**ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY**

# INVESTIGATION OF THE EFFECT OF DIFFERENT ORGANIC SOLVENTS ON SOLID SUPPORTED LIPID BILAYERS VIA ATOMIC FORCE MICROSCOPY

**M.Sc. THESIS**

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**Department of Advanced Technologies**

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**İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ**

# FARKLI ORGANİK ÇÖZÜCÜLERİN YÜZEY DESTEKLİ LİPİT KATMANLAR ÜZERİNE ETKİLERİNİN ATOMİK KUVVET MİKROSKOBU İLE ARAŞTIRILMASI

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***To my family,***

***Emine***

***Mehmet Emir***

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**ABBREVIATIONS**

**(v/v)** : Volume/Volume
**ACE** : Acetone
**AFM** : Atomic Force Microscopy
**BLMs** : Black Lipid Membranes
**EtOH** : Ethanol
**f** : Resonance frequency (Hz)
**F-D** : Force-Distance Curve
**IPA** : Isopropanol
**LMV** : Large Multilamellar Vesicles
**LUV** : Large Unilamellar Vesicles
**MLV** : Multilamellar Vesicles
**MVV** : Multivesicular Vesicles
**OLV** : Onion-like Vesicles
**PBS** : Phosphate Buffered Saline
**PC** : Phosphatidylcholine
**SLBs** : Solid Supported Lipid Bilayers
**SUV** : Small Unilamellar Vesicles
**Tc** : Transition Temperature

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# INVESTIGATION OF THE EFFECT OF DIFFERENT ORGANIC SOLVENTS ON SOLID SUPPORTED LIPID BILAYERS VIA ATOMIC FORCE MICROSCOPY

## SUMMARY

Biological membranes are selectively permeable systems which cover surfaces of living cells, and provide transfer of molecules between intracellular and extracellular regions. Due to their complex structure and their duties on the cell, it is really difficult to observe them in their biological environment. Therefore, those structures should be isolated from each other to observe each individual component of the membranes. Those isolated membranes and the components of these membranes should also be stabilized on an arbitrary surface to analyze them in detail.

Model membrane systems (artificial lipid membranes) have been developed to increase their reviewability. Studies on artificial lipid membranes are vital topic for understanding intermolecular interactions and discovering possible biotechnological applications in future.

Artificial lipid membranes are getting significant attention by researchers due to their uniqueness, and their usability in fundamental and applied research. Surface supported biomimetic lipid layers are very crucial systems which allow to investigate the fundamental functions of lipid and protein molecules. Those systems have been utilized for a model to investigate the molecular delivery, the results of enzymatic catalysis experiments, and the cell adhesion extensively. Nowadays, drug-screening platforms, which use membrane as a base, could be considered as possible practical application of those systems.

Atomic force microscopy (AFM) is a powerful tool to characterize the surface within nanoscale. It can be used in different medium like in liquid, vacuum or air. As used in the liquid medium, it has grown into an important provider to physiological and biological cell research. A thin needle is incorporated into AFM systems and through that needle, the surface is scanned and its topography is attained.

Understanding the mechanisms ruling the biomembrane's resistance to solubilization by organic solvents are crucial in biochemical research. In this thesis, the AFM imaging used to visualize the actions of a model supported lipid bilayer in the presence of different organic solvents at two different concentrations. AFM provides a unique tool to monitor the nanostructure of supported lipid bilayer membrane (SLBs) in their physiological conditions. It can probe the real-time events, like dissolution process of lipid bilayers.

The composition of SLBs on the mica surface and deformation /desintegration of SLBs by organic solvents were observed by AFM. Ethyl alcohol (EtOH), isopropyl alcohol (IPA), chloroform and acetone (ACE) were used in this study. In order to observe the effects of solvents on lipid membranes, each organic solvent was used at two different concentrations (10% and 25% v/v) and results were obtained by AFM.

In this study, single type phospholipid, phosphatydylcholine (PC), was used. Constructed bilayer was in the liquid phase at room temperature and it was observed that sweeping out the movement of lipids within the membrane was possible by scanning probe (Cantilever). In order to compose the PC lipid membrane, liposomes were produced by thin lipid/extrusion approach, and then, they were transferred on the mica surface where the lipid bilayers were formed, and analyzed with AFM.

During the analysis of mica surface by AFM, many challenges were faced. Firstly, due to the rapid evaporation of some organic solvents, such as chloroform, liquid volume and concentration of the medium changed, which led to the deviation and shift in laser position on the photodetector. Therefore, any effect of chloroform on bilayer desintagration could not be observed. This problem might occur due to the sudden evaporation of chloroform which leave PBS, a non-solvent of lipids, in the medium.

In conclusion, in this study, the composition of supported lipid bilayer on mica surface and also its desintagration/dissolution by organic solvents were monitored, step by step. Conducted analysis during this research revealed that, while some of the organic solvents had more pronounced effects on dissolution of supported lipid bilayer, others had no significant effect.

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## ÖZET

Biyolojik membranlar, canlı hücrelerin dış yüzeyini saran, seçici geçirgen, hücre içi ve dışı arasındaki iletişimi sağlayan özel bölgelere sahip membran sistemleridir. Biyomembranların bu karmaşık yapı ve görevlerinden dolayı kendi biyolojik ortamlarında incelenmesi oldukça zordur. Dolayısı ile, membran veya membran bileşenlerinin incelenebilmesi için bu yapıların izole edilmiş olması gerekmektedir. İzole edilen membran veya bileşenlerinin incelenebilmesi ve analizlerinin yapılabilmesi için bazı yüzeylere sabitlenmesi gerekebilmektedir. İşte bu noktada membranların incelenebilir olması için model membran sistemlerinin (yapay lipid membran) geliştirilmesine ihtiyaç duyulmuştur. Yapay lipid membran çalışmaları, biyolojik zarların yapısında yer alan moleküler olayları kavramak ve olası biyoteknolojik uygulamaları keşfetmek için büyük önem taşımaktadır.

Yapay lipit membranlar benzersiz özellikleri nedeniyle, her geçen gün daha fazla ilgi çekmekte olup, temel ve uygulamalı araştırmalarda kullanılmaktadır. Yüzey destekli biyomimetik lipid katmanlar; biyolojik membranlar ve onları oluşturan lipid ve protein moleküllerinin temel özelliklerini araştırmak için önem arz eden model membran sistemleridir. Bu sistemler, yaygın olarak biyolojik membranların özelliklerinin incelenmesi, moleküllerin tanınması, enzimatik kataliz deneyleri, hücre yapışması ile ilgili işlemlerin özelliklerini araştırmak için bir model olarak kullanılır. Ayrıca son zamanlarda membran proteinlerini temel alan ilaç görüntüleme platformları oluşturma gibi çok sayıda pratik uygulamanın olabilirliğini de bizlere sunmaktadır.

Atomik kuvvet mikroskobu (AKM), yüzey görüntüleme ve karakterizasyonu için geliştirilmiş çok önemli bir araçtır. AKM; hava, sıvı, vakum ortamı gibi çeşitli ortamlarda kullanılabilir. AKM yöntemi yalıtkan örneklerin özelliklerini bozmadan inceleyebilmeye olanak sağlayan yöntemlerden biridir. AKM ile örnekleri sıvı ortamda incelemek mümkündür. Sıvı ortamda kullanılabilmesinin biyolojik araştırmalar bakımından önemi oldukça fazladır. Çünkü bu yöntem, biyolojik yapıların kendi fizyolojik ortamlarındaki aktivitelerinin yüksek çözünürlükte incelenmesine olanak sağlayan tek yöntemdir. Son gelişmelerle birlikte yüksek kuvvet hassasiyeti ile yüksek hızda ortamı 3 boyutlu olarak analiz etmek AKM yöntemi ile mümkün olmaktadır. Ayrıca AKM yöntemi ile biyolojik moleküllerin dinamik yapı değişimini ve etkileşimlerini incelemekte mümkündür. AKM yönteminin bu özellikleri nedeniyle sıvı ortamda biyolojik yapıları inceleyen bilim insanlarını heyecanlandırmaktadır.

AKM tekniklerinde, sivri bir iğne bulunur ve bu iğne ile yüzey taranarak yüzeye ait topografi ve çeşitli özellikler (iletkenlik, esneklik, 3 boyutlu görüntü vb.) elde edilir.

AKM iğnesi yüzey üzerinde gezdirildiğinde örnek yüzeyi topoğrafisindeki değişiklikler, fotodedektör sensörde elde edilen sinyalin değişimine sebep olur. AKM tekniğinde yüzey ızgara (raster) tarama tekniği ile taranır. Yay ile yüzey arasındaki etkileşim kuvveti sabit tutulacak şekilde sensör dikey olarak hareket ettirilir ve bu hareket 2 boyutlu bir dizide tutularak her bir veriye karşılık renk değeri atandığında 3 boyutlu yüzey topoğrafi görüntüsü elde edilmiş olur

Bu çalışmada tek çeşit lipit (fosfatidilkolin-PC) kullanılmıştır. Kullanılan lipidin geçiş sıcaklığı (Tc) -10ºC olup oda sıcaklığında (22-24ºC) sıvı fazdadır. PC lipid membranların oluşturulmasında, lipozom (100 nm) yapıları içeren tampon çözeltisi, ince lipit film/ekstrüzyon yöntemi ile üretildikten sonra mika yüzeyine transfer edilmiştir. Mika yüzeyine transfer edilen çözelti, yüzeyde yüzey destekli lipid katman (SLBs) yapılar oluşturmuş olup analizlerde kullanılmıştır.

Bu çalışmada, mika yüzeyinde SLBs membranlar oluşturularak, bazı organik çözücülerin SLBs membranlar üzerine etkileri incelenmiştir. Mika yüzeyinde oluşturulan SLBs membranların tekrar organik çözücüler aracılığı ile çözünmesi esnasında ve sonrasında AKM tekniği ile yüzey 3 boyutlu olarak görüntülenmiştir. Deneylerde kullanılan organik çözücüler etil alkol (EtOH), izopropil alkol (IPA), kloroform ve asetondur (ACE). Bu organik çözücülerin, yapay lipid membranlar üzerine etkilerinin araştırılmasında her organik çözücü için farklı iki konsatrasyon (%10 ve %25) denenmiş olup deney sonuçları AKM ile görüntülenmiştir.

Çalışmalara ilk olarak SLBs membrane oluşumunun görüntülenmesi ile başlanmıştır. Lipozom içeren fosfat tampon çözeltisi (PBS), temiz mika yüzeyine damlatıldıktan hemen sonra AKM mikroskobu ile mika yüzeyi taranmaya başlanmıştır. Tarama 128 dakika boyunca sürmüş olup her 8 dakikada bir götüntü alınmıştır. Ilk 48 dakika boyunca yüzeyde herhangi bir SLBs oluşumu gözlemlenmezken 64. dakika ile 88. dakika arasında taranan alanın hemen hemen tümünün SLBs ile kaplandığı görülmüştür. İlk 48 dakika boyunca mika yüzeyinde SLBs membran oluşmamasının iki farklı nedeninin olabileceği düşünülmektedir. Bunlardan biri, oluşturulan lipozomların PBS içerisinde askıda kaldığı ve zamanla mika yüzeyine inerek yüzeyde SLBs membran oluşturduğu fikri, diğeri ise tarama esnasında AKM iğnesi ve mika yüzeyindeki etkileşimden dolayı tarama alanında SLBs membran oluşum sürecinde AKM iğnesi ile tekrar süpürülerek yüzeyden atılması fikridir. 48. dakikadan sonraki zaman diliminde ise yüzeyde fazla miktarda lipozom yapısının parçalanması dolayısı ile SLBs membran oluşumu söz konusu olmuştur.

Mika yüzeyinde SLBs membran oluşumu esnasında görüntüleme yapılmasının ardından organik çözücülerin (EtOH, IPA, kloroform ve ACE), SLBs membran çözünmesi üzerine etkileri ayrı ayrı araştırıldı. Bunun için her deneyde ilk olarak mika yüzeyinde SLBs membran oluşup oluşmadığı AKM ile görüntülendi. Yüzeyde SLBs membran oluşumunun görülmesinin ardından ortama her deney için ayrı konsatrasyonda organik çözücü (%10 ve %25) eklenerek yüzeyler tarandı.

AFM ile mika yüzeyindeki lipidlerin analizi sırasında birçok problem ile karşılaşılmıştır. Bunların başında, fosfat tampon çözeltisi (PBS) içerisine eklenen

organik çözücülerin tarama esnasında buharlaşması sonucunda, sıvı ortamın yoğunluğu sürekli değişerek lazer ışığında sapmalara/veya kaymalara neden olmuştur. Bu da alınan görüntülerin kalitelerini etkilemiştir.

Bu çalışmada mika yüzeyinde oluşturulan SLBs membran yapıları bazı deneylerde hemen hemen tüm mika yüzeyini tamamen kaplarken bazı deneylerde ise adacıklar halinde mika yüzeyini kapladığı görülmüştür. Bu durumun nedeni araştırıldığında boş mika yüzeyindeki alt etki alanlarının veya delik/defektlerin olmasından dolayı adacıklar halinde SLBs membran yapıların oluştuğu görülmüştür.

Organik çözücülerin SLBs membranları çözme mekanizmaları; fosfolipid molekülü temel yapısında bulunan su seven (hidrofilik) baş ve su sevmeyen (hidrofobik) hidrokarbon zinciri ana grupları arasına girerek, bu aradaki etkileşimi kesmesi sonucunda SLBs membranda yapısal bir bozukluğua neden olarak organik çözücü içerisinde çözünmesi şeklindedir.

Sonuç olarak, bu çalışmada, lipozomların mika yüzeyinde SLBs oluşturması ve organik solventler ile çözünmesi adım adım görüntülenmiştir. Yapılan analizler sonucunda bazı organik solventlerin SLBs çözünmesi üzerine çok etkili olduğu görülmüştür.

Yapılan bu çalışma neticesinde organik çözücülerin SBLs membran yapılar üzerinde etkili olduğu görülmüştür. Kloroformun PC çözünmesi üzerinde etkili bir çözücü olduğu bilinmesine rağmen bu çalışmada, kloroformun etkileri eklenen çözücünün ortamdan hemen buharlaşması veya PBS içerisinde çözünememesi nedeniyle kloroform-SLBs etkileşiminin olmamasından dolayı gözlenememiştir. Diğer üç organik çözücü (ETOH, IPA, ACE) incelendiğinde, her üç çözücününde SBLs çözünmesi üzerinde çözme etkisinin olduğu görülmüştür.

Yapılan analizler sonucunda SLBs çözünmesi üzerine en etkili organik çözücü ACE olup en az etkiye sahip çözücünün ise EtOH olduğu gözlemlendi. Kloroform PC molekülünü çözen temel çözücülerden biri olmasına ragmen kloroformun etkileri ise gözlemlenemedi.

## 1. INTRODUCTION

Nanotechnology is a multidisciplinary field, that covers a vast and diverse array of devices from physics, engineering, biology, and chemistry (Sahoo et al., 2006). It is the procedure of controlling matter at the scale of atom and particles (Drexler et al., 1986). In 1974, Norio Taniguchi utilized the expression "nanotechnology" surprisingly while portraying an ion sputter machine. The expression "nano" was initially came from Greek word.

Nanotechnology works with materials, devices, and other structures with at least one dimension sized from 1 to 100 nanometres. Nanometer is defined as one billionth of a metre that is equivalent to the length of ten hydrogen atoms. Nowadays nanotechnology has a global interest. As mentioned in environment and green nano, the term nanotechnology embraces various fields and specialities including green nanotechnology, wet nanotechnology, nanoengineering, nanobiotechnology.

The nanoparticle is structural and functional unit of nanotechnology. It is characterized as a small object which behaves as a whole unit with respect to its transport and properties. Particles are further classified into three group based on their diameter; which are coarse particles (10,000-2,500 nm), fine particles (100-2500 nm) and ultrafine particles (1-100 nm) (Granqvist et al., 1976). For example, liposomes are a form of nanoparticles prepared from phosphatidylcholine (PC) and PC is classified as fine particle.

The phospholipids are the main components of all cell membranes. They form lipid layers. Phospholipids are a kind of lipid mainly consisting of diglyceride and phosphate group. The structure of the phospholipid molecule consists of hydrophilic head and hydrophobic tails. Phosphatidylcholine from egg yolk was the first discovered phospholipid. Other common phospholipids are phosphatidic acid, phosphatidyethanolamine, phosphatidylserine, phosphoinositides, etc. Phospholipid synthesis is formed in the cytosol which is adjacent to endoplasmic reticulum (Fahy et al., 2009).

The narrative of achievement of liposomes was started by Bangham and his coworkers in the mid 1960s. They observed that smears of egg lecithin responded with water to frame entirely amazing structures. They were dissected by electron microscopy demonstrating that a huge number of vesicles were shaped suddenly. These pretty much homogenous lipid vesicles were initially called smectic mesophases (Bangham, 1972). Later on, a partner of Bangham termed them liposomes (Sessa and Weissmann, 1968).

Liposome is an artificial microscopic single vesicle, consisting of an aqueous core enclosed in one or more phospholipid layers. They are used for drug delivery sytems, gene delivery, convey vaccines, enzymes etc. to target cells or organs (Lawerence et al., 1986). Liposomes are a form of nanoparticle prepared from phosphatidylcholine. They are microscopic/nanoscopic and concentric bilayered vesicles.

**Purpose of Thesis**

This study is focused on the development of Atomic Force Microscopy (AFM) methods to investigate the supported lipid bilayers (SLBs) with different organic solvents during the formation and deformation. These biological structures (SLBs) have found wide application area as models of cellular membranes. They consist of phospholipid molecules that can be self organise into bilayer structures containing phase-separated microdomains, which play an important role in many biological processes. SLBs are well defined and stable under a variety of conditions, allowing characterisation with a broad range of physical methods.

Liposomes are now most clinically established nanometer scale delivery systems by the outstanding profile over the other systems due to their biocompatibility, biodegradability, reduced toxicity and capacity for size and surface manipulations (Al-Jamal and Kostas, 2007).

Biological membranes are crucial in cell life as being selectively permeable barriers and having special sites of communication between the inside and outside of the cellular worlds. Because of the complexity of biomembranes, there is a clear need to develop model membrane systems, where one or a few membrane components can be isolated and studied. Therefore, artificial lipid membrane studies are crucial to comprehend the molecular events taking place at biological membranes and to explore

possible biotechnological applications. These studies must sustain the structure, fluidity of the lipid bilayer and the physiological reality, as well as the structure and function of reconstituted membrane proteins (Richter et al., 2003; Volker et al., 2008).

The current knowledge of the molecular processes occurring at biological membranes mainly based on the studies with the biological membrane models including liposomes, giant vesicles in solution, lipid monolayers, black lipid films, lipid bilayers between two aqueous phases and solid supported membranes (Richter et al., 2003).

## 2. BACKGROUND INFORMATION

### 2.1 Cells & Biological Membranes

All living organisms consist of cells and they are surrounded by a membrane. The membranes physically separate the interior components of the cell from its environment. Single cell organisms, which are arguably the smallest units of life, include bacteria and achaea, while multi cellular organisms, such as plants and animals, consist of billions of cells. The shape, size and composition of cells vary according to their function, but one ubiquitous structure is the plasma membrane. Plasma membranes consist of different phospholipids arranged in a bilayer, which encapsulates the cytoplasm. In eukaryotic cells, the cytoplasm contains different functional sub compartments known as organelles. The bilayer matrix of the plasma membrane contains vital components such as cholesterol as well as integral proteins. Additionally, the surface of some plasma membranes are decorated with glycolipids and glycoproteins that are involved in a variety of cellular processes such as cell adhesion, cell signalling and ion conductivity. Thus, the plasma membrane is a quite complex system, a "mosaic" of different molecules, and owing to lateral fluidity of the bilayer matrix it is commonly referred to as a" fluid mosaic".

The lipid membrane, consists of a double layer of lipids, known as a phospholipid bilayer. It separates and protects the cell from its surrounding environment (Singer and Nicolson, 1972). Lipids are hydrocarbon containing organic compounds and there are two types, saturated and unsaturated. When combined in a bilayer, these different types of lipids organise themselves into phase separated areas known as microdomains (Edidin, 1997). These regions play a central role in the workings of a cell membrane. They feature in many biological processes, have been associated with membrane proteins. They are possible entry points for toxins (Simons and Ikonen, 1997; Engelman, 2005). For this reason, it is a great interest to study the properties of different lipids and learn more about their form.

## 2.2 Phospholipids & Lipid Bilayers

Phospholipids are a kind of lipids which are a major component of all cell membranes. Due to their amphiphilic characteristic, they can form lipid bilayers. The structure of the phospholipid molecule generally incorporates into two hydrophobic fatty acid "tails" and a hydrophilic phosphate "head", joined together by a glycerol molecule. The phosphate groups can be fitted with simple organic molecules such as choline (Figure 2.1).

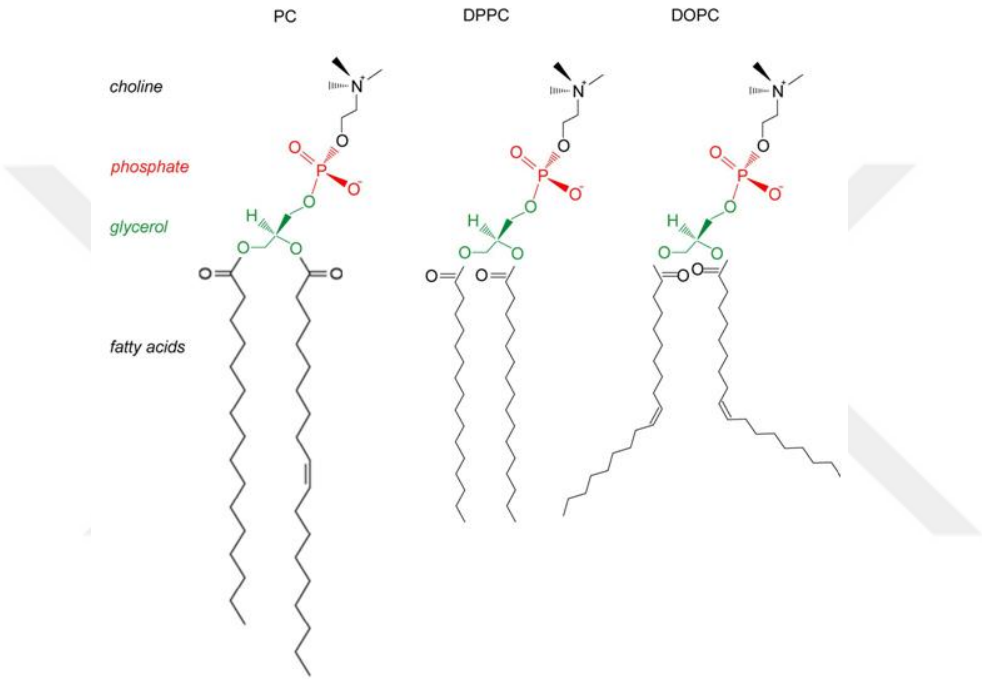


**Figure 2.1 :** Chemical structure of some neutral phospholipids

(Adapted from Sweetenham, 2011).

In the case of phospholipids, the ‘head’ contains a negatively charged phosphate group and glycerol making it highly polar and hydrophilic. Conversely, the ‘tail’ is very hydrophobic and usually consists of two long fatty acid chains. The tails are repelled by water and forced to aggregate. These specific properties allow phospholipids to play an important role in the phospholipid bilayer. In biological systems, the phospholipids often exist with other molecules (e.g., proteins, glycolipids, sterols) in a bilayer such as a cellular membrane.

Forces such as van der Waals, electrostatic, hydrogen bonds and non-covalent interactions all contribute to the formation of a lipid bilayer. However, hydrophobic

interactions are the major driving force in this process. The assembly of a lipid bilayer under these interactions gives the cellular membrane its protective nature. The arrangement of hydrophilic 'heads' and hydrophobic 'tails' in a lipid bilayer means that it has very low permeability for ions and most polar molecules. It prevents many polar solutes such as amino acids, nucleic acids, carbohydrates, proteins and ions from diffusing across the membrane, whilst allowing the passive diffusion of small hydrophobic molecules. This gives the cell the ability to regulate the movement of these substances via transmembrane protein complexes such as pores and gates. The fluidity of the bilayer means that these structures are free to move about laterally.

The most typical natural phospholipid is the phosphatidylcholine (PC), which is an amphipathic molecule, and isolated mostly from animal (chicken egg yolk). Its primary role is to provide a structural framework for the membrane and maintain the permeability barrier with some other proteins. It also plays a role in cell signaling.

Supported lipid bilayers (SLBs) provide a biologically relevant model for cell membranes. The ability to manipulate and modify the makeup and environment of these lipid structures enables a fair representation of the cellular system being depicted. Recently, SLBs have became a 'hot topic' for scientific research. It has been studied with a wide variety of microscopy and spectroscopy methods. On the other hand, the physical and chemical properties of lipid bilayers and microdomains are still unclear (Engelman, 2005).

### 2.2.1 Investigation of SLBs

One of the foremost techniques used for sample characterisation in a range of research fields is atomic force microscopy (AFM). The AFM can measure atomic forces between a probe and surface to provide high resolution images and mechanical information at the nanoscale. AFM has been used extensively to analyse lipid bilayers (El Kirat et al., 2010). It has been used to visualise the formation of SLBs from vesicles (Schönherr et al., 2004) and study their structure and stability (Hui et al., 1995) mechanical (Steltenkamp et al., 2006; Schneider et al., 2000) and electrical properties (Jeuken, 2008). AFM has been used to monitor the inclusion of biomolecules and proteins (Kumano et al., 2010) and the effect of external agents (Dekkiche et al., 2010). A number of studies have also focused on the existence of microdomains (Giocondi et al., 2010). Electron microscopy techniques such as scanning electron microscopy

(SEM) and transmission electron microscopy (TEM) have also been used to image SLBs (Lee et al., 2002) often combined with other methods such as AFM (Ruspantini et al., 2001). However, these microscopy techniques offer no chemical specificity. On the other hand, mass spectrometry has been employed to analyse the chemical composition of lipid bilayers (Hebling et al., 2010; Gunnarsson et al., 2010), but this requires invasive procedures. Nuclear magnetic resonance (NMR) spectroscopy is often used to investigate the structure of lipid bilayers and also their interaction with proteins (Lee et al., 2008).

### 2.2.2 Self organization of biomimetic membranes

Due to the amphiphilic nature of phospholipid molecules, a fast and automatically association of the molecules occur when they are transferred into a watery solution. Thus, when they are put in water or water based solution, phospholipids spontaneously arrange to point that their ‘tails’ inwards to water and their ‘heads’ outwards from the water, so that a membrane is formed. That self-organization of the phospholipids results in the formation of the bilayer structure (Figure 2.2) (Gazit, 2007).

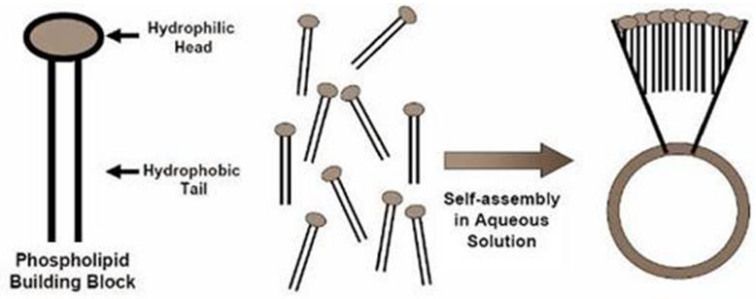


**Figure 2.2 :** The formation of liposome membranes by the self-assembly of phospholipids in aqueous solution.

### 2.3 Lipid Vesicles

In a watery system, depending on the concentration, chemical structure and experimental conditions of the lipid, clusters of lipids form monolayer, micelle, lipid bilayer and liposome structures. Phospholipids with two or more alkyl chains do not form micelles but organize into bilayer structures. Liposomes are artificial lipid vesicles defined as lipid bilayers surrounding one or more internal aqueous compartments (Figure 2.3) (Zhang and Guihua, 2011).

- **Monolayer:** A single layer film of lipids at an air-water interface formed by compression in a Langmuir-Blodgett (LB) trough (a);
- **Micelle:** A colloid of lipids in an aqueous suspension that is hydrophilic on the outside and hydrophobic on the inside (b);
- **Bilayer:** The natural, energetically-favoured form that lipids take in an aqueous environment (c);
- **Liposome, or Vesicle:** The spherical form of a bilayer with an aqueous core (d).

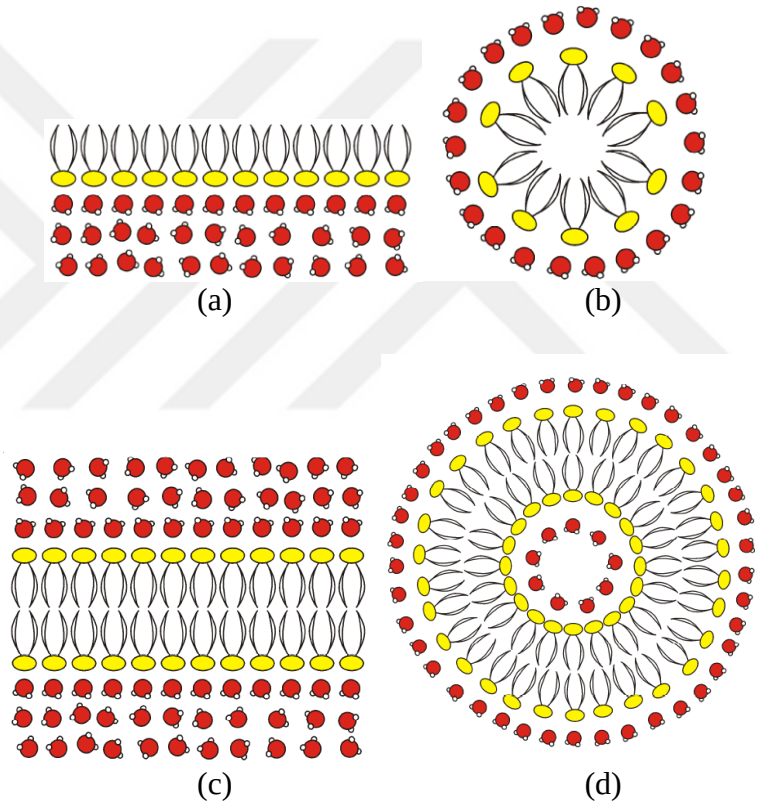


**Figure 2.3 :** Different arrangements of phospholipids

(a) Monolayer, (b) Micelle, (c) Bilayer, (d) Liposome or vesicle (not to scale) (Adapted from Sweetenham, 2011).

### 2.3.1 Liposome preparation

Altough there are many different properties of lipid formulations, depending on the composition (cationic, anionic, neutral lipid species) (Table 2.1), same preparation method can be used for all lipid vesicles in any case of composition. The general elements of the procedure involve preparation of the lipid for hydration, hydration with agitation, and sizing to a homogeneous distribution of vesicles (URL-1).

Liposomes can be prepared with a diversity of phospholipids (natural, modified or synthetic). The most widely used lipids are phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS). They are used either individually or in combination with cholesterol. Cholesterol allows the close packing of phospholipids in bilayers even above the transition temperature (Tc). It also reduces the permeability by filling up the holes or disruptions of the bilayers to the encapsulated compounds. Cationic lipids, like stearylamine, are usually used in order to introduce a surface charge to the liposomes (Vasant and Ranade, 2004).

**Table 2.1 :** Some of the lipids used in vesicle preparation (Wagner, 2011).

| | |
|---|---|
| **Positively charged (Anionic)** | 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) |
| | 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) |
| **Negatively charged (Cationic)** | Phosphatidylserine (PS) |
| | Phosphatidylinositol (PI) |
| | Phosphatidylglycerol (PG) |
| | Phosphatidic acid (phosphatidate) (PA) |
| **Neutral** | Phosphatidylcholine (PC) |
| | Phosphatidylethanolamine (cephalin) (PE) |
| | 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) |
| | Dipalmitoyl phosphatidylcholine (DPPC) |
| | 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) |

There are many different methodes for the preparation of liposomes. The methodes can be classified into three main groups (Table 2.2). Further methods, such as freeze

thawing, freeze drying, and extrusion, are all based on preformed vesicles (Andreas and Karola, 2011).

The lipid film method is still the easy method for the liposome formation but it is limited because of its low encapsulation efficiency. In this method, phospholipids dissolved in hydrophobic organic solvent. Usually this process is carried out using chloroform or chloroform: methanol mixtures. The solvent is removed to yield a lipid film. After production of dried thin lipid film layer, it must be rehydrate again. The hydration of the dry lipid film is accomplished simply by adding an aqueous medium to the container of dry lipid and agitating the lipid film. So that "onion-like" (OLV), larger micron-scaled multilamellar vesicles (LMV) are formed. These "multi-walled" structures can be transformed in to small unilammelar vesicles (SUV) of single bilayers with sizes that range from few hundreds of nm to 50 nm, upon sonication or extrusion (Figure 2.4) (Gazit, 2007).

**Table 2.2 :** Liposome preparation methods (Wagner, 2011).

| **Method** | **Vesicles** |
|---|---|
| | |
| ***Mechanical methods*** | |
| Vortex or hand shaking of phospholipid dispersions | MLV |
| Extrusion through polycarbonate filters at low or medium pressure | OLV, LUV |
| Extrusion through a French press cell "Microfluidizer" technique | Mainly SUV |
| High-pressure homogenization | Mainly SUV |
| Ultrasonic irritation | SUV |
| | |
| ***Methods based on replacement of organic solvent(s) by aqueous media*** | |
| Removal of organic solvent(s) | MLV, OLV, SUV |
| Use of water-immiscible solvents: ether and petroleum | MLV, OLV, SUV |
| Ethanol injection method | LUV |
| Ether infusion (solvent vaporization) | LUV, OLV, MLV |
| Reverse-phase evaporation | |
| | |
| ***Methods based on detergent removal*** | |

| | |
|---|---|
| Gel exclusion chromatography | SUV |
| "Slow" dialysis | LUV, OLV, MLV |
| Fast dilution | LUV, OLV |
| Other related techniques | MLV, OLV, LUV, SUV |

In ultrasonication method, aqueous dispersion of phospholipids is disrupted with an either bath or a probe sonicator. This procedure will usually yield SUVs with diameters down to 15-25 nm. Similar to the ultrasonication methods, homogenization techniques have been used to reduce the size and number of lamellae of multilamellar liposomes (Andreas and Karola, 2011).

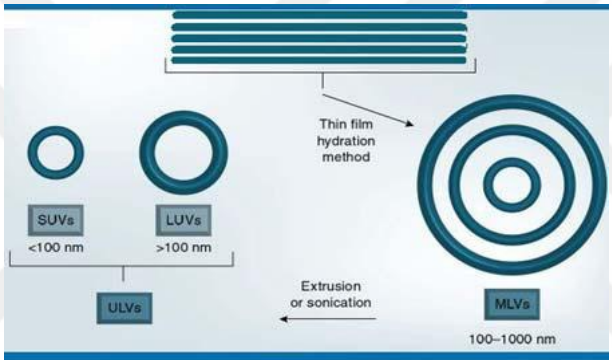


**Figure 2.4 :** Operation and production steps to generate various types of liposomes.

Extrusion technique is the most prominent scalable downsizing method. With this methode the lipid suspension is forced through a polycarbonate filter with a defined pore size to yield uniformed particles. As it is in all procedures for downsizing LMV dispersions, the extrusion process should be done at above the transition temperature (Tc) of the lipid. Below Tc, the lipids have a tendency to form crystalline structures "gel phase" that cannot pass through the membrane pores. Extrusion through filter membrane with 100 nm pores typically yields large, unilamellar vesicles (LUVs) with a mean diameter of 120-140 nm. Mean particle size also depends on lipid composition (Figure 2.5) (URL-1).

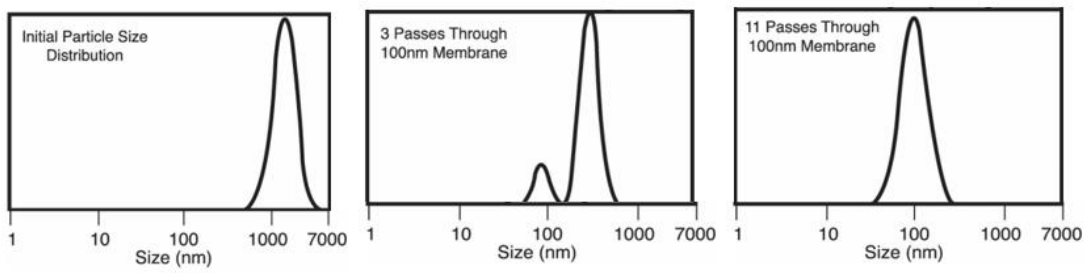


**Figure 2.5 :** Downsizing of liposomes by extrusion method.

A common practice is to subject MLVs to freeze-thaw cycles prior to extrusion, which increases the proportion of unilamellar vesicles in preparations. The freezing and thawing cycle has been shown to cause internal lamellae of MLVs to separate and to vesiculate. This cycle probably decreases the number of closely associated bilayers forced through pores together, thus reducing the formation of oligolamellar vesicles (Gregory, 2007; Kılıc, 2012).

### 2.3.2 Liposome classification

Liposomes can be characterized based on their size, lamellarity, lipid creation, surface charge and usefulness. In this study, one of them is specified.

**Based on their size and lamellarity**

The liposome sizes can be varying between 25 nm to few micrometers. The thickness of a single lamella is around 4 nm. Multivesicular vesicles (MVVs) carry several smaller liposomes inside one big vesicle. Multilamellar vesicles (MLVs), in an "onion-like" arrangement consists of several (up to 14) lipid layers separated from one to another by a layer of aqueous solution. These vesicles are more than hundred nanometers in diameter. Small unilamellar vesicles (SUVs) are surrounded by a single lipid layer and they are 25–100 nm in diameter. Large unilamellar vesicles (LUVs) include very heterogenous group of vesicles as like the SUVs. These are surrounded by a single lipid layer. The diameter of LUVs is very extensive, from 100 nm up to cell size (giant vesicles) (Figure 2.6) (Zhang and Guihua, 2011; Kozubek et al, 2000).

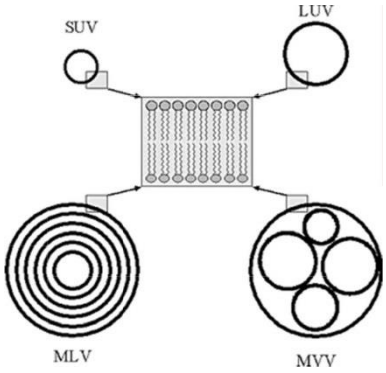


**Figure 2.6 :** Liposomes with different size and number of lamellae.

### 2.3.3 The applications of liposomes

Liposomal vesicles are prepared from various lipid types. The discovery of liposomes leads the scientists to use them as cell membrane models. Also, the ability of liposomes to encapsulate hydrophilic solutes in their internal aqueuos spaces and/or incorporate the hydrophobic molecules within the lipid bilayer was led to the idea of loading and carrying antitumor/antifungal/antimicrobial drugs, genes, enzymes, proteins, vitamins, vaccines, as well as imaging agents into liposomes or polymers or targeting ligands (e.g. antibodies) on their surface (Kozubek et al., 2000; Al-Jamal and Kostas, 2007; Al-Jamal and Kostas, 2007).

Liposomes are now most clinically situated nanometer scale delivery systems by the selected profile over the other systems due to their biocompatibility, biodegradability, reduced toxicity and capacity for size and surface manipulations (Al-Jamal and Kostas, 2007). They are appealing delivery mechanisms in a number of useful applications. They are especially useful for increasing the lifetime of drugs in the blood, targeting and delivering very potent and very toxic drugs to given target locations (David, 2004).

## 2.4 Biomimetic Lipid Membranes

Biological membranes are essential in cell life as being selectively permeable barriers. The membranes having special sites for communication between the inside and outside of the cellular worlds. Due to the complexity of biomembranes, model membrane systems must be developed. Hence, artificial lipid membrane studies are crucial to understand the molecular events taking place at biological membranes and to explore

possible biotechnological applications. These studies must sustain the structure, fluidity of the lipid bilayer and the physiological reality, as well as the structure and function of reconstituted membrane proteins (Richter et al., 2003; Volker et al., 2008).

The current knowledge of the molecular processes occurring at biological membranes are mainly based on the studies with the biological membrane models including liposomes, giant vesicles in solution, lipid monolayers, black lipid films, lipid bilayers between two aqueous phases and solid supported membranes (Figure 2.7) (Richter et al., 2003).

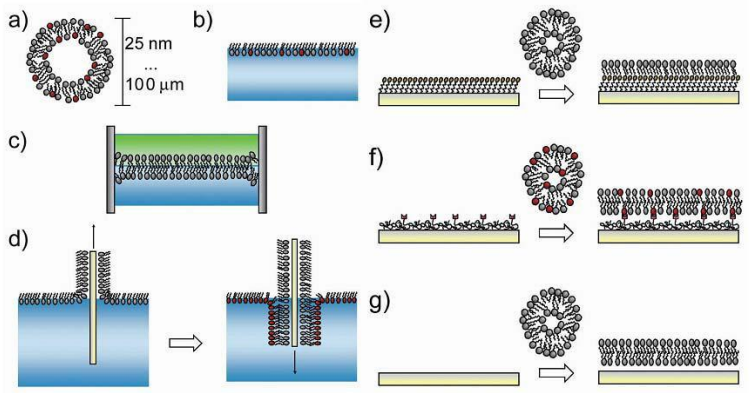


**Figure 2.7 :** Models of biological membranes.

(a) liposomes; (b) lipid monolayers at the air-water interface; (c) black lipid membranes suspended over an aperture between two aqueous phases; (d) Langmuir-Blodgett method allowing the transfer of lipid mono- and multi-layers from the air-water interface to a solid support; (e) self-assembled monolayer on glass/gold/silica surface; (f) polymer cushioned bilayer; (g) spontaneous spreading of liposomes on surface.

**Supported lipid bilayers (SLBs)**

SLBs are flat, two-dimensional and outspread bilayers of the same arrangement as vesicles, that adsorbed on a solid surface such as mica, silicon, silicon dioxide, polymer and quartz surface. SLBs are common studied membrane models which are mimicking the cell membrane. They are formed spontaneously when liposomes in solution encounter a solid and smooth surface like mica.

When the liposomes reach the surface, they follow vary different pathways (Figure 2.8).

Adsorption is the first step for the consequent deformation of liposome (1). If deformation is big enough, some liposomes might rupture (2-5) to form a continuous lipid bilayer on the solid surface. Either vesicles can tear individually (2) or after interaction with other vesicles (3, 4). After coalescence of bilayer patches, SLBs can form a continuous bilayer (5).

The interaction conditions such as smoothness and charge of the solid support between liposomes and solid support are important parameter for formation of SLBs.

Also the composition of the aqueous solution such as charge, size of the liposome pH and ionic strength are important (Richter et al., 2003; Reimhult et al., 2009)

Supported lipid bilayers are highly stable so that long-term experiments are possible with SLBs (Purrucker et al., 2001)

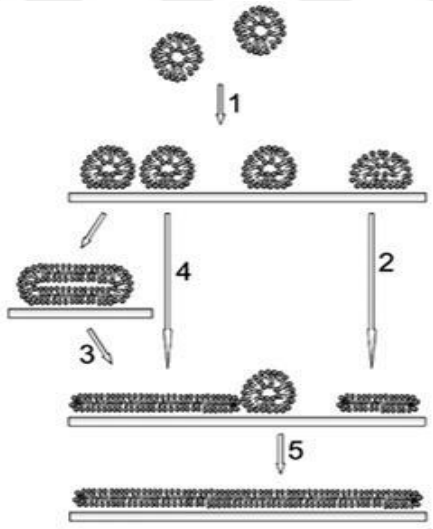


**Figure 2.8 :** Step by step formation of SLBs

(1) adsorption, (2-5) rupture of liposomes, followed by SLBs.

## 2.5 Atomic Force Microscopy for Analyse the SLBs

Atomic force microscope (AFM) is a characterization tool that is very useful to analyse of materials and surfaces. It is a direct imaging tool for visualizing SLBs on a substrate. Analyse of biopolymers is also implement by AFM successfully (Figure 2.9). Not only

AFM technique could be used analyze of topographical changes but also determining a range of biomechanical properties makes possible by using AFM. There has been a great deal of interest recently in planar supported lipid bilayers as model systems, compose of either single or multiple component lipids. It has different preparation methods which are vesicle fusion (Dufrene et al., 1997; Seantier et al., 2008), Langmuir–Blodgett and Langmuir–Schaefer deposition method (Petty, 1996; Picas et al., 2010).

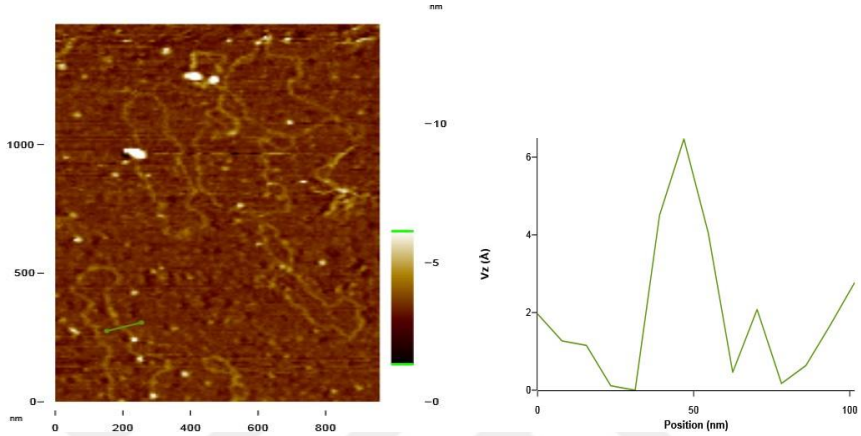


**Figure 2.9 :** AFM images of plasmid DNA (left) and crossection of DNA (right).

In literature, there are two major approaches to work these systems with AFM. Firstly, AFM imaging may be performed by scanning the AFM probe through the surface of a lipid bilayer, which gives information about the topographical characteristics of the supported lipid bilayer, For example, the lateral extent of domains, roughness and height of patches relative to the substrate. After that, supplement of an effector molecule of interest, the surface topography may be re-assessed. It can be also determined the timescale of the interaction by imaging the surface after increasing time steps and at each point assess the changes. It can be given examples of these types studies contain lipid interactions with anesthetic halothane (Leonenko and cramb, 2004; Leonenko et al., 2006), ethanol (Leonenko and cramb, 2004), antibiotic azithromycin (Merino et al. 2003; Berquand et al., 2004), immunodeficiency peptide (El kirat et al., 2006), peptide gramicidin (Leonenko et al., 2000), amyloid beta (Choucair et al., 2007; Quist et al., 2005). Other approaching is to implement force spectroscopy to assess the biomechanical changes due to some effector molecule. The mean or most probable rupture force has demonstrated to be a fingerprint for the intrinsic properties of the bilayer. The impact of pH (Hui et al., 1995), ionic strength of medium (Dekkiche et al., 2010), temperature (Benz et al., 2004), deposition pressure

(Fang and Yang, 1997) and head/tail group composition (Garcia-Manyes et al., 2010) on membrane structure and function have all been worked. Otherwise, the impact of several proteins and drugs have also been worked, containing myelin based protein (Mueller et al., 2000; Mueller et al., 1999), cytochrome-c (Mueller et al., 2000), bax protein (Garcia-Saez et al., 2007), cholesterol (Chiantia et al., 2006; An et al., 2010; Sullan et al., 2010), synapsin I (Pera et al., 2004), general anesthetic halothane (Leonenko et al., 2006) and antibiotic azithromycin (Berquand et al., 2004). This requirement of defect free supported lipid bilayers covers extended areas. In all of these works an essential prerequisite is a well improved protocol that can usually be used to prepare lipid bilayers (Mingeot-Leclercq et al., 2008).

### 2.5.1 Basic principles of the AFM

First atomic force microscopy was invented by Gerd Binnig, Quate and Gerber in 1987 (Binning et al., 1986). A spring is made by very tiny gold foil, and at the edge of it very small needle made by diamond was inserted. Thorough approaching this spring to the surface sufficiently spring deviation was sensed by scanning tunneling microscopy which was inserted at the back side of spring. By this method force mapping between diamond needle and ceramic surface was attained. In current AFM systems in order to get surface topography the interaction forces between needle and sample are used. One edge of the spring is connected to very sharp needle. The other edge of the spring is connected to the body of AFM. Surface is scanned through tapping of sharp needle on the surface. With a closed feed back loop, force between needle and sample decides by user is getting stabilized then surface topography is attained. Typical components of AFM are denoted in Figure 2.10.

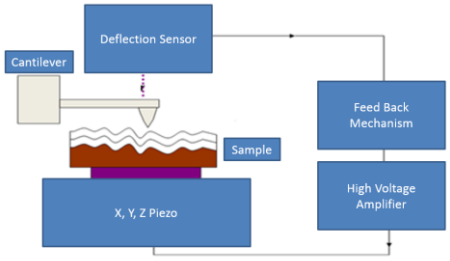


**Figure 2.10 :** Typical Components of AFM.

Main principle of all AFM techniques are measuring a specific property of material through bringing a probe to in close proximity with its surface and scanning across it in a raster pattern. Through utilization of a feedback loop to get high resolution image of scanned area interaction between the surface of the material and the probe is stabilized. It is also feasible to investigate some physical properties as well as chemical structure, charge distribution, capacity and conductivity of materials by AFM (Wiesendanger, 1994)

AFM has many significant advantages over scanning electron microscopy (SEM). SEM is a popular technique in which surface scanning of a material is made by high energy beam of electrons. However, at AFM without any extra sample preparation or treatment it is feasible to get surface analysis in three dimensional (3D) format. While AFM works perfectly on either air or liquid, SEM needs a vacuumed environment to operate properly. Furthermore, by AFM high resolution results could be attained rather than SEM

### 2.5.2 The probe (cantilever)

The probe of an AFM is crucial to its performance. Because it is the force between probe and the surface of a material which is used to create topographical images of the surface as fine as collecting a plethora of mechanical information related about material. The AFM probe could be considered as a spring, oscillating up and down as it is moved across the surface. While it scans, the impels between tip and the material cause the spring to deform; these deformations are measured to produce a mapping of the surface. AFM generally detects forces on the order of $10^{-6}$-$10^{-12}$ N. To be able to show a measurable reaction to forces on this scale, the probe must be highly sensitive. On the other hand, at the same time, mechanical vibrations must be minimized. Therefore, the spring system must be as soft as possible in order to get maximum deflection with a resonant frequency high enough to decrease the effects of noise. The balance of these two factors arises from the equation for a spring,

$$\omega_0 = \left(\frac{k}{m}\right)^{1/2}$$

where $\omega_0$ is the resonant frequency of the system, k is its spring constant and m is the effective mass. Therefore, it is clear that for a system to have high sensitivity to small

forces, small value of k is required also in order to achieve minimal noise and a large value of $\omega_0$ is needed.

In the case of AFM, used probe being must be as small as possible. An AFM probe consists of a cantilever with a tiny, and preferably very sharp tip (Figure 2.11). The forces between tip and the surface of a material cause the cantilever to bend and deflection is measured and converted into a topographical image of the surface.

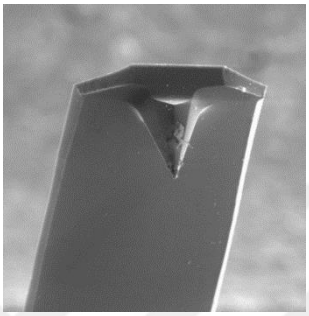

**Figure 2.11 :** SEM image of a typical AFM cantilever.

### 2.5.3 Detection methods and feedback

There are several methods to detect the deflection of the cantilever in AFM systems. It was measured by the difference between tunnel current of an STM in the original version and it is located from the rear side of the cantilever (Binnig et al., 1986). This method base on different techniques such as optical interferometry and capacitive sensing. However, this regulation causes many difficulties. On the other hand, laser beam deflection method is the simplest detection method to implement and consequently which is used in modern AFM systems (Figure 2.12).

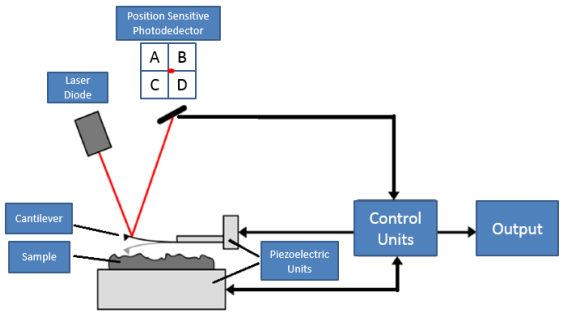


**Figure 2.12 :** Schematic of a typical AFM operating system.

In this laser beam deflection method, the rear side of the cantilever has a reflective coating in which a laser beam is focused and then reflected back onto a position sensitive photodetector. If there is any deflection on the cantilever, it causes a movement and it can be reflected in laser beam, where located on photodetector. Changing in position of the signal recorded by detector can be used to measure the deflection of cantilever. This method has many advantages comparing to other methods due to the fact that it has extreme simplicity and high sensitivity. Furthermore, impressed force on the cantilever by the laser beam is negligible.

Positioning of the sample related to the tip which is controlled by combination of piezoelectric crystals, typically one set in the sample stage and another in the cantilever holder. Oscillation of the cantilever is caused by piezoelectric crystals in cantilever holder when AFM is dynamic mode. These piezoelectric crystals in the sample stage are used to produce scanning motion of sample relative to the tip and to change the probe surface distance in response to changes in the deflection of the cantilever. Piezoelectric drive units are accompanied by feedback mechanism in all of other scanning probe microscope techniques. In AFM, this mechanism works to adjust the probe surface distance in order to maintain a constant force between the tip and the sample. When a reference value for the deflection of the cantilever is given, it is established before scanning commences. This is also known that setpoint voltage and it is chosen to be just below the 'free air' amplitude voltage of the cantilever, this is the point at cantilever starts to feel the presence of the surface (Figure 2.13) (Sweetenham, 2011). In the process of scanning, changes in the structure of the surface cause deflection of the cantilever to vary. In return for this piezoelectric crystals in the sample stage raise or lower the sample in an effort to return the deflection to this setpoint value. The number of the piezoelectric crystal has to raise or lower the stage to accomplish this is used as a direct measurement of the surface topography.

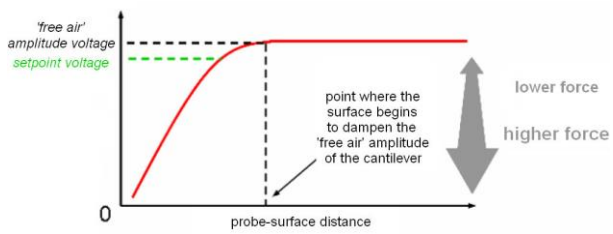


**Figure 2.13 :** A qualitative, conceptual sketch of amplitude voltage vs. distance, illustrating the choice of setpoint voltage.

### 2.5.4 Modes of operation

There are three major imaging modes for AFM operation which are called contact mode, noncontact mode, and tapping mode. The modes of operation imply the probe to surface attractions constituted a topographical mapping of the surface (Figure 2.14).

In contact mode, the probe always contacts with the sample during scanning. Contact mode indicates the short range repulsive forces that appear between the tip and the surface while they are in contact. Although imaging under contact mode operation yields quite a high resolution, it can cause a considerable amount of damage to the sample surface.

In non contact mode, the probe scans the sample without any contact and only the intermolecular forces between the probe and the sample can be detected. Therefore, non contact mode operation does not damage the sample at all, but it is not suitable for imaging sample surfaces with large changes in height.

In tapping mode, the cantilever oscillates at a resonant frequency and its probe intermittently contacts the sample during scanning. Imaging under intermittent contact mode does not cause any appreciable damage to the sample, and the image resolution is sufficiently high. Therefore, this operation mode is frequently used for imaging biological samples.

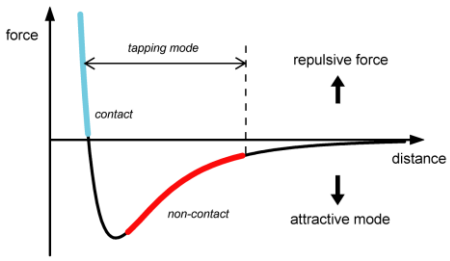


**Figure 2.14 :** Representation of graphic a typical force-distance relation between an AFM probe and a surface.

(The regions that correspond to contact, non contact and tapping mode operation are highlighted) (Sweetenham, 2011).

But, there are various handicaps for these static modes of operation. Contact mode shows probe or sample degradation effects which causes disadvantages effect for

AFM. Because it includes large lateral forces that are exerted by the probe on the surface of the sample. It plays a crucial role to obtain imaging soft samples such as biological materials, which could be shifted or even damaged by these forces.

Non contact mode is made possible solution for the risk of destroying the sample. Therefore, this leads decrease of resolution. In proportion large distances between tip of the probe and the surface greatly reduce the magnitude of forces that behave on the cantilever.

Further than developments came with the development of a hybrid mode of AFM between that of contact and non-contact mode, as known as tapping mode (Figure 2.14) (Zhong et al., 1993). This mode is used for an oscillating cantilever, but positions is much closer to the surface than the probe gently taps the surface at the "bottom end" of its oscillation. This tapping of the surface drops the amplitude of oscillation of the cantilever from its 'free air' value and causes an alteration in its resonant frequency. Once again, decreased amplitude of oscillation is resumed at a fixed value by a feedback mechanism.

Tapping mode AFM apparently compounds the advantages of unique two modes; short contact times between the tip and the surface riddle the disruptive lateral forces present in contact mode, whilst retention its perfect resolution. Therefore, tapping mode is a famous choice for imaging in modern AFM and is main for number of important applications.

### 2.5.5 Force spectroscopy

Apart from imaging, there are a multitude of applications of AFM. One of them is force spectroscopy, that provides a direct measurement of probe surface interaction forces as a function of distance between the tip and sample. Deflection of the cantilever determines these forces, which is monitored as the AFM probe is extended towards and retracted from the surface by the piezoelectric drive units. Over this approach and retract cycle, divergences in the cantilever deflection arise because of elastic properties of the sample and interactions between the surface and the tip such as rupture, binding and adhesion. Force spectroscopy measurements could be seen as a force curve (Figure 2.15).

Usually, force curves consist of an approach and a retract trace, which each have a diagonal region where the probe is in contact through the surface and a flat region where the probe is away from the surface. Force curves have also some number of essential features that are specific to a particular sample and reveal a wealth of mechanical information about it. A preeminent aspect of the approach trace is the point at which the tip first comes into contact with a sample. This is where the tip is close enough to the surface of the sample to experience attractive van der Waals forces, which cause the tip to 'snap' to the surface. This is shown as a sudden decrease in the deflection of the cantilever as it bends towards the sample. Then, the piezoelectric crystal driving the cantilever is extended further to move the cantilever into the surface, causing its deflection to increase. Then at full extension of this crystal it begins to retract. The retract trace of a force curve initially follows the same path as the approach trace. However, the point at which the tip eventually leaves the surface of the sample diverts from this trace and is of great interest. In many cases there is some adhesion experienced between the tip and the sample due to the nature of the sample. For instance, at room temperature, water coats surface, because of attractive capillary forces. Thus, while tip is pulled away from the surface, the cantilever is bent as the tip is held in contact with the surface. At the same time, depending upon the adhesive quality of the sample or the thickness of the water layer, the probe is retracted enough that the tip jumps free and moves away from the surface.

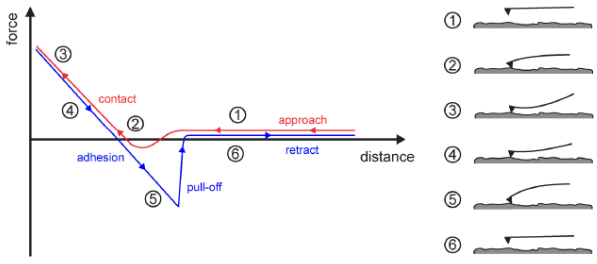


**Figure 2.15 :** A typical approach and retract cycle, or force curve.

(Numbered points on the curve and corresponding illustrations highlight key interactions between an AFM probe and a surface) (Sweetenham, 2011).

Force spectroscopy is very useful for a broad range of research areas to analyze the physical properties of surfaces. Also, according to the recent studies, it is possible to manipualate single molecules. Mechanical resistance of chemical bonds is the major

implimentation of AFM techniques. The tip of an AFM probe provides to functionalise by a molecule which binds to a molecule on a surface. Litarature includes the study of many biological systems such as antibody-antigen, protein-protein and protein-cell interactions (Kuhner et al., 2004; Ratto et al., 2004; Merkel et al., 1999). Yet another application of force spectroscopy is to understand the elasticity of materials, adsorbed to a surface and then stretched by the AFM probe is the method to undersand the elasticity of materials or biopolymers.

### 2.5.6 Imaging in liquids with AFM

Due to the fact that in physical sciences, AFM mostly dominates many other devices, which is used to study for solid samples. It has also become a crucial research tool for studying materials of a biological enviroment. These materials denote new challenges for the technique, hence in many of situations they are very soft and it is obligatory to keep those hydrated at all times. AFM is needed for using in liquid with both the sample and probes are totally depth into fluid. This diversity on the traditional setup introduces new forces and effects that stem from the presence of liquid around the system. In this case extra consideration must be given to many point of views setting up the AFM, including the mode of operation, the selection of cantilever and the values of most imaging parameters. In order to study samples in liquid, tapping mode AFM is always the first choice (Basak and Ramana, 2007).

This can be prevented by using non contact mode, as the tip is scanned at relatively large distances above the surface. In spite of the movements in the fluid within this space would make it almost impossible to obtain good sensitivity and resolution. Tapping mode can achieve the resolution of contact mode alongside the gentleness of non contact mode, making it ideal for studying biological materials. An oscillating cantilever is always required when imaging with tapping mode; on the other hand, when working in liquid the selection of cantilever is especially crucial. Usually, long triangular silicon nitride cantilevers with low spring constants are used in liquid AFM, mainly by the cause of they are soft and flexible and not affected by the fluid dynamics in the system. First and most importantly, the cantilever demands special tuning to determine its resonance frequency. In liquid AFM, a thermal tune is required for the purpose of identifying the region in which the resonant frequency of the cantilever lies,

as it can be difficult to locate among the random resonances of the cavity of liquid surrounding it.

Before getting near to the surface a set point voltage is chosen that is very close to the ‘free air’ amplitude of the cantilever, by this way there is minimum force experienced between the tip and the sample. This value can be slowly decreased with imaging to obtain good resolution. It is also most efficient to scan at a slower rate than in air, to abate movements in the fluid surrounding the system.

# 3. MATERIALS & METHODS

Phosphatidylcholine, L-α-phosphatidylcholine (Egg PC), was purchased from Avanti Polar Lipids (Alabaster, AL) and used without any extra processing. Ethanol, isopropanol, chloroform and acetone were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, DE).

Mica sheets were purchased from USA MICA (USA). Mini-Extruder kit and support membranes were purchased from Avanti Polar Lipids (Alabaster, AL). All chemicals and materials can be find at Appendix A and B.

## 3.1 Preparation of Unilamellar Liposomes with Extrusion Method

### 3.1.1 Preparation of stock solutions

In the current work, only one protocol has been developed to prepare fluid phase planar bilayers on mica for the use in AFM studies. Phosphatidylcholine (PC) has a transition temperature of −10ºC (Al-Jamal, and Kostas, 2007), and therefore exists in the fluid like liquid crystalline state at room temperature. So in this phase, phospholipid bilayer is mobile at all times.

Phosphatidylcholine (Figure 3.1) lipids were dissolved in chloroform at 1 mg/mL final concentration at +4°C as a stock solution, and than aliquoted and stored at -20°C.

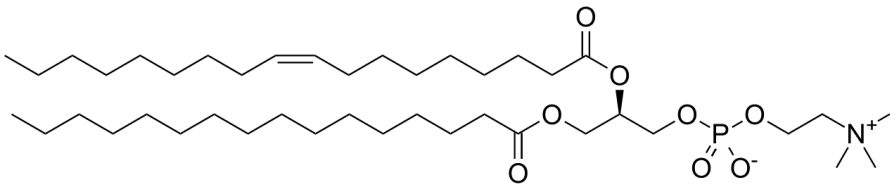


**Figure 3.1 :** Chemical structure of phosphatidylcholine.

### 3.1.2 Preparation of the lipid vesicle suspension solution

Multilamellar vesicle (MLVs) solution was obtained by appropriate amounts of EggPC lipids in chloroform stock solution (100 µL). It was poured into a round bottom flask and evaporated by manually rotating the flask using a dry nitrogen stream in a fume hood. The formed layers were left overnight +4 °C under vacuum to ensure the removal of all chloroform residues.

### 3.1.3 Hydration of lipid film

To hydrate the dried lipid film layer, 400 µL of 0.1 M phosphate buffered saline (PBS, pH 7.4) was added to the flask to get final lipid concentration of 0.025 mg/mL. At that point, the flask was agitated vigorously for 10-15 min using a vortex. During the hydration, the lipid film was gradually stripped off layer by layer from the wall of flask, and formed cloud like floaters in the PBS solution. After that, the flask was sonicated for 10 min with sonicator.

### 3.1.4 Extrusion with extruder

Hydrated and vortexed lipid arrangements form large and multilamellar vesicles (MLVs), firstly an extruder (Avanti Mini-Extruder) was used to produce unilaminar liposomes. To do this, the obtained multilamellar liposomes were extruded 15 times with the assistance of two syringes through a 100 nm polycarbonate film (Figure 3.2). Obtained SUVs were gathered in a eppendorf tube and put away at +4 °C until use. The syringes of extruder were washed by isopropanol and flushed with plenty of water. Teflon parts were cleaned by ethanol and put away in ethanol.


**Figure 3.2 :** Extruder for liposome preparation.

### 3.2 Imaging of Liposomes with Optical Microscopy

A drop of multilammellar vesicle suspension acquired after the sonication was put on a slide, covered with a coverslide and than observed under 50x objective. Acquired small unilammellar vesicles were also observed with optical microscopy.

### 3.3 Preparation of Lipid Bilayers On Mica Surfaces

In order to analyse the surface of the lipids, mica plates were sticked onto the surface of the AFM sample holder (Figure 3.3). 100 µL lipid solution was droped to freshly cleaved mica surfaces and it had been left in humidity chamber for overnight. After this procedure, SBLs formed on the mica surface.


**Figure 3.3 :** Mica sticked on a sample holder.

### 3.4 AFM Operation

AFM imaging and force measurement were conducted using a AQUA nc-AFM atomic force microscope from Nanomagnetics Instruments (Oxford, UK) (Figure 3.4). The AFM system has a scan area of 40 µm$^2$. The scanner was calibrated following the standard procedures provided by Nanomagnetics Instruments with calibrating grating.

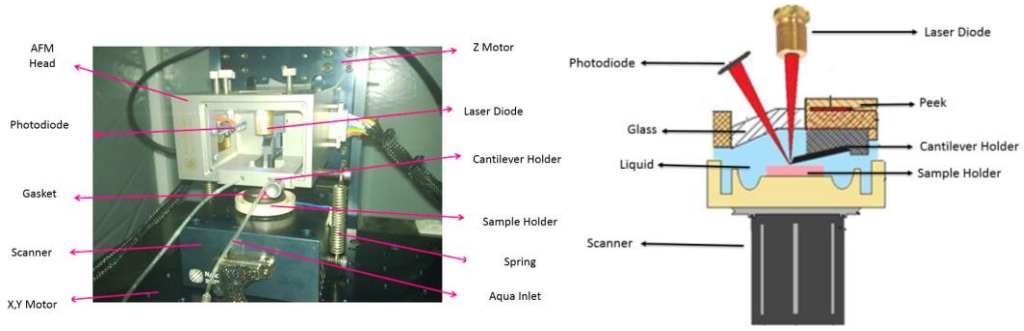


**Figure 3.4 :** General (Left) and schematic (Right) illustration of AFM head.

The aqua head with an open cell (Figure 3.5) was washed with ultrapure and particle free deionized water, ethanol, and deionized water again before each experiment. Before starting the measurement, freshly cleaved mica was sticked onto a stainless steel sample holder with an epoxy. After the epoxy dried up, the mica surface was cleaved again, and then 100 µL PC vesicle solution was dropped on the freshly cleaved mica surface at the room temperature. For the formation of the supported lipid bilayer, the PC vesicle solution was allowed to incubate in a humidity chamber on mica at room temperature during overnight.

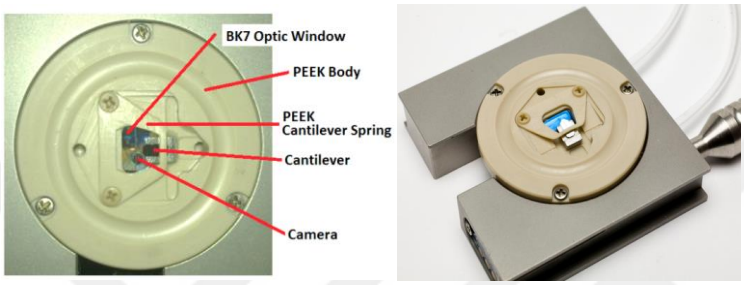


**Figure 3.5 :** Liquid head and cantilever holder.

Excessive amount of vesicles was removed by flushing the aqua head open cell with 1x PBS solution. All images were obtained in 1x PBS solution.

The AFM was allowed to equilibrate thermally for 15 minutes before imaging. Free amplitude was adjusted in the range of 0.8 – 1V, and the scanning range was set to 0.4 – 0.5 V (Figure 3.6).

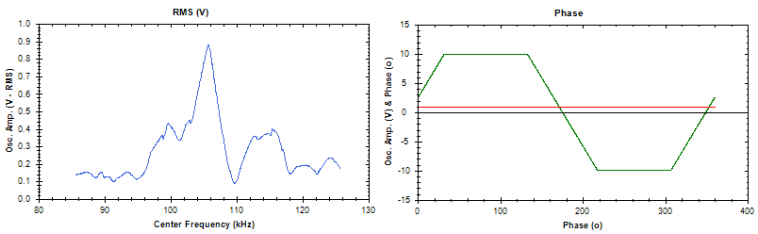


**Figure 3.6 :** Cantilever frequency (left) and phase (right) tuning image.

Room temperature was maintained at 22 ± 1˚C. Images were recorded either in tapping mode or non-contact mode by using standard and commercially available silicon based tips (Nanosensors), mounted on the cantilevers with spring constant of 10 N/m, length of 125 µm and width of 30 µm.

# 4. RESULTS AND DISCUSSION

## 4.1 Optical Microscopy of Liposomes

Vortexed and sonicated lipid solutions initially formed micron or nano scaled multilamelar (MLV) and “onion-like” (OLV) vesicles. These vesicles were analysed under 50x objective with optical microscopy (Figure 4.1). It was seen that their sizes vary between 1-10 μm diameter. These micron scale multilamelar vesicles can be reduced to small unilamellar vesicles by the help of the extruder. It has been reported in literature (URL-1) that it is possible to obtain the liposomes with diameters between 100 nm and 1 μm by the extrusion method.

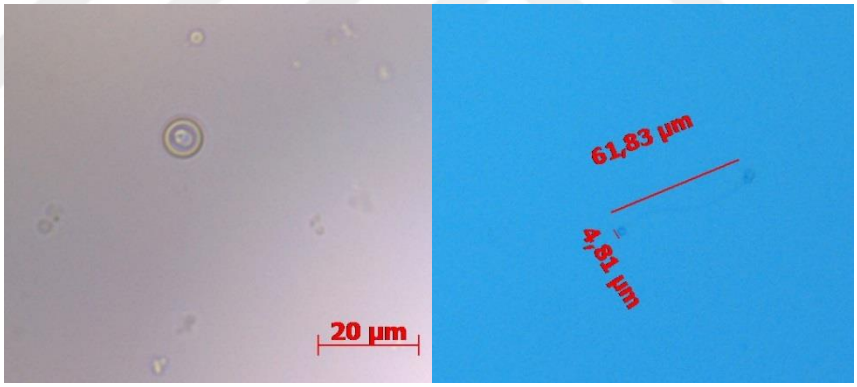


**Figure 4.1 :** Multilamelar vesicles observed under optical microscope.

## 4.2 Force-Distance (F-D) Curve Spectroscopy

The mechanical properties of phospholipid layers can be identified by AFM (Svetina et al., 1996). Bending modulus is a characteristic property of the vesicles, which is firmly identified with the examination of liposomes and the gel-liquid phase transition of a liposome’s bilayer membrane. Various experimental strategies have been intended to quantify the bending rigidity of the membrane.

AFM is a special system that can directly test the flexibility properties of numerous surfaces by utilizing force plots (Weisenhorn et al., 1993; Butt et al., 1995), where the AFM tip moves up and down over a point on the specimen surface in order to extract the elastic and adhesive properties of the materials (Ikai et al., 1997; Laney et al., 1997; Lee and Marchant, 2000; Radmacher et al., 1996; Radmacher et al., 1994).

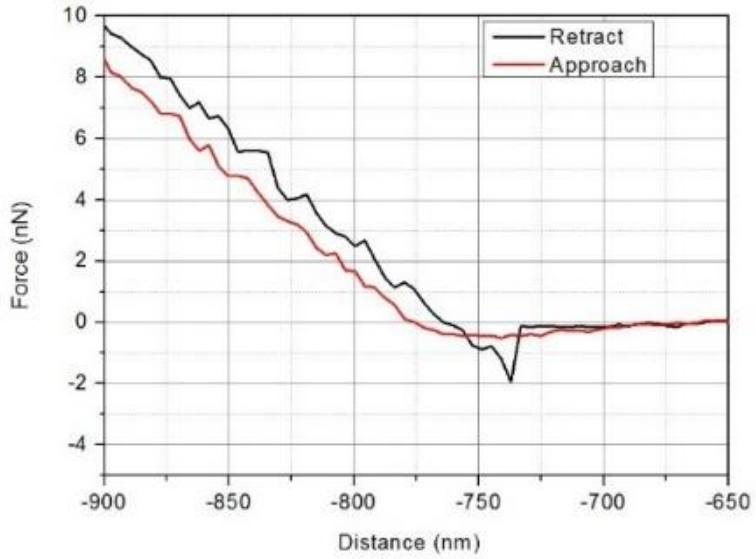


**Figure 4.2 :** Force-Distance Curve for SLBs on mica.

It is possible to obtain F-D curve by moving the AFM tip up-down over a point on the SLBs surface. F-D curve has been showed in Figure 4.2 where red curve corresponds to approaching cantilever to the mica specimen surface and the black curve corresponds to retracting of the cantilever from the specimen. During the approaching procedure, the cantilever penetrates into the SLBs and touches to the solid mica surface which results in a rapid increase of the applied force on the cantilever. After approaching procedure, a negative force (2 nN) which is caused by stiction of SLBs to the cantilever can be clearly seen during the retracting from the surface of the mica.

### 4.3 Formation of SLBs

At Figure 4.3, AFM image related about the composition of SLBs on the mica surface is shown. Following the subsequent addition of 100 µL of prepared liposome solution, which has just cleaved, the AFM liquid head was approached to the surface. After that process, arrangements of laser diode and photo detector were done. After waiting for

15 min to let the system reach its thermal equilibrium, cantilever was tuned at liquid environment (PBS), and than resonance frequency of the cantilever was found.

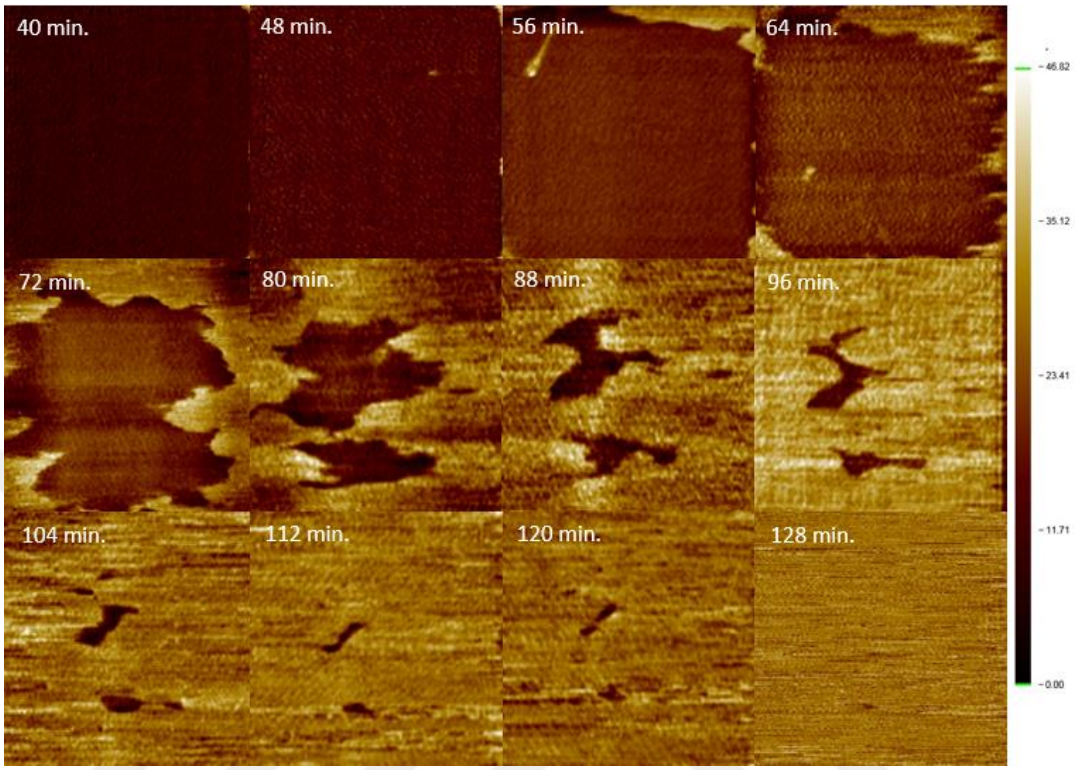


**Figure 4.3 :** AFM Phase images during the SLBs formation.

(Area: 10μm x 10μm, Scan speed: 5 μm/s)

As can be seen from Figure 4.3, formation of SLBs was monitored for 128 min and it was observed that suspended liposomes in PBS solution was not precipitated in the first 48 min. After 56 min, precipitated liposomes led SLBs to form at the surface, and in the 128$^{th}$ min, the mica surface was fully covered with SLBs. At Figure 4.4, percentage of SLBs composition as a function of time was shown. As can be seen from that graph, composition percentage does not increase linearly, most of the surface were coated between 64$^{th}$ min and 88$^{th}$ min.

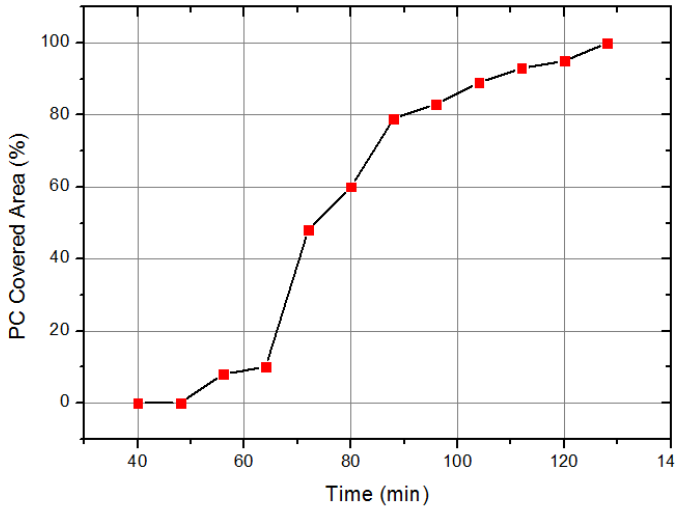


**Figure 4.4 :** Time depended PC coverage on mica surface.

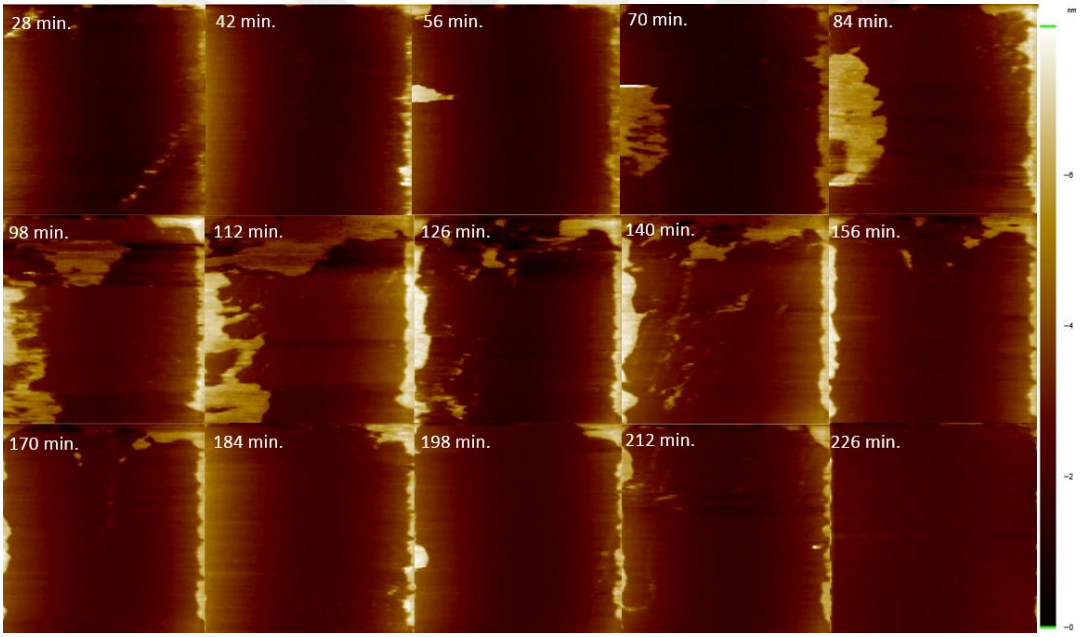


**Figure 4.5 :** Lipid deformation during AFM scanning.

(Area: 20µm x 20µm, Scan speed: 1 µm/s)

Figure 4.5 shows slowly scanned images. This clearly shows that SLBs begin to form after the 56$^{th}$ minute, but they were kept away from the surface by the cantilever. This could be because cantilever was too close to the surface and therefore, the formation of SLBs on mica surface was blocked by interactions between surface and cantilever.

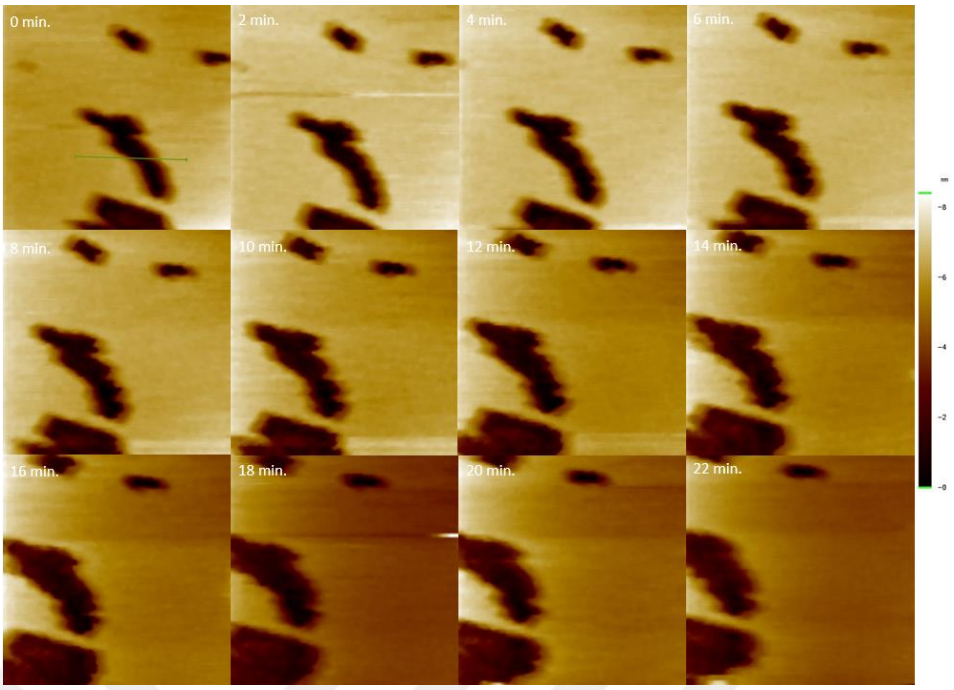


**Figure 4.6 :** Lipid migration during AFM scanning.

(Area: 2μm x 2μm, Scan speed: 20 μm/s)

Phosphatidylcholine has a transition temperature of −10ºC, and therefore it exists in the fluid like liquid crystalline phase at room temperature. This situation can lead lipids to be mobile. At temperatures above the transition temperature, the bilayer is in the liquid crystalline phase which consequently led the lipid molecules move laterally.

At Figure 4.6, AFM images, which were taken for 22 min with 2 min time interval, were depicted. As can be seen from the figures, size of the regions where composition of SLBs was not observed (dark region), varies by time. This situation expresses that SBLs moves laterally. On mica surface, the thickness of SLBs measured at the natural defects was 4 ± 0.5 nm. This indicated that a single bilayer of SLBs was composed on the mica surface with a thin layer of water between the mica surface and the bilayer.

### 4.4 Dissolution of PC Lipid Bilayer

#### 4.4.1 Effect of ethanol on SLBs

##### 4.4.1.1 10% ethanol solution

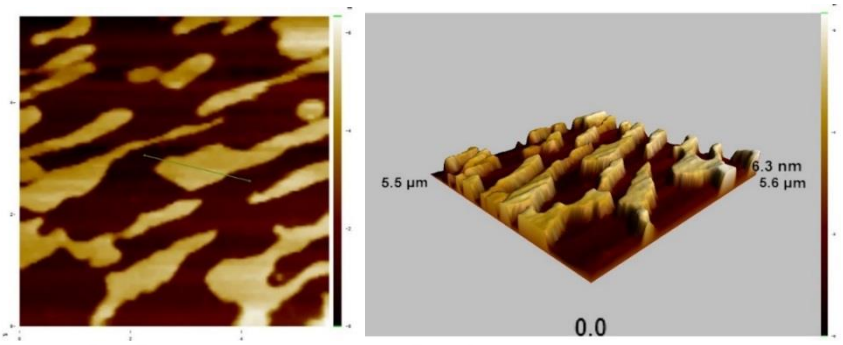


**Figure 4.7 :** SLBs formation on mica (Before 10% ethanol treatment).

(Area: 5µm x 5µm, Scan speed: 10 µm/s)

At Figure 4.7, AFM topography and the 3D image of the SLBs formed on the mica surface were shown. It is easy to see that the surface was not covered completely. These SLBs surfaces naturally have defects either in the form of lower domains or holes exposing the bare mica. After composition of SLBs on mica surface, 10% (v/v) ethanol was introduced to the environment, whose effect on the SLBs surface can be seen at Figure 4.8.

As can be seen from Figure 4.8, complete dissolution of lipids at the surface was not attained, rather they tended to form small island by changing their shape.

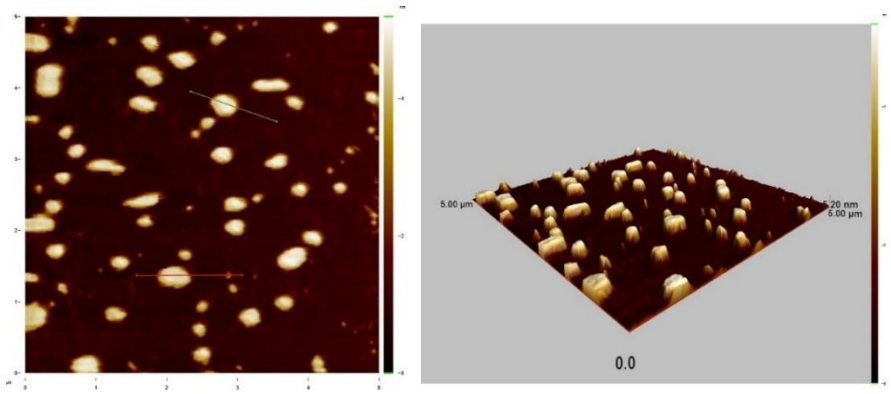


**Figure 4.8 :** SLBs dissolution on mica (After 10% ethanol treatment).

(Area: 5µm x 5µm, Scan speed: 10 µm/s)

#### 4.4.1.2 25% ethanol solution

At Figure 4.9, AFM topography and 3D image of composed SLBs at mica surface was shown. As can be seen from Figure 4.9, almost all the surface was covered with SLBs. The structure of composed layer varied between different trials even though those experiments repeated under the same conditions so more work has to be done to understand the important parameters in the construction mechanism.

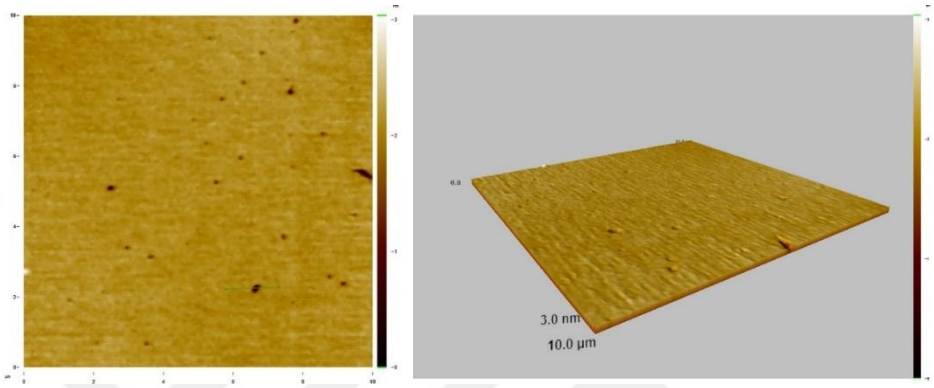


**Figure 4.9 :** SLBs formation on mica (Before 25% ethanol treatment).

(Area: 10µm x 10µm, Scan speed: 20 µm/s)

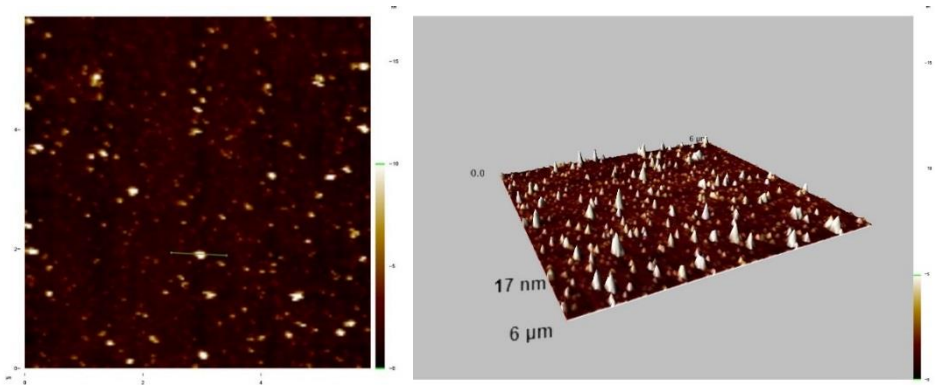


**Figure 4.10 :** SLBs dissolution on mica (After 25% ethanol treatment).

(Area: 5µm x 5µm, Scan speed: 10 µm/s)

After the construction of SLBs on mica surface, 25% (v/v) ethanol was introduced to the environment. This treatment was monitored by AFM topography and 3D images of the surface (Figure 4.10).

At high concentration of ethanol, formation of small cone-shaped aggregates and clusters was observed on the surfaces of mica in the liposome contained PBS solution.

Those cone-shaped clusters were analysed by AFM, the height was measured as 15 nm which was higher than SLBs.

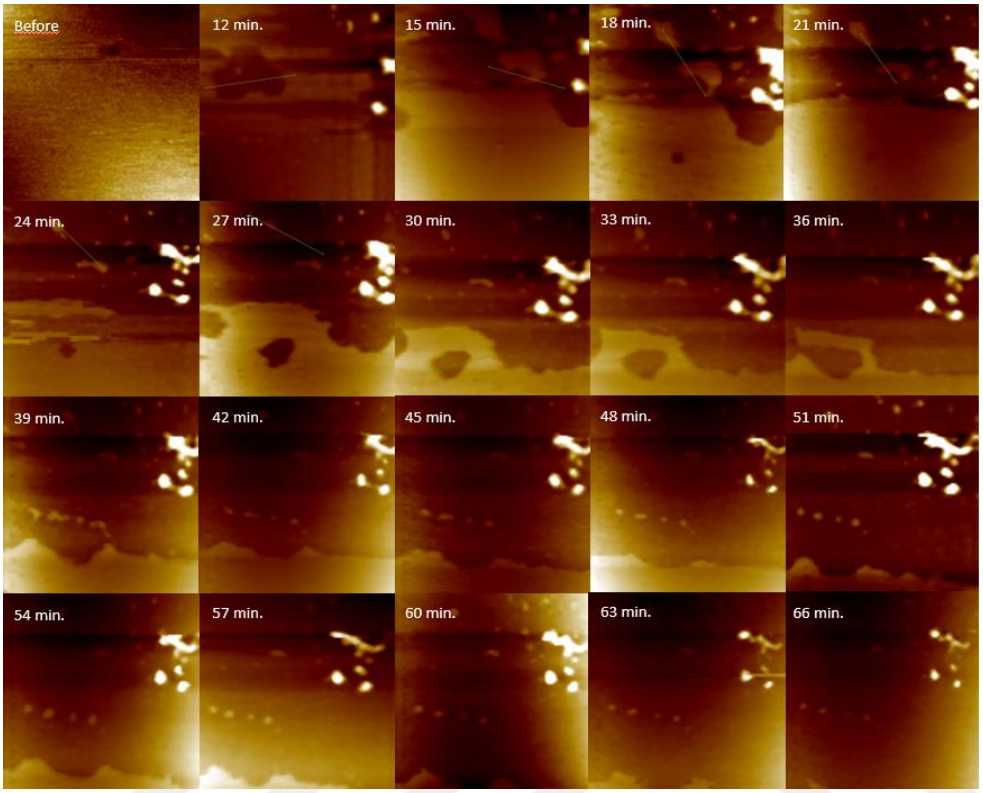


**Figure 4.11 :** SLBs dissolution on mica (After 25% ethanol treatment).

(Area: 4μm x 4μm, Scan speed: 20 μm/s)

After SLBs formed on mica surface, ethanol was added on environment with concentration of 25% and the surface was scanned by AFM. Figure 4.11 shows that scanning took 66 min. In Figure 4.11, "before" represents the surface topography without alcohol. After adding alcohol, first scanning was taken at the $12^{th}$ min because of calibration of laser diode and photodetector and maintained until the $66^{th}$ min. PC molecules lost their SLBs structure and they aggregated in different region.

### 4.4.2 Effect of isopropanol (IPA) on SLBs

#### 4.4.2.1 10% isopropanol solution

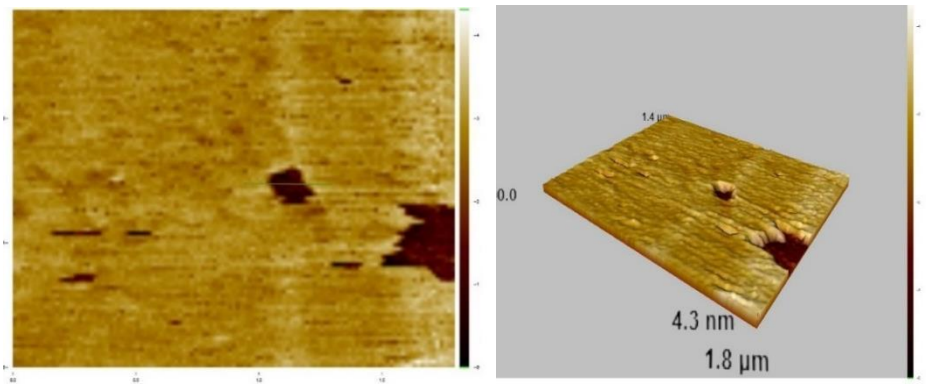


**Figure 4.12 :** SLBs formation on mica (Before IPA treatment).

(Area: 2µm x 2µm, Scan speed: 10 µm/s)

Figure 4.12 demonstrates surface topography and 3D image which were scanned by AFM. It can be seen that SLBs cover almost all the surface of mica.

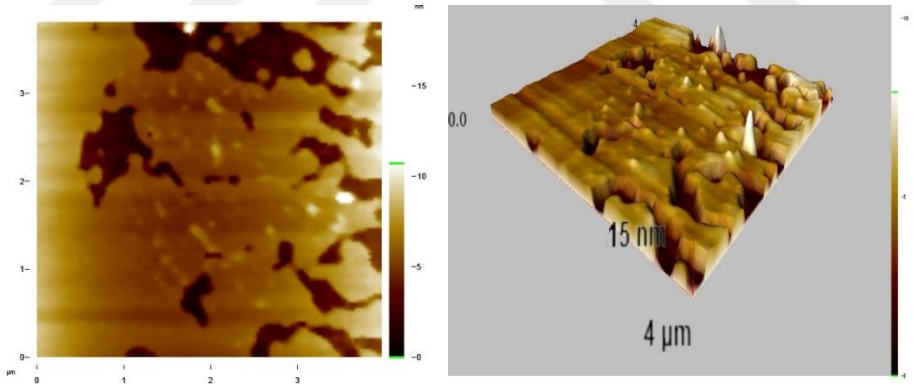


**Figure 4.13 :** SLBs dissolution on mica (After 10% IPA treatment).

(Area: 4µm x 4µm, Scan speed: 10 µm/s)

Figure 4.13 shows AFM images of the SLBs surface which was treated by IPA. After formation of SLBs, IPA with the concentration of 10% (V/V) was added in the solution. Subsequent to IPA addition, SLBs on surface were dissolved partially. Disolved SLBs molecules led the random cluster formation on the surface. These clusters were of the height of 5-15 nm.

#### 4.4.2.2 25% isopropanol solution

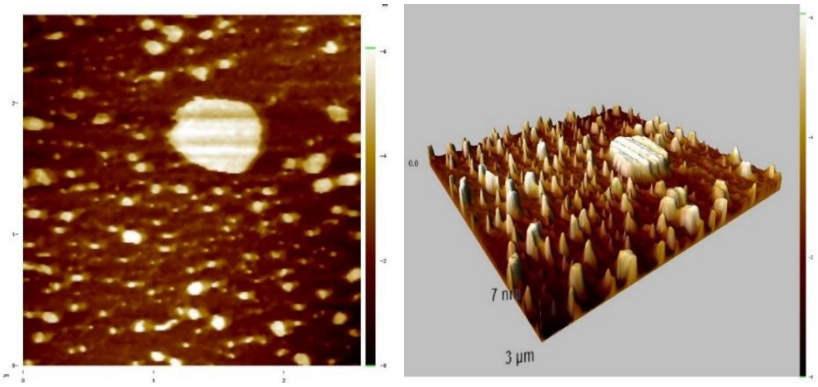


**Figure 4.14 :** SLBs formation on mica (Before IPA treatment).

(Area: 3µm x 3µm, Scan speed: 6 µm/s)

Figure 4.14 demonstrates AFM images of surface before IPA treatment. It can be seen that SLBs partially covered the surface of mica.

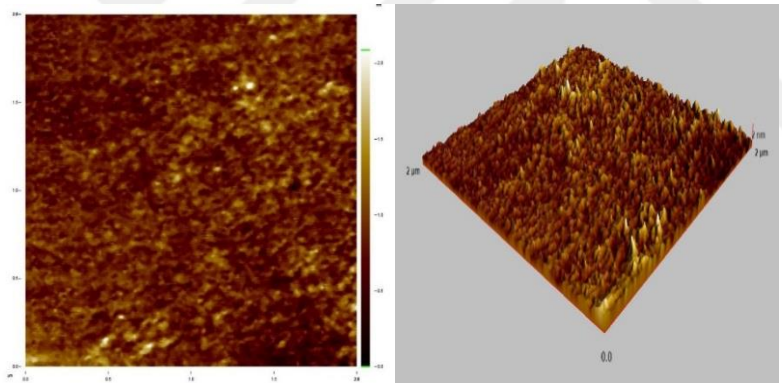

**Figure 4.15 :** SLBs dissolution on mica (After 25% IPA treatment).

(Area: 2µm x 2µm, Scan speed: 4 µm/s)

Figure 4.15 demonstrates AFM images of surface after treatment with 25 % IPA (V/V) As can be seen, SLBs did not appear on surface after adding high concentration of IPA to the enviroment.

### 4.4.3 Effect of acetone (ACE) on SLBs

**10% acetone solution**

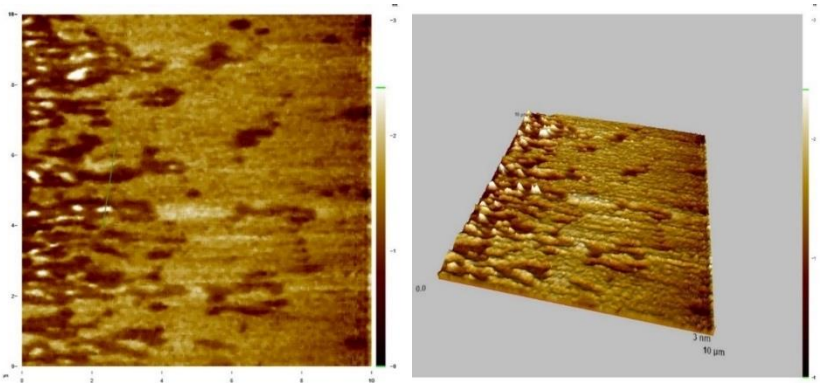

**Figure 4.16 :** SLBs formation on mica (Before ACE treatment).

(Area: 10µm x 10µm, Scan speed: 20 µm/s)

Figure 4.16 demonstrates 3D image and surface which was scanned by AFM. It can be seen that SLBs covered a great majority of the mica surface.

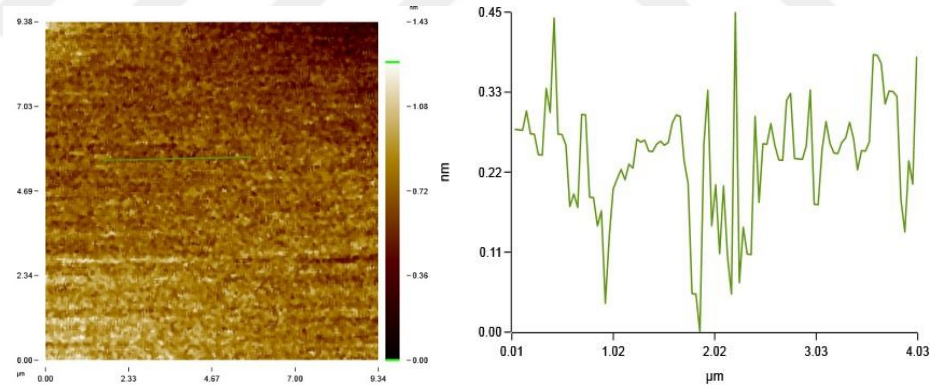


**Figure 4.17 :** SLBs dissolution on mica (After 10% ACE treatment).

(Area: 10µm x 10µm, Scan speed: 20 µm/s)

Figure 4.17 demonstrates AFM images of SLBs on mica surface which was treated with 10% ACE (V/V). It is clear to see that SLBs disolved completely. Surface roughness was measured less than 0.5 nm on crossection area which approximately equals to rougness of mica surface.

### 4.4.4 Effect of chloroform on SLBs

**10% chloroform solution**

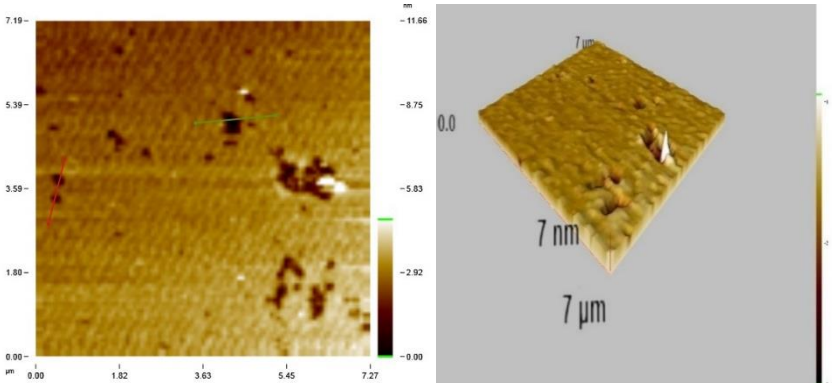


**Figure 4.18 :** SLBs formation on mica (Before chloroform treatment).

(Area: 7 µm x 7 µm, Scan speed: 10 µm/s)

The Figure 4.18 demonstrates 3D image and surface topography of SBLs surface on mica. SLBs covered great majority of the surface.

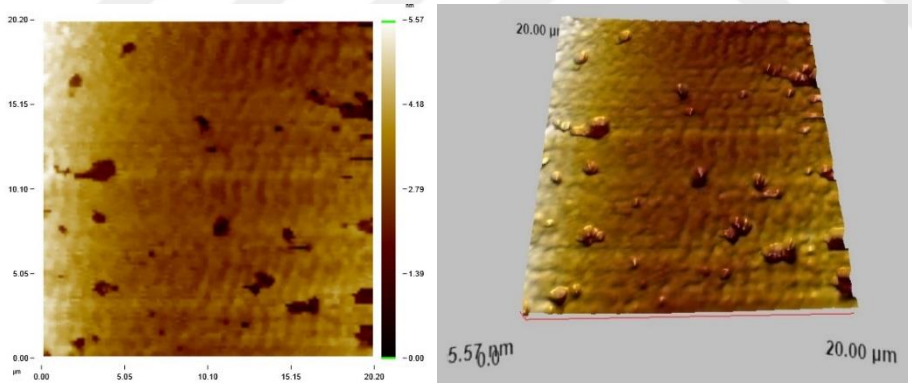


**Figure 4.19 :** SLBs dissolution on mica (After 10% chloroform treatment).

(Area: 20µm x 20µm, Scan speed: 20 µm/s)

Figure 4.19 demonstrates 3D image and surface topography of SBLs surface on mica scanned by AFM. When 10 % chloroform was introduced to surface, SLBs structure did not change considerably. This was probably due to the rapid evaporation of chloroform added into the environment, which led the change of the concentration and the liquid volume of the medium, which subsequently deviated and shifted the laser.

Therefore, any effect of chloroform on bilayer dissolution could not be observed due to the volatile nature of chloroform.

### 4.4.5 Reformation of SLBs on mica

When the SLBs formed on the mica surface was dried at room temperature, PC structure accumulated together (Figure 4.20). These structures formed hills with a height of more than 600 nm.

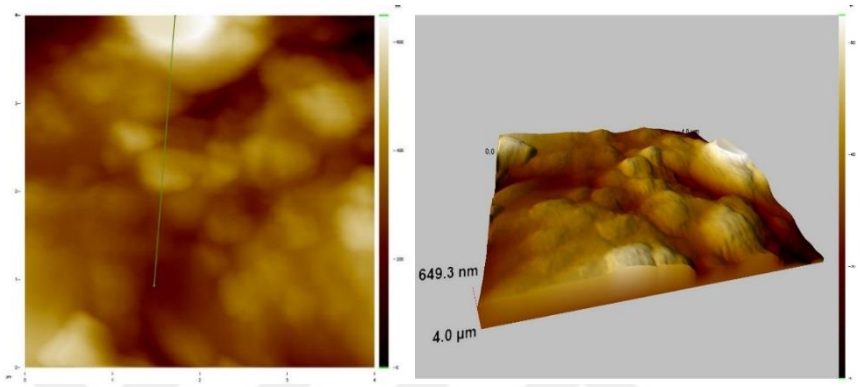


**Figure 4.20 :** Dried SLBs on mica AFM image.

(Area: 4µm x 4µm, Scan speed: 8 µm/s)

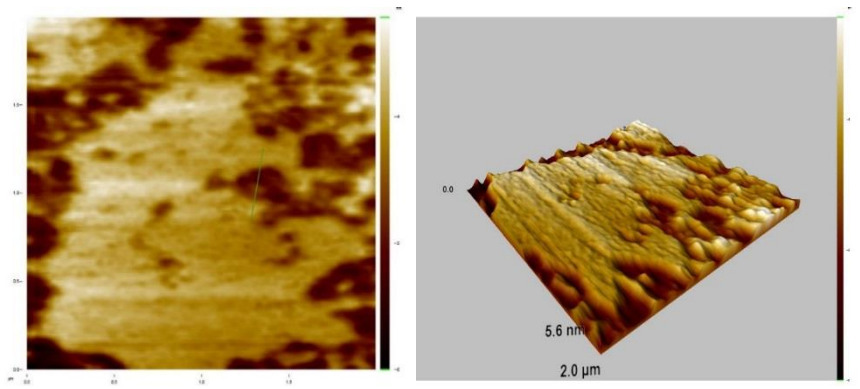


**Figure 4.21 :** Reformation of SLBs on PC dried mica AFM image.

(Area: 2µm x 2µm, Scan speed: 4 µm/s)

As can be seen in Figure 4.21, when the dried mica surface was treated with 1 x PBS solution, formation of SLBs occurs again on the mica surface. Those results may suggest that liposome preparation is not necessary to form SLBs on the surface.

## 5. CONCLUSION

The formation of SLBs has been explored in depth, not only because of their importance in mimicking to the cell membrane, but also due to their potential in biotechnological applications such as MEMS, biosensors and immunoassays.

The self assembly of SLBs depends critically on solvent environments. Therefore, if the interactions between supported bilayers and organic solvents are well understood, organic solvents can be used for manipulating the properties of supported membranes. It is known that some organic solvents mainly reside in the head group region of lipid bilayers, with the hydrocarbon chains pointing toward or inserting into the hydrocarbon core of membranes. For this reason, SLBs can be solved or manipulated with some organic solvents.

AFM is a powerful system for investigating lipid bilayers and it can give detailed information about the surface of nano structures. The AFM system can provide information about the physical properties of the lipid bilayer (viscoelastic behavior) and it can be used for real time imaging during the formation or dissolution of lipid bilayer.

In this study, effect of organic solvents on artificial SLBs was investigated using AFM spectroscopy methods. In literature, no systematic studies exist to solve their stability and interactions with organic solvents. Therefore, this work will be valuable in filling the gap and broadening the potential of supported membranes.

Results in this study are given as follows:

It was hard to obtain a continuous lipid bilayer on the surfaces and while in some cases, they covered almost all over the surface, some other structures were observed in other cases. The reasons behind this could be lower domains or holes on bare mica.

Mica covered by SLBs was applied with 10% and 25% EtOH, and after each treatment, surfaces were analysed by AFM. It was observed that SLBs were dissolved partially when 10% EtOH was used, while undissolved parts formed small islands. At

high EtOH concentrations (25% v/v), the bilayer structure could no longer be maintained and small globular structures appeared. It was clearly observed that when the EtOH concetration was very high in the solution, EtOH started to disrupt membranes by binding within the phospholipid bilayer.

Moreover, IPA, which is slightly larger than ethanol in terms of molecular weight with one more methyl group, similar interdigitated domains was induced in PC bilayers. For this, mica surfaces covered by SLBs were treated with %10 and %25 IPA, and surfaces were measured by AFM after each experiment. When the concentration of IPA was increased, more SLBs were dissolved. Additionally, there was no lipid left when 25% IPA was applied. It could be said that IPA was much more effective solvent on PC SLBs compared to EtOH.

Mica covered by SLBs was also treated with 10% ACE and no SLBs was found on mica surface after the experiments. Therefore, more than 10% ACE was not applied for further analysis.

Finally, mica surfaces covered by SLBs were treated with 10% chloroform, and surfaces were measured by AFM after each experiment. A moderately polar solvent, chloroform, was expected to dissolve the PC phospholipids, easily. Although chloroform is considered to be effective solvent for PC, no effect was observed in this study. This is probably because, lipid and chloroform can not interact together due to its sudden evaporation from the solution.

In sum, it was observed that organic solvents were effective on manipulating SLBs. The intercalation of the organic solvents might disrupt the interactions between the headgroups and the hydrocarbon chains of the lipids.

When all three organic solvents (EtOH, IPA, ACE) were considered, all of them were found to have a dissolution effect on SLBs, but ACE was determined as the most effective solvent among them.

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**APPENDICES**

**APPENDIX A:** Laboratory Equipment

**APPENDIX B:** Chemicals

## APPENDIX A

### Laboratory Equipment

**Pipettes** (Eppendorf 10µl, 100 µl, 1000 µl, 2500 µl, 5000 µl)

**pH meter** (Hanna Instruments, HI 9124)

**Magnetic stirrer** (Cole-Parmer, Stable Temp)

**Bath Sonicator** (Transsonic TP 690)

**Vortex** (Heidolph, REAX top)

**Optic Microscopy** (Olympus)

**Filter Support** (10mm Filter Supports, 610014 Avanti Polar)

**PC Membranes** (0.1µm Polycarbonate Membranes, 610005 Avanti Polar)

**Mini-Extruder** (Avanti Polar Lipids/Hamilton Company, 610017)

**Extruder membranes** (Whatman, Nuclepore Track-Etch Membrane 0.1 um, 800309)

**Atomic Force Microscopy:** Nanomagnetics Instruments AQUA nc-AFM

**Cantilever** (Nanosensors PPP-NCHR-W)

**Mica sheets** (USA MICA (USA).

## APPENDIX B

### Chemicals

**PC** (Egg PC), L-α-phosphatidylcholine, (Egg, Chicken), (Avanti Polar Lipid, 840051P)

**PBS** (Phosphate buffered saline) (Sigma, 79382-50TAB)

Dissolve 1 tablet in 200 mL water to obtain 10 mM phosphate buffer solution (pH 7.4 at 25 °C) (137 mM NaCl, 2.7 mM KCl)

**Distilled water** (Sigma, 7732-18-5)

**Ethanol** (Sigma, 32221)

**Isopropanol** (Sigma, 24137)

**Chloroform** (Sigma, 32211)

**Acetone** (Sigma, 179124)

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