Studies on Liposome Membrane Design for Selective Adsorption of Amino Acids and Its Application

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Studies on Liposome Membrane Design for Selective Adsorption of Amino Acids and Its Application

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PREFACE

This dissertation work was conducted under the supervision of Professor Hiroshi Umakoshi at Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University from 2009 to 2016.

The objective of this thesis is to establish the methodology to design the liposome membranes for the selective adsorption of amino acids and its application. The selective adsorption of amino acids on liposome membranes and its mechanism are investigated, especially focusing on the surface property of liposome membranes, in order to understand the key factors for efficient molecular recognition.

The author hopes that this research would contribute to the design of the liposome membrane for the application of efficient separation processes. The methodology established in this study is expected to contribute to the understanding of the function induced in self-assembled interfaces and its application.

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Summary

Self-assembly system is known to exhibit the high molecular selectivity with variation of its configuration. Actually, the function of recognition and regulation of biological molecules can be induced by the self-assembled liposome membranes, which form ordered interfaces. In this study, the selective adsorption of amino acids is investigated by liposome membranes in order to establish the design of liposome membranes for the efficient molecular recognition systems.

In chapter 1, the partition behaviors of amino acids are compared among different kinds of systems including phospholipid assemblies. The selective adsorption of L-tryptophan (Trp) is observed on liposome membranes, despite of non-selective Trp partition in emulsion systems, indicating the importance of both highly-ordered membranes and hydrophilic interface for selective adsorption. In addition, the liposome membranes can show the molecular recognition at the surface hydrophilic region. It is thus required to investigate the detail characterization of membranes, and to understand the mechanism of selective adsorption in liposome membranes.

In chapter 2, the liposome membrane property during adsorption is evaluated based on the combination of several analyses. The adsorption of amino acids is assumed to progress in the surface region of liposome membranes together with the variation of membrane property. It is thus suggested that the detailed understanding of the membrane surface property can contribute to design of the liposome membrane for the efficient selective adsorption.

In chapter 3, the surface property and the adsorption behavior of histidine (His) are investigated in the liposomes containing cholesterol. The enhancement of His adsorption is associated with the correlation diagram of two surface properties such as surface polarity and surface fluidity, indicating that the liposomes with high surface hydrophilicity or the domain formation in heterogeneous liposomes can induce the higher efficiency for adsorption. These findings can propose the strategy of the liposome membrane design for efficient molecular recognition.

In chapter 4, the application of liposome membranes is examined by the case studies such as the polycondensation reaction and separation process. The oligomerization of His was enhanced by the adsorption of L-His on liposome membranes. In addition, the immobilized liposome membranes embedding in hydrogels showed the chiral resolution of Trp. These results are expected to contribute to the design strategy of efficient process by using liposome membranes.

Based on the findings in this study, the selective adsorption of amino acids induced by liposome membranes is evaluated by the understanding of adsorption mechanism and of the effect of surface property of liposome membranes, proposing the design of liposome membranes for the induction of efficient molecular recognition function.

Contents

General Introduction

In chemical process, the separation technique is an important unit operation since the purification and concentration of target material is essential for recovering the chemical products. The separation techniques are in general classified based on their principle, such as equilibrium separation and rate-governed separation (**Table 1**). Equilibrium separation is carried out based on the difference of characteristics of a system consisting of two different phases under the equilibrium condition, for example, distillation, liquid-liquid extraction, adsorption in solid phases, gas absorption and supercritical extraction. On the other hand, rate-limiting separation is based on the difference of the flux of the molecular transport that are derived from several driving forces, such as the difference of concentration and temperature and the difference of other kinds of potentials. The membrane separation, electrophoresis and centrifugation are exampled as these kinds of separation. Since the above traditional separation techniques require the certain kind of difference of physicochemical property in the system, it is unsuitable for the highly-selective separation of quite similar constituents, such as stereoisomers. Moreover, since the formation of different phases in a system and their phase transition are essential during the separation in many kinds of methods, a lot of energy are required to be consumed in their traditional separation techniques (enthalpy-driven aspect). To overcome such problems, the use of chromatography or micro-reactor

Table 1 List of separation techniques

	Principles of Separation	**Phase**	**Method**
Equilibrium separation	Different property between two phases	Gas / Liquid	Gas absorption
		Liquid / Liquid	Liquid-liquid extractions
		Liquid / Solid	Adsorption
	Phase transition	From liquid to gas	Distillation
		From liquid to solid	Crystallization
Rate-governed separation	Concentration difference	Gas	Gas permeation
		Liquid	Dialysis
	Pressure difference	Liquid	Ultrafiltration
	Potential difference	Liquid	Electrophoresis
	Centrifugal force	Liquid	Centrifugation

process has been proposed. Although these methods developed the performance of separation by means of the increase of the number of theoretical plates or the introduction of stereospecific structure, the low efficiency of separation was remained as an inevitable problem in terms of the cost.

Chiral isomers are often obtained in the chemical synthesis of the chemicals that have asymmetric carbons in their structure and are formed by identical composition but non-superposable mirror image configurations, which shows reverse optical rotatory (Pasteur et al., 1848). Many kinds of chiral isomers sometimes show the different effects for the living systems, i.e., the side effect of teratogenic was found by only in one side of thalidomide enantiomer. Hence, the separation of one enantiomer from the other is important techniques for fine chemical products. Typical methods of separating chiral molecules are shown in **Table 2**. Although chiral column chromatography is in general utilized as the analytical methods (Kaida et al., 1994; Yang et al., 1993), the cost of equipment in its use is quite high because of the requirement of constructing chiral stationary phase and its maintenance. While the crystallization methods can be carried out in lower cost (Wu et al., 2012; Martín et al., 2007; Takahashi et al., 2002), there are some difficulties in the optimization of the operational condition to obtain pure crystal of one side of enantiomers and to maintain the crystal quality during a long-term process in pharmaceutical industry. In the case of some rate-governed separations through the asymmetrically specific binding with proteins (Ghanem et al., 2004), low efficiency in the recovery yield is still remained. While chiral selective synthesis techniques

Table 2 Chiral separation methods by several chiral selector

Reference	Selector	Method
Kaida et al., 1994	Polysaccharide carbamates	Chromatography
Yang et al., 1993	Human serum albumin	Chromatography
Gumí et al., 2005	*N*-Hexadecyl-L-hydroxyproline	Capillary electrophoresis
Wu et al., 2012	Enantiomeric tartaric acid	Separation of diastereomer salt
Martín et al., 2007	Methylbenzylamine	Salt formation in supercritical carbon dioxide
Takahashi et al., 2002	Crystal of $NBMe_3$	Preferential enrichment
Ghanem et al., 2004	Lipase enzyme	Dynamic kinetic resolution
List et al., 2000	L-Proline catalyst	Synthesis of enantiomer

have been also developed by the design of catalysts (List et al., 2000), the cost of catalyst is still high.

Amino acid is one of the basic biological molecules, which are known as the building block to construct proteins. From the viewpoint as for the physicochemical property, amino acids are known to be regarded as zwitterionic molecules that possess both amine and carboxyl group at the α-carbon, together with other functions called as "side chains" that can be classified as about 20 species (**Fig. 1**). Based on the characteristics of side chains, the tertiary structures are constructed when the amino acids are polymerized to peptides or proteins. In the practical use, amino acid monomers are produced for the nutrient or seasoning. In addition, dipeptides such as carnosine are treated as the drug for recovery from exhaustion. One of the important features of amino acids is forming chiral compounds as L-form and D-form. Among these enantiomers, it is known that there is the difference in their biological function, such as the sense of taste. In biological system, amino acids in only L-form among

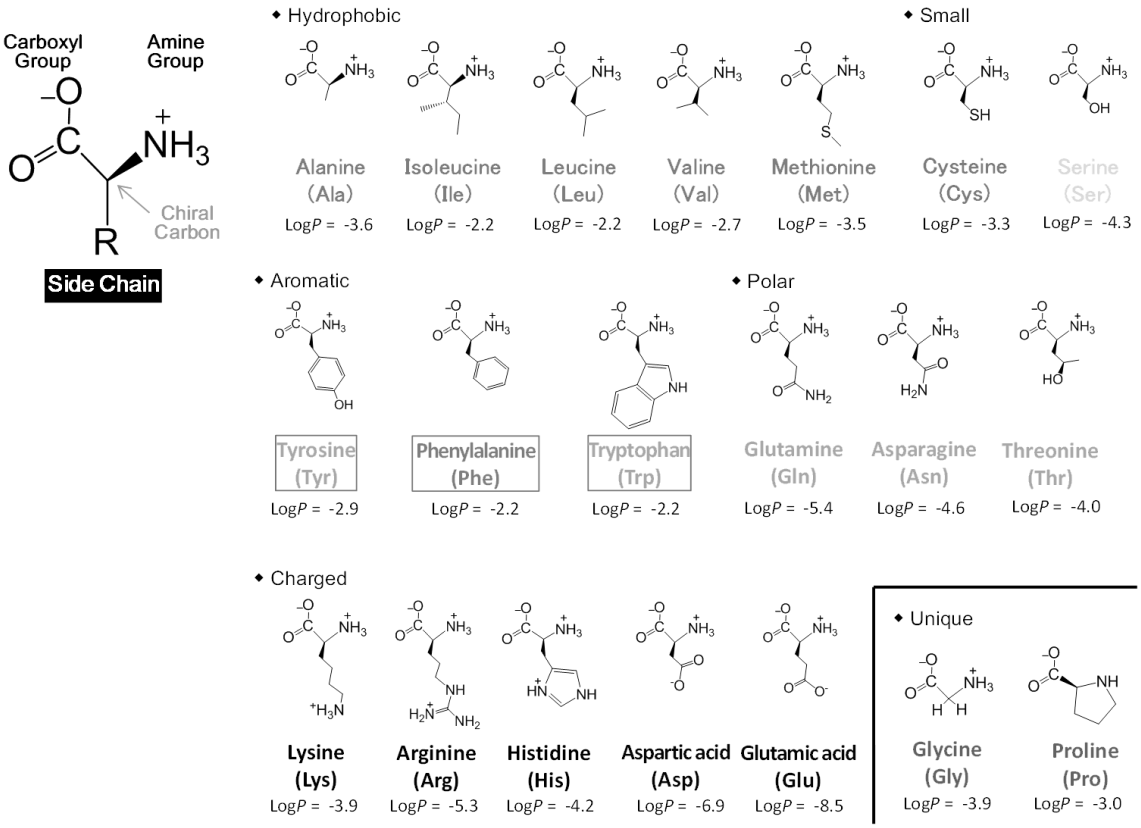


Fig. 1 Chemical structures of amino acids.

their enantiomers are selected as the building block of the biomacromolecules or the key material of their derivatives. Besides, biopolymers such as proteins and DNA also exhibit homochirality. The origin of, reason for, and properties of homochirality have been still attracting many researchers and have been widely investigated (Bada et al., 1995). On the other hand, previous study revealed the asymmetric autocatalysis in the enantiomeric amplification (Kawasaki et al., 2009). Based on these findings, it is also possible that the chiral amplification is induced by utilizing the self-organizing system that consists of biological system.

In contrast to the problems underlying in the conventional separation process, several kinds of biomacromolecules in biological system achieves the efficient and precise recognition. In biological cells, several unit processes including the separation process are regarded to be operated in the small and well-organized compartment at micro to nano-meter scale. Such hypothetical separation process in a biological cell has been carried out through the extremely selective molecular recognition, inducing the regulation of complicated functions. There are many examples of the selective separation in the biological system, such as the control of mass transfer by the specific channels of membrane protein, the signal transduction through the receptor proteins and the enzymatic activity for the specific substrates. In actual, these great functions of biological system are also utilized in the practical applications as the bio-separation techniques (**Table 3**). In these methods, efficient molecular recognition that exceeded against the traditional techniques can be performed by the induction of the functions of biomolecules themselves immobilized in artificial materials

Table 3 Separation or recognition techniques by utilizing biological system

Reference	Biomolecules	Separation
Guo et al., 2006	DNA aptamer	Isolation of mesenchymal stem cells from bone marrow
Voller et al., 1978	Antigens	Enzyme immunoassays (ELISA)
Lee et al., 2002	Antibody	Enantiomeric drug separations
Kohli et al., 2004	DNA-functionalized nanotube membranes	Recognition of single-base mismatch in hybridized DNA
Higuchi et al., 2003	DNA immobilized membrane	Chiral separation of phenylalanine
Lee et al., 2009	Albumin imprinted membrane	Adsorption of albumin from bovine serum
Lundahl et al., 1991	Liposomes immobilized gel beads	Separation for quaternary structure of proteins
Shimanouchi et al., 2010	Liposome immobilized ITO-electrode	Intermembrane interaction of liposomes

(Voller et al., 1978; Lee et al., 2002; Kohli et al., 2004; Higuchi et al., 2003). However, there is a problem in the cost of preparing such systems owing to the difficulty in the maintenance of the structure and function of biomolecules during their use. Therefore, the understanding of the insight about the biological system and its application are expected to be applied to the efficient design of artificial separation system for practical use, that is, "bio-inspired system".

Recently, self-assembled materials have attracted many interests of researchers in various research fields. Depending on the property derived from chemical structure and/or surrounding environment, some kinds of amphiphilic molecules can be automatically assembled to form the supramolecular structures. Amphiphilic molecules, for example, can form the self-assembly by several driving forces, such as electrostatic interactions and van der Waals interactions in hydrophobic regions. Apart from the typical polymers formed by covalent bonds, such self-assembly structures can show dynamic property despite of the increment of entropy, which is regarded as fluctuation in non-equilibrium state. These phenomena are described as the theory of dissipative systems (Prigogine et al., 1967), which contributes to research about the birth of living system. According to the biomolecules, various organisms such as cells is formed by the assemblies, called as "self-organization" system. Microtubule which is one of the organelle is constructed by the self-organization of tubulin proteins, forming the long and rigid fibers. Hence, they are enabled to transport proteins in the cell. As for the other example, the protein of actin can organize to filament structure and form the actomyosin complex with another protein of myosin, inducing the active movement of muscle fiber due to the flexible organization (Geeves et al., 2005). In more microscopic view, proteins or DNA is formed by the conformation of polymers of amino acids or nucleic acids (**Fig. 2**). The appropriate self-organization of such structures induces several functions by regulating the dynamic changes of its conformation. Furthermore, cell membranes, which exist in the boundary of cells, are composed by amphiphilic phospholipids. The dynamic changes of cell membranes such as endocytosis are induced by the rearrangement of phospholipid components of outer or inner membranes (Farge, 1995). These dynamic regulations of assembled states may possibly play important role for the flexible recognition of target materials.

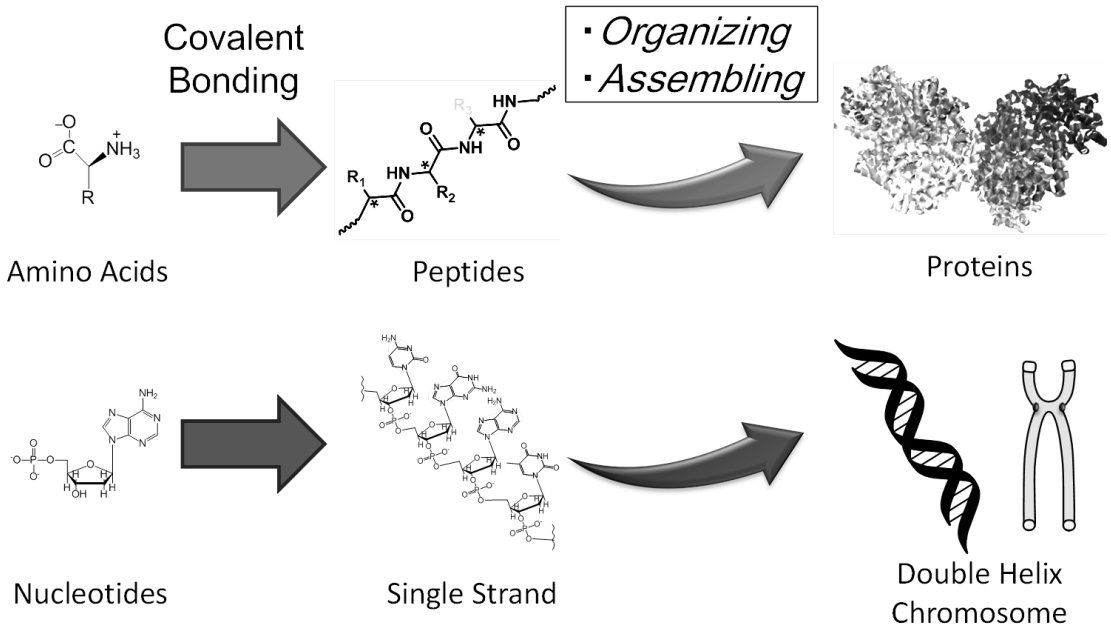


Fig. 2 Self-organization of biological molecules.

In reference to the knowledge of self-organization, the molecular recognition system using self-assembly system has been recently developed for efficient processes, such as separation (**Table 4**). Some studies revealed the high performance in the separation of chiral molecules by means of chiral monomers. Since self-assembly system can easily form the interactions with foreign molecules with "low energy consumption", In addition, it is expected that the formation of highly-ordered structure required for asymmetric recognition is automatically induced in the self-assembly systems (Lee et al., 2002; Mohanty et al., 2005; Kamata et al., 2015). In actual, it has been reported that the amphiphilic monomers possessing L-glutamic acid in their hydrophilic headgroup can form the self-assembled nanotube structures, which act as the platform of asymmetric synthesis (Jin et al., 2011). The micelle-phenylalanine interaction has been applied to cascading ultrafiltration to achieve enantiomer separation (Overdevest et al., 2002). Self-assembled monolayers at the air-water interface have also revealed selective partitioning of enantiomers of the amino acids of valine, leucine, and phenylalanine, depending on the surface pressure of the monolayer (Michinobu et al., 2011).

Phospholipid molecules are known to form several kinds of self-assemblies in aqueous or organic solvents (**Fig. 3**). In particular, liposome membranes are constructed by forming the bilayer lamellar phase of a closed phospholipid assembly and can be regarded as

Table 4 Self-assembly systems formed by artificial monomers

Reference	Monomer and its application
Lee et al., 2002	Bio-nanotube membranes for enantiomeric drug separations
Ziserman et al., 2011	Helical nanotube formation from self-assembly of amphiphiles
Eliseev et al., 1994	Molecular recognition of some biomolecules by aminocyclodextrins
Mohanty et al., 2005	SDLV vesicles as pseudo-stationary phase for enantiomer separation
Liu et al., 2015	Lyotropic liquid crystals for extractant of biomolecules
Jin et al., 2011	Catalytic reaction by nanotube containing bola-amphiphilic amino acid
Kameta et al., 2015	Enantiomer-sensitive vesicle formation by fluorescent glycolipid amphiphiles
Makino et al., 2012	Vesicle formation from poly-L-lactic acid utilized in drug carrier

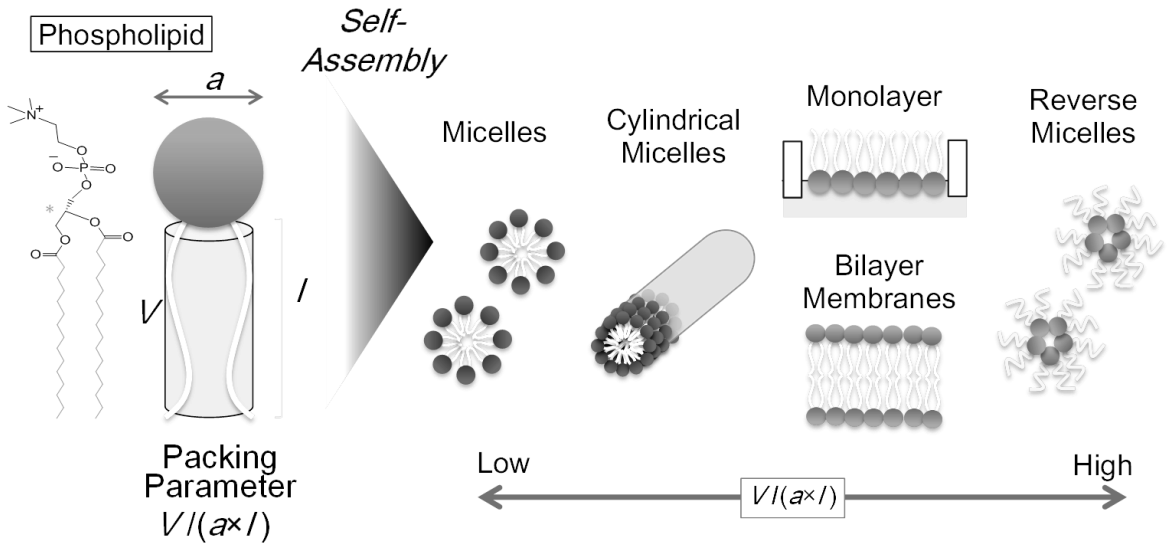


Fig. 3 Self-assembled structures derived from phospholipids.

the model of biomembranes. Moreover, these membranes also have the ordered alignment of phospholipids, inducing the increasing anisotropy of several steroid molecules. This feature is expected to contribute to the formation of stereospecific interactions. Furthermore, it is known that the liposome membranes are characterized by the degree of lateral diffusion as the membrane fluidity. Based on the thermodynamic analyses, the phase transition can be observed in specific temperatures, where the endothermic transition of enthalpy is induced with dynamic changes. Hence, the interaction with guest molecules may induce the change of assembled states of phospholipids in liposome membranes with easily formation. From another point of view, micelles or liposomes in aqueous phase can include the hydrophobic regions, resulting that it can induce the several reactions of hydrophobic substrates even in the

aqueous phase (**Table 5**). In this case, this approach for reactions is useful due to the reduction of solvent and the regulation of reaction rate by the assembled states. In addition, the reverse micelles may also develop the infinitely-connected structure to give the fiber network in some kinds of solvent conditions, resulting in the formation of the organogels containing emulsion phases (Scartazzini et al., 1986). This finding means that phospholipids can form the specific materials that can include the hierarchical assembly in their material structure. The several findings about the phospholipid assemblies indicate the emergence of functions and materials with varying systems dynamically (Walde et al., 2014).

Based on the above findings, the development of liposome membrane systems is important for the efficient separation techniques with high molecular recognition. In the previous investigation, Liposomes have been developed as a functional platform for recognition of biomolecules: proteins and enzymes (Umakoshi et al., 2010), amyloid β fibrils (Shimanouchi et al., 2013), and single-stranded RNA (Suga et al., 2013). These recognition events were significantly affected by the liposomal membrane properties, such as fluidity and polarity. The phospholipid used to make the liposomes is usually consisted of L-enantiomers; thus, the liposomal membranes potentially possessing chiral specificity. It is therefore expected that not only chemical properties of amphiphilic molecules, but also the physicochemical properties of self-assembled membranes are possible clues to develop a novel, flexible recognition site ("Lock") on the membranes. Based on the characterization of physicochemical properties of membranes, an ideal membrane surface for molecular recognition can be designed. Furthermore, in the surface region of liposome membranes, it is possible to form the highly-ordered alignment of charged functions and to induce the domain

Table 5 List of reactions induced in hydrophobic region of self-assembly

Reference	Assembly	Reaction
Manabe et al., 2002	Emulsion droplets formed by DBSA	Several dehydrative conversion
Monti et al., 2002	CTAB and Mn-derived amphiphiles	Epoxidation reactions
Peng et al., 2003	SDS micelles	Aldol reaction catalyzed by L-proline
Mase et al., 2005	Assembly of catalyst with the reactants	Direct asymmetric aldol reactions
Otto et al., 1998	SDS, CTAB micelles or vesicles	Diels–Alder reactions
Li et al., 2012	Micellar assembly of amphiphilic catalysts	Asymmetric reduction of aliphatic ketones

formation by plural components. As for the recognition of RNA in liposome membranes, the domain size is regarded as the key factor of the design of recognition (Suga et al., 2013).

The final purpose of this thesis is to establish the methodology to design the liposome membranes for the selective adsorption of chiral small molecules such as amino acids by utilizing the liposome membranes as the platform of recognition and conversion processes. To design the molecular recognition, it is required to understand the membrane property in several aspects and to investigate the mechanism of bindings between liposome membranes and guest molecules. The framework and flow chart of the present study are schematically shown in **Figs. 4** and **5**, respectively.

In chapter 1, the partitioning and adsorption behavior of amino acids were investigated in two kinds of self-assembled states of phospholipids, *e.g.*, emulsion layer at the water-solvent interface or liposome membranes. In the liquid-liquid extraction system, the partitioning of the tryptophan (Trp) enantiomer in emulsion layer of phospholipids was investigated in different ratio of chloroform/methanol mixture as the solvent. In addition, the partitioning behavior of the Trp in liposome membranes was investigated by adsorption of L- or D-form amino acids dissolved in water phase. Based on the adsorption behaviors of several amino acids possessing hydrophilic or hydrophobic side chains and the adsorption isotherms, chiral recognition function by L-enantiomer of phospholipids was induced by the formation of highly-ordered assembly such as lamellar phase.

In chapter 2, the variation of liposome membrane property was investigated by employing the adsorption of amino acids as targets. Time course of surface hydrophobicity was first evaluated by 8-anilino-1-naphthalenesulfonic acid (ANS) fluorescent probes during the adsorption process of amino acids. This analysis for membrane surface property was also examined by analyzing dielectric dispersion spectra at the higher frequency region. As the evidence of multiple interactions, direct observation of adsorbed Trp or histidine (His) molecules was also carried out by Raman spectroscopic analysis. In addition, thermodynamic analysis for this adsorption was measured by differential scanning calorimetry (DSC), comparing the interaction behavior by the evaluating compensation of enthalpy and entropy. Each measurement mentioned above was conducted in both enantiomers of amino acids in order to discuss the mechanism of chiral recognition of the liposome membranes.

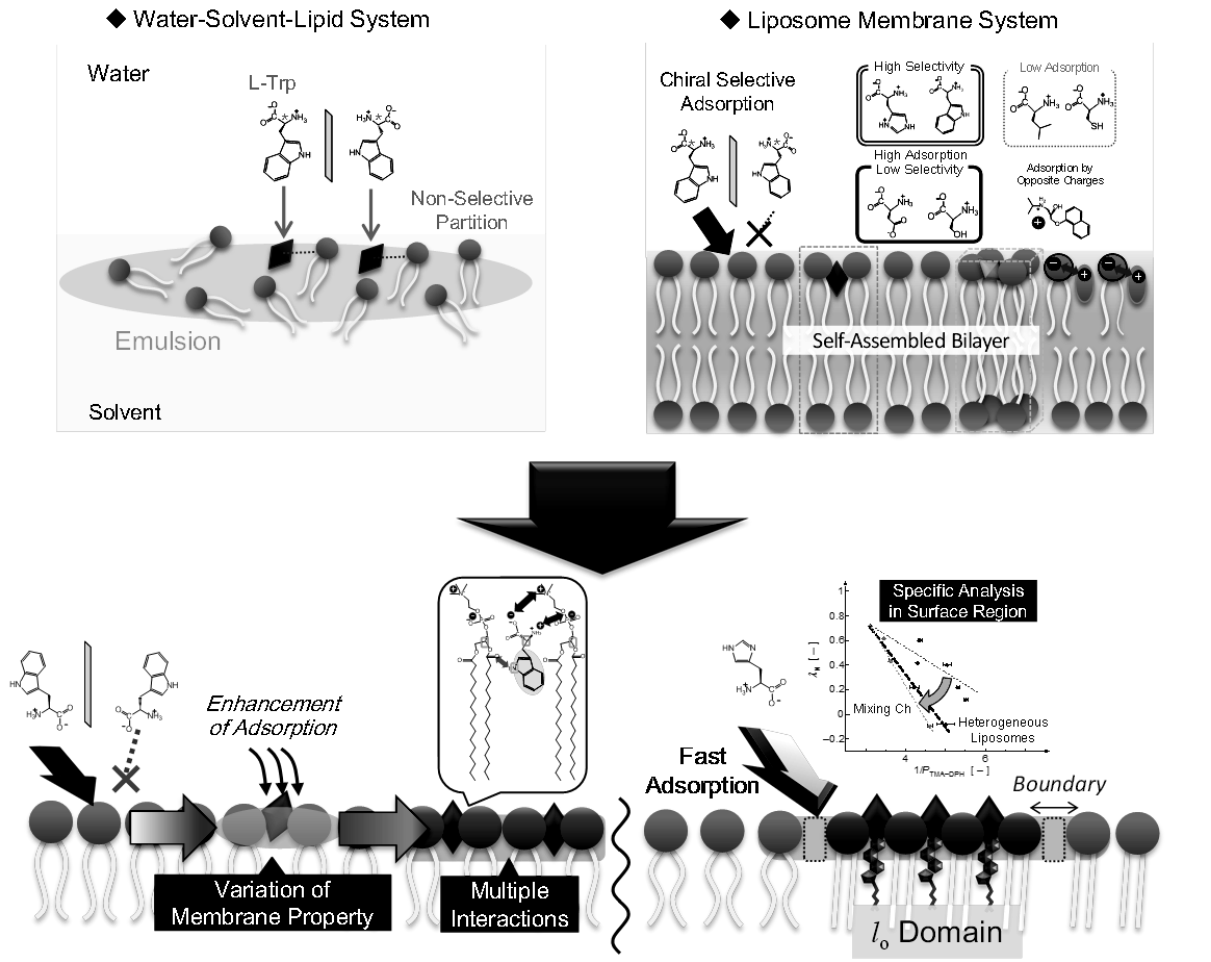


Fig. 4 Framework of the present study.

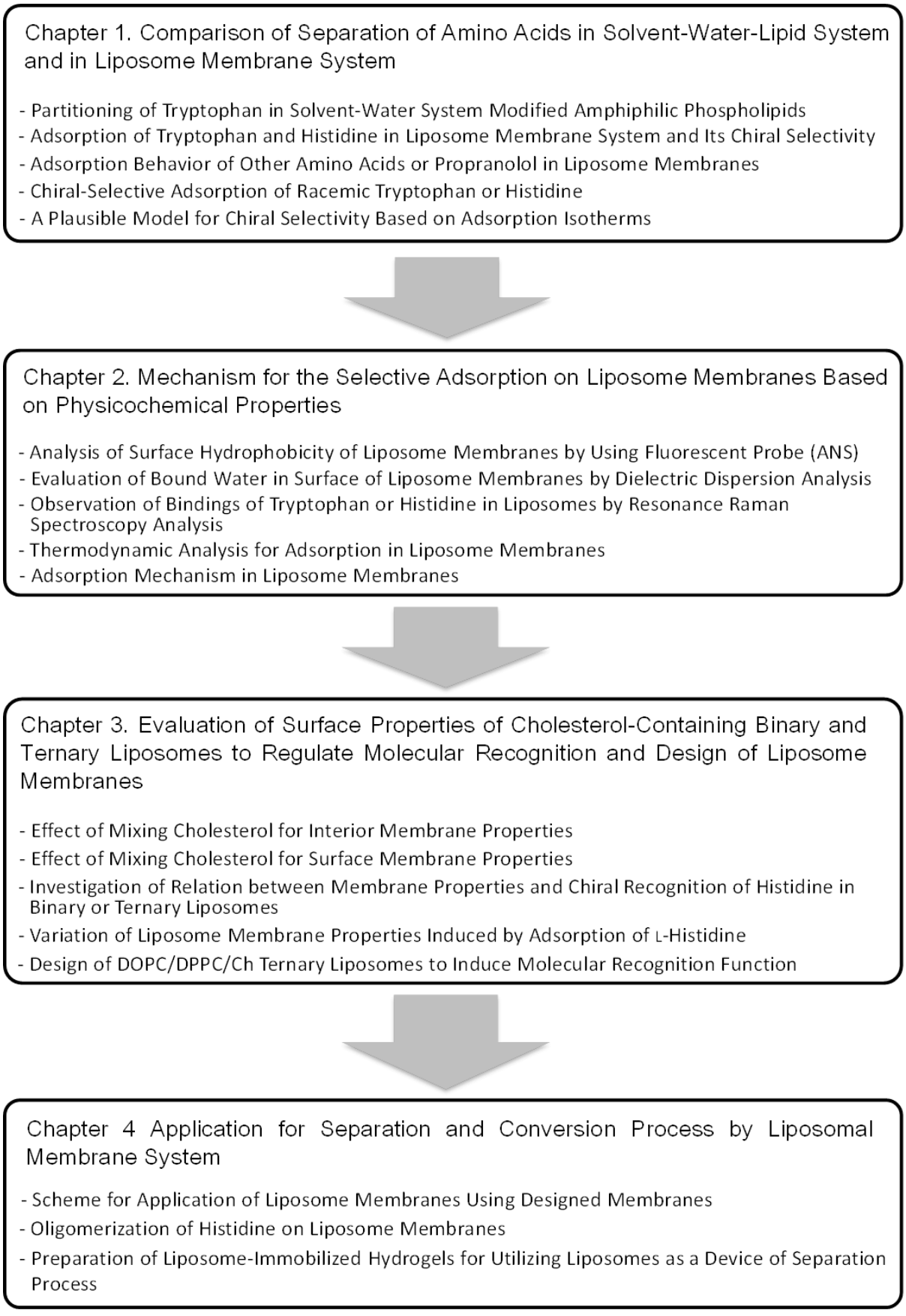


Fig. 5 Flow chart of the present study.

In chapter 3, the effect for chiral recognition of His is investigated by using liposomes of several components contained cholesterol (Ch). At First, the methodology of evaluating the surface property of the liposome membranes is developed by the combination of two fluorescent probes that can be localized at the surface edge of the membrane. By means of the comparison of Cartesian diagram for interior property, it is expected to evaluate the liposome membrane property in detail. According to the relation between such properties and the adsorption of L- or D-form of His, the effect of mixed Ch in liposome membranes is discussed. The transition of membrane property after the adsorption is also investigated to understand the behavior of liposome membranes. Furthermore, the membrane properties of ternary liposomes are observed based on their phase state, and then, the relation with chiral recognition of His are investigated. By comparing the domain states relating to the line tension, the contribution of domain is considered for understanding the effect of existing domain "edges".

In chapter 4, based on the molecular recognition in liposome membranes described in chapters 1, 2, and 3, the application of liposome membrane systems is demonstrated in the conversion reaction and optical resolution. The promotion of oligomerization of amino acids was investigated by partition in liposome membranes with molecular recognition of L-His. In this reaction, the behavior of conversed substrates was considered in reference to previous reports about the aqueous reactions (Kunishima et al., 2005). As for the application for separation process, continuous process is examined by liposomes accumulated in the ultrafilter. Besides, the immobilization of liposomes is developed by the embedding in several hydrogels, which is analyzed by the direct observation and the Raman spectroscopy. Adsorption behavior and chiral separation of Trp are evaluated in prepared liposome immobilized hydrogel (LI-gel).

The results obtained in this work are summarized in the General Conclusions section. Suggestions for Future Works are described as extension of the present thesis.

Chapter 1

Comparison of Separation of Amino Acids in Solvent-Water-Lipid System and in Liposome Membrane System

1. Introduction

Extraction is one of the separation methods based on the partitioning behavior of the molecules among the immiscible two-phase system. The operational conditions of this process have been usually optimized by the following factors: (i) physicochemical property of two solvent system, (ii) characteristics of the target material, and (iii) property of the interface formed in solvent-water two-phase system. Two-phase extraction process has a merit of low energy consumption because of no requirement of phase transition unlike the distillation. On the other hand, experimental knowledge is required in each separation process to optimize the separation condition. Therefore, the synthetic ligands that can specifically interact with target molecules (so called as "extractants") are usually applied in the extraction in order to achieve highly-selective separations (**Fig. 1-1**). Calix[4]arene derivatives, for example, have been used as the extractants of Chromium (VI) with higher selectivity, where the extraction can be controlled by varying pH of the aqueous phase (Ediz et al., 2004). In other cases, the extraction processes of Am (III) in organic phase extraction have been investigated by using the extractant, *N,N,N'N'*-tetraoctyl-3-oxapentanediamide (Panja, et al., 2012). Chiral host molecules, such as hydrophilic-β-cyclodextrin (Wang et al., 2014) and L-tartaric acid derivatives (Ren et al. 2014), have been also used to perform the chiral recognition for pantoprazole and ibuprofens, respectively.

In recent years, there have been some reports on the usage of surfactants as extractant in liquid–liquid extraction of the silica refinary (Kusaka et al., 1998), and in biomass recovery (Pursell et al., 2009). In general, the surfactant molecules are known to be distributed at the liquid-liquid interface due to their amphiphilic nature and, after that, they can enhance the emulsification of one liquid phase into another phase. These behaviors or both partitioning and emulsification have been reported to depend on the structure of surfactant, such as charges,

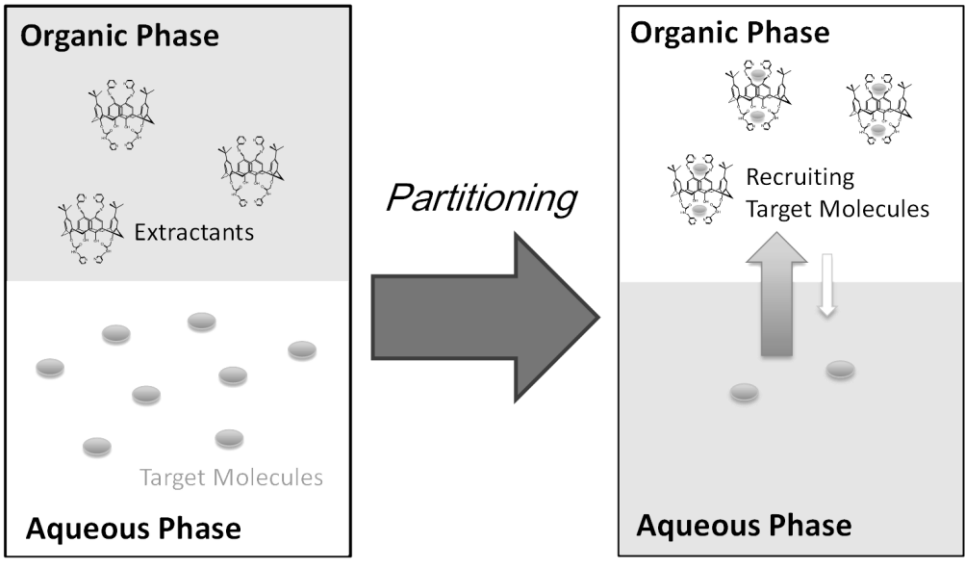


Fig. 1-1 Schematic illustration of extraction using extractants.

hydrophilicity of headgroups and length of acyl chains. In addition, the surfactants and some amphiphilic molecules can form several kinds of self-assembly structures, depending on their geometry and solvent environment. In particular, micelles and lamellar structures are typical forms of amphiphilic surfactant or lipid (**Fig. 1-2**). Owing to the dynamic equilibrium nature (including their deformation under various temperatures or concentrations), the micelles can control the partitioning of micelle-constituting molecules and also guest molecules, resulting in the less stability of the host-guest complex. In contrast to the micelle, the lamellar structure forms relatively stable bimolecular phase at the high-ordered state in horizontal direction. Lamellar membranes can thus orientate the materials such as proteins (White, 1999) and several sterols (Biruss, et al. 2007).

Phospholipid molecules are common amphiphilic molecules in L-enantiomer derived from biological system. Therefore, the lamellar membranes formed by phospholipids can partition several biological molecules in the hydrophobic interior and hydrophilic surface. Besides, the biological molecules can be recomposed in phospholipid membranes by the molecular order. Based on these features, there have been several researches focusing on the up- or down-regulation of function of gene expression machinery (Bui et al., 2008), and the conformational change of RNA (Suga, et al., 2011), peptide (Tuan, et al., 2008) and enzyme (Umakoshi, et al., 2012; Suga, et al., 2015) on the phospholipid membranes. In addition, the enantiospecific interaction of the amino acid dimers has been observed in the phospholipid

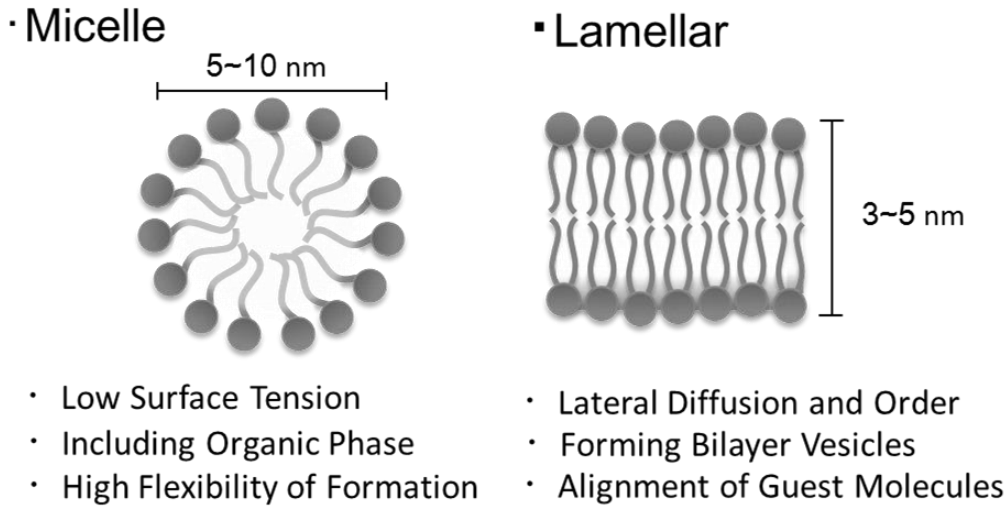


Fig. 1-2 Illustration of micelle and lamellar about specific features.

bilayers. At the phospholipid liposomal or micellar surface, ^{1}H NMR analysis indicated that dipeptide enantiomers at different conformations were interacted with their surface, in which the distance and dihedral angle of Trp-Trp differed between the enantiomers (Cruciani, et al., 2006; Bombelli, et al., 2004). The possible of chiral recognition by the phospholipid membranes are also reported by using some liposomes (Yamada, et al., 2006; uniono et al., 2011; Pathirana et al., 1992). It is expected that the use of the highly-ordered structure of phospholipid membrane could provide us some benefits on the chiral selectivity in the recovery of biomolecules.

In this chapter, the partitioning and adsorption behavior of amino acids were investigated in two kinds of self-assembled states of phospholipids, *e.g.*, emulsion layer at the water-solvent interface or liposome membranes (**Fig. 1-3**). In the liquid-liquid extraction system, the partitioning behavior of the tryptophan (Trp) enantiomer in emulsion layer of phospholipids was investigated in different ratio of chloroform/methanol mixture as the solvent. In addition, the partitioning behavior of the Trp in liposome membranes was investigated by adsorption of L- or D-form amino acids dissolved in water phase. Based on the adsorption behaviors of several amino acids possessing hydrophilic or hydrophobic side chains and the adsorption isotherms, the interaction model in liposome membranes was finally proposed.

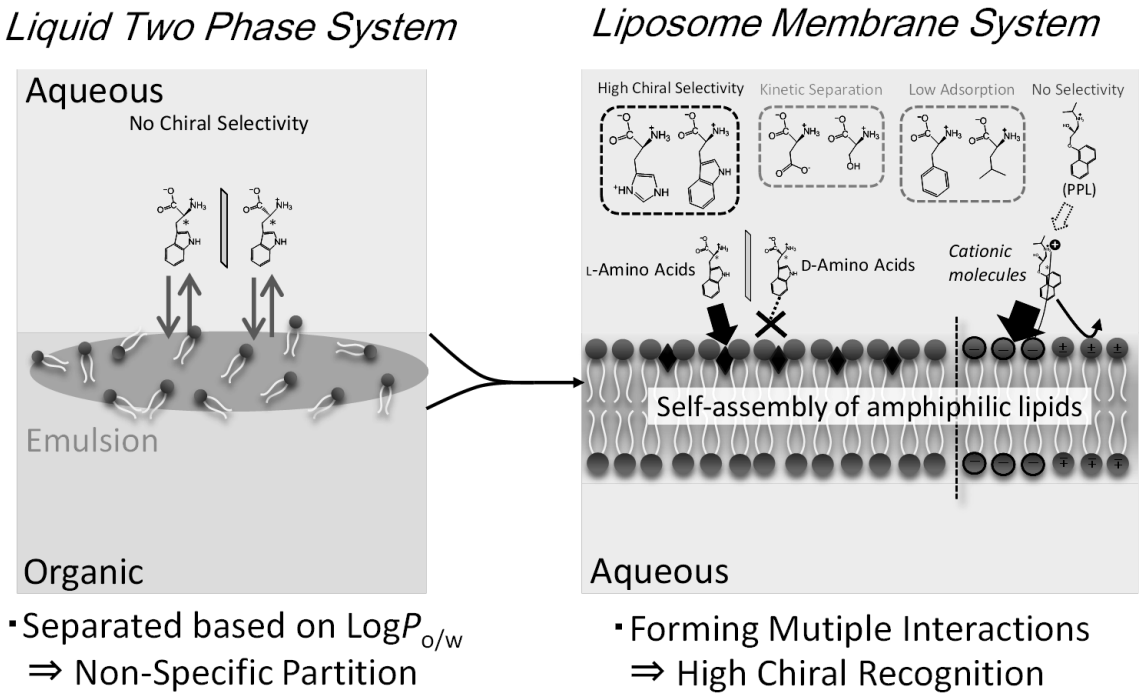


Fig. 1-3 Conceptual illustration of chapter 1.

2. Materials and Methods

2.1 Materials

A zwitterionic phospholipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC; carbon number/unsaturated bond = 16:0), and negatively-charged phospholipids 1,2-dimyristoyl-*sn*-glycero-3-phosphatidic acid (DMPA; 14:0), 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine (DMPS; 14:0) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). L-Trp, D-Trp and other amino acids were purchased from Peptide Institute (Suita, Osaka, Japan). All the amino acids were over 98% purity of enantiomers. Chloroform, methanol and other chemicals were purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan) and used without further purification.

2.2 Liposome Preparation

A solution of phospholipids in chloroform was dried in a round-bottom flask by rotary evaporation under vacuum. The resulting lipid films were dissolved in chloroform and the solvent was evaporated twice. The lipid thin film was kept under high vacuum for at least 3 h, and then hydrated with ultrapure water at room temperature. The vesicle suspension was frozen at -80 °C and then thawed at 50 °C to enhance the transformation of small vesicles into larger multilamellar vesicles (MLVs). This freeze-thaw cycle was repeated five times. MLVs were used to prepare large unilamellar vesicles (LUVs) by extruding the MLV suspension 11 times through two layers of polycarbonate membrane with mean pore diameters of 100 nm using an extruding device (Liposofast; Avestin Inc., Ottawa, Canada).

2.3 Evaluation of Partition Behavior of L-Trp in Solvent-Water System

With regard to solvent-water systems, chloroform and its mixtures with methanol were used as solvent phases. First, DPPC was dissolved in solvent phase in 27 mM of concentration. In the case of chloroform/methanol mixtures, the ratio of methanol (x_{met}) varied from 0.05 up to 0.25. Secondly, L-Trp or D-Trp was dissolved in aqueous phase as 3 mM solution. Then, the distribution ratio (D) of Trp at each enantiomer ($D_{L\text{-Trp}}$ for L-Trp and $D_{D\text{-Trp}}$ for D-Trp) was calculated from the decrease of Trp in aqueous phase by following equation:

$$D = (C_{ini} - C_{eq}) / C_{ini},$$

where C_{ini} and C_{eq} represent the initial and equilibrium concentration of Trp in aqueous phase. The Trp concentration in water phase was measured by UV absorbance of 280 nm, based on their calibration curves.

2.4 Evaluation of Adsorption Behavior of L-Trp in Liposome Membrane System

The liposome suspensions (lipid: 4.5 mM) were mixed with L-Trp or D-Trp, and other amino acids (0.5 mM). They were incubated at 25 ^{o}C for 48 hours, to be equilibrium of adsorption. After the incubation, liposomes and adsorbed amino acids were separated by ultrafiltration membrane with the molecular cut of 50,000 Da (USY-5; Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The concentration of filtered amino acids (C_{flt}) was measured by the absorbance by using UV spectrometer (UV-1800; Shimadzu, Kyoto, Japan), and by the fluorescence of fluorescamine (Ex: 390 nm, Em: 475 nm) (Stein et al., 1973) by spectrofluorometer (FP-8500; JASCO, Tokyo, Japan). The concentration of adsorbed amino acids (C_{ads}) and adsorbed amount of amino acids per lipid amount (q) were calculated by using following equations:

$$C_{ads} = C_{ini} - C_{flt}$$

$$q = C_{ads} / c_{lip} ,$$

where C_{flt} represent the concentration of amino acids in leakage of ultrafiltration and c_{lip} represents the concentration of lipid (liposomes). The adsorption isotherms were evaluated by the plot of q versus C_{flt} in 48 hours incubation (regarded as equilibrium concentration) at the same lipid concentration (4.5 mM). Especially, Langmuir isotherms were described by following equations:

$$q = q_{max} K C_{flt} / (1 + K C_{flt}) ,$$

where q_{max} and K represent the maximum of q and a binding constant. The correlation for Langmuir isotherms and q_{max} and K values were estimated by the plot of C_{flt} versus C_{flt}/q_{max}. From the C_{ads} of L-amino acids and D-amino acids, separation parameter ($S_{L/D}$) was calculated by following equations:

$$S_{L/D} = C_{ads\ (L\ form)} / C_{ads\ (D\ form)} ,$$

where $C_{ads\ (L\ form)}$ and $C_{ads\ (D\ form)}$ represent the C_{ads} values of L-amino acids and that of D-amino acids, respectively.

2.5 Circular Dichroism Spectroscopy Analysis of Racemic Amino Acids

In order to analyze the concentration ratio of L- and D-amino acids, circular dichroism (CD) spectra were measured by JASCO J-820 SFU spectropolarimeter (JASCO, Tokyo, Japan). The CD spectrum from 300 nm to 200 nm was measured with a quartz cell (0.1 cm path length) at a scan speed of 100 nm per minute and a width of 2 nm. Three scans excluding water background signals were obtained at 25 ºC, and the data was calculated as molar ellipticities. In the case of racemic solutions, the initial concentrations of total amino acids were 1.0 mM. The racemic mixtures of L- and D-Trp or L- and D-His were incubated with DPPC liposome (4.5 mM) for 48 hour at 25 ºC, and the liposomes and adsorbed amino acids were removed by filtration shown in above.

2.6 Statistical analysis

Results are expressed as mean ± standard deviation. All experiments were performed at least three times. The distribution of data was analyzed, and statistical differences were evaluated using the Student's t-test. A P-value of <5% was considered significant.

3. Results and Discussion

3.1 Partitioning of Tryptophan in Solvent-Water System Modified Amphiphilic Phospholipids

Although Trp is known as a hydrophobic amino acid due to the hydrophobic side chains (Tanford, 1962), it has a difficulty in its partitioning to organic solvent (**Fig. 1-4(a)**) because of its relatively hydrophilic nature that can be described in the negative value of Log*P* (-2.2). In order to investigate the partitioning of Trp, the effect of additives, such as the amphiphilic phospholipid (DPPC) and methanol, was first evaluated in the water-chloroform extraction system. When chloroform (dielectric constant ε = 5) was used as the organic phase, a thin cloudy layer was formed in chloroform phase on mixing the two phases (**Fig. 1-4(b)**) due to the emulsification mediated by DPPC molecules. This was probably caused by the formation of phospholipid aggregates at the interface on the solvent phase. When methanol (ε = 33) was added to the chloroform phase, the formation of such emulsion phases was enhanced with an increase in the ratio of methanol (**Fig. 1-4(c)**).

The partitioning behaviors of L- and D-Trp in water-DPPC-solvent phase are shown in **Fig. 1-5**. The distribution ratio of Trp, D_{Trp}, was determined by measuring the Trp concentration in water phase, which can be therefore regarded as an indicator of the Trp

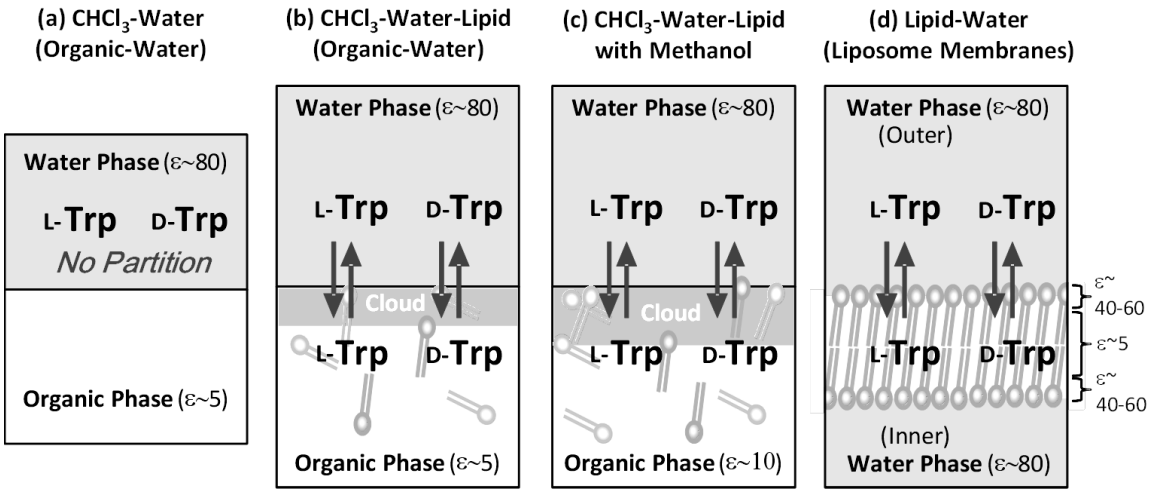


Fig. 1-4 Schematic illustration of the partitioning behavior of L- or D-tryptophan (Trp) in chloroform-water system with phospholipids. (a) Water-chloroform extraction system. (b) Formation of emulsion after mixing as the cloudy layer. (c) Increasing thickness of cloudy layer by addition of methanol. (d) Liposomes prepared by thin film of phospholipids in the aqueous phase.

partitioning in the water/organic two-phase systems (including the emulsion layer). The D_{Trp} value was found to become larger as the increase of methanol ratio with expanding the emulsion phase, while the D_{Trp} value was small (approximately 0.2) at the lower methanol ratio (x_{met} = 0 ~ 0.1). Finally, the D_{Trp} value reached a plateau at a methanol ratio above 0.2. Incidentally, at methanol ratios over 0.25, the emulsion phase disappeared on mixing due to the reduction in the difference in the dielectric constant between the two phases. This behavior indicated that the D_{Trp} value did not always depend on the dielectric constant of the organic solvent because the variation of the ε values was slight in available range of forming emulsion (**Fig. 1-5**). It is thus considered that the enhancement in the formation of the emulsion layer is promoted by the efficient interaction of DPPC and Trp molecules. It is thus suggested that the partitioning behavior of Trp was enhanced through the strong binding between the phospholipid and Trp molecules in the emulsion layer containing lipid aggregation. The chiral selectivity of Trp partitioning was also investigated by determining the ratio of the D_{Trp} value of L-Trp to that of D-Trp ($D_{L\text{-}Trp}/D_{D\text{-}Trp}$). As the x_{met} increased, the $D_{L\text{-}Trp}/D_{D\text{-}Trp}$ value reached to approximately 1 (x_{met} = 0.15), while the $D_{D\text{-}Trp}$ became slightly larger at lower methanol ratio. From the viewpoint of actual recovery of amino acids, the partitioning behaviors were

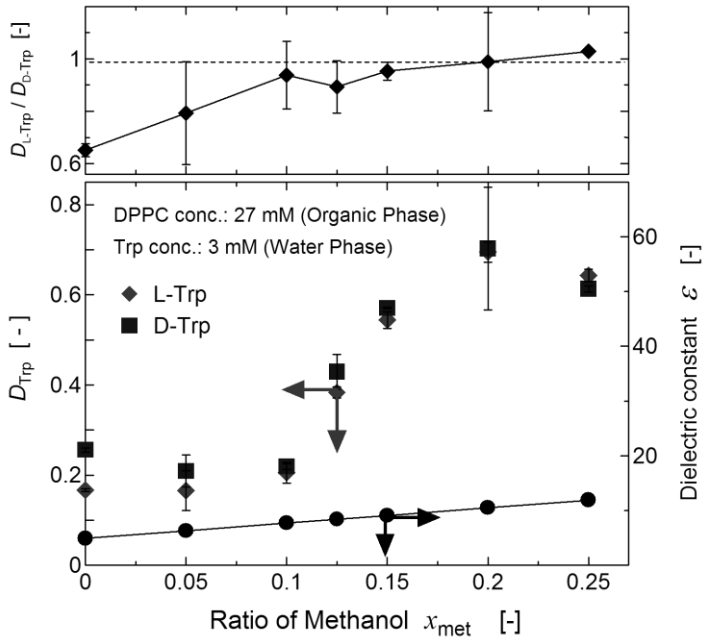


Fig. 1-5 Distribution ratio of L- or D-Trp (D_{Trp}) and its chiral selectivity ($D_{L\text{-}Trp}/D_{D\text{-}Trp}$) in different ratio of methanol in chloroform solvent (x_{met}). Dielectric constant (ε) of solvent were shown as the black keys and line.

thus improved with phospholipids and methanol. The distribution ratio of Trp can be predicted by 0.006 at the interface of water and organic solvent system based on the Log*P* value of Trp (-2.2). On the other hand, the D_{Trp} value increased to approximately 0.2 by the addition of phospholipids that can induce the formation of emulsion phase. Furthermore, the increase of ε in organic phase was also found to induce the increase of the D_{Trp} up to 0.7. It was thus found that the specificity of enantiomers in partitioning Trp was not observed in the liquid-liquid two phase systems with the DPPC extractant.

3.2 Adsorption of Tryptophan and Histidine in Liposome Membrane System and Its Chiral Selectivity

DPPC liposome bilayer membranes have ordered structures owing to molecular alignment and can also be characterized as gradient polarity layers at nano-meter scale as shown in **Fig. 1-4(d)**. As a preliminary experiment, Trp dissolved in the aqueous phase was partitioned to the liposome membranes by mixing them for 48 hours. In this liposome membrane system, the yield of L-Trp recovery, calculated by its partitioning behavior from the aqueous phase to the phospholipid phase, was found to be high, while that of D-Trp was

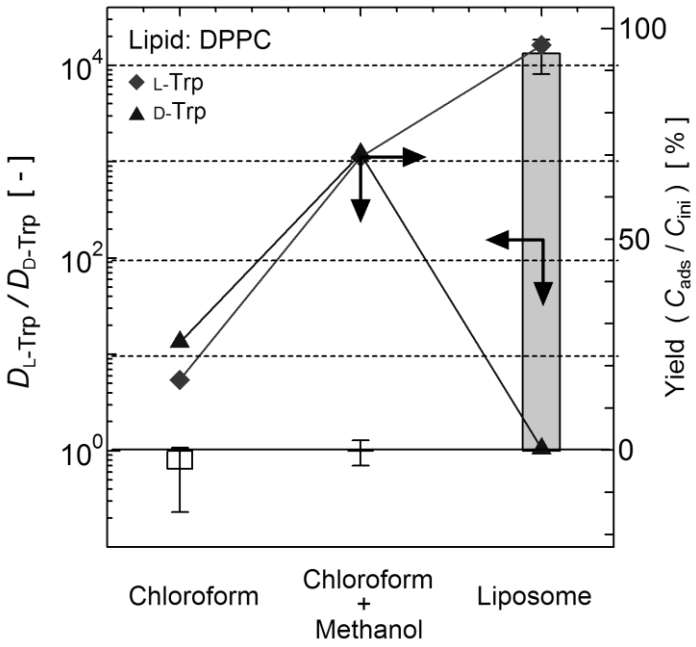


Fig. 1-6 $D_{L\text{-}Trp}/D_{D\text{-}Trp}$ and the yield of Trp (ratio of concentration between in water and in lipid phase) at the solvent of chloroform, chloroform mixed methanol and liposome membranes corresponding to **Fig. 1-4**.

extremely low (**Fig. 1-6**). As for the chiral selectivity, the liposome system showed an extremely high value (~10^4) as compared with other solvent extraction systems. It is thought that the liposome membrane could provide a suitable environment for the partitioning of the hydrophilic Trp, where some interactions (i.e. electrostatic interaction, and hydrogen bond) neighboring to the chiral carbon of the lipid molecules could be related to its chiral recognition. For further investigation to the liposome membranes, the partitioning behavior of Trp and another amino acids (His) were investigated by analyzing the adsorption behaviors from aqueous solution. **Figure 1-7(a)** shows the time course for the adsorption of Trp on DPPC liposomes. The adsorbed amounts of L-Trp gradually increased after 16-hour incubation,

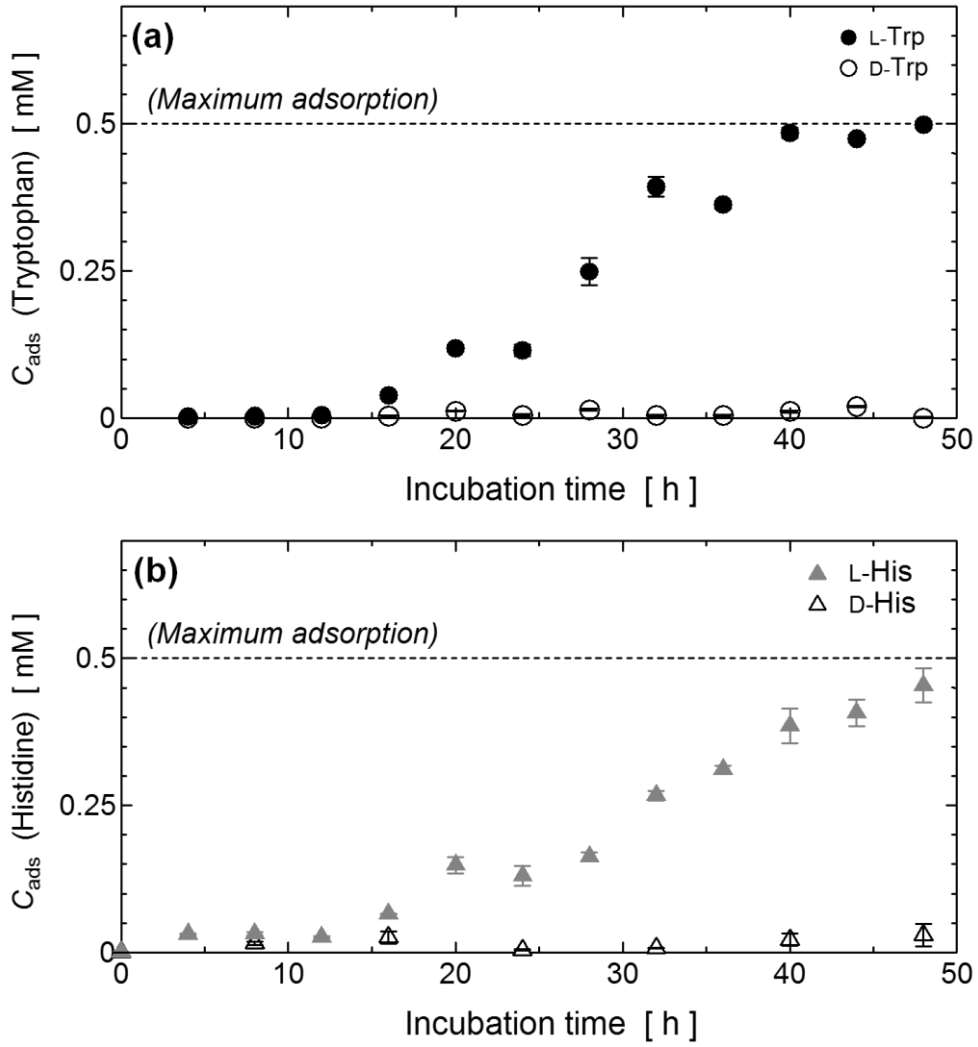


Fig. 1-7 Time course of adsorbed concentration (C_{ads}) of L or D-forms of amino acids. (a) C_{ads} of L-Trp (filled) and D-Trp (open). (b) C_{ads} of L-His (filled) and D-His (open).

and reached to be an equilibrium after 48 hours, where almost all of the amino acids adsorbed on

DPPC liposomes. On the other hand, negligible amounts of D-Trp and D-His adsorbed on the DPPC liposomes even at 48 hours. As a result, extremely high chiral selectivity in the Trp adsorption on the DPPC liposome was observed at the final stage of the adsorption process, while such behaviors were not found in the solvent-water system modified with DPPC (**Fig. 1-6**). As shown in **Fig. 1-7(b)**, similar tendencies for the time course of the adsorption and for the chiral selectivity were observed in the case of His. It was also found that the adsorption kinetics of both L-Trp and L-His were sigmoidal with a lag time (no adsorption from 0 to 16 hours). This result was also imply that the binding of L-amino acids on the liposome structure can be very weak at the initial stage of adsorption, considering the previous findings on the adsorption in supported lipid bilayer (Sarangi, et al., 2012). This is because the amino acids preferably exist in the aqueous phase rather than in liposome membranes, judging from the negative values of Log*P*. It is thought that the membrane property could be varied after the accumulation of amino acids on the membranes at the initial stage: such varied membrane properties could recruit the additional L-Trp partitioned to the membrane at the latter step. In comparison with the adsorption kinetics in L-Trp and L-His, it is assumed that there could be the similar steps to promote L-His adsorption.

3.3 Adsorption Behavior of Other Amino Acids or Propranolol in Liposome Membranes

Natural amino acids possess unique surface characteristics, depending on their side chains, such as hydrophobic and hydrophilic. In order to investigate the effect of side chain property in the chiral selective adsorption on liposomes, the C_{ads} values for 10 kinds of amino acids were analyzed after 48 hours incubation (**Fig. 1-8**). For almost all amino acids (except for Ser), the adsorption of the L-form was dominant, indicating that DPPC liposomes could be widely applied to recognize the chirality of amino acids. The chiral selectivity ($S_{L/D}$) was calculated from the ratio of C_{ads} of L- and D-amino acids. The highest $S_{L/D}$ was obtained with Trp and His ($S_{L/D}$ >1000). Relatively high $S_{L/D}$ was also observed in the case of Tyr and Pro ($S_{L/D}$ >100), owing to the hydrogen donors or acceptors in their side chains, indicating that the formation of hydrogen bonds provided from the aromatic structure could play a crucial role in their high L-amino acid selectivity. In contrast, amino acids possessing hydrophobic (non-polar)

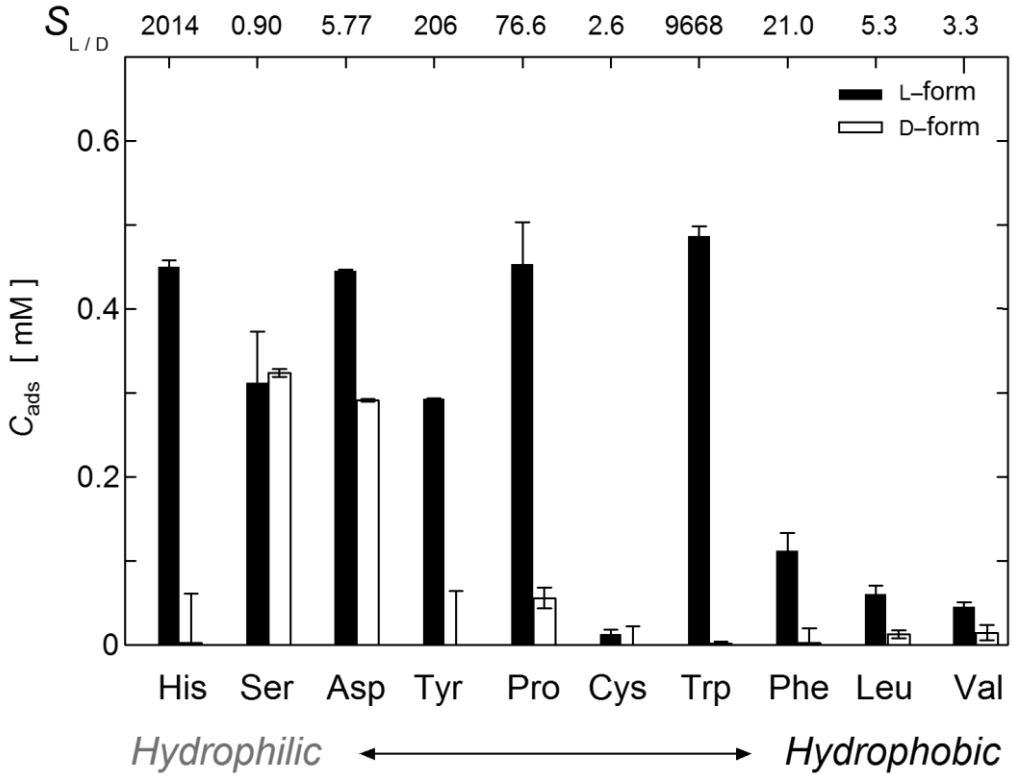


Fig. 1-8 Adsorbed concentration (C_{ads}) of L- or D-forms of 10 amino acids on DPPC liposomes. Amino acids is listed by the hydrophobicity (Tanford, 1962) from left to right. Separation parameter ($S_{L/D}$) of each amino acid is described above the corresponding bars. In all samples, the incubation temperature was 25 ºC.

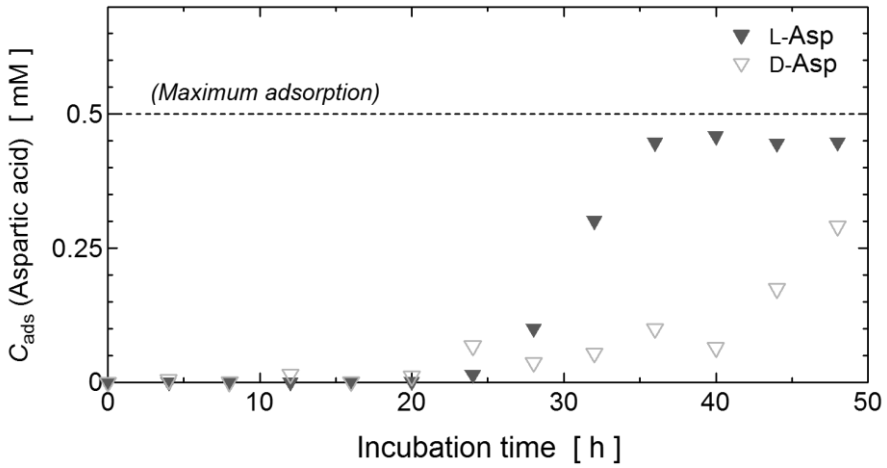


Fig. 1-9 Time course of C_{ads} of L-Asp (filled) and D-Asp (open) in DPPC liposomes.

side chains (Leu and Val) showed lower L-selectivity. Moreover, no L-selectivity was observed for Ser. These results indicate that the hydrophobicity of the side chains might not

be an important factor for inducing asymmetric recognition. The contribution of small side chains is also negligible in stereospecific recognition due to the difficulties in forming certain binding to phospholipid. Interestingly, Asp showed high chiral selectivity in adsorption kinetics at 0-40 hours, although the low $S_{L/D}$ values were obtained after 48 hours (**Fig. 1-9**). It is possibly explained that Asp molecules (even in D-form) easily form the hydrogen bonds with phospholipids due to possessing the polar and small side chains, resulting in decreasing L-selectivity in long time incubation.

3.4 Chiral-Selective Adsorption of Racemic Tryptophan or Histidine

In many case of chiral separation methods, enantiomer excess (ee) from racemic state is evaluated as the efficiency of chiral selectivity. As mentioned above, the chiral recognitive adsorption was observed only in enantio-pure solution, thus, the performance of chiral recognition of liposome membranes was evaluated by studying the adsorption of racemic Trp or His in CD spectroscopic analysis.

In the CD spectra of aqueous solution of Trp or His, the peak intensities at 222 nm (Trp) and 213 nm (His) corresponded to the ratio of L- and D-amino acids at the same total concentration of amino acids (**Fig. 1-10**). From this result, the molar ratio of L-amino acids against D-form (X_L) can be calculated. **Figure 1-11** shows the CD spectra of racemic Trp and His solutions, before and after the treatment of ultrafiltration. The deracemization of both Trp and His solutions were observed in the ultrafiltrated solution, which indicates the removal of L-amino acids from the solution due to the binding of L-Trp and L-His to DPPC liposomes. The molar fractions of L-amino acids (X_L) for Trp and His were 0.140 and 0.306, respectively. In the previous reports, a monolayer of cholesterol-armed cyclen Na^+ and a cellulose acetate polymer imprinted with enantiomer glutamic acids resulted in the enantiomeric ratios of 0.36 (for Trp) and 0.13 (for His), respectively (Michinobu, et al., 2011; Sueyoshi, et al. 2010). Although it is difficult to directly compare these values with our results, our results show a higher chiral selectivity for L-Trp and L-His. The above results indicate the preferential adsorption of L-Trp and L-His on DPPC liposomes in racemic solutions; however, the adsorbed amounts were not as large as with the enantio-pure (L-form) solutions (**Fig. 1-7**). It is hypothesized that the racemic amino acid solutions resulted in the inhibition of the adsorption

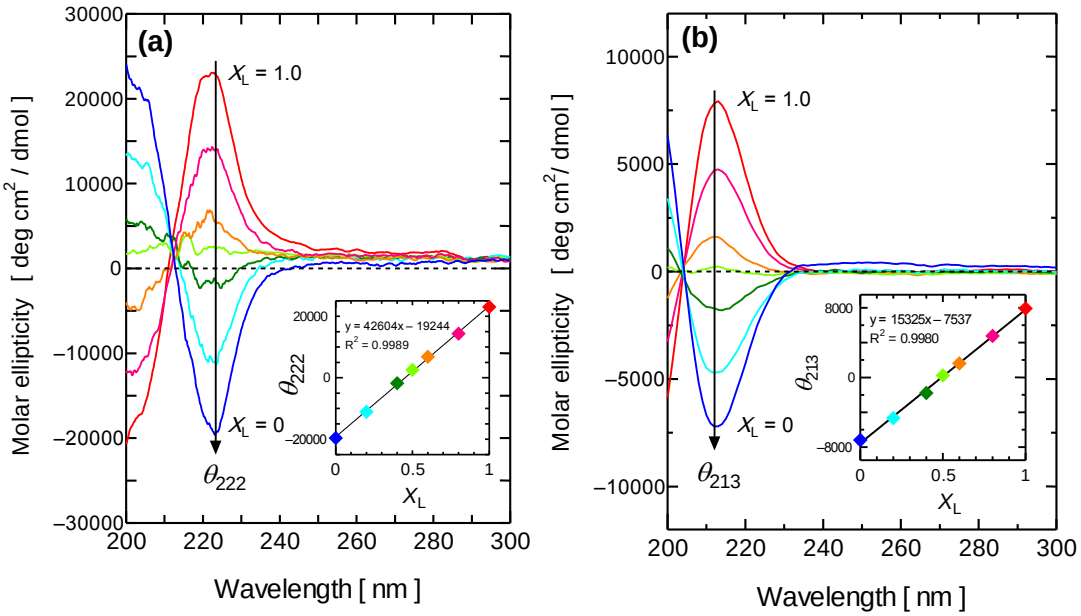


Fig. 1-10 (a) CD spectra of Trp solutions including L- and D-forms at different ratios (X_L = 1.0 - 0). Inset shows the plot of X_L versus θ_{222}. (b) CD spectra of His solutions including L and D forms at different ratios (X_L = 1.0 - 0). Inset shows the plot of X_L versus θ_{213}. The total concentrations of amino acids are 1.0 mM.

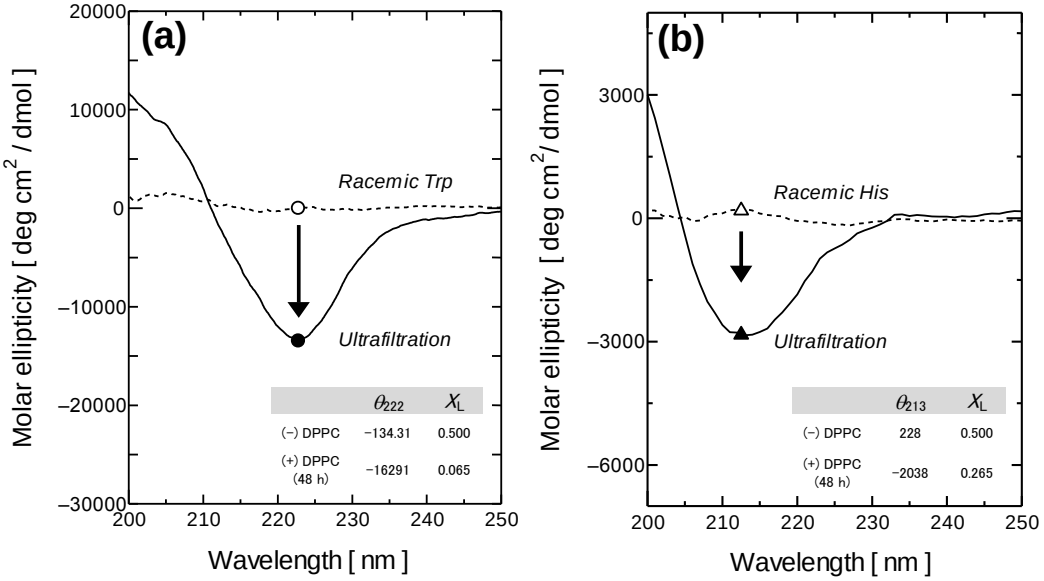


Fig. 1-11 CD spectroscopic analysis of racemic amino acid solutions before or after adsorption in DPPC liposomes. (a) CD spectra of initial racemic (dotted line) and filtered (solid line) solutions of Trp. The inset shows the X_L calculated by θ_{222}. (b) CD spectra of initial racemic (dotted line) and filtered (solid line) solutions of His. The inset shows the X_L calculated by θ_{213}. In all samples, the incubation time and temperature were 48 h and 25 ºC, respectively.

of L-forms. Although D-form Trp and His showed little adsorptions on DPPC liposomes, there still exists a possibility that D-form amino acids weakly interact with DPPC membranes, resulting in the occupancy of the amino acid binding site of L-forms. In the case of the enatio-pure samples, the interactive moieties (carboxyl group, amine group, side chain) in L-form amino acid molecules could fully interact with DPPC liposome, while those in D-forms did partially, and then, resulted in the enantioselective adsorption.

3.5 A Plausible Model for Chiral Selectivity Based on Adsorption Isotherms

The analysis of adsorption isotherms is known to be effective to investigate the adsorption type for several adsorbent. It could be analyzed by investigating the adsorption behavior in various initial concentrations of adsorbent and target molecules. In accordance with the profile of fitting curves, the adsorption type, such as Henry, Langmuir, Freundlich type, and so on, can be assessed. The adsorptive behaviors of L-amino acids on liposomal membranes can be, in general, regarded as those of guest molecules onto host materials (e.g., molecular imprinted membrane), according to the previous report for the molecular imprinted membrane (Yoshikawa et al., 2003).

To estimate the adsorption type of Trp and His, the adsorbed amount (q) versus the equilibrium concentration of amino acids (C_{flt}) was plotted at against the different molar ratios of amino acids and DPPC (**Fig. 1-12**). A linear correlation between equilibrium and adsorbed concentrations, as revealed by the Langmuir plot (**Fig. 1-13)**, suggesting that the adsorption isotherms of L-Trp and L-His with DPPC liposomes are Langmuir type. It has been reported that the adsorption of L-arginine on 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) or 1,2-dimyristoyl-*sn*-glycero-3-phosphoethanolamine (DMPE) liposomes was also correlated with Langmuir isotherms (Bouchet et al., 2010). The other report also revealed that the adsorption of propranolol (PPL) could be correlated with Langmuir isotherms in the case of the negatively-charged liposomes (Kubo et al., 1986). In the case of D-amino acids, reliable correlations were not obtained for C_{flt} vs. q, possibly due to their negligible adsorption on DPPC. According to general theory, a monolayer adsorption to the surface site is known as Langmuir model. These results therefore suggest that the membrane structure of DPPC liposome forms a "uniform adsorption site" for L-Trp and L-His molecules due to the rearrangement

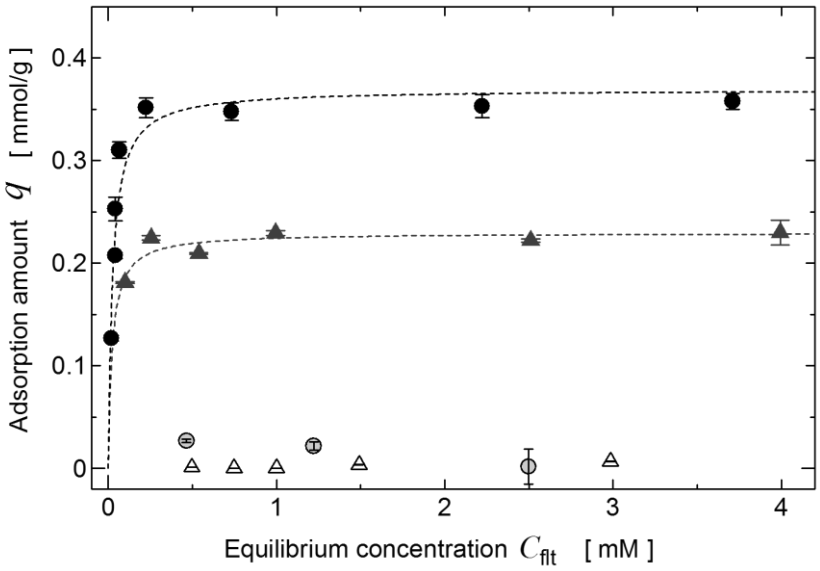


Fig. 1-12 Evaluation of adsorption isotherms of L-Trp (filled circles), L-His (filled triangles), D-Trp (open circles), D-His (open triangles) on DPPC liposomes (4.5 mM). The fitting curve of Langmuir isotherms are shown by dotted line.

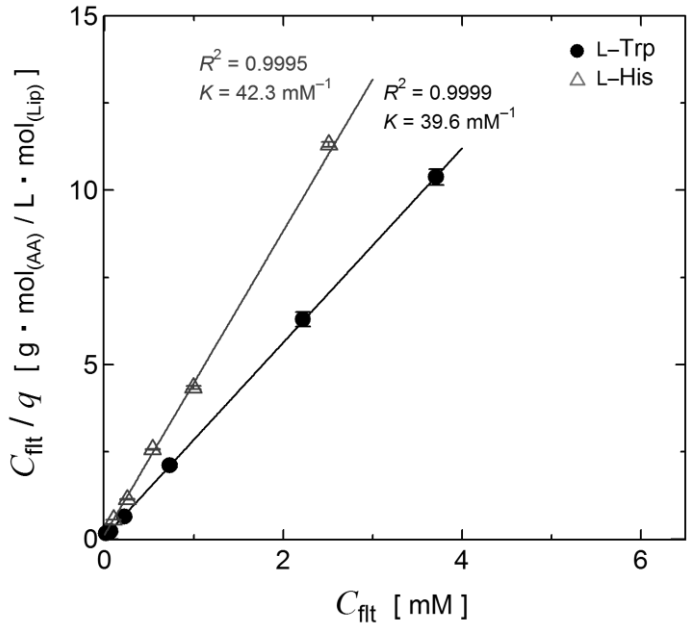


Fig. 1-13 Langmuir plot of L-Trp (Circles) and L-His (Triangles) on DPPC liposomes. Linear relationships between C_{flt} and C_{flt}/q were obtained both for L-Trp (R^2 = 0.9999) and for L-His (R^2 = 0.9995).

of phospholipid molecules, to decrease the binding free energy to achieve a local minimum.

From the fitting of Langmuir equation, the binding constants for L-Trp and L-His to DPPC liposomes were calculated as 39.6 and 42.3 mM^{-1}, respectively. These values suggest a slightly strong binding of L-Trp to DPPC liposomes compared with the case of L-His. The ratio of DPPC per adsorbed L-Trp and L-His was about 3.9 and 8.8, respectively, indicating that the chiral recognition sites were composed of multiple DPPC molecules. In the other case of PPL adsorption in negatively-charged liposomes, those values became approximately 1, indicating that the strong binding between opposite charged molecules provided one-to-one interaction, resulting in non-chiral selectivity due to the lack of stereospecific formation of interactions by plural phospholipids. The plausible interaction model is shown in **Fig. 1-14**. It was therefore investigated whether the self-assembled membrane structure of the DPPC liposomes showed a higher chiral recognition for L-Trp and L-His.

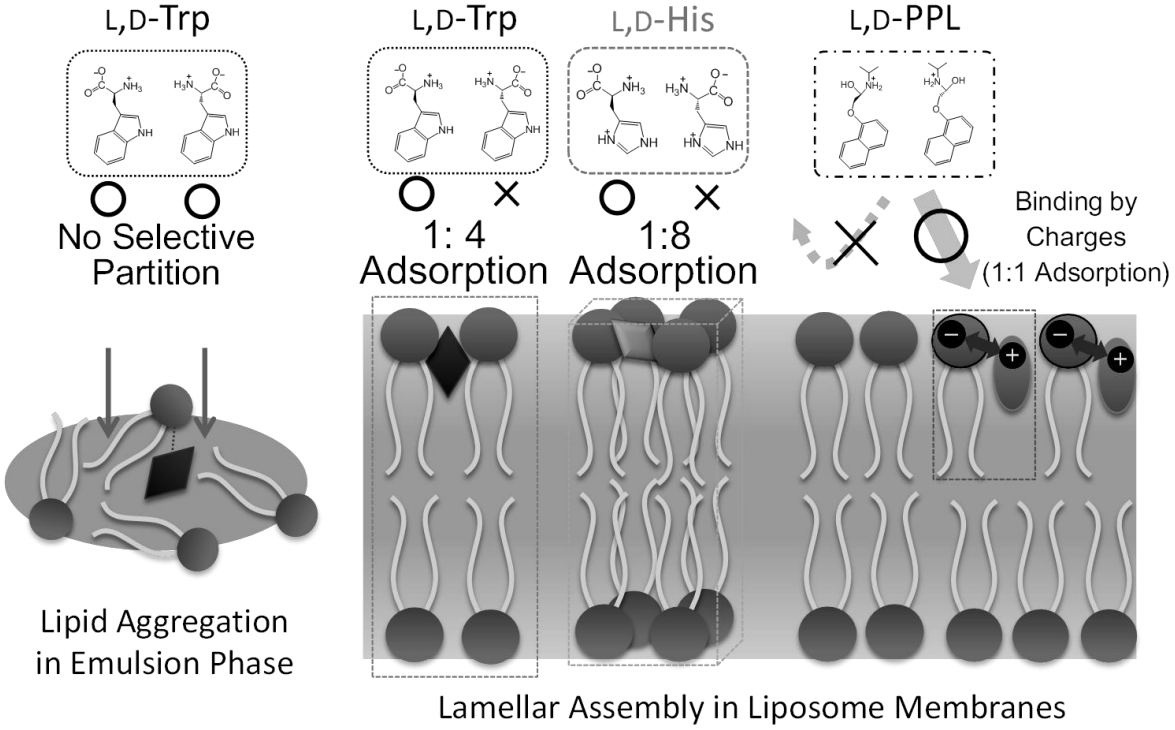
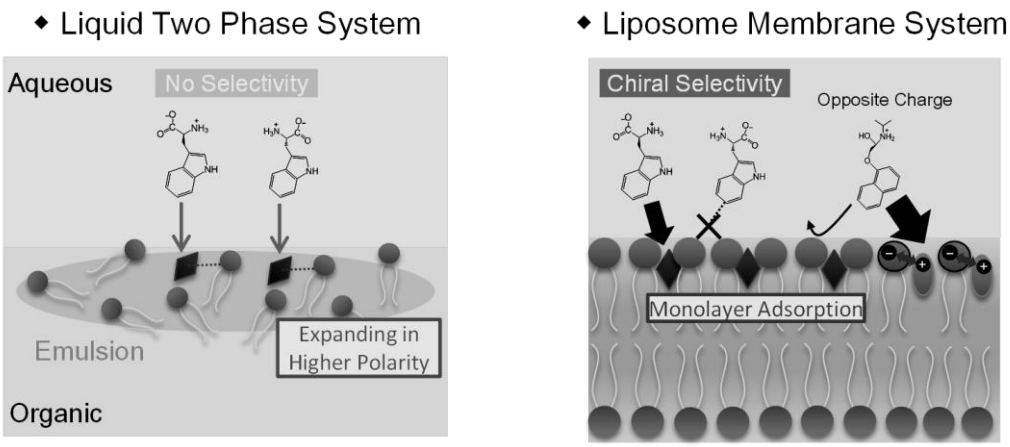


Fig. 1-14 The plausible adsorption model of each molecule on phospholipid assemblies.

4. Summary

The assembly structures formed by phospholipids were shown to play an important role to promote their interaction with target molecules. In the liquid-liquid interface, the emulsion phase formed by phospholipids became expanded by the decrease of dielectric constant of the solvent, resulting in the increase of the partitioning of Trp from aqueous phase to organic phase. Because amphiphilic molecules (e.g. phospholipids) are known to decrease the interfacial tension, the formation of emulsion layers was promoted by the lipid aggregation in the organic phase. In the case of Trp partitioning, it is considered that electrostatic interactions were induced between phospholipids and Trp molecules owing to their zwitterionic group. Although it is indicated that the certain degree of phospholipids can be useful as the extractant to improve the recovery yield, the chiral selectivity lies in low level because of the formation of "disordered" aggregation. On the other hand, highly-selective recognition of L-Trp and L-His was observed in their partitioning in liposome membranes by using the same phospholipid DPPC. In comparison with above results, it was found that the chiral recognition function by L-enantiomer of phospholipids was induced by the formation of highly-ordered assembly bilayer membrane in gel phase. The comparison of partition behavior in solvent-water system and liposomes membranes system are shown in **Fig. 1-15**. This chiral recognition of liposomes was also induced in racemic amino acid solution.

Although the chiral selective adsorption in liposome membranes was also observed in other amino acids in part, its efficiency depended on the chemical structure of side chain. Amino acids possessing polar side chain showed high chiral selectivity, wherein those with hydrophobic side chains or with low-molecular weight side chains remained lower adsorption amounts and lower chiral selectivity. From these results, it is considered that the hydrogen bonds were very important to form chiral selective adsorption rather than electrostatic interactions, which strongly affected on the direction and the distance. This assumption could be regarded as one of the factors to increase the adsorption of L-amino acids, although it requires very long time as compared with general adsorption strategies. From other viewpoints, it was shown that the adsorption of opposite-charged systems, such a negatively-charged liposome and a positively-charged PPL molecule while no adsorption of PPL occurred in the zwitterionic liposomes. Because amino acids and PPL have hydrophilic moieties, their bindings were possibly carried out at the surface region around the headgroup



	Assembly	Interaction	Selectivity
Solvent-Water-Lipid System	Emulsion phase	Partition	· Inducing partition of hydrophilic molecule · No chiral selectivity
Liposome Membrane System	Lamellar phase	Adsorption	· Separating amino acids · Selective adsorption of L-amino acids · Specific adsorption of charged molecules

Fig. 1-15 The overview in the comparison of the molecular recognition in phospholipid assemblies.

of phospholipids. As for the molar ratio adsorbent against phospholipid, L-Trp and L-His were calculated as over 4, while that of PPL was approximately 1, speculating the interactions with plural phospholipids. From these results, it is suggested that chiral molecules is adsorbed to the liposome surface which forms the binding site regarded as the "lock-and-key" model.

Based on the investigation in this chapter, it is obvious that detail analysis for the physicochemical properties of the membrane is required for chiral recognition function in order to understand the mechanism. The variations in the membrane properties during amino acid adsorption were investigated in the following chapter (chapter 2). Besides, because the adsorption by using liposomes required very long time, this methodology might be unsuitable for the practical application for industrial processes. To overcome such disadvantages, the design of lipid composition of liposomes is an important strategy to improve the adsorption behavior as well as the selection of target molecules. Design of the liposome membranes for more effective chiral recognition, and the development of analyzed method is described in chapter 3.

Chapter 2

Mechanism for the Selective Adsorption on Liposome Membranes Based on Physicochemical Properties

1. Introduction

It is important to analyze the interaction of the molecules for the deeper understanding of its mechanism. There have been several kinds of previous reports on the evaluation of inter-molecular interactions (**Table 2-1**). Fluorescent probe molecules are generally used for the evaluation of the specific interaction or environmental property based on the degree of Stokes shift. The fluorescent prove method has been usually applied not only the detection of calcium ions or reactive oxygen species (Minta et al., 1989; Umezawa et al., 1999), but also characterization of lipid membranes, such as polarity (Parasassi et al., 1991), protonation degree (Zuidam et al., 1997) and membrane fluidity (Lentz, 1989). From another aspect, fluorescence resonance energy transfer has been also developed for the investigation of membrane fusion or peptide localization by energy transfer among the two fluorescent probes that can be closely interacted (Düzgünes et al., 1987; Persson, et al., 2004).

Table 2-1 List of analyzing method for characterization of liposome membranes

Method	Objective	Features
Fluorescent probe molecules	Membrane hydrophobicity/polarity	Easily measurement
		Indirect information of liposomes
Dynamic light scattering (DLS)	Size of vesicles	Good detection for aggregation process
		Limited information about shape
NMR spectroscopy	State of each molecule in liposomes	Detailed analysis per molecules
		Different detection in fluid membranes
IR/Raman spectroscopy	State of each functional group	Direct analysis for liposome membranes
		Many interferences from bulk solution
Dielectric dispersion analysis	Dipole moment of components	Good detection of dynamics of headgroup
		Very weak signals
Differential scanning calorimetry	Internal energy in phase transition	Giving the thermodynamic information
		Coarse information about lipid membrane

Although the fluorescent probes can be employed in the characterization of liposome membranes because of its comparative ease, the obtained results are not derived from the membrane itself and can provide us only indirect information. Thus, the combination of different kinds of the analyses can be important for interpretation of information of the properties of liposome membranes.

Infrared spectroscopy (IR) and Raman spectroscopy analysis are useful tools for the direct detection of the liposome membrane properties. IR and Raman spectra are gained by the analyses of penetrating light and scattering light, respectively. In the IR spectra, the energy absorbance derived from each molecular vibration mode is observed as the peaks, which can be applied for evaluation of the interactions as each functional group. However, it is difficult to measure weak signals in a dispersion sample such as liposome suspension because of the obstruction from the bending vibration of water molecules. On the other hand, Raman spectra are obtained by observing difference of wavelength between incident and scattering light. Raman spectra are complement with IR spectra, and there have been advantages in the measurement of liposome suspension using confocal Raman spectroscopy. Recently, the enhanced Raman spectra induced by surface plasmon of nanoparticles was developed for the detection of liposome membranes to overcome the disadvantage about feeble scattering light (Suga et al., 2015). Incidentally, some electrochemical approaches are also available to analyze the characteristics of liposome membranes, such as surface charge and hydrophilicity. Zeta potential of liposomes provides the information about the surface charge and, furthermore, dielectric dispersion analysis (DDA) is also effective methodology of measuring the liposome membrane properties. In this measurement, assigned frequency becomes higher from center to surface of liposome membranes because of hydrophilicity. Actually, the rotation of headgroup or acyl chain moieties can be evaluated by dielectric dispersion spectra (Shimanouchi et al., 2014; Hayashi et al., 2013).

By using the methods recommended above, the interactions of phospholipid membranes with foreign molecules have been evaluated. In the case of hydrophobic molecules such as cholesterol, the alignment in lipid assembly and the accumulation in hydrophobic core can be observed by the measurements of membrane polarity and fluidity evaluated by fluorescent probes (M'Baye et al., 2008; Suga et al., 2013). On the contrary, hydrophilic molecules with smaller molecular weight or hydrophilic polymers are interacting

with the headgroup regions of liposome membranes. Arginine, which is one of the amino acids, is adsorbed on the surface of some zwitterionic liposomes as well as negatively-charged ones (Bouchet et al., 2010). This adsorption induced the increase of membrane polarity and fluidity, indicating the effect in inner region of liposomes. From another viewpoint, the phospholipid monolayer membranes formed by PE phospholipids have reported to adsorb the tartaric acids at the headgroup region of the membrane (Petelska et al., 2002). Furthermore, adsorption of poly-L-lysine on anionic liposomes induces the marked changes of the phase transition temperature, depending on the polymerization degree of poly-L-lysine (Schwieger et al., 2007). These results were considered to be caused by the effect that these polymers could interact only in surface charged region small molecular weight and, in the case of larger polymers, the insertion into interior of membranes occurs by means of secondary structure of α-helix.

Furthermore, thermodynamic properties of the liposome membranes are helpful to evaluate the adsorption of guest molecules on the liposome membrane. Differential scanning calorimetry (DSC) can evaluate the variation of internal energy by measuring the change of temperature with heating constantly. In measurement of liposome membranes, a dramatic response appears around the phase transition temperature, which provides the thermodynamic effect due to forming interactions. In addition, isothermal titration calorimetry (ITC) can also evaluate the thermodynamic behavior of molecular adsorption such as difference of enthalpy and entropy, which is used for assignment of ligand of ciguatoxin fragment from thermodynamic aspects (Ui et al., 2008). Furthermore, the relation between enthalpy and entropy is also important for understanding the several host-guest interactions. In general, the change of enthalpy shows compensation to that of entropy, enabled to assume the degree of conformational changes and the dehydration from the slopes and the intercept of regression line (Rekharsky et al., 2007). As for the assembly of phospholipids, these relations are considered similar in the case of micellar formation (Sugihara et al., 1999), implying that it could be a good way of investigating the interactions of liposomes and amino acids.

In this chapter, the variation of liposome membrane property was investigated by employing the enantioselective adsorption of amino acids as case study. Time course of the surface hydrophobicity was first evaluated by using ANS fluorescent probes during the

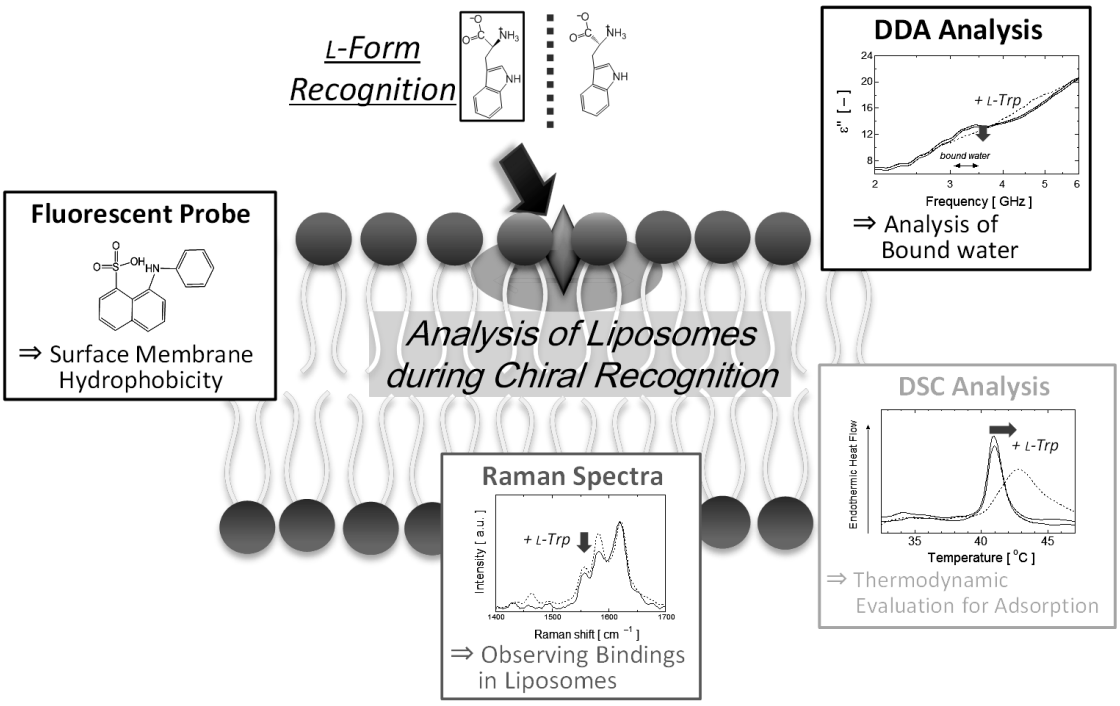


Fig. 2-1 Conceptual illustration of chapter 2.

adsorption process of amino acids. This analysis for membrane surface property was also examined by analyzing dielectric dispersion spectra at the higher frequency region. As the evidence of multiple interactions, direct observation of adsorbed Trp or His molecules was also carried out by using Raman spectroscopic analysis. In addition, thermodynamic analysis for this adsorption was measured by DSC, comparing the interaction behavior by the evaluating compensation of enthalpy and entropy. Each measurement mentioned above was conducted in both enantiomers of amino acids in order to discuss the mechanism of chiral recognition of the liposome membranes.

2. Materials and Methods

2.1 Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). L-Trp, D-Trp, L-His and D-His amino acids were purchased from Peptide Institute (Suita, Osaka, Japan). All amino acid reagents were over 98% purity of enantiomers. The fluorescent probe, 8-anilino-1-naphthalenesulfonic acid (ANS), was purchased from Sigma Aldrich (St. Louis, MO, USA). 1,4-Dioxane and other chemicals were purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan) and were used without further purification.

2.2 Liposome Preparation

A solution of phospholipids in chloroform was dried in a round-bottom flask by rotary evaporation under vacuum. The resulting lipid films were dissolved in chloroform and the solvent evaporated twice. The lipid thin film was kept under high vacuum for at least 3 h, and then hydrated with distilled water at room temperature. The vesicle suspension was frozen at -80 °C and then thawed at 50 °C to enhance the transformation of small vesicles into larger multilamellar vesicles (MLVs). This freeze-thaw cycle was repeated five times. MLVs were used to prepare large unilamellar vesicles (LUVs) by extruding the MLV suspension 11 times through two layers of polycarbonate membrane with mean pore diameters of 100 nm using an extruding device (Liposofast; Avestin Inc., Ottawa, Canada). Liposomes with different compositions were also prepared by using the same method.

2.3 Characterization of Fluorescent Probes

The fluorescent probes 8-anilinonaphthalene-1-sulfonic acid (ANS) was excited at 350 nm respectively. Fluorescent spectra in water/dioxane solutions were monitored using FP-6500 or FP-8500; JASCO, Tokyo, Japan).

2.4 Hydrophobicity Analysis of the Membrane Surface by ANS

The local hydrophobicity of the liposomal membrane was characterized by using an environmentally sensitive probe; ANS (Kachel et al., 1998). ANS dissolved in ethanol were

added to the liposome suspension or the pre-incubated mixture of liposome and amino acids. The final concentrations of lipid, ANS, and amino acids were 100 μM, 1 μM, and 10 μM, respectively. The fluorescence spectra of ANS were measured by using a fluorescent spectrometer after incubation for 30 minutes. ANS was excited at 350 nm, and the emission spectra were measured from 375 nm to 600 nm.

2.5 Dielectric Dispersion Analysis (DDA) for Analysis of Bound-Water at Liposome Surface

The dielectric loss (ε'') was measured at the frequency range from 1.0 GHz and 6.0 GHz, by using a network analyzer (Keysight Technologies, PNA-X N5245A, 10 MHz to 50 GHz). The measurements were performed at 25 ºC and the concentrations of lipid or amino acids of liposome suspension were 100 mM and 15 mM, respectively.

2.6 DSC Analysis of Liposomal Membranes

A differential scanning calorimeter (DSC-60; Shimadzu, Kyoto, Japan) was used for calorimetric measurements of liposomes. Liposome suspensions (100 mM) with or without amino acids (15 mM) were incubated for 48 hours at 25 ºC before DSC measurements. The 20 μl sample solution was sealed in an alumina hermetic pan. Thermograms were obtained with a heating and cooling rate of 2 ºC/min between 25 ºC and 50 ºC. There were no significant differences between the thermograms in heating and in cooling processes for one sample. At least three cycles of heating/cooling were repeated in each experiment, and the accumulated data was used for the calculation of the enthalpy variation (ΔH) and transition temperature (T_m) values.

2.7 Raman Spectroscopy Analysis of Trp and His

UV resonance Raman spectra of Trp and His were measured by a confocal Raman microscope (LabRAM HR-800; HORIBA, Ltd., Kyoto, Japan) at a excitation wavelength of 266 nm, with laser power of 50 mW. In addition, Raman spectra of liposomes were measured by using a confocal Raman microscope (LabRAM HR-800; HORIBA, Ltd., Kyoto, Japan) at a wavelength of 532 nm, with laser power of 100 mW and a total data accumulation time of

30 s. For each sample, the background signal of the solution was removed, and then the baseline was corrected.

2.8 Statistical Analysis

Results are expressed as mean ± standard deviation. All experiments were performed at least three times. The distribution of data was analyzed, and statistical differences were evaluated using the Student's t-test. A *P*-value of <5% was considered significant.

3. Results and Discussion

3.1 Analysis of Surface Hydrophobicity of Liposome Membranes by Using Fluorescent Probe, ANS

The properties of liposomal membranes during amino acid adsorption were characterized by using the fluorescence probe, 8-anilino-1-naphthalenesulfonic acid (ANS). ANS is known as a polar environment-sensitive probe (Slavík, 1982), which has been extensively applied to the analysis of membrane hydrophobicity. In addition, the localization in surface region of liposome membranes was also considered by the quench of xanthene dye probes attached in phospholipids by covalent bond (Kachel et al., 1998). **Figure 2-2(a)** shows the fluorescent spectra of in the mixture of water and 1,4-dioxane. As the increase of dielectric constant, blue shifts of the spectra and the increase of intensity were observed. In reference to previous report about dielectric constant of the water and 1,4-dioxane mixtures (Critchpield et al., 1953), the relation of dielectric constant and ANS fluorescence was estimated by calculating the fluorescence intensity ratios (I_{474}/I_{518}) of ANS (**Fig. 2-2(b)**). Except for the plot in the lowest dielectric constant values, a linear correlation was obtained, indicating the I_{474}/I_{518} of ANS fluorescence can monitor the hydrophobicity in the surface region of liposomes.

Figure 2-3 shows the time course of the I_{474}/I_{518} of ANS in the DPPC liposome with L- or D-forms of Trp and His. In the presence of L-Trp, the I_{474}/I_{518} values increased after 20 hours of incubation, while the opposite tendencies were observed in the presence of L-His (**Fig. 2-3(a)**). These results imply that the adsorption of amino acids induces the changes in the hydrophobicity of the surface region of liposome membranes. The adsorption of L-Trp resulted in the exclusion of water molecules from the DPPC liposomes, whereas L-His induced the hydration of the membrane surface, possibly because His is less hydrophilic than Trp (Tanford, 1962). As for the kinetics of surface hydrophobicity, these changes are corresponding to the adsorption behaviors of amino acids (**Fig. 1-6**). It is possible that the change of surface property is one of the essential factors for increasing adsorption. Conversely, there were no significant changes of I_{474}/I_{518} values in the presence of D-Trp and D-His, even after 48 hours (**Fig. 2-3(b)**), indicating the importance of the change of surface hydrophobicity for the enantioselective adsorption, that is to say, chiral recognition.

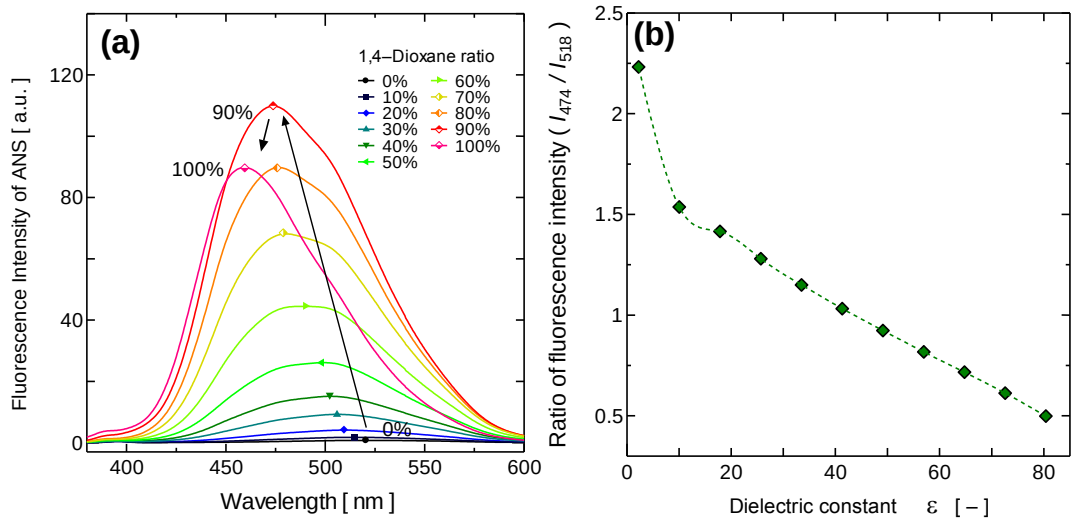


Fig. 2-2 Fluorescent spectra of ANS in water/1,4-dioxane. (a) Fluorescence spectra of ANS at the excitation of 350 nm. (b) The ratio of fluorescence intensity (I_{474}/I_{518}) of ANS in the different dielectric constant.

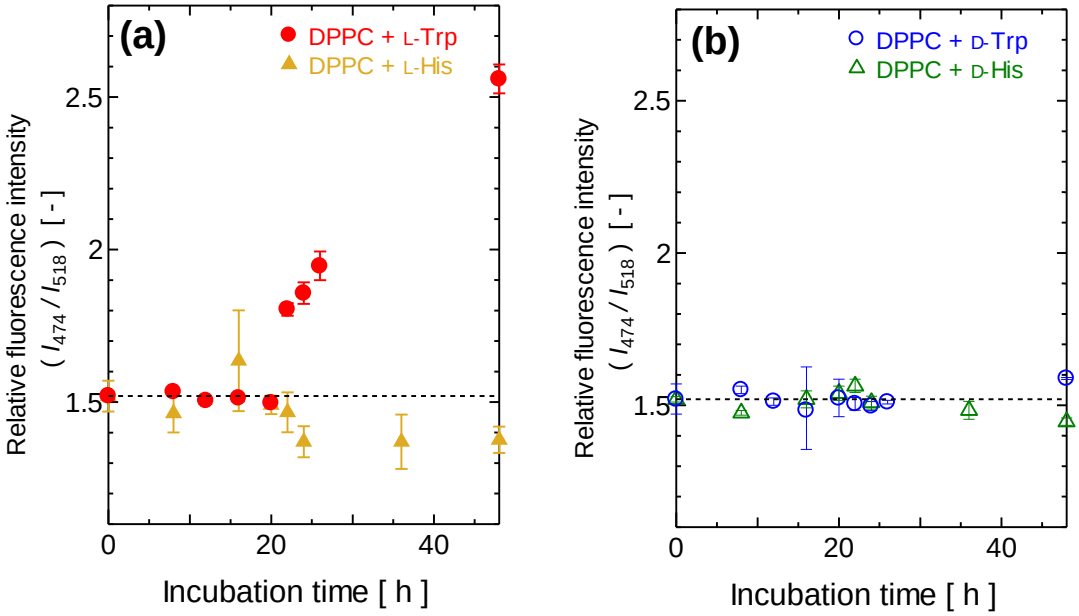


Fig. 2-3 Time course of the relative fluorescence intensity of ANS with amino acids. The ratio of fluorescence intensities at 474 nm and 518 nm (I_{474}/I_{518}) are measured in the presence of (a) L-Trp (filled circles) and L-His (filled triangles), (b) D-Trp (open circles) and D-His (open triangles). In all samples, the concentrations of amino acid and DPPC were 0.1 mM and 1.0 mM, and the incubation temperature was 25 ºC before measurements.

3.2 Evaluation of Bound Water in Surface of Liposome Membranes by Dielectric Dispersion Analysis

To investigate the hydration states of liposome membranes, dielectric dispersion analysis (DDA) is a useful tool because this is the direct analysis of bound water molecules. According to the previous studies (Noda et al., 2006; Takada et al., 2012), the peaks of dielectric loss around 0.1-5 GHz is assigned to bound water in liposome membranes, towards that around 20 GHz means the bulk water. **Figure 2-4** shows the dielectric spectra of DPPC liposomes with or without L- or D-forms of Trp and His. In this figure, the mixing of L-Trp induced the suppression of the shoulder peak from bound water unlike the mixing of D-Trp. The suppression of this shoulder peaks is corresponding to diminishing the dielectric relaxation by insertion of cholesterols or proteins (Takada et al., 2012). It is thus considered that the adsorption of L-Trp resulted in the exclusion of water molecules from the membrane structure of the DPPC liposomes, while L-His induced the hydration of the membrane surface. Considering this result and ANS measurement, it is suggested that the chiral recognition of amino acids induced the changes in the membrane surface properties.

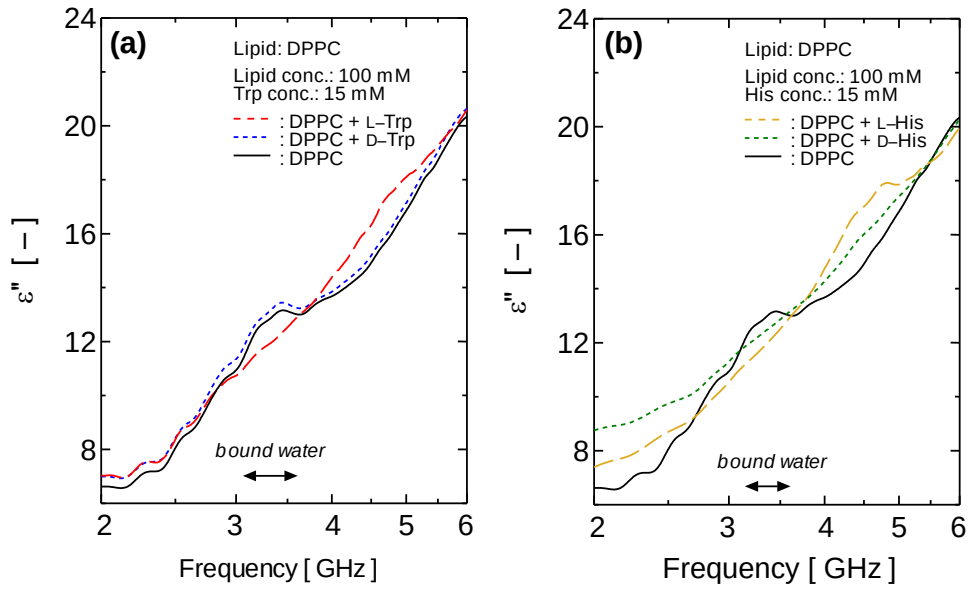
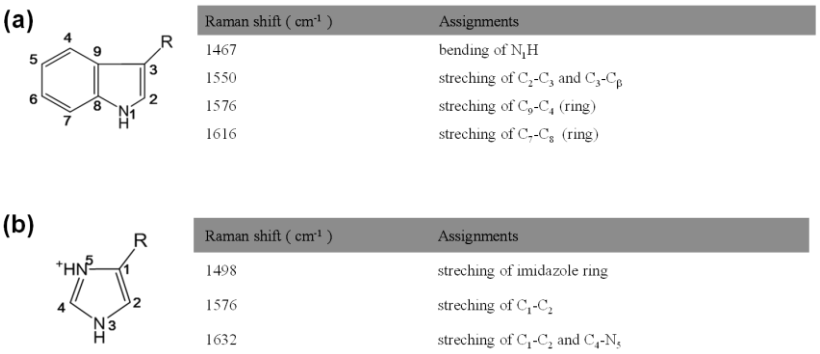


Fig. 2-4 Dielectric dispersion analysis of DPPC liposome membranes with amino acids. (a) Dielectric dispersion spectra of DPPC mixed L-form (dashed line) or D-forms (dotted line) of Trp. (b) Dielectric dispersion spectra of DPPC mixed L-form (dashed line) or D-forms (dotted line) of His. The dielectric loss (ε'') were analyzed in frequencies of 2-6 GHz. All samples were incubated by 25 ^{o}C in 48 hours before measurements.

3.3 Observation of Bindings of Tryptophan or Histidine in Liposomes by Resonance Raman Spectroscopy Analysis

A possible model for interaction of amino acids by liposome membranes was investigated based on UV resonance Raman spectroscopic analysis. UV resonance Raman signals of Trp and His can be distinguished based on their constituents (**Fig. 2-5**),; for Trp, the peaks at 1467, 1550, 1576, and 1616 cm^{-1} were assigned as N_1-H bending, C_2-C_3 or C_3-C_β stretching, respectively (Hirakawa et al., 1978; Miura et al., 1989; Wei et al., 2007); for His, the peaks at 1498, 1576 and 1632 cm^{-1} were assigned as stretching mode of the imidazole ring, C_1-C_2 stretching mode, and C_4-N_5 stretching mode, respectively (Marques et al., 2013). **Figures 2-6(a)** and (**b)** showed the UV resonance Raman spectra of L-Trp and D-Trp in the presence of DPPC liposomes after 48 hours of incubation. In the presence of DPPC liposomes, the peak intensities at 1467, 1550, 1576 cm^{-1} of L-Trp decreased, while no significant changes was observed in the case of D-Trp. A decrease in the peak intensities of L-His was also observed, but not in the case of D-His (**Figs. 2-6(d)** and **(e)**). These results suggest that L-Trp and L-His interacted with the DPPC liposomes. In addition, the decrease in the peak intensities was proportional to the lipid concentration (**Figs. 2-6(c)**, **(f)**). The Raman peak intensity of Trp is sensitive to environmental hydrophobicity (Nagatomo et al., 2013). It has been reported that the adsorption of cysteine ethyl ester on DPPC liposomal membranes resulted in a decrease in its Raman peak intensities, in which the interaction between the phospholipids and amino acid side chains could be induced by the replacement of water molecules, with a consequent weakening of the vibrational force constants (Arias et al., 2015). Furthermore, nucleobases,



Raman shift (cm^{-1})	Assignments
1467	bending of N_1H
1550	streching of C_2-C_3 and C_3-C_β
1576	streching of C_9-C_4 (ring)
1616	streching of C_7-C_8 (ring)

Raman shift (cm^{-1})	Assignments
1498	streching of imidazole ring
1576	streching of C_1-C_2
1632	streching of C_1-C_2 and C_4-N_5

Fig. 2-5 Peak assignments of side chains of amino acids. (a) Assignment of Trp. (b) Assignment and His. Atom number about carbon and nitrogen were described in chemical formula.

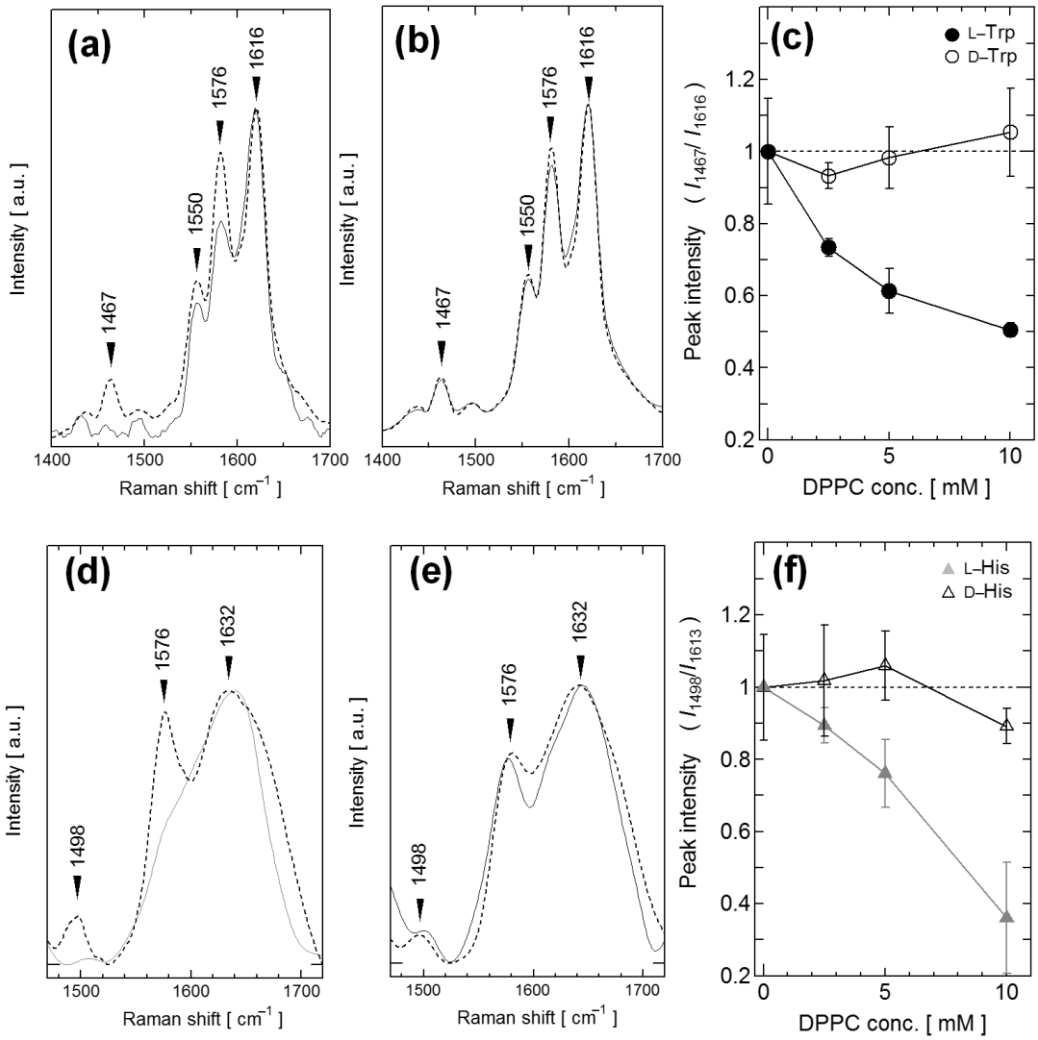


Fig. 2-6 UV resonance Raman spectroscopic analysis of Trp and His with DPPC liposomes. Raman spectra of each amino acids are measured without (dotted line) or with DPPC liposomes (solid line): (a) L-Trp, (b) D-Trp, (d) L-His and (e) D-His. (c) Relative Raman peak intensity of Trp (1467cm^{-1}) with DPPC liposomes. The similar analysis for His (1498 cm^{-1}) was shown in (f). All samples were incubated by 25 ºC in 48 hours before measurements.

which are hydrophobic moieties in RNA molecules, can interact with the liposome membranes via hydrophobic and hydrogen bonding interactions (Suga et al., 2013). The log*P* values of the indole ring (Trp) and imidazole ring (His) are 2.1 and -0.1, respectively (cf. amino acid back bone (Gly): -3.9)), and the hydrogen bond donors and acceptors are accumulated at the hydrophobic-hydrophilic interface of membranes. Taken together, these data suggest that the side chains of L-amino acids interacted with the hydrophobic regions of the liposome membranes, in which hydrogen bonds could be formed between L-amino acids

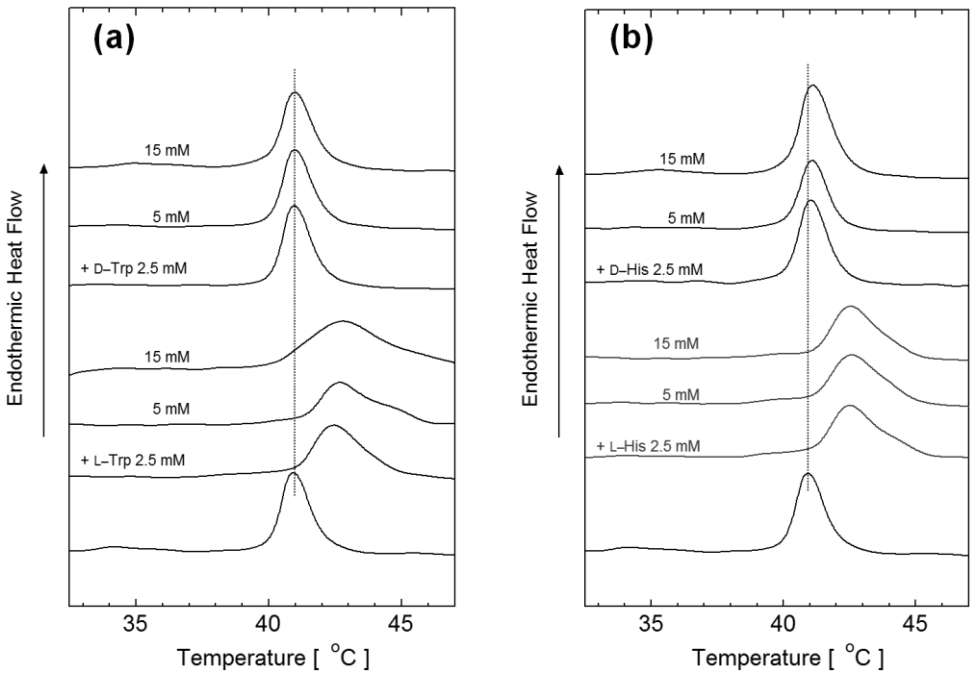
and DPPC molecules.

3.4 Thermodynamic Analysis for Adsorption in Liposome Membranes

Based on the above results of amino acids, the "surface region" of liposomes was found to be significant in the adsorption of amino acids. To discuss the adsorption mechanism, thermodynamic analysis of adsorption energy is useful for understanding the behavior of liposomes as the adsorbent of amino acids.

3.4.1 Evaluation of Phase Transition by DSC Analysis

The phase transition of the DPPC liposomes was analyzed by DSC, and pre-transition and main transition peaks were observed at 34.69 ºC and at 41.09 ºC, respectively. The effect in phase transition is investigated by mixing L- or D-forms of Trp and His (**Fig. 2-7**). The main phase transition temperature (T_m) slightly increased by the addition of L-Trp or L-His, whereas no change was induced by D-amino acids (**Figs. 2-7(a)** and **(b)**). Such a variation of DPPC liposomes in T_m is corresponding to the previous report about the incorporation of cationic lipid, owing to the electrostatic interaction between lipid molecules (Troutier et al., 2005). In the case of anionic liposomes, T_m was increased by the addition of poly(L-lysine) or poly(L-arginine), owing to the stabilization of the gel phase via the restriction of electrostatic repulsion between neighboring phospholipid molecules (Schwieger et al., 2007; Schwieger et al., 2009). Considering our results, the slight increases in the T_m values indicate that the electrostatic interactions between L-amino acids and liposome membranes induced the stabilization of the membrane packing. The adsorption of L-amino acids is also suggested by the suppression of the pre-transitions to a ripple gel phase only in the presence of L-amino acids. The enthalpy (ΔH) was also calculated for DPPC liposomes incubated with the amino acids (**Fig. 2-7(c)**). The ΔH values slightly increased with the addition of L-amino acids, but no significant increase was observed in the presence of D-amino acids. Furthermore, the increase in ΔH values was proportional to the amino acids concentration (**Fig. 2-8**). These results suggest that such variations in T_m and ΔH correspond to the adsorption of the L-enantiomers on DPPC liposome membranes. It has been suggested that these endothermic changes could be related to the stabilization of acyl chain packing via interactions



(c)

	T_m [°C]	ΔH [kJ/mol]
(control)	41.09±0.03	38.31±0.25
L-Trp	42.64±0.08	41.78±0.40
D-Trp	41.02±0.04	38.07±0.27
L-His	42.57±0.03	39.98±0.64
D-His	41.09±0.03	38.55±0.69

Fig.2-7 DSC analyses of DPPC liposome membranes with or without amino acids. The thermograms of pure DPPC liposomes are shown as black lines. The main transition of DPPC liposomes (41.09 ºC) is shown as dotted line. (a)DSC thermograms of DPPC liposomes mixed with L-Trp (lowers) or D-Trp (uppers) at the different concentrations. (b)DSC thermograms of DPPC liposomes mixed with L-His (lowers) or D-His (uppers) at the different concentrations. (c)Table of phase transition temperatures (T_m) and enthalpies (ΔH). All samples were incubated by 25 ºC in 48 hours before measurements.

with L-amino acids (Zhao et al., 2007; Marques et al., 2013). Therefore, it was investigated whether the enantioselective adsorption of L-Trp and L-His on DPPC liposomes induced the membrane to become more ordered, in which electrostatic and van der Waals interactions are possible driving forces of their interaction.

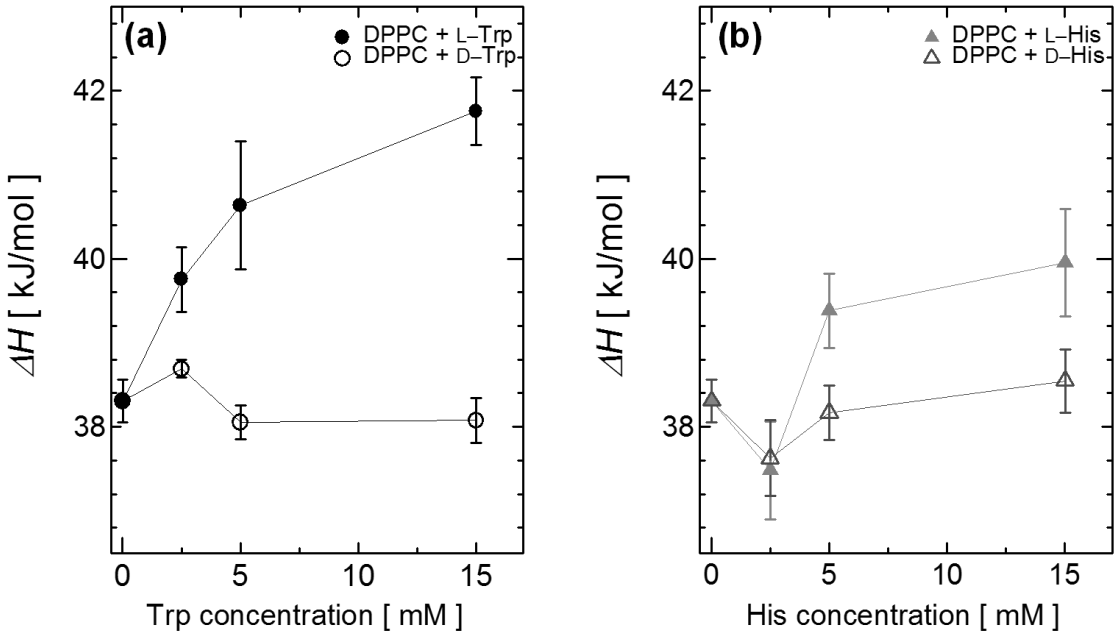


Fig. 2-8 Variations of the main transition enthalpy (ΔH) in different concentrations of (a) L- or D-forms of Trp and (b) L- or D-forms of His, respectively. All samples are incubated by 25 ºC in 48 hours before measurements.

3.4.2 Discussion of Adsorption in Relation between Enthalpy and Entropy

The compensation analysis between enthalpy and entropy is important to evaluate the adsorption of amino acids on liposome membranes. Since the entropy changes (ΔH) are a state function, it can be estimated from the DSC analysis of phase transition behavior. In addition, entropy changes ($T\Delta S$) can be calculated from the free energy changes obtained by the distribution constant of amino acids (K_{lip}) as the following equations:

$$T\Delta S = \Delta H + \mathrm{R}T_m \ln K_{lip}$$

$$K_{lip} = C_{flt}\, V_{lip} / \{(C_{ini} - C_{flt})\, V\}$$

where C_{ini} and C_{flt} represent the concentration of amino acids in the initial solution and the ultrafiltrated dolution, V and V_{lip} represent the volume of the bulk solution phase and the liposome membrane phase, R means the gas constant. In the case of D-Trp or D-His, compensation relationship could not be discussed because of the positive value of free energy changes, which is caused by no adsorption. On the other hand, the adsorption of L-Trp or L-His showed the linear correlation of enthalpy changes and entropy changes (**Fig. 2-9**), indicating the compensation relationship. These plots lay in the upper-left area of diagram similarly in the case of the ligand bindings (Muralidhara et al., 2007) and distribution of water

in reverse micelle (Khougaz et al., 1997), implying that thermal changes by adsorption were relatively large compared to the structural change of whole system. As a result, these adsorptions were regarded as the high affinity of binding.

To compare with general examples of interactions, the slopes (σ) and intercepts ($T\Delta S_0$) of plot of enthalpy changes and entropy changes were summarized in **Table 2-2**. When the $T\Delta S_0$ values are positive, the entropy-driven adsorption can be applicable in general. Almost all the cases (including this study) can be regarded as the entropy-driven adsorption. As compared with other cases, the σ value is relatively small in this study. From this result, the energy required in the structural change of the liposome membrane is assumed to be the

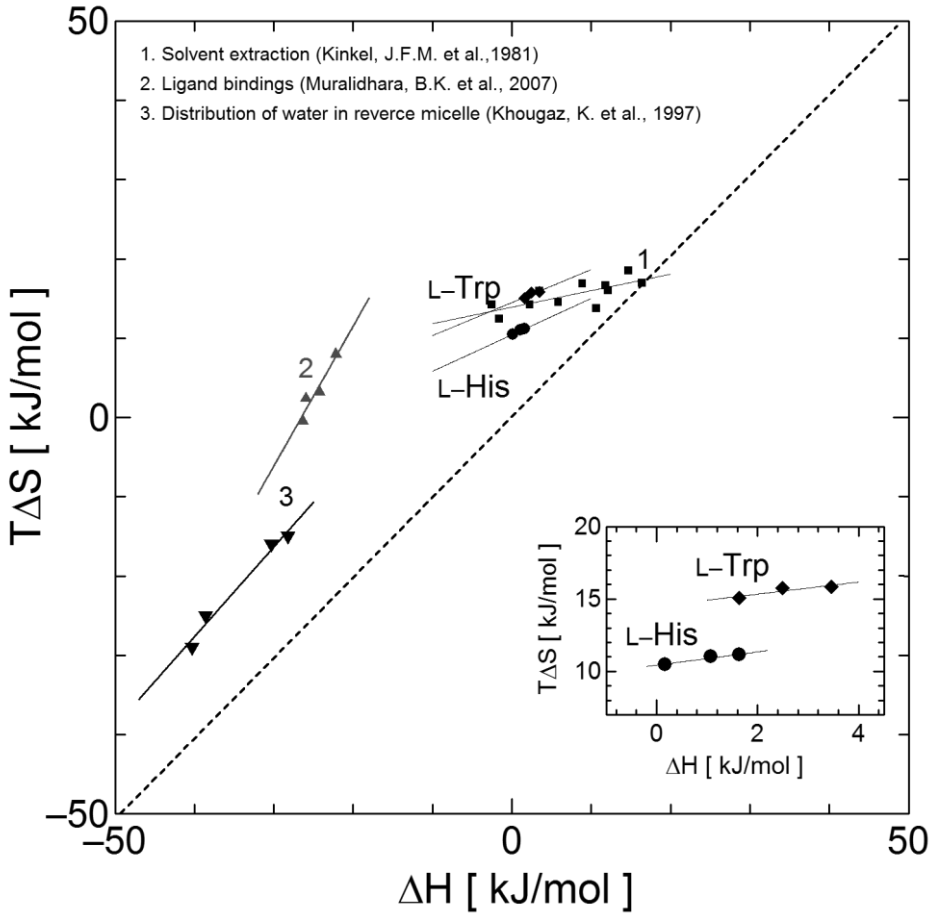


Fig. 2-9 The relation between enthalpy changes and entropy changes in several systems. In addition to the L-amino acid adsorptions, the profile of solvent extraction (1), ligand bindings (2) and distribution of water in reverse micelle (3) were shown. Dotted line represents the proportional line whose slope and intercept are 1 and 0 kJ/mol, respectively. The detailed plots of adsorption of L-Trp and L-His were shown in the inset.

Table 2-2 List of thermodynamic parameters in several types of interactions

Reference	Interaction type	σ [-]	$T\Delta S_0$ [kJ mol^{-1}]
Bojesen et al., 2003	Fatty acid binding to BSA from water	1.4	68.8
	Fatty acid binding to BSA from hexane	1.01	0.13
Khougaz et al., 1997	Distribution of water in reverse micelle	1.13	17.6
Kojima et al., 2001	Distribution of carboxylic acids in solvent-water system	1.05	-3.16
	Dimerization of carboxylic acids	0.91	20
Haroun et al., 2005	Binding Trp in chiral-recognitive ligand	1.02	22
Kinkel et al., 1981	Solvent extraction between phosphate buffer and 2,2,4-trimethylpentane	0.21	13.9
Nagatoishi et al., 2009	Ligand binding to TATA-box	0.856	53.3
Muralidhara et al., 2007	Allosteric binding to Cytochrome P450eryF	1.76	46.8
Richieri et al., 1997	Fatty acid (PA) bindings by Mutant proteins	0.864	-1.2
This work	Adsorption of L-Trp on DPPC liposomes	0.416	14.5
	Adsorption of L-His on DPPC liposomes	0.459	10.4

smaller than that of solvation or ligand bindings, which implies that the energy required in the adsorption and the relating change of hydrophobicity at the surface region are not so drastic as compared with that in the structural changes of liposomes: thus, the membrane fusion or disruption are hardly occurring. From the thermodynamic investigations, the liposome membranes were shown to act as the host adsorbents that recruit the guest molecules in entropy-driven manner.

3.5 Adsorption Mechanism in Liposome Membranes

Based on the above results, a plausible mechanism of chiral recognition is suggested as schematically shown in **Fig. 2-10**. In the early stage of adsorption, the adsorption amount of L-amino acids was still retained at the lower level till 12 hours of incubation, indicating that the relatively weak interactions were induced by the contribution of electrostatic interactions (**Fig. 2-10**). On the contrary, PPL adsorption in opposite charged-liposomes was saturated in short-times incubation. The contributions of electrostatic interactions are thought to be very important to improve the final recovery yield in the adsorption. The adsorption amount of L-Trp obtained was lower in acidic and basic pH conditions, wherein the L-Trp surface was positively- and negatively-charged, respectively (data not shown). During their interaction

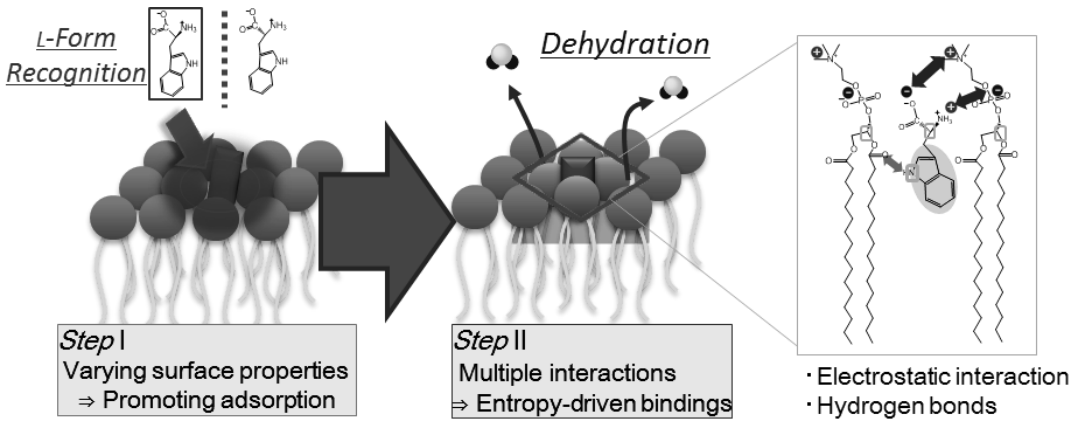


Fig. 2-10 Schematic illustration of the mechanism of chiral recognition on liposomal membrane.

process, the membrane property, such as hydrophobicity, was varied, especially at the surface region of the membranes. These variations were well corresponding to the increase of the adsorbed amount of L-amino acids, indicating that the adsorption can be promoted by the variation of the surface membrane properties. It is therefore implied that the surface property of liposome membranes transition to appropriate states for high adsorption. In the case of the adsorption of amino acids, it is also suggested that there are several steps in the adsorption mechanism as the variation of surface property induce the enhancement of the binding between amino acids and liposome membranes. At the step of variation in surface property, the liposome membranes are considered to exhibit the chiral recognition function, judging from the results of remarkable increase of the L-enantiomer. Finally, the adsorption of L-amino acids was saturated, resulting in the binding of plural phospholipids and one amino acid molecule, which means that the assembled state at the membrane surface is quite important for the chiral recognition. In addition, the possibility of more than three kinds of interactions was also evidenced by Raman spectroscopy analysis. Because the concept of three-points interaction is important for chiral recognition (Davankov, 1997), the suprastructure of liposome membranes may effectively induce such multiple interactions for chiral recognition. Furthermore, DSC thermograms revealed the increase of the phase transition temperature for the DPPC liposomes adsorbed with of L-amino acids, indicating that the recognized amino acid can be a member of the assembled membranes. In the viewpoint of adsorption energy, the slight conformational change is considered to be induced, and then the chiral recognitive interaction is gradually carried out to achieve a “local

minimum” of free energy. Based on the aforementioned adsorption mechanism, the high recognition function is induced on the liposome membranes. Considering the results in chapter 1, such adsorption steps are possibly suggested in the case of the adsorption of other amino acids, as shown in **Fig. 2-11**.

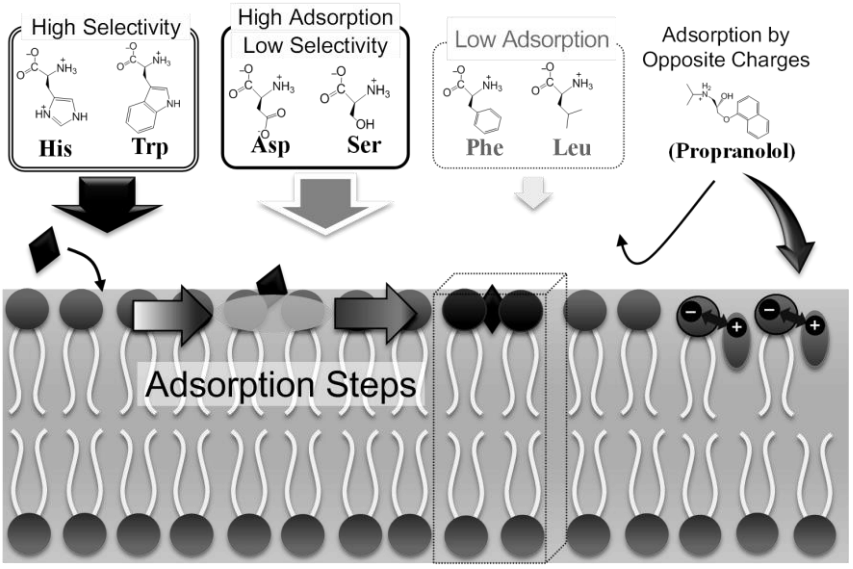


Fig. 2-11 List of adsorption behavior in several amino acids (picked up) and propranolol.

4. Summary

The chiral recognition of amino acids on the liposome membranes was found to be induced through the interactions especially at the surface region. The fluorescent probe analysis using surface-localized fluorescent probe, ANS, indicated the specific changes of hydrophobicity by mixing of L-Trp and L-His after 20 hours incubation, while no changes by mixing of D-Trp or D-His. The variations were also observed in dielectric dispersion spectra as the shift of the relaxation of peaks relating to "bound water" at the surface region of liposomes, indicating that the effect in membrane property can be corresponding to the amino acid adsorption. It is therefore important to form the interactions between the side chain of amino acids and lipid headgroups, which is evidenced by the decrease of peak intensity in resonance Raman spectra. In addition, the increase of the phase transition temperature occurred in liposomes that adsorbed L-Trp and L-His, implying the decrease of electrostatic dispersion in headgroup region of liposomes. Considering the relation of enthalpy and entropy changes, it is thought that this adsorption induces dehydration with slight effect of conformational changes in phospholipid assembly.

According to the above results, the mechanism of chiral recognition can be explained as "step-by-step" adsorption. In the first step, the partitions derived from electrostatic interactions are dominant. In the case of amino acids, stereospecific interactions are induced by the formation of multiple interactions. After that, the increase of adsorption was induced by the rearrangement of surface assembly as the change of membrane property. The effect on bound water existing in the membrane surface region is possible key factors for the binding of amino acids. Finally, multiple bindings were formed between plural phospholipids and one amino acid, according to the results about adsorption isotherms investigated in chapter 1. In the energetic viewpoint, it is considered that the molecular adsorption can be induced with gradually transfer of the total free energy to a local minimum in organizing the self-assembled membrane composed with phospholipid and "adsorbed" amino acid.

Aforementioned mechanism required longer time to complete adsorption process, which is one of the disadvantages for the practical application for industrial processes. Toward this, it is possible that the design of liposome membranes focusing on initial hydrophobicity or fluidity controls the adsorption behavior. Because such chiral recognition occurred in surface region of liposome membranes, the specific characterization for surface property is

important to design the chiral recognition function. The investigation of detailed surface property in liposomes for efficient chiral recognition will describe in the following chapter 3.

Chapter 3

Evaluation of Surface Properties of Cholesterol-Containing Binary and Ternary Liposomes to Regulate Molecular Recognition and Design of Liposome Membranes

1. Introduction

According to the results in the previous chapter, the analysis of the surface property of liposome membranes is important in order to design the optimal liposome membranes for recognition of small molecules. As mentioned in chapter 2, the fluorescent probe analysis can provide the membrane property in specific region of liposome membranes. Actually, it has been investigated that the dielectric constants of liposome membranes decrease with the sigmoid curve from surface to interior (Cevc, 1990). Thus, the location of fluorescent probes in liposome membranes depends on the polarity of the probe molecule: such behaviors can be investigated by the quencher bound to phospholipids in designated location (Kachel et al., 1998; Jurkiewicz et al., 2006; Kaiser et al., 1998; Asuncion-Punzalan et al., 1998). The locations of several fluorescent probes were summarized in **Fig. 3-1**. In contrast to ANS, Laurdan and DPH are stably localized in inner regions, and they indicate the whole property of liposome membranes. Among the surface-localized fluorescent probes, the detail localizationcan be analyzed by the distinction of fluorophore location. That is why the combination of multiple fluorescent probes is good way to evaluate the changes in the membrane properties in various regions. In addition, such a technique is also applied for evaluating the phase state of liposomes. Suga et al. have developed the evaluation method for the phase state of liposome membranes by Cartesian diagram evaluated by plotting the membrane polarity and fluidity derived from Laurdan and DPH, respectively (Suga et al., 2013).

Cholesterol (Ch) is known to regulate the membrane property because of the steroid skeletons which induce high order in liposome membranes. Besides, Ch also induces the increase

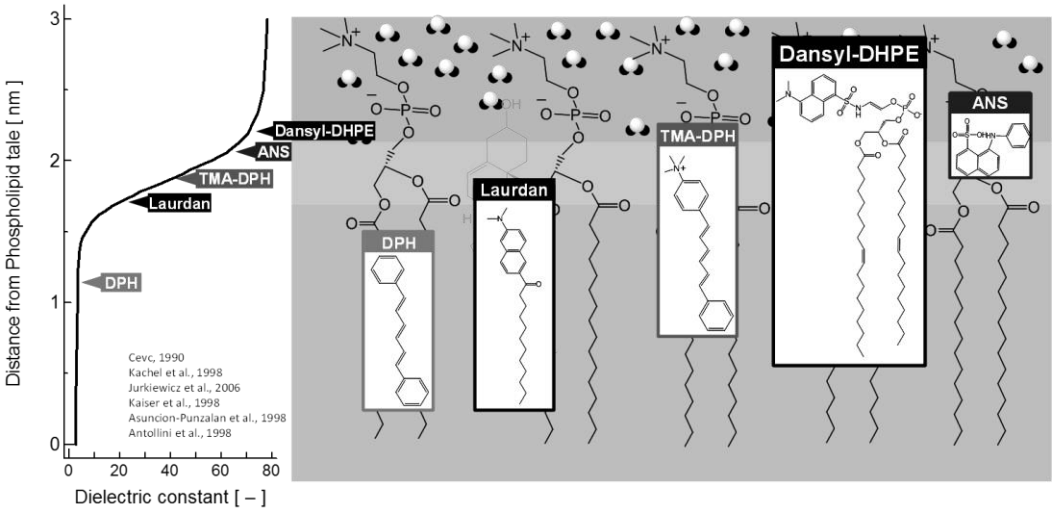


Fig. 3-1 Summary of the depth of fluorescent probes in liposome membranes.

of membrane packing via the hydrogen bonds between hydroxyl groups of Ch and headgroups of phospholipids. As a result, disordered liposome membranes show the decrease of membrane fluidity by mixing Ch. On the contrary, the fluidity of quite rigid liposome membranes increased in mixing Ch by disturbing the membrane packing. Actually, the liposomes containing Ch resulted in the specific effects via the adsorption of some peptides (Shigematsu et al., 2007; Gopal et al., 2013). Furthermore, heterogeneous liposomes containing Ch indicate the regions of low fluidity, such as microdomains or “rafts” (Scolari et al., 2010; Björkbom et al., 2007).

Microdomains mean the separated phases in liposome membranes, which are mainly induced by the difference of lateral diffusion in each phase and hydrophobic interactions. Such domains actually play significant roles in the regulation of protein and enzyme functions in the cell membranes (Litt et al., 2009; Anderson et al., 2002; Carozzi et al., 2002). In giant unilamellar vesicles (GUV), domains can be formed in micrometer order, and are directly observed in microscopic images by fluorescent labeling (Veatch et al., 2003; Stottrup et al., 2004; Cicuta et al., 2007). In addition, the membrane property of domains has been evaluated by thermodynamic procedures (Svetlovics et al., 2012; Garidel et al., 2000), Raman, and nuclear magnetic resonance spectroscopy (de Lange et al., 2007; Davis et al., 2014). On the other hand, the analysis of domains for small liposomes (nanometer order) is difficult due to the limit of measurable scale in optical microscope, nevertheless, domain formation in liposome membranes can be analyzed by excimer/monomer ratios of pyrene probes and their

derivatives even in nano-order (Lagane et al., 2002; Arrais et al., 2007). The estimation of nanodomain formation (<5 nm) has been also carried out by the fluorescent quench method using TEMPO probe (Suga et al., 2013). Moreover, the line tension is generated in the edge of domains due to the boundary of different phases. Actually, the previous report revealed that the HIV fusion peptide preferentially targeted to l_o-l_d boundary regions and promoted full fusion at the interface between ordered and disordered lipids (Yang et al., 2015). As the increase of domain size, the contributions of such a line tension is thought to decrease (Tolpekina et al., 2004). Therefore, it is possible that the detailed investigation of the phase behaviors in heterogeneous liposomes contributes to understanding about the formation of interactions in liposome membranes.

In this chapter, the effect for chiral recognition of His is investigated by using the liposomes with multiple components contained Ch (**Fig. 3-2**). At first, the methodology for evaluating the surface property of the liposome membranes was developed by the combination of two types of fluorescent probes that can be localized at the surface edge of the membrane. By means of the comparison of Cartesian diagram for interior membrane properties, it is expected to evaluate the liposome membrane property in details. According to the relation between such properties and the adsorption of L- or D-form of His, the effect of mixed Ch in liposome membranes is discussed. The variation in the membrane property after the His adsorption was also investigated to understand the behavior of liposome membranes. Furthermore, the membrane properties of ternary liposomes were evaluated based on their phase behaviors, and then, the relation with chiral recognition of His was investigated. By comparing the domain states relating to the line tension, the contribution of domain is considered for understanding the effect of existing domain “edges”

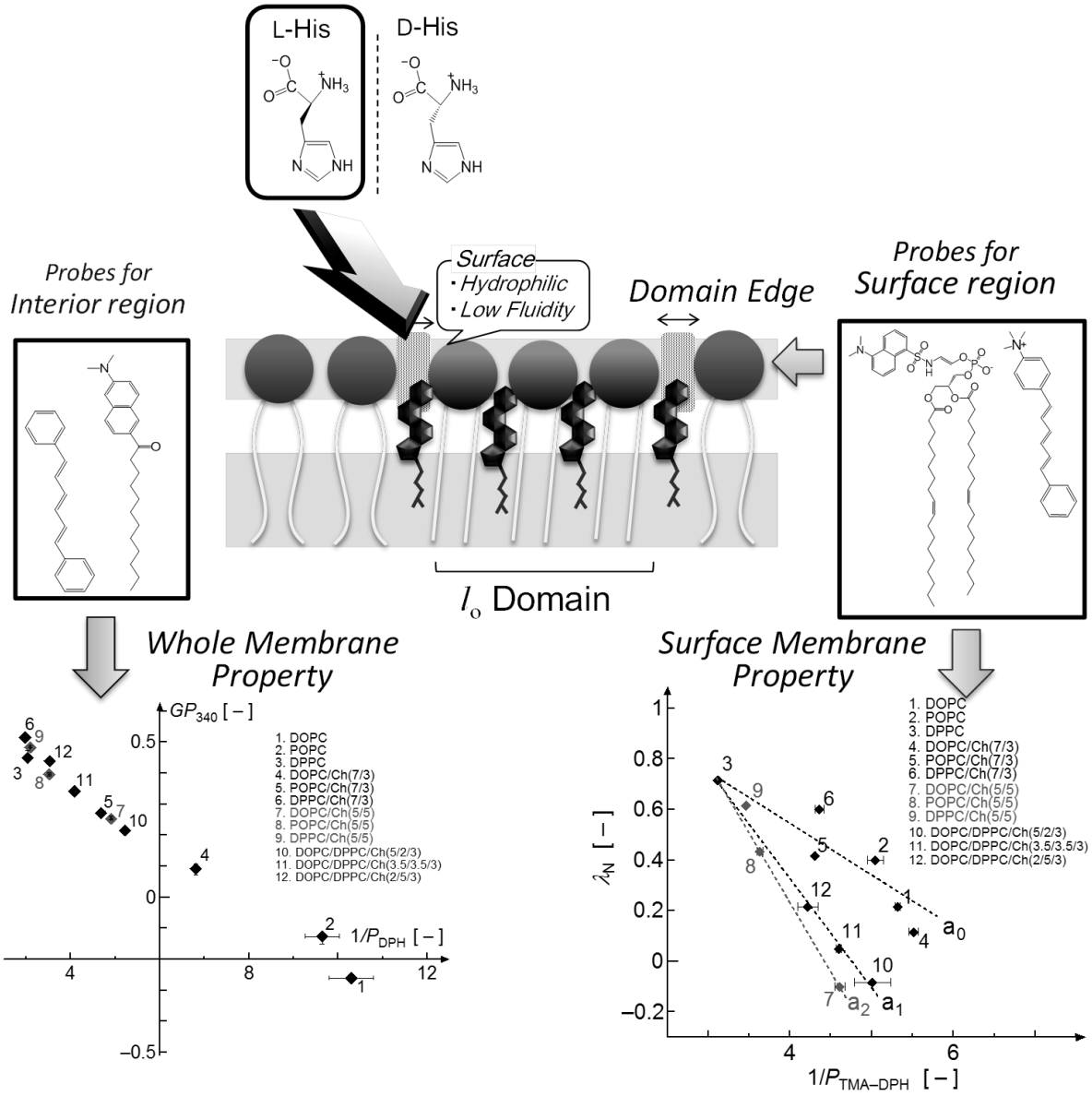


Fig. 3-2 Conceptual illustration of chapter 3.

2. Materials and Methods

2.1 Materials

Several phospholipids such as 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). A fluorescent probe, *N*-(5-dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Dansyl-DHPE) was also purchased from Avanti Polar Lipids, Inc., and 1,6-diphenyl-1,3,5-hexatriene (DPH), (1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene (TMA-DPH), 6-lauroyl-2-dimethylaminonaphthalene (Laurdan) were obtained from Sigma Aldrich (St. Louis, MO, USA). Cholesterol (Ch) was purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan), and L or D-form of histidine (His) were purchased from Peptide Institute (Suita, Osaka, Japan). Other chemicals were purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan) and were used without further purification.

2.2 Liposome Preparation

A solution of phospholipids in chloroform was dried in a round-bottom flask by rotary evaporation under vacuum. The resulting lipid films were dissolved in chloroform and the solvent evaporated twice. The lipid thin film was kept under high vacuum for at least 3 h, and then hydrated with ultrapure water at room temperature. The vesicle suspension was frozen at -80 °C and then thawed at 50 °C to enhance the transformation of small vesicles into larger multilamellar vesicles (MLVs). This freeze-thaw cycle was repeated five times. MLVs were used to prepare large unilamellar vesicles (LUVs) by extruding the MLV suspension 11 times through two layers of polycarbonate membrane with mean pore diameters of 100 nm using an extruding device (Liposofast; Avestin Inc., Ottawa, Canada). Liposomes with different compositions were also prepared by using the same method.

2.3 Evaluation of Membrane Properties by Fluorescent Probes

Membrane properties of liposomes can be characterized in Cartesian diagram by the plot of the membrane fluidity versus polarity evaluated by DPH and Laurdan, respectively (Suga et al., 2013). The fluidity in the interior of the liposome membrane was evaluated by

measuring the fluorescence anisotropy of the DPH incorporated in the vesicles using the fluorescence spectrophotometer FP-6500 (JASCO, Tokyo, Japan). 10 μL of 100 μM DPH in ethanol was added into 1 mL of liposome suspension (lipid: 0.25 mM). The samples were excited with vertically polarized light (360 nm), and emission intensities both perpendicular ($I_{\perp}$) (0°, 0°) and parallel ($I_{/\!/}$) (0°, 90°) to the excited light were recorded at 430 nm. The polarization (P) of DPH was then calculated by using the following equations (Hayashi et al., 2011):

$$P = (I_{/\!/} - GI_{\perp}) / (I_{/\!/} + GI_{\perp}) ,$$

$$G = \mathrm{i}_{\perp} / \mathrm{i}_{/\!/} ,$$

where $i_{\perp}$ and $i_{/\!/}$ are the emission intensities perpendicular to the horizontally polarized light (90°, 0°) and parallel to the horizontally polarized light (90°, 90°), and G is the correction factor. The membrane fluidities were evaluated based on the reciprocal of polarization, $1/P$. The membrane fluidities were measured at room temperature. The fluorescent probe Laurdan is sensitive to the polarity around itself, which allows the membrane polarity of liposomes to be determined. Laurdan emission spectra exhibit a red shift caused by dielectric relaxation. Thus, emission spectra were calculated by measuring the general polarization (GP_{340}) for each emission wavelength as follows (Parasassi et al., 1991):

$$GP_{340} = (I_{440} - I_{490}) / (I_{440} + I_{490}) .$$

Laurdan excited with 340 nm light at 20 ºC. The fluorescent spectrum of each sample was normalized. The total concentrations of amphiphilic phospholipid and Laurdan in the test solution were 1000 and 10 μM, respectively.

Besides, the surface membrane property could be characterized by the similar diagram analyzed by partially-hydrophilic molecular probes. TMA-DPH could be used for characterization of the surface membrane fluidity ($1/P_{\mathrm{TMA\text{-}DPH}}$) by analyzing anisotropy with the same manner to DPH. As for surface membrane polarity, dansyl-DHPE was used as a probe molecule: it was mixed with a liposome suspension in final concentrations of lipid and dansyl-DHPE were 100 and 1.0 μM, respectively. The fluorescence spectra were analyzed by the excitation light (336 nm) for observing the wavelength of maximum fluorescence.

2.4 Measurement of Adsorption to Liposome Membranes

The liposome suspensions (Lipid: 3.0 mM) were mixed with L-histidine (His) (0.5 mM) and then they were incubated at 20 ºC for 24 or 48 hours. After incubation, liposomes and adsorbed L-His were separated by ultrafiltration with the 50,000 Da of molecular weight cut off (USY-5; Toyo Roshi Kaisha, Ltd., Tokyo, Japan). After filtration, the leaked concentration (C_{flt}) of L-His was measured by the absorbance of UV spectrometer (UV-1800; Shimadzu, Kyoto, Japan). The adsorbed concentration (C_{ads}) and adsorption amount of L-His ($q_{L\text{-His}}$) or D-His ($q_{D\text{-His}}$) in several liposome membranes were calculated by following equations:

$$C_{ads} = C_{ini} - C_{flt}$$

$$q_{L\text{-His or D-His}} = C_{ads} / c_{lip} ,$$

where C_{ini} and c_{lip} represent an initial concentration of adsorbates and liposomes, respectively. To evaluate the selectivity of His enantiomer, percent based enantiomer excess (ee) were calculated by following equation:

$$ee = (C_{ads(L\text{-His})} - C_{ads(D\text{-His})}) / C_{ini} .$$

2.5 Statistical Analysis

Results are expressed as mean ± standard deviation. All experiments were performed at least three times. The distribution of data was analyzed, and statistical differences were evaluated using the Student's t-test. A P-value of <5% was considered significant.

3. Results and Discussion

3.1 Effect of Mixing Cholesterol for Interior Membrane Properties

The properties of the interior of liposome membranes were first evaluated based on the previous report (Suga et al., 2013). As shown in **Fig. 3-3**, the general polarity (GP_{340}) of Laurdan and the fluidity evaluated by DPH ($1/P_{DPH}$) for several liposomes containing Ch were plotted in a Cartesian diagram, indicating the regression of the plotting data in a single line. POPC (T_m: −5 ºC) and DOPC (T_m: −20 ºC) liposomes were in the disordered (l_d) phases, and an increase in both properties in the upper-left of the diagram along with increased Ch ratios, while DPPC (T_m: 41ºC) liposomes were in the solid-ordered (s_o) phases, and only a slight increase in both properties were observed. These results agree with the previous findings showing that Ch induced the phase transition of liposomes from l_d to liquid-ordered (l_o) phase caused by membrane orientation (Arrais et al., 2007; Walde et al., 2014). In the case of ternary DOPC/DPPC/Ch liposomes, their surface properties were similar to those of liposomes with corresponding Ch concentrations, indicating that there was no clear difference among

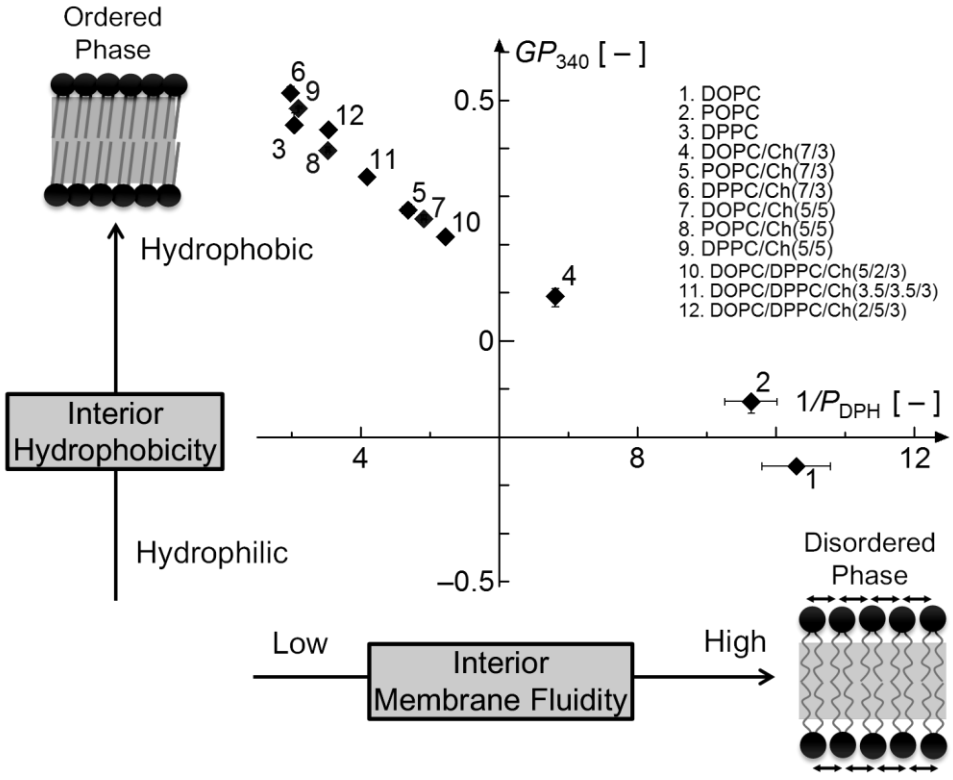


Fig. 3-3 Cartesian diagram of membrane polarity (GP_{340}) and fluidity ($1/P_{DPH}$) for binary and ternary liposomes containing Ch. Numbered points are defined in the inset.

liposomes showing similar T_m in the plot of **Fig. 3-3**. Therefore, these results show that the Cartesian diagram, characterizing membrane polarity and fluidity, can be utilized for the understanding of overall membrane properties in the case of Ch-modified liposomes, owing to the composition of phospholipids or Ch.

3.2 Effect of Mixing Cholesterol for Surface Membrane Properties

It is important to evaluate the behavior of the surface region of liposome membranes in order to understand the interactions between hydrophilic molecules (at polar moieties) and liposomes. Detail analysis of the phase state of liposomes is also required. As the increase of Ch molar ratio, the liposomes mixed Ch in 50 mol% showed the transition of phase state in DPPC, DOPC and POPC (Fritzsching et al., 2013; de Almeida et al., 2003). Furthermore, some of DOPC/DPPC/Ch ternary liposomes formed the segregated l_o phase regarded as domains. However, the aforementioned probes, such as Laurdan and DPH, are basically categorized as hydrophobic fluorescent probes and, thus, are not suitable to monitor membrane properties at the surface region. Besides that, Cartesian diagram remains the evaluation of whole properties derived from each ratio of lipid components. As for the surface property, ANS fluorescent probe molecules could be used for the analyses of before or after adsorption of amino acids as described in chapter 2. Nevertheless, because this probe molecule is difficult to be inserted in ordered phase, it is not appropriate to compare the surface membrane properties among the liposomes especially in secondary and ternary systems. Hence, dansyl-DHPE is known as the fluorescent probe capable of localizing to hydrophilic regions at the surface of liposomal membranes (Asuncion-Punzalan et al., 1998), and can be used for the estimation of membrane hydrophobicity via the emission wavelength sift in the liposomes (Takechi et al., 2011). As shown in **Fig. 3-4(a)**, the fluorescent spectra of dansyl-DHPE were measured in mixtures of water and 1,4-dioxane as solvents, showing a blue shift in the peak wavelength with decreased ratios of water. This behavior was caused by environmental changes around the fluorophore of the dansyl moiety. The shift in the peak wavelength was plotted against the dielectric constant of the solvent (**Fig. 3-4(b))**, resulting that a linear correlation in wavelengths was observed from 512 nm to 527 nm, except for the data plot in the dielectric constant value of 57. Hence, the surface-membrane hydrophobicity

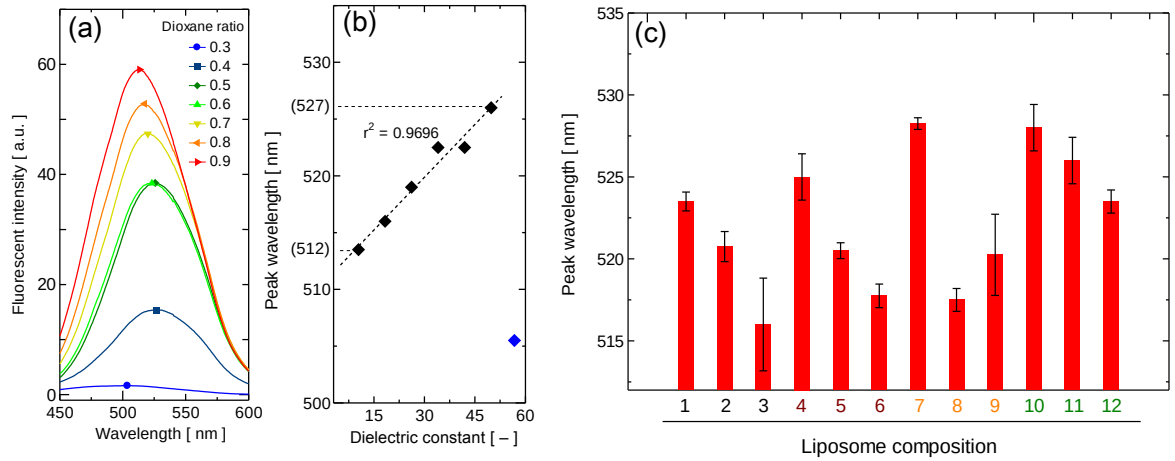


Fig. 3-4 Peak wavelength analysis of dansyl-DHPE surrounding in solvent or liposome membranes. (a) Fluorescent spectra of dansyl-DHPE (Ex.: 336 nm) in several ratio of water/1,4-dioxane solvent. (b) Peak wavelengths versus dielectric constant. Dielectric constant values were calculated the ratio of water (ε = 80.1) and 1,4-dioxane (ε = 2.2). (c) Peak wavelength of dansyl-DHPE inserted in several liposome membranes. Numbered points are correlated in the inset of **Fig. 3-3**.

can be evaluated by the normalized value of the surface hydrophobicity (λ_N) by using the following equation:

$$\lambda_N = (\lambda - \lambda_1) / (\lambda_0 - \lambda_1)$$

where λ_0 and λ_1 represent the fiducial peak wavelengths in hydrophobic (512 nm) and hydrophilic (527 nm) condition, respectively.

Based on these preliminary results, the peak wavelength of dansyl-DHPE fluorescence in the liposome was measured (**Fig. 3-4(c)**). The peak wavelength of highly ordered DPPC liposomes was found to be lower as compared with those in disordered membranes, such as DOPC and POPC, indicating that the DPPC liposome membrane was displaying a hydrophobic (dehydrated) surface. With the increase in Ch ratio, DOPC/Ch and DPPC/Ch liposomes displayed a red shift in the fluorescence spectrum, while no shifts were observed in the binary POPC/Ch liposomes. In the case of ternary liposomes, the wavelength of dansyl-DHPE spectra remained to be similar to those of hydrophilic surfaces, implying that the profile of surface hydrophobicity was not exactly characterized by the GP_{340} measured by using Laurdan.

With reference to the Cartesian diagram (**Fig. 3-3**), **Fig. 3-5** shows the evaluation of the properties at the surface of various liposomes by the plot of λ_N versus TMA-DPH fluidity

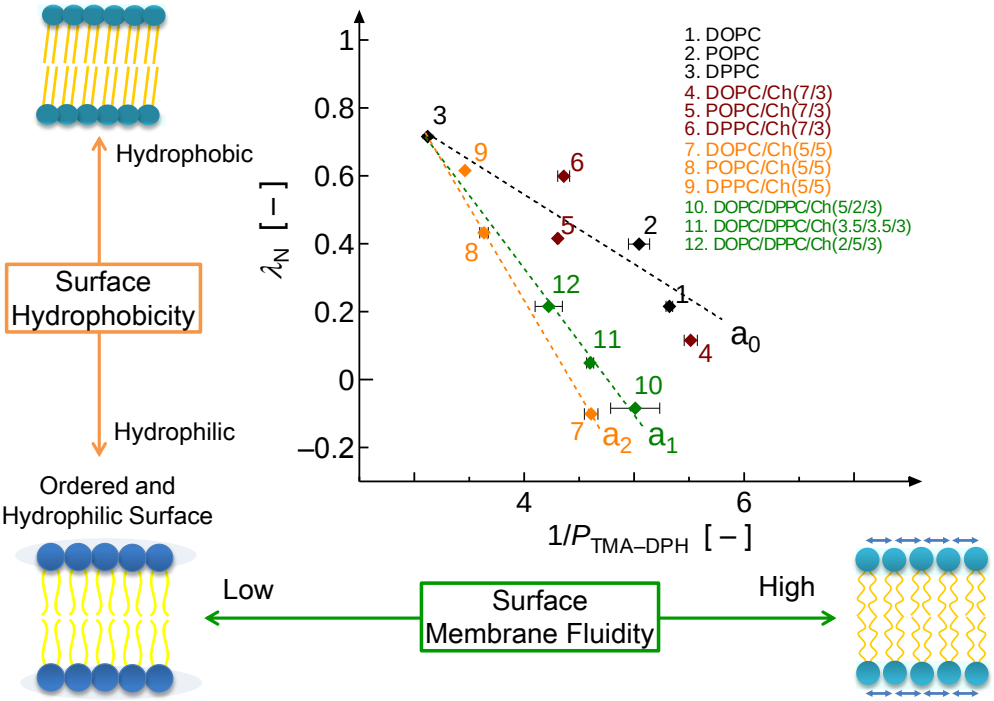


Fig. 3-5 Correlation diagram evaluated by surface membrane fluidity ($1/P_{TMA\text{-}DPH}$) and surface membrane hydrophobicity (λ_N) in several binary and ternary liposomes containing Ch. Numbered points are defined in the inset. Dotted line a_0 represents the trend in pure or binary liposomes containing 30% of Ch; dotted line a_1 and a_2 also represent that in 50% Ch ratio liposomes and ternary liposomes, respectively.

($1/P_{TMA\text{-}DPH}$). In the case of liposomes with single components (DOPC, POPC, and DPPC) or those with 30% Ch, the data were clustered on the right-downward trend line (**a_0**), indicating that the surface hydrophobicity decreased with increased surface fluidity. However, in the case of binary liposomes at 50% Ch, the data were clustered in another trend line (**a_2**) that demonstrated lower value of its slope, resulting in the discrimination of transitioned phase caused by Ch. The variation of these parameters was also plotted against the Ch ratio in order to discuss the features of membrane properties (**Fig. 3-6**). The λ_N value of various liposomes, except for POPC, decreased depending upon the Ch ratio, with the $1/P_{TMA\text{-}DPH}$ values indicating only a slight decrease, while GP_{340} and $1/P_{DPH}$ reached values similar to those observed with DPPC liposomes. As increasing the Ch ratio, the liposome membranes change in accessible surface capable of permitting water-molecule invasion (Stein et al., 2015), resulting in membrane variation toward hydrophilicity only at the surface regions of liposomes modified with Ch. The change in λ_N became larger than that of $1/P_{TMA\text{-}DPH}$, since

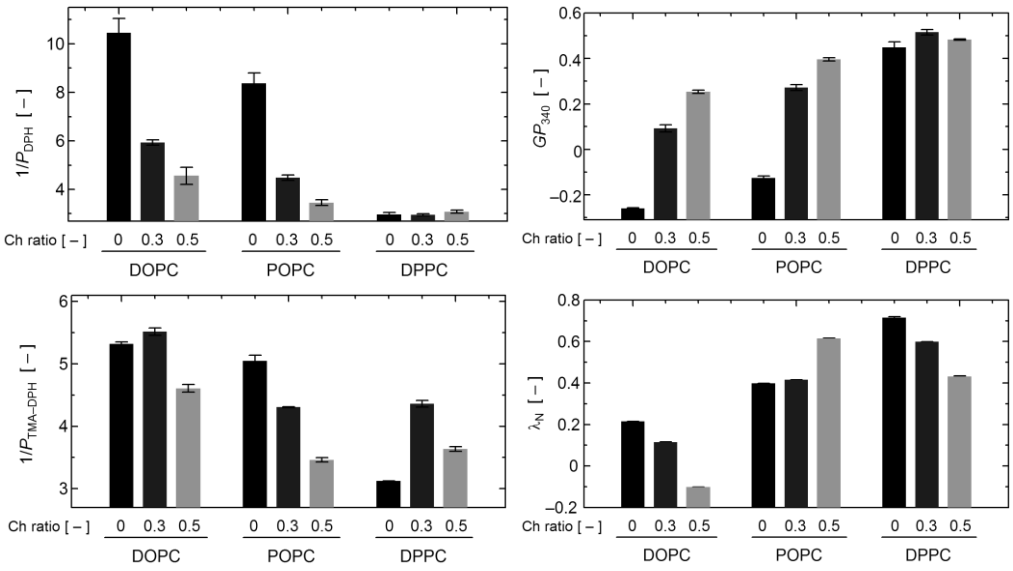


Fig. 3-6 Transition of several membrane properties by increase of Ch molar ratio in three liposomes. Composition of liposomes represented below each bar.

the water exclusion from the inner membrane to the surface region can be induced by insertion of Ch molecules (M'Baye et al., 2008). From these results, it is considered that membrane properties derived from fluorescent probes located at the surface region can be utilized for the evaluation of intrinsic properties of the liposome surface.

The above characterization method was also applied to ternary liposomes. Although the Ch ratio was unchanged and kept at 30%, the data were found to be clustered on another trend line ($\mathbf{a}_1$). It has been reported that liposomes at these components could form the heterogeneous surfaces of l_o and l_d phases (Cicuta et al., 2007), resulting in hydrophilic liposome surfaces. Although previous methods evaluating membrane properties provided general information relating to phase separation, the proposed method for determining membrane properties at surface regions can discriminate between heterogeneous or Ch-rich liposomes. Based on these results, it is expected that variations in membrane properties before and after adsorption of hydrophilic guest molecules, such as amino acids, will be capable of more detailed study.

3.3 Relation between Membrane Properties and Chiral Recognition of Histidine in Binary or Ternary Liposomes

Highly-selective adsorption of L-His molecules was induced in a DPPC liposome formed in the ordered (s_o) phase, where the surface membrane hydrophobicity decreased during the adsorption of L-His and the adsorption behavior of His was affected by initial membrane properties. The His adsorption behavior was studied by selecting various liposomes classified in different categories (**a_1**, **a_3**, and **a_2**; **Fig. 3-5**). **Figure 3-7(a)** shows the

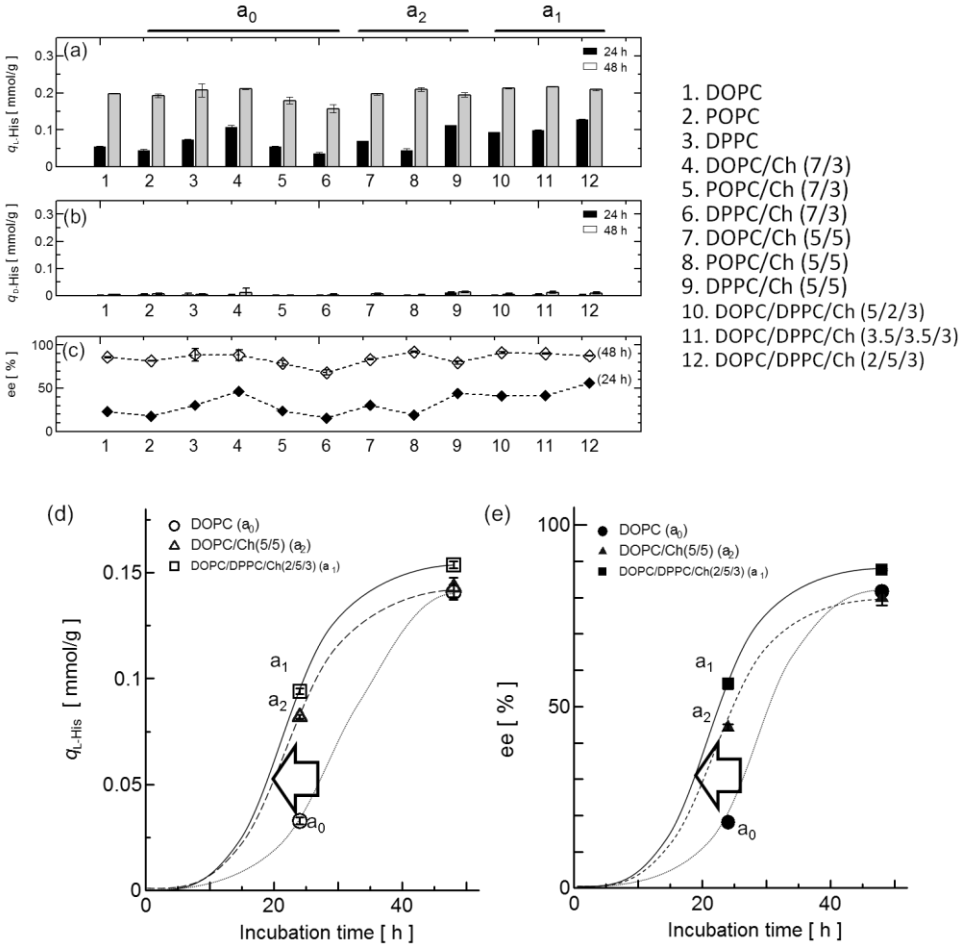


Fig. 3-7 Adsorption and chiral recognition behavior in several liposomes. (a) Adsorption amount of L-His ($q_{\text{L-His}}$) in 24 or 48 hours of incubation. (b) Adsorption amount of D-His ($q_{\text{D-His}}$) in 24 or 48 hours of incubation. (c) Percent of His enantiomer excess (ee) in several liposomes. Closed and open keys show ee values in 24 and 48 hours of incubation. (d) Time course of $q_{\text{L-His}}$ in the three kinds of components categorized in **a_0** (dotted line), **a_2** (dashed line) and **a_1** (solid line). (e) Time course of $q_{\text{L-His}}$ in the three kinds of components categorized in **a_0** (dotted line), **a_2** (dashed line) and **a_1** (solid line). **a_0**, **a_2** and **a_1** represent the trend lines described in **Fig. 3-5**.

adsorption levels of L-His ($q_{\text{L-His}}$) in binary and ternary liposomes containing Ch. In the case of several liposomes categorized in $\mathbf{a_0}$, except for DPPC, the $q_{\text{L-His}}$ value increased after 24 and 48 hours incubations, while the λ_{N} value decreased. Specifically, the liposome of DOPC/Ch 7/3 indicated the larger $q_{\text{L-His}}$ after a 24- and 48-h of incubation in contrast to DPPC liposomes, resulting in more efficient adsorption as compared to results in previous chapter 1. This result was due to the promotion of the insertion of L-His molecules, resulting in high levels of surface fluidity and hydrophilicity. However, higher $q_{\text{L-His}}$ values over 24 h were obtained in several liposomes categorized as $\mathbf{a_2}$, despite the indications of lower surface-membrane fluidities. In comparison with the adsorption to liposomes categorized as $\mathbf{a_0}$, this result indicates that the liposomes categorized along the trend line with lower slope can have potentials capable of increasing $q_{\text{L-His}}$. The conditions enabling fast and efficient adsorption can be estimated by the evaluation of surface-membrane properties, suggesting that the changes in membrane properties toward hydrophilic surfaces could be an important step in L-His adsorption as mentioned in chapter 2. Furthermore, high $q_{\text{L-His}}$ values observed over 24 h were obtained in all liposomes containing ternary components categorized in $\mathbf{a_1}$, indicating that the heterogeneous phase preferably influenced L-His adsorption in phase kinetics. To investigate the chiral recognition function of the above liposomes, the $q_{\text{D-His}}$ value was also shown in **Fig. 3-7(b)**. In all liposomes, the $q_{\text{D-His}}$ value remained at level <0.01, even after a 48-h incubation, resulting in the high enantiomer excess (**Fig. 3-7(c)**).

3.4 Variation of Liposome Membrane Properties Induced by Adsorption of L-Histidine

In chapter 2, the changes of membrane properties by adsorption of L-amino acids were investigated by several procedures. To compare these changes among several phases of liposomes, the above evaluation is conducted after adsorption of L-His.

As shown in **Fig. 3-8(a)**, the variation of surface properties of the whole membrane was not significant before and after L-His adsorption in all components. This result may imply that the adsorption of L-His molecules had less effect on properties of the inner region of liposomes as mentioned in previous results involving dielectric dispersion spectra or anionic-probe hydrophobicity due to the difficulty of insertion of highly polar molecules. However, the surface-membrane properties were changed in the fixed areas of 0–0.4 in λ_{N} and

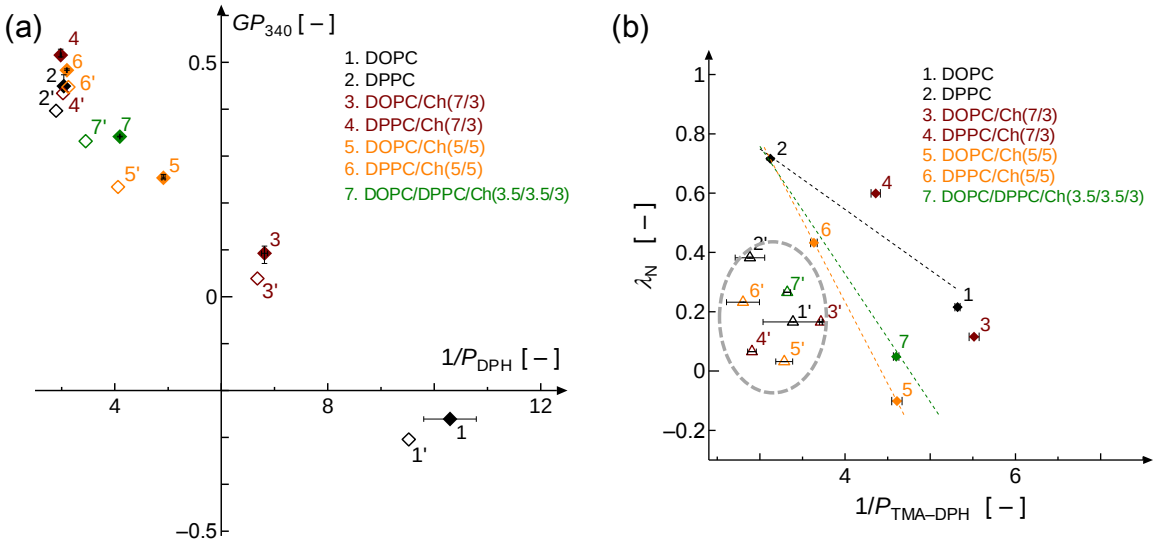


Fig. 3-8 Transition of membrane properties by the adsorption of L-His. (a) Cartesian diagram analyzed by Laurdan and DPH before or after adsorption of L-His. (b) Correlation diagram of surface membrane properties before or after adsorption of L-His. The dotted circle showed the area of plots after adsorption. In both diagrams, numbered plots before and after adsorption of L-His are defined in the inset, and the plots after adsorption are described in primed numbers.

2.8–3.8 in $1/P_{TMA\text{-}DPH}$ after L-His adsorption, even in the initial surface properties (**Fig. 3-8(b)**). This result revealed that L-His binding at the surface region of liposome membranes could induce the convergence of surface-membrane properties despite the initial phase state. Therefore, our results supported the previously-suggested hypothesis that L-His adsorption occurred in two steps (insertion and enhancement) during the simultaneous change of membrane property. Furthermore, it is considered that the variation of surface hydrophobicity is more effective than that of surface fluidity because $q_{L\text{-}His}$ indicates larger values in liposome membranes which show the λ_N in the fixed area of 0-0.4. This suggestion can imply the adsorption model that the surface hydrophobicity of liposome membranes is regulated by the slight partition of amino acids at first, and then, the effective "trapping" of amino acids results in the increase of adsorption with decrease of surface membrane fluidity (**Fig. 3-9**). As for the phase state of liposome membranes, high ee value after 24 hand 48 h was obtained in ternary liposomes with separated l_o phases as dispersed microdomains on the membrane surface (**Fig. 3-10**).

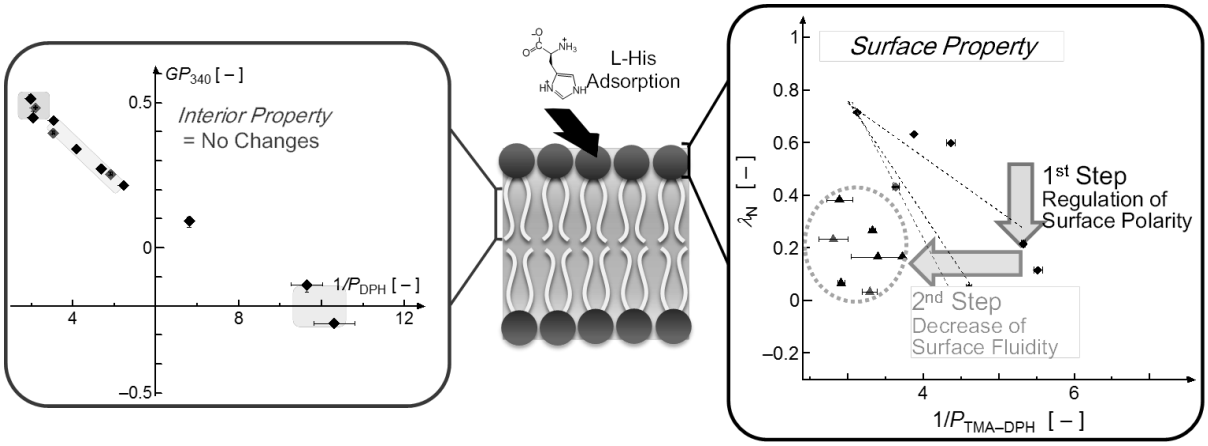


Fig. 3-9 Adsorption steps described in the diagrams of interior and surface membrane properties.

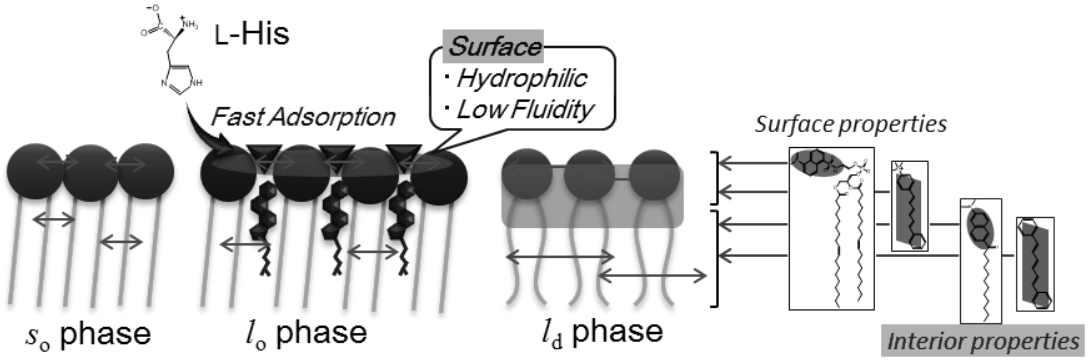


Fig. 3-10 Conceptual illustration of fast L-His adsorption in Ch containing phase.

3.5 Design of DOPC/DPPC/Ch Ternary Liposomes to Induce Molecular Recognition Function

Based on the above results, detailed investigation of ternary liposomes can play an important role for the understanding of molecular recognition function of liposome surface. The method for possible design of the liposome membranes with heterogeneous ones was further investigated based on the phase diagram of the ternary components membrane. In reference to the previous report (Cicuta et al., 2007), a phase diagram of DOPC/DPPC/Ch ternary liposomes is shown in **Fig. 3-11**. The detailed investigations is conducted focusing on the $l_o + l_d$ phases and its boundary.

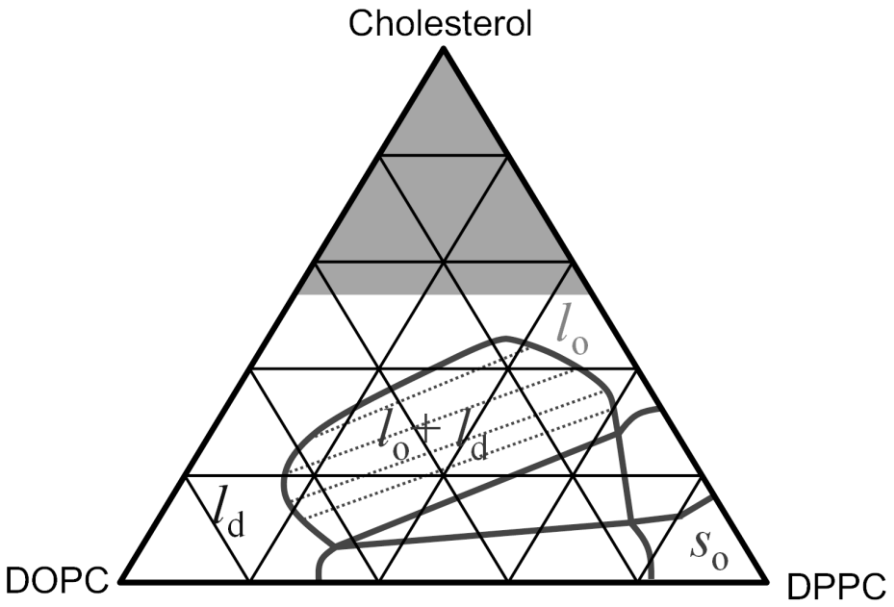


Fig. 3-11 Phase diagram of DOPC/DPPC/Ch ternary liposomes. Solid line shows the phase boundary and dotted lines showed the tie line in reference to the previous report (Cicuta et al., 2007).

3.5.1 Inner and Surface Membrane Properties in Ternary Liposomes

Figure 3-12(a) shows the phase diagram of DOPC/DPPC/Ch liposomes at 20 ºC, describing the phase boundaries as purple lines. In this diagram, ternary liposomes prepared in the above experiments (A, B, and C; **Fig. 3-12(a)**) existed at l_o+l_d heterogeneous phases, while all binary liposomes existed at homogeneous-ordered (s_o) or disordered phases. Although there were other findings that micro-domains at nanometer sizes could be formed in some binary liposomes, such as DOPC or DPPC liposomes (Suga et al., 2013), the Cartesian diagram of the membrane properties was similar to previous results. **Figure 3-12(b)** also indicated the existence of other ternary liposomes with different components in several phase states (D: l_d phase; E: l_o phase; F: l_o+l_d phase; **Fig. 3-12(a)**), showing that they must have the corresponding properties with the phase state. It has been already shown that the plot of surface properties revealed the specific distribution of several ternary liposomes, i.e., those categorized as $\mathbf{a_1}$ with another slope of the trend line (**Fig. 3-12(c)**). In this diagram, D and E were distributed regarding B as the intersection point, which was involved in the parallel direction of tie line. Additionally, F was plotted on line $\mathbf{a_1}$ due to the similarity of the phase ratio as a vertical direction of the tie line. It was found that surface properties of ternary liposomes depended upon the ratio of each phase, as well as Ch ratio.

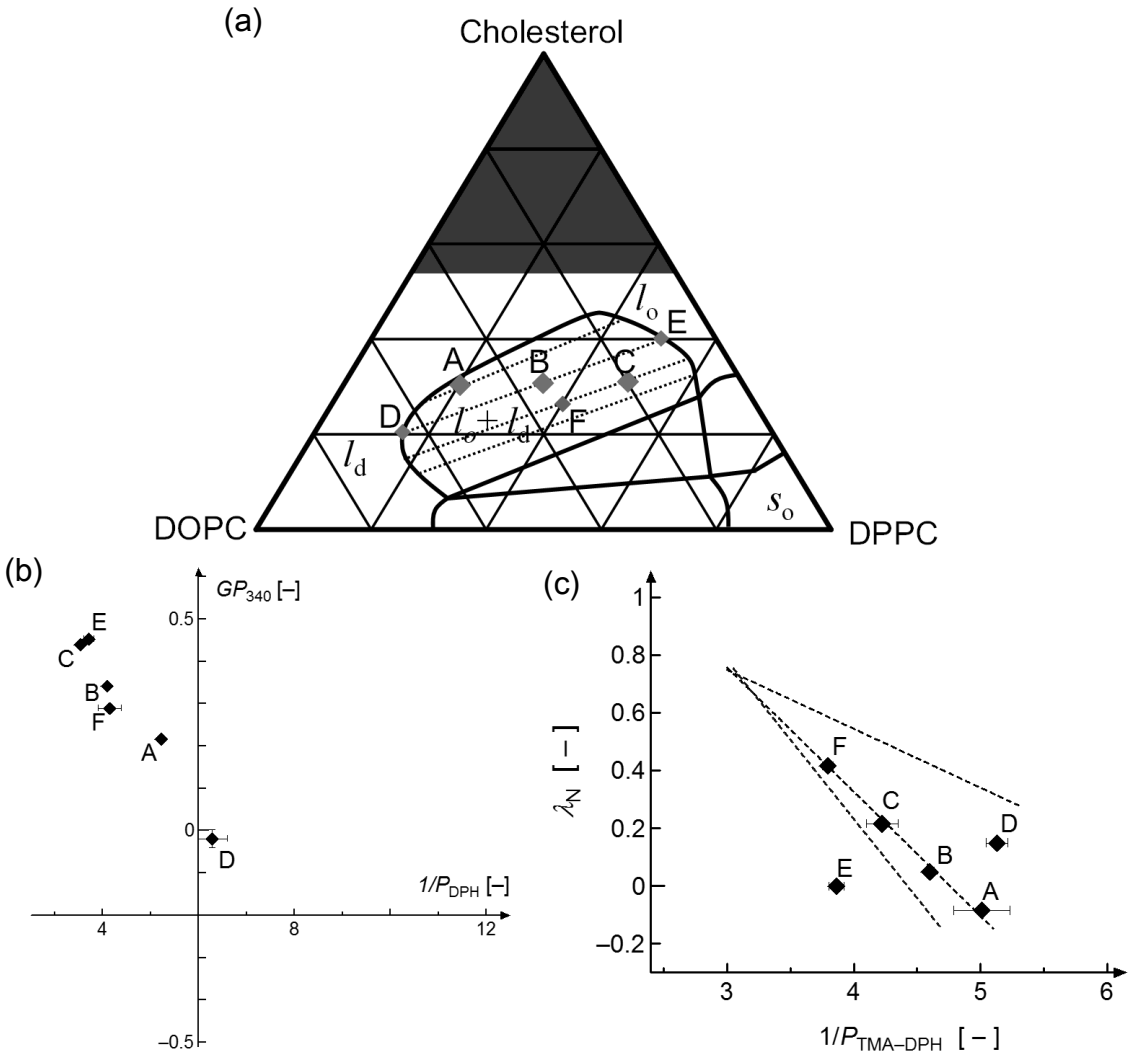


Fig. 3-12 Evaluation of membrane properties in ternary liposomes. (a) Phase diagram of DOPC/DPPC/Ch ternary liposomes. (b) Cartesian diagram analyzed by Laurdan and DPH in several ternary liposomes. (c) Correlation diagram of surface membrane properties. All capital indicators are corresponded to components described in phase diagram.

3.5.2 Effects of Phase State for Adsorption in Ternary Liposomes

Figure 3-13(a) shows the $q_{\text{L-His}}$ values in several ternary liposomes, indicating the low adsorption of His for the liposomes in D and E, on the contrary, high adsorption of His for the liposome in F. Although single-phase liposomes in D and E, showed the lower $q_{\text{L-His}}$ values after 24- and 48-h incubation relative to heterogeneous-phase liposomes, L-His adsorption in l_o phase (E) was more efficient than that observed in l_d phase (D), indicating that the ordered phase preferably influenced on the adsorption of Ch-containing liposomes. In addition, because the $q_{\text{D-His}}$ values in the corresponding liposomes was negligible (**Fig. 3-13(b)**),

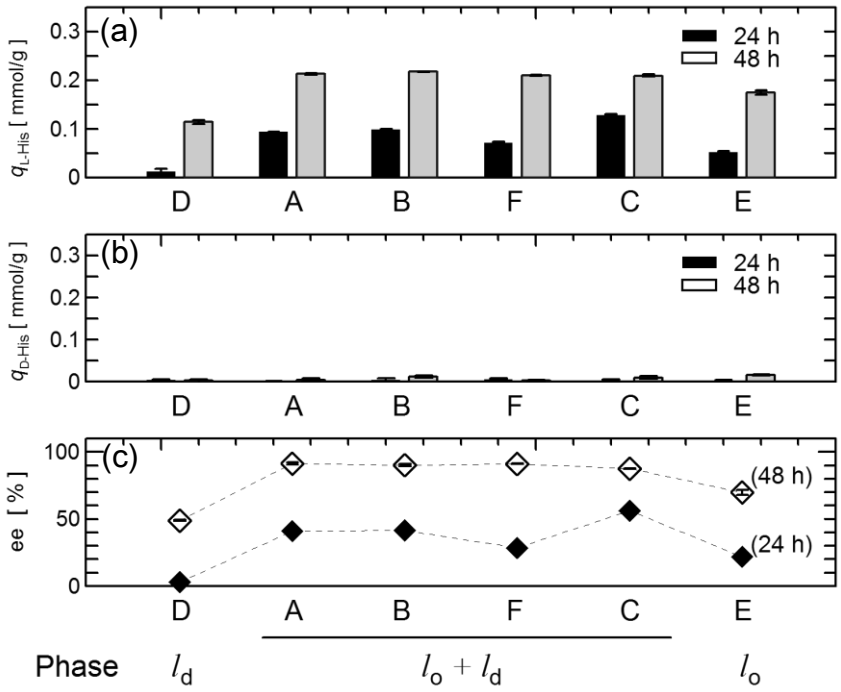


Fig. 3-13 Adsorption and chiral recognition behavior of His in several ternary liposomes mentioned in **Fig. 3-12**. (a) Adsorption amount of L-His ($q_{L\text{-}His}$) in 24 or 48 hours of incubation. (b) Adsorption amount of D-His ($q_{D\text{-}His}$) in 24 or 48 hours of incubation. (c) Percent of His enantiomer excess (ee) in several liposomes. Phase state described below the diagrams was estimated from phase diagram in **Fig. 3-12(a)**.

such differences in $q_{L\text{-}His}$ can directly reflect the ee after 24- and 48-h incubation (**Fig. 3-13(c)**). The ee values in the liposomes forming micro-phase segregation were found to be higher, indicating that the high chiral selectivity of hydrophilic molecules can be attained in heterogeneous liposomes. In heterogeneous liposomes, a phase boundary includes the line tension derived from topological discontinuity. In terms of a spherical shape of liposomes, the mismatch of each phase also occurs in the phase boundary (**Fig. 3-14**). Such a mismatched region is thought to induce the attraction of L-His molecules because of the effect of line tension, resulting in the fast adsorption and high chiral recognition. Therefore, the design of domain formation is effective in controlling the phase boundary that can induce efficient adsorptions, rather than in controlling the phase state itself.

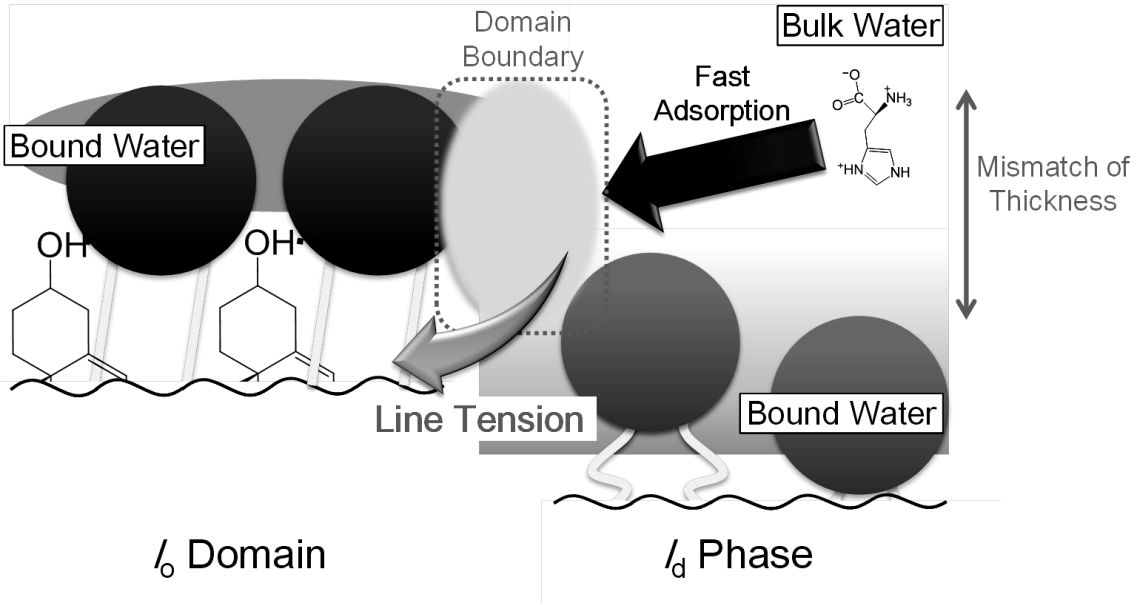


Fig. 3-14 Conceptual illustration of domain boundary formed by separated phases in liposome membranes.

3.5.3 Effects of Domain Edge in Adsorption and Chiral Recognition

In terms of phase state, heterogeneous liposomes commonly indicated high $q_{\text{L-His}}$ value after 24 h incubation. As for the micro-domain formation, the diameters of the micro-domain on prepared heterogeneous liposomes (d_{domain}) were estimated by the fluorescent resonance energy transfer (FRET) between 1-myristoyl-2-[12-[(5-dimethylamino-1-naphthalenesulfonyl)amino]dodecanoyl]-snglycero-3-phosphocholine (DAN-PC) and dehydroergosterol (DHE) in the previous report (Brown et al., 2007). Therefore, the domain length per liposome perimeter (L_{domain}) could be calculated from following equation:

$$L_{\text{domain}} = 4 \times X_{lo} \times (d_{\text{lip}} / d_{\text{domain}}),$$

where d_{lip} and X_{lo} represent a diameter of liposomes (100 nm) and an area ratio of l_o phase estimated by tie-lines in **Fig. 3-12(a)**, respectively. The above equation can further be transformed into the following equations:

$$l = L_{\text{domain}} / d_{\text{lip}} ,$$

that show the specific boundary length (l) regarded as the total domain length per liposome surface area. Considering this equation, l can be regarded as the parameter analogized to the specific surface area with the decrease of one order. The estimation of l is supported by the reported results that domain size may increase as the ratio of l_o phase in liposomes of 100 nm

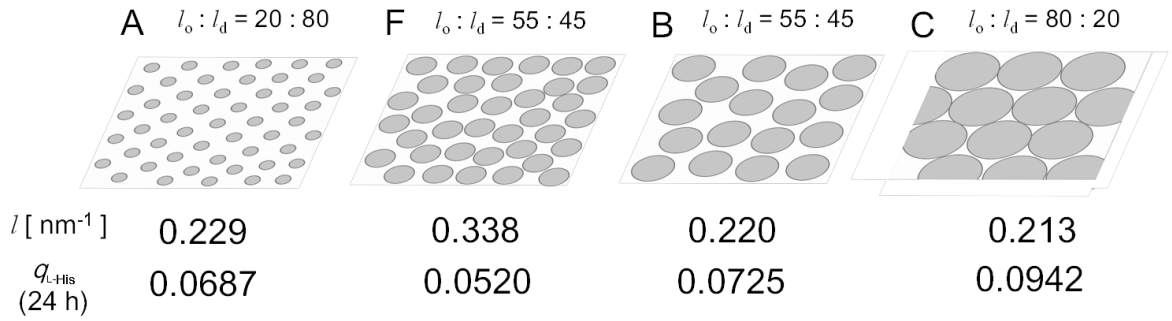


Fig. 3-15 Illustration of domain formation and profiles in several ternary liposome membranes. A, F, B and C indicating the composition described in **Fig. 3-12(a)**. Circle area and background represent the l_o and l_d phase, respectively.

diameter (Suga, et al., 2013). In comparison with the l value of A, B and C (**Fig. 3-15**), it is possible that the adsorption process becomes faster with the decrease of l value while the adsorption was low at the condition F with higher l value. As compared with the $q_{L\text{-His}}$ of l_d or l_o phase with no domains, it is possible that the existence of line tension can contribute to the adsorption in earlier stages (**Fig. 3-16**). However, in the situations of smaller or larger

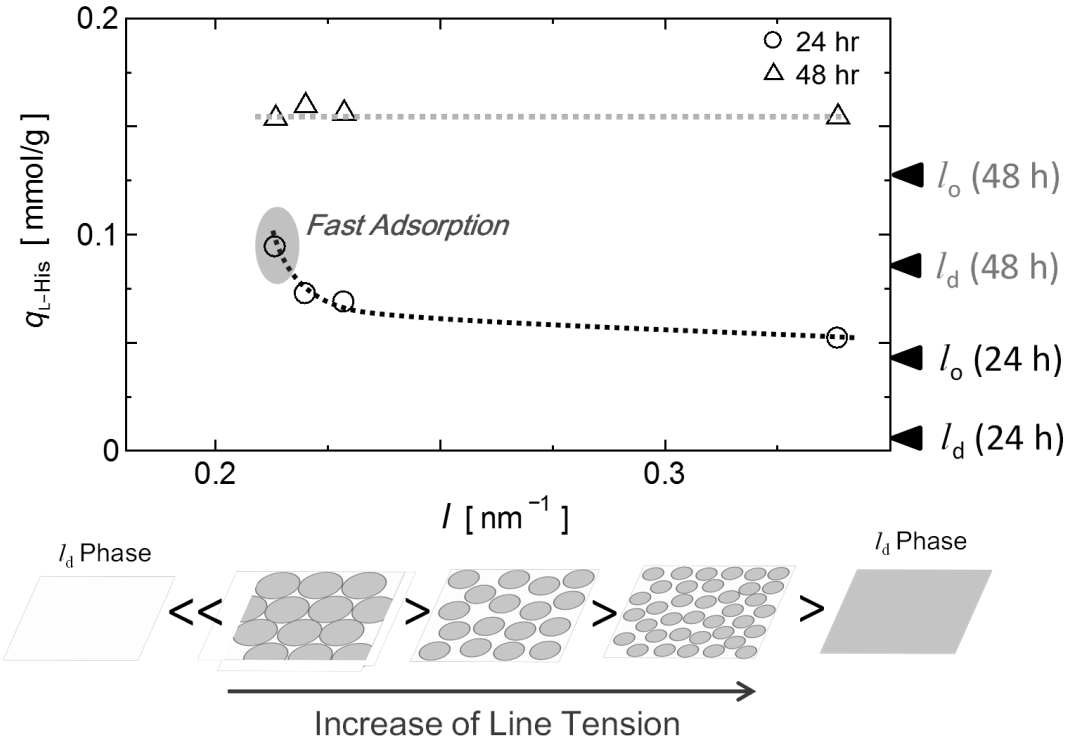


Fig. 3-16 The $q_{L\text{-His}}$ of several l values in 24 h. The comparison of $q_{L\text{-His}}$ with each phase state is illustrated below the diagram.

values of *l*, the liposome membranes become unstable state because of the larger line tensions, resulting in the decrease of $q_{\text{L-His}}$ in 24 h. Considering the relationship between *l* and $q_{\text{L-His}}$ in 24 h, it is implied that there is the suitable range of *l* inducing the fast adsorption of L-His, that is, efficient molecular recognition. Considering this previous findings and our results mentioned above, it is hypothesized that binding of L-His can be stabilized "at the inter-phase" of larger domains bearing weak line tension, inducing the efficient adsorption. Therefore, these findings can contribute to the understanding the formation of electrostatic interactions among the dipoles at the phosphate region of lipid molecules and may afford the hydrophobic environment of the membrane surface for small molecules.

Based on the results of adsorption behavior in several liposomes, the design of liposome membranes for the enhancement of the adsorption is suggested (**Fig. 3-17**). The highly hydrophilic surface in the initial property of liposome membranes can induce the fast adsorption because of the preferable partition of amino acids in liposome membranes. Provided that the liposomes in l_{d} phase show the low adsorption due to the unstable formation of the interactions derived from high membrane fluidity. Liposomes containing Ch are suitable for the enhancement of adsorption because Ch molecules can change the surface region into hydrophilic state. In the surface region of liposome membranes, membrane

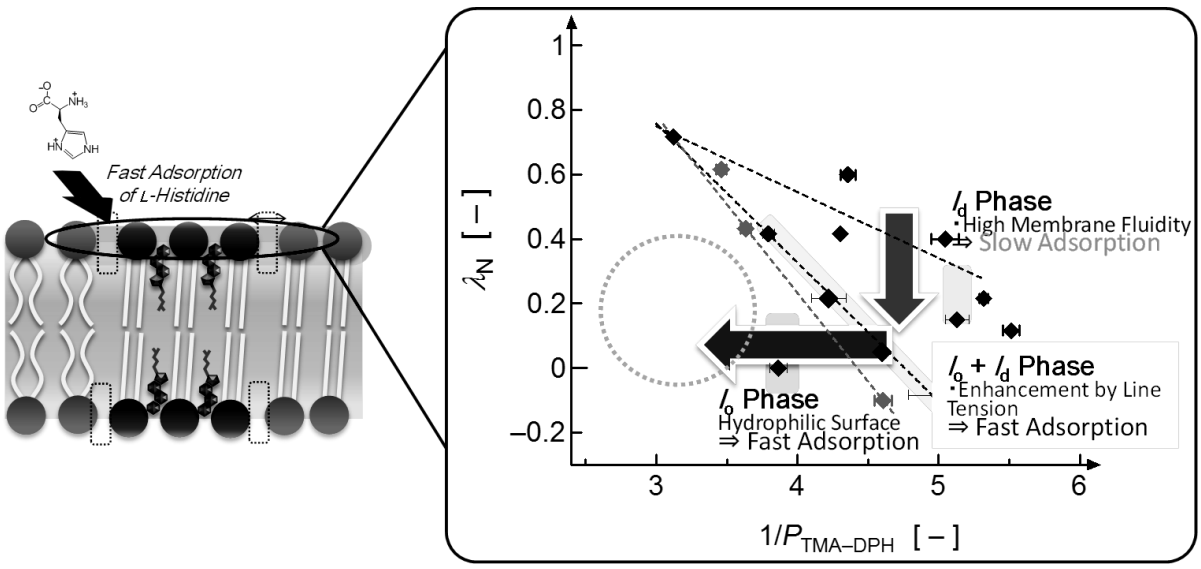


Fig. 3-17 Overview of liposome membrane design for efficient molecular recognition on the basis of the diagrams about interior and surface properties.

fluidity decrease as the increase of adsorption of amino acids. It is considered that such variation may be derived from the formation of interactions with the rearrangement of phospholipid assemblies, resulting in the adsorption formed by plural phospholipids with high molecular recognition. In the case of liposome membranes forming domains, faster adsorption is induced by the effect of line tension in addition to the specific surface property in the boundary of domains. Since the appropriate domain size is implied in the above result, the length of domain in liposome membranes should be considered in the design of liposomes for efficient adsorption.

4. Summary

The analysis of surface membrane property provided valuable information relating to the molecular recognition in liposome membranes. The methodologies for membrane property analysis are summarized in **Table 3-1**. While Catesian diagram of interior membrane property indicated the averaged information of phase state in whole liposome membranes, newly-developed diagram of the correlation of two surface properties showed the discrimination of liposomes that contain Ch in large ratio or that form heterogeneous phase. These specific properties also influenced on the adsorption of L-His, resulting in the induction of fast adsorption on the liposomes with low membrane fluidity and polarity at the surface region. Besides, the surface properties were found to be changed to be such states after the adsorption of L-His and these phenomena may imply the significant role of surface membrane property. Especially in the case of ternary liposomes containing Ch, the fast adsorption was induced only in the lipid composition forming heterogeneous phased liposomes. Among them, a faster adsorption was observed by some kinds of liposomes and was not affected by line tension, but the edge of domains, implying the relation to interactions forming at the boundary of different phases on the liposome membranes.

Table 3-1 The comparison of several methods for membrane property analysis

Target	Methods	Analysis
Surface Membrane Property	Correlation diagram of surface hydrophobicity (Dansyl-DHPE) and membrane fluidity (TMA-DPH)	Evaluation of the effect for molecular recognition of hydrophilic molecules in liposome membranes (This work)
Interior Membrane Property	Cartesian diagram of membrane hydrophobicity (Laurdan) and fluidity (DPH)	Evaluation of phase states including phase separation in nano-order liposomes (Suga et al., 2013)
Bound Water in Membranes	Dielectric dispersion analysis in 0.1-5 GHz	The depth of inserted proteins in liposome membranes and its effect on hydration (Takada et al., 2013)
Phase Transition Behavior	Differential scanning calorimetry	The effect of mixing cholesterol in liposome membranes for phase transition temperature or enthalpy (Arrais et al., 2007)

The scheme of liposome membrane design for the molecular recognition is shown in **Fig. 3-18**. The adsorption and chiral selectivity of several amino acids was observed on the liposome membranes with the relation in the property of its side chains, assuming the high molecular recognition through the multiple interactions with phospholipid molecules. Such a process that the guest molecule form interactions with host can be specifically discriminated from the non-selective adsorption, for example, the case of the PPL adsorption governed by electrostatic interactions with opposite-charged phospholipids. As for the mechanism of selective adsorption of amino acids in liposome membranes, the variation of membrane property, especially at the surface region, is suggested during the progress of the adsorption process, implying the possibility of the rearrangement of assembled states. The above plausible model is also involved in the result that no chiral selective partition of Trp is observed in the emulsion phase formed by solvent-water-lipid system. Although the chiral selective adsorption of amino acids requires the long-time incubation due to the stepwise adsorption, the regulation of surface property of liposome membranes, such as mixing of Ch, shows the possibility of inducing the faster adsorption. Furthermore, the enhancement of adsorption is also observed in the liposome membranes possessing segregated phases, which were characterized based on the relationship between the phase state and the surface property of liposome membranes. The effect of forming domains is notable in the fast adsorption of L-His. In spite of the positive effect of existing domains, it is assumed that the contribution of line tension induces the negative effect in the adsorption of L-His. While the phase boundaries of liposome membranes affect the adsorption via the specific assembly, such as the lateral mismatch of phospholipids and Ch molecules, it is possible that large line tension induces the instability of L-His bindings. Based on the comprehensive consideration of findings in chapter 1, 2 and 3, consequently, a scheme to design the liposome membranes for the selective recognition of various target molecules can finally be proposed (**Fig. 3-18**).

The mechanism of the molecular recognition induced by the liposome membranes is suggested based on several aspects of investigations. This molecular recognition is important in the application of liposomes for optical resolution (chiral separation) processes. The design of continuous process is reasonable because such a molecular recognition induces the dynamic behavior in adsorbed molecules and membrane property itself. It is therefore expected that the liposome membrane with adsorbed amino acids can be also applied for the

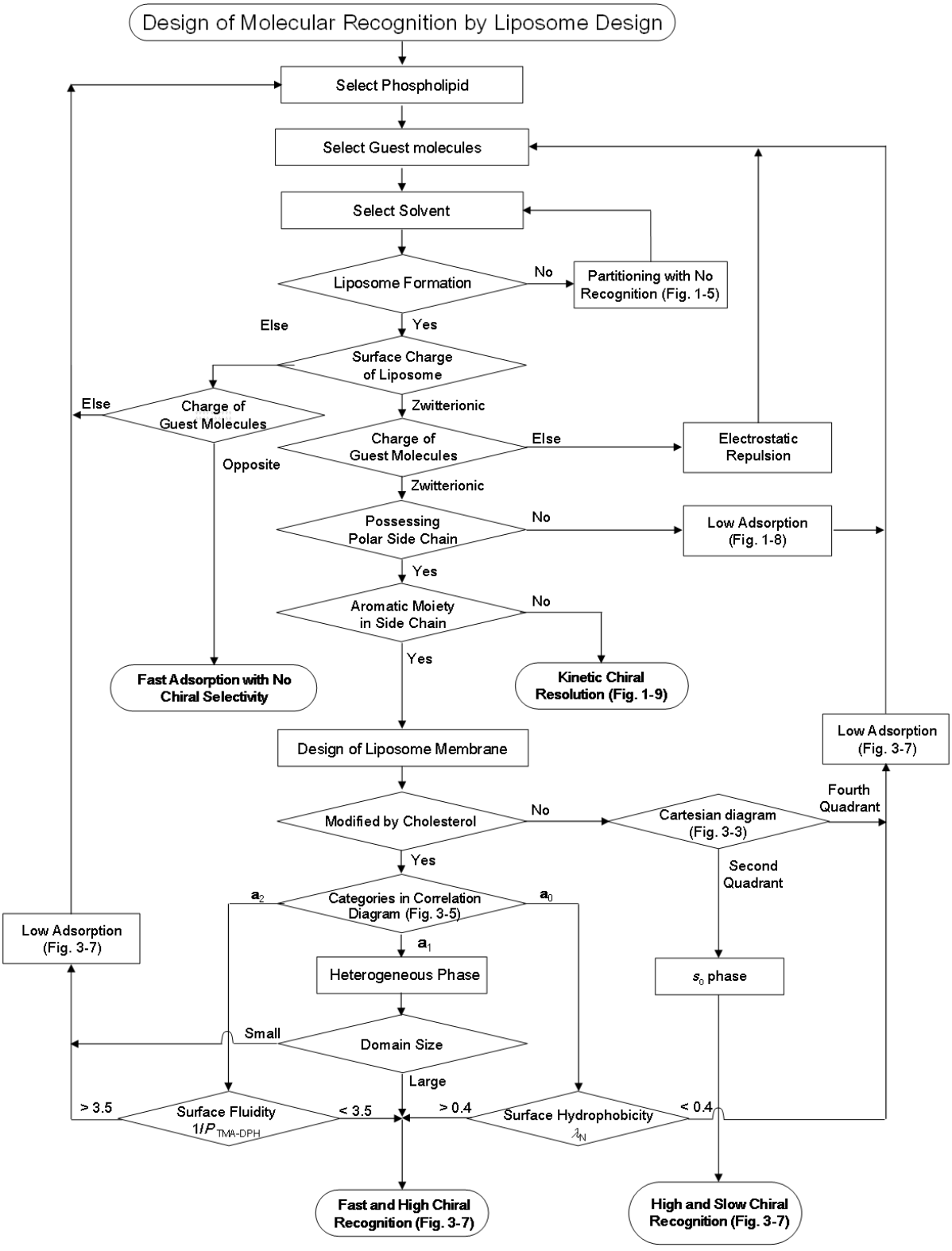


Fig. 3-18 Scheme of design of the molecular recognition.

conversion process in aqueous solution. Despite the advantages of the liposome membrane for the chiral recognition, the liposomes suspended in aqueous phase are regarded as unstable, as compared to the functional ligands immobilized in the solid surface. Hence, it is required to develop the techniques of immobilizing liposomes to fully utilize its functions. The case studies as an extension of the proposed strategy on membrane design will be described in the following chapter 4.

Chapter 4

Application for Separation and Conversion Process by Liposomal Membrane System

1. Introduction

There are several reports on the use of liposome membranes as a platform of chemical conversion. In such reports, the main purpose of using the liposomes is to solubilize the hydrophobic substrates in water phase and to induce asymmetric catalyst function by utilizing the ordered chiral environment of the membranes. The previously-reported results revealed the possible functions of liposome membranes as the platform of molecular recognition. In addition to the recognition function, the specific hydrolysis for L-form enantiomer has been performed in lipid vesicles (Ueoka et al., 1986). It is generally known that the "recognition" of substrate on the active site of the enzyme is the most important step for the effective catalytic reaction because it varies the energy profiles of the reactant system and, as a result, the reduction of activation energy. It is therefore considered that the molecular recognition function of liposome membranes could be extended to the effective chemical reaction process on their surface, with a similar way of enzyme strategy.

One of possible extensions to the chemical conversion is the homochiral polymerization of amino acids on the membranes. In biological system, amino acids form peptides and proteins through their polycondensation in the ribosomal apparatus. In this case, it is known that all of such products are constructed by L-amino acids. Based on the results obtained in previous chapters, it is considered that the selective adsorption of L-amino acids could be carried out in liposome membranes. It is also expected that the appropriate design of the liposome membranes could provide us the improvement of the effective polymerization of amino acids in liposome membranes in reference to the effect of enhancing reaction in the interface of lipid assemblies.

From the viewpoint of practical application, the immobilization of liposome membranes in various carriers is required to effectively utilize the "membrane platform" for

the molecular recognition and conversion as an intact self-assembly structures. Various immobilization methods are classified, focusing on their immobilization principle as shown in **Fig. 4-1**. Depending on the binding strength, the functions of liposome membranes can be regulated. For practical use of the liposomes, the liposome-incorporated matrices become useful candidates: liposomes-immobilized in porous gel (Lundahl et al., 1991; Yang et al., 1998; Yamanaka et al., 1997; Khaleque et al., 2003; Yang et al., 1988), liposomes-entrapped hollow fiber (Sugaya et al., 2009), and liposomes-incorporated hydrogel (Liu et al., 2012; Ditizio et al., 1998). In these cases, the liposomes are immobilized through the covalent bonding, antigen-antibody interaction, avidin-biotin specific interaction, hydrophobic interaction, and physical entrapment. Although these preparation methods have been developed, there are still some technical problems, such as lower amounts of immobilized liposomes, multistep operation including chemical reactions, poor separation efficiency, and employment of derivatized lipid.

According to the findings clarified in previous chapters 1, 2 and 3, the mechanism of molecular recognition induced at the surface region of liposome membranes suggests the strategy of suitable process of recognition and separation by liposome membranes. Especially in the case of the recognition of amino acids, the variation of surface hydrophobicity was observed during the adsorption of L-amino acids. In addition, since the variation of such properties

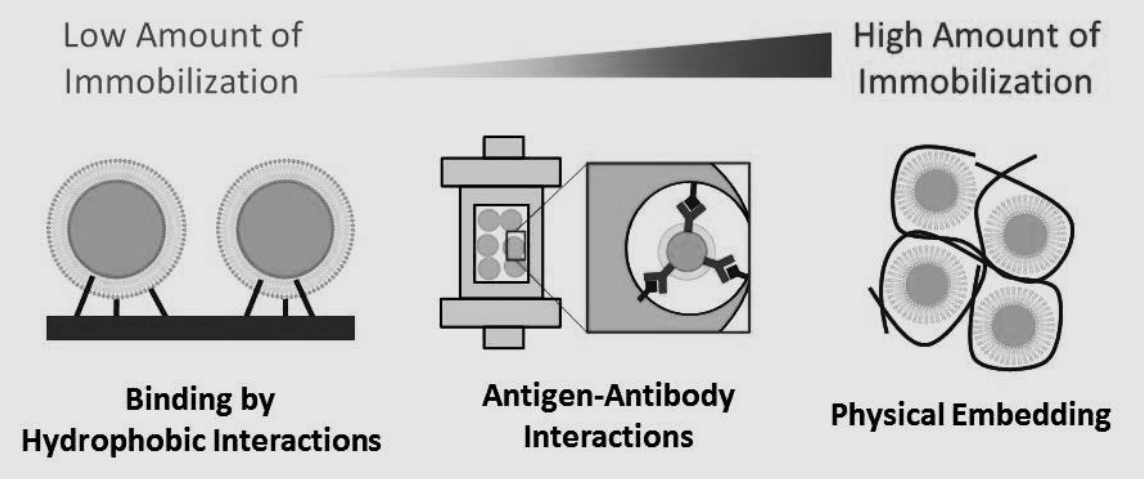


Fig. 4-1 Several methods about immobilization of liposomes.

was assumed to remain the slight effect in surface regions according to the thermodynamic analysis, it is possible that the liposome membranes can preferably promote the reaction in hydrophilic regions. Meanwhile, to achieve the utilization of the above liposomal function, the moderate effects for surface property or bound water are important for the immobilization of liposomes. The necessity of this condition is also indicated by the results that the interactions in high molecular recognition are relatively weak. Consequently, direct embedding methods is suitable for the application of liposomes for molecular recognition, and then, the development of adsorption amount is required for the practical use.

In this chapter, the application of liposome membrane systems is demonstrated in the conversion reaction and optical resolution, based on the molecular recognition of the liposome membranes described in chapter 1, 2, and 3, (**Fig. 4-2**). The promotion of oligomerization of amino acids was investigated by partition in liposome membranes with molecular recognition of L-His. In this reaction, the behavior of conversed substrates was considered in reference to previous reports about the aqueous reactions (Kunishima et al., 2005). As for the application for separation process, a continuous process is examined by liposomes accumulated in the ultrafilter. Besides, the immobilization of liposomes is developed by the embedding in several hydrogels, which is analyzed by the direct observation and the Raman spectroscopy. Adsorption behavior and chiral separation of Trp were evaluated in prepared liposome immobilized hydrogel (LI-gel).

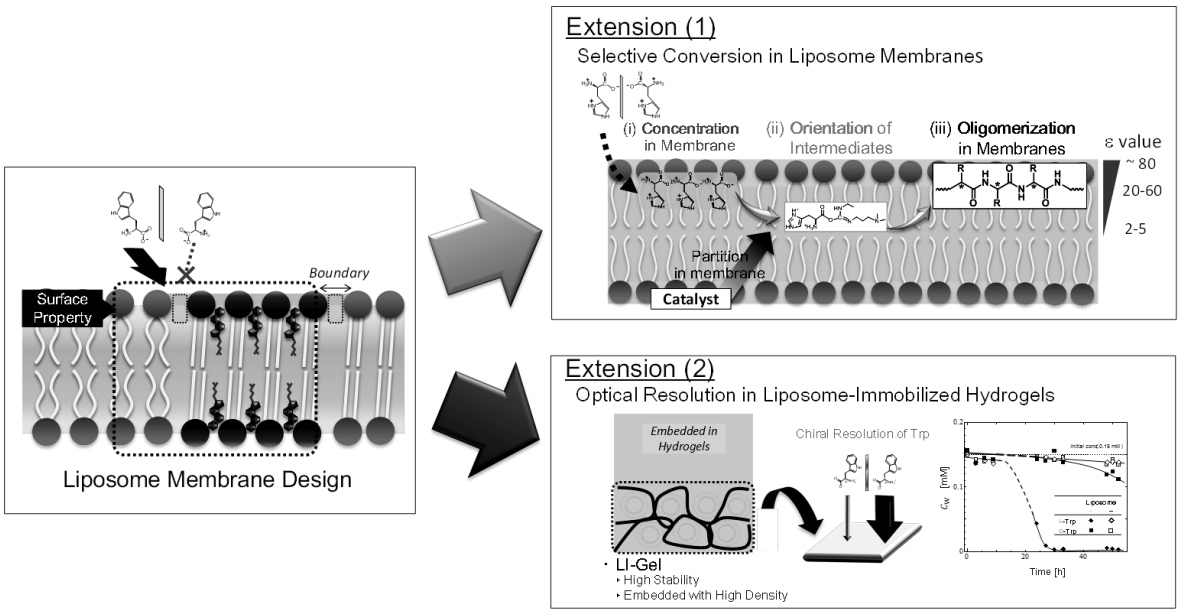


Fig. 4-2 Conceptual illustration of this chapter.

2. Materials and Methods

2.1 Materials

Several phospholipids, such as 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Amino acids, such as L-Trp, D-Trp, L-His and D-His, were purchased from Peptide Institute (Suita, Osaka, Japan). All amino acid reagents were over 98% purity of enantiomers. 1-Hydroxybenzotriazole (HOBt) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). For the preparation of LI-gel, acrylamide (AAm), *N,N'*-methylenebisacrylamide, ammonium persulfate, calcein, poly(oxyethylene *p*-*t*-octylphenyl ether) (Triton X-100), and other chemicals were also purchased from Wako Pure Chemicals Industries, Ltd. (Osaka, Japan). Agarose was purchased from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals were used without purification. Ultrafiltration membranes (molecular weight cut off: 50 kDa) were purchased from Toyo Roshi Kaisha, Ltd. Ultrapure water was produced using a Direct-Q 3 UV system (Merck, Darmstadt, Germany).

2.2 Liposome preparation

Liposomes were prepared by using a freeze–thaw extrusion method. Briefly, a chloroform solution of lipids was dried in a round-bottomed flask under vacuum with a rotary evaporator to prepare a lipid thin film. The thin film was hydrated with ultrapure water at room temperature to prepare a vesicle suspension. The vesicle suspension was frozen at −80 °C and thawed at 50 °C to enhance the transformation of small vesicles to large multilamellar vesicles (MLVs); this freeze–thaw cycle was performed five times. The MLVs were used to prepare smaller unilamellar vesicles by extruding the MLV suspension 11 times through two layers of polycarbonate membranes, with mean pore diameters of 100 or 200 nm, using an extruding device (Liposofast; Avestine Inc., Ottawa, ON, Canada). The obtained unilamellar vesicles were concentrated by centrifugation at 135,000 ×g for 2 h at 4 °C. In preparation of calcein-entrapped POPC liposomes, a calcein solution (1 mM) was added in the hydration of thin lipid film.

2.3 Adsorption of His on POPC Liposome Membrane

L-His or D-His (40 mM) and POPC liposomes (50 mM lipid) were mixed in ultrapure water and incubated at 25 °C for 72 h. The suspension was then diluted 100-fold and filtered with an ultrafiltration membrane. The His concentration on the liposome membrane was determined using UV spectroscopy (UV-1800, Shimadzu, Kyoto, Japan). A calibration curve was obtained by plotting absorbance vs. His concentration and the amounts of adsorbed L-His (Q_L) and D-His (Q_D) were calculated using the following equation:

$$Q_L \text{ or } Q_D = (C_{ini} - C_{flt}) \times V$$

where C_{ini} is the initial His concentration, C_{flt} is the equilibrium concentration of His in the filtered solution, and V is the volume of the filtered solution.

2.4 Oligomerization of His in Presence or Absence of POPC Liposomes

A mixture of POPC and L-His or D-His was incubated for 72 h, and then His oligomerization was performed at 25 °C for 48 h by adding HOBt (1.0-fold against substrate) and EDC (5.0-fold against substrate) to the POPC suspension, as shown in **Fig. 4-3**. After oligomerization, the supernatant and POPC were separated using an ultrafiltration membrane. The oligomerization of His in the absence of a liposome membrane was conducted under the same conditions but without POPC. The molecular weights of oligo(His) in the reaction using matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) using a Voyager-DE STR instrument (Applied Biosystems, Framingham, MA, USA) equipped with a N_2 laser (337 nm), in linear mode. The locations of His and activated

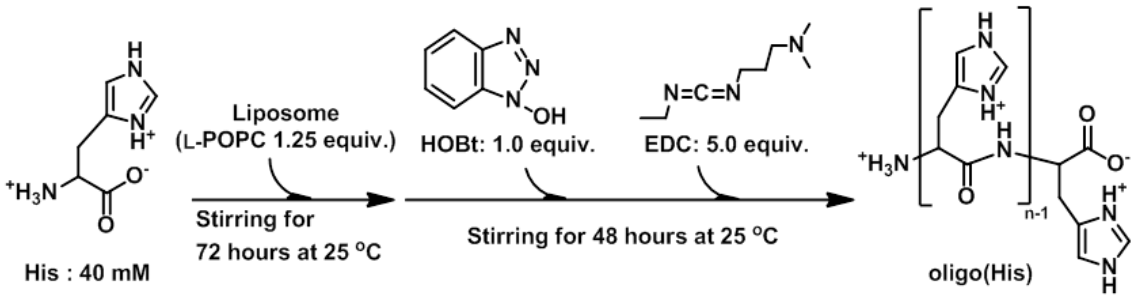


Fig. 4-3 Scheme of adsorption and oligomerization of His with POPC Liposomes. The chemical structures of His and oligo-(His) are given with the expected dominating states of protonation/deprotonation.

intermediates in the POPC membrane were predicted from the log*P* values, which were calculated using ChemBioDraw 12.0.2 (CambridgeSoft Corporation, MA, USA). It is assumed for estimation of log*P* values that the carboxyl group of His is deprotonated and one of nitrogen atom at imidazoyl group would be protonated.

2.5 Preparation of LI-gel

Liposomes of diameter 100 or 200 nm were used to prepare LI-gels. An LI-gel based on poly(AAm) was prepared as follows. Liposomes (lipid: 100 mM) and AAm (1.15 M) were mixed in ultrapure water and degassed for 1 h. This solution was heated at 50 °C and polymerized overnight by the addition of ammonium persulfate (0.48 mM) and tetramethylethylenediamine (2.0 mM). An LI-agarose gel containing 6.0 wt% agarose and liposomes (lipid: 100 mM) was prepared by heating and cooling a mixture of agarose and liposomes. Prepared LI-gel was cut into a rectangle-shape of 20 × 20 mm with 0.75 mm thickness.

2.6 Scanning Electron Microscopy (SEM) Observations Using Plasma Replica Method

The sample structures were examined using the plasma replica method, as previously reported (Iida et al., 2005). A small amount of agarose gel and liposomes immobilized in agarose gel were injected into a cellophane-tape cylinder of diameter 2 mm. The sample was vitrified by dipping in liquid nitrogen (−80 °C). The quenched sample was cracked under vacuum with a cutter, and immediately replicated by plasma polymerization of methane and ethylene (NL-OP50SF, Laser Techno Co., Ltd., Nagoya, Japan). After osmium coating to make it electrically conductive, the replica was examined using SEM (S-5000, Hitachi, Ltd., Tokyo, Japan).

2.7 Investigation of Dynamic Properties Using Raman Spectroscopy

DMPC liposomes were used to determine the dynamic properties of liposomes in a poly(AAm) gel using Raman spectroscopy. The phase transition temperature of DMPC liposomes is 23 °C. The Raman spectra of the liposomes were recorded using a confocal Raman microscope (LabRAM HR-800, Horiba, Ltd., Kyoto, Japan) at 532 nm (YAG, 50

mW), with a 600 grooves/mm grating and a total data accumulation time of 30 s. For each sample, the background signal of the solution was removed and then the baseline was corrected. The packing density of the lipid membrane (Huang et al., 1983), R, was determined using the following equation:

$$R = I_{2880} / I_{2850,}$$

where I_{2880} and I_{2850} are the peak intensities at 2880 and 2850 cm^{-1}, respectively.

2.8 Adsorption of Trp on Liposome Membrane in the Hydrogel

The amount of Trp adsorbed on the liposome membrane in the hydrogel was determined, using 100 mM DPPC liposomes of diameter 200 nm immobilized in a poly(AAm) gel, by incubation in ultrapure water (10 mL) containing L-Trp or D-Trp (0.15 mM) for 48 h at room temperature under stirring. The concentration of non-adsorbed Trp was determined using ultraviolet spectroscopy, based on a calibration curve at 280 nm. The distribution ratio of Trp was determined by assuming that three phases are present, i.e., a lipid membrane phase, hydrogel phase, and bulk water phase. For the hydrogel without liposomes, the distribution constant (K_Z) of Trp between the hydrogel and bulk ultrapure water is calculated using the following equations:

$$K_Z = C_H / C_W ,$$

$$C_{ini}\, V_W = C_W\, V_W + C_H\, V_H .$$

These equations are combined to give

$$K_Z = (C_{ini} - C_W)\, V_W / (C_W\, V_H) ,$$

where C_W is the Trp concentration in the bulk water phase, C_H is the Trp concentration in the hydrogel phase, C_{ini} is the initial Trp concentration, V_W is the volume of the bulk ultrapure water phase, and V_H is the volume of the hydrogel phase. In the case of a hydrogel containing liposomes, the distribution constant (K_{lip}) of Trp between the liposome membrane and bulk ultrapure water is calculated using the following equations:

$$K_{lip} = C_{(lip)} / C_W ,$$

$$C_{ini}\, V_W = C_W\, V_W + C_H\, V_H + C_{lip}\, V_{lip} .$$

These equations are combined to give

$$K_{lip} = (C_{ini}\, V_W / C_W - K_Z\, V_H - V_W) / V_{lip} ,$$

where $C_{(lip)}$ is the Trp concentration in the liposome membrane phase, V_{lip} is the volume of the liposome membrane phase in the LI-gel, and V_H is the volume of the hydrogel phase. The separation factors of L-Trp against D-Trp in the bulk water phase ($\alpha_{(L/D)}$) and on the liposome membrane phase ($\beta_{(L/D)}$) are calculated using the following equations.

$$\alpha_{(L/D)} = C_{W(L\text{-}Trp)} / C_{W(D\text{-}Trp)} ,$$

$$\beta_{(L/D)} = K_{lip(L\text{-}Trp)} / K_{lip(D\text{-}Trp)} ,$$

where $C_{W(L\text{-}Trp)}$ and $C_{W(D\text{-}Trp)}$ are the concentrations of L-Trp and D-Trp in the bulk water phase, respectively, and $K_{lip(L\text{-}Trp)}$ and $K_{lip(D\text{-}Trp)}$ are the distribution ratios of L-Trp and D-Trp, respectively, between the liposome membrane and bulk ultrapure water.

For optical resolution of Trp, a racemic solution of Trp (1 mM) was incubated in ultrapure water (10 mL) with DPPC liposomes immobilized in poly(AAm) gel. The decrease in the Trp concentration in the bulk water phase was determined using circular dichroism spectroscopy (JASCO J-820 SFU spectropolarimeter, JASCO, Tokyo, Japan) at 222 nm.

2.9 Statistical analysis

Results are expressed as mean ± standard deviation. All experiments were performed at least three times. The distribution of data was analyzed, and statistical differences were evaluated using the Student's t-test. A P-value of <5% was considered significant.

3. Results and Discussion

3.1 Scheme for Application of Liposome Membranes Using Designed Membranes

The design scheme of the application of liposome membranes is shown in **Fig. 4-4**. The appropriate selection on the characteristics of the target materials is required to utilize the hydrophobic region of the membrane and highly ordered structure constructed in liposome membranes. The effective suggestions are expected in the application of liposome membranes

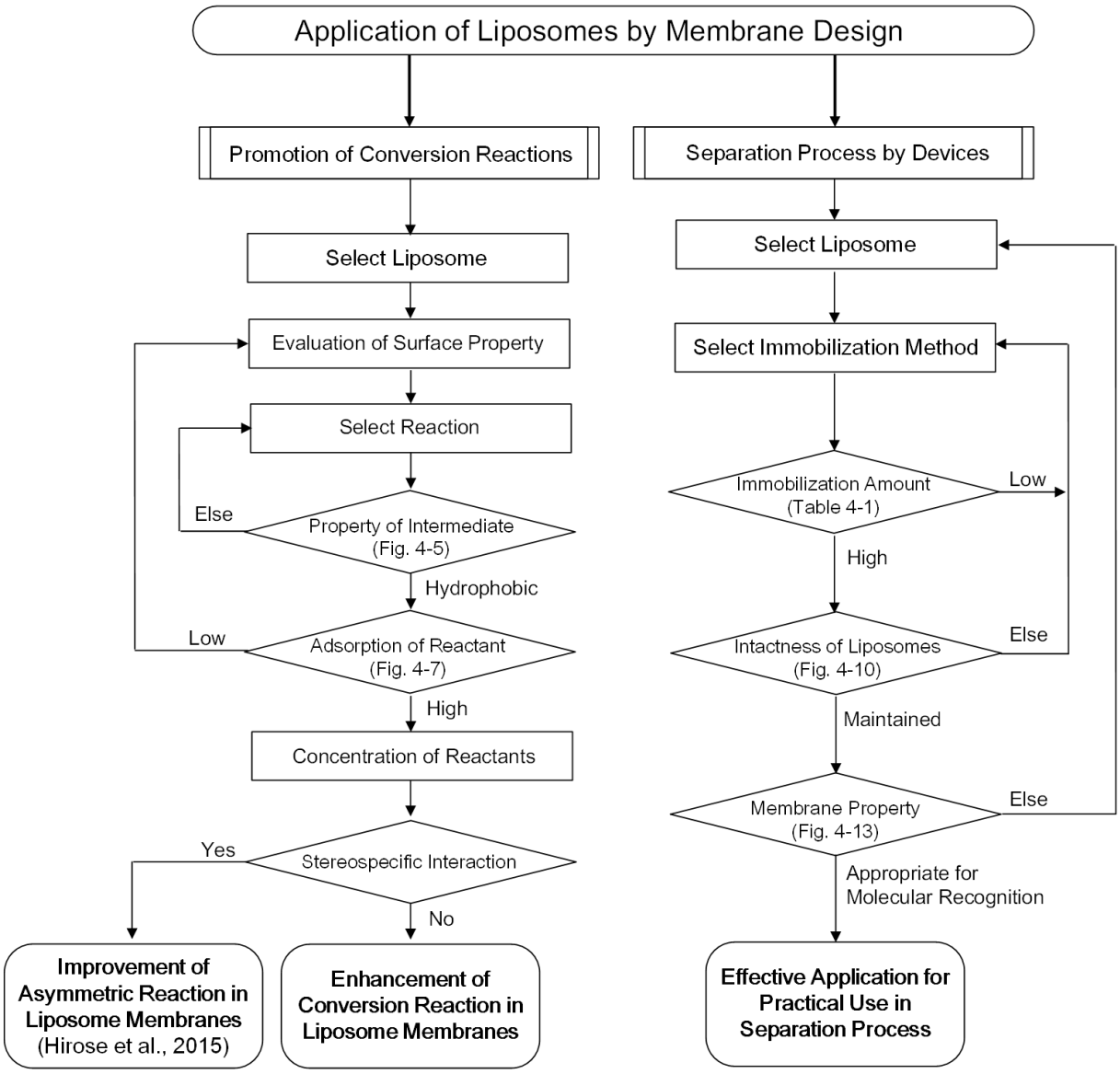


Fig.4-4 Design scheme for the application of liposome membranes.

by means of utilizing the findings about the mechanism of molecular recognition and about the surface properties of liposome membranes.

As for the selective adsorption of several hydrophilic molecules described in the previous chapters, some advantages are expected by using the liposome membranes as the platform of reactions. In the process of polycondansation of amino acids, the polarity of the molecule of reaction intermediate is known to be varied during the reaction. In spite of the reaction conducted in aqueous solution, the Log*P* values of intermediates indicate the positive values, which means less hydrophobic nature of intermediates. By means of utilization of liposome membranes, the formation of such intermediates is considered to enhance their localization at the hydrophobic region of the membrane, as a result, promoting the polycondensation reaction (**Fig. 4-5**). The design of selective conversion is also expected by the specific interactions against the chemical structures of target molecules, such as the accumulation of ionic function by the electrostatic interactions, and as the organized alignment of ring moieties. Thus, the membrane property is thought to be important for the control of polycondensation reactions. In previous chapters, it was revealed that the formation of domains and the concomitant line tension could be key factors to promote the adsorption of L-His. In addition, the surface fluidity and hydrophobicity are considered to be involved in the formation of intermediates because of the transition of polarity to be hydrophobic. Toward the change of characteristics in conversion process, liposome membranes may induce the rearrangement of the lipid ordering in membrane surface based on the findings that the surface property changes during adsorption with several steps.

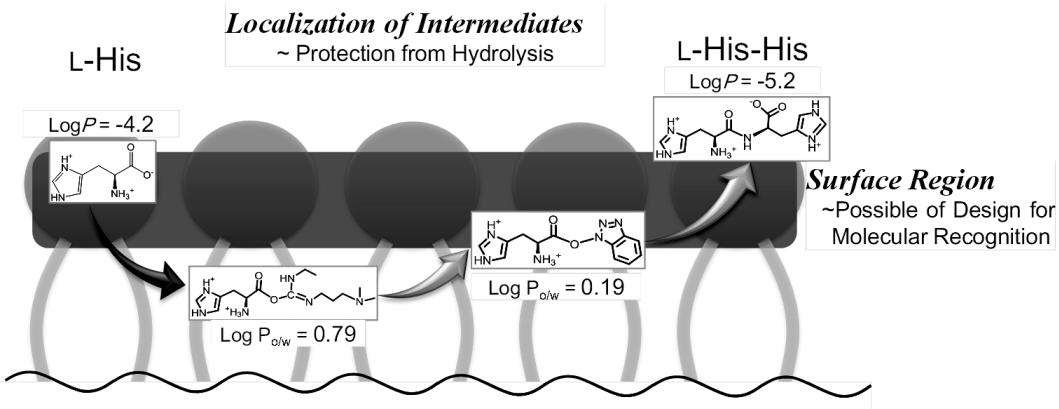


Fig. 4-5 Variation of polarity of molecules during their oligomerization process.

Considering direct embedding of liposomes, the hierarchical structure of the liposome immobilized materials is suggested as shown in **Fig. 4-6**. Such materials are expected to show the effective function in molecular recognition and conversion, because the interactions between phospholipids and amino acids are stereospecific in assembled structures. Hence, the performance of prepared devices can be predicted by that of liposome membranes. In particular, the performance of this device in the recognition of hydrophilic molecules can be controlled by means of the evaluation of surface property of liposomes. On the other hand, the effect of immobilization in liposome membranes can be estimated via the evaluation of membrane characteristics and of adsorption behavior. In reference to the effect of poly(L-lysine) in anionic liposome membranes (Schwieger et al., 2007), the embedding in hydrogels derived from hydrophilic monomer can affect the surface property of liposome membranes, which mean the possibility of the effect of interactions with amino acids. Therefore, the design in surface hydrophobicity or line tension is required for the effective performance of the device.

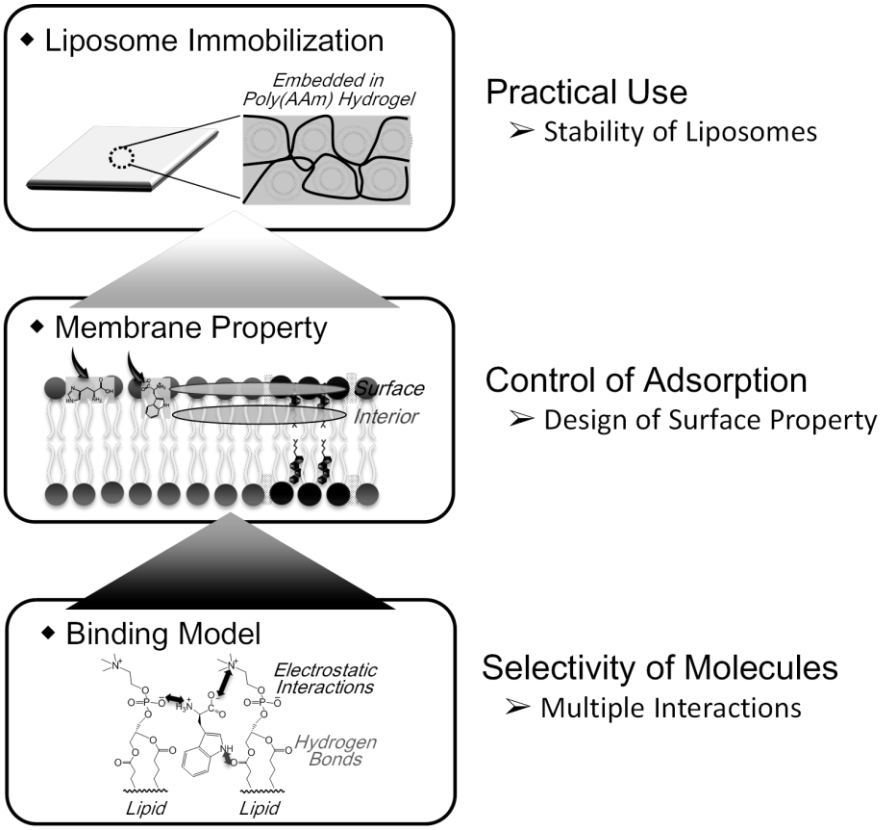


Fig. 4-6 Conceptual illustration of hydrogel matrix immobilizing liposomes.

3.2 Oligomerization of Histidine on Liposome Membranes

To demonstrate the polycondensation of the His adsorbed in liposome membranes, the adsorption behavior of L- or D-form of His was investigated in the high concentration of amino acid as compared with previous experiments. In these conditions, the polycondensation was examined with water soluble initiators. In order to invade reactants easily, liposomes were prepared by POPC phospholipids that form l_d phase in room temperature.

3.2.1 Adsorption and Condensation of Histidine on Liposome Membranes

Figure 4-7 shows the amount of L- or D-form of His adsorbed on the POPC liposomes after incubation for 72 h was 5.0×10^{-6} mol. In contrast, negligible amount of D-His were adsorbed on the POPC liposomes. The total volume of POPC liposome membranes in the sample solution was 5.9 vol%, wherein the adsorbed L-His was condensed. From these results, the concentration of L-His on or within the POPC liposome bilayer membrane ($C_{\text{on membrane}}$) after incubation for 72 h was estimated to be 84.3 mM, using the following equations:

$$C_{\text{ads (on membrane)}} = n_{\text{ads}} / V_{\text{lip}}$$

$$V_{\text{lip}} = (4\pi/3) \times (r_{\text{out}}^{\ 3} - r_{\text{in}}^{\ 3})$$

where V_{liposome} is the volume of POPC liposomes (100 nm diameter), n_{ads} is the amount of His

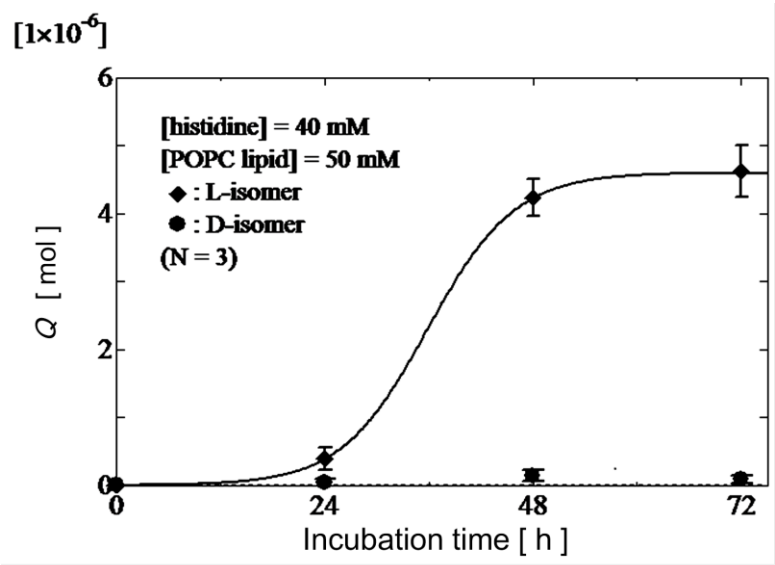


Fig. 4-7 Time-course of adsorption of L-His (Diamonds) or D-His (Circles) on POPC liposomes.

adsorbed on the POPC liposome membrane (5.0×10^{-6} mol), and r_{in} (~46 nm) and r_{out} (~50 nm) are the inner and outer radius, respectively, of the POPC liposomes. Although the adsorption of His in POPC liposomes required long time of incubation, the result also showed high selectivity of L-His as shown in chapter 3. Furthermore, the concentration of His on the POPC membrane was twice as large as that in the bulk solution, i.e., POPC liposomes enantioselectively concentrated L-His on the liposome membrane. These specific properties of the POPC liposomes suggest that they could be used for the specific oligomerization of L-His.

3.2.2 Polymerization Degree of Adsorbed L- or D-Histidine

The oligomerization of L/D-His in the presence and absence of POPC liposomes were determined by using MALDI-TOF MS. **Figures 4-8(a)**, **(b)** and **(c)** show that the oligomerization degrees of oligo(L-His) in the reaction solution including POPC liposomes reached 13 mer. After the reaction solution was treated with ultrafiltration to remove the

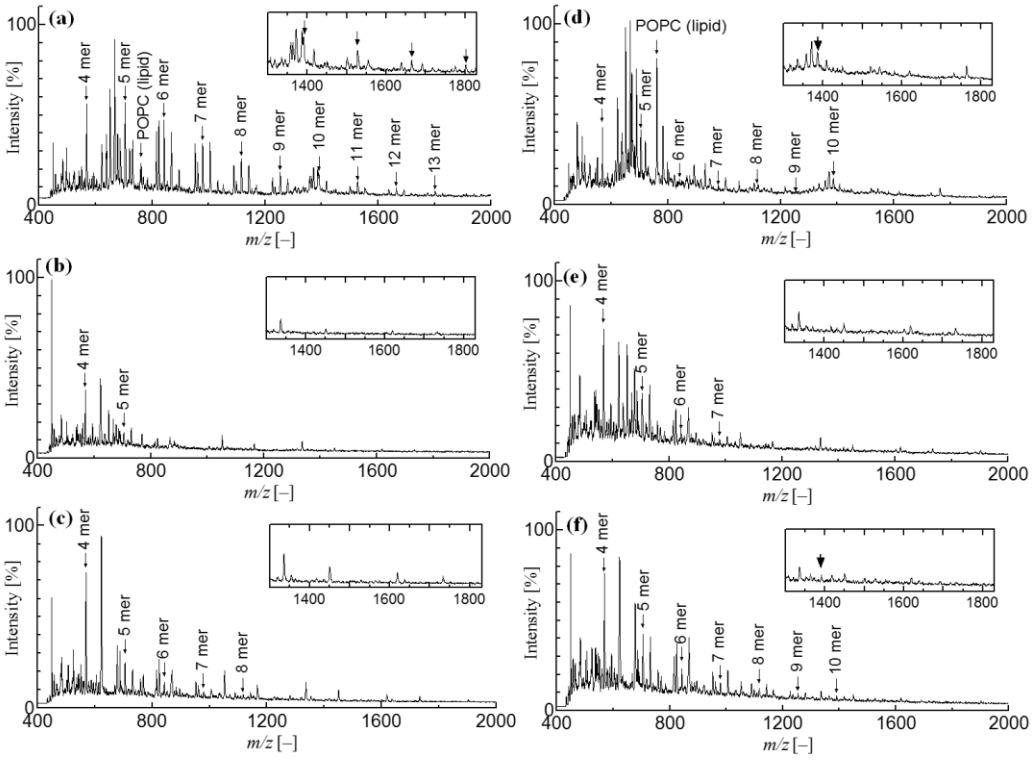


Fig. 4-8 MALDI-TOF mass spectra of oligo(L-His) and oligo(D-His). (a), (d); in reaction solution containing POPC liposomes, (b), (e); in supernatant, (c), (f); in absence of POPC liposomes.

liposome-binding oligomer, the oligomerization degree of oligo(L-His) in the supernatant reached 5 mer. The oligomerization degree in the absence of POPC reached 8 mer. These results indicate that POPC liposomes can promote the oligomerization of L-His and enable longer peptide production. Similar effects of the liposomes have been seen in the previous findings of the oligomerization of Trp in the presence of POPC liposomes (Hitz et al., 2001). In contrast, the oligomerization degrees of oligo(D-His) in the reaction solution, in the supernatant, and in the absence of POPC reached 10 mer, 7 mer and 9 mer, respectively, as shown in **Figs. 4-8(d)**, **(e)** and **(f)**. The lack of significant differences among the oligomerization degrees shows that the POPC liposomes did not greatly affect the oligomerization of D-His, because D-His has no specific interactions with the POPC liposomes and is not adsorbed on them.

3.2.3 Mechanism of Inducing Reaction on Liposome Membranes

The following theoretical model of L-His oligomerization with POPC liposomes was employed to understand the details of the liposomal effect. A theoretical study of the polycondensation of amino acids (Orgel et al., 1998) showed that the average chain length $\overline{n}$ depends on the balance between the rate of chain elongation and the rate of hydrolysis, as shown in following equations:

$$\bar{n} = \sqrt{\Lambda / k_{\mathrm{h}}}$$

$$\Lambda = k_{\mathrm{e}} C_{\mathrm{mono}}$$

where Λ is the elongation rate, k_{h} is the hydrolysis rate constant, k_{e} is the elongation rate constant, and C_{mono} is the concentration of the activated monomer. A longer polymer chain can where Λ is the elongation rate, k_{h} is the hydrolysis rate constant, k_{e} is the elongation rate constant, and C_{mono} is the concentration of the activated monomer. A longer polymer chain can be therefore obtained by using a sufficient concentration of reactants, suppression of hydrolysis, and removal of water from the reaction field. In this oligomerization system with liposomes, the emergent properties of the liposomes, such as enantioselectivity, hydrophobic environments, and fluidity (Walde et al., 2014), are favorable for the formation of longer peptide chain; L-His is enantioselectively concentrated and the fluidity enables molecular interactions, in contrast to solid-phase reactions (Ferris et al., 1996). A hydrophobic environment

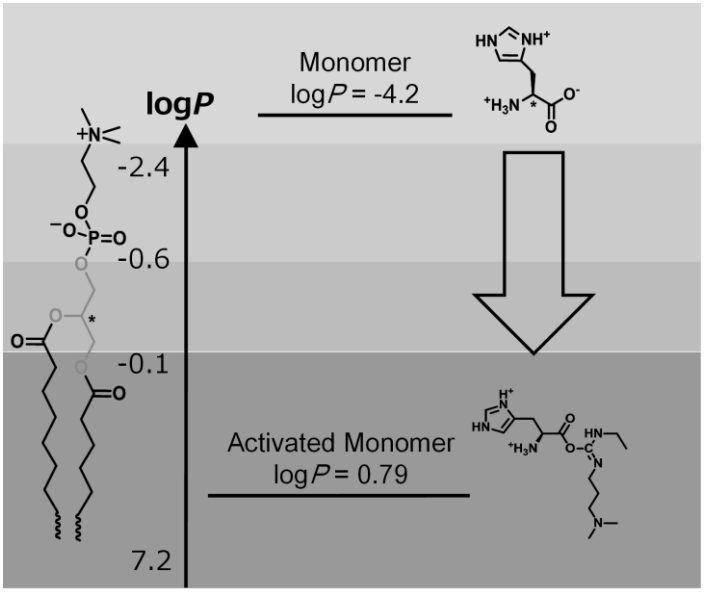


Fig. 4-9 Predicted locations of His monomer and activated monomer in POPC liposome membrane. Please note that the pK_a values of the acidic groups present in the molecules may change in the vicinity of the liposome membrane. The log*P* calculations were made for the chemical structures given.

prevents hydrolysis (Kunishima et al., 2005) and removes the produced water. The use of the liposome membrane system can therefore enhance to synthesize of L-form peptides with longer chain lengths than the D-form peptide synthesis. The L-His concentration increased from 40 mM in the bulk solution to 84.3 mM in the POPC membrane area. Furthermore, the estimated log*P* values of the monomer and activated intermediates suggest that the activated intermediates are located in the center of the bilayer and protected from hydrolysis, as shown in **Fig. 4-9**.

The superior properties of the liposomes in our oligomerization system, such as enantiomer selectivity, a hydrophobic environment, and fluidity, therefore enable efficient oligomerization of L-amino acids rather than D-amino acids. The system has potential applications in homochiral oligomerization from racemic monomers.

3.3 Preparation of Liposome-Immobilized Hydrogels (LI-gel) for Utilizing Liposomes as a Device of Separation Process

In order to apply the function of liposomes for separation, the condensed liposomes were immobilized within hydrogels. By adopting this method, the stability of liposomes in

solid matrices could be enhanced with high amount of immobilized liposomes, which permits an easy separation of liposomes from sample. The methodologies of liposome immobilization have been developed, while the liposome immobilized hydrogels reduce the operation steps for immobilization, and need neither chemical reaction between liposomes and matrices nor derivatized lipid, with keeping the potential properties of liposome membranes. To access this method, the membrane property of immobilized liposomes was investigated by Raman spectra, and then, their chiral separation efficiencies were determined.

3.3.1 Observation and Evaluation of Liposomes Embedded in LI-gel

In immobilization of liposomes in a gel matrix for use of the liposome membrane for separation or analysis, it is important that the liposomes remain intact. The immobilization and intactness of the liposomes were examined by eye (visually) and by using SEM. Visual observations showed that the liposomes were successfully immobilized both in the poly(AAm) and in agarose gels, because the gels became cloudy, as a result of light scattering by the liposomes, as shown in **Figs. 4-10(b)** and **(d)**, whereas both gels were transparent without liposomes, as shown in **Figs. 4-10(a)** and **(c)**. Although these figures showed the results of immobilization of DMPC liposomes, similar immobilization was observed in spite of the phase state of liposomes. The detail structures of the immobilized liposomes were furthernore examined by SEM observations of the gels, using the plasma replica method. A comparison of **Figs. 4-10(e)** and **(f)** indicates that intact spherical liposomes were present in the LI-agarose (6 wt%) gel. For calcein-trapped liposomes, the intactness of the immobilized liposomes, i.e., their retention of DMPC liposomes, similar visual of immobilization was observed in spite of the phase state of liposomes. The detailed structures of the immobilized liposomes were examined by SEM observations of the gels, using the plasma replica method. A comparison of **Fig. 4-10(e)** and **(f)** shows that intact spherical liposomes were present in the LI-agarose (6 wt%) gel. For calcein-trapped liposomes, the intactness of the immobilized liposomes, i.e., their retention of the inner aqueous phase, was confirmed. After washing the LI-gel with 10 mM phosphate buffer containing 143 mM NaCl (pH 7.4), the color of calcein still remained in the hydrogel, indicating the integrity of liposomes (lipid bilayer structure) in hydrogel. The volume fraction of liposomes against the total volume of the hydrogel can be

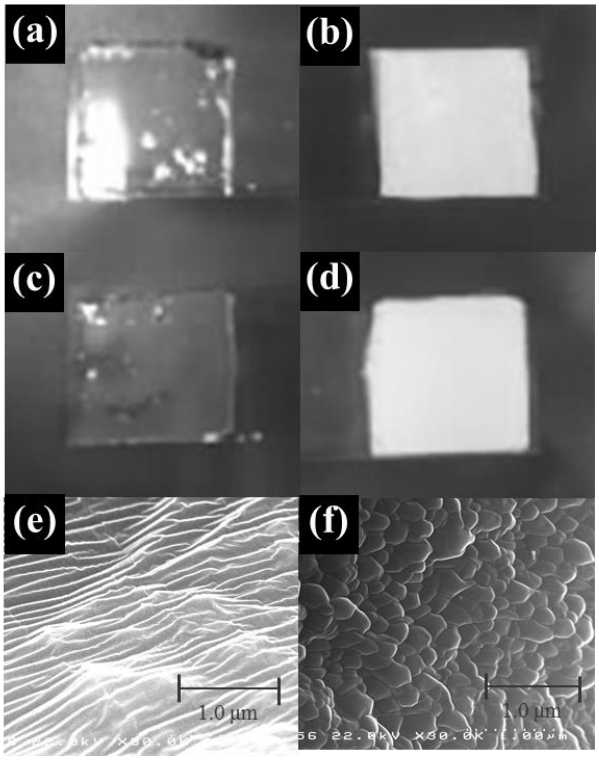


Fig. 4-10 Visual and SEM observations of poly(AAm) and agarose hydrogels: (a) poly(AAm) gel without liposomes, (b) poly(AAm) gel containing DMPC liposomes, (c) agarose gel without liposomes, (d) agarose gel containing DMPC liposomes, (e) SEM image of agarose gel without liposomes, and (f) SEM image of agarose gel containing DMPC liposomes.

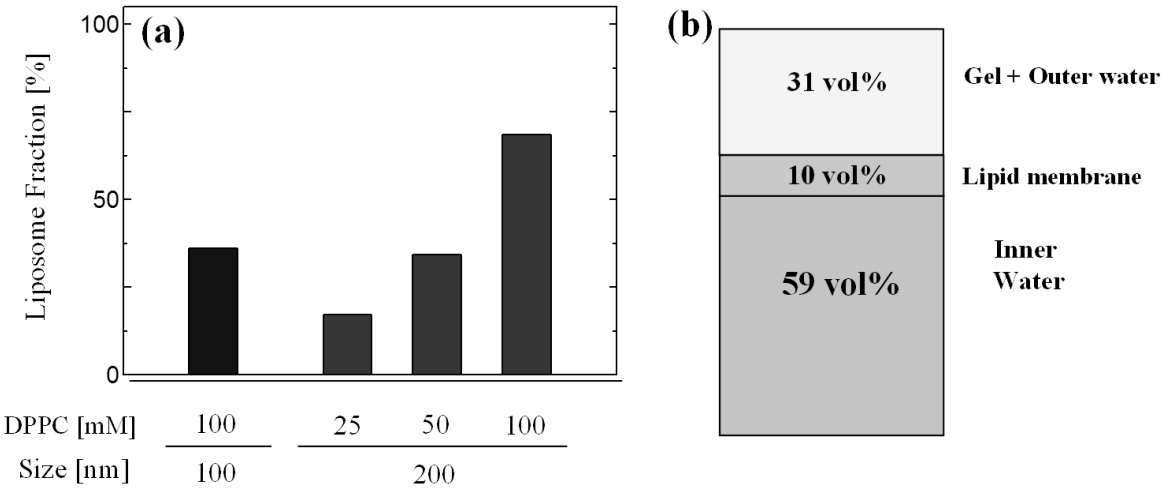


Fig. 4-11 (a) Calculated volume fractions of liposomes prepared with different concentration and diameter in poly(AAm) hydrogel. (b) Volume fraction of lipid membrane, hydrogel and inner water phase of liposome, which lipid concentration and diameter were 100 mM and 200 nm, respectively.

Table 4-1 Comparison of amount of immobilized liposome and its stabilities at some liposome immobilized system

References	Matrix Type	Immobilization Method	Amount of Immobilized Lipid [μmol -lipid/ml -matrix]	Volume Fraction of Liposome [%]
Yang et al., 1998	Sepharose® gel	Avidin-biotin binding	37.6	14.6
Yang et al., 1998	TSK® gel	Covalent bond	34.2	13.3
Yang et al., 1999	Cellulose gel beads	Covalent bond	14.5	5.8
This work	Acrylamide hydrogels	Radical polymerization	100.0	68.6
This work	Agarose hydrogels	Non-covalent bond	100.0	68.6

estimated from the lipid concentration, liposome diameter, area occupied by lipid molecules, and thickness of the lipid membrane. **Figure 4-11(a)** shows the theoretical volume fractions of DPPC liposomes in hydrogels prepared using liposomes of various sizes and concentrations. In the case of 100 mM liposomes of diameter 200 nm, the volume fraction of immobilized liposomes in the hydrogel was estimated to be 68.6% and the combined volume of the hydrogel and bulk water phase was 31.4 vol% (**Fig. 4-11(b)**). These results indicate that this method immobilizes larger amounts of liposomes than other methods do, as shown in **Table 4-1**.

3.3.2 Analysis of Embedded Liposomes by Using Raman Spectroscopy

In the use of LI-gels in separation or analysis, the surface properties of the liposome membrane, especially the membrane fluidity, surface charge, and microdomain formation, are important for controlling the interactions between liposome and target molecule. Among these properties, the dynamic nature of the liposome membrane is particularly important, because selective interactions of target molecules with the liposome membrane can be achieved through rearrangement of the membrane molecules. In LI-gels, the flexible surface of the liposome membrane must be preserved after immobilization. The dynamic properties of liposomes was determined by using Raman spectroscopy, focusing on the symmetric and asymmetric vibrations of C–H bonds in the hydrocarbon tail region of the lipid, where Raman

peaks at 2850 and 2880 cm^{-1} are assigned to CH_2 symmetric and asymmetric oscillations, respectively. **Figure 4-12(a)** shows the Raman shifts of DMPC liposomes immobilized in poly(AAm) gel at various temperatures (15–35 °C). The results show that the intensity of the Raman peak at 2880 cm^{-1} decreased with increasing temperature, and reached to the minimum value above 30 °C. This is because the asymmetric vibration becomes similar to the symmetric one as a result of enhancement of lipid molecular motion at higher temperatures and in a disordered phase. The hydrocarbon-packing density inside the liposome membrane was determined from the ratio of I_{2880} to I_{2850} (Huang et al., 1983). The DMPC liposomes in aqueous solution and the poly(AAm) LI-gel showed similar phase transition behaviors, where the phase transition from gel (ordered) phase to liquid-crystalline (disordered) phase was found at around 23 °C, while the packing densities of DMPC in chloroform solution were constant independent to temperatures, as shown in **Fig. 4-12(b)**. The reversible changes in the packing densities were confirmed by performing heating and cooling cycles between 15 and 35 °C. **Figure 4-13** shows that the packing densities of the LI-gel could be controlled based on the successive temperature shifts, and those values in gel phases (15 °C) or those values in liquid-crystalline phases (35 °C) remained at similar levels (constant). These results suggest that polymer chains do not interfere with the dynamic behaviors of liposomes, indicating the

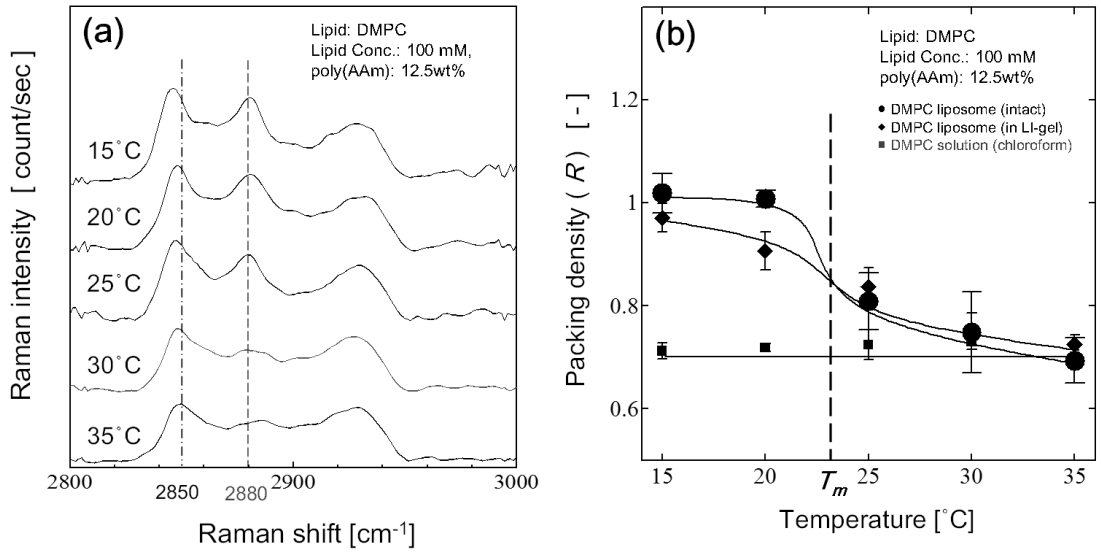


Fig. 4-12 Raman spectroscopic analysis of LI-gels: (a) DMPC liposomes immobilized in poly(AAm) hydrogels at various temperatures, (b) temperature dependence of packing density of DMPC liposome membrane immobilized in poly(AAm) hydrogel.

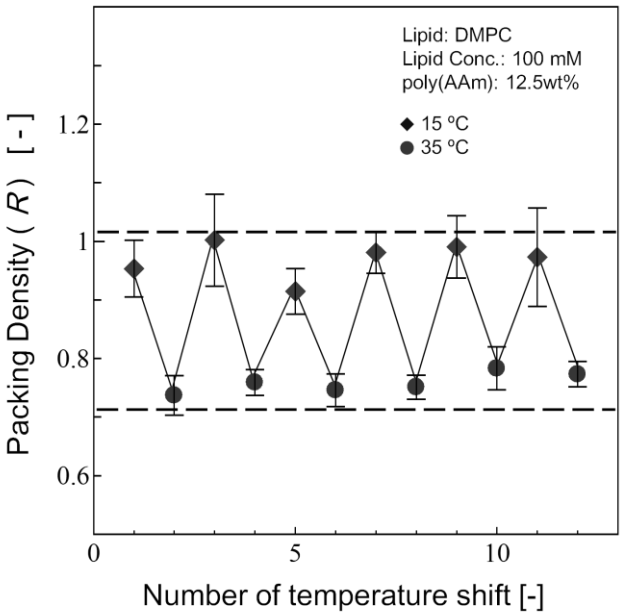


Fig. 4-13 Reversibility of packing density of DMPC liposome membranes.

liposomes immobilized in a hydrogel possess their original dynamic properties that are necessary for their emergent functions (e.g. selective adsorption of amino acids).

3.3.3 Optical Resolution of Tryptophan in LI-gel

The adsorption behaviors of L-Trp and D-Trp on DPPC liposomes immobilized in poly(AAm) gel were investigated to determine whether LI-gels have this function. **Figure 4-14(a)** shows the time courses of Trp adsorption on poly(AAm) gels with or without liposomes. In the absence of liposomes, little amounts of L-Trp and D-Trp were entrapped (or adsorbed) in the poly(AAm) gel. In contrast, L-Trp adsorption on the LI-gel increased and reached a plateau at approximately 0.009 mM (99% of the initial concentration) after incubation for 20 h, but D-Trp adsorption was negligible. These results suggest that the chiral selective separation of amino acids can be achieved using the LI-gel. In **Fig. 4-14(a)**, a time lag before the adsorption occurring was observed, which is similar to the case of free DPPC liposomes in chapter 1. After incubation for 27 h, the amount of L-Trp adsorbed on liposomes in the poly(AAm) hydrogel turned to be greater than that on bulk liposomes at the same lipid concentration, as shown in **Fig. 4-14(b)**. One possible reason for this enhancement is the concentration difference between Trp in bulk water and the hydrogel, which accelerates the

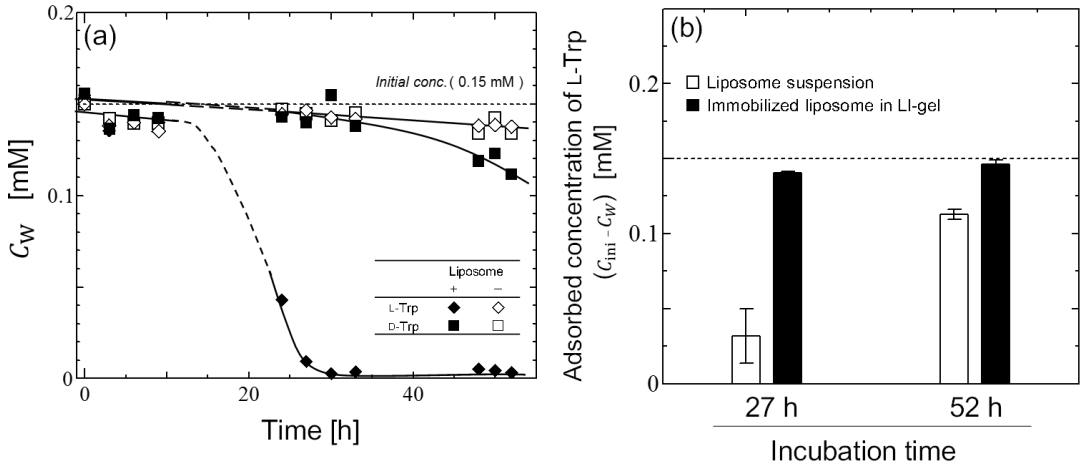


Fig. 4-14 (a) Time course of Trp concentration in bulk water phase coexisting with poly(AAm) LI-gel. (b) The adsorbed concentration of L-Trp (C_{ini}-C_w) in liposome suspension or LI-gel.

adsorption. Another is the hydrophobic environment around the liposomes in the poly(AAm) hydrogel. In this case, a dehydration of the liposome membrane surface was induced by strong binding of water molecules to the poly(AAm) side chains (Sekine et al., 2014). The Trp concentration difference and liposome dehydration cause faster adsorption of Trp in the LI-gel in the early stage.

The separation efficiency was estimated for the distribution ratios of L-Trp and D-Trp between the lipid membrane and bulk water phases. The separation factors of L-Trp against D-Trp on the liposome membrane phase ($\beta_{(L/D)}$) of L-Trp and D-Trp were calculated as shown in **Fig. 4-15**. The distribution ratio of L-Trp and $\beta_{(L/D)}$ for the LI-gel was higher than that for the liposome-free hydrogel. The separation factors of L-Trp against D-Trp in the bulk water phase ($\alpha_{(L/D)}$) for the current and other separation methods are summarized in **Table 4-2**.

The data show that the separation factors achieved using the method described here are higher than those obtained using other methods such as protein-captured polymer membranes (Yong et al., 2010) and β-cyclodextrin glutaraldehyde crosslinked membranes (Singh et al., 2012). The selective adsorption of L-Trp in a racemic solution was investigated. **Figure 4-16(a)** shows the time course of the Trp concentration in the racemic solution; the Trp concentration gradually decreased after 20 h, and was reduced to the half of the total Trp concentration in the racemic solution after incubation for 40 h. Circular dichroism

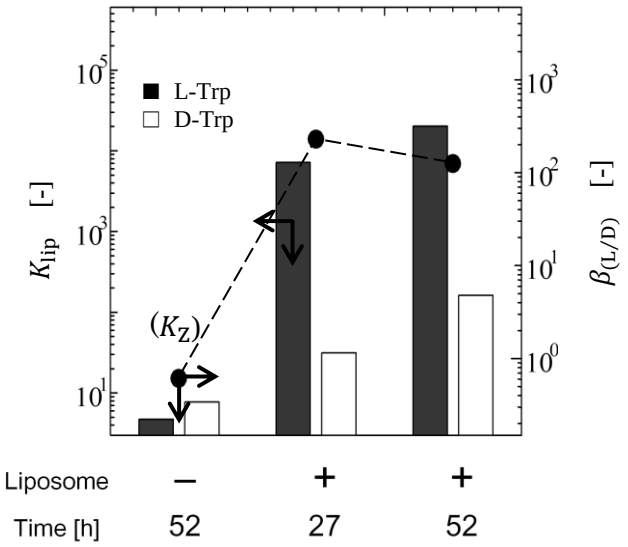


Fig. 4-15 Distribution ratios (K_{lip}) and separation factors ($\beta_{(L/D)}$) of L-Trp and D-Trp in liposome membrane phase.

Table 4-2 Comparison of separation factors achieved using several methods

References	Materials		Separation Factor ($\alpha_{(L/D)}$)
Yong et al., 2010	Bovine serum albumin captured polymer membranes		3.8
Singh et al., 2012	β-CD glutaraldehyde cross-linked membranes		5.8
This work	Poly(AAm) hydrogel		0.77
This work	Liposomes immobilized poly(AAm) hydrogel	27 h	15
		52 h	32

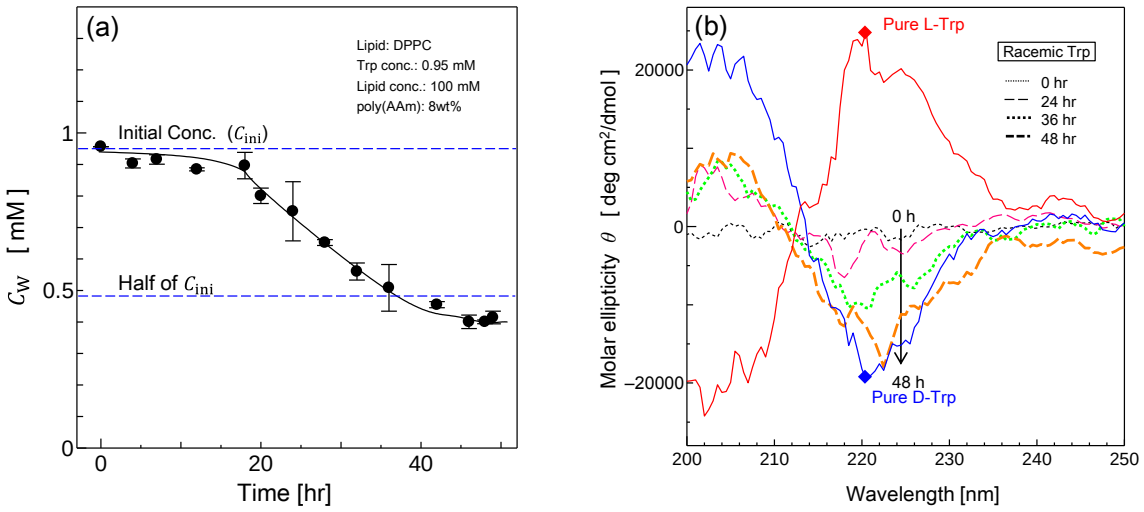


Fig. 4-16 Chiral separation of racemic Trp solution by LI-gel: (a) time course of racemic Trp concentration (C_W) and (b) circular dichroism spectrum of bulk water phase with LI-gel.

spectroscopy clearly shows that mainly D-Trp remained in the chamber, because of the selective adsorption of L-Trp, after incubation for 46 h (**Fig. 4-16(b)**). It is therefore concluded that LI-gels can be used as novel optical resolution agents for amino acids.

4. Summary

In this chapter, some possible extensions of the design scheme described in previous chapter and described by selecting (i) homochiral oligomerization on designed membranes and (ii) optical resolution using designed membranes embedded in hydrogel, by selecting amino acid recognition as a core phenomenon. The developments of chiral resolution and oligomerization for amino acids are induced by utilizing the functions of liposome membranes to selectively accumulate the molecules at the membrane surface and to induce the chiral recognition.

By means of the adsorption of amino acids on liposome membranes, the oligomerization of L-His was found to be promoted as compared with that in bulk solution. In the case of selective adsorption of L-His, the hypothetical concentration increased in the liposome membranes. This situation induced the longer oligomers than bulk L-His in polycondensation conducted by water-soluble initiators such as EDC and HOBt. The effect of the adsorption into liposome membranes is revealed by no elongation of D-His oligomerization in the presence of the liposomes. With regards to the reaction process of oligomerization, there are some relatively hydrophobic intermediates. Hence, the localization of reactants in liposome membranes leads to the protection of such intermediates against the hydrolysis.

The above results indicated that the promotion of conversion reaction is conducted in surface region of liposome membranes. Therefore, the design of more efficient reaction is expected by the analysis of surface membrane property developed in chapter 3. It is possible that mixing Ch in liposome membranes may induce the interference of conversion reaction by hydrolysis. However, the heterogeneous liposomes possibly induce the accumulation or orientation of reactants due to the specific assembled states around phase boundaries, which results in the promotion of conversion reaction in liposome membranes. In addition, the above oligomerization is examined by His molecule that possesses hydrophilic side chain. Thus, the evaluation of the surface membrane property is expected to contribute to the supposition of the effect of liposome membranes for the conversion of other molecules such as Trp that have hydrophobic side chain.

The immobilization of liposomes was examined by the embedding in poly(AAm) and agarose hydrogels. In these hydrogels, it was observed that DPPC liposomes embedded in

high concentration, which was monitored by the microscopy and SEM images. The immobilization of liposomes encapsulating calcein was also observed, indicating that the vesicle structure was still maintained during immobilization in hydrogels. As for the membrane property of liposomes, the packing density of immobilized liposome membranes was characterized by Raman spectra. The embedded DMPC liposome membranes showed the similar phase transition temperature to the DMPC liposomes in water, and the phase transitions were reversibly, implying that the effect of interactions with the branch of poly(AAm) was not so large. In the case of investigation of Trp adsorption in hydrogel prepared above, high selectivity for L-forms was shown after 27 hours of incubation. The adsorption performance of this method is at least equal to or more than other separation membranes. The optical resolution of racemic Trp was also demonstrated in the hydrogels immobilized liposomes.

According to the L-Trp adsorption in LI-gel, the incubation time for adsorption became shorter than the bulk condition with same liposomes, which means that the adsorption steps suggested in chapter 2 is induced efficiently. The enhancement of adsorption was also observed by the mixing of Ch based on the results in chapter 3, indicating that the understanding of the surface property plays an important role in the design of liposome membranes for the selective adsorption of amino acids. Although the use of fluorescent probes is very difficult to apply for the characterization of the solid materials embedded liposomes, LI-gel systems are expected to be utilized by designing and easily-characterization of the liposomes membrane properties optimized for separation processes. This examination treated DPPC as the liposome components, nevertheless, the mixing of Ch may be available for the design of LI-gel in the case of the negligible interference of polymerization. Furthermore, the above results about selective adsorption of Trp show the great significance in the development for various polar molecules including amino acids.

As a summary of this chapter, it is found that liposome membranes, as a self-assembly system, can induce the selective adsorption of amino acids, suggesting its plausible mechanism and the membrane design for performing its functions. Based on the case studies focusing on the separation and conversion processes, the application of liposome membranes is expected to contribute for the development of practical separation techniques.

General Conclusions

The method to design the liposome membranes for selective adsorption of amino acids was established in order to develop innovative separation processes. Based on the previous findings of the recognition functions that can be performed by some biological molecules, the utilization of liposome membranes is expected to be extended for the separation process that can perform the prominent recognition of amino acids. From the viewpoint of the self-assembly system, the variation of the surface properties of liposome membranes, together with their physicochemical state, was observed during the above-described recognition step and can also play an important role to elucidate the highly selective adsorption. A scheme for the liposome membrane design was proposed based on the obtained results on the characterization of the liposome membrane, its adsorption function and their relationship. Consequently, some extensions of the proposed scheme were examined through two kinds of case studies.

In chapter 1, the selective interaction of target molecules was examined by some assembled structures of phospholipids. In a solvent-water system, emulsion layer formed by phospholipids at the interface in the solvent phase became expanded by the decrease of dielectric constant of solvent, resulting in the increase of the partitioning of Trp from aqueous phase to organic phase with low enantiomer discrimination in the formation of "disordered" aggregation. On the other hand, highly selective recognition of L-Trp and L-His was observed in their partitioning in the liposome membrane prepared by the same phospholipids; DPPC. Such a difference between the solvent-water system and the liposome membrane system can be caused by the formation of highly-ordered assembly, wherein the certain orientations of the membrane-constituting lipid molecules and of the guest molecule interacting at the hydrophilic-hydrophobic interface were organized. In addition, the liposome membranes also induced the discrimination of charge in guest molecules and the enantioselective adsorption in racemic solution. Considering the assumption of monolayer adsorption by the adsorption isotherms, it is proposed that some recognition sites for L-amino acid could be formed on the liposome membranes.

In chapter 2, the variation of liposome membrane property was investigated by

employing the adsorption of amino acids as targets. In the case of selective adsorption of L-amino acids, the formation of interactions from side chain of amino acids was evidenced by the decrease of peak intensity in resonance Raman spectra. In addition, the variation of surface hydrophobicity of liposome membranes was observed by fluorescent spectra of ANS fluorescent probes and, also, by dielectric dispersion analysis. Therefore, the selective adsorption could be carried out by accompanying with the subtle changes in the surface property on the liposome membranes. This assumption was supported by the thermodynamic analysis i.e. the relation of enthalpy and entropy in L-amino acid adsorption. These findings suggest the mechanism of step-by-step adsorption in the surface region of liposome membranes. It was thus found that the detailed evaluation of surface property of liposome membranes could be important to design the recognition performance for L-amino acids.

In Chapter 3, the design of liposomes containing Ch was investigated based on the surface membrane property analysis and His adsorption. The methodology of evaluating the surface property of the liposome membranes was developed with the combination of two fluorescent probes that can be localized at the surface edge of the membrane. The diagram obtained by this methodology enabled to characterize the variation by selective adsorption of L-His, indicating the convergence of the surface property by adsorption of L-His. Furthermore, in the lipid composition forming heterogeneous phase, the fast adsorption of L-His was shown to correspond with the increase of the line tension derived from domain boundary. These results indicate that the understanding of the surface property leads to the important contribution in the design of liposome membranes for the efficient and selective adsorption for amino acids.

The above findings revealed that the liposome membranes could perform high selective adsorption of L-amino acids. The design of electrostatic interactions in hydrophilic region could control the adsorption behaviors of amino acids, resulting in the selectivity of chiral molecules. The highly ordered structures of phospholipid assemblies could play a beneficial role in inducing the stereochemical selectivity through multiple interactions. In addition, the design of membrane property induced by surrounding adsorbed molecules was also important for enhancement of adsorption process. The formation of interactions in adsorption was induced together with the changes of membrane property and of assembled states in surface region of liposome membranes. It is thus considered that such changes could

be promoted by the design of initial states of liposome membranes, resulting in the induction of fast adsorption. Besides, the flexibility on the heterogeneously-segregated liposome membranes could provide suitable environments for the adsorption in the energetic aspect. According to the above findings, the strategy of liposome membrane design for efficient performance of chiral separation was proposed as utilizing the function of liposomes, i.e., asymmetric recognition of amino acids.

In Chapter 4, some extensions of the design scheme of the liposome membrane have been investigated through two case studies, such as the conversion reaction followed by the amino acid recognition, and optical resolution of racemic solution of amino acids by using the liposome immobilizing hydrogel. The adsorption of L-His molecules could induce their condensation and localization in hydrophobic region of the liposome membranes. This behavior was utilized for the efficient oligomerization of L-His assisted in liposome membranes, resulting in the elongation of poly(L-His), but not poly(D-His). It is thus proposed that the liposome membranes could be utilized as the platform of prominent conversion process of hydrophilic molecules due to the combination with its function of selective adsorption. Furthermore, a development of immobilization method of liposomes can contribute to the practical use of the functions of liposome membranes. The embedding of liposomes in hydrogels could achieve the immobilization in high concentration with unimpaired property of liposome membranes. Based on the results about the chiral separation by liposome-immobilized hydrogels, the expansion of the selective adsorption of amino acids is expected by designing the processes utilizing liposome membranes.

The scheme of the design of liposome membranes for molecular recognition was thus established based on the analysis of surface membrane property, and on the mechanism of adsorption of target molecules. These findings are expected to contribute to the approach for the separation processes and for the unit process in practical use such as fine chemicals.

Suggestions for Future Works

To expand the findings obtained in this work, the following studies are recommended as future work.

(1) Further Investigation on the Design of Molecular Recognition by Regulating External Condition

In order to design the property of liposome membranes, the external condition such as temperature, pH, salt concentration, are important factors. For the practical use of liposome membranes, the design and characterization of liposome membranes through such conditions is valuable because of the easier handling and regulation. From the viewpoint of surface property of liposome membranes, the addition of the other molecules such as small organic acids or metal-affinity ligands can be used to assist the molecular recognition. In addition to the effect of membrane property, the formation of clusters with target molecules is expected to affect the regulation of molecular recognition. For the development of recognition processes by the above methods, it is useful to employ the methodology of evaluating surface membrane property proposed in this study.

(2) Extension of Selective Adsorption of Liposome Membranes for Various Processes

In this study, the application for separation or conversion processes was examined by employing some case studies. From these findings, it is expected to apply for various processes. On liposome membranes, the condensation of amino acids could be induced with high chiral selectivity. This result is considered to be developed for the chiral crystallization of amino acids on the liposome membranes. Furthermore, because the location of inserted molecules can be regulated by the design of liposome membranes, the effective production such as self-reproduction can be induced by not only controlling the localization of reactants but also the dynamic changes of assembled structures derived from alignment of product.

(3) Expansion of Target Molecules for Asymmetric Recognition of Liposome Membranes

The molecular recognition function of liposome membranes was performed by employing small hydrophilic molecules such as amino acids in this study. In actual, the liposome membranes are considered to be applied for molecular recognition in the wide range of target molecules because of the recognition functions for several biological molecules. According to the chiral selectivity, the application of liposome membranes for hydrophobic chiral molecules is important in addition to hydrophilic molecules. It is possible to achieve chiral recognition by the features of liposome membranes with ordered structures in acyl chains and stacked rings derived from cholesterols. In this case, the design strategy of liposome membranes suggested in this study can be applied by the several evaluations for both interior and exterior membrane properties.

(4) Approach of Inversed Stereochemistry by Utilization of D-Liposomes

In biological system, all kinds of phospholipids are produced in only the L-form enantiomer. A novel understanding can be therefore obtained about the chiral recognition in liposome membranes by means of the investigation in liposomes formed by D-phospholipids. In reference to this study and previous findings, self-assembly systems are expected to perform chiral recognition function based on the ordered structures of the components. It is thus required to consider the effect of the chirality of the phospholipid molecules for chiral recognition induced by liposome membranes. Such a finding possibly contributes to excellent control such as chiral switching.

Nomenclatures

C_{ads}	= concentration of adsorbent on liposome membranes	[mM]
$C_{ads\ (on\ membrane)}$	= concentration of adsorbent per liposome membrane volume	[mM]
D	= distribution ratio in emulsion phase	[-]
ee	= enantiomer excess	[-]
G	= correction factor	[-]
GP_{340}	= general polarization calculated at exciting light at 340 nm	[-]
I_{474} / I_{518}	= fluorescence intensity of ANS	[-]
K	= binding constant	[mM^{-1}]
K_Z	= distribution constant in hydrogel phase	[-]
K_{lip}	= distribution constant on liposome membranes	[-]
l	= specific boundary length	[nm^{-1}]
L_{domain}	= domain length per liposome perimeter	[-]
$1/P$	= membrane fluidity	[-]
P	= fluorescence polarization of probes embedding in membranes	[-]
q	= amount of adsorbent per lipid amount	[mmol/g]
Q	= amount of adsorbent on liposome membranes	[-]
R	= packing density of lipid membrane	[-]
$S_{L/D}$	= separation parameter	[-]
X_L	= molar ratio of L-amino acids against D-form	[-]
X_{lo}	= area ratio of l_o phase	[-]
$\alpha_{(L/D)}$	= separation factor in the bulk water phase	[-]
$\beta_{(L/D)}$	= separation factor on the liposome membrane phase	[-]
λ_N	= normalized surface hydrophobicity	[-]

List of Abbreviations

AAm	Acrylamide
ANS	8-Anilino-1-naphthalenesulfonic acid
Asp	Aspartic acid
BSA	Bovine serum albumin
CD	Circular dichroism
CTAB	Cetyltrimethylammonium bromide
Ch	Cholesterol
Cys	Cysteine
DBSA	Dodecylbenzenesulfonic acid
DDA	Dielectric dispersion analysis
DLS	Dynamic light scattering
DMPA	1,2-Dimyristoyl-*sn*-glycero-3-phosphate
DMPC	1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine
DMPE	1,2-Dimyristoyl-*sn*-glycero-3-phosphoethanolamine
DMPS	1,2-Dimyristoyl-*sn*-glycero-3-phospho-L-serine
DOPC	1,2-Dioleoyl-*sn*-glycero-3-phosphocholine
DPH	1,6-Diphenyl-1,3,5-hexatriene
DPPC	1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine
DSC	Differential scanning calorimetry
Dansyl-DHPE	*N*-(5-Dimethylaminonaphthalene-1-sulfonyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride
ELISA	Enzyme-linked immunosorbent assay
Em	Emission wavelength
Ex	Excitation wavelength
GUV	Giant unilamellar vesicle
HIV	Human immunodeficiency virus
^{1}H NMR	Proton nuclear magnetic resonance

HOBt	1-Hydroxybenzotriazole
His	Histidine
IR	Infrared resonance
ITC	Isothermal titration calorimetry
ITO	Indium tin oxide
l_d	Liquid disordered
LI-gel	Liposome-immobilized hydrogels
l_o	Liquid ordered
LUV	Large unilamellar vesicle
Laurdan	6-Lauroyl-2-dimethylamino naphthalene
Leu	Leucine
MALDI-TOF MS	Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry
MLV	Multilamellar vesicle
POPC	1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine
PPL	Propranolol
Phe	Phenylalanine
Pro	Proline
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
Ser	Serine
s_o	Solid ordered
TEMPO	(2,2,6,6-Tetramethylpiperidin-1-yl)oxyl
TMA-DPH	1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene
TNS	6-(*p*-Toluidino)naphthalene-2-sulfonate
T_m	Phase transition temperature
Triton X-100	Poly(oxyethylene *p*-*t*-octylphenyl ether)
Trp	Tryptophan
Trp-Trp	Ditryptophan
Tyr	Tyrosine
UV	Ultraviolet-visible
Val	Valine

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List of Publications

[Papers]

1. Takaaki Ishigami, Hiroshi Umakoshi, Comparison of Partitioning Behaviors of L-/D-Trp in Solvent-Water System and Liposome Membrane System. *Solvent Extr. Res. Dev., Jpn.*, **2013**, *20*, 213-217.

2. Takaaki Ishigami, Keishi Suga, Hiroshi Umakoshi, Chiral Recognition of L-amino Acid on Liposome Prepared by L-Phospholipid. *ACS App. Mater. Interfaces* **2015**, *7*, 21065-21072.

3. Takaaki Ishigami, Kazuma Sugita, Keishi Suga, Yukihiro Okamoto, Hiroshi Umakoshi, High Performance Optical Resolution with Liposome Immobilized Hydrogel. *Colloid Surf. B* **2015**, *136*, 256-261.

4. Takaaki Ishigami, Yoshinori Kaneko, Keishi Suga, Yukihiro Okamoto, Hiroshi Umakoshi, Homochiral Oligomerization of L-Histidine in the Presence of Liposome Membranes. *Colloid Polym. Sci.* **2015**, *293*, 3649-3653.

[Related Papers]

1. Hye Sung Cho, Junsoo Kim, Keishi Suga, Takaaki Ishigami, Hyunchul Park, Jung Won Bang, Soonmin Seo, Mansoo Choi, Pahn-Shick Chang, Hiroshi Umakoshi, Ho-Sup Jung, Kahp-Yang Suh, Microfluidic Platforms with Monolithically Integrated Hierarchical Apertures for the Facile and Rapid Formation of Cargo-Carrying Vesicles. *Lab Chip* **2015**, *15*, 373-377.

2. Masanori Hirose, Takaaki Ishigami, Keishi Suga, Hiroshi Umakoshi, Use Liposome Membrane as a Platform of L-Pro Catalyzed Michael Addition Reaction of *trans*-β-Nitrostyrene and Acetone. *Langmuir* **2015**, *31*, 12968-12974.

[International Conference (Proceeding)]

1. Hiroshi Umakoshi, Takaaki Ishigami, Chiral Recognition of L-/D-Amino Acid by Liposome, *9th International Conference on Separation Science and Technology (ICSST11)*, Jeju, Korea, November, (2011)

[International Conference / Symposium]

1. Takaaki Ishigami, Hiroshi Umakoshi, Chiral Recognition of L-/D- Amino Acids by Liposome Membrane, *JST Japan-India Joint Workshop on "Biomedical Research"*, Tokyo, Japan, February (2012)

2. Takaaki Ishigami, Hiroshi Umakoshi, Asymmetric Recognition and Synthesis on L-Liposome Membrane, *The 7th Conference of Aseanian Membrane Society*, Busan, Korea, Jury (2012)

3. Takaaki Ishigami, Kazuma Sugita, Keishi Suga, Hiroshi Umakoshi, Chiral Recognition of Amino Acids by "Polymer Membrane" Immobilizing "Liposome Membrane", *The 8th Conference of Aseanian Membrane Society*, Xi'an, China, Jury (2013)

4. Takaaki Ishigami, Hiroshi Umakoshi, Use Liposome Membrane as a Platform of Asymmetric Recognition and Conversion, Next Symposium "Membranome" for "Bio-Inspired Chemical Engineering", Osaka, Japan, September (2013)

5. Takaaki Ishigami, Hiroshi Umakoshi, Analysis of Chiral Recognition Induced by Liposome Membrane; Effects of Surface Polarity at Initial Adsorption Step, *Next Symposium "Membranome" for "Bio-Inspired Chemical Engineering"*, Osaka, Japan, September (2013)

6. Takaaki Ishigami, Hiroshi Umakoshi, Asymmetric Recognition of L-/D-Amino Acid by Liposome Membrane, *2013 AIChE Annual Meeting*, San Francisco, U.S.A., November (2013)

7. Hiroshi Umakoshi, Takaaki Ishigami, Optical Resolution of Racemic Compounds by W/O/W Emulsion with Nano-Hydrophobic Interface (Liposome), *20th Regional Symp. on Chemical Engineering*, Bohol, Philippine, November (2013)

8. Takaaki Ishigami, Keishi Suga, Yukihiro Okamoto, Hiroshi Umakoshi, Roles of Physicochemical Properties of "Nano-Platform on Membrane" in Asymmetric Recognition Function of Liposome Membrane, *2014 AIChE Annual Meeting*, Atlanta, U.S.A., November (2014)

9. Takaaki Ishigami, Keishi Suga, Hiroshi Umakoshi, Characterization of Selective Adsorption Behavior of L-Amino Acid on Liposome Membrane, *10th Int'l Conference on Separation Science and Technology*, Nara, Japan, October (2014)

10. Takaaki Ishigami, Kazuma Sugita, Keishi Suga, Yukihiro Okamoto, Hiroshi Umakoshi, Characterization of Selective Adsorption Behavior of L-Amino Acid on Liposome Membrane, *The 20th Symposium of Young Asian Biochemical Engineers' Community, Chiayi*, Taiwan, July (2014)

11. Takaaki Ishigami, Yoshinori Kaneko, Keishi Suga, Yukihiro Okamoto, Hiroshi Umakoshi, Recognition of L-Amino Acid at Liposome Membrane Surface and Its Application for Homochiral Polymerization, *2015 AIChE Annual Meeting*, Salt Lake City, U.S.A., November (2015)

[Patent]

1. Hiroshi Umakoshi, Takaaki Ishigami, Kazuma Sugita, Toshinori Shimanouchi, New Optical Resolution Method, JP 5743595, July 1st (2015)

[Article / Review]

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