Interactions of Engineered Silica Nanoparticles with Cell Membrane Models

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This thesis titled
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ABSTRACT

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Interactions of Engineered Silica Nanoparticles with Cell Membrane Models

Director of Thesis: Amir M. Farnoud

The ubiquity of engineered nanomaterials in industrial and biomedical applications has led to higher chances of exposure, raising concerns regarding their cytotoxicity. In this thesis, interactions of engineered silica nanoparticles (104 ± 5 nm), coated with different surface functional groups [hydroxyl, amine, and polyethylene glycol (PEG) 2K, PEG 5K, PEG 20K], with lipid monolayer and lipid bilayer membrane models are investigated. The membrane models are comprised of an equimolar mixture of sphingomyelin, cholesterol, and 1,2-dioleoyl-sn-glycero-3-phosphocholine. Surface tension, surface topology, dye leakage, and fluorescence anisotropy were monitored to elucidate the role of particle surface properties in their interactions with membrane models. Monolayer studies demonstrated that PEGylated particles penetrate the lipid monolayer, regardless of PEG molecular weight, while amine and hydroxyl-coated particles did not affect monolayer surface properties. In contrast, amine and hydroxyl-coated particles induced a time-dependent leakage in lipid bilayer model, demonstrating a loss of its integrity. Taken together, these results indicate that while nanoparticle surface properties play an important role in their interactions with lipids, clear differences exist between nanoparticle interactions with lipid monolayers and lipid bilayers of the same composition. These results will also help predict the potential toxic effects of engineered nanoparticles on the cell membrane.
DEDICATION

to my parents and my sister who have selflessly supported me throughout my life.
ACKNOWLEDGMENTS

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CHAPTER 1: INTRODUCTION*

Nano, a prefix that represents the factor of $10^{-9}$ and is derived from a Greek root, νᾶνος (meaning dwarf), has become an irrefutable part of our everyday life. As Richard Feynman, the physics Nobelist, foresaw the future of nanotechnology in 1959: “There's plenty of room at the bottom”. The National Nanotechnology Initiative (NNI) in early 2000 categorized materials with at least one dimension between 1 and 100 nm as nanomaterials. This definition also considers materials that function uniquely at lower than 1 nm or slightly higher than 100 nm still in the nanoscale range.

*Part of this work has been published as an abstract at the American Institute of Chemical Engineers and the Biomedical Engineering Society 2017 Annual Meetings and is being submitted for a peer-reviewed journal publication, co-authored by Saeed Nazemi Dashtarjandi, Addy Kruse, Katherine Cimatu, Alexander Kelly, Allan David, and Amir M. Farnoud.

Figure 1-1. The classification of nanomaterials based on their dimension (first approach). Materials with only one dimension less than 100 nm are 1D (a), with two dimensions less than 100 nm are 2D (middle) and with all the three dimensions less than a 100 nm are 3D nanomaterials.

Nanomaterials (NMs) following NNI framework can be categorized in many different manners. Firstly it should be noted that nanomaterials can be either fixed...
Different classification of nanomaterials can be based on dimension or composition. Dimension wise, there are two approaches for categorizing the NMs. The first approach is based on the number of dimensions that are in the nanoscale range (Figure 1-1), and according to it, NMs can be either one dimensional such as nanolayers (only one dimension which is the thickness is in the nanoscale range), two dimensional such as nanowires and nanotubes, or three dimensional such as nanoparticles (quantum dot, silica, and fullerene). The second dimensional classification approach categorize nanomaterials based on their number of dimensions out of the nanoscale. Composition wise, there are again two approaches to sort NMs. One is based on the number of materials the NM is made of including single material or composites. The other is based on the chemical composition through which nanomaterials are categorized into carbon based NMs, inorganic NMs (such as metal oxides) or the combination.

Since the start of the NNI program, nanotechnology has been developing very rapidly, and we are currently facing a great variety of nanomaterials being developed and used in different fields. The Nanotechnology Consumer Products Inventory (CPI), developed in 2005 by the Woodrow Wilson International Center for Scholars and the Project on Emerging Nanotechnologies, reported the use of nanomaterials in 1814 consumer products in its updated version in 2013. In addition, vast industrial, diagnostic and therapeutic applications have been reported for nanomaterials, from electronics, food, paints and textile, to bio imaging and drug delivery, which confirm their paramount importance in human life. A class of nanomaterials which are intentionally
synthesized and preferentially modified for meeting specific purposes, are referred to as engineered nanomaterials (ENMs).\textsuperscript{13} To develop nanomaterials for diverse applications, researchers have utilized surface functionalization techniques in order to yield ENMs with higher biocompatibility,\textsuperscript{12,14} colloidal stability,\textsuperscript{15} and blood circulation half-life.\textsuperscript{16}

Among ENMs, engineered silica nanoparticles (SiO\textsubscript{2}) have found vast incorporation in everyday life.\textsuperscript{17–19} A recent survey on the use of engineered nanoparticles in Swiss industry has introduced SiO\textsubscript{2} as one of the few nanoparticles with more than an extraordinary total of 1000 kg/year that companies have used or produced.\textsuperscript{9} The applications reported for engineered silica nanoparticles varies from cosmetics, dental and pharmaceutical applications to wide use in paints, textiles and ceramics.\textsuperscript{9} The increasing incorporation of engineered silica nanoparticles into everyday life has inevitably increased the risk of exposure of human body to such particles, raising concerns regarding nanomaterial health and safety.\textsuperscript{20} Such concerns have particularly been raised in the case of ENMs of wide use such as silica.\textsuperscript{21,22} When inside the body, nanoparticles interact with living cells, which are protected by a plasma membrane. This is the core reason for intensive research in the area of ENM-cell interaction.\textsuperscript{23–25}

However, before discussing the ENM-cell interaction in detail, it is necessary to know more about the other end of this interaction; the cell membrane.

The Cell Membrane

Mammalian cells are surrounded by a plasma membrane which surrounds the cytoplasm and not only protects the intracellular components, but also mediates transport of materials in and out of the cell.\textsuperscript{26} Our knowledge of the cell membranes has been well
developed through the past 50 years. Currently, it is generally accepted that the mammalian cell membrane is comprised of two major components: lipids and proteins, and has a complex and dynamic structure. The four main functions regulated by the cell membrane can be summarized in: separation, exchange, integration and metabolism. The cell membrane is a barrier that separates the intracellular component from extracellular matrix. Protein transporters which are incorporated in the membrane, along with membrane pores and tunneling complexes, are in charge of exchanges through the membrane. The cell membrane is also known to mediate communications with other cells and a portion of metabolic pathways. In order to better understand the cell membrane structure it is useful to understand the characteristics of one of its main components; lipids. Lipids are molecules that have a hydrophobic, non-polar tail and a

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**Figure 1-2.** Lipids are amphiphilic molecules with a hydrophilic polar head and a hydrophobic non polar tail. Phospholipids (left) including saturated, and unsaturated lipids, sphingolipids (middle) and sterols such as cholesterol (right) are the main classes of lipids.
hydrophilic polar head (Figure 1-2). Although many different lipids exist in the membrane which makes it an intricate structure, cell membrane lipids can be categorized into three main classes: phospholipids, sphingolipids and sterols. Phospholipids can be saturated (Figure 1-2 left) or unsaturated. Presence of at least a double bond in one of the fatty acyl chains makes the lipid unsaturated (Figure 1-2 middle). The amount of different lipids present in different cell membranes differs, but reports show that plasma membrane contains 30-40 mol% cholesterol and 10-20% sphingomyelin along with 20-30% phosphatidylcholine. This intrinsic feature of lipids, having hydrophilic and hydrophobic parts, will enable them to form self-assembled bilayer structures in aqueous medium with polar head facing the inner and outer aqueous medium and non-polar tails facing together, forming a lipid bilayer. However, the structure of the cell membrane is beyond a simple lipid bilayer. Proteins are the other major component of the cell membrane which can also be classified to integral and peripheral proteins. The complex and dynamic functions of proteins in the cell membrane, including transport, signaling and enzymatic activities add to the span of cell membrane intricacies.

The understanding on the structure of the cell membrane reached a new level by the emergence of the fluid-mosaic membrane model (F-MMM), developed by Singer and Nicolson in 1972. F-MMM was proposed as a replacement to previously accepted membrane structure models such as the one theorized by Davson and Danielli, which was a trilayer composed of a lipid bilayer with two protein layers on each side of it. F-MMM considered the lipid bilayer as a fluid bed for the integral proteins (mosaic) that
enable them to localize preferentially. However, the F-MMM had the assumption of homogenous lipid bilayer membrane with randomly distributed components. Along with the development of fluid-mosaic model in 1970s, Stier and Sackmann postulated the presence of rigid lipid clusters around reductase proteins in the microsomal membrane of rabbit liver cell. Meanwhile, it took no later than 1980 that this hypothesis was formalized by Klausner et al., who proved the presence of lipid domains based on the heterogeneities observed in fluorescence life time measurements of 1,6-diphenyl-1,3,5-hexatriene (DPH). Ever since, researchers have elucidated different aspects of these lipid domains, as of today, it has been further proved that sterols and saturated lipids have a tendency to aggregate and form ordered lipid domains in the bed of disordered domains made of unsaturated lipids. These ordered domains are also known as lipid rafts, which have been suggested to play a crucial role in protein sorting and cell function. For instance, Brown and Rose have reported the localization of GPI-anchored proteins in glycosphingolipid-cholesterol rich domains which were also detergent resistant.

Taken together, cell membrane is a complex and dynamic structure, composed of various types of lipids, and proteins that are known to preferentially move and localize in the membrane plane. These complexities have challenged investigations on the effect of xenobiotics on such complex structure.

ENM-Cell Membrane Interactions

The increasing incorporation of engineered nanoparticles in industrial and biomedical applications has led to increased chance of exposure, raising concerns about their cytotoxicity. When inside the body, nanoparticles will interact with living cells. A
great body of literature has demonstrated that widely used ENMs induce toxicity to cell lines. Studies on silica nanoparticles with different doses and sizes (10-500 μg/ml and 16, 60, and 104 nm) interacting with A549 cells (rat alveolar cells) revealed that the nanoparticles decrease the cell viability of the test cells, mostly due the significant reactive oxygen species (ROS) that are generated.\textsuperscript{22,49,50} This ROS generation is shown to be accompanied by the reduced amount of glutathione (GSH) levels and the elevated levels of LDH which attributes to lipid peroxidation and membrane damage.\textsuperscript{51} However, a later study claimed that the cytotoxicity of silica nanoparticles are mostly due to ROS generation rather than GSH depletion.\textsuperscript{52} Therefore, while it is known that engineered silica nanoparticles are capable of exerting deleterious effects on mammalian cells,\textsuperscript{49,50,53–57} mechanisms of nanoparticle-induced cytotoxicity are still not completely understood, leading to inconclusive and sometimes conflicting observations.\textsuperscript{53,58} These discrepancies call for more detailed investigations on the interaction of ENMs, specially engineered silica nanoparticles, with cells.

Upon interaction of ENMs with cells, the plasma membrane, a lipid bilayer that surrounds all mammalian cells, is the first cellular entity that comes into contact with exogenous particles. It has been suggested that the cell membrane plays a major role in ENM induced cytotoxicity.\textsuperscript{23,59} Evidenced by various studies investigating nanotoxicity of different ENMs,\textsuperscript{60,61} it is been demonstrated that the interactions (attachment) of ENMs to the membrane of microorganisms is the crucial starting point of the toxic effects. For instance, carbon based nanomaterials antimicrobial activities have been shown to be due to strong nanoparticle-membrane interaction which leads to bacterial cell
death. Moreover, similar activity has been observed by inorganic ENMs. The attachment of silver nanoparticles to the membrane of Escherichia coli (E. coli) is also shown to play the main role in nanotoxicity of the particle by formation of holes in the membrane. Moreover, internalization of ZnO nanoparticles via damaging the E. coli membrane was visualized using transmission electron microscopy (TEM). In addition, ENMs have been shown to induce toxic effect on the membrane of mammalian cell lines and primary cells, which serve as another strong evidence to prove the irrefutable role of plasma membrane in ENMs toxicological pathway. For instance, membrane disruption has been demonstrated to be the major toxic effect of silica nanoparticles on HIH/3T3 cells. Silica particles have been also reported to disrupt and rupture rabbit red blood cells by Shinto et al., while Joglekar et al. only observed deformation in human red blood cells membrane.

While cell membranes are proved to be the initial site for occurrence of nano toxic effects, there are numerous limiting factors in studying nanoparticle-cell membrane interactions using live cells, which hinders uncovering the mechanism of NP-cell membrane interactions. The plasma membrane does not lend itself well to mechanistic studies due to its complex and dynamic structure, composed of various types of lipids and proteins, which might change over time and differ from one cell to the other. On the contrary to the complexities of the cell membrane, membrane lipids composition and behavior have been well studied and defined throughout the years. Utilizing this feature, a number of plasma membrane models, with known characteristics, such as lipid monolayers, supported lipid monolayers and bilayers, and lipid bilayer
vesicles, have been developed to model the cell membrane behavior and study nanoparticle effects on the cell membrane structure and function. Membrane models provide a simple, yet biologically-relevant system, which has provided valuable information on the mechanisms of nanomaterial-cell membrane interactions. However, membrane models that are being utilized for studying ENM-membrane model interaction might in themselves be an artifact, and there are still lack of correlation between results obtained from different membrane models. In addition, to the best of our knowledge, except a few studies, role of membrane phase segregation have been overlooked in ENM-cell membrane model interactions.

Objectives

Interactions of ENMs with the cell membrane have received increasing attention as of late. However, despite a number of fundamental studies, the role of nanoparticle surface properties in altering membrane structure and integrity remains ill-defined. This is in part because different membrane models have been used to mimic the cell membrane in mechanistic studies, while the role of the membrane model itself in regulating nanoparticle-membrane interactions is unclear. The current study investigates the interaction of silica nanoparticles (104 ± 5 nm) with different surface-functional groups: hydroxyl, amine, and polyethylene glycol (PEG, with molecular weights of 2K, 5K and 20K Daltons) with lipid monolayers and bilayers of the same composition, which is an equimolar mixture of sphingomyelin (SM), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and cholesterol (Chol), in an effort to elucidate how changes in
the particle surface properties and the membrane model system might affect the resulting interactions.

We hypothesize that particle-membrane interaction is regulated by particles surface properties. In addition, the characteristics of monolayer and bilayer membrane models interacting with the particle model might also have an impact on the obtained results. To this end, the following aims will be addressed:

Aim 1: Investigate the role of nanoparticle surface properties in their interactions with lipid monolayers.

Aim 2: Investigate the role of nanoparticle surface properties in their interaction with lipid bilayers.
CHAPTER 2: CELL MEMBRANE MODELS CHARACTERIZATION

Introduction

As discussed in the previous chapter, cell membrane is a complex and dynamic lipid bilayer containing proteins, which does not lend itself well to mechanistic studies. Due to all the complexities and lack of control over the membrane function and mechanistic behavior, researchers have utilized lipid membrane models to study the membrane in simpler yet biologically relevant system.

Lipid monolayer and bilayer model membranes were developed thanks to contributions of Langmuir\textsuperscript{71} in 1920s and Bangham and Horne\textsuperscript{72} in 1960s. In the years since, cell membrane models continued to be developed and improved for better resemblance to the mammalian cell plasma membranes resulting in the recent novel membrane models that have incorporated proteins,\textsuperscript{73} phase segregation,\textsuperscript{68,74} and asymmetry\textsuperscript{75} in the structure of the membrane. Figure 2-1 depicts some of the widely used cell membrane models including Langmuir monolayers, supported lipid monolayer and bilayers, and vesicles. Langmuir monolayers are created by spreading lipid at an air water interface and can be utilized as a simple membrane model mimicking the outer

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{cell_membrane_models.png}
\caption{Cell membrane models (left to right): A monolayer at the air water interface, supported lipid monolayer (top) and bilayer (bottom), and a vesicle.}
\end{figure}
leaflet of the plasma membrane. Supported lipid monolayers and bilayers are developed by depositing one or two layers of lipid on a solid support; respectively. These models have been proven to be useful tools for microscopy purposes with more degree of control compared to living cells for mechanistically probing their topology and microstructure changes. Vesicles are self-assembled lipid bilayers that can be considered as a more complex membrane models compared to lipid monolayers. This is mostly because they are spherical lipid bilayer models which mimics cell membrane shape and structure in a more realistic way. Different type of vesicle used for mechanistic studies of cell membrane includes small unilamellar vesicles (SUVs), multilamellar vesicles (MLVs) and giant unilamellar vesicles (GUVs).

Membrane models that have been studied so far are mostly made out of one phospholipid. While these models have been useful in uncovering interactions of the membrane with exogenous materials, they do not mimic some of the complexities of cell membranes of living cells. For instance, one of the important features of the cell membrane, as discussed in chapter 1, is segregation of membrane lipids. The binding affinity of sphingolipids and sterols results in formation of phase segregated domains also known as liquid ordered (Lo) phase. Phase segregated domains have been proven to be crucial for protein sorting. Although functions and structure of phase segregated membrane models have been well studied, phase segregation has mostly been neglected in studies investigating the interaction of ENMs with cell membrane models.

In this chapter, we firstly characterize a single lipid monolayer model and then move to introduction and characterization of ternary lipid mixture monolayer and bilayer
models which mimic phase segregation and can be employed as a more complex model for investigating NP-cell membrane interactions.

Materials and Methods

Commercial Reagents

Brain sphingomyelin (SM), Cholesterol (Chol), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Lipid stock solutions at a concentration of 1 g/L were prepared by dissolving the lipids in 99% HPLC grade chloroform and methanol (4:1) purchased from Fisher Scientific (Hampton, NH). Acetone and ethanol 200 proof used for cleaning purposes in the experiments were purchased from Fisher Scientific and Decon Labs, Inc. (King of Prussia, PA) respectively. Purified water used for all the experiments was obtained from ELGA PURELAB classic (High Wycombe, UK) having the resistivity of 18.2 mΩ·cm. Fresh 10x phosphate-buffered saline (PBS) solutions were prepared by dissolving prepared powder packs (containing 98.8 g PBS powder) obtained from Fisher BioReagents (Fair Lawn, NJ) in 1 liter of purified water. Fluorescent probes 5(6)-carboxyfluorescein (CF) and 1,6-diphenyl-1,3,5-hexatriene (DPH), and Triton X-100 were purchased from Sigma Aldrich (St. Louis, MO).

Tensiometric Studies

Equimolar ternary lipid mixture of SM/DOPC/Chol with a final concentration of 1.32 mM was prepared by mixing proper amounts of each lipid from stock solutions, which were previously prepared at concentration of 1 g/L by dissolving the lipids in 99%
HPLC grade chloroform and methanol (4:1). The amount (in µL) of each lipid required to make an equimolar ternary lipid mixture was calculated using equation 2-1, with \( C_1 \) being the molar concentration of the stock solution, \( V_1 \) the volume to be pipetted from each of the stock solutions in order to make the ternary mixture, \( C_2 \) the molar concentration of the final solution divided by 3, and \( V_2 \) the final volume of the ternary mixture solution. After

\[
C_1 V_1 = C_2 V_2
\]  

(2-1)

*Figure 2-2. KSV NIMA Langmuir-Blodgett trough and its different parts: (1) body, (2) barrier, (3) trough, (4) pressure sensor, (5) LB dipping mechanism and (6) Wilhelmy plate.*

\( V_1 \) micro liters of each stock solution are mixed together, proper amount of chloroform/methanol (4:1) mixture is added to the solution in order to increase the volume to \( V_2 \), and thus the equimolar ternary lipid mixture is prepared.
Interfacial experiments were carried out using a KSV NIMA Langmuir-Blodgett (LB) trough (Biolin Scientific, Finland) (Figure 2-2). This apparatus is equipped with a trough (364 mm × 75 mm × 4 mm, surface area = 273 cm², subphase volume = 176 mL) made of hydrophobic polytetrafluoroethylene (PTFE), having a dipping well (20 mm × 56 mm × 60 mm) in the middle, and two hydrophilic movable barriers made of Derlin, which enable symmetric compression and expansion at the air-subphase interface. The effective surface area of the trough is 243 cm² when barriers are at their highly expanded position.

The trough and the barriers were washed carefully prior to each experiment (Figure 2-3). The washing process started by washing the trough and the barriers with Alconox solution in water (Fisher Scientific), and then rinsing them with purified water. The next step was spraying and brushing the trough and barriers with 70% ethanol and then rinsing them with purified water once more. To start an experiment, purified water was poured in the trough as the fresh subphase, and was given 10 minutes to equilibrate.
Figure 2- 4. The front view of the Wilhelmy plate used for surface tension measurements (a). The force equilibrium which lead to calculation of surface tension is shown in (b). The width of the Wilhelmy plate is negligible and its weight is zeroed out at the start of the experiment.

with room temperature (24.3 ±1.1 °C). Using a vacuum pump, surface of the purified water was aspirated while compressing the surface in order to remove any dust or contamination at the interface. Afterwards, surface tension was measured in real-time using a platinum Wilhelmy plate (W: 19.62 mm, H: 38 mm, T: 0.10 mm) (Figure 2-4a), which was hung from the pressure sensor hook. The Wilhelmy plate yielded the value of 72.3 ± 0.3 mN/m as the pure water surface tension after aspiration, which is in agreement with reported values in the literature.Using a Wilhelmy plate surface tension can be calculated by a force balance (Figure 2-4b) shown in equation 2-2,

\[ \gamma = \frac{F}{2L \cos \theta} \]  

(2-2)

where \( \gamma \) is the surface tension (mN/m), \( F \) is the vertical force applied to the plate (mN), \( L \) is the length of the Wilhelmy plate (m), and \( \theta \) is the angle between the plate and the liquid.
The weight of the Wilhelmy plate was zeroed using the KSV NIMA LB software after getting connected to the hook and prior to being placed in water. Surface pressure was calculated accordingly, by subtracting the surface tension of pure water from the surface tension measured by the device after the lipids were added using equation 2-3,

\[
\Pi = \gamma_{pure} - \gamma_{lipid}
\]

(2-3)

where \( \Pi \) is the surface pressure (mN/m), \( \gamma_{pure} \) is the surface tension of pure water and \( \gamma_{lipid} \) is the surface tension after lipids are spread.

Afterward, 32 µL of 1.32 mM lipid solution was spread at the air-water interface using a Hamilton micro-syringe (Hamilton Company, Reno, NV) and was given 15 minutes for the organic solvents to evaporate. Due to the intrinsic feature of the lipids, having a hydrophobic head and a hydrophobic tail, a monolayer is self-assembled at the air-water interface (Figure 2-5e inset, size of the lipids are exaggerated). Surface pressure of the monolayer model was measured and recorded while the surface was being compressed by two barriers. Previously, the Langmuir trough had Teflon barriers, which have been replaced with hydrophobic Delrin barriers. Hydrophobic Delrin barriers are shown to reduce the chances of lipid leakage out of the trough surface area.
Figure 2-5. The Langmuir trough experimental procedure, a) The clean trough is placed in the experiment setup, b) barriers are placed on top of the trough, c) the trough is filled with purified water, d) the Wilhelmy plate is hanged from the pressure sensor hook (shown in Figure 2-1-4), e) lipids are spread using a Hamilton micro syringe, f) lipid monolayer is formed at the air water interface.

Surface pressure – surface area or mean molecular area - the mean area occupied by a surfactant molecule- also known as Π -A isotherms were generated in real-time upon surface compression. A summary of the experimental procedure is demonstrated in Figure 2-5.

Atomic Force Microscopy (AFM) of Supported Lipid Monolayer

Langmuir-Blodgett deposition was used to generate supported lipid monolayers for AFM. For these experiments a hydrophilic cover glass (VMR®, Randor, PA) made of borosilicate with the thickness of 0.13 mm and diameter of 32 mm was used as the substrate. The cover glass was washed using acetone, ethanol, and purified water and dried carefully prior to each experiment. After washing and drying, the cover glass was put in the dipper sample clip holder, and the dipper position was zeroed when the cover glass was barely touching the surface of the subphase. The dipper was then inserted
inside the subphase until it was fully immersed in the subphase (-26 mm vertical displacement). The SM/DOPC/Chol (1:1:1) solution was then spread while the cover glass was immersed in the subphase. At the desired surface pressures (5 mN/m, 20 mN/m and 30 mN/m), the cover glass was pulled up with the speed of 5 mm/min allowing for the deposition of the monolayer while the barriers kept the surface pressure constant.

Atomic force microscopy (AFM) was performed to study the topology of the monolayers deposited on the cover glass using Asylum Research MFP-3D™ AFM system (Santa Barbara, CA). The device was equipped with a silicon 7 nm cantilever tip with spring constant of $k = 26$ N/m. Measurements were carried out in tapping mode.

*Vesicle Preparation*

Large unilamellar vesicles were synthesized following the methods of Zhang et al.\(^8^6\) Briefly, 10 mM equimolar lipid mixture of SM/DOPC/Chol was prepared by dissolving proper portion of lipids in chloroform. The calculations for the amount of each lipid were done using equation 1. The mixture was dried under nitrogen gas (2 psi pressure) for 15 minutes. The sample was then placed inside a desiccator overnight in order for the extra chloroform to evaporate. The lipid film was then rehydrated using 1x PBS (PH=7.4) at 70 °C, above the melting temperatures of all three lipids. The obtained solution was vortexed for 5 minutes so that multi lamellar vesicles (MLVs) were self-assembled. The MLVs were then exposed to 7 cycles of freeze and thaw using an acetone bath and a water bath (70 °C) to produce large unilamellar vesicles (LUVs).\(^8^7,^8^8\) Dynamic light scattering was used in order to measure the hydrodynamic size of the vesicles using a 90 Plus particle sizer (Brookhaven Instruments, Holtsville, NY). In order to reproduce
the literature, sphingomyelin only and DOPC only MLVs were also produced using the above mentioned method.

**DPH Fluorescence Anisotropy**

**Anisotropy Measurement Theory and Concepts**

Fluorescence anisotropy was employed to shed light on the fluidity of the bilayer membrane models. Rotational motion of the fluorophore molecules can be detected by their polarized emission upon their excitation. Anisotropy, a dimensionless number, is the ratio of the difference in intensity of linearly polarized emission components to the whole

![Figure 2-6](image-url) The L-shaped spectrofluorometer used for anisotropy measurements. Lamp, monochrometers, polarizers, sample compartment and detector are the main parts of the spectrofluorometer. Excitation light passes through the monochromator and light with a specific wavelength will enter the excitation polarizer (P1) and gets vertically polarized. Polarizers can rotate and produce vertical or horizontal components. Emission light will again pass through the emission monochromator to obtain a specific wavelength. Emission light will then pass through vertically and horizontally rotated emission polarizer and both V and H components of emission will be measured by the detector (only V position is shown).
fluorescence intensity of the emission. The measurements were carried out using a Fluorolog®-3 spectrofluorometer. As shown in Figure 2-6, the device is L-shaped and include a light source, two polarizers, the sample holder and the detector. Monochromic light initially passes through the first polarizer (excitation polarizer) and in case oriented in vertical direction, only vertically polarized light will pass and hit the sample. Afterwards, the fluorophores that are in the same orientation with the polarized light will get excited. However, their rotational movements, which changes with respect to how fluid or rigid its environment is, will result in an emission which is not in the same orientation as the vertically polarized excitation light. The second polarizer (emission polarizer), which is alternately rotating between vertical and horizontal modes, is in charge of polarizing the emitted light in both vertical and horizontal directions. The detector then measures the intensity of the vertically and horizontally polarized components of the emitted light and anisotropy (r) will be calculated accordingly using equation 2-4,

$$ r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} $$

(2-4)

where $I_\parallel$ is the intensity of the polarized emission parallel to the excitation polarization and $I_\perp$ is the intensity of the polarized emission perpendicular to excitation polarization. This equation is the theoretical definition of anisotropy. However, in experiment condition due to dissimilar sensitivity of the detecting compartment to the vertically and horizontally polarized light, correction factors should be calculated to reduce device error in calculations of anisotropy. It is been shown that the emission that passes through the monochromometer has different sensitivities to vertical (Sv) and horizontal (Se) emissions.\textsuperscript{89}
Therefore, the intensities that would be detected by the device will be different than the ideal intensities. Knowing that we have vertical excitation polarization,

\[ I_{VV} = kS_V I_l \]  \hspace{1cm} (2-5)
\[ I_{VH} = kS_H I_l \]  \hspace{1cm} (2-6)

where \( k \) is other instrumental factors that might affect both polarizations similarly. \( I \) is the fluorescence intensity and the first subscript in all terms stands for the orientation of the excitation polarizer and the second subscript for the orientation of emission polarizer. \( I_{VV} \) is the vertically polarized emission (that has been caused by vertical excitation) detected by the device and \( I_{VH} \) is the horizontally polarized emission (that has been caused by vertical excitation) detected by the device. Therefore, dividing the two equations, the ratio of the measured intensities should be multiplied a factor \((1/G)\) to be equal to the actual polarized intensity ratio (equation 2-7),

\[ \frac{I_\parallel}{I_\perp} = \frac{1}{G} * \frac{I_{VV}}{I_{HV}} \]  \hspace{1cm} (2-7)

\[ G = \frac{S_V}{S_H} \]  \hspace{1cm} (2-8)

In order to be able to determine the actual linear intensities we only need to calculate \( G \), the grating factor, since \( k \) is already canceled out. This can be achieved easily, by horizontal excitation. Since the fluorophore will be in the direction of the detecting axis when horizontally excited, its linear components in the plane perpendicular to the observation will be equal. This is because the electrical field around observation axis is
Figure 2-7. The fluorophores are excited by horizontally polarized excitation. The horizontal and vertical components are equal prior to passing through the monochrometer. The emission polarizer detect the experimental horizontal and vertical polarized intensities.

symmetrically distributed. Therefore both vertical and horizontal component will be equal and perpendicular to the horizontal excitation \( I_\perp \) (Figure 2-7). Therefore, G is calculated by the intensity of the polarized lights ratio:

\[
\frac{I_{HV}}{I_{HH}} = \frac{S_v \cdot I_\perp}{S_h \cdot I_\perp} = G
\]  

(2-9)

Finally, r can be calculated by substituting \( \frac{I_H}{I_\perp} \) with \( \frac{1}{G} \cdot \frac{I_{VV}}{I_{HV}} \) in the equation 2-4.

\[
r = \frac{I_{VV} - G \cdot I_{VH}}{I_{VV} + 2G \cdot I_{VH}} = \frac{I_{VV} \cdot I_{HH} - 1}{I_{VV} \cdot I_{HH} + 2}
\]  

(2-10)

When the experiments are set to be conducted by vertical excitation polarization at fixed wavelengths, the G factor is calculated by the device prior to the experiment by rotating the excitation polarizer and calculating \( I_{HV} \) and \( I_{HH} \). Since the excitation and emission wavelengths remains the same, the calculated G is applicable through the whole experiment. This approach for measuring anisotropy has been addressed the literature.\(^{90,91}\)
Sample Preparation

In our study, determining changes in the membrane fluidity is achieved by monitoring the rotational movements of the lipophilic fluorophore, diphenyl hexatriene (DPH), in the SM/DOPC/Chol (1:1:1) lipid bilayer with which the alterations of the lipid ordering due to changes in the temperature or particle entrapment can be examined accordingly. For sample preparation, the lipophilic fluorescent probe DPH was dissolved in HPLC grade acetone and was added to the vesicle solutions at a small amount (0.2 mol %). LUVs or MLVs were diluted in quartz cuvettes (Hellma Analytics, Germany) with a total volume of 2 mL. Anisotropy measurements were carried out versus temperature (from 10 ºC to 60 ºC) at each 5 ºC intervals for 500 μM sphingomyelin only, DOPC only and SM/DOPC/Chol, each with 0.2 mol % DPH vesicles. Samples were given 5 minutes to equilibrate with the environment’s temperature at each interval after the sample holder reached the desired temperature.

Vesicle Leakage Assay

CF loaded LUVs were prepared with the same procedure as LUVs preparation explained using the method of Zhang et al., except 80 mM CF solution in 1x PBS (PH=7.4), 1M NaOH solution at 70 ºC, was added in the rehydration step. In order to remove the excess unloaded CF from the medium, disposable PD-10 desalting column containing 8.3 mL of SephadexTM G-25 medium was used (GE Healthcare, Sweden). The porosity of the SephadexTM G-25 gel inside the column retains the smaller CF particles and loaded liposomes pass through it. The 3.5 mL elute containing CF loaded LUVs was diluted to a lower concentration for the leakage measurements. Leakage
experiments were carried out using Fluorolog®-3 spectrofluorometer, (Horiba Instruments Inc., Edison, NJ), equipped with FluorEssence™ Software. CF excitation and emission wavelengths are 492 nm (λex) and 517 nm (λem), respectively. Emission spectra versus excitation measurements were carried out to confirm that the excitation peak for emission at 517 nm was at 492 nm. LUVs were diluted to 50 µM in quartz cuvettes with a total volume of 2 mL. The cuvettes were then placed inside the instrument sample holder. Spectra experiments versus emission were conducted at λex=492 nm with λem ranging from 500 nm to 535 nm. The fluorescence intensity at λem =517 nm was monitored every 150 seconds over the course of 60 minutes for all samples. CF fluorescence is self-quenched when encapsulated at 80 mM; the release of the dye from the liposomes, caused by membrane disruption, leads to its dilution below the self-quenching limit, resulting in an increase in fluorescence. Triton X-100 with a concentration of 0.2% (v/v) was added after 60 minutes to wash away the lipids and release the encapsulated CF completely. The fluorescence intensity from fully disrupted detergent treated vesicles was considered as 100% leakage. CF leakage percentage is calculated and normalized utilizing equation 2-11,

\[
\text{% Leakage} = \frac{F_T - F_0}{F_{100} - F_0}
\]  

(2-11)

where \( F_T \) is the fluorescent intensity measured at each interval, \( F_0 \) is the fluorescent intensity of the control before particles are added, and \( F_{100} \) is attributed to the fluorescence of vesicles that are fully disrupted by detergent.
Results and Discussion

DPPC Monolayer Isotherm and Comparison to the Literature

Surface pressure isotherms of DPPC dissolved in chloroform were generated to ensure that the protocol led to results that were similar to those reported in the literature. Prior to that, the surface activity of water and chloroform alone were obtained in order to check the background and make sure that there is no surface activity for the subphase.

Figure 2-8. Surface activity of (a) water and (b) chloroform at the air water interface.

As shown in Figure 2-8, no surface activities were observed for either water or chloroform as evidence by maximum surface pressure values of 0.04 mN/m and 0.05 mN/m at the end of compression; a requirement for moving to the next step which was addition of lipids at the air water interface. The surface activity of DPPC monolayers have been well studied in the literature; thus, DPPC was chosen for
preliminary experiments and comparison to the literature. DPPC monolayer is known to experience different phases along with compression. As shown in Figure 2-9, good agreement with the literature can be observed. Our result demonstrate the phase changes in monolayer model in agreement with the literature, as the monolayer firstly experiences a low surface pressure phase (near zero) which is known as gas phase, then enters the liquid expanded phase in which surface pressure increase linearly. Then the monolayer experiences a plateau in which both liquid expanded and liquid condensed phases coexist. Afterwards, there is an exponential rise in the surface pressure followed by a plateau which denote liquid condensed phase and membrane collapse respectively.

After reproducing the single lipid monolayer model membrane successfully, we can proceed to the synthesis and characterization of the SM/DOPC/Chol (1:1:1) monolayer model which is later employed as one of the membrane models interacting

Figure 2-9. Surface pressure – surface area isotherm of DPPC at room temperature compressed to mean molecular area of 20 (Å²/molecule) (a), which demonstrates a similar shape and trend compared to the previously reported isotherm by Farnoud and Fiegel (b). Reprinted with permission. Error bars demonstrate the standard deviation from at least three independent experiments.
with ENMs. Having three lipids in its structure, ternary SM/DOPC/Chol monolayer model is definitely a step forward in monolayer model complexity.

**SM/DOPC/Chol Monolayer Isotherm and Topography and Comparison to the Literature**

The monolayer used as a model membrane in our study, is an equimolar mixture of sphingomyelin, 1,2-dioleoyl-sn-glycero-3-phosphocholine and cholesterol, SM/DOPC/Chol (1:1:1), dissolved in chloroform and methanol (4:1). The lipid mixture includes one saturated lipid (SM), one unsaturated lipid (DOPC), and one sterol (Chol), and has been widely used as a cell membrane model to study membrane structure and function. The molecular structure of the lipids is demonstrated in Figure 2-10. Prior to generating the isotherm, chloroform and methanol need to be examined to ensure that they are not surface active. The lack of surface activity for chloroform was shown in Figure 2-8a earlier in this chapter. Methanol was also examined when spread at

![DOPC](image1.png)

![SM](image2.png)

![Chol](image3.png)

*Figure 2-10. Molecular structure of the lipids in the membrane model. Images obtained from Avanti Polar Lipids website.*

...
the air-water interface to ensure the lack of surface activity, which was confirmed by the observation that a maximum surface pressure of 0.10 mN/m was achieved when methanol was compressed to a surface area of 52 cm$^2$ (Figure 2-11). The surface pressure isotherm of the SM/DOPC/Chol (1:1:1) ternary lipid model was obtained as the control.

Figure 2-11. Surface activity of methanol.

Figure 2-12. Surface pressure isotherms for SM/Chol/DOPC (1:1:1) was similar to the literature. The reproduced SM/Chol/DOPC (1:1:1) in the lab (a), showed agreement with the SM/DOPC and 33% of Chol (curve with the red circles) generated by Yuan et al. (b). The graph shows the effect of addition of cholesterol to a SM/DOPC (1:1) binary system and the resulting shift to the left. Red circles were not present in the original version and are added for facilitating the comparison. Reprinted with permission. Error bars demonstrate the standard deviation from at least three independent experiments.
for monolayer experiments and later interactions of this model membrane with silica nanoparticles. The results were compared with the literature (Figure 2-12) to ensure the accuracy of the isotherm. In this isotherm, the surface pressure started from a value close to zero and remained almost constant (0.33 ± 0.30 mN/m) in the early stages of compression (A=243 cm$^2$ to 191 cm$^2$ corresponding to mean molecular areas of 92 to 73 Å$^2$/molecule). At this range, the lipid mixture is known to be in the gas phase and lipid molecules have free lateral movement with respect to each other. Afterwards, the surface pressure underwent an exponential rise, which is a transition from gas phase to a mixture of liquid ordered and disordered phase until the monolayer collapses at the

*Figure 2-13.* The control isotherm (left) and the phase evolution of the phase segregate SM/Chol/DOPC monolayer shown by AFM. The emergence of liquid ordered domains at surface pressure of 20 mN/m and their consequent merging upon compression to 30 and 40 mN/m.
surface pressure of 45 mN/m (surface area of A=110 cm²). This phase evolution can be visually observed utilizing AFM of supported monolayers. AFM records the force variations and deflection that occurs at the head of a cantilever tip using a laser beam which is emitted to the tip, to measure the height difference on the model and image the surface at the scale of nanometers. As shown in Figure 2-13, ordered domains were formed and merged together upon compression, representing phase segregation in the SM/DOPC/Chol (1:1:1) membrane model. This domain formation behavior for the SM/DOPC/Chol is expected. It has been well demonstrated that in ternary lipid mixtures with similar lipid composition, ordered domains are formed in the fluid bed of unsaturated lipids (DOPC here) due to the binding affinity of SM and Chol, 68,81,82,98 and merged together upon compression.67,81

*LUVs Leakage Upon Addition of Detergent (Triton-X)*

Vesicle leakage can provide valuable insights on the effects of exogenous materials interacting with vesicles as a cell membrane models. We were able to synthesize large unilamellar vesicles with the average size of 1.1 um following the conventional procedure. Leakage assay was performed for the SM/DOPC/Chol (1:1:1) LUVs and the vesicles were shown to maintain their integrity over the course of one hour demonstrated by percentage leakage which at its maxima was 2.41± 0.09 % leakage (Figure 2-14). The 100% leakage was achieved by adding small amount of Triton X detergent (0.2% v/v) after 60 minutes which disrupt the liposome and completely release the encapsulated CF.
Figure 2-14. Leakage percent of the CF loaded SM/DOPC/Chol (1:1:1) LUVs. The result is reported as percentage of vesicle leakage, calculated as the ratio of CF fluorescence after the time zero, over fluorescence after the addition of Triton X-100. Error bars demonstrate the standard deviation from at least three independent experiments. Error bars that are not visible have small values.

**DPH Anisotropy of LUVs**

DPH anisotropy was used as a method to shed light on the fluidity and lipid order of the bilayer membrane model over increase in the temperature. DPH anisotropy increases with an increase in lipid order. Utilizing this technique one can predict the ordering of lipids and the effects of exogenous particles being entrapped in the lipid bilayer, which is expected to reduce the area available to lipid molecules, thus increasing their order, which is expected to increase DPH anisotropy. To examine lipid ordering, the
fluorescence anisotropy of vesicles was examined after incorporation of DPH. Firstly, the DPH anisotropy of the single lipid models was measured. DPH anisotropy of 500 uM sphingomyelin and 500 uM DOPC large MLVs were obtained and compared to the previously obtained data, showing good agreement (Figure 2-15). Furthermore, the DPH

Figure 2-15. Anisotropy measurements vs. temperature for sphingomyelin only and DOPC only MLVs (a) and comparison to the previously obtained data by my adviser, Dr. Farnoud (b). Error bars demonstrate the standard deviation from at least three independent experiments.

Figure 2-16. Anisotropy measurements vs. temperature for 500 uM SM/DOPC/Chol (1:1:1) LUVs. Measurements was carried between 10 ºC to 60 ºC at each 5 ºC interval. Equilibrium time at each interval was 5 minutes. Error bars demonstrate the standard deviation from at least three independent experiments.
anisotropy of the main cell membrane model in our study, SM/DOPC/Chol (1:1:1) LUVs (Figure 2-16), was obtained which demonstrated similar trend to a similar but not exactly the same model (POPC/SM/Chol).\textsuperscript{87} It was demonstrated that increasing the temperature reduced anisotropy which is expected as increasing the temperature reduces the lipid order in the vesicles.\textsuperscript{87,91,99}

Conclusion

In this chapter, synthesis and characterization of monolayer and bilayer model membranes with single and ternary lipid composition were presented. Monolayer models have been widely used to mimic lung surfactant behavior,\textsuperscript{83,94,100} and the outer leaflet of the cell membrane,\textsuperscript{66} while lipid bilayer vesicles have been mostly used to mimic cell membrane structure and function.\textsuperscript{25,40,41,69,87,91} Starting with a single lipid monolayer model, DPPC monolayer was successfully characterized and its surface pressure isotherm was obtained and compared with the literature,\textsuperscript{94} demonstrating good agreement. More importantly, the ternary monolayer and bilayer models composed of SM/DOPC/Chol were synthesized and characterized, utilizing various methods. Surface pressure isotherm of the SM/DOPC/Chol demonstrate good agreement with the literature.\textsuperscript{81} AFM of the SM/DOPC/Chol supported lipid monolayers revealed the presence of ordered domains at high surface pressures. In addition, LUVs were prepared and characterized using dynamic light scattering, leakage assay and DPH anisotropy. The anisotropy values were also compared to a not exact but similar model and showed similar trends. All in all, the presented results in this chapter provide a strong basis for continuing to the NP-membrane model interaction studies.
CHAPTER 3: INTERACTIONS OF SILICA NANOPARTICLES WITH
MONOLAYER MEMBRANE MODELS

Introduction

Langmuir monolayers, named after the chemistry Nobelist, Irwing Langmuir, are dispersion of an insoluble layer of amphiphilic molecules at the air-liquid interface.97 One of the well-known Langmuir monolayers is the lipid monolayer at the air-water interface. Having their nonpolar hydrophobic tails facing the air and the polar hydrophilic head on water, these monolayers can mimic the outer layer of the plasma membrane. Although a simple model far from the plasma membrane actual structure, monolayer membrane models provide great information on lipid packing, microstructure and topology of the membrane model upon interaction with exogenous materials.101 Therefore, extensive research has been conducted investigating the interaction of nanoparticles with various lipid monolayer models.66,67,93,94,102

Researchers have extensively looked at the effects of nature and surface properties of ENMs on their interactions with lipid monolayers, mostly comprised of single or binary lipid mixtures. For instance, Guzmán et al.,93 have investigated the effect of two nanoparticles on a lipid monolayer model consisting of a single phospholipid, dipalmitoylphosphatidyl choline (DPPC), and found that the silica nanoparticles had more pronounced effects on the mechanical properties of the monolayer, such as phase behavior, in comparison with the other particle, carbon black. The single lipid monolayer system has been also used to study the effects of other engineered particles such as polystyrene, Polymer capped magnetic nanoparticles and polyorganosiloxane.
Monolayer systems were later improved by the addition of other lipids, such as the unsaturated phospholipid dioleylphosphatidyl choline (DOPC), saturated dipalmitoylphosphatidyl glycerol (DPPG), and cholesterol. However, except for a few studies, the presence of sterols, and sterol-mediated ordered lipid domains in the monolayer membrane models has been overlooked. Sterols promote the formation of phase segregated domains due to their affinity to saturated lipids which is an important phenomena for the proteins to be able to function properly. Moreover, the role of membrane model itself in the interaction and its correlation to other model membranes has not been well addressed before. Therefore, the extrapolation of such data to other models or living cells seems to be conflicting.

In this chapter, we try to address this shortcoming by investigating the interaction of silica nanoparticles, which have broad applications in the biomedical research, food industry, as well as in cosmetics industry, with lipid monolayer membrane models consisting of three lipids, mimicking phase segregation. The results presented in this chapter, elucidate the mechanisms of interaction between engineered particles and the SM/DOPC/Chol monolayer membrane models. These results will help predict the potential cytotoxic effect of silica nanoparticles with different surface properties and can be correlated to bilayer model membrane investigations.
Materials and Methods

Figure 3-1. Particle model used for nanoparticle cell membrane model interaction. From left, hydroxyl, amine, PEG 2K, 5K and 20K Da coated silica nanoparticles

Five differently coated silica nanoparticles (hydroxyl, amine, PEG 2K, PEG 5K and PEG 20K) were used as the particle model in this study. Particles were prepared and partially characterized by Dr. Allan David’s group, Chemical Engineering Department, Auburn University, following the procedure discussed in the appendix. A schematic of the particle model is demonstrated in Figure 3-1.

Commercial Reagents

The information regarding the commercial reagents used in this chapter are already provided in Chapter 2.

Exposure of the Particle to Monolayer Models

In order to study the effect of silica nanoparticles on the SM/DOPC/Chol (1:1:1) surface pressure isotherm, obtained by LB trough apparatus, the monolayer model was exposed to silica nanoparticles with different coatings at three different concentrations (0.0001 g/L, 0.001 g/L and 0.01 g/L). Briefly, the through was carefully washed and filled with fresh purified water at room temperature. The SM/DOPC/Chol lipid mixture was spread at the air-water interface and was given 15 minutes to ensure the evaporation
Figure 3-2. The procedure for nanoparticle-lipid monolayer interaction experiments: Clean trough is placed on the LB setup (a), purified water is poured as the subphase (b), lipids are spread using a Hamilton syringe (c), and particles are added into the subphase beneath the monolayer (d).

of organic solvents. Meanwhile, using a Hamilton micro-syringe, silica nanoparticles at desired concentration were injected into the subphase, while surface pressure was being monitored to ensure the monolayer was not disturbed by inserting the syringe into it. The schematic of the experimental procedure can be seen in Figure 3-2. The experiment was started five minutes after the injection to provide the particles with some time for equilibration and dissipation. Each surface pressure isotherms were obtained at least three times. The AFM sample preparation was performed as described in Chapter 2. The only difference was adding the particles in the abovementioned manner before pulling up the dipper.

Results and Discussion

Particle Characterization

Nanoparticle characterizations were partially carried out by Dr. Allan David’s group (Chemical Engineering department, Auburn University) and the results are provided in the appendix.
In addition, we also performed TEM analysis for PEGylated particles (Figure 3-3) and the mean diameter of PEG 2K, PEG 5K and PEG 20K were $103.71 \pm 3.83 \text{ nm}$, $103.88 \pm 5.23 \text{ nm}$, $104.24 \pm 5.20 \text{ nm}$ with a mean value of approximately $104 \pm 5 \text{ nm}$. Due to the lower standard deviation of our measurement compared to what was measured by Dr. David group for amine and hydroxyl coated particles, $104 \pm 5 \text{ nm}$ was presented as the representative bare nanoparticle size throughout the thesis.

**Effect of Silica Nanoparticles on SM/DOPC/Chol Surface Pressure Isotherms**

Lipid monolayers have been a popular model in studies of nanoparticle-biomembrane interactions, mimicking the outer leaflet of the cell membrane $^{66,67,93,94,102,103,106,107}$. The effects of silica nanoparticles on the lateral packing of the lipid
monolayer model were examined by monitoring the surface pressure isotherm of lipids after injection of the particles in the subphase. Equimolar mixture of sphingomyelin, 1,2-dioleoyl-sn-glycero-3-phosphocholine and cholesterol, SM/DOPC/Chol (1:1:1), was used as the membrane model. As explained earlier, this lipid mixture includes one saturated lipid (SM), one unsaturated lipid (DOPC), and one sterol (Chol), and not only has been widely used as a cell membrane model to study membrane structure and function\textsuperscript{73,75,81}, but also has recently gained attention for investigating NP-cell membrane interactions.\textsuperscript{67,68,108} Using a Langmuir-Wilhelmy apparatus, which allows real-time measurement of surface tension, effect of particles on the surface pressure of the lipid monolayer upon compression was investigated. In this setup, penetration of particles in the lipid monolayer can be monitored based on changes in the surface pressure isotherm as particle penetration in the monolayer is generally expected to increase the lateral packing of the lipids\textsuperscript{109,110}, thereby increasing the surface pressure. The control isotherm was previously discussed in chapter 2 (Figure 2-12), which was obtained in the absence of particles, and demonstrated agreement with the ternary SM/DOPC/Chol lipid mixture isotherms previously reported in the literature.\textsuperscript{81}
Figure 3-4. The effects of different concentrations (0.0001 to 0.01 g/L) of amine coated (a), and hydroxyl coated (b), silica nanoparticles on the surface pressure-surface area (Π-A) isotherms of SM/DOPC/Chol (1:1:1). Along with the surface activity of the highest concentration of amine coated (c), and hydroxyl coated (d), silica nanoparticles. Highest values of surface activity were 0.04 ± 0.02 and 0.01 ± 0.01 mN/m for amine and hydroxyl coated silica particles. For surface pressure isotherms, particles were injected into the purified water subphase after the lipid monolayer was spread. The surface was compressed at a rate of 10 mm/min between 243 and 53 cm². Error bars show the standard deviation from at least three independent experiments.

Addition of silica particles to the subphase after spreading the lipid monolayer resulted in slight changes in the surface pressure isotherm of the SM/DOPC/Chol (1:1:1) monolayer, depending on particle surface properties. Surface pressure isotherm of the monolayer was not altered by addition of different concentrations of anime and hydroxyl coated particles (0.0001 to 0.01 g/L) into the subphase (Figure 3-4 a and b). In addition, amine and hydroxyl coated particles did not alter the surface pressure of pure water at the
Figure 3-5. The effects of different concentrations (0.0001 to 0.01 g/L) of PEG 2K (a), PEG 5K (b), and PEG 20K (c), coated silica nanoparticles on the surface pressure-surface area (Π-A) isotherms of SM/DOPC/Chol (1:1:1), along with the surface activity of the highest concentration of the PEG 2K (d), PEG 5K (e), and PEG 20K (f), coated silica nanoparticles. For surface pressure isotherms, particles were injected into the purified water subphase after the lipid monolayer was spread. The surface was compressed at a rate of 10 mm/min between 243 and 53 cm². Error bars show the standard deviation from at least three independent experiments.

The air-water interface demonstrating no surface activity which can be correlated to their no alternation of surface pressure isotherms. On the contrary, PEGylated particles increased
the packing of lipids at the early stages of compression in a dose-dependent manner, 
evidenced by the shift in the surface pressure isotherm in surface areas of 243 cm$^2$ to 170 
$\text{cm}^2$ (Figure 3-5 a-c), suggesting that particles are incorporated in the monolayer high 
surface areas, but are then squeezed out of the monolayer as the surface pressure 
increases to a certain values. This is in direct correlation with PEG surface activity as 
PEGylated particles showed minor surface activity (maximum surface pressure of 2.56 ± 
0.14, 4.02 ± 0.77 and 8.16 ± 0.18 mN/m for 2K, 5K and 20K PEG; respectively) when 
added in the absence of lipids (Figure 3-4 d-f). Despite the fact the molecular weight of 
the PEG coatings did not play a role in the interactions of particles with the monolayer 
model as the particles coated with PEG 2K, 5K, and 20K all demonstrated similar effects 
on the surface pressure isotherm of the monolayer, in terms of surface activity, PEG 20K 
had more surface activity than PEG 5K and 2K (significantly different evidenced by t-test 
results, P<0.0001). This is in agreement with tensiometric studies on pure-PEG or PEG-
modified lipids, reporting that high molecular weight PEG molecules are more surface-
active compared to low high molecular weight PEG molecules.$^{111,112}$ These results 
suggest that the surface-activity of the PEGylated particles dominate their interaction 
with lipid monolayers allowing the particles to penetrate the air-water interface at high 
mean molecular areas before being squeezed out upon compression.

The effect of PEGylated particles on the surface pressure isotherm of the lipid 
monolayer, suggested that particles are incorporated in the monolayer at low surface 
pressure, but are squeezed out of the monolayer at high surface pressure values. This
possibility, which has been previously suggested for other particles\textsuperscript{110}; was further investigated using atomic force microscopy (AFM).

*Penetration of Silica Nanoparticles into SM/DOPC/Chol Supported Lipid Monolayers*

The incorporation of particles into the monolayer was further investigated by studying the topology of deposited monolayers at different surface pressure values (5 mN/m, 20 mN/m, and 30 mN/m) using AFM. AFM analysis of supported lipid monolayers and bilayers has served as a valuable tool for studying the effect of particles on membrane topography.\textsuperscript{81,98,113} Various studies have visualized particle penetration to the membrane model utilizing AFM,\textsuperscript{67} while some studies have demonstrated the effect of nanoparticles on the lipid topography.\textsuperscript{94} As shown in Figure 2-9, in the previous chapter, ordered domains were formed and merged together upon compression, representing phase segregation in the SM/DOPC/Chol (1:1:1) membrane model, which is well discussed in the literature.\textsuperscript{68,81,82,98} AFM studies were performed on lipid monolayers deposited on glass substrates using the Blodgett technique, after the particles were injected beneath the monolayer into the subphase at 0.01, to examine whether nanoparticles penetrated the monolayer.
Figure 3-6. Surface topography of deposited SM/DOPC/Chol (1:1:1) monolayers characterized by atomic force microscopy at 5 mN/m (left column), 20 mN/m (middle column), and 30 mN/m (right column) without the presence of particles (a), and after exposure to 0.01 g/L of amine (b), hydroxyl (c), PEG 2K (d), PEG 5K (e), and PEG 20K (f), coated silica nanoparticles. At least two experiments were carried out for each microscopy.
AFM images demonstrated that amine and hydroxyl coated silica particles do not penetrate the lipids at the air-water interface, as observed by the height range (Figure 3-6). However, AFM images obtained for the monolayer deposited in presence of PEGylated particles, demonstrated significant incorporation of particles into the monolayer at 5 mN/m, confirming the trends observed in monolayer studies (Figure 3-5 a-c). The height differences of more than 100 nm (Figure 3-6 f left) suggest that some of the particles penetrated the monolayers as aggregates. Interestingly, none of the PEGylated particles were present in the deposited lipid monolayers at higher surface pressure values of 20 mN/m and 30 mN/m, and no significant changes to the domain formation was observed (Figure 3-6 e-f middle and right columns). These findings were in good agreement with the surface pressure isotherm results, which demonstrated no effects for amine and hydroxyl coated particles and presence and subsequent elimination of PEGylated particles from the monolayer with increase in the surface pressure. In contrary to our finding, penetration of 15 nm negatively charged silica particles in DPPC/DOPC/Chol (55:34:11) ternary membrane model has been previously reported in the literature, and it was claimed that particle penetration reduced the size of ordered domains. These differences could be due to the smaller size of the particles used in that study (15 nm vs. 105 nm in this study), higher dose of exposure (10 g/L vs. 0.1 g/L in this study), and differences in the composition of the membrane model. The findings of the AFM experiments (Figure 3-6) were in good agreement and support the observations from the surface pressure isotherms (Figure 3-4 and 3-5). Taken together, these studies indicated that PEG-modified silica particles penetrated the monolayer and were
subsequently squeezed out, while the hydroxyl- and amine-modified particles did not penetrate the monolayer.

Conclusion

In this chapter, utilizing surface pressure isotherms and AFM the interactions of differently coated silica nanoparticles with lipid monolayers model at the air-water interface and supported lipid monolayers were investigated. Surface pressure isotherms of SM/DOPC/Chol after exposure to particles revealed surface property dependency in silica nanoparticles interaction. While, PEGylated silica particles demonstrated a shift in the isotherm at high surface areas which is attributed to PEGs surface activity, amine and hydroxyl-coated particles did not alter the surface pressure isotherms. PEGylated particles were also present in the supported lipid monolayers obtained at 5 mN/m (Figure 3-6 e-f left) while no particle was observed at 20 mN/m (Figure 3-6 e-f middle) and 30 mN/m (Figure 3-6 e-f right). This observation is in agreement with the surface pressure isotherm. On the other hand, amine and hydroxyl coated silica nanoparticles were not present in the AFM results which again correlates to no effects observed in the surface pressure isotherms for these particles.

These observations revealed that particles interactions with monolayer models are dependent to the surface activity of the surface properties and can be regulated by them. This is important as it strengthen the hypothesis that nanoparticles surface properties can play a role in their cytotoxicity. To further investigate the effects of surface property of the particles on their interaction with membrane models, and to find the relevance of membrane models, same particle system was studied with lipid bilayer which will be
discussed in the next chapter. Bilayer models provide fruitful information on the effect of particles on membrane fluidity and integrity. Therefore, the penetration of PEGylated particles in monolayer models can be investigated in bilayer models by monitoring changes in the anisotropy and leakage in bilayer models.
CHAPTER 4: INTERACTIONS OF SILICA NANOPARTICLES WITH A BILAYER MEMBRANE MODEL

Introduction

One of the widely used model membranes to mimic mammalian cell membrane are lipid bilayer models. Also known as liposomes or vesicles, these spherical lipid bilayers form a cell membrane like structure that separates the aqueous medium inside from the aqueous environment outside\(^5\) (Figure 4-1). Spherical lipid bilayers were first introduced by Bangham and Horne in 1965 by transmission electron microscopy of negatively stained Lecithin dispersions, and were named as concentric lamellae of lipids.\(^7\) The name “liposome”, derived from two Greek words meaning “fat body”, was given to these lipid bilayer structures by Gerald Wiessman in early 1970.\(^11\) Not much after liposomes discovery, they were introduced as biological cell membrane models by Bangham et al.\(^11\) Moreover, liposomes ability to encapsulate and carry drugs in their

*Figure 4-1. Categorization of vesicles based on lamellarity, demonstrating a SUV, LUV, MLV and GUV from left.*
aqueous medium was also introduced in the same era (1970s) by Gregory Gregoriadis.\textsuperscript{116,117} Ever since, vesicles are being used as a valuable and cost effective model membrane to study the possible cytotoxicity of xenobiotics (foreign to the body)\textsuperscript{118} and as a drug carrier for targeted drug delivery.\textsuperscript{119,120} Vesicles can be categorized based on their structural features. For instance, based on lamellarity (number of lipid bilayer layers), vesicles can be categorized to two different categories: unilamellar vesicles including small, large and giant unilamellar vesicles, and multilamellar vesicles (Figure 4-1).

Vesicles have been widely employed as a model membrane to study the interaction of xenobiotics including ENMs.\textsuperscript{25,68,118} This is mainly due to the cost effectiveness and vast degree of control over experimental parameters compared to the cell membrane of living cells. Using vesicles as the model membrane, nanoparticle internalization, membrane disruption\textsuperscript{25}, particle entrapment\textsuperscript{90} and membrane phase reconstruction\textsuperscript{110} can be monitored utilizing Cro-TEM, dye leakage assay, fluorescence anisotropy and Förster resonance energy transfer (FRET); respectively. The leakage assay which was firstly developed in 70s for liposome cell interactions;\textsuperscript{121} however, it was not until 2004 that the assay was employed to study both nanoparticle effect on vesicle leakage.\textsuperscript{122} Both anionic and cationic gold nanoparticle interactions with neutral L-R-Stearoyloleoyl-phosphotidylcholine (SOPC) and negatively charged SOPC and L-R-stearoyl-oleoyl-phosphotidylserine (SOPS) were investigated, and electrostatic interaction was introduced as the main mechanism of leakage induced by particles. Other more recent studies have also presented the electrostatic interaction as the main
mechanism of vesicle rupture.\textsuperscript{25,68,69,108} Recently, few studied have employed more complex, phase segregating vesicle and have shown that the interaction of ENMs are regulated by their surface charge.\textsuperscript{68,108} However, despite the body of literature investigating the interactions of ENMs with vesicles, to the best of our knowledge, the role of ENMs surface properties, specially PEGylation molecular weight, on a phase segregated bilayer model have not been well defined. Moreover, a correlation between monolayer and bilayer models of the same composition interacting with the same particle model has not been presented.

In this chapter, utilizing vesicle leakage assay and fluorescence anisotropy, effect of the different surface properties of the silica nanoparticles on LUVs integrity and fluidity will be investigated. The results of this investigation will be correlated to the monolayer studies and reveals the relevance of membrane models in studying ENMs disruptive interactions.

Materials and Methods

Large unilamellar vesicles (LUVs) were prepared following Zhang et al.\textsuperscript{86} procedure which has been explained in detail in Chapter 2. Vesicles size were 1.1 µm.

\textit{DPH Anisotropy of LUVs in Presence of Particles}

Silica nanoparticles were added to the prepared LUVs at 0.01 g/L and the final molarity of the vesicles was maintained at 500 µM in a final volume of 2 mL. DPH anisotropy experiments were carried out using the same phenomenon and procedure explained in chapter 2. Anisotropy versus temperature was measured at 20 °C intervals.
from 20 °C to 60 °C. Samples were given 10 minutes to equilibrate with sample holder temperature at each step.

Leakage Assay in Presence of Particles

Vesicles were exposed to each particle and changes in fluorescence intensity were monitored. Experiments were performed with three different concentrations (0.0001, 0.001 and 0.01 g/L) of particles for at least three times and the final molarity of 50 µM was maintained for vesicles in 2 mL of medium for all the experiments. The fluorescent intensity was measured in every 150 seconds and Triton X-100 was added to all samples after 60 minutes to release all the CF and yield the highest fluorescent intensity. CF fluorescence is self-quenched when encapsulated at 80 mM; the release of the dye from the liposomes, caused by membrane disruption by the particles, leads to its dilution below the self-quenching limit, resulting in an increase in fluorescence. Triton X-100 with a concentration of 0.2% (v/v) was added after 60 minutes to wash away the lipids and release the encapsulated CF completely. The fluorescence intensity from fully disrupted detergent treated vesicles is considered as 100% leakage. CF leakage percentage is calculated and normalized utilizing equation 2-11.

Results and Discussion

Silica Nanoparticles Induced Surface Property Dependent Leakage in LUVs

Nanoparticle effects on lipid vesicles were investigated by evaluating the integrity of the vesicles after interaction with the particles using a vesicle leakage assay. For this assay, the self-quenching, water-soluble fluorescent probe, 5(6) carboxyfluorescein (CF), was loaded in the vesicles at a concentration of 80 mM according to the methods of
Figure 4-2. Effects of different concentrations (0.0001 to 0.01 g/L) of engineered silica nanoparticles coated with amine (a), hydroxyl (b), PEG 2K (c), PEG 5K (d), PEG 20K (e), on the leakage of carboxyfluorescein (CF) from vesicles. Triton X-100 was used as positive control for complete disruption of vesicles. All results are reported as percentage of vesicle leakage, calculated as the ratio of CF fluorescence after the addition of silica particles over fluorescence after the addition of Triton X-100. Error bars demonstrate the standard deviation from at least three independent experiments.

Zhang et al. CF self-quenches at high concentrations; however, its dilution below the self-quenching limit results in an increase in fluorescence, providing a simple method to
monitor the integrity of vesicles based on the leakage of the fluorescent probe. CF-loaded vesicles were exposed to various concentrations of nanoparticles (0.0001-0.01 g/L, the same concentration range used in monolayer studies) and the leakage of CF from vesicles upon interaction with the particles was monitored by recording CF fluorescence over the course of one hour.

Silica nanoparticles affected the bilayer models in a different manner compared to the monolayer model. Highly charged amine and hydroxyl coated silica nanoparticles, resulted in significant vesicle leakage (Figure 4-2a and b), even though they had not disrupted the monolayer. Several studies have reported that the opposite electrostatic charge of the nanomaterials and vesicles plays a key role in inducing vesicle leakage. Hirano et al. and Moghadam et al. proposed electrostatic interaction to be the primary reason by which nanoparticles get adsorbed to the membrane model and induce vesicle leakage, demonstrating that positively charged, but not negatively charged, nanoparticles induce significant leakage in negatively charged vesicles through pore formation. In agreement, Chen et al. demonstrated that graphene oxide nanoparticles cause leakage by forming pores in the bilayer without rupturing the vesicles. Besides, Melby et al. reported the same phenomenon showing that unlike negatively charged gold nanoparticles, positively charged particles attached significantly to negatively charged DOPC/SM/Chol (60:20:20) vesicles. Attachment of positively charged gold nanoparticles were reduced drastically as the negative charge of the vesicles was reduced by decreasing the molar percent of the negatively charged lipid (DOPC) in the lipid composition. More recently, Lai et al. have also shown that kinetics of membrane
leakage is regulated by the opposite charge of the particles with respect to the vesicle through pore formation and nonspecific electrostatic interactions. They have reported that the rapid leakage in negatively charged DPPC LUVs is caused by positively charged CdSe/ZnS QDs functionalized with 3-mercaptopropionic acid (MPA), which also increase the fluidity of the membrane while the negatively charged CdSe/ZnS QDs functionalized with cysteamine hydrochloride (CA) induce a minimal leakage in the same system.

However, despite the importance of electrostatic interactions, the net vesicle and particle charge might not be the only deciding factor in particle binding to vesicles. For example, positively charged core-shell, maghemite-silica particles have been reported to bind to negatively charged, inert, and positively charged vesicles.\textsuperscript{123} A similar, but adverse, phenomenon is observed in the current study were both negatively charged hydroxyl-modified particles (-47 mV) and positively charged amine-modified and PEG-20K-modified particles (18 mV and 8 mV, respectively) were capable of inducing leakage in negatively charged vesicles. A confounding factor in interpreting such interactions is the fact that phospholipids, DOPC and sphingomyelin in this study, are zwitterionic and contain both positively charged and negatively charged groups. The positively charged trimethyamine groups in these lipids are still available to the hydroxyl groups (and potentially free silanol groups) on the surface of the particles for binding even if particles and vesicles do not have a net opposite charge and might explain the strong leakage induced by hydroxyl particles on negatively charged vesicles. The observed trend for silica nanoparticle induced vesicle leakage in our study is in agreement with previously reported cellular uptake of the surface functionalized quantum
dots (QDs) by skin cells (QD-COOH > QD-NH2 > QD-PEG).\textsuperscript{124} It is also worth noting that our results, showing a lack of leakage with PEG2K- and 5K-modified nanoparticles (Figure 4-2c and d) is in agreement with two very recent reports in the literature: Gal et al.\textsuperscript{125} verified that PEGylation (5K) of iron oxide nanoparticles (3–8 nm) suppress the nanoparticles interactions with zwitterionic POPC supported lipid bilayers, as the particle gets bigger in size and thus PEGylation becomes denser. In addition, Aldrian et al.\textsuperscript{108} reported that modification of cell penetrating peptides by modification using PEG 2K, reduced their effects on the leakage from DOPC/SM/Chol (2:2:1) vesicles. It remains to be seen whether modification of peptides and other nanoparticles with higher molecular weight PEG groups will result in leakage effects.

It is also important to note that the disruptive effects caused by the particles on the bilayers is in contrast to what was observed in monolayers. Amine and hydroxyl coated particles, which did not alter the surface pressure to a substantial degree, significantly disrupted the bilayer. In addition, while all PEGylated particles penetrated the lipid monolayer regardless of the PEG molecular weight, a clear dependence on the PEG molecular weight was observed in bilayer studies (Figure 4-2 c-e). This effect is likely due to the fact that while both lipid monolayers are used to investigate particle-membrane interactions, different mechanisms drive the interactions in these systems. In monolayer studies in the Langmuir trough, the particles are suspended in the subphase; thus, positively charged particles that are highly hydrophilic are unlikely to penetrate the surface and disrupt the monolayer. Instead, particle surface-activity regulates particle-lipid interactions in this system. While the amine- and hydroxyl-modified silica
nanoparticles are not surface active, addition of PEG groups to particle surfaces increases their surface activity (Figure 3-4). Particle surface-activity increases as the molecular weight of PEG is increased. This is in agreement with tensiometric studies on pure-PEG or PEG-modified lipids, reporting that high molecular weight PEG molecules are more surface-active compared to low high molecular weight PEG molecules.\textsuperscript{111,112} The surface-activity of the PEGylated particles will then dominate their interactions with lipid monolayers allowing the particles to penetrate the air-water interface at high mean molecular areas before being squeezed out upon compression. However, in studies with vesicles, particles and vesicles are both suspended and are thus particle interactions with vesicles do not depend on particle surface-activity. Instead, particle surface charge appears to regulate the interactions in this system, as evidenced by the fact that only charged particles induce vesicle leakage. Taken together, these results depict a clear difference between in particle interactions with lipid monolayers compared to lipid bilayers.

\textit{Nanoparticle Effects on Membrane Fluidity}

The vesicle leakage studies reveal the release of CF from inside the vesicles. However, these studies do not reveal whether the leakage is caused by particles disrupting the vesicles or particles being entrapped inside the bilayer, thus causing pores in the vesicles that result in leakage without vesicle disruption. The latter possibility is likely as it has been graphene oxide nanoparticles cause leakage by forming pores in the bilayer without rupturing the vesicles.\textsuperscript{78} To investigate this possibility, DPH anisotropy experiments were employed. It was hypothesized that particle entrapment inside a vesicle
Figure 4-3. Effects of 0.01 g/L of engineered silica nanoparticles on DPH Anisotropy of SM/DOPC/Chol (1:1:1) at different temperatures. Particles are coated differently with amine (a), hydroxyl (b), PEG 2K (c), PEG 5K (d), and PEG 20 K (e). All experiments were performed with a lipid concentration of 500 μM using 0.2 mol% DPH to measure anisotropy. Error bars show the standard deviation from at least three independent experiments.

is likely to affect the lipid packing (fluidity) of the bilayer, as has been previously reported. Thus, particle entrapment in the bilayer should result in alterations in
anisotropy. DPH is a small hydrophobic molecule, which localizes at the core of the bilayer. An increase in DPH anisotropy suggests the presence of a more rigid bilayer, while a reduction in anisotropy suggests a more fluid bilayer in which lipids have more lateral mobility. DPH anisotropy of vesicles was examined before and after exposure to nanoparticles. Studies were performed over a range of temperatures since temperature has been shown to affect the anisotropy of vesicle-nanoparticle mixtures. In the absence of particles, increasing the temperature reduced anisotropy (Figure 4-3). This trend is expected as increasing the temperature reduces the lipid order in the vesicles. However, no significant changes were observed in DPH anisotropy versus temperature after exposure to any of the surface-engineered silica nanoparticles (Figure 4-3a-e). Since the change in the temperature already affects the ordering of lipids and therefore anisotropy, the particle exposure effect to the vesicles might be hard to notice. As a result, DPH anisotropy of vesicles was also measured at a constant temperature of 25 ºC, versus time, in order to better monitor the effect of particles. However, no significant changes in the anisotropy of vesicles exposed to the nanoparticles was observed (data not shown). These observations suggest that, although some vesicles were disrupted based on the leakage assays, changes in membrane fluidity are insignificant. Using silver nanoparticles (3-4 nm) and DPPC vesicles, Park et al. reported a temperature-dependent change in DPH anisotropy after exposure to nanoparticles. Later, Bothun demonstrated the opposite, that by increasing the weight ratio of silver nanoparticles (5.7±1.8 nm) entrapped inside DPPC bilayers, the anisotropy values at lower temperatures (gel phase) decreased significantly, indicating more fluidity in the bilayer.
However, in both cases the size of the particles was significantly smaller than the size of the vesicles. In this study, the particles are of a much larger size (104 ± 5 nm), thus particle entrapment in vesicles appears less likely, as confirmed by anisotropy experiments.

**Conclusion**

In this chapter, the interactions of silica nanoparticles with a bilayer membrane model was examined. The effect of differently coated silica nanoparticles on the integrity and fluidity of LUVs was investigated, utilizing vesicle leakage assay and DPH anisotropy. It was shown that particles surface properties mediate the vesicle leakage effect caused by the particles as the highly charged particles induced the most dye leakage. While hydroxyl and amine-coated particles which were highly negatively and positively charged, and PEG 20K coated silica particles which were slightly positively charged induced leakage in accordance to their charge, PEG 2K and PEG 5K particles which are almost neutral did not cause any leakage. Further investigation was carried out on the membrane fluidity, using DPH anisotropy, to find out whether particle entrapment is one of the reasons behind vesicle leakage. No changes were observed in DPH anisotropy that can be attributed to the facts that either particle entrapment in the bilayer has not occurred or due to the coexistence of ordered and disordered phases and compensation by one phase when the other phase has been affected. The surface property dependent vesicle leakage effect provide fruitful guidelines on choice of surface property for achieving a biocompatible and safe particle, which can be low molecular PEG based on our results.
CHAPTER 5: CONCLUSION AND FUTURE WORK

Nanoparticles have a plethora of applications in industrial and biomedical settings\textsuperscript{20}, with over 1,800 consumer products reported to use nanoparticles in their formulation, and a lot more applications being constantly developed.\textsuperscript{6} The increasing assimilation of engineered nanomaterials (ENMs) into daily life has inevitably increased the risk of exposure to such particles leading to concerns regarding nanomaterial health risks and safety. Such concerns have particularly been raised in the case of engineered nanomaterials (ENMs).\textsuperscript{21} While the ability to functionalize the surface of nanomaterials has been instrumental in the development of new applications, despite intensive research\textsuperscript{23–25} it is still unclear how changes in ENM surface properties alter their interactions with mammalian cells and biomolecules.

In this thesis, utilizing different membrane models comprised of a ternary lipid mixture, we demonstrated that silica nanoparticles interact with lipid monolayers and lipid bilayers differently, dependent on their surface properties. Most importantly, we demonstrated that the characteristics of the membrane model affect the NP- membrane interaction. Monolayer studies revealed the incorporation of PEGylated particles to the membrane model, regardless of PEG molecular weight, at low surface pressures. Particle incorporation in the monolayer was evidenced by a rise in surface pressure at the early stages of compression in (Π-A) isotherms, which was due to increased lipid packing, and penetration of the PEGylated particles into the supported lipid monolayer at low surface pressures, visualized by AFM. In addition, no significant effects were observed by negatively charged hydroxyl and positively charged amine coated particles in the
monolayer studies. On the contrary, studies with lipid vesicles showed that highly charged particles (hydroxyl- and amine-coated) induce significant time dependent vesicle leakage after one hour, which was attributed to electrostatic interactions and pore formation in the vesicles. Moreover, in contrast with monolayer studies, the molecular weight of PEG plays a prominent role in inducing vesicle leakage since PEG 2K- and 5K-coated silica particles demonstrated negligible leakage effect while PEG 20K-coated silica particles resulted in substantial leakage. DPH anisotropy of vesicles was also measured versus temperature to monitor changes in membrane fluidity, however, no significant changes were observed due to coexistence of the ordered and disordered domains and the fact that change in the ordering of part of vesicle can be compensated by change in the ordering of another part.

In summary, this study demonstrates that engineered silica nanoparticles interact differently with lipid monolayers than they do with lipid bilayers. Nanoparticle interactions with lipid monolayers are primarily determined by particle surface-activity, resulting in monolayer penetration by PEGylated particles. However, nanoparticle surface charge appears to play the main role in nanoparticle interactions with lipid bilayers, with highly charged particles causing the most significant vesicle disruption. These results can have significant implications for mechanistic studies on nanoparticle-cell membrane interactions as both lipid monolayers and bilayers are very commonly used as cell membrane models.
Future Directions

To further study the effects of membrane models characteristics on NP-cell membrane interactions, other models including SUVs, GUVs and supported lipid bilayers can be employed. Our laboratory findings demonstrate that even different bilayer models can yield dissimilar results when interacting with the same particle model. Effect of the particles on membrane integrity and fluidity can be investigated using GUVs which are a different system compared to LUVs and allow for fluorescence visualization. The topology of SUVs deposited on a solid support as a supported lipid bilayer can also shed more light on the effect of membrane models characteristics on the changes in topology due to particle interactions. Furthermore, other methods including QCM-D can be employed to monitor particle attachment to membrane models. In another route, the effects of membrane composition on the NP-cell membrane model interactions can be investigated. Changing the mol% of SM in the ternary lipid mixture model can better elucidate the effects of phase segregation on the disruptive effect of nanoparticles. In addition, effect of lipid molecular structure can also be investigated by changing the sterol from cholesterol to coprostanol or ergostrol.

All in all, the multifarious ENMs with many different surface functionalities have increased the variable parameters of related studies tremendously, leaving us researchers with no other ways than employing various models and methods to gain control over them, and provide guidelines on toxicity of such materials.


(61) Sondi, I.; Salopek-Sondi, B. Silver Nanoparticles as Antimicrobial Agent: A Case Study on E. Coli as a Model for Gram-Negative Bacteria. *J. Colloid Interface Sci.* **2004**, *275*, 177–182.


(92) 5(6)<WBR> Carboxyfluorescein 21877


APPENDIX: PARTICLE PREPARATION AND CHARACTERIZATION

Particle preparation and characterization has been carried out in Dr. Allan David's laboratory (Chemical Engineering Department, Auburn University). The following is a description of materials and methods and characterization results as provided and authored by Alexander Kelly, a graduate student in Dr. David's research group.

Hydroxyl coated (Lot: ECP1044) and amine modified (Lot: JEA0116) silica nanoparticles were purchased from nanoComposix (San Diego, CA). N-Hydroxysuccinimide (NHS) functionalized methoxy polyethylene glycol (m-PEG-NHS) polymer in 2K, 5K and 20K Da molecular weights were purchased from Nanocs, Inc. (New York, NY). Fluorescamine, p-methylaminophenol sulfate (99%), ethanolamine (98%), sodium hydroxide (NaOH), oxalic acid anhydrate (98%), ammonium molybdate tetrahydrate (99%) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 99%) were purchased from Alfa Aesar (Ward Hill, MA). Silica standard was purchased from Ricca Chemical (Arlington, TX). Dimethyl sulfoxide (DMSO), sulfuric acid, and 37% hydrochloric acid (HCl) were purchased from BDH (VWR, Randor PA). Anhydrous sodium sulfite was purchased from Amresco (Solon, OH).

Pegylated silica nanoparticles were prepared by reacting m-PEG-NHS at a 10:1 molar excess ratio to reactive amines on the silica particles. Silica was added to m-PEG-NHS dissolved in 100 mM HEPES buffer at pH 7.5 and incubated overnight at room temperature using a RotoFlex tube mixer (Argos Technologies, Inc., Elgin, IL). Following functionalization, pegylated particles were washed three times to remove unreacted PEG from suspension by centrifuging at 16,000g for 10 minutes and
resuspending in water with a probe sonicator (2% amplitude) for 3-5 seconds.
Nanoparticle diameter, polydispersity index (PDI), and surface charge (zeta potential)
were determined by dynamic light scattering and laser Doppler electrophoresis using a
Zetasizer Nano ZS (Malvern Instruments, Inc., Westborough, MA). Measurements were
performed with nanoparticles dispersed in water. Nanoparticle morphology was
characterized using transmission electron microscopy (TEM) on a Zeiss EM10
microscope.

Silica nanoparticle concentration was determined using a modified blue
silicomolybdic assay.\textsuperscript{104} Briefly, acidified ammonium molybdate was prepared by adding
0.3 mL of concentrated HCL to 10 mL of 1\% (w/v) ammonium molybdate. Reducing
reagent was prepared by mixing 0.12 g anhydrous sodium sulfite, 0.4 g p-
methylaminophenol sulfate in 10 mL water with 6 mL oxalic acid (10\% w/v), 6 mL 50\%
(v/v) sulfuric acid, and 8 mL water. Silica nanoparticle samples were dissolved in 1 M
NaOH overnight and diluted with water before analysis. Two hundred microliters of
acidified ammonium molybdate reagent was mixed with 500 µL of sample and allowed
react for 10 minutes at room temperature. Finally, 300 µL of reducing reagent was added
and the mixture and incubated at room temperature for 2.5 hours before reading the
absorbance at a wavelength of 810 nm using a SpectraMax i3 instrument (Molecular
Devices, Sunnyvale, CA). A standard solution of silica was used to determine
nanoparticle concentration.

Nanoparticle suspensions received from nanoComposix were analyzed with a
fluorescamine assay to determine reactive surface amine concentration on the particles.
Fluorescamine is nonfluorescent until bound with a primary amine, which permits quantification of surface amine groups that are available for functionalization. Amine concentration was determined to be $0.48 \pm 0.14 \mu\text{mol amine/mg silica}$. Additionally, a modified blue silicomolybdic assay was used to determine silica concentration. As received silica suspensions were found to be $12.94 \pm 0.13 \text{ mg/mL}$. This suspension was diluted to $10 \text{ mg/mL}$ for all experimental purposes. PEG molecules were reacted with primary surface amine groups to form PEGylated nanoparticles. Silica particles were characterized with transmission electron microscopy (TEM) and dynamic light scattering (DLS). TEM analysis yielded a mean nanoparticle diameter of $113 \pm 13$ and $119 \pm 17 \text{ nm}$ for the amine and hydroxyl coated particles, respectively. Table 1 shows the hydrodynamic diameter and zeta potential of the nanoparticles.

Table 1
Hydrodynamic diameters and ζ-potential values of silica nanoparticles

<table>
<thead>
<tr>
<th>Coating</th>
<th>ζ-potential (mV)</th>
<th>Hydrodynamic Diameter (nm)</th>
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</thead>
<tbody>
<tr>
<td>Hydroxyl</td>
<td>-47.03</td>
<td>154.7</td>
</tr>
<tr>
<td>Amine</td>
<td>18.33</td>
<td>160.4</td>
</tr>
<tr>
<td>PEG 2K</td>
<td>-2.90</td>
<td>170.6</td>
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<tr>
<td>PEG 5K</td>
<td>-1.14</td>
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<tr>
<td>PEG 20K</td>
<td>8.09</td>
<td>190.2</td>
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