Studies on Proline-Catalyzed Reactions on Lipid Membrane in Aqueous Media

Masanori Hirose

MARCH 2019

Studies on Proline-Catalyzed Reactions on Lipid Membrane in Aqueous Media

A dissertation submitted to

THE GRADUATE SCHOOL OF ENGINEERING SCIENCE

OSAKA UNIVERSITY

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY IN ENGINEERING

BY

Masanori Hirose

MARCH 2019

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 2012 to 2019.

The objective of this thesis is to establish the methodology to design the vesicle membranes for the control of the C-C bond reaction enhanced at the interface of the vesicle membrane suspended in aqueous media. The interactions of the reactants and catalyst (L-Pro) molecules are investigated especially, focusing on the stability of their reaction intermediate at the interior of the liposome membrane in order to clarify their key factors for high yield and selectivity.

The author hopes that this research would contribute to the design of the liposome membrane for its application to the control of the chemical reaction. The methodology established in this study is expected to contribute to the regulation of the chemical reaction at the hydrophobic-hydrophilic interface.

Masanori Hirose

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

Abstract

The final purpose of this thesis is to reveal the potential nature of liposome membrane to trap the "*enamine*" intermediate at the hydrophobic/hydrophilic interface by selecting L-Pro catalyzed reaction as a target. First, the method to detect the "*enamine*" intermediate as a fluorescence intensity was established and a strategy to apply it for the chemical conversion has been proposed. The proposed scheme was applied for the effective conversion (aldol reaction) and for the chiral conversion (Michael addition reaction) as the case studies of the proposed scheme. As the further extension of the above strategy, L-Pro was replaced by simple primary amine to apply the liposomal platform for the stabilization of its enamine intermediate.

In Chapter 1, some reported works relating to the general concepts of self-assembly, together with model examples of the chemical conversion, will be introduced to shed the light on the key concepts in this study.

In Chapter 2, the kinetics of the L-Pro catalyzed Michael addition reaction of BIPM and acetone were analyzed by using fluorescence spectroscopy. The composition of liposomes could be a key factor to control this reaction: zwitterionic DPPC liposome showed highest reaction rate constant among the liposomes tested in this work. The obtained results provide us the easy-detectable method to assess the enamine intermediate of the L-Pro in the membrane platform, which can be detected by using BIPM as donor reactant in L-Pro catalyzed Michael addition. Based on the obtained data, a scheme for the stabilization of the enamine intermediate at the hydrophobic/hydrophilic interface was proposed.

In Chapter 3, Aldol reaction catalyzed by L-Pro was herewith investigated as an extension of the strategy described in Chapter 2, aiming at the improved conversion of the reaction product in C-C bond formation of the ketone and *p*-nitrobenzaldehyde (*p*NBA). As described in previous chapter, the enamine intermediate of L-Pro and acetone was first formed at the interface of the lipid bilayer membrane and then it was applied to the reaction with the aldehyde group of the second substrate (*p*NBA). It was shown that the liposome membranes provided a hydrophobic region for the aldol reaction of *p*NBA and acetone, which was catalyzed by L-Pro adsorbed onto liposomes.

In Chapter 4, as an extension of the strategy described in Chapter 2, the L-Pro-catalyzed reaction on membrane was applied to the enantioselective conversion of C-C bond formation of the ketone and *trans*-β-nitrostyrene (*t*βNS). After the enamine intermediate of L-Pro and acetone was formed at the interior of the lipid bilayer membrane, it was reacted with the unsaturated bond of the second substrate (*t*βNS). It was found that the L-Pro-catalyzed reaction could proceed in "water" using a liposome membrane. Specifically, when the liposome membrane surface was modified in the dehydrated state, i.e., having "*ordered"* (s_o) *phase* and a "*positively-charged*" surface, the normalized reaction rate constant in the DOPC/DPTAP and DPPC/DPTAP systems, calculated based on the amount of L-Pro adsorbed, was almost equal to that in the DMSO.

In Chapter 5, as the further extension of the L-Pro utilizing strategy described in previous chapters, a primary amine was used for the conversion of C-C bond formation of the ketone and *trans*-β-nitrostyrene (*t*βNS) on the liposome membrane. The Michael addition reaction of acetone and *t*βNS was achieved by using stearylamine (SA) as a possible catalyst in liposome systems. It was found that the above-mentioned reaction was induced in "water" by liposome membrane with simple catalytic molecule (SA).

It was thus proposed that the liposome membrane could also be utilized as a "*platform*" to concentrate both catalyst and substrate molecules and, especially, to trap the "*reaction (enamine) intermediate*" of both molecules. The results obtained in this work are summarized in General Conclusions, and Suggestions for Future Work are described as extension of this Thesis.

Contents

Chapter 1

General Introduction

There are many reports focusing on the significance of the self-assembly as a platform of the molecular recognition and chemical conversion [Walde *et al*., 2014, Serrano-Luginbühl *et al*., 2018]. Fundamental comprehension of the self-assembly and its structure are essential for the development of novel kinds of functional materials. In this chapter, some of previous works relating to the general concepts of self-assembly, together with model examples of the chemical conversion, are introduced to shed the light on the key concepts in this study.

1. Fundamental Characters of Self-Assembly System ~Micelle, Vesicle, and so on~

The hydrophilic-hydrophobic interfaces formed in self-organizing systems, such as micelles and vesicles (liposomes) (**Fig. 1-1**), can be used as a platform for organic synthesis or molecular recognition in water [Dwars *et al*., 2005, Zhang *et al*., 2009, Zayas *et al*., 2013]. A liposome is a vesicle that is constructed by self-assembly of phospholipids in aqueous solution [Yeagle *et al*., 2011]. In recent years, the assembly of amphiphiles has led to certain emergent properties, where the "*ordered state*" of the self-assembly surface could be a key factor for achieving membrane function, i.e., molecular recognition [Tuan *et al*., 2008], porphyrin assembly [Umakoshi *et al*., 2008], polymerization of amino acid derivatives [Blocher, *et al*.,

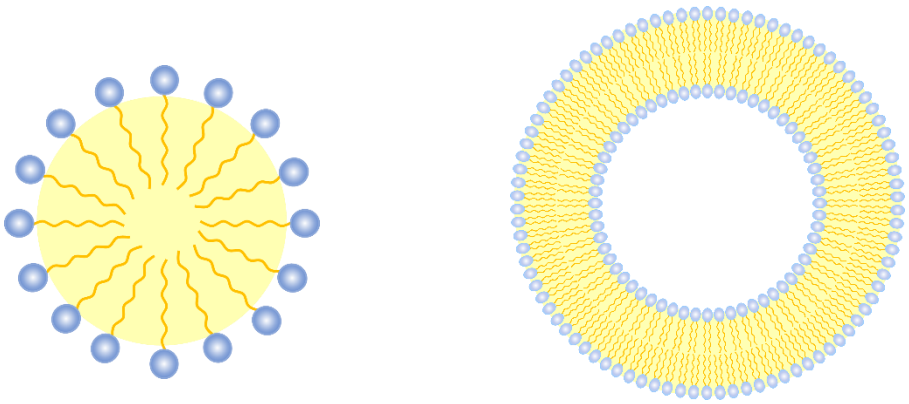


Figure 1-1 Schematic Illustration of Micelle and Vesicle

1999, Hitz *et al*., 2001], antioxidative function [Yoshimoto *et al*., 2007], *etc*. Some recent findings demonstrate the importance of evaluating the physicochemical membrane properties, such as the membrane fluidity and polarity. Membrane fluidity and polarity, which are often used for evaluation of lipid order or hydrophobicity, are evaluated by fluorescence measurement with fluorescent probe molecules. Several methodologies have been developed for probing the "*microscopic*" environment at the lipid membrane surface, for example TEMPO quenching method and membrane surface-enhanced Raman spectroscopy (MSERS) [Suga *et al*., 2013, Suga *et al*., 2015].

2. Emergent Properties of the Liposome

Lipid molecule has an asymmetric carbon in the glycerol backbone (**Fig. 1-2**). It is therefore expected that the liposome has the function as a platform of chiral selection or chiral conversion. In recent years, the ability of liposome as chiral selector of amino acids has been

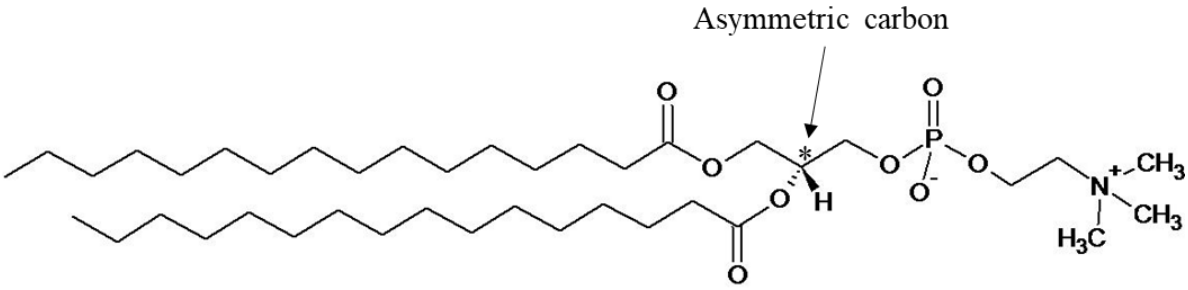


Figure 1-2 Structural formula of the lipid (ex. DPPC)

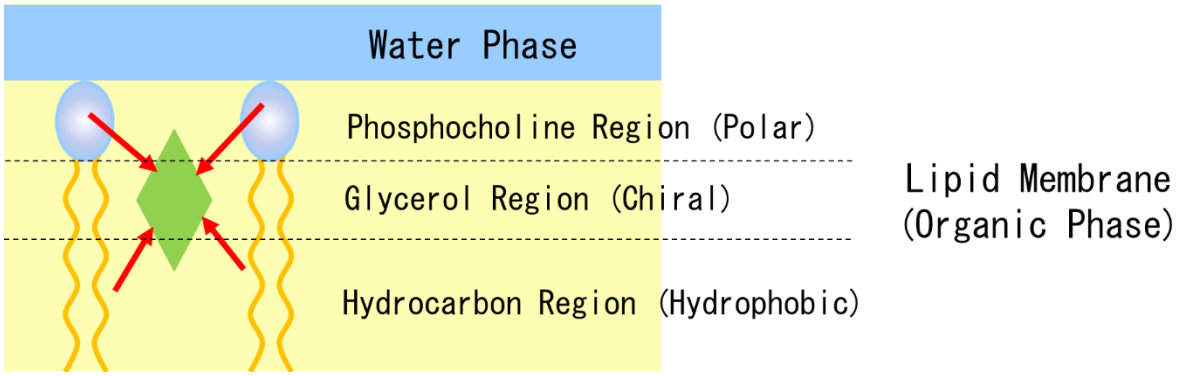


Figure 1-3 Image of the interaction between lipid membrane and substrate

reported [Ishigami *et al*., 2015]. The use of the liposome membrane as a chiral selector for amino acids is one of advantageous approach; for example, the adsorption of L-Proline (L-Pro) on the 1,2-palmitoyl-*sn*-glycero-3-phosphocholine (DPPC, solid-ordered phase at room temperature) liposome was much higher than that of D-Proline (D-Pro) on DPPC, whereas the adsorption behaviors of L-Pro and D-Pro on liposomes in the liquid-disordered phase have not been clarified. Liposome membranes in the "*ordered*" phases provide a hydrophobic environment in which water molecules are excluded, and they can selectively interact with L-Pro [Ishigami *et al*., 2015]. It is therefore expected that the liposome membrane can be used as a platform for molecular conversion in aqueous media, owing to the following features: (1) enantiospecific substrate (or catalyst, intermediate) interaction at "*ordered*" membranes, (2) conversion of hydrophobic molecules (e.g., log*P* >1) in aqueous media, and (3) easy separation (purification) of chiral products. However, the chiral ability of liposome membranes is still not well understood, and the reaction utilizing the chiral environment of liposomes has not been reported. It is expected that liposome membrane can stereo-selectively recognize molecules by multipoint interaction and can induce enantiospecific reaction (**Fig. 1-3**).

3. Liposome Membrane as a Platform of Chemical Conversion

In recent studies, the use of self-assembly systems in organic synthesis is also focused, because self-assemblies enable insoluble materials dispersed in aqueous phase [Palmqvist, 2003]. The hydrophilic-hydrophobic interface formed in self-organized systems, such as micelles and vesicles (liposomes), can be used as a platform for organic synthesis or molecular recognition in water [Dwars *et al*., 2005, Zhang *et al*., 2009, Zayas *et al*., 2013]. Chemical and biochemical reactions on vesicle membrane surfaces have been evaluated in several studies [Walde *et al*., 2014, Urabe *et al*., 1996, Ueoka *et al*., 1988, Umakoshi *et al*., 2008, Yoshimoto *et al*., 2007, Luginbühl *et al*., 2018] (**Table 1-1**). It has been recently reported that the pseudo-interphase of the liposome membrane can promote the 1,3-dipolar cycloaddition reaction in

aqueous solution [Iwasaki *et al*., 2015]. The liposome membrane (self-organizing system) has recently attracted many interests of scientists and engineers, because of their emergent properties that are different from the conventional materials in bulk materials. Thus, the liposome membrane can be utilized as a platform of organic synthesis in aqueous media [Iwasaki *et al.*, 2017].

Table 1-1 Examples of chemical and biochemical reactions on vesicle membrane surfaces

References	Reaction (Product)
Urabe *et al*., 1996	Stereoselective hydrolysis reaction
Ueoka *et al*., 1988	Hydrolysis of amino acid esters
Yoshimoto *et al*., 2007	Decomposition of hydrogen peroxide
Umakoshi *et al*., 2008	Antioxidative function
Walde *et al*., 2014	Reaction by artificial amphiphiles
Luginbühl *et al*., 2018	Aldol reactions

To control the organic synthesis in liposomes, there are some beneficial points, especially, in the "*designability*", where the liposome surface property can be designable simply by modifying lipid compositions [Suga *et al.*, 2016, Suga *et al.*, 2013, Bui *et al*., 2016, Suga *et al.*, 2017]. The combined use of small molecules with liposome would lead a "*cooperative*" alteration in the membrane properties, which can contribute to provide improved functions [Okamoto *et al.*, 2016, Ishigami *et al.*, 2016, Han *et al.*, 2017]. The use of the liposome could shed a light in the research field of the chemical synthesis that could provide us new kinds of "*quality*" as compared to conventional strategy. However, reaction utilizing the asymmetric environment of lipid membranes have not been reported yet. If the catalyst and reactive substrate molecules were accumulated in the chiral environment of the lipid membrane, an enantioselective reaction could be induced and modulated based on the membrane properties.

4. L-Pro: Natural Chemical for Catalytic Reaction

There are many reports on organocatalysts that can achieve an asymmetric conversion, such as phase transfer catalyst (Maruoka catalyst) [Ooi *et al*., 1999], MacMillan catalyst [Ahrendt et al., 2000], and Proline catalyst [List *et al*., 2000] as summarized in **Table 1-2**. Among these organocatalysts, L-Proline (L-Pro) can interact with lipid membrane and can be adsorbed on lipid membrane. The proline-catalyzed reaction is therefore considered to be suitable for model reactions on liposome membranes.

L-Pro is an L-amino acid having a five membered ring conjugating with a nucleophilic secondary amine and carbonic acid (Brønsted acid). In a biological system, L-Pro is known to induce various kinds of physiological or bio-functions because of its reactive secondary amines [Williamson, 1994]. L-Pro is one of well-recognized organic catalysts for the aldol reaction, Michael addition, etc [List *et al*., 2000, List *et al*., 2001, List *et al*., 2002, Zandvoort *et al*., 2012]. The catalytic ability of L-Pro has originally been reported as an asymmetric Robinson cycloaddition reaction [Eder *et al*., 1971, Hajos *et al*., 1974], although many researchers are mainly focusing on the organo-metal catalysts and pay almost no attention on L-Pro catalyst in 1970's. Recently, there have been several reports of improved reactivity, reaction rate, and yield achieved with L-Pro derivatives [Lu *et al*., 2012, Rodríguez-Lansola *et al*., 2010, Mase *et al*., 2006]. The L-Pro-catalyzed reaction is usually achieved though the formation of "*enamine*" intermediate, between L-Pro and the substrate with ketone group (**Figure 1-4**). It is known that most of reported reactions were carried out in rather less-polar solvent (i.e. DMSO), but not in high-polar solvent (i.e. water), owing to the stability of the enamine intermediate. From the viewpoints of difficulty in the separation of the solvent from the target product and, also, possible risks of the contamination of product solution, a new strategy to stabilize the enamine intermediate should be established to utilize the L-Pro and its derivatives in effective method to synthesize the valuable chemical products. Using the L-Pro catalyst in a non-solvent system

could broaden the horizons for its application in the development of environmentally-harmonized asymmetric synthesis processes.

Table 1-2 Examples of organocatalytic process

References	Catalyst	Reaction (Product)
Dolling *et al*., 1984	Phase-Transfer Catalyst	Catalytic Asymmetric Alkylations
Ooi *et al*., 1999	Maruoka catalyst	Catalytic Asymmetric Alkylations
Ahrendt *et al*., 2000	MacMillan catalyst	Asymmetric Diels-Alder reaction
Heine *et al*., 2001	Class I aldolase enzymes	Aldol raction
Hajos *et al*., 1974	L-Pro	Robinson annulation
List *et al*., 2000	L-Pro	Aldol reaction
List *et al*., 2001	L-Pro	Michael addition
Notz *et al*., 2004	L-Pro	Mannich reaction
Ishii, *et al*., 2004	L-Pro derivative	Michael addition

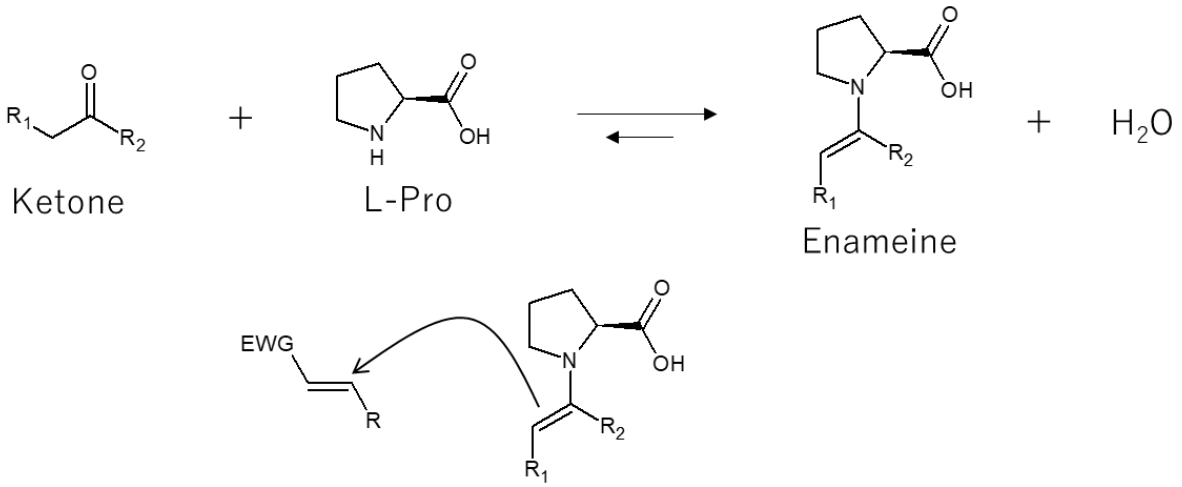


Fig 1-4 Enamine intermediate of L-Pro and ketone group.

Many researchers have previously studied the extractive conversion in (multi-)liquid phase systems (i.e. oil-water system) modified with the rather hydrophobic or amphiphilic catalysts [Grassert *et al*., 1998, Lo *et al*., 2009]. It is expected that the combination of such L-Pro catalysts with the solvent extraction system enables us to develop the chiral conversion process. Among the possible multi-phase systems, there have been several reports on the use of the L-Pro derivatives in the self-assembly systems (i.e. micelles, vesicles, etc.) [Lu *et al*., 2012, Rodríguez-Llansola *et al*.,2010, Hayashi *et al*., 2006, Huerta *et al*., 2013]. Importantly, the self-assemblies enable insoluble and hydrophobic compounds, i.e. substrate, catalyst, reaction intermediate, to be dispersed in aqueous solution.

Table 1-3 Examples of Pro-derivative catalyst

References	Catalyst	Reaction field	Reaction
Hayashi *et al*., 2006	Combined Proline-Surfactant Organocatalyst	Emulsion in Water	Aldol reaction
Rodríguez-Llansola *et al*.,2010	L-Proline-L-valine dipeptide derivatives	Supramolecular Gel in Organic Solvent	Michael addition
Lu *et al*., 2012	L-proline functionalized polymeric nanoreactors	Michelle in water	Aldol reaction
Huerta *et al*., 2013	L-proline functionalized Water-Soluble Polymer	Folding in water	Aldol reaction

5. Concept of the Present Study

In recent years, the assembly of amphiphiles has led to certain emergent properties, where the "*ordered state*" of the self-assembly surface could be a key factor for achieving membrane function. It was reported that the lipid membrane (liposome) could be utilized as a platform of organic synthesis in aqueous media [Iwasaki *et al.*, 2015, Iwasaki *et al.*, 2017]. The highly-ordered assembly of phospholipid molecules can provide a nano-scaled hydrophobic environment, which brings high-designability of the membrane surface [Ishigami *et al.*, 2013,

Umakoshi *et al.*, 2013]. The liposome surface property can be designed by modifying lipid compositions [Suga *et al.*, 2016, Suga *et al.*, 2013, Bui *et al.*, 2016, Suga *et al.*, 2017]. It is a promising strategy to integrate the "L-Pro" and the above liposome membrane for development of novel chemical conversion system.

In the case of lipid membranes, they have a hydrophilic hydrophobic interface in which the glycerol backbone with an asymmetric carbon atom is concentrated. The lipid membrane is therefore expected to have function as chiral selector or asymmetric reaction field. The use of the liposome membrane as a chiral selector for amino acids has been reported [Ishigami et al., 2015]. However, there is no research of the role of the chiral region at the hydrophilic/ hydrophobic interface of liposome membrane in asymmetric reaction.

The final purpose of this thesis is to reveal the potential nature of liposome membrane to trap the "*enamine*" intermediate at the hydrophobic/hydrophilic interface by selecting L-Pro-catalyzed reactions as target. First, the method to detect the "*enamine*" intermediate as a fluorescence intensity was established and a strategy to apply it for the chemical conversion has been proposed. The proposed scheme was applied for the effective conversion (aldol reaction) and for the chiral conversion (Michael addition reaction) as the case studies of the proposed scheme. As further extensions of the strategy, L-Pro was replaced by the primary amine which is applied on the liposomal platform to stabilize enamine intermediates. The flamework and the flow chart of the present study are shown in **Figure 1-6** and **Figure 1-7**, respectively.

In Chapter 1, some previous works relating to the general concepts of self-assembly, together with model examples of the chemical conversion, were introduced to shed the light on the key concepts in this study.

In Chapter 2, the kinetics of the L-Pro catalyzed Michael addition reaction of *N*-[*p*-(2-benzimidazolyl)-phenyl]maleimide (BIPM) and acetone were analyzed by using fluorescence spectroscopy. The composition of liposomes could be a key factor to control this reaction: zwitterionic DPPC liposome showed the highest reaction rate constant among the liposomes

tested in this work. The obtained results provide us the easy-detectable method to assess the apparently existing enamine intermediate of the L-Pro in the membrane platform, which can be detected by using BIPM as donor reactant in L-Pro-catalyzed Michael addition. Based on the obtained data, a scheme for the stabilization of the enamine intermediate at the hydrophobic/hydrophilic interface.

In Chapter 3, the aldol reaction catalyzed by L-Pro was herewith investigated as an extension of the strategy described in Chapter 2, aiming at the improved conversion of the reaction product in C-C bond formation of the ketone and *p*-nitrobenzaldehyde (*p*NBA). As described in previous chapter, the enamine intermediate of L-Pro and acetone was first formed at the interior of the lipid bilayer membrane and then it was applied to the reaction with the aldehyde group of the second substrate (*p*NBA). It was shown that the liposome membranes provided a hydrophobic region for the aldol reaction of *p*NBA and acetone, which was catalyzed by L-Pro adsorbed onto liposomes.

In Chapter 4, as an extension of the strategy described in Chapter 2, the L-Pro-catalyzed reaction on membrane was applied to the enantioselective conversion of C-C bond formation of the ketone and *trans*-β-nitrostyrene (*t*βNS). After the enamine intermediate of L-Pro and acetone was formed at the interface of the lipid bilayer membrane, it can react with the unsaturated bond of the second substrate (*t*βNS). It was found that the L-Pro-catalyzed reaction could proceed in "water", by using a liposome membrane. Especially, when the liposome membrane surface was modified in the dehydrated condition, i.e., having "*ordered"* (s_o) *phase* and a "*positively-charged*" surface, the normalized reaction rate constant in the DOPC/DPTAP and DPPC/DPTAP systems, calculated based on the amount of L-Pro adsorbed, was almost equal to that in the DMSO.

In Chapter 5, as the further extension of the L-Pro utilizing strategy described in previous chapters, a primary amine was used for the conversion of C-C bond formation of the ketone and *trans*-β-nitrostyrene (*t*βNS) on the liposome membrane. The Michael addition reaction of

acetone and tβNS was achieved by using stearylamine (SA) as a possible catalyst in liposome systems. It was found that the above-mentioned reaction was induced in “water” by liposome membrane with simple catalytic molecule (SA).

It was thus proposed that the liposome membrane could also be utilized as a “*platform*” to concentrate both catalyst and substrate molecules and, especially, to trap the “*reaction (enamine) intermediate*” of both molecules. The results obtained in this work are summarized in General Conclusions, and Suggestions for Future Works are described as extension of this thesis.

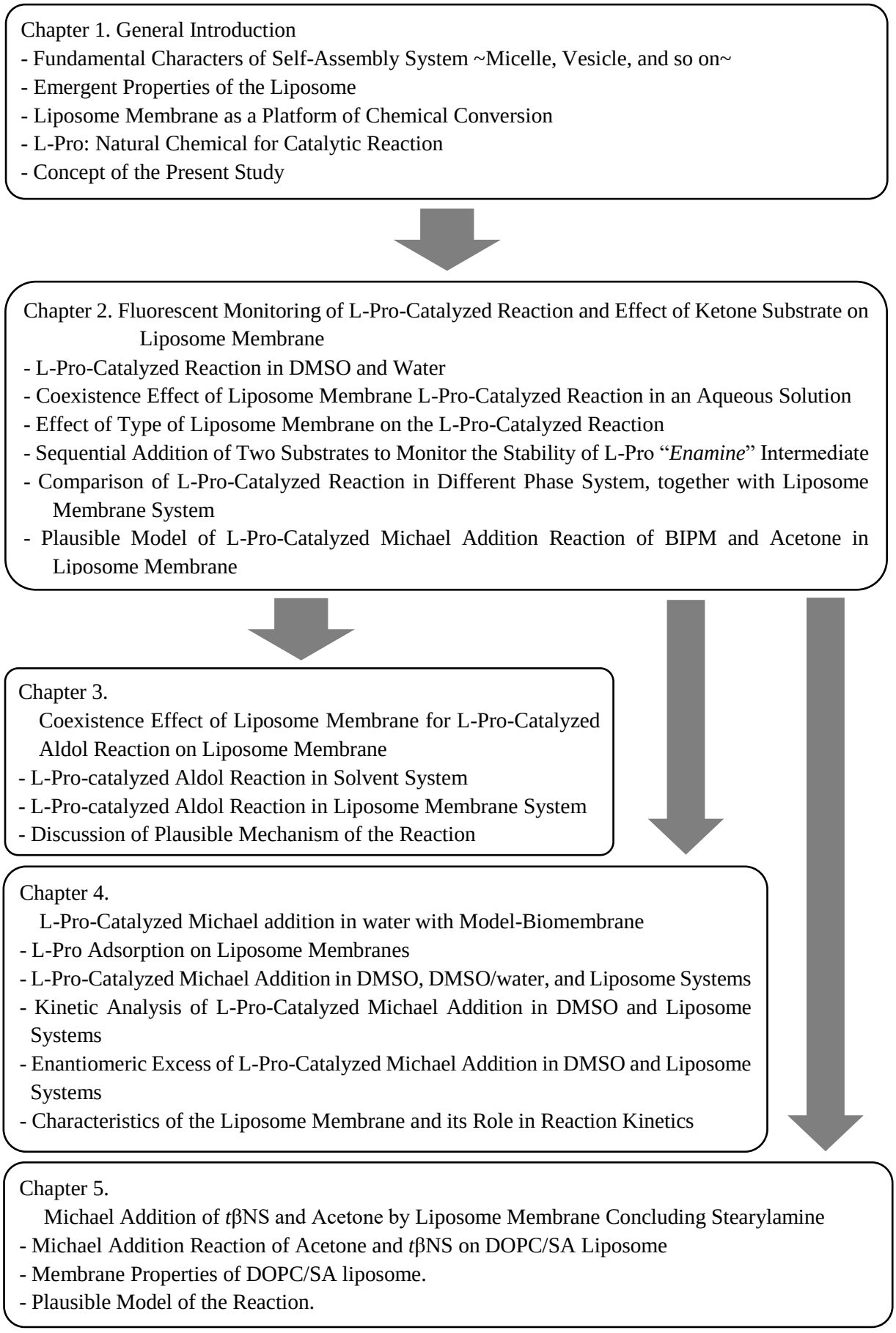


Figure 1-6 Flow chart of the present stud

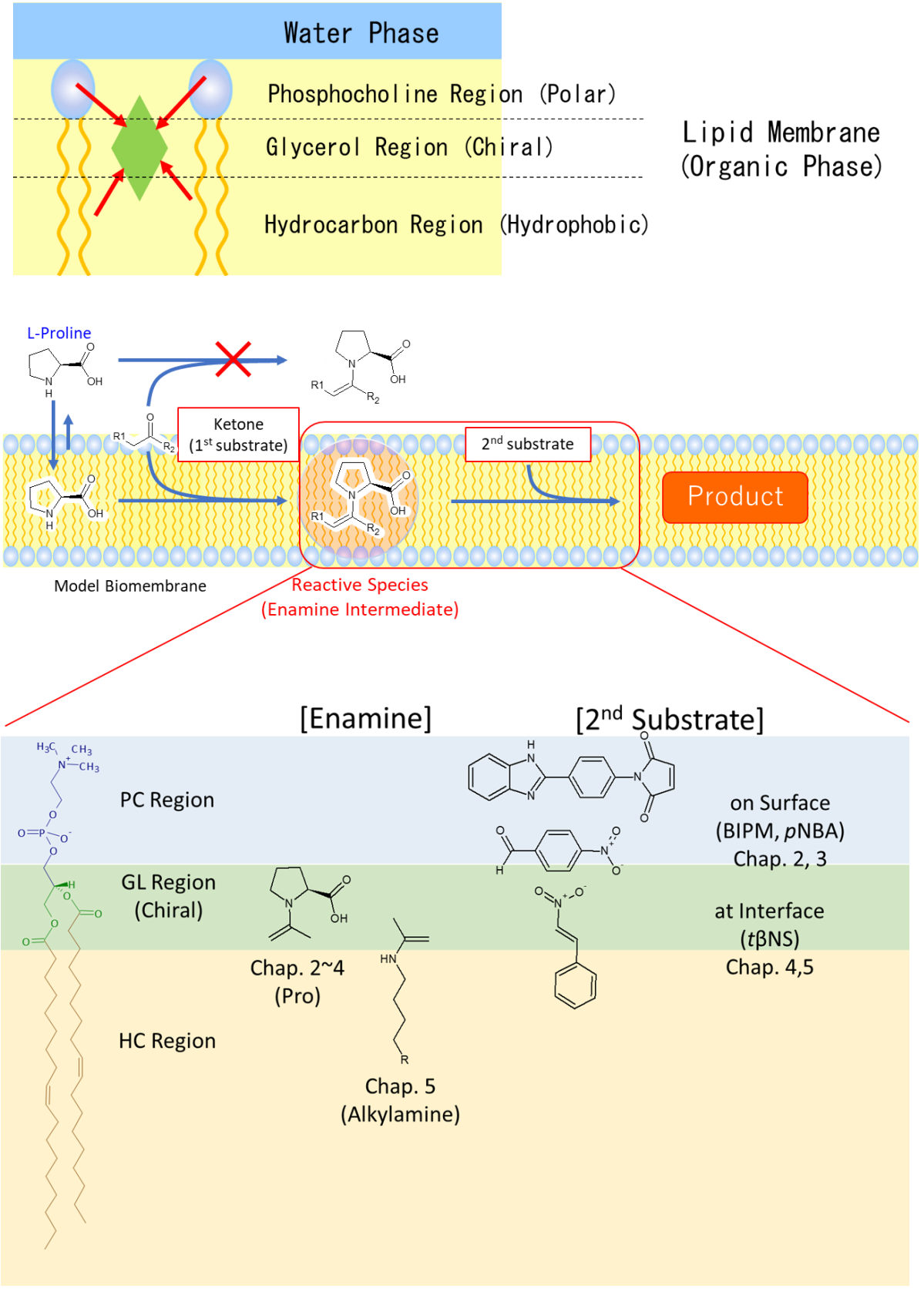


Figure 1-7 Framework of the present study

Chapter 2

Fluorescent Monitoring of L-Pro-Catalyzed Reaction and Effect of Ketone Substrate on Liposome Membrane

1. Introduction

L-Proline (L-Pro) is one of the principle amino acids that are the building blocks of the protein structure. L-Pro is known to elucidate various kinds of physiological activities though its interference in the metabolic pathway, such as the enhancement of growth of epidermal cells, the activation of collagen *biosynthesis*, the repair of the damaged collagen, and the moisture of the stratum corneum [Li *et al*., 2018]. As another important aspect of L-Pro, it is attracting attention as organocatalyst [List *et al*., 2000, List *et al*., 2001]. Because of the advantage of safety and high efficiency, the use of L-Pro will provide a benefit in the industrial processes, such as medical and food. Barbas and co-workers have originally pioneered a strategy to utilize the L-Pro in many kinds of catalytic reactions, especially focusing on the view point of enantioselectivity [Mase *et al.*, 2006, Sakthivel *et al.*, 2001, Bui *et al.*, 2000]. The L-Pro catalyzed reaction is usually achieved though the formation of "*enamine*" intermediate that could be formed via the conjugation of L-Pro and the substrate with ketone group. Conventionally, most of reported reactions are known to proceed in polar solvent (i.e. DMSO, dielectric constant, $\varepsilon = 46.7$), but not in water ($\varepsilon = 80$). In order to keep higher stability of the enamine intermediate, less polar environments are required, while L-Pro shows low solubility in nonpolar solvent (L-Pro: log*P*= -2.4). There are also some problems in the removal of polar solvents due to high boiling point (b.p. (DMSO, b.p. = 189 ℃)). Thus, the solvent must be well considered for the aforementioned L-Pro catalyzed reaction processes.

Many researchers have previously studied the extractive conversion in (multi-)liquid phase systems (i.e. oil-water system) modified with the rather hydrophobic or amphiphilic catalysts [Grassert *et al.*, 1998, Lo *et al.*, 2009]. It is expected that the combination of such L-Pro catalysts with the solvent extraction system enables us to develop the chiral conversion process. Among the possible multi-phase systems, there have been several reports on the use of the L-Pro derivatives in the self-assembly systems (i.e. micelles, vesicles, etc.) [Lu *et al*., 2012, Rodríguez-Llansola *et al.*, Hayashi *et al.*, 2006]. Importantly, the self-assemblies enable insoluble and hydrophobic compounds, i.e. substrate, catalyst, reaction intermediate, to be dispersed in aqueous solution. Some recent studies have therefore also focused on the use of self-assembly systems in organic synthesis [Palmqvist *et al.*, 2003].

Among self-assembly systems, the liposome can also be utilized as a platform of organic synthesis in aqueous media [Iwasaki *et al.*, 2015, Iwasaki *et al.*, 2017]. Liposome, a vesicular envelope composed by phospholipid bilayer membrane, is known as model of biological membranes. The liposome surface property can be designable by modifying lipid compositions [Suga *et al.*, 2016, Suga *et al.*, 2013, Bui *et al.*, 2016, Suga *et al.*, 2017]. The combined use of small molecules with liposome would lead a cooperative alteration in the membrane properties, which can contribute to provide improved functions [Okamoto *et al.*, 2016, Ishigami *et al.*, 2016, Han *et al.*, 2017]. The highly-ordered assembly of phospholipid molecules can provide a nano-scaled hydrophobic environment, which brings high-designability of the membrane surface [Ishigami *et al.*, 2013, Umakoshi *et al.*, 2013].

It has been previously investigated that the L-Pro catalyzed reactions can be proceeded on the liposome membranes in aqueous media. However, there are still some difficulties in direct monitoring of the kinetics of L-Pro catalyzed reactions in aqueous medium, because the product is basically analyzed after extraction and purification of the products. Recently, the method for direct observation of L-Pro-catalyzed reaction has been developed [Tanaka *et al.*, 2003], wherein the fluorescence of substrate (*N*-[*p*-(2-benzimidazolyl)phenyl]maleimide

(BIPM)) can be enhanced by C-C bond formation with ketone. This reaction system seems to be suitable to compare the effect of reaction medium on the L-Pro-catalyzed reaction.

In this chapter, the L-Pro catalyzed Michael addition reaction was selected as a target reaction, wherein the above fluorescent product of ketone and BIPM was employed because of its easy-detective nature. First, the effect of liposome membranes on this reaction was kinetically analyzed by using fluorescence spectroscopy of the reaction product in order to investigate the possible use of L-Pro catalyst incorporating with the liposome membrane. Based on the obtained results with precious findings, a plausible model of the side reaction of was finally discussed (**Fig. 2-1**). Secondly, the L-Pro catalyzed reaction was compared with (a) DMSO system and (b) liposome membrane system, to investigate the effectiveness of the liposome platform as a catalytic reaction of L-Pro-based reaction (**Fig. 2-2**). It was shown that "*enamine*" intermediate entrapped at the interface of lipid membrane could be a key material to regulate the L-Pro catalyzed reaction in given environment. Based on the obtained results, a strategy to conserve the enamine intermediate of L-Pro at the interior of the liposome membrane was finally proposed as a scheme.

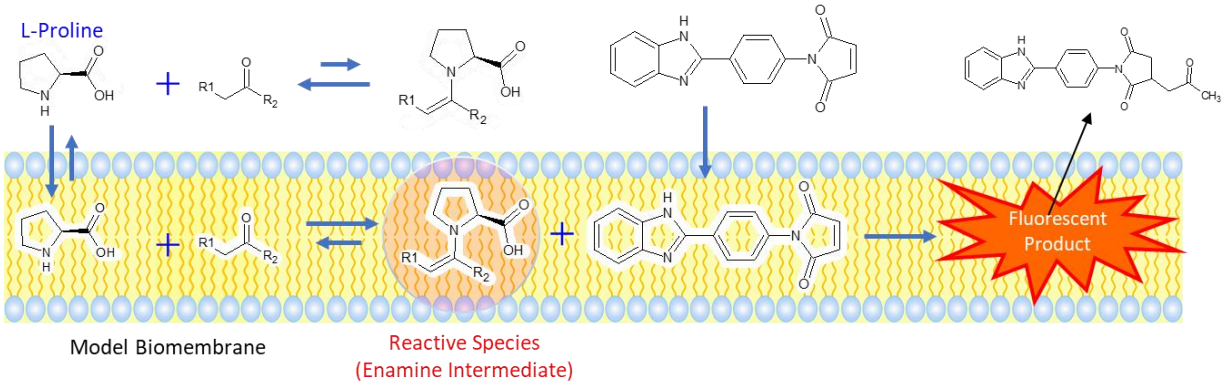


Figure 2-1 Schematic illustration of L-Pro-catalyzed C-C bond formation on liposome membrane system.

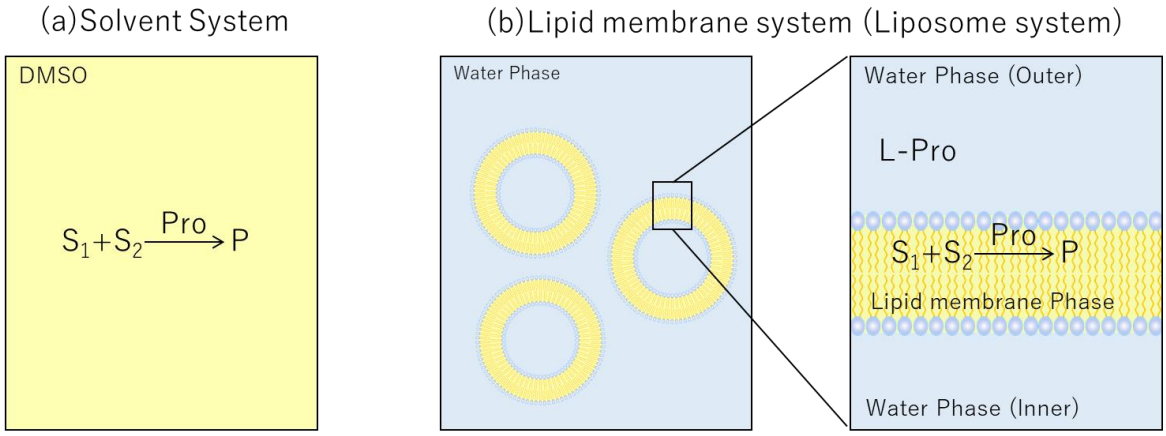


Figure 2-2 Schematic illustration of L-Pro-catalyzed C-C bond formation in different systems. (a) DMSO solution system (single phase), (b) Liposome membrane system.

2. Materials Methods

2.1 Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC: C16:0, zwitterionic), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC: C18:1, zwitterionic) and 1,2-dipalmitoyl-3-trimethylammonium-propane (DPTAP: C16:0, cationic) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). L-Proline (L-Pro) was purchased from Peptide Institute (Osaka, Japan). *N*-[*p*(2-benzimidazolyl)phenyl]maleimide (BIPM) was purchased from Tokyo Chemical Industry co., Ltd. (Tokyo, Japan). Other chemicals were purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan) and were used without further purification.

2.2 Liposome preparation.

A chloroform solution of lipids (DOPC, DPPC, etc.) was dried in a round-bottom flask by rotary evaporator under a vacuum. The lipid films obtained were dissolved in chloroform and the solvent was removed. These operations were repeated twice or more. The lipid thin film was kept under a high vacuum for at least three hours, and then hydrated with ultrapure water at above the transition temperature (T_m). The vesicle suspension was frozen at -80 °C and thawed at 60 °C to enhance the transformation of small vesicles into multilamellar vesicles (MLVs). This freeze-thaw cycle was performed five times. MLVs were used to prepare the large unilamellar vesicles (LUVs) by extruding the MLV suspension 11 times through two layers of polycarbonate membranes with mean pore diameters of 100 nm using an extruding device (Lipofast; Avestin Inc., Ottawa, Canada).

2.3 Kinetics of L-Pro-catalyzed reaction in DMSO/Water system.

L-Pro-catalyzed Michael addition of BIPM (BIPM: 28.9 μg (0.1 μmol)) with acetone (0.2 mL (2.7 mmol)) was conducted in DMSO or aqueous solution. L-Pro catalyzed reaction was initiated by adding 10 μl of stock solution of BIPM (10 mM) in CH_3CN/DMSO (1/1) mixture, 10 μl of stock solution of L-Pro in water (0 ~ 8 mM) and acetone (200 μl) to 780 μl of DMSO or aqueous solution. The reaction was carried out in a quartz cell for measurement without stirring. Fluorescence spectra were measured by the fluorescence spectrophotometer FP-8500 (JASCO, Tokyo, Japan) at 25 °C. The samples were excited with 315 nm and measured at 362 nm. The reaction kinetic was considered by first-order kinetics, and the reaction rate constant, k, was determined by following equation:

$$-\ln\left(1-\frac{A_{\mathrm{p}}}{A_{\mathrm{pmax}}}\right)=kt \quad , \tag{2-1}$$

wherein, A_{p} and A_{pmax} represent the fluorescence at arbitral time (t) and the final fluorescence intensity, respectively.

2.4 The L-Pro-catalyzed reaction in liposome membrane

L-Pro-catalyzed reaction in liposome membrane was initiated by adding 10 μl of stock solution of BIPM (5 mM) in CH_3CN/DMSO (1/1) mixture and acetone (200 μl) to a 790 μl of mixture of Liposome and L-Pro. A mixture of Liposome and L-Pro was pre-incubated by mixing 780 μl of liposome membrane (5 mM) and 10μl of L-Pro stock solution of water (100 mM). The reaction was carried out in 5 ml of screw vial with stirring and light shielding. A sample for fluorescence measurement was prepared by diluting the reaction solution 50-fold with water.

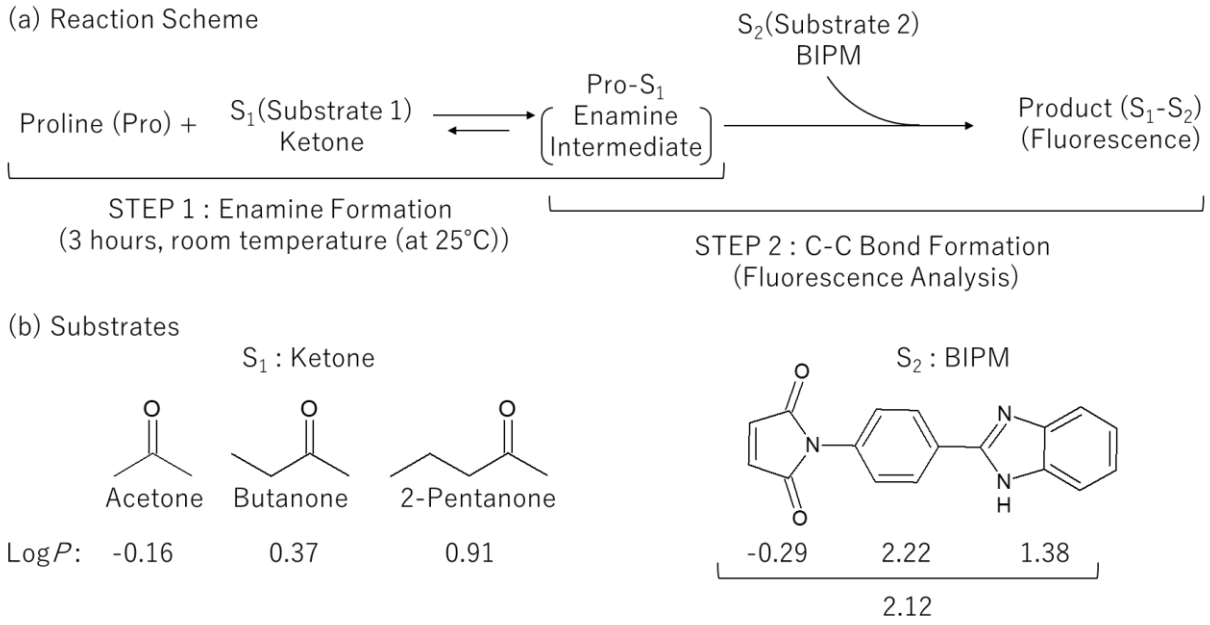


Figure 2-3 Schematic illustration of a target reaction. (a) L-Pro-catalyzed C-C bond formation of the first substrate with keto group was performed through two step operations. (b) Chemical structure of the first substrates with keto group and the second substrate (BIPM).

2.5 L-Pro-catalyzed reaction in DMSO and liposome system

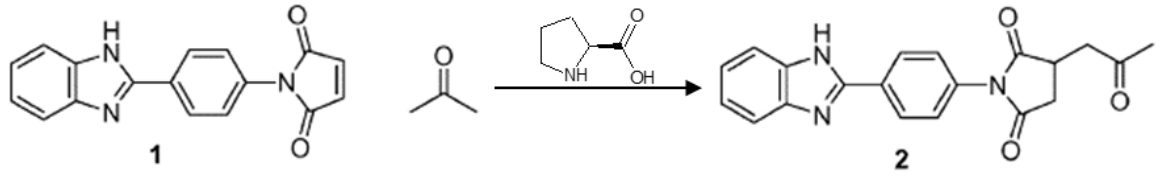
L-Pro-catalyzed Michael addition of BIPM (28.9 μg (0.1 μmol)) with acetone or other substrates (50 μL (0.675 mmol)) was conducted in (a) DMSO solution and (b) liposome membrane system. The L-Pro-catalyzed reaction is schematically shown in **Fig. 2-3**. Based on the previous findings, the above reaction was performed through two step operations; the first substrate with keto-group was pre-incubated with L-Pro for three hours (25 ℃) in the above systems to form the enamine intermediates (Step 1 in **Fig. 2-3(a)**) and, then, the formation of the fluorescent product was initiated by the addition of the second substrate (BIPM; with no fluorescence). The reaction was initiated by adding BIPM into the reaction medium. The fluorescence intensity of the product (λ_{ex} = 315 nm, λ_{em} = 370 nm) was recorded after 60 or 180 min from the begging of reaction. The effect of the first substrate with keto group (acetone, butanone, and 2-pentanone in **Fig. 2-3(b)**) on the final product formation in the above systems

was investigated. Among the systems in which the above reaction was performed, the DOPC liposome was selected as the liposome membrane system because of its capability to keep the stability of the enamine intermediate of the L-Pro-catalyzed reaction. The DOPC liposome was prepared according to the previous report. The log*P* value of each molecule was referred from Chemspider [Pence *et al.*, 2010] or from computational calculation (ChemBioDraw (Cambridge Soft Corporation, MA, USA)).

3. Results and Discussion

3.1 L-Pro-Catalyzed Reaction in DMSO and Water

BIPM is reported as a good molecular probe to monitor the L-Pro-catalyzed reaction because its product shows fluorescence. First, the L-Pro catalyzed Michael addition of BIPM and acetone (**Scheme 2-1**) was performed in DMSO solution and in water. **Figure 2-4** shows the fluorescence spectra of the reaction products in DMSO solution. The emission peak wavelength of the product could be dependent on the solvent: the emission peaks in DMSO and in water were 360 nm and 368 nm, respectively. The dependence of catalyst amount in this reaction was then investigated by varying the amount of L-Pro. **Figure 2-5a** shows the time course of the fluorescence intensity at different concentration of L-Pro in DMSO. The reaction rate constants (*k*) were calculated based on the first-order kinetics (eq. 2-1) (**Figure 2-5b**), suggesting that the reaction could be proceeded by following a first-order kinetics, because the reaction rate and L-Pro (catalyst) amount were in a linear relationship.



Scheme 2-1 Michael reaction of BIPM and acetone. 1: BIPM, 2: product.

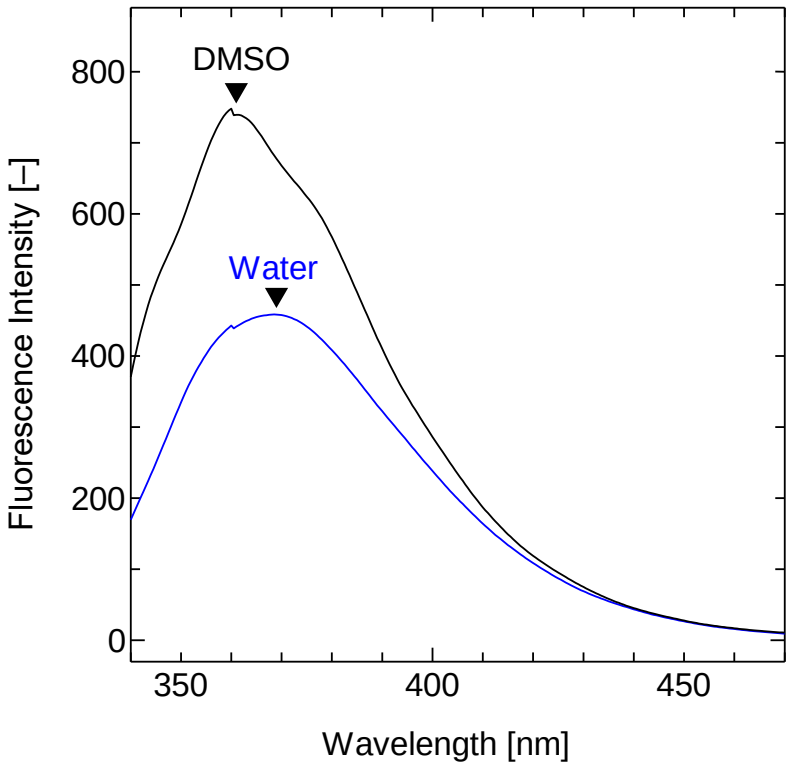


Figure 2-4 Fluorescence spectra of the product of L-Pro catalyzed Michael addition reaction of BIPM and acetone in DMSO.

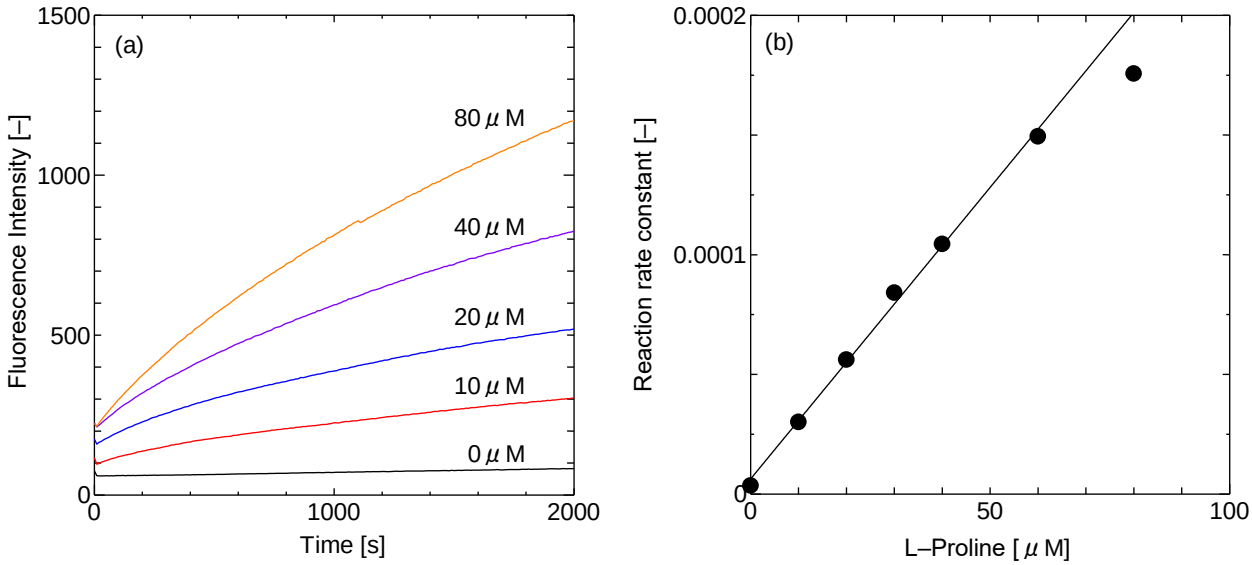


Figure 2-5 (a) Time course of the fluorescent intensity from the reaction product, at different L-Pro concentrations. (b) Relationship between L-Pro concentration and reaction rate constant.

To investigate the effect of solvent polarity, the reaction was furthermore conducted in the mixture of DMSO and water. **Figure 2-6** shows the time course of the fluorescence intensities, wherein the fluorescence intensities were monitored at 360 nm. The presence of water significantly decreased the reaction rate. In general, the enamine formation can be inhibited by the solvent water [Mase *et al.*, 2006]. It is suggested that water molecules also play an inhibitory role in this reaction. In common to the reaction mechanisms of the L-Pro catalyzed reactions, the formation of the enamine intermediate is a key in efficiency and enantioselectivity [List *et al.*, 2000, List *et al.*, 2001, List *et al.*, 2002], whereby the water molecules could decrease the stability of the enamine intermediate. The above results clearly indicate that the efficiency of the L-Pro catalyzed reaction of BIPM and acetone can be inhibited in aqueous solution, due to the lack of hydrophobic (dehydrated) environment.

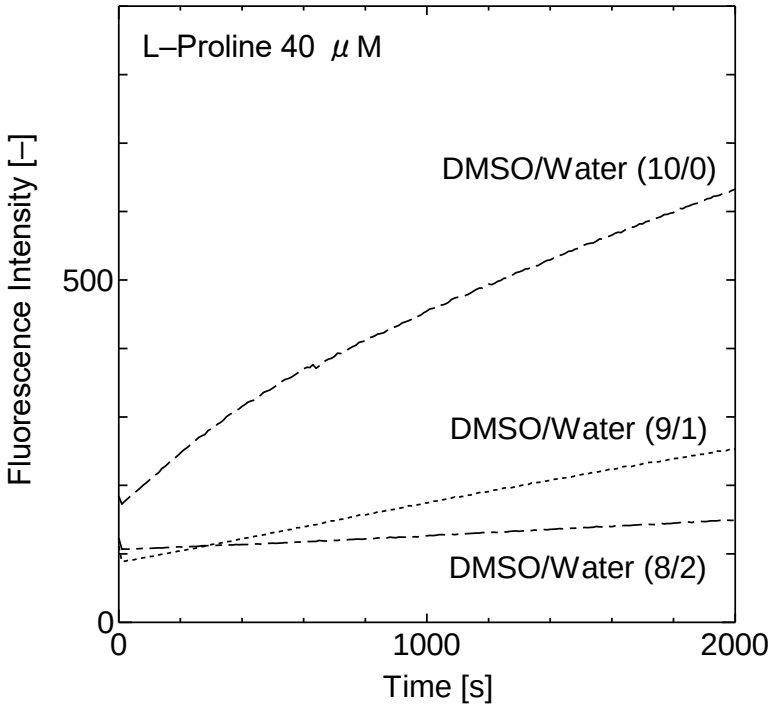


Figure 2-6 L-Pro-catalyzed Michael addition reaction of BIPM and acetone in DMSO/water solutions.

3.2 Coexistence Effect of Liposome Membrane L-Pro Catalyzed Reaction in an Aqueous Solution

The coexistence effect of the liposome membrane on the L-Pro catalyzed reaction of BIPM and acetone was investigated in water. Although L-Pro is a water-soluble molecule, it has been reported that L-Pro can be adsorbed onto the liposome surface by incubation for 48 hours [Ishigami *et al.*, 2015]. After L-Pro was pre-incubated with DPPC liposome for 24 hour or 48 hours (**Fig. 2-3**), the Michael addition reaction was initiated by adding BIPM and acetone. **Figure 2-7a** shows the fluorescence spectra of the products at different incubation times. In the case of 24-hour pre-incubation, the spectra were not changed significantly, indicating that the reaction was not proceeded. On the contrary, after 48-hour pre-incubation, the fluorescence intensities were drastically increased, showing that the L-Pro catalyzed reaction of BIPM and acetone was achieved in aqueous medium when the liposome was coexisting. **Figure 2-7b** shows the time course of the fluorescence intensity, showing that the k value at 48-hour pre-incubation was five times higher than that of 24-hour pre-incubation. It has been reported that the L-amino acid adsorption was not proceeded until 24 hours, and then dramatically proceeded within 24 to 48-hour pre-incubation [Ishigami *et al.*, 2015], in which the liposome surface property can be varied by the adsorption of L-amino acid, and then the concerted binding can be initiated. The obtained results show that the Michael addition reaction of BIPM and acetone was promoted by the L-Pro that adsorbed onto the membrane surface. Considering the facts that 1) water molecules principally inhibit the L-Pro-catalyzed reaction and 2) the liposome membrane provides a hydrophobic environment in aqueous medium, the L-Pro catalyzed reactions can be carried out in the aqueous medium, by the presence of the liposome membrane. The potential roles of the liposome membrane are to carry hydrophobic reactant (BIPM) at the membrane interior region, and to accumulate reactants and catalyst in the dehydrated environment.

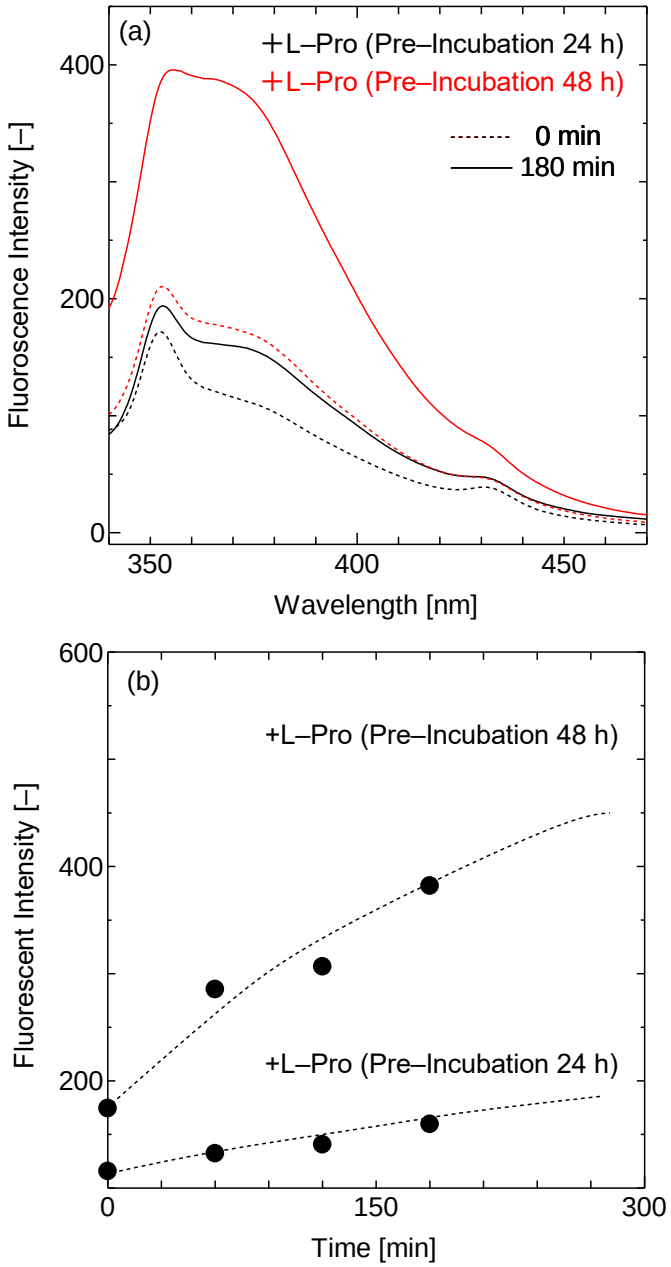


Figure 2-7 Coexistence effect of DPPC liposomes on L-Pro-catalyzed Michael addition reaction of BIPM and acetone. (a) Fluorescence spectra of products, (b) Time course of fluorescence emission peaks at 370 nm. L-Pro was pre-incubated with DPPC liposome for 24 hours and 48 hours, before initiation of the reaction.

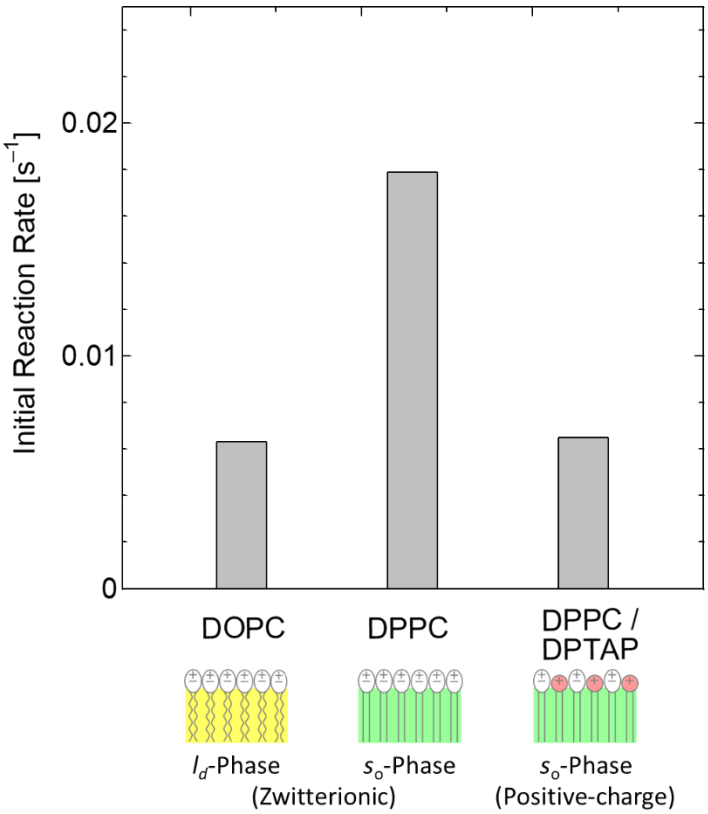


Figure 2-8 Comparison of liposome phase state and initial reaction rate.

3.3 Effect of Type of Liposome Membrane on the L-Pro-Catalyzed Reaction

Biological membranes are consisted of various kinds of lipids and proteins. Among the naturally occurring zwitterionic phospholipids, the DOPC and DPPC were selected to investigate the effect of membrane properties on this reaction. At room temperature, DOPC and DPPC liposomes are in fluid state (liquid-disordered (l_d) phase) and in rigid state (solid-ordered (s_o) phase)), respectively. It is also reported that cationic liposome DPPC/DPTAP can improve the L-Pro catalyzed Michael addition reaction. After the L-Pro pre-incubation for 48 hours, the reaction of BIPM and acetone was carried out in the presence of above liposomes (**Figure 2-8**). The initial reaction rate was herein analyzed by slope of approximate straight line of plot of fluorescence intensity at 370 nm against time since the fluorescence intensity increased proportionally with time. The initial reaction rate in DPPC (s_o phase) was 2.5 times higher than

that in DOPC (l_d phase). Furthermore, the addition of cationic DPTAP decreased the initial reaction rate, although both of DPPC and DPPC/DPTAP liposomes are in lipid membrane states (s_o phases).

The membrane surface of DPPC liposome is known to be in dehydrated state as compared to that of DOPC membrane [Suga et al., 2013], which could be favorable to stabilize the enamine intermediate. In addition, the "ordered" state of DPPC membrane could contribute to promote the adsorption of L-amino acid (including L-Pro), while the L-amino acid adsorption can be decreased when the membrane is in disordered sates [Ishigami *et al.*, 2015]. In our previous results, the modification of DPPC liposome with DPTAP slightly lead the membrane hydrophilic [Ishigami *et al.*, 2015], but the membrane property differences between DPPC and DPPC/DPTAP could be negligible. Thus, the inhibitory effect of the DPTAP on the reaction could be due to the positively-charge of the membrane.

3.4 Sequential Addition of Two Substrates to Monitor the Stability of L-Pro "*Enamine*" Intermediate

The L-Pro-catalyzed reaction was performed through the two-step operation (**Fig. 2-3(a)**) by employing two types of substrates described in **Fig. 2-3(b)**. **Figure 2-9** shows a typical example of the fluorescent spectra of the final product of the above reaction in DMSO solution. The fluorescence of product was monitored by the maximal emission peak at 360 nm. While no fluorescence was derived just after the addition of BIPM, the fluorescence gradually increased during incubation, as shown in **Fig. 2-9(a)**. The fluorescence intensity at 370 nm was plotted against the incubation time (**Fig. 2-9(b)**), where the fluorescence intensities reached to a plateau 180 min after the BIPM addition. As reported previously, the generation of reaction product.

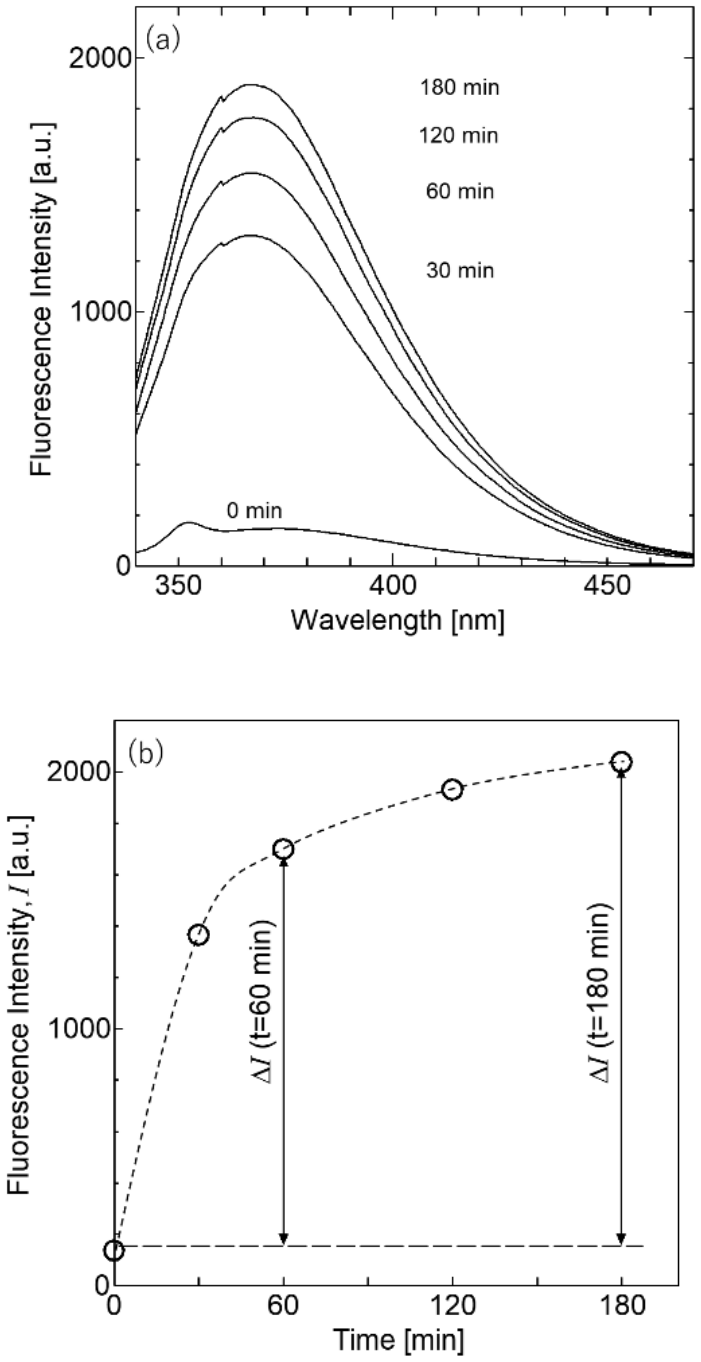


Figure 2-9 Time-dependent variation of fluorescence intensity during L-Pro-catalyzed reaction of acetone and BIPM. (a) Fluorescence spectra and (b) Time course of the maximal fluorescence of the reaction product.

of acetone and BIPM, catalyzed by L-Pro, was slow enough, thus the reaction kinetics can be monitored by fluorescence. Recently, the above reaction system has been applied to monitor the L-Pro catalyzed reaction in the lipid membrane system [Tanaka *et al.*, 2003]. It is therefore considered that the above reaction could be applied as an effective detection method to monitor the progress of the L-Pro catalyzed reaction in different systems described in **Fig. 2-2**. As described in **Fig. 2-9(b)**, the total conversion of the substrate molecules was assessed as the increment change of fluorescence intensity at 370 nm after 180 min incubation, $\Delta I_{t=180}$, and the value of $\Delta I_{t=60}$ was used to assess the conversion at initial stage of the reaction in the following.

3.5 Comparison of L-Pro-Catalyzed Reaction in DMSO solution and Liposome Membrane System.

L-Pro catalyzed reactions of ketone (acetone, butanone, 2-pentanone: first substrate) and BIPM (second substrate) were performed in different kinds of liquid phase systems: (a) DMSO system and (b) DOPC liposome membrane system. The values of $\Delta I_{t=180}$ and $\Delta I_{t=60}$ for all the conditions were shown in **Fig. 2-10**. The highest conversion of the L-Pro-catalyzed reaction was observed in the case of the DMSO solution. A similar value in $\Delta I_{t=180}$ was obtained for all the substrates tested here, while the $\Delta I_{t=60}$ was slightly reduced in the case of 2-pentanone with rather hydrophobic nature.

In the case of DOPC liposome, the increment of the fluorescence was increased with the increase of the incubation time ($\Delta I_{t=60}$ and $\Delta I_{t=180}$) for all the substrate while the maximal value, $\Delta I_{t=180}$, was retained to 10% of that in DMSO system. The above results show that L-Pro-catalyzed reaction could occur in the liposome membrane system although the reaction was performed in an aqueous solution. It has already been reported that the preferential adsorption of amino acids, including L-Pro, on the DOPC liposome membrane after long incubation time [Ishigami *et al.*, 2015]. Different from the solvent extraction system, the liposome membrane

system could induce the accumulation of the above small molecules through the multiple interaction at the membrane surface [Ishigami *et al.*, 2015]. Although some of molecules in this reaction system are rather hydrophilic, all the molecules could be accumulated in the membrane environment, considering the partial hydrophobicity (hypothetical log*P* value of partial structure exampled in **Fig. 2-10(b)**). Furthermore, the possible enamine intermediate could be stabilized at the hydrophobic environment of the interior of the hydrocarbon region of the lipid membrane. However, the chemical structure of the second substrate is not suitable for its stable orientation at the membrane surface because of rather hydrophobic nature of the partial groups at its head and tail. Actually, there is a report on the L-Pro-catalyzed reaction by selecting the second substrate as other chemicals that possess well-balanced chemical structure [Zhong *et al.*, 2007]. It is thus considered that, although the reaction yield is not so high, the hydrophobic environment of the liposome membrane system could be utilized as a platform of the L-Pro catalyzed reaction.

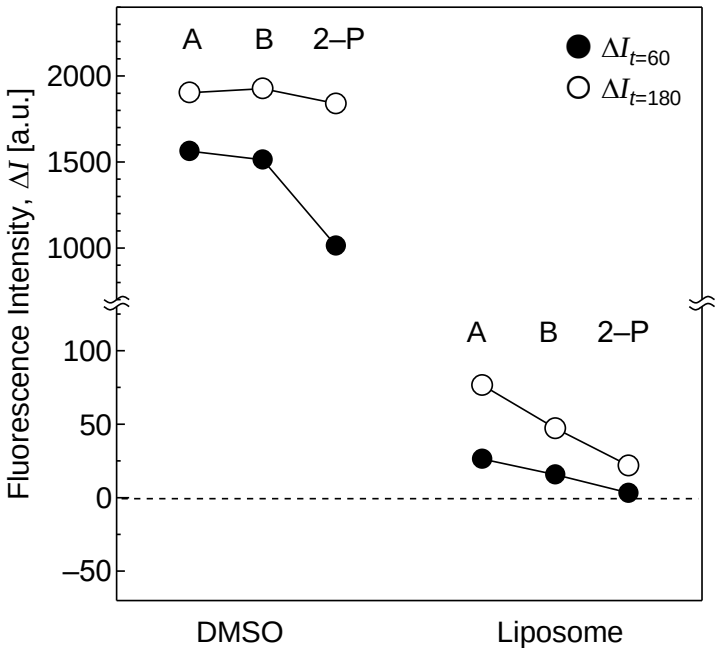


Figure 2-10 Summary of the $\Delta I_{t=}180$ and $\Delta I_{t=60}$ values in different kinds of the systems (from left to right: DMSO system, chloroform/water system, water solution, and DOPC liposome membrane system). A, B, and 2-P indicate acetone, butanone, and 2-pentanon, respectively.

3.6. Plausible Model of L-Pro Catalyzed Michael Addition Reaction of BIPM and Acetone in Liposome Membrane

Based on above results, a plausible model of this reaction is discussed in this section. To assess the location of the product in liposome membrane, the peak shift of the emission fluorescence of the reaction product was first analyzed in various kinds of solvents with different polarity. **Figure 2-11** shows the emission peak wavelength of the reaction product in 1,4-dioxane/water solution, indicating that the peak gradually blue-shifted in dose-dependent to 1,4-dioxane ratio. The emission peak of the product obtained in the DPPC liposome was rather red-shifted, indicating that the product could exist in more hydrophilic site at the liposome surface, although the emission peaks were broader in liposome systems. Possible locations of the molecules relating in this reaction are summarized in **Fig. 2-12**. The obtained results imply that L-Pro, enamine intermediate, and acetone could be basically localized at the interface region neighboring to phosphate and glycerol groups and therefore, the enamine

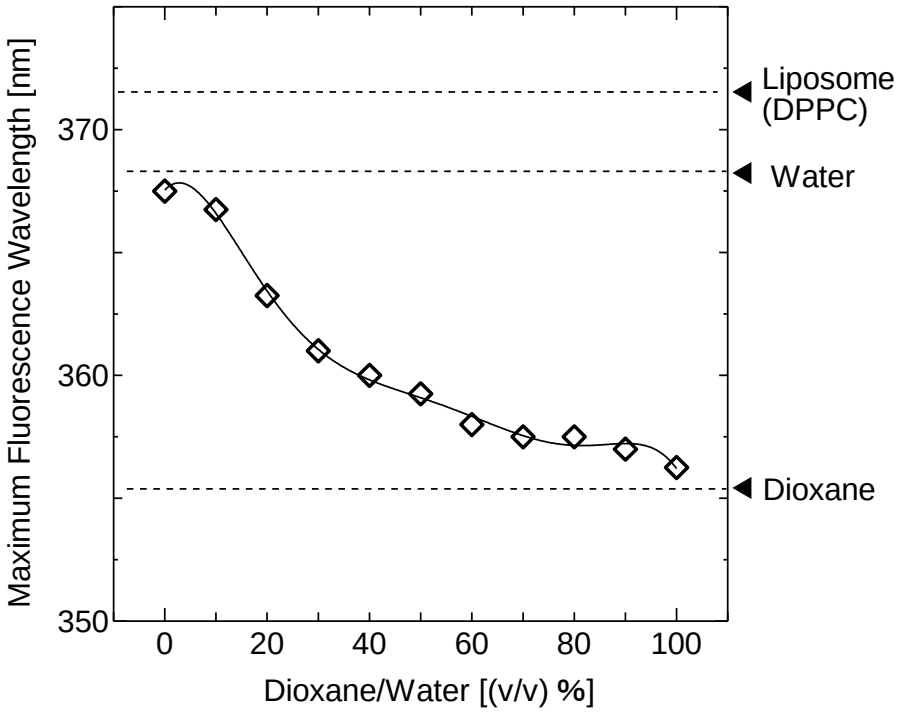


Figure 2-11 Emission peak wavelength of the reaction products in 1,4-dioxane/water mixtures.

intermediate could be stably trapped there. The reaction could be completed by the attack of the enamine intermediate to another reactant, BIPM, which could be localized in more hydrophobic region near hydrocarbon chains of lipids. Similarly, the reactants, *trans*-β-nitrostyrene (*t*βNS) and *p*-nitrobenzaldehyde (*p*NBA), can be estimated to be located around the hydrophobic site, while the *t*βNS could be localized at the glycerol region because of its zwitterionic nature of nitro group. The obtained results and previous findings suggest that the enamine intermediate, composed of L-Pro and acetone, could be preferentially localized at the hydrophobic/hydrophilic interface of the liposome membrane, which successfully promote the L-Pro catalyzed reactions. The efficiency and enatioselectivity can be varied, depending on the localized property of reactants, where *p*NBA at non-chiral environment could show the high conversion with low selectivity and *t*βNS at chiral environment could show the high enantioselectivity. For BIPM, although the conversion was lower, the reaction kinetics was directly analyzed in aqueous medium, revealing that the L-Pro catalyzed Michael addition of BIPM and acetone was surely performed in the liposomal membrane.

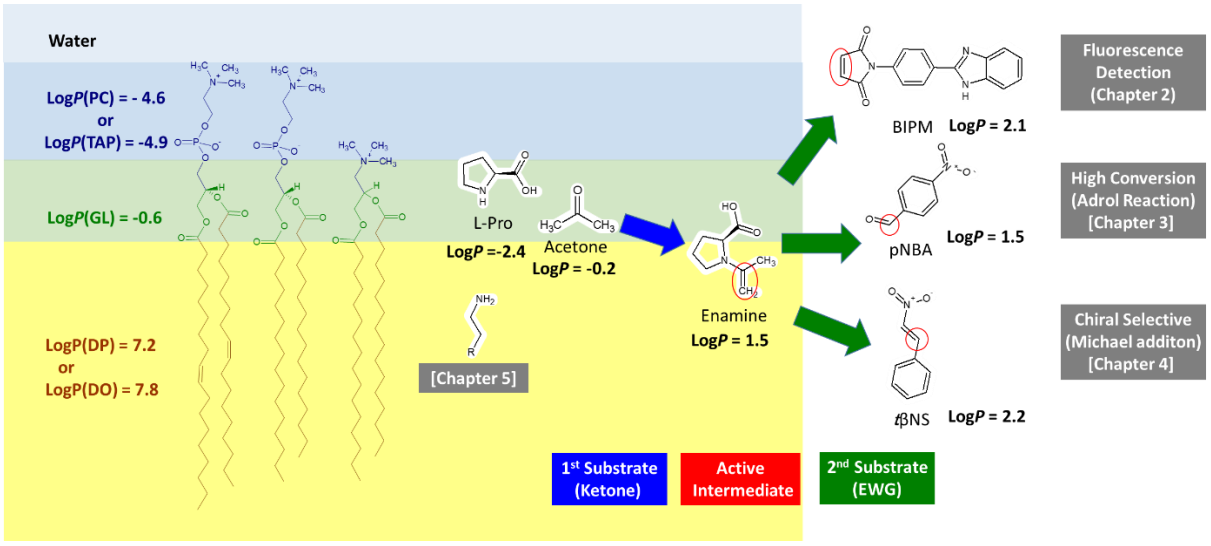


Figure 2-12 Schematic illustration of the location of the molecules relating to the L-Pro catalyzed Michael addition reaction in liposome membranes.

Figure 2-14 shows the schematic drawing the whole reaction on Michael addition of BIPM and acetone. It is assumed that the "*reaction (enamine) intermediate*" at the hydrophobic/ hydrophilic (chiral) interface of the lipid membrane could be an important factor to govern L-Pro catalyzed reaction on the liposome membrane because this reaction cannot be carried out in an aqueous solution owing to the instability of the enamine intermediate in water solution. BIPM, the second substrate of the reaction, is herewith considered to be effective for the simple and easy monitoring of the L-Pro-catalyzed reaction because it can be detectable as "fluorescence" of the reaction product.

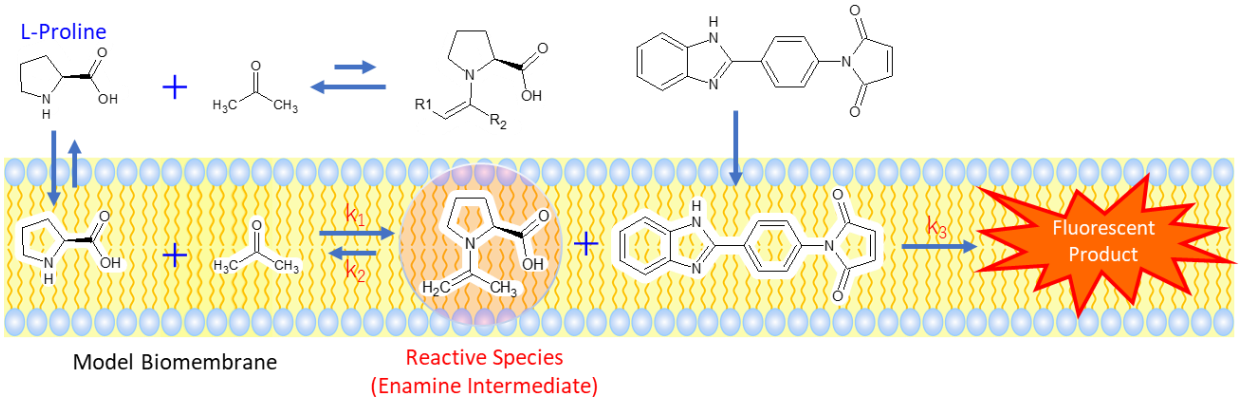


Figure 2-14 Schematic illustration of the L-Pro-catalyzed Michael addition reaction in liposome membranes.

In addition, its fluorescence can be proportional to the amount of enamine intermediate that exist at the interior of the membrane. It is therefore considered that the kinetic analysis of the increase of fluorescence of the reaction product could enable us to monitor the enamine intermediate at the interior of the membrane. The reaction rate detected as its "fluorescence" can herewith be described in the following equation relating to the concentration of enamine and BIPM.

$$\frac{dI}{dt} = k_3[Enamine][BIPM] \qquad (2\text{-}2)$$

It can furthermore rewritten as the following equation.

$$k_3[Enamine] = \frac{1}{[BIPM]} \cdot \frac{dI}{dt} \tag{2-3}$$

It is considered that the amount of the enamine can be estimated based on the reaction rate and the concentration of second substrate (BIPM) because k_3 is herewith be constant.

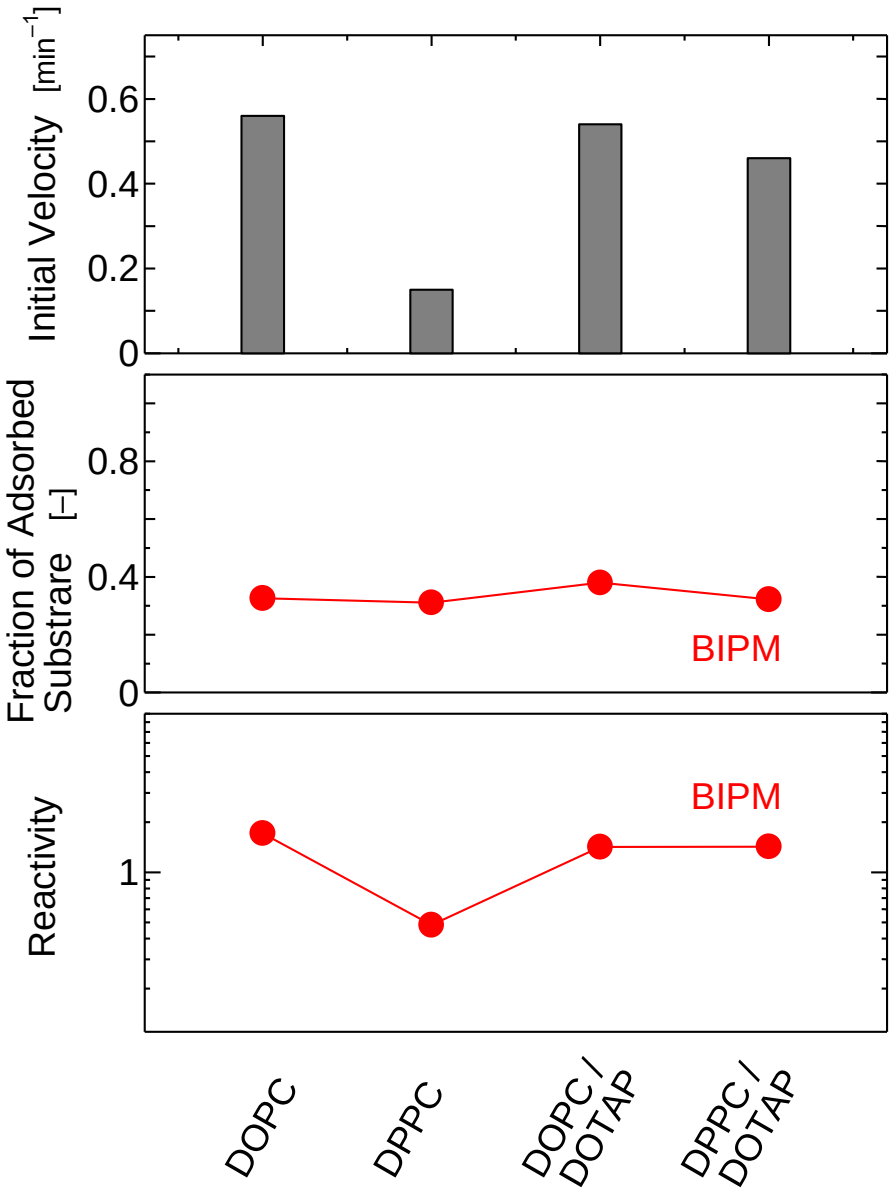


Figure 2-15 Proposal to Estimate the Reactivity on Various Membrane (c), based on (a) Initial Velocity of the Reaction and (b) Adsorption of BIPM after 30 min.

Figure 2-15(a) shows the reaction rate of the Michael addition of BIPM and acetone at the various membranes, such as DOPC (l_d phase), DPPC (l_o phase), DOPC/DOTAP (l_d phase with positive surface charge), and DOPC/DPTAP (l_d and *lo* phases with positive surface charge). The reaction rate was varied depending on the type of lipid membrane. The fraction of the adsorbed substrate on the lipid membrane was shown in **Fig. 2-15(b)**, showing that the concentration of BIPM at the membrane was almost constant. By using the equation (2-3), the amount of enamine was estimated, as enamine stability, based on the above-obtained results as shown in **Fig. 2-15(c)**. Judging from the value, it was found that the lipid membrane at l_d phase (DOPC) could provide the better environment to stabilize the enamine intermediate in contrast to that at *lo* phase (DPPC). If the DOPC membrane was modified with positively-charged lipid (DOTAP or DPTAP), its stability was similar with that of original intermediate. The above results could imply that the proposed value could be utilized to estimate the enamine intermediate on lipid membrane environment.

Based on the above results obtained in this chapter, a general scheme for the L-Pro-catalyzed reaction on "*lipid membrane*" is shown in **Fig. 2-16**. After the target membrane composition was selected, L-Pro adsorption (**Fig. 4-1**) and reaction intermediate (**Fig. 2-15**) should be suitably modulated to perform the whole reaction. The additional key factor is the orientation of the second substrate of the reaction (**Fig. 2-12**), resulting in its strong relation with the "*quantity*" and "*quality*" of the whole reaction. If the substrate could be localized on the (non-chiral) membrane surface (i.e. BIPM or *p*NBA), relatively high conversion could be obtained. If the second substrate could be located at the hydrophobic/hydrophilic (chiral) interface of the membrane via multiple interactions, resulting in the rather high chiral products (i.e. *t*βNS). The reaction was achieved in the following chapters based on this proposed scheme.

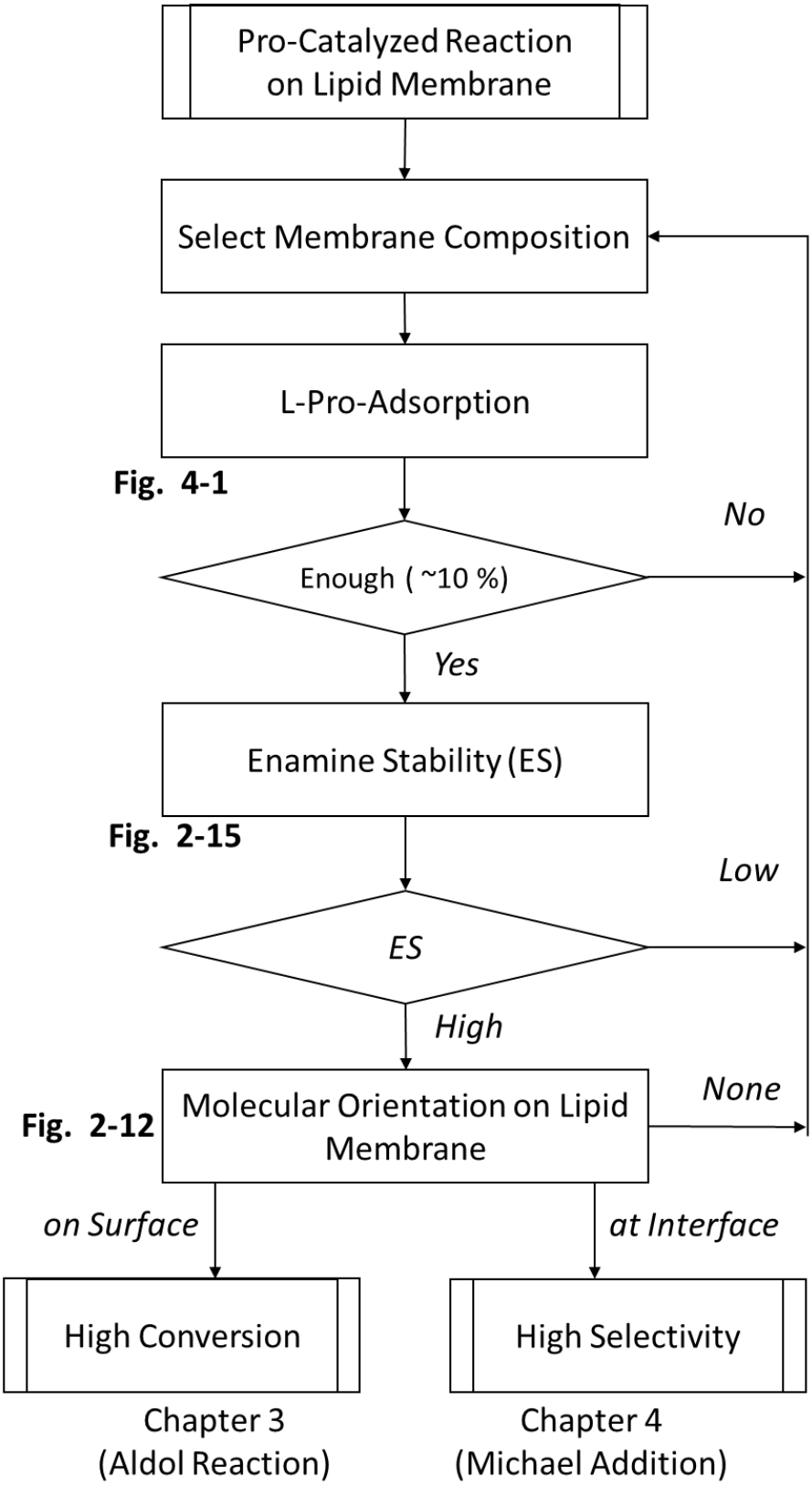


Figure 2-16 A proposed scheme for the L-Pro-catalyzed reaction on "*lipid membrane*"

4. Summary

The kinetics of the L-Pro catalyzed Michael addition reaction of BIPM and acetone were analyzed by using fluorescence spectroscopy. The composition of liposomes could be a controlling factor in this reaction: zwitterionic DPPC liposome showed highest reaction rate constant within the liposomes tested in this work. The obtained results provide us the easy-detectable method to assess the reactivity of the L-Pro at the membrane interface, which can be detected by using BIPM as donor reactant in L-Pro catalyzed Michael addition. Various systems were compared by selecting L-Pro catalyzed reaction to yield a fluorescent product at target reaction. DMSO system gives the highly efficient conversion of the reaction. In the case of DOPC liposome membrane system, the L-Pro catalyzed reaction was proceeded in spite of aqueous environment, while the conversion was very low in contrast to DMSO system. It could be related to the orientation of the second substrate (BIPM) at the membrane interface. Based on the results obtained in this chapter, a general scheme for the L-Pro-catalyzed reaction on "*lipid membrane*" was finally proposed, considering the L-Pro adsorption, enamine stability and orientation of the second substrate at the membrane interface. Among the possible regions of lipid bilayer membrane, the glycerol region that provides chiral environment was found to be strongly related with the enantiomeric selectivity of the product. It is thus considered that the liposome membrane platform would be utilized for the L-Pro catalyzed reaction after careful selection of the second substrate and, also, the beneficial aspects of the obtained reaction.

Chapter 3

Coexistence Effect of Liposome Membrane for L-Pro-Catalyzed Aldol Reaction on Liposome Membrane

1. Introduction

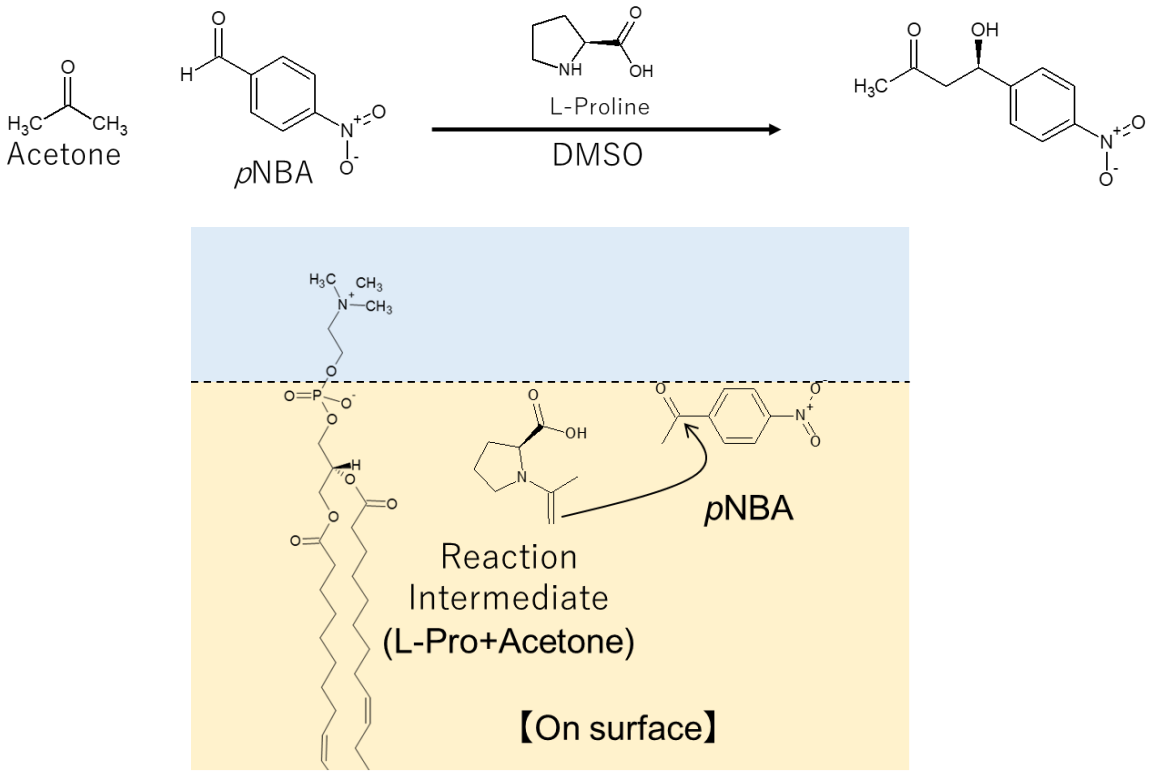
The advantages of micellar catalysts have been the focus of recent attention: in aqueous medium, the formation of micelle structures can promote the condensation of both the catalyst and reactant [Zhang *et al*., 2009, Zayas *et al*., 2013]. To achieve the aldol reaction in aqueous media, Li et al. have used calix[4]arene-based catalysts, as a hydrophobic environment exists on the inside of the host molecule [Li *et al*., 2017]. One of the aims of this study was to establish an aqueous reaction system for organocatalytic reactions, by using self-assembling structures, such as liposomes. The potential functions of liposomes (phospholipid self-assembly) as reaction media in water was investigated [Iwasaki *et al*., 2015, Iwasaki *et al*., 2017]. Also, the L-type amino-acids, such as L-Pro, L-Tryptophan, and L-Histidine, could adsorb onto the liposome membrane surface [Ishigami *et al*., 2015].

Organocatalysts, such as L-proline (L-Pro), have attracted attention as a third type of catalyst for promoting the aldol reaction (e.g., **Scheme 3-1** [List *et al*., 2000]), Michael addition, *etc*. [Gruttadauria *et al*., 2008, List *et al*., 2001, List *et al*., 2002, E. Zandvoort *et al*., 2012]. In general, polar organic solvents such as DMSO have been preferentially used as reaction media owing to the solubility of the reactants (substrates). For example, the L-Pro-catalyzed Michael addition of acetone and *trans*-β-Nitrostyrene (*t*βNS) in DMSO resulted in >97% conversion and 7% enantiomeric excess (e.e.) [Mukherjee *et al*., 2007, Hirose *et al*., 2015]. L-Pro or its derivatives have been utilized for the productions of fine chemicals (e.g., tamiful [Ahrendt *et al*., 2000, Fukuta *et al*., 2006].). Considering these pharmaceutical applications, a process

without the use of organic solvents would be preferable.

L-Pro-catalyzed reactions progress via an enamine intermediate [Gruttadauria *et al*., 2008, List *et al*., 2000, List *et al*., 2001, List *et al*., 2002, E. Zandvoort *et al*., 2012]. List *et al.* reported that L-Pro could catalyze aldol reactions in DMSO, wherein the intermediate complex, present in the hydrophobic (dehydrated) environment was effectively converted to R product. In contrast, the presence of water drastically inhibited the reaction [Dickerson *et al*., 2002, Dickerson *et al*., 2004]. Though Gruttadauria *et al.* reported that water could play important roles in achieving both higher activity and selectivity of organocatalysts, they pointed out that excess amounts of water could promote the hydrolysis of enamine intermediates, which would then inhibit the catalytic reaction [Gruttadauria *et al*., 2009].

Herein, the effects of water on this reaction was determined by using DMSO/water mixtures as solvents. Finally, the effects of liposomes with different membrane compositions were investigated by comparing the obtained conversion and enantiomeric excess (e.e.).



Scheme 3-1 L-Pro-catalyzed aldol reaction of acetone and *p*NBA.

2. Materials Methods

2.1 Materials

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC: C16:0, zwitterionic), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC: C18:1, zwitterionic) and Shingomyerin (brain porsin, bSM) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Cholesterol (Ch) was purchased from Sigma-aldrich. L-Proline (L-Pro) was purchased from Peptide Institute (Osaka, Japan). *p*-Nitrobenzaldehyde (*p*NBA) was purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan). Other chemicals were purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan) and were used without further purification.

2.2 Preparation of Vesicles.

A chloroform solution of lipids (DOPC, SM), or of their mixtures (DOPC/SM, DOPC/SM/Ch) was dried in a round-bottom flask by rotary evaporator under a vacuum. The lipid films obtained were dissolved in chloroform and the solvent was removed. These operations were repeated twice. The lipid thin film was kept under a high vacuum for at least three hours, and then hydrated with ultrapure water at a temperature above the transition temperature (T_m). The vesicle suspension was frozen at -80 °C and thawed at 60 °C to enhance the transformation of small vesicles into large multilamellar vesicles (MLVs). This freeze-thaw cycle was performed five times. MLVs were used to prepare the large unilamellar vesicles (LUVs) by extruding the MLV suspension 11 times through two layers of polycarbonate membranes with mean pore diameters of 100 nm using an extruding device (Lipofast; Avestin Inc., Ottawa, Canada).

2.3 Conversion of L-Pro-Catalyzed Aldol reaction in DMSO/Water system.

The L-Pro catalyzed aldol reaction of acetone (0.2 ml (2.7 mmol)) and *p*NBA (15.1 mg (0.1 mmol)) was conducted in DMSO/water systems (0.8 ml; ratio of DMSO/water varied from 8/0 to 0/8). The total concentration of L-Pro was 40 mM (4.6 mg (0.040 mmol)). Conversion of substrate was analyzed by high-performance liquid chromatography (HPLC), using CHIRALPAK QN-AX (diameter, 4.6 mm: length, 15 cm) (DAICEL, Osaka, Japan). Enantiomeric excess (e.e.) of the reaction product was analyzed by CHIRALPAK AD-3 (diameter, 4.6 mm: length, 15 cm) (DAICEL, Osaka, Japan).

2.4 HPLC analyses of L-Pro-Catalyzed Aldol reaction in DMSO/Water system

The reaction conversion values and enantiomeric excess were analyzed by HPLC (SHIMAZU). When conversion values were analyzed by HPLC, the peak area of *p*NBA was monitored (UV adsorption at 260 nm). Mobile phase was Methanol/acetic acid (9/1) (pH = 7, by adding 30 % ammonia water) and the flow rate was set at 0.3 ml/min. HPLC sample was made by diluting reaction mixture tenfold with mobile phase.

For the reaction mixture obtained in DMSO/water systems, the enantiomeric excess (e.e.) values were analyzed by using HPLC with a CHIRALPAK AD-3. As a pre-treatment before the chromatographic analysis, the reaction mixture (1 ml) was extracted by ethyl acetate. Ethyl acetate (1 mL) and saturated NH_4Cl solution (1 mL) were added into the reaction mixture and the aqueous layer was extracted with ethyl acetate (3 x 10 mL). The combined organic layers were dried by $MgSO_4$, filtered, and evaporated by rotary evaporator. The obtained sample was solubilized in hexane/isopropanol (IPA) = 8/2 or 9/1. After centrifugation to remove the insoluble precipitate, the sample solution was applied to the above HPLC system. The Hexane/IPA = 8/2 or 9/1 was used as a mobile phase and the flow rate was set at 0.5 ml/min. The absorbance at 260 nm was recorded (**Figure 3-1**).

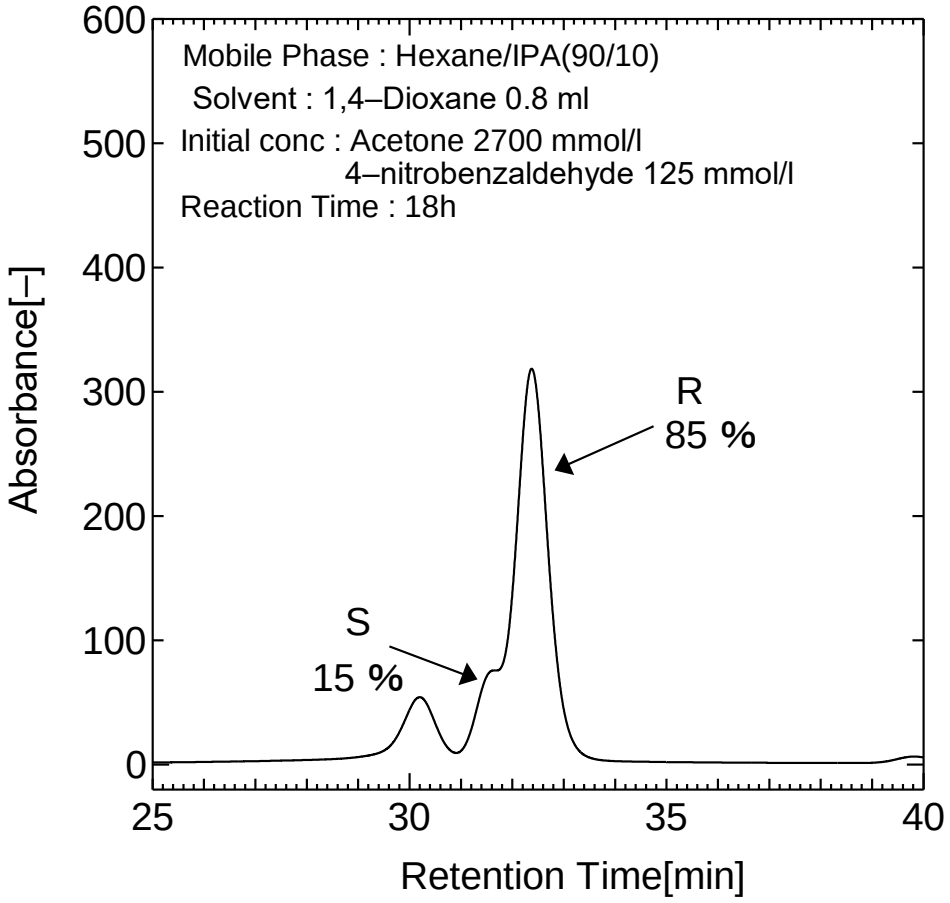


Figure 3-1 HPLC chart of Products by CHIRALPAK AD-3 (R product: 31.5 min, S product: 32.3 min.

2.5 Conversion of L-Pro-Catalyzed Aldol reaction in the liposome suspensions.

L-Pro-catalyzed aldol reaction of acetone (0.4 ml (5.4 mmol)) and *p*NBA (15.1 mg (0.1 mmol)) was conducted in the liposome suspensions (1.6 ml) at room temperature. The total concentrations of L-Pro and lipid were 20 mM (4.6 mg (0.040 mmol) and 16 mM (total lipid: 0.032 mmol), respectively. The reaction conversion values were analyzed by the HPLC, using CHIRALPAK IA (diameter, 4.6 mm: length, 15 cm or 25 cm) (DAICEL, Osaka, Japan). Enantiomeric excess (e.e.) of the reaction product was analyzed by CHIRALPAK IA (diameter, 4.6 mm: length, 15 cm or 25 cm) (DAICEL, Osaka, Japan).

2.6 HPLC analyses of L-Pro-Catalyzed Aldol reaction in the liposome suspensions.

The reaction conversion values and enantiomeric excess were analyzed by HPLC (Waters 1500 HPLC System, Waters, Milford, MA, USA). When conversion values were analyzed by HPLC, the peak area of *p*NBA was monitored (UV adsorption at 264 nm). Mobile phase was IPA/Water (8/2) and the flow rate was set at 0.5 ml/min. HPLC sample was made by diluting reaction mixture 100 times with mobile phase.

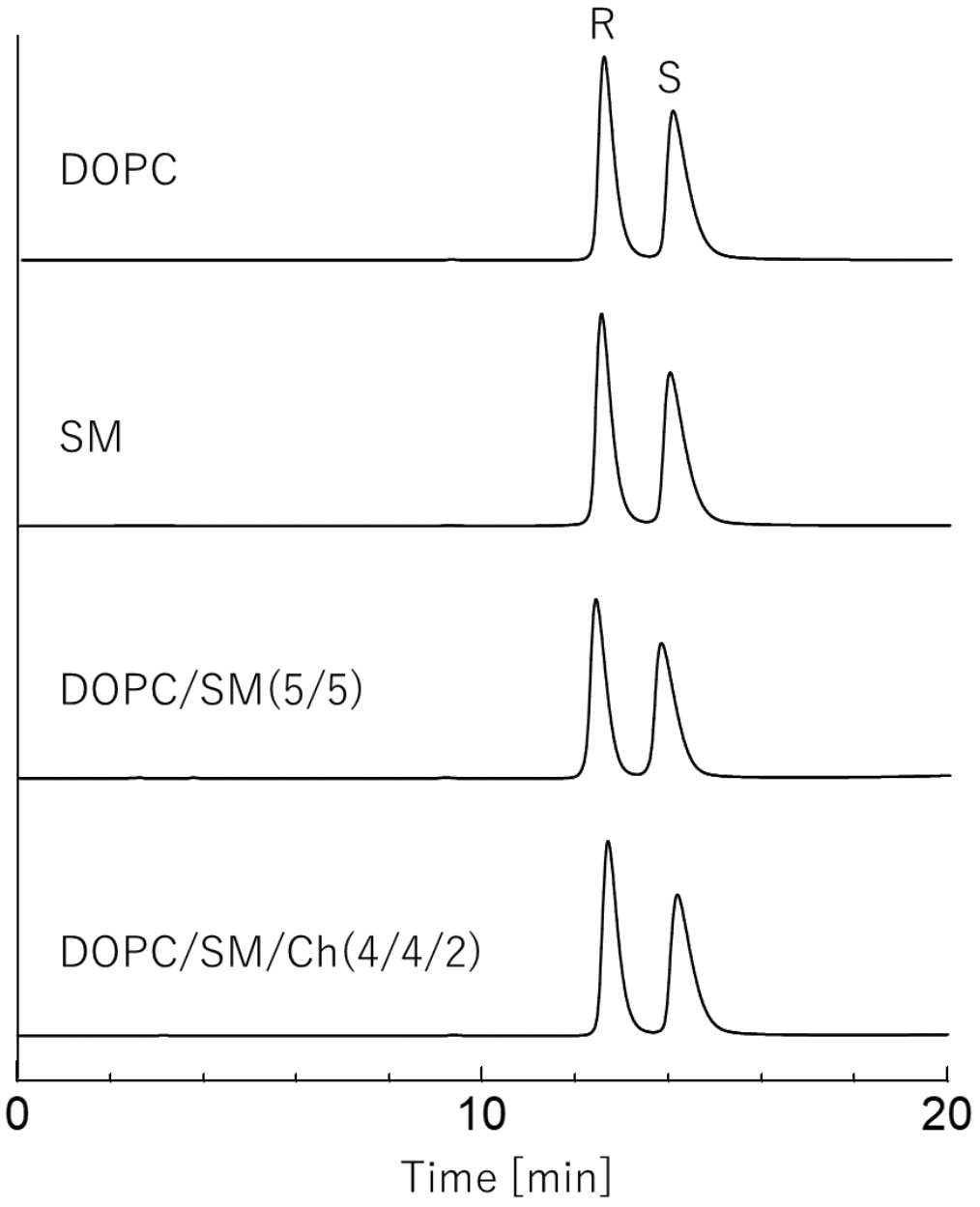


Figure 3-2 HPLC chart of Products by CHIRALPAK IA column (diameter, 4.6 mm: length, 25 cm) (R product (12.5 min) and S product (14.1 min))

The e.e. values were analyzed by using HPLC with a chiral column CHIRALPAK IA. As a pre-treatment before the chromatographic analysis, the reaction mixture (1 ml) was first mixed with methanol (water in the case of DMSO) and, then was mixed with methanol/chloroform (1/1) of twice volume (2 ml). The additional chloroform (1 ml) and water (1 ml) were added to the solution. The chloroform phase recovered was evaporated and the obtained sample was solubilized in acetonitrile (ACNT)/water (3/7). After centrifugation to remove the insoluble precipitate, the sample solution was applied to the above HPLC system. The ACNT/water (3/7) was used as a mobile phase and the flow rate was set at 1 ml/min. The absorbance at 273 nm was recorded by 489 UV/Visible Detector (Waters, Milford, MA, USA). The e.e. values were determined by the peak area of products (R = 12.5 min, S=14.1min) (**Figure 3-2**).

3. Results and Discussion

3.1 L-Pro-Catalyzed Aldol Reaction in Solvent System

The effect of solvent polarity on the L-Pro-catalyzed aldol reaction was first investigated by using DMSO/water mixtures as reaction media. The conversion and e.e. values decreased as the amount of water increased (**Table 3-1**, **Figure 3-3**). In DMSO solvent, a high conversion (conv. >90%) value was obtained after 18 h incubation, while the conversion value decreased to ~34% in DMSO/water=1/7 solvent after 8 d incubation. Basically, amino-acid catalyzed aldol reactions are slow, requiring several days to complete the reaction [Dickerson *et al*., 2002, Córdova *et al*., 2005]. In the reaction pathway, an iminium carboxylate was derived from a hemiaminal between L-Pro and acetone, after dehydration [Andreu *et al*., 2011]. At this step, the presence of excess water shifts the reaction equilibrium and then leads the reaction slowly. Recently, a cyclic intermediate of enamine, iminium, and oxazolidinone was investigated [Seebach *et al*., 2007]. Isart *et al.* reported that the oxazolidinone itself did not appear to play any productive role in the catalysis, but accelerated the aldol reaction [Isart *et al.*, 2008]. In DMSO/water solvent systems, the hydrophobicity of each solvent can be described using the

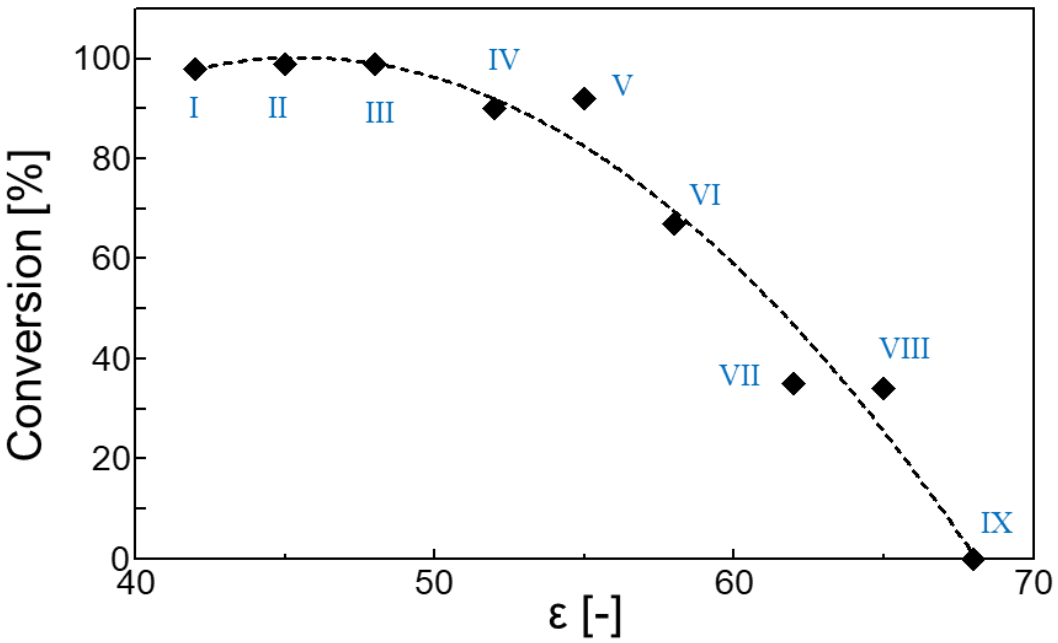


Figure 3-3 Conversion of reaction in DMSO/water systems.

dielectric constant, ε [Luzar *et al*., 1990, Yang *et al*., 2009]. As shown in **Figure 3-3**, the aldol reaction of acetone and *p*NBA could be efficiently conducted in hydrophobic environments (ε <50).

Table 3-1 Results of reaction in DMSO/water systems.

DMSO/Water [vol%/vol%]	ε [-]	Reaction time [h]	Conversion [%]
80/0	42	18	98
70/10	45	48	99
60/20	48	48	99
50/30	52	48	90
40/40	55	120	92
30/50	58	120	67
20/60	62	192	35
10/70	65	192	34
0/80	68	192	*trace*

*1 Determined by HPLC with CHIRALPAK QN-AX (column length: 15 cm, Methanol/acetic acid (9/1) (pH = 7) as mobile phase).
*2 Determined by HPLC with CHIRALPAK AD-3 (column length: 15 cm, hexane/IPA (9/1) or (8/2) as mobile phase).
*3 n.d.; not detected.

3.2 L-Pro-catalyzed Aldol Reaction in Liposome Membrane System

Naturally occurring amphiphiles, DOPC, bSM, and Chol, form a lipid bilayer vesicle structure (liposome) (**Figure 3-4**). The interior region of lipid bilayer is hydrophobic (ε ~5), whereas the surface of the liposome membrane is hydrophilic and surrounded by water (ε ~78) [Cevc 1990]. Herein, we employed liposome suspensions as reaction media, in which the lipid molecules (total lipid conc.:16 mM, ca. 1.2 wt%) formed condensed hydrophobic regions in aqueous environments. The total lipid volume was estimated as 1-2% of the solution (lipid head group area: 0.72 nm^2, lipid length: 2 nm). The L-Pro catalyzed aldol reaction of acetone and

*p*NBA was conducted in the presence of DOPC liposomes (**Figure 3-5**). The reaction proceeded slowly, with the conversion values with liposomes reaching >90%, after 8 d (**Figure 3-5b**). This could be caused by the viscous environment in the membrane. The microviscosity of the liposome interior regions can be varied, depending on the liposome composition [Iwasaki *et al*., 2015]. Such a viscous environment could decrease the diffusion of reactants. DOPC liposomes, which exist in a liquid-disordered phase (fluid), efficiently conducted the reaction in two days (conv. >90%). However, the reaction in the presence of bSM liposomes, which exist in a solid-ordered phase (gel phase), was slow, but finally reached the conversion value of 90%, after 8 d. The difference between the reaction rates of DOPC and bSM liposomes could be also affected by membrane polarities. DOPC liposomes (liquid-disordered (l_d) phase (fluid phase)) form relatively hydrophilic membranes (apparent dielectric constant value, ε~25), while bSM liposomes (solid-ordered (s_o) phase) can form dehydrated membranes (apparent ε~5) [Parasassi *et al*., 1998, Suga *et al*., 2013]. In addition, DOPC liposomes have higher fluidity, suggesting that the reactant can be distributed to the hydrophobic interior easily. The results also suggest that the hydrophobic interior regions of the liposome membranes surely act as a platform for the L-Pro catalyzed aldol reaction.

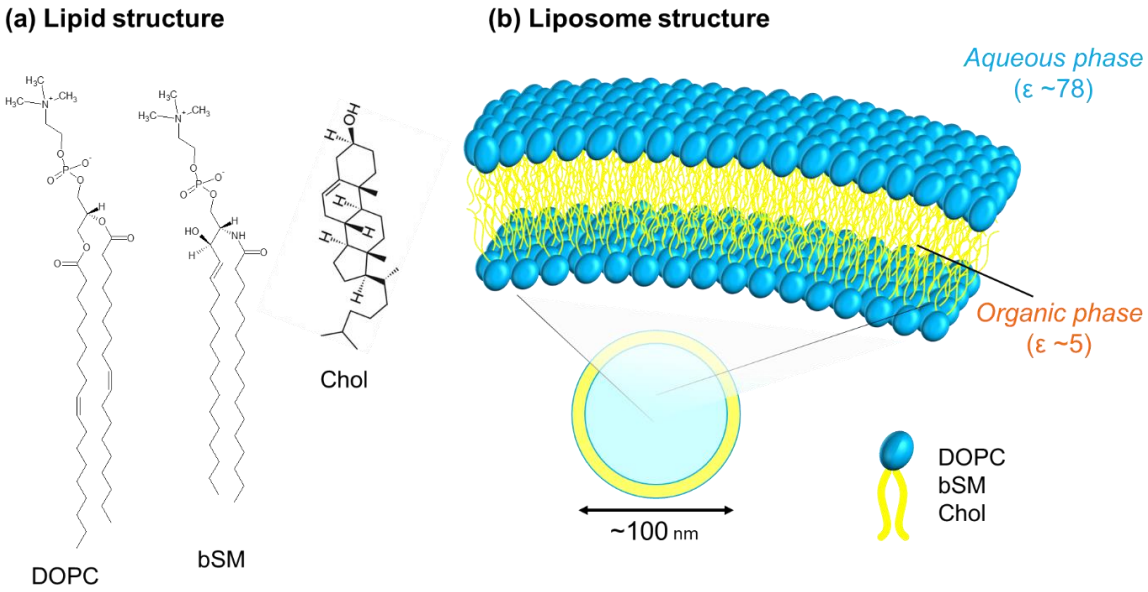


Figure 3-4 (a) Chemical structures of DOPC, bSM, and Ch. (b) Schematic illustration of liposome.

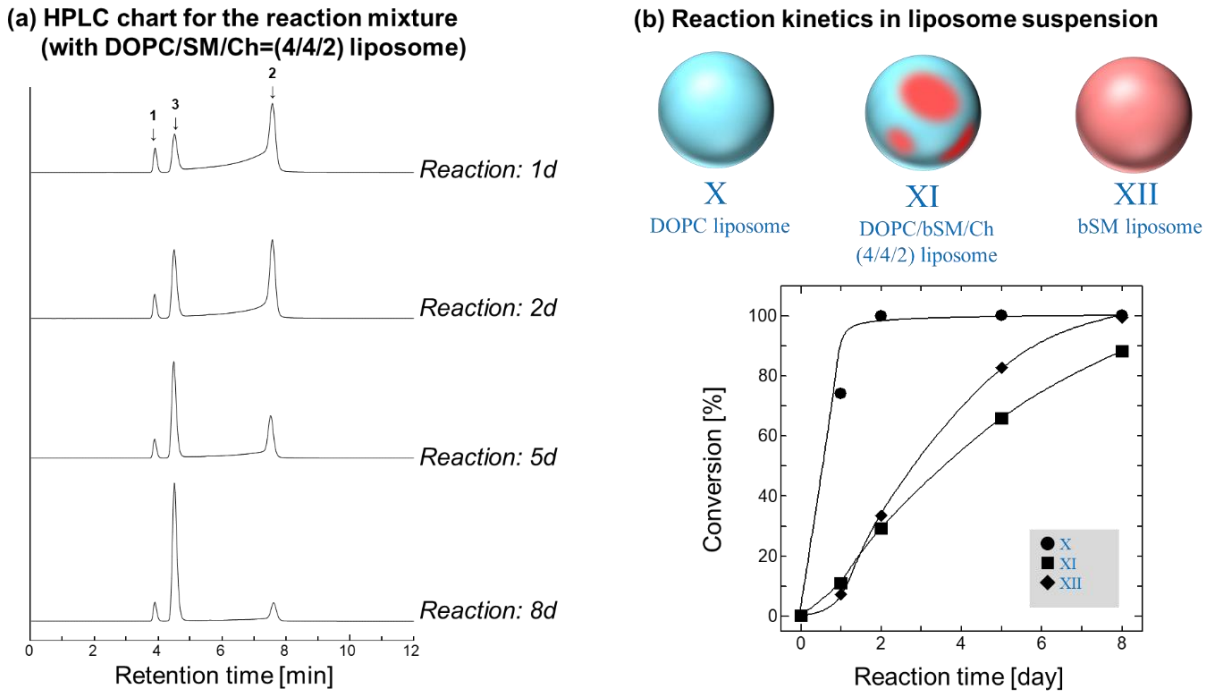


Figure 3-5. (a) HPLC chart of the reaction mixture (DOPC/bSM/Chol=4/4/2) analyzed by CHIRALPAK IA (column length: 15 cm, IPA/water (8/2)). (b-top) Schematic illustration of the liposome. The disordered (l_d) and ordered (s_o or l_o) domains are drawn with blue and red, respectively. (b-bottom) Time-course of conversion values in liposome suspensions.

The membrane properties of liposomes, i.e. membrane polarity and fluidity, can vary depending on the phase state, which can be controlled by the membrane composition [Okamoto *et al*., 2016, Suga *et al*., 2017, Han *et al*., 2017]. Veatch *et al*. investigated the phase behaviors of DOPC/SM/Chol, and reported that the phase states can be described as a single liquid phase (l_d, liquid-ordered (l_o)), two liquid phase (l_d+l_o), liquid and solid phase (l_d+s_o), and a single solid phase (s_o) [Veatch *et al.*, 2005]. Several kinds of liposomes were prepared by mixing DOPC, bSM, and Chol, for the aldol reactions to be carried out in **Table 3-2**. With an increasing ratio of DOPC, the membrane fluidity increased as the membrane became hydrophilic. These conversion values could be related to the membrane properties: in the membranes with higher fluidity and polarity, the reaction became faster with higher conversion (>90%).

The e.e. value of the reaction in DMSO solution was 74%, while it deceased to 20% in the case of DMSO/water =5/3 solvent. In the presence of liposomes, e.e. values decreased to ~1% (**Table 3-2**). Considering the hydrophobicity of the liposome membrane (ε ~5-25), water molecules hardly exist in the membrane interior regions. The enamine intermediate is generated by a dehydration reaction and reverse reaction; the presence of water could shift the reaction equilibrium to not promote the generation of the enamine intermediate. Moreover, the surrounding water can disturb the intermolecular steric interaction via hydrogen bonding. When the substrates are incorporated into the membrane, the possible interactions, such as electrostatic, hydrophobic, and hydrogen bond interactions, are formed between substrates and lipids. *p*NBA possesses a positively charged nitro moiety, which could interact with a negatively charged phosphate group in DOPC (or bSM). As a result, the aldehyde group of *p*NBA could migrate into the hydrophobic-hydrophilic interface region of the membrane.

Table 3-2 Summary of aldol reaction with liposomes

System	Reaction time [h]	Conversion[*1] [%]	e.e. [%]
DMSO	18	98	74[*2]
DMSO/water (75/25)	48	99	22[*2]
DOPC	48	99	1[*3]
DOPC/bSM/Ch (4/4/2)	192	90	1[*3]
bSM	192	99	1[*3]
DOPC/bSM (5/5)	192	98	1[*3]

*1 Determined by HPLC with CHIRALPAK IA (column length: 15 cm, IPA/water (8:2) as mobile phase).

*2 Determined by HPLC with CHIRALPAK AD-3 (column length: 15 cm, hexane/IPA (9/1) or (8/2) as mobile phase).

*3 Determined by HPLC with CHIRALPAK IA (column length: 25 cm, acetonitrile/water 3:7).

3.3 Discussion of Plausible Mechanism of the Reaction.

A plausible mechanism is shown in **Figure 3-6**. Considering the location of substrate, catalyst, and intermediate, enamine and oxazolidinone [Isart *et al.*, 2008] intermediates could be stabilized at the hydrophobic region in the liposome. In homogeneous reaction media (e.g., in DMSO), the enantioselectivity can be decided in the step of carbon-carbon bond formation, wherein intermolecular hydrogen bond plays a key role. The stereoselectivity can be explained based on Zimmerman-Traxler model: the enamine preferentially attacks to *Re*-face of reactant, to reduce steric hindrance. In our case, the liposome membrane provides a gradient of hydrophobicity in vertical axis, thus the systems were considered as heterogeneous. Herein, the location of the molecule could be roughly estimated according to its log*P* value. The obtained products were almost racemic, independent to the type of liposome. This suggests that not only *Re*-face but also Si-face of *p*NBA can be attacked by enamine at the liposome interface. In other point of view, the lipid molecules also act as hydrogen bond donor or acceptor, which could interrupt the intermolecular hydrogen bond between enamine and *p*NBA. As a result, the enantioselectivity of the product was decreased by conducting the reaction in liposome.

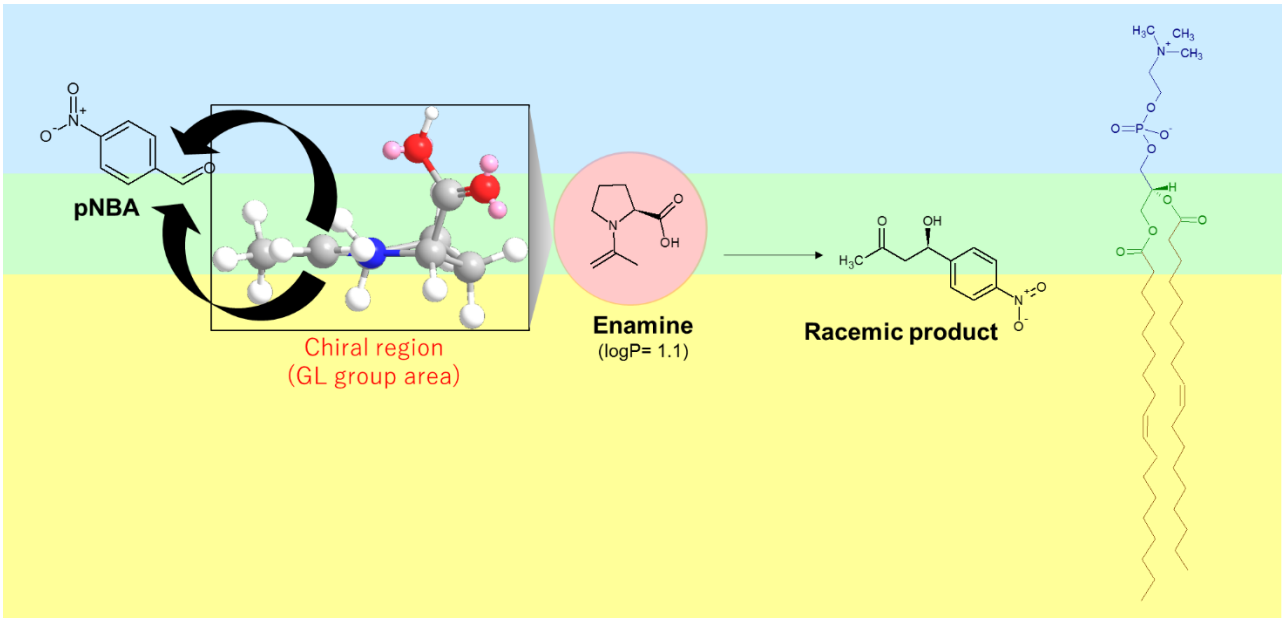


Figure 3-6 Plausible mechanism of the reaction.

4. Summary

Aldol reaction catalyzed by L-Pro was herewith investigated as an extension of the strategy described in Chapter 2, aiming at the improved conversion of the reaction product in C-C bond formation of the ketone and *p*NBA. As described in previous chapter, the enamine intermediate of L-Pro and acetone was first formed at the interior of the lipid bilayer membrane and then it was applied to the reaction with the aldehyde group of the second substrate (*p*NBA). Because the *p*NBA is not deeply inserted into the interior of the hydrophobic region of the membrane, it is expected that the reaction could occur with the higher efficiency. It was shown that the liposome membranes provided a hydrophobic region for the aldol reaction of *p*NBA and acetone, which was catalyzed by adsorbed L-Pro onto liposomes. The conversion and e.e. values of this reaction could be controlled, depending on the solvent properties, by using DMSO/water mixtures as reaction media. Thus, the membrane polarity (i.e., localized hydrophobicity) and membrane fluidity could be controlling factors of this reaction. These findings will contribute to developing environmental-friendly organocatalytic reaction systems in aqueous media.

Chapter 4

L-Pro-Catalyzed Michael Addition in Water with Model-Biomembrane

1. Introduction

In recent years, organic catalysts have attracted attention as a third type of catalyst, providing an alternative to metal catalysts and biocatalyst [Raj *et al*., 2009]. Because organic catalysts do not require a metal ion, their utility as catalysts for the asymmetric synthesis of pharmaceuticals has gained prominence [Ishikawa *et al*., 2009, MacMillan *et al*., 2008]. L-Proline (L-Pro) is an amino acid and a well-recognized organic catalyst for the aldol reaction, Michael addition, etc [List *et al*., 2000, List *et al*., 2001, List *et al*., 2002, Zandvoort *et al*., 2012]. Recently, there have been several reports of improved reactivity, reaction rate, and yield achieved with L-Pro derivatives [Lu *et al*., 2012, Rodríguez-Lansola *et al*., 2010, Mase *et al.*, 2006]. Despite the interest in the use of L-Pro and its derivatives for organic synthesis, there are some drawbacks in that the reactions usually require the use of organic solvents. Dimethylsulfoxide (DMSO) is typically used as the solvent for the conventional L-Pro catalyzed reaction; however, this solvent requires a large amount of energy for separation from the reaction mixture after conversion of the substrate [Jacob *et al*., 1986]. Using the L-Pro catalyst in a non-solvent system could broaden the horizons for its application in the development of environmentally-harmonized asymmetric synthesis processes.

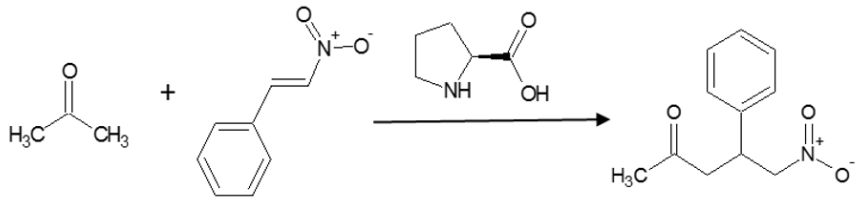
The hydrophilic-hydrophobic interface formed in self-organized systems, such as micelles and vesicles (liposomes), can be used as a platform for organic synthesis or molecular recognition in water [Dwars *et al*., 2005, Zhang *et al*., 2009, Zayas *et al*., 2013]. A liposome is a vesicle that is constructed by self-assembly of phospholipids in aqueous solution [Yeagle *et*

al., 2011]. Chemical and biochemical reactions on vesicle membrane surfaces have been evaluated in several studies [Walde *et al*., 2014, Urabe *et al*., 1996, Ueoka *et al*., 1988, Umakoshi *et al*., 2008, Yoshimoto *et al*., 2007]. In a previous study, we reported that the pseudo-interphase of the liposome membrane can promote the 1,3-dipolar cycloaddition reaction in aqueous solution [Iwasaki *et al*., 2015]. In recent years, the assembly of amphiphiles has led to certain emergent properties, where the "ordered state" of the self-assembly surface could be a key factor for achieving membrane function, i.e., molecular recognition [Tuan *et al*., 2008], porphyrin assembly [Umakoshi *et al*., 2008], polymerization of amino acid derivatives [Blocher, *et al*., 1999, Hitz *et al*., 2001], antioxidative function [Yoshimoto *et al*., 2007], *etc*.. Our previous findings demonstrate the importance of evaluating the physicochemical membrane properties, such as the membrane fluidity and polarity. Several methodologies [Suga *et al*., 2013, Suga *et al*., 2015] have been developed for probing the "microscopic" environment at the lipid membrane surface. The use of the liposome membrane as a chiral selector for amino acids is one advantageous approach [Ishigami *et al*., 2015]; for example, the adsorption of L-Pro on the 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, solid-ordered phase) liposome was much higher than that of D-Proline (D-Pro) on DPPC, whereas the adsorption of L-Pro and D-Pro on liposomes in the liquid-disordered phase has not been clarified. Liposome membranes in "ordered" phases provide a hydrophobic environment in which water molecules are excluded, and liposome membrane can selectively interact with L-Pro [Ishigami *et al*., 2015]. It is therefore expected that the liposome membrane can be used as a platform for molecular conversion in water, owing to the following features: (1) enantiospecific substrate (or catalyst, intermediate) interaction at "ordered" membranes, (2) conversion of hydrophobic molecules (e.g., log*P* >1) in aqueous media, and (3) easy separation (purification) of chiral products.

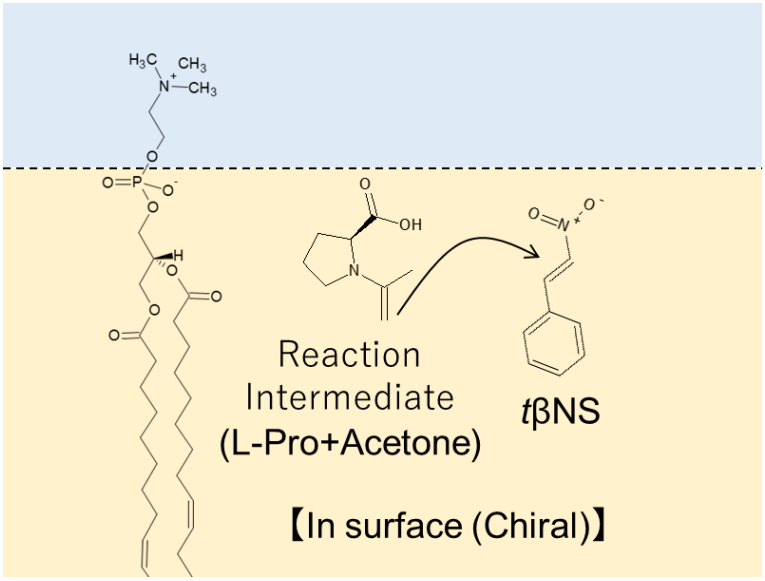
In chapter 4, the effect of the coexistence of liposome membranes on the L-Pro-catalyzed Michael addition of *trans*-β-nitrostyrene (*t*βNS) with acetone (**Scheme 4-1(a)**) is investigated. By selecting four kinds of lipids as liposome components, the membrane properties, such as

phase state (disordered/ordered) and surface charge (positively-charged/neutral), are tuned to control the reaction.

(a)



(b)



Scheme 4-1 L-Pro-catalyzed Michael addition of *t*βNS and acetone

2. Materials and Methods

2.1 Materials

Materials. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC; carbon number/unsaturated bond = 16:0), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC; 18:1), 1,2-dipalmitoyl-3-trimethyl-ammonium-propane (DPTAP; 16:0), and 1,2-dioleoyl-3-trimethyl-ammonium-propane (DOTAP; 18:1) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Chemical structures of lipids are shown in supporting information. L-Pro (>98 % purity of enantiomers) was purchased from Peptide Institute (Osaka, Japan). The fluorescent probes, 1,6-diphenyl-1,3,5-hexatriene (DPH) and 6-lauroyl-2-dimethylamino naphthalene (Laurdan), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals were purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan) and were used without further purification.

2.2 Preparation of Vesicles.

A chloroform solution of lipids (DOPC, DPPC, DPTAP, or DOTAP) or of their mixtures (DOPC/DPTAP, DPPC/DPTAP, DOPC/DOTAP, or DPPC/DOTAP, 50 mol% for each) was dried in a round-bottom flask by rotary evaporator under a vacuum. The lipid films obtained were dissolved in chloroform and the solvent was removed. These operations were repeated twice. The lipid thin film was kept under a high vacuum for at least three hours, and then hydrated with ultrapure water at a temperature above the transition temperature (T_m). The vesicle suspension was frozen at -80 °C and thawed at 60 °C to enhance the transformation of small vesicles into large multilamellar vesicles (MLVs). This freeze-thaw cycle was performed five times. MLVs were used to prepare the large unilamellar vesicles (LUVs) by extruding the MLV suspension 11 times through two layers of polycarbonate membranes with mean pore diameters of 100 nm using an extruding device (Lipofast; Avestin Inc., Ottawa, Canada).

2.3 Adsorption of L-Pro to liposome membranes.

The liposome suspensions (total lipid: 0.032 mmol) were mixed with L-Pro (0.040 mmol), and they were incubated at 25 °C for 48 hours to reach an equilibrated state. After incubation, the liposomes with adsorbed L-Pro 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 L-Pro (C_{flt}) was determined by ninhydrin reaction method by analyzing the absorbance using UV spectrometer (UV-1800; Shimadzu, Kyoto, Japan). The concentration of adsorbed L-Pro (C_{ads}) and the percentage of L-Pro adsorption (Q_{ads}) were determined from the mass balance of L-Pro before and after the ultrafiltration operation as follows:

$$C_{ads} = C_{ini} - C_{flt} \quad (4\text{-}1)$$

$$Q = (C_{ads} / C_{ini}) \times 100 , \quad (4\text{-}2)$$

where C_{ini} represent the initial concentration of L-Pro before adsorption treatment.

2.4 Kinetics of L-Pro Catalyzed Michael addition.

L-Pro-catalyzed Michael addition of *t*βNS (14.9 mg (0.1 mmol)) with acetone (0.4 ml (5.4 mmol)) was conducted in 1.6 ml of DMSO, DMSO/water (3/1) mixture, and in the liposome suspensions at room temperature. The total concentrations of L-Pro and lipid were 20 mM (4.6 mg (0.040 mmol)) and 16 mM (total lipid: 0.032 mmol), respectively. The reaction was kinetically analyzed by monitoring changes in the intensity of the UV spectrum of *t*βNS (310 nm). The reaction was assumed to be governed by first-order kinetics and the reaction rate constant, *k*, was determined by using following equation:

$$\ln \frac{C_{A0}}{C_A} = kt \quad , \quad (4\text{-}3)$$

where C_{A0} is the initial concentration of *t*βNS, C_A is the *t*βNS concentration at any time *t*, and *k* is the reaction rate constant. The pH of sample solutions was 5.5-6.5 for water and liposome

systems. In these conditions, L-Pro, DOPC, and DPPC were zwitterionic, and DOTAP and DPTAP were cationic. The normalized reaction constant (*k*') was calculated as follows:

$$k' = k / w_i \quad (i = ini \text{ or } ads) \qquad (4\text{-}4)$$

where w_{ini}[g-Pro] and w_{ads}[g-Pro], respectively, indicate the initial amount of L-Pro in DMSO system and the adsorbed amount of L-Pro on liposome membrane in aqueous solution.

2.5 HPLC Analysis of products of the L-Pro-catalyzed Michael addition.

The final products of the L-Pro catalyzed reaction of *t*βNS with acetone in DMSO solution and in liposome suspensions were analyzed by using chiral column chromatography (Waters 1500 HPLC System, Waters, Milford, MA, USA) equipped with CHIRAL PAK IA column (diameter, 4.6 mm: length, 25 cm) (DAICEL, Osaka, Japan). As a pre-treatment before the chromatographic analysis, the reaction mixture (1 ml) was first mixed with methanol (water in the case of DMSO) and, then, methanol/ chloroform (1/1) with twice volume (2 ml). Additional chloroform (1 ml) and water (1 ml) were added to the above solution. The chloroform phase recovered was evaporated and the obtained sample was solubilized in hexane (HEX)/isopropanol (IPA) (9/1). After centrifugation to remove the insoluble precipitate, the sample solution was applied to the above HPLC system. The HEX/IPA (9/1) was used as a mobile phase and the flow rate was set at 1 ml/min. The absorbance at 210 nm was recorded by a 489 UV/visible detector (Waters, Milford, MA, USA).

2.6 Measurement of membrane fluidity.

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). A sample of 10 μL of 100 μM DPH in ethanol was added into 1 mL of 0.25 mM liposome suspension. The samples were incubated at least 30 min in the dark. 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:

$$P = (I_{//} - GI_{\perp}) / (I_{//} + GI_{\perp}) \quad (4\text{-}5)$$

$$G = i_{\perp} / i_{//} , \quad (4\text{-}6)$$

where $i_{\perp}$ and $i_{//}$ are emission intensities perpendicular to the horizontally polarized light (90 °, 0 °) and parallel to the horizontally polarized light (90 °, 90°), respectively, 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.

2.7 Evaluation of the membrane polarity by Laurdan.

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:

$$GP_{340} = (I_{440} - I_{490}) / (I_{440} + I_{490}) , \quad (4\text{-}7)$$

where I_{440} and I_{490} are the emission intensities of Laurdan excited with 340 nm light at room temperature. 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.

3. Results and Discussion

3.1 L-Pro Adsorption on Liposome Membranes.

L-Pro was initially adsorbed on various liposome membranes based on the previously described procedure [Ishigami *et al*., 2015]. After mixing of L-Pro (0.040 mmol) with the liposome suspension (total lipid: 0.032 mmol), the solution (1.6 ml) was incubated for 48 h to complete the adsorption process. The percentage of the L-Pro adsorbed was determined from the mass balance of L-Pro concentration measured after adsorption and ultrafiltration. **Figure 4-1** shows the percentage of L-Pro adsorbed on various liposomes. L-Pro adsorption was approximately 8.4-14.3 %, while D-Pro adsorption was negligible (<1 %, data not shown) for the liposomes used in this study. Among the zwitterionic liposomes (DPPC and DOPC), a slight increase in the percentage of adsorbed L-Pro was observed in the case of DPPC, which is in the solid-ordered (s_o) phase. L-Pro adsorption on DOPC liposomes (in the liquid-disordered (l_d) phase) was improved by modification with positively-charged DOTAP and DPTAP lipids. The maximum adsorption of L-Pro (14.3 %) was achieved with DPPC modified with positively-charged DOTAP. The binary lipid membrane system reportedly shows phase-separation at the membrane surface, and the phase behavior is sensitive to the acyl chains of the lipids (unsaturated: l_d phase, saturated: s_o phase) [Suga *et al*., 2013]. We previously reported the enantioselective recognition of amino acids on the liposome membrane; membranes in the "ordered" phase (i.e. DPPC) can be advantageous for the adsorption of L-amino acids [Ishigami *et al*., 2015]. The L-Pro/D-Pro adsorption ratio was approximately 9 in the case of DPPC [Ishigami *et al*., 2015]. In addition, because the carboxyl group of L-Pro is deprotonated and is negatively-charged at neutral pH, the electrostatic interaction between the carboxyl group of L-Pro and the trimethylammonium group of the cationic lipid could contribute to L-Pro binding on the membrane. Although L-Pro adsorption on the liposome membrane is not as efficient (8.4-14.3 %), the adsorption of L-Pro was dependent on the phase state as well as on the surface charge of the membrane. The more rigid the membrane, the more L-Pro is bound to the

membrane (compare the data for DPPC, T_m=41 °C) and for DOPC, T_m<0 °C, in **Fig. 4-1**). Positively charged domains could further improve the adsorption of L-Pro. The positively-charged domain could improve the adsorption of L-Pro.

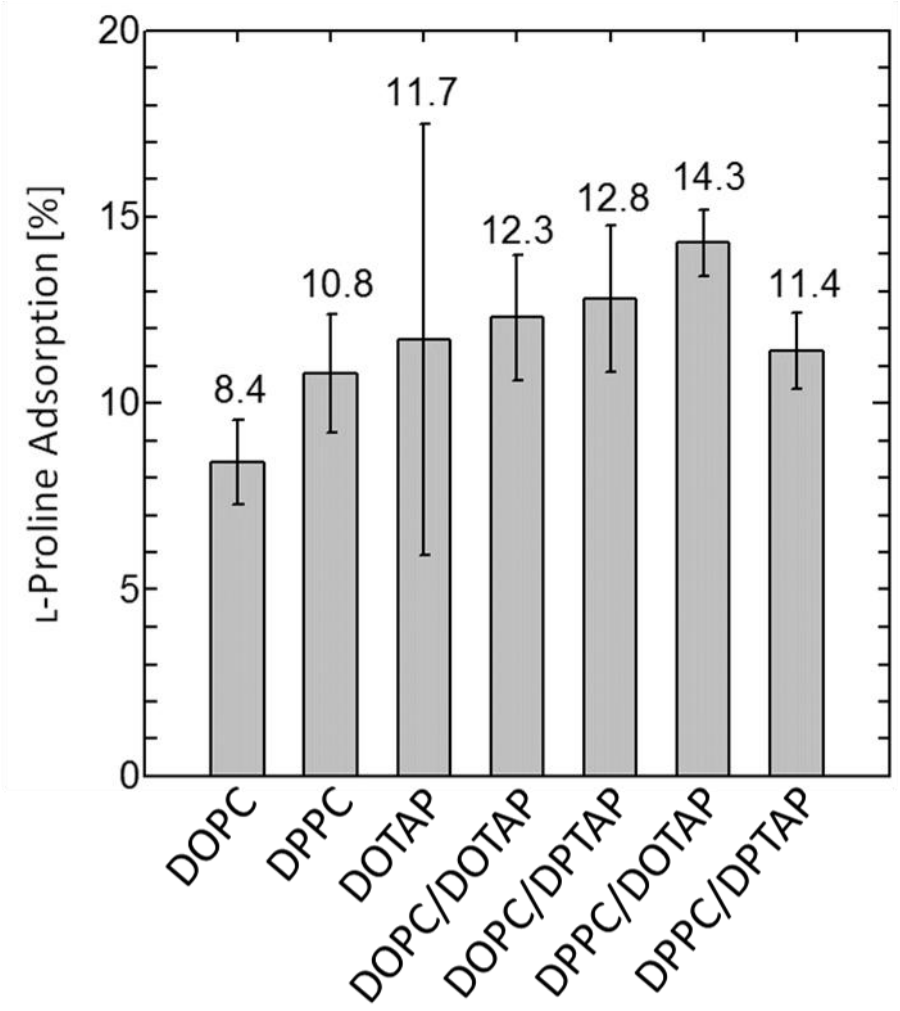


Figure 4-1 Adsorption of L-Pro onto liposomes. The solution including each liposome and L-Pro was incubated at 25 °C for 48 hours. The initial concentrations of lipid and L-Pro were 20 mM (0.032 mmol) and 25 mM (0.040 mmol), respectively.

3.2 L-Pro-Catalyzed Michael Addition in DMSO, DMSO/water, and Liposome Systems.

As previously reported, we performed the L-Pro catalyzed Michael addition reaction of *t*βNS and acetone in the DMSO solution system [List *et al*., 2001]. The time course of the UV absorbance of *t*βNS at 310 nm in DMSO, in DMSO/water (3/1), and in liposome suspensions was measured (**Fig. 4-2**). **Figure 4-3** shows the reaction kinetics. In the case of the DMSO solvent system, the reaction proceeded with pseudo-first-order kinetics, at least during the 0-120 min incubation period. The initial reaction rate constant was found to be proportional to the L-Pro concentration. Contrastingly, the presence of water significantly reduced the reaction rate, resulting in a 100-fold reduction of the rate constant. Thus, it is suggested that water molecules could play an inhibitory role in this reaction. There are several reports on the reaction mechanism for the L-Pro-catalyzed reaction, especially focusing on the "*enamine*" intermediate (**Scheme 4-1(b)**) [Mukherjee *et al*., 2007]. whereby water molecules could reduce the stability of the enamine intermediate [Mukherjee *et al*., 2007]. The above results and these previous findings clearly indicate that the efficiency of the L-Pro-catalyzed Michael addition reaction is lower in aqueous solution than in DMSO solution.

Next, we performed the L-Pro-catalyzed Michael addition of *t*βNS and acetone using the liposome membranes as the reaction platform (**Fig. 4-2**). Before the reaction, L-Pro was adsorbed onto the liposome membranes by incubation for 48 h, as shown in **Fig. 4-1**. Although the reaction rate was slower than that in DMSO, conversion of *t*βNS also proceeded in the liposome suspensions, where the reaction kinetics conform to a pseudo-first reaction within the conversion range from 0 to 70 % in the liposome suspensions (**Fig. 4-3**). Considering to the fact that (1) water molecules inhibit the L-Pro-catalyzed reaction and (2) the liposome membrane provides an environment for condensing the L-Pro (and *t*βNS), it was found that the hydrophobic environment of the liposome membrane could play an important role as a platform for the condensation and conversion of the catalyst and substrate molecules. Because not all of the L-Pro (0.040 mmol) in the medium was adsorbed on the liposomes, the initial reaction rate

decreased as compared to that in DMSO solution. It is proposed that the L-Pro molecules which adsorbed on the liposome membrane act as catalyst for this reaction. The net efficiency of L-Pro adsorption in the liposome suspensions is discussed in the following section.

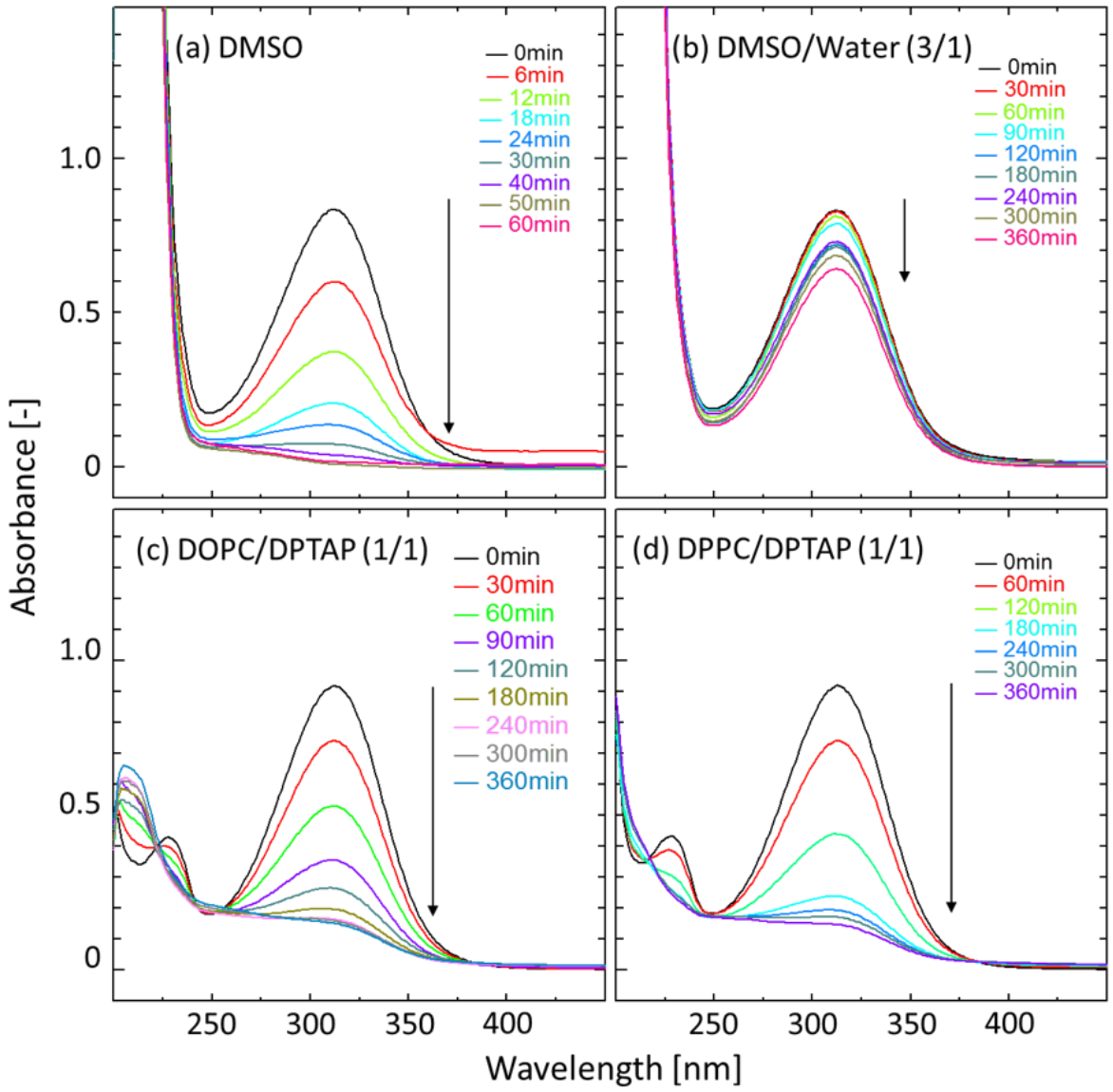


Figure 4-2. UV absorbance spectra of L-Pro-catalyzed Michael addition. (a) DMSO, (b) DMSO/water (3/1), (c) DOPC/DPTAP (1/1), and (d) DPPC/DPTAP (1/1). L-Pro (0.040 mmol) and liposome (t otal lipid: 0.032 mmol) were preliminary incubated at 25 °C for 48 hours. After the incubation, *t*βNS (0.10 mmol) and acetone (5.4 mmol) were added to the sample solution, and the reaction was conducted at room temperature.

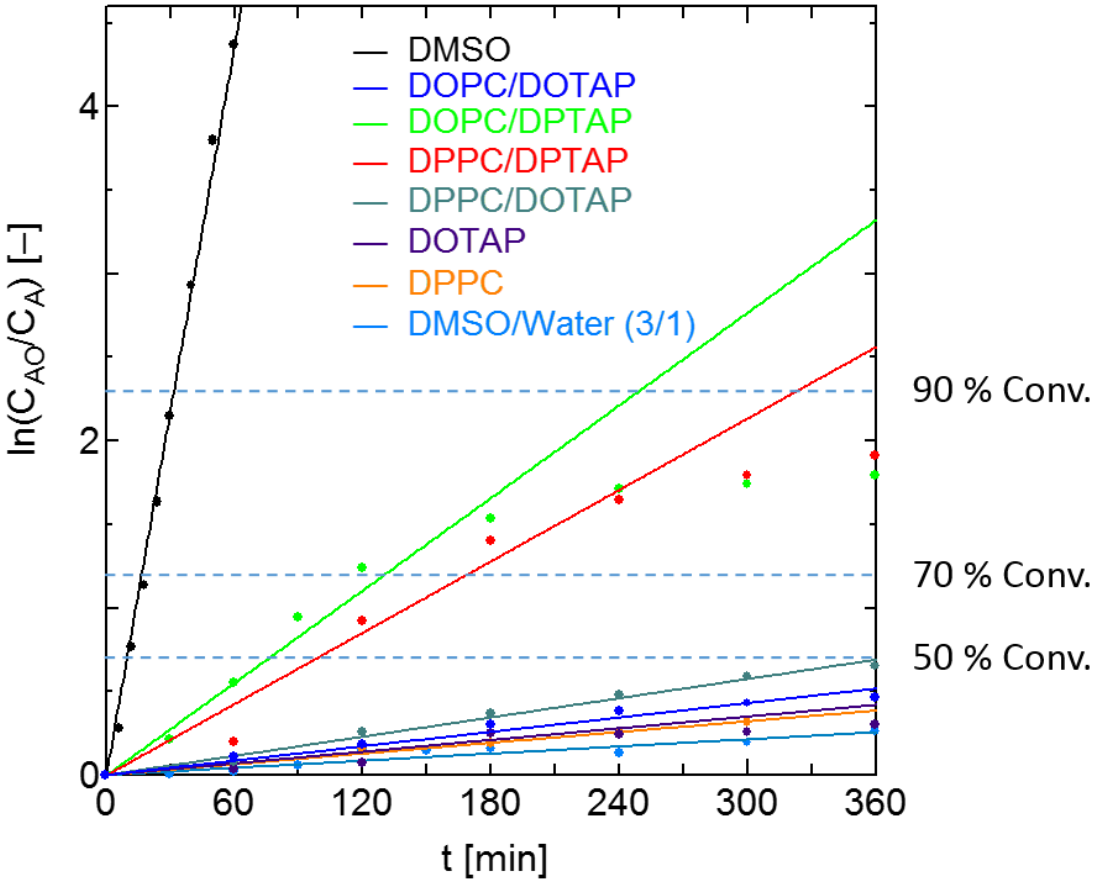


Figure 4-3 Kinetic analysis of L-Pro catalyzed Michael addition. The concentration of *t*βNS was calculated based on the UV absorbance at 310 nm. L-Pro (0.040 mmol) and liposome (total lipid: 0.032 mmol) were preliminary incubated at 25 °C for 48 hours. After the incubation, *t*βNS (0.10 mmol) and acetone (5.4 mmol) were added to the sample solution, and the reaction was conducted at room temperature.

3.3 Kinetic Analysis of L-Pro-Catalyzed Michael Addition in DMSO and Liposome Systems.

In order to assess the L-Pro-catalyzed reaction within the initial period, the apparent reaction rate constant, k, and the normalized reaction rate constant, k' (= k/w_{ads}), w_{ads} [g]: the amount of L-Pro adsorbed), were plotted presented in **Fig. 4-4**. For the DMSO solution, the k value was significantly higher than that achieved under the other conditions evaluated herein, while no reaction was observed in water, which clearly shows the high-efficiency of the reaction in DMSO. The k values in the DOPC/DPTAP and DPPC/DPTAP liposomes system were found to be higher than those in the other liposome systems. As shown in **Fig. 4-1**, not all of the L-Pro in the system (each liposome suspension) was adsorbed on the liposome membrane: only a small portion of L-Pro (approximately 8.4-14.3 %) was adsorbed. Based on the results obtained for the DMSO/water and water systems, L-Pro in the bulk aqueous cannot act as a catalyst. In contrast, L-Pro adsorbed on the liposome membrane can act as an "effective" catalyst in aqueous media. Based on the amount of L-Pro adsorbed, the calculated k' value, which represents the reaction constant of L-Pro adsorbed on the liposome, was comparable to the k value in DMSO as a good medium for this reaction. The k' values for the DOPC/DPTAP and DPPC/DPTAP liposome systems were almost equivalent to that obtained in DMSO. The turn-over number of the reaction, the number of moles of substrate that a mole of catalyst (L-Pro) could convert [mol-substrate/mol-Pro], was estimated as 15-18 based on the conversion data. The above results suggested that the L-Pro catalyst can also function in aqueous medium when the liposome membrane provides a hydrophobic environment.

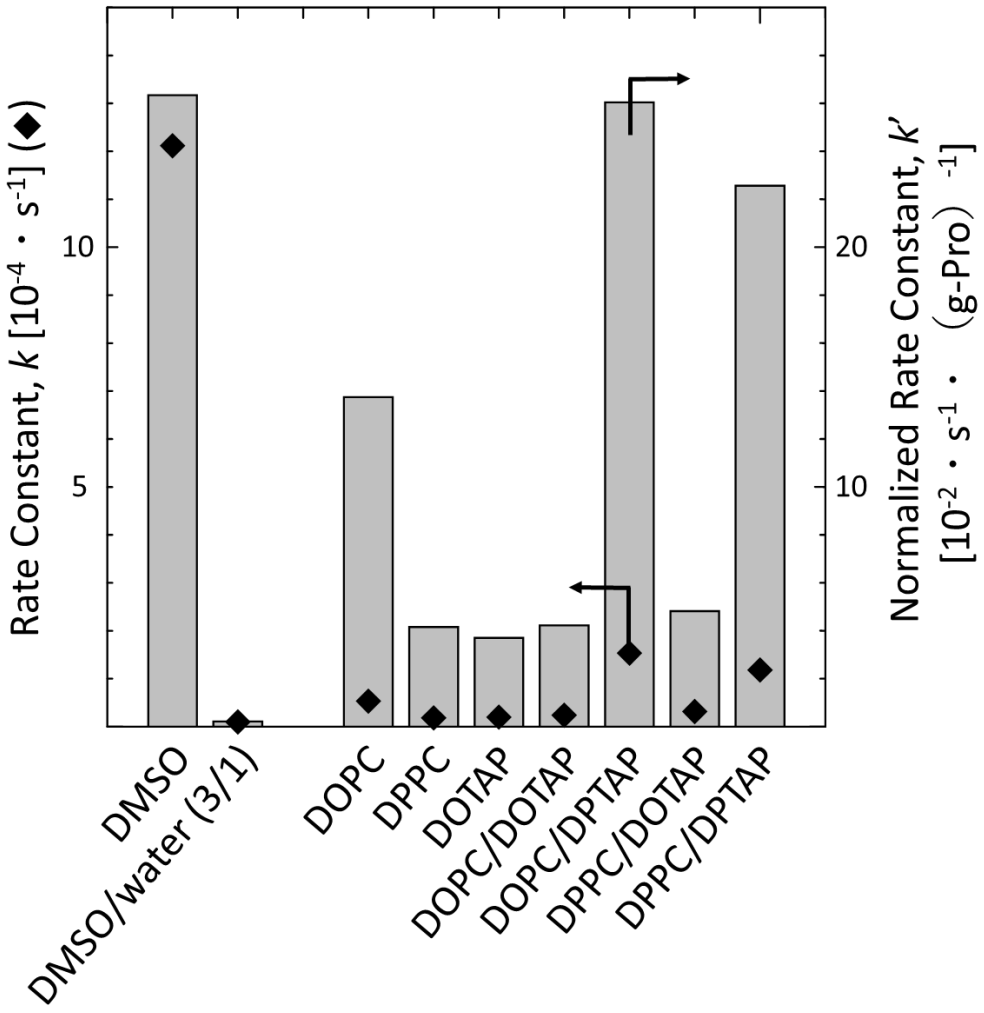


Figure 4-4 Reaction rate constant *k* (*closed diamond*) and normalized rate constant *k*' (*filled bar*). The *k* values were calculated based on the eq. (3). The *k* values obtained were normalized as following equation: $k' = k/w_{ads}$. For DMSO system, the *k'* value was calculated as $w_{ads} = w_{ini}$.

3.4 Enantiomeric Excess of L-Pro-Catalyzed Michael Addition in DMSO and Liposome Systems.

The products of the L-Pro catalyzed reaction were further analyzed by high performance liquid chromatography (HPLC) with a chiral column. **Figure 4-5** shows the chromatogram of the products obtained in (a) DMSO solution, (b) DOPC/DPTAP liposome solution, and (c) DPPC/DPTAP solution. Twin peaks at 10 min, which could be attributed to the product, were observed in the DMSO solution (control condition). According to a previous report, left peak was S-product and right peak was R-product, respectively [Gu *et al*., 2009]. Higher conversion (ca. 99 %), but lower enantioselectivity (enantio excess (ee): 10 ee%), was obtained for the L-Pro catalyzed reaction of *t*βNS with acetone in DMSO solution. The conversion and enantioselectivity data are summarized in **Table 4-1**. The results obtained for the DMSO system are consistent with previous report [List *et al*., 2001]. On the other hand, the obtained peaks for the R-product had higher abundance in the case of DOPC/DPTAP and DPPC/DPTAP liposomes. Although the conversion in the liposome systems was not significantly higher than that in DMSO, higher enantioselectivity was obtained in the liposome systems. Thus, it is demonstrated that liposome membrane system with certain compositions such as DOPC/DPTAP or DPPC/DPTAP, can improve the conversion and enantioselectivity of the L-Pro catalyzed reaction in aqueous media.

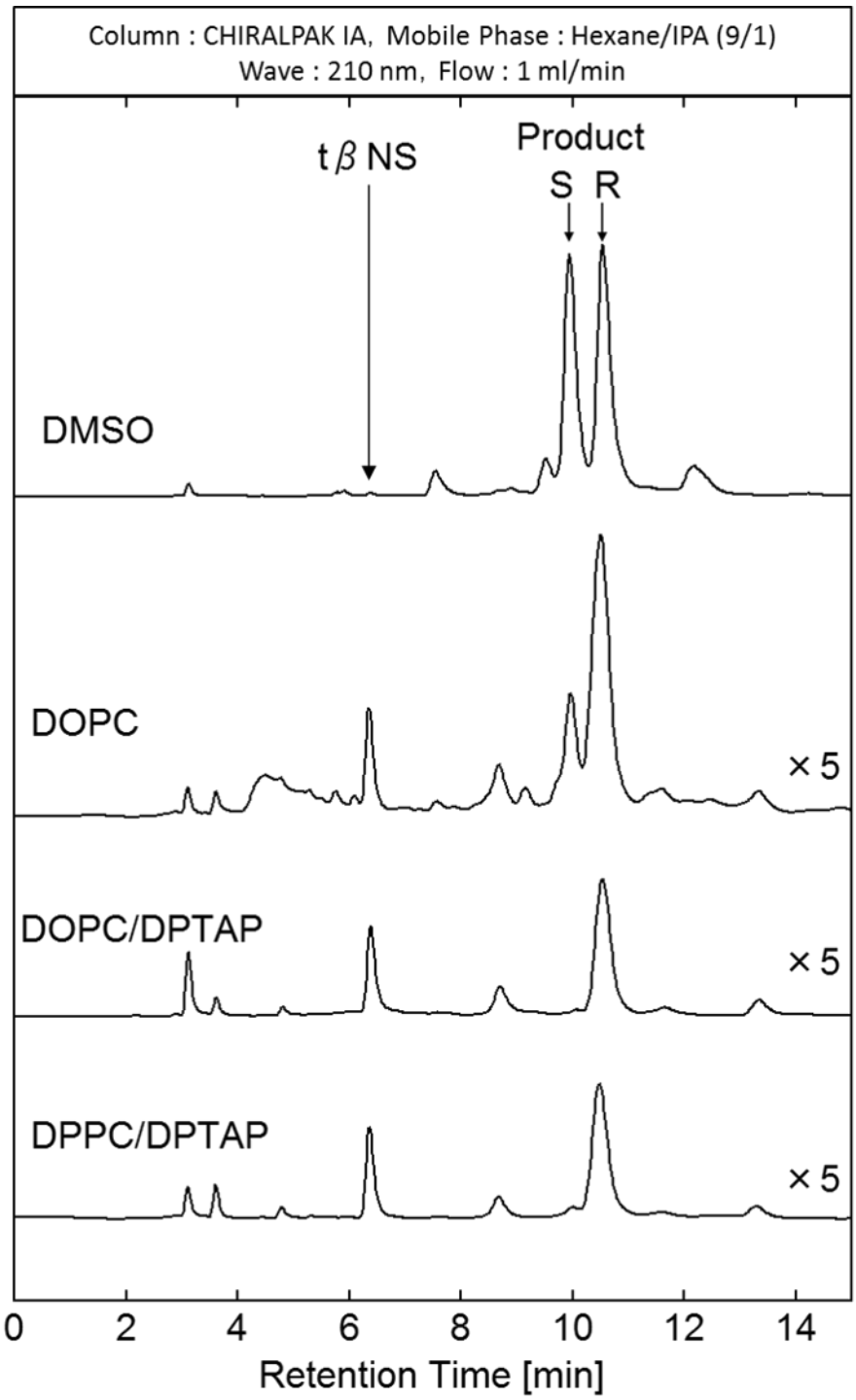


Figure 4-5 HPLC analysis of the product. CHIRALPACK IA was used as column, and the amount of the *t*βNS and product was detected by UV absorbance at 210 nm. For peak assignments, see Gu *et al*.

3.5 Characteristics of the Liposome Membrane and its Role in Reaction Kinetics.

The liposome membrane was found to affect the adsorption and reactivity of the catalyst and substrate, depending on the composition of the liposome. It was recently reported that the 1,3-dipolar cycloaddition reaction of benzonitrile oxide and *N*-ethylmaleimide was promoted in the liposome membrane [Iwasaki *et al.*, 2015]. Previously, DPH and Laurdan were employed as fluorescence probes to characterize membrane fluidity and membrane polarity, respectively [Suga *et al.*, 2013]. Herein, all data were plotted on a Cartesian plot (**Fig. 4-7**), where the cross point of *x*-axis and *y*-axis are set at the thresholds of the phase transition in terms of membrane fluidity and membrane polarity, respectively (**Fig. 4-6**) [Suga *et al.*, 2013]. As schematically shown in **Fig. 4-8**, all of the molecules (i.e., the substrates (*t*βNS and acetone), catalyst (L-Pro), and their intermediate complexes (enamine complexes)), could exist at the surface region of the liposome membrane, indicating that the aforementioned membrane properties had a direct impact on the Michael addition reaction in the interior of the nano-platform created by the lipid self-assembly.

The following four factors associated with the membrane properties may be significant for the promoting effect on the L-Pro-catalyzed reaction: (1) *heterogeneity*: among all the conditions evaluated, the data for the DOPC/DPTAP and DPPC/DOTAP liposomes, plotted in the first quadrant of the Cartesian diagram, showed that their membranes can be considered as heterogeneous surfaces having an ordered (s_o) phase domain (DPPC- or DPTAP-domain). (2) *Hydration*: water is considered a strong inhibitor of the L-Pro catalyzed Michael addition employed in this study. Similarly, in the case of the reaction in aqueous solution, the L-Pro catalyzed reaction may be affected by the "*wetness*" at the interface of the liposome membrane, which implies that "*bound water molecules on the membrane*" could reduce the stability of the enamine intermediate during the L-Pro catalyzed reaction (**Scheme 4-1(b)**) [List *et al.*, 2000, Mukherjee *et al.*, 2007]. In fact, the reaction rate constants were lower for the liposomes with lower GP_{340} values (i.e., DOTAP and DOPC/DOTAP), as shown in **Fig. 4-4**. (3) *Substrate*

accessibility: the membrane fluidity (1/*P*) can also be considered an important factor for condensation of the substrate molecules (*t*βNS and acetone) on the membrane, considering the ease-of-penetration of the molecules into the ordered self-assembly. (4) *Positive charge*: in this study, the negatively charged molecule (*t*βNS) was selected as a substrate in the L-Pro catalyzed reaction. Although the variation in the membrane properties of DPPC and DPPC/DPTAP was not significant (**Fig. 4-7**), a dramatic improvement in the *k* values was observed with the use of liposomes possessing positive charge and ordered (s_o) phases, which reveals the impact of the positively-charged domain on the membrane in promoting the reaction kinetics. As described in the previous section, higher *k* values were obtained in DOPC/DPTAP and DPPC/DPTAP systems. It seems that the positively charged surface (factor 4) is the most important of the aforementioned factors affecting the L-Pro catalyzed reaction. In addition to the positive charge effect, hydrated water (factor 2) could promote the L-Pro-catalyzed reaction in the case of DPPC/DPTAP; moreover, the effect of the positively-charged ordered domain (factors 1-3) could promote the reaction in the DOPC/DPTAP system. Although the amount of the product in the liposomes systems was less than that in generated DMSO, it is assumed that an enantioselective conversion process can be designed (**Table 4-1**), by utilizing the liposome membranes as a reaction media.

Therefore, the ordered (s_o) domain with positive charge on the heterogeneous membrane can provide a better platform for the L-Pro-catalyzed reaction in aqueous solution because of the high stability of the enamine intermediate during the reaction. Although further investigation is needed, the enantioselectivity of the obtained product is thought to be improved because of the chiral environment in the interior of the liposome membrane surface (glycerol backbone region possessing an asymmetric carbon).

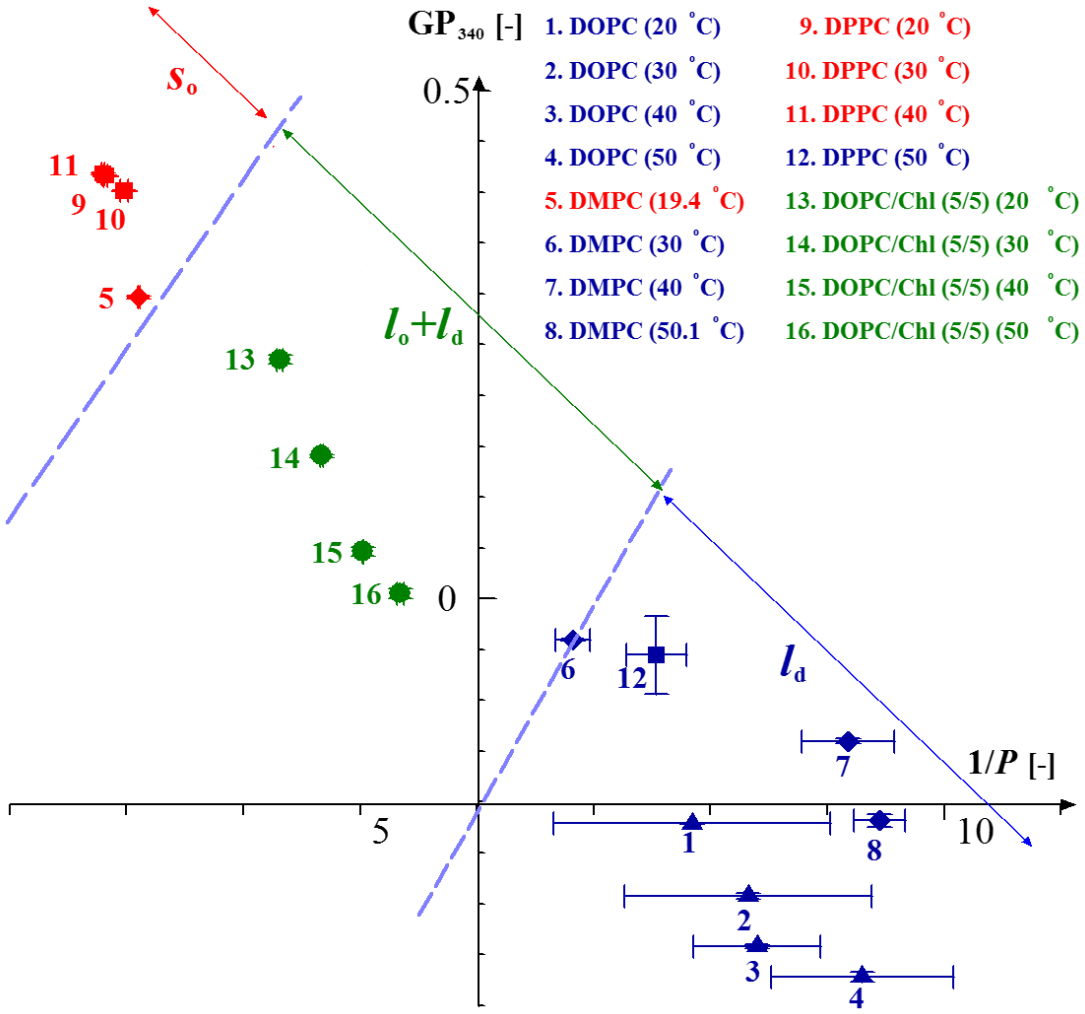


Figure 4-6 Cartesian diagram shown the membrane fluidity (1/*P*, X axis) and polarity (GP_{340}, Y axis) between 19-50 °C. It was found that by using DPH fluorescent probe, the 1/*P* value between disordered and ordered phases is estimated to be 1/*P* = 6, while membrane polarity determined by Laurdan shown that the threshold between disorder and order phases is GP_{340} = -0.2. Therefore, the threshold point in the Cartesian diagram is considered with 1/*P* = 6.0 and GP_{340} = -0.2. Liposomes are plotted in the first quadrant being heterogeneous phase, in the second quadrant being order phase and in the fourth quadrant being disordered, while no liposome distributed in the third quadrant [Suga *et al.*, 2013].

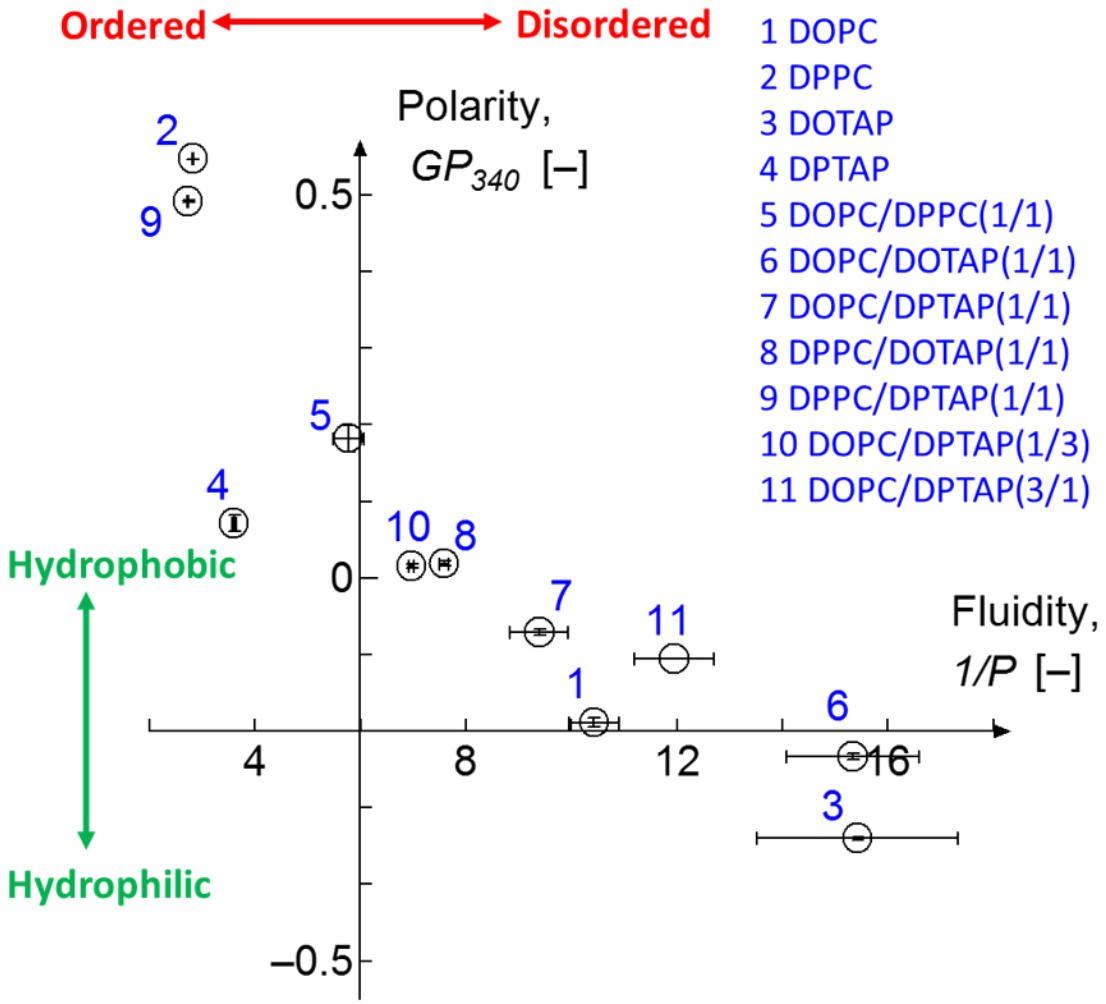


Figure 4-7 Cartesian diagram of liposomes. The membrane fluidity (1/*P*) and membrane polarity (GP_{340}) were measured at room temperature. Bars show error on individual data (at least n=3).

Table 4-1 Conversion, yield, and e.e. values of L-Pro-catalyzed Michael addition

Condition	Conversion [%] (*1)	Yield [%] (*2)	e.e.[%]
DMSO	>99 (at 1 h)	>99	10
DMSO (Ref.[4])	—	97	7
Water (*3)	n.d.	n.d.	n.d
DOPC	77.3	56.9	81.9
DPPC	73.6	36.2	40.7
DOPC/DPTAP	89.1	70.1	97.6
DPPC/DPTAP	92.8	75.5	88.7

*1 Determined by UV spectrum peak of tβNS at 310 nm, after 24 h conversion.

*2 Determined by the peak areas of tβNS and products (R and S) in chromatogram using HPLC equipped with chiral column (**Fig.4-5**).

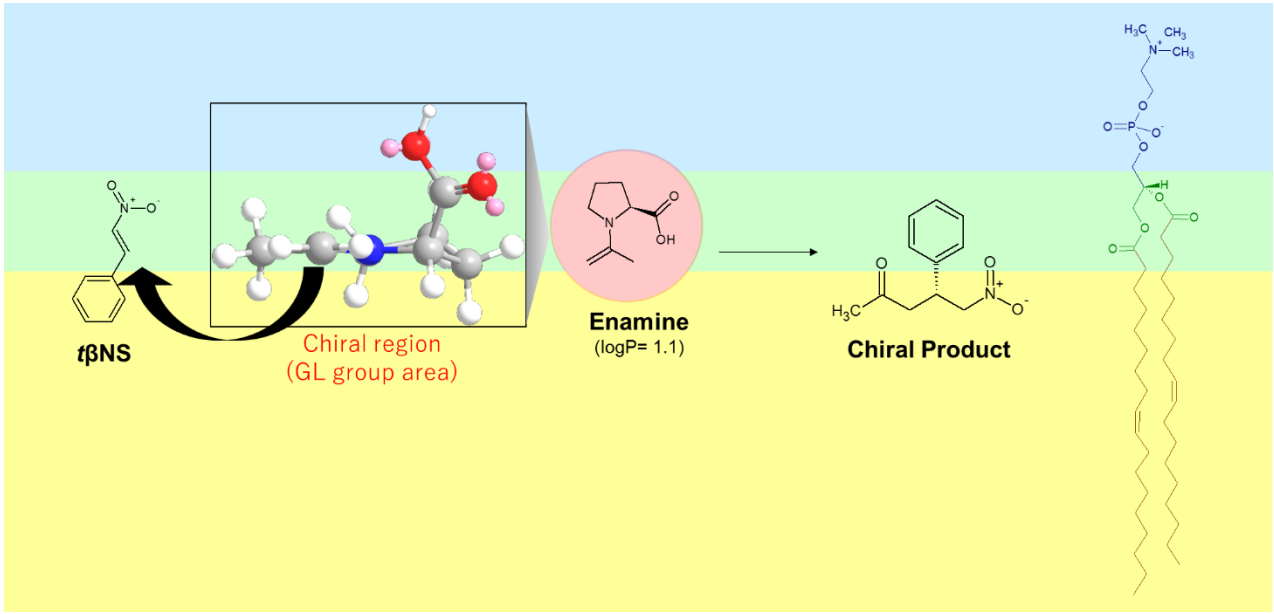


Figure 4-8 Plausible mechanism of the reaction.

4. Summary

As an extension of the strategy described in Chapter 2, the L-Pro catalyzed reaction on membrane was applied to the enantioselective conversion of C-C bond formation of the ketone and *t*βNS. After the enamine intermediate of L-Pro and acetone was formed at the interior of the lipid bilayer membrane, it will react with the unsaturated bond of the second substrate (*t*βNS). It is therefore considered that the orientation of the *t*βNS at the membrane could be important to improve the enantioselectivity of the product. In other words, it is considered that reaction at chiral region of lipid membrane is important to enantioselective reaction. It was found that the L-Pro catalyzed reaction could proceed in "water" using a liposome membrane. Specifically, when the liposome membrane surface was modified in the dehydrated state, i.e., having "*ordered"* (s_o) *phase* and a "*positively-charged*" surface, the normalized reaction rate constant in the DOPC/DPTAP and DPPC/DPTAP systems, calculated based on the amount of L-Pro adsorbed, was almost equal to that in the DMSO. It is proposed that the liposome membrane can be utilized as a "*platform*" to concentrate both catalyst and substrate molecules and to effectively convert the substrate.

Chapter 5

Michael Addition of *t*βNS and Acetone on Liposome Membrane Modified with Simple Primary Amine

1. Introduction

Pro is an amino acid and a well-recognized organic catalyst for the aldol reaction, Michael addition and so on [List *et al*., 2000, List *et al*., 2001, List *et al*., 2002, Zandvoort *et al*., 2012]. A key in the L-Pro catalyzed reaction is "*enamine intermediate*". As the first step of the reaction, an enamine intermediate of L-Pro and ketone by amine and ketone was formed and, then, it attacks to the receptor molecules with electric withdrawing group [List *et al*., 2000]. It is therefore considered that the "*amine group*" of the L-Pro could play a key role in the formation of the enamine intermediate at the primary step of the whole reaction. Actually, there are many reports on the L-Pro derivative catalysts that are designed to retain the amino group in their chemical structure [Torii *et al*., 2004, Mase et al., 2004, Hayashi *et al*., 2006]. It has been reported that the chemical compounds harboring "*primary amine*" in their structure could act in a catalytic reaction of C-C bond formation, similarly in the case of L-Pro catalyst [Ho *et al*., 2009].

The enamine intermediate is known to be destabilized in an aqueous environment, so the L-Pro catalyzed reaction is often achieved in DMSO environment [List *et al.*, 2000]. In the previous chapters, it has been shown that (i) enamine intermediate of L-Pro and ketone is stabilized at the hydrophobic interior of the lipid membrane, (ii) the L-Pro-catalyzed reaction can be achieved in an aqueous environment, and also, (iii) the conversion and (enantio-)selectivity of the reaction could be regulated in depending manner on the location of the second substrate at the hydrophilic/hydrophobic interface of the lipid membrane. This is

because of the “recognitive function” of the lipid membrane. There are recent several reports on the recognition functions of liposome membranes, such as chiral selective adsorption of L-amino acids (Ishigami *et al.*, 2016) and ibuprophen (Okamoto *et al.*, 2017). Multiple interactions of the several functional group of the molecules that constitute the liposome membrane (i.e. phospholipid, cholesterol, *etc*.). In previous chapters, it has been described that the enamine intermediate of L-Pro and ketone could be stably trapped at the hydrophilic/hydrophobic interface via the multiple interactions similarly in the case of the above-mentioned recognitive function of the liposome. It is therefore considered that the possible intermediate formed from simpler functional group (i.e. primary amine) could also be stabilized at the liposome interface.

In this chapter, the stearylamine (SA), which consists of single alkyl chain and a primary amine at its edge, was selected to prove the above hypothesis based on the possible precognitive function that could occur at the liposome membrane. The SA molecule is known to be trapped at the hydrophobic interior of the liposome membrane as one of the membrane-constituting molecules and the primary amine group could locate at the surface edge of the liposome membrane. After the SA modified liposome was prepared, the C-C bond formation of acetone and *t*βNS was carried out by using the primary amine as the possible catalyst.

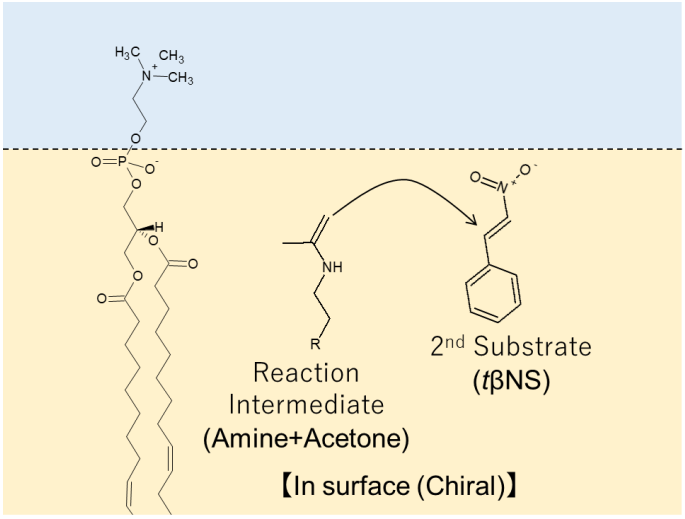


Figure 5-1 Schematic illustration of this chapter

2. Materials and Methods

2.1 Materials

1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC; carbon number/unsaturated bond = 18:0), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC; 18:1) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). *trans*-β-Nitrostyrene (*t*βNS) and the fluorescent probes, 1,6-diphenyl-1,3,5-hexatriene (DPH) and 6-lauroyl-2-dimethylaminonaphthalene (Laurdan), were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other chemicals were purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan) and were used without further purification.

2.2 Preparation of Vesicles.

A chloroform solution of lipids (DOPC, DSPC, etc.) and stearylamine was dried in a round-bottom flask by rotary evaporator under a vacuum. The lipid films obtained were dissolved in chloroform and the solvent was removed. These operations were repeated twice or more. The lipid thin film was kept under a high vacuum for at least three hours, and then hydrated with ultrapure water at above the transition temperature (T_m). The vesicle suspension was frozen at -80 °C and thawed at 60 °C to enhance the transformation of small vesicles into large multilamellar vesicles (MLVs). This freeze-thaw cycle was performed five times. MLVs were used to prepare the large unilamellar vesicles (LUVs) by extruding the MLV suspension 11 times through two layers of polycarbonate membranes with mean pore diameters of 100 nm using an extruding device (Lipofast; Avestin Inc., Ottawa, Canada).

2.3 Kinetics of L-Pro-Catalyzed Michael addition.

Kinetics and conversion of SA-catalyzed Michael addition. SA-catalyzed Michael addition of *t*βNS (14.9 mg (0.10 mmol)) with acetone (0.4 mL (5.4 mmol)) was conducted in the liposome suspensions at room temperature. The total concentrations of SA and lipid were 4 or 2 mM (0.008 or 0.004 mmol)) and 16 mM (total lipid: 0.032 mmol), respectively. The reaction was kinetically analyzed by monitoring changes in the intensity of the UV spectrum of tβNS (310 nm). The reaction was assumed to be governed by first-order kinetics, and the reaction rate constant, *k*, was determined by using following equation:

$$\ln\frac{C_{A0}}{C_A} = kt \quad , \qquad (5\text{-}1)$$

where C_{A0} is the initial concentration of *t*βNS, C_A is the *t*βNS concentration at any *t*, and *k* is the reaction rate constant.

2.4 Measurement of membrane fluidity.

Measurement of membrane fluidity. 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). A sample of 10 μL of 100 μM DPH in ethanol was added 1 mL of 0.25 mM vesicle suspension. The samples were incubated at least 30 min in the dark. The samples were excited with vertically polarized light (360 nm), and emission intensities both perpendicular (I⊥) and parallel (I∥) to the excited light were recorded at 430 nm. The polarization (P) of DPH was then calculated by using the following equations:

$$P = (I\parallel - GI\perp) / (I\parallel + GI\perp) \qquad (5\text{-}2)$$

$$G = i\perp / i\parallel \quad , \qquad (5\text{-}3)$$

where i⊥ and i∥ are emission intensities perpendicular and parallel to the horizontally polarized light, respectively, 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.

2.5 Evaluation of the membrane polarity by Laurdan.

Evaluation of the membrane polarity by using Laurdan. 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 (GP340) for each emission wavelength as follows:

$$GP_{340} = (I_{440} - I_{490}) / (I_{440} + I_{490}) \quad , \qquad (5\text{-}4)$$

where I_{440} and I_{490} are the emission intensities of Laurdan excited with 340 nm light at room temperature. 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.

3. Results and Discussion

3.1 Michael Addition Reaction of Acetone and *t*βNS on DOPC/SA Liposome.

The Michael addition reaction of acetone and *t*βNS was achieved by using SA as a possible catalyst in liposome systems. DOPC liposome was selected as a basal platform of the lipid membrane environment. DOPC was modified with the SA at different molar ratio and the Michael addition reaction was performed in the presence of SA-modified DOPC liposome. **Figure 5-2** shows the typical example of the time course of the Michael addition reaction in the conditions of DOPC/SA ratio at 8/1 and 4/1. In spite of the aqueous environment, the reaction was found to occur in the presence of DOPC/SA liposome. In both cases, it was found that the fraction of the conversion was linearly increased upon incubation time in semi-log plot, showing that the first order kinetic can be applied to this reaction. Furthermore, the rate constant of the reaction in the DOPC/SA liposome system was found to depend on the amount of SA, where the reaction rate in DOPC/SA at 4/1 was twice higher than that at 8/1. These time course

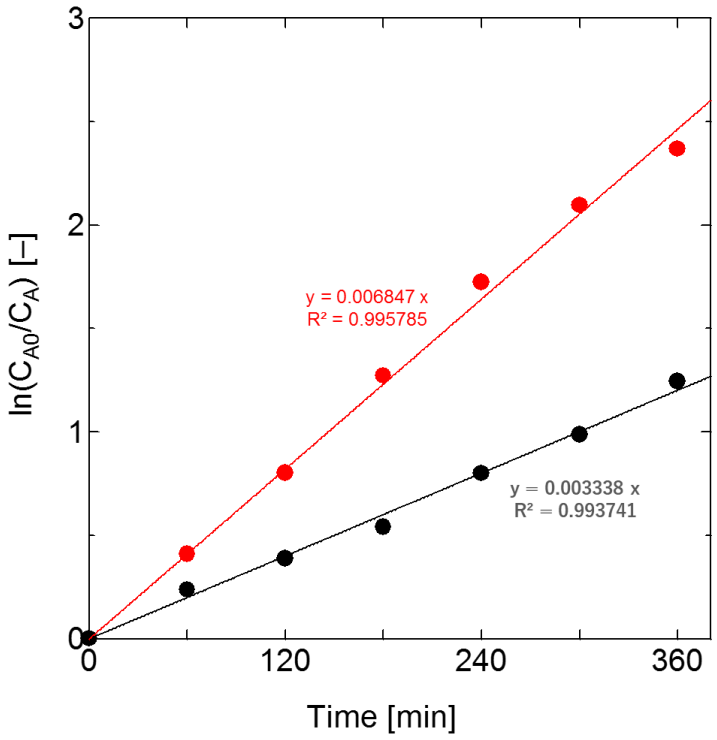


Figure 5-2 Time course of the Michael addition reaction of acetone and tβNS in the presence of the DOPC/SA liposomes (Black; 8/1, Red; 4/1) (25 ℃).

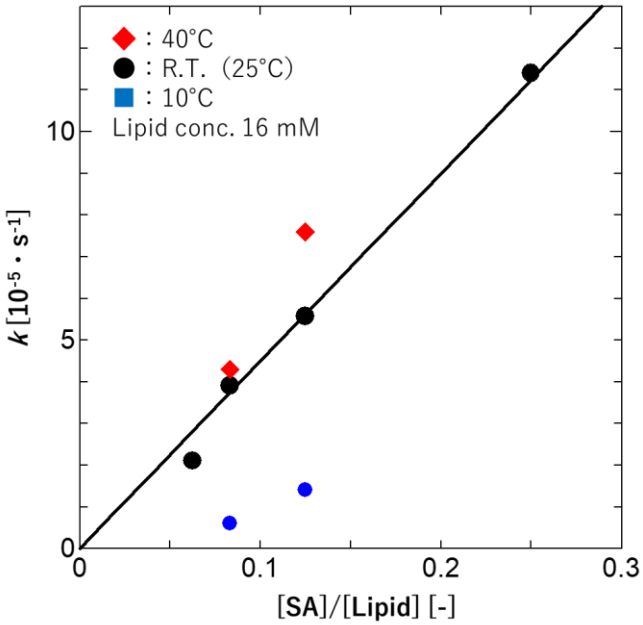


Figure 5-3 Relationship between the SA/lipid ratio and kinetic constant, *k*, on the Michael addition reaction of acetone and *t*βNS in the presence of the DOPC/SA liposomes.

measurements were performed at various conditions that differs in molar ratio of SA. The rate constant, *k*, was determined based on the Eq. (5-1). The obtained *k* value was shown in **Figure 5-3**. It was found that the *k* value was linearly dependent on the SA molar ratio, showing that the SA could be key material in this reaction. Chemical and biochemical reactions on vesicle membrane surfaces have been evaluated in several studies [Walde *et al*., 2014, Urabe *et al*., 1996, Ueoka *et al*., 1988, Umakoshi *et al*., 2008, Yoshimoto *et al*., 2007, Luginbühl *et al*., 2018]. It has recently been reported that the asymmetric conversion can be achieved by the tertiary amine that act as a phase transfer catalyst [Iwasaki *et al*., 2017], indicating that the reaction substrate could be trapped at the membrane interface through multiple interactions of functional groups because of the highly-ordered molecular assembly. As described in the previous chapters of this thesis, the stabilization of "*enamine intermediate*" of L-Pro and ketone at the membrane interface is important factor to achieve the L-Pro-catalyzed reaction. The results obtained above clearly indicated that the "simple" primary amine could also induce the Michael addition reaction, strongly suggesting that the "*enamine intermediate*" of primary amine and acetone

could also be stabilized at the surface of DOPC liposome similarly in the case of L-Pro catalyzed reaction. The liposome environment could be a key factor to regulate the above reaction catalyzed by primary amine on the DOPC liposome surface. These reactions were also performed at different temperature as shown in **Fig. 5-3**. Although only a little increase in the *k* value was observed at the higher temperature (40 ℃), a significant reduction in the *k* value was observed at the lower temperature (10 ℃). The above results imply that the membrane properties could be important to regulate the above reaction.

The obtained kinetic constants and the conversion after 24 hours were summarized in **Table 5-1**, together with those of L-Pro described in Chapter 4. A comparison of the standardized rate constant, *k*', normalized the amount of effective catalyst (amine) show that the reaction ability is equivalent to the L-Pro catalyst in liposome membrane and in DMSO environment and, in almost all cases, the conversion was found to be reached to a plateau at the higher level more than 90%. It was thus shown that the simple primary amine could also be utilized for the Michael addition reaction by way of its enamine intermediate on the surface of liposome membrane. Herein, enantiomeric excess of SA catalyzed Michael reaction was lower than that of L-Pro catalyzed Michael addition, while the value was improved by deducing the temperature (10 ℃).

Table 5-1 Kinetic constant, *k*, and the conversion of the Michael addition reaction of acetone and *t*βNS in the presence of the DOPC/SA liposomes

Condition	k_{rev} [10^{-5} ▪ s^{-1}]	*k*' [s^{-1}/ mol-effective cat.]	24 h conv. [%]
DOPC/SA (4/1)	11.4	14.3	93
DOPC/SA (8/1)	5.6	13.9	93
DOPC/SA (12/1)	3.9	14.6	90
DOPC/SA (16/1)	2.1	10.5	83
DOPC (with L-Pro)	5.3	15.8	94

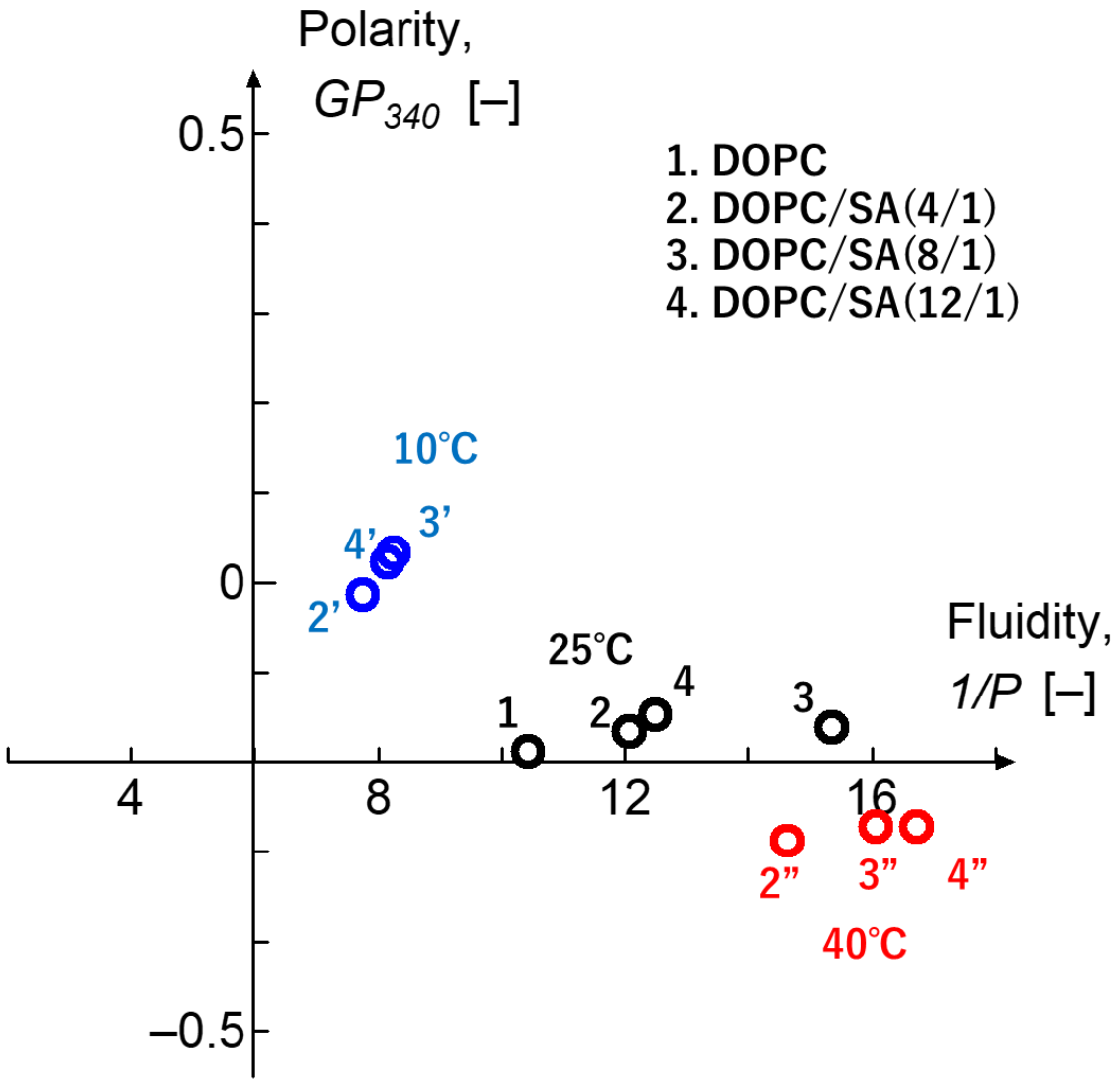


Figure 5-4 Cartesian diagram of liposomes. The membrane fluidity (1/*P*) and membrane polarity (GP_{340}) were measured at 10, 25, 40 ℃.

Table 5-2 Comparison of enantiomeric excess of Michael addition of *t*βNS and acetone

Condition	*k*' [s^{-1}/ mol-cat.]	24 h conv. [%]	e.e. [%]
DMSO (L-Pro)	30.3	>99	7*
DOPC (L-Pro)	15.8	94	82
DOPC/SA (8/1)	13.9	93	0~10 (≦30 at 10 ℃)

3.2 Membrane Properties of DOPC/SA Liposome.

The reaction was found to be dependent on the membrane properties of DOPC/SA liposome. Previously, DPH and Laurdan were employed as fluorescence probes to characterize membrane fluidity and membrane polarity, respectively [Suga *et al.*, 2013]. Herein, all data were plotted on a Cartesian plot (**Fig. 5-4**), where the cross point of *x*-axis and *y*-axis are set at the thresholds of the phase transition in terms of membrane fluidity and membrane polarity, respectively. In focusing on the data at 25 ℃, there is not so significant change in the *GP* value upon the variation of the SA molar ratio, while rather large variation was observed in membrane fluidity (1/*P*). However, all the data are plotted around the x axis, where the liposomes at disordered phase (l_d phase). In the case of the data at 40 ℃, the membrane fluidity was more increased, showing that they are at l_d phase. Only in the case of the lower temperature at 10 ℃, the plotted data were shifted to left-upper side. According previous data in relation to this Cartesian plot, this plotting regions of the data at 10 ℃ show that the membrane surface could be at the heterogeneous state of both l_o phase and l_d phase. Considering the results obtained above, these tendencies of the membrane properties were thought to be related with the Michael addition reaction. **Figure 5-5** shows the schematic illustration of the orientation of the molecules relating to this reaction. The substrates (*t*βNS) and the enamine intermediate

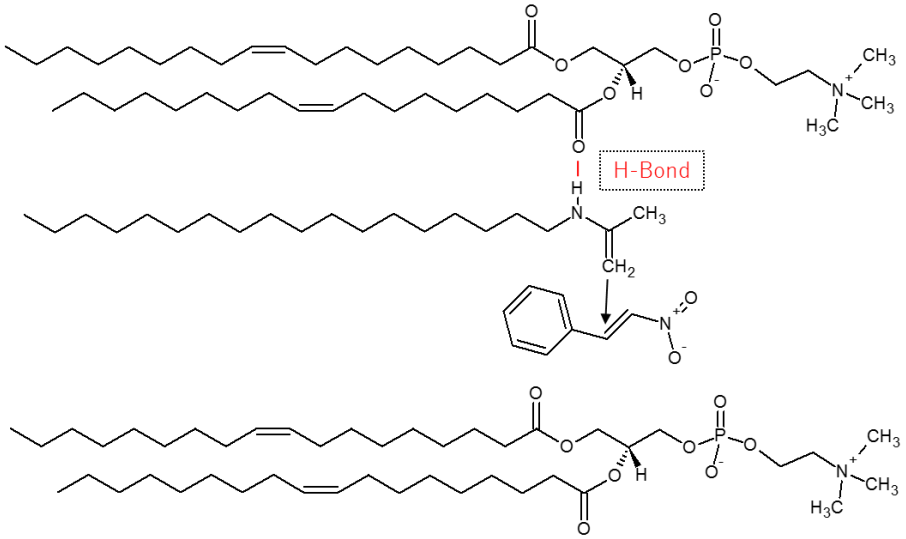


Figure 5-5 Schematic illustration of molecular orientation of enamine intermediate of primary amine and acetone in DOPC membrane surface.

complexes (stearic amine and acetone) could exist at the surface region of the liposome membrane, indicating that the aforementioned membrane properties had a direct impact on the Michael addition reaction in the interior of the nano-platform created by the lipid self-assembly, while the above tendency was rather different from that of L-Pro described in the previous chapter.

3.3 Plausible Model of the Reaction.

A plausible model of the SA catalyzed reaction on liposome membrane was discussed based on the obtained results. In the case of L-Pro, the enamine intermediate can be localized at interfacial region that possess the chiral environment, resulting in the induction enantiomeric selective C-C formation with the second substrate localizing at the chiral environment (*t*βNS). The enamine intermediate of SA and acetone can be localized around membrane, but a little shifted to (non-chiral) hydrocarbon region, resulting in the lower selectivity. In other words, a key factor to induce the enantiomeric selectivity is the chirality of the environment where the enamine intermediate and second substrate localized at the membrane interface.

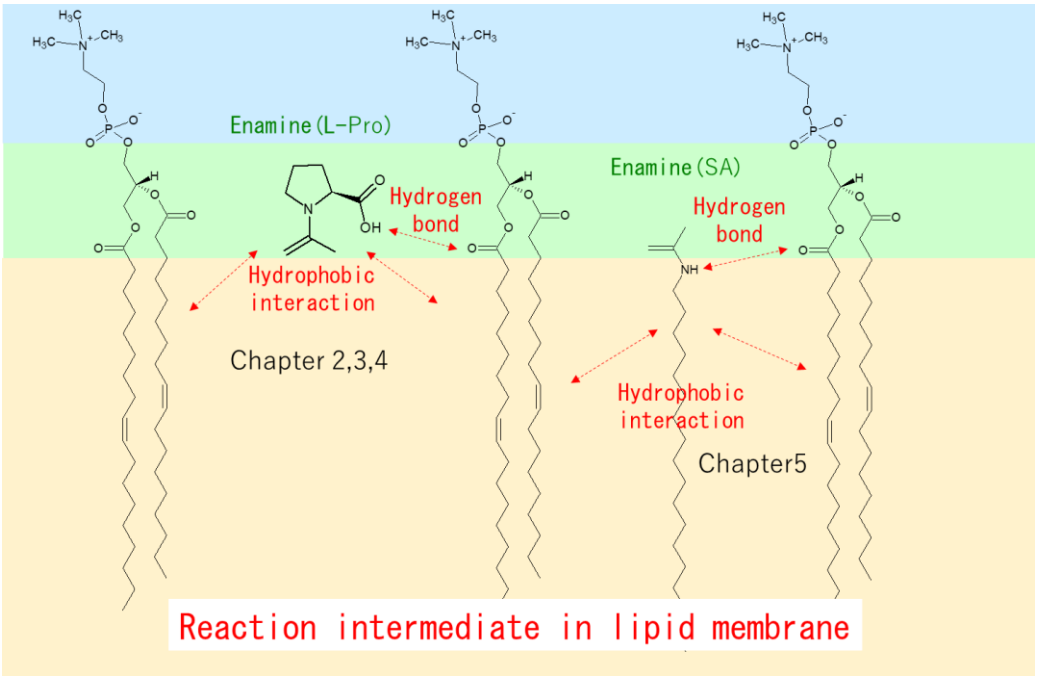


Figure 5-6 Illustration of enamine intermediate in lipid membrane

4. Summary

As further extension of the L-Pro utilizing strategy described in previous chapters, a primary amine was used for the conversion of C-C bond formation of the ketone and *t*βNS on the liposome membrane. The Michael addition reaction of acetone and *t*βNS was achieved by using stearic amine as a possible catalyst in liposome systems. After the enamine intermediate of steric amine and acetone was formed at the interior of the lipid bilayer membrane, it will react with the unsaturated bond of the second substrate (*t*βNS). It is found that the above-mentioned reaction was induced by the simple chemical structure of the catalytic molecule (steric amine) in "water" using a liposome membrane. Especially, when the DOPC liposome membrane at l_d *phase* was used as a platform of the reaction, calculated based on the amount of amine, was almost equal to that of L-Pro in DMSO and liposome membrane. It is proposed that the liposome membrane can also be utilized as a "*platform*" to concentrate both catalyst and substrate molecules and, especially, to trap the "*reaction (enamine) intermediate*" of both molecules.

Chapter 6

General Conclusions

The kinetics of the L-Pro-catalyzed Michael addition reaction of BIPM and acetone were analyzed by using fluorescence spectroscopy. The composition of liposomes could be a controlling factor in this reaction: zwitterionic DPPC liposome showed highest reaction rate constant within the liposomes tested in this work. The obtained results provide us the easy-detectable method to assess the reactivity of the L-Pro at the membrane interface, which can be detected by using BIPM as donor reactant in L-Pro-catalyzed Michael addition. Various systems were compared by selecting L-Pro-catalyzed reaction to yield a fluorescent product at target reaction. DMSO system gives the highly efficient conversion of the reaction. In the case of DOPC liposome membrane system, the L-Pro-catalyzed reaction was proceeded in spite of aqueous environment, while the conversion was very low in contrast to DMSO system. It could be related to the orientation of the second substrate (BIPM) at the membrane interface. Based on the results obtained in this chapter, a general scheme for the L-Pro-catalyzed reaction on "*lipid membrane*" was finally proposed, considering the L-Pro adsorption, enamine stability and orientation of the second substrate at the membrane interface. Among the possible regions of lipid bilayer membrane, the glycerol region that provides chiral environment was found to be strongly related with the enantiomeric selectivity of the product. It is thus considered that the liposome membrane platform would be utilized for the L-Pro-catalyzed reaction after careful selection of the second substrate and, also, the beneficial aspects of the obtained reaction.

Aldol reaction catalyzed by L-Pro was herewith investigated as an extension of the strategy described in Chapter 2, aiming at the improved conversion of the reaction product in C-C bond formation of the ketone and *p*NBA. As described in previous chapter, the enamine intermediate of L-Pro and acetone was first formed at the interior of the lipid bilayer membrane

and then it was applied to the reaction with the aldehyde group of the second substrate (*p*NBA). Because the *p*NBA is not deeply inserted into the interior of the hydrophobic region of the membrane, it is expected that the reaction could occur with the higher efficiency. It was shown that the liposome membranes provided a hydrophobic region for the aldol reaction of *p*NBA and acetone, which was catalyzed by adsorbed L-Pro onto liposomes. The conversion and e.e. values of this reaction could be controlled, depending on the solvent properties, by using DMSO/water mixtures as reaction media. Thus, the membrane polarity (i.e., localized hydrophobicity) and membrane fluidity could be controlling factors of this reaction. These findings will contribute to developing environmental-friendly organocatalytic reaction systems in aqueous media.

As an extension of the strategy described in Chapter 2, the L-Pro catalyzed reaction on membrane was applied to the enantioselective conversion of C-C bond formation of the ketone and *t*βNS. After the enamine intermediate of L-Pro and acetone was formed at the interior of the lipid bilayer membrane, it will react with the unsaturated bond of the second substrate (*t*βNS). It is therefore considered that the orientation of the *t*βNS at the membrane could be important to improve the enantioselectivity of the product. It was found that the L-Pro catalyzed reaction could proceed in "water" using a liposome membrane. Specifically, when the liposome membrane surface was modified in the dehydrated state, i.e., having "*ordered"* (s_o) *phase* and a "*positively-charged*" surface, the normalized reaction rate constant in the DOPC/DPTAP and DPPC/DPTAP systems, calculated based on the amount of L-Pro adsorbed, was almost equal to that in the DMSO. It is proposed that the liposome membrane can be utilized as a "*platform*" to concentrate both catalyst and substrate molecules and to effectively convert the substrate.

In chapter 5, as a further extension of the L-Pro utilizing strategy described in previous chapters, a primary amine was used for the conversion of C-C bond formation of the ketone and *trans*-β-nitrostyrene (*t*βNS) on the liposome membrane. The Michael addition reaction of

acetone and *t*βNS was achieved by using stearic amine as a possible catalyst in liposome systems. It is found that the above reaction was induced in spite of the simple chemical structure of the catalytic molecule (steric amine) in “water” using a liposome membrane.

Suggestion for Future Works

1. Using reaction substrate other than those used in this study to understand the function of the lipid membrane as a reaction field in more detail.

2. Using L-amino acids as a catalyst other than L-Proline to understand the function of the lipid membrane as a reaction field in more detail.

Nomenclatures

A	= fluorescence intensity	[-]
C_A	= concentration of reaction substrate	[mM]
C_{ads}	= concentration of adsorbent on liposome membranes	[mM]
D	= distribution ratio in emulsion phase	[-]
ee	= enamtiomeric excess	[-]
ε	= relative permittivity	[-]
G	= correction factor	[-]
G	= correction factor	[-]
GP_{340}	= general polarization calculated at exciting light at 340 nm	[-]
k	= observed reaction rate constant of the reaction	[s^{-1}]
k'	= normalized reaction rate constant	[$s^{-1} \cdot g^{-1}$]
Log$P$	= partition coefficient	[-]
Log$P$	= partition coefficient	[-]
$1/P$	= membrane fluidity	[-]
P	= fluorescence polarization of probes embedding in membranes	[-]
w_{ads}	= amount of the adsorbed L-Proline	[g]

List of Abbreviation

BIPM	*N*-[*p*-(2-benzimidazolyl)phenyl]maleimide
CTAB	Cetyltrimethylammonium bromide
Chol	Cholesterol
DBSA	Dodecylbenzenesulfonic acid
DDAB	Dilauryldimethylammonium bromide
DLS	Dynamic light scattering
DMSO	Dimethyl sulfoxide
DOPC	1,2-Dioleoyl-*sn*-glycero-3-phosphocholine
DOTAP	1,2-Dioleoyl-3-trimethylammonium-propane
DPH	1,6-Diphenyl-1,3,5-hexatriene
DPPC	1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine
DPTAP	1,2-Dipalmitoyl -3-trimethylammonium-propane
DSPC	1,2-Distearoyl-*sn*-glycero-3-phosphocholine
EWG	Electron Withdrawing Group
Em	Emission wavelength
Ex	Excitation wavelength
HPLC	High-performance liquid chromatography
L-Pro	L-Proline
LUV	Large unilamellar vesicle
Laurdan	6-Lauroyl-2-dimethylamino naphthalene
MLV	Multilamellar vesicle
NMR	Nuclear magnetic resonance
POPC	1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine

PTC	Phase transfer catalysis
SA	Stearylamine
SDS	Sodium dodecyl sulfate
TBAH	Tetrabutylammonium hydrogensulfate
TEM	Transmission electron microscope
TNS	6-(*p*-toluidino)naphthalene-2-sulfonate
T_m	Phase transition temperature
cac	critical aggregate concentration
cmc	critical micelle concentration
cvc	critical vesicle concentration
e.e.	Enantiomer excess
l_d	Liquid disordered
l_o	Liquid ordered
*p*NBA	p-Nitrobenzaldehyde
s_o	Solid ordered
*t*βNS	*trans*-β-Nitrostyrene

References

Ahrendt, K. A.; Borths, C. J.; MacMillan, D. W. C. New Strategies for Organic Catalysis: The First Highly Enantioselective Organocatalytic Diels−Alder Reaction, *J. Am. Chem. Soc.* **2000**, *122 (17)*, 4243-4244.

Andreu, C.; Varea, T.; Asensio, G. Inhibition of imidazolidinone intermediate formation in the aldol reactions catalyzed by zinc–prolinamide complexes. *Tetrahedron* **2011**, *67 (45)*, 8705-8709.

Berkessel, A.; Koch, B.; Lex, J. Proline-derived N-sulfonylcarboxamides: Readily available, highly enantioselective and versatile catalysts for direct aldol reactions. *Adv. Synth. Catal*. **2004**, 346, 1141-1146.

Blocher, M.; Liu, D.; Walde, P.; Luisi, P. L. Liposome-Assisted Selective Polycondensation of α-Amino Acids and Peptides. *Macromolecules* **1999**, *32*, 7332-7334.

Bui, T. T.; Suga, K.; Umakoshi, H. Roles of Sterol Derivatives in Regulating the Properties of Phospholipid Bilayer Systems. *Langmuir* **2016**, *32(24)*, 6176-6184.

Bui, T.; Barbas III, C. F. A. Proline-Catalyzed Asymmetric Robinson Annulation Reaction. *Tetrahedron Letters*, **2000**, *41*, 6951-6954.

Cevc, G. Membrane electrostatics. *Biochim. Biophys. Acta Rev. on Biomembr.* **1990**, *1031 (3)*, 311-382.

Córdova, A.; Zou, W.; Ibrahem, I.; Reyes, E.; Engqvist, M.; Liao, W.-W. Acyclic amino acid-catalyzed direct asymmetric aldol reactions: alanine, the simplest stereoselective organocatalyst. *Chem. Commun.* **2005**, *28*, 3586-3588.

Dickerson, T. J.; Janda, K. D. Aqueous Aldol Catalysis by a Nicotine Metabolite. *J. Am. Chem. Soc.* **2002**, *124*, 3220-3221.

Dickerson, T. J.; Lovell, T.; Meijler, M. M.; Noodleman, L.; Janda, K. D. Aqueous Aldol Catalysis by a Nicotine Metabolite. *J. Org. Chem.* **2004**, *69*, 6603-6609.

Dolling, U.-H.; Davis, P.; Grabowski, E.J.J. Efficient catalytic asymmetric alkylations. 1. Enantioselective synthesis of (+)-indacrinone via chiral phase-transfer catalysis. *J. Am. Chem. Soc.* **1984**, *106 (2)*, 446–447.

Dwars, T.; Paetzold, E.; Oehme, G. Reactions in Micellar Systems. *Angew. Chem. Int. Ed.* **2005**, *44*, 7174– 7199.

Eder, U.; Sauer, G.; Wiechert, R. New Type of Asymmetric Cyclization to Optically Active Steroid CD Partial Structures. *Angew. Chem. Int. Ed*., **1971**, 10, 496-497

Fukuta, Y.; Mita, T.; Fukuda, N.; Kanai, M.; Shibasaki, M. De Novo Synthesis of Tamiflu via a Catalytic Asymmetric Ring-Opening of meso-Aziridines with TMSN3, *J. Am. Chem. Soc*., **2006**, *128 (19)*, 6312-6813.

Grassert, I.; Schinkowski, K.; Vollhardt, D.; Oehme, G. Amphiphilic proline and prolylproline derivatives in the rhodium(I) ‐ catalyzed asymmetric hydrogenation in water: Chiral induction as indication of the location of reactants within the micelle, *Chirality*, **1998***, 10(8)*, 754-759.

Gruttadauria, M.; Giacalone, F.; Noto, R. Water in Stereoselective Organocatalytic Reactions. *Adv. Syn. Cat.*, **2009**, *351(1-2)*, 33-57.

Gruttadauria, M.; Giacalone, F.; Noto, R. Supported Proline and Proline-Derivatives as Recyclable Organocatalysts. *Chem. Soc. Rev.*, **2008**, *37(8)*, 1666-1688.

Gu, Q.; Guo, X.-T.; Wu, X.-Y. Highly enantioselective Michael addition of acetone to nitroolefins catalyzed by chiral bifunctional primary amine-thiourea catalysts with acetic acid. *Tetrahedron* **2009**, *65*, 5265-5270.

Hamasaka, G.; Uozumi, Y.; Cyclization of Alkynoic Acids in Water in the Presence of a Vesicular Self-Assembled Amphiphilic Pincer Palladium Complex Catalyst. *Chem. Commun.* **2014**, *50,* 14516-14518.

Han, J.; Suga, K.; Hayashi, K.; Okamoto, Y.; Umakoshi, H. Multi-Level Characterization of the Membrane Properties of Resveratrol-Incorporated Liposomes, *J. Phys. Chem. B*, **2017**, *121(16)*, 4091-4098.

Hajous, Z. G.; Parrish, D. R. Synthesis and Conversion of 2-Methyl-2-(3-oxobutyl)-1,3-cyclopentanedione to the Isomeric Racemic Ketols of the [3.2.1] Bicyclooctane And of the Perhydroindane Series, *J. Org. Chem*., **1974**, 39, 1612-1615.

Hayashi, Y.; Aratake, S.; Okano, T.; Takahashi, J.; Sumiya, T.; Shoji, M. Combined Proline-Surfactant Organocatalyst for the Highly Diastereo- and Enantioselective Aqueous Direct Cross-Aldol Reaction of Aldehydes. *Angew. Chem. Int. Ed.* **2006**, *45*, 5527-5529.

Hein, A.; De Santis, G.; Luz, J. G.; Mitchell, M.; Wong, C.-H.; Wilson, I. A. Observation of covalent intermediates in an enzyme mechanism at atomic resolution. *Science* **2001**, *294*, 369-374.

Hitz, T.; Blocher, M.; Walde, P.; Luisi, P. L. Stereoselectivity aspects in the condensation of racemic NCA-amino acids in the presence and absence of liposomes. *Macromolecules* **2001**, *34*, 2443–2449.

Ho, M.-C.; Menetret, J.-F.; Tsuruta, H.; Allen, K. N. The origin of the electrostatic perturbation in acetoacetate decarboxylase. *Nature*, **2009**, 459, 393-397.

Huerta, E.; Stals, P. J. M.; Meijer, E. W.; Palmans, A. R. A. Consequences of Folding a Water-Soluble Polymer Around an Organoccatalyst. *Angew. Chem. Int. Ed.* **2013**, 52, 2906-2910.

Isart, C.; Burés, J.; Vilarrasa, J. Seebach's Oxazolidinone is a Good Catalyst for Aldol Reactions. *Tetrahedron Lett.* **2008**, *49(37)*, 5414-5418.

Ishigami, T.; Suga, K.; Umakoshi, H. Chiral Recognition of L-Amino Acid on Liposome Prepared by L-Phospholipid. *ACS Applied Materials and Interface* **2015**, *7(38)*, 21065-21072.

Ishigami, T.; Tauchi, A.; Suga, K.; Umakoshi, H. Effect of Boundary Edge in DOPC/DPPC/Cholesterol Liposomes on Acceleration of L-Histidine Preferential Adsorption, *Langmuir*, **2016**, 32(24), 6011-6019.

Ishigami, T.; Umakoshi, H. 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.

Ishii, T.; Fujioka, S.; Sekiguchi, Y.; Kotsuki, H. A new class of chiral pyrrolidine-pyridine conjugate base catalysts for use in asymmetric Michael addition reactions, *J. Am. Chem. Soc*., **2004**, 126, 9558

Ishikawa, H.; Suzuki, T.; Hayashi, Y. High-yielding synthesis of the anti-influenza neuramidase inhibitor (-)-oseltamivir by three "One-Pot" operations. *Angew. Chem. Int. Ed.* **2009**, *48*, 1304-1307.

Iwasaki, F.; Suga, K.; Okamoto, Y.; Umakoshi, H. Liposomes Can Conduct Enantioselective C-C bond formation of an α-Amino Acid Derivative in Aqueous Media," *ACS Omega*, **2017**, *2(1)*, 91-97.

Iwasaki, F.; Suga, K.; Umakoshi, H. Pseudo-Interphase of Liposome Promotes 1,3-Dipolar Cycloaddition Reaction of Benzonitrile Oxide and N-Ethylmaleimide in Aqueous Solution," *J. Phys. Chem. B*, **2015**, *119(30)*, 9772-9779.

Jacob, S. W.; Herschler, R. Pharmacology of DMSO. *Cryobiology* **1986**, *23*, 14-27.

Kumar, A.; Gupta, M.K.; Kumar, M.; Saxena, D. Micelle Promoted Multicomponent Synthesis of 3-Amino Alkylated Indoles via a Mannich-Type Reaction in Water, *RSC Advances*, **2013**, *3*, 1673-1678.

Kunishima, M.; Yamamoto, K.; Watanabe, Y.; Hioki, K.; Tani, S. Unusual Rate Enhancement of Bimolecular Dehydrocondensation to Form Amides at the Interface of Micelles of Fatty Acid Salts. *Angew. Chem. Int. Ed.* **2005**, *44*, 7254-7257.

Li, P.; Wu, G. Roles of dietary glycine, proline, and hydroxyproline in collagen synthesis and animal growth, *Amino Acids*, **2018**, *50*, 29-38.

Li, Z.-Y.; Chen, Y.; Zheng, C.-Q.; Yin, Y.; Wang, L.; Sun, X.-Q. Highly enantioselective aldol reactions catalyzed by reusable upper rim-functionalized calix[4]arene-based l-proline organocatalyst in aqueous conditions, *Tetrahedron*, **2017**, *73(1)*, 78-85.

List, B.; Lerner, R. A.; Barbas III, C. F. Proline-Catalyzed Direct Asymmetric Aldol Reactions, *J. Am. Chem. Soc.*, **2000**, *122*, 2395−2396.

List, B.; Pojarliev, P.; Biller, W. T.; Martin, H.J. The Proline-Catalyzed Direct Asymmetric Three-Component Mannich Reaction: Scope, Optimization, and Application to the Highly Enantioselective Synthesis of 1,2-Amino Alcohols, *J. Am. Chem. Soc.*, **2002**, *124*, 827−833.

List, B.; Pojarliev, P.; Martin, H. J. Efficient Proline-Catalyzed Michael Additions of Unmodified Ketones to Nitro Olefins, *Org. Lett.*, **2001**, *16*, 2423−2435.

Lo, C.-M.; Chow, H.-F. Structural Effects on the Catalytic, Emulsifying, and Recycling Properties of Chiral Amphiphilic Dendritic Organocatalysts. *J. Org. Chem.*, **2009***, 74 (15)*, 5181-5191.

Lu, A.; Cotanda, P.; Patterson, J. P.; Longbottom, D. A.; O'Reilly, R. K. Aldol reactions catalyzed by L-proline functionalized polymeric nanoreactors in water. *Chem. Commun*. **2012**, *48*, 9699–9701.

Luzar, A.; Stefan, J. Dielectric Behaviour of DMSO-Water Mixtures. A Hydrogen-Bonding Model. *J. Mol. Liq.*, **1990**, 46 (C), 221-238.

MacMillan, D.W.C. The advent and development of organocatalysis. *Nature* **2008**, *455*, 304-308.

Manabe, K.; Iimura, S.; Sun, X. -M.; Kobayashi, S. Dehydration Reactions in Water. Brønsted Acid-Surfactant-Combined Catalyst for Ester, Ether, Thioether, and Dithioacetal Formation in Water. *J. Am. Chem. Soc.* **2002**, *124*, 11971-11978.

Mase, N.; Watanabe, K.; Yoda, H.; Takabe, K.; Tanaka, F.; Barbas III, C.F. Organocatalytic Direct Michael Reaction of Ketones and Aldehydes with β-Nitrostyrene in Brine. *J. Am. Chem. Soc.* **2006,** *128*, 4966–4967.

Mukherjee, S.; Yang, J. W.; Hoffmann, S.; List, B. Asymmetric Enamine Catalysis. *Chemical Reviews* **2007**, *107*, 5471-5569

Notz, W.; Watanabe, S.; Chowdari, N. S.; Zhong, G.; Betancort, J. M.; Tanaka, F.; Barbas III, C.F. The Scope of the Direct Proline‐Catalyzed Asymmetric Addition of Ketones to Imines *Adv. Synth. Catal*., **2004**, 346, 1131-1140

Okamoto, Y.; Kishi, Y.; Ishigami, T.; Suga, K.; Umakoshi, H. Chiral Selective Adsorption of Ibuprofen on a Liposome Membrane, *J. Phys. Chem. B*, **2016**, *120(10)*, 2790-2795.

Ooi, T.; Kameda, M.; Maruoka, K. A new class of chiral pyrrolidine-pyridine conjugate base catalysts for use in asymmetric Michael addition reactions, *J. Am. Chem. Soc.*, **1999**, *121*, 6519-6520

Palmqvist, A. E. C. Synthesis of Ordered Mesoporous Materials Using Surfactant Liquid Crystals or Micellar Solutions, *Current Opinion in Colloid and Interface Science*, **2003**, *8(2)*, 145-155.

Parasassi, T.; Krasnowska, E. K.; Bagatolli, L.; Gratton, E. Laurdan and Prodan as Polarity-Sensitive Fluorescent Membrane Probes. *J. Fluoresc.,* **1998**, *8*, 365-373.

Pence, H. E.; Williams, A. ChemSpider: An Online Chemical Information Resource, *J. Chem. Educ.*, **2010***, 87*, 1123−1124.

Raj, M.; Singh, V. K. Organocatalytic reactions in water. *Chem. Commun.* **2009**, 6687-6703.

Rodríguez-Llansola, F.; Miravet, J. F.; Escuder, B. Supramolecular Catalysis with Extended Aggregates and Gels: Inversion of Stereoselectivity Caused by Self-Assembly, *Chem. Eur. J.* **2010**, *16*, 8480-8486.

Sakthivel, K.; Notz, W.; Bui, T.; Barbas III, C. F. Amino Acid Catalyzed Direct Asymmetric Aldol Reactions: A Bioorganic Approach to Catalytic Asymmetric Carbon-Carbon Bond-Forming Reactions, *J. Am. Chem. Soc.*, **2001**, *123*, 5260-5267.

Seebach, D.; Beck, A. K.; Badine, D. M.; Limbach, M.; Eschenmoser, A.; Treasurywala, A. M.; Hobi, R. Are Oxazolidinones Really Unproductive, Parasitic Species in Proline Catalysis? - Thoughts and Experiments Pointing to an Alternative View, *Helvetica Chim. Acta*, **2007**, *90(3)*, 425-471.

Serrano-Luginbühl, S.; Ruiz-Mirazo, K.; Ostaszewski, R.; Gallou, F.; Walde, P. Soft and dispersed interface-rich aqueous systems that promote and guide chemical reactions, *Nature Reviews Chemistry*, **2018**, 2(10), 306-327

Suga, K.; Akizaki, K.; Umakoshi, H. Quantitative Monitoring of Microphase Separation Behaviors in Cationic Liposomes Using HHC, DPH, and Laurdan: Estimation of the Local Electrostatic Potentials in Microdomains, *Langmuir*, **2016**, *32(15)*, 3630-3636.

Suga, K.; Tauchi, A.; Ishigami, T.; Okamoto, Y.; Umakoshi, H. Preferential Adsorption of L-Histidine onto DOPC/Sphingomyelin/3ß-[N-(N',N'-dimethylaminoethane)-carbamoyl]cholesterol Liposomes in the Presence of Chiral Organic Acids, *Langmuir*, **2017**, *33(15)*, 3831-3838.

Suga, K.; Umakoshi, H. Detection of Nano-sized Sized Ordered Domains in DOPC/DPPC and DOPC/Ch Binary Lipid Mixture Systems of Large Unilamellar Vesicles Using a TEMPO Quenching Method, *Langmuir*, **2013**, *29(15)*, 4830-4838

Suga, K.; Yoshida, T.; Ishii, H.; Okamoto, U.; Nagao, D.; Konno, M.; Umakoshi, H. Membrane Surface-Enhanced Raman Spectroscopy (MSERS) for Sensitive Detection of Molecular Behaviour of Lipid Assemblies. *Anal. Chem.* **2015**, *87*, 4772-4780.

Tang, Z.; Jiang, F.; Cui, X.; Gong, L.; Mi, A.; Jiang, Y.; Wu, Y. X Enantioselective direct aldol reactions catalyzed by L-prolinamide derivatives. *Proc. Natl. Acad. Sci. U.S.A.*, **2004**, 101, 5755-5760

Tanaka, F.; Thayumanavan, R.; Barbas III, C. F. Fluorescent Detection of Carbon-Carbon Bond Formation, *J. Am. Chem. Soc.*, **2003**, *125*, 8523-8528.

Torii, H.; Nakadai, M.; Ishihara, K.; Saito, S.; Yamamoto, H. Asymmetric direct aldol reaction assisted by water and a proline-derived tetrazole catalyst *Angew. Chem. Int. Ed.* **2004**, 43, 1983-1986

Tuan, L.Q.; Umakoshi, H.; Shimanouchi, T.; Kuboi, R. Liposome -Recruited Activity of Oxidized and Fragmented Superoxide Dismutase. *Langmuir* **2008**, *24*, 350-354.

Ueoka, R.; Matsumoto, Y.; Moss, R.A.; Swarup, S.; Sugii, A.; Harada, K.; Kikuchi, J.; Murakami, Y. Membrane Matrix for the Hydrolysis of Amino Acid Esters with Marked Enantioselectivity. *J. Am. Chem. Soc*. **1988**, *110*, 1588–1595.

Umakoshi, H.; Morimoto, K.; Ohama, Y.; Nagami, H.; Shimanouchi, T.; Kuboi, R. Liposome Modified with Mn−Porphyrin Complex Can Simultaneously Induce Antioxidative Enzyme-like Activity of Both Superoxide Dismutase and Peroxidase. *Langmuir* **2008**, *24*, 4451-4455.

Umakoshi, H.; Suga, K. Use Liposome as a Designable Platform for Molecular Recognition ~ from “Statistical Separation” to “Recognitive Separation”, *Solvent Extr. Res. Dev., Jpn.*, **2013**, *20*, 1-13.

Urabe, K.; Usui, S.; Sagawa, T. The role of the membrane-assisted hydrophobic interaction between di-, tri-, or tetrapeptide catalysts and amino acid esters for the enhancement of stereoselective hydrolysis reactions. *Macromol. Rapid Commun*. **1996**, *17*, 109–116.

Veatch, S.L.; Keller, S. L. Miscibility Phase Diagrams of Giant Vesicles Containing Sphingomyelin, *Phys. Rev. Lett.*, **2005**, *94 (14)*, 148101-1-14801-4.

Walde, P.; Umakoshi, H.; Stano, P.; Mavelli, F. Emergent Properties Arising from the Assembly of Amphiphiles. Artificial vesicle membranes as reaction promoters and regulators. *Chem. Comm.* **2014**, *50*, 10177-10197.

Wang, F.; Wen, M.; Feng, K.; Liang, W.-J.; Li, X.-B.; Chen, B.; Tung, C.-H.; Wu, L.-Z. Amphiphilic Polymeric Micelles as Microreactors: Improving the Photocatalytic Hydrogen Production of the [FeFe]-Hydrogenase Mimic in Water. *Chem. Commun.* **2016**, *52*, 457-460.

Williamson, M. P. X The structure and function of proline-rich regions in proteins, *Biochem. J.* **1994**, 297, 249-260

Yang, L.-J.; Yang, X.-Q.; Huang, K.-M.; Jia, G.-Z.; Shang, H. Dielectric Properties of Binary Solvent Mixtures of Dimethyl Sulfoxide with Water, *Int. J. Mol. Sci.*, **2009**, *10(3)*, 1261-1270.

Yeagle, P. in "*The structure of Biological Membranes, 3rd Ed.*" (CRC Press) **2011**, *Chapter 4*, 19-91.

Yoshimoto, M.; Miyazaki, Y.; Umemoto, A.; Walde, P.; Kuboi, R.; Nakao, K. Phosphatidylcholine Vesicle-Mediated Decomposition of Hydrogen Peroxide. *Langmuir* **2007**, *23*, 9416-9422.

Zandvoort, E.; Geertsema, E. M.; Baas, B. -J.; Quax, W. J.; Poelarends, G. J. Bridging between Organocatalysis and Biocatalysis: Asymmetric Addition of Acetaldehyde to β-Nitrostyrenes Catalyzed by a Promiscuous Proline-Based Tautomerase. *Angew. Chem. Int. Ed.* **2012**, *51*, 1240 –1243.

Zayas, H. A.; Lu, A.; Valade, D.; Amir, F.; Jia, Z.; O'Reilly, R.K.; Monteiro, M.J. Thermoresponsive Polymer-Supported L-Proline Micelle Catalysts for the Direct Asymmetric Aldol Reaction in Water. *ACS Macro lett.* **2013**, *2*, 327-331.

Zhang, H.; Zhao, W.; Zou, J.; Liu, Y.; Li, R.; Cui, Y. Aldol Reaction Catalyzed by Hydrophilic Catalyst in Aqueous Micelle as an Enzyme Mimic System. *Chirality,* **2009**, *21*, 492–496.

Zhong, L.; Gao, Q.; Gao, J.; Xiao, J.; Li, C. Direct catalytic asymmetric aldol reactions on chiral catalysts assembled in the interface of emulsion droplets, *J. Catal.*, **2007***, 250(2)*, 360-364.

List of Publications

[Papers]

1. Masanori Hirose, Shigenori Sugisaki, Keishi Suga, Hiroshi Umakoshi, Detection of L-Proline Catalyzed Michael Addition Reaction in Model Biomembrane. *J. Chem*., 2019 in press

2. Masanori Hirose, Shinpei Fujiwara, Takaaki Ishigami, Keishi Suga, Yukihiro Okamoto, Hiroshi Umakoshi, Liposome Membranes Assist the L-Proline-Catalyzed Aldol Reaction of Acetone and p-Nitrobenzaldehyde in Water. *Chem. Lett*., 2018, 47, 931-934

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

[Related Papers]

1. Masanori Hirose, Keishi Suga, Hiroshi Umakoshi, Michael addition of *trans*-β-Nitrostyrene and Acetone in Liposome membrane with Long-chain alkylamine. to be submitted

[International Conference / Symposium]

1. Masanori Hirose, Takaaki Ishigami, Hiroshi Umakoshi, L-Proline-Catalyzed Michael Addition on Liposome Membranes in Water.
   Next Symposium “Membranome” for “Bio-Inspired Chemical Engineering", Osaka, Japan, September (2013)

2. Masanori Hirose, Takaaki Ishigami, Keishi Suga, Yukihiro Okamoto, Hiroshi Umakoshi, Coexistence Effect of Liposome Membrane on L-Proline Catalyzed Michael Addition. 10th Int'l Conference on Separation Science and Technology, Nara, Japan, October (2014)

3. Masanori Hirose, Takaaki Ishigami, Keishi Suga, Yukihiro Okamoto, Hiroshi Umakoshi, L-Proline Catalyzed Michael Addition Reaction Conducted at Liposome Interface. Pacifichem 2015, Hawaii, United States, December (2015)

4. Masanori Hirose, Keishi Suga, Yukihiro Okamoto, Hiroshi Umakoshi, L-Proline Catalyzed Reactions Conducted at Liposome Interface. The 10th Conference of Aseanian Membrane Society (AMS10), Nara, Japan, July (2016)

5. Masanori Hirose, Keishi Suga, Yukihiro Okamoto, Hiroshi Umakoshi, Design of Lipid Membrane Surfaces as Organocatalyst for Michael Reactions in Aqueous Media.AIChE Annual Meeting, Minneapolis, United States, 10.29-11.03 (2017) (Poster exhibition only)

6. Masanori Hirose, Keishi Suga, Yukihiro Okamoto, Hiroshi Umakoshi, Characterization of Lipid Membrane Surfaces as Catalytic Platform in Aqueous Media. 11th International Conference on Separation Science and Technology, Busan, Korea, November (2017) (Poster exhibition only)

[Article / Review]

1. Masanori Hirose, Catalytic reaction by L-Proline adsorbed to liposome membrane (in Japanese), *Bunri Gijutsu*, **2016**, *46*, 5.

Acknowledgements

The author is greatly indebted to Prof. Dr. Hiroshi Umakoshi (Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University), for his excellent guidance and helpful advice and supports throughout this work. The author is thankful to Prof. Dr. Koichiro Jitsukawa, Prof. Dr. Norikazu Nishiyama, Assoc. Prof. Dr. Yukihiro Okamoto (Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University) for a number of valuable comments and suggestions during the completion of this thesis. The author would like to offer my special thanks to Assist. Prof. Dr. Keishi Suga (Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University) for a huge support on my research. The author also would like to offer special thanks to Assoc. Prof. Dr. Takato Mitsudome (Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University), Assoc. Prof. Dr. Toshinori Shimanouchi (Division of Environmental Science, Graduate School of Environmental and Life Science, Okayama University), and for his valuable comments, and helpful advises. The author would like to express one's thankfulness to Ms. Keiko Fukumoto for her kind support during this work.

The author would like to show my greatest appreciation to Prof. Dr. R. Kuboi, Prof. Dr. K. Ohgaki, Prof. Dr. Y. Inoue, (Honored Professor of Osaka University). The author is thankful to Prof. Dr. M. Taya, Prof. Dr. Y. Okano, Prof. Dr. M. Nakano, Prof. Dr. T. Hirai, Prof. Dr. N. Matsubayashi, Prof. Dr. S. Sakai, and all the staff of Division of Chemical Engineering, Graduate School of Engineering Science, Osaka University for their kind cooperation during my research.

The author wishes to thank for Prof. Dr. P. Walde (Institute for Polymer, ETH, Zurich), Prof. Dr. B. Higgins (Department of Chemical Engineering and Materials Science, University of California, Davis), Prof. Dr. P. Alexandridis (Department of Chemical Engineering, University at Buffalo), Prof. Dr. Ho-Sup Jung (Department of Mechanical and Aerospace Engineering, Seoul National University), Prof. Dr. S. Ichikawa (Graduate School of Life and Environmental Sciences, University of Tsukuba), Prof. Dr. H. Nakamura (Department of Chemical Engineering, National Institute of Technology, Nara College), Prof. Dr. M. Konno (Department of Applied Chemistry, Tohoku University) for their comments and suggestions during this work. The author is grateful for the advice given by Assoc. Prof. Dr. A. Heyden (Department of Chemical Engineering, University of South Carolina), Assoc. Prof. Dr. K. Kato (Department of Materials Science and Biotechnology, Graduate School of Science and Engineering, Ehime University), Prof. Dr. K. Shiomori (Department of Applied Chemistry, University of Miyazaki), Prof. Dr. D. Nagao (Department of Applied Chemistry, Tohoku University), Assoc. Prof. Dr. M. Yoshimoto (Department of Applied Molecular Bioscience, Yamaguchi University), Assoc. Prof. Dr. S. Morita (Department of Material Science, Wakayama National College of Technology), Assist. Prof. Dr. N. Yoshimoto (Graduate School of Medicine, Yamaguchi University), Assoc. Prof. Dr. H. Ishii (Yamaguchi University), Senior Lecturer Dr. K. Hayashi (National Institute of Technology), Dr. V. T. Huong (Hanoi National University of Education), Dr. H. Sugaya (Toray Industries, inc.), Dr. Y. Yamada (Kao corporation).

Special thanks are given to following colleagues for their experimental collaboration: Dr. T. Ishigami, S. Tanaka, T. Tatsui, T. Yokoi, J. Chinzaka, T. Hinoyama, N. Maruyama, K. Sugita, Dr. F. Iwasaki, M. Kiriishi, D. Kondo, Y. Takaya, Y. Kaneko, T. Bando, T. Katsura, S. Taguchi, K. Goshima, A. Hamasaki, M. Kota, Y. Tsujimoto, T. Yoshida, T. T. Bui, Y. Kishi, K. Akizaki, Y. Higashie, T. Ikeda, N. Watanabe, R. Matsuba, Y. Mine, S. Sugisaki, Y. Otsuka, Y. Shinozuka, A. Tauchi, D. Wada, J. Han, K. Midogochi, T. Wakita, R. Ito, Y. Ooe, R. Kawakami, R. Kawakami, R. Nishino, K. Yoshida, K. Tanimura, Y. Iimure, Y. Goto, K. Kitagawa, Y. Murata, M. Faried, M. Amau, N. Ikushima, R. Ueno, K. Kojima, Y. Seno, Y. Hasunuma, D. Matsui, S. Matsushita, R. Murazawa, M. S. Chern, C. Lishi, C. Tran, A. Ajaikumar, R. Wakerlin, Y.-C. Lai, W. H. Shi, M. Sikorski, D. Etwaru, H. J. Kim, B. S. Kang, and all the member in Bio-Inspired Chemical Engineering Laboratory.

The author would like to thank his parents Shigenori Hirose and Hatsumi Hirose and his sister Ayumi Hirose for their continuous encouragements and kind support throughout this work.

The author gratefully acknowledges the financial support of this work by the Sasakawa Scientific Research Grant from The Japan Science Society (29-336).