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ELECTROFORMATION OF HYDROGEL –STAMPED PROTEIN AND LIPID DEPOSITS FOR THE CREATION OF GIANT VESICLE ARRAYS

A Thesis in

Bioengineering

by

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ABSTRACT

The cellular membrane is a vital barrier between the cell and its extracellular environment, providing mechanical support, allowing growth and movement, regulating transport, and organizing communication. However, ascribing specific biological functions to particular membrane compositions remains difficult because of the difficulty of precisely controlling composition or measuring function in cells. Giant vesicles made of lipids and proteins offer a simplified and controllable platform for simulating natural cell membranes and can be used to study biological processes such as vesicle budding and protein-lipid interactions. The formation of giant vesicles arrays, in turn, can permit high-throughput investigation of these membrane processes. In this thesis, a simple technique to form arrays of giant vesicles from hydrogel-stamped lipid and protein deposits is described. First, topographically-patterned agarose stamps were applied that could absorb and transfer aqueous solutions of lipids, proteins, and cell membrane fragments to an indium tin oxide substrate. Next, these patterned deposits were used to produce giant vesicles through electroformation. Through the variation of stamp feature size, the size and number of vesicles formed at each stamped location could be prescribed. The versatility of this technique was demonstrated by forming vesicle arrays from stamped proteoliposome solution, stamped membrane fragment, and in physiologically relevant solutions. Furthermore, protein-lipid interactions were studied by observing the binding of fluorescently-labeled proteins to phosphatidylserine in vesicles. Finally, the future outlook of this technique is discussed and potential future studies that take advantage of its simplicity are suggested.

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Chapter 1

Introduction

Model membranes are key platforms for the simplified analysis of the many complex interactions and processes that occur naturally in cells. As many diseases are caused by or inflict changes on cellular membrane structure and interactions, it is vital to study these changes in the hope of better diagnoses, imaging, treatment, and disease prevention. Model membranes are ideal for the isolation of cellular membrane phenomena of interest due to their controllable compositions and structures, which are readily analyzed with quantitative microscopy techniques. Of the model membrane systems available, giant vesicles have proven to be particularly valuable due to their simple production and similarities to cell size, membrane curvature, and membrane structure. Specifically, giant vesicle arrays are high-throughput platforms that yield improved statistical analysis over detached vesicles in solution. These surface-attached vesicles are simpler to locate, observe, and analyze than detached vesicles. Giant vesicle arrays are also located in a controllable and compositionally homogenous experimental environment.

In this thesis we introduce several model membrane systems, focusing on giant liposomes as simplified representations of the cellular membrane. Common methods to form vesicles are detailed, with particular emphasis on the development of electroformation as a process to form giant unilamellar vesicles (GUVs). We look at the advantages of arrays of lipid bilayers and giant liposomes, noting that the increase in analyzable, distinct samples improves statistical analysis. Importantly, uniformity of vesicles within an array contributes to further applications involving the detachment and screening studies. Patterning strategies are discussed, with an emphasis on the use of microcontact printing to produce patterned arrays of biomolecules. Finally, we describe the

significance of the stamp material selected for use in microcontact printing, noting that its material properties greatly influence the range of compatible ink materials, particularly affecting the incorporation of functional biomolecules. In the interest of understanding the significance of the hydrogel stamping and electroformation technique described in this thesis, an overview of cell membrane structure and function, model membrane systems, micropatterning techniques, and relevant previous work in this area are discussed in this chapter.

1.1 Background

1.1.1 The Cellular Membrane

The plasma membrane of the cell is a barrier between the exterior environment and the interior components and cytosol of the cell. More than a simple, passive boundary, the membrane serves as a regulator of transport into and out of the cell, a flexible structure that allows movement and growth, and an organizational platform for cellular processes and communication (Nelson and Cox 2008). In order to study the functions of the plasma membrane, it is important first to understand its structure and components. Consisting of phospholipids, sterols, proteins, and carbohydrates, the plasma membrane forms a bilayer surrounding the volume of the cell (Figure 1-1).

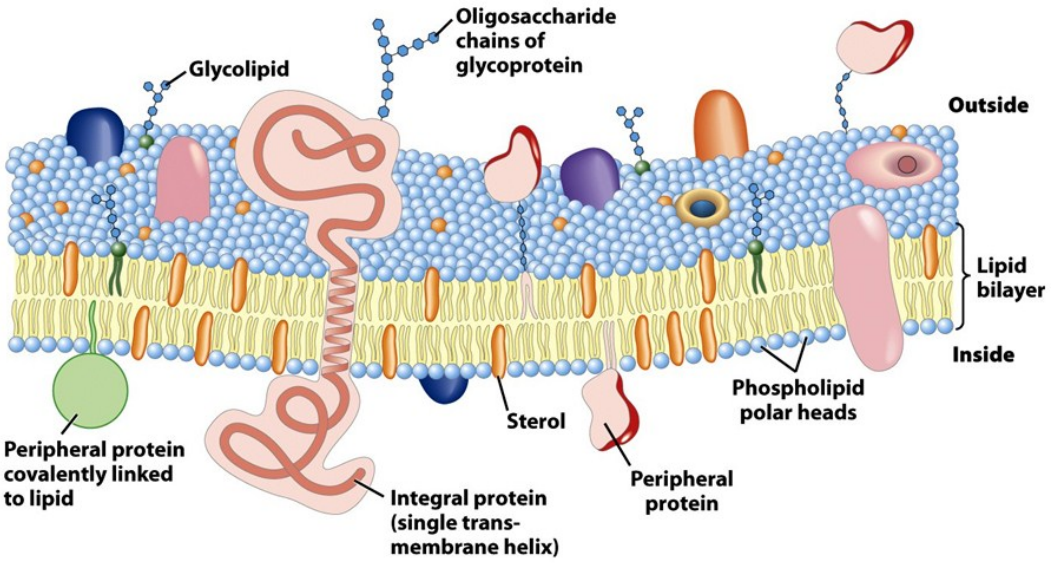

Figure 1-1. Components and structure of the membrane. The membrane is composed of a phospholipid bilayer where both lipids and proteins freely diffuse in lateral directions but are restricted in transbilayer movement. Carbohydrates are attached to the membrane as parts of glycolipids and glycoproteins. Figure adapted from Nelson and Cox, 2008.

Because the membrane primarily is composed of phospholipids, the structure of these components plays a significant role in the overall bilayer structure. Phospholipids contain a glycerol backbone, hydrophobic acyl tails, and a hydrophilic head group that determines the properties and identity of the phospholipid (Caplan 2009) as exemplified in Figure 1-2.

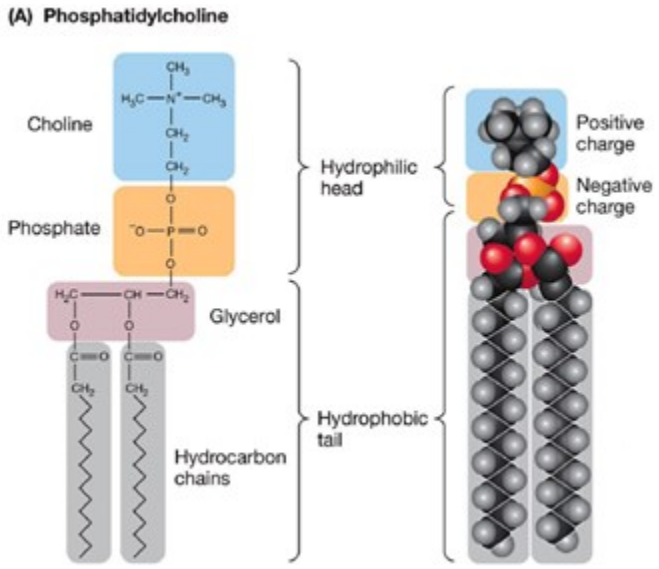

Figure 1-2. Structure of phosphatidylcholine. The glycerol backbone connects to fatty acid acyl chains via ester linkage. Another hydroxyl group of the glycerol backbone is attached to a phosphate group, which is subsequently attached to the choline head group. Figure adapted from Sadava *et al*., 2009.

Phospholipids are amphipathic and, therefore, are oriented in such a way that their hydrophobic fatty acid tails avoid aqueous contact while their polar head groups interact favorably with the aqueous environment. The structures that these phospholipids spontaneously form depend on their concentration in the aqueous environment, ranging from a monolayer at low concentrations, micelles at higher concentrations, and finally to bilayers at the highest concentrations (Caplan 2009).

Bilayers consist of two parallel leaflets of lipids arranged where hydrophobic acyl tails face each other and hydrophilic head groups face outward. The width and packing of the bilayer is influenced by the number of carbons in the acyl chains, ranging from 14-24, and the degree of unsaturation of the chains (Gennis 1989). At 5-8 nm in thickness, the lipid bilayer leaflets exhibit an asymmetrical distribution of both protein and lipid components. Proteins may be attached to the membrane specifically on one side or span the membrane in a defined orientation, leading to functional differences between inner and outer leaflets of the bilayer (Op den Kamp 1979). For

example, it has been found that phophatidylserine, normally located on the inner leaflet, is an indicator of cell apoptosis when it translocates to the outer leaflet (Fadok et al. 2001).

Another consequence of heterogeneity of lipid composition is the formation of distinct lipid phases, which may be formed due to hydrophobic interactions of acyl chains that differ in length and degree of lipid tail saturation, as well as head group electrostatic behavior. The main phase transition denotes the point at which a temperature increase transforms the bilayer from an ordered solid gel phase (s_o) to a liquid disordered phase (l_d) (Gennis 1989; Simons and Vaz 2004). Sterols, specifically cholesterol, have rigid planar structures that induce order among neighboring lipids, creating a liquid ordered phase (l_o) that coexists with the l_d phase in liquid phase membranes. These cholesterol- and sphingomyelin-rich domains have been shown to form a platform for protein interactions and signal transduction and are termed “lipid raft” regions (Edidin 2003; Simons and Vaz 2004).

One purpose of the cell membrane is to transport material between the cell and its extracellular environment. While a few nonpolar molecules are able to cross the plasma membrane without assistance, ions and polar compounds require the aid of membrane transport proteins. Facilitated diffusion is one method by which ions and polar molecules may traverse the membrane. In this method a transporter binds to the substrate and provides the interactions to counteract the energy required for the substrate to lose its hydration shell as it crosses the membrane. In active transport, energy provided by a chemical reaction drives the transport of a substrate against its concentration gradient either directly (primary active transport) or through coupled transport (secondary active transport) (Nelson and Cox 2008). For extremely large or hydrophilic molecules, endocytosis and exocytosis allow particle movement through the budding or fusion of vesicles, respectively, with the plasma membrane.

The types, quantities, and orientation of lipids are tightly regulated by the cell and enable signaling pathways and provide cell structure (Márquez et al. 2012). Breakdown of theses

regulatory pathways can sometimes lead to diseased states. For example, defects in the hydrolysis of sphingolipids in lysosomes and late endosomes (Kolter and Sandhoff 2005) have been implicated in several lysosomal storage diseases, such as Fabry, Niemann-Pick type A and B, Sandhoff, and Tay-Sachs diseases (Maxfield and Tabas 2005). The accumulation of lipids from hydrolysis defects is most significant in brain tissue, where the subsequent death of cells can have severe neurological consequences (Maxfield and Tabas 2005). Alzheimer's disease is characterized by the formation of amyloid beta peptide aggregates, which are influenced by cholesterol levels (Abad-Rodriguez et al. 2004). It is not fully understood whether the accumulation of lipids leads to the aggregation of amyloid beta peptides or whether aggregated proteins lead to lysosomal dysfunction and membrane lipid accumulation. It is likely that both processes occur in a cycle that leads to further neurodegeneration in Alzheimer's patients (Van Echten-Deckert and Walter 2012), as summarized in Figure 1-3.

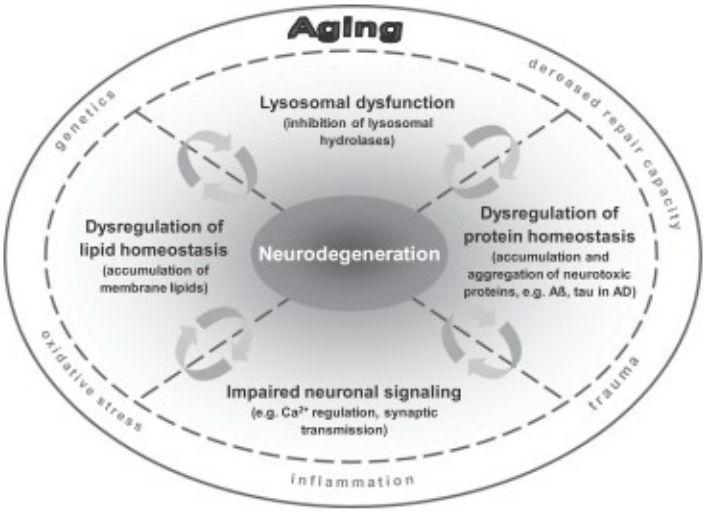

Figure 1-3. Summary of the possible defects leading to neurodegeneration in diseases such as Alzheimer's. Lipid and protein accumulation at membranes potentially influence each other in a detrimental cycle leading to further neurodegeneration. Figure adapted from Van Ecten-Deckert and Walter, 2012.

Examining the biophysical interactions in cellular membranes and understanding the cellular basis of diseases is fundamental in improving and simplifying future diagnoses, treatments, and prevention strategies. However, the complexity of cell membrane composition and molecular interactions makes it difficult to delineate the precise molecular mechanisms of disease. Model membranes are attractive platforms for the elucidation of specific cellular interactions due to their simple and controllable nature and by virtue of the fact that they enable quantitative analysis.

1.1.2 Model Membrane Systems

The significant role that the cellular membrane plays in biological function has prompted efforts to create model systems in which specific phenomena may be studied in a controllable and quantifiable manner. Model membranes are designed to contain all of the essential lipid and protein components of a system of interest, yet are simple enough to isolate and analyze specific interactions. One particular advantage of model membrane systems is the ability to employ sensitive microscopy techniques to determine membrane behavior. Utilizing detection techniques such as epifluorescence microscopy, Förster resonance energy transfer (FRET), and fluorescence recovery after photobleaching (FRAP), it is possible to monitor and quantify membrane interactions.

The type of model membrane system selected for a study often reflects the most practical, yet accurate system to express and evaluate a particular biological phenomenon. Common model membrane systems include lipid monolayers, planar lipid bilayers, supported lipid bilayers, and lipid vesicles (Figure 1-4).

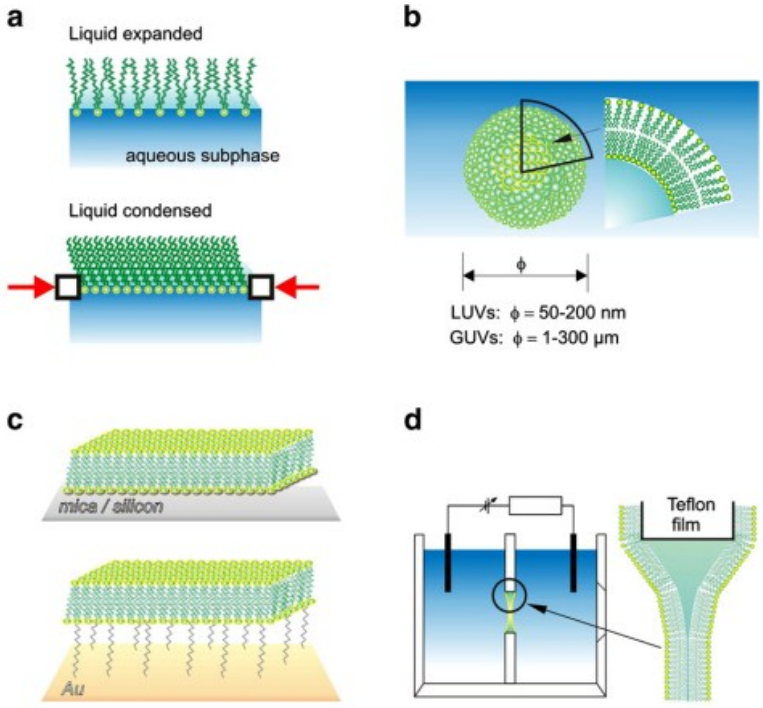

Figure 1-4. Common model membrane systems. A) Lipid monolayers are formed at the water-air interface of a Langmuir trough. B) Lipid vesicles model the bilayer structure and shape of cells. C) Supported lipid bilayers maintain fluidity on a stable solid support. D) Planar lipid bilayers span a break in a solid support structure. Figure adapted from Amado and Kressler, 2011.

Lipid monolayers, representing one leaflet of the cellular membrane, are formed at the water-air interface of a Langmuir trough equipped with a Wilhelmy balance to monitor surface pressure. Substrates that are immersed in and removed from the trough become coated with a lipid monolayer. While simple to create and control, monolayers do not faithfully represent the orientation of transmembrane proteins in natural membranes, nor do they possess natural membrane asymmetry (Castellana and Cremer 2006; Tien and Ottova 2001).

Planar lipid bilayers consist of a bilayer spanning the orifice of a solid support, surrounded by an aqueous environment. These membranes are produced by painting organic lipid solution across a submerged orifice or by immersing and removing a gapped substrate in a lipid-filled trough, allowing the folding of two monolayers into a bilayer at the gap (Montal and Mueller 1972). While useful for examining ion transport and membrane electrical properties,

membranes become unstable when spanning diameters greater than 100 μm and have limited lifetimes for analysis (Amado and Kressler 2011).

Supported lipid bilayers are mechanically stable atop a solid support structure with a thin (~ 1 nm) layer of hydration between the substrate and bilayer. Common techniques to form supported lipid bilayers include Langmuir-Blodgett/Langmuir-Schaefer transfer, vesicle fusion, and the Langmuir-Blodgett/vesicle fusion method. The accessibility to many surface-sensitive techniques (such as atomic force microscopy), simple preparation, and robust nature of supported lipid membranes are several advantages offered by this model membrane system. If integral membrane proteins are to be included, however, a polymer cushion between the bilayer and substrate may be necessary to keep proteins from denaturing and losing mobility (Castellana and Cremer 2006; Chan and Boxer 2007).

While other membrane systems have a planar structure, lipid vesicles are hollow, spherical bilayers mimicking the curvature of cells. Small vesicles of 15 to 200 nm are produced by extruding or sonicating a mixture of rehydrated lipid films (Amado and Kressler 2011). Methods for producing larger vesicles and their particular significance are discussed in detail later.

Giant unilamellar vesicles (GUVs) are generally larger than 10 μm in diameter, thus accurately modeling cell size and membrane curvature (Deamer 2005). Consisting of a bilayer in a hollow, spherical form, GUVs are large enough to be readily resolved using optical microscopy techniques. Simple to form and accessible to surface analysis, GUVs have been utilized in studies ranging from phase separation (Bacia et al. 2005; L. a Bagatolli and Gratton 2000; Veatch and Keller 2003) to membrane curvature (Bacia et al. 2005), and from channel protein studies (Doeven et al. 2005; Kreir et al. 2008; Varnier et al. 2010) to membrane fusion (P. Yang et al. 2009). Giant vesicles are controllable in size and composition, enabling the inclusion of desired lipid and protein species and focusing on the membrane components and interactions of interest.

High-throughput studies are of particular interest, as they allow uniformity in vesicle size, a homogenous experimental environment, and the simultaneous production of many compositionally similar samples. In the interest of high-throughput studies, liposome arrays have been utilized, along with fluorescence microscopy techniques, to study single molecule reactions (Bolinger et al. 2004), membrane transport (Stamou et al. 2003), fusion (Chan et al. 2007; Yoon et al. 2006), and membrane curvature (Hatzakis et al. 2009). The liposome array format provides a platform to observe and analyze the membrane binding interactions of many samples in a controllable environment.

There are several methods for preparing giant liposomes, though some such as swelling (Evans and Needham 1987) and rapid evaporation (Moscho et al. 1996) often lead to the formation of multilamellar vesicles. Electroformation, a technique developed by Angelova and colleagues (Angelova et al. 1992; Dimitrov and Angelova 1988), is generally preferred to achieve unilamellar liposome formation. In the original method, dry lipid films are deposited on a conductive platinum electrode and rehydrated. Then, a DC electric field is applied, enabling the growth of giant liposomes from the rehydrated lipid films (Angelova and Dimitrov 1986). Notably, lipid films are deposited on the electrode from chloroform-based lipid solvents. However, more recent changes to the electroformation procedure have allowed the use of aqueous-based lipid solvents for depositing lipid films (Pott et al. 2008). Further, the use of transparent, conductive substrates, such as indium tin oxide, facilitates sample analysis and visualization. To improve the fusion of small liposomes formed in the beginning of the electroformation process, alternating electric fields are employed (Angelova et al. 1992), as shown in Figure 1-5.

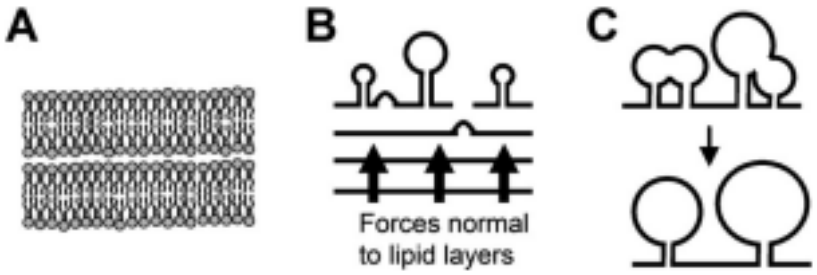

Figure 1-5. Electroformation of lipid mixtures to form giant liposomes. Lipid films (A) on an electrode surface (B) form vesicles upon application of an AC electric field due to forces normal to the surface. Adjacent liposomes (C) fuse to form larger giant liposomes. Figure adapted from Horger *et al.*, 2009.

Overall, membrane separation and formation of vesicles stems from electrostatic and osmotic forces. The applied electric field must oppose the attractive van der Waals forces between membrane layers and between the membrane deposit and the electrode surface to create separation (Angelova and Dimitrov 1988). Recently, Pott *et al.* modified the original electroformation procedure to allow electroformation of not only lipids, but also integral membrane proteins. While electroformation was originally limited to the use of organic lipid solvents, the adapted procedure forms proteoliposomes from aqueous deposits of lipids and proteins (Pott et al. 2008). Others have used electroformation to form giant vesicles from native membranes (Montes et al. 2007) and to create monodisperse giant vesicles by controlling the size and thickness of lipid deposits prior to electroformation (Estes and Mayer 2005; Taylor et al. 2003). Monodisperse giant vesicles are desirable for ease of statistical analysis and homogenous characterization. Controlling the size of initial lipid and protein deposits through patterning allows the formation of monodisperse giant vesicles and is explored in the following section.

1.1.3 Arrays of Model Membranes

Arrays of giant vesicles are useful for analysis due to the spacing of vesicles, which provides clear separation of neighboring vesicles and limits undesirable background fluorescence. Ideally, arrays should be attached to a surface to allow simple location and observation of the vesicles. In a work by Yoshina-Ishii and Boxer, oligonucleotide tethers enabled the attachment of giant vesicle to a supported lipid bilayer, as shown in Figure 1-6 (Yoshina-Ishii and Boxer 2003). However, the complex chemistry involved in array fabrication is not widely available and suggests the need for a simple, more accessible method.

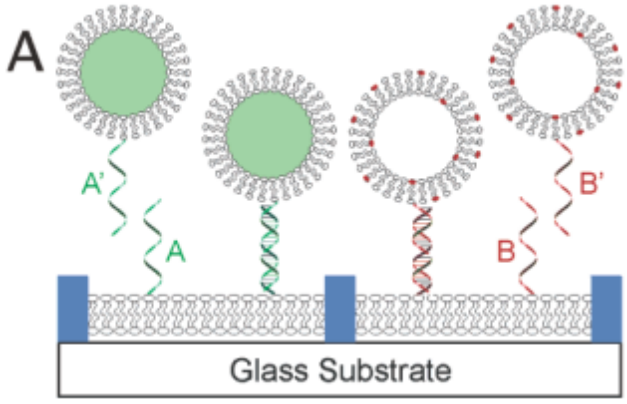

Figure 1-6. Mobile tethered vesicle array. Oligonucleotides attached to lipid headgroups specifically link vesicles to an underlying fluid supported lipid bilayer. Figure adapted from Yoshina-Ishii and Boxer, 2003.

The patterning of lipid films offers control over the size of resultant giant vesicles following electroformation of the spatially defined deposits. This control over deposit size promotes the formation and fusion of small vesicles during the initial stages of electroformation to form monodisperse giant vesicles. Additionally, these vesicles can remain attached to the substrate surface, providing ideal access for analysis techniques. Several microfabrication techniques have been utilized for the production of lipid films, including microfluidic channels (Kam and Boxer 2000; Stroumpoulis et al. 2007; T. Yang et al. 2001), lipid deposition onto substrates with micropatterned barriers (Diguet et al. 2009; Groves and Boxer 2002; Kung et al.

2000), dip-pen nanolithography (Lenhert et al. 2007), and microcontact printing (Hovis and Boxer 2001; Majd and Mayer 2005; Nafday et al. 2012; Taylor et al. 2003). In particular, microcontact printing is a simple and readily-accessible technique capable of simultaneously patterning many lipid deposits (Castellana and Cremer 2006). Traditionally, the stamps utilized in microcontact printing consist of poly(dimethylsiloxane) (PDMS) but the hydrophobic nature of this material (Perl et al. 2009) restricts the use of aqueous solvents as inks unless the surface is modified using plasma oxidation. However, the plasma oxidation process only induces a temporary hydrophilic surface layer that limits stamp usage time and undermines the reuse of the stamp (Perl et al. 2009).

In 2003, Taylor *et al.* reported the successful electroformation of PDMS-stamped patterned lipid deposits as evident in Figure 1-7 (Taylor et al. 2003). This group aimed to create monodisperse vesicle arrays through changes to lipid deposit size and spacing (Taylor et al. 2003). However, PDMS is not compatible with chloroform, and therefore ethanol solvents were used for the stamping of lipid deposits. Ethanol solvents, however, limit the selection of lipid components to those soluble in ethanol and exclude membrane proteins from the patterned arrays due to denaturing in the non-aqueous environment. The use of aqueous solvents is desirable, as lipid and proteins assemble into structures that shield non-polar regions from the polar environment and preserve the function of integral membrane proteins.

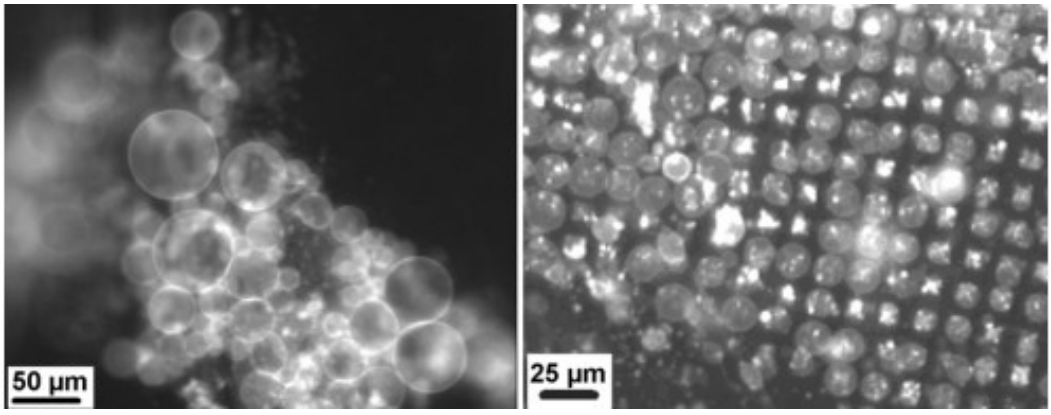

Figure 1-7. Giant liposomes formed from stamped lipid deposits. Right panel fluorescence micrograph displays GUVs produced from an unpatterned 1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine (DMPC) lipid film. Left panel fluorescence micrograph shows an array of liposomes formed from PDMS-stamped DMPC lipid deposits. Figure adapted from Taylor *et al.*, 2003.

In an effort to expand microcontact printing to a greater variety of inks, such as polar lipids and integral membrane proteins, Majd *et al.* and others have employed hydrogel stamps to pattern lipids (Majd and Mayer 2005; Majd et al. 2006), soluble proteins (Coq et al. 2007; Mayer et al. 2004), integral membrane proteins (Majd and Mayer 2008), cell fragments (Majd and Mayer 2008), bacteria (Weibel et al. 2005), and whole cells (Stevens et al. 2005). Hydrogels, which are generally more than 20% water by weight (H. Park and K. Park 1996), provide an ideal hydrated environment for the absorption and transfer of fragile biomolecules and functional membrane proteins. Hydrogels, due to their porous nature, are capable of storing material and stamping multiple times as displayed in Figure 1-8, thus conserving valuable inking material (Majd and Mayer 2005). In this study we present a hydrogel stamping technique for the creation of arrays of giant liposomes and proteoliposomes. This technique takes advantage of the attractive aqueous hydrogel environment to transfer small liposomes, small proteoliposomes, and native membrane fragments to a conductive substrate.

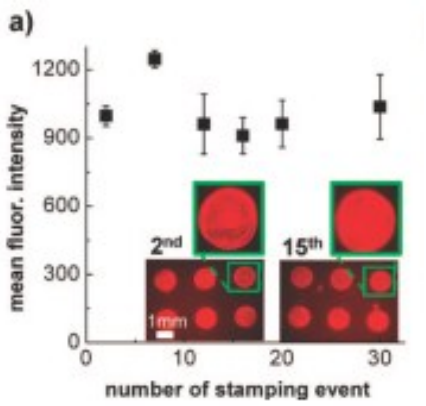

Figure 1-8. Production of multiple stamped integral membrane protein arrays from once-inked hydrogel stamp. a) The mean fluorescence of antibodies to the human tissue factor protein is plotted versus the corresponding number of stamping events (error bars represent standard error). Fluorescence micrographs display the 2nd and 15th stamping events. Figure adapted from Majd and Mayer, 2008.

1.2 Motivation of Present Study

Arrays of giant vesicles have become a popular research platform for biosensing and interaction studies due to their controllable composition, accessibility to quantitative surface analysis techniques, and high-throughput format. Researchers have developed several innovative methods for the creation of giant vesicle arrays, yet some obstacles must be overcome to design a highly versatile and robust array system. The work of Taylor *et al.* addresses the need for a simple technique by employing the common microcontact printing method to form size-controlled arrays of GUVs (Taylor et al. 2003). However, the use of PDMS as a stamping material excludes the use of lipids in chloroform and limits the use of aqueous lipid solutions. Our study addresses the limitations of this method by utilizing an agarose stamping material. This hydrated stamp that can support the transferal of aqueous lipid and protein solutions by promoting the formation of structures to sequester hydrophobic regions from the polar environment. In addition, hydrogels address the lack of storage that PDMS stamps exhibit by

effectively storing functional biomolecules and transferring them to substrates multiple times per inking.

Changes to allow the electroformation of aqueous lipid and protein deposits to produce giant proteoliposome arrays (Pott et al. 2008) expand the possible range of giant vesicle membrane studies. In the work of Pott *et al.*, physiological buffer is utilized in the electroformation procedure to better model the cellular environment and accommodate functional proteins. Using various lipid and protein compositions, the method successfully forms giant liposome and proteoliposome arrays in physiologically significant buffer solutions (Pott et al. 2008). These changes, along with thin film-specific modifications to increase electroformation electric field strength and frequency (Politano et al. 2010), may be applied to patterned lipid and protein deposits to promote giant vesicle formation. Taking advantage of the modifications of Pott *et al.* (Pott et al. 2008) and Politano *et al.* (Politano et al. 2010), the present study is aimed at addressing the inking and stamping limitations of PDMS stamping techniques. This motivation has led to the selection of agarose hydrogel stamps to effectively transfer lipids, integral membrane proteins, and native membrane fragments to conductive substrates and form GUV arrays. Furthermore, we aim to utilize the modified electroformation procedure to achieve successful electroformation of patterned protein and lipid deposits, forming giant liposome and proteoliposome arrays.

1.3 Outline of this Thesis

In this thesis, we present a method that utilizes micropatterned agarose hydrogel stamps to absorb and transfer aqueous lipid and protein solution to conductive substrates. Upon application of a modified electroformation procedure, we formed size-controlled arrays of monodisperse giant unilamellar vesicles. The next chapter details the experimental methods,

including materials, procedures, and analysis techniques. The following chapter shows the results achieved by the presented method and discusses these findings. In the final chapter, potential applications of this method that utilize its advantages are described.

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Chapter 2

Experimental Design

In this chapter, the experimental design, materials, and procedures utilized in this work are detailed. First, the process of hydrogel stamping to pattern lipid and protein deposits on conductive substrates is described. Next, steps to perform electroformation on the patterned deposits are explained, including specific parameters used to form giant vesicle arrays. The main concepts of this method are displayed in Figure 2-1. Following the explanation of experimental design, the next chapter discusses experimental results and conclusions.

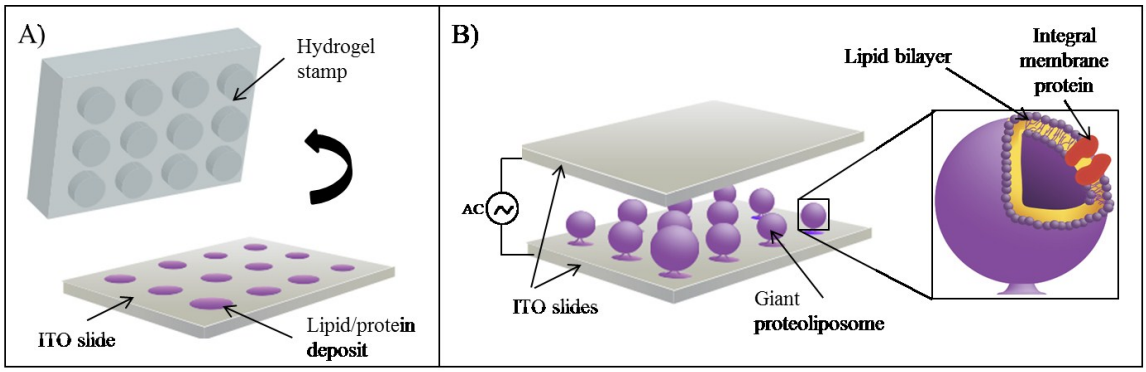

Fig 2-1. Main concept of the presented hydrogel stamping method for the formation of vesicle arrays. A) micropatterned agarose hydrogel stamps absorb and transfer lipid and protein solution to a conductive substrate. B) Patterned deposits are electroformed to create arrays of giant liposomes and proteoliposomes.

2.1 Materials

High gel-strength agarose powder was acquired from OmniPur (Merck, Darmstadt, Germany). All lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL): L-α-phosphatidylcholine (egg PC), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (sodium salt) (DOPS), and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B

sulfonyl) (ammonium salt) (14:0 Liss Rho-PE). Chloroform was acquired from Omnipur. Secondary TRITC-labeled goat polyclonal anti-rabbit IgG (H+L) antibodies were obtained from KPL (Gaithersburg, MD) and 6-carboxyfluorescein powder was purchased from Sigma-Aldrich Corporation (St. Louis, MO). Cell membrane fragments containing human nicotinic acetylcholine receptors (7 mg/mL) were purchased from PerkinElmer, Inc. (Waltham, MA) and α-bungerotoxin Alexa Fluor 555 conjugate powder was purchased from Molecular Probes (Eugene, OR). Annexin-V FITC conjugate (50 μg/mL) was purchased from Biotium, Inc. (Hayward, CA). For buffer solutions, phosphate buffered saline (PBS) tablets (Amresco, Solon, OH), Tris (hydroxymethyl) aminomethane (OmniPur), calcium chloride powder (Acros, Geel, Belgium), glycerol (≥ 99 %) (Acros), sodium hydroxide (NaOH) (VWR International, West Chester, PA), and hydrochloric acid (VWR International, West Chester, PA). For the flow chamber polydimethylsiloxane (PDMS) frame, a Sylgard 184 elastomer kit was used (Dow Corning Company, Midland, MI).

2.2 Methods

2.2.1 Fabrication of Hydrogel Stamps

Hydrogel stamps were prepared according to a protocol previously reported by Majd *et al.* (Majd and Mayer 2005). Briefly, a solution of 4% (w/v) of high-gel strength agarose was heated in deionized water to its boiling temperature in a microwave and cast it into a mold containing the PDMS master with a selected feature size. Depending on the desired feature dimensions of the agarose stamps, PDMS masters with a variety of feature sizes were used to cast the hydrogel stamps. The PDMS master for stamps with posts of 1 mm diameter was a replica (positive) of a PDMS replica (negative) of a standard 1536-well plate (polystyrene) with flat

bottoms. For stamps with features smaller than 1 mm, photolithographic techniques were employed to prepare patterned silicon wafers for fabrication of the PDMS masters. The mold filled with the hot agarose solution was immediately placed in a vacuum chamber drawing 600-700 mmHg for 2-3 seconds in order to degas the solution and remove air bubbles from PDMS wells (this step was repeated at least two times after a slow release of the vacuum chamber). The gel-containing mold was placed in the refrigerator and left to gel for about an hour. Once firmed, the agarose gel stamp was carefully peeled from the PDMS master and trimmed to the desired size using a razor blade. To remove the excess moisture from the posts of the hydrogel, the stamp was partially immersed in deionized water with the posts upwards and above the water level, for up to 30 minutes. 2.2.2 Preparation of Small Liposome Solutions

2.2.2 Preparation of Small Liposome Solutions

In order to handle the mixtures of lipids in organic solvents, glass Hamilton syringes (Hamilton, Reno, NV) were used. Lipid mixtures used to prepare small liposomes (in mol percentage) were: A) 99.2 % egg PC and 0.8 % Rho-PE, B) 100 % egg PC, C) 95% egg PC and 5% DOPS, and D) 90% egg PC and 10% DOPS. All mixtures were combined in chloroform to a final lipid concentration of 10 mg/mL. The lipid mixture was transferred to a Chemglass pear-shaped flask that was attached to a Heidolph Collegiate G1 rotary evaporator (Heidolph Instruments, Schwabach, Germany) for at least one hour to remove the organic solvent from the lipid mixture and to form a uniform film of lipids. Next, the lipid film was hydrated by adding deionized water to achieve a final lipid concentration of 6 mg/mL. This mixture was vortexed and then sonicated using a tip-sonicator (Sonic Dismembrator, Model 100, Fisher Scientific) for ~ 8-10 min. To avoid overheating the solution during sonication, the flask was immersed in an ice

bath and the sonication was carried out for 10 minutes with 30 second breaks in between one minute intervals. The final small liposome solution was stored at 4 ^{o}C until use.

2.2.3 Inking and Stamping

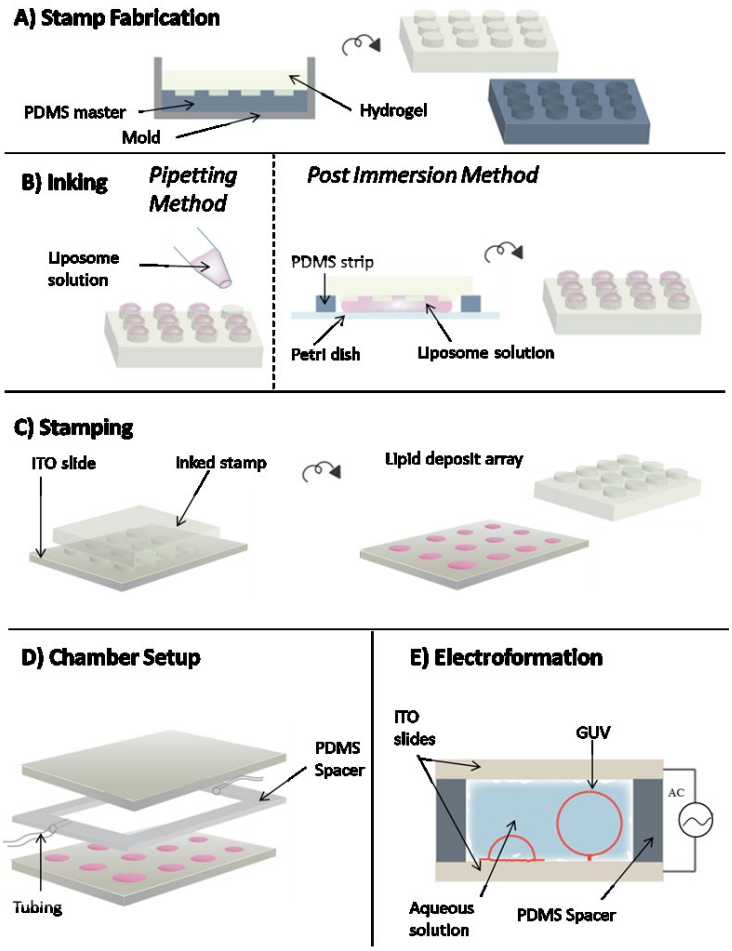

Figure 2-2. Schematic representation of electroformation of giant liposomes from hydrogel-stamped lipid and protein deposits. (A) Patterned hydrogel stamp is fabricated by casting agarose gel solution on a patterned PDMS master. (B) Agarose stamp is inked with lipids/proteins aqueous dispersions either by direct pipetting the solution onto posts or by immersing the posts in solution. (C) Inked stamp is brought into short contact with conductive surface of an indium-tin oxide (ITO)-coated slide to pattern an array of lipid/protein deposits. (D) The patterned ITO slide is used to assemble an electroformation flow chamber; a PDMS frame with inlet and outlet tubing is sandwiched between two conductive faces of sample and counter ITO slides. (E) After introduction of solution to the chamber, an AC electric field is applied between two ITO slides, leading to electroformation of giant vesicles from lipid/protein deposits.

The hydrogel stamp was inked either by the pipetting method or post immersion method, as depicted in Figure 2-2B. The pipetting method was used for membrane fragment experiments and involved pipetting of 5-10 μL of membrane fragment solution onto a region of the stamp three times with 5 min between each inking to allow the stamp to absorb the solution. In all other experiments, the post immersion method of inking was performed. For this procedure, the stamp was placed (with posts facing downwards) in a petri dish with two PDMS stripes (which served as spacers) placed about 1 cm apart. Approximately 40 μL of liposome solution was pipetted between the two spacers and the stamp was placed on top of the spacers and left in contact with lipid solution for up to 30 min. The stamp was then placed in a petri dish partially filled with deionized water with its posts facing upwards and above the water level for 30-40 min to allow the partial drying of excess solution. Next, the inked hydrogel stamp was used to pattern the lipids on indium-tin oxide (ITO) (15 Ω) slides. Slides were cleaned prior to stamping by rinsing with ethanol and deionized water. To stamp onto the ITO slides, a rolling motion with even pressure was employed with the stamp contacting the conductive side of the ITO slide (Figure 2-2C). Patterned slides were left to partially dehydrate before electroformation. For lipid deposits containing integral membrane proteins, this step was carried out in a humidity chamber with saturated salt solution (~ 75% relative humidity) to prevent complete dehydration (Pott et al. 2008).

2.2.4 Flow Chamber Setup and Electroformation

The electroformation chamber was assembled as detailed previously by Majd *et al.* (Majd et al. 2006) and depicted in Figure 2-2D. Briefly, a homemade PDMS flow chamber with built-in inlet and outlet tubing was placed between two ITO slides with their conductive faces inward.

The bottom ITO slide contained the lipid and protein deposits for the electroformation. An AC electric field was applied between two electrodes attached to ITO slides using an Agilent 33120A function/arbitrary waveform generator while introducing the buffer solution to the chamber. Solution flow rate (750 μL/hr – 1 mL/hr) was controlled using a Kd Scientific Legato 100 syringe pump with a 3 mL Becton Dickinson plastic syringe. After applying an initial frequency of 200 Hz and an electric field of 1 mVpp during the filling of the flow chamber, the electric field was increased to 1.6 Vpp at a rate of 100 mVpp/min. After ramping the electric field, the voltage was held at 1.6 Vpp and electroformation was allowed to occur for 1-2 hours. Resultant vesicle arrays were then observed and imaged using phase and fluorescence microscopy while the electric field was held at 1.6 Vpp.

2.2.5 Imaging and Microscopy

To observe and analyze the giant vesicles, electroformation was performed directly on the stage of an inverted Zeiss Axio Observer Z1 microscope equipped with an X-Cite Series 200 fluorescence lamp (EXFP Life Sciences, Ontario, Canada) and a CoolSNAP CCD camera (Photometrics, Tucson, AZ). We used AxioVision 4.8.2 software (Carl Zeiss Microscopy, Oberkochen, Germany) for image acquisition. Phase contrast images were obtained from 10x (4.5 mm working distance), 20x (7.9 mm working distance), and 40x (2.9 mm working distance) objectives in phase-contrast mode. Fluorescent micrographs are displayed in false color. For rhodamine-labeled lipids, TRITC-labeled antibodies, and Alexa Fluor 555-labeled bungerotoxin, a TRITC filter set was utilized and for 6-carboxyfluorescein and FITC-labeled annexin-V imaging, a GFP filter set was used.

2.2.6 Membrane Structure and Solution Transport Analysis

Membrane structure and solution transport were assessed using the 6-carboxyfluorescein dye. Giant vesicles composed of egg PC and 1% Rho-PE were formed in NaOH solution according to the above electroformation procedure. After electroformation, carboxyfluorescein (10 mg/mL) was added to the electroformation chamber and allowed to diffuse for 30 minutes, while maintaining the electric field at 1.6 Vpp. Fluorescence micrographs of vesicles and carboxyfluorescein solution were performed to determine transport of solution into the vesicles and potential GUV structure.

2.2.7 Giant Liposomes in Physiological Solutions

To form GUVs in physiological solutions, small liposome solution was prepared and stamping was executed as described earlier. Solution introduced to the electroformation chamber consisted of phosphate buffered saline (PBS) (1x). Electroformation parameters were changed to enable GUV formation as follows: frequency was increased to 500 Hz, electric field originated at 1 mVpp, and electric field ramping was increased to a 4 Vpp maximum at a rate of 130 mVpp/min.

2.2.8 Aquaporin Proteoliposome Preparation and Analysis

Aquaporin Z (AqpZ) is a channel protein that allows water movement through cell membranes (Calamita et al. 1995). Aquaporin Z-containing small proteoliposomes were utilized after preparation by Mustafa Erbakan from Prof. Manish Kumar and Prof. Wayne Curtis' groups in the Penn State University Chemical Engineering Department. These proteins were included to

display the ability of this system to transfer functional membrane proteins from hydrogel stamps to patterned protein and lipid deposits. Ultimately, the electroformation of these deposits was successfully performed. His-tagged AqpZ (Kumar et al. 2007) was expressed in Escherichia coli and was purified and reconstituted into small liposomes using a detergent dialysis technique as previously reported by Borgnia et al. (Borgnia et al. 1999). The lipids in these liposomes were composed of egg PC and porcine brain PS in a 4:1 molar ratio. The lipid concentration was 6 mg/mL and the lipid to protein mass ratio was 100:1. AqpZ-containing proteoliposomes were used to ink the hydrogel stamp through the post immersion method. Corresponding stamped deposits were electroformed in deionized water under the same conditions as egg PC/Rho PE liposomes. After 1-2 hours of electroformation, primary rabbit anti-his antibody was added to the chamber (at a final concentration of 2.48 μg/mL) and allowed 1 hour to incubate before rinsing the chamber 2-3 times with deionized water. Next, secondary TRITC-labeled goat anti-rabbit antibody was introduced into the flow chamber (at a final concentration of 0.01 mg/mL) and allowed 1 hour to incubate before washing the chamber 2-3 times with deionized water. Imaging was performed with a TRITC filter set.

2.2.9 Giant Liposome Formation from Membrane Fragments

Membrane fragments with overexpressed human nicotinic acetylcholine receptors (AChRs) were received at a concentration of 7 μg/μL in 50 mM Tris-HCL (pH 7.4), 0.5 mM EDTA, 10 mM $MgCl_2$, and 10% sucrose. The physiological role of these plasma membrane neuromuscular receptors is to cause muscle contraction upon stimulation by the binding of their ligands, acetylcholine or nicotine. These cell membrane fragments were utilized to demonstrate the capability of our technique to effectively transfer and electroform intact cellular protein and lipid components. After arrival, this solution was diluted to a final concentration of 3.5 μg/μL in

50 mM Tris-HCl (pH 7.4). Membrane fragment solution was immediately aliquoted and frozen until usage. The solution of membrane fragments was used to directly ink the stamp via pipetting the solution onto individual posts. The inking was repeated three times with ~ 5 min intervals for material to be absorbed. Electroformation was carried out in deionized water with a flow rate of 1 mL/hr, 100 Hz frequency, and field strength ramped from 0.1 to 1 Vpp after the chamber was filled with solution. After 1 hour of electroformation, fluorescently-labeled bungerotoxin (at a final concentration of 0.1 mg/mL in the chamber) was added, incubated for 30 min, and washed by 2-3 times the chamber volume of deionized water prior to imaging.

2.2.10 Analysis of Annexin-V Binding to Giant Liposomes

Small liposomes containing 0%, 5%, or 10% (mol %) DOPS were prepared as described above. The stamp was inked with the liposome solution by post immersion. The PS-containing liposomes were electroformed in a solution of 1 mM Tris and 300 mM glycerol at a frequency of 200 Hz and field strength of 1.6 Vpp for 1-2 hours. After the vesicle growth period, a solution of 1 mM Tris, 300 mM glycerol, and 2 mM $CaCl_2$, was added to the chamber. Next, a solution of $CaCl_2$ buffer solution with 50 μg/mL FITC-labeled annexin-V was introduced to the chamber to give a final chamber concentration of 2 μg/mL annexin-V. Annexin-V is a membrane protein associated with apoptosis and binds to phophatidyserine in membranes. This protein-lipid binding system serves as a model for the biophysical interactions that this method can be applied to analyze. Imaging of fluorescent liposomes was performed with a GFP filter set.

2.3 References

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Pott, T., Bouvrais, H., and Méléard, P. (2008). "Giant unilamellar vesicle formation under physiologically relevant conditions." *Chemistry and Physics of Lipids*, 154(2), 115–119.

Chapter 3

Experimental Results

This chapter describes the characterization of lipid deposits and resultant vesicle arrays using the method developed in the experimental section. We focus on the achievement of single vesicle arrays through the reduction of stamp feature size. Vesicle structure is later assessed through solution transport studies. We find that by adapting the electroformation procedure, vesicles can be formed in physiological buffer solutions. We then demonstrate the versatility of this method by forming giant vesicle arrays from stamped small proteoliposome solutions and natural membrane fragments. Finally, we study protein-lipid interactions by analyzing the binding of annexin-V to phosphatidylserine in GUVs. The details of these studies are elaborated in this chapter.

3.1 Characterization of Liposome Arrays Electroformed from Stamped Deposits

To produce arrays of giant liposomes, we first employed an agarose stamp that was inked with a solution of small unilamellar vesicles (SUVs) to pattern an array of lipid deposits on an ITO-coated slide. During this step, a short contact between SUV-loaded agarose stamp and the substrate resulted in deposition of a thin film of lipids on areas of contact. Lipid deposits were smaller in diameter than the post features that were used to transfer the material, as shown in Figure 3-1. In addition to a decrease in lipid deposit diameters, the size distribution of deposits narrowed as stamp post diameters were decreased. The reduction in diameter from stamp post size to deposit size was potentially due to the relative hydrophobicity of the ITO-coated glass slide. During stamp removal from the ITO-coated slide, lipid solution likely clung to the hydrophilic stamp as it was lifted due to favourable surface interactions and formed a “beaded”,

dome-like structure on the substrate. The contact angle of water on ITO-coated glass has been shown to be 80º (Patel et al. 2010), demonstrating relative hydrophobicity when compared to the extremely hydrophilic agarose stamp. The beading effect of aqueous SUV solution may have resulted in a decrease in lipid deposit diameter in comparison to the diameter of the patterning post.

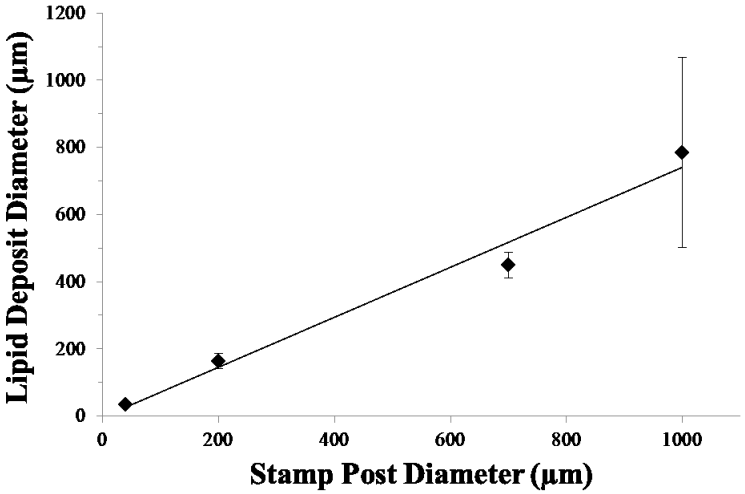

Figure 3-1. Lipid deposit diameter variation with changes in hydrogel post diameter. As hydrogel post diameter is increased from 40 µm to 1000 µm, post diameter likewise increases, but remains smaller than the original patterning hydrogel post. Error bars represent standard deviation (n = 7).

We then employed electroformation to form GUVs from these patterned lipid deposits. As expected, upon rehydration and exposure to an AC electrical field during electroformation, each patch of lipid deposits generated giant unilamellar liposomes with compositions similar to that of initial SUVs. Using epifluorescence and phase contrast microscopy, we monitored the formation of GUVs and characterized the resultant GUV arrays. Figure 3-2A demonstrates a small representative area of an array of GUVs formed from SUV deposits composed of PC and a small fraction of fluorescent-labeled PE lipids. In this experiment, a stamp with 200 µm feature size was applied to pattern lipids on an ITO slide. Upon electroformation, each of the deposited spots resulted in the formation of several giant liposomes (Figure 3-2A).

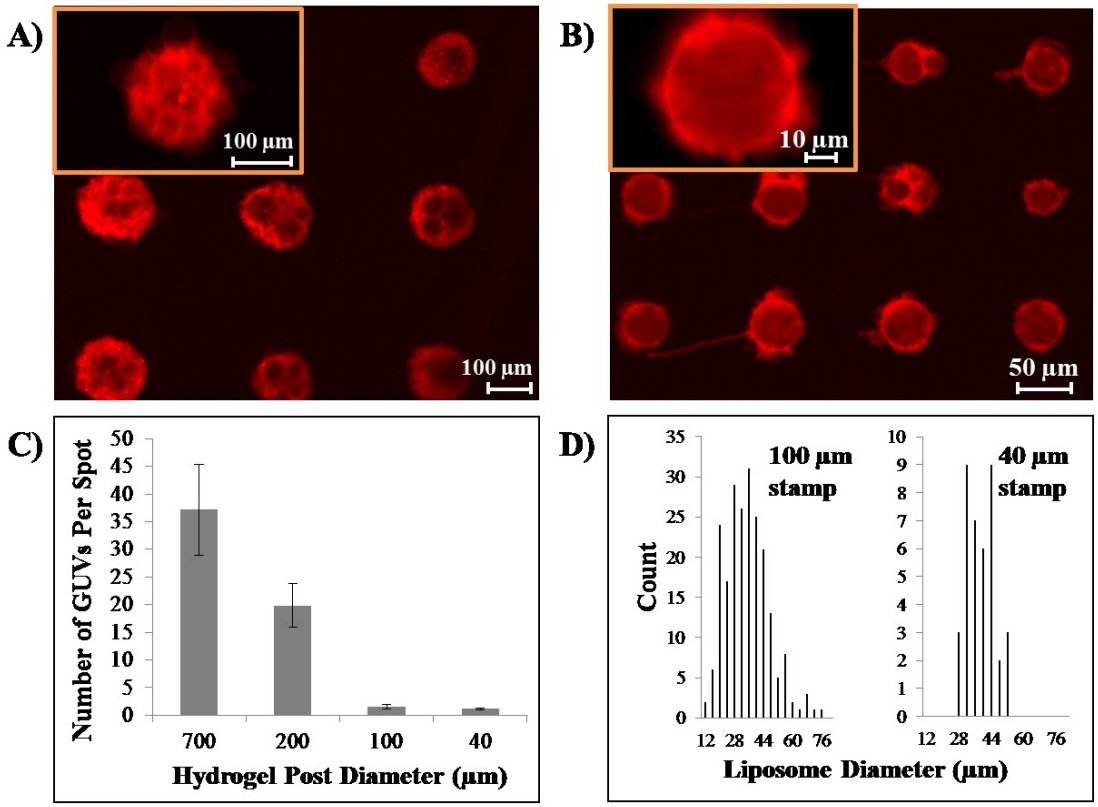

Figure 3-2. Electroformation of arrays of giant liposomes from hydrogel-stamped lipid deposits of various sizes. Fluorescent micrographs of arrays of vesicles composed of egg PC/ 1% Rho-PE electroformed from lipid deposits that were stamped by a stamp with post size of (A) 200 µm and (B) 40 µm diameters. The insets show a magnified representative spot from these arrays. (C) The bar graph displays the average number of GUVs formed per lipid deposit as a function of the post diameter of stamp used to pattern the array. Error bars represent the standard error of the average number of GUVs per stamped spot (n = 7). (D) Histograms represent the size distribution of liposomes formed from lipid deposits patterned by stamps with 100 µm and 40 µm diameter posts. Fluorescence micrographs are contrast enhanced to enable better visualization of discrete liposomes.

We hypothesized that changing the size of lipid deposits on the electrode would affect the number of giant liposomes that formed per deposit. To examine this hypothesis, we employed stamps with different feature sizes, ranging from 100 µm to 1 mm, to create arrays of lipids for electroformation. Lipid deposits transferred from stamps with large features produced many liposomes at each location in the array. However, lipid deposits produced by 100 µm featured-stamps formed only a few GUVs at each location, as illustrated in Figure 3-2C. With a reduction

in the size of lipid patches, the resultant vesicles became more uniform in size. Therefore, we reduced the stamp feature size further to 40 μm in order to achieve single-vesicle arrays (Figure 3-2B).

Next, we investigated size distribution of vesicles formed at each stamped location with changes in stamp feature size. On lipid patches transferred from stamps with ≥ 200 μm features, electroformed GUVs had relatively broad size distribution. Histograms in Figure 3-2D illustrate the size distribution of the GUVs formed on deposits from 100 μm and 40 μm stamp features where the average size of GUVs in the array are 33 ± 11 μm and 37 ± 7 μm respectively (mean ± standard deviation). The slight increase in average GUV diameter from 100 μm to 40 μm arrays may stem from the smaller surface area of lipid deposits resulting in greater proximity of liposomes growing on each deposit and therefore, better conditions for vesicle fusion. We noted that the uniform size of vesicles formed from 40 μm hydrogels may be advantageously utilized, through detachment and harvesting, to rupture and form continuous membranes over nanowells in future studies.

3.2 Vesicle Structure and Solution Transport

To assess the structure of the membranes formed by this method, we studied transport of solution through vesicle membranes with the addition of 6-carboxyfluorescein (CF). The dye, CF, is fluorescent and membrane impermeable, allowing its use as a visible marker for the transport of solution following electroformation. Giant vesicles were electroformed from stamped lipid deposits of egg PC/ 1% Rho-PE and held at an electric field strength of 1.6 Vpp for the duration of the experiment. After the addition of CF to the electroformation chamber, images were acquired for the visualization CF, the vesicle membrane, and merged images of both the CF solution and membrane (Figure 3-3).

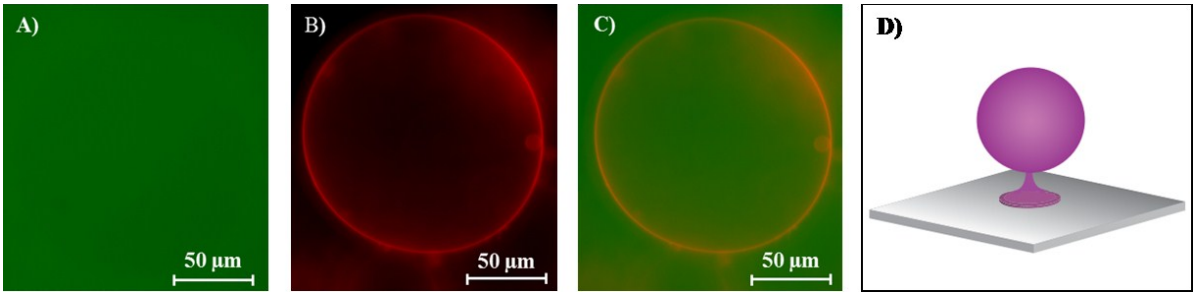

Figure 3-3. Membrane structure analysis with carboxyfluorescein solution. Vesicles were electroformed from egg PC/ 1% Rho-PE stamped deposits, after which the electroformation chamber was incubated with 6-carboxyfluorescein. The fluorescent membrane-impermeable dye was imaged with a GFP filter set (A) and the vesicle was imaged with a TRITC filter set (B). An image (C) was acquired with both filter sets, revealing that dye enters the vesicle. The attached, "open vesicle" structure (D) is the likely configuration that the GUVs form upon electroformation.

In the images, CF is not completely washed from the chamber and vesicles are expected to have a dark interior if their membranes are impermeable to the CF solution. However, as shown in Figure 3-3C, we observe that CF fluorescence intensity is similar both inside and outside the vesicle membrane. We interpret these similar fluorescence intensities to be a result of vesicle membrane permeability. A likely explanation for this transport of solution is that CF entered vesicle membranes at the attachment point with the substrate, where membranes may not have complete enclosure. This entrance of solution has led to the development of a structural model for the attached, "open vesicle" configuration depicted in Figure 3-3D. This structure is characterized by the attachment of spherical giant vesicles to a region of stamped lipid deposit on the substrate surface by a thin, neck-like structure.

3.3 Giant Liposomes in Physiological Solutions

Cellular processes proceed in aqueous environments with approximately 200 mM ionic strength. In order to apply GUVs as model membranes for physiologically relevant studies, it is critical to form these vesicles in solutions that mimic physiological environment using, for example, phosphate buffered saline (PBS) with an ionic strength of 162.7 mM (Lloret et al. 2013). While formation of GUVs in high ionic strength solutions was initially believed to be possible only using spontaneous swelling techniques that require the incorporation of negatively-charged lipids (Akashi et al. 1996), more recent studies have shown that the electroformation method may be adapted to physiological solutions (Estes and Mayer 2005; Horger et al. 2009; Montes et al. 2007; Pott et al. 2008). Here, we confirmed that the present technique also affords formation of GUVs in PBS solution, as seen in Figure 3-4. When formed in PBS solutions, however, giant vesicles composed of PC and 1% w/w Rho-PE were smaller in diameter and more numerous per lipid patch compared to those formed in deionized water under otherwise identical experimental conditions. This reduction in vesicle size under high ionic strength conditions is in agreement with previous reports of electroformation of GUVs in high salinity buffers (Pott et al. 2008).

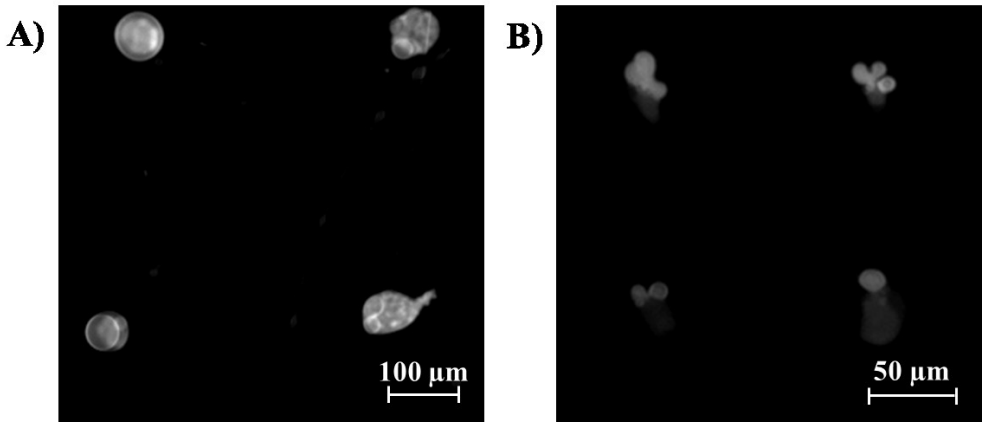

Figure 3-4. Formation of liposome arrays in physiologically relevant solution from hydrogel-stamped lipids. Fluorescence micrographs of a representative area of GUVs composed of egg PC/ 1% Rho-PE, electroformed in a flow chamber filled with (A) deionized water or (B) PBS.

3.4 Formation of Proteoliposome Arrays

To better represent natural cell membranes in studies of membrane-associated processes such as membrane binding interactions and phase separation, GUVs should ideally contain both proteins and lipids. In the approach presented here, hydrogels absorb, store, and transfer aqueous dispersions of small lipid vesicles onto the substrate for subsequent electroformation of GUVs. Agarose stamps provide a hydrated and biocompatible environment for fragile lipids and proteins and are also able to absorb and deliver small proteoliposomes onto surfaces (Majd and Mayer 2005, 2008). We hypothesized that inking hydrogel stamps with proteoliposome solutions would enable direct formation of giant proteoliposomes upon electroformation of lipid/protein deposits. We assessed this hypothesis using aquaporin Z (AqpZ)-containing small liposomes as a model system. Aquaporins are transmembrane pore-forming proteins that provide pathways for water to cross lipid membranes (Kumar et al. 2007, 2012). For these experiments, solutions of AqpZ (Calamita et al. 1995)-containing proteoliposomes (Borgnia et al. 1999) were used to ink hydrogel stamps that later delivered these materials onto ITO-coated glass slides. Upon

electroformation, these stamped lipid/protein deposits produced arrays of single giant liposomes that contained AqpZ (Figure 3-5A).

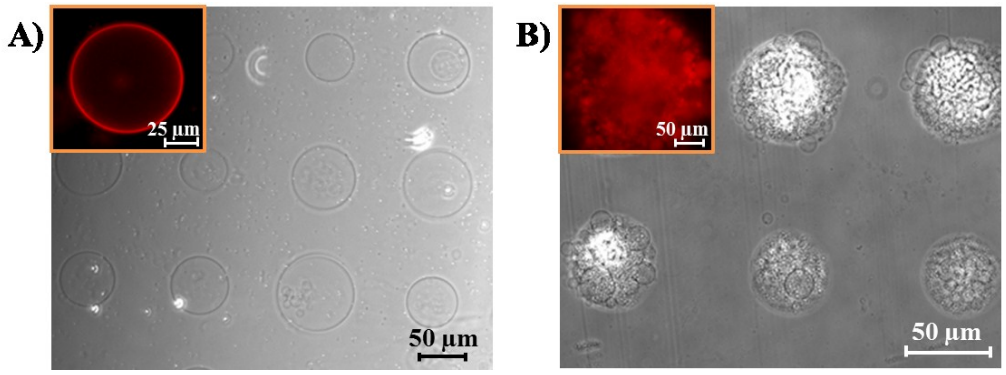

Figure 3-5. Proteoliposome arrays formed from hydrogel-stamped deposits of small proteoliposomes and cell membrane fragments. Phase-contrast images of arrays of vesicles electroformed from stamped deposits of (A) small proteoliposomes with reconstituted aquaporin channels and (B) membrane fragments containing nicotinic acetylcholine receptors (AChR). The inset in (A) shows a fluorescent micrograph of a magnified representative giant proteoliposome from the array upon incubation with a primary antibody against aquaporin and a fluorescently-labeled secondary antibody, confirming the presence of aquaporin in membrane. The fluorescence micrograph is contrast enhanced to enable better contrast of the liposome against background. The inset in (B) displays a fluorescent micrograph of a representative spot within the array after exposure to a fluorescently-labeled bungerotoxin that binds to AChR, confirming the inclusion of this protein in the vesicles.

To confirm the presence of AqpZ in the resulting GUVs, we conducted an immunofluorescence assay. The inset in Figure 3-5A demonstrates that the his-tagged AqpZ was detected through binding of a rabbit anti-his primary antibody and a fluorescently-labeled goat polyclonal anti-rabbit secondary antibody. In a control experiment, GUVs formed from PC lipid with no proteins, showed no fluorescence signal when exposed to these antibodies under the same experimental conditions. The resulting giant proteoliposomes were, interestingly, larger than those formed from PC lipids. This difference in size of GUVs may stem from different lipid formulation used in small aquaporin-containing liposomes.

Furthermore, we explored the capability of the present method to form giant proteoliposomes directly from natural cell membrane fragments (Montes et al. 2007; Pott et al.

2008). If possible, this approach would eliminate the protein reconstitution step for preparation of ink materials since hydrogel stamps can be inked with membrane fragments with desired proteins. Majd *et al.* previously demonstrated that agarose stamps can deliver and pattern membrane fragments (Majd and Mayer 2008). Here, we applied dispersions of membrane fragments containing the human acetylcholine receptor (AChR) to agarose stamp and, after stamping these materials on ITO-coated slides, electroformed giant liposomes in deionized water. These membrane fragments were too large to penetrate the pores of the hydrogel stamp and remained concentrated at the surface of the hydrogel posts. Transferal of these cell fragments was only possible one time per stamp inking, as was noted previously (Majd and Mayer 2008). Figure 3-5B shows a representative area of an array of GUVs electroformed from membrane fragment deposits. Binding of fluorescently-labeled bungerotoxin, a neurotoxin that binds selectively to AChRs, to these vesicles confirmed the inclusion of AChR (inset in Figure 3-5B). This fluorescently-labeled toxin showed no detectable binding towards control PC vesicles formed under identical conditions. As shown in Figure 3-5B, electroformation of the stamped membrane fragments resulted in growth of many large to giant (5-70 μm) unilamellar vesicles containing AChR at each stamped spot. The stamped deposits of membrane fragments were observed to be dense and thick in comparison to stamped SUV deposits. Electroformation of the stamped membrane fragments resulted in the growth of many vesicles at each stamped location. It is thought that this growth stems from the larger amount of material present in these stamped deposits relative to the amount of lipid deposited using stamped SUV solution. These results are in good agreement with our previous findings that large membrane fragments, unlike small vesicles, do not diffuse into the hydrogel and instead become pre-concentrated on stamp posts leading to transfer of very thick (> 10 μm) (Majd and Mayer 2008) lipid and protein deposits. However, formation of single GUV arrays from these membrane fragments proved challenging

because the application of stamps with 100 μm features did not decrease the size of deposits, presumably due to the large size of these intact fragments (Majd and Mayer 2008).

3.5 Analysis of Protein-Lipid Interactions in Giant Liposome Arrays

Examining the interactions between proteins and lipids in model membrane systems is key to understanding how membrane constituents are organized. To explore the potential of the present method for studying protein-lipid interactions, we examined the binding interactions of protein annexin-V to DOPS-containing GUVs. Annexin-V is a membrane-associated protein that plays a role in coagulation cascade disruption (Tait and Gibson 1992) and is also commonly used as a marker for apoptotic cells (Fadok et al. 2001). This protein has a high affinity for the negatively-charged DOPS lipid and in the presence of calcium ions, binds to this lipid (Tait and Gibson 1992). For this study, we formed arrays of GUVs with various molar percentages of DOPS and monitored the binding of FITC-labeled annexin-V to the GUVs through the flow chamber. Arrays of GUVs composed of PC lipids with 0%, 5%, and 10% (mol %) DOPS were prepared in a buffer solution of 1 mM Tris and 300 mM glycerol (≥ 99%). After electroformation, we exposed these GUVs to fluorescently labeled annexin-V by flowing a solution of this protein into the electroformation chamber. The replacement buffer solution contained 2 mM calcium. As Figure 3-6 illustrates, the fluorescence intensity of GUVs, due to the bound annexin-V, increased with increasing DOPS content in GUVs. With an increase in DOPS content from 5% to 10%, there was a subsequent 65% increase in the average fluorescence intensity of vesicle membranes ($n = 6$). This detectable increase in fluorescence intensity may be used to determine vesicle component content and binding efficiency. These results confirmed that the present technique is capable of forming GUVs from variety of lipid and protein-lipid compositions and establishes the potential of these GUV arrays for studying lipid-protein interactions.

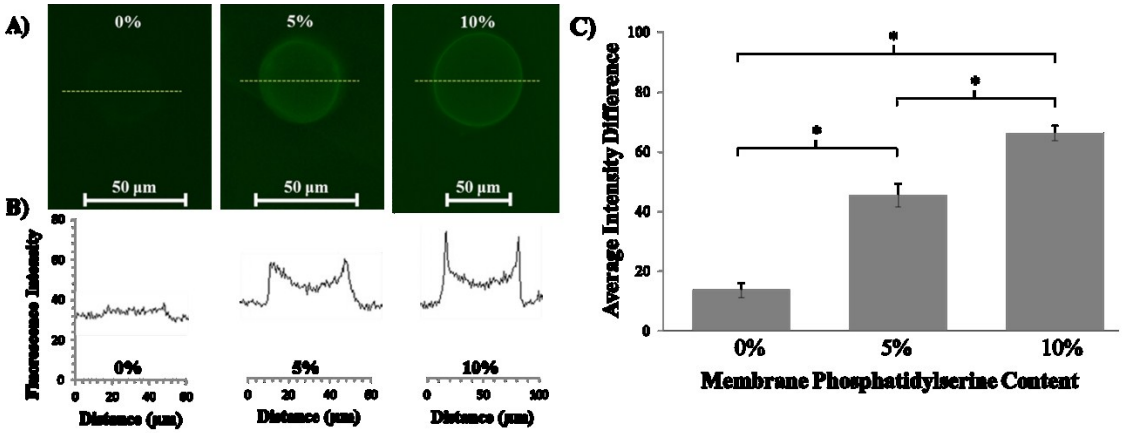

Figure 3-6. Binding of the FITC-labeled annexin-V protein to electroformed PS-containing giant liposomes. (A) Fluorescent micrographs display a representative vesicle from arrays of vesicles composed of egg PC and 0% (mol %), 5%, and 10% DOPS after incubation with FITC-labeled annexin-V in the presence of 2 mM calcium. (B) Fluorescence intensity profiles measured across the dashed lines shown in panel (A) illustrate that the amount of bound annexin-V to vesicles increases with increasing PS content in the membrane. (C) The differences of mean fluorescence intensity above background (in relative fluorescence units) from line scans of GUVs increase with increasing DOPS content (error bars represent standard deviation, n = 4 for 0%, n = 6 for 5% and 10%). The asterisks indicate significant differences ($p < 0.05$).

3.6 Summary

Here, we present a versatile approach for the preparation of arrays of giant liposomes and proteoliposomes by combining hydrogel-based microcontact printing with the commonly-used technique of electroformation. Wet hydrogel stamps are particularly suitable for storage and delivery of fragile lipids and proteins onto substrates and therefore, enable electroformation of GUV arrays from deposits of lipids, reconstituted membrane proteins, and cell membrane fragments. Using this approach, we created arrays of liposomes from different lipid compositions including negatively-charged lipids, and two different membrane proteins, an aquaporin channel and an acetylcholine receptor. This technique is capable of forming arrays of mostly single giant vesicles with a narrow size distribution. It provides a simple tool for preparation of a large

number of uniformly-sized giant liposomes or proteoliposomes quickly and without the need for any specialized equipment. The resulting giant liposomes can be used while attached to the surface in an array format or can be detached for additional screening steps. We examined the applicability of these single vesicle arrays for monitoring lipid-protein and protein-protein binding interactions.

Agarose gel stamps are also capable of adsorbing and storing small liposomes and proteoliposomes and can pattern multiple copies of an array of lipids/protein upon one-time inking (Majd and Mayer 2005). In combination with electroformation, this capability would make it possible to rapidly create multiple copies of an array of GUVs using minute amounts of lipids and proteins. We expect that the combination of hydrogel-stamping and electroformation for the production of giant vesicle arrays to be useful in biotechnology and biosensing applications as well as the analysis of membrane-binding interactions and functional membrane-protein assays. The future outlook of this technique is evaluated in the following chapter.

3.7 References

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Chapter 4

Future Directions

The COPII complex is a protein coat that initiates the budding of cargo transport vesicles from the endoplasmic reticulum to the Golgi apparatus. While it is known that Sar1p, a GTPase, along with Sec23/24p and Sec13/31p are involved in the release of vesicles (Bacia et al. 2011), the exact mechanism and binding interactions in this process are not well understood. It is believed that membrane deformation as a result of the binding of coat proteins is a key step in the release of budding vesicles from the endoplasmic reticulum (Manneville et al. 2008).

This chapter presents a potential future study that applies the techniques developed in this thesis. In this potential study, we intend to assemble the COPII complex on a surface-attached array of giant unilamellar vesicles (GUVs) and assess the resultant changes in membrane fluidity and phase separation. First, GUVs will be electroformed from hydrogel-stamped lipid deposits to form a single vesicle array. Purified COPII subunits (Barlowe et al. 1994) will then be added sequentially and paired with FRET fluorophores to confirm the COPII coat assembly mechanism. Next, we will perform FRAP on vesicles with various subunits of the coating complex to assess changes in membrane fluidity. Membrane phase partitioning due to the binding of proteins and potential organization of the membrane into phases will be monitored through fluorescence microscopy techniques. This introductory section serves to provide the necessary background and motivation for the proposed study, and is detailed in the following sections.

4.1 Background

Early cellular secretory pathways are critical processes that may be particularly amenable to analysis using the presented method. In cells, proteins are transported from the endoplasmic

reticulum to the Golgi apparatus before further sorting and export to specific locations. This protein transport occurs in vesicles that bud from organelle membranes with the aid of coating molecules. Essential steps in this budding process include cargo capture, coat assembly, membrane deformation, and finally release of the vesicle with its contained protein cargo (Hughes and Stephens 2008). Further knowledge of the mechanisms and interactions in this pathway is necessary to understand and potentially treat the trafficking defects that occur in diseased states.

A specific coating protein complex, COPII, is known to be present on the surface of vesicles participating in anterograde transport from the endoplasmic reticulum to the Golgi apparatus (Sato and Nakano 2005). The formation of vesicles requires the assembly of three coating subunits, Sar1p, Sec23/24p, and Sec13/31p, and a guanine exchange factor, Sec12p (Matsuoka et al. 1998). Upon assembly of this complex, membrane deformation of the endoplasmic reticulum occurs until a vesicle buds off. Hydrolysis of GTP by Sar1p leads to the dissociation of the coat complex, which occurs prior to vesicles docking and fusing to the Golgi membrane. A summary of the vesicle budding from the endoplasmic reticulum is presented in Figure 4-1.

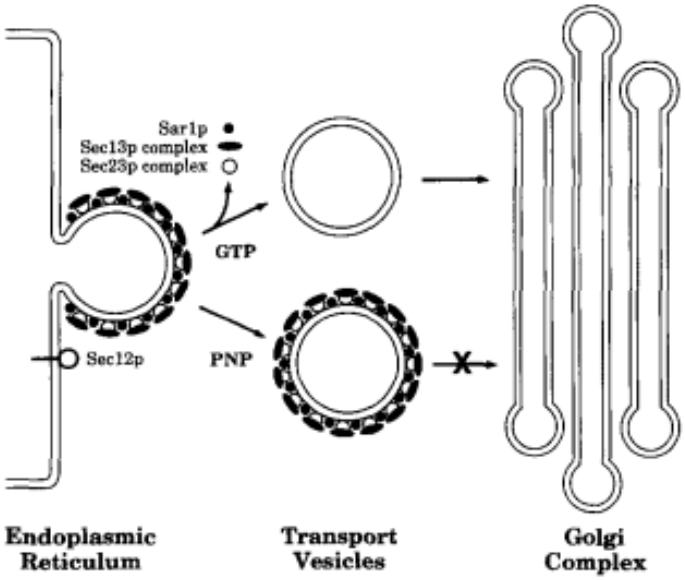

Figure 4-1. Anterograde transport and COPII complex. A guanine exchange factor, Sec12p, exchanges GDP with GTP on Sar1p. Afterwards, Sar1p recruits Sec23/24p and Sec13/31p to form the coating complex, COPII. After membrane deformation and vesicle budding, GTP hydrolysis results in coat disassembly. However, when nonhydrolyzable GTP analogs (GMP-PNP) are included in coat assembly, dissociation fails and vesicle docking and fusion does not occur. Figure adapted from Barlowe *et al.*, 1994.

The study of the COPII complex is relevant in diseases resulting from defects in protein trafficking components. While many diseases are due to protein folding and assembly issues, several diseases such as cranio-lenticulosutural dysplasia and chylomicron retention diseases are attributed to mutations of COPII machinery (Hughes and Stephens 2008). Moreover, viruses and bacteria are capable of "hijacking" the COPII pathway, leading to pathogenic conditions (Hughes and Stephens 2008).

In previous studies, fluorescence microscopy techniques have been applied to reconstituted COPII liposomes to study subunit assembly and disassembly. In a study by Sato and Nakano, a real-time FRET-based assay is used to gather kinetic information about coat assembly and disassembly, while a tryptophan fluorescence study monitors Sar1p GTP hydrolysis (Sato and Nakano 2005). This study focuses on the interaction of Sec23/24p with the complex as Sar1p

undergoes GTP hydrolysis. Timescales of fluorescence suggest some stability of the Sec23/24p subunit even after hydrolysis (Sato and Nakano 2005). A study by Bacia *et al.* uses confocal microscopy and cryo-electron microscopy to visualize COPII responses to membrane curvature and assembly structure (Bacia et al. 2011). Interestingly, binding of the COPII complex resulted in the formation of multibudded tubules. This result led to a hypothesis that vesicle fission is dependent on changes in Sar1p interactions and bilayer deformation (Bacia et al. 2011).

4.2 Motivation for this Study

Due to the importance of cargo trafficking, it is important to understand the impact of defects in these pathways and how these defects can lead to diseased states. A fundamental step in comprehending the effects of coating protein defects is to determine the role that the protein binding and assembly plays in healthy membranes. The COPII system is known to involve several coating proteins, but the precise manner in which they interact with each other and initiate vesicle release from the endoplasmic reticulum is not clearly understood. While direct observation of cells is the most biologically accurate system to study this pathway, the complexity of cellular membrane components and signaling pathways hinders the isolated study of the pathway of interest. Model membranes offer a controllable and simplified alternative to observing cells directly. This study aims to utilize this beneficial model membrane platform, along with sensitive fluorescence techniques, to elucidate the behavior of coating proteins in the COPII complex.

The results of a study by Bacia *et al.* show that mutations to Sec23/24p hinder the formation of the COPII complex (Bacia et al. 2011). This work, along with the evidence that the related COPI complex affects membrane fluidity by imposing order and partitioning in budding membranes (Manneville et al. 2008), leads to questions about the assembly of COPII complex

and its effects on membrane partitioning. Previous studies focus mainly on the effects of GTP hydrolysis in reconstituted COPII liposomes, but do not emphasize binding order or changes in membrane partitioning. With evidence by Bacia *et al.* that membrane deformation is an important factor in COPII vesicle fission (Bacia et al. 2011), there are still questions as to the significance of changes in membrane structure in this destabilizing process. The proposed study aims to determine the relationships between the binding coating proteins, their significance toward the aggregation of the COPII complex, and the effects of protein binding on membrane partitioning.

4.3 Methods and Analysis

4.3.1 Assessment of COPII Coat Assembly in Giant Vesicle Arrays

After formation of giant vesicle arrays by our presented method, COPII assembly will be studied. The lipid compositions of these vesicles will be prepared as detailed by Matsuoka *et al.* (Matsuoka et al. 1998). To study COPII assembly, we will employ FRET to visualize interactions of COPII subunits separated by less than 10 nm. The FRET pair cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) will be inserted into vectors to express labeled COPII subunits according to Sato and Nakano (Sato and Nakano 2005). We will first perform FRET with CFP-Sar1p-incubated GUVs and YFP-Sec23/24p to establish binding between these first subunits. We expect that FRET efficiency will increase with the addition of YFP-Sec23/24 in accordance with **(4-1)**.

$$\varepsilon = \frac{YFP_{post} - YFP_{pre}}{YFP_{post}} \qquad \textbf{(4-1)}$$

The dependence of Sec23/24p for Sec13/31p binding will be determined by performing FRET on GUVs incubated with 1) CFP-Sar1p, and YFP-Sec13/31p and 2) Sar1p, CFP-Sec23/24p, and YFP-Sec13/31p. We expect to observe a higher FRET efficiency in the second case than in the first, as previous studies have shown limited COPII complex assembly when Sec23/24p has been truncated through mutation, as shown in Figure 4-2 (Bacia et al. 2011).

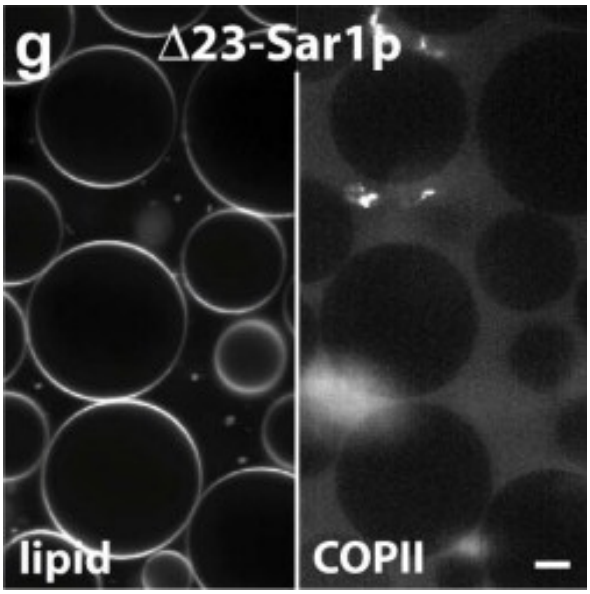

Figure 4-2. Assembly of COPII with mutated Sec23/24p. Fluorescently-labeled lipids are visible in the left panel to define the GUV membranes. In the right panel, GFP-labeled Sec13/31p does not colocalize with GUV membranes when COPII subunit Sec23/24p is truncated. Figure adapted from Bacia *et al.*, 2011.

4.3.2 Influence of COPII Coat Assembly on Fluidity and Partitioning of the Membrane

Coating proteins are responsible for structural membrane changes, such as deformation, that lead to the eventual budding and export of protein-laden vesicles. Therefore, it is beneficial to understand the biophysical interactions between COPII subunits and how the assembly of these proteins affects membrane fluidity and domain formation. In a recent study, Manneville *et al.* have reported that COPI coating proteins self- assemble into liquid disordered phases of GUVs, as displayed in Figure 4-3 (Manneville et al. 2008). This ordering is observed to reduce

membrane fluidity, as is evident in FRAP studies. Interestingly, vesicle with only Arf1 bound exhibited similar membrane mobility to control vesicles with no protein bound, while addition of coatomer to Arf1 induced the formation of COPI lattices and diminished membrane fluidity (Manneville et al. 2008).

Similarities between Arf1 and Sar1p have led to comparisons between mechanisms of membrane deformation in COPI and COPII pathways. However, it is not known if COPII assembly coincides with or leads to the formation of membrane phase domains as has been observed for COPI assembly. In this study, we aim to investigate membrane fluidity of giant vesicles with no coating subunits, only Sar1p, Sar1p and Sec23/24p, and the entire COPII complex. To quantify the membrane fluidity, we propose the use of FRAP on GUVs incubated with each of these coating protein combinations. By photobleaching regions of fluorescently labeled GUVs, diffusion coefficients can be determined from the recovery of fluorescence.

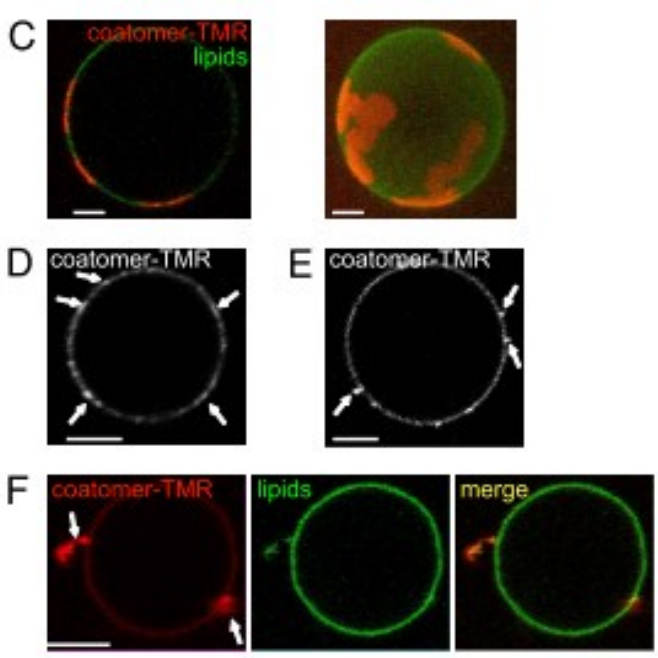

Figure 4-3. Domain formation induced by COPI assembly. Fluorescently-labeled coatomer marks domains formed by the COPI complex. Domains may (C) appear static and cover up to a 5 μm diameter, (D) form small patches on the membrane, or (E) partition with bundled membrane deformation regions. F) coatomer also colocalizes with highly curved or tubular membrane extensions. Figure adapted from Manneville *et al.*, 2008.

4.4 Expected Outcomes and Findings

In COPII assembly studies, we expect that FRET analysis will provide evidence of the necessary binding subunits in the COPII complex. As previous studies have noted that mutations in Sec23/24p lead to low levels of COPII complex assembly (Bacia et al. 2011), we predict that Sec23/24p will be necessary in the complex formation and that FRET will have low efficiency without the inclusion of this coating protein.

For membrane partitioning and fluidity studies, we anticipate that, in a similar fashion to the COPI mobility study (Manneville et al. 2008), mobility will not be completely impaired until addition of the full COPII complex. It is possible that upon partial assembly of the COPII complex, there will be intermediate levels of mobility reduction due to higher levels of membrane

organization. This mobility reduction due to COPII assembly can be correlated to membrane deformations and instability that may indicate budding and fission events. Further, we hope to discover if changes in membrane organization leads to increased curvature, and consequently, more COPII complex assembly.

This potential study, along with other model membrane studies may be used to investigate biological phenomena. Specifically, this technique has potential in modeling diseased states and investigating the effects of defective membrane compositions and binding interactions. We expect this technique to impact the field in biophysical sensing studies and functional membrane protein assays.

4.5 References

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Sato, K., and Nakano, A. (2005). "Reconstitution of Cargo-Dependent COPII Coat Assembly on Proteoliposomes." *Methods in Enzymology*, 404(05), 83–94.

VITA

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EDUCATION

The Pennsylvania State University — August, 2011 - Present
Ph.D. in Bioengineering — University Park, Pennsylvania

University of Notre Dame — August, 2007 – May, 2011
Bachelor of Science in Chemical Engineering — Notre Dame, Indiana

RECENT AWARDS AND HONORS

- Biophysical Society Educational Travel Award, Fall 2012, Penn State University
- William Martin Evans Scholarship, Fall 2010, University of Notre Dame
- Richard E. Duerr Scholarship, Fall 2010, University of Notre Dame
- John F. Dunne Scholarship, Fall 2009, University of Notre Dame
- Frank Huisking Scholarship, Fall 2009, University of Notre Dame
- Dean's List, Spring 2008, University of Notre Dame
- National Merit Finalist, 2007

PLATFORM PRESENTATIONS

1) **Richards, E.E.**, Wostein, H. S. and Majd, S.* "Giant Vesicle Arrays Electroformed from Hydrogel-Stamped Lipid and Protein Deposits". Bioengineering Research Symposium, April 2013, University Park, PA.

POSTER PRESENTATIONS

1) **Richards, E. E.**, Nie, H., and Majd, S.* "Micropatterned Hydrogel Stamping for the Production of Giant Vesicle Arrays to Model Natural Membranes". Biomedical Engineering Society Annual Meeting, October 2012, Atlanta, GA.

2) **Richards, E. E.** and Majd, S.* "Electroformation of Hydrogel-Stamped Lipid and Proteolipid Deposits for the Creation of Giant Vesicle Arrays". Biophysical Society Pennsylvania Network Meeting, September 2012, Lehigh, PA.

3) **Richards, E. E.** and Majd, S.* "Hydrogel-Based Microcontact Printing of Conductive Polymers". Materials Research Society Electronic Materials Conference, June 2012, University Park, PA.

PROFESSIONAL EXPERIENCE

DePuy Orthopaedics May 2010 – August 2010
Johnson & Johnson New Bedford, MA
Manufacturing Engineer Intern

-Researched materials and methods for the design and manufacture of single-use surgical equipment
-Coded videos of several hip replacement surgeries to allow for future study and comparison of techniques and equipment use
- Observed cadaver surgeries for testing of knee replacement instruments

U.S. Army Weapons Development & Integration May 2009 – August 2009
May 2008 – August 2008
Engineering Aid Redstone Arsenal, AL

- Designed models of human brains to determine the effects of explosions
- Modeled parts, equipment, and buildings using Solid Works
- Assisted in engine testing for unmanned aviation vehicles
- Conducted research on the use of nanodiamonds as additives to improve thermal conductivity of polymers

Notre Dame Dean's Office August 2008 - May 2011
Office Assistant Notre Dame, IN

- Aided in communications and organization in the engineering department
- Gained exposure to communication skills vital for efficiency and clarity

SOCIETY MEMBERSHIP

- Biophysical Society (BPS), 2012
- Biomedical Engineering Society (BMES), 2012
- Society of Women Engineers (SWE), Notre Dame undergraduate chapter (2007-2011)

MENTORSHIP EXPERIENCE

- Training and supervising undergraduate researchers
  - Harrison Wostein (August 2012- present), Samantha Corber (June 2012-August 2012), Kayla Schnarrs (March 2012-May 2012)
- Training and supervising graduate researchers
  - Nrutya Madduri (June 2012- October 2012)

RECENT ACTIVITIES AND SERVICES

- Multiple Sclerosis Walk for funding of multiple sclerosis research
- Knights of Columbus fundraising for local Catholic charities
- Project Pink auction for funding cancer research
- 12 Hours of Ultimate fundraiser for a community tutoring center
- Tours of Notre Dame STEM facilities for elementary school students