixing.

space for lateral diffusion of lipid molecule under the condition of high calcein leakage. From the above consideration, it is suggested that the membrane perturbation (calcein leakage) was followed by the lipid mixing, which is consistent with the time dependency of both calcein leakage (~10 sec, **Fig. 2-4**) and lipid mixing (~30 min, **Fig. 2-9**). However, it is not meant that the intense promotion of lipid mixing induce the perfect fusion of vesicles because **Fig. 2-15** suggests the hemifusion as a possible mechanism. Therefore, the relationship between membrane perturbation and lipid mixing, as the hemifusion process, will be discussed in the following.

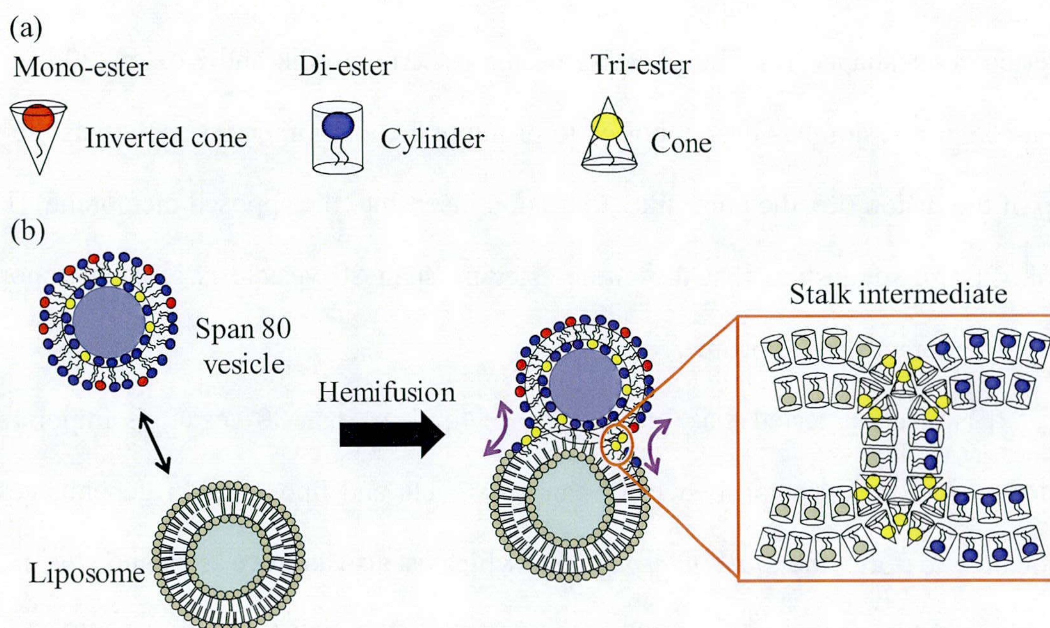


Fig. 2-15 Possible scheme of hemifusion model via *Stalk intermediate*. (a) Span 80 vesicle contain some kinds of esters, such as mono-, di-, and tri-ester. The relative size of the headgroup and the acyl chain region is relatively different. The shapes of mono-, di-, and tri-ester are likely to be inverted cone, cylinder and cone structure, respectively. (b) Hemifusion process. Cone–shaped lipids are able to form a *stalk intermediate* with negative curvature.

The membrane perturbation is the initiating factor for hemifusion process. From the results obtained before, the Span 80 vesicle is a promising system to induce the membrane perturbation. In addition, the molecular mobility of lipid in membrane would depend on the structure of lipid molecule. Therefore, hemifusion model will be discussed by exemplifying Span 80 vesicle. It has been reported that Span 80 vesicle contains mono-ester, di-ester, and tri-ester (Kato et al., 2006). Based on previously-reported definition (Chernomordik and Kozlov, 2008), mono-ester, di-ester, and tri-ester correspond with an inverted cone structure, a cylinder structure, and a cone structure, respectively **(Fig. 2-15(a))**. It is suggested that the mono-ester in Span 80 vesicle can form a point-like protrusion, which can typically be observed in the case of inverted cone–shaped lysophosphatidylcholine (Chernomordik and Kozlov, 2008). The point-like protrusion has been reported to promote the membrane interaction as the first step of the fusion (i.e. the point-like protrusion insert into the apposed membrane) (Efrat et al., 2007), suggesting that the mono-ester in Span 80 vesicle is also an important factor to interact with liposome.

It is also suggested that the structure of lipid in Span 80 vesicle is important to understand the hemifusion between Span 80 vesicle and liposome. In general, vesicle associates to form the *stalk intermediate*, which is also known as *hemifusion* model (Chernomordik and Kozlov, 2008, Hafez and Cullis, 2001, Siegel, 1999). In the associated region of different vesicles, lipid membranes deform to a bilayer membrane structure, as shown in the zoomed frame of **Fig. 2-15(b)**. In the process of hemifusion, the geometric structure of the lipid (e.g., inverted cone, cylinder, cone **(Fig. 2-15(a))** is important, as well as the lipid mixing. Especially, a lipid with a cone structure is useful for the generation of a negative curvature, which can contribute to the formation of the

stalk intermediate. When a *stalk intermediate* is formed, the lipids in the liposome diffuse into the Span 80 vesicle, a process which is called lipid mixing. This is compatible with the obtained result on a high %ET value in Span 80 vesicle / DPPC liposome system (**Fig. 2-9(b)**).

3.4. Design of the Surfactant-Vesicle Membrane for the Efficient Drug Capsulation in to the Drug Carrier Based on the Membrane Polarity.

The effect of membrane properties was investigated, focusing on the membrane interaction not only with surfactant vesicle membrane, but also with drugs. The efficient encapsulation of drugs into the drug carrier is important to accumulate drugs into the disease site. In this section, the encapsulation of doxorubicin hydrochloride (DOX) by the remote loading method was investigated, since this method is conventionally used as the advantageous method for the drug delivery using surfactant vesicle (Lasic et al., 1995). The remote loading of DOX was performed by the transmembrane pH gradient (**Fig. 2-16**). Surfactant vesicle was prepared by buffer solution at low pH (ammonium

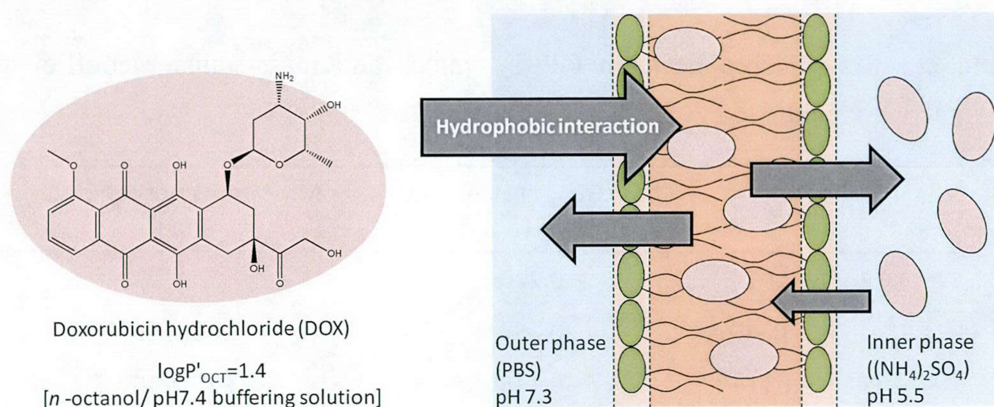


Fig. 2-16 Encapsulation mechanism of DOX. DOX inserts into the membrane dependent on the hydrophobicity.

sulfate), followed by adding a more basic solution to raise the pH of external solution pH (PBS). When DOX molecule passes through the surfactant vesicle membrane, DOX molecule becomes protonated intravesiculary in the inner phase at low pH. The positively charged DOX is difficult to pass the bilayer, resulting in the trapping of DOX into the inner aqueous phase of surfactant vesicle.

In the first series of experiments, the time course of DOX present at the external aqueous phase of liposome was monitored to estimate the mass transfer coefficient, according to **Section 2-9**. The obtained mass transfer coefficient was summarized in **Table 2-3**. Overall, the order of mass transfer coefficient was $10^{-7} \sim 10^{-6}$ m/s under the temperature condition of 60 °C. DPPC/cholesterol liposome gave the maximal mass transfer coefficient. Based on the previous finding that the permeation coefficient of calcein could be correlated with the membrane fluidity (Shimanouchi et al., 2009), DOPC or DMPC liposomes should give the higher mass transfer coefficient than DPPC liposome. Then, to reveal the above ambiguous feature, the mass transfer coefficient was compared with the physiochemical property of membranes.

Table 2-3 Relationship between $GP_{(340)}$ values and mass-transfer coefficient. These liposome were evaluated at 60 °C

Lipid mixture	$GP_{(340)}$ value [-]	Mass-transfer coefficient [m/s]
DLPC (12:0 PC)	-0.220	7.02×10^{-10}
DMPC (14:0 PC)	-0.154	1.06×10^{-9}
DPPC (16:0 PC)	-0.115	2.25×10^{-9}
DPPC:ch (9:1)	-0.024	1.35×10^{-6}

The relationship between $GP_{(340)}$ value and mass-transfer coefficient at 60 °C was summarized in **Table 2-3**. Considering the $GP_{(340)}$ value (Parasassi et al., 1994) and previous report (Koynova and Caffrey, 1998), these liposome have membrane at liquid crystalline phase at 60 °C (Liquid crystalline phase: $GP_{(340)} < -0.3$, Gel phase: $GP_{(340)} > -0.3$). The increase of $GP_{(340)}$ value increased the accelerated-mass-transfer coefficient. In order to investigate the relationship between $GP_{(340)}$ values and mass-transfer coefficient in detail, the above experiment was also carried out at lower temperature (20 °C or 40 °C). These results are plotted in **Fig. 2-17**. In the case of liposome at liquid

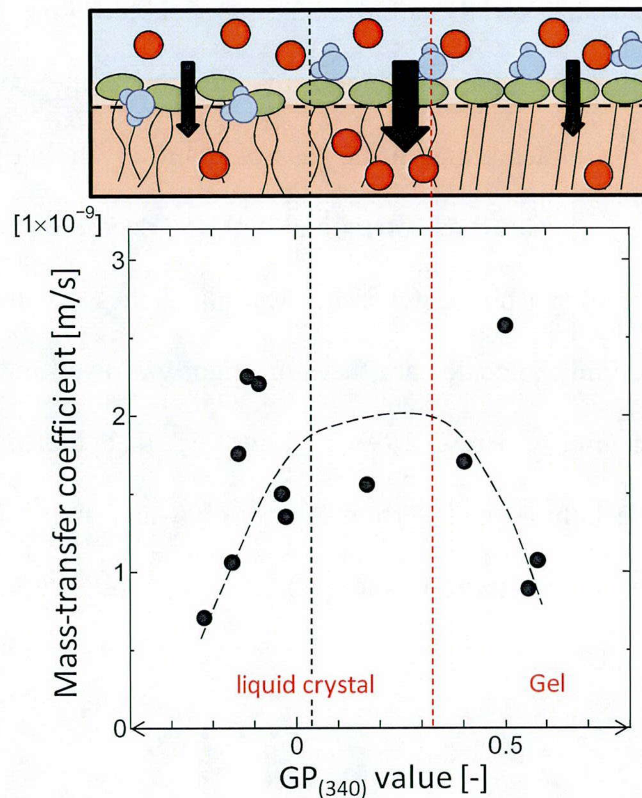


Fig. 2-17 Effect of membrane polarity and fluidity for the mess-transfer coefficient.

crystalline phase (liquid crystalline phase: $GP_{(340)} < -0.3$), the increase of $GP_{(340)}$ value increased the accelerated-mass-transfer coefficient, being independent of temperature. On the other hand, the mass-transfer coefficient was decreased in match with the increase of $GP_{(340)}$ value in the range of $GP_{(340)} > -0.3$ (Gel phase).

These results suggest that the mass-transfer coefficient is dependent on the membrane polarity. DOX is hydrophobic molecule ($\log P'_{OCT}=1.4$ [*n*-octanol/ pH7.4 buffer solution]), suggesting the easier distribution the hydrophobic phase. In addition, in the range of $GP_{(340)} < -0.3$ (Liquid crystalline phase), the vesicle has the higher membrane fluidity (**Table 2-3**). The membrane with high fluidity can afford the space not only for lipid molecule to laterally diffuse on the membrane but also for DOX to distribute into membranes. Such a space on the lipid membrane should give the hydrophobic environment. Therefore, the hydrophobicity of the membrane contributed to the insertion of DOX into the membrane in the case of liquid crystalline phase. However, the increase of the $GP_{(340)}$ value was correspond with the decrease of the mass-transfer coefficient in the case of gel phase. Overall, these liposomes had the low membrane fluidity. The phospholipid molecules are therefore tightly arrayed on the membrane at gel phase (Koynova and Caffrey, 1998), suggesting that the tightly arrayed of phospholipid molecules inhibit the insertion of not only water molecule, but also DOX. Therefore, it is likely that the hydrophobic membrane at liquid crystalline phase could effectively encapsulate DOX.

4. Summary

From the experiments of the calcein leakage and the FRET method, it has been demonstrated that the Span 80 vesicle with intense headgroup mobility as a possible drug carrier can easily interact with the liposome as a model cell membrane. There are three steps during the interaction between different vesicles, such as perturbation, hemifusion, and fusion which is the final step of the interaction of the "hetero"-vesicle. Span 80 vesicle aggregates (calcein leakage), and hemifuses (FRET method) with liposome, though further investigation on the fusion between Span 80 vesicle and liposome is needed (the aqueous content mixing assay) (Kato et al., 2008). As an interaction mechanism, it is revealed that the difference of membrane properties (especially, head group mobility) between vesicles (called as "membrane potential") and lipid structure (Inverted cone structure) in Span 80 vesicle can act as the trigger for the interaction between vesicles (**Section 3.1**), which is advantageous for the enhancement of the lipid mixing process (**Section 3.2**). The membrane-membrane interaction (membrane perturbation, and lipid mixing) was strongly induced when the value of "membrane potential" of "*fluid*" "*flexible*" and "*wet*" was large. It is necessary that the value of "membrane potential" was overpassed the threshold of the differences in membrane properties. The increase of ⊿ $1/P$ ("*fluid*") and ⊿ $\ln f_{ci}$ ("*flexible*"), and/or the decrease of ⊿ $GP_{(340)}$ ("*wet*") induce the membrane-membrane interaction. Although, the relationship between membrane-membrane interaction and "membrane potential" has not been clarified in terms of molecule level, this relationship is important to completely understand the mechanism of membrane-membrane interaction. After the interaction between Span 80 vesicle and apposed vesicle, resulting in the alteration of their membrane properties (**Section 3.3**). The above phenomena were

different from the case of the ideal hemifused vesicle, suggesting the existence uncompleted hemifusion in the case of Span 80 - liposome system. It was also observed that Span 80 vesicle could hemifuse with liposomal membranes without the use of fusogenic proteins (*e.g.* SNARE protein (Martens and McMahon, 2008)). While the liposome differs from the plasma membrane from the view point of the lipid component, diameter size, curvature, and existence of scaffold protein, these results are helpful to clarify the drug delivery mechanism of Span 80 vesicle and are useful for the further development of Span 80 vesicle as drug carriers.

Encapsulation efficiency of DOX was controlled by the membrane polarity (**Section 3.4.**). The hydrophobicity of DOX improves the insertion of DOX into the surfactant vesicle surface by the hydrophobic interaction. Therefore, more hydrophobic membrane effectively recruits DOX into the surfactant vesicle. However, it is difficult for DOX to be inserted into the surfactant membrane at gel phase, in spite that surfactant membrane at gel phase is more hydrophobic than that at liquid crystalline phase. Tight array of surfactant molecules on the membrane can inhibit the insertion of DOX into the membrane. These results suggest that the moderate membrane dynamics and hydrophobicity of the surfactant vesicle are needed for the effective encapsulation of DOX into the surfactant vesicle.

The interaction with the membrane (membrane-membrane or membrane-drug) is intricately related with the membrane properties, "*fluid*", "*flexible*", and "*wet*". It is expected that these interaction can be controlled by the design of drug carrier based on these membrane properties. In relation to Span 80 vesicle, Span 80 vesicle with intense headgroup mobility easily interacts with the lipid membrane like a plasma membrane. However, the dynamic membrane of Span 80 vesicle allows the insertion of the water

molecule into the Span 80 vesicle membrane, resulting in the hydrophilic membrane. It is disadvantage for the encapsulation of DOX by remote loading method. Cholesterol insertion into the Span 80 vesicle does not affect the headgroup mobility of Span 80 vesicle, while the increases the hydrophilicity of the Span 80 vesicle membrane. Therefore, Span 80 vesicle can efficiently encapsulate the DOX by remote loading method, with keeping the higher interaction activity for the lipid membrane by insertion of cholesterol into the Span 80 vesicle membrane.

Form the obtained results, the interaction between membranes (binding/internalization/fusion) could be separated to each fundamental step: (i) a membrane perturbation; (ii) a lipid mixing/hemifusion; (iii) a diffusion of lipids on apposed membranes, as shown in **Fig. 2-18**.

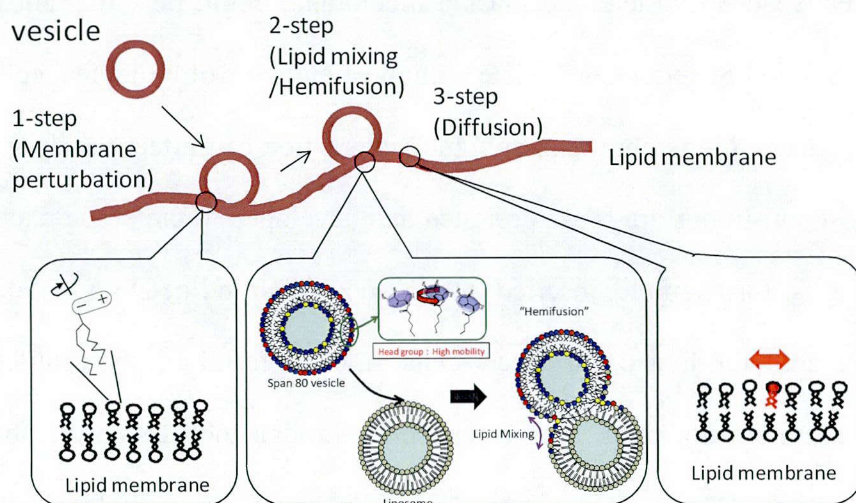


Fig. 2-18 Membrane-membrane interaction composing of fundamental steps.

Chapter 3 Control of Membrane-Membrane Interaction between Vesicle and Cell Based on Membrane Properties

1. Introduction

"Membrane-membrane" interaction is dependent on the membrane properties. As described in previous chapter, Span 80 vesicle with the intense mobility of its headgroup easily binds to the liposome membrane and also perturbs the surface of the liposome. Liposome has been conventionally employed as a model of plasma membrane, although the plasma membrane is more complex than liposome. The plasma membrane is composed by various lipids (phospholipid, cholesterol, sphingolipid, etc), containing membrane proteins and "raft" structure. Therefore, the expected "membrane-membrane" interaction based on the membrane properties (**Chapter 1**) (e.c. Fusion between Span 80 vesicle and plasma membrane) could be important factor to control the interaction of surfactant vesicle with the membrane of biological cells.

The pattern of "membrane-membrane" interaction can affect the drug delivery efficiency. Most anti-tumor drugs are designed and developed to target the nucleus. The anti-tumor drug is, for example, inserted into the double strand of DNA, inhibiting the DNA synthesis and cell division of cancer cells. In order to inhibit the proliferation of cancer cells, an anti-tumor drug is needed to pass through the plasma membrane and nuclear membrane. Drugs encapsulated into liposome are usually uptaken by endocytosis into the cytoplasm, in some cases, resulting in the elimination the encapsulated drug by exocytosis from biological cells. Therefore, it seems that the "membrane fusion" is one of the most effective drug delivery pathways to directly deriver the drug into the cytoplasm without the escape from exocytosis mechanism.

The contribution of membrane properties for the "membrane-membrane" interaction was investigated in this caption by using cancer cells as a target. The scheme of the design of drug carrier was established based on the membrane characterization as described in detail in results and discussion section. The first step of the interaction between drug carrier and plasma membrane is contributed by the headgroup mobility. The surfactant vesicle with high-intense headgroup mobility can easily fuse with the plasma membrane. In the case of the surfactant vesicle with high fluidity, the surfactant diffuses on the plasma membrane after the fusion with the plasma membrane. The diffusing surfactant on the plasma membrane alters the membrane properties of the plasma membrane, resulting in the cell death at an appropriate condition. The cytotoxicity of the fusing surfactant vesicle is dependent on the type of the cell line or applied dose. The control of the cytotoxicity of the fusing surfactant vesicle enables us to develop a novel drug delivery targeting an intracellular organelle by using vesicle-in-vesicle system, as described below. Moreover, the surface of the surfactant vesicle is capable of the modification of the substance, such as antibody (Kesharwani et al., 2012). These proteins can specifically bind to cancer cells. There is a difference in the plasma membrane surface between normal cells and cancer cells. It has been reported that the epidermal growth factor receptor (EGFR) (Thomas, 2003), or folic acid receptor (Ross et al., 1994) are more displayed on the plasma membrane of the cancer cells as compared with the normal cells. The anti-EGFR antibody or folic acid are applicable for the active targeting by the modification for the surface of the surfactant vesicle (Kesharwani et al., 2012, Ross et al., 1994). While it is important to understand the binding mechanism between cancer cells and the surfactant vesicle modified with substance (anti-EGFR antibody or folic acid), the mechanism has not been clarified in

detail. It is expected that membrane characterization could contribute to the understanding of the behavior on the membrane, such as protein-protein interaction on the membrane. The feature of the plasma membrane of cancer cells also helps to design the drug carrier. The lipid composition of plasma membrane and expression of the plasma protein are different from cancer cells and normal cells. After the investigation using a design scheme to control the "membrane-membrane" interaction based on "membrane properties", some kinds of drug delivery systems were proposed as a case study of proposed scheme.

2. Materials and Methods

2.1. Materials

Sorbitan monooleate (Span 80), polyoxyethylene sorbitan monooleate (Tween 80), and doxorubicin hydrochloride (DOX), and ammonium surface were purchased from Wako Pure Chemical Industries (Osaka, Japan). Cholesterol was obtained from Sigma-Aldrich (St. Louis, MO, USA). Phospholipids such as 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) were purchased from the NOF Corporation (Tokyo, Japan). 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (rhodamine-PE; (Rh-PE)) was purchased from Avanti Polar lipids (Alabaster, AL, USA).

2.2. Preparation of liposome

Phospholipids and cholesterol were dissolved in a chloroform solution. The organic solvent was removed by evaporation in a rotary evaporator. The residual lipid film, after drying under a vacuum overnight, was hydrated with the inner-phase liquid (155 mM $(NH_4)_2SO_4$ or PBS; 137mM NaCl, 2-9mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4; pH 7.3). The suspension was subjected to five cycles of freezing and thawing and then extruded. The lipid concentration was determined by phosphorous analysis.

2.3. Preparation of Span 80 vesicle.

Span 80 vesicle was prepared by the two-step emulsification method (Kato et al., 2006). 6 ml of n-hexane solution containing Span 80 and cholesterol was added to 0.6 ml of inner-phase liquid (155mM $(NH_4)_2SO_4$ or PBS), followed by the first emulsification for 6 min at 17,500 rpm using a micro-homogenizer NS-310E 2

(Microtec Co., Ltd., Funabashi, Japan). The solvent obtained from the water-in-oil emulsion was evaporated in a rotary evaporator at2-9 °C under reduced pressure, yielding a water-lipid emulsion to which 6 ml of outer-phase liquid (PBS) containing 96 mg of Tween 80 (and 85.2 mg DSPE-PEG_{2000} (NOF Corporation)) was added, followed by mixing with the homogenizer for 2 min at 3500 rpm to obtain the heterogeneous Span 80 vesicle suspension. The heterogeneous vesicle suspension was stirred with a magnetic stirrer for 3 hours at room temperature, followed by storage overnight at 4 °C. The vesicle was then purified by ultracentrifugation (50,000 rpm at 4 °C for 120 min) in a Himac centrifuge CR15B (Hitachi Koki Co., Ltd., Tokyo, Japan). The purified Span 80 suspension was passed through 100-nm nucleopore track-etch polycarbonate membranes and purified twice by ultracentrifugation (When Span 80 vesicle were prepared by $(NH_4)_2SO_4$, Outer liquid phase was exchanged from $(NH_4)_2SO_4$ to PBS).

2.4. Remote loading method.

Doxorubicin hydrochloride (DOX) was loaded into POPC liposome and Span 80 vesicle by a remote loading method (Haran et al., 1993, Madden et al., 1990). In short, surfactant vesicle suspension (50 mM) prepared by $(NH_4)_2SO_4$ was then mixed with a DOX saline solution (1 mM). To accelerate a loading of DOX, the sample was then heated to 60 °C for each time. Afterwards, the unloaded material was removed by using a gel permeation chromatography (SepharoseTM4B).

2.5. Cells and cell culture.

Murine osteosarcoma cell line (LM8 cell), murine rectal cancer cell line (Colon26 cell), human hepatocellular carcinoma cell line (HepG2 cell) were obtained

from RIKEN (RIKEN BRC Cell Bank, Ibaraki, Japan). These cells were grown in Eagle's minimal essential medium (E-MEM) (Wako Pure Chemical Industries; Osaka, Japan) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific; Waltham, MA, USA) in the incubator (a humidified atmosphere consisting of 5 % CO_2 at 37 °C). Human osteosarcoma Takase (OST) cells were offered by Dr. Katsuro Tomita (Department of Orthopaedic Surgery, Kanazawa University School of Medicine, Japan). OST cells were cultured in Dulbecco's Modified Eagle Medium (D-MEM) (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 10 % of fetal bovine serum (FBS) at 37 °C in a humidified atmosphere consisting of 5 % CO_2.

2.6. Evaluation of the cellular uptake of doxorubicin hydrochloride (DOX).

Colon26 Cells were seeded at 2×10^5 cells/ml in a cell culture dish in complete medium, E-MEM with 10% FBS for Colon26 cells. Colon26 cells were then incubated for 24 hours in a humidified atmosphere of 5 % CO_2 and at a temperature of 37 °C. Then, test samples (PBS, DOX, DOX encapsulated liposome, and DOX encapsulated Span 80 vesicle) were added to the cells and were further incubated for another 1 hour under 5 % CO_2 atmosphere and 37 °C. After incubation, cells were removed by EDTA-Trypsin and washed by PBS twice. Colon26 cells were analyzed by flowcytometry (Life Technologies, Gaithersburg, MD, USA). Fluorescence intensity excited at 488 nm was monitored with BP filter (575/24 nm).

2.7. MTT assay.

MTT assay was performed by using CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega; Fitchburg, WI, USA). The cells (LM8 cells, Colon26

cells, and HepG2 cells) were seeded on the 96 well cell culture plate (100 μl, 2.0×10^5 cells/ml). The cells were cultured for 24 hours in E-MEM supplemented with 10% FBS in the incubator. The culture medium was exchanged by the new medium (100 μl) containing Span 80 vesicle or DOX- loaded vesicles. The cells were incubated for 24 hours (37 °C, 5% CO_2). The culture medium was exchanged by new medium (100 μl) once more. The 15 μl of dye solution was added into the new medium. After incubation for 4 hours in the incubator, the 100μl of solubilization solution/stop Mix was added into the new medium. After incubation for 1 hour at room temperature, the absorbance of each well was measured by microplate spectrophotometer, xMark^{TM} (BIO-RAD; Hercules, CA, U.S.A.). The ratio of cell viability was calculated from the following equation;

$$\text{Ratio of cell viability: } \frac{(A_{570(x)}-A_{630(x)})}{(A_{570(0)}-A_{630(0)})} \tag{3-1}$$

$A_{570(x)}$ and $A_{630(x)}$: Absorbance at 570nm and at 630nm, when the cells were added into vesicle suspension

$A_{570(0)}$ and $A_{630(0)}$: Absorbance at 570nm and at 630nm, without Span 80 vesicle

The 50% inhibitory concentration was estimated from the dependence of the cell viability on the vesicle concentration.

2.8. Observation of blebbing formulation.

The cells (LM8 cells, Colon26 cells, and HepG2 cells) were seeded on the 96 well cell culture plate (100 μl, 2.0×10^5 cells/ml), and were cultured for 24 hours in the E-MEM supplemented with 10% FBS in the incubator. The culture medium was exchanged by a new medium (90 μl). 10 μl of Span 80 vesicle (final concentration: 1.25

mM) was added to the cell. The cells were observed at room temperature by fluorescence microscopy, IX51 (Olympus; Tokyo, Japan).

2.9. Detection of the alteration of plasma membrane properties.

The alteration of plasma membrane properties was detected by double staining method with the combination of Annexin 5 and 7-ADD, and with the combination of F2N1S and SYTOX®. Both experiments were performed by using PE Annexin V Apoptosis Detection Kit I (Becton Dickinson Biosciences; Franklin Lakes, NJ, U.S.A.) and Violet Ratiometric Membrane Asymmetry Probe/Dead Cell Apoptosis Kit (Life Technologies, Inc.; Carlsbad, CA, U.S.A.), respectively.

LM8 cells were seeded on the 6 well cell culture plate (2.0 ml, 2.0×10^5 cells/ml). LM8 cells were cultured for 24 hours in E-MEM supplemented with 10% FBS in the incubator. The culture medium was exchanged by a new medium (2.0 ml) containing PBS, staurosporine (0.43 mM), or Span 80 vesicle (1.25 mM). After the incubation for 3 hours, the cells were treated with EDTA-Trypsin and washed by PBS twice.

When double staining of F2N1S and SYTOX® was achieved, LM8 cells were re-suspended in 500 μl PBS, 1 μl F2N12S and SYTOX® AADvanced™ dead cell stain solution, and where incubated for 5 min. LM8 cell suspension diluted by PBS was analyzed by Attune® Acoustic Focusing Cytometer.

When double staining of Annexin 5 and 7-ADD was performed, LM8 cells were re-suspended in 500 μl of 1× binding buffer. 100 μl of the cell suspension was added 5 μl Annexin 5 -PE and 7-ADD, followed by incubation for 15 min. LM8 cell suspension was added more 400μl of 1× binding buffer, and was analyzed by Attune®

Acoustic Focusing Cytometer.

2.10. Annexin 5 -PE staining (Observation by fluorescence microscopy).

LM8 cells were seeded on the 96 well cell culture plate (100 μl, 2.0×10^5 cells/ml), and were cultured for 24 hours in the E-MEM supplemented with 10% FBS in the incubator. The cell culture medium was exchanged by the new medium (100 μl) containing PBS, staurosporine (0.43 mM) or Span 80 vesicle (1.25 mM). After the incubation for 3 hours, the culture medium was removed. 30 μl binding buffer and 5μl Annexin 5 -PE were added for each well. After incubation for 30 min at room temperature, these cells were observed by fluorescence microscopy, IX51, after exchanging the binding buffer for PBS.

2.11. Observation of F-actin

F-actin was observed by using Phalloidin, Alexa Fluor®488 Conjugate (Life Technologies, Inc.; Carlsbad, CA, USA). The cells were seeded on the coverslip in the 6 well cell culture plate (2.0 ml, 2.0×10^5 cells/ml). The cells were cultured for 24 hours in E-MEM supplemented with 10% FBS in the incubator. The culture medium was exchanged by a new medium (2.0 ml) containing PBS, or Span 80 vesicle (1.25 mM). After the incubation for 3 hours, the cells were washed by PBS twice. The cells were fixed by in 4.0 % formaldehyde solution in PBS for 10 min at room temperature. The fixed cells were washed by PBS twice. The cells were incubated with 0.1% Triton®X-100 in PBS for 3 min. The cells were stained with the fluorescent phallotoxin (5μl fluorescent phallotoxin was diluted by 200μl PBS) which was added 1% bovine serum albumin in order to reduce nonspecific background for 20 min at room

temperature. The stained cells were observed by the confocal fluorescence microscopy, BX50 (Olympus; Tokyo, Japan).

2.12. Modification of ESA on the vesicle membrane

ESA was extracted from the red alga *Eucheuma serra*, by means of ethanol precipitation, followed by purification with fast protein liquid chromatography (FPLC), using a 10 mM sodium phosphate buffer (pH = 7.4) (Kawakubo et al., 1997). ESA was anchored to 1,2-dioleoyl-*sn*-glycero3-phosphoethanolamine-N-(succinyl) (SuPE). 100 μL of a SuPE solution (1.25 mg/mL in chloroform) were added to a test tube. A thin film of SuPE formed after evaporation of chloroform under a stream of nitrogen gas. Afterwards, 2.5mL of an ESA solution (0.675 mg/mL) were added to the film to react with SuPE in 0.15M sodium carbonate buffer (pH9.0) at room temperature. The reaction mixture was incubated for 2 hours with vortexing for a few seconds every 30min, followed by letting the suspension stand at 4 °C overnight. Residual SuPE in the buffer solution was removed by gel filtration with a PD-10 column packed with Sephadex G-25 (GE Healthcare; Buckinghamshire, England). ESA-SuPE conjugation was added at second emulsification (**Section 2.3**).

3. Results and Discussion

3.1. Design Scheme of Drug Carrier for Effective Drug Delivery

The importance of membrane property to control the "membrane-membrane" interaction was shown in the previous section (**Chapter 2**) by employing the lipid- and detergent-vesicles as target materials. The relationship between the membrane properties of the surfactant vesicle and their membrane-membrane interaction can contribute to the rational design of drug carrier to achievement of the effective drug delivery. The design scheme of drug carrier to control the interaction of the vesicle, as a drug carrier, with the biological cells is herewith shown in the **Fig. 3-1**, based on the findings obtained in **chapter 1** and **chapter 2**.

It is expected that headgroup mobility of surfactant vesicle (**Membrane property: "Flexible"**) is an important key factor to affect the uptake pathway of the drug carrier into the cells. The headgroup mobility of phosphatidylcholine liposome was inhibited by the electrostatic interaction of headgroup region, resulting in the reduction of the binding of phosphatidylcholine liposome to the plasma membrane. This weak interaction between phosphatidylcholine liposome and a plasma membrane is expected to induce the uptake of drug carrier by the cell-dependent uptake mechanism, endocytosis. In contrast, intensively-high head mobility of the headgroup of Span 80 vesicle improves the binding of Span 80 vesicle to the plasma membrane. The dynamics of Span 80 molecule on the Span 80 vesicle membrane induces the membrane fusion between Span 80 vesicle and the plasma membrane after the interaction with the plasma membrane.

After the fusion with the plasma membrane, Span 80 molecule diffuses on the plasma membrane from their binding region to other region. Span 80 molecule

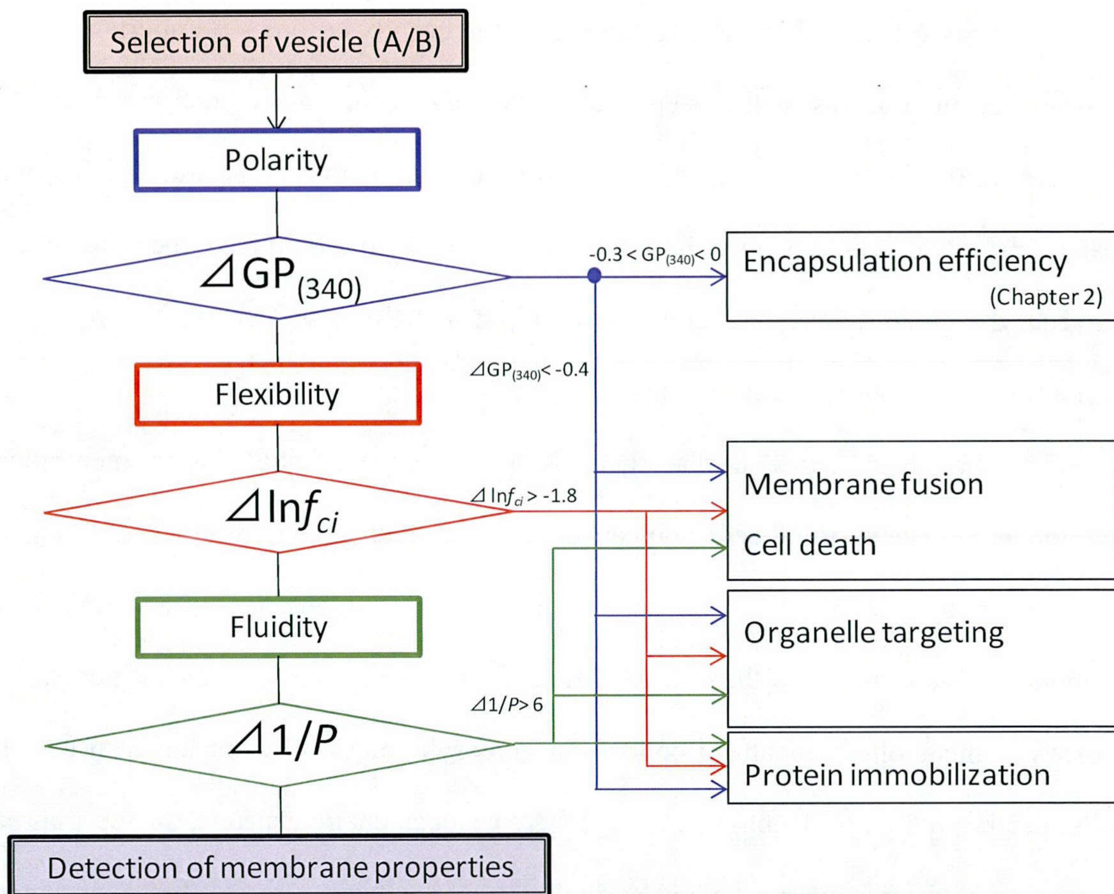


Fig. 3-1 Design scheme for effective drug carriers to control the interaction between detergent-based membrane and phospholipid-based membrane.

immediately diffuse on the plasma membrane, considering the high membrane fluidity (**Membrane property: "*Fluid*"**) of Span 80 vesicle. The immediate diffusion of Span 80 molecule on the plasma membrane alters the membrane properties of the plasma membrane, resulting in the induction of cell death.

The membrane polarity (**Membrane property: "Wet"**) is also important to encapsulate the drug efficiency. In the case of remote loading method, DOX is encapsulated across the membrane of the surfactant vesicle membrane. The existence of water molecule on/in the surfactant vesicle membrane prevents the insertion of the DOX. Therefore, "*wet*" membrane is not appropriate for the encapsulation of DOX.

The targeting of intracellular organelle has recently become an important issue, with the recent progress in the deeper understanding of the case of cellular diseases. Considering the "*Flexible*", "*Fluid*", and "*Wet*" natures of the surfactant vesicles, the vesicle-in-vesicle was designed based on the scheme to minimized the interaction between the Span 80 vesicle and phospholipid vesicle with keeping their higher interaction ability with plasma membrane.

It has been reported that there is a difference of the plasma membrane component between normal cells and cancer cells. In order to increase the selectivity of drug carrier against the cancer cells, the modification of antibody (i.e. anti-EGFR antibody or folate) is one of the effective strategies for the high efficiency drug delivery. Moreover, metabolic mechanism containing glycosylation is different between cancer cells and normal cells, resulting in the different sugar chain structure on the plasma membrane between cancer cells and normal cells. It has been reported that sugar chain binding protein, lectin, is also effective for the cancer-targeting ligand on the surface vesicle membrane, although the modification condition for the lectin has not been optimized yet. The method to design an appropriate membrane surface of the surfactant vesicle was also shown based on the design scheme.

3.2. Direct Drug Delivery Based on "Flexible" Nature of Span 80 Vesicle.

The importance of headgroup mobility in the interaction of the vesicles with the plasma membrane was shown as the first step of the scheme of drug carrier design (**Fig. 3-1**). The intense mobility of Span 80 molecule on its vesicle surface can realize an effective drug delivery to the cancer cells, and can also induce the fusion with plasma membrane. In this section, Span 80 vesicle was added into the cholesterol (23.1 mol%)

to reduce the drug permeability from Span 80 vesicle membrane with the reduction of membrane fluidity (Shimanouchi et al., 2009). DOX was first entrapped in the Span 80/cholesterol vesicle by a remote loading method. The DOX-entrapped Span 80/cholesterol vesicle was incubated with Colon26 cells, to investigate the role of Span 80/cholesterol vesicle. **Figure 3-2** shows the amount of DOX uptaken by Colon26 cells, determined from the mass balance of DOX fluorescence. Almost all the Span 80/cholesterol vesicles entrapping DOX was uptaken by Colon26 cells in contrast to the case of POPC liposome. It seems that the intense mobility of headgroup of Span 80 molecule on the vesicle surface could induce their easy insertion into the plasma

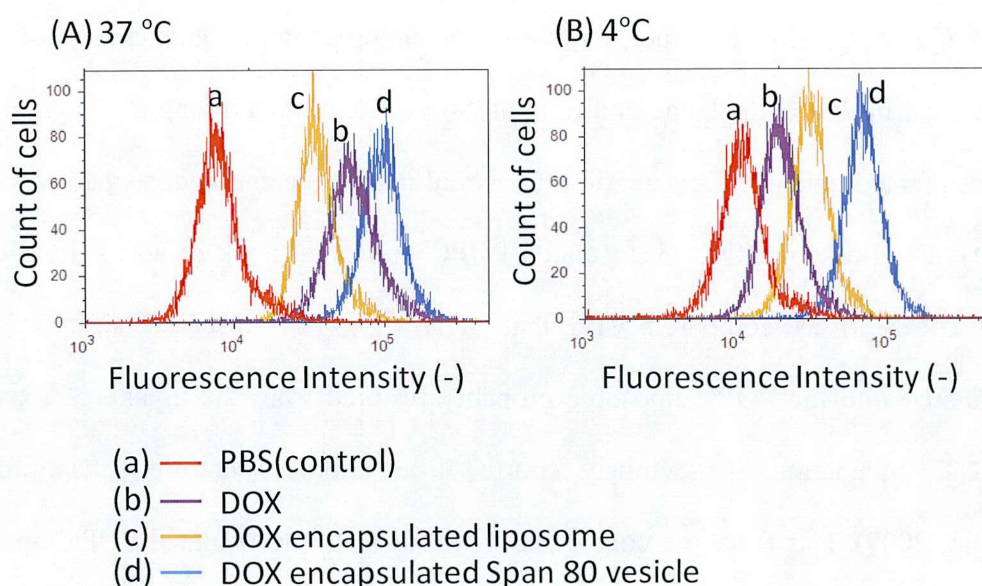


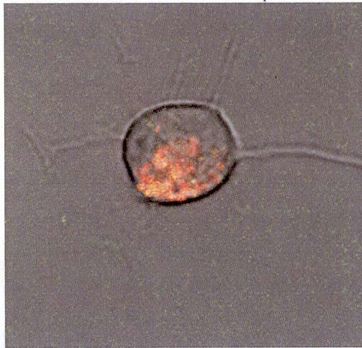
Fig. 3-2 Flowcytogram of Colon26 cells at (a) 37 °C and (b) 4 °C. Colon26 cells were treated with (a) PBS (control) (—), (b) DOX (—) (c) DOX encapsulated liposome (—), or (d) DOX encapsulated Span 80 vesicle (—). Other detailed experimental conditions were referred to experimental section. Fluorescence intensity was monitored with the condition of ex: 488 nm/ em. 575 nm (575/24 nm BP filter).

membrane that is similar to liposome membrane (**Chapter 2**) as compared with the case of POPC liposome. These results suggest that the dynamics of membrane surface is important to control the interaction with the plasma membrane rather than inner membrane properties, considering the value measured by DPH (**Figs. 1-5** and **1-6**). The intense of headgroup mobility of the Span 80 vesicle (f_{ci} > 200 MHz) is strong enough to interaction with the plasma membrane.

There are some possibilities to explain the cellular uptake mechanism of the drug delivery using surfactant vesicles. After interaction with the plasma membrane, POPC liposome is commonly known to be uptaken by endocytosis, while the uptake pathway of Span 80 vesicle is considered to be different from POPC liposome in the case of Colon26 cells. In order to clarify the internalization mechanism of DOX encapsulated in POPC liposome and in Span 80 vesicle, their binding to Colon26 cells was investigated under 4 °C-incubation that could inhibit the endocytosis pathway (**Fig. 3-2 (B)**). The peak of DOX encapsulated POPC liposome (peak c) was shifted to the control (peak a) as compared with that at 37 °C. The observed shift of DOX encapsulated into the POPC liposome probably resulted from the uptake of the DOX under 4 °C -incubation, as previously reported in the another literature (Elbayoumi and Torchilin, 2007). It is therefore considered that the DOX-encapsulated POPC liposome used here was internalized via an endocytosis pathway. In contrast, the peak of DOX encapsulated Span 80 vesicle (peak d) was not obviously shifted to the peak at 4 °C. The DOX encapsulated Span 80 vesicle was thus found to be up-taken by the different pathway from the endocytosis observed in the DOX encapsulated POPC liposome.

Moreover, the uptake mechanism of Span 80 vesicle by Colon26 cells was confirmed by the confocal laser scanning microscope. **Figure 3-3** shows the image of

(a)POPC Liposome



(b) Span 80 vesicle

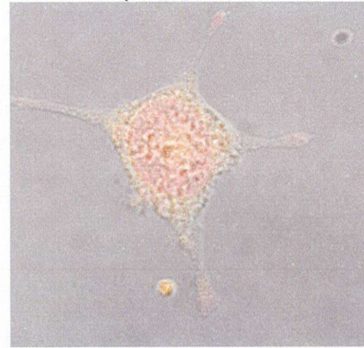


Fig. 3-3 Confocal laser microscopic images of DOX encapsulated (a) POPC liposome and (b) Span 80 vesicle. Green fluorescence: NBD-PE; red fluorescence: DOX.

Colon26 cells treated with DOX encapsulated in (a) POPC liposome, or in (b) Span 80 vesicle. The dotted green (NBD-PE) and red (DOX) fluorescence in cytosol of Colon26 cells could be observed at the same region in the case of POPC liposome. This observation is a definite evidence that the DOX encapsulated into the POPC liposome was internalized by the endocytosis pathway. On the other hand, green fluorescence derived from NBD-PE was observed at the plasma membranes, as shown in **Fig. 3-3**. This observation implies the lipid mixing between the Span 80 vesicle and plasma membrane. Red fluorescence derived from DOX was also observed at the inside of Colon26 cell cytoplasm, implying the direct delivery of DOX into the cytoplasm of Colon26 cells. From this double-staining experiment, it is considered that the Span 80 vesicle delivered the DOX into the cytoplasm of cells via membrane fusion of Span 80 vesicle with the plasma membrane. These interaction mechanisms are controlled by the flexibility of the surface, the intense of headgroup mobility of the Span 80 vesicle (f_{ci} > 200 MHz) is enough to fuse with the plasma membrane (**Fig. 3-4**).

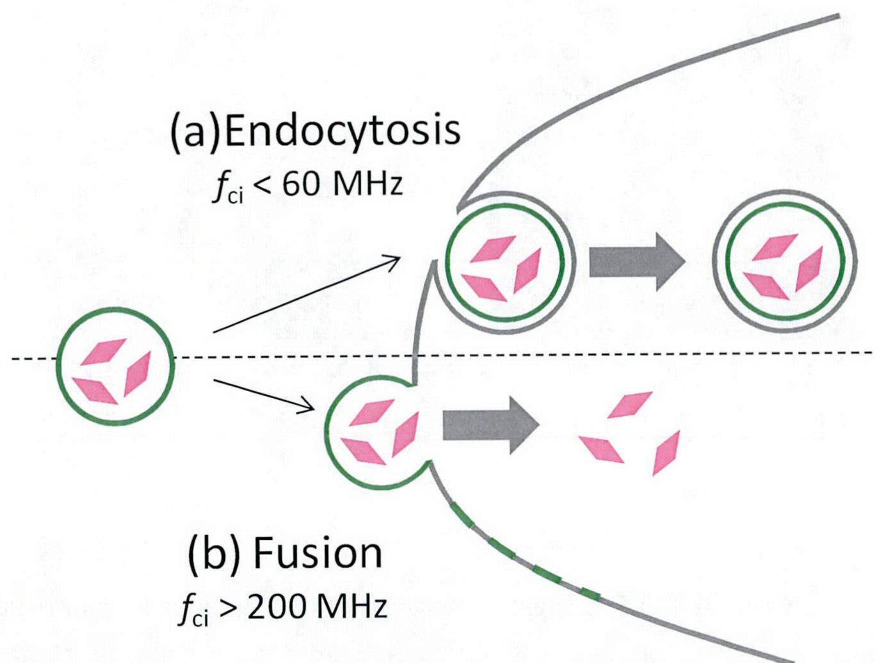


Fig. 3-4 Scheme of the mechanism of interaction between surfactant vesicle and plasma membrane. (a) The surfactant vesicle with low-intense headgroup ($f_{ci} < 60$ MHz) on the membrane was uptaken by endocytosis pathway. (b) The surfactant vesicle with high-intense headgroup ($f_{ci} > 200$ MHz) on the membrane was uptaken by membrane fusion.

3.3. Partial Transformation of Plasma Membrane to induce Cellular Response through the Fusion of Span 80 vesicle ~Induction of "Pseudo-Apoptosis"~

After the fusion with the plasma membrane, Span 80 vesicle/molecule diffuses on the plasma membrane, considering the lipid mixing. The results of **chapter 2** suggest that the diffused Span 80 vesicle/molecule could alter the membrane properties of the plasma membrane. According to the scheme (**Fig. 3-1**), the "*fluidity*" ($1/P$ value) is the key factor for the cytotoxicity. The plasma membrane was highly regulated to operate the cellular mechanism, such as signaling (Allen et al., 2007, Ostrom and Insel, 2004),

or substance transport (Eckford and Sharom, 2008, Razani et al., 2002), possibly together with the variation of their membrane properties. The alteration of membrane properties is thus one of the important issues for the control of membrane function. In this section, the effect of Span 80 vesicle for the cells was investigated after its insertion into the plasma membrane.

(1) General Cellular Response after Fusion of Span 80 vesicle

The cytotoxicity of Span 80 vesicle/molecule was evaluated after its fusion with the plasma membrane. It has been shown that, after its interaction with the plasma membrane, Span 80 molecule could diffuse on the plasma membrane as described above (**Section 3.2.**). The above case study implies that the plasma membrane of biological cell can be partially transformed through the membrane fusion of "foreign" vesicle that differs from lipid vesicle in relation to their nature. It has been reported that the membrane-related phenomena can be induced by modifying the membrane properties by adding some reagents or by change the environmental condition. It is considered that the cellular response can also be induced through the fusion of Span 80 vesicle with *fluid*, *flexible*, and *wet* surface. Various cellular responses were observed after the fusion of Span 80 vesicle. From the microscopic observation, blebbing was observed after the cells were treated with Span 80 vesicle. After incubation, Span 80 vesicle finally induces the cell death for these cells. In order to investigate the effect of cell line, LM8 cells, Colon26 cells, and HepG2 cells were treated with Span 80 vesicle and were incubated for 3 hours, resulting in the observation of blebbing by microscopic observation (**Fig. 3-5(a)**). The sensitivity of the Span 80 vesicle is dependent on the cell line. Among the cells tested here, LM8 cells induced the blebbing more frequently as

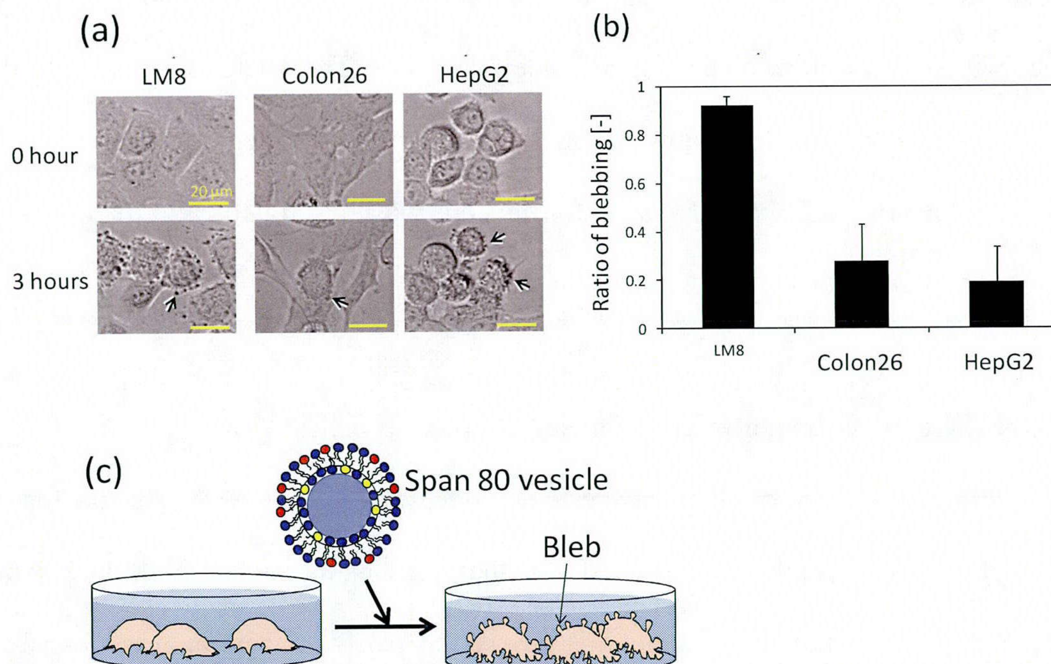


Fig. 3-5 The observation of cellular blebbing, and evaluation of cytotoxicity of Span 80 vesicle for cells. (a) The microscopic image of LM8 cells, Colon26 cells, and HepG2 cells. These cells were treated with Span 80 vesicle, resulting in blebbing after 3 hours. (b) The frequently of blebbing was evaluated for these cells. LM8 cells formed bleb most frequently than other cells. (c) The scheme of the cells with bleb on the plasma membrane by treated with Span 80 vesicle.

compared with Colon26 cells or HepG2 cells (**Fig. 3-5(b)**). 92.2 % of LM8 cells in population were found to induce the blebbing, while 27.6 % and 19.3 % of Colon26 cells and HepG2 cells were population with bleb. The LM8 cells were also sensitive for the cytotoxicity of the Span 80 vesicle as compared with other cell lines through the MTT assay (**Fig. 3-6**). **Figure 3-6(b)** shows the 50% inhibitory concentration (IC_{50}) of Span 80 vesicle which was determined by **Fig. 3-6(a)**. The IC_{50} value of the Colon26 cells was 1.82 times more, and that of HepG2 was 3.99 times more than that of LM8 cells. In the following experiment, the LM8 cell line was selected as a target because of its higher sensitivity.

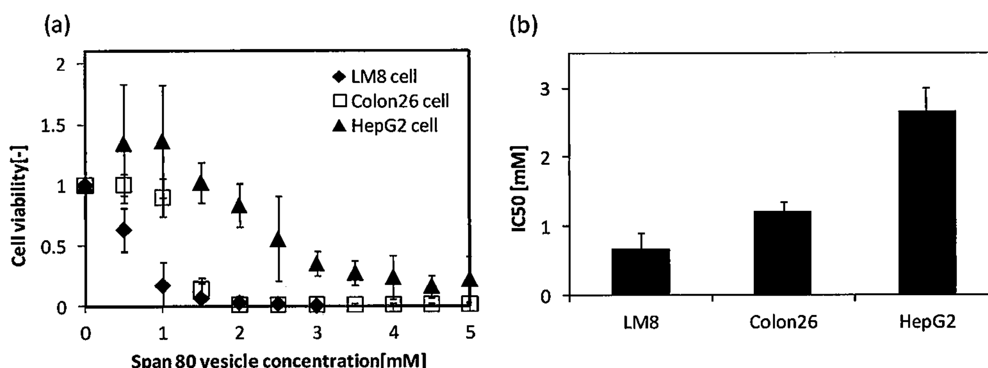


Fig. 3-6 Span 80 vesicle finally induced the cell death. (d) Cell viability of these cells treated with Span 80 vesicle was measured by MTT assay, and (e) the 50% inhibitory concentration (IC_{50}) was calculated.

(2) Bleb formulation by treatment with Span 80 vesicle is related with actin filament.

The mechanism of blebbing induced by Span 80 vesicle was further investigated, focusing on the "actin" which is the scaffold protein on the plasma membrane. Actin is well-known as one of the cytoskeleton protein, which can regulate the cellular adhesion (Parsons et al., 2010), or the cell movement (Salbreux et al., 2012). The actin can also regulate the cellular shape (Salbreux et al., 2012). It has been reported that there is a relationship between bleb and actin filament (Charras and Paluch, 2008). Two kinds of models on the bleb formulation have previously been proposed (**Fig. 3-7**): Bleb initiation can result from a local detachment of the cortex from the membrane or from a local rupture of the cortex (Charras and Paluch, 2008), while it is not clear about the blebbing mechanism.

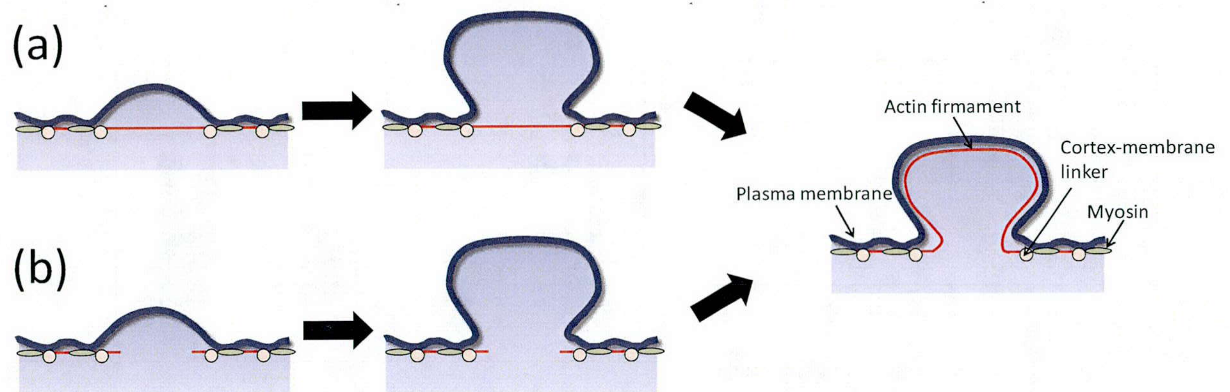


Fig. 3-7 Mechanism of bleb formation. (a) Local detachment of the cortex from the membrane. (b) Local rupture of the cortex.

Blebs of LM8 cells induced by Span 80 vesicle is also related with actin. LM8 cells treated with Span 80 vesicle were fixed by formalin, and were stained with Phalloidin, Alexa Fluor®488 Conjugate. **Figure 3-8** shows the confocal macroscopic image of LM8 cells. When LM8 cells were treated with PBS as control, actin filament was observed in the side of basolateral membrane. When LM8 cells with bleb by treated with Span 80 vesicle were observed, actin filament was found to be extended around the inside of bleb of LM8 cells. This result suggests that the blebbing initiated by the addition of Span 80 vesicle was not formed as non-biological (such as, physical) response, but as biological response (cellular response).

Blebbing formation is related with the cell death mechanism. Apoptosis and necrosis are well known as main categories of the type of cell death (Leist and Nicotera, 1997). The difference of cellular response between apoptosis and necrosis has been reported, together with many features, such as the alteration of the plasma membrane, nucleus structure, expression of protease, and so on (**Table 3-1**) (Leist and Nicotera, 1997). Apoptotic process, well-known as the program cell death, is also one of the

cellular responses correlated well with the plasma membrane, apoptotic bleb (Kepp et al., 2011, Orrenius et al., 2011). On the other hand, the mechanism of the necrosis has not been clarified, especially, in relation to the response of plasma membrane, in contrast to apoptosis. The lysis of the plasma membrane was only recognized as the feature of necrosis (Kepp et al., 2011, Melino et al., 2005). Considering the difference of membrane feature between apoptosis (apoptotic bleb) and necrosis (Lysis), LM8 cells were shown to induce the apoptotic cell death by their treatment with Span 80 vesicle. On the other hand, it has recently been reported that the blebbing is observed when the cells induce necrosis on a particular occasion. Blebs have thus been regarded as unconventional necrotic feature. In another previous report, necrotic blebs induced by H_2O_2 appear much later as compared with apoptotic blebs (Barros et al., 2003). The mechanism of blebbing is different between apoptosis and necrosis, and necrotic blebs are not regulated by ROCK-1, MLCK, and p38MAPK differently from apoptotic bleb (Barros et al., 2003). However, almost all necrosis was observed as only lysis of plasma membrane. It is therefore considered that Span 80 vesicle should induce apoptosis for the cells.

(3) Pseudo-Apoptotic Response; the Induction of Blebbing and Cell Death for Cancer Cells by Treated with Span 80 Vesicle.

Considering the alteration of the membrane properties of liposome by lipid mixing with Span 80 vesicle (**Chapter 2**), it is suggested that the plasma membrane fluidity is increased by its interaction with Span 80 vesicle. It is considered that the high membrane fluidity of Span 80 vesicle is advantageous to injure the plasma membrane. The increase of the membrane fluidity of plasma membrane is expected to be induced

by the disruption of the regulation of membrane properties, such as asymmetry of lipid component. The blebbing and cell death induced by Span 80 vesicle were further investigated, focusing on the mechanism whether apoptosis or necrosis. The observation of phosphatidylserine exposure was employed as the method of the detection of apoptosis in this study (Kepp et al., 2011). The phosphatidylserine was usually located at inner leaf of the plasma membrane (Leventis and Grinstein, 2010). However, the phosphatidylserine is exposed when cells induce apoptosis (Stowell et al., 2009). The phosphatidylserine exposure can be detected by fluorescence probe (F2N12S) (Shynkar et al., 2007), or phosphatidylserine binding protein (Annexin) (Gerke and Moss, 2002) (**Fig. 3-9**).

The double staining of F2N12S and SYTOX® was performed in order to clarify the alteration of the plasma membrane when LM8 cells were treated with Span 80 vesicle. F2N12S and SYTOX® were employed as the marker of the alteration of the

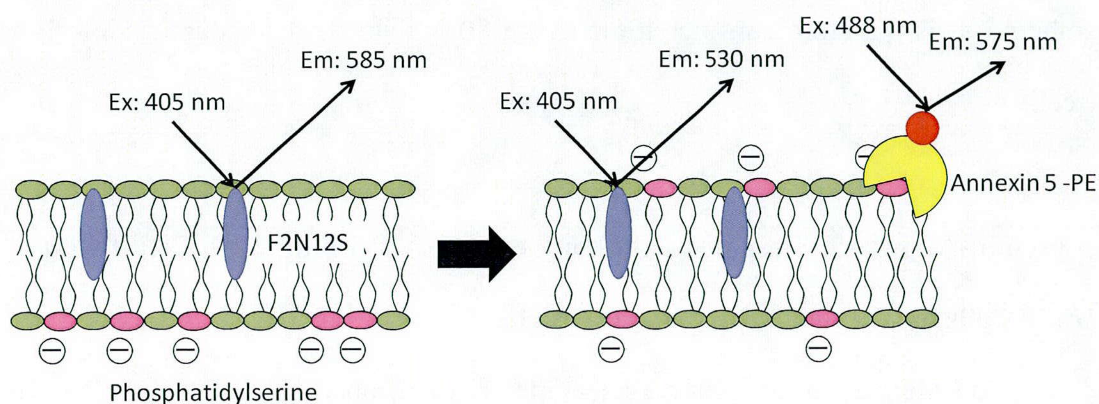


Fig. 3-9 Schematic illustration of phosphatidylserine exposure. The fluorescence of F212S is altered from 585 nm to 530 nm by exciting at 405 nm by phosphatidylserine exposure. Annexin 5-PE binds to the phosphatidylserine on the outer leaflet when cell induce the phosphatidylserine exposure. The fluorescence at 575 nm is observed by exciting to the annexin-5 PE at 488 nm.

plasma membrane properties and dead cells, respectively. F2N12S®, which is known as environmentally-sensitive fluorescence probe and can make a response to the changes in membrane asymmetry, increased the membrane fluidity and the negative charge on the surface together with the progress of apoptosis (Shynkar et al., 2007). X-Axis and Y-axis shown in **Fig. 3-10(a)** indicate [F2N12S ratio 585 nm/530 nm] and [SYTOX® fluorescence intensity], respectively. R4, R3, and R1 regions show living cells, apoptotic cells, and dead cells, respectively. The 46.3% of the cellular population treated with staurosporine and the 76.0% of the cellular population treated with Span 80 vesicle were observed at R3 region. This result suggests that the membrane properties of cells were transformed to those of apoptotic cell by the treatment with Span 80 vesicle. In order to evaluate the plasma membrane property, the double staining of Annexin 5-PE and 7-ADD was also performed. Annexin 5-PE and 7-ADD were employed as the indicators of the exposure of PS, and dead cells, respectively. The 15.1% of the cellular population treated with Span 80 vesicle were shown at R4 (Annexin 5-PE +/7-ADD -) by flow cytometry analysis (**Fig. 3-10(b)**). The fluorescence of the Annexin 5-PE was also observed by microscopy, when LM8 cells were treated with Span 80 vesicle (**Fig. 3-11**). These results suggest that the exposure of PS by its treatment with Span 80 vesicle, i.e. LM8 cells induced apoptotic like cell death by its treatment with Span 80 vesicle.

3.4. Vesicle Delivery Considering the Targeting to the Organelle Inside Cells

In the above section, the pharmacokinetics of drugs encapsulated into surfactant vesicles was described. In this section, an alternative approach to achieve the

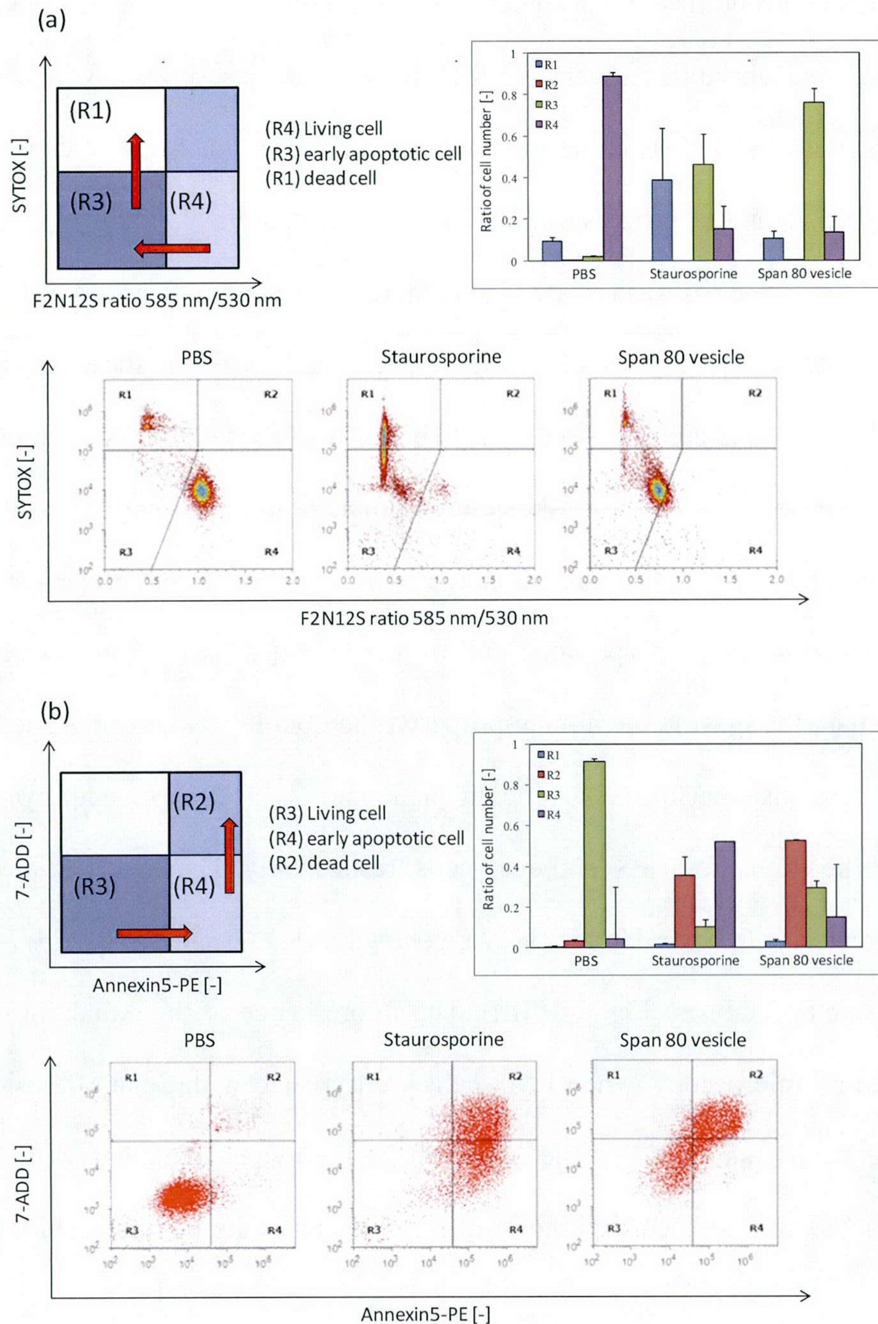


Fig. 3-10 Alteration of the plasma membrane of LM8 cells by treated with Span 80 vesicle. (a) Nature of LM8 cell membrane was altered like as early in apoptosis by treated with Span 80 vesicle. Percentage of LM8 cells in R1 and R3 was increased in both case of treated with staurosporine and Span 80 vesicle, compared with PBS treatment. (b) Exposure of PS was also observed by double staining of Annexin5-PE and 7-ADD. Exposure of PS was observed accompany with increasing of uptake of 7-ADD by treated with Span 80 vesicle.

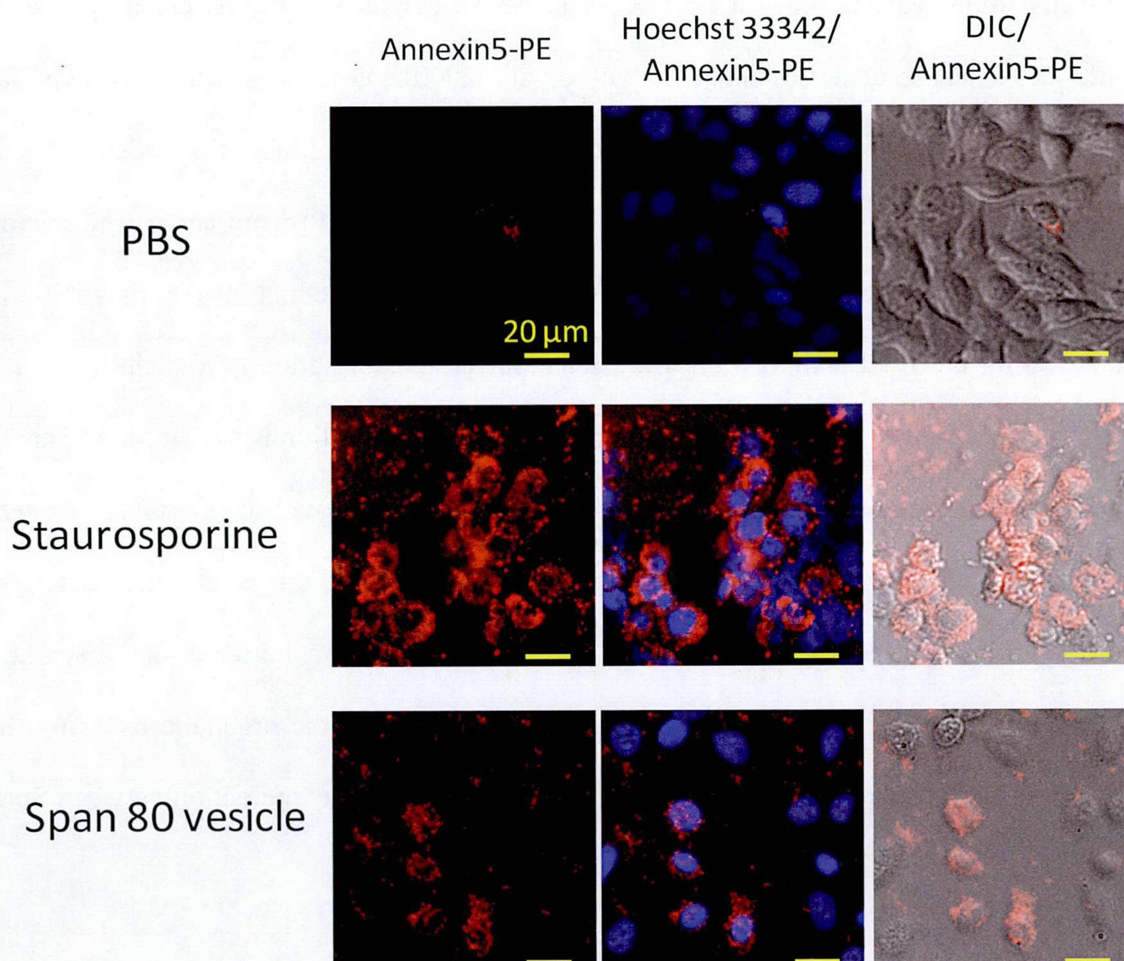


Fig. 3-11 Image of the fluorescence microscopy. Exposure of PS was also observed when LM8 cells adhered on cell culture plate. (Red: Annexin5-PE, Blue: Hoechst 33342 (nuclear))

controlled release of drug was proposed based on the control of different membranes such as Span 80 vesicle, DPPC liposome, and plasma membrane by selecting the encapsulation of liposome-based drugs by the liposomes as case study. The most promising strategy is the *vesicle-in-vesicle system*, where vesicles prepared from one type of amphiphile were encapsulated in another vesicle prepared from a different amphiphile (McPhail et al., 2000). It has been demonstrated that the vesicle-in-vesicle could achieve the controlled release because the second inner vesicle, entrapped inside

the first outer vesicle, gave a certain permeation resistance to the model drug loaded into the encapsulated vesicle (McPhail et al., 2000). Vesicle-in-vesicle system has another beneficial point that the second vesicle could reduce the side-effect of liposome-based drugs, due to the compartment effect of lipid membrane of the second vesicle. Meanwhile, the preparation using a hydration method has a difficulty in controlling the lipid composition of vesicles during the formation of vesicle-in-vesicle system (McPhail et al., 2000). The major objective in this section is to combine Span 80 vesicles with conventional vesicle-in-vesicle system for a novel drug delivery system (**Fig. 3-12**). It seems that the first outer vesicle can release the second inner vesicle to the cytoplasm of biological cells with the membrane fusion between the first outer vesicle and plasma membrane, resulting in the delivery for the organelle by using the second inner vesicle. In order to pursue the above purpose, the interaction between Span

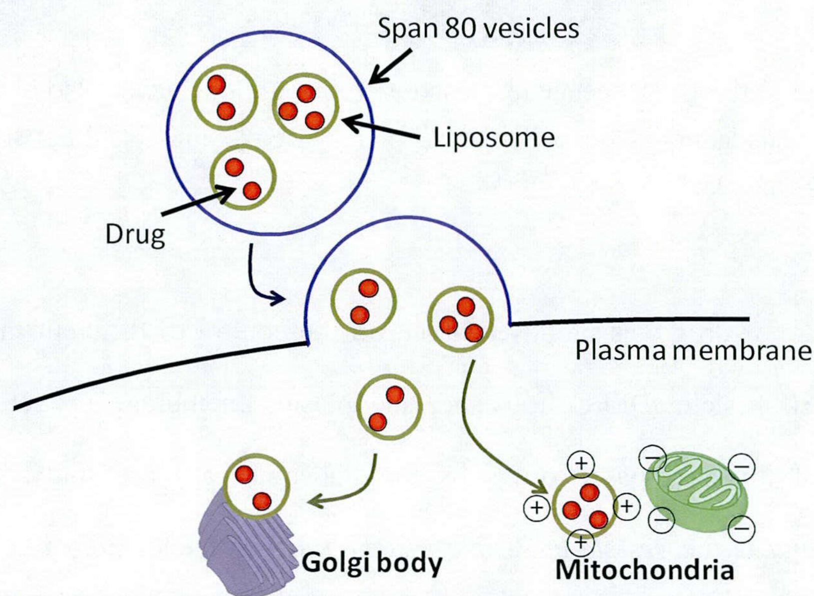


Fig. 3-12 Scheme of the strategy of organelle targeting by using vesicle-in-vesicle system.

80 vesicle and DPPC liposome should be minimized to avoid the fusion of themselves and, the interaction between the Span 80 vesicle and plasma membrane should be maximized from efficient delivery. The characters for two kinds of vesicles were optimized based on the scheme (**Fig. 3-1**).

In short, the method to prepare the vesicle-in-vesicle system was described below. The vesicle-in-vesicle was prepared by the two-step emulsification method, as described above. In this case, the liposome suspension was added into the Span 80 solution (n-hexane) as a inner phase at first emulsification (**Fig. 3-13**). As a result, the Span 80 vesicle entrapping the phospholipid vesicle can be obtained as a final product.

For the drug delivery into the intracellular organelle, the cytotoxicity of Span 80 vesicle for the cells needs to be prevented. Considering the sensitivity of the cytotoxicity of Span 80 vesicle for the cell line, HepG2 cells and Span 80 vesicle were employed as target. According the scheme (**Fig. 3-1**), Span 80 vesicle and DPPC liposome were used as the first outer vesicle and the second inner vesicle, respectively. An interaction of the vesicle-in-vesicle system with HepG2 cell membrane was then investigated. First, DPPC liposome, stained by rhodamine-PE (Rh-PE), was used to

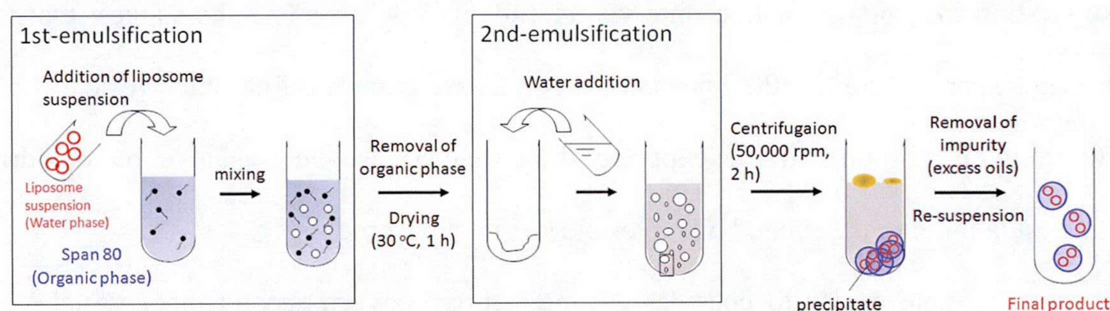


Fig. 3-13 Preparation method of the vesicle-in-vesicle system.

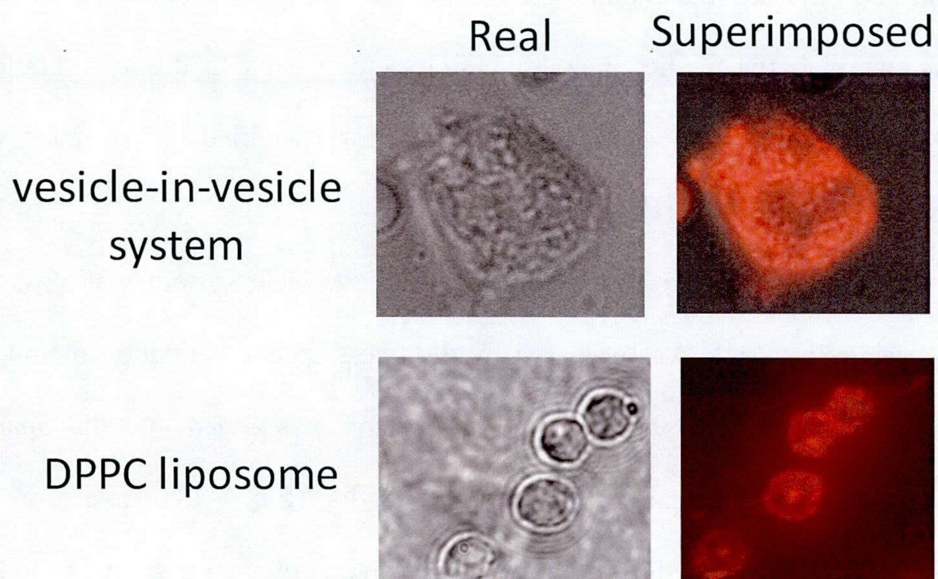


Fig. 3-14 Fluorescence microscopic images of HepG2 cells treated by Rhodamine-stained (a) vesicle-in-vesicle system and (b) DPPC liposome.

prepare the Rh-stained vesicle-in-vesicle system (Rh-LVs). This vesicle-in-vesicle system was added into HepG2 cells. **Figure 3-14** shows the confocal laser microscopic images. In the case of vesicle-in-vesicle system, red fluorescence originated from rhodamine was observed inside the HepG2 cells. In contrast, the addition of DPPC liposomes stained by Rh-PE resulted in the dotted red fluorescence inside the cells, suggesting the up-take of liposome via an endocytosis. These results suggest that the second inner vesicle (DPPC liposome) was released from the first outer vesicle (Span 80 vesicle), resulting in the expected drug delivery to the organelle by the drug encapsulation into the second inner vesicle (DPPC liposome).

It is necessary to consider the interaction between second inner vesicle and organelle membrane. Organelle membrane is also composed by the various kinds of phospholipids, the component of organelle membrane is different from each organelle,

such as golgi body, or mitochondria (van Meer et al., 2008). The design of not only the first outer vesicle but also second inner vesicle is therefore needed. In the case of mitochondria targeting drug delivery, there is a previous report on vesicle-in-vesicle system (MITO-Porter) based on the membrane properties (Yamada et al., 2008). Mitochondrial membrane contains more anionic phospholipid, phosphatidylethanolamine (van Meer et al., 2008). The second inner vesicle of MITO-Porter contains cationic lipids, where this vesicle easily interacts with anionic mitochondrial membrane. Therefore, the design of the surface charge of the surfactant vesicles is important for the case of organelle targeting drug delivery.

3.5. Design of Antibody – Displayed vesicle ~ the Modification of Span 80 Vesicle Membrane by Novel Lectin *Eucheuma serra* Agglutinin ~.

The modification of vesicle membrane with proteins, peptides, and small molecules on the liposome membrane enhances the selectivity for the cancer cells. However, a general strategy on the modification of the surfactant vesicle with dynamic membrane has not been reported yet. The membrane properties could be presented by the optimal modification of membrane by protein according to the design scheme (**Fig. 3-1**). The amount of protein modifying the membrane surface of surfactant vesicle was regulated in order not to affect the membrane properties. In this section, the activity of protein was evaluated on the Span 80 vesicle membrane, by using *Eucheuma serra* Agglutinin (ESA) as a model protein. ESA is the lectin derived from marine red alga (Kawakubo et al., 1997) and, specifically, binds to the high mannose sugar chain structure (Hori et al., 2007). It has been reported that the ESA can specifically bind to the sugar chain on the membrane surface of cancer cells, without binding to the normal

fibroblast cells (Sugahara et al., 2001). After the Span 80 vesicle was modified with the ESA on the surface of the vesicle membrane, the ESA-modified Span 80 vesicle was added to the OST cells.

Figure 3-15 shows the affinity of ESA-modified Span 80 vesicle to the OST cells. The fluorescence intensity of OST cells was more increased by the treatment with ESA modifying Span 80 vesicle as compared with that in the case of unmodified Span 80 vesicle. It is suggested that ESA modified on the Span 80 vesicle bind to the high mannose sugar chain of the OST cell membrane, resulting in the selective binging to the cancer calls by using Span 80 vesicle (**Fig. 3-16**). Moreover, ESA modifying pegylated Span 80 vesicle was also evaluated in relation to its affinity for OST cells. Pegylation of

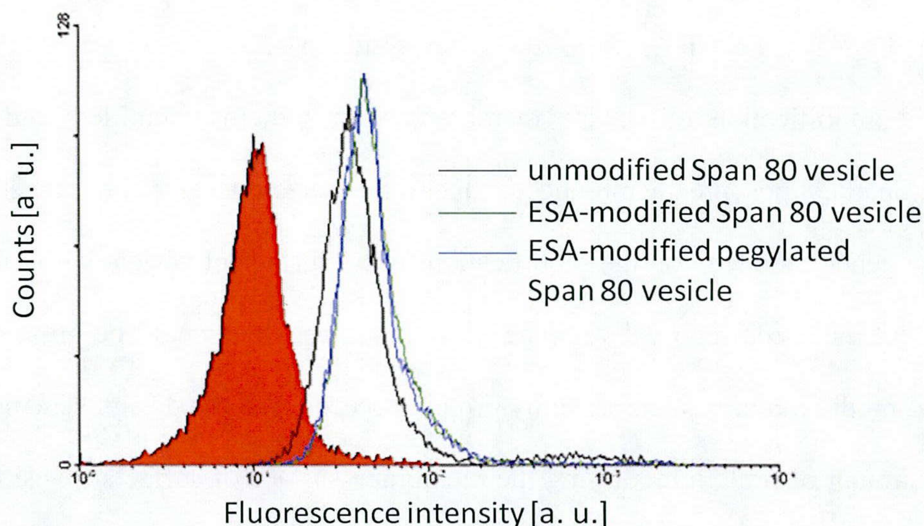


Fig. 3-15 Flow cytometric analysis of the interaction between OST cells and different types of Span 80 vesicles containing entrapped FITC: unmodified Span 80 vesicle (—), Span 80 vesicles with immobilized ESA (—), and pegylated Span 80 vesicle with immobilized ESA (—). Before analysis, the OST cells were incubated with the vesicles during 15 min at 37 ℃ in a humidified atmosphere of 5 % CO_2. PBS was added to OST cells as control (red fill).

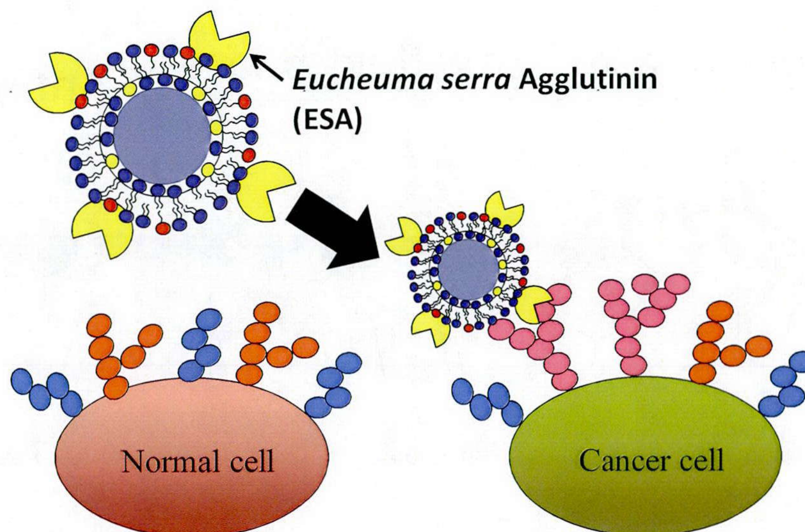


Fig. 3-16 *Eucheuma serra* Agglutinin (ESA) specifically bind to the high mannose type sugar chain on the cell surface. It is expected to the selective binding to the cancer cells.

surfactant vesicle like Doxil® is expected to be escaped from the natural immunity by the hydration layer as described above (**General Introduction**), while it is needed to consider the inhibition of protein activity by hydration layer or steric hindrance. ESA modifying pegylated Span 80 vesicle was found to bind to OST cells as same as ESA modifying Span 80 vesicle. This result shows the capability of multi modification on the Span 80 vesicle membrane.

On the other hand, a certain level of unmodified Span 80 vesicle also bound to OST cells. This binding affinity was only contributed to "membrane-membrane" interaction. These results suggest that not only modified protein but also the membrane properties of surfactant vesicle are important for the binding to the plasma membrane. However, it is needed to consider the protein behavior on the surfactant vesicle membrane in order to clarify the contribution of protein to the binding for cancer cells. ESA is known to possess four active sites in the signal molecule. It is considered that

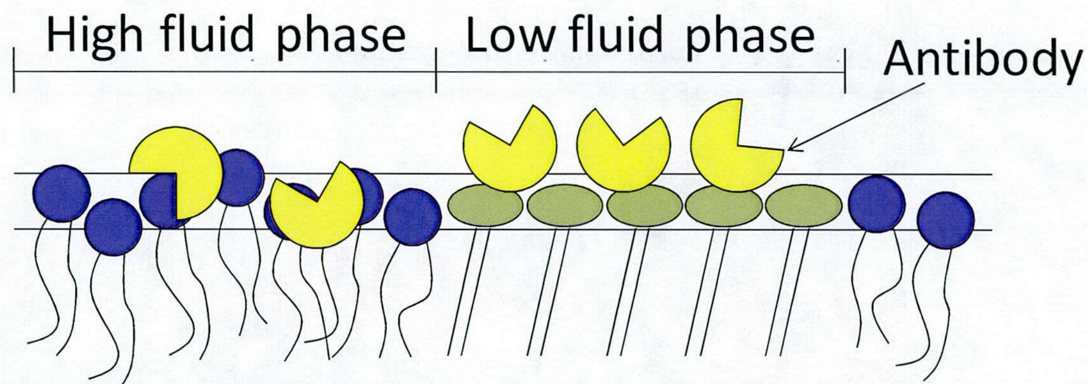


Fig. 3-17 Scheme of the strategy of organelle targeting by using vesicle-in-vesicle system.

the dynamic membrane of Span 80 vesicle could mask the active sites of ESA. When the active site of ESA can be hidden in the Span 80 vesicle membrane, it is needed to expose the active site of the antibody to the outside of the vesicle membrane. The hybrid vesicle was proposed as an idea (**Fig. 3-17**). The hybrid vesicle was prepared by mixing with Span 80 molecule and saturated lipid, resulting in the formation of the heterogeneous (liquid crystalline - gel phase) vesicle. The part of membrane with gel phase is expected to prevent the insertion of protein into the surfactant vesicle membrane. It has been confirmed that the active sites of the antibody molecule can be preserved in the hybrid vesicle modified with another kind of antibody (Concanavalin A) (data not shown). The design of membrane of the surface vesicle should be effective for the important of protein binding activity on the membrane.

4. Summary

The design of drug carrier based on the interaction mechanism is important to develop the efficient drug delivery. For example, the liposome is with less-interacting nature with the plasma membrane, resulting in the inefficient drug delivery to the cells. In order to increase the interaction with the plasma membrane, the dynamics of membrane surface need to be improved. Considering the results of **chapter 2**, the design scheme of drug carrier was established **(Fig. 3-1)**. According to this scheme, the headgroup mobility was shown to be an important key factor to improve the drug delivery efficiency. The surfactant vesicle with low intense headgroup mobility is uptaken by the endocytosis. Endocytosis pathway is disadvantageous for the drug delivery, resulting in the elimination of the delivered drug by the exocytosis. On the other hand, the surfactant vesicle with high intense headgroup mobility can be uptaken by the "membrane fusion", resulting in the direct and effective drug delivery.

After the fusion with plasma membrane, the surfactant molecules of vesicle diffuse on the plasma membrane, resulting in the alteration of the plasma membrane properties. The plasma membrane properties were highly related to the cellular regulation, which is important to control the membrane function such as membrane traffic or cellular signaling. It is therefore considered that the alteration of the plasma membrane properties can induce the damage to the cells. As expected, the cells induced the cell death with the bleb formation after the treatment with Span 80 vesicle. The membrane fluidity of Span 80 vesicle is higher as compared with the plasma membrane as previously reported (Kaur and Sanyal, 2010). The cell death mechanism by the disruption of the membrane composition was demonstrated by observation of phosphatidylserine exposures. The phosphatidylserine is usually located at inner leaflet

of the plasma membrane, while phosphatidylserine transported to the outer membrane by induction of apoptotic cell death. Therefore, the treatment of Span 80 vesicle for the plasma membrane induces the alteration of membrane properties similarly in the case of apoptotic cell death.

Moreover, the scheme of drug carrier is useful for more complex drug delivery, such as organelle targeting drug delivery. The vesicle-in-vesicle system was herewith proposed as a case study. DPPC liposome (second inner vesicle) was encapsulated into the Span 80 vesicle (first outer vesicle). Span 80 vesicle fuses with the plasma membrane, and releases DPPC liposome from the Span 80 vesicle. In this system, the strong interaction with the plasma membrane is important to carry out an effective drug delivery to the cells. The diffusion of Span 80 vesicle on the plasma membrane induces the cell death as described above. In the case of the drug delivery targeting an organelle by using the vesicle-in-vesicle systems, the cytotoxicity of Span 80 vesicle for the cells can be reduced, resulting in the prevention of the induction of cell death.

The membrane properties are also altered by the modification of the protein, ESA. ESA was modified on the membrane of Span 80 vesicle. It has been reported that ESA is lectin specifically binding to the high mannose sugar chain structure. ESA can specifically bind to the sugar chain on the cancer cell membrane. The binding affinity of Span 80 vesicle for the cancer cells was increased by the modification of ESA on the Span 80 vesicle membrane. Moreover, pegylation of ESA-modified Span 80 vesicle did not affect the binding affinity for cancer cells, suggesting no effect for the ESA binding activity by pegylation.

General Conclusion

A general strategy to control the interaction between different kinds of membranes has not been established yet, because there is less information on the physicochemical properties of "molecular assembly" itself. In this thesis, the author mainly focus on the establishment of the systematic design of the interaction between the different membranes of surfactant vesicles (i.e. detergent vesicle and phospholipid vesicle) based on the key properties of their membrane (as a "molecular assembly"). As the first step of this investigation, new kinds of membrane properties of the surfactant vesicles have been characterized in addition to the conventional property (i.e. "membrane fluidity"), where the "*flexible*", "*fluid*", and "*wet*" natures of the membrane surface were shown to be important to understand the character of the membrane from the systematic viewpoint **(Chapter 1)**. Secondly, the "*membrane-membrane*" interaction was further studied through both calcein leakage and lipid mixing analyses, mainly focusing on its relationship with the above-characterized membrane properties, by employing the detergent vesicle (Span80 vesicle) and phospholipid vesicle (phosphatidylcholine liposome) as target membrane to be mutually interacted **(Chapter 2)**. Based on the above findings, a design scheme to control the membrane-membrane interaction based on the membrane properties was finally proposed, together with its extension for some kinds of applications, by employing (i) drug delivery via direct fusion using detergent vesicle, (ii) induction of response on the cellular membrane via designed vesicle, (iii) targeting of intracellular organelle using vesicle-in-vesicle, and (iv) active targeting using antibody-displayed vesicle as case studies of the scheme.

In chapter 1, various kinds of surfactant vesicles were characterized, focusing on the membrane properties that differ in their principle. The spontaneous aggregation

of surfactants in an aqueous solution can induce the formation of lipid bilayer, resulting in the unique properties (membrane properties) that are different from the properties of sole molecule. The surfactant vesicles were quantitatively characterized in relation to different kinds of membrane properties, such as "*fluidity*", "*flexibility*", and "*wetness*". It was found that the inner membrane properties of Span 80 vesicle, which was composed by the non-ionic surfactant (sorbitan Monooleate; Span 80), were similar to those of the POPC liposome composed by the phospholipid harboring a dipolar structure at the hydrophilic head group. Both surfactant vesicles (Span 80 and POPC) have headgroup and acyl chain structure and it is common that the inner phase of these vesicles is composed by the similar acyl chain structure (unsaturated acyl chain). The inner membrane properties of Span 80 vesicle are thus similar to those of the POPC liposome in contrast to those of the DPPC liposome with saturated acyl chain. Both Span 80 vesicle and POPC liposome have a membrane with liquid crystalline phase, while DPPC liposome has a membrane with gel phase. The above character of the membrane can herewith be regarded as "*fluid*" nature of the membrane. In addition, the difference of the structure between Span 80 vesicle and POPC liposome also affected the headgroup interaction, where the headgroup of POPC liposome interacts with each other by the electrostatic interaction while that of Span 80 vesicle is composed by the sorbitol structure to give a relatively weak interaction. By using the dielectric dispersion analysis, the Span 80 vesicle was shown to have a dynamic membrane with high intense headgroup mobility, which can be regarded as "*flexible*" nature. By using the hydrophobicity-sensitive molecular probe (ANS), it was shown that the water molecule could be easily inserted into the dynamic Span 80 vesicle membrane, which can be regarded as "*wet*" nature. The membrane surface properties of surfactant vesicles, such

as Span 80 vesicle and phospholipid vesicle, were thus characterized systematically along with the different axis on "*fluid*", "*flexible*", and "*wet*" natures.

In chapter 2, the "*membrane-membrane*" interaction was evaluated by investigating the membrane-related phenomena (i.e. calcein leakage and lipid mixing) by using various surfactant vesicles characterized by the membrane properties. There are three steps during the interaction between vesicles, perturbation, hemifusion, and fusion which is the final step of the interaction of "*hetero*"-vesicles. Span 80 vesicle was found to aggregate (calcein leakage), and hemifuse (FRET method) with the liposome. Although further investigation on the fusion between Span 80 vesicle and liposome is needed (the aqueous content mixing assay), it is suggested that the difference of membrane properties between vesicles ("membrane potentials") is the important for the "membrane-membrane" interaction. After the interaction with the liposome, Span 80 vesicle was found to perturb the liposome membrane, resulting in the induction of its hemifusion with the liposome membrane. These results suggest that the surfactant vesicle with dynamic membrane surface is advantageous for the interaction with the plasma membrane. After the interaction to the liposome membrane, Span 80 vesicle was found to diffuse on the liposome membrane. Span 80 vesicle diffusing on the liposome membrane alters the liposome membrane more dynamic. The liposome membrane hemifused with Span 80 vesicle was more "*wet*" as compared with the intact liposome membrane. On the other hand, the dynamic membrane was disadvantageous for the encapsulation of DOX.

The encapsulation efficiency of the DOX was employed by using remote loading method. The existence of water molecule into the membrane ("*wet*" membrane) was shown to prevent the insertion of DOX. It is therefore needed to design the

surfactant vesicle membrane with the high intense headgroup mobility and low existence of water molecule (e.c. Span 80 vesicle inserted cholesterol into the membrane).

In chapter 3, the design scheme for the novel surfactant vesicle to control the membrane-membrane interaction was finally established based on the findings described in chapter 1 (i.e. membrane properties) and chapter 2 (i.e. membrane-membrane interaction). It is suggested that this design scheme could also be applicable for the plasma membrane (biological cells) because it partially consists of phospholipid bilayer. It is generally known that the liposome is with less-interacting nature with the plasma membrane, resulting in the inefficient drug datively to the cells. If one could succeed in the enhancement of the liposome interaction with the plasma membrane, the cellular-uptake of liposome through the endocytosis could incredibly be promoted because one could employ the direct fusion strategy without the elimination by exocytosis. The conventional strategy in drug delivery system, it is needed to increase the intense of headgroup mobility according to the scheme of the drug carrier design. Span 80 vesicle with high intense headgroup mobility was shown to fuse with the plasma membrane, resulting in the diffusion of Span 80 vesicle on the plasma membrane. After the Span 80 interaction, the plasma membrane was found to become the heterogeneous membrane, where different domains of phospholipids and Span 80 molecules could be formed on a same membrane. Considering the problems to be solved in the conventional DDS design, the following case studies of the proposed scheme were investigated. [1] Direct fusion via detergent vesicle: According this scheme, headgroup mobility was shown to be an important key factor to the drug delivery efficiency. The surfactant vesicle with low intense headgroup mobility was

found to be uptaken by the endocytosis. On the other hand, the surfactant vesicle with high intense headgroup mobility was shown to be uptaken by the membrane fusion, resulting in the direct and effective drug delivery. [2] Induction of cellular membrane response via designed vesicle: The alteration of the plasma membrane properties prevent to the regulation of function on the membrane, resulting in the induction of apoptotic like cell death (phosphatidylserine exposure). The plasma membrane properties are important to control the membrane-relating functions, such as membrane traffic or cellular signaling. It is therefore considered that the alteration of the plasma membrane properties could induce some damages to the cells. As expected, the cells were found to induce the cell death, accompanied with bleb-formation, by the treatment of the cells with Span 80 vesicle. Moreover, this scheme was applied for the design of complex drug delivery strategy, such as vesicle-in-vesicle system or modification of protein on the surfactant membrane. [3] Targeting of intracellular organelle using vesicle-in-vesicle: The vesicle-in-vesicle system is expected for the capability of the organelle targeting drug delivery according the scheme of the drug carrier design. The second inner vesicle is encapsulated into the first outside vesicle. The first outside vesicle fuse with plasma membrane. The second inner vesicle is released from the first vesicle, delivering the drug to the organelle directly. In order to the design the drug carrier based on the membrane properties according to the design scheme, it is important to unchange the membrane properties of the surfactant vesicle. It was found that the effective drug delivery could be carried out by using the vesicle-in-vesicle. [4] Enhancement of DDS efficiency using antibody-displayed vesicle: It has been reported the modification of the surfactant vesicle of substance (such as antibody) can improve the its availability, especially, in relation to its delivery efficiency. However, the effect

of modification for the membrane properties has been hardly investigated in the previous reports. It is important to clarify the behavior of the substance modified on the membrane of the surfactant vesicle to increase the binding activity for the plasma membrane. It was shown that the suitable membrane to modify the antibody could enhance the targeting of surfactant vesicle.

The scheme to control the "membrane-membrane" interaction based on the "membrane properties" was thus established, by employing its possible application in drug delivery system as a case study. It is expected that this research could contribute the rational design of not only the drug carrier design, together with the deeper the understanding of all phenomenon relating to the membrane.

Suggesting for Future Work

1. Application of the membrane characterization for the surfactant vesicle

It is considered that all the surfactant vesicles have their individual membrane properties, from phospholipid vesicle (liposome) to non-ionic surfactant vesicle (niosome). In this thesis, these membrane properties were systematically categorized along with "three" major categories, together with the analysis of "five" properties, (i) inner membrane fluidity (DPH), (ii) membrane surface fluidity (TMA-DPH), (iii) headgroup mobility (dielectric dispersion analysis), (iv) inner polarity (Laurdan), and (v) membrane surface polarity (ANS). Various kinds of drug carriers have been conventionally investigated since 1980's. In the previous investigation, these drug carriers were evaluated, only focusing on the "phenomena", such as drug leakage, retention time in the blood vessel, accumulation into the disease site, although the evaluation of the function and nature of these drug carriers was only limited to "case-by-case" trials. Therefore, these drug carriers have not been evaluated in a systematic manner. However, this problem could be solved by the characterization of the membrane of the drug carrier. These membrane properties, described here, can contribute to the prognosis of the drug carrier behavior. As described in chapter 2, "*membrane-membrane*" interaction is well related with the membrane dynamics, especially, the headgroup mobility of the surfactant vesicle membrane. The surfactant vesicle with high-intense headgroup mobility easily inserts into and perturbs the plasma membrane. After the interaction with the membrane, the surfactant vesicle fuses with the membrane. The fusing surfactant vesicle diffuses on the membrane, resulting in the alteration of the membrane properties of the plasma membrane. On the other hand, the surfactant vesicle with low-intense headgroup mobility interacted weakly with the

plasma membrane as compared with that with high-intense headgroup mobility. This surfactant vesicle does not perturb the plasma membrane, resulting in the uptake by endocytosis pathway.

In this study, these characterizations of the surfactant vesicle membrane were used for the design of the drug carrier. However, these membrane properties are fundamental nature of the membrane, the characterization method can be also used for other studies related to the membrane. It is expected that these knowledge is useful not only the design of the drug carrier, but also the understanding of the phenomenon on the membrane. For example, it is very difficult to analyze the protein behavior on the lipid bilayer as compared with that in the solution. The membrane protein is related to various cellular functions (such as signaling, membrane transfer, and uptake), while the behavior of these membrane proteins has not been clarified. It has been already reported that the plasma membrane can work as a "foothold" for the membrane protein, especially on raft structure. Therefore the information of membrane properties contributes to the clarification of the mechanism of the work of membrane proteins.

2. Induction of membrane-membrane interaction by the difference of membrane properties

Membrane properties of the vesicle were shown to control the interaction with the apposed vesicle. Vesicles were evaluated by various kinds of membrane properties using fluorescence probes and dielectric dispersion analysis, as described above. The membrane properties analysis by DPH (fluidity), dielectric dispersion analysis (flexible), and polarity analysis by Laurdan (wet), respectively, show the membrane properties of the headgroup region, boundary region, and acyl chain region, respectively. When the

calcein leakage was evaluated, the increase of the difference of fluidity (Δ $1/P$) and flexible (Δ $\ln f_{ci}$) between vesicles was shown to induce the perturbation of the membrane. Alternatively, the decrease of the difference of wet ($GP_{(340)}$) induce the perturbation of the membrane. Moreover, lipid mixing was also related with the difference of the membrane properties between vesicles. Especially, the flexibility is proportionality related with the %ET. In this way, the concept of "Membrane Potential" can be suitable for the explanation of the membrane-related phenomena. However, the relationship between membrane properties and membrane-membrane interaction has not been fully clarified yet in terms of molecule level. Further experiments are required to refer the molar behavior in/on the membrane. The elucidation of the molar behavior in/on the membrane is expected to improve the modulation of the self-assemble properties itself and, also, membrane-membrane interaction in detail.

3. Design of the drug carrier ~ more complex drug delivery strategy ~

In this study, the scheme of the drug carrier was established based on the membrane properties described above. It has been reported that membrane fluidity is important for the interaction of the vesicle on liposome membranes with the biomembrane of the cells. However, it has been clarified that the headgroup mobility is strongly related with the interaction with plasma membrane as shown in this scheme. Moreover, the headgroup mobility was shown to be well correlated with the fusion capacity. The fusion capacity can be applied for design of the complex drug delivery, such as vesicle-in-vesicle system. Recently, the cause of various diseases has been gradually clarified gradually, focusing on its relation with the intracellular organelles that differ in the cellular function. It is thus necessary to deliver the drug to the each

organelle, such as nuclear, golgi body, and mitochondria. The vesicle-in-vesicle system is expected to contribute to fulfill these expectations relating to this issue.

Moreover, the membrane characterization is considered to control the behavior of the surfactant vesicle modified with substance (such as antibody). It has been reported that the modification of substance on the surfactant vesicle enhances the accumulation of drug into the disease site, especially in the research area of cancer chemotherapy. However, the use of these modified surfactant vesicles has a difficulty in the complete inhibition of the tumor growth. In previous conventional reports by the behavior of the substance on the surfactant vesicle membrane has not been investigated. For example, it is important to expose the active site on the outer surface of the surfactant vesicle in case of protein. When the active site can be hidden in the membrane, the surfactant vesicle cannot be effectively bound to the cancer cells. The protein can be deeply inserted into the surfactant vesicle membrane, especially in the case of the membrane with high-intense headgroup mobility. The more headgroup mobility is increased, the more the binding of protein can be inhibited by the membrane. However, the reduction of the headgroup mobility of the surfactant vesicle prevents the fusion with plasma membrane. This problem is solved by the strategy based on the membrane properties. The heterogeneous membrane containing both membrane properties is capable for the more effective drug delivery. The protein, modified on the membrane with low fluidity, and the surfactant vesicle, which can interact with the plasma membrane in the highly-fluid phase, should be selected for the design of the high performance DDS.

Nomenclature

Symbol		Description	Unit
$1/p$	=	Membrane fluidity estimated by DPH or TMA-DPH	[-]
f_{ci}	=	Characteristic frequency at around 60 MHz (phospholipid), and 200 MHz (Span 80)	[-]
$GP_{(340)}$	=	General polarity into the surfactant vesicle membrane	[-]
RT	=	Ratio of the calcein fluorescence intensity (resolution from self-quenching)	[-]
%ET	=	Ratio of fluorescence resonance energy transfer	[-]
N	=	material flow rate	[$mol \cdot m^{-2} \cdot s^{-1}$]
C_{out}	=	Concentration of the DOX in the outer phase	[$mol \cdot m^{-3}$]
S	=	Total surface area of vesicle	[m^2]
V_{out}	=	Total volume of vesicle	[m^3]
K	=	Mass-transfer coefficient	[$m \cdot s^{-1}$]
IC_{50}	=	50% inhibitory concentration	[mM]

List of Abbreviation

ANS	8-Anilino-1-naphthalenesulfonic acid
Ch	cholesterol
DDS	Drug delivery System
DLS	light scattering photometer
DMPC	1,2-dimirystoyl- *sn*-glycero-3-phosphocholine
DOX	doxorubicin hydrochloride
DPH	1,6-diphenyl-1,3,5-hexatriene
DPPC	1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine
DSC	differential scanning calorimeter
EGFR	epidermal growth factor receptor
ERFR	Fluorescence resonance energy transfer
ESA	*Eucheuma serra* Agglutinin
GP	generalized polarization
NBD-PE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)
PBS	phosphate-buffered saline
PEG	poly(ethylene glycol)

POPC	1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
PS	phosphatidylserine
Rho-PE	1,2-dioleoyl-*sn*-glycero-3-phosphoethanol-amine-N-(lissamine rhodamine B sulfonyl)
SuPE	1,2-dioleoyl-*sn*-glycero3-phosphoethanolamine-N-(succinyl)
TMA-DPH	N,N,N-trimethyl-4-(6-phenyl-1,3,5-hexatrien-1-yl) phenylammonium p-toluenesulfonate

References

Alipour, M., Halwani, M., Omri, A. and Suntres, Z. E., Antimicrobial effectiveness of liposomal polymyxin B against resistant Gram-negative bacterial strains. *Int. J. Pharm.*, **355**, 293-298 (2008).

Allen, J. A., Halverson-Tamboli, R. A. and Rasenick, M. M., Lipid raft microdomains and neurotransmitter signalling. *Nat. Rev. Neurosci.*, **8**, 128-140 (2007).

Alves, I., Staneva, G., Tessier, C., Salgado, G. F. and Nuss, P., The interaction of antipsychotic drugs with lipids and subsequent lipid reorganization investigated using biophysical methods. *Biochim. Biophys. Acta*, **1808**, 2009-2018 (2011).

Ashley, C. E., Carnes, E. C., Phillips, G. K., Padilla, D., Durfee, P. N., Brown, P. A., Hanna, T. N., Liu, J., Phillips, B., Carter, M. B., Carroll, N. J., Jiang, X., Dunphy, D. R., Willman, C. L., Petsev, D. N., Evans, D. G., Parikh, A. N., Chackerian, B., Wharton, W., Peabody, D. S. and Brinker, C. J., The targeted delivery of multicomponent cargos to cancer cells by nanoporous particle-supported lipid bilayers. *Nat. Mater.*, **10**, 389-397 (2011).

Bárány-Wallje, E., Gaur, J., Lundberg, P., Langel, U. and Graslund, A., Differential membrane perturbation caused by the cell penetrating peptide Tp10 depending on attached cargo. *FEBS Lett.*, **581**, 2389-2393 (2007).

Baba, M., Matsumoto, Y., Kashio, A., Cabral, H., Nishiyama, N., Kataoka, K. and Yamasoba, T., Micellization of cisplatin (NC-6004) reduces its ototoxicity in guinea pigs. *J. Control. Release*, **157**, 112-117 (2012).

Bagatolli, L. A., Parasassi, T., Fidelio, G. D. and Gratton, E., A model for the interaction of 6-lauroyl-2-(N,N-dimethylamino)naphthalene with lipid environments:

implications for spectral properties. *Photochem. Photobiol.*, **70**, 557-564 (1999).

Bakht, O., Pathak, P. and London, E., Effect of the structure of lipids favoring disordered domain formation on the stability of cholesterol-containing ordered domains (lipid rafts): Identification of multiple raft-stabilization mechanisms. *Biophys. J.*, **93**, 4307-4318 (2007).

Barros, L. F., Kanaseki, T., Sabirov, R., Morishima, S., Castro, J., Bittner, C. X., Maeno, E., Ando-Akatsuka, Y. and Okada, Y., Apoptotic and necrotic blebs in epithelial cells display similar neck diameters but different kinase dependency. *Cell Death Differ.*, **10**, 687-697 (2003).

Borenstain, V. and Barenholz, Y., Characterization of liposomes and other lipid assemblies by multiprobe fluorescence polarization. *Chem. Phys. Lipids*, **64**, 117-127 (1993).

Carrillo, C., Teruel, J. A., Aranda, F. J. and Ortiz, A., Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. *Biochim. Biophys. Acta*, **1611**, 91-97 (2003).

Charras, G. and Paluch, E., Blebs lead the way: how to migrate without lamellipodia. *Nat. Rev. Mol. Cell Biol.*, **9**, 730-736 (2008).

Chernomordik, L. V. and Kozlov, M. M., Mechanics of membrane fusion. *Nat. Struct. Mol. Biol.*, **15**, 675-683 (2008).

Chonn, A., Semple, S. C. and Cullis, P. R., Association of blood proteins with large unilamellar liposomes in vivo. Relation to circulation lifetimes. *J. Biol. Chem.*, **267**, 18759-18765 (1992).

Cornec, M., Mackie, A. R., Wilde, P. J. and Clark, D. C., Competitive adsorption of beta-lactoglobulin and beta-casein with Span 80 at the oil-water interface and the

effects on emulsion behaviour. *Colloid Surf. A*, **114**, 237-244 (1996).

Düzgüneş, N. and Nir, S., Mechanisms and kinetics of liposome-cell interactions. *Adv. Drug Deliv. Rev.*, **40**, 3-18 (1999).

Dimitrova, M. N., Matsumura, H., Dimitrova, A. and Neitchev, V. Z., Interaction of albumins from different species with phospholipid liposomes. Multiple binding sites system. *Int. J. Biol. Macromol.*, **27**, 187-194 (2000).

Eckford, P. D. and Sharom, F. J., Interaction of the P-glycoprotein multidrug efflux pump with cholesterol: effects on ATPase activity, drug binding and transport. *Biochemistry*, **47**, 13686-13698 (2008).

Efrat, A., Chernomordik, L. V. and Kozlov, M. M., Point-like protrusion as a prestalk intermediate in membrane fusion pathway. *Biophys. J.*, **92**, L61-L63 (2007).

Elbayoumi, T. A. and Torchilin, V. P., Enhanced cytotoxicity of monoclonal anticancer antibody 2C5-modified doxorubicin-loaded PEGylated liposomes against various tumor cell lines. *Eur. J. Pharm. Sci.*, **32**, 159-168 (2007).

Fadeel, B. and Xue, D., PS externalization: from corpse clearance to drug delivery. *Cell Death Differ.*, **13**, 360-362 (2006).

Flanagan, M. T. and Hesketh, T. R., Electrostatic interactions in the binding of fluorescent probes to lipid membranes. *Biochim. Biophys. Acta*, **298**, 535-545 (1973).

Gerke, V. and Moss, S. E., Annexins: from structure to function. *Physiol. Rev.*, **82**, 331-371 (2002).

Hafez, I. M. and Cullis, P. R., Roles of lipid polymorphism in intracellular delivery. *Adv. Drug Deliv. Rev.*, **47**, 139-148 (2001).

Hao, Y. M. and Li, K., Entrapment and release difference resulting from hydrogen

bonding interactions in niosome. *Int. J. Pharm.*, **403**, 245-253 (2011).

Haran, G., Cohen, R., Bar, L. K. and Barenholz, Y., Transmembrane ammonium sulfate gradients in liposomes produce efficient and stable entrapment of amphipathic weak bases. *Biochim. Biophys. Acta*, **1151**, 201-215 (1993).

Hayashi, K., Shimanouchi, T., Kato, K., Miyazaki, T., Nakamura, A. and Umakoshi, H., Span 80 vesicles have a more fluid, flexible and "wet" surface than phospholipid liposomes. *Colloids Surf. B*, **87**, 28-35 (2011).

Hiemenz, J. W. and Walsh, T. J., Lipid formulations of amphotericin B: recent progress and future directions. *Clin. Infect. Dis.*, **22**, S133-144 (1996).

Hirt, U. A. and Leist, M., Rapid, noninflammatory and PS-dependent phagocytic clearance of necrotic cells. *Cell Death Differ.*, **10**, 1156-1164 (2003).

Hori, K., Sato, Y., Ito, K., Fujiwara, Y., Iwamoto, Y., Makino, H. and Kawakubo, A., Strict specificity for high-mannose type N-glycans and primary structure of a red alga Eucheuma serra lectin. *Glycobiology*, **17**, 479-491 (2007).

Ipsen, J. H., Karlstrom, G., Mouritsen, O. G., Wennerstrom, H. and Zuckermann, M. J., Phase equilibria in the phosphatidylcholine-cholesterol system. *Biochim. Biophys. Acta*, **905**, 162-172 (1987).

Kato, K., Chin, K., Yoshikawa, T., Yamaguchi, K., Tsuji, Y., Esaki, T., Sakai, K., Kimura, M., Hamaguchi, T., Shimada, Y., Matsumura, Y. and Ikeda, R., Phase II study of NK105, a paclitaxel-incorporating micellar nanoparticle, for previously treated advanced or recurrent gastric cancer. *Invest. New Drugs*, **30**, 1621-1627 (2012).

Kato, K., Walde, P., Koine, N., Ichikawa, S., Ishikawa, T., Nagahama, R., Ishihara, T., Tsujii, T., Shudou, M., Omokawa, Y. and Kuroiwa, T., Temperature-sensitive nonionic vesicles prepared from Span 80 (sorbitan monooleate). *Langmuir*, **24**,

10762-10770 (2008).

Kato, K., Walde, P., Koine, N., Imai, Y., Akiyama, K. and Sugahara, T., Molecular Composition of Nonionic Vesicles Prepared from Span 80 or Span 85 by a Two - Step Emulsification Method Emulsification Method. *J. Dispersion Sci. Technol.*, **27**, 1217 (2006).

Kaur, J. and Sanyal, S. N., Alterations in membrane fluidity and dynamics in experimental colon cancer and its chemoprevention by diclofenac. *Mol. Cell. Biochem.*, **341**, 99-108 (2010).

Kawakubo, A., Makino, H., Ohnishi, J., Hirohara, H. and Hori, K., The marine red alga Eucheuma serra J. Agardh, a high yielding source of two isolectins. *J. Appl. Phycol.*, **9**, 331-338 (1997).

Kepp, O., Galluzzi, L., Lipinski, M., Yuan, J. and Kroemer, G., Cell death assays for drug discovery. *Nat. Rev. Drug Discov.*, **10**, 221-237 (2011).

Kesharwani, P., Gajbhiye, V. and Jain, N. K., A review of nanocarriers for the delivery of small interfering RNA. *Biomaterials*, **33**, 7138-7150 (2012).

Komizu, Y., Matsumoto, Y. and Ueoka, R., Membrane targeted chemotherapy with hybrid liposomes for colon tumor cells leading to apoptosis. *Bioorg. Med. Chem. Lett.*, **16**, 6131-6134 (2006).

Koynova, R. and Caffrey, M., Phases and phase transitions of the phosphatidylcholines. *Biochim. Biophys. Acta*, **1376**, 91-145 (1998).

Kratz, F. and Elsadek, B., Clinical impact of serum proteins on drug delivery. *J. Control. Release*, **161**, 429-445 (2012).

Krijnen, P. A., Sipkens, J. A., Molling, J. W., Rauwerda, J. A., Stehouwer, C. D., Muller, A., Paulus, W. J., van Nieuw Amerongen, G. P., Hack, C. E., Verhoeven, A. J.,

van Hinsbergh, V. W. and Niessen, H. W., Inhibition of Rho-ROCK signaling induces apoptotic and non-apoptotic PS exposure in cardiomyocytes via inhibition of flippase. *J. Mol. Cell. Cardiol.*, **49**, 781-790 (2010).

Kuboi, R., Mawatari, T. and Yoshimoto, M., Oxidative refolding of lysozyme assisted by negatively charged liposomes: relationship with lysozyme-mediated fusion of liposomes. *J. Biosci. Bioeng.*, **90**, 14-19 (2000).

Kuboi, R., Shimanouchi, T., Yoshimoto, M. and Umakoshi, H., Detection of protein conformation under stress conditions using liposomes as sensor materials. *Sens. Mater.*, **16**, 241-254 (2004).

Lasic, D. D., Doxorubicin in sterically stabilized liposomes. *Nature*, **380**, 561-562 (1996).

Lasic, D. D., Čeh, B., Stuart, M. C. A., Guo, L., Frederik, P. M. and Barenholz, Y., Transmembrane gradient driven phase-transitions within vesicles - lessons for drug-delivery. *Biochim. Biophys. Acta-Biomembr.*, **1239**, 145-156 (1995).

Leist, M. and Nicotera, P., The shape of cell death. *Biochem. Biophys. Res. Commun.*, **236**, 1-9 (1997).

Lentz, B. R., Use of Fluorescent-Probes to Monitor Molecular Order and Motions within Liposome Bilayers. *Chem. Phys. Lipids*, **64**, 99-116 (1993).

Leventis, P. A. and Grinstein, S., The distribution and function of phosphatidylserine in cellular membranes. *Annu. Rev. Biophys.*, **39**, 407-427 (2010).

Mackenzie, A. B., Young, M. T., Adinolfi, E. and Surprenant, A., Pseudoapoptosis induced by brief activation of ATP-gated P2X7 receptors. *J. Biol. Chem.*, **280**, 33968-33976 (2005).

Madden, T. D., Harrigan, P. R., Tai, L. C. L., Bally, M. B., Mayer, L. D., Redelmeier, T.

E., Loughrey, H. C., Tilcock, C. P. S., Reinish, L. W. and Cullis, P. R., The accumulation of drugs within large unilamellar vesicles exhibiting a proton gradient - a survey. *Chem. Phys. Lipids*, **53**, 37-46 (1990).

Marsden, H. R., Tomatsu, I. and Kros, A., Model systems for membrane fusion. *Chem. Soc. Rev.*, **40**, 1572-1585 (2011).

Martens, S. and McMahon, H. T., Mechanisms of membrane fusion: disparate players and common principles. *Nat. Rev. Mol. Cell Biol.*, **9**, 543-556 (2008).

Maxfield, F. R. and Tabas, I., Role of cholesterol and lipid organization in disease. *Nature*, **438**, 612-621 (2005).

McPhail, D., Tetley, L., Dufes, C. and Uchegbu, I. F., Liposomes encapsulating polymeric chitosan based vesicles--a vesicle in vesicle system for drug delivery. *Int. J. Pharm.*, **200**, 73-86 (2000).

Melino, G., Knight, R. A. and Nicotera, P., How many ways to die? How many different models of cell death? *Cell Death Differ.*, **12**, 1457-1462 (2005).

Moghimi, S. M. and Patel, H. M., Serum opsonins and phagocytosis of saturated and unsaturated phospholipid liposomes. *Biochim. Biophys. Acta*, **984**, 384-387 (1989).

Morse, M., Technology evaluation: Rexin-G, Epeius Biotechnologies. *Curr. Opin. Mol. Ther.*, **7**, 164-169 (2005).

Mross, K., Niemann, B., Massing, U., Drevs, J., Unger, C., Bhamra, R. and Swenson, C. E., Pharmacokinetics of liposomal doxorubicin (TLC-D99; Myocet) in patients with solid tumors: an open-label, single-dose study. *Cancer Chemother. Pharmacol.*, **54**, 514-524 (2004).

Nagano, T., Yasunaga, M., Goto, K., Kenmotsu, H., Koga, Y., Kuroda, J., Nishimura, Y.,

Sugino, T., Nishiwaki, Y. and Matsumura, Y., Antitumor activity of NK012 combined with cisplatin against small cell lung cancer and intestinal mucosal changes in tumor-bearing mouse after treatment. *Clin. Cancer. Res.*, **15**, 4348-4355 (2009).

Nagata, S., Hanayama, R. and Kawane, K., Autoimmunity and the clearance of dead cells. *Cell*, **140**, 619-630 (2010).

Okamura, E. and Yoshii, N., Drug binding and mobility relating to the thermal fluctuation in fluid lipid membranes. *J. Chem. Phys.*, **129**, 215102 (2008).

Omokawa, Y., Miyazaki, T., Walde, P., Akiyama, K., Sugahara, T., Masuda, S., Inada, A., Ohnishi, Y., Saeki, T. and Kato, K., In vitro and in vivo anti-tumor effects of novel Span 80 vesicles containing immobilized Eucheuma serra agglutinin. *Int. J. Pharm.*, **389**, 157-167 (2010).

Orrenius, S., Nicotera, P. and Zhivotovsky, B., Cell death mechanisms and their implications in toxicology. *Toxicol. Sci.*, **119**, 3-19 (2011).

Ostrom, R. S. and Insel, P. A., The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: implications for molecular pharmacology. *Br. J. Pharmacol.*, **143**, 235-245 (2004).

Papahadjopoulos, D., Poste, G. and Schaeffe, B. E., Fusion of mammalian-cells by unilamellar lipid vesicles - influence of lipid surface charge, fluidity and cholesterol. *Biochim. Biophys. Acta*, **323**, 23-42 (1973).

Parasassi, T., Di Stefano, M., Loiero, M., Ravagnan, G. and Gratton, E., Influence of cholesterol on phospholipid bilayers phase domains as detected by Laurdan fluorescence. *Biophys. J.*, **66**, 120-132 (1994).

Parasassi, T. and Gratton, E., Membrane lipid domains and dynamics as detected by

laurdan fluorescence. *J. Fluoresc.*, **5**, 59-69 (1995).

Parasassi, T., Krasnowska, E. K., Bagatolli, L. and Gratton, E., LAURDAN and PRODAN as polarity-sensitive fluorescent membrane probes. *J. Fluoresc.*, **8**, 365-373 (1998).

Parsons, J. T., Horwitz, A. R. and Schwartz, M. A., Cell adhesion: integrating cytoskeletal dynamics and cellular tension. *Nat. Rev. Mol. Cell Biol.*, **11**, 633-643 (2010).

Poste, G. and Papahadjopoulos, D., Lipid vesicles as carriers for introducing materials into cultured-cells - influence of vesicle lipid-composition on mechanism(s) of vesicle incorporation into cells. *Proc. Natl. Acad. Sci. U. S. A.*, **73**, 1603-1607 (1976).

Pugh, E. L., Bittman, R., Fugler, L. and Kates, M., Comparison of steady-state fluorescence polarization and urea permeability of phosphatidylcholine and phosphatidylsulfocholine liposomes as a function of sterol structure. *Chem. Phys. Lipids*, **50**, 43-50 (1989).

Razani, B., Woodman, S. E. and Lisanti, M. P., Caveolae: from cell biology to animal physiology. *Pharmacol. Rev.*, **54**, 431-467 (2002).

Rivera, E., Liposomal anthracyclines in metastatic breast cancer: clinical update. *Oncologist*, **8**, 3-9 (2003).

Ross, J. F., Chaudhuri, P. K. and Ratnam, M., Differential regulation of folate receptor isoforms in normal and malignant tissues in vivo and in established cell lines. Physiologic and clinical implications. *Cancer*, **73**, 2432-2443 (1994).

Salbreux, G., Charras, G. and Paluch, E., Actin cortex mechanics and cellular morphogenesis. *Trends Cell Biol.*, **22**, 536-545 (2012).

Shimanouchi, T., Ishii, H., Yoshimoto, N., Umakoshi, H. and Kuboi, R., Calcein permeation across phosphatidylcholine bilayer membrane: effects of membrane fluidity, liposome size, and immobilization. *Colloids Surf. B*, **73**, 156-160 (2009).

Shimanouchi, T., Sasaki, M., Hiroiwa, A., Yoshimoto, N., Miyagawa, K., Umakoshi, H. and Kuboi, R., Relationship between the mobility of phosphocholine headgroups of liposomes and the hydrophobicity at the membrane interface: A characterization with spectrophotometric measurements. *Colloids Surf. B*, **88**, 221-230 (2011).

Shynkar, V. V., Klymchenko, A. S., Kunzelmann, C., Duportail, G., Muller, C. D., Demchenko, A. P., Freyssinet, J. M. and Mely, Y., Fluorescent biomembrane probe for ratiometric detection of apoptosis. *J. Am. Chem. Soc.*, **129**, 2187-2193 (2007).

Siegel, D. P., The modified stalk mechanism of lamellar/inverted phase transitions and its implications for membrane fusion. *Biophys. J.*, **76**, 291-313 (1999).

Slavik, J., Anilinonaphthalene sulfonate as a probe of membrane-composition and function. *Biochim. Biophys. Acta*, **694**, 1-25 (1982).

Stepniewski, M., Pasenkiewicz-Gierula, M., Rog, T., Danne, R., Orlowski, A., Karttunen, M., Urtti, A., Yliperttula, M., Vuorimaa, E. and Bunker, A., Study of PEGylated lipid layers as a model for PEGylated liposome surfaces: molecular dynamics simulation and langmuir monolayer studies. *Langmuir*, **27**, 7788-7798 (2011).

Stowell, S. R., Karmakar, S., Arthur, C. M., Ju, T., Rodrigues, L. C., Riul, T. B., Dias-Baruffi, M., Miner, J., McEver, R. P. and Cummings, R. D., Galectin-1

induces reversible phosphatidylserine exposure at the plasma membrane. *Mol. Biol. Cell*, **20**, 1408-1418 (2009).

Struck, D. K., Hoekstra, D. and Pagano, R. E., Use of resonance energy-transfer to monitor membrane-fusion. *Biochemistry*, **20**, 4093-4099 (1981).

Sugahara, T., Ohama, Y., Fukuda, A., Hayashi, M., Kawakubo, A. and Kato, K., The cytotoxic effect of Eucheuma serra agglutinin (ESA) on cancer cells and its application to molecular probe for drug delivery system using lipid vesicles. *Cytotechnology*, **36**, 93-99 (2001).

Suzuki, J., Umeda, M., Sims, P. J. and Nagata, S., Calcium-dependent phospholipid scrambling by TMEM16F. *Nature*, **468**, 834-838 (2010).

Thewalt, J. L. and Bloom, M., Phosphatidylcholine: cholesterol phase diagrams. *Biophys. J.*, **63**, 1176-1181 (1992).

Thomas, M., Epidermal growth factor receptor tyrosine kinase inhibitors: application in non-small cell lung cancer. *Cancer Nurs*, **26**, 21S-25S (2003).

Uchiyama, K., Nagayasu, A., Yamagiwa, Y., Nishida, T., Harashima, H. and Kiwada, H., Effects of the size and fluidity of liposomes on their accumulation in tumors - a presumption of their interaction with tumors. *Int. J. Pharm.*, **121**, 195-203 (1995).

van Meer, G., Voelker, D. R. and Feigenson, G. W., Membrane lipids: where they are and how they behave. *Nat. Rev. Mol. Cell Biol.*, **9**, 112-124 (2008).

Wiener, M. C. and White, S. H., Structure of a fluid dioleoylphosphatidylcholine bilayer determined by joint refinement of x-ray and neutron diffraction data. III. Complete structure. *Biophys. J.*, **61**, 434-447 (1992).

Yamada, Y., Akita, H., Kamiya, H., Kogure, K., Yamamoto, T., Shinohara, Y., Yamashita,

K., Kobayashi, H., Kikuchi, H. and Harashima, H., MITO-Porter: A liposome-based carrier system for delivery of macromolecules into mitochondria via membrane fusion. *Biochim. Biophys. Acta*, **1778**, 423-432 (2008).

Zhang, J. A., Xuan, T., Parmar, M., Ma, L., Ugwu, S., Ali, S. and Ahmad, I., Development and characterization of a novel liposome-based formulation of SN-38. *Int. J. Pharm.*, **270**, 93-107 (2004).

List of Publications

[Papers]

1. K. Hayashi, T. Shimanouchi, K. Kato, T. Miyazaki, A. Nakamura, H. Umakoshi, Fluid, Flexible, and "Wet" Surface of Span80 Vesicle, Compared with Phospholipid Liposomes, *Colloid Surf. B*, **87(1)**, 28-35 (2011)

2. K. Hayashi, P. Walde, T. Miyazaki, K. Sakayama, A. Nakamura, K. Kameda, S. Masuda, H. Umakoshi , K. Kato, Active Targeting to Osteosarcoma Cells and Apoptotic Cell Death Induction by the Novel Lectin *Eucheuma serra* Agglutinin (ESA) Isolated from a Marine red Alga, *J. Drug. Delivery*, **2012**, Article ID 842785, 11 pages (2012)

3. K. Hayashi, T. Tatsui, T. Shimanouchi, H. Umakoshi, Enhanced Cytotoxicity for Colon 26 Cells Using Doxorubicin-Loaded Sorbitan Monooleate (Span 80) Vesicles, *Int'l J. Biol. Sci.*, **9(2)**, 142-148 (2013)

4. K. Hayashi, T. Tatsui, T. Shimanouchi, H. Umakoshi, Membrane Interaction between Span80 Vesicles and Liposome: Span80 Vesicles Perturb and Hemifuse with Liposomal Membrane, *Colloid Surf. B*, in press (2013)

[Proceeding]

1. K. Hayashi, T. Shimanouchi, T. Tatsui, K. Kato, T. Miyazaki, H. Umakoshi, Fluid, Flexible, and "Wet" Surface of Span80 Vesicle, Compared with Phospholipid Liposomes, *Proc. 1st Int'l. Symp. on Multiscale Multiphase Process Engineering (MMPE)*, Kanazawa, Japan, October (2011).

2. K. Hayashi, T. Shimanouchi, K. Kato, T. Miyazaki, A. Nakamura, H. Umakoshi, Characterization of Span 80 Vesicles Membrane for Its Application to Novel DDS, *Proc. 9th Int'l. Conf. on Separation Science and Technology (ICSST11)*, Jeju, Korea, November (2011).

Acknowledgement

The author is deeply indebted to Prof. Dr. Hiroshi Umakoshi (Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University), for all his excellent supervision and helpful supports throughout this work. The author is thankful to Prof. Dr. Masahito Taya (Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University), Prof. Dr. Norikazu Nishiyama (Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University), and Associate Prof. Dr. Toshinori Shimanouchi (Graduate School of Environmental Science, Okayama University) for their valuable comments and suggestions during the completion of this thesis.

The author is also indebted to Prof. Dr. Keiichi Kato (Department of Materials Science and Biotechnology, Graduate School of Science and Engineering, Ehime University), Prof. Dr. Peter Walde (Department of Materials, ETH, Zürich, Switzerland) and Associate Prof. Dr. Tatsuhiko Miyazaki (Department of Pathogenomics, Graduate School of Medicine, Ehime University) for numerous suggestions and discussion, and kind supports through this work.

Many thanks to Dr. Kenshi Sakayama (Graduate School of Medicine, Ehime University), Kenji Kameda (Integrated Center for Science Shigenobu Station, Ehime University), Atsushi Nakamura (Graduate School of Medicine, Ehime University), and Seizo Masuda (Integrated Center for Science Shigenobu Station, Ehime University) for their kind cooperation during my research. The author would also like to thank all the professors and all the staff of the Division of Chemical Engineering, Graduate School of Engineering Science in Osaka University for their kindness and helpful cooperation during my research.

The author is also grateful to Prof. Dr. Sosaku Ichikawa (Graduate School of Life and Environmental Science, University of Tsukuba), Assoc. Prof. Dr. Kenichi Morigaki (Research Center for Environmental Genomics, Kobe University), Prof. Dr. Makoto Yoshimoto (Yamaguchi University), Prof. Dr. Seiichi Morita (Wakayama National College of Technology), Prof. Dr. Noriko Yoshimoto (Yamaguchi University), Assoc. Prof. Dr. Koichiro Shiomori (Miyazaki University), Dr. Hiroyuki Sugaya (Toray), Dr. Yasushi Yamada (Kao), and Prof. Dr. Hidemi Nakamura (Nara National College of Technology).

Special thanks are given to the following cooperation for their helps and experimental collaboration: T. Tatsui, N. Maruyama, and M. Kiriishi at Bio-Inspired Chemical Engineering laboratory, R. Teel (Univ. of South Carolina) and Dr. K. Suga, T. Ishigami, S. Tanak, T. Yokoi, K. Sugita, J. Chinzaka, T. Hinoyama, F. Iwasaki, T. Katsura, D. Kondo, Y. Takaya, M. Hirose, T. Bando, and other members at Bio-Inspired Chemical Engineering laboratory. Thanks are due to Ms. Keiko Fukumoto (Secretary at Bio-Inspired Chemical Engineering laboratory) for good effort to keep the research environment.

The author would like to thank his parents Tadanori Hayashi and Michiko Hayashi, and his brother Syota Hayashi for their continuous encouragements and kind supports throughour work.

The author gratefully acknowledges the financial support of this work by the fellowship of the Japan Society for the Promotion of Science for the Japan Junior Scientist and the fellowship of Global COE program “Bio-Environmental Chemistry” and, also, the fellowship of NEXT program “Innovative Biotechnology based on Membranome”.