ULTRATHIN FILMS OF BIOMOLECULES WITH WELL-CONTROLLED NANOSTRUCTURES

DISSERTATION

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Assembling amphiphilic molecules at air/liquid interfaces with Langmuir-Blodgett (LB) techniques has been applied in a wide variety of fields. Surface compression and subsequent transfer of the floating film onto a solid substrate provide a convenient way to fabricate supported ultrathin films with the thickness of single or multiple layers of molecules. There are many factors that can be monitored to vary the resulting film structures. However, the sensitivity of the molecular arrangement in the film to environmental disturbances and possible transfer defects makes it difficult to realize reproducibility and long-range uniformity for the supported thin film, which is critical for further commercial applications.

Ultrathin films containing phosphatidylethanolamines (PEs), the most important phospholipid species present in many biological membranes, and an extracellular matrix (ECM) protein, collagen, are investigated in the current study to determine how regularly patterned films can be prepared in a manner that preserves the bioactive properties of the biomolecules. With a novel delivery method, collagen can be successfully incorporated into a lipid matrix at an air/water interface for the first time. Surface pressure-area compression isotherms of the composite films measured using the Wilhelmy plate method provide information about the way lipids interact with collagens. Composite
films transferred onto freshly cleaved mica by LB technique are characterized by atomic force microscopy (AFM). The resulting LB monolayer films exhibit various hierarchical structures of collagen, similar to those observed in vivo, that are patterned laterally by phase separated binary lipid domains. The film composition is the most important factor in determining how the collagen is laterally distributed within the lipid matrix and presents a narrow range over which collagen forms intricately structured assemblies. Selective control of the surface patterning in a reproducible way is realized by optimizing the fabrication procedure, with a long-range uniformity extending throughout the entire area of sample surface (typically 1cm²). The adhesion and growth of Chinese hamster ovary (CHO) cells on the patterned films demonstrate the biocompatibility of these composite films. Collagen features with preferential surface localization in the monolayer film are applied to template the growth of inorganic silicate structures two-dimensionally. Exploring bilayer films consisting of PE lipids and collagen provides insight into how collagen is incorporated in a lipid matrix. Based on the experimental phenomena and the understanding of fundamental interactions involved, a mechanism of the co-self-assembly of phospholipids and collagen is proposed in the current study.

Thin films of synthesized porphyrin compounds with highly-ordered arrangement are fabricated and investigated due to their potential applications in molecular devices, gas sensors and biorecognition surfaces. LB films, cast films and self-assembled monolayers (SAMs) of a series of amphiphilic phenyl-amidophenyl-substituted
porphyrins with one, two, three, and four $\text{C}_{15}\text{H}_{31}$ tails are synthesized on various solid substrates to study the self-assembly process of those porphyrin compounds in different circumstances. The morphologies of the thin films are visualized and the film thicknesses were estimated with AFM. Spectroscopic studies of the porphyrin films provide information about molecular orientation and packing within the thin films. UV-vis absorption and fluorescence spectra of the films appear different from the corresponding solution spectra. The direction and extent of the spectral shifts indicate the overall arrangement of porphyrin molecules in the supported films. A mechanism for the self-assembly procedure is proposed to explain the influence of solid substrate, deposition surface pressure, and film fabrication method on the structures of the ultrathin films containing derived porphyrin molecules.
Dedicated to my parents
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CHAPTER 1

INTRODUCTION

1.1 Ultrathin Films with Nanostructures

Ultrathin films composed of a single layer or multilayers of biomolecules with highly organized nanostructures on solid surfaces have been drawing increased attention because of their potential applications in a broad range of fields, such as chemical or biosensors, drug delivery, catalysis and nanoelectronic devices, and their important role in the fundamental investigation of complex interactions in natural systems. The nanostructures, either two dimensional (2-D) surface features or three dimensional (3-D) inner alignments, presenting in the artificial biosystems can yield interesting novel chemical and/or physical properties or provide templates for fabricating new materials.

Amphiphilic molecules, such as common type of lipids, proteins and porphyrin molecules that are composed of both hydrophilic and hydrophobic components have the tendency to self-assemble to form numerous interesting structures both in vivo and in vitro. Monolayer or bilayer lipid films have been of special interest to the investigation
of cell membrane structure and biofunctions. A mixture of lipids with different molecular structures, especially with respect to hydrophobic tail length and conformation, exhibits the formation of domains under phase separation, which can be mainly monitored by controlling lateral packing density of the lipid molecules and molar fractions between those species for a given system. The existence of a double bond in the lipid tails renders a kinked tail structure for lipids with unsaturated hydrocarbon tails. Lateral phase separation has been commonly observed for the composite monolayer consisting of lipids with saturated straight tails and those with unsaturated kinked tails. Both the miscibility of hydrocarbon tails and the interaction between the polar head groups play an essential role in the 2-D domain development. The shape and size of the lipid domains are the result of the competition between repulsive interactions between lipid molecules and line tension that is related to the unsaturation of intermolecular interactions along the periphery of domains. Water insoluble lipid species with interesting phase separation have significant advantages in templating proteins at an air/water interface in the cases, where the penetration of proteins in the lipid composite film will not exert significant disturbances to the originally formed surface patterns.

One of the most common extracellular matrix (ECM) proteins, collagen, has been observed to associate with lipids in both normal and pathological tissues in nature. Compared with normal tissues, the collagens exhibit excess aggregation in pathological tissues, which leads to the appearance of scar. The environment provided by the lipid matrix has been believed to have an influence on the degree of collagen polymerization. New discoveries in lipid/collagen association will shed light on new biomedical treatments, such as scar control and skin transplants. The co-self-assembling behavior of
lipids and collagen has also been utilized in drug delivery systems. Collagen has been found to increase the stability of lipid vesicles. The way that lipids interplay with collagen plays an essential role in the design of drug delivery systems, although this is not fully understood. The study of the co-self-assembly behavior of lipids and collagen has been in the very initial stage. Monolayer and bilayer of lipids can serve as suitable model systems to investigate the collagen/lipid interactions. This mimetic system of lipids provides an oriented molecular array or a similar environment as the membrane surface for collagen to interact with. Traditionally, information on the assembling behavior of lipids associated with collagen has been obtained by incorporating water soluble collagen molecules beneath the floating lipid monolayer, followed by the measurement of the surface pressure change to estimate the extent of collagen penetrating into the lipid matrix at an air/water interface. In this type of characterization, it is difficult to estimate the amount of collagens that bind with the lipid monolayer and to get direct visualization of surface features composed of collagen and lipids. Since the penetration is a highly dynamic process, it is hard to obtain reproducible experimental results. In the current study, the collagens will be introduced into the lipid matrix in a novel way to ensure the convenient characterization of the film structure of the composite films and also the reproducibility of the fabrication procedures.

In addition to the fundamental investigation, collagen/lipid thin films with surface patterns are also demanded by new advances in biosensor development. Patterned collagen plates have the potential to be further developed as cell-based sensors to detect the existence of many types of species, such as toxicity and inflammatory agents, in vitro. Collagen-patterned solid surfaces have been explored to localize the culture of
anchorage-dependent cells, on which the cell responses to target species can be observed directly. The spatially directed attachment and shape control of cells can lead to the manipulation of cell function by varying the cell density and film surface morphology. Biocompatibility of the previously immobilized proteins and the sterility of the whole template are among the primary requirements for cell-based biosensors; therefore, the template film composed of collagen/lipid aggregated structure has to exhibit sufficient stability and durability after undergoing the sterilizing procedure. The presence of lipids may help maintain the biocompatibility of proteins during the film formation due to the reduced surface tension in the presence of surface active lipids.

Ultrathin films of porphyrin derivatives with well-defined molecular organization and film thickness have been under intensive investigations because of their potential applications in chemical sensors, especially in gas sensors, which can detect a variety of gases, such as ammonia, NO, CO₂, O₂ and chloride. Thin films of porphyrins have showed high sensitivity and promising selectivity for a given species once exposed to the sensing gas with significant changes in absorption spectra. The design of an efficient sensing system based on a structured film of porphyrin derivatives focuses mainly on the choice of porphyrin species with inherent stability and required optical responses, the adjustment of molecular orientation and organization within the film and also the environmental conditions under which to conduct the sensing.

The interactions involved in the assembled structures of the supported films may include covalent bonds, electrostatic interactions, hydrophobic forces and van der Waals forces. The construction of artificial features in those films is primarily based on weak intermolecular interactions; therefore, the stability of the film structure in terms of time
and environmental conditions and also the long-range uniformity of the molecular organization have been difficult to achieve for developing commercial products. Any disturbance coming from the existence of contaminations, substrates with heterogeneous surface features or the fabrication procedures can lead to the failure to maintain the highly organized local structure at a large length scale. Intricate assemblies are usually produced in a non-reproducible way. The intrinsic interactions between the self-assembled molecules, combined with various film fabrication techniques and different solid substrates, can result in numerous surface architectures.

1.2 Film Fabrication Techniques

Complex molecular systems in the form of thin films can be built up through two pathways: bottom-up and top-down. Lithographic fabrication techniques that are of the top-down type are based on a mask with pre-designed features, which have consequent disadvantages in terms of high cost for mask preparation and the limitation on the dimension of distinguishable structural features. Compared with the top-down route, the bottom-up fabrication methods can yield surface patterns of sizes below the micron level at a lower cost and in a more convenient manner.

Based on the bio-inspired self-assembling behavior of amphiphilic biomolecules, bottom-up is a category of techniques that can fabricate thin films with nano-scaled structures from the fundamental building blocks (usually individual molecules). To date, the bottom-up procedures still have had many restrictions on realistic commercial applications. Since the self-assembling process is mainly based on weak binding forces, the resulting structure is very sensitive to even tiny environmental disturbances. Compared with other commonly utilized bottom-up techniques, such as casting and dip-
coating, Langmuir-Blodgett (LB) technology provides additional control of the formation of lateral structures in addition to the principle of self-assembly and also a feasible way to transfer the fabricated film from an air/liquid interface onto a solid surface, forming a monolayer or multilayer film. An enhanced degree of molecular order in the fabricated films is realized by controlling the packing density of the biomolecules along the interface, which may in turn increase the bioactivity of the biomolecules. The LB approach has also been applied to incorporate multiple microbiomolecules (protein, enzyme, etc.) or inorganic compounds (hydroxyapatite, silicate) into the membrane-like structure by inducing them into the liquid subphase or spreading them on top of the initially spread monolayer.

1.3 Scope and Objectives of Research

In the present study, most of the research has been focused on developing novel fabrication techniques to obtain ultrathin films composed of lipids, proteins and porphyrin derivatives. Following the principle of self-assembly, long-range uniformity and reproducibility of the fabrication procedure are achieved for those composite films supported by various solid substrates, although the spontaneous formation of well organized supramolecular architectures is a highly dynamic process. Biocompatibility and stability of the synthesized films are required for further applications. In systems with multiple components, the co-self-assembly mechanism based on primary interactions is investigated.

2-D lipid matrixes with phase separation are applied to provide adjustable environments for collagen to polymerize and anchor on the surface. To successfully pattern collagen in a lipid matrix, the challenges include finding a proper lipid matrix
with stable and well organized surface patterns, optimizing the fabrication procedure to successfully fabricate collagen-containing films at an air/water interface and finally realizing the long-range uniformity of the composite film and the reproducibility of the fabrication procedure. Collagen is delivered onto the previously spread lipid monolayer in a specific way to prevent collagen from penetrating into the aqueous subphase. After transferring the lipid/collagen film onto mica, intricate hierarchical assemblies of collagens at various levels are fabricated in a controllable way. Systematic experiments are performed to explore the effects of the adjustable factors of the fabrication procedures on the resulting surface features and collagen associates. Biocompatibility of collagen in the film is demonstrated by attaching and growing cells on its surface. Excess charges along the collagen surfaces in certain pH regions motivate the exploration of growing inorganic compounds templated by the characteristic fibril structure of collagen with confined dimension and surface localization.

We have a particular interest in the way that collagens interact with lipids in the aggregated structures and the mechanism of co-self-assembly in the collagen/lipid system that determines the degree of polymerization of collagen monomers in the lipid matrix. To obtain more insight into the co-self-assembly process, bilayer films are also produced by multilayer deposition. Since collagen has a molecular size that has a different magnitude than that for lipids, possible protrusion of collagen assemblies throughout the bilayer film is investigated. The orientation and packing density of alkyl chains of lipids at the air/water interface are manipulated to develop a qualitative understanding of the collagen polymerization in a bilayer lipid matrix.
Ultrathin films of newly synthesized porphyrin compounds with various numbers of substituted long aliphatic chains are produced to investigate the possibility to anchor those porphyrin species on a solid support with well-defined film structures. This study is motivated by the requirements of developing novel chemical sensors that are mostly multilayered thin films. It is crucial to understand the factors ruling the molecular alignment and orientation on a solid surface and also in a multilayered assembly. The effects of the tail numbers, solid surface properties, fabrication techniques and experimental conditions on the film structures are investigated in this study.

1.4 Outline

This dissertation includes six chapters. Chapter 1 (introduction) and Chapter 2 (literature review) give an overview of this study and the most recent investigations in the field of fabricating ultrathin films of biomolecules with well-defined nanostructures. Chapter 3 covers the development of novel techniques to fabricate collagen/lipid monolayer films in a highly-controlled way at an air/water interface and the investigation of a co-self-assembly mechanism in this system. Based on the composite monolayer of collagen/lipid, Chapter 4 focuses on fabricating bilayered structure and exploring the possible applications of the synthesized films as a surface template. In Chapter 5, ultrathin films consisting of a series of newly synthesized porphyrin compounds are produced on various solid substrates. The variety of the assembled film structures are explored under different fabrication techniques. Finally, Chapter 6 lists the major conclusions and addresses some work that is worthy of future consideration.
CHAPTER 2

LITERATURE REVIEW

2.1 LB Films of Phospholipids

2.1.1 Phospholipids in Biomembranes

Phospholipids, the main constituents of a wide variety of biomembranes, have been intensely studied in recent years. Self-assembly of amphiphilic and water-insoluble phospholipids at an air/water interface provides a way to reconstruct membrane structures \textit{in vitro}. Based on those simplified models for biological membranes, the fundamental mechanism of membrane function and the detailed interactions between membrane components can be investigated. Since the membrane constituents are mostly heterogeneously distributed in the membrane,\textsuperscript{1,2} the ultrathin film consisting of binary or multiple mixtures of phospholipids is a more realistic model to give information about the relation between the film stacking structure and its biofunction. Different types of lateral domains formed by mixed phospholipids have been related to functional specialization of different regions of membranes.\textsuperscript{3,4}
Phosphatidylcholines (PC) and phosphatidylethanolamines (PE) are quantitatively among the most important phospholipid species found in biological membranes, in which these zwitterionic (having both hydrophobic and hydrophilic portions) phospholipid molecules self-assemble into the well organized lipid structures. The mixture of various PCs and/or PEs containing saturated or unsaturated acyl chains with various lengths exhibit interesting 2-D patterns. The films with mixed PC and PE components have been investigated by analyzing their surface compression isotherms, the shape and size of the domains and the interactions involved in the self-assembly process. DPPC(1,2-dipalmitoyl-sn-glycero-3-phosphocholine), DLPC (1,2-dilauroyl-sn-glycero-3-phosphocholine), POPC(1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), DOPC(1,2-dioleoyl-sn-glycero-3-phosphocholine), DMPE(1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine), DPPE(1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine), DSPE(1,2-distearoyl-sn-glycero-3-phosphoethanolamine) and DOPE(1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) are among the common types of lipids applied to study heterogeneous composite films.

2.1.2 LB Technique

The LB technique provides a surprisingly effective means of controlling the assembly of molecules at an interface and therefore the formation of surface patterns. In a typical LB trough, water-insoluble lipids are spread at an air/water interface to form a floating monolayer that can be compressed to control the surface molecular density. The lipid tails lie flat on the surface at extremely low surface coverage due to the weak interactions between the gas phase and the tails. As the concentration of lipid molecules
on the surface increases, the tails orient more perpendicularly to the surface because of the stronger interactions between the tails. Due to the change of molecular orientation and the interactions between adjacent molecules, domains with various sizes and shapes have been developed for pure lipids and also for their mixtures when phase separation is present. LB technology allows transferring the well-organized monolayer from an air/water interface onto a solid support or fabricating multilayered film by repeating the deposition process, which has promising applications in microelectronics, sensors, catalysis and life science.

Since the 1930s, the LB technique has been developed extensively and even applied to fabricate films with complicated molecular architecture and designed functions. However, a few existing limitations prevent the realization of the potential benefits of the LB approach predicted by Langmuir and Blodgett in the 1930s. First, only amphiphilic and self-assembled molecules are capable of forming LB films at a gas/liquid interface. Second, the defects resulting from the transfer and drying process make it difficult to realize reproducibility and long-range uniformity, which are critical for commercial applications. The interactions between the solid support and the thin film might change the original molecular organization exhibited at gas/liquid interfaces and affect the long-term stability of the supported LB film in various environments. Third, due to the lack of understanding of the fundamental principles of self-assembly found in biological systems, it is difficult to design and fully control the final structure of the film. Finally, the interactions involved in self-assembly are generally multiple weak interactions, therefore, any impurity or other disturbance can induce additional uncertainty into the film architecture. Because of the weak interactions, multilayered
films have a limitation on the number of monolayers that could be deposited onto a solid support, keeping the original morphology intact. Despite all the restrictions mentioned above, the LB technique has demonstrated its feasibility with successful applications in the fundamental investigations of biological structures and the fabrication of novel organic thin film devices. It is promising to incorporate the LB technique into more complicated “molecular engineering” in the future.

2.1.3 Lipid LB Film Characterization

The structures and properties of lipid LB films at the air/water interface and on solid substrates can be characterized by numerous modern tools. For the monolayers floating at the air/water interface, fluorescence microscopy, Brewster angle microscope (BAM) and X-ray Reflectivity are very powerful for studying the surface morphology, phase transition, film thickness and the interactions of the monolayer with ions or biomolecules from the water subphase.

Solid supported LB films have been mainly investigated by X-ray powder diffraction (XRD), scanning tunneling microscopy (STM), scanning electron microscopy (SEM) and atomic force microscopy (AFM). XRD is used to determine the inner structure of the film and the alignment of mesostructures, but it lacks the ability to describe the local defect and morphology of the film. SEM can reveal the 3-D structures of the LB films with nanoscale resolution, though the approach is restricted to be operated at low temperature. To obtain the 2-D morphology of thin LB films at room temperature, STM and AFM are two ideal options. Based on the phenomenon of electron tunneling, STM can provide atomic resolution when exploring the surface morphology.
STM is only applicable to the conductive substrate coated with either a conductive thin film or an insulating one with the thickness lower than a few angstroms. AFM has no limitation for the substrate properties and has been applied to all kinds of LB films either in air or in liquid. AFM gives less lateral resolution than STM due to the size limitation of the AFM tip (usually above 5 nm). The horizontal dimensions of the structures revealed by AFM are usually larger than their actual values when the surface feature size is comparable to the tip size. AFM offers extremely high vertical resolution that is below 1 nm, which is especially useful to reveal surface patterns with tiny height or composition difference.

2.1.3.1 Compression Isotherms at Air/liquid Interfaces

The isotherms for pure lipids or their mixtures are obtained by compressing the surface with floating monolayer in a LB trough, as the temperature of the subphase is kept constant. The surface pressure, defined as the decrease of water surface tension due to the presence of the monolayer on it, shows strong dependence on the area per molecule, usually called Π–A isotherms. The monolayer experiences various stages during the compression of the surface, which could be related to the change of the isotherm curvature. Initially, at an infinitely dilute surface concentration, surface pressure is not affected by the compression. At this state, molecules are far away from each other and do not interact, which is referred to as gas phase. When the molecules are approaching and interacting with each other, the isotherm displays a lift-off and the surface pressure begins to increase above 0 mN/m. Upon further compression, the isotherm exhibits a liquid phase followed by a solid phase, characterized by more sharp
increase in the surface pressure for a given decrease in surface area. Further compression results in the collapse of the monolayer and the formation of multilayer structures at the air/water interface.  

Isotherms of pure lipids and their mixtures can be studied qualitatively or quantitatively to explore their structural properties, compressibility and miscibility. The lipids with the same PE or PC head groups give different isotherms due to the difference in the structures of their hydrocarbon tails. The lipids with saturated and straight alkyl chains form a highly ordered and tightly packed monolayer, which is indicated by a lift-off at a fairly high surface molecular density and a sharp increase in the surface pressure upon further compression. For the lipids with unsaturated hydrocarbon tails, the double bonds existing in the middle of the tails enhance the steric repulsion and result in isotherms with a much less absolute slope and a more loosely packed monolayer. On the other hand, with the same tail structure, PCs give isotherms that are typical of less condensed monolayers than those for PEs because more bulky PC groups lead to more repulsive interactions between the heads. The organization of the monolayer can also be quantified by the calculation of compressibility modulus from the isotherm. The compressibility modulus ($C_s^{-1}$), defined as $C_s = -\frac{1}{A} \cdot \frac{\partial A}{\partial \pi}_T$, gives quantitative measurement of the compressibility of the monolayer, where $A$ is the surface area, $\pi$ is the surface pressure and $T$ is the subphase temperature. The larger the value, the more rigid and less compressible the monolayer.
There are wealth studies of the mixing behavior of PEs and PCs based on the analysis of average area, collapse pressure and phase transition presenting in isotherms. Binary monolayers formed by totally immiscible components or ideal miscible components (i.e., 1-2 interactions are identical to the 1-1 and 2-2 interactions) follow the ideal additive relation:

\[ A = X \times A_1 + (1 - X) \times A_2, \]

where \( A \), \( A_1 \) and \( A_2 \) are the average molecular area for the mixture and the pure components obtained from isotherms at a specific surface pressure, and \( x \) is the molar fraction of component 1 in the mixture.

At a surface pressure of 40 mN/m, DOPE and DPPE mixtures displayed total immiscibility by fitting the above linear correlation. Any deviation presented for a mixed monolayer implies possible miscibility in the film. Positive deviations are related to stronger repulsive interaction between the two components in their mixture than that among pure lipid molecules, while negative ones are corresponding to more attractive forces between the molecules. However, a non-ideally miscible film might confirm the same correlation due to the experimental error, therefore other investigations were required to demonstrate any conclusion. When two lipids are immiscible, mixed monolayer collapsed at the same surface pressure, not varying with the monolayer composition; while for the miscible monolayer, the collapse pressures showed a linear dependence on the composition. Some lipids, such as DPPC, have a plateau region in their isotherms indicating the transition from a liquid-expanded phase to a liquid-condensed phase. When one compound with a phase transition was mixed with another one with two liquid phases indistinguishable, immiscibility was implied if the phase transitions for both components were present.
2.1.3.2 AFM and Other Microscopes

AFM and other types of microscopy have been used to visualize the domain formation of lipids and their mixtures at air/liquid interfaces or on solid supports. At an air/water interface, fluorescence microscopy revealed the existence of stripes and spots in the monolayer consisting of DPPC and a tiny amount of dye lipids as the surface pressure was in the region of the liquid-expanded phase.\(^{17}\) Those domains with diameters of several tens of microns became less evident at higher surface pressures and the film appeared more uniform under further surface compression. Györvary, etc. obtained a similar morphology of DPPC monolayer at an air/water interface with BAM.\(^{18}\) Without the disturbance of fluorescence dye lipids, pure DPPC monolayer showed a clear phase transition at the surface pressure of around 13 mN/m, corresponding to the transition from a liquid-expanded phase to a liquid-condensed phase (the plateau region of the isotherm). At a surface pressure as high as 30 mN/m, the BAM image gave a smooth and uniform film structure. Pure monolayers of DMPE and DPPE exhibited different morphologies under BAM due to the difference in their tail lengths. A similar phase transition as that for DPPC was observed for DMPE, while only the liquid-condensed phase was visualized for DPPE before the homogeneous monolayer was obtained at higher surface pressure. As the surface pressure was approaching the collapse pressure, DMPE monolayer broke down and the appearance of fractures indicated the formation of multilayers. By introducing enough DPPE-EMC (biofunctionalized linker lipid) into the monolayer of DMPE/DPPE at an air/water interface, the mixed monolayer did not show a homogeneous organization during the whole compression.\(^{18}\) The visible domains packed closer during the compression but never disappeared, indicative of the miscibility of
DMPE and DPPE with the linker lipid. However, DPPC/DPPE-EMC matrixes were found totally miscible by reaching a homogeneous monolayer after the compression. The investigations that have been performed on the mixing behavior of different types of phospholipids revealed the significant effects of both lipid structure and surface pressure on the surface features of their composite monolayers.

After the monolayer of lipids is transferred onto a solid substrate, AFM is an ideal tool to carry out visual investigation either in air or in liquid phase. DPPE monolayer was deposited onto hydrophilic mica at surface pressures in solid phase. The topographical images of the film obtained under contact mode in air appeared densely packed and highly ordered with only a few defects (dented holes) at the micron scale, which is consistent with the results obtained under BAM at an air/water interface. The depth of the holes was measured to be 2.8 nm and corresponded to the thickness of a single monolayer of DPPE. The double tails of DPPE were visualized to be almost vertically oriented relative to the mica surface in nanometer scale images. However, homogeneous films could not survive in air for the DPPE monolayer deposited onto hydrophobic highly ordered pyrolitic graphite (HOPG). Monolayer, bilayer and trilayer organizations were found co-existing due to the rearrangement during the drying process. The rearrangement also occurred for the DPPE bilayer deposited onto mica and air-dried at ambient temperature, leading to the formation of domains consisting of monolayer and trilayer. It is expected that the reorganization should be avoided when the DPPE bilayer on mica was kept under water; however, a mixture of monolayer and bilayer structures was observed by AFM under water. DPPC monolayer on mica was first investigated by Yang with AFM in the repulsive mode and friction mode in air. The AFM images gave
a more clear visualization of the phase transition at low surface pressures. More vertical molecular orientations during compression and finally more densely packed films were observed than the features revealed by fluorescence microscope and BAM. DPPC bilayer on mica in air\textsuperscript{21} exhibited more significant reorganization than that for DOPE, because the trimethyl group in the polar headgroup of DPPC reduces the hydrophilicity.

The LB films consisting of a binary mixtures of DPPC and Ceramide showed considerable miscibility when deposited onto mica at a surface pressure of 10 mN/m, which was in good agreement with the negative deviation from the ideal curve (more attractive interactions in the mixture).\textsuperscript{23} The comparison of area fractions in AFM images and the average areas in the isotherm demonstrated that the well-defined elliptical domains with diameters of around 2.5 nm were resulted from the phase separation between the liquid-expanded phase and the liquid-condensed one, in which the two components are miscible. However, the DOPE/DSPE\textsuperscript{11} and DOPE/DPPE\textsuperscript{14} monolayers formed domains caused by complete phase separation of one component from the other. Solid-like DSPE and DPPE formed domains embedded into a matrix consisting of liquid-like DOPE. It is well known that the incorporation of cis double bonds into hydrocarbon tails increases the cross-sectional area of the lipid molecule dramatically. The unsaturated tails of DOPE molecules were predicted to lead to more sterical repulsions between DOPE than those between DSPE or between DPPE. The shape and the size of the domains were found to be affected by the monolayer composition, surface pressure and the surface chemistry of the solid substrates. There was no phase separation or domain formation observed for DPPE/DPPC at all the molar fractions,\textsuperscript{14} which could be attributed to the same straight tail structures. It is expected that the difference in tail
structures is one key point that determines the miscibility behavior of binary phospholipids mixtures, which was confirmed by the partial miscibility of the DPPC/DOPE system. Döfler found complete or partial miscibility in binary monolayers relied on the film state (liquid-expanded or condensed) and their difference in the chain lengths and chain-chain interactions. Bilayer depositions of DPPE/DOPE and DSPE/DOPE onto mica exhibited irregular and inhomogeneous domains with significant defects. It is difficult to maintain the integrity of the monolayer domains after the transfer of the second layer either in air or in water.

2.1.4 Theoretical Study of Lipid Domain Formation

The formation of 2-D domains of phospholipids and their miscibility have been theoretically studied by analyzing the interactions involved in mixed monolayers. Simple mathematical models have been developed to predict the domain shape and size in either pure lipid film or multiple component lipid film at an air/water interface. The equilibrium domain shape and size are determined by a competition between interfacial line tension (which favors isotropic circular domains) and long-range dipole-dipole repulsion (which favors elongated domains). The balance between these factors can lead to undulations and branching of domains (chiral domain shapes). In the mixed lipid films, the compositions around the domain boundaries were found to be different from the bulk lipid compositions, which was found to contribute to stabilizing the domain formation.

In those simplified models, many factors that influence the domain formation have not been taken into account, which include van der Waals forces between the
hydrophobic tails, various steric forces and most importantly the hydration forces existing in the film.\textsuperscript{29-32} Hydration effects are crucial to the fragile interplay of the weak forces and often dominate at small molecular separations. Hydration of PE and PC lipids with saturated and unsaturated tail structures was investigated by preparing cast thin films with lipids on mica and exposing them to a gas phase with various relative humidities.\textsuperscript{31,32} The existence of a dense hydrogen bonding network formed between the phosphate and NH\textsubscript{3}\textsuperscript{+} groups of PE heads led to the phenomenon that PC lipids adsorbed more water than PE lipids under the same conditions. The lipids with unsaturated tails took up more water than the saturated lipids, which was attributed to a larger cross-sectional area of the unsaturated tails.

\textbf{2.2 Collagens and Their Polymerized Hierarchical Structures}

Collagen is the most abundant structural protein in the ECM of connective tissues, such as tendon, bone and skin. Collagen is a family of over 19 distinct proteins sharing a common structural element---a triple helix. More types of collagen might to be found during further investigations of biological molecules. The monomeric unit of collagen, tropocollagen, can self-assemble to form fibrils that are the fibrous scaffold maintaining the integrity of tendons, ligament and bones; or aggregate in the form of network structures in basement membrane. The self-assembled and highly ordered fibrils or thin filaments subsequently aggregate into fiber bundles and other structures to fulfill a variety of biological functions. This following aggregation has been found to be regulated by the cell-mediated information and the presence of other important matrix macromolecules.\textsuperscript{33-35} Collagen plays a crucial role in the evolution of complex organisms
and tissues in vivo by providing a stable and insoluble scaffold for the attachment of cells, macromolecules, glycoproteins and inorganic ions. The biocompatible and degradable collagen has a wide range of therapeutic applications, such as bone implants, skin repair and drug delivery. On the other hand, in vitro, acid-soluble collagen self-assembles forming interesting 2-D and 3-D organizations, which promises the potential applications of collagen in biosensor design, cell patterning and the synthesis of inorganic or inorganic/organic materials with highly ordered mesostructures.

2.2.1 Molecular Composition and Structure

All types of collagens comprise a triple helix consisting of three helically wound polypeptide chains. The amino acid composition of all collagen types is high in glycine (Gly), proline and hydroxyproline, which together account for over fifty percent of the total amino acid content. For type I collagen, one third of the residues are Gly and proline or hydroxyproline, occupying around 10% for each. Additional amino acid types and the ratio of the amino acids in each polypeptide chain vary from type to type.

Through a dehydration reaction, amino acids polymerize into a polypeptide chain of approximately 1,000 amino acids with the repeat sequence of Gly-X-Y, where X and Y can be any amino acid but are frequently proline and hydroxyproline. The three-residue helix is a left-handed twist, which is introduced and stabilized by the contribution of proline through its conformation-directing pyrroline ring, because polyproline has been found to form a left-handed helix similar to the helix of one chain in collagen molecule in aqueous solution. In each turn of the helix, Gly residues are positioned along the same outside of a polypeptide chain. The absence of a side chain on Gly is critical for
packing the three chains tightly with all the Gly residues in the middle of the triple helix. To accommodate Gly residues in different chains into the superhelix, adjacent chains are staggered by one residue and coiled right-handed. The supercoiled structure of tropocollagen is partially stabilized by the hydrophobic interstrand interactions between the apolar residues in different chains. The highly ordered hydration network further stabilizes the 3-D molecular conformation by the formation of interchain and/or intrachain hydrogen bonding. In water mediated hydrogen bonding, hydroxyproline plays a special role to bind water molecules and serves as the hydrogen bond acceptor and donor. The high content of hydroxyproline is essentially unique for collagen. The stereochemical restriction of the imino acid rings in hydroxyproline leads to its frequent appearance at the Y position. The hydroxyl and carbonyl groups in hydroxyproline participate in the formation of water-bridged hydrogen bonds.

With most of the side chains extending outside of the supercoil, numerous hydrophobic and charged residues are available on the outmost surface of collagen for self-associating or interacting with other molecules. Uneven distribution of hydrophobic residues and net charges along the collagen monomer surface leads to a unique and highly ordered way for collagen to self-assemble, which will be discussed in detail in the following section. Collagen plays a crucial role in specific binding of many different kinds of proteins and cells, such as collagenase and platelet. Linear sequences lining along the triple-helix provide the binding sites, which is determined by the basic triple helical structure of collagen. In the repeating Gly-X-Y helix, all residues in X and Y positions are possibly exposed to solvent and accessible for binding other molecules.
Fibril-forming collagens, such as type I collagen, are synthesized as a soluble precursor collagen (procollagen) through intracellular processing. The procollagen contains helix domains in the middle and telopeptide and propeptide groups attached to the end subsequently. The amino terminal propeptide (N-propeptide) comprises a short helical region and intrachain disulfide bonds, folding back onto the main triple helix. The carboxyl terminal extension (C-propeptide) has both intrachain and interchain disulfide bonds and displays a clustered conformation on the other end. During extracellular processing, the procollagen is converted into collagen by specific enzymatic cleavage of those propeptides by the procollagen metalloproteinases. The resulting collagen monomer consists of two short non-triple helical groups (N-telopeptide and C-telopeptide) positioned on both ends of a long triple helix. The length of the helical region and the properties of nonhelical portions vary for different types of collagen. The fibril-forming collagen has a triple helix of approximately 300 nm in length and 1.5 nm in diameter. Type I collagen has two identical $\alpha_1$(I) and one $\alpha_2$(I) chains, in which $\alpha_2$(I) chain has shorter terminal groups than $\alpha_1$(I) chain and the order of the three chains has not been known for certain.

2.2.2 Polymerization in Nature

Fibril is the most common supramolecular structure formed by the association of collagen monomers (tropocollagens) both in vivo and in vitro. The collagen fibril is the main source of flexibility and mechanical strength in connective tissues and provides a template for the anchorage of other macromolecules from the ECM and cells. Fibril formation is intrinsically a self-assembling process, which is to a large extent determined
by the inherent properties of the collagen molecules and also influenced by cell-mediated
procedures in vivo. From the point of view of thermodynamics, the assembly of collagen
molecules into fibrillar structure is driven by the loss of collagen-bound solvent
molecules, leaving more free solvent molecules and lowering energy of the bulk solvent.
This entropy-driven process results in cylindrical fibril assemblies with diameters ranging
from 20 nm to 500 nm. The broad principle of collagen fibril assembly is based on end-
to-end and side-by-side interactions.\textsuperscript{52} The lateral fusion between collagen molecules
may be mediated mainly by a water-bridge, while the direct van der Waals contacts
between adjacent molecules are restricted to the longest side chains. As collagen
molecules are packed together, there should be a rearrangement of the hydration network.
In contrast to the side-by-side combination, the axial molecular stagger packing is likely
determined by direct interactions involving long side chains.

Tropocollagen molecules exhibit a specific D-staggered arrangement in the
assembled fibrils, where D is 67 nm, the characteristic axial periodicity of collagen.
Along the 300 nm long collagen molecule, the polar regions appear with the periodicity
of 67 nm, dividing the monomer into 4.4D unites.\textsuperscript{50} The repeated hydrophobic clusters
and hydrophilic residues direct the monomers to associate in the way that one molecule is
staggered by one D with respect to its laterally neighboring molecules. The information
required to assemble molecules in the D-staggered association is built in the helical
sequence of the tropocollagen.\textsuperscript{45} Hydrophobic and electrostatic interactions exhibit
maxima at stappers corresponding to integral D space, however, 1-D staggering\textsuperscript{53} or 4–D
staggering\textsuperscript{49,54,55} turned out to be the most possible way to stabilize the fibril
conformation. Since the number of D-units is non-integral for each monomer, gap regions
(0.6 D in length) and overlap regions (0.4 D in length) exist to maintain a configuration in register, which has been visualized by a periodic banded appearance in the electron micrograph. The gap between the C-terminal end of one molecule and the N-terminal end of the next molecule in a row was likely to induce the nucleation and crystallization of hydroxyapatite along the surface to collagen fibrils during bone formation.\textsuperscript{50}

The detailed investigation of how the fibril is developed from monomeric precursors to aggregations with desired dimensions and structures has major implications for the tissue growth and repair. In the early stage of fibril growth, two kinds of fibrils have been observed: unipolar (with all molecules aligned exclusively in one direction) and bipolar (in which a switch in molecular pointing direction occurs at a single location along the fibril).\textsuperscript{56} The fibrils found \textit{in vivo} are not all unipolar, in which fibrils are ended with one N-terminal group and one C-terminal group. N-N bipolar fibrils (with two N-terminal groups at both ends of fibrils) were also observed in developing vertebrate tendon and echinoderm ligament and dermis.\textsuperscript{56-58} \textit{In vitro}, the reconstructed fibrils from acid-soluble collagen extracted from animal tissues appeared unipolar with characteristic D-period.\textsuperscript{59} Solely bipolar fibrils can be obtained by the cleavage of purified type I procollagen with C-proteinase. Both unipolar and bipolar fibrils are developed in two ways: end-to-end accretion of monomers into long and thin filaments and side-by-side aggregation of those filaments into broader fibrils. The growth of fibrils examined \textit{in vitro} turns out to be a multi-step process in which the formation of linear association precedes lateral growth.
It is evident that some kind of control must be exerted over the size and length of the fibrils in vivo, though the underlying mechanism has not been well known. In young tissues, the fibril bundles have a typically sharp diameter distribution.\textsuperscript{50} Although with the maturation of the tissues, the diameter and length distributions become broader, individual fibrils in vivo tend to be fairly uniform in diameter (except at the ends). The regulation of fibril length and diameter has not been observed for collagen regenerated from pure solution in vitro, which might be contributed to the extraction process. The terminal residues in either procollagen or collagen molecules might play an important role in the control over the fibril growth.\textsuperscript{52,56} It is suggested that the cleavage of the C-propeptides from procollagen initiates the fibril self-assembly and the remaining N-propeptides at the fibril surface restrain the further lateral growth. Fibril formation is prevented by complete removal of both telopeptide ends. During the aggregation, the hydrophobic residues in the C-telopeptides are condensed into a hydrophobic cluster, which promote both the early linear assemblies and further lateral association of linear filaments; while the N-telopeptide is crucial for the formation of a staggered alignment.

\textbf{2.2.3 Cross-link and Extraction}

The assembly of tropocollagens into fibril is accompanied by the formation of inter- and intra-molecular covalent cross-links between monomers.\textsuperscript{50} Although the hydrophobic and electrostatic interactions can stabilize the assembled fibrils, the high tensile strength and mechanical strength needed for tissue integrity are essentially coming from the cross-links. Without covalent cross-links, the fibrils are easily degraded and dissociated. The cross-links are based on enzymatic conversion of lysine or
hydroxyllysine residues in the telopeptide terminal regions to allysine or hydroxyllysine that participates in the further condensation reactions to form covalent cross-links intra-molecularly or inter-molecularly. The regions that are available to be cross-linked along the tropocollagen are specifically in the telopeptide regions or 87 and 930 residues in the helical chain. The hydroxyl group of hydroxyllysine serves important functions in the formation of interchain cross-links. The degree of cross-linking increases during the maturation process.

Type I collagen is usually extracted from young animal tissues, especially skin and tendon, where the cross-links are not as extensive as those in mature tissues. The extraction can be operated either in dilute acid (eg, 0.5 M acetic acid) or in old 0.5-1 M NaCl at neutral pH. The collagen extracted by neutral salt solution contains varying proportions of monomers and higher molecular weight aggregates with cross-links, which can be cleaved by acidic extraction. Pepsin-extraction yields a much greater amount of soluble collagen with a higher ratio of monomer than the salt- or acid- extracted collagen, though the collagen is slightly truncated, loosing the telopeptide ends in most cases. The extracted collagen solution consisting of monomers, dimers, trimers and higher aggregates is purified mainly by precipitation based on the solubility in aqueous solution that is dependent on ionic strength, pH and highly on temperature around a neutral pH. Under the right conditions, the solubility of collagen aggregates containing a few molecules is dramatically reduced, resulting in the separation of monomeric collagen.
2.2.4 Reconstituted Collagen Structures

2.2.4.1 Hierarchical Structures in Bulky Solutions

All the collagen features of fibrillar shape observed \textit{in vivo} have been observed in reconstituted fibrillar collagen \textit{in vitro} from bulk solutions, except the partial control of diameter and length that is particularly found \textit{in vivo}.\textsuperscript{56} Under some conditions, the fibrils reconstructed from solutions have a very similar or even identical structure to that of the native ones.\textsuperscript{60} A diversity of polymorphic assemblies in addition to the native fibril has also appeared for poorly understood reasons. Fibrous long spacing collagen (displaying a band with periodicity longer than D=67 nm) and segmental long spacing collagen (bloke-like aggregates) can be induced by incorporating $\alpha_1$-acid glycoprotein and adenosine triphosphate to monomeric collagen solution \textit{in vitro}.\textsuperscript{61-63} Those unusual variations of fibrous collagens have been utilized to explore the mechanism of self-assembly and the fundamental properties of various collagen monomers.

There are many factors that possibly affect collagen fibrillogenesis \textit{in vitro}, including ionic strength, pH and temperature of the solution and the presence of macromolecules (eg, proteoglycans, glucose, alkylureas and free fatty acids).\textsuperscript{50,56} For the cold, acidic solution of type I collagen, the fibrillar assembly can be induced by neutralizing and/or warming up the solution to be near neutral pH with low ionic strength at a temperature of 20-37 °C. At near neutral pH, the degree of association shows an inverse dependence on temperature. The neutralized solution granted fibrils with diameters in the range of 20-70 nm at 34 °C and broader fibrils with diameters of up to 200 nm at 20 °C.\textsuperscript{64} The fibrils formed at higher temperatures exhibit longer and more
flexible conformations than those formed at a lower temperature. The order of warming and neutralizing of collagen monomeric solution was critical to the initial fibril formation and finally the resulting fibril conformation.\textsuperscript{59} Warming followed by neutralization led to the formation of early fibrils; neutralization followed by warming resulted in the accumulation of filaments. The pH and temperature of the precursor solution had a drastic effect on the thermal stability and mechanical stability of the fabricated collagen fibrils by influencing the aggregated structures.\textsuperscript{65,66}

2.2.4.2 Hierarchical Structures in Supported Thin Films

Collagen thin films supported by solid substrates with highly ordered structures are of major importance in drug delivery, biosensors and biocompatible implant materials. The supported ultrathin films have been fabricated by various coating technologies\textsuperscript{64,67,68} or adsorption from monomeric solution of collagens\textsuperscript{69-71} onto different solid substrates. Collagen molecules concentrated on the surface formed a variety of structures influenced essentially by the surface properties of the solids, the drying procedure and the solution conditions, which promises a way to control the aggregation process, the degree of association and inherently the resulting mesostructures of the film.

Collagens in bulk aqueous solution generally assemble with most of the hydrophobic amino acids in the core of the aggregates and the charged/polar amino acids at the exterior surface.\textsuperscript{50} Once the solid surface participates in the self-assembling process, the general principle of collagen adsorption at the solid/liquid interface interplays with the intrinsic properties of collagen, leading to the polymorphism of the supported film. When collagen molecules are approaching the solid surface and
dewetting in air, redistribution of charged groups, changes of hydration state and rearrangement of collagen molecules play a major role in determining the final morphology of the thin film.\textsuperscript{72}

Monomeric and fibrillar type I collagen was visualized under AFM after the adsorption of collagen onto the hydrophilic mica.\textsuperscript{69} Thin films of monomeric collagen adsorbed from a cold (21 °C) and acidic collagen solution showed a homogeneous and felt-like morphology at higher concentration and individual monomers or fine filaments at concentrations as low as 0.22 \(\mu\)g/ml. Fibrillar collagen structures were observed in the adsorbed film from warm (37 °C) and neutralized collagen solutions during or after the 30 min fibrillogenesis process, resulting in the formation of straight and long bundles of collagen fibrils with D banding and finer fibrillar materials without D spacing, respectively. Dupont-Gillan et al investigated the adsorbed type I collagen film on the surface of various hydrophobic polymers.\textsuperscript{70} AFM revealed the surface organization of the collagen films absorbed onto poly methyl methacrylate (PMMA) from collagen solutions at different concentrations at 37 °C followed by either a fast or slow air-drying. Both the solution concentration and the drying procedure affected the film structure considerably. Fast drying yielded a continuous and dense film with filamentous structures, the size of which was larger when the collagen solution concentration was higher. The water contact angle measured on the homogeneous film was lower than that for PMMA, implying that there are numerous charged/polar groups exposing at the outmost surface of the air-dried collagen film. On the contrary, network assemblies were formed after slow drying, where collagen self-organized to be the threads of a net leaving PMMA exposed at the surface in the hole regions with diameters of 30-120 nm and 100-150 nm for low concentration
samples and high concentration samples, respectively. The heterogeneous surface organization was assigned to the cooperation of collagen-collagen interactions, collagen-substrate interactions and the change of hydration during dewetting. Polycarbonate, polyethylene terephthalate (PET) and polyvinylidene difluoride (PVdF) with different surface roughness and substratum hydrophobicity were applied as the basically hydrophobic substrates to support the adsorbed collagen films. Holes that are 4-5 nm deep were visualized by AFM in the films on PVdF and PET. The treatment of the substrata with oxygen plasma discharge reduced the hydrophobicity of the surfaces and gave rise to the formation of densely packed uniform films on treated PET and PVdF. Surface pattern formation in collagen films prepared by spin-coating or dip-coating involves collagen self-assembly, solution drop spreading on solid surface and dewetting in air simultaneously, which makes the duplication of surface patterns difficult to achieve. Dip-coated and spin-coated collagen films on hydrophilic mica displayed similar mesh-like structure under high solution concentrations and isolated monomers under low solution concentrations. Net-like structures were observed for collagen films spin-coated onto hydrophobic HOPG in ambient condition, which resembles the film organization adsorbed on PMMA, although the diameters of the open holes were increased to be 380 nm. Under the same conditions, the hole structure was not observed on mica. This was attributed to the difference in wetting behavior of the substrata. Reducing the drying speed led to the formation of a more highly developed polygonal network with a more narrowly distributed pore size. On a more hydrophobic surface, assembled collagen structures undergo stronger forces coming from the surface tension of water and are ruptured to a certain degree, especially after slow air-drying.
2.3 Development of 2-D Protein Patterns

2.3.1 Comparison of Commonly Used Techniques

Immobilized multiple biomolecules on solid substrates with well-defined patterns and specific biofunctions are of great importance in the development of biosensors, biologically integrated devices and in the fundamental study of cellular behavior. There have been a wide variety of techniques used to localize proteins on solid surfaces and to form 2-D patterns with resolution from the micron to the nanometer scale. To date, all of the commonly utilized patterning methods, including photolithography, photochemistry, microcontact printing, patterning by microfluidic channels, laminar flow patterning and SAMs, are only capable of giving well-controlled patterns of single proteins on the micron level and within two dimensions. The conventional photolithography is a highly developed technique that can be applied to produce protein arrays of size below 1 µm. The most apparent obstacle for photolithography is the high cost correlated to the required equipment and the clean environment during fabrication, especially when the resolution requirement is below 1 µm. Protein activity might be jeopardized through the exposure to the residual solvents and photoresists, which can be minimized by selecting proper solvents, buffers and reactants for photochemical methods. Microcontact printing, microfluidic channels and laminar flow patterning have the advantages of low cost, convenient operation and applicability to non-planar substrates, though their resolution can not go lower than micron. Except SAMs, all the other patterning technologies are associated with microfabricated masks, stamps or fluidic channels, which makes the resolution of the patterned features unable to overcome
the limitation of several hundreds of nanometers. Possible UV irritation utilized by most of the patterning methods may denature the proteins and decrease their surface activities.\textsuperscript{73}

Compared with the techniques discussed above, self-assembling of proteins and/or other biomolecules at an well-defined interface is a much easier way to organize and immobilize proteins forming surface features with nanometer size. The self-associated patterns are primarily determined by the intrinsic properties of those biomolecules and their co-assembling mechanism in a mixture.\textsuperscript{76} The fabrication procedure and condition can partially influence the final protein localization. Those biologically derived systems, mainly based on weak interactions, are sensitive to any disturbance from the processing steps, such as slight contaminations. The spontaneous assembling leads to a fast, easy and low-cost procedure that can retain the protein bioactivity and promise the possibility of including multiple proteins in the patterned films, although this method suffers from the lack of long-range uniformity and high reproducibility.

Self-assembled protein or composite monolayers are usually immobilized onto solid substrates by casting method and LB deposition.\textsuperscript{7,77} Casting methods have limited control over the protein film structure by adjusting the contents and the condition of the cast protein solution or the drying process. The advantage of LB deposition over casting is that the fabrication of thin films at an air/water interface and the transfer of films onto solid surfaces can be preformed in a well-defined way.\textsuperscript{7} There are many factors that can be varied to flexibly control the protein patterning, including the composition of the
spread solution, the way to introduce protein, the pH, ionic strength and temperature of the subphase, 2-D molecular density and therefore the way that the molecules interact and orient on the surface and finally the method of deposition. Another apparent advantage of LB technology is retaining the bioactivity of proteins by controlling the protein orientation either at air/water interfaces or on solid surfaces.\textsuperscript{77} For example, in immunodiagnostics, the optimum bioactivity, the highest surface binding ability and therefore the sensitivity of antibodies can be obtained by aligning the antibodies in a highly ordered and specifically oriented way.

2.3.2 LB Films of Proteins and Lipids

SAMs of proteins embedded in lipids, especially phospholipids, have been intensely studied with the LB technique to explore the biofunctions of proteins and the interactions in the cell membranes.\textsuperscript{77} In addition to serving as a model for a biological membrane, co-self-assembled monolayers of phospholipids and proteins provides an encouraging way to pattern proteins two-dimensionally. Phospholipids composed of two aliphatic chains connected by a hydrophilic head group orient themselves at an air/water interface with chains pointing toward the air and heads toward the aqueous subphase with long-range order. The specific alignment and possible microdomain formation of phospholipids render a promising method to incorporate proteins at interfaces and to induce the formation of a wide variety of 2-D protein configurations mainly through hydrophobic and electrostatic interactions between lipids and proteins. Moreover, lipids can significantly reduce the strong forces exerted on the proteins by surface tension of water that might denature the proteins.\textsuperscript{78} The transfer of patterned thin films from
air/water interfaces to solid surfaces can be realized by various deposition methods. Successful fabrication of the LB films requires keeping the original lateral features generated at air/water interfaces and the bioactivity of biomolecules intact to a certain extent.

There are two common ways to introduce proteins into a LB composite monolayer. Typically, a tiny amount of lipid solution in organic solvent is firstly spread on an aqueous surface and the protein of interest is injected under the lipid monolayer and allowed to adsorb onto or penetrate into the lipid monolayer. The surface pressure will increase significantly if vast amounts of proteins can insert themselves between the lipid molecules and interact with the lipids. Another way is to spread protein solutions with lipids or on top of an already self-assembled lipid monolayer, which is only feasible for the system that can keep the majority of the protein molecules staying at the interface, instead of going into the subphase.

Alexandre et al introduced FtsZ, a key protein in cell division, and guanosine 5’- triphosphate (GTP) beneath the previously spread DPPE monolayer on a buffered aqueous subphase at pH of 7.\textsuperscript{19} In the LB trough, the surface pressure was controlled by moving the barrier to enhance the incorporation of proteins from the subphase. After transferring the composite monolayer onto mica by horizontal deposition, AFM revealed interesting lateral patterns of the protein. FtsZ alone assembled with DPPE forming a homogeneous film with granular surface and randomly scattered FtsZ aggregates. In the presence of GTP, notable ring-shaped or fibrillar domains of FtsZ were observed embedded in the lipid background, which resembles the Ftsz pattern found \textit{in vivo} during
the early step of cell division in bacteria. Proteins and lipids can also be introduced simultaneously in a vesicle solution as the subphase, which leads to the formation of a mixed monolayer when the vesicles are in equilibrium with the air/water interface under proper conditions. Antibody fragments, Fab’, cross-linked with DMPC formed a monolayer in the LB trough from a vesicle solution. Fluorescence microscopes were applied to explore the surface morphology of the composite monolayer at an air/water interface. There was no domain formation in the uniform and fluidic film of Fab’ and DMPC as the surface pressure was low and the subphase temperature was high. When the surface pressure was increased above 30 mN/m by compressing the surface, remarkable and well-defined circular domains were observed, which was the protein-rich phase evenly distributed in a continuous phase composed of pure lipid. The domains were well preserved in the transferred film. Introducing proteins from the subphase requires a long time, generally over one hour, to reach equilibrium with the air/water interface and has the problem of controlling the amount of proteins that participate in the film formation.

For the proteins that are water insoluble, it is fairly easy to spread the mixed solution of proteins and lipids on the surface of the aqueous subphase. Gramicidin A (GRAM), a channel-forming protein in membranes, mixed with methyl stearate (MS) and ethyl palmitate (EP) in chloroform was spread on the subphase of a buffer solution at pH of 5.6 in a LB trough. The compression isotherms exhibited ideal miscibility in those two binary component films. Under certain conditions, it is possible to introduce the partially water soluble proteins by simply spreading the protein solutions on top of a floating lipids monolayer. The degree of preventing the proteins going into the aqueous subphase is mainly determined by the interactions between the lipids and proteins, the
subphase compositions and even the solvent selected for the protein solution. Tazi et al successfully delivered an aqueous ethanol solution of Cytochrome f (Cyt f) (a component of the b\textsubscript{6}/f protein complex) at many locations on top of a floating Monogalactosyldiacylglycerol (MGDG) monolayer that was previously spread from its chloroform/ethanol solution onto the surface of a Tris-HCl buffer (pH 8.1) and was allowed to evaporate the solvent completely. The mixed film at the air/water interface was compressed to reach the surface pressure of 26 mN/m before it was transferred by vertical dipping onto modified mica on which a monolayer of C\textsubscript{20} was deposited. AFM observation revealed the coexistence of individual Cyt f molecules and protein assemblies as an octamer dispersed in between the lipid irregular domains. Since pure Cyt f monolayer showed domains comprised of well connected assemblies, it was suggested that the presence of lipids can inhibit the protein aggregation.

2.3.3 Protein Patterned by Lipid Template

2.3.3.1 Interactions Involved

The underlying principle that governs the pattern formation in a composite protein/lipid monolayer is complex interactions between lipids and proteins which can be influenced by the properties of the subphase and the solid surface.\textsuperscript{76,77}

Weak attractive interactions and/or entropic forces tend to lead to non-specific binding of protein to a lipid monolayer where the protein exhibits indiscriminate adhesion to the lipid matrix.\textsuperscript{73} Electrostatic forces dominate the initial attachment of the protein to the film surface. The packing status of the lipid film and the hydrophobic mismatch

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between the protein and lipid determine whether proteins can penetrate into the lipid film and participate in the lateral pattern formation. It is a general finding that protein prefers penetrating into a lipid monolayer in the liquid-expanded phase to that in the liquid-condensed phase. As the lipid molecules are packed more densely, the binding and dispersion of proteins in the lipid film are confined significantly. This phenomenon has been explained that the highly ordered liquid-condensed phase leaves less free space for protein to penetrate and the protein is taken as a defect in this unfavorable environment. Proteins tend to aggregate at the interface to reduce the extra energy introduced by incorporating proteins. For the systems containing protein and lipids with functional groups, specific bonding (such as antibody bonding) is of more interest than a non-specific one. In this case, the non-specific effect can be inhibited typically by either applying a tightly packed lipid monolayer as the reference matrix or introducing another ‘block’ species to cover the undesired locations before the protein adhesion.

2.3.3.2 Protein Surface Pattern Formation

Both non-specific and specific interactions between the proteins and lipids can be applied to selectively adsorb proteins onto a patterned and deposited LB film of lipids, which is another method based on LB technique that has been widely utilized to form protein features two-dimensionally. Moraille and Badia successfully patterned two blood plasma proteins, human serum albumin (HSA) and human γ globulin (HGG), on previously patterned DPPC/DLPC LB monolayer and bilayer supported by mica. At an air/water interface, when the surface pressure is above 18 mN/m, it was proposed that DPPC/DLPC experienced a phase separation into an array of circular DPPC domains
embedded in a continuous DLPC matrix, where DPPC was in condensed phase while DLPC was in a fluidic phase.\textsuperscript{83,84} During the vertical deposition process, the DPPC domains coalesced and rearranged at the three-phase contact line between the water subphase, mica and air, leading to a periodic change of contact angle and meniscus height. The above process resulted in the formation of parallel stripes and/or circular domains of condensed DPPC embedded in a fluid-like DLPC matrix, where the lines were always perpendicular to the dipping direction whatever the orientation of the mica sheet was. The molar ratio of DPPC/DLPC, the surface pressure and the dipping speed were applied to adjust the distribution, the size and the type of the formed surface patterns. The protein of HAS and HGG that contacted with the mica-supported DPPC/DLPC monolayer from their aqueous solutions showed selective adsorptions directed by the stripe-like patterns of lipids. HGG was primarily attached to the more expanded DLPC matrix with the 10 nm long protein molecules lying flat on the DLPC matrix. Since the hydrophobic interaction is the key factor leading to the adsorption of HGG, the origin of the selective adsorption is the difference in packing density of alkyl chains and/or the surface energies between the condensed DPPC and less ordered DLPC phases. The preference of HSA localization was similar as that of HGG at low surface pressures, but showed dependence on the phase state of DPPC lines. This phenomenon was ascribed to the fact that HSA interacted directly with the alkyl chains, not just the top of an alkylated surface, when the alkyl chains of lipids were packed neither too tightly nor too loosely.\textsuperscript{82}

Similar stripe-like patterns were fabricated by two vertical depositions of DPPC/DLPC at an air/water interface on a mica surface.\textsuperscript{84} They proposed that the first
patterned layer served as a template for the deposition of the second layer. Due to the partial desorption of the first layer during the transfer of the second layer, circular bilayer defects were dispersed in the bilayer film. HGG showed preferential absorption into the circular holes and attached onto mica, which was assigned to the inhibition of the zwitterionic PC head groups to the protein adsorption.

2.3.3.3 Hydrophobic Mismatch

In vivo, hydrophobic interactions play a major role in stabilizing the biological structure of membranes and keeping the fluidic organization of proteins and lipids. In 3-D protein/lipid bilayer systems, hydrophobic mismatch, that is the difference between the hydrophobic length of intrinsic membrane protein and the bilayer thickness, has been intensely investigated experimentally and theoretically. In general, as the hydrophobic part of the protein is much larger than the bilayer thickness, the protein might self-assemble, tilt or change its molecular conformation to reduce its effective hydrophobic length; while the hydrocarbon chains of the lipids could adopt a more stretched and straight structure to reduce the original mismatch. The mismatch will affect the protein activity and stability by adjusting the protein aggregation state and the molecular conformation and will also modify the bilayer lipid organization by disrupting the localization of lipid chains. It is reasonable to anticipate that the presence of hydrophobic mismatch in the 2-D protein/lipid co-self-assembly at an interface is of great importance in the pattern formation and bioactivity preservation, especially for the systems in which the molecular sizes of the proteins and lipids are of different magnitude.
2.3.4 Collagen/lipids at Air/water Interfaces

A typical method that has been used to pattern collagen molecules at 2-D is introducing an acidic solution of collagen monomers onto a solid substrate through adsorption or coating processes. When the substrate surface properties, the collagen solution concentration, the pH and temperature of the solution and the method to dry the sample are properly selected, the self-assembled fibrillar or net-like patterns of collagens can be fabricated in an evenly distributed way on the surface of the solid. Those conventional techniques that are mainly based on the interactions between collagen and the solid surface lack the ability to control the lateral distribution of collagen molecules and the degree of collagen association.90 It is also difficult to obtain the pattern variety, long-range uniformity and high reproducibility of the fabricated ultrathin films.

Phase separated phospholipid monolayers or bilayers floating at air/water interfaces or immobilized on solid substrates can serve as templates for patterning macromolecules at the nanometer scale. It promises a potential way to form various patterned collagen features in a well-controlled way by incorporating collagens in a suitable phospholipid system using the LB technique. To the best of our knowledge, no prior investigation exists in patterning collagen with the aid of phospholipids and the LB technique. There have only been very limited studies of pure collagen or collagen/phospholipid LB film at air/water interfaces or on a solid support. At 25 °C, 0.1 wt% acidic solution of type I collagen (pH 3.0) was spread onto the surface of ultrapure water in a LB trough and successfully transferred onto silicon-coated glass by horizontal deposition.91 Ghannam et al used a different way to include collagen at air/water
interfaces by injecting a collagen acidic solution into the water subphase.\textsuperscript{92} After waiting 30 min, the collagen spread at the interface yielded a compression isotherm similar to those for the insoluble monolayer. It was concluded that collagen is a good surface-active molecule and can penetrate the air/water interface spontaneously. To study the interactions between the collagen and PC lipids and to explore the mechanism by which collagen can increase the stability of liposome vesicles, collagen was placed in the subphase after the floating DPPC or DMPC monolayer was compressed to reach liquid-expanded phase.\textsuperscript{92} The less ordered package of PC molecules provided collagen enough space to participate in between the lipid molecules resulting in a remarkable shift towards larger occupation area in the isotherm. Collagen molecules were expected to penetrate into the lipid monolayer orienting parallel to the monolayer and interact with the alkyl chains of DPPC or DMPC with the hydrophobic residues on the collagen molecule surface.

Those previous studies of collagen at interfaces with or without lipids have a problem estimating and controlling the amount of collagen that was delivered to the interfacial region. Although sufficiently robust proteins may be spread as a monolayer alone or with lipids, the partial water-soluble collagen possibly went into the aqueous subphase after its acidic solution was spread on the water surface.\textsuperscript{93} It is even more difficult to evaluate the actual amount of collagen incorporated in the floating monolayer after the equilibrium between the interface incorporation and subphase solubility. A way to keep the majority of collagen molecules staying at the interface is required to meet the principle requirement of the LB technique.
2.4 Applications of Patterned Protein Films

2.4.1 Fabrication and Localization of Inorganic Structures

Semiconductor or metallic nanoparticles have been applied in a wide variety of fields acting as building blocks for devices at the nanometer scale. Nanoparticles or quantum dots, with a size in the range of 2-10 nm, exhibit substantial variation of electronic and optical properties from those of individual molecules and those of bulk substance.\textsuperscript{94} Since the physical properties of the materials composed of nanoparticles are essentially determined by the length scale corresponding to the structure and organization of the particles, the application of nanoparticles is highly size-dependent and alignment-dependent.\textsuperscript{95}

The fabrication of nanoparticles has been mostly performed in a bulky suspension from corresponding salt aqueous solutions. Many attempts have been made to arrange the fabricated particles one- or two- dimensionally. Au was aligned linearly directed either by an electric field or a molecular surface template. An electric field was applied by Schumann to trap Au clusters from a solution.\textsuperscript{96} The resulting nanowire consisting of up to 10 Au clusters on an SiO\textsubscript{2} surface displayed quantum dot characteristics and single electron transistor behavior. In Hoeppener’s study, a template layer with long chain fatty acids forming a highly ordered structure of stripes on HOPG was utilized to direct the assembly of Au clusters.\textsuperscript{97} Strands consisting of different numbers of Au clusters with a diameter of 2.1 nm were visualized by STM, which resulted from the substitution of template molecules with a Au clusters. Genetically engineered viruses,\textsuperscript{98} double-helical DNA molecules, lipids\textsuperscript{99}, polymers\textsuperscript{100}, and proteins\textsuperscript{82} were also applied to anchor
quantum dots at 2-D relying on the self-assembly properties of those template organic molecules. The techniques mentioned above are based on attractive electronic interactions between the nanoparticles or relevant ions to the template surface by immersing the pre-patterned surface in a solution with component ions or fully developed particles. In those cases, without additional control over the surface organization and/or nanoparticle growth on the surface except for the self-assembling and electrostatic attractions, no well-defined nanoparticle pattern was present.

The LB technique has the ability to control the 2-D organization of the biological template and therefore the surface alignment of inorganic particles directed by the template. One feasible way to incorporate the inorganic particles in patterned LB films is generating the particles at an interface with a partially or fully developed monolayer template. The nucleation and growth of nanoparticles can be realized by introducing the synthesized elements from the subphase or directly at the interface. Another way to arrange nanoparticles is introducing a pre-fabricated nanoparticle suspension in the subphase under a template film or immersing the deposited template film in the nanoparticle solution. Patterning Au and CdTe nanoparticles with a controllable 2-D alignment was realized with the LB technique, in which the reorganization on various solid substrates and the deposition method were emphasized.

Mesoporous silica films were grown by employing a surfactant template at an air/water interface. Collagen has not been applied to template the silica film formation at an interface, but was utilized in one study to direct the hollow silica fiber growth in bulky solution. In acidic and neutral pH regions, the collagen surface has net positive
charges and the anionic silicate tends to accumulate and aggregate along the collagen surface. This study promises a method to grow silica particles or aggregated structures on the surface of a collagen containing film once the collagen surface pattern could be developed with preferential surface localization.

2.4.2 Topographical Control of Cell Behavior

Many studies in vivo and in vitro have revealed that the behavior of cells exposed to a bioactive surface, including attachment, migration, proliferation and differentiation, is mainly affected by the topographical and chemical properties of the surface. The cell responses to a synthesized surface have been explored intensely in vitro due to their importance in both theoretical and practical points of view. Cell-surface interactions play an important role in tissue development, the mechanism of which is still ambiguous. Attaching and culturing cells on a designed surface in vitro can provide important information on understanding the cell-cell communication and the interactions between cells and biomolecules, such as ECM proteins and phospholipids, in vivo. Spatial organization of cells with controlled shape and location can be realized in vitro on a pre-patterned surface, which has essential applications in cell-based biosensors, medical implants and diagnostic tests.

2.4.2.1 Cell Behavior on Heterogeneous Surfaces

Cells can react to adjacent cells, surrounding biomolecules and surface topography on a patterned heterogeneous surface. Various types of cells exhibit complicated behaviors corresponding to the changes in surface geometry and surface
chemistry, one of which exerts dominant influence in some cases. Cells can detect the surface discontinuity in terms of chemistry, such as surface charge, molecular composition or package and hydrophilicity, and in terms of topography, such as groove, ridge and cliff. For example, on a surface patterned with protein patches where the step at the joint region is less than a molecule in height, cells selectively attach to the domains consisting of protein that the cell has more affinity to. The extending cells appear to be positioned more frequently at the junction region if the cell has similar affinity to all the protein patches. As the width of the topographical feature is comparable to the size of the cell and the height difference is big enough, the topographic effect overrides the chemical cue and mainly controls the adhesion, orientation and activation of the cells on a surface.\textsuperscript{104} There have been many reports that cells prefer attaching to more hydrophilic surfaces, though cells did exhibit growth on hydrophobic surfaces in some cases. In addition, the surfaces with gross and random roughness promote the cell adhesion. Three types of cell lines were employed by Xie Y. et al\textsuperscript{105} to demonstrate the effect of surface roughness on the cellular response. A PET surface with nanoscale roughness produced by oxygen plasma etching showed moderate influence on the cell adhesion, proliferation and differentiation, which was attributed to the possible leveling up of the surface through the adhesion of ECM proteins.

2.4.2.2 Cells with ECM Proteins

In the cell culture study, an optimal substrate for cell function should provide a surface interacting with the cells in a similar way as that which occurs \textit{in vivo}. A thin layer of ECM proteins has been normally applied on the solid substrates, such as glass,
silica and polystyrene (PS) cell culture plate, to enhance the cell adhesion and support the cell growth. Self-assembled ECM proteins containing multiple adhesion sites for various types of cells, such as collagen and fibronectin, have been applied to form a uniform layer on a solid substrate by different techniques.

Abdelghani et al \(^{41}\) employed the indium-tin-oxide electrodes functionalized with an adsorbed type IV collagen thin layer to realize sufficient adhesion of endothelial cells, which was applied as a cell-based biosensor for the inflammatory agent detection. Fibrillar type I collagen adsorbed on glass demonstrated a highly adhesive surface for the platelet attachment.\(^{106}\) Smooth muscle cell exhibited different spreading shape and area after adhesion to a self-assembled monolayer of natural collagen and heat-denatured collagen.\(^{121}\) On a natural collagen monolayer with a lower density of large fibrils, the well spread smooth muscle cells demonstrated larger morphology due to closer interactions between cells and large natural collagen fibrils. Type I collagen was covalently immobilized by Yamamoto et al \(^{107}\) on a hydrophobic PET substrate to enhance the bioactivity and hydrophilicity of the polymer surface. Osteoblast cells showed significant attachment to the collagen-treated surface, but very weak adhesion to the bare hydrophobic polymer surface. Layer-by-Layer assemblies of type I collagen thin films were synthesized by adsorbing oppositely charged polymers and collagen onto various commonly applied substrates with highly connected fibers.\(^{108}\) The biocompatibility of the outmost surface of the composite film was demonstrated by remarkable adhesion and growth of myoblast cells and pheochromocytoma cells with uniform distribution over the entire sample.
García et al. have shown that the fibronectin-enhanced attachment of osteosarcoma cells stem from not only the multiple cell adhesion sites presenting on fibronectin that improve the cell adhesion strength in a concentration-dependent way, but also the conformational changes of fibronectin in the adsorbed layer on pre-treated glass. Upon the adsorption to modified bioactive glass, the conformational changes of fibronectin might lead to more cell binding sites exposed to the cell-receptor and available for cell attachment.

The adhesion of cells to the matrix of ECM proteins is mediated by the interactions between cell-surface receptors and the cell adhesion sites distributed along the ECM protein surface. Cells recognize the ECM proteins, either body fluid soluble fibronectin or cross-linked collagen, by those surface receptors called integrins that are heterodimetric glycoproteins consisting of two categories of units, α and β, various types of which associate to form different pairs to mediate the cell-surface binding. For example, integrin α1β1, α2β1, α3β1 are involved primarily in cell-collagen adhesion. Several specific regions located along the triple helix of collagen of various types have been known to promote the cell adhesion and spreading, named as cell adhesion sites, which was identified mostly by the synthesis of peptide representing amino acid sequences that were proposed to be the binding sites and the subsequent test of the cell response to the synthesized peptide. Type V collagen showed a heparin binding region within α1(V) chain that promoted the attachment of Chinese hamster ovary (CHO) cells and the type IV collagen α1 chain contains 1263-1277 residues that enhanced the melanoma cell adhesion and spreading. Type I collagen exerted promotion on the adhesion and migration of a wide range of cells, such as hepatocytes, keratinocytes,
fibroblasts, melanoma, and neural crest cells. Several sequences localized within α1(I) chain behaved as the binding sites for various types of cells, which induced the cell adhesion and spreading in a conformation-dependent way with the unique triple helical structure. William et al revealed a tetrapeptide sequence within the α1(I)-CB3 fragment of type I collagen that acts as a binding site for the α2β1 integrin on platelets and other cells. At least six peptide sites in fibronectin can participate in the adhesion and migration of most cells by binding a specific polypeptide sequence within fibronectin to corresponding cell surface receptor. For example, the sequence RGDS in the cell-binding site of fibronectin was assigned to be responsible for binding cells via the integrin α5β1.

2.4.2.3 Cells with Phospholipids

Amphiphilic phospholipids, especially PC and PE, are the major membrane components of many cell types. The interactions between the cell surface and phospholipid are mainly electrostatically mediated in the lipid-based gene delivery system, where the cationic lipids were applied to incorporate DNA inside of the lipid vesicles. Cell-cell communication is mediated in large part by membrane-associated proteins. Substrate-supported lipid bilayer offers a unique system to study the interactions of cells with membrane proteins and potentially to utilize these biomolecules to modulate and investigate cellular function. Supported lipid bilayers consist of two leaflets of phospholipids in close association with a hydrophilic surface such as glass. However, there have been few studies exploring the cell behavior on a planar surface with well-organized phospholipids, because the cells appear to have restrained affinity to the lipid
coated surfaces. Lewis employed a surface coated with amphiphilic PC-containing polymers that have a polar PC head group and hydrophobic alkyl chains to investigate the protein adsorption and cellular interactions. The organized bilayer of the PC-based polymer suppressed the adsorption of water soluble proteins, which was assigned to the mobility of the PC groups and the presence of a large hydration layer. The PC treated surface demonstrated reduced adhesion and activation of a wide variety of cells, such as platelets \textit{in vitro}. The fluidity of phospholipid bilayers might contribute to the inability of cell binding to the surface. The hindrance of cellular response by the PC-based lipids was confirmed by Orth et al.’s study. They micropatterned a silicon surface with DPPC or POPC bilayer patches. As the lipid patches and spacing distance were larger than the size of mast cells (around 10 µm for a round cell), the mast cells preferred to localize on the bare silicon surface leaving the lipid patches intact.

2.4.2.4 Spatial Localization of Anchorage-dependent Cells

One area of interests in tissue engineering and biosensor design has been the spatial organization of cells in predetermined locations and arrays on a surface with defined separating distance and dictated shape. The cellular microstructure responding to the heterogeneous surface is determined by cell type, cell-surface interaction, surface feature and cell surface density. Interfacing anchorage-dependent cells such as CHO cells, hepatocytes, endothelial cells, and neurons spread out upon attaching to a synthetic surface. The flattened shape was believed to be the criteria for the cell growth and proliferation. In addition to the regulation of cell spatial distribution, successful control of the cell extension degree could result in enhanced cell function, differentiation, and
proliferation of the anchorage-dependent cells. ECM proteins, synthetic peptides with cell adhesion or growth factors and membrane-related phospholipids have been intensely employed to form surface patterns at micron scale to guide the cell anchorage and growth. Lithography, microcontact printing and other micropatterning techniques, combined with SAMs in some cases, are the typical methods to immobilize the cell-adhesion active molecules.\textsuperscript{127-129} The engineered surface consists of areas either promoting or inhibiting the cell adhesion, thus the cells are localized in a well-defined manner, leading to the formation of specific cell arrays and patterns. Parallel rows of proteins that are 10-100 \( \mu m \) wide are the most common surface topology, which can confine the cell attachment mostly in the protein tracks.

The presence and immobilization of vitronectin,\textsuperscript{130} laminin,\textsuperscript{127} collagen and fibronectin promoted the attachment, spreading and proliferation of a wide variety of anchorage-dependent cells. Miyamoto et al prepared a quartz surface patterned with type I collagen stripes 50 \( \mu m \) wide and 200 \( \mu m \) in separation with conventional photolithography technique.\textsuperscript{42} Hepatocyte cells cultured on the pre-patterned surface were found to be selectively adhered onto the collagen stripe regions with elongated and well-spread shapes, as compared with the round cell shape on a bare quartz surface. A similar confinement of cells was observed on a plate featured with collagen circles (50 \( \mu m \) in diameter and 100 \( \mu m \) for intervals), having comparable dimension to that of the hepatocyte cells. The appearance of individual cells promised the application of the spatially controllable cellular behavior in toxic sensing because it had the advantage of measuring the response and activity of single cell. Thakar et al's study provided more detailed information about the relation between the surface topography composed of
collagen stripes and the cell morphology. Vascular smooth muscle cells (SMCs) were cultured on micropatterned typed I collagen strips with the width of 20-, 30- and 50 µm on a glass or a PS surface with soft lithography. The fluorescence microscope revealed that the cells were aligned exclusively in the regions covered by collagen strips and the spreading direction was the same as that of the collagen stripes. The number of cells that could line up perpendicularly to the strips and the spreading area of cells decreased with the width of the collagen strips, suggesting narrower protein tracks led to more elongated cell morphology and a decreased proliferation for SMCs in vitro. A surface template consisting of collagen or fibronectin features on a polymer surface was also employed to control the position of hepatocyte cells. The underlying protein arrays 20 µm in width and other geometries with sizes comparable to that for a single hepatocyte cell served as a good template for the cell adhesion and spreading, which in turn might influence the long-term function and activity of cells. The behavior of another anchorage-dependent cell, the endothelial cell, was studied on a heterogeneous surface micropatterned with lipids and fibronectin on glass. Cells selectively binded to the fibronectin grills or squares, leaving the lipid bilayer regions untouched, which is consistent with the conclusions from other studies that cells exhibited affinity to many ECM protein surfaces, but much less adhesion onto the lipid structures. The control of cell morphology with different degrees of spreading could be achieved by varying the geometry and dimension of the fibronectin feature. The maximum spreading was obtained, as the fibronectin squares were 20 µm in length and 5 µm apart.

The behavior of a CHO cell, a typical anchorage-dependent cell line, has been intensely studied in cell culture and adhesion spatial control. The plasma membrane of
CHO cells contains approximately half of the total phospholipids, in which PC and PE are two major types. CHO cells can bind to ECM proteins, such as fibronectin, collagen, laminin, or galectin, through an integrin-mediated pathway and well spread out on the surfaces coated with ECM proteins. Mitchell et al realized the spatial organization of CHO cells by using a surface with variance in hydrophobicity. Tissue culture polystyrene dishes (TCPS) were patterned with a plasma polymer that remarkably increased the surface hydrophobicity. CHO cells had weak binding with the modified hydrophobic polymer surface presenting a round shape, while strong interactions with the untreated TCPS surface were indicated by significant adhesion and flat morphology. The sensitivity of CHO cells to the surface hydrophobicity was applied to confine their attachment predominantly along a copper grid positioned on a modified TCPS with elongated shape. The dimension of the micron size grid and the cell incubation time were varied to control the size and shape of the CHO cells in certain degree.

The controlled cellular adhesion and proliferation at the interface of biomaterials have been commonly achieved by micropatterning a solid surface with a cell adhesive ligand. The micropatterning methods relying on lithography and mask have the disadvantages of high cost, low speed and resolution limit of above micron. A sufficient number of binding sites on the ECM protein surface exposed to cells is the principle requirement for the cell attachment onto an ECM protein coated surface. The random or loosely packed protein layers produced by micropatterning methods lack the ability to align the protein functional groups in a controllable way.138
The LB technique combined with self-assembling phenomena of the common ECM proteins provides a promising way to pattern a surface with well-controlled packing density and orientation of the protein molecules with the expectation of exhibiting higher bioactivity and more functional groups available for the cell binding.\textsuperscript{139} Higuchi et al compared the adhesion and growth of normal human skin cells on type I collagen films produced by the LB technique and casting method.\textsuperscript{91} The cell density on the LB films was found to be higher than that on the cast film, which was assigned to the enhanced density and degree of order of collagen molecules in the LB films. A more complex ECM-mimetic surface with controlled surface fluidity and molecular density was fabricated by Dori et al with the LB technique.\textsuperscript{140} The supported bilayer had the outmost layer consisting of polyethylene glycol lipids and synthesized amphiphiles containing an amino acid sequence found in collagen IV as head groups, which were found to prevent and enhance the cell adhesion respectively by themselves. The mixed surface exhibited either promotion or hindrance for the adhesion of human melanoma cells, which was determined by the relative height difference between the lipids and the peptide amphiphiles. When the lipid was much shorter than the peptide, the peptide ligands that are fully accessible to the cell recognition led to dramatic attachment and spreading of cells. In the contrary case, the peptide ligands were completely buried under the lipids with no way to interact with cell receptors. Through the use of LB technique, the surface can be engineered with pre-determined spatial organization of cell adhesive groups that are available for the cell recognition and binding by adjusting the surface density and subphase conditions. Therefore, the cell-surface interactions can be investigated in a well-controlled environment.
2.5 Ultrathin Films of Porphyrin

Porphyrin and related derivatives have been synthesized to explore their potential applications in numerous chemical, photochemical and photobiological systems. Those porphyrin compounds synthesized with well-designed molecular structures play an important role in developing sensors, catalysts and optical devices with desirable properties. Porphyrin compounds can be applied to form highly ordered ultrathin films with the thickness from monolayer to hundreds of layers of molecules on the surface of conducting or semiconducting solid substrates by various techniques. Those supported films with well-organized inner structures provide a possible way to convert the energy from excitation directly into photocurrents. The molecular stability and synthetic versatility of porphyrin compounds promises the development of molecule-based devices with functions determined by both the molecular structure of porphyrin derivatives and the supramolecular alignment of porphyrin molecules in the assembled structures.

2.5.1 Porphyrin Structures and Derivatives

The basic structure of a porphyrin or metalloporphyrin is a cyclic conjugated tetrapyrrole chromophore, in which four pyrrole units are bound by four methane bridges. Four nitrogen atoms inside the porphyrin ring point towards the center, which plays an important role in determining the polarity of the ring, especially for a metal-free one. The porphyrin ring is very stable to concentrated acids, and can behave both as an acid and a base. Strong bases can remove the two protons on the inner nitrogen atoms of a porphyrin resulting in a dianion; while the two free pyrroline nitrogen atoms can be protonated easily with strong acids. There are $18 \pi$ electrons that participate in delocalization.
pathway in the electronic core of a porphyrin. The optical spectra of the porphyrins and corresponding derivatives can be related to this electronic core and different chemical modifications to the basic structure. There are two types of derivatives of porphyrin: \( \alpha \)-substituted and \( \beta \)-substituted. Porphyrins with substituents connected to pyrrole rings have generally been found in natural systems, known as \( \alpha \)-substituted porphyrins. Synthesized porphyrin compounds with substituents presenting at methylene bridging positions are known as meso- or \( \beta \)-substituted porphyrins, such as tetrakis(phenyl)porphyrins (TPP) and tetrakis(pyridyl)porphyrins (TPyP). Individual porphyrin without substituents positioned at an interface mostly exists in near-planar conformation, which varies to be ruffled or extended for porphyrin macrocycles in TPP derivatives.

Typically, porphyrins with small substituents do not form stable films at an interface; however porphyrin derivatives with long hydrocarbon tails can self-assemble to form well-defined intricate supramolecular structures under the same conditions.\(^{141}\) Those amphiphilic porphyrins often lie flat on the surface with a low molecular density and reach much higher tilting angles with a higher molecular density at the interfaces, forming stacked dimers or trimers in some cases. The effects of varying the number of substituted alkyl chains on the resulted structure of the assembled thin films have been investigated in many studies. The number of long alkyl chains and also the substituted positions of those tails would influence the geometry of the overall porphyrin molecules and thus decide the way that the derived porphyrin molecules interact and aggregate under a given circumstance. The hydrophobic interactions between those hydrocarbon
tails may play a key role in the alignment of porphyrin molecules, including the orientation of both the substituted tails and the tilting angle of the ring planes with respect to an interface.

2.5.2 Highly Structured Films of Porphyrin Compounds

Prophyrin compounds, either with free base or metal substituted one, have been assembled on solid surfaces with well-controlled surface morphologies and highly-ordered inner structures, which practically or potentially could be applied especially in the field of sensors, catalysts and molecular sieves. To develop ultrathin films of porphyrin, the main techniques that have been utilized include LB deposition, cast coating and SAM with dip coating. Glass, quartz, silicon, metal and graphite are among the commonly utilized solid supports.

2.5.2.1 LB Monolayer and Multilayer

Amphiphilic porphyrins substituted with various functional groups can spread spontaneously and form stable monolayers at an air/water interface. The molecular structure of the porphyrin derivatives and the interactions between porphyrins and/or water subphase are the main factors that determine the organization within the floating monolayer, which could be characterized by compression isotherms\textsuperscript{142} and UV-vis absorption spectra\textsuperscript{143} at an air/water interface. Four types of meso-substituted porphyrins at 5,15 positions were spread on pure water and their compression isotherms revealed fairly low mean molecular areas at the point of lift-off, indicating almost perpendicular orientation of the porphyrin macrocycles.\textsuperscript{143} Compared with tetra-substituted porphyrins,
the significantly reduced mean molecular area was assigned to less steric hindrance. There was an obvious difference between the UV spectra of solution and monolayer for those porphyrin compounds that had a chance to interact strongly with adjacent porphyrin rings at an air/water interface. Significant red shift of Soret bands in UV spectra for one species was explained as the result of a possible interactions between its hydrophilic portion and water subphase. Johannes and Sudhölter found the presence of similar interactions with an aqueous subphase and the tilting orientation of porphyrin macrocycles of a series of pyridyl((hexadecyloxy)phenyl)porphyrins. Upon monolayer compression, the monolayer underwent the transition from separated domains of porphyrin clusters to a homogeneous coverage of water surface, and finally the growth of multilayers indicated by the collapse of isotherms. High collapse surface pressures for the synthesized porphyrin compounds and the surface visualization via BAM revealed the formation of stable monolayers at an air/water interface. A stack-of-cards organization in the compressed monolayer was ascribed to a balance between the ring-ring interplay, the interactions between polar pyridyl groups and the water subphase and the sterical repulsions between the attached long tails. The number of alkyl tails exerted a significant effect on the intermolecular distance between individual molecules, and therefore determined the extent that porphyrin rings interact with each other and the strength of steric repulsions between the tails. Unlike the porphyrins with one or two hydrocarbon chains, three- and four- chain porphyrins can form a highly structured monolayer at air/water interfaces in this case, however, the asymmetrical three-tail one did not show the stable organization at surface pressures higher than 10 mN/m once the monolayer was transferred onto the glass surface.
Ultrathin films consisting of a monolayer or multilayers of the organized porphyrin molecules assembled at an air/water interface can be transferred onto a solid surface by LB deposition. In addition to the effect of the molecular structure of the porphyrin compounds and the deposition pressure, the surface properties of the solid support are of great importance for the resulting film structures.

Substituted TPyP monolayer and multilayer were successfully transferred onto glass slides by LB vertical up/down depositions.\textsuperscript{144-147} Those synthesized porphyrin species followed the same trend that increasing the number of aliphatic tails led to more stable monolayers due to a higher degree of intermolecular stacking as what was observed at air/water interfaces,\textsuperscript{144,146} although another group has reported the absence of correlation between the number of tails and the porphyrin macrocycle orientation both at an air/water interface and on glass.\textsuperscript{148} Resembling spectral characteristics and good transfer ratios suggested there was no significant alternation of the molecular arrangement after the transfer of the monolayer.\textsuperscript{145} Typically, increasing the deposition surface pressure gave only a rise in the absorption intensity, but no considerate shift of the Soret band.\textsuperscript{146,147} Surface compression was expected to induce the mergence of those isolated domains of porphyrin molecules, instead of the change of the package of individual molecules in those domains. In a multilayered film, both the lateral interactions of the porphyrin rings\textsuperscript{144} and the interplay between adjacent layers\textsuperscript{147} could lead to the change of spectra features due to the enhanced interactions between TPyP molecules in the deposited films. More detailed study of aggregation and orientation of porphyrin rings in multilayered LB films of 5-(4-N-octadecyl-pyridyl)-10,15,20-tri-p-tolyporphyrin (named as 338a) deposited on glass and CaF\textsubscript{2} were conducted by Zhang et
Successful transferring of a porphyrin monolayer compressed to 20 mN/m previously was demonstrated by a transfer ratio larger than unity for the up/down strokes of monolayer transfer and the linear correlation between the intensity of UV absorption and the number of transferred layers. Compared to the Soret band of chloroform solution, red shifts of 10 nm and 20 nm exhibited for multilayers of 338a and its mixture with stearic acid, respectively. It was concluded that the porphyrin rings adopted a nearly flat orientation with respect to the solid surface despite the number of layers and the excitonic interactions between the rings made a primary contribution to the red shift of the Soret band. A red shift appeared in UV-vis spectra was in good agreement with a similar shift present in the fluorescence spectra.\textsuperscript{150,151}

In addition to glass, quartz is another of the most frequently utilized hydrophilic solid substrate to support thin films of porphyrin derivatives. After deposited onto the quartz surface, mixed monolayers of porphyrin substituents and surfactants showed the influence of the number and the length of the hydrocarbon tails and the incorporation of various surfactants into the monolayer on the organization of porphyrin macrocycles, but not the orientation of the ring, which was related mainly to the interactions between the hydrophilic portion of the porphyrin compounds and solid surface and/or the interactions between porphyrin rings.\textsuperscript{152-154} For a hydrophilic solid surface, increasing the hydrophilicity would reduce the tilting angle of the porphyrin macrocycles with respect to the solid surface. Red shifts in spectra for most of those mixed systems suggested a J-type aggregation in the film with small tilting angle of the rings, while blue shifts observed in some cases, especially for the LB films deposited at high surface pressures, implied H-type assembly with more perpendicular orientation of macrocycles. Chordhury et al
reported the comparison investigation of LB monolayers of two series of carboxyporphyrins.\textsuperscript{155} For porphyrin derivatives with a longer chain length, the increased red-shift and narrowed Soret bands in characteristic spectra of their LB monolayers on quartz indicated closer and more ordered package in the monolayer, which was directly related to stronger hydrophobic attractions between individual molecules. Freshly cleaved mica with extremely smooth surface were used by Phadke et al to deposit a monolayer of amphiphilic metalloporphyrin (PEPP-Zn) with a vertical descent method,\textsuperscript{156} in which the water subphase was allowed to drain slowly and the surface compression was realized by descending the monolayer in a conical trough. The measurement of surface pressure in this unique system followed the same procedure as that for a typical LB trough. A mica plate was positioned parallel to the air/water interface in the subphase, on which the PEPP-Zn monolayer landed during the seepage of the subphase.

2.5.2.2 Cast Film and SAM

Simply dropping a droplet of organic solution of porphyrin compounds onto a solid surface yields a cast thin film composed of porphyrin molecules assembled in a way that is mainly determined by the interactions of porphyrin-porphyrin and porphyrin-solid. Bai’s group has investigated the molecular alignment of ((tetradecyloxy)phenyl) porphyrin (TTPP) on both hydrophobic\textsuperscript{157,158} and hydrophilic\textsuperscript{159} solid supports. In cast films of TTPP on silicon, XRD revealed perpendicular orientation for porphyrin macrocycles and parallel arrangement for molecular columns with respect to the solid surface. Due to natural oxidation, the surface of the silicon plate is hydrophilic once exposed to air. On hydrophobic graphite, the ring plane positioned parallel to the solid
surface was expected to pile to be a perpendicular column, which was explained as the result of self-assembling behavior of TTPP and van der Walls forces between TTPP and hydrophobic graphite. The predominant effect of peripheral long alkane chains on the close package of TTPP and the structure stability of the cast films was also demonstrated in LB films of porphyrin derivatives, in which two types of porphyrin species without the attachment of aliphatic tails failed to form stable monolayers. Cast films of two tetraphenylporphyrins with disulfide and thiol end-groups on gold were compared with SAMs of those two species by immersing a glass slide into their diluted solutions. The diameter of the tetraphenylporphyrin ring was measured to be 1.76 nm under STM. Based on the UV spectra of the films prepared with different methods, random distribution of porphyrin molecules were found in cast films with both the side-by-side and face-to-face organization, while significant red shifts of Soret bands for SAMs revealed the dominant arrangement of porphyrin planes in a side-by-side way.

Adsorption of porphyrin derivatives from solutions and self-assembling on a solid surface allows the development of SAMs, producing thin films on a solid support with more well-organized structures compared with those in cast film. The substitutes on the porphyrin ring, the solid substrates and the immersing time in the solutions are adjustable factors to monitor the resulted molecular alignment inside the SAMs. Zhang et al fabricated SAMs of 5,10,15,20-tetra(N-10-carboxydecyl-pyridinium-4-yl)porphyrin (TCPyP) on both Au and mica. Film thicknesses of 1.5 nm and 1.9 nm were obtained for the SAMs on mica and Au, respectively. On both solid surfaces, the TCPyP macrocycles with the thickness of 0.5 nm and diameter of 1.8 nm were anchored nearly parallel to the solid surface and the COOH end-groups bound to the surface of solids. A
nearly flat orientation of porphyrin rings with respect to the solid surface was also detected for SAMs on hydrophobic graphite\(^\text{163}\) and hydrophilic glass, quartz and silicon surfaces.\(^\text{164}\) Chain number and chain length effects play important roles as well in molecular organization of SAMs. For free base methyl pyridinium porphyrins adsorbed on negatively charged substrates, the difference in molecular orientation was significant for compounds with various numbers of side chains.\(^\text{164}\) In a proposed model for adsorption, four-tail porphyrins adopted a flat-lying orientation due to predominant binding of positively charged pyridinium groups to the oppositely charged solid surfaces, while two- and three-tail ones had random alignment as van der Waals forces became more considerable relative to solvation forces for a decreasing number of side chains. SAMs of porphyrins bearing disulfides on a gold surface demonstrated that increasing the length of alkyl chains led to more highly organized structures in the thin films.\(^\text{165}\) Monolayers and multilayers (up to 30 layers) of porphyrin derivatives were also produced on quartz and glass through covalent binding or electrostatic interactions.\(^\text{166-169}\)

### 2.5.3 Porphyrin Film Characterization

For a transferred film, either a monolayer or multilayer, of porphyrin derivatives, AFM can reveal the topographical feature of the outmost surface and also the thickness of the film by scratching a hole down to reach the solid surface with AFM tips. The inner structure, including the organization of individual porphyrin molecules and the orientation of porphyrin macrocycles can be investigated by UV-vis absorption and fluorescence spectroscopy by comparing the spectra for a diluted solution of a porphyrin compound and those for deposited films.\(^\text{170,171}\) The solutions used for comparison should
be diluted enough to ensure the existence of only porphyrin monomers. A red shift of the characteristic bands in absorption spectra appears when the solution concentration is above the critical one and aggregations of porphyrin molecules are present in the solution, which was demonstrated by the broadening of Soret bands at decreasing solution concentrations.164

The UV absorption spectra of metal free porphyrins typically contain a most prominent Soret band with the highest intensity located around 420 nm and a series of weaker Q bands (450-700 nm). The wavelength corresponding to the Soret band, the origin of a second singlet excited state, can be influenced by the structure and the environment of the porphyrin macrocycles.172-177 Based on molecular exciton model developed by Kasha et al172,173, a red-shift of the Soret band for a porphyrin system indicates J-type aggregation or edge-to-edge alignment of the porphyrin plane, which suggests the presence of in-line transition dipoles, usually having a tilting angle smaller than 54.7° with respect to the interface plane. Coplanar arrangement or porphyrin rings with transition dipoles parallel to each other leads to a blue-shift in absorption spectra, which has been mentioned as H-type aggregation or face-to-face organizations. The extent of the shift depends on several factors including the degree of aggregation and the interplanar separation distance. The broadening of the bands has been observed for the assembled films that are not homogenous with irregular orientation of porphyrin molecules. Varying the peripheral aliphatic chains attached on the porphyrin rings mostly leads to the change of molecular package in the films and consequently the absorption spectra to a certain degree.
Fluorescence spectroscopy is one of the most sensitive instrument techniques, because the intensity of the emission is measured above a low background level. With the sensitivity 1000 times of that for absorption spectroscopy, the detectable concentration of a sample can be fairly low. Compared with the rate at which an electron is excited from a ground state to a higher energy state and then emits radiation, fluorescence emission occurs at a much lower rate. In solution, the excess energy coming from the electronic excitation is converted and released in form of heat through collision between excited solute and solvent molecules. In a deposited film of porphyrin compounds, the immobilized molecules interact in a different way from that in solution, which varied with the packing mode inside the film. The excited state of localized molecules gets deactivated in several ways, including internal conversion, vibrational relaxation and external conversion and intersystem crossing. Since the excited electron can go back to any one of the vibrational levels of the ground state, the fluorescence band is located at a wavelength longer than that for the absorption band and different types of shifts from that for diluted solution are indicative of different way of molecular packing in the films.
CHAPTER 3

LIPID/COLLAGEN MONOLAYERS WITH WELL-CONTROLLED HIERARCHICAL COLLAGEN ASSEMBLIES

3.1 Introduction

Phosphatidylethanolamines (PE) are among the most important components found in biological membranes. These zwitterionic phospholipid molecules self-assemble into well organized bilayer structures in nature. When adsorbed at air/liquid interfaces, monolayers composed of mixtures of different PEs containing saturated or unsaturated acyl chains with various lengths often exhibit interesting 2D patterns.\(^{11,14,18}\) For example, mixtures of DOPE (1,2-dioleoyl-\(sn\)-glycero-3-phosphoethanolamine) and DPPE (1,2-dipalmitoyl-\(sn\)-glycero-3-phosphoethanolamine) form heterogeneous monolayers that exhibit distinct DOPE-rich and DPPE-rich domains.\(^{14}\)

LB technology provides a way to transfer a self-assembled monolayer from an air/liquid interface onto a solid surface. Multilayered structures can be fabricated by repeating the deposition process. LB films supported by a solid substrate have many applications, including microelectronics, sensors, catalysis, and coatings. In comparison
to evaporation or spin-coating methods, the LB technique provides an additional level of control over the molecular organization and therefore a more precise way to control surface pattern formation by adjusting the surface molecular density and the temperature and pH of the subphase. Since weak interactions are typically involved in the self-assembly process, self-assembled structures are sensitive to small changes in these and other variables, such as a trace of contaminations. Film defects incurred during the transfer process are often difficult to avoid. Bottom-up strategy for fabricating ultrathin films has had the limitations to realize the reproducibility and long-range uniformity that are critical for commercial applications.

Collagen is a triple stranded peptide that is the most abundant structural protein in the extracellular matrix of connective tissues. Uneven distribution of hydrophobic regions and charged residues along the surface of a collagen monomer leads to a unique and highly ordered way for collagen to self-assemble, forming complex fibrous network structures in vivo. Collagen plays a crucial role in specific binding of many different types of proteins and cells, such as fibronectin and platelets. Collagen thin films supported by solid substrates with well-defined surface patterns and highly-ordered inner structures are of major importance in drug delivery, biosensors and biomedical implant materials due to the biocompatibility of collagen.

Supported ultrathin films of collagen have previously been fabricated by introducing acidic solution of collagen monomers onto a solid substrate through adsorption from solutions or cast coating. When the substrate surface properties, the concentration, pH and temperature of the collagen solutions and the method utilized
to dry the supported film were properly selected, collagen fibril or network coatings were fabricated evenly distributed on the surface. These conventional techniques are primarily based on the interactions between collagen and the solid surface of the substrate, and also are dependent on the dewetting mechanism, which generally limits the ability to control both the lateral distribution of collagen molecules at the nano-scale and the degree of collagen polymerization.  

There have been very few prior studies of pure collagen or collagen/lipid LB films at an air/liquid interface or on a solid support. At 25 °C, 0.1 wt% acidic solution of type I collagen (pH 3.0) was spread onto the surface of ultrapure water in a LB trough and successfully transferred onto silicon-coated glass by horizontal deposition. Ghannam et al used a different method to incorporate collagen at the air/water interfaces by injecting a collagen solution in 0.5 M acetic acid into the aqueous subphase with or without a phospholipids monolayer spread on the surface. After waiting 30 min, the collagen spread at the interface yielded a compression isotherm similar to the water-insoluble monolayers, indicating that collagen is a good surface-active molecule that can penetrate the air/water interface and interact with the lipid monolayer spontaneously. The previous studies of collagen at interfaces with or without lipids have not addressed the problem of accurately controlling the amount of collagen delivered to the interface. Although amphiphilic proteins may be spread as a monolayer alone or with lipids, acid-soluble collagen molecules dissolve into the subphase if spread by themselves on an aqueous subphase. It’s even more difficult to evaluate the actual amount of collagen incorporated in the floating lipid monolayer after the equilibrium between the interface incorporation and subphase solubility is reached. A new method that effectively prevents
the participation of collagen into the subphase is presented in this chapter. The ability to spread water-soluble macromolecules such as collagen at an interface in this manner provides a valuable technique to prepare ultrathin composite films.

In the current study, the domains observed in heterogeneous phospholipid monolayers or bilayers floating at an air/water interface are expected to serve as templates for patterning macromolecules at the nanometer scale. This approach can potentially be used to form various collagen architectures in a well-controlled way by incorporating collagen in a properly selected lipid matrix based on the LB technique. Patterning collagen assemblies in the presence of phospholipids has not been reported. The current study focuses on developing a new procedure to fabricate collagen-containing ultrathin films with well-defined 2D patterns and various surface morphologies, in which DOPE/DPPE monolayers serve as templates to localize collagen features and control the degree of collagen self-assembly. This novel procedure successfully realizes the long-range uniformity of the film structure in a reproducible manner. The co-self-assembly mechanism for collagen and lipids in the film will be investigated, which will shed light on the way that collagens interact with lipids either in natural tissues or in artificial drug delivery systems.

3.2 Experimental

3.2.1 Materials

DOPE and DPPE were purchased from Avanti Lipids (Alabaster, AL) and used without further purification. DPPE was dissolved in chloroform, DOPE and the mixtures
of DOPE/DPPE were soluble in chloroform/methanol (4/1 vol/vol). The total lipids concentration in the spreading solutions was 0.7 mg/ml. Type I collagen extracted from calf skin was supplied by Sigma-Aldrich (St. Louis, MO). In 1.0 M acetic acid, collagen solution at the concentration of 0.1 mg/ml was stirred for at least four hours at ambient temperature until the solution was transparent.

3.2.2 Film Fabrication and Characterizations

Surface pressure isotherms for lipid only and lipid/protein mixtures with various molar ratios were measured at 25 °C using a LB trough from Nima Technology (Coventry, England). Surface pressure was measured using the Wilhelmy plate method. The subphase was an aqueous buffer solution of potassium dihydrogen phosphate, sodium hydroxide, and sodium bicarbonate at pH of 7.2. Highly purified water (Milli Q, electrical resistivity > 18 MΩ cm) was used in all experiments.

To obtain a lipid LB film on a solid support, a volume of 40 µl DOPE/DPPE solution was first spread dropwise on the subphase using a microsyringe with the trough barriers set at the maximum area of 280 cm². After spontaneous spreading on the subphase, the monolayer was kept for 10 min to allow complete evaporation of the volatile solvents in the spreading solution. The monolayer was then compressed in a stepwise quasi-static mode using area increments of 1 cm², wait interval of 16 s, and barrier speed of 25 cm²/min. Upon the surface pressure reaching a target value (typically 25 mN/m or higher), the surface pressure was maintained at a constant value by the LB trough by automatic adjustment of the barrier position as necessary. Typically, the surface area continued to slowly decrease during this phase, compressing the film to
maintain a constant surface pressure, indicating that the film was not in steady state due to molecular reorganization within the film. When the barrier stopped moving, the LB films were transferred onto freshly cleaved mica using vertical up deposition with a lifting speed of 1 mm/min. The surface pressure was kept constant throughout the deposition step.

Attempts to prepare a pure collagen monolayer or to simultaneously spread both lipids and collagen were unsuccessful due to the dissolution of collagen into the subphase. It was observed that collagen could be deposited at the interface and prevented from partitioning into the subphase if a sequential deposition process was used. The lipid monolayer was first spread and then compressed to non-zero, but still very low, surface pressures. At this point the collagen solution was delivered on top of the lipid matrix. In the experiments reported here, collagen was delivered after the DOPE/DPEE monolayer had been compressed to a low surface pressure, \( \Pi_S \), mentioned as spreading pressure. The collagen solution in acetic acid at a concentration of 0.1 mg/ml was spread drop by drop using a microsyringe. During the spreading process, the surface pressure was maintained constant at \( \Pi_S \) by moving the barrier and increasing the surface area. The composite film was allowed to age for 15 min after spreading the collagen and was then compressed until reaching the desired deposition pressure, \( \Pi_D \), generally 25 mN/m or higher. The LB film was held at \( \Pi_D \) until the barrier stopped and then transferred onto mica by vertical up deposition. The size of the supported film is typically 1x1 cm.

The LB films were air-dried for 24 hours before the observation using tapping mode AFM. A Nanoscope III AFM from Digital Instruments (Santa Barbara, CA) was
used for investigating the surface morphology of the film in air and at ambient temperature. Tapping mode imaging using silicon cantilevers was performed at a low spring constant of 0.3 N/m.

3.3 Results and Discussions

3.3.1 DOPE/DPPE Monolayer

The behavior of DPPE/DOPE monolayers was observed to be very sensitive to the pH of the subphase due to NH₃⁺-containing phosphoethanolamine (PE) lipid head groups. The protonation (charge) of this group is determined by a pH-sensitive dissociation equilibrium, so the monolayer structure and the resulting film organizations are expected to also be sensitive to the pH of the subphase. For studies around neutral pH, the subphase for this system was buffered at pH 7.2.

The surface pressure (Π) vs area per molecule (A) isotherms for the DPPE/DOPE system at 25 °C were investigated. These are the two dimensional analogy of pressure-volume isotherms of compressible three dimensional bulk phases. As shown in Figure 3.1, there is no apparent phase transition for single-lipid isotherms for either DPPE or DOPE. The DPPE isotherm is typical of a highly condensed film while the DOPE isotherm suggests a more loosely packed film. The isotherms of their mixtures with various molar ratios are intermediate between those of the two pure components and the area at which Π starts to increase above zero decreases with the proportion of DPPE in the mixture. The double carbon bond existing in the DOPE tails enhances the steric repulsions between adjacent molecules and increases the average area occupied by an
Figure 3.1 Π-A isotherms for PE lipids: (a) DPPE; (b) DOPE; DOPE/DPPE mixtures with molar ratios of DOPE:DPPE at: (c) 1:4; (d) 2:3; (e) 1:1; (f) 3:2; (g) 4:1 at 25 °C on buffer solution (pH=7.2).
individual molecule in the mixed monolayer. The collapse pressures do not show
dependence on the molar proportions in the mixed films, implying at least partial
immiscibility of the two lipids in all proportions studied.

To further investigate the miscibility of DOPE and DPPE in the binary film, the
area per molecule at surface pressures of 25 mN/m and 40 mN/m are plotted against the
molar fraction of DOPE in the mixture (Figure 3.2). The dotted lines shown in Figure 3.2
represent the ideal additive relation: \( A = X * A_{DOPE} + (1 - X) * A_{DPPE} \), where \( A \), \( A_{DOPE} \)
and \( A_{DPPE} \) are the average molecular areas for the mixture and the pure components and \( X \)
is the molar fraction of DOPE in the mixture. For both surface pressures the area curves
exhibit positive deviations from the ideal relation at DOPE molar ratio below 0.8,
indicating stronger repulsive interactions between DPPE and DOPE in their mixtures
than between pure lipid molecules. Since DOPE and DPPE have the same head groups,
the additional repulsive interactions can be mainly attributed to the steric effect of the
kinked DOPE tails. At a slightly lower pH, in which pure water (pH=6.8) was used as the
subphase, there are additional electrostatic repulsions between PE head groups, which
results in larger average areas of 0.72 nm\(^2\) and 0.42 nm\(^2\) occupied by DOPE and DPPE,
respectively, at a surface pressure of 40 mN/m.\(^2\) Ideal additivity was observed for all
DOPE proportions.\(^{14}\) Significant deviation from area additivity suggests possible
miscibility in the mixture; such deviation was not observed in this study, so DOPE and
DPPE are assumed to be totally immiscible, which will be demonstrated further by AFM
topographic study.
Figure 3.2 Comparison of ideal additive relation (dashed line) and actual relation (solid line) between average area and film composition for DOPE/DPPE monolayer compressed to surface pressures of: (a) 25 mN/m; (b) 40 mN/m.
The films consisting of DOPE/DPPE mixtures with various molar compositions were deposited onto mica at a surface pressure of 25 mN/m. The morphology of these binary monolayers as characterized by AFM in air is illustrated in Figure 3.3. Phase separation is clearly observed at all molar ratios, with topographically higher closed domains embedded in a lower continuous phase. A previous study reported similar domain formation for the supported DOPE/DPPE monolayer transferred from water subphase at 40 mN/m, where the domains were assigned to pure DPPE.14 The area fraction occupied by DPPE calculated from isotherms at 25 mN/m is in good agreement with the area fraction shown in Figure 3.3 for all molar ratios. The relative height difference of 1 nm between the domains and the background is in good agreement with the expected heights of DOPE and DPPE monlayers (1.8 nm and 2.8 nm, respectively) leading to the conclusion that the domains are composed of nearly pure DPPE and that DPPE is excluded from the continuous matrix consisting of DOPE.

The shape and size of the DPPE domains can be adjusted by varying the molar ratio of DOPE to DPPE. With an increasing proportion of DOPE in the mixture, the DPPE domains vary from well-defined circular shapes to more elongated snow flake-like shapes. In Figure 3.3a, the film with the lowest DOPE ratio at 0.2 exhibits well defined circular domains with diameters ranging from a couple of microns to more than 10 microns. Upon further addition of DOPE in the mixture, the domain shape becomes less rounded, changing to multi-lobed patches (Figure 3.3b and 3.3c) and finally rosette patterns (Figure 3.3d) as the DOPE ratio increases to 0.8. Though at various molar ratios, the DPPE domains vary distinctly in shape, they are relatively homogeneous in average size. However, the size distribution and the domain localization appear to be more
Figure 3.3 AFM images of DOPE/DPPE LB monolayers on mica deposited at 25 mN/m at various molar ratios of DOPE:DPPE: (a) 1:4, (b) 2:3, (c) 1:1, (d) 3:2; (e) 4:1.
uniform by increasing the fraction of DOPE, which could be ascribed to more scattered DPPE domains and weaker interactions between separated DPPE domains. The surfaces were reexamined after air-drying for up to 10 days and no visible difference in morphology was observed, indicating the stability of the supported lipid film in ambient environment.

For binary lipid film supported by solid substrate, lipid/lipid and lipid/solid surface interactions coexist. Since DOPE and DPPE have the same PE head group, the negatively charged mica surface exhibits similar binding to the positively charged NH$_3^+$ head group of PE, which should not be an essential factor influencing the phase separation. Lipid/lipid interactions include electrostatic repulsions between PE head groups, steric repulsions between alkyl tails and attractive van der Waals forces between hydrophobic tails. At the surface pressure of 25 mN/m, the average area per molecule obtained from the compression isotherms of binary monolayer is less than 0.64 nm$^2$. When the distance between molecules is in the short range of a few angstroms, the electrostatic repulsions between head groups and steric-type repulsions between long alkyl chains dominate the interactions between lipid molecules. As the proportion of DOPE to DPPE varies, there would be no significant change of electrostatic repulsions involved within the film because of the same PE head for both DOPE and DPPE. However, introducing more DOPE with kinked tails into the lipid mixture will increase the steric hindrance of the lipid molecule motion dramatically. Double chain lipids with small headgroups, such as PE, tend to exhibit the “inverted cone” shape. The average molecular area of 0.64 nm$^2$ at surface pressure of 25 mN/m is comparable to the cross sectional area of two saturated and straight hydrocarbon chains (0.4 nm$^2$), indicating
even DPPE molecules with two straight alkyl tails undergo almost the tightest packing at the deposition pressure. DOPE with unsaturated and kinked double chains occupies an even larger space than DPPE under the same surface pressure. The restriction of the possible molecular configurations and possible overlap of the kinked tails yield strong entropy related steric repulsions. With the increase of the proportion of DOPE in the film, the steric repulsions between lipid tails for the whole system are of more significance.

Although the interactions within the film are of course 3D, the deposited film has the thickness of only one layer of molecules and the interactions can to a first approximation be thought of as confined within a 2D plane. The intermolecular interactions for the molecules located along the periphery of the DPPE domains gives rise to a 2-D line tension. Along an air/water interface, the attraction between the water molecules is stronger than in air phase, but much weaker than in water bulk phase, which results in the surface tension of water along the interface. By analogy to the origin of surface tension, the line tension comes from the difference between the intermolecular interactions of the molecules in the bulk (DPPE domain or continuous DOPE matrix) and the molecules positioned in the interfacial area (mixture of DOPE and DPPE molecules). The bigger the difference between net attractive interactions existing in the bulk phase and interfacial region, the stronger the line tension. At various molar fractions, the DOPE/DPPE mixtures exhibit positive deviations from the ideal mixing behavior, indicating a stronger repulsive interaction between DPPE and DOPE in their mixtures than that between pure lipid molecules. Therefore, the overall net attractions between lipid molecules follow the rank of: DPPE/DPPE > DOPE/DOPE > DOPE/DPPE.
During the domain development, line tension is counteracted by repulsions between lipid molecules. A balance between interfacial line tension and the overall repulsive interactions, which induces the formation of circular domains and elongated domains respectively, mainly determines the shape and the size of the 2-D lipid domains. When the DOPE proportion is much less than that of DPPE, the difference between net attractions of DPPE/DPPE in DPPE domains and DOPE/DPPE along the interfaces contributes mainly to the origin of line tension. At lower proportions of DOPE and therefore less steric repulsions in the mixture, the dominating line tension leads to the formation of nearly circular domains, such as the domains formed at DOPE:DPPE=1:4 molar ratio. With the increase of DOPE fraction in the system, line tension comes more from the difference between net attractions between DOPE/DOPE in the continuous matrix and DOPE/DPPE along the periphery, which is less than the difference between DPPE/DPPE and DOPE/DPPE attractions. Reduced line tension is taken over by increased steric repulsions coming from the kinked hydrocarbon tails of DOPE, leading to more elongated domain shapes, such as multi-lobed domains developed at DOPE:DPPE=4:1 molar ratio.

The synthesis of collagen assemblies in the form of natural fibrils has been reported on microfabricated surfaces with defined micron-sized features, achieved by adsorption of collagen from monomeric solutions.\textsuperscript{42,131,132} This method provides no control over the degree of collagen association and results in irregular spatial localization of collagen with features typically on the scale of 100 µm. Based on self-assembly behavior and the LB technique, collagen assemblies with well-defined 2-D patterns at the nano-scale templated by lipids have not been previously reported.
3.3.2 Collagen LB Films at Air/water Interface

To accomplish collagen polymerization in the fluid environment provided by the self-assembled lipid matrix at an air/liquid interface, the first challenge is to deliver collagen molecules at the interface and to prevent their dissolution into the aqueous subphase, which is an obvious prerequisite for reproducible and systematic formation of films by the LB deposition. One prior study reported that collagen behaved as an insoluble monolayer at an air/water interface based on only one compression Π-A isotherm; however, this is not sufficient enough to demonstrate that collagen molecules remain at the interface. In this study, collagen monolayer compression isotherms were obtained when different amounts of collagen were delivered at the air/water interface (Figure 3.4). As the amount delivered was increased, the isotherm shifted to the left, indicating a lower average area per molecule. At a specific surface pressure, the actual average area per molecule should be the same regardless of the delivery amount if all collagen remains at the interface. The reduced average area calculated based on the total delivery amount indicates a significant amount of collagen penetrating into the subphase.

The actual surface concentration of collagen is difficult to determine and to control due to the partial solubility of collagen in the aqueous subphase. If a lipid monolayer floating at an air/water interface can effectively prevent the dissolution of collagen monomer into the subphase, the first key step to pattern polymerized collagen at an air/water interface in a well-controlled way is obtained.
Figure 3.4 Π-A isotherms for collagen with delivery amounts of: (a) 1.5 mg; (b) 2.0 mg; (c) 3.0 mg at 25°C on ultrapure water (pH=6.8).
3.3.3 Collagen/PE-lipid Binary LB Films

Two dimensional lipid matrices floating at an air/water interface may provide an oriented molecular array to prevent the dissolution of collagen into the subphase due to possible electrostatic and hydrophobic interactions between collagen and lipid molecules. Amphiphilic lipid molecules adopt different orientations under various surface densities, which may be applied to manipulate the environment for the collagen monomers to polymerize in between and the collagen assemblies to localize with spatial selectivity. When the lipids spread spontaneously at an air/water interface, those water-insoluble molecules preferentially lie flat on the water surface when the molecular density is fairly low. During the compression of the lipid monolayer, the initially floating molecules adopt more vertical orientation with the hydrophobic tails pointing toward the air phase. Varying the package of lipid molecules at an air/water interface may have a significant effect on the dispersion and polymerization of collagen monomers, which provides a possible way to inhibit the penetration of collagen into the aqueous subphase.

DOPE and DPPE were investigated to form the supporting monolayers for collagen at an air/water interface due to the difference in their tail structures that may influence the mixing behavior with collagen. Unlike lipid molecules with the size of a few nanometers, collagen monomers with the length of 300 nm can not spread spontaneously at the interface. Thus, a proper procedure to deliver the collagen onto the lipid template should be developed, which can ensure the successful stay and uniform distribution of collagen at the air/water interface.
The collagen acidic solution was first spread on a widely dispersed DOPE monolayer. Initially, the surface pressure increased dramatically, indicating the disturbance of bulky collagen molecules to the lipid monolayer. However, the surface pressure fell down to 0 mN/m in a few minutes after the collagen delivery, suggesting that a significant amount of collagen molecules penetrate through the lipid monolayer and dissolve into the subphase. The compression isotherm of the composite film looks similar to the one for pure DOPE and no trace of collagen feature is shown in the AFM images of the film deposited onto mica at a surface pressure of 25 mN/m. Taken together, the DOPE lipid monolayer with widely spread molecules can not prevent the dissolution of collagen into the subphase. A slightly compressed lipid monolayer with the surface pressure just above 0 mN/m was tried to serve as an anchorage matrix for collagen at an air/water interface because the restrained distance between lipid molecules and moderate interactions of lipid/collagen would provide collagen an environment to disperse and aggregate at the interface.

As collagen solution was delivered with fixed surface area and increasing surface pressure after moderate compression of the DOPE monolayer, a very bumpy surface with isolated huge aggregates was obtained under AFM for the transferred DOPE/collagen film on mica. The increasing density of collagen molecules at the interface during the introducing process prevents the dispersion of collagen molecules within the lipid monolayer. Collagen monomers may concentrate and self-assemble around the randomly distributed lipid molecule clusters, forming the observed huge bumps. To provide more space for continuously introduced collagens to scatter, the composite film was expanded during the collagen delivery by moving the barrier when necessary to keep the surface
pressure constant at the same low value as the one at which the lipid monolayer was initially compressed to. The speed of the barrier was set at the maximum value to give a quick response to the surface pressure change.

Collagen displayed completely different mixing behavior with DOPE and DPPE. In the transferred films of DOPE/collagen at proper molar ratios, interesting collagen supramolecular structures similar to those observed in nature spread out evenly on top of the DOPE monolayer. However, when incorporated with DPPE under the same conditions, whatever the molar ratio between lipid and collagen is, no trace of collagen monomer and associated hierarchical structure has been noticed. The loosely packed monolayer of DOPE acts as an effective 2-D solvent for collagen, while the DPPE monolayer, more densely packed due to their straight hydrocarbon tails, excludes collagen. At a given surface pressure, the kinked DOPE tails have a larger cross sectional area for collagen molecules to localize and associate in between. Some of the hydrophobic clusters along collagen molecules would partially insert into the tail layer and bind with the hydrophobic tails of DOPE, and other hydrophobic residues have the tendency to extend to the air phase either at the air/water interface or on the solid support. The tightly packed DPPE tails provide limited space to incorporate collagen molecules within the matrix. Low conformational freedom of collagen monomers in the DPPE monolayer prohibits their aggregation to form polymerized structures to a higher degree, because collagen assemblies are developed in two specific ways: end-to-end accretion of monomers into long and thin filaments and side-by-side aggregation of those filaments into broader fibrils. The solubility of collagen aggregates contains a few molecules in aqueous solution at neutral pH is dramatically reduced compared with tropocollagens.
Thus, the collagen molecules staying at the monomeric status have higher chances to dissolve into the subphase during the initial spreading and also the subsequent compression compared with the partially associated collagen fragments dispersed in more loosely packed DOPE matrix.

The molar ratio of DOPE to collagen was monitored in the range of 130:1 to 10:1 to investigate the optimum range in which collagen can be successfully incorporated into the DOPE monolayer at an air/water interface and co-self-assemble with DOPE forming naturally hierarchical structures. Addition of a small amount of collagen to DOPE can not induce the formation of intricate collagen aggregations within the DOPE matrix. The composite film of DOPE and collagen, molar ratio 130:1, deposited at 25 mN/m on mica shows barely a smooth surface of the DOPE monolayer without any trace of collagen associations. As the content of collagen is increased to 100:1 (molar ratio of DOPE:collagen), a variety of interesting collagen features with different sizes is present on top of the DOPE supporting layer. AFM scan focusing on randomly selected regions throughout the sample surface reveals different types of collagen hierarchical structures, including partially connected fine filaments, network structures and highly condensed elongating associations (Figure 3.5a-c). The fine filamentous structures of collagen protrude from the background with the height of at most 3 nm that is comparable to the diameter of two tropocollagen molecules, indicating that collagen monomers spreading evenly within the DOPE matrix with their long axes parallel to the solid surface associate mostly in the end-to-end way to form long and thin filaments. Another type of network assembly of collagen exhibits a broader distribution of thread wideness and pore size, which was ascribed to the rupture of the collagen film by the water meniscus during
Figure 3.5 AFM images of collagen/DOPE LB monolayers deposited at 25 mN/m on mica with molar ratios of: (a)-(c) DOPE:collagen=100:1; (d) DOPE:collagen=80:1.
dewetting, when the interwoven sheet of collagen with high density that covers the DOPE monolayer was obtained by immersing a less hydrophilic PS substrate into a collagen monomeric solution. Increasing the collagen molar ratio to 1:80 (collagen:DOPE) leads to a much more identical surface feature of polymerized collagens. In Figure 3.5d, collagen fibers appear connected throughout the film surface with branches at different levels prolonging on top of the DOPE monolayer and extending into the lipid layer. This conclusion is supported by the measured vertical height of the collagen branches protruding from the continuous background. The main collagen threads are of the height of up to 15 nm that is equivalent to the size of 10 collagen monomers agglomerating side-by-side; however, fine branches at the far end display a height of only 1 nm that is even smaller than the diameter of one tropocollagen, implying the partial insertion of collagen molecules into the lipid matrix. Upon raising the collagen fraction in the composite film subsequently to reach a DOPE/collagen molar ratio of 10:1, no intricate collagen structures have been observed within the DOPE matrix, though isolated and irregular agglomerations of collagen molecules appear somewhere.

The molar ratio between DOPE and collagen turns out to be a critical factor that determines the formation of the variety of collagen polymerized features in the DOPE monolayer. The phenomena observed during the collagen solution spreading onto the floating DOPE layer provide important information to explain the relation between the successful incorporation of collagen into the DOPE layer and the molecular fraction between these two components. For greater amounts of collagen, molar ratio in the range of 10:1 to 50:1 (DOPE:collagen), the introduction of the collagen monomeric solution onto a slightly compressed DOPE monolayer resulted in a dramatic increase of surface
pressure within a minute. Although the surface area was controlled to increase corresponding to the surface pressure rise, the final surface pressure was above 5 mN/m because of reaching the maximum area limit of the LB trough, suggesting significant participation of collagens into the DOPE layer at an air/water interface. In the case when the least amount of collagen within the investigation range was included, molar ratio of 130:1 (DOPE:collagen), it is interesting to notice that similar quick surface pressure increase occurred and no trace of collagen features were observed when collagen content is low, while irregular collagen aggregates were obtained for higher amounts of collagen. It is reasonable to postulate the collagen features interpenetrating into the DOPE matrix after delivering at an air/water interface should be different for the films with fairly low or high collagen contents. Lower concentrations of lipids would restrict the scattering of collagen monomers, leading to the formation of a local polymerization of collagens after the delivery. During the subsequent compression of the surface, most of the huge collagen clusters formed initially might be squeezed out of the floating lipid film and get into the subphase. In the case with less collagen, widely dispersed collagen molecules may participate in the lipid film mainly in the form of monomer and dissolve into the subphase during the surface compression due to their solubility in water, which would be the reason that no collagen features were noticed after deposition onto mica. As the molar ratio of collagen to DOPE is falling into a critical range, molar ratio of DOPE/collagen 80:1~100:1, the interactions between these two species allow the homogeneous distribution of collagen molecules within the lipid matrix and a moderate degree of association before the surface compression. The partially polymerized collagen structures may bind with the lipid monolayer by partial insertion into the fluidic DOPE matrix and
establish interconnection further with each other during the compression, which is in good agreement with the negligible surface pressure change during the collagen spreading and the height analysis of the composite films.

The difference in the compatibility of collagen with DOPE and DPPE and the phase separation in the DOPE/DPPE binary system allow us to exploit patterning collagen assemblies at an air/water interface templated by the DOPE/DPPE monolayer.

3.3.4 Collagen/DOPE/DPPE Ternary LB Films

The patterning procedure developed in this study is based on the co-self-assembly of collagen and PE lipids by introducing collagen in a previously spread lipid monolayer on an aqueous surface. Restricting collagen molecules to the interface region is the first key step in this method. If significant amounts of monomeric collagen dissolve into the subphase, the reproducibility of polymerized collagen patterns and the long-range uniformity of the sample are impossible to realize, a major challenge for all surface patterning methods based on self-assembly.

The fabrication of the composite collagen/DOPE/DPPE monolayer was performed in a LB trough on top of an aqueous subphase with pH at 7.2 and temperature at 25 °C. The compression isotherm for a DPPE/DOPE/collagen composite film (DOPE:DPPE/collagen=80:20:1 in molar) at 25 °C was investigated. As shown in Figure 3.6, the collagen/DOPE/DPPE isotherm is positioned to the right of the isotherms for single lipids and lipid mixtures, suggesting a more loosely packed film due to the incorporation of collagen molecules that have much bigger molecular size than the lipids.
Figure 3.6 Π-A isotherms for: (a) DPPE; (b) DOPE; (c) lipid mixture with DOPE: DPPE=4:1; and (d) lipid/collagen mixture with DOPE:DPPE:collagen=80:20:1 in molar at 25 °C on buffer solution (pH=7.2).
In addition to the intrinsic information contained in collagen amino acid sequences that leads to the polymerization of collagen monomers, a novel technique based on LB film preparation was developed in the current study, which provides a promising way to influence and control the degree of collagen association by adjusting the phospholipid matrix floating at an air/water interface. Spreading collagen onto a previously spread lipid monolayer was believed to prevent the dissolution of collagen molecules into the subphase and selectively anchor the collagen structures at the interface, thereby providing the key for patterning polymerized collagen features two-dimensionally and in a well-controlled way. Although the patterning technique developed is based on the co-self-assembly of collagen and PE lipids, many factors of the fabrication procedure may affect the resulting surface structure of the composite film, among which, the molar ratio of those components, the way to deliver multiple species and the surface pressure for deposition were demonstrated to be the most important ones. Fabrication of uniform collagen/lipid composite films with well-defined collagen patterns was realized by optimizing these key factors. In the current study, systematic investigation of the influence of those critical factors on the growth of collagen features is conducted. Detailed analysis and comparison of various collagen polymerized structures at different levels and the phenomena observed during the film preparation build up the foundation for a proposed mechanism of lipid/collagen co-self-assembly at an interface.

Collagen assemblies were obtained by spreading its acidic solution on a slightly compressed DOPE/DPPE mixed monolayer at an air/water interface. The extent of lipid monolayer compression before introducing the collagens determined the successful incorporation of collagen into the lipid matrix at proper molar ratios of collagen to PEs.
3.3.4.1 Effect of Spreading Surface Pressure

On a fully compressed DOPE/DPPE monolayer (at surface pressure of 25 mN/m), the collagen solution was spread and the composite film was transferred onto mica at the same surface pressure. Whatever the molar ratio between the PE lipids is, multilayered structures were produced, in which collagen aggregations were observed on top of the lipid monolayer, as shown in Figure 3.7a and 3.7b. Lipid domains underneath the collagen features appear similar to those observed in the pure lipid binary films, implying the penetration of collagen through the interface and the disturbance to the lipid monolayer are trivial, which is also demonstrated by the moderate area change during the collagen delivery. By keeping the surface pressure constant at 25 mN/m, the slight area increase that resulted from the collagen spreading suggests the highly ordered DOPE/DPPE monolayer serves as a dense hydrophobic surface composed of tightly packed alkyl chains. The affinity of collagen to the DOPE matrix exhibited in the collagen/DOPE film is confined by the dense organization of long tails after the surface is compressed to solid state. As the surface pressure to which the lipid monolayer was compressed before introducing the collagen (mentioned as spreading pressure in the following part) was decreased to 20 mN/m, it is interesting to observe an area decrease after the collagen solution was dispersed and the absence of collagen assemblies in the AFM images of transferred films. The area decrease corresponding to the diffusion of collagen into the aqueous subphase and the rearrangement of binary lipid monolayer was more considerable at the spreading pressure of 10 mN/m. This phenomenon was consistent with the observation for the binary DOPE/DPPE monolayer that the reorganization at lower surface pressure is more significant than that at higher surface pressure.
pressure. When the spreading pressures are intermediate between the deposition pressure of 25 mN/m and the fairly low surface pressure just above the lift-off point in the isotherm, it seems that the package of the lipid matrix and the orientation of collagen molecules do not allow sufficient binding of collagen features to the floating lipid matrix before most of the collagens, either as monomers or assemblies at different levels, come across the interface and disperse into the subphase.

Support of a collagen film was also tried on a fully expanded PE monolayer at an air/water interface with the surface pressure staying at 0 mN/m. For the lipid monolayer with DOPE contents more than 20 % (molar %), regardless of the collagen fraction, AFM images of the composite film exhibit no trace of collagen structures within the lipid monolayer. The isolated lipid molecules may give collagen enough space to disperse into the subphase before the collagens can polymerize due to the solubility of collagen monomers in aqueous solution. Weak interactions between widely spread lipids and collagens could not keep the collagen molecules at the interface. This explanation is supported by the fact that the inclusion of collagen did not result in an obvious increase of surface pressure from 0 mN/m. However, as DPPE is dominant in the mixed lipid matrix (molar ratio as high as 4:1 of DPPE:DOPE), the expanded lipid monolayer showed a selective anchoring function for collagen features in the DOPE matrix with proper contents of collagen (Figure 3.7c). Participation of collagen structures in the lipid matrix was indicated by a rapid surface pressure increase upon the delivery of collagen and stable surface pressure remaining at a high level after aging the composite film at an air/water interface for half an hour. A majority of DPPE in the lipid matrix reduces the average area per molecule in the binary monolayer at a specific surface pressure as well
as the surface fraction covered by DOPE monolayer that has affinity to collagen molecules, and therefore, it decreases the migration of collagen molecules. The collagen molecules confined in limited regions that are covered by the DOPE monolayer have a greater opportunity to polymerize and form water-insoluble surface structures.

Spreading pressure in the range of 0.2~1.4 mN/m was found to effectively restrict collagen at the interface for all the lipid compositions investigated in this study. When introducing the collagen solution with the surface pressure maintained constant at a specific value in the above range, a dramatic increase in surface area was observed. In the case that the maximum area was reached during the collagen delivery, the surface pressure increased subsequently and remained at a level without significant dropping after the composite film was aged at an air/water interface for half an hour. Figure 3.7d shows the well developed collagen fibril structures preferentially localized in the DOPE phase and completely excluded by the DPPE domains. These phenomena indicate the protrusion of collagen molecules or fragments into the lipid matrix and the successful holding of collagens at the interface. Within this lifting-off (isotherm beginning to raise above 0 mN/m) range of surface pressures, a binary lipid monolayer would be compressed to the extent that the individual molecules begin to interact, leading to the change of molecular orientation from the lying position to a more vertical one. In the moderately compressed monolayer, the distance between lipid molecules and the alignment of lipid molecules at an air/water interface may be optimum for the collagen monomers to distribute evenly and interact with the lipid matrix in a way that promotes the formation of water insoluble collagen assemblies before the monomeric collagens penetrate the interface and disperse into the aqueous subphase.
Figure 3.7 AFM images of DOPE/DPPE/collagen LB monolayers deposited at 25 mN/m on mica with various molar ratios of DOPE:DPPE:collagen and spreading pressures: (a) 8:2:1, 25 mN/m; (b) 2:8:1, 25 mN/m; (c) 2:8:1, 0 mN/m; (d) 80:20:1, 0.2 mN/m.
The accurate measurement of low surface pressure would be affected significantly by the resolution limitation of the Whihelmy balance and various possible disturbances coming from air flow, trace of contaminations and other resources. Although the amount of lipids spread at the interface can be controlled precisely, it is difficult to reach the same surface area and also the same surface density at a specific spreading pressure in the lifting-off range. The collagen, therefore, could not be introduced into a lipid matrix with well-defined alignment by monitoring the surface pressure. Since the quantity of lipid molecules was measured with the accuracy of 0.01 mg and the lipid solution was spread by microsyringe, the average molecular surface concentration calculated from the delivery amount and surface area would be a better way to decide the point to incorporate collagen. For example, for the DOPE/DPPE monolayer with molar ratio of 4:1, the optimum spreading surface concentration is in the range of 100 Å²/molecule to 120 Å²/molecule. Compared with the controlling spreading pressure, manipulating the spreading surface concentration of lipid molecules was demonstrated to get a better chance to spread collagens and obtain collagen surface patterns in a more reproducible way.

3.3.4.2 Effect of Lipid/collagen Molar Ratio

As collagen is incorporated into the DOPE/DPPE film at the optimum spreading surface concentration, the existence of a collagen lateral anchorage and the type of collagen aggregations are very sensitive to the molar ratio between lipids and collagen in the composite film. Regardless of the components of the templating lipid matrix, collagens preferentially bind with DOPE-rich regions and are totally excluded from the
more condensed DPPE domains under proper molar ratios of collagen to lipids. A variety of polymerized collagen structures at different levels was observed between the DPPE domains, from fine branching bundles evenly dispersed throughout DOPE matrix to highly condensed and interwoven sheets that completely cover the DOPE phase. The degree of collagen polymerization and the resulting type of surface features can be mainly controlled by adjusting the molar ratio between the three components. A wide range of lipids and collagen compositions has been investigated with the lipid molar ratio fixed at 4:1, 1:1 and 1:4 (DOPE: DPPE).

Collagen assemblies formed on the binary lipid monolayer with a DOPE/DPPE molar ratio of 4:1 are of similar branched fibril structures. For example, at molar ratio 80:20:1 (DOPE:DPPE:collagen), branching assemblies of collagen are extensively dispersed within the DOPE phase and are completely excluded by DPPE domains (Figure 3.7d). The main collagen branches are around 5.6nm higher than the bottom DOPE phase and 4.8 nm higher than the DPPE domains, which is consistent with the height difference of 1.0 nm between the DOPE and DPPE monolayers. This indicates the underneath DOPE monolayer remains intact after the growth of collagen fibers. Since the collagen monomer is 300 nm in length and 1.5 nm in diameter, collagen molecules should be aligned with their long axis parallel to the surface. The collagen features protrude from the DOPE monolayer less than 6nm. There should be at most four layers of collagen molecules packed along the direction vertical to the surface plane. Filaments polymerized at lower degrees are also observed extending from the major collagen branches, some of which exhibit the height lower than 1nm, even smaller than the monomeric collagen.
Figure 3.8 AFM images of DOPE/DPPE/collagen LB monolayers on mica with fixed DOPE/DPPE molar ratio of 4:1 and various DOPE:DPPE:collagen molar ratios of: (a) 80:20:1; (b) 100:25:1; (c)-(d) 160:40:1 (spreading pressure: 0.2 mN/m; deposition pressure: 25 mN/m).
Within the range of pH investigated in the current study, the integrity of the collagen triple helix can be ensured and no degradation of the collagen monomer would occur. Thus, the finest collagen feature suggests the insertion of collagen molecules or segments into the DOPE matrix. The horizontal width of the main collagen branches appears above 60 nm in AFM images. Although the measurement of horizontal length of surface features by AFM could be enlarged due to the finite size of the AFM tip (around 20 nm), the width of the collagen branches is still much larger than their height after taking the possible image distortion into account. The “flattened” collagen bundles may result from the influence of the DOPE layer on the assembling sequence of collagen monomers or the dewetting effect during the slow air-dry process. As shown in Figure 3.8a, the growth of collagen fibril structures spreading out on top of DOPE phase stops upon reaching the periphery of DPPE domains, where thinner branches point towards the DPPE domains. The size of the collagen bundles is confined by the existence of the DOPE matrix and the surface localization is realized by the DPPE exclusion. The shape and the size of the DPPE domains from which collagen is excluded are similar to those formed in a binary lipid monolayer, indicating the introduction of collagen does not result in a considerable rearrangement of the lipid lateral organization in this case.

At the same molar ratio of DOPE:DPPE 4:1, decreasing the collagen contents to 1:125 (collagen:PEs in molar) results in a similar distribution of collagen branching features within the DOPE matrix (Figure 3.8b). However, the evenly dispersed collagen fibril assemblies have reduced vertical height of up to 4.8 nm and decreased horizontal size compared with the collagen fibers formed at the molar ratio of 1:100 (collagen:PEs). The lesser degree of polymerization and lateral interconnection could be simply
attributed to fewer contents of collagen in the composite film. DPPE domains surrounded by collagen fibrils exhibit less elongated shape and more uneven surface distribution compared with the previous case with a higher amount of collagen. Further reduction of the collagen ratio in the mixture to 1:200 (collagen:PEs) gives rise to isolated collagen fibril aggregations randomly distributed on the DOPE monolayer without lateral connection (Figure 3.8c). It is interesting to notice the formation of duplicated collagen bundles with similar surface morphologies, but different heights in the range of 3 to 6 nm as exhibited by Figure 3.8d. Incorporating collagen into the binary lipid monolayer induces the formation of DPPE domains with a two-lobed shape and smaller size compared with those in the binary lipid film. Images at a smaller scale disclose the presence of patches randomly scattered in the background. Based on the 1 nm height and the exclusion of the collagen assemblies from the patches, it is reasonable to conclude that those patches consist of mainly DPPE. In the cases when collagen can successfully form polymerized structures at an air/water interface, significant participation of collagens into the lipid matrix can be demonstrated by a significant increase of surface area during the spreading of collagen solution at the interface. There could be a competition between the interactions of collagen/DOPE, collagen/DPPE and collagen/collagen, which is governed by the molar ratio of the three components and the distance between those molecules. Thus, it is not surprising to find the amount of collagen that can be supported by a given floating lipid matrix falls into a narrow range.

DOPE/DPPE/collagen composite films with the molar ratios that are out of the range of 80:20:1~160:40:1 did not reveal the existence of collagen lateral aggregations localized on top of the lipid monolayer. For ternary films with a lesser amount of
collagen, moderate surface increase during the collagen spreading implies the failure of incorporating collagen into the lipid matrix. The AFM images with a surface morphology the same as the one of the mere DOPE/DPPE monolayer further suggests the delivery of collagen onto a lipid monolayer did not affect the lipid domain formation as a majority of collagens went into the subphase, implying sufficient collagen molecules are required to form polymerized structures and bind with the DOPE monolayer. However, when the collagen ratio exceeds the critical value of 1:100 (collagen:PEs) in molar, for example at 1:50, tiny collagen features can be found somewhere in the region covered by the DOPE phase. Although the dramatic increase of surface area during the collagen spreading in this case suggests strong binding of collagen into the lipid monolayer initially, the subsequent compression may exclude the already formed collagen aggregations from the interface into the subphase. The collagen polymerization and penetration through the interface have a striking effect on the formation of DPPE domains that display many irregular shapes than those in pure lipid film. Excess contents of collagen did not lead to polymerized structures at a higher degree, implying there might be a limitation of collagen molecules that could be supported by a slightly compressed lipid monolayer.

Ternary composite films templated by lipid monolayer with equimolar DOPE and DPPE exhibit an even narrower critical range of collagen ratios to PE lipids. Under the investigated molar ratios of DOPE:DPPE:collagen that are above the critical range of 70:70:1~80:80:1, there is no sign of collagen structures supported by lipids, and the deposited films exhibit the same surface morphology as that for the equimolar lipid monolayer. It is noticeable that the collagen delivery was followed by a considerable increase of surface area; therefore, the participation of collagen into the lipid monolayer
Figure 3.9 AFM images of DOPE/DPPE/collagen LB monolayers on mica with fixed DOPE/DPPE molar ratio of 1:1 and various DOPE:DPPE:collagen molar ratios of: (a) 80:80:1 (mesh); (b) 80:80:1 (sheet); (c) 70:70:1 (mesh); (d) 70:70:1 (sheet); (e) height analysis of (d); (f) 70:70:1 (sheet) phase image (spreading pressure: 0.2 mN/m; deposition pressure: 25 mN/m).
before surface compression is not negligible and the absence of collagen features would occur mainly during the surface compression. The AFM images of DOPE/DPPE/collagen films composed of 70:70:1 and 80:80:1 molar mixtures display the coexistence of collagen meshwork structures located between DPPE domains and interwoven sheets that almost completely cover the DOPE phase (Figure 3.9). The highly connected collagen meshwork is 4~9 nm above the DOPE monolayer, which is a bit higher than the collagen branches formed in the lipid template with DOPE:DPPE=4:1 in molar. The decrease of the DOPE component in the lipid template reduces the available space for collagen to disperse, and therefore, polymerized collagens yield assemblies at a higher degree. The densely interlaced collagen sheet exhibiting a porous structure at a smaller scale is unique in terms of its possible applications as a porous membrane or molecular filter. The collagen sheets exhibit rims along the DPPE peripheral regions that are 3 nm higher than the middle portion of the sheet (Figure 3.9d-e). The excess concentration of collagen molecules along DPPE domain edges could result from the prevention of collagen dispersion along the surface by the DPPE phase. The AFM phase image in Figure 3.9f reveals the presence of tiny DPPE islands with irregular shape surrounding the periphery of DPPE domains. The development of a collagen sheet around DPPE domains may exert influence on DPPE migrations and therefore have an effect on the formation of DPPE domains. The less condensed meshwork architecture resembles the collagen structure formed by immersing a hydrophobic polymer substrate in a collagen solution and drying slowly in air, which was assigned to the rupture of the film by the moving water meniscus during the detwetting process. Since the DPPE domains with “melting” edges did not appear in the case when collagen meshwork presents, a different pathway of
collagen participation into the lipid monolayer and self-association would give better explanations to the formation of a collagen net, instead of driven apart during air-drying. In general, compared with 80:80:1 mixture, the 70:70:1 film has collagen architectures with 2 nm less thickness distributed between more circular DPPE domains. The degree of collagen self-assembling could be related to the resulting shape of DPPE domains enveloped by collagen aggregates.

Within the DOPE/DPPE lipid matrix at a molar ratio of 1:4, polymerized structures of collagen at a higher level can be synthesized when the molar compositions are in an optimum range. As the collagen ratio exceeds the optimum range, only irregular collagen clusters could be produced. Homogeneous collagen structures start to appear from the molar ratio of 3:12:1 (DOPE:DPPE:collagen) (Figure 3.10a) in the form of a flat sheet. The collagen sheet developed between DPPE domains is 1.7 nm higher than the DPPE domains, suggesting the formation of at most two-layer stacks along the vertical direction. Figure 3.10b displays the formation of honeycomb-like collagen networks spreading out along the restricted area covered by the DOPE monolayer at a molar ratio of 4:16:1 (DOPE:DPPE:collagen). The threads of the collagen net exhibit the largest collagen bundles obtained in this study with the vertical height of 19 nm and the horizontal width of over 300 nm. The appearance of huge lumps indicates the constrained dispersion of collagen molecules in the area adjacent to two DPPE domains. Surrounded by those collagen assemblies, the circular DPPE domains do not show a considerable difference from those formed in a binary lipid monolayer. However, a slight reduction of collagen contents in the film results in both a significant change of DPPE domains and the collagen structures. The 6:24:1 (DOPE:DPPE:collagen) composite film gives
Figure 3.10 AFM images of DOPE/DPPE/collagen LB monolayers on mica with fixed DOPE/DPPE molar ratio of 1:4 and various DOPE:DPPE:collagen molar ratios of: (a) 3.12:1; (b) 4:16:1; (c) 6:24:1; (d) 8:32:1 (spreading pressure: 0 mN/m; deposition pressure: 25 mN/m).
more elongated DPPE domains embedded in a dense collagen sheet that is on top of the DOPE phase as shown in Figure 3.10c. The collagen sheet has a two-layered structure, where the porous bottom layer, 3 nm higher than the DPPE domains, completely covers the area previously possessed by DOPE, and the top layer with the height difference of 10-12 nm occupies the region away from the boundary of the DPPE domains. As the collagen proportion is decreased to 8:32:1 (DOPE:DPPE:collagen), the previously distinguished DPPE domains begin to merge with each other, and isolated collagen fibril agglomerates can be seen scattered on DOPE islands (Figure 3.10d). Further reduction of collagen compositions leads to the disappearance of collagen structures and similar surface morphologies as that for a pure lipid binary film.

3.3.4.3 Effect of Deposition Pressure

The optimum range of spreading surface concentration of lipids (mentioned as spreading concentration in the following part) and molar ratio of lipid/collagen are two key factors that determine the successful incorporation of collagen molecules in the floating lipid monolayer and control the collagen polymerization with preferential surface localization. In addition to these two factors, the influence of the deposition surface pressure on the resulting collagen structures was also explored. The deposition pressure itself shows a moderate effect on the collagen structures in the composite film, but no influence on the surface anchorage of collagen aggregates. However, the deposition pressure combined with the spreading concentration can induce the formation of collagen assemblies in a more reproducible way.
For example, to prepare a ternary film of DOPE:DPPE:collagen=80:20:1, at the same spreading concentration, collagen solution was introduced onto the lipid monolayer. The target surface pressure at which the LB films were transferred onto the mica surface was investigated from 25 mN/m to 30 mN/m. To some extent, the size of collagen bundles is sensitive to the deposition pressure, but no variety of assembly types has been observed under different deposition pressures (figure not shown here). The deposition pressure itself did not show significant influence on the collagen aggregation type and surface localization. At the same molar ratio, collagen was spread at the interface when the mixed lipid monolayer was compressed to the surface concentration of 100 Å²/molecule. The composite film that was deposited at 30 mN/m showed a more condensed meshwork-like collagen architecture in a highly reproducible manner. Spreading the collagen onto a more loosely packed lipid matrix at the surface concentration of 120 Å²/molecule and transferring the film at a lower surface pressure of 25 mN/m lead to less aggregated collagen filaments dispersed evenly in DOPE phase.

At a given lipid/collagen composition, manipulating deposition pressure together with spreading concentration at optimum values can fabricate collagen structures with long-range uniformity and enhanced reproducibility. Generally, a higher spreading concentration of lipids combined with lower deposition pressure results in less aggregated and more dispersed collagen features, such as fine filaments, while lower spreading concentration with higher deposition pressure leads to more condensed collagen structures such as continuous collagen sheets fully covering the DOPE region. It is consistent with the hypothesis that a more loosely packed lipid matrix provides a better template for collagen monomers to disperse, participate and self-assemble.
3.3.5 Co-self-assembling Mechanism of Lipids and Collagen

Our exploration of fabricating collagen hierarchical structures at various levels at an air/water interface templated by a previously spread binary lipid matrix indicates there exist critical points where collagen can interact with lipid molecules, DOPE and/or DPPE, in a specific way that allows the collagen to stay at the interface and polymerize on top of DOPE phase. Compared with the liquid condensed phase, the preferential adsorption of proteins onto a loosely packed liquid-expanded lipid monolayer or an expanding lipid film has been attributed to the available space between lipid molecules for proteins to insert into. However, the difference in the package of DOPE tails and DPPE tails itself could not explain the phenomena that the initial spreading of collagen at a surface pressure lower than 1.5 mN/m is critical to the successful incorporation of collagen into the lipid matrix and selective localization of collagens within the DOPE phase. At the spreading pressures that are just above the lift-off point, both the DOPE and DPPE molecules adopt a randomly tilted orientation and there is no significant difference in the tail package of DOPE and DPPE that may lead to the preferential anchorage of collagen. Since the non-comparable size of collagen and PE lipid molecules would restrict the migration of collagen even at the fairly low spreading pressure, the further surface compression could not induce the collagen pattern formation.

There are three questions to be answered for the successful incorporation of collagen into the binary lipid monolayer at an air/water interface: 1) Why do collagens show affinity exclusively for the DOPE phase? 2) How do collagens interact with the DOPE and/or DPPE monolayer? 3) Why can collagen polymerized features only be
formed under spreading concentrations and molar ratios that are in a narrow range? The co-self-assembly mechanism of collagen and lipids proposed below will give explanations to the above questions according to our observations and experimental results.

Figure 3.11a illustrates the case when collagen was delivered onto a widely spread DOPE or DPPE monolayer, in which the penetration of collagens through the interface was considerable due to weak interactions between lipids and collagen; therefore, a denser alignment of lipid molecules is required to keep collagen at the interface. After the lipid monolayer was compressed to the point where the lipids begin to change their orientation from lying flat to tilting with small angles on the water surface, attractive interactions between collagens and DOPE can prevent collagen dissolving into the subphase (Figure 3.10b). At the same optimum spreading pressure, the average area occupied by DPPE (0.65 nm²) is only half of that occupied by DOPE (1.3 nm²) due to the kinked tail structure of DOPE. The much smaller area per molecule of DPPE reduces the possibility that charged segments along the collagen surface interact and bind with PE head groups that contact with the water surface. In Figure 3.10c, most of the collagens dissolve into the subphase without significant attractions between collagen and DPPE molecules. More loosely packed lipid tails of DOPE provide more space for collagen segments to insert partially into the DOPE monolayer and interact with PE heads. Exposing polar PE head groups to collagen molecules might be a key step to anchor the collagen at an air/water interface and promote the polymerization.
Figure 3.11 Schematic illustration of co-self-assembly mechanism of collagen and lipids
This hypothesis can be further supported by the phenomena observed during the investigation of spreading pressure for a ternary lipid/collagen film. A spreading pressure as high as 25 mN/m gives rise to a tightly packed lipid film, providing a mere hydrophobic surface composed of hydrocarbon tails. Although the dense lipid monolayer can prevent collagens going into subphase, no selective localization of collagen structures can be formed in this case (Figure 3.11d). Under the lower spreading pressures of 20 and 10 mN/m, there was no trace of collagen assemblies in the deposited film. A possible explanation is that the still restricted motion of the lipid alkyl chains reduces the chance of binding collagens with PE heads and therefore weakens the affinity of collagens to the lipid monolayer (Figure 3.11e). It seems that the initial insertion of collagen subgroups into the lipid monolayer and the electrostatic interactions between PE heads and charged groups along collagens are decisive for the self-assembly of collagen monomers at an air/water interface. It is reasonable to expect that without considerable polymerization, the dispersed collagens, most in the form of a monomer, are squeezed out of the lipid support and dissolve into an aqueous subphase. When DOPE contents in the lipid template are prominent (DOPE:DPPE=4:1 and 1:1 in molar), despite of the collagen fractions, the ideal spreading surface pressure is just above the lift-off point of the isotherm. At this point, the distance between individual DOPE molecules is ideal for collagens to partially participate into the lipid film and interact with PE heads, which ensures enough time for the polymerization of collagen monomers and interconnection of initially formed assemblies before the collagens penetrate through the interface. On the lipid template with predominant components of DPPE (DOPE:DPPE=1:4), collagen was successfully delivered onto a widely spread lipid monolayer with a surface pressure at
0 mN/m and polymerized in confined regions occupied by DOPE. The decreased area per molecule at a specific surface pressure would allow collagen to be supported by the lipid film and the diminished available area for collagen to anchor would promote the formation of collagen assemblies at higher level, both of which inhibit the dissolution of collagen monomers.

Figure 3.11f and 3.11g illustrate the striking effect of molar ratios between lipids and collagen on the formation of polymerized collagen features at an air/water interface. For the lipid template with the majority of DOPE, the critical molar ratios of DOPE to collagen are the same as those observed for collagen/DOPE films (80:1 to 100:1 in molar). At the highest composition of DOPE, collagen would exhibit a wider and more uniform dispersion within the DOPE phase; therefore collagen hierarchical structures with a lower degree of polymerization and smaller size could develop and interconnect within DOPE phase as demonstrated by Figure 3.11f. Since collagen has a specific staggered way to aggregate side-by-side, the growth of collagen fibril bundles relies on the conformational freedom of collagen monomers before the polymerization to satisfy the specific alignment in the assemblies. The absolute majority of DOPE in the lipid template allows the spread of collagen monomers to the extent that a relatively small amount of collagen monomers is enough for collagen to form polymerized and interconnected structures. However, as the collagen content is lower than that critical molar ratio, the interactions between collagens are not strong enough to ensure the polymerization of collagen monomers. The absence of collagen features in the composite film would be ascribed to collagens in the form of monomers dissolving into the aqueous subphase. On the other hand, excess concentrations of collagen, at molar ratios higher
than the critical one, would result in the formation of huge collagen clusters with randomly packed inner structure and wide distribution of size due to the restricted dispersion of collagen monomers in the lipid matrix. Those collagen clusters that could not build up a strong connection with the DOPE phase and also with each other would be squeezed out of the lipid template monolayer and finally disappear on the deposited film. At equal molar concentrations of DOPE and DPPE, a bit more collagen is needed to form polymerized insoluble aggregates without extensive spreading and interacting with the DOPE phase as the lipid monolayer contains more DOPE, thus a more aggregated collagen meshwork and dense sheet could be fabricated at higher ideal collagen compositions (collagen:DOPE=70:1 to 80:1 in molar). Decreasing the DOPE molar fraction to 20% in the lipid template significantly increases the collagen proportion in the ternary film required to polymerize at the interface that is as high as one third of the lipid contents. The restricted motion of collagen monomers intermediate between DPPE domains weakens the attractive interactions between PE heads and charged groups along collagens, which could be counterbalanced by spreading collagens onto the lipid monolayer with a lower surface density (spreading pressure at 0 mN/m) and incorporating many more collagen molecules to form more aggregated features as shown in Figure 3.11f. The successful development of collagen assemblies at the highest level in the current study was observed in confined space occupied by DOPE. The incorporation of vast amount of collagens into the binary lipid monolayer exerts a considerable effect on the formation of distinct and circular DPPE domains.
Figure 3.12 Optical micrograph of CHO cells cultured on a DOPE/DPPE/collagen (molar ratio 80:20:1) film supported by mica after 4 hours attachment and 24 hours incubation (100X, each grid is 50 × 50 µm).
3.3.6 Biocompatibility of Collagen-containing Films

Successful adhesion and growth of CHO cells on the patterned film surfaces demonstrate the biocompatibility of these composite films. Figure 3.12 illustrates the spreading morphology of CHO cells grown on a collagen/lipid composite film with a molar ratio of DOPE:DPPE:collagen at 80:20:1, as observed by optical microscopy. The composite film is sufficiently stable to survive the sterilization and cell culture procedures.

3.4 Conclusions

The studies reported here form the basis for examination and fabrication of more complex collagen-containing structures of biological importance in a more controllable way. Monolayers of phospholipids can be used to pattern ECM proteins twodimensionally. Phospholipids orient themselves at an air/water interface with chains pointing toward the air and heads toward the aqueous subphase with long-range order. The specific alignment and possible microdomain formation of phospholipids can be exploited to incorporate proteins at an interface and to induce a wide variety of 2D protein configurations through hydrophobic and electrostatic interactions between lipids and proteins. The stability, long-range uniformity and biocompatibility of the fabricated composite films and the reproducibility of the fabricating procedure are of great importance for future applications.

In the co-self-assembly mechanism investigation, the penetration of collagen through the lipid monolayer and the interactions between PE heads and charged groups
along the collagen surface are believed to play a crucial role in successfully incorporating collagen polymerized hierarchical structures in a binary lipid monolayer at an air/water interface.
CHAPTER 4

COMPLEX FILMS BASED ON WELL-DEFINED LIPID/COLLAGEN STRUCTURES

4.1 Introduction

Supported phospholipid bilayers have been extensively investigated as biomembrane models, providing information on the structure-function correlations in cell membranes and protein/lipid interactions. The fluidity of the bilayered structure plays an important role in cell functions. In general, artificial lipid bilayers have been rebuilt on a solid surface by fusion of unilamellar vesicles and LB deposition. The LB technique has the advantage of fabricating surface templates with well-controlled chemically or physically different surface patterns with the dimension at micron to submicron scale. During the transfer of the second layer, defects in the film structure were found due to the peeling off of the first layer from the solid substrates. Rearrangement of the bilayer structure of lipids has been observed after exposing the transferred LB bilayers to the air phase, leading to the formation of domains of monolayers and trilayers, which did not occur if the lipid bilayer was kept in water.
during the AFM investigation. The phase separated lipid bilayer provides proteins a
good templating matrix in which the proteins can selectively bind with one specific lipid
phase. Bilayered thin films containing PE lipids and collagen are developed and
explored intensively in this chapter to get sight into the way that collagen is incorporated
in a bilayer lipid matrix. On the other hand, inspired by the previous study on fabricating
silica fiber on the surface of collagen assemblies in bulky solution, the possibility of
templating the growth of silica structures is explored on the surface of a well-structured
lipid/collagen monolayer.

4.2 Experimental

4.2.1 Materials

DOPE, DPPE and collagen were coming from the same source as that is
demonstrated in Chapter 3. Silicate solution was prepared by stirring mixed tetraethyl
orthosilicate (TEOS), highly pure water and HCl solution for over an hour until the
solution became completely one phase and then diluted with pure water to get \(1 \times 10^{-3}\) M
silicate solution at pH of 6.3.

4.2.2 Bilayer Fabrication and Characterizations

The fabrication of the composite lipid/protein monolayer was based on the LB
technique mentioned in Chapter 3 and the collagen was delivered after the mixed
DOPE/DPPE (4:1 in molar) monolayer was compressed to a moderate surface pressure.

The bilayer films were fabricated by passing the mica sheet twice through the
water surface with the monolayer floating on it. The first layer was deposited by the same
method used in Chapter 3 and air-dried for 2 hours to 6 days before the delivery of the second layer. The solid substrate covered by one layer of film was either dipped into (vertical down) the subphase or lifted from (vertical up) the subphase to transfer the second layer, which was determined by the surface properties of both layers. In the former case, the surface was cleaned by vacuum sucking up before taking the film out of the water; in the later case, the monolayer supported by mica was firstly lowered into the subphase at the highest speed of 43 mm/min and lifted up with the lowest speed of 1 mm/min. Since the lipid molecules diffuse along an air/water interface at a speed of around 0.6 mm/min, the immersing of the monolayer film before the deposition of the second layer at a much higher speed of 43 mm/min would not result in considerable deposition of molecules onto the monolayer film. The deposition pressure is 26 mN/m for the films with collagen and 25 mN/m for the films without collagen.

The LB films were air-dried for 24 hours before the observation using tapping mode AFM to investigate the surface morphology of the film in air and at ambient temperature. Tapping mode imaging using silicon cantilevers was performed at a low spring constant of 0.3 N/m.

4.2.3 Silica Structure Growth

Two methods were utilized to grow silica structures. In the first one, a ternary monolayer of DOPE/DPPE/collagen with molar ratio of 80:20:1 supported by mica sheets was fabricated and air-dried for one day and then immersed into the silicate solution at room temperature for 2 hr, 12 hr 1, 2, 5, 7 and 14 days, respectively, without stirring. In the second method, the ternary monolayer was delivered on the subphase
composed of silicate aqueous solution with pH of 6.3 and temperature of 25 °C, following the procedure described in the previous chapter and compressed until reaching the surface pressure of 25 mN/m. The compressed film was kept still at the interface for 1 and 2 hours, during which the surface pressure dropped significantly. The aged LB films were deposited onto mica at the final surface pressure, typically lower than 10 mN/m, with vertical up method. The resulting films were air-dried for a day before the characterization using tapping mode AFM.

4.3 Results and Discussions

4.3.1 Bilayer Films Containing Lipid/collagen Assemblies

4.3.1.1 Bilayer Supported By DPPE Monolayer

A DPPE monolayer deposited at 30 mN/m and 40 mN/m on mica exhibits different surface morphologies. There is a phase transition from liquid condensed phase to solid phase at surface pressure around 38 mN/m for the DPPE monolayer. A monolayer compressed to 30 mN/m displays irregular plateau regions with 1 nm height randomly dispersed on the surface that could be initially formed lipid domains in a solid phase, while the 40 mN/m film has a homogeneous and smooth surface (figures not shown here). The lateral arrangement of DPPE molecules in the bottom layer and the surface properties of DPPE film could be affected by deposition pressure and air-drying time, which is expected to influence the structure of the soon to be deposited second layer.

To investigate how collagen is incorporated into the lipid monolayer and bilayer, a control film without collagen was first fabricated. A top layer with the lipid mixture of
DOPE/DPPE (4:1 in molar) was deposited onto a DPPE monolayer that was deposited at 30 mN/m and air-dried for only 2 hours before the second layer transfer. During the vertical down deposition of the DOPE/DPPE monolayer, the DPPE monolayer exhibited an obvious hydrophobic surface according to the water meniscus shape along the interface. Though the down path is typical for the transfer of a lipid monolayer onto a hydrophobic surface, the transfer ratio was fairly low in this case, implying possible desorption of DPPE molecules from the first layer. The air-dried bilayer of DOPE/DPPE on top of DPPE shows a heterogeneous surface morphology with domains around 1nm above the base, corresponding to the height difference between the DOPE monolayer and DPPE monolayer (Figure 4.1a). The down path deposition and the subsequent drying did not lead to the typical film composed of domains of monolayers and trilayers, but a composite monolayer of DOPE/DPPE. The partial peeling off the first DPPE monolayer and the rearrangement during the air-drying in order to present the hydrophobic tails of PE lipids to the air phase could be the explanation for the monolayer formation.

On the same DPPE base layer, transferring another film of collagen/DOPE/DPPE with the lipid molar ratio of DOPE/DPPE fixed at 4:1 results in collagen structures that are different from those observed for the corresponding monolayers on mica. At a molar ratio of 1:80:20 (collagen:DOPE:DPPE), the uniform collagen branches evenly dispersed within the DOPE phase obtained for the monolayer supported by mica is replaced by collagen bundles and a highly condensed sheet with random surface distribution along the surface of the fabricated bilayer film as shown in Figure 4.1b. Without extensive dispersion of collagen monomers within the lipid matrix, the assembled collagen
Figure 4.1 AFM images of bilayers supported by mica, the first layer was air-dried for 2 hours before the transfer of the top layer with vertical down deposition: (a) DOPE/DPPE (4:1 in molar) on top of DPPE (deposition pressure (DP)=30mN/m); collagen/DOPE/DPPE with molar ratios of (b) 1:80:20; (c) 1:48:12; (d) 1:100:25 on top of DPPE (DP=30mN/m); (e) collagen/DOPE/DPPE with molar ratio of 1:80:20 on top of DPPE (DP=40mN/m) (for collagen/DOPE/DPPE, DP=26mN/m).
structures have a much larger vertical size of 15 nm compared with the ternary monolayer. The multi-lobed DPPE domains that appeared in the monolayer of collagen/DOPE/DPPE did not show up in the bilayer film, but irregular aggregates protruding from the background phase are observed. Those aggregates exhibit a height difference of 1 nm and exclude the scattered tiny collagen strings and clusters, leading to the conclusion that DPPE is the main component of those aggregates and DOPE phase locates intermediate between DPPE aggregates. As the collagen content is increased to 1:48:12 (collagen:DOPE:DPPE) or decreased to 1:100:25 (collagen:DOPE:DPPE) in the top layer, the bilayer films give a similar lateral structure that contains isolated collagen bundles and lumps widely dispersed between the irregular assemblies that are believed to be DPPE islands (Figure 4.1c-d). The reorganization of PE lipids in the top layer strongly affected the collagen self-assembly and surface localization, which could be attributed to the hydrophobic and heterogeneous surface properties of the bottom DPPE monolayer deposited at 30 mN/m.

Varying the bottom layer to DPPE monolayer deposited at higher surface pressure of 40mN/m, the more densely packed DPPE film in solid phase provides a more smooth surface composed of highly ordered hydrocarbon tails, on which a collagen/DOPE/DPPE (1:80:20 in molar) monolayer was transferred with vertical down method. Figure 4.1e shows a granular surface feature including rearranged DPPE domains and isolated collagen clusters excluded by the DPPE phase, which is different from the one for the bilayer supported by the DPPE monolayer that is under deposition pressure of 30 mN/m. Successful incorporation of collagen into the bilayer lipid matrix and the formation of self-assembled collagen hierarchical structures may be determined by the alignment of
lipid molecules in both layers. The DPPE monolayer compressed to solid phase (40 mN/m) provides less space for collagen molecules to insert into the bottom layer and thus reduced the chances that collagen polymerized structure could be developed during the reorganization of DOPE/DPPE matrix.

To explore the influence of dewetting of the first layer of a bilayer structure, DPPE monolayers air-dried for 2 days were also applied to deposit a second collagen/DOPE/DPPE composite film. All the collagen/DOPE/DPPE films mentioned in the following part are of the molar ratio 1:80:20. On the two-hour air-dried DPPE films that are still partially wet, the subsequent deposition could be conducted by vertical down dipping. However, as the two-day air-dried DPPE film was lowered into the subphase passing through the floating collagen/DOPE/DPPE film, the surface area did not decrease as usual to compensate the removal of molecules from water surface to solid substrate. The moderate increase of surface area indicates partial desorption of DPPE films from the mica surface to the air/water interface, which is also demonstrated by AFM investigation of the resulted film. Vertical up deposition turns out to be the feasible way to deliver the collagen/DOPE/DPPE film on completely dried DPPE monolayer.

The bilayer on top of the DPPE film that was deposited at 30mN/m and air-dried for 2 days displays 1nm deep holes embedded in a smooth surface and bumpy collagen assemblies mostly located in the regions covered by holes (Figure 4.2a). Based on the depth of the randomly located holes and the preferential attachment of collagen assemblies, the homogeneous background should be a DPPE monolayer and the holes regions could be covered by the DOPE monolayer, where the original DPPE film
Figure 4.2 AFM images of bilayers supported by mica, the first layer was air-dried for 2 days before the transfer of the top layer with vertical up deposition: collagen/DOPE/DPPE with molar ratios of 1:80:20 on top of (a) DPPE (DP=30mN/m); (b)-(c) DPPE (DP=40mN/m) (for collagen/DOPE/DPPE, DP=26mN/m).
was peeled off. There is no evidence of lipid patches that are typical for bilayers on wet bottom layers, indicating that the dewetting of the first layer combined with the transfer direction for the second collagen-containing film are critical factors that determine the incorporation of collagen assemblies in the bilayer lipid matrix. Increasing the deposition pressure of the bottom DPPE film to 40 mN/m leads to totally different surface structure of the resulted bilayer. Without the appearance of lipid patches and dented holes, Figure 4.1b and 4.1c exhibit highly connected collagen fibril features that are supported by a smooth surface. In the absence of a DOPE/DPPE surface template in the bilayer, collagen structures spread throughout the sample surface that consists of the densely packed hydrophobic DPPE hydrocarbon tails.

4.3.1.2 Bilayer Supported by DOPE Monolayer

The surface morphology of the DOPE monolayer deposited at either 30mN/m or 40mN/m on mica appears smooth and defect free (figures not shown here). However, the AFM images of DOPE/DPPE (4:1 in molar) monolayer that was transferred on top of a two-hour dried DOPE monolayer (deposition pressure 30 mN/m) with vertical down method exhibited a heterogeneous surface covered by tiny holes with high density, which could be assigned to the desorption of DOPE molecules from the originally flat monolayer (Figure 4.3a). The peeling off during the vertical down deposition is more significant when a collagen/DOPE (1:100 in molar) monolayer is transferred onto a DOPE monolayer deposited at 40 mN/m and air-dried for days. In Figure 4.3b, collagen branches that can be developed throughout the DOPE phase in collage/DOPE monolayers are replaced by irregular collagen clogs that locate between scattered dents. Those
Figure 4.3 AFM images of bilayers supported by DOPE monolayer on mica: (a) DOPE/DPPE (4:1 in molar) on top of DOPE (DP=30mN/m; vertical down deposition; first layer air-dried for 2 hours); (b) collagen/DOPE (1:100 in molar) on top of DOPE (DP=40mN/m; vertical down deposition; first layer air-dried for days); collagen/DOPE/DPPE (1:80:20 in molar), vertical up deposition, first layer air-dried for days, on top of DOPE (c) DP=30mN/m; (d) DP=40mN/m (for collagen/DOPE and collagen/DOPE/DPPE, DP=26mN/m).
patches with the smooth surface and 1.8 nm lower than the continuous background should be an exposed mica surface. Randomly dispersed collagen clusters bind mostly with the DOPE monolayer surface and leave the hydrophilic mica surface intact, suggesting specific binding between collagens and DOPE film. The downward transfer could not ensure the integrity of the DOPE monolayer and therefore fails to maintain the collagen structures already grown at an air/water interface. The down stroke could neither lead to the successful delivery of a collagen/DOPE/DPPE ternary film onto a DOPE monolayer supported by mica and was replaced by vertical up lifting. In spite of the deposition pressure of the DOPE monolayer onto the mica sheet, the wet (two-hour dried) first layer could not induce interesting collagen feature formation in the bilayer structure except for isolated collagen clusters. Therefore, bilayers supported by DOPE films were fabricated after drying the first layer for several days.

The bilayer on top of a 30 mN/m DOPE monolayer has a similar collagen surface assembly and localization as those of collagen/DOPE/DPPE monolayer on mica. The interconnected meshwork of collagens preferentially anchors in the regions with DOPE coverage as described in Figure 4.3c. Unlike the ternary monolayer on mica, DPPE domains in a bilayer film appear more circular and collagen features exhibit more variety in terms of dimension and interconnection of polymerized assemblies. The essential collagen/DOPE/DPPE film structure formed at an air/water interface is maintained after being transferred to a DOPE covered surface with the vertical up method, although the DOPE monolayer surface is obviously hydrophobic. Possible extension of collagen from the upper layer to the underneath DOPE layer could occur during the second layer deposition, which may strengthen the binding between two adjacent layers and preserve
the ternary film structure initially formed at an air/water interface. In another bilayer film, the collagen 2-D pattern in lipid matrix is kept nearly intact when a DOPE monolayer deposited at a higher surface pressure of 40 mN/m serves as the first layer (Figure 4.3d). Collagen bundles excluded by multi-lobed DPPE domains are of the height over 10nm. Compared with the ternary collagen/DOPE/DPPE monolayer on mica, the collagen branches formed in the bilayer are polymerized to a higher degree and the bilayer film surface appears bumpier, suggesting additional aggregation due to the presence of the bottom DOPE layer.

The DOPE monolayer anchored on mica acts as not only a smooth hydrophobic surface to support the collagen/DOPE/DPPE film, but also a flexible environment to incorporate collagen molecules or segments due to the affinity between collagen and DOPE. The interactions between adjacent layers could be enhanced by partial insertion of collagen structures into the bottom DOPE layers, which leads to the presence of the preferential collagen surface localization in the DOPE/DPPE matrix.

4.3.1.3 Bilayer of Collagen/DOPE and Collagen/DOPE/DPPE

Since introducing collagen into a lipid matrix can strongly influence the hydrophilic character of monolayers and impact deposition of subsequent layers, films with collagen in the bottom layer are expected to interact differently with the second layer from what was observed for bilayers supported by pure lipid monolayers. It is worthy to note that deposition of either collagen/DOPE monolayer or collagen/DOPE/DPPE monolayer on mica could only be conducted successfully through two subsequent upward passes, suggesting the upper surface of the mixed film of lipids
and collagen contacting with air is partially hydrophilic even after days of air-drying, although the mica surface should be much more hydrophilic than that of films of collagen/lipid mixtures.

Bilayers of collagen/DOPE consisting of a 3-day air-dried bottom layer show two types of morphologies coexisting: evenly distributed collagen fine filaments with relative uniform size protruding from the background matrix (Figure 4.4a) and branching structures buried inside the continuous phase (Figure 4.4b). The defects, shown as dark spots in Figure 4.4a around 5.6 nm lower, reveal the bilayer organization of DOPE molecules forming the background phase. The vertical dimension of collagen filaments aggregated at various levels is within the range from less than 1 nm to 4 nm. The presence of collagen structures with the size even smaller than its monomeric configuration indicates that collagens in the top layer interact with and penetrate inside the bottom layer. Collagens form regular branched patterns similar to those observed in collagen/DOPE monolayer, while the penetration of collagen molecules through the DOPE bilayer results in a less connected network and lower degree of polymerization compared with collagen surface features developed in monolayer. The dented branching shades that are 2-3 nm lower could be assigned to the vacancy formed after the desorption of collagen assemblies in the top layer during the up stroke and similar collagen branches extending above the surface could be the remaining collagen features after the partial peeling off.

To explore the influence of the bottom layer dewetting on the bilayer structure of collagen/DOPE/DPPE, the mica supported ternary monolayers were air-dried for 2 hours
Figure 4.4 AFM images of bilayers deposited on mica at surface pressure of 26mN/m with vertical up method: (a)-(b) collagen/DOPE (1:100 in molar), first layer air-dried for 3 days; (c) collagen/DOPE/DPPE (1:80:20 in molar), first layer air-dried for 3 days; (d)-(f) collagen/DOPE/DPPE (1:80:20 in molar), first layer air-dried for 2 hours.
and 3 days before the transfer of the top film. On a 3-day dried monolayer, Figure 4.4c demonstrates a heterogeneous surface morphology with the most common fibril collagen assemblies embedded in a lipid matrix. The insertion of collagen features into a bilayer template, similar as what was observed for collagen /DOPE bilayer, can be clearly seen. There is no evidence of lipid phase separation on the surface and exclusion of collagen by DPPE domains. A bilayer structure of lipids is suggested by the fairly high roughness of background matrix, instead of the smooth one that appears in the ternary monolayer. Although the bottom collagen/DOPE/DPPE monolayer was dried for days, interplay between adjacent layers, especially the collagen assemblies penetrating through the bilayer lipid matrix, can still occur in the resulting bilayers, which may diminish the phase separation of DOPE/ DPPE and therefore selective collagen surface localization. The bilayer of collagen/DOPE/DPPE that was fabricated by immediate double vertical up depositions (two-hour air-drying for the first layer) retains typical aggregation and surface pattern of collagens to a certain degree and displays a variety of surface morphologies. Collagen branches are developed as an intermediate between DPPE domains that are of a circular or multi-lobed shape (Figure 4.4d). Unlike the collagen/DOPE/DPPE monolayer, the collagen bundles present a much higher vertical size of 12nm and DPPE domains show less asymmetry in shape and less uniformity in size. DPPE domains exhibit granular surfaces at some locations, where highly agglomerated collagen fibril structures can be observed with random surface distribution (Figure 4.4e). At a smaller scale, Figure 4.4f reveals the existence of bilayer lipid matrix beneath the collagen assemblies, appearing as 3-4nm deep holes embedded in a continuous film.
4.3.1.4 Discussions

To investigate various ways to incorporate collagens into lipid bilayers, DOPE and DPPE monolayers deposited at different surfaces were applied to support the collagen/DOPE/DPPE composite films. The reorganization of the DPPE film during the transfer of the second layer and the highly ordered alignment of DPPE tails give rise to a bilayer film that does not show the interesting collagen patterns templated by the DOPE/DPPE 2-D matrix. When DOPE is applied as the bottom layer, the original collagen assemblies and selective surface localizations could be kept intact in a resulting bilayer film. The penetration of collagens through the bilayer lipid matrix could be the key to ensure the formation of collagen structures that resemble those observed for ternary monolayers on mica. The insertion of collagens into the bilayer matrix can be demonstrated more clearly in a collagen/DOPE bilayer. On a collagen/DOPE/DPPE monolayer, there might be a surface distribution of hydrophobic and hydrophilic regions due to the surface charges existing along collagen monomers. Therefore, in the bilayer of collagen/DOPE/DPPE, collagen penetration would be induced by both hydrophobic attractions with DOPE in the bottom layer and electrostatic interactions between collagen molecules from both layers.

Direction (up/down) of the solid surface movement through the interface during LB deposition of the second composite layer with collagen shows correlation with the bottom layer properties. In the case where DOPE serves as the supporting layer, either wet or air-dried for several days, all the transfer for the second collagen composite film can only be successfully conducted with the vertical up method. If a typical Y type
bilayer is formed, the DOPE monolayer immobilized on mica shows a hydrophobic surface and a down path is the feasible way to deposit another lipid monolayer. The formation of a collagen polymerized structure within the lipid matrix and insertion of collagen into the bottom DOPE layer due to the affinity between collagen and DOPE may lead to the formation of a Z type bilayer in terms of the alignment of PE lipids, in agreement with the upward path for the second layer transfer. On the DPPE monolayer, the wet and dry films require vertical down and up deposition for the subsequent layer, respectively. Within the wet (two-hour dried) DPPE monolayer, a thin water film existing between the mica surface and PE headgroups maintains the hydrogen bonding between PE heads and mica surface, which could prevent considerable desorption of DPPE from the bottom layer during the downward path. The integrity of the DPPE film ensures the hydrophobicity of the first layer. Since collagen is excluded by the highly ordered DPPE film, the DPPE monolayer provides merely a hydrophobic surface for the subsequent transfer of the collagen/DOPE/DPPE monolayer without significant interactions between the two layers. A Y type bilayer structure could be formed through vertical down deposition of the second layer and the irregular lipid patches might be developed during the drying process to expose the hydrophobic lipid tails to the air phase. However, the complete air-dried DPPE monolayer has a much weaker binding with the mica surface due to the absence of hydrogen bonds and undergoes considerable peeling off of the DPPE monolayer when it is passing through the air/water interface. The exposure of the bare mica surface increases the surface hydrophilicity and therefore requires an upward path to transfer the second layer.
4.3.2 Silica Structures Developed on Collagen/DOPE/DPPE Films

4.3.2.1 Immersing Collagen/DOPE/DPPE Films in Silicate Solution

Mesostructured silica films were fabricated by using collagen fibril structures as a template in bulky solution. The precipitation of silica occurred on the surface of collagen assemblies due to the opposite charges that silicate and collagen possessed at neutral pH. After the deposition of the collagen/DOPE/DPPE monolayer on mica, the film surface should have a distribution of both hydrophobic and charged regions due to the presence of collagen assemblies and lipid tails. To investigate the possibility of fabricating and anchoring silica particles two dimensionally on the surface of the supported ternary film, the transferred films were immersed into silicate solutions for various periods of time. A collagen/lipid surface may act as a nucleation site and anchoring template for precipitated silica structures.

Submerging the ternary film anchored on mica for 2 hours results in the appearance of micron-sized domains with vague boundaries and granular surfaces that locate on a less rough surface and are 4nm higher than the continuous phase, as shown in Figure 4.5a. There is no evidence of collagen bundles with selective surface distribution. AFM images of the continuous phase at higher resolution (Figure 4.5b) reveal the presence of densely packed irregular patches that could be partially desorbed DOPE monolayers with loose package because of the 1 nm height difference and smooth surface of the dented gap. Increasing the immersing time to 12 hours, granular domains are exhibited in Figure 4.5c with both shapes and surface localizations resembling those for DPPE domains in the original composite film. Despite the dipping time, those domains
Figure 4.5 AFM images of silica structures developed by immersing mica-supported collagen/DOPE/DPPE (1:80:20 in molar) films in silicate solutions for: (a)-(b) 2 hours; (c) 12 hours; (d) 14 days; (e)-(f) 2 days.
that are of similar structure and 5 nm height difference were also observed for films dipped in silicate solutions for 1, 2, 5, 7 and 14 days. The immersed films supported by mica undergo adsorption of negatively charged silicate ions and possible desorption of collagen assemblies and PE lipids. The collagen assemblies developed initially could dissolve into the silicate solution due to the low pH of 6.3 and high surface tension of aqueous solution exerted on the film supported by mica. Focusing on the regions covered by those grainy domains, there exist irregular plateaus 1-2 nm above the continuous background and highly aggregated clusters with the height difference up to 7 nm positioning mainly on those plateaus (Figure 4.5d). Based on the surface features observed and height analysis, those smooth plateaus are mostly the reorganized DPPE domains and the clusters on top are the precipitated silicate aggregates developed during the incorporation of silicate species into the ternary film, although the overall multi-lobed shape of initially formed DPPE domains are still maintained to a certain degree. The selective anchorage of silicate precipitation on DPPE domains may result from the peeling off of collagen assemblies followed by partial desorption of the DOPE monolayer beneath the collagen branching network. Negatively charged silicate groups are repelled by the exposed mica surface, while the DPPE domains provide a surface for silicate to seed and precipitate with their neutral and hydrophobic tails. The initially formed silica particles bound on DPPE islands may stabilize the DPPE feature positioned on the mica surface, and therefore, the principle boundary of the DPPE multi-lobed domain can be kept after even 14 days immersing. It is interesting to notice that after 12 hours dipping the dimension of the silicate aggregation did not change considerably with further immersing, implying there is a confinement that silica particles can grow on top of DPPE
domains. The irregular patches presented on 2-hour immersed film could be also seen on other films under various immersing time. The blank area intermediate between those 1nm higher islands increases with the immersing time and becomes nearly smooth for the film with the longest dipping time of 14 days, consistent with the expectation that the DOPE monolayer was peeled off gradually during the submerging process, and finally, most of the mica surface was exposed.

Interesting silica precipitations templated by collagen fibril structures were observed for the 2-day immersed films (Figure 4.5e and 4.5f). Compared with the collagen branching assemblies appearing in the collagen/DOPE/DPPE monolayer, the silica covered bundles exhibit about half of the height protruding from the background surface, however a much higher horizontal dimension. The lower degree of interconnection and the reduced surface density of those bundles, together with the decreased vertical height, could be ascribed to partial decomposition of collagen assemblies formed previously and subsequent desorption of collagens into the bulky silicate solution. Those bundles have gnarled and fairly rough surfaces, resembling the hollow silica fibers that were fabricated in the collagen bulky solution.43

4.3.2.2 Developing Collagen/DOPE/DPPE Films on a Subphase of Silicate Solution

At the interface of the air/silicate aqueous solution, a film of collagen/DOPE/DPPE was compressed until the surface pressure reached 25 mN/m. Surface pressure was found dropping rapidly after stopping surface compression and decreased to 9 mN/m after one hour. The film deposited at 9 mN/m on the mica surface displays a surface morphology of distinct DPPE domains with unclear boundaries and
Figure 4.6 AFM images of collagen/DOPE/DPPE (1:80:20 in molar) LB monolayers developed on a subphase consisting of silicate solution with reaction time of: (a) 1 hour, deposited at 9mN/m on mica; (b) 2 hours, deposited at 7.4mN/m on mica.
holes inside (Figure 4.6a and 4.6b. Those domains are more circular and less symmetrical compared with the collagen/DOPE/DPPE monolayer fabricated at an air/water interface. There is no trace of collagen patterns or silica structures on the composite film. The dramatic decrease of surface pressure during the one hour reaction could be ascribed to significant dissolution of collagen molecules into the subphase.

As the reaction at the interface was prolonged for two hours, the surface drop occurred predominantly in the first one hour and leveled off in the second hour to be 7.4 mN/m. Deposition at the same surface pressure gives a composite film with a precipitated silicate structure in form of either clogs or long filaments as shown in Figure 4.6c and 4.6d. Most of the collagens initially floating on the surface undergo penetration into the subphase and the remaining collagens form long filaments and template the further precipitation of silicate. Because more silica is incorporated into the composite film, the DPPE domains appear less uniform in size and shape compared with those observed on the one-hour reacted film.

4.4 Conclusions

To investigate various ways to incorporate collagens into a lipid matrix, bilayer films consisting of a collagen/DOPE/DPPE monolayer on top of DOPE, DPPE, collagen/DOPE or collagen/DOPE/DPPE monolayer were fabricated. AFM images taken for the air-dried bilayer films reveal the key factors that affect the bilayer structures, including composition of each layer, direction of the surface movement through the monolayer at the air/water interface during LB deposition and the dewetting time for the bottom layer before the second layer transfer. All those factors are expected to influence
the surface properties of the first monolayer, such as roughness, hydrophobicity and composition, of the bottom layer and therefore affect the successful deposition of the top layer.

Bilayer studies provide insight into how collagen is incorporated in a lipid matrix, both mono- and bilayer ones. DOPE provides a comfortable environment for collagen to disperse and self-assemble, while DPPE excludes collagen molecules serving as insulating islands. In addition to the properties of the bottom layer surface, the possible penetration or insertion of collagen molecules or segments into the bottom layer should be an even more important factor that could promote the development of collagen assemblies and stabilize the bilayered film structure.

The attempts to develop silica structures on top or within the composite ternary monolayer with collagen yields interesting precipitated silica bundles templated by collagen fibril structures in the case of immersing the transferred ternary monolayer in a silicate solution for 2 days; and a filamentous structure of silica when silicates solution is applied as the subphase in a LB trough.
CHAPTER 5

THIN FILMS OF A SET OF COVALENTLY LINKED PORPHYRIN-BASED AMPHIPHILES

5.1 Introduction

Porphyrs, which are large conjugated organic compounds, have received attention due to their potential applications in molecular electronics,\textsuperscript{191-194} in photovoltaic cells\textsuperscript{195,196} and as gas sensors.\textsuperscript{197-200} An important consideration in such applications is assembling the porphyrin compounds into thin films by procedures such as spin coating, casting, vapor deposition or the Langmuir-Blodgett (LB) technique. The LB technique has the unique ability to build up monolayer assembly at the air-water interface from amphiphiles in a well-controlled process.\textsuperscript{144} The LB technique of monolayer transfer has often been used for construction of highly ordered ultra thin films. Recently, this method has been widely used to incorporate porphyrin into molecular assemblies with well-controlled compositions, structures, and thickness.\textsuperscript{201-205} Pure unsubstituted tetraphenylporphyrin complexes do not form a stable monolayer at the air/water interface.\textsuperscript{206} Long-chain amphiphilic substituents on the phenyl groups seem to be
necessary for the formation of stable monolayers.\textsuperscript{205,207} Functionalized ionic porphyrins containing long hydrophobic chains at the periphery of the conjugated $\pi$-electron system have been studied for the formation of a Langmuir monolayer at the air-water interface and LB film deposition onto a solid surface.\textsuperscript{155,208} Surfactant carboxyporphyrins have also been studied in Langmuir monolayers as well as LB films deposited on solid substrates.\textsuperscript{216} Fuhrhop et al have shown that the tetrakis[(bixinylamino)-o-phenyl] porphyrins form a stable monolayer on water surfaces.\textsuperscript{209} Incorporation of other ionic and non-ionic surfactants into these films is also of current research interest.\textsuperscript{151,154,210-214} Chou et al have first showed the effect of varying the number of the substituted alkyl chains for porphyrin on the LB film formation at the air/water interface.\textsuperscript{148} Several other studies have shown that the number and the length of alkylpyridyl groups on the porphyrin ring can influence the orientation and packing of porphyrin molecules.\textsuperscript{148,175,203}

In the current study, four novel (L\textsubscript{1}-L\textsubscript{4}) porphyrin amphiphiles with one (L\textsubscript{1}), two (L\textsubscript{2}), three (L\textsubscript{3}), and four (L\textsubscript{4}) palmitoyl chain(s) were synthesized from their parent amino porphyrin (Figure 5.1). The effect of varying the number of substituted alkyl chains on the absorption and fluorescence spectra is reported here for solutions and LB films of these materials. Moreover, detailed atomic force microscopic studies on the films fabricated using various procedures on different substrates was performed. Based on the porphyrin monolayer thickness obtained from both current AFM observation and previous theoretical prediction, the molecular orientation and arrangement within the film can be analyzed. The length of a fully extended alkyl chain with 15 carbon atoms is 2.05 nm.\textsuperscript{215} The thickness and diameter of tetraphenylporphyrin is 0.5 nm\textsuperscript{162} and 1.76 nm\textsuperscript{160} respectively. Thus, the estimated total length of the porphyrin compound is
Figure 5.1 Schematic graphs of porphyrin compounds synthesis.
2.55 nm for orientated with tails vertical and the porphyrin ring parallel to the surface and 3.81 nm for the one with both the tails and porphyrin ring vertical with respect to the surface.

5.2 Experimental

5.2.1 Materials

All the chemicals were obtained in India and used as such, unless otherwise stated. Palmitoyl chloride and 4-nitrobenzaldehyde were obtained from Aldrich (U.S), pyrrole, benzaldehyde, triethylamine and stannous chloride were received from S.D Fine chemicals (India). Solvents for reactions and various studies were used as received from Merck (India); unless stated otherwise. Pyrrole was distilled under reduced pressure prior to use. Tetrahydrofuran (THF) was dried prior to use. Synthesis of the nitro derivatives of porphyrin (1-4) were achieved by Dr. A. Das’s group (Analytical Sciences, Central Salt & Marine Chemicals Research Institute) in India.

5.2.2 Film Fabrication and Characterization

Surface pressure isotherms of the porphyrin amphiphiles at the air/water interface were measured at 20 °C using a 611 Langmuir-Blodgett trough from Nima Technology (Coventry, England). Surface tension was measured using the Wilhelmy plate method. A volume of 40 µl of the chloroform solution containing the porphyrin amphiphile was spread on the subphase, with the trough barriers set at the maximum area of 280 cm². Although the amount of porphyrin amphiphiles delivered to the air/water interface forming a Langmuir film is extremely small, this spreading process could be observed
visually because of the strong color of the porphyrin compounds. At the lowest surface
coverage, the spreading solution quickly formed a uniformly colored layer, though
immediately after delivery regions next to the trough sides, barriers, and Wilhelmy plate
were colorless in the case of L₁, L₂ and L₃. The uniformity of the initial spreading
increased with the increasing number of alkyl chains and therefore increasing the
hydrophobicity of the amphiphile. The layer was allowed to stand for 10 minutes to allow
complete evaporation of chloroform and thorough dispersion of amphiphile on the
surface. For L₁, L₂ and L₃, randomly scattered colorless domains were observed at this
point within the light yellow monolayer, suggesting that domain formation had occurred
even at the low surface coverage (i.e. high area/molecule). The monolayer was then
compressed in a stepwise quasi-static mode using area increments of 1 cm², wait interval
of 16 sec, and barrier speed of 25 cm²/min. In order to investigate the influence of
subphase pH on the isotherms, films were prepared on highly pure aqueous buffer
solutions consisting of potassium dihydrogen phosphate, NaOH, and sodium bicarbonate.

The LB films were transferred onto freshly cleaved mica, glass slides, and silicon
wafers using a vertical dipping method with the dipping speed 1 mm/min. During the
deposition at an air/water interface, the surface pressure was kept constant at a target
surface pressure. For comparison, self-assembled monolayers (SAM) and the cast thin
films of the amphiphiles were also fabricated by immersing a solid substrate in porphyrin
solution and evaporating a drop of porphyrin solution on solid surface, respectively,
under atmospheric conditions.
The supported films were air-dried for 24 hours before observation by tapping mode AFM. A Nanoscope III from Digital Instruments (Santa Barbara, CA) was used for AFM measurements in air at ambient temperature. Tapping mode imaging used silicon cantilevers with a low spring constant of 0.3 N/m. UV-vis absorption and fluorescence spectra of the deposited films were recorded on a Cary 100 spectrophotometer from Varian (Sugar Land, TX) and QM-1 spectrofluorometer from Photon Technology International (Lawrenceville, NJ) respectively at 298 K.

5.3 Results and Discussions

5.3.1 Compression Isotherms

Surface pressure isotherms at 20 °C for porphyrin amphiphiles at air/pure water interface are shown in Figure 5.2. The mean molecular areas of the four amphiphiles were obtained by extrapolating the first linear region of the isotherms to determine the areas at zero surface pressure, which correspond to the hypothetical state of an uncompressed close-packed layer where the molecules at the interface begin to interact with each other. The mean molecular areas are 37, 105, 140 and 246 Å² for L1, L2, L3 and L4 respectively. The mean area for L1 is much smaller than the area occupied by a perpendicular tetraarylporphyrin molecule of 90 Å²,147 which implies the formation of multilayer or a overlapped packing of L1 molecules. L4 shows a mean molecular area of 246 Å² that is slightly larger than the area of a flat tetraarylporphyrin molecule of 225 Å²,147 indicating that L4 molecules may float on the water surface with their porphyrin rings parallel to the surface when the interactions between the tails become significant.
Figure 5.2 Π-A isotherms for: (a) L₁; (b) L₂; (c) L₃; (d) L₄ monolayers on water at 20 °C.
The isotherm for L₁ is typical of an amphiphile that forms a tightly packed monolayer. Significant increases in surface pressure are observed only at low average area, after complete monolayer coverage has been attained. This monolayer is relatively incompressible, as indicated by the sharp increase in surface pressure upon further compression. Increasing the number of hydrophobic tails results in successively more loosely packed monolayers. For example, surface pressure liftoff occurs at a much higher average area for L₄ than that for L₁, indicating significant interactions between L₄ molecules adsorbed at the interface, even at relatively low coverage. At surface pressures below the plateau region in the isotherm of L₄, the absolute value of the slope of the isotherm is much less for L₄ compared with other compounds, indicating the L₄ film to be highly compressible. The plateau region at a surface pressure of 18-19 mN/m is likely due to a transition in the orientation of both the alkyl chains and porphyrin rings of L₄ molecules with respect to the interface normal. At low surface pressure, the porphyrin rings and the alkyl chains may lie flat on the water surface, while at higher surface pressure both adopt a more vertical orientation due to attractive tail-tail interactions between neighboring molecules. This hypothesis is supported by AFM analysis in the following section. Compared with other porphyrin amphiphiles, the L₃ monolayer collapses at a lower surface pressure of 28 mN/m. It is possible that the asymmetric structure of L₃ results in films that are unstable under compression especially at higher surface pressure. Within the sub-phase pH range of 5.8 to 10, no effect of pH on the isotherms was observed. Within this range, there is apparently no influence of the subphase pH on the hydrophilicity of the porphyrin ring.
5.3.2 Thin Films of L₁, L₂ and L₃ on Mica

Monolayers transferred onto solid substrates were investigated by AFM. We denote films formed from amphiphiles L₁, L₂, L₃, and L₄ as P₁, P₂, P₃ and P₄ films respectively. In the case of the P₁ film, randomly scattered domains could be seen clearly with the unaided eye at the air/water interface, while the P₄ film was found to be fairly uniform. The two extreme cases, P₁ and P₄, were studied more extensively.

The LB film of P₁ deposited on a mica at surface pressure of 20 mN/m presents visible macro-domains on the mica surface. The AFM image focusing on the region covered by a light yellow layer reveals a rough surface with irregular aggregates randomly scattered in a granular homogeneous background (Figure 5.3a). The image for the homogeneous background at a smaller scale displays mainly elliptical assemblies densely packed on mica with the RMS surface roughness of 1 nm (Figure 5.3b). The height of the elliptical domains is 3.6 nm, much higher than the height of the monolayer with porphyrin rings arranged in a side-by-side monolayer organization, suggesting the formation of aggregations with porphyrin rings overlapped with each other, which is consistent with the isotherm. A mean molecular area of 30 Å² at a surface pressure of 20 mN/m is obtained from the isotherm, which is much smaller than the molecular area of 225 Å² for a simple tetraarylporphyrin ring (macrocycle) lying parallel to the aqueous surface,\(^{147}\) and is even smaller than the molecule area of 90 Å² for the ring positioned perpendicular to the surface.\(^{147}\) When the deposition pressure was increased to 30 mN/m, no visible domains were observed in the deposited film. However, striped and rounded regions with flat surfaces embedded in a continuous and rough matrix are observed.
Figure 5.3 AFM images of P1 films on mica: LB monolayers deposited at (a)-(b) 20 mN/m; (c)-(d) 30 mN/m; (e)-(f) cast film by evaporating 35 µl of 0.4 mg/ml L1 chloroform solution.
In Figure 5.3c. The stripe regions with widths ranging from 0.5 to 5 µm and the circular domains with diameters of several micrometers are believed to be bare mica surface because of the extreme uniformity observed in both the height and phase images. The average height from the surface of the rough matrix to the mica surface is around 15 nm, indicating the formation of multilayers with the porphyrin rings stacked upon each other. The circular domains are found to contact each other with their boundaries connected by line-shaped assemblies with the average height above 15 nm as shown in Figure 5.3d. One possible explanation is that the development of round domains of bare mica surface during de-wetting prohibited the formation of a continuous P1 film and confined the L₁ molecules to aggregate within the boundary regions.

A cast film was prepared by evaporating 35 µl of 0.4 mg/ml L₁ chloroform solution on freshly cleaved mica. The L₁ molecules spread and self-assembled on the mica surface and formed a very uniform layer after the sample was air-dried for a day. In Figure 5.3e, leaf-like domains are seen evenly distributed on a flat surrounding phase. The surrounding phase has the RMS roughness of 0.5 nm and the average height of 1.4 nm above the surface of the substrate, indicating the formation of a tightly and homogeneously packed P1 film during the evaporation of solvent (Figure 5.3f). From the sectional and baring analysis, the leaf-shaped plateaus are approximately 4.5 nm higher than the smooth background, which is even higher than the length of a fully stretched L₁ molecule with both the porphyrin ring and the alkyl chain perpendicular to the surface, suggesting the multilayer assemblies were developed in the leaf-shaped plateaus. The elongated domain shape may be due to the highly asymmetric structure of L₁ molecules.
The P2 and P3 monolayers deposited at 25 mN/m appear densely packed and homogeneous on the surface of mica (figures not shown here), which demonstrates that L2 and L3 molecules are good film-forming amphiphiles, capable of forming a monolayer with sufficient integrity to survive the transfer process intact.

5.3.3 LB Films of L4 on Mica

LB films of P4 monolayer were deposited onto mica by keeping the surface pressure constant at various deposition pressures. The monolayer deposited at the point when the surface pressure began to increase above 0 mN/m (lift-off point) appears having irregular terraces with diameters from 100 nm to 400 nm, evenly distributed on the mica surface as shown in Figure 5.4a. Those terrace-shaped assemblies with blur peripheries and relatively rough surfaces are on average 0.52 nm above the mica surface, which is demonstrated by the peak-to-peak distance in the baring analysis. The height difference of 0.52 nm is consistent with the height of L4 molecules positioned on the mica surface with both the porphyrin ring and hydrocarbon chains lying flat on the surface. From the compression isotherm for the P4 monolayer at the air/water interface, the area per molecule corresponding to the lift-off point is 2.46 nm$^2$, and is in excellent agreement with the estimated area of 2.25 nm$^2$ for a simple tetraarylporphyrin ring. This implies that L4 molecules are packed densely with their rings side-by-side on the water surface. However, the AFM image for the monolayer transferred onto mica shows scattered assemblies only 0.5 nm above the partially covered mica surface, indicating low transfer efficiency typical of transfers attempted at low deposition pressure.
Figure 5.4 AFM images of P4 LB film on mica deposited at: (a) lift-off point in isotherm; (b)-(c) 10 mN/m; (d) 15 mN/m; (e) 18 mN/m; (f) 28 mN/m.
Figure 5.4b shows that at the deposition pressure of 10 mN/m, well-defined round domains set on a homogeneous background. The round-shaped domains with diameters less than 10 µm are about 1.8 nm below the continuous background and are believed to be bare mica regions, as evidenced by the extreme smoothness appearing on both the topological image and phase image. Scattered holes with diameters of approximately 300 nm within the continuous phase (Figure 5.4c) have the depths of 1~2 nm, which is consistent with the height of continuous background, implying that the observed holes are the bare mica surface. Compared with the height difference exhibited in Figure 5.4a, the homogeneous P4 film is composed of a monolayer in which the porphyrin rings and/or the alkyl chains adopt a more ordered package and more vertical orientation with respect to the mica surface. There are a number of relatively high aggregates appearing only at the periphery of the circular domains. The formation of those aggregates is most likely related to the development of the circular domains during air-drying. During the de-wetting of the film in air, circular domains developed while the molecules previously covering the bare mica regions agglomerated at the periphery regions and formed irregular multilayer assemblies.

When the surface pressure was increased to 15 mN/m, just slightly below the phase transition pressure at 18 mN/m, a similar topological pattern as the one at 1 mN/m was observed in Figure 5.4b. The thickness of the homogeneous film increases from 1.8 nm at 10 mN/m to 2.1 nm, implying a more vertical and ordered orientation of the hydrocarbon chains. The image at the smaller scale (Figure 5.4d) shows more densely packed holes with decreased diameters compared with those in the 10 mN/m film.
Upon further compression of the P4 monolayer to reach the plateau region of the isotherm, the area decreased significantly when the surface pressure was kept constant at 18 mN/m. With the decrease of the surface area, it is expected that L₄ molecules would reorganize and form more densely packed structures. Figure 5.4e reveals the coexistence of circular domains with 1~3 µm in diameter and worm-like aggregations localized mainly along the periphery of the circular domains and totally excluded by those domains. AFM topography and phase angle images suggest that the circular domains and the worm-like aggregations correspond to the liquid-expanded phase and the liquid-condensed phase, respectively. The smooth area between the worm-like aggregations is the bare mica surface. The circular domains show an average height difference above the mica surface of 1 nm that is in accordance with the height of a loosely packed monolayer of L₄ molecules. The worm-like aggregations have rough surfaces and an average height difference of 3~5 nm, indicating the formation of multilayers and/or monolayer consisting of L₄ molecules oriented with tilted porphyrin rings and densely packed long chains. With the development of highly packed aggregations, strong attractions between L₄ molecules would lead to the concentration of worm-like aggregates around the rounded-domains and the exposure of bare mica surface. At a surface pressure 28 mN/m, the observed pattern on mica (Figure 5.4f) is similar to that in Figure 5.4e. The worm-like aggregations are assembled in a more condensed way, leaving no apparent bare mica surface. The area per molecule is 0.76 nm² at this point, which is even smaller than the area occupied by a vertically oriented tetraarylporphyrin ring of 0.9 nm², which is consistent with the observed height difference of 5~8 nm for the highly packed worm-like aggregations, indicating the formation of multilayer structures.
To study the stability of P4 film deposited on mica and to investigate the interactions within the film, a 19-layer P4 LB film was deposited at the same surface pressure of 15 mN/m. The first layer was created using vertical up deposition onto the hydrophilic mica surface. Once the first layer was deposited, further layers were deposited on each subsequent pass of the substrate through the air/water interface. The transfer ratio was close to 1.5 for the up path, indicating that L₄ molecules were packed more densely on the hydrophilic solid substrate than at the air/water interface. The transfer ratio is approximately 0.5 for the down path, which could be the result of poor transfer efficiency onto the hydrophobic surface or desorption of the L₄ molecules deposited previously. When moving the surface downward through the interface to deposit the even-numbered layers, the outermost surface of the film appeared to be highly hydrophobic—judging from the curvature of the meniscus at the water/substrate interface boundary. This is attributed to tightly packed alkyl chains. The transfer ratios remained constant initially and began to decrease after the deposition of the 17th layer, suggesting that there is a limit for the number of the layers that could be deposited onto mica and form a stable film.

Figure 5.5a displays the morphology of the 19-layer film. Circular domains with the diameter about 6~7 µm could be seen embedded in a rough continuous background, where the regions around the edge of the domains appear much lower than the rest of the film. Assuming the internal structure of the 19-layer film to be uniform and the deposition mode to be the common “Y-type”, the average height of one bilayer structure calculated from the measured thickness of the film is 3.16 nm. This height is
Figure 5.5 AFM image of (a) 19-layer P4 LB film deposited at 15 mN/m on mica; (b) Schematic plot of porphyrin molecular organization in multilayer film.
consistent with the estimated height of a bilayer assembly in which the two porphyrin rings are parallel to the substrate surface and the alkyl tails from both layers are fully stretched and overlapping with each other. The strong steric repulsion between the huge head groups of L₄ molecules would prevent the hydrocarbon tails from being packed tightly within one layer. Since the L₄ molecules do not form stacked bilayers with a height of the addition of two monolayers (4.2 nm at surface pressure of 15 mN/m), it is reasonable to expect that the loosely packed tails from adjacent layers may insert into and overlap with each other as demonstrated by Figure 5.5b. The average height of a P₄ bilayer obtained in this study suggests the attractions between the hydrophobic tails and the space between the tails lead to the overlapping of the tails from adjacent layers and thus forms a more stable structure than a stacked one.

It is interesting that the rounded-shaped domains observed for the monolayer deposited at 15 mN/m still could be noticed after the transfer of 19 layers. A possible explanation could be as follows: After the first layer was deposited, the circular domains of the bare mica surface observed for monolayer did not fully develop due to the limited time interval between the depositions of the first layer and second layer. However, the dewetting effect reduced the attractive interactions between the mica surface and L₄ molecules existing inside the circular domains. During the down path to transfer the second layer, L₄ molecules inside the circular domains were peeled off and then rearranged into a bilayer structure, leaving the regions along the periphery blank because of the line tension. That is in agreement with the much lower transfer ratio for the downward path compared with the upward path. Additional layers of P₄ might cover those blank regions along the periphery of the circular domains during further deposition.
However, the number of the layers that could be deposited onto the periphery regions is limited by the steric repulsion, which could explain the circular periphery regions appear much lower on the topological images.

5.3.4 Cast Film and Self-assembled Monolayer of P4 on Mica

In Figure 5.6a, the cast film prepared by evaporating 35 µl of L₄ chloroform solution at 0.4 mg/ml on the mica surface shows randomly scattered patches that have smooth surfaces and an average height difference of 3~4 nm above the mica surface. The morphology of the film suggests a tightly packed organization of L₄ molecules in which the stacked porphyrin rings and the alkyl chains may form multilayers. In the present study, varying the concentration of the L₄ solution did not show an obvious effect on the morphology of the cast film, which implies the interactions between L₄ molecules and the mica surface dominates during film formation. After the mica sheet was immersed in 0.4 mg/ml L₄ chloroform solution at ambient temperature for 22 hours and then air-dried for one day, a SAM of L₄ molecules adsorbed onto the mica surface display a well-defined structure as shown in Figure 5.6b. Round-shaped assemblies with diameters ranging from 400 nm to 1.5 µm are connected by randomly dispersed patches of L₄ aggregates that have a similar size and height above the substrate surface as those observed in Figure 5.6a.
Figure 5.6 AFM images of: (a) P4 cast film on mica prepared by evaporating 35 µl of 0.4 mg/ml L₄ chloroform solution and (b) P4 self-assembled monolayer by immersing the mica substrate in 0.4 mg/ml L₄ chloroform solution for 22 hours.
5.3.5 P4 Films on Various Hydrophilic Substrates

To investigate the influence of various hydrophilic substrates on the assembling behavior of L₄ molecules, glass and silicon substrates were also used. Glass slides and silicon wafers were cleaned with detergent, distilled water, acetone, methanol and distilled water again in an ultrasonic bath. These were stored in pure water prior to use.

5.3.5.1 on Silicon

LB films could not be successfully fabricated on a silicon wafer at deposition pressures lower than 15 mN/m. The monolayer deposited at a surface pressure 15 mN/m on the silicon wafer (Figure 5.7a) exhibits similar patterns as those obtained for the film on mica sheet. At a higher surface pressure of 18 mN/m, which is in the plateau region of isotherm, the circular domains of bare silicon surface appeared at 15 mN/m remain intact as shown in Figure 5.7b. While the dispersed irregular assemblies of L₄ molecules replace the continuous and smooth phase, which is significantly different from the morphology obtained on mica at the same surface pressure. The assemblies are only 2~3 nm above the silicon surface, indicating L₄ molecules are packed more loosely than that on the mica surface deposited at the same surface pressure. This suggests that the attractive interactions between L₄ and silicon surface is weaker than that between L₄ and the mica surface, which results in the random agglomerating of L₄ molecules instead of a uniform and highly packed film. The difficulty in preparing a LB film at a surface pressure below 15 mN/m could also be attributed to the weaker attractions. Upon further compression up to 28 mN/m, Figure 5.7c shows the elongated regions of bare silicon
surface embedded in a rough matrix consisting of both a monolayer and multilayer structures, which are about 2 nm and 5~7 nm higher than the solid substrate, respectively.

The remarkable difference between the morphology of LB films deposited on mica and silicon wafer arises mainly from the difference in their surface chemistry properties. The mica used in this study is muscovite type with a unit cell of \(\text{KAl}_2\text{(Si}_3\text{Al})_\text{O}_{10}(\text{OH,F})_2\). The charge density (net charge per unit cell) for the mica surface is \(-1\), because one \(\text{Si}^{4+}\) is substituted by one \(\text{Al}^{3+}\) in a unit cell. The \(\text{--NH--}\) moiety in the \(\text{L}_4\) molecules and/or the porphyrin ring could be positively charged on a water surface. Columbic forces dominate the interactions between \(\text{L}_4\) molecules and the negatively charged mica surface. Upon exposing the silicon wafer to air, the surface is mostly oxidized to be silica and the cleaning procedure could not remove the oxidized layer. When the oxidized layer was dipped into the water subphase, surface \(\text{--Si-OH}\) groups are present, which are neutral but polar. The surface will therefore be hydrophilic. Both the positively charged porphyrin ring and the \(\text{--NH}_2^+\) groups in the \(\text{L}_4\) molecule have the tendency to interact with the silicon wafer surface, though the overall attractive interactions in this case are expected to be weaker than the Columbic forces between \(\text{L}_4\) and the mica surface. These differences in surface chemistry could explain why it is more difficult to create highly structured films of \(\text{L}_4\) on silicon.

A SAM of \(\text{L}_4\) on silicon wafer was also prepared by immersing the solid substrate in a 0.4 mg/ml \(\text{L}_4\) chloroform solution for 40 hours. The AFM images were taken after drying in air for one day. Figure 5.7d shows that the silicon surface is fully covered by a tightly packed P4 film in which circular or elliptical granular domains with diameters
Figure 5.7 AFM images of P4 film on silicon: LB films deposited at (a) 15 mN/m; (b) 18 mN/m; (c) 28 mN/m; (d)-(e) self-assembled monolayer prepared by immersing the substrate in 0.4 mg/ml L₄ chloroform solution for 40 hours.
ranging from 2~6 µm could be seen 5~7 nm above the film surface, indicating the development of multilayers. Compared with the well-organized patterns formed on mica, the domains formed here have a larger size, a more poorly defined periphery and a rougher surface, which could also be assigned to the weaker interactions between L₄ and the silicon substrate. The rough film in which the domains are embedded is composed of randomly dispersed patches that were also observed for the cast P4 film on mica by evaporating a drop of L₄ chloroform solution. Figure 5.7e reveals the detailed structure of the granular domains, where rings with diameters below 250 nm and their peripheries up to 9 nm above the substrate surface can be seen. This ring structure might be developed from those granular aggregates during the film de-wetting process.

5.3.5.2 on Glass

Although the surface of the glass microscope slide is not as smooth as the surface of mica and silicon wafer under AFM, it is sufficiently smooth to form uniform LB films. The common glass slide is not pure SiO₂, but silicate. When the cleaned glass slide is exposed to air or water, --Si-OH and --Si-O-- groups on the glass surface result in a relatively hydrophilic surface. Since the glass is not pure SiO₂, the overall interaction of L₄ molecules with a glass slide is weaker than that with a silicon wafer, and even weaker than that with mica. This is confirmed by the AFM images for LB films at various deposition pressures. Uniform and patterned LB films could not be obtained even when surface pressure was increased to 15 mN/m. Figure 5.8a, where the LB film was deposited at 18 mN/m, exhibits a similar film structure as observed in Figure 5.4e. Notably, the domains with the bare glass surface are elliptical and the long axial is along
Figure 5.8 AFM images of P4 LB films deposited on glass at: (a) 18 mN/m; (b) 28 mN/m.
the same direction as that the substrate was pulled out of the water during the deposition, implying that the weak interaction between L₄ and glass surface could not maintain the integrity of the film structure during the vertical deposition. At a higher deposition pressure of 28 mN/m (Figure 5.8b), the film displays irregular and large regions of bare glass and the previously separated domains finally merge together during the surface compression.

5.3.6 Spectral Study of L₄ Porphyrin

5.3.6.1 L₄ in Chloroform

Absorption spectra of a dilute L₄ solution (10⁻⁶~10⁻⁵ M) in chloroform shows strong Soret bands at the same wavelength (422 nm) for various concentrations. The peak intensity of the Soret band increases with concentration. While Q-bands appear between 450 nm and 600 nm, typical of a free base porphyrin, they are observed only for the more concentrated solutions. Red-shifted Q-bands were observed for more concentrated solutions due to the aggregation of the monomers in solution. However, at low concentrations, due to a low signal-to-noise ratio, Q-bands are difficult to detect and can not provide useful information. It is worth noting that the Soret bands broaden with decreasing concentration, which implies the formation of aggregates in more concentrated solutions.¹⁶⁴ At a lower concentration of 3x10⁻⁶ M, no obvious change in the Soret bands is observed upon further dilution of the solution, indicating the monomer conformation of L₄ in the solution from this point. This conclusion is confirmed by further fluorescence spectroscopy studies.
Figure 5.9 Fluorescence spectra for L₄ solution in chloroform at various concentrations at:
(a) 2.3e-5 M; (b) 3.0e-6 M; (c) 1.5e-6 M (with excitation of 422 nm).
Fluorescence is usually more sensitive than absorbance\textsuperscript{170}, therefore the spectrofluorimeter can more easily detect the existence of aggregations. The emission spectra of L\textsubscript{4} chloroform solutions excited at the peak wavelength of UV (422 nm) are shown in Figure 5.9. The Q-bands red-shift significantly and become broader by diluting the solution until the concentration is decreased to 3x10\textsuperscript{-6} M, where L\textsubscript{4} molecules exist in the solution as monomers and the primary Q-band is centered at 680 nm. The band shift is mainly due to the strong intermolecular interactions between macrocycles in L\textsubscript{4} aggregates.

5.3.6.2 P4 LB films Deposited on Glass

In Figure 5.10, the absorption spectra reveal a red shift of the Soret bands by 6 nm for P4 LB films deposited onto glass slides at various surface pressures (15 mN/m–28 mN/m) with respect to the solution spectra. According to the molecular excitation model\textsuperscript{177}, a red shifted absorption suggests either the in-line alignment of the transition dipole moments in L\textsubscript{4} molecules or the co-planar inclined transition dipoles (J-aggregation), where the angle between the center-to-center vector and the transition moment is less than 54.7°. Based on the qualitative prediction, L\textsubscript{4} molecules are positioned in the self-assembled LB films with their porphyrin rings either lying flat on the solid substrate or tilted at small angles with respect to the glass surface. The steric hindrance introduced by the long alkyl chains and the relative weak interactions between L\textsubscript{4} molecules and the glass surface are expected to make J-aggregations energetically more favorable than H-aggregations that lead to a blue shift of Soret bands. The red shift arises from a strong \(\pi-\pi\) interaction between porphyrin rings\textsuperscript{177}, therefore the overall
Figure 5.10 Adsorption spectra for L₄ in LB monolayers on glass deposited at: (a) 28 mN/m; (b) 18 mN/m; (c) 15 mN/m.
Figure 5.11 Fluorescence spectra for L$_4$ in LB monolayers on glass deposited at: (a) 28 mN/m; (b) 18 mN/m; (c) 15 mN/m.
magnitude of the shift is related to the degree of order and intermolecular distance. A 6-nm shift in this case implies a relative weak porphyrin-porphyrin interaction. Increasing the surface pressure results in higher absorption and a narrowed Q-band that is indicative of a higher degree of order in the aggregation.  

The Q-bands in the fluorescence emission spectra (excited at 422 nm) for LB films on glass exhibit substantial blue shifts of 20 nm (Figure 5.11) relative to that of the solution at 680 nm, inconsistent with the red shift seen in the UV spectra. It is well known that shifts in fluorescence and UV spectra generally are in the same direction.  

In this study, these shifts were in opposite directions. Although rare, this is not without precedence. The mechanism of fluorescence emission is much more complicated than simple release of energy that was absorbed. An excited molecule can return to its ground state in different ways, such as the vibrational relaxation due to the collisions between molecules and the internal conversion due to an overlap in vibrational energy levels. Therefore, a fluorescence spectrum may exhibit peak shifts that are quite different from those observed in the corresponding UV spectrum if other deactivation processes dominate the relaxation process. For monolayers deposited at surface pressures in the range of 15 mN/m to 28 mN/m, neither the peak position (20 nm blue shifted from the solution) nor the shape of the band is apparently affected by the change of surface pressure, suggesting the assemblies are similar in structure.
Figure 5.12 Fluorescence spectra for P4 films on mica: LB monolayer deposited at (a) lift-off point; (b) 15 mN/m and (c) 18 mN/m; (d) self-assembled monolayer prepared by immersing the substrate in 0.4 mg/ml L4 chloroform solution for 22 hours (e) cast film by evaporating 35 µl of 0.4 mg/ml L4 chloroform solution; (f) 19-layer LB film deposited at 15 mN/m.
5.3.6.3 P4 Films on Mica

Figure 5.12 shows the fluorescence spectra for the P4 films supported by a mica sheet. The highly ordered LB films deposited at the lift-off point, 15 and 18 mN/m, exhibit Q-bands red-shifted up to 11 nm from that of the solution. On contrast, the less organized 19-layer LB films deposited at 15 mN/m, the cast film and the SAM prepared by immersing the substrate in L4 chloroform solution, exhibit blue shifts as significant as 27 nm, indicating that L4 molecules are arranged in a different way from those within the highly ordered films. The monolayer deposited at 18 mN/m and the SAM displayed much higher absolute emission intensities because of the fairly high local concentration. However, the emission intensity of the 19-layer film and the cast film with higher molecular concentration is even lower than the monolayer. That could be explained as the fluorescence self-quenching in those films that are not highly ordered.

5.4 Conclusions

The bis-, tris- and tetra-substituted porphyrins are better LB film forming amphiphiles than the mono-substituted porphyrin, which forms irregular aggregates on the surfaces. AFM studies clearly indicate that the mica surface has stronger interactions with P4 LB films compared to silicon wafer and glass slides. Multilayer LB film formation from the P4 monolayer on mica support was also successfully prepared.
CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

Ultrathin films consisting of biomolecules were fabricated at interfaces with various techniques. The influence of various fabrication techniques as well as the properties of the solid substrate surface properties were investigated systematically on the resulted film surface morphology and inner structure. The self-assembly mechanism was explored based on the experimental results and fundamental interactions analysis.

Collagen was successfully incorporated into a binary lipid matrix at an air/water interface for the first time and formed intricate hierarchical structures in the lipid matrix. Preferential localization of collagens was demonstrated and controlled in the phase separated lipid monolayer. The degree of collagen polymerization was monitored by varying the film composition and experimental conditions. The long-range uniformity and procedure reproducibility were realized by optimizing the key factors that affect the film structures. The biocompatibility and stability of the mica supported composite films were demonstrated. Based on the collagen-containing monolayer, bilayered films were
also fabricated to get insight into how the collagens interplay with the lipid matrix. Silica structures were developed in monolayer films to investigate the possibility of using the collagen-containing film to template inorganic structure growth.

Newly synthesized porphyrin compounds with various numbers of long aliphatic chains were applied to form single component films. The number of substituted tails is crucial to the formation of stable solid supported thin films. Generally, increasing the number of tails benefits the formation of stable and highly structured thin films.

6.2 Recommendations

Attempts to manipulate collagen assembly formation could be conducted in other phase separated lipid systems. Phospholipids with various head groups, tail lengths and tail structures could be utilized as lipid templates to incorporate collagens. Fibronectin is another ECM protein that can replace collagen or bind to collagen forming well-defined 2-D patterns with membrane lipids. Cell adhesion and migration would be induced and promoted either by direct patterning fibronectin on a surface or by attaching fibronectin to a surface with collagen features.

The patterned collagen surface can also be applied to study the cell behavior on a chemically heterogeneous surface. Cells with an affinity to the collagen surface are expected to selectively attach to collagen patterns and the surface morphology of collagen is expected to influence the cell behavior.
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