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**DEVELOPMENT OF ROBUST PLATFORM FOR
BIOMATERIALS APPLICATIONS ASSEMBLY WITH AFM
MEASUREMENT**

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SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

2019

DEVELOPMENT OF ROBUST PLATFORM FOR BIOMIMETIC APPLICATIONS ASSEMBLY WITH AFM MEASUREMENT

HOKYUN CHIN

SCHOOL OF MATERIALS SCIENCE AND ENGINEERING

A thesis submitted to the Nanyang Technological University
in partial fulfilment of the requirement for the degree of
Doctor of Philosophy

2019

Statement of Originality

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Authorship Attribution Statement

This thesis contains material from two papers published in the following peer-reviewed journals / from papers accepted at conferences in which I am listed as an author.

Chapter 4 is published as **H Chin**, J J Gillissen, E Miyako, NJ Cho, “Microfluidic liquid cell chamber for scanning probe microscopy measurement application”, *Review of Scientific Instrument*, 90 (4), 046105.

The contributions of the co-authors are as follows:

- Prof. Cho provided the initial project direction and edited the idea.
- I prepared the manuscript drafts. The manuscript was revised by Dr. Gillissen and Dr. Miyako.
- I co-designed the study and performed all the laboratory work at Centre of Biological Sensor Science (CBSS). I also analyzed the data.
- All microscopy, including sample preparation, was conducted by me in the Facility for Analysis, Characterization, Testing and Simulation.
- I designed the actual microfluidic chamber using 3D software.
- Dr. Gillissen assisted in the numerical simulation of the fluidic channel.
- Dr. Miyako assisted in the collection of the necessary data and provide guidance in the interpretation of the whole paper design.

Chapter 5 is published as T Maekawa[#], **H Chin**[#], T Nyu, TN Sut, AR Ferhan, T Hayashi, NJ Cho, “Molecular diffusion and Nano-Mechanical Properties of Multi-Phase Supported Lipid Bilayers”, *Physical Chemistry Chemical Physics* 21 (30), 16686-16693. (# denotes equal contribution)

The contributions of the co-authors are as follows:

- Prof. Cho suggested the initial idea of the project and direction.

- I and Maekawa performed the all laboratory work at Centre of Biological Sensor Science (CBSS)) and Tokyo Institute of Technology (TIT). We also analyzed the data together.
- I revised the design of the microfluidic chamber for continuing experiment at TIT.
- Maekawa wrote the drafts of the manuscript. The manuscript was revised by me and Dr. Ferhan.
- Dr. Nyu conducted the AFM experiment and data analysis.
- Mr. Sut conducted QCM-D experiment and data analysis.

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Hokyun Chin

Abstract

Cellular membrane host a variety of crucial biological activities such as trafficking molecules in and out of cells, protecting the cell from external assaults, maintain homeostasis, segregate the internal composition and provide specificity. Improved capabilities to study the lipid membrane properties and to quantify their characteristics is an important scientific goal that would enable the better understanding of the very origins of life to the development of targeted therapeutics. To these artificial membrane organizations spanning planar to vesicular configurations have been used as a simplified mimic model of biological membranes. There is an ever-growing interest in generating planar bilayer as they are relatively easy to prepare, are stable and can provide a versatile platform through patterning. More importantly, a variety of surface sensitive techniques such as FRAP, QCM/QCM-D, SPR, and AFM can be used to extract useful information about their biophysical properties. In particular, the Atomic Force Microscopy (AFM) techniques have shown great promise in the successful characterization of membrane topography by offering high-resolution with non-contact imaging capabilities in the liquid environment that are biologically more relevant. Despite this, it remains a challenge in to determine useful parameters in a fluid condition using AFM. One of the main drawbacks comes from the lack of better sealing systems that would reduce not only the volume of liquid but also create more uniform flow, which is essential for bilayer formation. Moreover, there is often limited solvent exchange through the conventional AFM sample chambers. Exchange of solvent would enable more versatility in creating the custom environment for bilayer studies using AFM. To address these drawbacks, my proposed scheme of the Ph.D. program includes developing more versatile biophysical tools for studies using AFM to explore properties of biological materials. As a first step, I have designed fluidic chamber for AFM that addresses the drawbacks mentioned above of the conventional liquid sample chamber. The newly designed sample chamber is modular, uses the minimal sample solvent and enables the use of a wide variety of lipids reagents while measuring biophysical properties of the lipid bilayer. As a proof of concept, I investigated the morphological and mechanical properties of the supported lipid bilayer made from varying lipids. Using the mixed-lipids platform for planar bilayer formation is known to

form domains usually, in case of they have a different transition temperature. The new sample chamber, therefore, shows promising applications in the field of complex bilayer studies in a variety of context. As a final remark, a brief discussion on the ongoing work on the Extracellular Matrix (ECM) membrane studies combining with the AFM application is outlined.

Keywords: Model membrane, Lipid bilayer, Solvent-Assisted Lipid Bilayer, Atomic Force Microscope, Microfluidic Device, Force Spectroscopy

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Abbreviations

AFM	Atomic Force Microscope
AM-AFM	Amplitude Modulation AFM
BSA	Bovine Serum Albumin
CCD	Charge-Coupled Device
CFM	Chemical Force Microscopy
Chol	Cholesterol
DI	Deionized
DLS	Dynamic Light Scattering
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DP-NGPE	a free carboxylic acid (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(glutaryl))
DPPC	1,2-dipalmitoyl-sn-glycero-3-phosphocholine
ECM	Extracellular Matrix
EFM	Electrostatic Force Microscopy
F-D curve	Force Distance curve
FM-AFM	Frequency Modulation-AFM
FRAP	Florescence Recovery After Photobleaching
IOM	Inverted Optical Microscopy
IPA	Isopropanol
LSPR	Localized Surface Plasmon Resonance
MFM	Magnetic Force Microscopy
POPE	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
POPG	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)
PSPD	Position Sensitive Photo Detector
QCM-D	Quartz Crystal Microbalance Diagnostics
RMS (Rq)	Root Mean Square
SPM	Scanning Probe Microscope
SALB	Solvent-Assisted Lipid Bilayer
SCM	Scanning Capacitance Microscopy

SICM	Scanning Ion Conductance Microscopy
SLB	Supported Lipid Bilayer
SPM	Scanning Probe Microscopy
SPR	Surface Plasmon Resonance
SSRM	Scanning Spreading Resistance Microscopy
STM	Scanning Tunneling Microscope
SUV	Small unilamellar vesicle
TIRF	Total Internal Reflection Fluorescence
TRIC	Tetramethylrhodamine
UV	Ultraviolet

Chapter 1

Introduction

The biological membrane takes a significant role to understand not only the fundamental function which protects the inner components of the cell but also interacts with outer substances. To explore the properties of the natural membrane, the several fabrication methods of the model membrane have been developed. Also, over the years, various experimental techniques to study the model membranes properties have been established. The atomic force microscopy (AFM) has been a promising tool for nanoscale surface measurement, the application studies for the biological samples were challenging due to the difficulties of several controlling parameters in a liquid environment. In this chapter, the problem statement of the AFM application for model membrane platform will be overviewed. Additionally, the overall strategy of this dissertation will be introduced.

1.1 Problem of Statement

Biological membrane plays a crucial role in cellular protection as well as in control and the delivery of nutrients [1, 2]. Many mechanisms such as membrane fusion, cellular adhesion, enzymatic catalysis, and molecular recognition also occur at the biological membranes [3]. To explore the several mechanisms in cell membranes, the various techniques have been established to fabricate the artificial membrane platform, which is the simplified model of the nature membranes [4-6]. In particular, supported lipid bilayer (SLB) [7-9] is a representative robust cell membrane model basically consisted of the brief two lipid leaflets structure. As a promising artificial model of the biological cell membrane, SLB has been widely studied using a number of techniques such as quartz crystal microbalance with dissipation monitoring (QCM-D), [10-12] localized surface plasmon resonance (LSPR) [13] and AFM [12, 14].

The QCM-D and LSPR are the persuasive techniques that can measure the structural changes, including molecular interactions occurred on the surface without chemical labeling. However, these two techniques are not able to directly observe the morphological structures. Meanwhile, AFM is often used as an alternative tool to measure the direct structure of morphological and mechanical properties [15, 16] instead of QCM-D and LSPR. AFM offers promising imaging capabilities at the nanometer range that is well suited for investigating the dynamics and behavior of biological samples such as live cells, DNA, and soft materials such as lipid bilayers and proteins [15]. The non-contact AFM mode is extremely powerful as it enables super high-resolution images of surfaces detailing surface morphologies in the nanometer scale without destructing the sample. This level of resolution is attained through monitoring minute amplitude modulations that occur at the tip as a result of being in the vicinity of the sample [17]. Although AFM has been used to investigate biological samples for almost thirty years, the investigation of the properties of lipid bilayer has been challenging. Most of the techniques to obtain a bilayer are extremely time consuming, use high volumes of sample, and most importantly, the bilayer formed is not always uniform.

On the other hand, the vesicle fusion method is one of the most well-known technique to create bilayer structure using spontaneous rupture processes of the vesicles on the hydrophilic substrate such as glass and mica.[12, 18, 19] Unfortunately, the bilayer formation using this vesicle fusion with AFM is often not be uniformed on a substrate resulting in complicating data analyses to extract useful information. To overcome this problem, the solvent-assisted lipid bilayer (SALB) technique has been developed [11, 12, 18, 19]. The benefit of the SALB method is that it can be assembled with a different type of lipid components to form the planar bilayer without vesicle preparation. In addition, SALB can be used to form a bilayer in a wide variety of supported systems, including gel phase supported membrane. Although the procedure of SALB has been extensively investigated, utilizing this technique to AFM as a promising tool for bilayer investigation has not been sufficiently explored because the small volume of the reservoir and sealing chamber without solvent leakage are indispensable for the solvent exchange to perform the SALB. The commercial liquid chamber for AFM does not ensure this environment as a fluidic device; the vesicle fusion can be used for bilayer studies without solvent exchange. However, the bilayer formation using vesicle fusion is occasionally difficult to produce the uniform bilayer, especially containing heterogeneity in the compositions of vesicles [20, 21] that have higher transition temperatures and larger bending rigidities [22]. **(Figure 1.1)**

To address this issue, in this dissertation, the microfluidic chamber for AFM which have enabled to investigate the SLB properties using SALB formation technique, has been developed to fabricate complex bilayer platform. Applying the originally designed microfluidic chamber for AFM, The formation of planar bilayer containing two mixed-lipids which has different transition temperature will be demonstrated. Using the microfluidic chamber for SALB technique which can be combined with AFM enables to explain how the controlled environment parameters such as concentration ratio, temperature, and type of the solvent influence to the structural and mechanical properties of the mixed-lipids bilayer on the glass substrate as a statistical model. Also, the results from the AFM will be compared with the other experimental techniques such as fluorescence microscopy and QCM-D to strengthen understanding.

Figure 1.1 AFM images of intact vesicle consist of DOPC lipids containing 40% of cholesterol. Because of high rigidity, the vesicles are adsorbed to the surface without rupturing. (a): 10 μm images, (b) 5 μm image, (c) 2 μm image.

Table 1.1 Comparison of characterization techniques of SLBs

Technique	Application	Drawback
AFM Atomic force microscopy	Morphology, Thickness, Mechanical properties	Difficult to measure change in properties in short time scales
FRAP Fluorescence recovery after photobleaching	Lateral mobility	Requires labeling, Low resolution
LSPR Localized Surface plasmon resonance	Refractive index, Layer thickness	Cannot observe structural changes
QCM-D Quartz crystal microbalance with dissipation monitoring	Mass & viscoelasticity	Cannot observe structural changes

1.2 Objectives and Scope

The entire scope of this dissertation is to develop AFM approaches that enable to the understanding of the structural properties of SLBs in a mixed-lipids platform and can be utilized for probing how the environmental parameters such as temperature, concentration ratio and solvent influence to the morphological and mechanical properties in a molecular-level interactions. One major challenge in the AFM measurement for SALB is the lack of the proper sealing chamber with a small volume of reservoir area, which enables to perform the solvent exchange process without leakage.

Along this line, in my Ph.D. work, the first objective is the development of the microfluidic chamber device based on SALB technique for AFM measurement. For the successful formation of the uniform bilayer via SALB, the fluidic simulation of the solvent exchange from organic solvent to the aqueous solution with a specific flow rate that is satisfying the parameters for the bilayer formation should be performed under the perfect sealing environment. For that sense, the overall objective of the Ph.D. thesis is to investigate the multi-components composition in SLBs in structural and functional properties, including morphology, mechanical strength, and solvent charge influence using AFM with newly developed chamber. To test this objective, the solvent-assisted lipid bilayer (SALB) method will be employed as a fabrication technique for bilayer formation, which enables the controllable fabrication of SLBs with complex lipids composition, thereby establishing an experimental platform to systematically investigate the influence of the environment [14, 23].

In addition, as utilizing the originally designed microfluidic chamber device, the investigation of the multiple lipids composition bilayer properties using the various experimental techniques such as QCM-D, fluorescence microscopy will be compared with the results from the AFM. All experiments were conducted using the new designed microfluidic chamber for SALB.

In the first result part, the development of the originally designed microfluidic liquid

cell chamber for AFM will be introduced, which enables the SALB with its numerical fluidic simulation. Next part, the morphological and mechanical properties of mixed bilayer platform of DOPC and DPPC lipids has been investigated in different concentration ratio at the room temperature. Last part of this dissertation, the unusual topological property of Gram-negative bacterial membrane composition (POPE (85)): POPG (15), molar ratio) has been observed by using originally designed microfluidic chamber for AFM. For future work, the multi composed bilayer platform studies with SALB will be introduced based on AFM applications.

Towards this goal, I seek to achieve the following hypothesis and specific aims:

1.3 Hypothesis and Specific Aims

The overall hypothesis of this dissertation is that the SLB platform is can be utilized significantly to study the biological membrane as a representative model system. The SALB formation technique is the dominant method to fabricate the model membrane platform as a planar bilayer. This structure allows the approach to study of physiological conditions as a mimicking system of a cell membrane. To combine the research of the SLBs to the AFM studies as an experimental tool, the AFM has been a shown a lot of benefits to obtaining the local properties in nano-scale, such as morphological, mechanical and molecular interaction [15, 16].

Therefore, in order to confirm the hypotheses above, this dissertation aims to provide a novel platform to understand the model membrane platform using AFM. The following specific aims will provide the motivation for this work:

Aim 1: To develop a new microfluidic chamber device that supports the formation of SLB based on SALB formation method, which can be combined with the AFM system for its applications.

Aim 2: To investigate the morphological and mechanical properties of mixed bilayer platform of DOPC: DPPC lipids in different concentration ratio at room temperature.

Aim 3: To observe the unusual non-equilibrium membrane topology consisting of POPE and POPG phospholipids formed by SALB method

All experiments have been performed using the originally designed microfluidic chamber for AFM that supports the SALB formation method for bilayer fabrication.

1.4 Dissertation Overview

This dissertation has been organized into the following chapter:

Chapter 1 “Introduction” presents the overview of the experimental techniques for the bilayer research as a model membrane system. Also, the problem statement of the performing the AFM experiment for the model membrane is briefly introduced; afterward, the objectives and specific aims of the Ph. D. research is outlined.

Chapter 2 “Literature Review” describes the several techniques for the fabrication of the SLBs. The detailed review of the conventional method (vesicle fusions) and recent robust technique (solvent-assisted lipid bilayer) will be explained in detail. To perform the SALB formation method combined with the AFM system, the motivation and numerical studies of the microfluidic chamber are described. At the last part of the chapter, the AFM applications for biological samples are briefly introduced.

Chapter 3 “Experimental Methodology” lists the materials including lipid and buffer reagents and its preparation. It also records how to perform the solvent-assisted lipid bilayer process for bilayer fabrication in detail. Afterward, the experimental techniques that are employed throughout in this dissertation will be demonstrated.

Chapter 4 “Microfluidic device for solvent-assisted lipid bilayer for Atomic Force Microscopy measurement” presents the details of the motivation, fundamental principles and the design considerations for the development of an AFM sample chamber for bilayer studies based on SALB formation method.

Chapter 5 “Molecular diffusion and mechanical properties of mixed lipid bilayers of DOPC and DPPC fabricated by the solvent-assisted lipid self-assembly” reports the practical experimental results of the mixed bilayer platform of the DOPC/DPPC in different concentration ratio at room temperature. The microfluidic chamber which adopted the same principle for SALB formation technique designed in this chapter for different AFM model.

Chapter 6 “Unusual non-equilibrium membrane topology consisting of POPE and POPG phospholipids formed by Solvent Assisted Lipid Bilayer (SALB) method” elaborates the observation of the unusual morphological changes in a Gram-negative bacteria like membrane using the microfluidic chamber for AFM.

Chapter 7 “Conclusion and Future Outlook” draws the conclusion of the whole dissertation. The summaries of the work are introduced, including remarks output of the previous chapters. At last, the future expectations and plan for this research will be reviewed.

1.5 Main Findings and Outcomes of the Thesis

This dissertation led to several novel outcomes by:

1. Offering the platform by the development of originally designed microfluidic liquid cell chamber for AFM which enables to perform the solvent-assisted lipid bilayer formation based on numerical fluidic simulation.
2. Investigation of mixed bilayer platform with the DOPC/DPPC lipids in different concentration ratio, at room temperature. Applying the new microfluidic chamber

for AFM with SALB formation method, the successful structure of the bilayer was achieved; even the DPPC lipids exist in a gel-phase at room temperature. This offers the investigation method of properties of complex membrane platform while it contains the lipids with the high rigidity.

3. Providing the real-time observation of unusual topological changes occurring in Gram-negative bacteria membrane composition using the AFM and fluorescence microscopy technique in various environmental condition parameters.

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Chapter 2

Literature Review

The biological membrane has a significant function in the cellular structure. It does not only separate inner components from the outer environments, but it also mediates the overall passage of various cellular materials in and out of cells. However, because of its high complexity, containing the main structure of phospholipids and various proteins, it has been challenging to study the natural membrane specimens. Hence, several techniques have been developed to fabricate the model membrane platform. In this chapter, an overview of SLBs as a model membrane platform and fabrication technique will be introduced. In addition, it highlights the comparison between the conventional method (vesicle fusion) and recent novel technique which is the solvent-assisted lipid bilayer (SALB) method with the numerical microfluidic simulation. Finally, the preliminary studies for biological samples and its applications using the AFM technique will be reviewed.

2.1 Model Membrane Platform

The biological membrane takes the important role of not only the protecting the inner components from the outer environment, but also as the multi-functional sensing platforms for monitoring the local ion concentrations, sending and receiving transmitter molecules for communications between cells [1-5]. The biological membranes also govern cellular functions (cellular adhesion, cellular division, membrane fusion, etc.) via molecular recognition process among the biomolecules embedded in a lipid bilayer.[6-8] Also, in the cell membranes, lipid bilayers accommodate various biomolecules such as channel proteins, globular proteins, glycoproteins, glycolipids, cholesterol, etc. [9] However, because the biological membrane is highly complex with a lot of components, it is difficult to study these biological processes. Therefore in order to investigate the biological processes which take place on the membrane surface or within the cell, which is enveloped with the membrane lipid bilayer, many studies have been performed using the biomimetic model systems [1, 10].

Figure 2.1 Representative scheme of the biological cell membrane. Reproduced from Singer et al [5].

The biological cell membrane consists primarily of an amphipathic phospholipid which has a hydrophilic polar head and hydrophobic hydrocarbon chain. An example of other membrane component is the transmembrane protein that expands across the cell membrane. It plays a significant role in regulating and transporting biomolecules into and out of a cell [9]. As mentioned, the artificial model membrane is the simplified version of the biological membrane [10, 11]. Ideally, the model membrane must maintain the key components and properties while providing good accessibility so that it is easy to be investigated and characterized. The objective of developing a model membrane is not only to investigate these biological processes, but the model membrane also expands the range of applications by integrating organic and inorganic materials together. For example, by coating, an inorganic implant with lipid bilayer would improve cell recognition, cell adhesion or prevent unwanted protein adsorption on the implant [9-11].

As explained previously since the biological membranes have a complicated system, several model membranes have developed over the last century for investigating its properties. The famous biomimetic models of the membrane which are also commonly used are lipid monolayers, lipid vesicles, and the SLB [1, 9].

Lipid monolayers are a powerful model for characterizing the drug-lipid or lipid-lipid interactions at a molecular level [12]. The substrate can be prepared as hydrophilic or hydrophobic depending on the application. If the solid support is hydrophilic support, the lipid polar head is facing to the substrate [13, 14]. On the other hand, the hydrophobic support is used to transfer the lipid hydrocarbon chain via hydrophobic interaction [12, 15]. However, lipid monolayers consist of only one lipid leaflet that cannot explain the model biological membrane, which has two leaflets.

Another type of model membrane is lipid vesicle. Lipid vesicles are useful biomimetic model commonly used for investigating membrane properties such as membrane fusion, molecular recognition, and cell adhesion [16]. Additionally, the curvature of the vesicle can be adjusted by varying the diameter of vesicles. These vesicles enclose aqueous compartment which has been used as a delivery carrier for drug, proteins, enzymes, and DNA. Lipid vesicles can be formed by mechanical, chemical, or

electrochemical technique [17]. However, in general, lipid vesicle has poor long term stability, that means it may easily aggregate and lost its function.

SLB is constituted of two lipid leaflets where the polar head group of lipid facing outwards and hydrocarbon chain facing inwards. These bilayers are much more stable than lipid vesicles, and it can be the representative model of biological membranes because different from lipid monolayer, it has the same two lipids leaflets structure. These model membranes are confined to a solid substrate; therefore, they can be formed much more straightforward than the other free-floating model platform [18, 19]. Here in this chapter, SLB will be focused as a biomimetic model, and several techniques will be introduced to fabricate the uniform bilayer structure on the solid-substrate.

Table 2.1 Types of the solid-supported model membranes

<u>Monolayer</u>	<u>Intact Vesicle</u>	<u>Bilayer</u>
<ul style="list-style-type: none"> • Langmuir-Blodgett technique • Constituted of only one leaflet • Do not reflect the biological membrane • Commonly used to study drug-lipid or lipid-lipid interactions 	<ul style="list-style-type: none"> • Molecular delivery applications • Formed by sonication or extrusion • Poor long-term stability • Cell adhesion • Molecular recognition • Membrane fusion 	<ul style="list-style-type: none"> • Two lipid leaflets like biological membrane • More stable than lipid vesicles • Interface between inorganic and biological systems

2.2 Fabrication Techniques of SLBs

In this paragraph, the fabrication techniques will be outlined that lead to the development of the new microfluidic chamber for AFM studies on supported lipid bilayer systems. The primary motivation was the formation of SLBs using SALB technique that has several advantages over the conventional vesicle fusion method [20]. Since the motivation is the fabrication of uniform bilayers using SALB technique, and a principle description of this method is presented below.

2.2.1 Conventional methods for fabrication of SLBs

Several techniques have been established for the fabrication of SLBs including Langmuir-Blodgett deposition [21], dip-pen nanolithography [22], spin-coating [23], air bubble collapse [24], and vesicle fusion [25]. Each of these techniques may offer specific advantages. For example, Langmuir-Blodgett deposition allows the deposition of asymmetric bilayers [26, 27], while dip-pen nanolithography facilitates the parallel fabrication of biomolecular arrays [22]. The spin-coating technique enables the quick generation of highly oriented, homogeneous bilayer stacks with defined thickness [23]. However, most of these techniques require a specific skillset and good quality SLBs can often only be achieved with a considerable amount of training and practice. Furthermore, techniques such as dip-pen nanolithography and spin-coating require special instrumentations. Among these techniques, one of the most common method to deposit a bilayer on a substrate is the vesicle fusion method [15, 28]. Here, small unilamellar vesicles formed via extrusion using the filter with a particular pore size which determines the size of the vesicles, then the bilayer is formed on the surface by spontaneous rupture process [29, 30]. The nature of the deposited lipid layer can be characterized using QCM-D and optical techniques such as fluorescent microscopy [31]. The bilayer formed using this technique may not be uniform and can lead to challenging data analysis to extract useful information especially the vesicles contain the substance with the substantial bend rigidity such as a gel phase lipid [32]. As mentioned, vesicle fusion represents the most popular method to prepare SLBs prior to the development of SALB, since it can be performed with

minimal training and without the need for elaborate equipment setup. Nevertheless, the extrusion of vesicles in the vesicle fusion method is non-trivial and requires adequate practice in order to achieve high-quality SLBs. In addition, adsorbed vesicles only can be ruptured on a few of substrate support materials. The reliance on vesicle rupture also limits the applicability of vesicle fusion on certain surfaces such as Au, TiO₂, and Al₂O₃, and hampers the fabrication of SLBs with multiple-compositions, especially involving charged and gel phase lipids. On these surfaces, vesicles are typically remained in unruptured or difficult to absorbed to the surface [30].

Figure 2.2 The schematic of the vesicle fusion process. The free vesicles are adsorbed to the hydrophilic surface and merged each other. After spreading and propagation process, the bilayer structure is formed on the surface by lateral diffusion

The experimental attempt of the vesicle fusion method was challenging especially in case of the interaction energy which is needed to stabilize on hydrophilic surface is much smaller than the interaction energy which should be enough to occur the rupture process an adsorbed vesicle [33]. Only a few solid supports, such as silica-based materials, have

satisfied these parameters to induce the spontaneous rupture of adsorbed vesicles. As such, the vesicle fusion method requires the skills to prepare the vesicles and only can be worked with a small range of the material supports and lipid compositions.

2.2.2 Solvent-Assisted Lipid Bilayer (SALB)

To overcome the limitation of the conventional fabrication methods for SLBs, a new technique called the SALB had been developed [31, 34, 35]. The SALB process is a simple and robust technique where the phospholipids are dissolved in organic solvent-aqueous solution mixtures, and the buffer/water content is increased gradually until all the solvent has been replaced with the buffer/water. **(Figure 2.3)** The bilayer formation is interlinked to the lipid phase transition from inverted micelles, and monomers to vesicles and micelles where the bilayer usually nucleates just before the transition [36]. Since SALB formation is essentially driven by the self-assembly process, the room for human error is minimized, allowing high-quality SLBs to be easily reproduced [37, 38]. This is in contrast to the vesicle fusion method whereby the vesicle extrusion step is performed manually, and the quality of extruded vesicles and resulting SLBs are therefore prone to technical variability. Such drawback is exacerbated by the fact that vesicle properties such as size [39, 40], lipid composition [13, 41, 42], intrinsic curvature [43], osmotic pressure [44-46] and lamellarity [47], as well as bulk solution parameters such as pH [48], ionic strength [49, 50], and temperature [51, 52] all need to be strictly controlled in order to successfully trigger vesicle rupture and SLB formation on a particular substrate. As the SALB method totally eliminates the need for vesicle rupture, which in most cases represents the rate-limiting step for the transformation of adsorbed vesicles to SLBs, it is less sensitive to the substrate material and bulk solution conditions. In the preliminary study, SLB formation has been demonstrated via the SALB method on a wide variety of hydrophilic substrates including titanium oxide (TiO₂), aluminium oxide (Al₂O₃), gold (Au), indium tin oxide (ITO) and chromium (Cr), all of which are intractable to the vesicle fusion method [31, 34, 53]. Another interesting aspect of the SALB method is that the resultant SLBs have a high degree of reconfigurability in terms of lipid compositions. Lipid bilayers comprising of multiple lipid compositions with varying charge can be conveniently fabricated by directly

mixing the lipid components and introducing the cocktail to the solid substrate. Likewise, the incorporation of membrane components such as cholesterol, phosphoinositides, and oligosaccharides can be achieved in a similar fashion. Previously, our research group has shown that, compared to vesicle fusion, a higher fraction of cholesterol can be incorporated in the SLBs following the SALB method [54]. Furthermore, the method also enables the membrane components to be remodeled post-construction [55]. These possibilities highlight the promise of the SALB method to become an indispensable technique for the construction of SLBs with an unprecedented degree of biomimicry.

While the gradual solvent-exchange process is a simple approach to form SLBs, it is rather slow in the entire process, so recently the quicker and more robust procedure has been suggested from Ferhan et al. [56]. With the new developed SALB method, from the first step, the lipids-dissolved organic solvent is injected, which leads to more rapid solvent exchange to the aqueous solution in the entire procedure [31]. **(Figure 2.4)**. The direct transfer from organic solvent to aqueous buffer causes more sudden changes of the phase transition in a shorter time scale, (<10 min) that is more suitable for general fabrication. Surprisingly, the new approach of the SALB procedure enables to form SLBs on not only silica substrates but also gold surfaces. The gold substrate is one of the most challenging to form uniform SLBs with conventional fabrication methods [31]. Guided by this new protocol of the SALB, Seyed et al. showed that the successful formation of the SLB with a series of organic solvents, including isopropanol, methanol, ethanol, and n-propanol [20, 31, 34]. Additionally, the new approach of SALB procedure has reported that enables to form SLBs in various cases which were not successfully formed before with conventional fabrication methods.

Figure 2.3 The self-assembly process of phospholipid molecules in a different solvent. In the organic solvent phase, the phospholipid molecules exist as inverted micelles or monomers. By increasing the aqueous solution to the solvent, the phospholipid molecules change to normal micelles and vesicles; then it formed to the bilayer structure to the surface at the end. Adapted with the permission from [56]. Copyright 2019 Nature Protocol

In addition to silica and gold surfaces, SLBs can be formed on a several types of the substrates, including aluminum oxide, titanium oxide, nanoporous gold, chrome, graphene, and indium tin oxide [34, 57-59]. It also showed the successful cases to form SLBs using charged lipid compositions, including anionic lipids and monovalent [53] along with the different type of lipids such as phosphoinositides [55]. The SALB procedure also works with a mixed composition of fluid-phase and gel-phase lipids [4, 34], and can be used to fabricate cholesterol-enriched SLBs [54, 60, 61]. With conventional vesicle fusion method, when the lipid compositions including high cholesterol ratio, it was impossible to form the uniform SLBs so that it has been reported typically up to ~20 mol% of cholesterol [62]. In

contrast, by using the SALB method, Seyed et al. reported the successfully formed SALB with upper ~60 mol% of cholesterol [60]. The significant benefits of this method are that it can be assembled with a different type of lipids (e.g., bicelles, monomers, or inverted micelles, charged lipids, etc.) to form the planar bilayers without vesicle preparation. In addition, SALB can be used to form bilayers in a wide variety of supported systems including gel phase supported membranes; it allows the experiment can be performed by less controlling the temperature. The planar lipid bilayer is formed in about 30 min in the total procedure, making it less time- consuming.

Another significant parameter in the SALB process is the flow rate of the solvent-exchange. If the flow speed is too high or low, then the results of the SALB procedure can be unsuccessful in forming the uniform SLB because the lipids supply is not enough in a bulk reservoir area [31]. Also, the reservoir area should satisfy a very low volume to guarantee the complete solvent-exchange with a certain flow speed [63]. The numerical simulations of flow velocity and dimension of the reservoir area, which leads the motivation to design and develop the new microfluidic chamber for AFM will be underlined in the next chapter (**Chapter 2.3**).

As mentioned earlier, the advantages of the SALB technique stems from the fact that the lipid molecules are directly introduced to the substrate, enabling SLBs to be obtained on a wide variety of substrates and with greater reconfigurability in terms of lipid composition, since the dependence on vesicle-substrate and vesicle-vesicle interactions is greatly reduced. This also facilitates the incorporation of membrane components in the SLBs, compared to most other methods. Since SALB is performed entirely in the solution environment, it is also particularly advantageous for fabricating SLBs when the exposure of the sample to air should be completely avoided. In addition, SLBs obtained via SALB has excellent purity since it does not require intermediate steps involving other lipids or peptide-induced vesicle rupture. However, SALB is sensitive to lipid concentration and flow rate, since these factors govern the kinetics of the solvent-exchange step. It has been established that there exists a minimum threshold for the effective lipid supply (determined by the balance of bulk lipid concentration and the solvent-exchange rate) that would ensure complete bilayer formation.

Figure 2.4 Schematic of simplified SALB procedure. The aqueous solution such as buffer or water is filled up the reservoir area first; then it exchanges to the lipids-dissolved organic solvent (isopropanol, IPA). After injecting the buffer solution to the chamber again, the SLBs can be formed on the solid substrate. At the last step of the process, the chamber area should be washed with the aqueous solution to eliminate floating lipid molecules from the bulk solution. Adapted with the permission from [56]. Copyright 2019 Nature Protocol

2.3 The Design Concept of the Microfluidic Chamber for AFM

Characterization of the SLB can be performed by AFM, FRAP, SPR, and QCM-D techniques. Each technique has its advantages and limitations and the choice of technique depends on the measurement parameters. A brief summary of the uses, and drawbacks of the surface techniques are described in **Table 1.1**. This dissertation is focused on AFM based measurements on bilayer systems as it can provide detailed information of both morphological as well as mechanical properties of bilayers. Since bilayer formation for AFM measurements is conventionally performed in a Petri dish or commercial liquid cell chamber which significantly uses more sample, doesn't always form uniform bilayers, [64-67] the first objective of this thesis is centered around building an originally designed microfluidic device that can form uniform bilayer and uses less sample for AFM measurements. The design inspiration for building a sample chamber that can be modular, uses less sample volume, forms uniform bilayer with high repeatability and that can be used in combination with a wide range of techniques came from the fluidic chamber used for QCM-D studies (Gillissen et al. [63] as well as the commercially available microfluidic chamber. The design principles adapted from the commercial microfluidic chamber for optical microscope and QCM-D flow chambers are described below for the development of the AFM sample chamber for bilayer studies.

The SALB can be formed in the commercially available microfluidic flow chamber for fluorescence microscope or using the flow-fluidic chamber for the QCM-D. The principle of operation of both devices is very similar. The chamber consists of an inlet, an outlet and a channel where the bilayer deposits as the solvent are replaced with water. Successful formation of a uniform bilayer via solvent exchange depends on various parameters such as the flow rate, type of buffer, lipid, diffusion coefficients, and viscosity of water/isopropanol mixture, to name a few.

Figure 2.5 The drawing of the conventional microfluidic channel for fluorescence microscopy. The dimension of the reservoir area is $L = 48$ mm, $W = 5$ mm and $H = 0.1$ mm.

The properties of the flow conditions for a successful deposition of a bilayer in microfluidic and QCM-D chamber have been recently documented using theoretical simulations by Gillissen et al. [63]. Here, we used the same simulation results to calculate the dimensions of an AFM based flow chamber for forming uniform bilayers.

The microfluidic chamber for epi-fluorescence microscope has a rectangular microfluidic channel with dimensions of $L = 48$ mm of length, the width of $W = 5$ mm and a height of $H = 0.1$ mm. Since the flow duration of the solvent through the channel is relatively small (36s), the solvent is uniformly distributed along the streamlined direction of the chamber. For the given dimensions of the chamber, using a simple diffusion equation, we find that the flow is uniform along the y-axis as the penetration depth (δ) is larger than the channel height. Further, it is observed that along the corners of the channel, lipid bilayer adsorption was faster than the solvent displacement rate itself [63, 68].

With these assumptions, the diffusion/displacement of the water fraction can be expressed with following advection-diffusion equation.

$$\partial_t \phi + \nabla \cdot (u\phi) = D\nabla^2 \phi \quad (\text{Eq. 2.1})$$

For simulating the water fraction in a single channel, we integrated the polar coordinate

with azimuthal invariance in advection-diffusion equation as below [68].

$$\partial_t \phi + \partial_x (u_x \phi) = D(\partial_x^2 + \frac{1}{r} \partial_r r \partial_r) \phi \quad (\text{Eq. 2.2})$$

and under the fully laminar flow condition, the u_x can be written as

$$u_x = Q[1 - (r/R)^2] / (\pi R^2) \quad (\text{Eq. 2.3})$$

and the volumetric flow rate $Q = 0.68 \text{ mm}^3/\text{s}$. Assuming the inlet conditions of the first tube to be $\phi = 1$. Then the time-radial dependent ϕ -field at the outlet of the first tube can be considered as the same with the inlet condition for the next tube, and repeat this concept for the subsequent tubes. The $\phi(r, t)$ at the outlet of the fourth (last) tube is converted to $\phi(z, t)$ at the inlet channel using the mass conservation principle. When assuming the laminar flow with the constant viscosity, the analytical expression in a rectangular channel, which has a large aspect ratio with Width and Height (W/H), can be developed as below.

$$u(y, z) = \frac{3U}{2} \sum_{m=1,3,5,\dots} \frac{(-1)^{\frac{m-1}{2}}}{m^3} \left(1 - \frac{\cosh \frac{m\pi z}{H}}{\cosh \frac{m\pi W}{2H}}\right) \times \cos\left[\left(\frac{m\pi}{2}\right)\left(1 - \frac{2y}{H}\right)\right] \quad (\text{Eq. 2.4})$$

Using the above-mentioned assumptions and equation, the dimensions for an AFM is calculated to L, H should have millimeter order, and W should be less than 1 mm, for a flow rate of $0.3 \sim 1 \text{ mm}^3/\text{s}$. The total volume of the sample chamber is $9 \text{ mm} \times 9 \text{ mm} \times 0.5 \text{ mm}$. This is ten times orders of magnitude less compared to the traditional petri dish method of investigating bilayer properties.

Figure 2.6 The schematic of reservoir area of the microfluidic chamber. With a length L and a width W

Once the dimensions are calculated, the sealing system with the capability of solvent exchange and the use of probe were considered. The AFM chamber (**Figure 2.7**) consists of six components. The top cover of the chamber is tightly combined to the chamber part with four bolts to prevent the solvent leakage. Two O-rings made of rubber with different size (20mm, 25mm) absorb the pressure to the coverslip from the bottom cover. That act as a sealant for the chamber, the newly designed flow chamber and the structure which the actual chamber is placed contains the inlet and outlet for insertion of tubes. The overall dimensions of the chamber itself are 35 mm of diameter and 6 mm of the thickness, which can be easily mounted on a standard AFM XY stage. The design can be easily tweaked to fit specific sample stage for other AFM models.

Figure 2.7 The drawing of micro fluidic chamber for AFM. (1) 3D drawing of the all components, (b) Top view, (c) side view

2.4 Liquid applications for biological samples using AFM techniques

2.4.1 Scanning Probe Microscopy (SPM)

The Scanning Probe Microscopy technique has started from the invention of Scanning Tunneling Microscopy (STM) at 1981 by Binnig et al. [69, 70]. It takes a significant role in nanotechnology and the nano-science field since it was invented. Before the SPM techniques, it was a challenge to explore the nano-scale features of the sample surface because of many imaging artifacts [71, 72]. However, since SPM techniques have invented, the researchers enable to obtain and observe the morphological information in the nano-scale. In that sense, SPM is one of the most promising tools and important

technique for the nano-science field [72].

The STM is the first invented technique among SPMs [71]. It measures a tunneling current between probe and sample surface as an imaging signal due to the quantum mechanical phenomenon. The tunneling current, which is generated from the tunneling effect from the sample surface to the other materials, is used for the measuring parameter of STM. When the distance between the probe to sample surface is around to the order of less than a few nanometers, the tunneling current is changed. Detecting the change of the tunneling between the probe and samples, STM can obtain the sample surface image. However, in the earliest model of STM is hard to measure the atomic resolution because of the noise level of the system. The mechanical noise from the external vibration and, high electrical noise level made it difficult to detect the extremely small distance between probe and sample surface.

In 1981, Binnig and Rohrer success to measure the tunneling current in their experiment. They employed the feedback loop that enables to maintain the distance between probe and sample surface, and it was the critical trial to success to obtain the atomic-scale images. By applying feedback loop, they made a success to measure the atomic-scale topographic structure of silicon surface using STM [72].

The basic principle of all SPM techniques is commonly based on using the interaction between the probe and sample surface so that the probe should be with very sharpen shape due to atomic scale accuracy. In various application modes of SPM are using those certain signal in every single point as a local signal during the imaging from the sharp tip. Normally each of SPM techniques adopts a different type of probes, which are designed for its measuring signal as targeted materials properties. Thermal, electric, magnetic local properties of materials also can be obtained at the nano-scale level by using the proper mode of SPM and probes. For example, as mentioned above, due to using the tunneling current for its measuring parameter, the STM is using the sharp conductive probe in the vacuum condition. Similarly, a magnetic force microscope (MFM) measured the magnetic forces between probe and sample using the AFM cantilever, which has made with a

magnetic material such as magnetic thin film. The suitable selection of SPM mode and probe selection is the most important to investigate the appropriate material properties [73].

2.4.2 General principle of Atomic Force Microscope (AFM)

The Atomic Force Microscopy invented in 1986 by Binnig, Quate, and Gerber [74]. The STM has its limitation for mainly using the tunneling current for its detecting signal, the only conductive tip and conductive sample can be used in the vacuum condition. Although the STM cannot be applicable to the non-conducting sample, the AFM can adapt to the most kind of material in any environment such as vacuum, air, and liquid condition. As one of the applications of SPM, AFM moves over the sample surface with the sharp tip by detecting the deflection signal between tip and sample. Sensing the force depends on the distance between the tip and sample surface, there are two types of forces which are a repulsive force, and an attractive force is used to image. **Figure 2.8** shows the conventional configuration of most AFM system. The force between the AFM tip and the sample surface can be detected by obtaining the cantilever deflection signal. The reflected laser beam from the backside of cantilever drops to mirror first, afterward reach to the Position Sensitive Photo Detector (PSPD). Therefore the detector and AFM controller enable to follow the shape of the topography using the feedback loop. Unlike STM, the AFM can be used in various environments such as air, liquid, and vacuum. Also, it can measure both conductive and non-conductive sample without further sample preparation.

Table 2.2 List of SPM applications

SPM applications	Measuring interaction	Remarks
Electrostatic Force Microscopy (EFM) [75]	Electrostatic force	Can be immersed in non-conductive solution only
Magnetic Force Microscopy (MFM) [76]	Magnetic force	Use the magnetic sample and tip
Conductive AFM [77]	Current between tip and sample	Requires conductive tip and surface
Chemical Force Microscopy [78]	Van der Waals interaction	Complex tip preparation
Scanning Spreading Resistance Microscopy (SSRM) [79]	Spreading resistance between tip and sample	Requires conductive tip with high spring constant
Scanning Capacitance Microscopy (SCM) [80]	Capacitance between probe and sample	Requires conducting surface
Scanning Ion Conductance Microscopy (SICM) [81]	Ion conductance near from sample surface	Feasible for imaging biological sample in liquid condition

Figure 2.8 The schematic diagram of basic principle of the AFM. The deflection of the cantilever can be detected by the position of the laser spot on the photodiode. When the cantilever bends, the angle of the reflection changes and the laser spot reaches to the different position of the photodiode. By sensing this signal, the AFM controller gives the voltage signal using the feedback loop.

2.4.3 Scanning Ion Conductance Microscope (SICM)

The SICM invented in 1989 by Hansma group [81]. Different from other SPM techniques, SICM is designed for using a specific pipette as an AFM probe to investigate the morphological property of soft non-conductive biological sample such as cell or cell membranes [82, 83]. The principle of SICM is detecting the changes of ion conductance signal between two electrodes (**Figure 2.9**). The pipette which has a small hole with around 80 nm of the inner diameter (nano-pipette) approaches to the sample surface. Bias is applied to two electrodes, one is the nano-pipette, and the other is the conductive solution. The silver/silver chlorides are usually used for the electrode. While the nano-pipette is scanning on the sample surface, the current signal from the solution to the electrode has changed, thus record this signal as feedback control. Therefore, the morphological sample topography can be achieved. The most remarkable fact of the SICM is, the image can be

obtained without physical contact between nano-pipette and sample surface. Enable to maintain the non-contact condition; the SICM is one of the most powerful imaging application for the soft biological samples. Additionally, the nano-pipette, which is usually made of glass, can be filled with chemical substances or metal inside due to increasing its electrical signal sensitivity. For that sense, the SICM has a lot of potentials to improve its feedback stability, which is directly related to its scan speed. Kim et al. reported the feasibility of the improvement of SICM imaging speed by modifying the signal amplification circuit [84].

Figure 2.9 The schematic diagram of the feedback mechanism of the scanning ion conductance microscopy. (a) The ion current flows into the nano-pipette from a bath electrode, and the circuit starts sensing the current signal between electrode and sample. (b) The representative current-distance curve. When the pipette approaches the sample, the current value starts reducing in the very near from the sample surface. Adapted from Korchev et al. [85]

2.4.4 Bilayer studies using AFM applications

In the many biological applications using AFM, there are several approaches have been tried to investigate the bilayer's morphological and mechanical properties. One of the most remarkable technique in imaging mode is using the frequency modulation (FM) AFM mode. Although the AFM offering the high resolution with contact and non-contact

(Amplitude Modulation; AM-AFM) mode, for the directly visualize of the bilayer structure, the conventional mode does not have enough resolution [86]. However, FM-AFM modulates the tip with a very small amplitude, enables a super high-resolution image which can measure the detail feature morphology of the bilayer with non-destructive status [87-89]. Fukuma et al. have reported the super-high resolution of the biological samples such as imaging of the hydration layers, mobile ions structure, and success to observe subnanometer-scale imaging in the liquid condition, by optimizing the noise level of the FM-AFM system. [87, 90]. However, although the FM-AFM guarantees the super- high resolution, it is the imaging mode so not suitable to obtain the mechanical properties information of the sample at the same time.

To investigate the mechanical properties of the bilayer, the force-distance curve model can be employed. [91]. In this model, the AFM tip approaches and makes contact with the bilayer surface (**Figure 2.10(a)**), and the contact force (deflection) increases until when the bilayer is penetrated (**Figure 2.10(b)**). After penetrating the bilayer, the AFM tip reaches to the rigid substrate below the bilayer, and the contact force linearly increases again (**Figure 2.10(c)**). When the bilayer has ruptured by penetrated by AFM tip, the approach curve shows the clear peak area, which is usually defined as a break-through force. Also, derive the separation length from the substrate contact point to the peak of the break-through force, the bilayer depth can be obtained [86, 91]. The changes of experimental conditions such as the composition of lipids or chemical treatment to the bilayer can affect to the break-through force value so that the mechanical properties analysis can be determined by this force spectroscopy model. However, as the force spectroscopy only can measure the local properties of the sample surface, to obtain reliable statistic data, the robust platform of the bilayer is necessary. To address this issue, in this dissertation, the motivation of the development of microfluidic liquid cell chamber for AFM to make a robust SLB platform has been introduced. Basically, the new designed fluidic chamber can be combined to the AFM system and enable to form the uniform bilayer on the substrate using the SALB method, which is based on the lamellar flow of solvent. Utilizing the fluidic chamber and force-distance curve model, the biomechanical properties of bilayer can be widely studied with reliable results, including peptides or proteins influences.

Figure 2.10 The representative force-distance curve of the bilayer penetration model. (a) indicates the cantilever starts to approach to the sample surface, (b) starts make a contact between cantilever and sample, (c) is the contacting the substrate after penetration (breakthrough) of the bilayer

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Chapter 3

Experimental Methodology

In this chapter, the materials for various model membrane platforms and experimental techniques will be introduced, including the fundamental principle of instrumentations and protocols. To investigate the morphological and mechanical properties of the model membrane, several types of the lipids (DOPC, DPPC, POPE, POPG, cholesterol) are employed, and SALB formation method has been adopted for SLBs fabrication using originally designed microfluidic chamber for AFM. The detail of the new microfluidic chamber for AFM will be followed by the next chapter. The experimental techniques that are performed to monitor the dynamics of the SLBs, including AFM, fluorescence microscopy, and QCM-D, will be presented in detail in the last part of this chapter.

3.1 Rationale for selection

In this chapter, the experimental methodologies will be explained in detail, and it consists of three main sections; (i) main materials used in the thesis, (ii) Solvent-Assisted Lipid Bilayer method for bilayer formation, and (iii) major experimental and characterization techniques and its principle. In the first section, the materials will be described, including all type of phospholipids which are employed throughout the experiments, utilized buffer solution. All lipid reagents were used to form a model membrane platform as the mimetic system of a biological membrane, including various types of small unilamellar vesicles (SUV).

The second section, the experimental procedure of the Solvent-Assisted Lipid Bilayer (SALB) fabrication method will be introduced, which is the robust technique for bilayer formation with detailed protocols. The background of the development of this novel technique and comparison with the conventional methods in detail is reviewed in the previous chapter. The representative AFM data of the uniform bilayer (100% of DOPC) formed by SALB technique will be presented.

In the third section of this chapter, mainly employed experimental and characterization techniques are explained. The detail setup and concept of the Atomic Force Microscope (AFM) for biological applications is introduced for investigating the topology of the bilayers in nanoscale as a promised tool. The force spectroscopy technique that is using the force-distance (f-d) curve for measuring the mechanical properties of the surface is also presented in detail with the calibration method of the AFM tip. To make a robust data set by tracking the morphological dynamics, the fluorescence microscopy instrument, including fluorescence recovery after photobleaching (FRAP) technique were utilized. With analysis from the data images of FRAP, the diffusivity of SLBs can be obtained by capturing the morphological changes in a few interval seconds (2-3 sec). Lastly, quartz crystal microbalance with dissipation (QCM-D) measurement was performed to measure the surface dynamics, such as changes of mass and viscoelasticity during the SLBs formation.

3.2 Materials

3.2.1 Lipid Reagents

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG), cholesterol (ovine) (Chol), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (Ammonium salt) (Rh-PE) were purchased as diluted in chloroform from Avanti Polar Lipids (Alabaster, AL, USA). All lipid samples were kept at -20 °C freezer.

For chapter 4, to test originally designed microfluidic chamber for AFM device, the different ratio of cholesterol-enrich DOPC lipid was prepared using SALB technique to investigate morphological and mechanical changes due to cholesterol concentrations.

For chapter 5, to characterize the multi-phase (liquid and gel phase) SLBs, the mixed lipid bilayer with DOPC (transition temperature = -17 °C) and DPPC (transition temperature = 41 °C) were adapted which have different phase at the room temperature. The mechanical properties of the multi-phase bilayer are highlighted due to the change in the ratio of mixed lipids.

For chapter 6, mixed bilayer with POPE and POPG lipids were employed that is the mimetic system of the bacterial membrane. The unusual topology changes are observed with AFM and FRAP due to change of temperature and time-lapsed. All bilayer systems that need to be measured by fluorescence microscopy are mixed with rhodamine-PE as a fluorescence dye. All chemical structures of used materials are shown in **Figure 3.1**.

Figure 3.1. Chemical structures of used lipid-related materials

3.2.2 Buffer Reagents

For all aqueous solutions that are used in all performed experiments, deionized water was obtained from 18.2 M Ω ·cm MilliQ water (MilliPore, Oregon, USA). For all bilayer formations, including SALB method and vesicle fusion method using SUVs, Tris buffer (10 mM Tris, 150 mM of NaCl and pH 7.5) was utilized. Tris and NaCl were both obtained from Sigma-Aldrich.

3.3 Solvent-Assisted Lipid Bilayer (SALB) with the microfluidic chamber for AFM

For fabrication of the model membrane platform, the SALB technique was employed for the formation of uniform bilayer on the substrate. As explained in detail in the previous chapter, for this method, the vesicle preparation is not necessary so that the lipid powder was diluted to isopropyl alcohol directly to a concentration of (0.5 mg/ml). 15 μ L (wt ~ 0.5 %, dried by N₂ gas, 3 hours in a vacuum) rhodamine is mixed with 4 ml of 0.5 mg/mL lipid powder in IPA. The cover glass (shape depends on the microfluidic

chamber type, 0.15 – 0.17 mm of thickness) was washed with DI water (2 times) followed by 95% Ethanol (2 times) followed by a final wash with DI water (3 times) and dried completely under the nitrogen gas. To make glass surface hydrophilic, the glass was ozone plasma treated (Plasma Cleaner PDC-FMG, Harrick Plasma) for about 1-2 min. The inlet and outlet tubes were also washed with DI water followed by 95% ethanol, followed by DI water and dried thoroughly under a nitrogen stream. The tubes were connected to the inlet and outlet of the sample chamber. Flow the IPA at 100 ~ 200 $\mu\text{L}/\text{min}$ until the chamber is filled. Next, lipids containing IPA is placed at the inlet and is flowed through the channel at the rate of 100 $\mu\text{L}/\text{min}$ for 10~15 min. Next, the buffer (150 mM NaCl, 10 mM Tris, PH 7.5) is placed at the inlet and is flowed through the channel at the rate of 40~50 $\mu\text{L}/\text{min}$ for 30~40 min. This makes sure that the bilayer is now immersed in buffer. To remove the floating particles, 50 mL of the buffer has flowed through the channel at the rate of 300~500 $\mu\text{L}/\text{min}$.

Figure 3.2. (a) AFM bilayer image is formed with SALB method. The line profile (b) shows that the bilayer is uniform in an entire area of the image. The histogram (c) is calculated from roughness value of 256 lines.

Here, the bilayers (100% DOPC lipid) formed using the SALB technique using the originally designed AFM sample chamber was characterized. First, the surface morphology of DOPC bilayers was investigated (**Figure 3.2**). The surface roughness was calculated from the line profile at various places (select places are shown in red) using the inbuilt software. (XEI, Parksystems, Suwon, Korea) From the graph, the roughness of the bilayer formed using SALB method has a stable statistical distribution indicating that the morphological properties are quite uniform in the entire area of the bilayer. In this case, the surface roughness values are $0.35\sim 0.4\text{ }\mu\text{m}$. To characterize the bilayer formation, the square test by AFM (**Chapter 2-5**) has employed. From the result as **Figure 3.3**, the bilayer depth has measured an average of 5.8 nm , which shows the typical thickness of the bilayer.

Figure 3.3. Bilayer depth measured by square test. (a): AFM image after scratching, (b): The line profiles of square, (C): Histogram of each line profiles

3.4 Experimental / Characterization Techniques

3.4.1 Atomic Force Microscopy

As discussed in Chapter 2, AFM offers promising imaging capabilities at the nanometer range that is well suited for investigating the dynamics and behavior of biological samples such as live cells, DNA, and soft materials such as lipid bilayers and proteins.[1-5] The non-contact AFM mode is extremely powerful as it enables super high-resolution images of surfaces detailing surface morphologies in the nanometer range without destructing the sample. This level of resolution is attained through monitoring minute amplitude modulations that occur at the tip as a result of being in the vicinity of the sample [1]. Although AFM has been used to investigate biological samples for almost thirty years, the investigation of the properties of lipid bilayer has been challenging. Most of the techniques to obtain a bilayer are extremely time-consuming, use high volumes of sample, and most importantly, the bilayer formed is not always uniform. The application of AFM for membrane studies is further challenged because of the lack of a convenient platform for exchanging solvents while performing experiments. Exchange of solvent with a bilayer could open up various avenues to explore the effect of other biochemical molecules on lipid bilayer [6, 7]. A new sample chamber that has less sample volume is modular, can exchange any solvent conveniently and can form uniform bilayer consistently and reliably is highly desirable. The development of a new sample chamber addressing these challenges is the primary focus during the first objective as a Ph.D. project. This chapter presents the details of the motivation, fundamental principles, and the design considerations for the development of an AFM sample chamber for bilayer studies.

System Overview

All work is centered on the commercial AFM system (**Figure 3.4**, NX-Bio, Park Systems, Suwon, South Korea) combined with an Eclipse Ti optical microscope (Nikon, Tokyo, Japan). This setup has been used to for bilayer imaging in non-contact mode and to perform force spectroscopic studies. The AFM is located in the acoustic enclosure to

reduce the acoustic noise from the outside, and the experiment is performed at the room temperature (25 °C) in liquid condition with aqueous buffer solution (10 mM Tris, 150 mM NaCl, pH 7.5). The sample chamber assembled on an XY scanner. (100 μm $\times$ 100 μm scan range). The laser spot from the laser diode in AFM head fell on the backside of the cantilever and reflected the mirror. The reflected laser beam reaches the position-sensitive photodiode (PSPD) after passing through the optical system as a photon signal. The signal gets converted to the voltage the I-V converter in the AFM controller. The deflections in the cantilever as a result of the change in the position are detected via the changes in the reflected laser signal. In the contact mode imaging and force spectroscopy mode, the tip is in direct contact with the sample surface, the position of the laser spot can be calculated from the deflections resulting from the bending of the cantilever. In non-contact mode, the cantilever oscillates with its resonant frequency, the changes in the oscillation amplitude near from the sample surface are used for feedback signal. In this case, the amplitude sensitivity is considered as calibration constant. The calibration is performed with commercial software from AFM vender (XEP, Parksystem, Suwon, South Korea).

For non-contact imaging, an ultra-sharp silicon nitride Biolever mini cantilever (Olympus, Tokyo, Japan) has employed, which is the optimized for bio application. The spring constant of the cantilever is ~ 0.09 N/m, with ~ 110 kHz of the resonance frequency in the air (according to specification) is reduced to 30~50 kHz in liquid condition. For force spectroscopy, the gold-coated single crystal silicon cantilever NSG03 (NT-MDT, Moscow, Russia) has been used with 1.74 N/m of spring constant in order to penetrate the bilayer deposited on the glass substrate. The scan size is from 10 μm $\times$ 10 μm to 5 μm $\times$ 5 μm with 256 $\times$ 256, 16 $\times$ 16 pixel resolution with non-contact imaging and force spectroscopy mapping each. The details of the key parts of the AFM system used are described below. The AFM system consists of three primary components: (i) the AFM stage mounted on an optical microscope with both inverted and upright configurations capable of performing confocal (ii) fluorescence microscopy and (iii) the acoustic enclosure.

Acoustic enclosure

To observe the dynamics at the nanoscale region, isolation from the external noise coming from acoustic, vibrations and light is essential. To facilitate such an environment, we installed acoustic enclosure (Enhanced Acoustic Enclosures, Park Systems Corp, Suwon, Korea) that generates an environmentally sealed space for the SPM system. The dimensions of the structure is $1,000 \times 1,000 \times 1,400$ mm (outer) and it weighs 646 kg.

Figure 3.4. AFM experimental setup overview

Optical microscopy

The commercial inverted optical microscopy (Eclipse Ti microscope, Nikon, Tokyo, Japan) is combined with the AFM system. While the microscope can give useful information about the sample, particularly labeled samples, the AFM can extract nano-scale details of the same position. Since AFM is a label-free technique, the confirmation of the

presence of bilayer using fluorescent optical technique is crucial. The IOM consists of 4X, 10X, 20X, 40X, and 100X objective lenses and is equipped with laser (532 nm) for epifluorescence measurements.

AFM Cantilever

For lipid bilayer studies cantilever with a typical spring constant between (0.01 – 2 N/m) is used. For AFM experiment with the bilayer characterization, two important parameters of AFM cantilever are considered: one is the spring constant of the cantilever (k , N/m), the other is its resonant frequency (f , Hz). To investigate small forces on the soft biological material, such as protein interaction, very soft cantilever with low spring constant ($k < 0.1$ N/m) is required for contact mode imaging. Also, to minimize the thermal noise of cantilever due to Brownian motion, short (cantilever length < 100 μm) cantilever with low resonant frequency (bandwidth 0 – 60 kHz) is the most suitable. For considering these requirements, we used soft commercial AFM cantilever with a spring constant of 0.09 N/m for imaging and 1.74 N/m for force constant (**Figure 3.5**). A resonant frequency of cantilever for force spectroscopy is 90 kHz (NSG03, NT-MDT, Moscow, Russia). The maximum peak-to-peak noise level is 10 pN; therefore forces as small as 10 pN, which is at the smallest binding force for bio-molecules (20 – 240 pN), can be measured [4].

Figure 3.5. Schematic and specification of AFM cantilever (NSC03)

Piezo Stage

The Z scanner ($15\ \mu\text{m}$ scan range) is mounted on the AFM head allows the tip to maintain constant feedback conditions (deflection or amplitude) when raster scanning over the sample surface. The Z scanner range is up to $15\ \mu\text{m}$, and its resolution is around $0.2\ \text{nm}$. The voltage applied to the control unit, and the Z scanner determines the minimum accessible Z-direction pixel resolution. The noise level in Z-axis is acquired using zero-scan condition where an image is acquired without defining the scan size. The value obtained during zero scan can be used as an indicator of the resolution achievable in the vertical direction in experiments. The X-Y scanner is a Flexure Hinge (leaf-spring type) guided system, which is fabricated with a solid aluminum block. It has a $100 \times 100\ \mu\text{m}$ scan range with a resolution of $1.5\ \text{nm}$ with closed-loop and $0.3\ \text{nm}$ at open-loop in the X-Y direction.

Cantilever Selection and Calibration

The accurate cantilever calibration is significantly required for analysis of force spectroscopy measurement. Several techniques such as Sader method have been reported for calibration of cantilever's spring constant, the thermal noise analysis is most well-known for its convenience [8]. The main measuring parameter of this method is free fluctuation of the cantilever, which comes from the thermal energy from its environment, such as room temperature [9]. Usually, the amplitude of this fluctuation is very small; the cantilever can be considered as harmonic oscillator so that it can be written as below.

$$\frac{1}{2}k_c \langle q^2 \rangle = \frac{1}{2}k_B T, \quad k_c = \frac{k_B T}{\langle q^2 \rangle}, \quad (\text{Eq. 3.1})$$

Where k_c is the force constant of the cantilever, k_B is the Boltzmann constant, $\langle q^2 \rangle$ is the root mean square value of the amplitude, and T is the temperature. In this equation, the determination of $\langle q^2 \rangle$ is the main factor for cantilever's spring constant calibration. Although the cantilever has small amplitude, it is not the actual harmonic oscillator; the correction factor should be required. In here, if the shape of the cantilever is rectangle and assume that the length is longer than width, the mean square of the amplitude at the edge of the cantilever due to thermal energy can be calculated as below.

$$k_c = 0.817 \frac{k_B T}{\langle q^2 \rangle} \left[\frac{1 - \left(\frac{3D}{2L} \right) \tan \phi}{1 - \left(\frac{2D}{L} \tan \phi \right)} \cos \phi \right]^2 \quad (\text{Eq. 3.2})$$

Where D is the height, and L is the length of the cantilever. With this correlation factor, the range of errors (uncertainty) is usually 10-20%, which is a generally acceptable level for spring constant determination with thermal noise method.

Figure 3.6. Frequency sweep curve of AFM cantilever

AFM Imaging mode

In contact mode, the cantilever tip is in direct contact with the sample. The operation of imaging is conducted by the feedback of the repulsive force signal determined using the laser position recorded on a photon detector. The repulsive force is usually very small (less than $\sim$ nN order). In order to prevent the sample damage the cantilever with small spring constant is used (less than 1 N/m).

In the current AFM setup, the repulsive force is of the order of piconewtons. Consequently, the spring constant of the AFM cantilever is also appropriately small (contact mode cantilever, less than 0.1 N/m), thus allowing the AFM cantilever to be very sensitive to the changes in the sample position. The cantilever deflects upwards or downwards depending on the sample surface structure. The feedback loop uses the change in the cantilever deflection signal to image the topography of the sample surface. In order to obtain the sample topography, the piezo scanner with z-axis should keep the constant distance between the AFM tip and the sample by using the cantilever deflections signal as feedback. If the cantilever reaches a lower area, the z-scanner will move the cantilever

down by that distance, or back up if the cantilever begins rising. (**Figure 3.7 (a)**)

In non-contact mode (**Figure 3.7 (b)**), oscillating cantilever with its near from resonance frequency, using attractive force region. Basically, AFM tip moves very near to the sample surface, the destruction of the sample has hardly occurred. When the cantilever approaches to very near from sample surface, then the oscillating amplitude has changed in nano-scale level. Non-contact mode using the changes of amplitude as a feedback signal in contrast to contact mode AFM uses the “physical contact” between the tip and the sample surface that can damage the sample surface. However, when the sample height has a dramatically changed, such as the micro-level, the tip can be touch to the sample as well.

Figure 3.7. Imaging mode of AFM (Contact (a) and Non-contact (b) mode)

Force Spectroscopy

The AFM is widely used as a high-resolution imaging tool for the sample morphology; however, it is also a powerful tool that is widely employed to measure the mechanical properties such as stiffness, adhesion force and Young's modulus of the sample from the force-distance (f-d) curve [9, 10]. A force-distance curve contains the interaction between tip and sample surface by obtaining the force curve that responds to the distance of the tip from the surface. The tip moves directly towards the sample until it just touches the sample and then retracted again for measuring the interaction between the tip and sample. The first step of f-d curve measurement is the "approach (trace)" (**Figure 3.8.1**) process. The cantilever is positioned far from (several millimeters, depends on Z stage stroke) the sample surface where there is no interaction influencing the tip so no deflection changing that is used for trigger signal for feedback of the AFM. Next, the approach curve is obtained by recording the movement of the cantilever that is moving towards the sample surface and establishes a "contact" (**Figure 3.8.2**) condition. After approaching to the sample surface, the retract curve is obtained during the pulling up the Z scanner. In the retract curve, the adhesion force can be measured due to the interaction between the tip and the sample (**Figure 3.8.3**). The final step of the f-d curve is pull-off (separation) process which the distance between tip and sample is so far that it reaches to the critical point, there is no interaction between sample and tip anymore (**Figure 3.8.4**). Using the f-d curve, the various type of bonding force can be derived such as hydrogen bond and covalent bond from the retrace (separation) curve, and from the trace (approach) curve, the penetration force can be measured as explained in detail in the previous chapter. (**Chapter 2**) [11].

Figure 3.8. Typical diagram of force-distance curve. 1. Approach (trace), 2. Contact, 3. Adhesion, 4. Pull off (retrace).

3.4.2 Fluorescence Recovery After Photobleaching (FRAP)

To confirm the successful formation of the lipid bilayer that has mobility in a fluidic condition on the substrate, the fluorescence recovery after photobleaching (FRAP) measurement can be employed in order to derive the lateral diffusivity of the SLBs. Briefly, the SLBs are illuminated by high intensity of the laser light (in general, higher than 50-70 mW of laser intensity) then a small circular spot (around 20~30 μm of diameter) on the dyed-bilayer is photobleached by the excitation light. As time lapsed, the bleached area diffuses out to the non-bleached area, and non-bleached fluorophores diffuse into the bleached area as well. Hence, the overall fluorescence intensity of the bleached area is recovered back gradually to its initial intensity up to around 95%. Before performing the measurement, Rh-DPPE (0.5 % molar rate) was mixed to the lipids to track fluorescently dyed lipid molecules under an epi-fluorescence microscope. Subsequently, diluted lipid solution (IPA) which

was described in **Chapter 3.3** to form the SLBs on a treated as hydrophilic glass coverslip, placed in an originally designed microfluidic liquid cell chamber, a volume of 400 μL , fitted onto the AFM XY stage. The microfluidic liquid cell chamber mounted on the Eclipse TI-U microscope, and a 60x magnification (NA 1.49) oil immersion objective was selected for observation of the surface area from the bottom, in a camera pixel size of $0.267 \times 0.267 \mu\text{m}$. The measurements is conducted at the room temperature in general except in a special case. A TRITC filter was chosen to filter out the emission light and the excitation light from the rhodamine-PE dye during the measurements. Diode-pumped solid state lasers (Scherzo-532; Klastech GmbH, Germany) was adopted to bleach the fluorophores at the wavelength of 532 nm to bleach the dyed-SLBs. A fiber-coupled mercury lamp (Intensilight C-HGFIE; Nikon) was employed to monitor the recovery of the fluorescence intensity. Three pre-bleaching images were obtained first to compensate for non-uniform illumination. Then, the center of bilayer from the camera vision was bleached with the laser for five sec of the exposure time and following fluorescence recovery images will be collected for two minutes at either two sec or three sec intervals. At least three data set from the different bleached areas were collected to improve the statistics. The obtained images were analyzed using the Hankel transform method that is based on to calculate the diffusivity from the circular diffusion area and averaging of each recovered images resulting a reduced spatial noise from the data analysis [12]. By comparing the conventional Axelrod method with Hankel transform method, the Hankel Transform method can improve the accuracy of the diffusivity by compensating for temporal variation and drifts from illumination and natural bleaching effect during the imaging process.[12, 13] Using the Hankel transform method and stacked acquired image data in two minutes; diffusion coefficient values can be derived from the fluorescence intensity recovery plots. Following schematic diagram is briefly describing the basic principle of the FRAP measurements.

Figure 3.9. A brief schematic of FRAP experiment measured on dyed-lipid bilayers. (A) Experimental procedures of the photobleaching are presented. (B) Simplified fluorescence intensity recovery plot with corresponding FRAP micrographs.

3.4.3 Quartz Crystal Microbalance with Dissipation Measurement

Adsorption kinetics of the bilayers on the substrate via SALB were observed and analyzed using the quartz crystal microbalance with dissipation (QCM-D) monitoring on a Q-Sense E4 (Q-Sense AB, Gothenburg, Sweden). QCM-D is a powerful tool to measure the resonance frequency and energy dissipation on the surface simultaneously. [14, 15]. In principle, giving the alternating voltage potential to the quartz crystal located between two metal electrodes, the crystal is driven with a periodic oscillation with a certain frequency as the set voltage potential. The oscillation of quartz crystal at the resonance frequency (5 MHz) which is directly related to the surface thickness, any changes of mass on the surface

induces the changes of the resonance frequency. The relationship between absorbed mass and the resonance frequency is as followed from Sauerbrey equation[16, 17]:

$$\Delta m = \frac{C}{n} \times \Delta f \quad (\text{Eq. 3.3})$$

Where Δm is the adsorbed mass, Δf is the changes of the resonance frequency, n is the harmonic number, and C is the constant of the sensitivity value ($-17.7 \text{ ng/Hz} \cdot \text{cm}^2$) that is derived from the following equation:

$$C = \frac{t_q \times \rho_q}{f_o} \quad (\text{Eq. 3.4})$$

Where t_q is the thickness of the quartz crystal, and ρ_q is the density of quartz. QCM-D technique not only measures absorbed mass on the crystal, but it also can measure changes of viscoelastic properties of the surface layer by recording the damping in the free oscillations after the driving potential voltage to the crystal as shown in the **Figure 3.10**.

As for the measurement, silicon oxide coated QCM crystal chips (AT-cut quartz crystals) (Q-sense) was used for QCM-D measurements for SLBs formation as presented in the following schematic figure describing a brief working principle.

Before each measurement, the crystals were treated with oxygen plasma using a Plasma Cleaner (Harrick Plasma, Ithaca, N.Y., U.S.A.) at 29.6 W for 5 minutes. All measurements were recorded at the third overtone (15 MHz). A peristaltic pump was used to introduce the sample solution into the measurement chamber, with the flow rate fixed at 50 $\mu\text{L}/\text{min}$ for all measurements.

Figure 3.10. Schematic representation of QCM-D measurement. (A) A diagram of quartz crystal (B) Oscillating crystal driven by the voltage is shown

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Chapter 4*

Microfluidic device for solvent-assisted lipid bilayer for Atomic Force Microscopy measurement

In this chapter, the universal microfluidic liquid chamber device platform will be presented for atomic force microscopy (AFM), which enables to fabricate the uniform lipid bilayer on the hydrophilic surface using solvent-assisted lipid bilayer (SALB) formation method. Using this device enables to acquire the various properties of delicate soft matter, including morphological data, mechanical property measurements, using high-resolution AFM systems. The proposed technology is expected to provide an understanding of complicated biological materials.

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4.1 Introduction

Since the development of scanning probe microscopy (SPM) in 1981, the atomic force microscopy (AFM), the offspring of SPM techniques has been a successful tool to characterize morphological and mechanical properties of biological specimens in the liquid environment at molecular-level [1-5]. Further, exploring the nano-scale surface structures of biological materials [5, 6] is an important scientific and practical goal because many of significant biological processes are controlled at the nano-micro scales level.[5, 7-12] Although the AFM is the promising tool to explore the small scale of the biological samples, there are many drawbacks associated with conventional AFM liquid chamber system for biological applications; i.e., i) Lack of the stable liquid sealing chamber, ii) It does not support solvent exchange once it seals, iii) Evaporation controlling problem, iv) Uncontrollability of the flow speed, and v) Recyclability of samples in the chamber etc.

In fact, the AFM has been used as a tool for imaging the biological samples including supported lipid bilayers (SLBs) that is used as a representative and robust model of the biological cell membrane and comprises a two-lipid-leaflet structure.[10, 12-14] However, attempt to perform the measurement of SLBs in liquid condition was a challenge due to lack of the appropriate liquid chamber system. After all, a large volume of the conventional AFM chamber frequently causes time wasting for vesicle preparation and expensive consumable in addition to the formation of obstacle air bubbles which immediately hinder the AFM measurement.

The solvent-assisted lipid bilayer (SALB) formation method, [15, 16] which is the key technique for the simple one-step preparation of uniform SLBs at various interfaces without vesicle preparation, is not available in the conventional AFM chamber because there is no solvent exchange system in the chamber. In addition, the conventional chamber does not allow in situ multitask operations; flowing the solution with certain the flow rate, exchange the solvent using the peristaltic pump with tubes, availabilities of opening and closing of the top cover multi times for the AFM measurement in each experimental condition. Furthermore, the ordinary chamber cannot apply precision flow control by a

pump with tubes enables to measure the dynamic processes of functional biological molecules.

Meanwhile, liquid evaporation from the conventional chamber directly affects the AFM measurements. Indeed, liquid evaporation causes terrible signal drifts with drastic movements of the AFM cantilever. The pH changing and salting-out are also occurred by liquid evaporation, resulting in serious damages on sensitive biological samples such as cells and proteins. Much worse, the solvent cannot be easily compensated anymore into a chamber after or during liquid evaporation because adding solvent substantially accompanied with physical actions of pouring solutions and/or pipetting, even if these processes are gently manners, apparently disturb the biophysical signal.

Especially, controlling the flow speed takes a significant role in the formation of the uniform SLBs [12, 15]. The previous lipid bilayer formation for AFM imaging have shown the shortcomings with the conventional method, using the open-ended petri dish or liquid cell chamber with large volume. Unfortunately, due to the lack of robust fluidic chamber, the results without controlling the flow rate are often creating defects in SLBs that causes the complexity of the data analyses due to its signal instability, make it difficult to extract useful information for planar bilayer [16].

Further, conventional liquid cells are typically one time uses for filling the channel area with the solvent so that it was not possible to perform the multi-times solvent exchanges. Moreover, there is often a limitation for designing microfluidic chamber system with the conventional AFM because the reducing volume of the device hinders the approach of the AFM tip to the sample surface for imaging (**Figure. 4.1(a) and (b)**). For example, the channel dimension of the conventional microfluidic chamber for fluorescence microscope is $6\text{ mm} \times 45\text{ mm}$ with 0.1 mm height (**Figure. 4.1(c)**). However, it cannot be opened after assembling the coverslip as resulting in its disturbing the AFM tip approach to the surface. In addition, expanding the volume of the channel area of a conventional microfluidic chamber causes the generating of the air bubble that interferes the high resolution between AFM tip and soft matters.

In order to overcome these disadvantages of conventional AFM system, the microfluidic-based liquid cell chamber is originally developed that allows the constant flow in the reservoir area without the interference of AFM measurement. The designed chamber enabled to investigate the layer of molecules because of its perfectly sealing structure and reusable for multiple solvent exchange processes without solvent leakage to facilitate robust and easy of the experiment. The commercial liquid chamber for AFM does not ensure this environment it has served as a device for the only liquid environment. As a result, the vesicle fusion method, which uses the spontaneous rupture processes of vesicles for bilayer the formation should be used without solvent exchange.[17, 18] However, the vesicle fusion method requires the preparation of the vesicle sample, and it does not always form the uniform bilayer on a substrate, especially containing the different components of lipid [19]. In this chapter, alternative microfluidic chamber device will be introduced for model membrane application with the AFM system based SALB technique. For the successful formation of the uniform bilayer using SALB, these studies provide the fluidic simulation of the inner channel area of the chamber from an organic solvent to an aqueous solution at a certain flow rate that satisfies the various parameters for bilayer formation under a sealing environment. For the confirmation of the uniform bilayer formation, the AFM imaging of the SLBs on the bare glass substrate with containing the different concentration of cholesterol (20 and 40%) was performed. In addition, to compare the mechanical properties in each condition, the force-distance (f-d) curves and diffusivity of the lipid bilayer derived from the fluorescence recovery after photobleaching (FRAP) technique [20-22] are demonstrated with the proposed fluidic chamber.

4.2 Device Design and Experiment Method

4.2.1 Fluidic simulation and chamber design

Here, the outline of design requirements that lead to the development of the new microfluidic chamber for AFM studies on SLB will be introduced. The primary motivation is the formation of SLBs using SALB technique that has several advantages over the conventional vesicle fusion method [19, 22, 23]. The concept for building a microfluidic

chamber is that can be modular, uses less solvent volume, forms a uniform bilayer with repeatability, and that can be used in combination with the AFM system. The SALB can be formed in the commercially available microfluidic chamber for fluorescence microscope stage (**Figure. 4.1(c)**) or using the liquid cell of the quartz crystal microbalance with dissipation (QCM-D) [19, 24]. The principle of operation of both devices are similar; the chamber consists of an inlet, an outlet and a channel where the bilayer deposits as the solvent are replaced to an aqueous solution. Successful formation of a uniform bilayer via SALB depends on various parameters such as the flow rate, type of lipid and substrate, and the diffusivity of the lipid. Here, the simulation results to calculate the dimensions of an AFM based microfluidic chamber for forming uniform bilayers is explained.

For the preliminary experiments, the microfluidic chamber device for AFM can satisfy the perfect sealing condition, and it was manufactured as shown in **Figure 4.1(d)–(g)**. The top-cover of the chamber is tightly combined to the chamber part with bolts to prevent the solvent leakage. Two O-rings made of rubber with different size (20 mm and 25 mm) absorb the pressure to the coverslip from the bottom cover. That act as a sealant for the structure of the newly designed chambers where the actual solvent is placed contains inlet and outlet for the insertion of tubes. The overall dimensions of the chamber itself are 35 mm of diameter and 6 mm of the thickness, that can be mounted on an XY stage of the AFM system. The design can be easily tweaked to fit specific sample stage for other AFM models. The entire part of the chamber is consisting of six parts (**Figure. 4.1(d)**) and made from stainless except top-cover. The top cover is made from transparent acrylic material due to observing the solvent flow during the SALB process.

The optimal hydromechanics of the flow conditions needed to facilitate the successful deposition of a bilayer in a microfluidic channel was characterized using theoretical simulations (**Figure. 4.2**). In a previous study, the solvent exchange process was found to be acquired by diffusion and based on the time-dependent lipid concentration in the fluidic chamber [25, 26]. The maximum solvent exchange flow rate, Q_{max} , that still guarantees a complete formation of the bilayer was estimated as follows:

$$Q_{max} = \left(\frac{(1-\Phi_c)c_0V}{\rho_{LB}} \right)^{3/2} \frac{D_L}{LH} \quad (\text{Eq. 4.1})$$

where $\Phi_c = 0.5$ is the water fraction above which lipid micelles form in the water-alcohol-lipid mixture, [21, 24] $c_0 = 0.5 \text{ } \mu\text{g}/\text{mm}^3$ is the lipid concentration in the mixture, $V = L^2H = 0.4 \text{ mm}^3$ is the chamber volume, $L = 9 \text{ mm}$ is the chamber length (equal to the chamber width), $H = 0.5 \text{ mm}$ is the chamber height, and $D_L = 2 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ is the lipid diffusivity. D_L was estimated by fitting the diffusion-limited SALB formation model to experimental data [22]. Using these parameters, the Q_{max} value for the present system was calculated as $5.2 \text{ mm}^3 \text{ s}^{-1}$, which was well above the Q value of $0.83 \text{ mm}^3 \text{ s}^{-1}$ in the proposed SALB formation process; therefore, full SALB formation is expected. In deriving Eq. (4.1) it was assumed that the solvent exchange in the chamber is homogeneous [26]. To verify this assumption, a solvent-exchange simulation, similar to that in, [25] has been conducted, by numerically integrating the advection–diffusion equation, which describes the spatiotemporal water fraction, ϕ , in the chamber:

$$\partial_t \phi + \nabla \cdot (\mathbf{u}\phi) = D_s \Delta \phi \quad (\text{Eq. 4.2})$$

where $D_s = 5 \times 10^{-10} \text{ m}^2 \text{ s}^{-1}$ is the diffusivity of the solvent (a water-alcohol mixture)[26] and $\mathbf{u}$ is the fluid velocity in the rectangular channel and is defined as follows [27]:

$$u_x(y, z) = \frac{3U}{2} \sum_{m=1,3,5,\dots} \frac{(-1)^{\frac{m-1}{2}}}{m^3} \left(1 - \frac{\cosh\left[\frac{m\pi z}{H}\right]}{\cosh\left[\frac{m\pi W}{H}\right]} \right) \cos \left[\left(\frac{m\pi}{2} \right) \left(1 - \frac{2y}{H} \right) \right] \quad (\text{Eq. 4.3})$$

The velocity field described Eq. (4.3) is sketched in **Figure 4.2(d)**. In Eq. (4.3), $U = Q/(LH)$ is the mean flow velocity and x , y , and z are the streamwise distance to the channel inlet, the wall-normal distance to the channel centerline, and the spanwise distance to the channel centerline, respectively, as shown in **Figure 4.2(a)**. Eq. (4.2) is numerically solved using a finite-volume method, employing the first-order upwind scheme for the advection term and the second-order central finite difference scheme for the diffusion term.

Due to the symmetry of the geometry, it suffices to simulate only one quarter of the domain: $0 < x < L$, $0 < y < H/2$, and $0 < z < W/2$. The computational domain was discretized, into 100, 8, and 47 grid points in the x -, y -, and z -directions, respectively, corresponding to streamwise and wall-normal grid spacings of 100 μm and 25 μm , respectively. To improve the computational efficiency, the wall-normal grid spacing was increased by a factor of 1.05 for each grid cell away from the wall. The solvent exchange was simulated over a period of 1125 s using a time step of 12.5 ms, so there was a total of 9×10^4 computational time steps. Zero-flux boundary conditions were set at the channel walls and at the channel outlet. The channel was connected to the solvent reservoir by a tube with a length of 100 mm and a diameter of 1.6 mm. Hence, the water fraction boundary condition at the channel inlet is taken from the outlet conditions of a simulation, which models the solvent exchange in the tube, based on the polar coordinate version of Eq. (4.2).

Figure 4.2(b) shows the water fraction as a function of time at two fixed locations halfway down the channel: the center of the bottom wall and the side of the bottom wall, halfway in the channel. Since the water has to travel through the tube before reaching the channel, the solvent exchange starts after approximately 150 s. It was found that the solvent exchange was homogeneous in the x - and y - directions (not shown) while there is some inhomogeneity in the z -direction, as shown in **Figure 4.2(c)**. [24] Specifically, the solvent was fully exchanged after approximately 7 min in the center of the channel (**Figure. 4.2(b)**), but the solvent exchange was roughly twice as slow near the side walls. This inhomogeneity is not expected to impact the bilayer formation negatively; instead, a somewhat slower solvent exchange process provides more time for lipid deposition, [22] resulting in the formation of a full bilayer, that covers the entire channel walls after about 10 mins (**Figure. 4.2(b)**).

Figure 4.1. (a) Schematic of the bilayer structure comprising two lipid leaflets with the polar head groups facing outwards and the hydrocarbon chains facing inwards; the AFM tip is able to approach from the top to take measurements from the bilayer. (b) The conventional method for AFM using coverslip inside of the petri dish. (c) Disposable micro-fluidic chamber for fluorescence microscope. (d) Three-dimensional drawing of each part of the new designed fluidic chamber for AFM. (e) Top and (f) side views of the assembled chamber, (g) The designed fluidic chamber for AFM.

Figure 4.2. Schematic representation of the fluidic chamber and solvent displacement simulation. (a) The microfluidic channel has a length of $L = 9$ mm (x -direction), a width of $W = 9$ mm (z -direction) and a height of $H = 0.5$ mm (y -direction). The channel inlet and outlet correspond to $x = 0$ and $x = L$, the bottom and top walls correspond to $y = -H/2$ and $y = H/2$ and the channel side walls correspond to $z = -W/2$ and $z = W/2$. (b) The simulated solvent exchange from isopropanol ($\phi = 0$) to aqueous solution ($\phi = 1$) as a function of time t in the center of the bottom channel wall ($x = L/2$, $y = -H/2$, $z = 0$) and on the side of the bottom wall ($x = L/2$, $y = -H/2$, $z = W/2$). (c) Simulated water fraction ϕ as a function of the span-wise coordinate z and time t in the center of the bottom wall ($x = L/2$, $y = -H/2$). (d) The velocity distribution in the channel cross-section.

4.2.2 Bilayer Formation with Solvent Assisted Lipid Bilayer (SALB)

Bilayer formation was conducted using the solvent-assisted bilayer (SALB) formation method [19, 22, 24]. Briefly, 2 mg of 1,2-dioleoyl-sn-glycerol-3-phosphatidylcholine (DOPC) (Avanti Polar Lipids, Alabaster, AL, USA) powder was diluted in 4 ml of isopropanol to make a concentration of 0.5 mg mL^{-1} . Then, a round 25 mm-diameter coverslip with a thickness of 0.15–0.17 mm (Neuvitro, Vancouver, WA, USA) was treated with O_3 plasma (Plasma Cleaner PDC-FMG, Harrick Plasma, NY, USA) for about 1–2 min to make the surface hydrophilic immediately before use. The coverslip was then placed at the bottom of the chamber with the treated surface facing the inside of the chamber. After assembling the coverslip and connecting the tubes to the inlet and outlet of the sample chamber, pure isopropanol was flowed at $100\text{--}200 \text{ }\mu\text{L min}^{-1}$ until the chamber was filled. Next, the lipid-containing isopropanol was placed in the inlet and flowed through the channel at a rate of $100 \text{ }\mu\text{L min}^{-1}$ for 10–15 min. Finally, the Tris buffer (150 mM NaCl, 10 mM Tris, pH 7.5) was placed in the inlet and flowed through the channel at a rate of $40\text{--}50 \text{ }\mu\text{L min}^{-1}$ for 30–40 min (**Figure 4.3(b)** and **(c)**). This process ensured that the lipids aligned into a bilayer on the hydrophilic surface in the buffer. Finally, 20–30 mL of buffer was flowed through the channel at a rate of $300\text{--}500 \text{ }\mu\text{L min}^{-1}$ to remove the lipid suspended in the solution. To keeping the constant flow speed due to fill the reservoir with the solvent without generating an air bubble, a peristaltic pump (ISMATEC REGLO Digital ISM 833, Germany) was used.

4.2.3 Atomic Force Microscopy (AFM)

A commercial AFM system (NX-Bio; Park Systems, Suwon, South Korea) combined to an optical microscope (Eclipse Ti; Nikon, Tokyo, Japan) was used to investigate the morphological and mechanical properties of the materials in this experiment. An NSG03 cantilever (NT-MDT, Moscow, Russia) with a spring constant of about 1.7 N/m was used; its resonance frequency was about 90 kHz in the air but was reduced to 30–50 kHz in liquid. The experiments were performed at room temperature (25°C) in an aqueous Tris buffer solution (10 mM Tris, 150 mM NaCl, pH 7.5). Images were obtained at a

0.2–1.0 Hz scan rate to minimize the damage to the sample and the noise in the signal. To compare the differences of mechanical property in each case of experiment, (bare glass surface, DOPC bilayer, DOPC bilayer containing 20% of cholesterol, DOPC bilayer containing 40% of bilayer) the force-distance curves obtained after imaging. Furthermore, to confirm the thickness of the bilayer, a square test was performed: the height difference between the bilayer and an area from which the bilayer was scratched off was approximately 5nm.

4.2.4 Fluorescence Recovery After Photobleaching (FRAP)

Fluorescence recovery after photobleaching (FRAP) technique is employed in order to derive the diffusivity of the lipid bilayer which has formed on the hydrophilic surface. For obtaining fluorescence images, an inverted epifluorescence Eclipse TE 2000 microscope (Nikon, Tokyo, Japan) equipped with a 60× oil immersion objective (NA 1.49) and an iXon EMCCD camera (Andor Technology, Belfast, Northern Ireland) is used. The samples were measured by a Tetramethylrhodamine (TRITC) filter set with a mercury lamp (Intensilight C-HGFIE; Nikon Corporation). The size of the bleaching spot is around 20 μm diameter, and the bleaching process was performed with a 532 nm, 100 mW power of laser beam. The image traces of the recovery process were collected from minus 5 to 150 sec at two seconds intervals to monitor the fluorescence intensity signal. FRAP analysis was performed by Matlab software (MathWorks, Natick, MA, USA) in order to acquire the diffusion coefficients which are determined based on the Hankel transform method [28, 29].

Figure 4.3. The solvent exchange process of the designed microfluidic chamber device. (a) Combining the chamber device to AFM XY scanner, (b) Flow the buffer for the initial condition, (c) Exchange the solvent to the isopropanol with containing the lipids. The flow should be stopped during this process. (d) Open the top cover of the chamber for AFM imaging. (e) The approach of the AFM tip due to obtain the images. The cantilever should be placed the center of the reservoir area in order to contact to the glass surface. (f) Retract process of the AFM stage for solvent exchange, (g) Reassemble the top cover of the chamber, (h)-(j) Flow the solvent with certain flow rate again. After the exchange of the solvent, same as previous process, carefully reopen the top cover of the chamber and re-approach the AFM tip.

4.3 Experiment Results

The morphological and mechanical properties of the bilayer were recorded using the new designed microfluidic chamber for AFM. The AFM images and force-distance curves in the bare glass substrate and the bilayer using SALB technique are shown in **Figure 4.4**. The bilayer formation was also confirmed by measuring the thickness of the square after scratching. (**Figure 4.6**). Additionally, the bilayer properties of mixed lipid systems have been investigated. To differentiate and test the bilayer-forming capability of the microfluidic chamber for AFM, DOPC-cholesterol systems were tested. Cholesterol is known to alter the membrane organization in many lipid systems, [7, 30] at high cholesterol concentrations, the lipid bilayer becomes in-homogenous and forms micron-sized domains. The study of cholesterol-induced domains has been of great interest because of their roles in biological processes, [21, 30, 31] particularly in re-organizing membranes and regulating membrane trafficking [31, 32]. Apart from the biological implications, the cholesterol-lipid systems are ideal for AFM studies as the size and width of the domains are significantly different from those in the fluid lipid bilayer. In other words, cholesterol-lipid systems can serve as a representative sample for the morphologic studies that are required to test the proposed AFM sample chamber. Two compositional studies were performed on bilayers with varying cholesterol concentrations. To test the performance of the AFM microfluidic chamber, the properties of the bilayers formed by SALB technique with 0%, 20% and 40% of the cholesterol in DOPC were compared. At this concentration, domains (rafts and detergent-resistant membranes) were clearly visible (**Figure 4.4(c) and (d)**). Indeed, domains are typically formed in the presence of sphingomyelin and cholesterol [9, 10]. From the results of the f-d curves are shown the different peak value in each case with 0%, 20% and 40% of cholesterol, demonstrating similar trend of morphological data and viscoelastic property changes due to cholesterol contents (**Figure 4.4 (f), (g) and (h)**). In the case of the bare glass substrate, the f-d curve does not show the peak on it (**Figure 4.4 (a)**) that means it does not have specific interaction between tip and surface different from the case of bilayers. In general, bilayer formation is not reliable or reproducible under high cholesterol concentration using a vesicle fusion method because the SUVs often do not rupture on the substrate [33]. In contrast, surprisingly, the SALB method resulted in the

formation of a uniform bilayer with domains under this high cholesterol concentration (**Figure 4.4(d)**). The roughness values of the bilayers formed with different concentrations of cholesterol by SALB complement each other (**Figure 4.7**); however, the higher cholesterol concentrations led to higher roughness due to the formation of domains. Furthermore, the calculation of the diffusivity of the lipid bilayer has been derived from microscope-based fluorescence recovery after photobleaching (FRAP) experiment which is one of representative techniques to test the bilayer mobility using the newly designed chamber. In **Figure 4.5 (a), (b)** are captured at $t = 0$ and 2 min after photobleaching respectively. The intensity of the photobleached spot has near-completely recovered suggests that the uniform fluorescence intensity area is the formation of the lipid bilayer with laterally fluid phase. These results clearly indicate that the AFM equipped with the proposed microfluidic chamber designed based on the SALB process can provide a facile tool to form uniform bilayers on substrates even in the presence of high concentrations of cholesterol.

4.4 Conclusion

In this chapter, the optimized microfluidic liquid cell chamber was designed based on the Solvent-Assisted Lipid Bilayer (SALB) process for AFM imaging. The bilayer AFM images were taken after formation by the SALB at the different concentrations of cholesterol. Bilayers could be formed successfully in both concentration with 20% and 40% cholesterol. Additionally, the mechanical properties of cholesterol-enrich bilayer have investigated using force-distance curves and FRAP. The proposed AFM-compatible microfluidic device can be used to conduct detailed morphological and mechanical studies and extract other parameters, such as diffusivity, viscosity, etc. of soft biological systems. This system can provide a complete platform to extract many useful parameters for handling the fragile soft matter and understanding complicated biological membrane systems.

Figure 4.4. Comparison of AFM images of bare glass substrate in liquid condition (a) and the bilayer with containing different concentration of cholesterol ((b) pure DOPC lipid bilayer, (c) 20% of cholesterol, (d) 40 % of cholesterol). (e) f-d curve on the bare glass substrate, (f) on the pure DOPC bilayer, (g) with 20 % of cholesterol, (h) 40 % of cholesterol. The bilayer formation has performed via SALB method. From the images, the domain area has increased due to cholesterol concentration is higher (0%, 20%, 40%). On the bare glass substrate, the f-d curve does not show peak otherwise in the case of bilayer. The peak value is also increased when the cholesterol

concentration is higher.

Figure 4.5. FRAP screenshots of bilayer. Fluorescence microscopy images ($136\ \mu\text{m} \times 136\ \mu\text{m}$) of DOPC lipid bilayer containing 0.5 wt % Rho-PE lipid formed on glass surface using the new designed microfluidic chamber. (a) the screenshot right after photobleaching, (b) after recovery and (c) the normalized intensity time lapsed profile corresponds to circle area in (a), (d) the intensity profile of the bleached spots before (red) and after recovery (black) are presented. The measurements were repeated three times and the diffusion coefficient of lipid bilayer has calculated as $2.53 \pm 0.14\ \mu\text{m}^2/\text{s}$

Figure 4.6. Bilayer depth measured by the square test: (a) a $2 \times 2 \mu\text{m}^2$ segment showed the area from which the bilayer was removed using the AFM tip and (b) the corresponding height profile representing the thickness of the bilayer.

Figure 4.7. Histograms of the roughness values measured in the bilayers formed by the SALB method with different cholesterol concentrations: (a) 0%, (b) 20%, and (c) 40%.

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Chapter 5*

Molecular diffusion and mechanical properties of mixed lipid bilayers of DOPC and DPPC fabricated by the solvent-assisted lipid self-assembly

Understanding the physical properties of cell membranes is important in the fields of fundamental and applied biology. While the characterization of simplified biological membrane mimics comprising of liquid-phase lipids has been routinely performed due to the ease of fabrication, the characterization of more realistic membrane mimics comprising of multi-phase lipids remains challenging due to more complicated fabrication requirements. Herein, solvent-assisted lipid bilayer (SALB) formation method has been employed to fabricate mixed lipid bilayers comprising of liquid phase 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and gel phase 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) lipids at room temperature. The fabrication procedure was performed with a newly designed microfluidic chamber, which facilitated the subsequent characterization of the SLBs without exposure to air. The SLBs were then characterized via fluorescence microscopy, fluorescence recovery after photobleaching (FRAP), quartz crystal microbalance with dissipation monitoring (QCM-D), atomic force microscopy (AFM) and AFM-based force-distance measurements.

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5.1 Introduction

Cell membranes serve multiple functions including monitoring local ion concentrations, sending and receiving transmitter molecules for intercellular communications, [1, 2] and coordinating cellular processes (*e.g.*, cellular adhesion, cellular division, membrane fusion) via molecular recognition process that occurs within the lipid bilayer [3-5]. To execute these functions, the lipid bilayer accommodates various biomolecules, including channel proteins, globular proteins, glycoproteins, glycolipids, and cholesterol.[6] The composition and distribution of lipid molecules are not uniform, and various lipids are organized into nano-sized phase-separated domains with different morphological, mechanical, and chemical properties, which provide optimized environments for each type of membrane-associated biomolecule to perform its specific role [7].

Biosensing devices have been recently developed to reproduce the sensing function of cells by mimicking the structure of cell membranes [6, 8-10]. To investigate the complex membrane model, it is necessary to fabricate supported lipid bilayers (SLBs) comprising of various lipid compositions, which may exist in different phases, including the gel phase [11]. Conventionally, the vesicle fusion method is commonly used to form SLBs. In the vesicle fusion method, precursor phospholipid vesicles adsorb onto a support substrate and spontaneously rupture to form the SLB [12-16]. However, when the vesicles contain the phospholipids in the gel phase, which has a comparatively high transition temperature (*i.e.*, above room temperature), the large bending rigidities of the gel-phase phospholipids hinder the rupture process when vesicle fusion is attempted at room temperature, preventing the formation of uniform SLBs [17, 18]. Several works have circumvented this problem by raising the temperature (*i.e.*, above the transition temperature of the gel phase lipid) during the adsorption and vesicle rupture stages of the process and cooling the system down to room temperature only after the SLB has been completely formed [19-21]. Although these steps are rather straightforward within the context of SLB fabrication *per se*, the temperature changes greatly complicate SLB characterization using surface-based

techniques that are sensitive to the bulk environment due to large thermal fluctuations that make it difficult to extract reliable kinetic information.

To overcome the aforementioned limitation, the solvent-assisted lipid bilayer (SALB) formation method has been developed to fabricate SLBs on a support substrate in a flow channel following a solvent exchange process from organic solvent (*e.g.*, isopropanol, methanol, ethanol) to aqueous solvent (*e.g.*, water and buffer) [17, 22-25]. In organic solvents, the lipid molecules exist as monomers or form reverse micelles. During the solvent exchange step, the concentration of the organic solvent in the flow channel gradually decreases, leading to the transition of lipid molecules to form normal micelles and vesicles [26, 27]. This ultimately leads to the formation of SLB on the support substrate, when the solvent in the flow channel has been fully exchanged to the aqueous solution [27]. Most notably, the SALB method can form SLBs with complex components and comprising of gel phase phospholipids at the room temperature without requiring vesicles [28, 29].

The establishment of the SALB method, therefore, spurs us to investigate more complex SLBs comprising of lipids in different phases. To achieve this, it is imperative to utilize a fluidic chamber that enables the solvent exchange step to be performed with the precise control over the solution flow rate and with a perfect sealing environment at the micron level. At the same time, the dimensions of the chamber should allow optical access of microscopes and enable an atomic force microscope (AFM) tips to approach the SLBs. Commercially available liquid chambers or open-ended petri dish systems do not satisfy all of the conditions above and introduces several drawbacks such as the ease of forming defects by means of generating air bubbles on the bilayer surface due to the lack of proper sealing [13, 19, 30]. To address this issue, microfluidic chamber has designed that can be integrated with a commercial optical microscope (Eclipse TE 2000, Nikon) as well as an AFM (MFP-3D Bio, Oxford Instruments, USA) system. Conceptually, the novelty of our AFM-compatible microfluidic chamber lies in the ability to precisely control the flow rate inside the flow channel without hindering operations on the optical microscope and AFM setup [30]. In addition, the robust microfluidic chamber permits multiple solvent exchange steps without solvent leakage in a liquid environment.

In order to confirm the feasibility of forming multi-phase SLBs inside the microfluidic chamber, SLBs comprising of liquid phase 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, transition temperature = -17 °C) and gel phase 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC, transition temperature = 41 °C) (**Figure 5.1**) has fabricated. To this end, the formation of SLBs comprising of DOPC and DPPC under ambient conditions has been challenging using the vesicle fusion method since the transition temperature of DPPC is higher than room temperature [31, 32]. In order to form SLBs with a relatively high fraction of DPPC, the experiment would have to be performed above the transition temperature. As mentioned earlier, this would hamper effective characterization of the SLB since the high temperature would affect the signal-to-noise ratio in most analytical tools, including the AFM. Herein, the SALB formation method has employed to fabricate multi-phase DOPC-DPPC SLBs in our newly designed microfluidic chamber. A series of downstream characterization steps on the SLBs including fluorescence microscopy imaging, fluorescence recovery after photobleaching (FRAP), AFM visualization and AFM-based force-distance measurements were performed to compare the biophysical properties of pure liquid phase SLBs and multi-phase SLBs, and investigate the effect of increasing the gel phase composition on the biophysical property of the SLB.

Figure 5.1. Chemical structures of (a) DOPC and (b) DPPC.

5.2 Materials and Methods

5.2.1 Lipid solution preparation

1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rhod PE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Chloroform solutions of DOPC, DPPC, and Rhod PE were mixed in glass vials to prepare lipids solutions at different composition ratios. The composition ratios of the lipids mixtures are summarized in **Table 5.1**. The solution mixtures were then dried with a nitrogen gas stream, and the dried lipids films were placed in a vacuum chamber overnight. The dried lipids films were rehydrated in 2 mL Isopropanol (Fujifilm Wako Pure Chemical Corporation, Japan) to obtain a lipid concentration of 0.5 mg/mL.

Table 5.1 Composition ratios of the lipid mixtures used in the fabrication of SLBs by the SALB method.

DOPC (mol%)	DPPC (mol%)	Rhodamine-PE (mol%)
100	0	0.7
80	20	0.7
60	40	0.7
40	60	0.7
20	80	0.7

5.2.2 Microfluidic chamber design

To observe the lipid bilayer under the fluorescence microscope and seamlessly perform AFM measurements post-fabrication without exposure to air, a microfluidic chamber device has been designed wherein SLBs can be fabricated via the SALB technique

(**Figure 5.2**). All experiments were conducted using the new microfluidic chamber, which has a rectangular flow channel measuring 26 mm in length, 6 mm in width, and 0.5 mm in height. The entire chamber consists of six components. The top cover of the chamber is tightly fixed to the flow channel section with six bolts to prevent the solvent leakage. Two rubber O-rings of different sizes, absorb the pressure to the coverslip (22 mm $\times$ 50 mm) from the bottom cover, acting as a sealant for the chamber. There are inlet and outlet ports leading into and out of the flow channel for insertion of tubes. Overall, the chamber measures 62 mm in length and 33 mm in width and can be easily mounted and Microfluidic chamber design magnetically fixed onto the AFM stage.

Figure 5.2. (a) Three-dimensional drawing of the different parts of the microfluidic chamber. (b) Reverse (bottom-up) perspective of the three-dimensional drawing. (c) Cross-sectional view of the microfluidic channel area. (d) Photograph of the assembled microfluidic chamber for MFP-3D AFM.

5.2.3 Supported lipid bilayer fabrication

A coverslip (22 mm × 50 mm) (Fisher Scientific, USA) was ultrasonically cleaned for 20 min in DI water, followed by drying under a stream of nitrogen gas. The active surface of the coverslip (*i.e.*, where the SLB is fabricated on) was made hydrophilic by treating it to an oxygen plasma glow discharge using the hydrophilizing treatment device (HDT-400, JEOL Datum, Tokyo, Japan) for 1 min immediately before use. The microfluidic chamber was assembled with the inlet and outlet tubes and the coverslip. Tris buffer (10 mM Tris (Wako, Japan), 150 mM NaCl (Wako, Japan), pH 7.5) was first injected to the microfluidic chamber at a flow rate of 200 $\mu\text{L}/\text{min}$ using a peristaltic pump (Ismatec Reglo Digital ISM 833, Germany). After that, Tris buffer was replaced by introducing isopropanol at the same flow rate, phospholipids dissolved in isopropanol (4 mL) were injected into the microfluidic chamber at a flow rate of 100 $\mu\text{L}/\text{min}$ for 25 min. Finally, the solvent exchange step was performed by injecting Tris buffer into the chamber at a flow rate of 50 $\mu\text{L}/\text{min}$ for 30 min, resulting in the formation of SLB on the coverslip.

5.2.4 Epifluorescence microscopy and fluorescence recovery after photobleaching

An inverted epifluorescence Eclipse TE 2000 microscope (Nikon) equipped with a 60 $\times$ oil immersion objective (numerical aperture, NA = 1.49) and an Andor iXon+ EMCCD camera (Andor Technology, Belfast, Northern Ireland) was employed to observe the lipid film with Rhod PE after SLB formation is completed. All fluorescence micrographs were acquired from at least three different areas per sample. The resolution was 512 × 512 pixels with a scan area of 136 × 136 μm^2 . The samples were illuminated through the TRITC filter set by a mercury lamp (Intensilight C-HGFIE, Nikon, Tokyo, Japan). The fluorescence intensities of the micrographs were computed using the ImageJ software. Fluorescence recovery after photobleaching (FRAP) measurements were performed on the same platform. A laser beam (532 nm, 100 mW) was irradiated onto the SLB for 5 seconds, resulting in the photobleaching of a circular region $\sim 35 \mu\text{m}$ in diameter. The recovery of fluorescence intensity in the bleached region was recorded at every two-second interval

over a total duration of 3 minutes. The diffusivities of the SLBs were calculated in MATLAB using a Hankel transform method [33].

5.2.5 Atomic force microscopy

A commercial AFM instrument (MFP-3D Bio, Oxford Instruments, USA) was employed to perform the force spectroscopy experiments. Single crystal silicon cantilever (NSG03, NT-MDT, Moscow, Russia) was used with a nominal tip curvature of 6 nm in radius. The nominal spring constant is 2.0 N/m, which was calibrated by measuring the thermal noise [34]. All force measurements were performed in Tris buffer at room temperature (25 °C), and force maps were recorded in contact mode. The trace and retrace speeds of the cantilever was fixed at 4 mm/sec, and a maximum loading force of 30 nN was applied. The resolution was set to 100×100 points with a scan area of $10 \times 10 \mu\text{m}^2$, generating 10000 force curves for every single run of force mapping ($100 \times 100 \text{ nm}^2$ per point). To translate the deflection signal of the cantilever to the tip-surface separation, the separation of zero at the point was defined as the linearity in the constant compliance region began in the force-displacement curve [35]. To analyze the sample thickness, the point where the cantilever received a repulsive force is defined as the bilayer-probe contact point at which the value of force increased by three consecutive points and taking a value of force larger than a force detected by thermal fluctuation. The force curves were processed using the Igor Pro software.

5.2.6 Quartz Crystal Microbalance with Dissipation Monitoring Experiments

Quartz crystal microbalance with dissipation monitoring (QCM-D) experiments were conducted using a Q-Sense E4 instrument (Biolin Scientific AB, Stockholm, Sweden) to track the formation of supported lipid bilayers (SLBs). The QCM-D technique monitors changes in the resonance frequency (Δf) and energy dissipation (ΔD) of an oscillating, oxide film-coated quartz crystal as a function of time, corresponding to the mass and viscoelastic properties of the adsorbed lipid layer, respectively.[36] The 5 MHz quartz crystal sensor chips with a 50-nm-thick silicon oxide layer sputter-coated on the surface

were used. Before each experiment, the sensor chips were repeatedly washed with water and ethanol, and dried by nitrogen gas and then treated in an oxygen plasma chamber (PDC-002, Harrick Plasma, Ithaca, NY) for 1 min. Next, the chips were assembled in the measurement chambers and SLBs containing mixtures of DOPC/DPPC lipids were formed by the SALB technique as previously described.[22] A baseline recording was first established in the aqueous buffer, which was followed by the solvent exchange to IPA. Subsequently, 0.5 mg/mL lipid mixtures of DOPC and DPPC (50 mol% each) in IPA were added, and solvent exchange to the aqueous buffer was next performed, leading to SLB formation. The final Δf and ΔD values were -27.4 ± 0.5 Hz and $0.5 \pm 0.4 \times 10^{-6}$ (**Figure 5.4**), respectively, which are consistent with complete SLB formation. The temperature was maintained at 25.0 ± 0.5 °C and the data was collected at multiple odd overtones by the Q-Soft software package (Biolin Scientific AB). The reported data were collected at the 5th overtone and normalized according to the overtone number. Data processing was completed using Q-Tools (Biolin Scientific AB) and OriginPro (OriginLab, Northampton, MA) software programs.

5.3 Results and Discussion

5.3.1 Fluorescence microscopy and diffusion coefficient measurement analysis

Fluorescence microscopy images taken from SLBs comprising of different ratios of DOPC and DPPC lipids are shown in **Figure 5.3 (a)-(e)**. Since the DOPC lipids are dye-labeled, the fully liquid phase SLB comprising of only DOPC exhibits a uniform fluorescence intensity over the scanned surface (**Figure 5.3 (a)**). In the presence of gel phase DPPC lipids, which are unlabelled, distinct regions of lower fluorescence intensity (dark domains) start to become observable (**Figure 5.3 (b)**). As the fraction of DPPC increases, the dark domains grow in coverage (**Figure 5.3 (c)-(e)**).[37, 38] To confirm that the dark domains are regions of DPPC and not bilayer defects or “holes” within the SLB, the same SALB experiments were performed using a mixture of 50/50 mol% DOPC/DPPC lipids and tracked the SLB formation process in real-time via quartz crystal microbalance with dissipation monitoring (QCM-D) (**Figure 5.4**). The SLB formation kinetics resembled

has observed that of a standard single-composition liquid phase SLB (*e.g.*, 100% DOPC), which suggested that the SLB had formed properly (**Figure 5.4**). Most importantly, after the SLB formation was completed, a final frequency shift of -27.4 ± 0.5 Hz and a final dissipation shift of $0.5 \pm 0.4 \times 10^{-6}$ were obtained which are perfectly consistent with a complete SLB.[14, 15] Taken together, the QCM-D responses, therefore, confirmed the formation of a homogenous and complete multi-phase SLB via the SALB formation method. In other words, there are no regions where lipid is absent, and the dark domains shown in the fluorescence micrographs indeed represent areas enriched in DPPC.

The appearance of dark regions that become more apparent with an increasing amount of rigid phase constituent is consistent with our previous works where cholesterol-containing SLBs also demonstrate similar behaviors with increasing fraction of cholesterol.[17, 18] However, in this case, it is interesting to note that when the DPPC fraction was increased up to 40 mol%, the dark domains significantly grow in size while maintaining its spatial distribution (**Figure 5.3 (c)**). Upon further increase in DPPC fraction from 40% mol% to 60 mol%, the dark domains become dispersed into smaller circular regions that are rather homogenous in size and are uniformly spread over scanned surface (**Figure 5.3 (d)**). At the highest tested DPPC fraction of 80 mol%, the dark regions become merged, sprawling across the surface, resulting in a dramatic increase in lateral coverage (**Figure 5.3 (e)**).

Figure 5.3. Fluorescence micrographs of the SLBs fabricated following the SALB formation method. The DOPC:DPPC lipid composition ratios of the SLBs shown in the micrographs are (a) 100:0 mol%, (b) 80:20 mol%, (c) 60:40 mol%, (d) 40:60 mol%, and (e) 20:80 mol%, each containing 0.7 mol% fluorescent Rhod PE dye lipid. Each micrograph measures $136 \times 136 \mu\text{m}^2$. (f) Fluorescence intensities at different DOPC mole fractions in the precursor mixtures. (g) Molecular diffusivities evaluated by FRAP measurements. In (f) and (g), the error bars represent standard deviation ($n = 5$). Fluorescence micrographs immediately after photobleaching (left) and after a 3 min recovery period (right) from SLBs comprising of (h) 100% DOPC and (i) DOPC:DPPC = 60:40 mol%. (j) Recovery curves corresponding to the bright and dark areas. Each symbol correspond to the respective annotated area in (h) and (i).

Figure 5.4. QCM-D Monitoring of SLB Formation from 50/50 mol% DOPC/DPPC Mixtures. Changes in frequency (Δf) and energy dissipation (ΔD) were recorded as a function of time. SLB formation process entailed the following steps in sequence: stabilization in aqueous buffer, solvent exchange to IPA, addition of DOPC/DPPC lipids and solvent exchange to aqueous buffer.

To check the composition of lipids in the SLB has proceeded based on the average fluorescence intensity. Since the verification of the SLBs comprising of dye-labeled DOPC and unlabelled DPPC lipids are complete (*i.e.*, without any bilayer defects or “holes”), the fluorescence intensity will, therefore, be directly correlated to the fraction of DOPC lipid. In particular, the maximum fluorescence intensity will be observed for SLB comprising of 100 mol% DOPC lipid and zero fluorescence intensity will be observed for SLB comprising of zero mole percent DOPC lipid (*i.e.*, 100 mol% DPPC lipid). Conversely, in a plot of fluorescence intensity against DOPC mole fraction (**Figure 5.3 (f)**), the straight line connecting the origin to the experimentally determined maximum intensity originating from SLB comprising of 100 mol% DOPC lipid (*i.e.*, 2000 intensity a.u.) serves as a reference to obtain, the expected fluorescence intensity for a certain DOPC mole fraction in the SLB. Hence, by comparing the actual fluorescence intensity obtained using a certain DOPC mole fraction in the precursor lipid mixture to the expected fluorescence intensity in the formed SLB based on this linear correlation, the determination of the degree of compositional reproducibility during the SLB formation process can be defined. In particular, if the observed fluorescence intensity lies on the straight line, the DOPC mole fraction in the formed SLB perfectly matches the DOPC mole fraction in the precursor lipid mixture. If the observed fluorescence intensity lies below the straight line, the DOPC mole fraction in the formed SLB is lower than expected (*i.e.*, lower than the DOPC mole fraction in the precursor lipid mixture), and vice versa. Based on this, deduction from the plot in **Figure 5.3 (f)**, that the experimentally determined fluorescence intensities all lie close to the ‘Ideal intensity’ line, supporting that the composition of lipids in the precursor solution mixture is well reproduced on the substrate when the SLB is fabricated following the SALB formation method. It is worthy to note that the slight mismatch between the ratio of dark and bright regions to the lipid fraction can be attributed to the lack of spatial resolution of the optical microscope (*i.e.*, around 500 nm in lateral dimension), which is not sufficient to observe domains with dimensions in the 100-nm range.

The measurement of the membrane diffusivities of the SLBs comprising with different DOPC/DPPC compositions has also been proceeded to 1) evaluate the diffusivity of SLBs obtained via the SALB formation method in comparison to SLBs obtained via

vesicle fusion and more importantly 2) investigate the effect of increasing gel phase on the overall membrane diffusivity. The diffusivities calculation of the SLBs using the Hankel transform method, and the calculations were implemented in MATLAB. In brief, the Hankel transform method employs circular averaging of the analyzed data (in this case the fluorescence intensity of the photobleached area of the SLB) to obtain a mathematical curve fitting of the fluorescence recovery profile before the diffusivity value is extracted from the fitted curve [33]. Our calculations revealed a gradual decrease in the overall diffusivity of the membrane with an increasing fraction of DPPC (**Figure 5.3 (g)**), in agreement with previous observations [39, 40]. For fully liquid phase SLBs comprising of 100% DOPC lipids, the calculated diffusivity was 2.6 ± 0.1 , which agrees well with previously reported values [22, 41, 42]. Conversely, this further testifies that SLBs fabricated following the SALB formation method possess the same physicochemical properties (*i.e.*, molecular density and diffusivity) as SLBs prepared via the vesicle fusion method.

The high mobility of the lipid molecules in the film is one of the characteristics of liquid phase SLBs, which is difficult to observe for unordered aggregated or randomly adsorbed lipid molecules [43, 44]. Hence, to understand the effect of increasing gel phase fraction on the reduced overall membrane diffusivity, it is imperative first to prove that the dark domains are indeed regions of lipid bilayers in the rigid phase. The lipids in this region should still possess some degree of mobility, albeit considerably reduced. Following this line, the representative sets of raw fluorescence micrographs have been showed from photobleached SLBs comprising of 100% DOPC (**Figure 5.3 (h)**) and photobleached SLBs comprising of 60% DOPC and 40% DPPC (**Figure 5.3 (i)**), before and after fluorescence recovery. In the case of the latter, the photobleached area included a large dye-labeled region as well as a small portion of the dark region. Based on the fluorescence recovery profiles taken from the different regions (**Figure 5.3 (j)**), our results clearly show a recovery of fluorescence intensity in the area of mixed bilayer after the photobleaching, indicating that the lipid molecules freely diffuse within the film, which is an important characteristic of SLBs. It is worthy to note that this is also a testimony that a complete SLB has been formed and the dark regions are not bilayer defects or “holes.” However, while

the diffusivity of the dye-labeled region did not differ significantly between the two SLBs, the diffusivity of the dark region (*i.e.*, which is bleached) in the SLB comprising of 60% DOPC and 40% DPPC was significantly lower. The attribution of the differences in diffusivities to the fact that DPPC possesses two palmitic acid groups with a linear carbon chain. As such, they are packed at high density in the bilayer due to attractive van der Waals interaction between the chains. On the other hand, the chains of DOPC includes a carbon double bond, which leads to steric hindrance that deters them from packing densely. Therefore, the observed overall diffusivity of the SLB is lower when it contains higher fractions of DPPC.

5.3.2 Nano-mechanical properties of multi-phase supported lipid bilayers evaluated by AFM

To evaluate the morphological and nano-mechanical properties of the multi-phase lipid bilayer in detail, a set of quantitative nano-mechanical analysis by AFM has been conducted using the originally designed microfluidic chamber. The conventional raster scan on the SLBs was performed first to observe the surface morphology of the SLBs. The AFM images of the multi-phase SLBs with different ratios of DOPC and DPPC showed complete coverages regardless of the composition (**Figure 5.5**). To investigate the morphological structure of DOPC: DPPC mixed bilayer by AFM imaging as shown in **Figure 5.5**. An NSG03 cantilever (NT-MDT, Moscow, Russia) with a spring constant of about 1.7 N m^{-1} was used; its resonance frequency was about 90 kHz in the air but was reduced to 30–50 kHz in liquid. Images were obtained in contact mode over an area measuring $10 \mu\text{m} \times 10 \mu\text{m}$ at a 0.3–0.5 Hz scan rate to reduce the damage to the mixed bilayer and minimize signal noise. The experiment was conducted in different conditions to compare the changes in morphological structure due to the different ratios of DOPC and DPPC (pure DOPC, DOPC:DPPC = 80%:20%, DOPC:DPPC = 60%:40%). As expected, slight differences in surface morphology were observed due to the formation of phase domains, which confirmed the presence of a phase mixture. Then, the penetration force measurements have been conducted on the SLBs to evaluate changes in bilayer rigidity using the AFM probe tip.

Figure 5.5. Comparison of AFM height retracts and their corresponding cross-sectional height profiles across the surface, denoted by the red line, obtained from SLBs with different DOPC:DPPC compositions. The DOPC:DPPC lipid composition ratios of the SLBs shown in the height retracts are (a) 100 : 0 mol%, (b) 80 : 20 mol%, and (c) 60 : 40 mol%.

With reference to the force-distance (f-d) curve, the penetration force is defined as the amount of force required to disrupt the bilayer after the first point of contact by the AFM tip (**Figure 5.6 (a)**). In brief, when the tip comes into contact to the bilayer with typical separation depth of 5 to 10 nm, a repulsive force arises [45, 46]. Upon the further approach of the tip towards the substrate, the repulsive force increases as a result of bilayer compression. After compression of the bilayer, the film collapses at a separation of 2 to 3 nm, which leads to the tip jumping into hard contact with the underlying substrate [47]. The penetration depth is therefore defined as the distance between these two points - one is the point at which the repulsive force begins to rise after the initial contact between the tip and the bilayer, and the other is the point at which hard contact occurs between the tip and substrate [48-50]. Supported lipid bilayers are known to be formed on a hydration layer with a thickness of about 1 nm [49, 51]. As such, the penetration depth evaluated from the f-d curves is the sum of the thicknesses of the hydration layer and the bilayer, which is typically around 4-5 nm [52-54]. Representative force-distance curves obtained from SLBs clearly show differences in penetration force and penetration distance for SLBs with different DOPC/DPPC compositions (**Figure 5.6 (b)**), which is in the range of 5-7 nm, in close agreement with previous works.

The plot of calculated averaged penetration force against compositional ratio shows a direct relationship between these two parameters (**Figure 5.6 (c)**). With an increasing fraction of gel phase DPPC, the penetration force is higher, indicating that DPPC increases the rigidity of the bilayer. The increase in the rigidity at high fractions of DPPC can be explained by the intermolecular interactions that are strengthened by the presence of DPPC [32, 37]. At higher fractions of DPPC, the penetration depth of the bilayers is smaller compared to that of pure DOPC bilayer. As earlier discussed, the results from FRAP and force spectroscopy measurements suggest that the bilayer rigidity is higher and the diffusivity of the molecules in the bilayer decreases because of the strong intermolecular interactions between the DOPC and DPPC lipid molecules. As a consequence, the thermal undulation also decreases, resulting in lower penetration depths.

Figure 5.6. Definitions of penetration depth and penetration force in this work. (b) Representative force-separation curves recorded from SLBs fabricated following the SALB formation method in Tris buffer solution. (c) Average values of the penetration force observed from SLBs containing at different ratios of DOPC and DPPC. Error bars represent standard deviation ($n = 5$).

5.4 Conclusion

The successful fabrication of the SLBs comprising with both liquid phase DOPC and gel phase DPPC at different composition ratios has been shown following the SALB formation method inside a newly developed microfluidic chamber at room temperature. This enabled us to seamlessly perform a series of physical characterizations on the SLBs without exposure to air. Our multi-pronged analysis using the fluorescence microscope, QCM-D and AFM testified the ability of the SALB method to form uniform multi-phase SLBs even when they comprise of a significantly high fraction of gel phase lipids (*i.e.*, up to 80 mol% DPPC), which is challenging using the conventional vesicle fusion method. The composition of lipids in the precursor solution was well reproduced on the substrate, indicating that the SALB formation method allows the precise control of the lipid composition in the fabricated SLBs. Using the SLBs that have been obtained with precise control over its composition (*i.e.*, from zero up to 80 mol% DPPC, at 20 mol% intervals), the investigation of the effect of increasing the gel phase fraction has been performed on the biophysical properties of the SLBs. The results have shown that with increasing fraction of DPPC, the overall diffusivity of lipid molecules in the SLBs decreases and the rigidity of the films increases. This observation was attributed to differences in molecular structure, which governs the molecular organization and influences intermolecular interactions between liquid phase and gel phase lipid molecules. Taken together, the results in this work demonstrate that the SALB formation method represents a promising approach to fabricate high-quality complex multi-phase model membranes that mimic biological membranes of living systems. More importantly, it paves the way for more detailed qualitative and quantitative investigations on such biological mimics.

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Chapter 6*

Unusual non-equilibrium membrane topology consisting of POPE and POPG phospholipids formed by Solvent Assisted Lipid Bilayer (SALB) method

In this chapter, a supported lipid bilayer (SLB) membrane mimicking the Gram-negative bacteria membrane composition, consisting of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (sodium salt) (POPG) at the molar ratio of 85:15, was fabricated on silicon dioxide surface by using the solvent-assisted lipid bilayer (SALB) formation method. The fabrication procedure was conducted with an originally designed microfluidic chamber for AFM at different temperature (37 °C, 25 °C, 10 °C). To characterize the topological property of the mixed-bilayer, time-lapsed Atomic Force Microscopy (AFM) images in Tris-buffered saline, and deionized (DI) water was obtained. Also, to correlate the topological changes with lateral diffusivity, fluorescence recovery after photobleaching (FRAP) experiment was performed. The results illustrate the complex interplay between charged lipids, hydrophilic solid substrate, ionic strength, and temperature during the solvent exchange process in the SALB formation method, giving rise to an unusual non-equilibrium membrane topology.

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6.1 Introduction

Biological membrane plays a crucial role in the cellular protection from exogenous stresses as well as in the transport of nutrients [1, 2]. Many cellular mechanisms such as membrane fusion, cellular adhesion, enzymatic catalysis, and molecular recognition occur at the biological membrane interface [3]. However, the complexity of the native cell membrane hinders direct mechanistic biophysical understanding of the membrane functions. As such, model membrane systems represented by mixed bilayer platforms are used to recapitulate essential characteristics of the membrane and at the same time, isolate specific biophysical parameters [4]. Supported lipid bilayer (SLB) is one of the representative models of the simplified biological membrane [5-7]. The vesicle fusion method is the most commonly used technique for the fabrication of SLBs. In the vesicle fusion method, precursor small unilamellar vesicles are required. The vesicles adsorb onto a hydrophilic solid substrate and transform into the SLBs on the substrate by the spontaneous rupture process [8-12]. However, when the vesicles contain phospholipids with large bending rigidities such as gel phase lipids, the vesicle rupture process is hindered. Therefore, the formation of a uniform bilayer was challenging in the case where mixed-phospholipids are involved [13, 14].

The solvent-assisted lipid bilayer (SALB) technique has been developed as a robust method of forming the uniform lipid bilayer on hydrophilic substrates simply by a solvent exchange process in a microfluidic channel from organic solvents (isopropanol, methanol, ethanol, etc.) to aqueous solutions such as water and buffer [13, 15-18]. In the phase of organic solvents, the lipid molecules form the reverse micelles or monomer structure. When the organic solvents are exchanged to the aqueous solutions, the concentration of the organic solvent in the channel area is gradually decreased, and the lipids change the form of the assembly from monomer and reverse micelles to micelles and vesicles [19, 20]. Ultimately, a lipid bilayer is formed on the hydrophilic substrate when all the solvent in the channel area is fully filled up with the aqueous solution [20]. Therefore, the SALB method can be applicable to the formation of the bilayer, which contains complex components such as gel phase phospholipids at the room temperature without the

preparation of the vesicles [21-23]. Moreover, multicomponent SLB can exhibit two- and three-dimensional structures, depending on the lipid compositions and physicochemical parameters involved in forming and maintaining the SLB. The simplest two-dimensional structure includes lateral phase separation of lipids with different chemical structures. Coupling solvent, lipids and the solid supporting substrate, more complex non-equilibrium three-dimensional structures can be formed [24, 25]. Often, these structures, which differ in height, are modulated by the change in ambient temperature, which is directly related to the phase transition temperature of the lipids used [24, 26, 27].

A bacterial cell envelope usually consists of an inner and an outer membrane, of which their lipid composition varies depending on the class of the bacteria. The outer membrane of Gram-negative bacteria consists largely of lipopolysaccharide on the outer leaflet, whereas that of Gram-positive bacteria comprises of a thick peptidoglycan cell wall. On the inner leaflet of the outer membrane, as well as the inner membrane, the lipid composition is largely defined by phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) at different molar ratios (**Figure 6.1**). For instance, the membrane lipid composition of the Gram-negative *Escherichia coli* (*E. coli*), the most well-known bacterial host for the generation of membrane proteins, is commonly reported to be around 85:15 (PE: PG) [28]. Model membrane constructed based on this composition has been used to elucidate bacterial membrane related biophysical mechanism. For example, it has been used to model the *E. coli* membrane to address mechanisms leading to butanol tolerance [29, 30].

In this study, the SALB fabrication method was used to fabricate a bacterial mimicking model membrane in a physiological solvent on silicon dioxide (SiO₂) substrate and characterized based on the recently reported microfluidic chamber with AFM. The microfluidic chamber device is originally developed for solvent-assisted lipid bilayer (SALB) technique combined with AFM liquid applications [23, 31]. An unusual, non-equilibrium, three-dimensional structure was observed, and temporal changes of the membrane topography toward the equilibrium state were recorded real-time. The observation is discussed, in the context of a complex interplay between the charged lipid,

hydrophilic solid substrate, ionic strength, and temperature. Also, the fluorescence recovery after photobleaching technique was used to derive the diffusivity of the bilayer in each experimental conditions to compare the topological changes with the results of the AFM.

Figure 6.1. Chemical structure of (a) POPE and (b) POPG

6.2 Experimental Method

6.2.1 Solvent-Assisted Lipid Bilayer (SALB)

POPE and POPG lipid in chloroform solution were mixed in a molar ratio of 85:15 with 2 mg/ml of concentration. 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (Rhod-PE) (Avanti Polar Lipids, USA) in chloroform solution was added to POPE: POPG (85: 15) with 0.5% wt to acquire fluorescence images, and dried under nitrogen while rotating the glass vial. Afterward, the vial was then kept under the vacuum desiccator for drying overnight to ensure a more fully

dried and uniform layer of lipid film. 4 ml of isopropanol was directly added to glass vial to make the lipid mixed concentration of 0.5 mg/ml. The round type coverslip (25 mm-diameter coverslip with a thickness of 0.15–0.17 mm, Neuvitro, Vancouver, WA, USA) was treated with O₃ plasma (Plasma Cleaner PDC-FMG, Harrick Plasma, NY, USA) for making hydrophilic surface immediately before use. After assembling the coverslip with the microfluidic chamber for AFM [31], and connecting the tubes to the inlet and outlet, flow the Tris buffer (150 mM NaCl, 10 mM Tris, PH 7.5) and pure isopropanol subsequently with at 100–200 $\mu\text{L min}^{-1}$ until the chamber was filled. Next, lipids containing isopropanol was placed at the inlet for a formation of the mixed-bilayer on a substrate and was flowed through the channel at a flow rate of 100 $\mu\text{L/min}$ using the pump for 15 min. At the final stage, Tris buffer (10mM Tris, 150 mM NaCl, PH 7.5) or DI water were injected to from the inlet tube at the flow rate of 40–50 $\mu\text{L min}^{-1}$ for 30–40 min. This process made sure that the lipids aligned to the bilayer at the hydrophilic surface in the buffer [13, 15, 20]. After the successful formation of the SLBs on the substrate, flow the 20–30 ml of buffer or DI water through the channel at the rate of 300–500 $\mu\text{L min}^{-1}$ to remove the floating lipids [7]. Due to keep the environment temperature during the experiment, all solutions are controlled by using digital hotplate stirrer (IKA C-MAG HS hotplate stirrers, Merck KGaA, Germany) to 37 °C and 10 °C in each experiment. To controlling the constant flow rate due to filling up the chamber area without generating an air bubble, a peristaltic pump (ISMATEC REGLO Digital ISM 833, Germany) has been used.

6.2.2 Atomic Force Microscope (AFM)

A commercial AFM system (NX-Bio; Park Systems, Suwon, South Korea) combined to an optical microscope (Eclipse Ti; Nikon, Tokyo, Japan) was employed to observe the morphological dynamic change of the POPE: POPG (85: 15) bilayer in this experiment. A BioLever mini cantilever (Olympus, Tokyo, Japan) with a spring constant of about 0.09 N/m was used; its resonance frequency was about 110 kHz in the air but was reduced to 30–50 kHz in liquid. The experiments were performed at different temperature of (37°C and 10°C) in an aqueous Tris buffer solution (10 mM Tris, 150 mM NaCl, pH

7.5) and DI water. Images were obtained at a 0.3–0.5 Hz scan rate (tapping mode) to minimize the damage to the sample and the noise in the signal. To compare the differences of the topological changes in each experiment conditions, the all solutions which are used during the SALB process are controlled to 37°C of Tris buffer, 10°C of Tris buffer, 25°C of Tris buffer and 25°C of DI water.

In addition, to confirm the thickness of the POPE: POPG mixed bilayer, a square test was performed: Due to the E-coli like membrane (POPE: POPG lipid) is rigid to make a scratch on that, the cantilever with high spring constant (42N/m, NCHR, Nanosensors, Switzerland) was used for scratching and square area. To characterize the effect of the charge of the solution by time, inject the aqueous solution (Tris buffer or DI water) in the final step of the SALB to the chamber using the peristaltic pump, afterward measure the morphological changes right after stopping the flow at the same position where the scratched area by AFM tip in imaging mode. Taken the AFM images including the square area, the thickness differences can be shown in **Figure 6.3 (d), (e), Figure 6.4 (d), (e)**: the height difference between the bilayer and an area from which the bilayer was scratched off was approximately 5-6 nm.

6.2.3 Fluorescence Recovery After Photobleaching (FRAP)

Fluorescence recovery after photobleaching (FRAP) technique was conducted to derive the diffusion coefficient of the POPE: POPG (85:15) mixed-bilayer that has formed on the glass coverslip substrate. For obtaining fluorescence images, an inverted epifluorescence Eclipse TE 2000 microscope (Nikon, Tokyo, Japan) equipped with a 60 oil immersion objective (NA 1.49) and an Andor iXon + EMCCD camera (Andor Technology, Belfast, Northern Ireland) camera was employed. The sample was illuminated through the TRITC (Rhod-PE) filter set with a mercury lamp (Intensilight C-HGFIE; Nikon Corporation). Before measuring the diffusivity by the FRAP experiments, the solvent-assisted lipid bilayer (SALB) procedure was performed for the bilayer formation, which is explained in detail in the previous paragraph (**Chapter 6.2.1**). To compare the time-lapsed influence of the diffusivity of the POPE: POPG bilayer, the FRAP was performed in three

different experiment condition, right after the formation of the bilayer, after 15 min and 1 hour. The size of the circular bleaching spot is around 20~30 μm diameter and photobleached by 532 nm, 100 mW power of laser beam. The image traces of the recovery process were recorded from minus 3 to 180 sec at two seconds of interval time to monitor the recovery process of the fluorescence intensity signal. FRAP analysis was computed using Matlab software in order to acquire the diffusion coefficients that are determined based on the Hankel transform method [13, 14, 32].

Figure 6.2. The schematic process of POPE:POPG SLB formation by the SALB method. The lipids with red headgroup indicate POPG lipids and those with white headgroups indicate POPE. As aqueous solution fraction increases during the organic-aqueous solvent exchange process, the bilayer starts to form on the surface. When the aqueous fraction reaches 100%, four types of lipid configurations (PE-PG, PG-PE, PE-PE, PG-PG) may exist. When a salty aqueous solvent is used, the PG lipids that reside in the proximal leaflet move closer to the surface due to charge screening effect, from a “buckled” position.

6.3 Results and Discussion

The topological changes of the POPE: POPG (85:15) bilayer were first documented in Tris-buffered saline (10 mM Tris, 150 mM NaCl, pH 7.5) at 37°C. An uneven membrane topology with a maximum height variation of about 3 nm was observed (**Fig. 6.3 (a)**). Over the course of 1-2 hours in the same buffer condition, the uneven topology smoothened gradually without additional treatment (height roughness value (R_q) in entire area < 0.3 nm, **Fig. 6.3 (b-c)**). A simple scratch test confirmed that the resultant smooth topology consisted of a single bilayer (**Fig. 6.3 (d-e)**). This, together with additional experiments performed, can be understood as an interplay among three phenomena, described as follows.

First, in the SALB procedure, during the introduction of the Tris buffer into the mixture of phospholipids and isopropanol, already incubated on the silicon dioxide solid substrate, suspended lipid aggregates will be formed and subsequently bind to the surface, leading to a bilayer formation. Concomitantly, in contact with the aqueous media, the silica surface acquires a negative surface charge density, primarily due to the dissociation of terminal silanol groups. Because as charges repel, POPG, which has a net charge of -1, will experience electrostatic repulsion from the negatively charged surface. This repulsion is thought to cause an over-representation of POPG at the upper leaflet, oriented away from the negatively charged surface. Some POPG molecules, due to hydrophobically driven self-assembly process, will localize in the lower leaflet along with the POPE molecules. As a consequence of simple electrostatic repulsion of POPG-containing region from the surface, this likely leads to the formation of buckled membrane regions on the substrate, as supported by the height variation. The electrostatically induced membrane buckling is reduced over time in the presence of the 150 mM NaCl as a result of charge screening effect, giving rise to the topologically even SLB after equilibration of 1-2 h in the salty condition. (**Figure 6.2**). To support this mechanism, the experiment was repeated by replacing Tris buffer with deionized water. SLB was formed, and membrane topology with similar height variation was observed, albeit with a different morphology at the taller regions (**Figure 6.6, right**). Over the course of two hours, this topology did not change, consistent with the prediction that, in the absence of charge screening by salt, the proximal

POPG “domains” remains electrostatically repelled from the substrate. Screening only occurs in the Tris buffer. Also, this screening effect is well correlated to the result of the previous chapter, DPPC / DOPC mixed bilayer, which does not show the smoothing because it does not have a charge lipids [23].

Second, during the organic-to-aqueous solvent exchange step, lipid aggregates in the form of micelles are present in the bulk solution. The maximum height variation of 4 nm suggests that hole defects were also present at the earlier time points (**Fig. 6.3 (a)**), which were “healed” by the adsorption of micelles from the bulk. This temporal topographical change observed for the charged lipid composition suggests that the kinetics of the lipid adherence to the surface is influenced by the lipid composition. For lipids with like charges with the surface, the equilibrium time to achieve a smooth SLB appears to increase, as compared to zwitterionic lipids (**Chapter 5**).

To examine if the phase transition process is involved, two additional experiments at 10 and 25 °C were carried out (**Figure 6.4 and 6.5**). The former rendered the SLB in the gel phase for below 10 °C, and the latter was slightly above the gel-to-liquid crystalline phase transition temperature at 25 °C [33]. Observations at 25 °C were similar to that at 37 °C. However, at 10 °C, the maximum height variation was less (2-3 nm) than typical lipid bilayer height [34], along with a reduction in hole defects. The scratch test confirmed that a SLB was formed, even in the presence of defects. These additional results lend support to the proposed mechanism that the smoothening process involves partitioning of micelles from bulk to the surface [35], as the phase transition process is minimal at 10 °C [36]. In other words, this implies that at the higher temperature, the change in height variation involves phase transition of the SLB, which also implicates area-per-lipid – as the lipid headgroup is larger in the fluid phase as compared to that in the gel phase – leading to an areal expansion of the SLB.

Third, the observation of the buckled surface, presumably due to the charge-charge repulsion of POPG and the surface, suggests that POPG may not be homogeneously distributed in the proximal leaflet. The presence of two lipid species theoretically gives rise

to four configurations of lipid arrangement on the substrate (POPE: POPE, POPE: POPG, POPG: POPE and POPG: POPG; each refers to lipid on the distal leaflet: lipid at the proximal leaflet). Due to the surface electrostatic repulsion, only POPE: POPE and POPG: POPE will be enriched. This effectively leads to enrichment of POPG at the solvent-facing, upper (distal) leaflet to about 30 mol%. However, the complete depletion of POPG from the lower leaflet will remove the repulsive force needed to form a buckled membrane region. Thus, some POPG molecules, due to hydrophobically driven self-assembly process, will localize in the proximal leaflet along with the POPE molecules, as also reasoned above. As only POPG will experience the repulsion from the surface, this leads to variation in the vertical position between adjacent POPG and POPE molecules, which will result in a clustering of POPG molecules as will be expected from the line energy minimization effect [37]. Also, it is worthwhile to take into consideration that the similar critical packing parameter ascribed to POPE and POPG due to identical tail regions and similar-sized headgroups [38].

In addition, the integrity of the formed SLB at 37 °C was confirmed by FRAP (**Figure 6.8**) as a control experiment. Lateral diffusivity increased from about 1.8 to 2.6 $\mu\text{m}^2/\text{s}$, as a function of time (measured at 0, 30, and 60 min). Interestingly, bright speckles of rhodamine-conjugated-DPPE were observed at all the time points. As the SLB is formed by the SALB method, the presence of non-specifically adhered vesicles can be excluded. Furthermore, condensation of dye at defects of high local membrane curvature is also excluded, as shown supported by AFM images that a smooth surface (<0.3 nm height roughness value (R_q) in the entire area, **Figure 6.3**) is obtained.

Figure 6.3. Time lapsed AFM imaging of surface topology in Tris buffer (37°C). (a) right after formation of the bilayer, (b) after 30 min, (c) after 60 min. (d) scratched square area after 60 min. (e) Cross-section line profiles of the scratched area. The thickness of the bilayer is around 5~6 nm which is well-correlated value of the bilayer thickness.

Figure 6.4. Time lapsed AFM imaging of surface topology in Tris buffer (10°C). (a) right after formation of the bilayer, (b) after 30 min, (c) after 60 min. (d) scratched square area after 60 min. Different from the case of higher temperature, the dark spots are still detected on the surface, (e) Cross-section line profile of the scratched area. The thickness of the bilayer is around 6~7 nm.

Figure 6.5. Time lapsed AFM imaging of surface topology in Tris buffer (25°C). (a) right after formation of the bilayer, (b) after 30 min, (c) after 60 min. The smoothing process still occurs at 25°C. (d) scratched square area after 60 min. (e) Cross-section line profile of the scratched area. The thickness of the bilayer is around 5~6 nm.

Figure 6.6. Time lapsed AFM imaging of surface topology in DI water (25°C). (a) after 30 min, (b) after 3 hours. Different from the case of the buffer solution, the smoothing process has not occurred. The height difference between higher domain and lower area is around 2~3 nm. (c) scratched square area after 3 hours. (d) Cross-section line profile of the scratched area. The thickness of the bilayer is around 4~5 nm.

Figure 6.7. Comparison of the AFM images before smoothing process. ($t \sim 0$ min, at room temperature (25°C)). (a) in the Tris buffer solution, (b) in the DI water. The results show that the size of the dark domains have a different dimension (larger in Tris buffer) which has generated before time lapsed.

Figure 6.8. The FRAP experiment results in buffer solution. (a) right after formation of the bilayer, (b) after 30 min, (c) after 60 min. The bleached spot has recovered in every cases and the diffusivity value is slightly increased by time. (e) recovery curve of normalized fluorescence intensity of bleached area ((a)).

6.4 Conclusion

The fabrication of mixed SLB of POPE: POPG (85:15) in different temperature (37, 25 and 10°C) has been conducted by the SALB method using the newly developed microfluidic chamber that enables the whole process to be carried out and then imaged by time-lapsed AFM imaging without exposure to the air. The smoothening process of the SLB that occurs in Tris buffer (10 mM Tris, 150 mM NaCl, PH 7.5) is conspicuously absent when the aqueous phase is replaced by DI water. The presence of SLB is validated by scratch test and FRAP measurements. This observation can be explained due to the repulsive interaction between charged lipids (POPG) and the SiO₂ substrate, delayed surface absorption of lipids of like charge, and the clustering of charged lipids driven by line energy minimization. This complex interplay between charged lipid, hydrophilic solid substrate, ionic strength, and temperature during the solvent exchange process in the SALB method orchestrates an unusual non-equilibrium membrane topological transition.

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Chapter 7

Conclusion and Future Outlook

In this chapter, the conclusion of the findings in this thesis is summarized, and the future outlook will be proposed. In the first part of this dissertation, the originally designed microfluidic liquid cell chamber for AFM has been introduced, which enables solvent-assisted lipid bilayer (SALB) formation technique designed by its numerical fluidic simulation. Next part, the morphological and mechanical properties of mixed bilayer platform of DOPC and DPPC lipids has been investigated in different concentration ratio at the room temperature. Last part of this dissertation, the unusual topological property of Gram-negative bacterial membrane composition (POPE (85)): POPG (15)) has been observed by using originally designed microfluidic chamber for AFM. For the future work, Extracellular Matrix (ECM) – Functionalized Lipid Membrane platform studies with SALB method will be introduced and its expectations on AFM application.

7.1. Conclusion

In this dissertation, the significance of the supported lipid bilayer as a model membrane and several experimental techniques for studies its properties has been introduced. There are various measurement tools have been developed and established, such as QCM-D, LSPR, fluorescence microscopy, and atomic force microscopy (AFM) as a straight forward strategy for bilayer research. On the other hand, the solvent-assisted lipid bilayer (SALB) formation, which is the robust technique for fabrication of the supported lipid bilayer has been reviewed. By contrast with conventional vesicle fusion method, the procedures for SALB are easy to perform, mainly involving lipid dilution and mixing steps, and high-quality SLBs can be achieved even with minimal experience. Since the SALB technique enables to fabricate the mixed-lipids bilayer system, the application of AFM for membrane studies is further challenged because of the lack of a convenient platform for exchanging solvents while performing experiments. Exchange of solvent with a bilayer could open up various avenues to explore the effect of other biochemical molecules on lipid bilayer [1, 2]. A newly designed sample chamber in this dissertation has less sample volume, is modular, can exchange any solvent conveniently and can form uniform bilayer consistently and reliably, which is highly desirable. The development of a new sample chamber addressing these challenges is the primary focus during my Ph.D. based on numerical fluidic simulation.

In chapter 4, a microfluidic chamber device designed based on flow calculation of the SALB for AFM has been reported. This technology would provide the high-resolution AFM imaging systems with various useful information such as morphology, mechanical properties, and molecular recognition, for dealing with delicate soft matters and understanding complicated biological systems.

In chapter 5, solvent-assisted lipid bilayer (SALB) method to fabricate mixed lipid bilayers comprised of DOPC and DPPC has been reported, which has not been realized by a conventional vesicle fusion method. The designed a new microfluidic chamber to fabricate of the films by the SALB method successfully worked on characterizing the

mixed-lipids bilayer platform subsequently without exposure to the air. Our observation with a fluorescence microscope, measurements of fluorescence recovery after photobleaching (FRAP), and force measurements using AFM revealed that the SALB method enables to construct uniform bilayers at any composition ratio of DOPC and DPPC at room temperature even the DPPC lipids exist as a gel-phase due to it has a higher transition temperature. From these characterizations, we found that the composition ratio of DOPC and DPPC in the precursor solution is well reproduced on the mixed bilayer. Also, discovering the diffusion coefficient of the labeled DOPC in the mixed bilayer, a decrease of the thickness due to increasing the ratio of DPPC lipids indicates that the high ratio of gel-phase lipid revitalizes the lipid-lipid interaction results the low fluidity of the molecules and suppression of the thermal undulation of the bilayers. Through this work, we concluded that the SALB method is the robust method to fabricate lipid bilayers with precise control of complexed compositions of lipids for studies the function of biological membranes.

In chapter 6, a supported lipid bilayer (SLB) mimicking the Gram-negative bacteria membrane composition, consisting of POPE and POPG at the molar ratio of 85:15, was fabricated on silicon dioxide surface by utilizing the solvent-assisted lipid bilayer (SALB) formation procedure using the microfluidic chamber for AFM. When the SLB was formed in tris buffer (10 mM Tris, 150 mM NaCl) at 37°C, a membrane topology with a maximum height variation of about 3 nm was obtained. Over the course of 1-2 hours, this uneven topology was smoothened gradually without additional treatment. This can be understood as follows: In the SALB procedure, 150 mM NaCl was introduced into a mixture of lipid and IPA, already incubated on the silicon dioxide solid substrate. During the solvent exchange step, suspended lipid aggregates were formed and bind to the surface, leading to a bilayer formation. Concomitantly, in contact with the aqueous media, the silica glass surface acquires a negative surface charge density, primarily due to the dissociation of terminal silanol groups. Because like charges repel, POPG, which has a net charge of -1, will experience electrostatic repulsion from the negatively charged surface immediately after the SLB was formed. This repulsion leads to the formation of buckled membrane regions on the substrate. Over the course of 1-2 hours, because of charge screening from the neutral salt, the repulsion between POPG and the substrate is reduced, resulting in a

topologically even SLB. To support this mechanism, the experiment was repeated by replacing Tris buffer with water. SLB was formed and, membrane topology with similar height variation was observed, albeit with a different topology at the taller regions. Over the course of 2 hours, this buckled topology did not change, consistent with the prediction that in the absence of charge screening by salt, the proximal POPG “domains” remains electrostatically repelled from the substrate.

Consider an alternative case of POPG which constitutes 15 mol% of the total lipid and carries a net charge of -1 experienced electrostatic repulsion from the negatively charged surface, leads to depletion of POPG from the substrate-facing, lower (proximal) leaflet of the SLB. The presence of two lipid species theoretically gives rise to four configurations of lipid arrangement on the substrate (POPE: POPE, POPE: POPG, POPG: POPE and POPG: POPG; each refers to lipid on the distal leaflet: lipid at the proximal leaflet). Due to the surface electrostatic repulsion, only POPE: POPE and POPG: POPE will be enriched. This effectively leads to enrichment of POPG at the solvent-facing, upper (distal) leaflet to about 30 mol%. However, the depletion of POPG from the lower leaflet will remove the repulsive force needed to form a buckled membrane region. Thus, the explanation that a negatively charged surface “pre-selects” a certain lipid is unlikely. Also, it is worth to take into consideration that the similar critical packing parameter ascribed to POPE and POPG due to identical tail regions and similar-sized headgroups. Effectively, this also excludes the possibility that a certain lipid is locally condensed and deform due to its spontaneous curvature.

Taken together, this dissertation presented the new robust platform for complex bilayer system for AFM applications. The native biological membrane is complex to correct and characterize its properties. Therefore, researchers have been utilized the simple model system to mimic the biological membrane system, among the technique to assemble the bilayer, SALB is a powerful technique to be able to fabricate sophisticated bilayer platform, can be expected to form the natural membranes-like structure. There are several studies have been reported to investigate complex mimic membrane platforms such as response of anti-bacterial fatty acids [3], membrane attack complex formation [4], BSA

protein absorption properties [5], and its interaction with biological functional protein to identify the biological interface phenomena [6, 7]. Since the designed microfluidic chamber enables to explore these complex bilayer systems using AFM measurement, it can be applied to various research to investigate the more closed-composition to the natural membranes platforms by combining with fabrication technique in a nano-scale level. This can be expanded to the fundamental understanding of multi-composed lipid bilayer system and offers a new platform to characterize the mechanisms of the biological membrane combined with AFM system.

7.2. Future Outlook

The originally designed microfluidic chamber for AFM, which can be combined with the SALB fabrication technique has a strong potential that can be expanded to the multi-components bilayer studies. In this section, the future expectation of by utilizing this device for continuous research of complex SLB platform will be discussed and highlights the potential of AFM applications involve mechanical and morphological properties.

7.2.1 Main objective of the future outlook

Lipid microarrays offer a programmable cell membrane-mimicking interface to influence cell dynamics. In here, the extracellular matrix (ECM)-functionalized lipid membrane will be explained as a hybrid nanostructures [8-11]. Importantly, live single-cell imaging experiments demonstrate that lipid membrane platforms support reconstitution of dynamic cell behavior, including cell-mediated degradation and endocytosis of ECM proteins—features which are not observed with cell culture on glass. Because the ECM is intricately involved in many key cellular processes, the platform enables us to investigate the connection between cell dynamics and ECM degradation as well as diseases such as fibrosis, arthritis, and cancer which are related to abnormal ECM degradation [12, 13]. The main future outlook is that an extracellular matrix-lipid membrane hybrid biodevice can enable the reconstitution of cell dynamics associated with normal and irregular ECM

degradation behaviors. In order to test the hypothesis, we seek to achieve the following specific aims:

Aim 1. To develop an extracellular matrix-lipid membrane biodevice for tunable cell morphological behavior using SALB method

Aim 2. To correlate healthy versus abnormal ECM degradation with cell dynamic patterns.

Aim 3. To develop scanning probe microscopy for the physical assessment (morphological and mechanical properties) of cells and tissue with the originally designed microfluidic chamber for AFM

Based on these aims, the state-of-the-art lipid membrane fabrication tools will be employed along with corresponding surface-sensitive measurement techniques (e.g., quartz crystal microbalance, fluorescence microscopy) in order to establish the ECM-lipid membrane hybrid biodevice and determine effects of ECM composition and mechanical properties on cell dynamics. Furthermore, scanning ion conductance microscopy (SICM) and atomic force microscopy (AFM) using the originally developed microfluidic chamber will be utilized in order to monitor the dynamics of live, single cells, cell populations, and even tissue on the hybrid biodevice platform in conjunction with fluorescence microscopy. Taken together, this future outlook plan will establish a functional biodevice to reconstitute dynamic behaviors of cell-ECM interactions which require a lipid membrane interface. The main objectives of this research collectively focus on the development of engineered biomimetic interfaces for reconstitution of cellular dynamics as well as to explore noninvasive nanotechnology tools to characterize these interfaces, especially with regards to cellular interactions. Within this scope, a bottom-up approach will be employed to design biomaterials comprised of phospholipids and proteins with desired properties. Building on these platforms, a key focus is cell-ECM interactions which influence cell fate through a variety of biochemical and mechanical responses. The application of emerging tools to probe biological systems on the nanoscale is a complementary track to link attempts to control cell behavior with biophysical methods to establish correlations with functional

output. Hence, the collection of projects being pursued will support the development of methods for *in vitro* reconstitution and real-time monitoring of cell dynamics.

7.2.2 Extracellular Matrix (ECM) – Functionalized Lipid Membrane

The principal goal in this future outlook is to further optimize our protocol to prepare solid-supported model membranes functionalized with different ECM proteins using our recently developed solvent-assisted lipid bilayer (SALB) formation method [14-16], which does not require lipid vesicles. The SALB method involves lipid molecule deposition onto the solid surface in the presence of water-miscible organic solvents (*e.g.*, isopropanol) and subsequent solvent-exchange to aqueous buffer solution to induce spontaneous formation of a supported lipid bilayer. The various compositions of lipid mixtures has been tried and include a functional lipid which carries a free carboxylic acid to facilitate ECM protein conjugation. ECM proteins are classified as fiber-forming and non-fiber-forming molecules. The quartz crystal microbalance-dissipation (QCM-D) [17] and atomic force microscopy (AFM) [18, 19] will be used in order to verify the conformation of conjugated ECM components. When designing engineered biomimetic interfaces, one of the key challenges is to promote cell-ECM interactions that have a suitable combination of biochemical and mechanical cues. To this end, the strategies to create artificial ECM proteins with desired properties based on the modularity of protein domains will be outlined. Furthermore, cross-linking and mutation strategies will focus on tuning the mechanical properties of ECM protein thin films in order to not only promote cell attachment but also influence cell fate. The combination of improved cell adhesion and mechanical properties will prove particularly useful when these proteins are integrated within model membrane platforms to influence cell-material interactions.

7.2.3 Effect of ECM Remodeling on Stem Cell Growth and Differentiation, Morphogenesis, and Development

Another principal goal in this sub-project is to build model membrane platforms that induce and expand stem cells down a signaled pathway into stable, end-stage phenotypes

compatible with therapeutic needs. Because the ECM is known to influence chondrogenic differentiation by mesenchymal stem cells (MSCs) which are in turn sensitive to alterations in compressive elasticity, ECM layers will be fabricated with varying composition and elasticity and examine the effects of these mechanical properties on proliferative capacity and chondrogenic differentiation of MSCs [9, 12]. While existing tissue engineering platforms permit functionalization with extracellular matrix (ECM) proteins, these platforms do not allow mechanical properties of the collagen layer to be adjusted within a biologically relevant membranous environment. Our model membrane technology will provide the first platform to explore how the mechanical properties of membrane-associated ECM proteins are involved in regulating stem cell growth and differentiation, morphogenesis, and development. Importantly, these platforms take advantage of self-assembly fabrication and covalent immobilization so they can be adapted to meet high-throughput, large-scale production needs. In so doing, the expectation of this future outlook is to identify an optimal set of conditions for promoting efficient chondrogenic differentiation of MSCs in a commercially viable format.

Figure 7.1. ECM-functionalized membrane platform. (A) Schematic illustration of a supported bilayer prepared by the SALB method and coated with ECM matrix. (B) QCM monitoring of adsorption kinetics of collagen type I conjugation to a functionalized supported lipid bilayer containing various fractions of DP-NGPE lipid. (C) Standard curve of collagen binding capacity versus fraction of DP-NGPE. The linear correlation between the fraction of DP-NGPE included in

the bilayer and the amount of collagen allows easy control of the matrix coverage. (D) Fluorescence micrographs showing collagen coverage as a function of DP-NGPE percentage included in the SLB.

7.2.4 Live Cell Imaging and Analysis

The novel nanotechnology tools will be demonstrated how can be applied to characterize cellular phenotype, analyze the distribution of cell surface markers, and monitor the dynamics of cell surface projections. Whereas traditional methods to study cell dynamics occur after cell fixation or destruction during sample preparation, the scanning ion conductance microscopy (SICM) and atomic force microscopy (AFM) will be employed to noninvasively scan the surface of living stem cells and investigate cellular activities under physiologically relevant conditions with nanometer resolution using originally designed microfluidic device that enables SALB formation technique [18]. This capability is unmet by any other analytical technique available today, and is very important for quality control of cell-based therapies. In turn, the combination of morphological characterization of live cells by SICM and AFM combined with biochemical analyses will help monitoring the effect of ECM remodeling on cell fate as well as stem cell differentiation.

7.2.5 Novelty of the approach

SLBs provide a simple and robust model surface for studying the interaction between ECM proteins and cells [20]. SLBs can be prepared via several established techniques and the bilayer composition can be varied to control several factors such as electric charge and mobility. The lipid bilayer platform can be readily functionalized with various cell attachment ligands and membrane associated biomacromolecules such as proteins, peptides, or carbohydrates. In contrast to glass and tissue culture polystyrene, which promote nonspecific protein adsorption, SLBs made of natural lipids such as phosphatidylcholines (PC) are resistant to protein and cells [21]. Thus, SLBs enable studying selective interactions between cells and specific ECM components. One of the important benefits of employing SLB as a culture platform is that the density of matrix

proteins can be systematically controlled by modulating the degree of functionalization. Therefore, one can easily control the amount of absorbed ECM protein layers. Another advantage of SLB is to provide a platform that allows us to tune the stiffness of the conjugated ECM proteins without changing the stiffness of supported lipid bilayer platform which is one of the important factors to control stem cell fate. Additionally, this platform can be characterized by a wide range of surface-sensitive methods such as QCM-D, total internal reflection fluorescence microscopy (TIRF), and AFM. For instance, formation of SLB as well as the subsequent adsorption of ECM proteins to the SLBs can be monitored in real time by QCM-D. The structure of the ECM protein in the adsorbed state can be visualized by AFM with originally designed microfluidic chamber. TIRF can be used to evaluate the SLB properties such as homogeneity and fluidity and the subsequent changes in those properties upon ECM binding and cell attachment can be monitored. By using a combined experimental and theoretical approach to investigate how combination of biochemical and mechanical properties of ECM proteins influences mammalian cell fate, we will develop fundamental understanding of how to control pathways of assemblies of an underlying bilayer platform and in turn this will provide a general framework to design model membrane platforms on solid supports for cell culture and dynamic analysis applications.

7.2.6 Future applications and potential exploitations

Current technologies behind conventional cell culture platforms are a significant hurdle in the pharmaceutical drug development field in general due to the inability to study cell components in an authentic membrane environment. Recognizing the gap between current cell culture platforms and pressing market needs, our team has recently invented and patented a new ECM-functionalized cell culture platform that overcomes all these technical hurdles. It streamlines ECM-functionalized fabrication into a simple and quick process that is compatible with conventional cell culture platforms and enables programmable, high-throughput construction of array libraries. The potential expectation is by combining expertise with the analytical characterization capabilities and superior technical capabilities into the development of artificial ECM-functionalized cell membrane

platforms, which can be manufactured in a variety of configurations for different measurement approaches commonly applied in research and clinical fields.

The technical objectives towards commercialization are: i) to establish the generality of the microarray platform for molecular detection with common measurement readouts; and ii) to develop optimal procedures for the fabrication and storage of microarray platforms, including one-step assembly and long-term stability. Taken together, successful accomplishment will deliver an integrated platform technology that is ready for full-scale development and testing in operational settings. This future outlook plan will lead to the launch of a superior technology for artificial cell membrane microarrays that is superior in performance, with novel functionalities and easier manufacturing as compared to competitor technologies in the field. This technology will be widely applicable to scientific and clinical researchers in need of analytical platforms for investigating lipids, membrane proteins, and sterols—all ubiquitous elements in human health and disease. The commercial application of this project will be in the area of microarray technology for use in molecular diagnostics, drug screening and development, and profiling.

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List of Publications

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